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# Refining membrane penetration by a combination of steady-state and time-resolved depth-dependent fluorescence quenching

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ABSTRACT

Accurate determination of the depth of membrane penetration of a fluorescent probe, attached to a lipid, protein, or other macromolecule of interest, using depth-dependent quenching methodology is complicated by thermal motion in the lipid bilayer. Here, we suggest that a combination of steady-state and time-resolved measurements can be used to generate a static quenching profile that reduces the contribution from transverse diffusion occurring during the excited-state lifetime. This procedure results in narrower quenching profiles, compared with those obtained by traditional measurements, and thus improves precision in determination of the underlying depth distribution of the probe.



Depth-dependent fluorescence quenching is a method that allows estimation of the depth of membrane penetration of a fluorophore attached to a lipid, protein, or other molecule of interest from a series of measurements with the quenchers attached at different positions to the lipids [1–7]. The main idea is a rather straightforward one—the closer the respective depths of fluorophore and quencher, the stronger the quenching [8]—but the quantitative analysis requires careful considerations of the physical model of the process [9–12]. One of the challenges in accurate determination of the depth is the inherent disorder caused by thermal motion, which contributes to the broadening of the quenching profiles due to diffusion of both fluorophore and quencher [13]. Here, to resolve this problem, we use a combination of steady-state and time-resolved fluorescence measurements from which dynamic and static quenching profiles are calculated. The latter profile has a narrower width, which allows for a more accurate determination of the underlying transverse distribution of the fluorophore.

To evaluate the interplay of static and dynamic quenching in the depth-dependent measurements, we have measured steady-state and time-resolved fluorescence of 1,2-dipalmitoyl-*sn*-glycero-3-phospho-ethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD–PE)[2] (Fig. 1A), which has NBD fluorophore attached to the lipid head group (all details of data collection and fluorescence decay analysis were the same as described in Ref. [14]). NBD–PE is a popular model compound often used as a reference for fluorescence of membrane proteins site-selectively labeled with NBD [14–16]. NBD–PE was incorporated into 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) large unilamellar vesicles (LUVs) by coextrusion at 1 mol% with respect to the total lipid concentration. Quenching samples also contained various amounts of one of the following quenchers: Tempo–PC, 5-Doxyl–PC, 7-Doxyl–PC, 10-Doxyl–PC, 12-Doxyl–PC, and 14-Doxyl–PC. Examples of steady-state and time-resolved quenching with Tempo–PC, which has a quenching group attached to the lipid headgroup, are shown in Figs. 1A and B, respectively. Even from the raw data, it is obvious that the total loss in intensity is stronger than the reduction of the lifetime, which indicates a substantial static quenching.

The contribution of static quenching becomes even more apparent when reductions in intensity and lifetime are plotted in Stern–Volmer coordinates in Fig. 1C. Note that for quenchers localized in a lipid bilayer, the proper units of concentration are the fractions of quencher-labeled lipids in total lipid; therefore, the corresponding Stern–Volmer constants are dimensionless (they correspond to the reciprocal value of quencher concentration, resulting in a 50% reduction in fluorescence intensity or lifetime for total or dynamic quenching, respectively). The following Stern–Volmer constants correspond to intensity measurements (Fig. 1C, open symbols): 5.8 for Tempo–PC, 7.0 for 5-Doxyl-PC, 5.5 for 7-Doxyl-PC, and 2.6 for 12-Doxyl-PC. The dynamic Stern–Volmer constants for the same sequence of quenchers estimated from lifetime measurements (closed symbols) are 2.8, 3.8, 3.2, and 2.2, respectively. The difference between the two clearly indicates variable contribution of

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[2] *Abbreviations used:* NBD–PE, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-ethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl); POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; LUV, large unilamellar vesicle; Tempo-PC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(Tempo)choline; *n*-Doxyl-PC, 1-palmitoyl-2-stearoyl-(*n*-Doxyl)-*sn*-glycero-3-phosphocholine; DA, distribution analysis.

