Spin labeling of oligonucleotides with the nitroxide TPA and use of PELDOR, a pulse EPR method, to measure intramolecular distances

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Published online 12 April 2007; doi:10.1038/nprot.2007.97

In this protocol, we describe the facile synthesis of the nitroxide spin-label 2,2,5,5-tetramethyl-pyrrolin-1-oxyl-3-acetylene (TPA) and then its coupling to DNA/RNA through Sonogashira cross-coupling during automated solid-phase synthesis. Subsequently, we explain how to perform distance measurements between two such spin-labels on RNA/DNA using the pulsed electron paramagnetic resonance method pulsed electron double resonance (PELDOR). This combination of methods can be used to study global structure elements of oligonucleotides in frozen solution at RNA/DNA amounts of ~10 nmol. We especially focus on the Sonogashira cross-coupling step, the advantages of the ACE chemistry together with the appropriate parameters for the RNA synthesizer and on the PELDOR data analysis. This procedure is applicable to RNA/DNA strands of up to ~80 bases in length and PELDOR yields reliably spin–spin distances up to ~6.5 nm. The synthesis of TPA takes ~5 days and spin labeling together with purification ~4 days. The PELDOR measurements usually take ~16 h and data analysis from an hour up to several days depending on the extent of analysis.

INTRODUCTION

In this protocol we use two techniques: site-directed spin labeling of oligonucleotides and a pulsed electron paramagnetic resonance (EPR) method called PELDOR also known as double electron electron resonance (DEER). The combination of both the techniques enables the researcher to measure long-range distances on the nanometer scale in RNA/DNA in frozen solution. These distances can then be used to study the three-dimensional structure on the nanometer scale and how it changes upon binding of metal ions, small organic ligands or proteins.

Spin labeling

To be able to apply EPR spectroscopic methods, the biomacromolecules have to carry an unpaired electron spin. Such paramagnetic centers could be metal ions (e.g., Cu^{2+} or Mn^{2+}), metal cluster (e.g., FeS cluster) or organic radicals formed either by charge transfer steps in the functional cycle, by irradiation or by means of chemical redox agents. Another possibility is to covalently attach stable organic radicals, like nitroxides (see **Table 1**), to the biomacromolecule. This approach is called spin labeling and the organic radical is the spin label. Spin-labeling methods that allow placing the nitroxide at single and selected sites are called site-directed spin labeling. In contrast to other approaches where the spin label is attached randomly, site-directed spin labeling permits study of the structure and dynamics of specific and selected structure elements. Whereas site-directed spin labeling of peptides or proteins is well established[1], site-specific labeling of oligonucleotides is less common but of increasing interest. In particular, synthetic strategies had to be developed to overcome the problem posed by dealing with large numbers of repeats of only a few kinds of naturally occurring nucleotides. **Table 1** gives an overview of the possibilities described in the following.

One approach is to use spin labels that react only with the 3′-ends of the oligonucleotides[2,3] or with unusual bases within tRNAs[4,5]. As RNA or DNA seldom possesses unique groups, an alternative is to introduce a unique nucleotide at a specific site, which can then react selectively with an adequately functionalized spin label. Another method is to incorporate the already spin-labeled phosphoramidite into the oligonucleotide. Both latter approaches work only if the RNA/DNA can be chemically synthesized. In 1992, Nagahara *et al.*[6] described a method to label the phosphate backbone of DNA strands by incorporating an H-phosphonate at a specific position and making it react with 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amine. As the coupling yields reached only 70%, the unlabeled strand had to be separated by means of HPLC. An advantage of this method is that the H-phosphonate monomers and the nitroxide are commercially available. UV/visible and CD melting studies indicated that the spin label attached to the phosphorus does not disturb the B-form of the DNA double helix[6]; however, fairly strong deviations were observed in the CD spectra for the respective single strands[6]. This clearly shows that if a spin label does not appear to alter the structure in one instance or from the chosen perspective, here a double helix, it does not necessarily mean that all oligonucleotide structures will be equally unaffected. Thus, a careful evaluation of the influence of the spin label on the oligonucleotide structure is mandatory.

As shown by Hubbell and co-workers[7], the interconnecting phosphate group can also be used to site-specifically label RNA. Specifically, they used a 2′-deoxyribo-phosphorothioate instead of the H-phosphonate and coupled 2,2,5,5-tetramethylpyrrolin-1-oxyl-3-iodomethyl to the thiolate group. In this approach, yields are quantitative and the spin label, attached to the end of the stem of a GAAA tetraloop hairpin, does not severely disturb the secondary structure according to UV–visible melting studies and the formation of the GAAA tetraloop/receptor complex. A big advantage of this method is its sequence independence, but the substitution of the nucleotide on the 5′-side of the label site by a

TABLE 1 | Selection of different ways to label RNA/DNA.

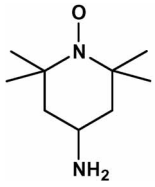
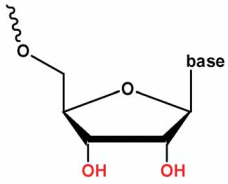
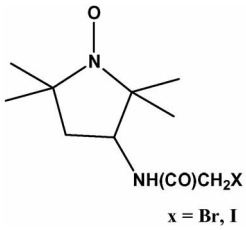
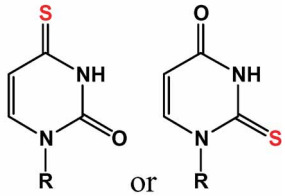
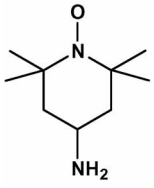
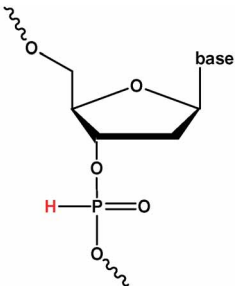
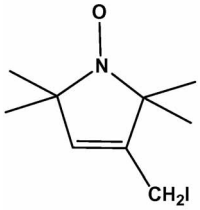
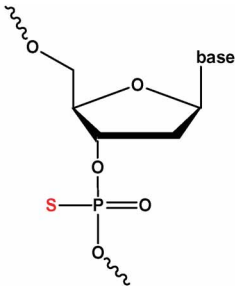
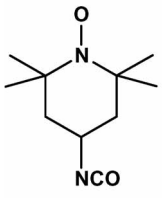
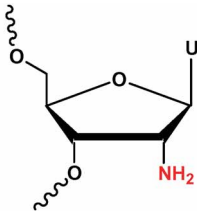
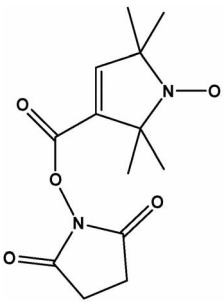
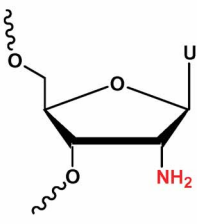
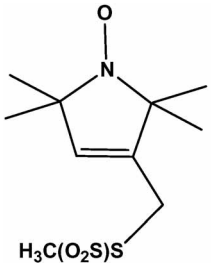
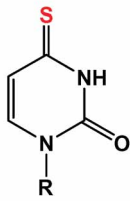
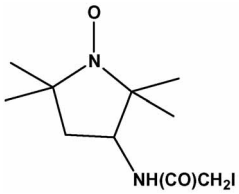
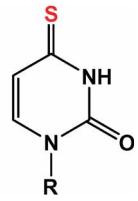
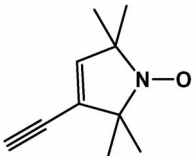
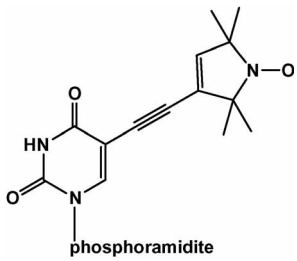
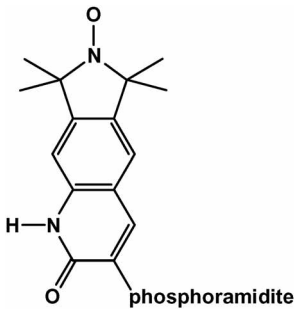
Nitroxide	Functional group	Advantage/disadvantage	Ref.
(A) Unique functional group available in RNA/DNA			
		Label commercially available Rough method (addition of $NaIO_4$) Applicable only to the 3′-end of RNA	2
		Mild reaction conditions Applicable only to these bases in tRNA Structural perturbations	4,5
(B) Introducing unique groups			
		Phosphoramidite/label commercially available Works sequence-independent for RNA and DNA 70% labeling yield Diastereoisomers have to be separated 2′-Deoxyribose has to be used to avoid phosphordiester bond cleavage	6
		High labeling yield Applied to RNA 2′-Deoxyribose has to be used to avoid phosphordiester bond cleavage Loss of negative charge	7
		Phosphoramidite commercially available High coupling yield >90%/RNA Applied only to the 2′-site of uridine Flexible linker	9
		Phosphoramidite and spin label commercially available Low coupling yields <50% for RNA Applied only to the 2′-site of uridine Flexible linker	10

TABLE 1 | Selection of different ways to label RNA/DNA (continued).

Nitroxide	Functional group	Advantage/disadvantage	Ref.
		Phosphoramidite/label commercially available High coupling yields/RNA Restriction to uridine, guanosine possible Labile disulfide bridge Structural perturbation due to loss of N3 proton	11
		As above, but more stable linker	12
	5-iodo-U-, 5-iodo-dU-, 5-iodo-C-, 2-iodo-A-	5-iodo-U/dU phosphoramidites are commercially available, 5-iodo-C and 2-iodo-A can be synthesized Cross-coupling on solid support High yield in labeled DNA/RNA Applicable to RNA/DNA Broad flexibility in label position Rigid and short label Minor local structural perturbation	17–19
(C) Labeled phosphoramidites			
		Rigid and short label Minor local structural perturbation Complicated and lengthy synthesis, low coupling yield Applied only to dU in DNA	13,14
		Very rigid Complicated synthesis Pairs only with 2-aminopurine	15
(D) For long RNAs use enzymatic methods			23–29

deoxyribonucleotide, to prevent strand cleavage, is a drawback if it leads to structural perturbations of the RNA. In a recent paper, Cai *et al.* [8] showed on duplex DNAs that even without separation of the two phosphorothioate stereoisomers R_P and S_P, clear PELDOR modulations are observable and that the measured distances correlate well with the distributions predicted by MD simulations on B-form duplex DNA.

In addition to the phosphate backbone, the sugar moiety can also be labeled. Sigurdsson and co-workers[9] achieved this for RNA by incorporating the commercially available 2′-amino-uridine-phosphoramidite during the automated solid-phase synthesis into the RNA sequence. The 2′-amino-modified RNA is then allowed to react with 2,2,6,6-tetramethylpiperidine-1-oxyl-4-iso-cyanate, which results in a urea linker between the nitroxide ring and the sugar. Yields are above 90% and structural perturbations for duplex and bulge regions are small, but the method was applied only to the 2′-sugar site of uridine, whereas the cytidine analog is also commercially available. DeRose and co-workers[10] used the commercially available 2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-succinimidyl-carboxylate instead of the isocyanate label, which leads to the formation of an amide linker. Depending on the length of the RNA, yields in this case are 20–50% lower. This spin label seems to lead in the case of duplex RNAs to severe structural perturbations, as melting studies indicated a destabilization by ~10 °C per spin label; bulge regions are less affected. A reason could be that the linker is too short, inducing sterical hindrance between the duplex RNA and the nitroxide ring.

