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Location of TEMPO-PC in Lipid Bilayers: Implications for Fluorescence Quenching

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The characterization of the behavior of lipid-attached spin probes in a bilayer is of fundamental importance for correct interpretation of the results of both EPR and fluorescence studies of protein-membrane interactions. The knowledge of the immersion depth of spin probes attached to lipid acyl chains or headgroups (e.g., Tempo-PC) is critical for the determination of the transverse location of fluorescence probes attached to proteins and peptides (London and Ladokhin 2002; Ladokhin 2014). In addition, Tempo-PC is a popular tool for probing solvation dynamics near lipid membrane interfaces. The question of bilayer penetration of Tempo moiety in Tempo-PC came into prominence in two recent studies of interfacial solvation. While Cheng, Han and co-authors (Cheng et al. 2015) assumed that Tempo was located well above phosphate groups, Lee and co-authors (Lee et al. 2016) used Molecular Dynamics (MD) simulations to confirm our earlier result that Tempo is located below the level of phosphates (Kyrychenko and Ladokhin 2013). In a subsequent Communication, Schrader and Han questioned the results of the MD simulations and argued that various indirect evidence indicates “that TEMPO indeed is partitioned at the bilayer surface at about 5 Å above the phosphate group” (Schrader and Han 2017). Here we examine the arguments on Tempo penetration using previously developed framework of the cross-validation of MD simulations and depth-dependent fluorescence quenching experiments (Kyrychenko et al. 2013; Kyrychenko et al. 2015). The analysis presented here confirms that Tempo in Tempo-PC penetrates below the level of phosphate groups.

Penetration of Tempo into lipid bilayer from MD simulations.

In our previous study we have used MD simulations to refine depth positions of a set of lipid-attached spin probes, including Tempo-PC (Kyrychenko and Ladokhin 2013). The snapshot from this simulation (Fig. 1A), illustrates that at 11% Tempo-PC (*red*), the Tempo group is located well below phosphates (*blue circles*). This result has been independently confirmed by Lee and co-workers for low concentration of labeled lipid (Lee et al. 2016).

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Compliance with Ethical Standards

Conflict of interest. The authors declare that they have no conflict of interest.

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The summary of the relative positions of Tempo and phosphate from these MD studies is presented Fig. 1B. At low concentrations the positions are well demarcated, while at the highest concentration of Tempo, perturbation at the interfaces results in overlap in transverse distributions. Nevertheless, all data indicate that Tempo is never exposed to the region above phosphates, as suggested by (Cheng et al. 2015) and (Schrader and Han 2017) (Fig. 1B, *dashed line*). Thus, the computational evidence from MD simulations published by two independent research groups strongly indicates that *in silico* Tempo, attached to the lipid headgroup, resides below the level of the phosphates. Next, we examine how the knowledge of the exact position of Tempo in the bilayer affects the analysis of fluorescence quenching experiments in membranes.

Analysis of fluorescence quenching by Tempo-PC.

In Figure 2, we present the original raw data on steady-state and time-resolved quenching of lipid-attached fluorescent probe NBD (NBD-PE) in bilayers that contain various amounts of Tempo-PC quencher (Fig. 2A–D) taken from (Kyrychenko et al. 2015). The NBD quenching measurements were also obtained with a series of lipids labeled with spin probes at various positions along acyl chains, that can be used to determine the transverse distribution of NBD using Distribution Analysis methodology (Ladokhin 2014). The details of data collection and analysis are presented in (Kyrychenko and Ladokhin 2014; Kyrychenko et al. 2015). Briefly, depth-dependent quenching profiles (QP's) were generated using measurements of fluorescence quenching of NBD with series of six lipids labeled with spin probes (5-, 7-, 10-, 12-, 14- doxyl-PCs and Tempo-PC) placed at different depth, h_m , defined as a distance from the bilayer center. The depth scale of these spin-labels were refined by our MD simulations (Kyrychenko and Ladokhin 2013), which recently were independently validated with the alternative MD force field (Laudadio et al. 2019). Both steady-state and time-resolved measurements were used to determine the intensities, $F(h)$, and lifetimes, $T(h)$, as a function of the quencher depth and the corresponding values in the absence of quenchers, F_0 and T_0 . Two different QP's were generated as follows: steady-state (or total) $QP(h) = (F_0/F(h)) - 1$ and dynamic $QP(h) = (T_0/T(h)) - 1$, respectively. Quantitative information on membrane penetration depth of NBD was extracted from these depth-dependent quenching data using the distribution analysis (DA) methodology (Ladokhin 1997; Ladokhin 2014), which approximates the transverse quenching profile (QP) of a fluorophore with a Gaussian function, $G(h)$ (Eq. 1):

