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# Determination of the depth of BODIPY probes in model membranes by parallax analysis of fluorescence quenching

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**Abstract**

The location of a series of lipophilic and lipid-attached BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene) membrane probes was analyzed by the quenching of BODIPY fluorescence by a series of nitroxide-labeled lipids in which the depth of the nitroxide group is varied. When attached to the polar headgroup of PE the BODIPY remained near the polar headgroup in depth. However, when attached at the end of free or phospholipid-attached fatty acyl chains, or when attached to two hydrocarbon chains, we observed two probe populations. One, usually dominant, population of BODIPY groups 'looped back' towards the surface, but a second population remained deeply embedded within the bilayer. When attached to a fatty acid or fatty acyl chain, the deep population appeared to locate at a depth related to its point of attachment to the acyl chain. In BODIPY linked to free fatty acids, the location of the deep population responded to the ionization of the carboxyl group. Because, unlike NBD (7-nitro-2,1,3-benzoxadiazol-4-yl) and most dansyl groups, acyl chain linked BODIPY groups can exist in a deeply buried form we conclude that BODIPY linked acyl chains are superior to NBD or dansyl linked acyl chains as membrane probes. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Liposome; Cholesterol; Membrane probe; Fluophore distribution; Spin-label

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Abbreviations: BODIPY, 4,4-difluoro-4-bora-3a,4a,diaza-*s*-indacene; BODIPY-PE, *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a,diaza-*s*-indacene-3-propionyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; C1-BODIPY-C12-COOH, 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoic acid; C8-BODIPY-C5-COOH, 4,4-difluoro-5-octyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoic acid; C10-BODIPY-C3-COOH, 5-decyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid; C1-BODIPY-C12-PC, 1-hexadecanoyl-2-(4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-*sn*-glycero-3-phosphocholine C8-BODIPY-C5-PC, 1-hexadecanoyl-2-(4,4-difluoro-5-octyl-4-bora-3a,4a,diaza-*s*-indacene-3-pentanoyl)-*sn*-glycero-3-phosphocholine; didecyl-BODIPY, 3,5-didecyl-4,4-difluoro-4-bora-3a,4a,diaza-*s*-indacene; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; ESR, electron spin resonance; methyl$_4$-BODIPY, 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a,diaza-*s*-indacene; MLV, multilamellar vesicles; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; OG, *n*-octyl-β-D-glucopyranoside (octyl glucoside); PC, 1,2 diacyl-*sn*-glycero-3-phosphocholine; 5-SLPC or 12-SLPC, 1-palmitoyl-2-(5- or 12-doxyl) stearoyl-*sn*-glycero-3-phosphocholine; SUV, small unilamellar vesicles; Tempocholine: 4-(*N*,*N*-dimethyl-*N*-(2-hydroxyethyl)) ammonium-2,2,6,6- tetramethyl piperidine-1-oxyl; TempoPC, 1,2-dioleoyl-*sn*-glycero-3-phosphotempocholine; TLC, thin-layer chromatography

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## 1. Introduction

One of the fundamental questions involved in the study of membrane structure and function is: what chemical and structural factors control the structure and depth of molecules in membranes? Fluorescent probes have proven a powerful approach to obtain answers to these questions. However, the interpretation of fluorescent probe behavior depends in large part upon where it is located in the bilayer, and for many probes this has been only poorly characterized. In fact, in more than one case, fluorescent probes have been found to localize in a region of the membrane other than that for which they were originally designed [1–3]. In most other cases, only a rough estimate of probe location has been available.

To help locate molecules in membranes we developed a fluorescence quenching technique called parallax analysis, which allows the depth of a fluorescent group in a lipid vesicle to be determined from the result of two quenching experiments [1,3,4]. In one experiment, the fluorescence intensity of a fluorophore in the presence of a nitroxide (spin)-labeled quencher located at a specific depth is measured. In a second experiment fluorescence is measured in the presence of a quencher carrying the nitroxide group at a different depth. The ratio of the two intensities is then substituted into a equation that allows calculation of depth at a high level of resolution. Parallax analysis has been applied to a large number of different fluorescent probes, polypeptides and proteins, and several tests confirming its accuracy have been made [1,3–11].