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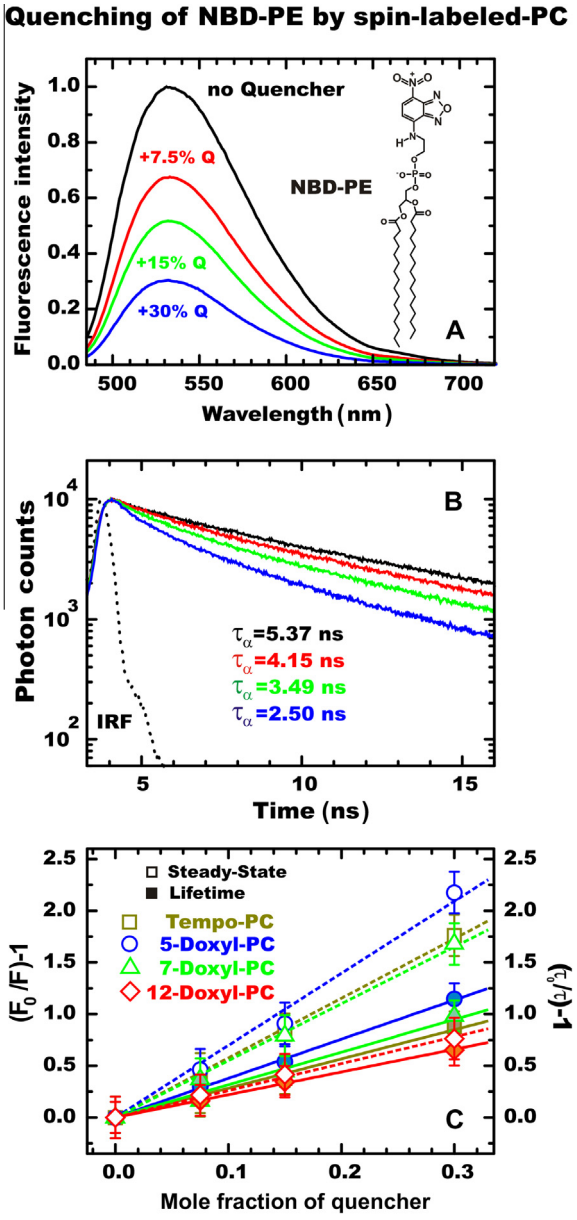


**Fig.1.** Depth-dependent fluorescence quenching of NBD–PE in LUVs. (A) Examples of the steady-state fluorescence quenching of NBD–PE coextruded with spin quencher Tempo–PC in LUVs composed of POPC lipids. The concentration of Tempo–PC was varied from 7.5 to 30 mol%, leading to a gradual decrease in the fluorescence intensity of the NBD fluorophore. The inset shows the molecular structure of NBD–PE. (B) The quenching of the steady-state fluorescence is accompanied by shortening of NBD fluorescence lifetime. The amplitude-averaged lifetimes ($\tau_\alpha$) estimated by double exponential fitting of the decay curves are shown for the samples containing (from top to bottom) no quencher (black) and in the presence of 7.5 mol% (red), 15 mol% (green), and 30 mol% (blue) Tempo–PC. The decays were acquired at 535 nm after pulse excitation with LED source emitting at 375 nm. Instrument Response Function, IRF, is shown as dotted line. (C) Stern–Volmer plots for the steady-state (open symbols and dotted lines) and lifetime (solid symbols and lines) quenching of NBD–PE in LUVs containing various spin-labeled lipids. $F_0/F$ and $\tau_0/\tau$ are the ratios of the fluorescence intensities and the lifetimes in the absence and presence of the quenchers, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

static quenching, which is further illustrated below with appropriate depth-dependent quenching profiles.

To determine the transverse position of the probe (i.e., its depth) in the lipid bilayer, one needs to construct a depth-dependent quenching profile, where quenching efficiency is plotted against the known depth of the quencher. We illustrate such profiles collected for the three quencher concentrations in Fig. 2A. The fitting lines through the data correspond to the application of the distribution analysis (DA) methodology, which we have developed for the purpose of extracting quantitative information on membrane penetration from depth-dependent quenching experiments [9–11]. DA fitting function is a pair of mirror-symmetric Gaussian functions (one for each leaflet) that reflects transverse distributions of lipid and protein moieties originating from the thermal motion in the bilayer [17,18]. Here, we represent the DA results by two parameters: the most probable depth of the fluorophore ($h_m$) and the width of the transverse distribution (FW). Recently, we validated this approach using Molecular Dynamics computer simulations [12]. Increasing quencher concentration results in more distinct quenching profiles, with a maximum indicating the most probable position of the fluorophore at approximately 14 Å from the bilayer center (Fig. 2A). Previously published studies gave somewhat conflicting results on the depth of the probe in NBD–PE, ranging from 14 to 20 Å [19–22]. In all of these studies, however, only two quenchers were used at a time, which inevitably undermines the reliability of depth estimates in comparison with fitting multiple data points [10,12], as illustrated in Fig. 2. Our results are also consistent with those of Molecular Dynamics simulations of NBD–PE in the POPC bilayer (A. Kyrychenko and A.S. Ladokhin, in preparation). The overall width of the quenching profile, which is determined by physical