Another possibility is to modify the base moiety. Mimicking nature, 4-thio-uridine was introduced during automated oligonucleotide synthesis into RNA and coupled to various methanethiosulfonate derivatives of 2,2,5,5-tetramethyl-pyrrolidine-1-oxyl (MTSL)[11]. This method led to almost quantitative yields (85–100%). NMR and UV–visible studies support the idea that the labeled thio-uridine base is still stacked within the loop and stem region of a tetraloop receptor and that the overall RNA structure perturbation is limited despite the loss of the N3 imino proton. However, this has to be verified carefully in each case as pointed out by Ramos and Varani[12]. Disadvantages of this method are the restriction to uridine sites and the possible cleavage of the disulfide bridge over time. The latter problem can be overcome at approximately the same yields using iodoacetamide-functionalized nitroxides, because in this case a thioether is formed instead of a disulfide bridge[12].

Instead of coupling the nitroxide to a unique group introduced into the RNA, it is also possible to synthesize an already spin-labeled phosphoramidite and to introduce this into the oligonucleotide using standard automated oligonucleotide synthesis. This approach was first applied by Hopkins and co-workers[13,14] to study the dynamics of DNA. They attached the ethynyl-substituted nitroxide TPA to the commercially available 5-iodo-2′-deoxyuridine via a palladium-catalyzed Sonogashira cross-coupling in solution and converted the labeled thymidine analog into the phosphoramidite. The major drawback is the lengthy and complex synthesis of the labeled phosphoramidite. As the label points into the major groove of duplex DNA, structural perturbations for duplex DNA are local and minor as confirmed by NMR, UV–visible and CD spectroscopy. To minimize structural perturbations by the nitroxide ring, a tether consisting of two ethynyl units was also developed, which however revealed a higher rotational freedom about the diyne axis[15]. An easier synthesis of a similar ethynyl-substituted six-membered ring nitroxide and synthesis of the labeled phosphoramidite was put forward by Gannett *et al.*[16].

In this protocol, we perform the Sonogashira cross-coupling on column during the solid-phase synthesis of nucleic acids. This approach has the following advantages: separation of the oligonucleotide from reagents and homocoupling product by a simple washing step, small amounts of spin labels introduced and cross-coupling yields of >95%[17]. We also demonstrated the applicability of this route to RNA[18] and its extension from 5-iodouridine to 5-iodocytidine and 2-iodoadenosine[19]. Using ACE instead of TBDMS chemistry increases the yield of labeled, isolated and purified RNA to 35–50% on a 0.2 μM scale[18]. Spin-labeled RNA/DNA strands can be synthesized up to 80 bases in length. Thus, this way of spin labeling combines the advantages of a fairly rigid and short label, enabling the observation of deep PELDOR modulations and easier translation of distance into structure, with a high-yield synthesis, broad flexibility with respect to the labeling site (major or minor groove, purine or pyrimidine, RNA or DNA) and only minor local perturbations to the structure.

An even more rigid nitroxide was placed into DNA by Hopkins and co-workers[20,21]. But, the synthesis of this labeled phosphoramidite is complicated and time consuming. Furthermore, this nucleotide forms a stable base pair with only 2-aminopurine and the effect of this base pair on the DNA structure is unknown.

Cekan and Sigurdsson[22] presented recently an extremely rigid nitroxide, which can also be transformed into a fluorophore with promising properties for the future. Such spin labels, which cannot rotate about the bonds in the linker, possess a decreased conformational flexibility and thus reduce the width of the distance distributions, which leads to PELDOR modulations less strongly damped and thus more clearly defined distances. In addition, the fixed relative orientation between the two labels might be resolved via orientation-selective PELDOR measurements. However, such extremely rigid labels might tend to induce severe structural perturbations of complex oligonucleotide folds other than B-form duplex DNA. Thus, to avoid structural disturbance in complex folds, more flexible and longer linker might be useful, but in this case the flexibility of the linker induces a broader distance distribution and makes a correlation of the measured distance with the RNA structure more complex. This shows that the spin-labeling strategy to use is a trade-off between several factors and that it has to be chosen according to the RNA and the problem to study.

The disadvantage of introducing unique groups during the automated synthesis is that the RNA/DNA has to be synthesized, meaning that the length of the oligonucleotides is limited to ~80 bases. This restriction in length can be avoided by enzymatic labeling within the sequence by Klenow filling in or by ligation of two already labeled oligonucleotides using a ligase. For example, already in 1973, a 2-thiocytidine[23] was attached with the help of tRNA nucleotidyl transferase to the 3′-end of $tRNA^{Phe}$ and the thio-group site-selectively allowed to react with 2,2,6,6-tetramethyl-piperidine-1-oxyl-4-(2-iodoacetamide)[24]. The reported reaction conditions are less drastic than the ones reported for the 3′-end labeling by Caron and Dugas[2], but require the enzymatic elongation of the tRNA. Shin and co-workers[25] described a method to label the 5′-end of RNAs. They transcribed the RNA using T7 RNA polymerase and an abundance of guanosine monothiophosphate (GMPS) in addition to the other NTPs in the reaction mixture.

The resulting RNA was a mixture of 5′-GTP and 5′-GMPS, only the latter of which was labeled with methyl-thiosulfonate spin-label. Although the labeling reaction was 90%, the yield of 5′-GMPS RNA from the transcription reaction may not be so high. Obviously, in this case no protection is needed as there is no nucleotide on the 5′-side of the label. Both methods are, however, restricted to the ends of the RNA. Bobst and co-workers[26,27] spin labeled uridine or cytidine at the 5-position via a large variety of flexible linker of different lengths and showed that these spin-labeled building blocks are accepted by the Klenow fragment. Their work is extensive and nicely summarized in ref. 28. Synthesizing a spin-labeled DNA/RNA fragment and filling the open sites using the Klenow fragment is another possibility[29]. Yields are, however, in both cases fairly low. Ligating two medium-sized and already labeled RNAs or DNAs together via a ligase has not been, to our best knowledge, performed yet.

Finally, it should be remembered that there is no general "best way" of spin labeling RNA/DNA, because the most suitable method depends on the system under study and the problem to solve. Thus, additional spin labels with different properties and further strategies to label RNA/DNA are required.

PELDOR

Once the oligonucleotide is labeled, EPR allows one to determine local and global dynamics ranging from picoseconds to milliseconds[30], to follow folding or unfolding[31] and to measure local pH values[32], solvent polarities[33] or viscosities[34]. In addition to EPR measurements, nitroxides can also be used for liquid-state NMR-based paramagnetic relaxation enhancement experiments[35]. If the biomolecule contains two spin labels separated by a distance r_{AB}, the dipolar coupling ω_{dd} between the two unpaired electrons can be measured by EPR spectroscopic methods and the distance r_{AB} can be calculated. With continuous-wave (cw) EPR spectroscopy, the dipolar coupling between two nitroxides was resolved up to distances of 20–25 Å (see ref. 36). Above this distance, the dipolar splitting vanishes within the line width and is not observable anymore. With PELDOR, the magnetic interaction between the two unpaired electrons can be resolved experimentally up to distances of 80 Å (see ref. 37). For biological samples in aqueous buffer solution, this distance is limited to about 60 Å owing to sample heterogeneity and fast T_2 relaxation times of the nitroxides.

Milov *et al.*[38] introduced first the three-pulse version of PELDOR shown in **Figure 1a** and Martin *et al.*[39] extended it to the dead time free four-pulse (**Fig. 1b**) version, now commonly applied and also described in detail in this protocol.

The pulse sequence $\pi/2-\tau_1-\pi-\tau_1-\text{echo}_1-\tau_2-\pi-\tau_2-\text{echo}_2$ (**Fig. 1b**) at the detection frequency ν_A creates a refocused echo (echo_2) from the spins on resonance, named in the following A spins. The position in time of the refocused echo does not change during the experiment, as the time intervals of the detection sequence are not changed. Introduction of the inversion pulse at pump frequency ν_B and in the time interval T flips spins, which are resonant with this second frequency, here defined as B spins. Thus, a coupling ω_{AB} between A and B spins causes a shift in the Larmor frequency of the A spins by ω_{AB} and therefore a change in the amplitude of the refocused echo. Incrementing the pump pulse from $t = 0$ to $t = T$ leads to an oscillation of the amplitude V of the refocused echo as a function of t.

The resulting PELDOR signal $V(t)$ can be considered as a product of two contributions:

$$V(t) = V(t)_{\text{intra}} V(t)_{\text{inter}} \quad (1)$$

$V(t)_{\text{intra}}$ describes all spins coupled intramolecularly, whereas $V(t)_{\text{inter}}$ takes into account the signal decay caused by intermolecular interactions between homogeneously distributed spins in the sample. Assuming that the spin orientations are not changed owing to spin diffusion or spin-lattice relaxation, $V(t)_{\text{intra}}$ can be described by equation (2)[40]

$$V(t)_{\text{intra}} = \frac{1}{n}\left\langle \sum_{A=1}^{n} \prod_{\substack{B=1 \\ B\neq A}}^{n} (1 - \lambda_B(1 - \cos(\omega_{AB}t))) \right\rangle \quad (2)$$

with

$$\omega_{AB} = \omega_{dd} + 2\pi J_{AB} \quad (3)$$

and

$$\omega_{dd} = -\frac{\mu_0 \hbar}{4\pi} \frac{\gamma_A \gamma_B}{r_{AB}^3} (3\cos^2\theta - 1) \quad (4)$$

where n is the number of radicals per biomolecule, λ_B is the fraction of B spins inverted by the pump pulse, t is the time delay of the pump pulse, ω_{dd} is the full dipolar splitting, μ_0 is the vacuum permeability, γ_A and γ_B are the magnetogyric ratios of the spins A and B, respectively, $\hbar$ is the Planck constant divided by 2π, r_{AB} is the distance between the spins, θ is the angle between the distance vector r_{AB} and the external magnetic field B_0, J_{AB} is the exchange

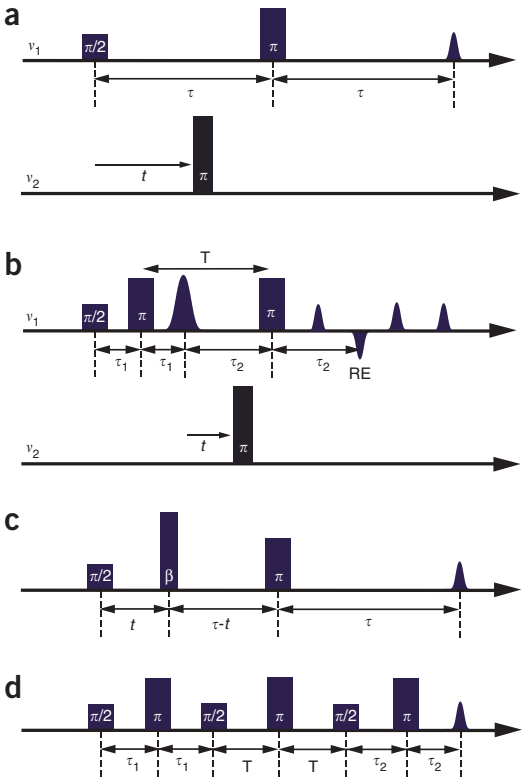


Figure 1 | Examples of EPR pulse sequences for distance measurements. (**a**) Three-pulse ELDOR, (**b**) four-pulse ELDOR (RE denotes the desired refocused echo), (**c**) "2+1" and (**d**) double quantum coherence. The pulse named β in **c** has to be adjusted in such a way that it flips the spin coupled to the spin detected by the $\pi/2-\tau-\pi$ sequence.

coupling in Hz and $\langle \cdots \rangle$ is the averaging over values of r_{AB}, J_{AB} and θ.