In this report, the location of BODIPY probes in membranes is examined. BODIPY probes are a recently introduced class of fluorescent probes that have excellent fluorescent properties. They have a high extinction coefficient, fluorescence quantum yield, and photostability [12,13]. They fluoresce in the visible, with excitation and emission spectra similar to those of the widely used xanthene dyes (fluorescein, rhodamine, eosin, Texas red, etc.) [12,13]. However, unlike xanthene dyes the BODIPY group carries no net charge and therefore can be used as a probe of membrane structure. Furthermore, it is believed that BODIPY groups are significantly more hydrophobic than previously used polar membrane probes such as NBD. As a result, BODIPY probes have been used in a number of studies of the structure and function of membranes and individual lipids [14–19]. In this study, we find BODIPY groups have a tendency to move toward the polar headgroup region of the bilayer. However, a considerable population of BODIPY molecules can remain buried within the lipid bilayer. Therefore, BODIPY appears to be a useful fluorescent analog for studies of lipid behavior.

## 2. Materials and methods

### 2.1. Materials

BODIPY probes were purchased Molecular Probes (Eugene, OR). The purity of the BODIPY probes was checked by TLC on silica gel plates (Silica gel H, Uniplate, Newark, DE) using solvent systems of: 10/30 $CHCl_3$/methanol (v/v) for C10-BODIPY-C3-COOH, C1-BODIPY-C12-COOH, and C8-BODIPY-C5-COOH; 32/25/2 $CHCl_3$/Methanol/$H_20$ (v/v) for C1-BODIPY-C12-PC, C8-BODIPY-C5-PC, and BODIPY-PE; 30/10 mixed hexanes/$CHCl_3$ (v/v) for didecyl-BODIPY; 20/20 mixed hexanes/$CHCl_3$ (v/v) methyl$_4$-BODIPY. All probes gave a single intensely colored spot.

### 2.2. Assay of spin-label content of spin-labeled lipids

Nitroxide-labeled PCs and DOPC were purchased from Avanti Polar Lipids (Pelham, AL). The purity of phospholipids was confirmed by TLC on silica gel plates as described previously [3]. The concentration of phospholipids was determined by phosphate assay subsequent to total digestion [3]. The actual nitroxide content of the nitroxide-labeled lipids was calculated from the intensities of the doubly integrated ESR spectra as described previously [1]. Alternatively, the concentration of the spin-labeled lipids was assayed with fluorescence quenching by determining the % of nitroxide-labeled lipid that had to be incorporated into vesicles to give the same quenching of anthroyloxy fatty acids as that found previously for vesicles containing 15% nitroxide-labeled lipids [3]. The ratio of nitroxide groups to lipid was generally found to be in the range 0.6–0.9.