$$QP(h) = G(h) + G(-h) = \frac{S}{\sigma\sqrt{2\pi}} \exp\left[-\frac{(h-h_m)^2}{2\sigma^2}\right] + \frac{S}{\sigma\sqrt{2\pi}} \exp\left[-\frac{(h+h_m)^2}{2\sigma^2}\right] \quad (1)$$

where, h_m is the most probable depth of the probe measured from the bilayer center; σ is dispersion of the transverse profile; S is total quenching efficiency. In order to reduce the quenching contribution from the transverse diffusion of a probe occurring during the excited-state lifetime, we used the “differential” $QP(h) = (F_0/F(h)) - (T_0/T(h))$, calculated by subtracting the “dynamic” lifetime quenching component from the total steady-state quenching (Fig. 2E) (Kyrychenko and Ladokhin 2014; Kyrychenko et al. 2015).

The key point is the comparison of the fluorescence quenching profiles, calculated under two different assumptions. Specifically, we used two different positions for the Tempo quencher to generate alternative profiles from the same original data. The alternation of the position of a single quencher (Tempo) in a set of six quenchers of different depth had a profound effect on the results of the analysis of fluorescence quenching (Fig. 2E). The assumption that Tempo is positioned at 5 Å above the phosphates result in the shift of the membrane depth of NBD up to 18.6 Å. The latter value is much further from the bilayer center than suggested by the independent MD calculations on NBD-PE in POPC bilayer (Kyrychenko et al. 2015).

Cross-validation of MD and fluorescence quenching of NBD-PE with Tempo-PC.

In order to extract valuable structural aspects and dynamics behavior of fluorescent lipids and fluorescent-labeled-proteins we have proposed a novel approach, which combines of fluorescence spectroscopy and MD simulations (Kyrychenko et al. 2013; Kyrychenko et al. 2014; Kyrychenko et al. 2015; Kyrychenko et al. 2017; Kyrychenko et al. 2018). The main idea is to refine the assumptions of fluorescence analysis by comparing experimental results to MD simulations. Here, we demonstrate this approach by reconstructing the distribution of lipid-attached NBD moiety from quenching experiments (under two different assumption of Tempo depth) and comparing it to NBD distribution from MD simulation (Kyrychenko et al. 2015).

The first step in applying this approach is to generate the probability profile for NBD transverse penetration into the bilayer from the experimental quenching profile. Both fluorophore probability and the quenching profile have the same main depth, but differ in the width of the distribution. Previously we have demonstrated that the dispersion of the quenching profile, $\sigma(QP)$, is always larger than the dispersion of the actual transverse distribution of the fluorophore, $\sigma(F)$ (Ladokhin 1997; Ladokhin 2014). The latter can be estimated from the following:

$$\sigma(F) = \sqrt{(\sigma(QP))^2 - (\sigma(Q))^2} \quad (2)$$

where $\sigma(Q)$ is the broadening dispersion introduced by the quencher. We have applied this procedure to fit the differential quenching profile of NBD under two assumptions of Tempo depth (Fig. 3). In both cases, for MD-based position of Tempo position (*red*) and for assumed Tempo above the level of phosphates (*green*), we applied the same empirical value of $\sigma(Q)=2.5$ Å in Eq. 2 and normalized the resulting profile to have the same area under the curve. Then these two experiment-based distributions of NBD were compared to the results of the computation-based estimate of NBD (*blue shaded profile* in Fig. 3). The latter was independently obtained from MD simulation of NBD-PE in POPC bilayer and completely coincides with the quenching-based profile with MD-based position of Tempo. In contrast, assuming that Tempo is located above the phosphates, would incorrectly suggest a much more water-exposed NBD. (The latter distribution is not only inconsistent with the MD data, but also with the independent observation that in the absence of quenchers, NBD-PE in

membranes has a long average lifetime of > 5 ns (for comparison, the water-exposed NBD has a lifetime of ~ 0.3 ns). The overlap between the red (*experimental*) and the blue (*simulated*) profiles in Fig. 3 is quite remarkable and clearly validates the deep penetration of Tempo below the level of phosphates into the lipid bilayer.

Cross-validation of Tempo position from comparison with quenching by brominated lipids.

