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Communication

DEER-Stitch: Combining three- and four-pulse DEER measurements for high sensitivity, deadtime free data

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ABSTRACT

Over approximately the last 15 years the electron paramagnetic resonance (EPR) technique of double electron electron resonance (DEER) has attracted considerable attention since it allows for the precise measurement of the dipole–dipole coupling between radicals and thus can lead to distance information between pairs of radicals separated by up to *ca.* 8 nm. The "deadtime free" 4-pulse DEER sequence is widely used but can suffer from poor sensitivity if the electron spin-echo decays too quickly to allow collection of a sufficiently long time trace. In this paper we present a method which takes advantage of the much greater sensitivity that the 3-pulse sequence offers over the 4-pulse sequence since the measured electron spin-echo intensity (for equal sequence lengths) is greater. By combining 3- and 4-pulse DEER time traces using a method coined DEER-Stitch (DEERS) accurate dipole–dipole coupling measurements can be made which combine the sensitivity of the 3-pulse DEER sequence with the deadtime free advantage of the 4-pulse DEER sequence. To develop the DEER-Stitch method three systems were measured: a semi-rigid *bis*-nitroxide labeled nanowire, the *bis*-nitroxide labeled protein CD55 with a distance between labels of almost 8 nm and a dimeric copper amine oxidase from *Arthrobacter globiformis* (AGAO).

1. Introduction

Double electron electron resonance, DEER (also known as PELDOR) utilises two microwave (mw) frequencies in order to detect the coupling (dipolar and exchange) between unpaired electrons. The initial 3-pulse (3p) sequence uses a 2-pulse (2p) primary (Hahn) echo sequence at one frequency (the observer frequency) and a π pulse at the second (the pump mw frequency) and was first introduced in the 1984 paper by Milov et al. [1]. The sequence was subsequently used by Larsen and Singel to investigate dipolar coupling between nitroxides in 1993 [2]. However, the start of the DEER time trace is distorted from the necessary application of the pump pulse at the same time as the first observer $\pi/2$ pulse in 3p DEER which means the proper shape of the dipolar spectrum is not recorded, which is particularly detrimental for samples with broad distance distributions [3,4]. Furthermore, this distorted data cannot be used reliably in routine analysis programs such as DeerAnalysis [3,5]. It is possible to remove 3p DEER deadtime problems by either using a second amplifier for the pump frequency or working in the linear TWT region but these options are more technically demanding and expensive than the standard DEER experimental set-up [6]. In 1998 Spiess and co-workers added an extra refocusing pulse into the observer frequency sequence so that the beginning of the resulting 4-pulse (4p) DEER time trace does not suffer from distortions due to pulse overlaps [7]. This improvement led to the technique becoming widely used for measuring distances *via* dipolar couplings in a range of materials, in particular for structural biology investigations [8].

Most of the published work using DEER has focused on measuring the dipolar coupling between nitroxide spin probes for materials or biological research. Nitroxides have a small g-anisotropy and an ^{14}N hyperfine splitting which makes them suitable for DEER measurements at X-band frequencies (~9.5 GHz) as mw pulses of *ca.* 12–32 ns excite a large fraction of the spins, the EPR spectrum is wide enough that pump and detection spins can be separately excited, and orientation selection effects are generally small or negligible. In the absence of orientation selection the dipolar time trace from nitroxide pairs can be routinely converted into a distance distribution with the aid of regularization methods [5]. 4p DEER has been used to measure distances between nitroxide probes from *ca.*1.8 to 7.5 nm [6,9–13]. It has also been applied to a range of other radical systems and at different EPR frequencies (*e.g.* Q-band and W-band) to measure both distances and relative orientations between spin centres [14–27].

In this paper we highlight the often large difference in the electron spin echo intensity in going from the 2p primary echo

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sequence to the 3p refocused echo sequence, a difference which depends upon the EPR spectrometer hardware (*e.g.* resonator B_1 field homogeneity) and the nature of the sample (e.g. diffusion and de-phasing mechanisms). It follows that these effects will result in poorer signal-to-noise from the 4p DEER experiment than the 3p DEER experiment even if the total measurement and DEER sequence length time of the two experiments are the same. Since the DEER time trace must be long enough to measure the dipolar modulation frequency for an accurate distance measurement, the phase memory time, T_m (related to spin–spin relaxation) needs to allow for the complete pulse sequence and echo detection. For example the typical T_m for a nitroxide attached to a protein in a deuterated glassy matrix at 50 K is *ca.* 5 μs, to measure an inter-spin distance of 7 nm would require a DEER time trace of greater than *ca.* 10 μs. From Fig. 1a it can be seen that a 10 μs time trace would only be obtained by measuring the echo approximately 20 μs after the initial $\pi/2$ pulse, a time which greatly exceeds the T_m [8]. Naturally, the longer the pulse sequence the smaller the echo intensity will be. In samples where the 4p DEER measurement is limited due to rapid loss of echo intensity with increasing sequence length, using the 3p DEER method with one less refocusing pulse potentially offers superior results. Unfortunately the 3p DEER trace suffers from distortions around the zero-time.

Here we show that by combining time traces from the 3p and 4p DEER sequences using a post-data-collection processing method which we call Stitch we can increase the sensitivity of a DEER measurement for a sample limited by T_m, without having to lose the valuable zero-time information on the dipolar modulation. We apply the DEER-Stitch (DEERS) method to three systems. The first is a model system and demonstrates the validity of the method using a short semi-flexible nanowire which has nitroxide moieties at either end. The second is a doubly-nitroxide-labeled protein which demonstrates that the method can be used to extend the distances measurable by DEER without otherwise changing the sample. Finally a DEERS time trace between copper centres in a protein is shown and compared to the 4p DEER measurement.