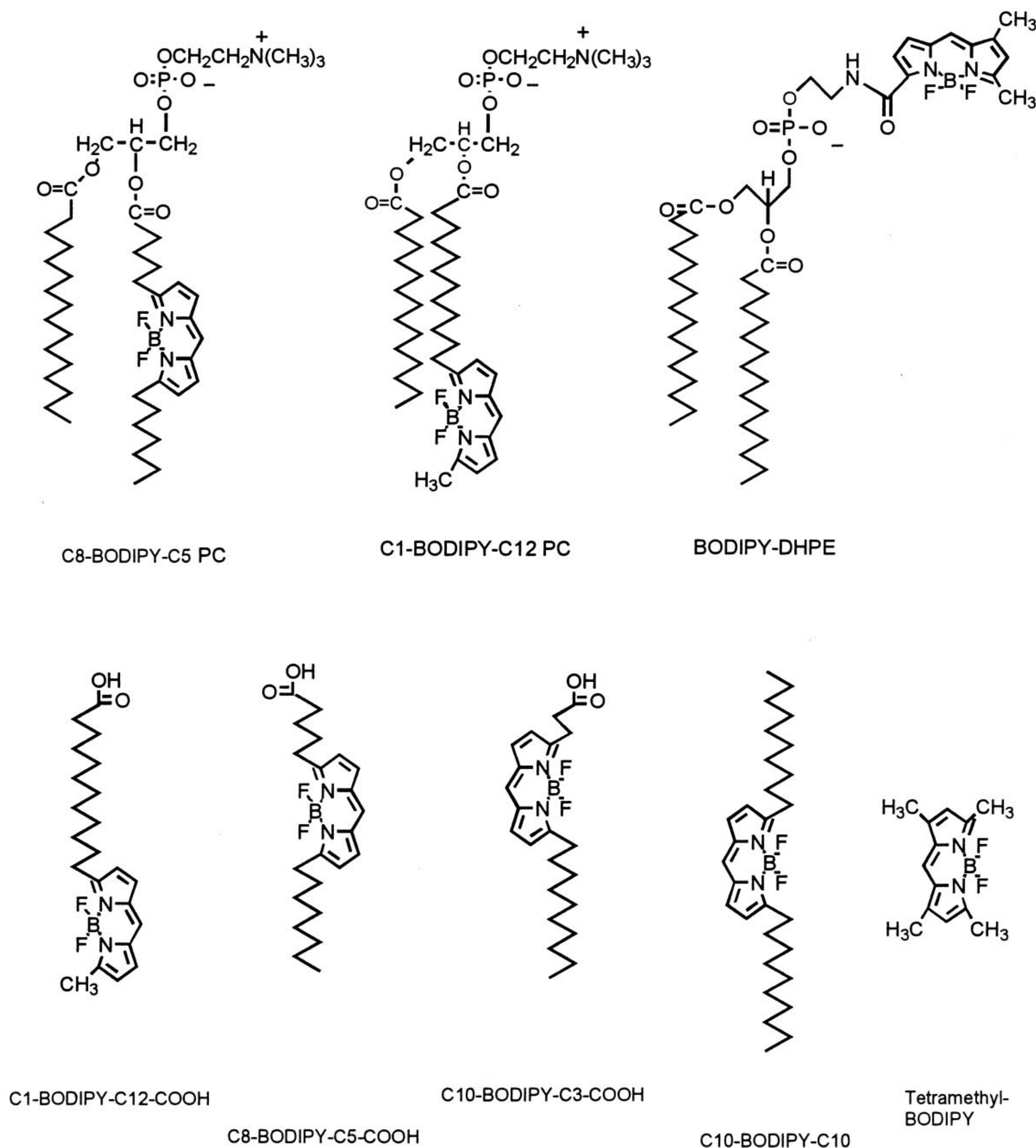


Fig. 1. Structure of the BODIPY probes studied in this report.

### 2.3. Preparation of samples for fluorescence measurements

Depth measurements were made on samples containing BODIPY derivatives incorporated into 200 μM SUVs prepared by octylglucoside (OG) dilution [20]. Solutions of 2.5 μM BODIPY derivative dissolved in 25 mM OG were prepared by drying aliquots of OG and BODIPY derivatives dissolved in ethanol with $N_2$ and then dissolving in $H_2O$. In separate tubes ethanol solutions containing 1 μmol of OG and 200 nmol of total phospholipid containing DOPC, or DOPC with 15 mol% 5-SLPC, 12-SLPC or TempoPC[1] were mixed and then dried with $N_2$. Then 40 μl of the BODIPY derivative in

[1] The nitroxide-labeled lipid contains some molecules that lack a nitroxide group. The amount of nitroxide-labeled lipid is adjusted to give 15% nitroxide-containing molecules and 85% other lipid molecules (DOPC plus inactive 'quencher').