In the following it is approximated that J_{AB} is negligible versus ω_{dd} and that there is no angular correlation between the nitroxides in a biomolecule. $V(t)_{inter}$ can be written in the form of a monoexponential decay function (equation (5)) (ref. 41), where c is the radical concentration in spins per m^3:

$$V(t)_{inter} = \exp\left(-\frac{2\pi\gamma_A\gamma_B\mu_0}{9\sqrt{3}\hbar}c\lambda_B t\right) \quad (5)$$

Thus, dividing $V(t)$ by $V(t)_{inter}$ leaves the frequency-modulated signal $V(t)_{intra}$ of the intramolecular interaction (equation (2)) (**Fig. 2a,b**). The frequency of this oscillation is $\nu_{AB} = \omega_{AB}/2\pi$ and can be obtained by cosine Fourier transformation of $V(t)_{intra}$. However, Fourier transformation yields not only a sharp single frequency, but the whole Pake pattern (**Fig. 2c**), because the molecules are randomly oriented with respect to B_0, and ω_{AB} depends on the angle θ between the distance vector r_{AB} and B_0. Condition for the observation of the full Pake pattern is that all orientations are excited. The two singularities of the Pake pattern correspond to $\theta = 90° = \theta_\perp$ and $\theta = 0° = \theta_{||}$. From the frequencies at which these singularities appear, the dipolar coupling constant ν_{Dip} and J_{AB} can be calculated using equations (6) and (7) derived from equation (3) (see also **Fig. 2c**)[42]:

$$\nu_{Dip} = (\nu(\theta_{||}) - \nu(\theta_\perp))/3 \quad (6)$$

$$J_{AB} = (\nu(\theta_{||}) + 2\nu(\theta_\perp))/3 \quad (7)$$

Knowing ν_{Dip} allows one to calculate r_{AB} parameter free applying equation (4).

In disordered samples, a broad distribution of ω_{AB} values exists. Therefore, the $\langle \cos(\omega_{AB}t) \rangle$ terms in equation (2) will interfere to zero for times $t \gg \omega_{AB}{}^{-1}$ and $V(t)_{intra}$ will tend to its limit V_λ, which is the amplitude of $V(t)_{intra}$ when all modulation is damped. Assuming that all B spins are of similar nature, V_λ can be written as follows[40]:

$$V_\lambda = (1 - \lambda_B)^{(n-1)} \quad (8)$$

From equation (8), it follows that the number of spins in a biomolecule is

$$n = \frac{\ln V_\lambda}{\ln(1 - \lambda_B)} + 1 \quad (9)$$

The only free parameter in the calculation of n is λ_B, which can be determined experimentally using a standard biradical. Thus, PELDOR can be applied not only to measure distances between two nitroxides[43] but also to determine the number of monomers in an oligomer provided each monomer is singly labeled[44]. In addition, it is not only applicable to nitroxides but also to cofactors in proteins[45], metal centers[46] or different combinations of these[47].

Comparison with other methods

In comparison with cw EPR measurements, PELDOR has the advantage of giving access to larger distances, and no calibrations with monoradicals are necessary. However, the concentrations required are slightly higher (cw X-band: 10^{12} spins or 10 µl of a 1 µM solution/pulse X-band: 10^{14} spins or 10 µl of a 0.1 mM solution) and cw EPR is capable of measuring shorter distances.

A pulse sequence called "2 + 1" (**Fig. 1c**) in which detection and pumping sequence are applied at the same frequency has the disadvantage that hyperfine coupling interactions contribute strongly to the spectrum, making an interpretation of the observed frequencies difficult[48]. In the PELDOR experiment, these hyperfine contributions are suppressed owing to the use of two non-phase correlated microwave sources and the frequency offset between them. The cleanest and, in principle, the most appropriate pulsed experiment to measure ω_{AB} is the double quantum coherence experiment of Freed *et al.*[49] (**Fig. 1d**). However, this method requires full excitation of the nitroxide spectrum, which demands pulse lengths as short as 2 ns. The corresponding microwave field strength B_1 is not achievable with commercial pulsed EPR spectrometers.

To obtain distances between slow relaxing spin centers like nitroxides and fast relaxing metal centers, relaxation measurements and disentanglement of the dipolar enhancement might be the best way to obtain r_{AB}[50].

Compared with fluorescence resonance energy transfer (FRET)[51], PELDOR has the following advantages: (i) If the whole Pake pattern is observed or if the orientation dependence is resolved in a 2D experiment varying $\Delta\nu$, the orientation-dependent term can be determined from the measurement, whereas assumptions have to be made for the orientation factor κ in FRET measurements. (ii) The spin label used here is much smaller and more rigid than the chromophores commonly used in FRET, leading to an easier correlation between measured distance and structure of the biomolecule and to minor local perturbation of the biomolecule. (iii) With PELDOR, the whole Pake pattern can be measured, which allows the separation of the two coupling mechanisms J_{AB} and ω_{dd} from the same measurement. In FRET, different mechanisms can lead to fluorescence quenching and have to be carefully deconvoluted using standard samples under identical conditions but without FRET and additional measurements.

On the other hand, FRET has the great advantage of being applicable in fluid solution at room temperature, whereas PELDOR is restricted to measurements in frozen solution or at least to motionally frozen samples (see e.g., cw EPR experiments or measurements in oriented membranes, which are carried out at room temperature). Furthermore, FRET can measure signals on the single molecule scale. In addition, FRET is able to yield real-time data, whereas PELDOR would have to employ freeze–quench techniques to yield snap shots of the biomolecular motion.

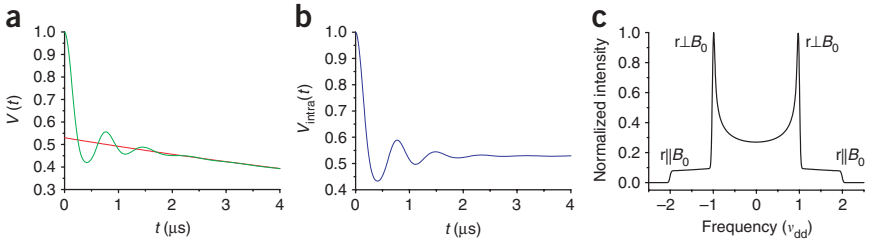


Figure 2 | Schematic representation of the PELDOR data processing. (**a**) The green curve shows the original PELDOR time trace $V(t)$. The red curve is the fitted monoexponential decay function, representing $V(t)_{inter}$. (**b**) $V(t)_{intra}$ after division of $V(t)$ by $V(t)_{inter}$. (**c**) Sketch of a dipolar Pake pattern.

NMR methods like REDOR[52] can also be used to measure long-range distances. However, owing to the smaller magnetic moment of the nuclei, the distance range is limited to roughly ≤1 nm. In summary, all these techniques are complementary to each other.

MATERIALS

REAGENTS

- 2,2,5,5-Tetramethyl-3-pyrrolin-1-oxyl-3-carboxyl (Acros, cat. no. 420640050)
- Red-Al, sodium-bis(2-methoxyethoxy)-aluminum hydride, 65% (w/w) in toluene (Sigma-Aldrich, cat. no. 19,619-3; usually received in portions of 100 g in Sure/Seal bottles for transfer via syringe (see Aldrich Technical Bulletin AL-134; http://www.sigmaaldrich.com/aldrich/bulletin/al_techbull_al134.pdf) ! CAUTION Red-Al is sensitive to moisture and reacts with water liberating extremely flammable gases (hydrogen).
- Toluene, puriss.; absolute; over molecular sieve (Fluka, cat. no. 89677)
- Dichloromethane, puriss.; absolute; over molecular sieve (Fluka, cat. no. 66749)
- Tetrahydrofurane, puriss.; absolute; over molecular sieve (THF; Fluka, cat. no. 87371)
- Dimethyl sulfoxide (DMSO), puriss.; over molecular sieve (Fluka, cat. no. 41647)
- Triethylamine, purum (NEt_3, Riedel de Haën, cat. no. 16304)
- Ethyl acetate, p.a. (Merck, cat. no. 1.09623.2500)
- *n*-Hexane, p.a. (Merck, cat. no. 1.04367.2500)
- Diethyl ether, p.a. (Merck, cat. no. 1.00921.1000)
- n-Butyllithium in *n*-hexane (*n*-BuLi; Merck, cat. no. 8.01660.0100). Usually, this is received in septum equipped bottles for syringe transfer ! CAUTION *n*-Butyllithium is sensitive to moisture and air, and reacts violently with water, liberating extremely flammable gases (butane, hydrogen). It is spontaneously flammable in air and causes burns. Wearing gloves during transfer of solutions containing *n*-butyllithium is highly recommended. For a Material Safety Data Sheet (MSDS), see http://www.emdchemicals.com/analytics/doc/msds/msds-display.asp?materialid=801660.
- Oxalyl chloride, purum (Fluka, cat. no. 75760) ! CAUTION Oxalyl chloride is toxic by inhalation and causes severe burns. Contact with water liberates toxic gases (hydrogen chloride). For an MSDS, see http://www.sigmaaldrich.com/catalog/search/AdvancedSearchPage for related information with cat. no. 75760.
- (Chloromethyl)triphenylphosphonium chloride, 97% (Sigma-Aldrich, cat. no. 377015-25G)
- Calcium hydride (Acros, cat. no. 199901000) ! CAUTION Calcium hydride is sensitive to moisture and reacts violently with water liberating extremely flammable gases (hydrogen). For an MSDS, see http://www.acros.com/DesktopModules/Acros_Search_Results/Acros_Search_Results.aspx?search_type=MSDS&SearchString=19990.
- Potassium tert-butoxide, 98% (KO^tBu; Acros, cat. no. 168881000)
- Silica gel; Merck, grade 60, 70–230 meshes, 60 Å (Sigma-Aldrich, cat. no. 39,148-4)
- Anhydrous sodium sulfate
- Dry ice
- Sodium hydrogencarbonate, aqueous solution (5% w/w)
- Sulfuric acid, aqueous solution (1% w/w)
- Ammonium chloride, aqueous solution (1 M)
- Sodium hydroxide, aqueous solution (5% w/w)
- DNA phosphoramidites from diverse sources, in particular Amersham Bioscience, 5-iodo-2′-deoxyuridine phosphoramidite (IUCE) from Glen Research
- Acetonitrile for DNA synthesis (Biosolve), also used for RNA (H_2O <0.003%; Biosolve, cat. no. 200-835-2)
- Standard solutions for the DNA synthesizer
  - Deblock (trichloroacetic acid (TCA) in CH_2Cl_2; Perseptive Biosystems, cat. no. GEN089894)
  - CAP A (*N*-methyl-imidazole; Proligo Biochemie, cat. no. L840020)
  - CAP B (acetic anhydride; Proligo Biochemie, cat. no. L850020)
  - Activator (dicyanoimidazole (DCI), 0.25 M in acetonitrile; Proligo Biochemie, cat. no. L080250 or tetrazole, 0.45 M in acetonitrile; Biosolve, cat. no. 20052401). Store it over molecular sieve
  - Oxidizer (iodine, 4.3 g in THF/pyridine/H_2O, v/v/v = 7/1/2; Biosolve, cat. no. 15182402) ! CAUTION For all reagents, see signs on bottles. It is advisable to use gloves during the replacement of the reagents on the synthesizer.
- Prepacked columns (clear pore glass (CPG) with the first base attached via a succinyl-linker, 1 μmol, 500 Å; Applied Biosystems, cat. no. 400948 for T, 400945 for dA, 400946 for dC and 400947 for dG)
- Ammonia (p.a. 32%; Grüssing, cat. no. 10350) mixed with p.a. methanol in a ratio of 3/1 ! CAUTION Ammonia causes burns. Wearing gloves is recommended.
- Buffer for the HPLC: 0.1 M triethylammonium acetate (TEAA) pH 7, acetonitrile, HPLC grade
- Phosphate buffer: 140 mM NaCl (>99%; Lancaster, cat. no. 13268), 5.77 mM $Na_2HPO_4 \cdot 2H_2O$ (p.a., Merck, cat. no. 6580 0500), 4.23 mM $NaH_2PO_4 \cdot H_2O$ (p.a., ACS Merck, cat. no. 6346.0500) pH 7
- RNA phosphoramidites of the natural bases and 5-iodouridine from Dharmacon, 5-iodocytidine and 2-iodoadenosine were synthesized[53]
- *S*-ethyl-tetrazole, purchased from Dharmacon (cat. no. R-002000-SE-200)
- Self-prepared standard solutions for the RNA synthesizer (see also REAGENT SETUP):
  - Deprotection of the first dimethoxytrityl (DMT) group: 3% dichloroacetic acid (puriss. 99%; Fluka, cat. no. 35810) in absolute CH_2Cl_2 over molecular sieve (Fluka, cat. no. 66749)
  - Deprotection of the 5′-*O*-silyl groups: hydrogen fluoride (HF, 48% in water; Fluka, cat. no. 47600), triethylamine (TEA, puriss. p.a. >99.5%; Fluka, cat. no. 47600), *N*,*N*-dimethyl-formamide (DMF, puriss. p.a. ACS >99.8%; Fluka, cat. no. 40250) ! CAUTION HF is extremely hazardous. Do not use without taking professional advice (please see also below).
  - CAP A: *N*-methylimidazole (redist. 99%; Aldrich, cat. no. 33,609-2), 10% in acetonitrile
  - CAP B: acetic anhydride (puriss. p.a. ACS >99%; Riedel-de Haën, cat. no. 33214), 10% in acetonitrile
  - Oxidizer: ^tBuOOH (purum, 70% in water; Fluka, 19995) in toluene (ACS; Fluka, cat. no. 32249) ! CAUTION Wear gloves for the preparation of all reagents. Especially be aware that hydrogen fluoride is extremely toxic and may be fatal if inhaled or ingested. It is readily absorbed through the skin and skin contact may be fatal as well. HF acts as a systemic poison and it causes severe burns; it is also a possible mutagen and reaction to it may be delayed. Any contact with this material, even minor, requires immediate medical attention. To handle HF, use rubber gloves, face-mask or safety glasses, apron, good ventilation. Do not work without calcium gluconate gel available to treat burns. Do not assume that gloves provide an impenetrable barrier to the acid. Do not work alone! Ensure that those working in the same laboratory are aware of how to treat hydrofluoric acid burns in an emergency. For an MSDS, see http://www.sigmaaldrich.com/catalog/search/AdvancedSearchPage for related information with cat. no. 47600.
- Prepacked columns (Dharmacon, aminomethyl-polystyrene with the first base protected with DMT attached via a succinyl linker, 0.2 μmol, cat. no. C-002020-rN-02, N = A, C, G or U)
- Deprotection of the amino groups: 40% methylamine in water (Aldrich, cat. no. 42,646-6)
- Deprotection of the methyl groups: 0.4 M disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate-trihydrate (S_2Na_2; purchased from Dharmacon, cat. no. R-001000-DP-50) in DMF/H_2O: 98/2
- Deprotection of the 2′-ACE groups: 100 mM acetic acid, adjusted to pH 3.8 with tetramethylethylenediamine (TEMED, >99%, for molecular biology; Fluka, cat. no. 87687)
- Buffer for the HPLC: 1 M LiCl (puriss p.a. ACS, >99.0%; Fluka, cat. no. 62478) pH 7
- Diethylpyrocarbonate-sterilized water (DEPC water), pH 8
- Phosphate buffer: 140 mM NaCl (>99%; Lancaster, cat. no. 13268), 10 mM $Na_2HPO_4 \cdot 2H_2O$ (p.a., Merck, cat. no. 6580 0500), 10 mM $NaH_2PO_4 \cdot H_2O$ (p.a., ACS Merck, cat. no. 6346.0500), pH 7 ! CAUTION TPA **5**, 2.1 mg; $Pd^{II}Cl_2(PPh_3)_2$ (purum, >98%; Fluka, cat. no. 15253), 2.0 mg; CuI (>99%, Riedel de Haën, cat. no. 03140), 9.5 mg; Dichloromethane over MS (Fluka, cat. no. 66749), 1.75 ml; TEA (puriss, p.a. >99.5%; Fluka, cat. no. 90340), 0.75 ml (used as received).