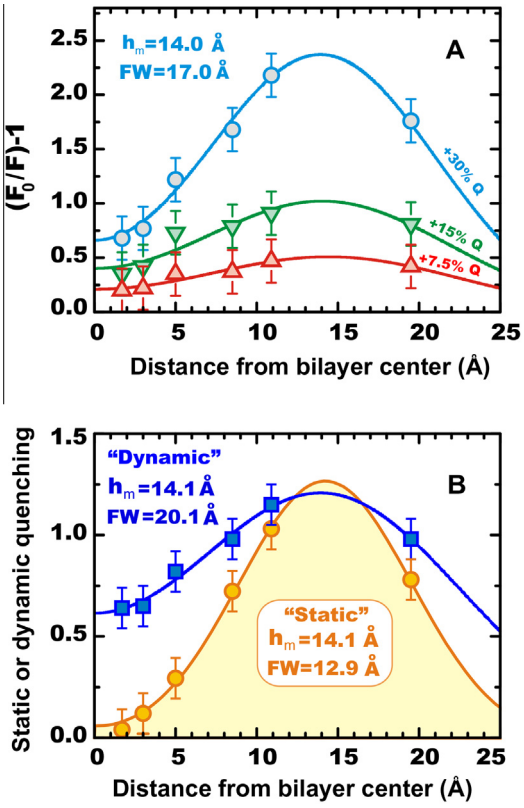


**Fig.2.** Distribution analysis (DA) [9,12] of depth-dependent fluorescence quenching profiles of NBD–PE obtained with a series of the spin-labeled lipids (Tempo–PC and $n$-Doxyl–PCs with $n$ = 5, 7, 10, 12, and 14). DA parameters for the most probable position of the fluorophore ($h_m$) and for the width of transverse distribution (FW) are shown on the graphs. (A) Application of DA methodology to steady-state fluorescence quenching of NBD–PE in LUVs containing (from bottom to top) 7.5, 15, and 30 mol% quenchers. (B) Application of DA methodology to lifetime quenching (solid squares) and static quenching (solid circles) calculated as the difference between total quenching (open circles in panel A) and dynamic (open squares in panel A) profiles. Accounting for dynamic broadening on the wings of the quenching profile produces better-defined, narrower "static" distributions of the depth of membrane penetration of the probe. See text for details.

sizes of the fluorophore and quencher, and widths of their thermal envelopes are quite large, which is typical for both experimental and simulated data [12].

In Fig. 2B, we present a deconvolution of the total quenching profile of NBD–PE obtained with a series of lipid-attached quenchers into dynamic and static components. A dynamic profile (solid squares) is defined as a quenching efficiency observed in a lifetime quenching experiment, whereas a static profile (solid circles) is the difference between total quenching efficiency (open circles in Fig. 2A) and the dynamic component. Although the average position is the same, the static distribution is much narrower than the dynamic profile. We suggest that this dynamic broadening reflects the nature of the quenching process in the bilayer. If the position of the quencher matches well with that of the excited fluorophore, then quenching occurs very fast and will be observed as static quenching in our experiments (for our experimental setup, any lifetime component shorter than 100 ps will be indistinguishable from the scattering caused by vesicles and will be excluded from the analysis [14,16]). Dynamic quenching is obviously never completely eliminated because of the lateral diffusion, which contributes even for samples containing high concentrations of quenchers [3]. Additional diffusion in direction normal to the bilayer occurs when the fluorophore and quencher are separated on the depth scale but come together during the excited state lifetime. This results in dynamic broadening of the quenching profile, clearly apparent in Fig. 2B.

Our results demonstrate how a combination of steady-state and time-resolved measurements can be used to generate a static depth-dependent quenching profile that reduces the contribution from the transverse diffusion occurring during the excited-state lifetime. As a result, we calculate narrower, better-defined quenching profiles compared with those obtained by traditional measurements of intensity or lifetime. This approach can be applied to studies of membrane proteins to improve the precision of the determination of bilayer penetration of the site-selectively attached probes.

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