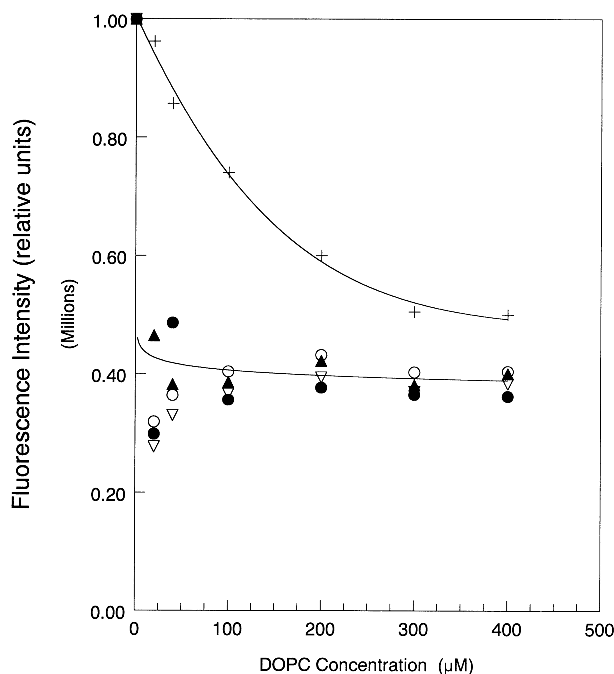


Fig. 2. Binding of BODIPY probes to model membrane vesicles. $F/F_o$ is the ratio of fluorescence in the presence of DOPC vesicles containing 15 mol% 5-SLPC to that in 100% DOPC vesicles. Binding is shown for: didecyl-BODIPY (▽) at pH 7; (+) methyl$_4$-BODIPY at pH 7; and (▲) C1-BODIPY-C12-COOH, (○) C8-BODIPY-C5-COOH, and (●) C10-BODIPY-C3-COOH at pH 10.

OG was added and the samples vortexed to dissolve all the components. Lastly, 960 μl of buffer (10 mM Na acetate/150 mM NaCl, pH 4.5; 10 mM Na phosphate/150 mM NaCl, pH 7 (PBS); or 10 mM glycine/150 mM NaCl, pH 10) was added to each tube and the tubes were vortexed for 30 s. In samples containing cholesterol, an additional 100 nmol cholesterol was included with the phospholipid. Final BODIPY concentration was 0.1 μM.

### 2.4. Fluorescence quenching measurements

Fluorescence was measured in 1 cm path length quartz cuvettes using a Spex Fluorolog spectrofluorimeter operating in ratio mode. The excitation and emission slits were set at 1.25 mm (2.3 nm bandpass) to prevent overloading of the photomultiplier tube. The excitation wavelength was set at 500 nm, and emission at 530 nm. Control experiments showed that fluorescence intensities were stable over several minutes, and that preincubation in the dark or in room light did not affect intensity. The fluorescence intensity from duplicate or triplicate samples containing fluorophore was averaged. The intensity of the background samples without fluorophore was found to be negligible ($<1\%$). All measurements were made at room temperature. The ratio of the fluorescence intensities in the presence ($F$) and in the absence ($F_o$) of the nitroxide-labeled lipids ($F/F_o$) was calculated and substituted into the parallax equation [1,3] to calculate BODIPY depth (see below).

To confirm the absence of reactivity between nitroxide-labeled lipids and BODIPY probes, in control experiments fluorescence was measured after 100 μl aliquots of each vesicle sample were dissolved in 900 μl ethanol. This abolished quenching and equal fluorescence intensities were observed in samples with and without nitroxide-labeled lipids.

Table 1
Nitroxide quenching and depth of BODIPY derivatives in DOPC at pH 7.0

| Fluorophores | $F_{tc}$ | $F_5$ | $F_{12}$ | $z_{cf}$ |
|---|---|---|---|---|
| Methyl$_4$-BODIPY | $0.53 \pm 0.03$ | $0.58 \pm 0.02$ | $0.57 \pm 0.03$ | $18.4 \pm 0.8$[a] |
| Didecyl-Bodipy | $0.20 \pm 0.03$ | $0.35 \pm 0.05$ | $0.32 \pm 0.03$ | $21.4 \pm 1.1$ |
| BODIPY-PE | $0.27 \pm 0.05$ | $0.41 \pm 0.04$ | $0.43 \pm 0.01$ | $20.3 \pm 0.9$ |
| C8-BODIPY-C5-PC | $0.26 \pm 0.04$ | $0.38 \pm 0.02$ | $0.40 \pm 0.02$ | $19.7 \pm 0.8$ |
| C1-BODIPY-C12-PC | $0.28 \pm 0.01$ | $0.40 \pm 0.01$ | $0.35 \pm 0.01$ | $19.4 \pm 0.4$ ($7.2 \pm 0.4$)[b] |

$F_{tc}$, $F_5$ and $F_{12}$ equal ratio of fluorescence intensity in the presence of the TempoPC, 5-SLPC or 12-SLPC, respectively, to that in the absence of quencher ($F_o$). Values shown are for the average of 4-8 samples ± S.D.

[a]This value was calculated after correction of fluorescence quenching values for probe (about 33%) not bound to membranes [6].

