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Communication

Light-induced dipolar spectroscopy – A quantitative comparison between LiDEER and LaserIMD

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ABSTRACT

Nanometric distance measurements with EPR spectroscopy yield crucial information on the structure and interactions of macromolecules in complex systems. The range of suitable spin labels for such measurements was recently expanded with a new class of light-inducible labels: the triplet state of porphyrins. Importantly, accurate distance measurements between a triplet label and a nitroxide have been reported with two distinct light-induced spectroscopy techniques, (light-induced) triplet-nitroxide DEER (LiDEER) and laser-induced magnetic dipole spectroscopy (LaserIMD). In this work, we set out to quantitatively compare the two techniques under equivalent conditions at Q band. Since we find that LiDEER using a rectangular pump pulse does not reach the high modulation depth that can be achieved with LaserIMD, we further explore the possibility of improving the LiDEER experiment with chirp inversion pulses. LiDEER employing a broadband pump pulse results in a drastic improvement of the modulation depth. The relative performance of chirp LiDEER and Laser-IMD in terms of modulation-to-noise ratio is found to depend on the dipolar evolution time: While LaserIMD yields higher modulation-to-noise ratios than LiDEER at short dipolar evolution times ($\tau = 2$ µs), the high phase memory time of the triplet spins causes the situation to revert at $\tau = 6$ µs.

1. Introduction

Pulsed dipolar EPR spectroscopy is a well-established technique to determine precise distance distributions between paramagnetic centers with distances ranging from around 1.6 nm up to 16 nm [1–3]. In combination with site-directed spin labeling, it is ideally suited for structural characterizations of macromolecules and complexes, and has emerged as valuable tool in structural biology [4–6]. Importantly, distance constraints can be obtained regardless of the overall size of the studied molecules, and measurements can be performed in the presence of membranes and even in cells [7–10]. Because the distance information is not encoded in signal intensity, but in the frequency domain, the result of a dipolar spectroscopy experiment is not a mean interspin distance, but a precise distance distribution reflecting structurally heterogeneous populations of macromolecules [1].

The most commonly employed dipolar spectroscopy technique is four-pulse double electron-electron resonance (DEER) [11] on nitroxide spin labels or metal complexes [12]. A very interesting recent addition to the collection of spin labels which are suitable for pulsed dipolar spectroscopy is the triplet state of porphyrins [13]. These chromophores are diamagnetic and thus EPR-silent in their ground state S_0, but absorption of light at a suitable wavelength leads to excitation of a fraction of the molecules to the first excited singlet state S_1, from which the paramagnetic triplet state T_1 with the spin quantum number $S_T = 1$ can then be formed by intersystem crossing (ISC). Next to being switchable by light, another attractive feature of porphyrins is their occurrence as endogenous prosthetic group e. g. in heme proteins [14] and photosynthetic complexes [15]. A further important phenomenon in this regard is optical spin polarization (OSP), that is, the fact that the initial population of the three triplet sublevels with $m_{S,T} = -1, 0, 1$ in high external magnetic fields does not correspond to a Boltzmann distribution immediately after ISC [16]. In consequence, the EPR spectrum of a spin-polarized triplet state shows both emissive and absorptive parts, and the signal intensity is significantly enhanced compared to systems in thermal equilibrium. The decay of the triplet EPR signal over time is governed by two mechanisms: spin-lattice relaxation between the triplet sublevels to thermal equilibrium, as well as the overall depopulation of the

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triplet state through ISC, non-radiative decay or phosphorescence, bringing the molecule back to the singlet ground state S_0 [16]. For porphyrins at cryogenic temperatures, these relaxation processes happen within milliseconds [14,17].

The first study that employed a porphyrin for light-induced dipolar spectroscopy was published by Di Valentin et al. [13]. In the experiment, the conventional 4-pulse DEER sequence was preceded by a laser flash to populate the triplet state in situ, which was then used as observer spin species, while the pump pulse was applied to the nitroxide. The pulse sequence of this technique, which will be called light-induced double electron–electron resonance (LiDEER) in the following, is schematically represented in Fig. 1(a). Potential advantages of LiDEER include a high observer signal intensity owing to OSP as well as clear spectral separation of the observed triplet from the pumped nitroxide [13].

A different approach for measuring the distance between a nitroxide and a porphyrin triplet state by light-induced dipolar spectroscopy was recently introduced by Hintze et al. [14]. In laser-induced magnetic dipole spectroscopy (LaserIMD), the most distinctive feature of the triplet state, i.e., the fact that its formation in situ can be controlled with high temporal precision with a laser pulse, is actively exploited in the pulse sequence. The basic idea is that instead of *changing* the dipolar interaction with a microwave inversion pulse during the echo sequence as it is done in (Li)DEER, the dipole-dipole coupling is *introduced* by triplet excitation at variable times during the observer pulse sequence [14]. The LaserIMD experiment is performed in practice by applying a Hahn echo sequence to the nitroxide, recording the primary spin echo and incrementing the position of this whole sequence relative to the (constant) position of a laser flash, as depicted in Fig. 1(b). It was shown in the original LaserIMD publication that as soon as the population of the triplet state with the laser flash occurs within the microwave pulse sequence at a time t before the refocusing of the nitroxide spin echo, the dipolar interaction of the nitroxide and triplet spins during this time t leads to a phase offset $\Delta\varphi_{dd} = m_{S,T}\omega_{dd}t$ at the time of echo formation if exchange coupling is neglected. The echo signal is thus modulated with $\cos(m_{S,T}\omega_{dd}t) = \cos(\omega_{dd}t)$ for $|m_{S,T}| = 1$, and the nitroxide-triplet distance distribution is extracted from the resulting form factor after background correction in an equivalent procedure to conventional DEER data [14,18].