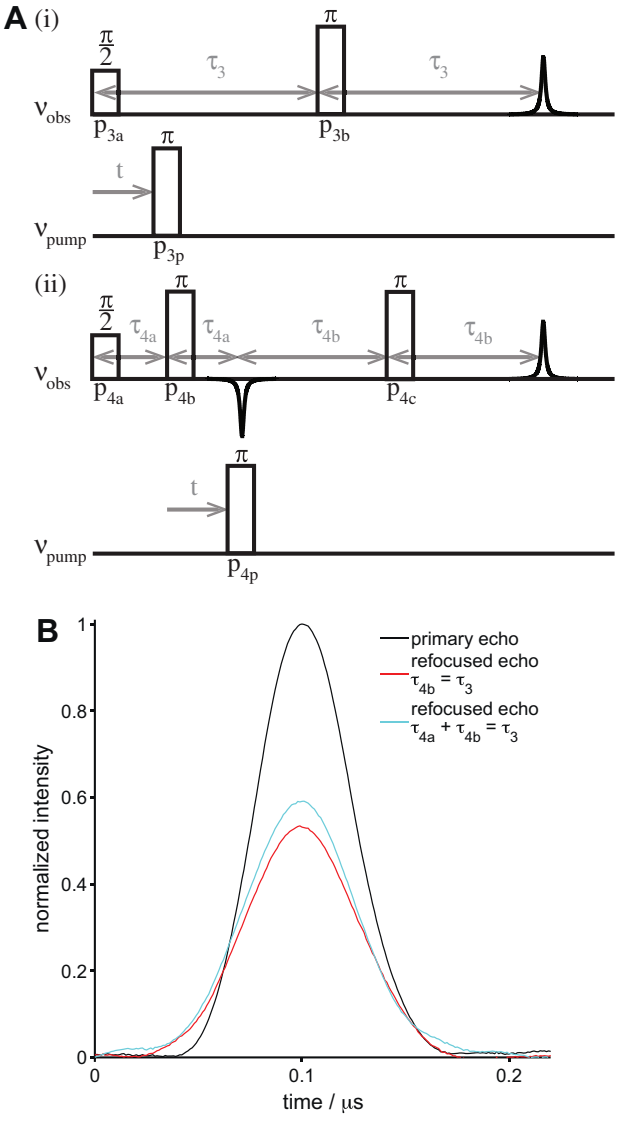

Fig. 1. (A) Pulse sequences with the primary and refocused echoes for (i) 3p DEER and (ii) 4p DEER with time delays and pulses labeled. In all experiments shown in this paper p_{3a} or p_{4a} are phase cycled to correct for signal intensity offsets. (B) Electron spin echoes from the observer pulse sequences for 3p DEER (*i.e.* 2p echo sequence) and 4p DEER (*i.e.* 3p refocused echo sequence). This corresponds to the maximum signal size in the DEER experiments. The echoes from the 3p sequence are displayed as having positive phase for comparison but were measured with negative phase. The duration of the pulses was 32 ns with τ_3 = 12 μs, τ_{4a} = 400 ns, τ_{4b} = 12 μs or τ_{4b} = 11.6 μs. The video gain was maintained at 39 dB. The sample measured was **P1** in d_{14}-oTP/BnPy.

2. Method

The proposed Stitch method will construct a DEERS time trace by taking the early part of the 4p DEER trace to replace the first part of the 3p DEER trace which is distorted due to overlap of the pump and observer pulses [3,4]. There are three major considerations to be made before beginning the procedure. The first is to define the zero-time in the 3p and 4p DEER traces. The second consideration is how much of the 3p DEER trace is distorted and needs to be replaced. The third factor is deciding how much of the 4p DEER trace is required to overlap with the 3p DEER trace in order to ensure that the resulting DEERS trace is a faithful representation of the desired undistorted time trace starting at time zero.

In defining the 3p and 4p DEER zero-times we assume that the pulse profiles are rectangular so that the centre of each echo can be related to the centre of the initial detection $\pi/2$ pulse (p_{3a} or p_{4a}, as defined in Fig. 1a). Thus, in the 3p DEER trace we take the zero-time of the dipolar modulations to be when the centre of the pump pulse is at the centre of the initial $\pi/2$ pulse. While in the 4p DEER experiment the zero-time is when the pump pulse centre coincides with the centre of the primary echo.

The second point to be addressed is to ascertain how much of the 3p DEER trace after the zero-time is distorted and needs to be replaced by the 4p DEER trace. It has been found in previous studies that while the pump and observer pulses, p_{3p} and p_{3a} respectively, are overlapping there may be distortions to the DEER time trace data [4]. Therefore, at least $(p_{3a}/2 + p_{3p}/2)$ ns of the data, after the zero-time, need to be replaced. We found that, with our spectrometer and cavity, it is advisable to include an additional 4–8 ns which presumably allows the TWT amplifier to recover after saturation due to the power demands of the two high power mw pulses overlapping. Therefore the minimum amount of data that will be replaced in the Stitch procedure is $(p_{3a}/2 + p_{3p}/2 + 8)$ ns which is 30 ns when p_{3a} = 32 ns and p_{3p} = 12 ns.

In a typical DEER experiment the pump-pulse will be stepped out by time intervals Δt, which is set to a value corresponding to a Nyquist frequency above any observed modulations to the echo, typically Δt = 8 ns ($\nu_{max} = 1/(2\Delta t)$ = 62.5 MHz). To allow full flexibility and accuracy when manipulating the DEER time traces the data points were re-sampled to give Δt = 1 ns using linear interpolation (as implemented by the Matlab function "interp1"). A similar procedure is also carried out in the DeerAnalysis software by Jeschke [5].