[b]The value in parentheses is the upper limit to $z_{cf}$ of the deep subpopulation of probes calculated from the ratio of fluorescence by 5-SLPC and 12-SLPC. See Section 2 for details.

Table 2
Nitroxide quenching and depth of BODIPY derivatives in DOPC/cholesterol at pH 7.0

| Fluorophores | $F_{tc}$ | $F_5$ | $F_{12}$ | $z_{cf}$ |
|---|---|---|---|---|
| Methyl$_4$-BODIPY | 0.59 ± 0.02 | 0.66 ± 0.01 | 0.64 ± 0.01 | 20.1 ± 0.6[a] |
| Didecyl-BODIPY | 0.32 ± 0.02 | 0.44 ± 0.03 | 0.41 ± 0.01 | 20.7 ± 0.8 |
| BODIPY-PE | 0.30 ± 0.01 | 0.43 ± 0.0 | 0.51 ± 0.003 | 21.4 ± 0.3 |
| C8-BODIPY-C5-PC | 0.30 ± 0.01 | 0.42 ± 0.003 | 0.48 ± 0.02 | 20.8 ± 0.3 |
| C1-BODIPY-C12-PC | 0.35 ± 0.05 | 0.48 ± 0.05 | 0.36 ± 0.03 | 20.7 ± 0.5<br>(7.0 ± 0.5)[b] |

[a]This value was calculated after correction of fluorescence quenching values for probe (about 33%) not bound to membranes [6].
[b]The value in parentheses is the upper limit to $z_{cf}$ of the deep subpopulation of probes calculated from the ratio of fluorescence by 5-SLPC and 12-SLPC. See Section 2 for details.

Table 3
Nitroxide quenching and depth of BODIPY derivatives of free fatty acids in DOPC

| Fluorophores | $F_{tc}$ | $F_5$ | $F_{12}$ | ]$z_{cf}$ |
|---|---|---|---|---|
| C1-BODIPY-C12-COOH pH 4.5 | 0.23 ± 0.02 | 0.39 ± 0.02 | 0.33 ± 0.04 | 20.6 ± 0.6<br>(7.1 ± 1.6) |
| C1-BODIPY-C12-COOH pH 10.0 | 0.32 ± 0.02 | 0.41 ± 0.01 | 0.43 ± 0.04 | 18.4 ± 0.8 |
| C8-BODIPY-C5-COOH pH 4.5 | 0.26 ± 0.05 | 0.37 ± 0.04 | 0.38 ± 0.01 | 19.5 ± 1.1 |
| C8-BODIPY-C5-COOH pH 10.0 | 0.29 ± 0.02 | 0.39 ± 0.01 | 0.44 ± 0.06 | 18.9 ± 1.1 |
| C10-BODIPY-C3-COOH pH 4.5 | 0.25 ± 0.05 | 0.38 ± 0.02 | 0.39 ± 0.01 | 20.4 ± 1.3 |
| C10-BODIPY-C3-COOH pH 10.0 | 0.24 ± 0.04 | 0.36 ± 0.02 | 0.41 ± 0.01 | 20.0 ± 1.3 |

### *2.5. Calculation of depth by parallax analysis*

Using $F/F_o$ values the distance of fluorophores from the center of the bilayer was calculated using the parallax equation [1,3]:

$$z_{cf} = L_{c1} + (-\ln(F_1/F_2)/\pi C - L_{21}^2)/2L_{21},$$

where $z_{cf}$ is the distance of the fluorophore from the center of the bilayer, $F_1$ is the fluorescence intensity ($F/F_o$) in the presence of the shallow quencher (quencher 1), $F_2$ is the fluorescence intensity ($F/F_o$) in the presence of the deeper quencher (quencher 2), $L_{c1}$ is the distance of the shallow quencher from the center of the bilayer, $L_{21}$ is the distance between the shallow and deep quenchers, and the $C$ is the concentration of quencher in molecules/Å$^2$=(mole fraction of nitroxide-labeled phospholipid/area per phospholipid)=(mole fraction nitroxide/70 Å$^2$) [1]. The quenching by the pair of quenchers (i.e. nitroxide-labeled lipid) that quench the most (i.e. the TempoPC/5-SLPC pair or 5-SLPC/12-SLPC pair) was used to calculate $z_{cf}$ [3]. The values used for the distances of the nitroxide group from the bilayer center were 5.85 Å for 12-SLPC, 12.15 Å for 5-SLPC, and 19.5 Å for TempoPC [1,3].