EQUIPMENT

- Teflon-coated magnetic stir bars
- Plastic syringes (all polypropylene)
- Reusable syringe needles (steel, 150 mm length, ∅ 1.2 mm) and disposable syringe needles (120 mm length, ∅ 0.8 mm)
- Rubber septa (∅ 15.9 mm)
- Glass-fritted funnels with medium porosity
- Fluted filter paper
- Buchner funnel with appropriate round paper filter
- Rotary evaporator (Buchi)
- Vacuum/inert gas manifold
- Hotplate
- Synthesize the oligonucleotides either on a commercial Perseptive Biosystems for DNA (program DNA 1 µmol) or a rebuilt ABI 392 from Dharmacon for RNA (program DRI-0.2 µmol) with the following main modifications: every glass part in particular the glass-flow restrictors must be replaced by Teflon to be resistant to HF, and TEA used for the deprotection of the 5′-silyl protecting group. A high-pressure regulator is also needed for the delivery of more viscous solutions like water, tBuOOH in toluene and HF, TEA[54]. The Gene Assembler Plus from Pharmacia does not require any modification
- For the Sonogashira cross-coupling on solid support, no special equipment is required except a vacuum/inert gas manifold (an oil-pump is advised to obtain a stable pressure) and argon. The 10 ml Schlenk flasks are not commercially available but can be home-made from 10 ml one-neck flasks (pear-shape or round-bottom flask). Reaction vials from Carl Roth (height: 4 cm, ∅: 8 mm, volume: 1 ml, ref. H-300.1)
- DNA purification: HPLC device from Merck/Hitachi, but any other low-pressure system with three solvents is also suitable; we used a reverse-phase semipreparative column Purospher RP-18e (5 µm, 250 × 10 mm) from Merck; flow: 3 ml min^{-1}
- RNA purification: HPLC device from JASCO in combination with an anion-exchange column preferentially NucleoPacTM PA 100 column, 250 × 9 mm from Dionex (advised owing to the very high quality and very good separation of n and n−1 oligonucleotides), flow: 5 ml min^{-1}
- Speed-vac (SC110 from Savant) to evaporate the probes and a thermostat for the deprotection steps
- Bruker ELEXSYS E580 pulsed X-band EPR spectrometer with a standard flex line probehead housing a dielectric ring resonator (MD5 W1, inner diameter 5 mm) equipped with a continuous flow helium cryostat (CF935) and temperature control system (ITC 502) both from Oxford instruments. The second microwave frequency was coupled to the microwave bridge by a commercially available setup (E580-400U) from Bruker. All pulses were amplified via a pulsed traveling wave tube amplifier (117×) from Applied Systems Engineering
- Liquid helium from AirLiquide
- Liquid nitrogen from Linde
- Gas tight 500 µl Hamilton syringe in combination with a Teflon tubing (outer diameter 1.96 mm, inner diameter 1.35 mm, length 30 cm) with a Kel-F hub on one end (available from Duratec)
- Suprasil quartz EPR tube, outer diameter 4 mm, available from Spintec
- 500 MHz Oscilloscope 9350AM from LeCroy

REAGENT SETUP

Triethylamine (NEt_3, purum, Riedel de Haën, cat. no. 16304) is dried with calcium hydride (3–5 g per 250 ml) by heating to reflux for 3 days and distilled under inert gas before use.

Toluene, dichloromethane, THF, DMSO (puriss.; over molecular sieve, Fluka, cat. nos. 89677, 66749, 87371, 41647). Usually obtained in dry cap flasks with septum for syringe transfer and used as received without further purification.

DEPC water Prepare a solution of 0.1% (1 ml) DEPC (purum, >97%, Fluka, cat. no. 32490) in 1 liter Millipore water. Let it react overnight and then autoclave for 40 min at 120 °C. After cooling to room temperature, adjust the pH to 8 with lithium hydroxide (microselect, >99%, Fluka, cat. no. 62528). The pH-electrode must be sterilized at least for 10 min with a solution of 2.5% formamide (Ultra for molecular biology, Fluka, cat. no. 47671) in Millipore water and then washed carefully with sterile water.

1 M LiCl Dissolve 42.4 g LiCl in 1 liter Millipore water (it does not need to be sterile).

0.1 M TEAA Under cooling mix 58 ml acetic acid (100%, p.a. Merck) and 140 ml TEA. Adjust to 1 liter with Millipore water (exothermic) with stirring on ice. Adjust the pH to 7 with acetic acid or TEA. Dilute 100 ml of this 1 M solution in 1 liter water to obtain the right concentration and check the pH.

TEMED/acetic acid 2.9 ml of acetic acid is added to sterile water (~500 ml) followed by 50–100 µl portions of TEMED to adjust the pH to 3.80 (sterile pH-electrode).

Phosphate buffer for DNA Dissolve 1.636 g NaCl, 205 mg $Na_2HPO_4 \cdot 2H_2O$ and 117 mg $NaH_2PO_4 \cdot H_2O$ in 200 ml Millipore water; check the pH.

Phosphate buffer for RNA Dissolve 1.636 g NaCl, 356 mg $Na_2HPO_4 \cdot 2H_2O$ and 276 mg $NaH_2PO_4 \cdot H_2O$ in 200 ml DEPC water (pH not adjusted to 8); check the pH.

Oxidizer ^{t}BuOOH in toluene Mix vigorously 36 ml *tert*-butylperoxide (70% in water) and 150 ml toluene in a separatory funnel and wait for 1 h. After separation, store the organic phase at −20 °C overnight to freeze the remaining water. ■ **PAUSE POINT** 16 h. ▲ **CRITICAL** The ice crystals are rapidly filtered over a glass-fritted funnel at 4 °C applying a slight vacuum and the solution collected in a pre-dried 200 ml bottle used for the RNA synthesizer. Do not filter until dryness to avoid ice crystals from melting. Argon is not necessary at this stage, but install this bottle as soon as possible on the synthesizer.

HF, TEA Needs to be prepared freshly before RNA synthesis, owing to problems with storage even at −20 °C and under argon. Mix under an argon atmosphere in the following order: to 120 ml DMF, add 80 ml TEA. Then add 7.8 ml HF (48% in water) dropwise and carefully. ! **CAUTION** HF is extremely toxic and must not be used without taking professional advice (see REAGENTS). Upon adding HF, a little bit of smoke evolves and the reaction is slightly exothermic. Transfer this solution directly into a 500 ml polyethylene glycol bottle (Nalgene) and install it immediately on the synthesizer.

S_2Na_2 Dissolve 200 mg of S_2Na_2 in 2 ml DMF/H_2O (v/v = 98/2) for each column; use an ultrasonic bath at 40 °C if the dissolution proceeds too slowly.

EQUIPMENT SETUP

RNA synthesizer For an optimal functioning, the RNA synthesizer must be prepared in the following way, in particular when it was not used during several weeks: The device must be cleaned very carefully with acetonitrile (use acetonitrile for DNA synthesis, Biosolve): 60 s for each nucleobase, 20–30 s for the other reagents. A program can be integrated to automatize the procedure. Use this cleaning step to check the solvent flow. The flow should be 7 ml in 3 min (for our setup). Install the reagents as indicated by the manufacturer. ▲ **CRITICAL** Always check the HF, TEA bottle at position 14. As the bottle is different from the usual ones (polyethylene instead of glass), it might not be tightly connected or could be damaged over time.

Install the phosphoramidites (0.1 M in acetonitrile): the oily nucleobases must be carefully and completely solved.

Before the Sonogashira cross-coupling reaction, dry all the necessary flasks, glass vials and spatulas overnight in an oven at 50 °C.

EPR spectrometer The setup for the pulsed EPR spectrometer is as commonly installed by Bruker.

PROCEDURE

Synthesis of TPA ● **TIMING** 5 days

Synthesis of 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-hydroxymethyl 2 ● **TIMING** ~1.5 days

1| The following synthesis Steps 1–23 are a modification of the procedure described by Hildeg *et al.*[55]. A 1,000 ml three-necked round-bottom flask containing a magnetic stir bar and equipped with a reflux condenser and a rubber septum is evacuated and flame-dried. Allow the flask to cool down to room temperature and flush it with inert gas. Repeat the procedure three times.