Additional validation for Tempo penetration comes from the comparison of the two types of depth-dependent quenching: one with the set of lipid-attached spin labels (including Tempo-PC) and another with bromine atoms attached at acyl chains. Because NBD fluorescence is not sensitive to bromines, we will use another model fluorophore, tryptophan octyl ester (TOE), which serves as a useful reference for analyzing tryptophan fluorescence of membrane protein (Ladokhin and Holloway 1995; Kyrychenko et al. 2013). We apply DA procedure to the raw data for quenching of TOE fluorescence with three spin labels taken from (Abrams and London 1993) and with four bromolipids (Ladokhin and Holloway 1995). In the latter experiments TOE was found to be located 11.3 Å from bilayer center (Ladokhin and Holloway 1995). The DA fitting of the fluorescence quenching of TOE in membranes by three spin-labeled lipid quenchers (Tempo-PC, 5-doxyl-PC and 12-doxyl-PC) resulted to estimation of the much shallower immersion depth 17.1 Å from bilayer center, when Tempo is assumed to be above the level of phosphates. A closer estimate 13.0 Å is obtained when MD-based positioning is used for Tempo depth. The latter value for TOE depth is also consistent with independent MD simulation of TOE in POPC bilayer, which indicates that the most probable position of the center of weight of the fluorophore is 13.3 Å from the bilayer center (Kyrychenko et al. 2013).

Conclusions.

Taken together, the results presented in this *Brief Communication* clearly demonstrate that proper analysis of fluorescence quenching requires the use of Tempo position below the level of phosphate groups. Failure to do so will result in substantial systematic errors in determining the penetration of the labeled site on a membrane protein of peptide.

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Figure 1. Distribution of depth of Tempo vs that of phosphate groups from MD simulations (Kyrychenko and Ladokhin 2013).

(A) A MD snapshot of a lipid bilayer composed of Tempo-PC and POPC with a ratio of 12:116 that correspond to 11 mol % of bilayer spin-labeling, respectively. The POPC phosphorus atom are shown as *blue balls*. POPC and Tempo-PC are shown in stick representations in *olive* and *red*, respectively, with the Tempo moieties shown using van der Waals representation. (B) Distances of the Tempo label (*squares*) are compared to the distances of the phosphate group (*circles*) for three different concentrations of 3.7 mol %, 11 mol % and 28 mol %, respectively. The data clearly indicate that even at these relatively high Tempo contents, utilized in fluorescence quenching experiments, the depth of Tempo resides below the level of the phosphates. This conclusion also holds for comparison of our MD simulations (*solid symbols*) with those performed at a lower Tempo content of 3.7 mol % (*empty symbols*) (Lee et al. 2016). A dotted line shows the suggested level of a Tempo moiety above the phosphates.

Figure 2. Fluorescence quenching of NBD-PE by Tempo-PC in lipid bilayer (Kyrychenko and Ladokhin 2014).

(A) Molecular structure of NBD-PE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) and Tempo-PC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(TEMPO)-choline) lipids. Addition of 7.5 to 15 and to 30% molar concentration of Tempo-PC resulted in reduction of the fluorescence intensity (B) and shortening of its excited-state lifetime (C). The resulting Stern-Volmer plots (D) exhibit no deviation from linearity. The difference between the steady-state and dynamic quenching is due to the contribution of static quenching. The difference between total and dynamic quenching can be used for a more reliable determination of the depth of the probe (Kyrychenko and Ladokhin 2014). (E) A depth-dependent profile for "differential" quenching (*circles*) calculated as the difference between total and dynamic quenching (Kyrychenko et al. 2018). The five solid symbol correspond to the data points obtained with the doxyl-labeled PC, in which the quencher is attached to acyl chains at the following positions (left to right on the depth scale): 14, 12, 10, 7 and 5. Two open symbols represent alternative depth for TEMPO quencher. A green dotted profile corresponds to the DA fitting of the depth-dependent profile obtained when position of Tempo is assumed to reside at 26 Å from bilayer center (*i.e.*, above the phosphate level). The solid orange line corresponds to the fit obtained under assumption that TEMPO is located below phosphate level, as indicated by MD results (Fig. 1). The latter fit suggests that the most probable depth for NBD $h_m = 14.7$ Å, which is consistent with independent MD simulations of NBD-PE (see Fig. 3 and (Kyrychenko et al. 2015) for details).

Figure 3. Comparison of the MD-simulated distribution of a NBD moiety (Kyrychenko et al. 2015) (*shaded area*) with those estimated from experimental data (*red and green lines*). Briefly, the Distribution Analysis methodology (Ladokhin 2014) was applied to the “differential” quenching data obtained with a series of six spin quenchers, including Tempo-PC (Kyrychenko and Ladokhin 2014). The experimental quenching profiles were adjusted for the various broadening factors (e.g., size of probe and quencher) using an empirical parameter of $\sigma(Q)=2.5$ Å, estimated by Eq. 2, as described in (Ladokhin 2014). *Red profile* corresponds to the result obtained when position of Tempo is taken from our MD simulation (Kyrychenko and Ladokhin 2013), while *green profile* to that when Tempo is assumed to reside above the phosphate level, as suggested by Schrader and Han (Schrader and Han 2017). The profiles were normalized for the same area.