In model membranes containing cholesterol, the (lateral) concentration of nitroxide-labeled lipid and the depth of the quenchers in the membranes are altered. These values were calculated by assuming that 33 mol% cholesterol results in a 10% increase in the width of the acyl chain region of the bilayer, and that a cholesterol molecule occupies a lateral area of 32 Å$^2$ [21,22]. The resulting value for $C$ is (mole fraction of nitroxide-labeled phospholipid relative to total lipid including cholesterol)/[0.67(70 Å)+0.33(32 Å)]=(mole fraction nitroxide/57.5 Å$^2$), and the values for the distances of 12-SLPC, 5-SLPC, and TempoPC nitroxides from the bilayer center are, 6.45, 13.4 and 21 Å, respectively[2].

[2] There is very little difference when the corrections for quencher depth and for concentration in the presence of cholesterol (see Section 2) are omitted. Therefore, the results are not sensitive to these corrections.

Table 4
Nitroxide quenching and depth of free fatty acid BODIPY derivatives in DOPC/cholesterol

| Fluorophores | $F_{tc}$ | $F_5$ | $F_{12}$ | $z_{cf}$ |
|---|---|---|---|---|
| C1-BODIPY-C12-COOH pH 4.5 | 0.31 ± 0.01 | 0.45 ± 0.01 | 0.37 ± 0.04 | 21.2 ± 0.3 (7.4 ± 1.1) |
| C1-BODIPY-C12-COOH pH 10.0 | 0.35 ± 0.03 | 0.47 ± 0.03 | 0.46 ± 0.05 | 20.4 ± 0.5 |
| C8-BODIPY-C5-COOH pH 4.5 | 0.33 ± 0.01 | 0.45 ± 0.02 | 0.44 ± 0.02 | 20.6 ± 0.3 |
| C8-BODIPY-C5-COOH pH 10.0 | 0.35 ± 0.01 | 0.45 ± 0.004 | 0.51 ± 0.02 | 19.8 ± 0.3 |
| C10-BODIPY-C3-COOH pH 4.5 | 0.31 ± 0.01 | 0.43 ± 0.03 | 0.46 ± 0.03 | 20.8 ± 0.5 |
| C10-BODIPY-C3-COOH pH 10.0 | 0.30 ± 0.004 | 0.43 ± 0.02 | 0.52 ± 0.01 | 21.1 ± 0.5 |

### 2.6. Upper limit of the depth of minor deep subpopulations

Consider the case of a fluorophore that has distinct shallow and deep subpopulations. In such a situation there are three different parameters that can be used to describe depth: the average depth of all fluorophores $z_{cf}$ average; the average depth of the deep fluorophore subpopulation, $z_{cf}$ deep; and the average depth of the shallow fluorophore population, $z_{cf}$ shallow. We are concerned with the case where the shallow population is near the headgroup and the deep one much closer to the center of the bilayer. Where the major subpopulation is shallow, as observed for BODIPY probes, the depth calculated from the parallax equation using the TempoPC and 5-SLPC quenching will be close to $z_{cf}$ average [20], and is reported as such. In contrast, the depth calculated from the parallax equation using the 5-SLPC and 12-SLPC quenching will be an upper limit to the depth of the minor deep population ($z_{cf}$ deep) [20], and is reported as such in those cases where an appreciable deep population was detected. A more detailed discussion is given in reference [20].

### 2.7. Binding curves

Two sets of OG dilution SUVs with increasing amounts of lipid were prepared. Samples in both sets ranged from 0 to 400 μM lipid. The first set contained duplicate samples of DOPC and the second set contained duplicate samples of 15 mol% 5-SLPC mixed with DOPC. Both sets contained 0.1 μM BODIPY. To prepare these samples the appropriate amount of lipids was dried together with 1 μmol of DG and then the 40 μl of BODIPY/OG solution was added and diluted as described above.