▲ CRITICAL STEP Air and water have to be kept out of the reaction flask as they react with Red-Al (see safety hints in REAGENTS).

2| Add 15.8 g (85 mmol) of 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxyl **1** (**Fig. 3**) to the flask and evacuate it once more. Flush the apparatus carefully with inert gas.

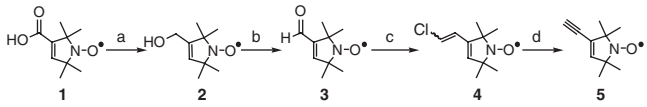


Figure 3 | Reaction scheme for the synthesis of TPA **5**. (**a**) Red-Al, toluene. (**b**) $Cl_2C_2O_2$/DMSO/NEt_3, CH_2Cl_2, −60° C. (**c**) [$ClCH_2(PPh_3)$]Cl/*n*-BuLi, THF. (**d**) KO^tBu, THF.

3| Turn the magnetic stirrer on and dissolve the solid in 250 ml dry toluene.

4| Using a 20 ml plastic syringe fitted with a steel needle (∅ 1.2 mm), add dropwise a total amount of 95 ml (312 mmol) of Red-Al to the yellow solution. The onset of hydrogen gas evolution is observed immediately.
! CAUTION Red-Al is sensitive to moisture and reacts with water liberating extremely flammable gases (hydrogen).
? TROUBLESHOOTING

5| After complete addition of the Red-Al, heat the mixture to 55 °C for 90 min.
▲ CRITICAL STEP Do not heat to reflux, otherwise the yield would be very low as the nitroxide itself is suspected to get reduced.

6| Allow the solution to cool to room temperature and add carefully about 200 ml of NaOH (5% w/w) to quench excess Red-Al.
! CAUTION Red-Al reacts with water, liberating extremely flammable gases (hydrogen).

7| Transfer the mixture to a separatory funnel, separate the phases and extract the aqueous phase with toluene. Up to 2 liters of toluene can be necessary for this extraction. Any unreacted 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxyl **1** will remain in the aqueous phase.

8| Combine the organic phases and dry the solution by adding ~30 g anhydrous sodium sulfate per 500 ml toluene and stir the solution for 5 min.

9| Filter the mixture through a fluted filter paper on a glass funnel to remove the sodium sulfate and collect the filtrate in a flask. Wash the sodium sulfate with additional toluene.

10| Evaporate the toluene using a rotary evaporator (50–60 °C per 80–120 mbar) to obtain 13.4 g of yellow crystals. Maybe it could further purified by recrystallization from diethyl ether/*n*-hexane. Yield: 92%.
? TROUBLESHOOTING

Synthesis of 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carbaldehyde 3 ● TIMING **1 day**

11| Place 7.8 ml (86.4 mmol) oxalyl chloride and about 100–150 ml dry CH_2Cl_2 in a dry (see Step 1) 500 ml three-necked round-bottom flask equipped with a dropping funnel, a rubber septum, a Teflon-coated magnetic stir bar and an inert gas inlet.

12| Transfer a solution of 13.4 g (78.5 mmol) 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-hydroxymethyl **2** in 80 ml CH_2Cl_2 into the dropping funnel under inert gas flow.

13| Cool the reaction flask to −60 °C with an acetone/dry ice bath and turn the magnetic stirrer on.

14| With a syringe, add a solution of 13.4 ml dry DMSO in 40 ml dry CH_2Cl_2 through the rubber septum dropwise. Let the mixture react for several minutes.

15| Within 10 min, add the solution of **2** from the dropping funnel to the reaction mixture. The color of the solution will turn from yellow to orange-brown. Continue stirring for further 15 min. This Swern-oxidation is more reliable than oxidation with manganese(IV) oxide as described by Hideg *et al.*[55].
! CAUTION During the reaction, dimethyl sulfide is formed and this has an intense sulfurous, boiled cabbage smell. Work in a well-ventilated hood.

16| Via a syringe, add 55 ml of dry NEt_3 and maintain the temperature at −60 °C for 5 min; then allow the reaction mixture to warm to room temperature.

17| The reaction mixture is quenched with the same amount of water. Separate the phases using a separatory funnel and extract the aqueous phase several times with CH_2Cl_2.

18| Wash the combined organic phases by extracting with brine (250 ml), H_2SO_4 (1% w/w, 125 ml), water (125 ml) and finally $NaHCO_3$ (5% w/w, 125 ml).

19| Dry the organic solution with the addition of ~30 g anhydrous sodium sulfate per 500 ml CH_2Cl_2. Stir the solution with a magnetic stir bar for 5 min.

20| Filter the mixture through a fluted filter paper on a glass funnel to remove the sodium sulfate and collect the filtrate in a flask. Wash the sodium sulfate with additional CH_2Cl_2.

21| Remove the solvent using a rotary evaporator (40 °C per 470 mbar).

22| Dissolve the obtained residue in a sufficient amount (~30 ml g^{-1}) of hot ethyl acetate/*n*-hexane (1:3, vol/vol) and separate the hot solution from insoluble by-products by rapid filtration. Place the recrystallization flask in a cooling bath (ice/sodium chloride) or in a refrigerator at −20 °C.
! CAUTION Both ethyl acetate and hexane are flammable solvents; do not use open flames to heat them.
■ **PAUSE POINT** For recrystallization, the flask should be left in the cooling bath for at least 1 h. It can be left in a refrigerator overnight.

23| Collect the yellow crystals by filtration with a Buchner funnel. Wash the crystals with a small amount of cold ethyl acetate/*n*-hexane (1:3, vol/vol) and finally with cold *n*-hexane. From the mother liquor, an additional amount of the product can be obtained. Usually, the yield of 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carbaldehyde **3** reaches between 65% and 75%. The first fraction of crystals (8.39 g, 49.8 mmol, 64%) is sufficiently pure for the next reaction step. Repeat recrystallization if necessary. The product can also be purified thoroughly by means of vacuum sublimation (50 °C, 2×10^{-3} mbar).

Synthesis of 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-(2-chloroethenyl) 4 ● TIMING 1 day

24| This procedure (Steps 24–43) is a modification of the synthesis described by Spaltenstein *et al.*[14]. A 500 ml three-necked round-bottom flask equipped with a rubber septum, a reflux condenser, an inert gas inlet and a magnetic stir bar is evacuated and flame-dried (see Step 1).

25| Place 19.6 g (55 mmol) (chloromethyl)triphenylphosphonium chloride in the flask, evacuate the apparatus and flush it again with inert gas carefully.
▲ **CRITICAL STEP** Air has to be thoroughly expelled from the flask; see also CAUTION to Step 27.

26| Suspend the solid in 125 ml dry THF under inert gas flow.

27| Cool the suspension with a cooling bath (ice/sodium chloride) and add 34.3 ml (55 mmol) *n*-BuLi dropwise with a syringe via the rubber septum. The color of the solution will turn yellow and finally dark red.
! CAUTION *n*-BuLi is pyrophoric (ignites in air).

28| After complete addition of *n*-BuLi, allow the mixture to warm to room temperature and continue stirring for 45 min.

29| Cool the reaction mixture again with an ice bath and add a solution of 8.39 g (49.8 mmol) 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carbaldehyde **3** in 100 ml dry THF via a syringe. Remove the cooling bath and continue stirring for 2 h.

30| Using a water bath, warm the mixture slowly to 50 °C and continue stirring at this temperature for 1 h to complete the reaction.

31| After cooling to room temperature, add a small amount of water (2 ml) and remove the solvent using a rotary evaporator (45 °C per 470 mbar).

32| Redissolve the residue in diethyl ether (300 ml) and wash the organic solution at least twice with the same amount of water.

33| Separate the phases and dry the organic phase with ~30 g anhydrous sodium sulfate. After stirring for about 5 min, remove the solid sodium sulfate by filtration.

34| Using a rotary evaporator (35 °C per 800 mbar), reduce the diethyl ether solution to one-half of its volume and filter the solution through a short column of silica gel (h × ∅: 6 × 10 cm). Wash the pad of silica gel with several portions of diethyl ether thoroughly (at least 500 ml).

35| Collect the organic solution in a flask and remove the diethyl ether using a rotary evaporator (35 °C per 800 mbar). The yellow solid consists mainly of a mixture of E- and Z-isomers of **4** and can be used for the elimination step after drying *in vacuo*.
? TROUBLESHOOTING

Synthesis of TPA, 5 ● TIMING **1–2 days**

36| Place 10 g of the solid obtained from Step 35 in a dry (see Step 1) round-bottom flask equipped with a reflux condenser, inert gas inlet and magnetic stir bar. With a flow of inert gas, add 200 ml THF to dissolve the solid completely.

37| Weigh roughly 2.5–3 equivalents of KO^{t}Bu in terms of molar quantity of the aldehyde **3** used in Step 29. Weigh 2.75 equivalents of KO^{t}Bu (15.36 g, 137 mmol) into a beaker and add it with a flow of inert gas to the reaction flask.

38| After complete addition of the base, heat the reaction mixture under inert gas to 55 °C for 2 h.

39| Allow the solution to cool to room temperature and add 10 ml of 1 M NH_4Cl aqueous solution for hydrolysis. Remove the organic solvent by using a rotatory evaporator (45 °C per 470 mbar).

40| Redissolve the residue in diethyl ether (250 ml). Transfer the solution to a separatory funnel and extract the organic phase three times with 100–150 ml water and finally brine.

Function	Mode	Amount	Time (s)	Description
$Deblocking	Arg1/Arg2			
144 /*Index Fract. Coll.	*/ NA	1	0	"Event out ON"
0 /*Default	*/ WAIT	0	1.5	"Wait"
141 /*Trityl Mon. On/Off	*/ NA	1	1	"START data collection"
16 /*Dblk	*/ PULSE	10	0	"Dblk to column"
16 /*Dblk	*/ PULSE	50	49	"Deblock"
38 /*Diverted Wsh A	*/ PULSE	40	0	"Flush system with Wsh A"
141 /*Trityl Mon. On/Off	*/ NA	0	1	"STOP data collection"
38 /*Diverted Wsh A	*/ PULSE	40	0	"Flush system with Wsh A"
144 /*Index Fract. Coll.	*/ NA	2	0	"Event out OFF"
$Coupling				
1 /*Wsh	*/ PULSE	5	0	"Flush system with Wsh"
2 /*Act	*/ PULSE	5	0	"Flush system with Act"
24 /*7 + Act	*/ PULSE	6	0	"Monomer + Act to column"
24 /*7 + Act	*/ PULSE	1	8	"Couple monomer"
2 /*Act	*/ PULSE	4	32	"Couple monomer"
0 /*Default	*/ WAIT	0	300	"Wait"
2 /*Act	*/ PULSE	5	0	"Flush system with Act"
24 /* 7 + Act	*/ PULSE	6	0	"Monomer + Act to column"
24 /* 7 + Act	*/ PULSE	1	8	"Couple monomer"
2 /*Act	*/ PULSE	4	32	"Couple monomer"
0 /*Default	*/ WAIT	0	300	"Wait"
1 /*Wsh	*/ PULSE	7	56	"Couple monomer"
1 /*Wsh	*/ PULSE	8	0	"Flush system with Wsh"
$Capping				
12 /*Wsh A	*/ PULSE	20	0	"Flush system with Wsh A"
13 /*Caps	*/ PULSE	8	0	"Caps to column"
12 /*Wsh A	*/ PULSE	6	15	"Cap"
12 /*Wsh A	*/ PULSE	14	0	"Flush system with Wsh A"
$Oxidizing				
15 /*Ox	*/ PULSE	15	0	"Ox to column"
12 /*Wsh A	*/ PULSE	15	0	"Flush system with Wsh A"
$Capping				
13 /*Caps	*/ PULSE	7	0	"Caps to column"
12 /*Wsh A	*/ PULSE	30	0	"End of cycle wash"

Figure 4 | Modified DNA cycle. The modifications are given in red. IUCE is placed at position 7.