The considerations outlined above are not only valid for the case where the laser flash occurs during echo refocusing, which is termed the "forward trace". Analogous considerations apply for the "reverse trace", where the laser flash comes between the two observer pulses, and the full LaserIMD trace thus has an approximately symmetric shape, where the reverse trace mirrors the forward trace [14].

Compared to nitroxide-nitroxide DEER, an increase in both sensitivity and modulation depth is expected for LaserIMD: Only one microwave frequency is needed which can be set to the center of a critically coupled resonator, yielding short observer pulses that are applied to the maximum of the nitroxide spectrum. Overall, this should maximize the number of excited observer spins, and thus the signal-to-noise ratio (SNR). Moreover, a temporal overlap of the laser flash with the microwave pulses or the acquisition trigger is not a problem, which allows the acquisition to be set on a primary echo, rather than the refocused echo as in the dead-time free DEER sequence. This leads to a further enhancement in SNR, since signal losses due to incomplete excitation by the refocusing pulse as well as transverse relaxation during the longer pulse sequence for a refocused echo are reduced. The modulation depth on the other hand depends on the light excitation and the OSP, as it denotes the fraction of all *observed* molecules where the porphyrin is excited to a triplet sublevel with $|m_{S,T}| = 1$. Therefore, the modulation depth can be maximized by optimizing the conditions for triplet excitation [14].

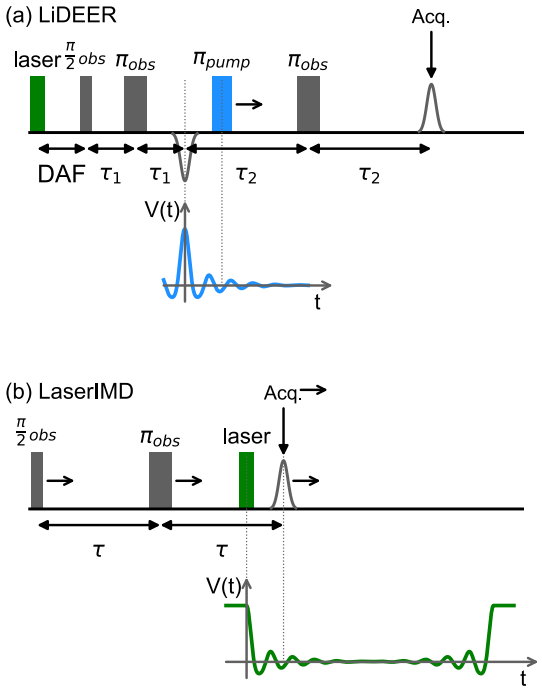

Fig. 1. Laser (green) and microwave pulse sequences and simulated dipolar evolution traces V(t) for (a) LiDEER and (b) LaserIMD. Horizontal arrows (→) in the pulse sequences indicate elements with incremented positions during the experiment. (a) In LiDEER, observer pulses (grey) are applied to the triplet, the pump pulse (blue) is applied to the nitroxide. The acquisition trigger is set to the refocused echo, with the position of the primary echo indicated with a negative Gaussian shape in the sequence. (b) In LaserIMD, the microwave pulses (grey) address the nitroxide, and acquisition is set to the primary Hahn echo. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In principle, for a porphyrin-nitroxide pair, both LiDEER and LaserIMD have been demonstrated to yield precise distance distributions [14,19]. As the attractive features of triplet-forming chromophores make these switchable spin labels very promising for future applications of pulsed EPR spectroscopy, the question that immediately arises is which of the two techniques would be preferable for studying a given system. In the present study, we set out to address this question by exploring the performance of LiDEER and LaserIMD under comparable conditions. To this end, we employ two of the spectroscopic rulers introduced recently by Di Valentin et al. as model compounds, with predicted distances of 2.3 nm (**1**) and 3.8 nm (**2**), respectively [19]. The structures of the model peptides are given in Fig. 2(a). Both LiDEER and LaserIMD were performed at Q band and with an optimized triplet excitation wavelength using a tunable laser system on identical samples to ensure maximal comparability. The resulting traces were analyzed with regard to the respective modulation-to-noise ratio. Furthermore, we demonstrate that in analogy to published results for DEER on other spin systems [20,21], the modulation depth of triplet-nitroxide LiDEER is enhanced dramatically when the rectangular pump pulse is replaced by a fast-passage chirp pulse.

2. Materials and methods

For the comparison of LiDEER and LaserIMD, the spectroscopic rulers introduced by Di Valentin et al. for X-band LiDEER constitute