### 2.8. Fluorescence vs. pH; p$K_a$

A buffer of 10 mM Na acetate/150 mM NaCl, pH 5.0, was titrated to pH 3 with a small amount of 1 M HCl. Three sets of 1.7 ml OG dilution SUV samples containing 200 μM lipid and 0.1 μM C8-BODIPY-C5-COOH were prepared in this buffer as described above. One set contained DOPC while the other sets contained 15 mol% 5-SLPC or 12-SLPC mixed with DOPC. The samples were placed in a 1 cm quartz cuvette, and both fluorescence and pH was measured. Successive aliquots of base ($NH_4OH$) were added to increase pH. Fluorescence was measured 1–2 min later. The final fluorescence values were corrected for dilution.

## 3. Results

### 3.1. Binding of BODIPY probes to model membrane vesicles

We applied fluorescence quenching to determine the membrane location of a series of BODIPY probes (Fig. 1) incorporated into model membrane vesicles. The binding of BODIPY probes was first examined in order to determine the best conditions for the quenching experiments. This was done by measuring the quenching detected when the probes bound to vesicles which contained a nitroxide-labeled lipid (Fig. 2). With the exception of the free methyl$_4$-BODIPY probe which appears to bind half-maxi-

mally near 100–150 μM, the BODIPY probes were all fully bound at 20 μM lipid[3].

### 3.2. Measuring depth by fluorescence quenching

To measure the depth of the BODIPY probes their fluorescence was measured in unilamellar vesicles[4] containing DOPC or DOPC mixed with 15% of shallow (TempoPC), medium (5-SLPC), or deep (12-SLPC) nitroxide-labeled lipids (Tables 1–4). Strong fluorescence quenching was observed for all BODIPY derivatives. The maximum amount of quenching obtained (70–75%) was about the same for all but the $methyl_4$-BODIPY probe, which was only partly bound to the vesicles under the conditions of these experiments. The amount of quenching obtained with the different nitroxide-labeled lipids was then used to calculate the distance of the fluorophores from the center of the bilayer ($z_{cf}$) using the parallax equation (see Section 2. In the case of the $methyl_4$-BODIPY probe this was done after correction for the amount of fluorescence arising for unbound molecules [6]).

### 3.3. Behavior of free and decyl linked BODIPY probes

As shown in Tables 1 and 2 the $methyl_4$-BODIPY probe has a predominately shallow location within the polar headgroup region of the bilayer ($z_{cf}$=18–20 Å). This can be seen qualitatively by the fact that its is quenched most strongly by the shallow TempoPC probe (Tables 1 and 2). However, the quenching by the medium depth 5-SLPC and deep 12-SLPC is very similar, which is consistent with a minor subpopulation of deeper BODIPY molecules or a very broad distribution of BODIPY depths.

Didecyl-BODIPY also is quenched most strongly by TempoPC and has a predominantly shallow population ($z_{cf}$=21 Å). Presumably this molecule is oriented with its BODIPY group near the membrane surface and its acyl chains penetrating the bilayer aligned with the lipid fatty acyl chains. There is also a subpopulation of didecyl-BODIPY molecules with deep BODIPY groups, as shown by the slightly stronger quenching by 12-SLPC than 5-SLPC[5].

### 3.4. Behavior of BODIPY attached to phospholipids

A more localized BODIPY depth is seen for BODIPY-PE, in which the BODIPY group is attached to the polar headgroup amino group of PE. In this case, the BODIPY probe clearly has a shallow location ($z_{cf}$=20–21 Å), and the fact that quenching by 12-SLPC is significantly weaker than that of the 5-SLPC suggests there is little if any deep subpopulation (Tables 1 and 2).

The behavior of BODIPY-PE is not surprising since the fluorescent moiety is attached to the headgroup of a PE, designed so that it would be a very shallow fluorophore. However, a similar predominantly shallow location is observed for BODIPY derivatives of PC in which the BODIPY group is attached to one of the lipid acyl chains at a deep (C1-BODIPY-C12-PC) or intermediate position (C8-BODIPY-C5-PC) along the fatty acyl chain ($z_{cf}$=19–21 Å). This implies that the BODIPY group has a sufficient affinity for the polar region of the membrane to induce the fatty acyl chain to loop up towards the surface, as has previously been seen for polar derivatives of fatty acids [1–3, 20].