41| Separate the organic phase and dry the solution with stirring by adding ~15 g anhydrous sodium sulfate. Remove the solid sodium sulfate by filtration after 5 min.

42| Evaporate the diethyl ether using the rotary evaporator (35 °C per 800 mbar). The residue is dried *in vacuo*.

43| Transfer the solid into a sublimation apparatus. The desired TPA **5** is efficiently purified by sublimation at 47–51 °C (2×10^{-3} mbar) and is obtained as yellow solid or shiny yellow crystals in about 50% yield over the last two steps. Following the procedure described here, 4.05 g (25 mmol, 50% referred to 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carbaldehyde **3**) of TPA **5** is prepared. TPA is stable when pure and can be stored at −20 °C for 1 year or more.

DNA/RNA synthesis (the time for the synthesis depends on the length of the oligonucleotide: ● TIMING **5 min per base for DNA, 6.5 min per base for RNA)**

44| Type the DNA or RNA sequences into the respective synthesizer and start the syntheses according to the respective technical manual. Stop the syntheses after incorporation of the iodinated phosphoramidites 5-iodo-2′-deoxyuridine for DNA and 5-iodouridine, 5-iodocytidine and 2-iodoadenosine for RNA. The last 5′-*O* protecting group must not be cleaved either in DNA or RNA (instruction DMT on even with the Dharmacon synthesizer). In the case of 5-iodo-2′-deoxyuridine, modify the DNA cycle to achieve a total coupling time of 12 min. For a better efficiency, the same quantity of base and activator was added to the column a second time after 6 min of coupling as shown in **Figure 4**. In the case of the RNA, do not change the cycle. For a detailed cycle, see the manual from Dharmacon. ? TROUBLESHOOTING

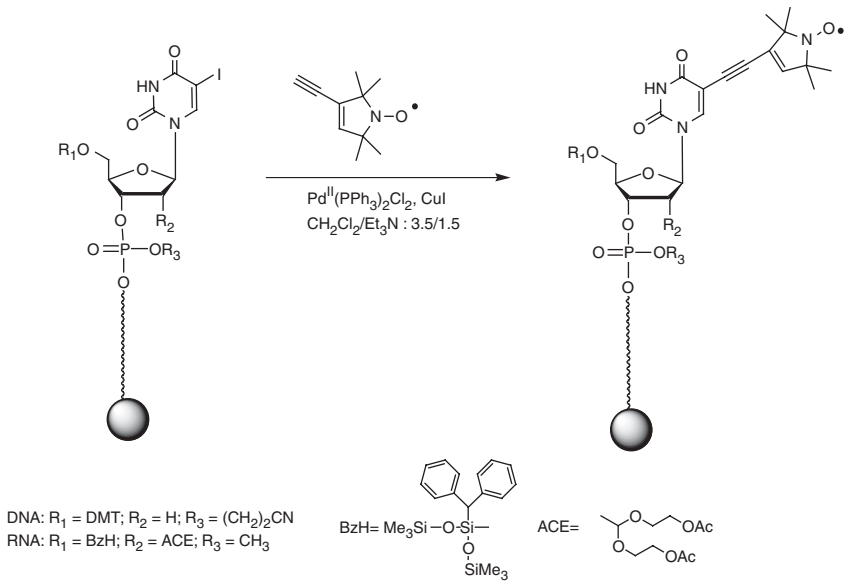


Figure 5 | Sonogashira cross-coupling of TPA **5** to 5-iodouridine on solid support (also applicable to 5-iodocytidine and 2-iodoadenosine).

Sonogashira cross-coupling on solid support (Fig. 5)
● TIMING **6 h**
45| Dry the TPA for 1 h *in vacuo*.

46| Take the column from the synthesizer and attach it to a 1 ml syringe and place it in a 100 ml Schlenk flask flushed with argon. Use the syringe to pull and push argon through the column (**Fig. 6a**). Another 1 ml syringe equipped with a needle (7 cm long) was purged several times with argon and placed in the same 100 ml flask (**Fig. 6b**).
! CAUTION Owing to a partial solubility of certain syringes in dichloromethane, syringes from Terumo (ref. 612-0106 by VWR) are advised.

47| Weigh precisely 2.0 mg $PdCl_2(PPh_3)_2$ and 2.1 mg TPA, each in a separate small glass reaction vial (Roth). Add TPA into the glass vial containing Pd(II). Put the glass vial with the mixture into a 10 ml Schlenk flask (**Fig. 6c**), already purged with a continuous flow of argon and purge the Schlenk flask with the vial inside. Also at this stage weigh 9.5 mg CuI into a 1.5 ml Eppendorf vial. The reaction scheme for the sonogashira cross-coupling is shown in **Figure 5**.

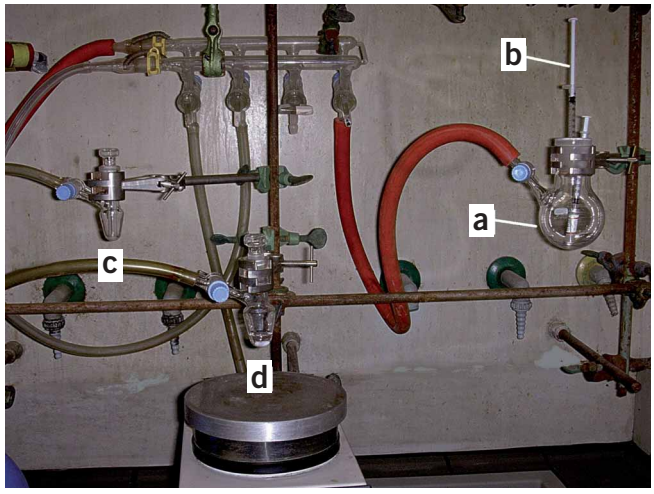


Figure 6 | Setup as used for the Sonogashira cross-coupling on column. (**a**) Column attached to a 1 ml syringe (argon atmosphere); (**b**) syringe full of argon; (**c**) glass vial containing Pd(II) and CuI in a 10 ml Schlenk flask under argon; (**d**) stirred solution of CuI in CH_2Cl_2/TEA in a flask under argon.

48| In another 10 ml Schlenk flask under argon (**Fig. 6d**), mix 1.75 ml of absolute methylenchloride and 0.75 ml of TEA.
▲ CRITICAL STEP The deoxygenation of this solution must be efficient and rapid at the same time because of the volatility of CH_2Cl_2. For this purpose, perform rapid but several vacuum/argon cycles (around 20). Choose polyethylene tubes to avoid dissolution of the tube in CH_2Cl_2. It might be helpful to cool the solution. Do not wait more than 1 min until the next step to avoid evaporation of the CH_2Cl_2.

49| Add 9.5 mg CuI to the CH_2Cl_2/TEA solution. This must not take longer than a few seconds. Do not wait until CuI is dissolved; otherwise, the solution will turn black after the addition of Pd^{II} and TPA and the reaction will stop (**Table 2**). The black color originates from Pd^0 in "*statu nascendi*," which is formed by reduction of Pd^{II} by TPA. Transfer immediately 200 µl of this suspension to the glass vial containing Pd^{II} and TPA using the 1 ml syringe previously placed in the 100 ml flask. Mix the reagents rapidly by pulling and pushing the solution twice back and forth using the syringe. Keep the solution in the syringe. The color of the solution thus obtained can range from yellow to brown, depending on the amount of CuI that managed to get solubilized, but the efficiency of the coupling is not affected by the actual concentration of CuI. Eject the bubble of argon from the syringe, which is important to be able to immerge the whole solid support with the solution. Remove the needle and attach the syringe to the column. Inject the solution into the column carefully with the help of both syringes and move the solution in the column back and forth. The solution turns yellow-orange.
▲ CRITICAL STEP The procedure from the addition of CuI to the addition of the solution to the column must not exceed 1 min. Every single operation must be performed quickly.
? TROUBLESHOOTING

50| Place the column, with the two syringes attached, in a dark drawer, and allow a reaction time of 2.5 h.

51| Take the two syringes off and wash the column with 10 ml of dry CH_2Cl_2 using a syringe. Dry the column for 10 min under vacuum in the 100 ml flask and purge with argon and repeat coupling (Steps 47–50) by following the same procedure.

Resumption of DNA/RNA synthesis ● TIMING depends on the length of the oligonucleotide
52| Wash the column with 10 ml absolute CH_2Cl_2, dry it carefully *in vacuo*, place it on the synthesizer and resume the DNA/RNA synthesis (instruction DMT off for the last 5′-*O*-protecting group).

Oligonucleotide purification ● TIMING 1 day
53| Perform the purification of oligo-DNAs following the directions in method A. For purification of oligo-RNA, follow instead the directions in method B.
Method A (DNA purification)
(i) Transfer the CPG beads into a 4 ml vial. Add a solution of 1.5 ml ammonia (32%) and 0.5 ml methanol to the vial to effect cleavage of labeled oligonucleotides from the CPG material along with deprotection of the amino and phosphate groups.
■ PAUSE POINT Incubate the mixture for 24 h at room temperature.

(ii) Filter the solid support with special filters (0.2 μm), wash the beads with Millipore water and evaporate the solvents by means of a speed-vac at room temperature. At this stage, the sample can be analyzed by means of ESI mass spectrometry to estimate the yield of the coupling step.

(iii) Perform DNA purification by reverse-phase HPLC applying the following gradient: 0.1 M TEAA/acetonitrile from 100/0 to 70/30 in 40 min. The DNA should elute between 15 and 25 min. However, these gradients and the retention time depend on the efficiency of the column. A major side reaction consists in the reduction of TPA to the corresponding amine. The difference in polarity is such that it is possible to separate both products very easily. The difference in the retention time is more than 1 min, allowing one to readily separate the products (**Fig. 7**). In contrast, if the Sonogashira cross-coupling is not complete, it is very difficult to separate the 5-iodo-2′-deoxyuridine-modified DNA from the TPA-modified one. Analyze DNA by ESI mass spectrometry.

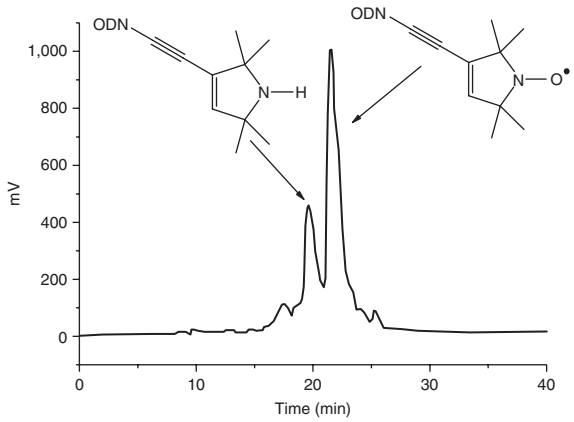


Figure 7 | HPL chromatogram of 3′-GCTGACTATATAGTCAGC.

Method B (RNA purification)

(i) First cleave the methyl groups on the phosphate with the RNA on column by slowly adding a 0.4 M solution of S_2Na_2 over 30 min. For this purpose, move the yellow solution back and forth with a system of two syringes.

(ii) Wash the column with 5 ml HPLC or Millipore water and 5 ml acetonitrile.

(iii) Dry the column for at least 10 min under vacuum.

(iv) Pour the beads into a 4 ml vial and cleave the oligonucleotides from the solid support and deprotect them by adding 2 ml methylamine (40% in water).

■ **PAUSE POINT** Incubate the reaction mixture for 12 h at room temperature.

(v) Filter the solution and evaporate under vacuum with a speed-vac (Dharmacon technical manual).

(vi) Purify the RNA by reverse-phase HPLC applying the following gradient: H_2O/1 M LiCl from 100/0 to 40/60 for 12–30-mers or to 30/70 for 40-mers, or 0/100 for larger RNAs. The RNA should elute between 20 and 25 min. However, these gradients and the retention time depend on the efficiency of the column. In the case of RNA, TPA reduction is minimized due to the use of ^tBuOOH/toluene instead of I_2/H_2O/pyridine.

