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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 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 to those obtained by traditional measurements, and thus improves precision in determination of the underlying depth distribution of the probe.

Keywords

static and dynamic fluorescence quenching; bilayer penetration; Distribution Analysis; lipid-attached quenchers

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, in order 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.

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In order to evaluate the interplay of static and dynamic quenching in the depth-dependent measurements, we have measured steady-state and time-resolved fluorescence of NBD-PE (Fig. 1a), which has NBD fluorophore attached to the lipid head group (all the details of data collection and fluorescence decay analysis were the same as described in (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 POPC large unilamellar vesicles (LUV) by co-extrusion 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. The examples of steady-state and time-resolved quenching with Tempo-PC, which has a quenching group attached to the lipid headgroup, are shown in Fig. 1 panels *a* 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 static quenching, which is further illustrated below with appropriate depth-dependent quenching profiles.

In order 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), which 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: most probable depth of the fluorophore, h_m , and the width of the transverse distribution, FW. Recently we have validated this approach using Molecular Dynamics computer simulations (12). Increasing quencher concentration results in more distinct quenching profiles with a maximum indicating most probable position of the fluorophore at about 14 Å from 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 to 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 POPC bilayer (Kyrychenko and Ladokhin, in preparation). The overall width of the quenching profile, which is determined by physical 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 Figure 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. Dynamic

profile (squares) is defined as a quenching efficiency observed in a lifetime quenching experiment, while a static profile (solid circles) is a difference between total quenching efficiency (open circles in Fig. 2a) and the dynamic component. While 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 quencher matches well to 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 fluorophore and quencher are separated on the depth scale, but come together during 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 which reduces the contribution from the transverse diffusion occurring during the excited-state lifetime. As a result, we calculate narrower, better-defined quenching profiles, compared to 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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ABBREVIATIONS

NBD-PE	1,2-dipalmitoyl- <i>sn</i> -glycero-3-phospho-ethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
Tempo-PC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phospho(TEMPO)choline
<i>n</i>-Doxyl-PC	1-palmitoyl-2-stearoyl-(<i>n</i> -Doxyl)- <i>sn</i> -glycero-3-phosphocholine
DA	Distribution Analysis
h_m	most probable depth of the fluorophore calculated by DA
FW	full width of the quenching profile measured on half-height
LUV	extruded large unilamellar vesicles.

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Quenching of NBD-PE by spin-labeled-PC

Figure 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 mol % to 30 mol%, leading to a gradual decrease in the fluorescence intensity of the NBD fluorophore. Insert shows molecular structure of NBD-PE (b) The quenching of the steady-state fluorescence is accompanied by shortening of NBD fluorescence lifetime. The amplitude-averaged lifetime τ_a estimated by double exponential fitting of the decay curves are shown for the samples containing (top-to-bottom) no quencher (*black*), and in the

presence of 7.5 (*red*), 15 (*green*), and 30 mol% (*blue*) Tempo-PC, respectively. The decays were acquired at 535 nm after pulse excitation with LED source emitting at 375 nm. **(c)** Stern-Volmer plots for the steady-state (open symbols and dotted lines) and lifetime (solid symbols and lines) quenching of NBD-PE in LUV containing various spin-labeled lipids. F_0/F and τ_0/τ are the ratios of the fluorescence intensities and the lifetimes in the absence and in the presence of the quenchers, respectively.

Figure 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 $n=5, 7, 10, 12, 14$). DA parameters for 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 LUV containing (bottom-to-top) 7.5, 15, and 30 mol% quenchers. **(b)** Application of DA methodology to lifetime quenching (squares) and static quenching (circles) calculated as the difference between total quenching (circles in Fig. 2a) and dynamic (squares) 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.