(vii) Desalt RNA with PD10 Sephadex columns from GE Healthcare.

(viii) Analyze the RNAs with MALDI and cleave the ACE groups under sterile conditions with 400 μl TEMED/acetic acid buffer over 30 min at 60 °C. Evaporate the solution until dryness and store at −20 °C.

? TROUBLESHOOTING

EPR sample preparation ● **TIMING** **DNA ~ 0.5 day, RNA ~ 1.5 day**

54| (Applies only to RNA; for DNA, go directly to Step **56**) Sterilize quartz EPR tubes and a Hamilton syringe by filling the tube completely with a 2.5% formamide solution in Millipore water (no air bubble must remain inside the tube).

■ **PAUSE POINT** Allow the solution to sterilize the glassware by incubating for at least 3 h or overnight.

55| Clean the tube carefully with DEPC water using sterile tips. Dry the tube overnight in an oven at 80 °C.

56| Prepare an EPR sample as a 50 μM (100 μM in spins) solution of the respective duplex in the appropriate phosphate buffer (see REAGENTS and REAGENT SETUP) with 20% (v/v) ethylene glycol. For this purpose, dissolve the required amount of DNA or RNA (measured with UV) in 100 μl of the aqueous phosphate buffer (without ethylene glycol) and transfer the solution into a cuvette, adapted for a UV spectrometer equipped with a thermostat. Denaturate the duplex by heating to 70–80 °C and anneal the oligonucleotides by cooling down to 5–10 °C (rate: 5 °C min^{-1}). Transfer the probe into a vial, evaporate to dryness and dissolve the residue in a 20% (v/v) ethylene glycol solution in water (Millipore or DEPC).

Transfer this solution into a quartz EPR tube using a Hamilton syringe with Teflon tubing (allow the glassware to cool down to room temperature before use). Freeze the sample as soon as possible by immersing the tube quickly into liquid nitrogen. The sample can be conserved in liquid nitrogen for months. In case a split-ring resonator is used instead of a dielectric ring resonator, the sample volume can be reduced to 10 μl.

▲ **CRITICAL STEP** It is important to find a good compromise between large echo intensity and weak intermolecular interactions. Generally, a spin concentration of 100 μM is well suited. Ethylene glycol was added to avoid crystallization of the water and to assure a homogeneous sample distribution.

PELDOR experiment ● TIMING ~17 h

57| Switch on the pulsed EPR spectrometer with the two microwave (mw) excitation sources for pump and probe spins. Cool the sample cryostat down to a working temperature of 40 K. If your AFC and/or frequency stabilizer system is in conflict with the two-frequency excitation, turn them off.

58| Couple the resonator critically and insert the frozen sample tube. The optimum position corresponds to the maximal frequency shift of the resonator.

59| Adjust Q of the cavity: overcouple the resonator to a Q-value of approximately 50. Readjust the mw frequency to the center of the resonator frequency.

60| Switch on the traveling wave tube amplifier and apply a two-pulse Hahn-echo sequence with mw pulses of a length of 32 ns, pulse separation of 180 ns and pulse repetition time of 7.5 ms to the cavity. Set the detection video bandwidth to 25 MHz. Note that a slight distortion of the echo at this bandwidth has no influence on the PELDOR experiment, as only echo amplitude changes are monitored. With full mw power, no cavity ringing should be observable after the protection switch in the mw receiver channel.
? TROUBLESHOOTING

61| Optimize echo signal: sweep the magnetic field until an echo signal is visible on the oscilloscope. Optimize the echo intensity by attenuating the mw power and by adjusting the phase of the detection channels. Increase the repetition rate until the echo signal starts to decrease. Sweep the magnetic field to the maximal signal position. Readjust the sample position in the cavity to maximize the echo intensity. Take care that no air penetrates into the cavity, for example, by switching off the vacuum pump.
? TROUBLESHOOTING

62| Optimize the PELDOR pump inversion pulse: Place a 12 ns pulse from the second mw source (pump pulse) with the same frequency as the probe frequency 500 ns before the probe Hahn-echo sequence optimized in Step 61. Optimize the inversion of the Hahn-echo by changing the attenuation of the inversion pulse.
▲ CRITICAL STEP It is most critical for the deepness of the PELDOR modulation to adjust the ELDOR pump pulse in such a way that the inversion of the echo is optimal. The modulation amplitude will be weaker otherwise. A shorter inversion pulse will lead to a stronger overlap of detected and pumped spins and thus to a decreased signal to noise ratio (S/N) and might introduce artifacts. A longer inversion pulse leads to a decreased modulation amplitude and potentially stronger orientation selections.
? TROUBLESHOOTING

63| Set the probe pulse frequency and optimize the probe pulses: Turn off the high-power pulses. Increase the frequency of the probe pulses by 70 MHz. Switch off the pump inversion pulse. Place into the +x-channel three pulses with a length of 32 ns and separation times of 300 and 1,200 ns between the first and second and second and third pulses, respectively. Optimize the stimulated echo by attenuating the power within this channel. The power in the +x-channel is now such that 32 ns pulses are $\pi/2$ pulses. Set up a two-pulse Hahn-echo sequence with pulse lengths of 32 ns and a pulse separation of 200 ns. Use the already optimized +x-channel for the 32-ns $\pi/2$ pulse and for the π pulse the y-channel. Keep the amplitude of the $\pi/2$ pulse and optimize the amplitude of only the π pulse by changing selectively the amplitude of the y-channel until the Hahn-echo is maximum.
▲ CRITICAL STEP Longer detection pulses lead to a reduced echo amplitude and stronger orientation selection, whereas shorter pulses strongly promote nuclear coherence artifacts and overlap of detected and pumped spins. The frequency offset of 70 MHz is optimal to avoid overlap of detected and pumped spins and thus to suppress nuclear coherence artifacts. To study angular correlations, this offset can be varied systematically.

64| Adjust the phases for the two-step phase cycling: two $\pi/2$ pulses with a phase difference of 180° are needed for this two-step phase cycle. Thus, have one $\pi/2$ pulse in the +x-channel and optimize a second $\pi/2$ pulse in the $-$x-channel (see Step 63). Adjust the phases of the $\pi/2$ pulses until an addition of the transient echo signal from a $\pi/2$(+x)–π(y) sequence with a $\pi/2(-x)$–π(y) sequence gives zero echo signal.

65| Set up the four-pulse ELDOR sequence: choose as probe pulse sequence $\pi/2$–τ_1–π–T–π, using the pulse settings for $\pi/2$ and π pulses from Step 61/62. The pulse separation time T has to be chosen long enough to detect at least one oscillation frequency of the PELDOR signal (depending on the expected distance r_{AB}). The pulse separation time τ_1 can be either set to a fixed value (a typical value would be 136 ns, which suppresses hydrogen modulation) or varied over a range of values in a 2D experiment to strongly suppress hyperfine modulations (eight steps of 8 ns to suppress the free Larmor frequency of hydrogen). The pump inversion pulse (on resonance with the mw cavity) with a length of 12 ns and an amplitude determined in Step 60 starts with a delay $<2\tau_1$ but large enough not to overlap with the first probe–π-pulse and is swept within the experiment up to a delay of $\tau_1 + T$. The acquisition trigger is placed on the refocused echo, which is the echo whose phase is turned by 180° with respect to the other four unwanted echoes. The echo signals of the two-step phase cycle of the detection pulse sequence

of $\pi/2(+x)-\pi(y)-\pi(y)$ and $\pi/2(-x)-\pi(y)-\pi(y)$ have to be subtracted to get zero background signal. Note that running the experiment with this two-step phase cycle eliminates receiver offsets and allows one to read the real echo signal intensity at the end of the PELDOR time trace as V_λ, which is important for spin counting.

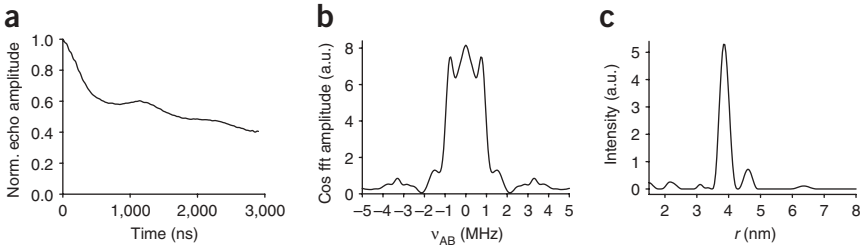


Figure 8 | As an example, this figure shows (**a**) the PELDOR time trace after time adjustment and normalization, (**b**) the Fourier transformation of **a** after background subtraction and (**c**) the Tikhonov regularization of **a**. The sample was a duplex RNA with the self-complementary sequence 3′GC**U**GAC UAUAGUCAGC (the bold U indicates the spin label site). Frequencies of 0.77 and 1.54 MHz, corresponding to $\theta_\perp$ and $\theta_{||}$, respectively, can be read from the Fourier transform spectrum. Substituting all constants into equation (4) and using the frequency for $\theta_\perp = 90°$ leads to $r = (52.04/0.77)^{1/3}$, which yields $r = 40.7 \pm 3$ Å. A slightly smaller mean distance of 38.7 ± 1.3 Å is obtained from the Tikhonov regularization. This distance corresponds to the distance expected for this RNA adopting an A-form, $r = 36.2 \pm 3.1$ Å based on MD simulations. This shows that duplex RNAs retain their A-form in frozen buffer solutions containing 20% ethylene glycol.

66| To start the experiment, set the pulse repetition time as determined in Step 61. Set the boxcar integration window to cover the whole echo. Step the pump pulse with an increment depending on the length of the oscillation period. A good starting point might be 12 ns. Repeat the experiment as many times as needed to obtain a good enough S/N of the PELDOR echo modulation trace (see also Steps 72 and 73 and **Fig. 8a**).
? TROUBLESHOOTING

Data analysis

67| Fit $V(t)_{inter}$ by a monoexponential decay function to the late part of the echo time trace.
▲ CRITICAL STEP The fitting of the background is the most critical step of the data analysis. A way to find out whether the fit was good is to look at the resulting Pake pattern (see Step 71). If a peak or a hole appears at the zero frequency, then the background was too shallow or too steep, respectively. If this procedure does not give good results, the time window was probably too small. In the case of a homogenously distributed sample, the monoradicals contribute only to the monoexponential decay. This decay is fitted and the time domain signal is divided by it during the data processing. However, controls using monolabeled oligonucleotides have to be performed, if specific aggregation is to be expected.
? TROUBLESHOOTING

68| Divide the experimental time trace by the monoexponential decay function determined in Step 67 to fit the intermolecular decay. The resulting time trace corresponds to $V(t)_{intra}$.

69| Adjust the t-axis of the experiment until the actual time point zero is at the maximum amplitude of the echo decay function. This is important to obtain a correct phase with the cosine Fourier transformation.

70| To determine n, first normalize the maximum of the time trace $V(t)_{intra}$ to 1. The signal $V(t)_{intra}$ should level off to a constant value, otherwise the time window was too short or the intermolecular decay function was not fitted correctly. To determine the number n of coupled spins in the biomolecule, read V_λ, which is the y-value at the end of the normalized time trace, and substitute it into equation (9). λ_B in equation (9) can be obtained by measuring a similar sample containing a structurally related standard biradical. Note that it is important that the sample be free of monoradical impurities, as n has to be 2. For the standard sample, n is known to be 2 and V_λ is read from the experiment, thus allowing calculation of λ_B. This λ_B can then be used to determine the number of spins n in the unknown sample.

71| Fourier transformation: subtract the value of V_λ from the time trace to remove the offset. Use a Hamming window function with parameters so that the noise at the end of the time trace is suppressed but the oscillations are not disturbed by the window function. Choose zero-filling to increase the points in Fourier space to obtain an appropriate resolution (not more than a factor of 2). Apply a cosine Fourier transform to the data set (imaginary time channel set to zero).

The resulting Fourier spectrum should show a Pake pattern if the intermolecular background was fitted correctly, if all dipolar vector orientations were excited and if the two radicals have a distinct single distance (**Fig. 8b**).
? TROUBLESHOOTING

72| Calculating r_{AB}: for nitroxides with $g_A = g_B = 2$, equation (4) can be written in the form

$$\nu_{Dip}\ (\mathrm{MHz}) = \frac{52.04}{r^3\ (\mathrm{nm})} \qquad (10)$$

Thus, reading the frequency at the singularity for $\theta_\perp$ and substituting it in MHz into equation (10) yields the spin–spin distance r_{AB} in nanometers. In systems that exhibit several distances or broad distributions, it is not always possible to extract all distances by Fourier transformation. In these cases, the extraction of the distance distribution function $P(r)$ in Step 73 is more efficient. At the current state, the separation of ν_{Dip} and J_{AB} can be performed only if both singularities $\theta_\perp$ and $\theta_{||}$ of the Pake pattern are observed in the Fourier transformed. The S/N required therefore depends on the following criteria: (i) With an increase in widths of distributions in r and J, the S/N has to be improved. (ii) The presence of an exchange coupling J complicates relating $\theta_{||}$ to the respective $\theta_\perp$. (iii) If differences in distances are small, a very high S/N is needed to resolve all distances. (4) With an increasing number of distances, the separation becomes more difficult and more prone to artifacts. (5) In the case of orientation selection, some $\theta_{||}$ and $\theta_\perp$ might not be observed in the Fourier transformed. In this case, the frequency offset has to be varied systematically.

73| Determination of the distance distribution function: it is difficult to extract the distance distribution function $P(r)$ from the time domain echo modulation traces. Several different distance distribution functions $P(r)$ may simulate equally good the experimental time traces within the given S/N. One way to analyze such mathematically ill-defined inversion problems is by Tikhonov regularization. Program packages based on this method and written in Matlab@ are provided by Professor G. Jeschke (DEER2006) and by the ACERT center of Professor J. Freed (DPD_Pkg). The optimization of the distribution function $P(r)$ is performed on the one hand by minimizing the deviation of the time domain simulation with respect to the experimental trace and on the other hand by searching for a smooth $P(r)$ distribution. The regularization parameter weighting these two contributions influences the solution strongly and can be optimized with the so-called L-curve. This can be performed either automatically or by manual inspection of the graph. The additional constraint that only positive r values can exist is implemented by either a constraint Tikhonov regularization or a maximum entropy constraint in a modified regularization functional.

Both program packages assume a simple integral kernel function, which describes a biradical without any orientational correlations. Application of this method to angular correlated systems will lead to additional artificial peaks in the distance distribution function $P(r)$ and might produce wrong distances. For the nitroxides used here, the angular correlation is small but the small peaks appearing in Tikhonov regularizations of the PELDOR time traces might be due to such correlations (**Fig. 8c**). In the same way, wrong results may occur for systems with more than two coupled radicals.

● TIMING

Synthesis of TPA: ~1 day per reaction step, a total of ~5 days (can be longer depending on the time allowed for sublimation of TPA)
Synthesis and labeling of oligonucleotides: ~1 day
Cleavage of the protecting groups and evaporation: ~1 day
Purification of labeled oligonucleotides, combination of fractions: ~2 days
Setting up of PELDOR experiment: ~1 h
Running the PELDOR experiment: ~16 h
Data analysis: couple of minutes to several days depending on the extent of analysis

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reasons	Solution
Synthesis of TPA			
4	Red-Al is difficult to fill into the syringe	High viscosity of the commercial solution	Use a needle with a wide diameter (1.2 mm); be patient
10	Low yield in the reduction step with Red-Al	Reaction temperature too high, poor quality of Red-Al	Repeat the reaction with fresh reagent. Ensure that the reaction is performed at temperatures not higher than 60 °C
35	Product from the olefination reaction is obtained as an oil	Impurities, residual solvent	Store the product in a refrigerator (−20 °C) overnight or redissolve in a small amount of diethyl ether and add *n*-hexane, store in a refrigerator
Synthesis and labeling of RNA/DNA			
44	DNA/RNA synthesis does not work	(1) Phosphoramidites are not in order	(1) Control the purity with a ^{31}P-NMR (check the presence of phosphonate)
		(2) Reagents are not in order	(2) Replace all the reagents

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reasons	Solution
		(3) Tubes of the apparatus are blocked	(3) Check the flow, the pressure, clean the tubes with acetonitrile
49	Solution becomes black during the Sonogashira cross-coupling	(1) Solution of CH_2Cl_2/TEA is not thoroughly deoxygenated	(1) Improve technique
		(2) Too long a time between the addition of CuI and the following steps	(2) Improve technique
53B (viii)	According to mass spectrometry, the Sonogashira coupling did not work or is not quantitative	(1) Catalyst is not in order	(1) Buy new catalyst
		(2) Solution of CH_2Cl_2/TEA is not thoroughly deoxygenated	(2) Training
		(3) Impure TPA	(3) Purify the TPA once more (HPLC) Try to purify DNA/RNA with the HPLC once more
PELDOR			
60	Substantial ringing after the protection pulse	(1) The receiver protection pulse is too short (2) Q of the cavity is too high	(1) Adjust the length of the receiver protection pulse; if you are in doubt, contact Bruker (2) Overcouple the resonator more. To determine the quality factor Q as a measure for the overcoupling, place a pulse of 400 ns into the +x-channel and read the time constant τ_M for the tune on or off of the pulse on the oscilloscope using the receiver monitor. Q is calculated as $Q = \pi\nu_0/\tau_M$ and should be about 50. If Q is considerably higher, the echo intensity will be larger for pulse sequences applied at ν_0, but the dead time of the spectrometer will be much longer (ringing) and the power of the TWT might not be enough to create a 32 ns π pulse at a frequency off-set of 70 MHz from ν_0 (probe frequency) where the PELDOR detection pulse sequence will be applied
61	No echo	Wrong settings on the spectrometer	For example, change the magnetic field to $g = 2$ and/or increase the Video Amplifier Gain
62	Not enough power to obtain an ELDOR π pulse	The overcoupling of the resonator is too strong	Reduce the coupling but check ringing and resonator width
66	The time trace shows no decay	The ELDOR channel has no power	Switch on the ELDOR power
66	The time trace shows not a full oscillation period	Time window τ_2 is too short	Change H_2O to D_2O. This extends the time window for the observation of a full period up to 6.5 μs, making distances of up to 6.5 nm accessible. Be careful with the data analysis if a full period is not resolved
67	The time trace shows only a monoexponential decay	There is no specific intramolecular coupling	A reason could be that the degree of labeling was very low or the spin–spin distance is too large (>8 nm)
67	The time trace shows only a bi- or multiexponential decay but no oscillation	(1) The degree of labeling is not high enough or	(1) Increase the label degree
		(2) The flexibility of the system is too high	(2) The flexibility of the system at room temperature translates into a wide distance distribution in the frozen state, which leads to negative interference of the dipolar oscillations. In general, an analysis of such time traces without visible oscillations should be treated with care. In these cases, the analysis is not

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reasons	Solution
			parameter-free anymore and several assumptions, which can be right or not, have to be made (orientation selection, fit of background, number of spins, and flexibility of the system versus low label degree)
67	The FFT spectrum shows a peak or a hole at the zero frequency	Fit of the intermolecular background was not optimal	Vary the extent of the qualifying region, so that more or less data points are fitted
71	The phase of the Fourier-transformed spectrum is not correct	Wrong zero time point	Adjust the zero time point better
71	Only part of the Pake pattern is seen	This will most commonly occur and results from orientation selection	The degree of freedom for the nitroxide used here is still high enough so that the peak seen corresponds usually to the $\theta_{\perp}$ singularity. However, if the two TPA labels happen to stand on top of each other, then placing the detection sequence +70 MHz from the pumping frequency (corresponding to selection of A_{zz}) results in the predominant excitation of the $\theta_{\|}$ singularity, because A_{zz} is with a high probability parallel to r_{AB}. Thus, reading this frequency as the dipolar coupling yields twice the frequency of the actual dipolar coupling. Whether this is the case can be solved by performing the experiment with $\Delta\nu$ ranging from +80 to +40 MHz. If the two TPAs are stacked on each other, the perpendicular orientation of the Pake pattern increases with smaller $\Delta\nu$. At this position, more of the A_{xx}, A_{yy} orientations are excited, which are in this case perpendicular to r_{AB}. This effect is even more pronounced with fully rigid labels. In any case, varying $\Delta\nu$ allows finding the best value for $\Delta\nu$ with respect to the deepest modulation and allows making a rough estimation about the relative orientations of the two nitroxides with respect to r_{AB}

ANTICIPATED RESULTS

TPA synthesis

Typical yields isolated

Usually, the amount of TPA **5** isolated is about 3–4 g starting from 15.8 g of the acid derivate. The yield of 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-(2-chloroethenyl) **4** is around 80% or higher, depending on the scale of the reaction but is not fully isolated in the synthesis sequence described here. At a smaller scale, the yields are usually higher.

Analytical data

2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-hydroxymethyl 2

MS (EI, 70 eV): (*m/z*) [M^+] calculated 170.12; found (%) 170 (26). Fractionation pattern, in found (%) [calculated] format: 140 (31) [$M - 2 \times CH_3$, 140.07]$^+$, 125 (23) [$M - 3 \times CH_3$, 125.05]$^+$, 107 (100) [$M - 3 \times CH_3 - H_2O$, 107.04]$^+$, 91 (18), 79 (13), 69 (21), 67 (20), 55 (26), 42 (43), 41 (43).

2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carbaldehyde 3

MS (EI, 70 eV): (*m/z*) [M^+] calculated 168.10; found (%) 168 (41). Fractionation pattern, in found (%) [calculated] format: 138 (60) [$M - 2 \times CH_3$, 138.06]$^+$, 123 (100) [$M - 3 \times CH_3$, 123.03]$^+$, 109 (24) [$MH - 4 \times CH_3$, 109.02]$^+$, 105 (26) [$M - 3 \times CH_3 - H_2O$, 105.02]$^+$, 95 (77), 91 (18), 81 (18), 79 (22), 77 (22), 67 (80), 55 (57), 53 (28), 42 (64), 41 (89).

TPA 5

MS (EI, 70 eV): (*m/z*) M^+] calculated 164.11; found (%)164 (35). Fractionation pattern, in found (%) [calculated] format: 149 (46) [$M - CH_3$, 149.08]$^+$, 134 (91) [$M - 2 \times CH_3$, 134.06]$^+$, 119 (100) [$M - 3 \times CH_3$, 119.04]$^+$, 105 (20) [$MH - 4 \times CH_3$,105.02]$^+$, 91 (78), 79 (25), 77 (24), 65 (15), 41 (52).

Synthesis and labeling of RNA/DNA

The yield for the Sonogashira cross-coupling is usually >95%.
Typical yield for labeled DNA: 5–10 OD for a 12-mer, depending on the degree of reduction of the nitroxide.
Typical yield for labeled RNA: unpurified 18–20 OD for a 12-mer, purified 8–10 OD (same range as indicated by Dharmacon for an unmodified 10–15-mer). With the labeled cytidine, the yields are always slightly less.

PELDOR

The PELDOR spectrum should show at least one full oscillation period (**Fig. 8a**). The S/N should be 50:1 or larger. The Fourier-transformed spectrum should show one strong peak for $\theta_{\perp}$ and a weaker one at twice this frequency for $\theta_{||}$. The peak at the zero frequency is due to a background decay not fitted perfectly (**Fig. 8b**). The Tikhonov regularization should reveal one dominant peak (**Fig. 8c**). Weaker peaks at larger distances may be due to orientation selection effects or stacking of duplexes on top of each other[56]. Further details are given in **Figure 8**.

ACKNOWLEDGMENTS We thank D. Margraf for helpful advice on this manuscript. This work was supported by the DFG within the SFB 579.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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