However, at least in the case of C1-BODIPY-C12-PC there is also a large population of BODIPY groups that is deeply buried. This is shown by the stronger quenching by 12-SLPC than 5-SLPC. The fact that quenching by the 12-SLPC is almost as strong as that by TempoPC suggests the deep population is a very considerable fraction of the total, and an upper limit to the depth of this population of

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[3] In most cases, there was a large increase in intensity in the presence of lipid compared to that in its absence. Since BODIPY fluorescence is ordinarily insensitive to polarity [13], we assume this reflected an aggregation of the probes in solution in the absence of lipid. Aggregation would result in BODIPY self-quenching, perhaps due to excimer formation [14].

[4] We have repeatedly found the depth of small fluorescent probes is not affected by the size or curvature of the model membrane vesicles used [1,5,6].

[5] It should be noted that it is difficult to pinpoint the depth of minor subpopulations using parallax analysis [20], and generally we have not attempted to do so. On the other hand when a second subpopulation is large, a useful limit to $z_{cf}$ can be calculated (see [20] and Section 2).

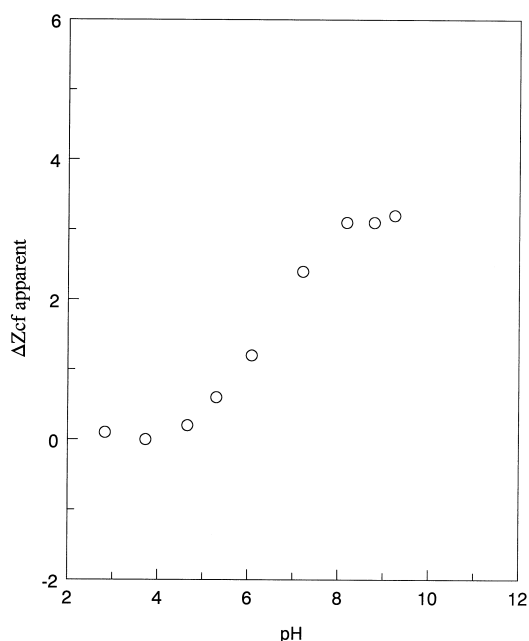


Fig. 3. The pH dependence of $z_{cf}$ apparent for the deep subpopulation of C8-BODIPY-C5-COOH. Note that $z_{cf}$ apparent is the upper limit to the true $z_{cf}$ of the deep population in this case (see Section 2). The change in $z_{cf}$ relative to that at pH 4 is shown on the $y$-axis.

$z_{cf}$=7 Å (Tables 1 and 2) can be calculated from the quenching of the 5-SLPC and 12-SLPC (see Section 2).

### 3.5. Behavior of BODIPY probes linked to free fatty acids: effect of fatty acyl ionization on depth

The behavior of free fatty acid BODIPY derivatives is similar to that of PC with BODIPY fatty acyl chains in many ways. The main population of BODIPY groups is located within the polar headgroup region for all three BODIPY fatty acids ($z_{cf}$=18–21 Å). This is true whether the BODIPY is attached close to (C10-BODIPY-C3-COOH), or far from (C1-BODIPY-C12-COOH) the COOH group.

In addition, there is clearly a deep subpopulation of BODIPY groups for C1-BODIPY-C12-COOH at low pH, similar to that noted above for the corresponding PC derivative. A distinct deep population, characterized by stronger 12-SLPC than 5-SLPC quenching, cannot be detected for the other BODIPY fatty acids. This could reflect either the lack of such a population, or alternately that the deep population is too close to the main population to be clearly discerned. In fact, the pH dependence of quenching suggests that a deeper subpopulation does exist for these BODIPY fatty acids as well. As in the case of C1-BODIPY-C12-COOH, there is a significant increase in the ratio of quenching by the 5-SLPC relative to 12-SLPC at high pH without an increase in quenching by TempoPC for both C8-BODIPY-C5-COOH and C10-BODIPY-C3-COOH (Tables 3 and 4). This would be predicted if there was a deep subpopulation with a depth that decreases upon COOH ionization and a shallow population that does not decrease in depth upon ionization.

In further support of this idea the pH dependence of the quenching of C8-BODIPY-C5-COOH fatty acid shows a change in depth at the pH at which the COOH group ionizes (i.e. p$K_a$) (Fig. 3). The population of molecules with a deep BODIPY group has COOH p$K_a$ close to 7 which is similar to that previously observed for a number of derivatives of free fatty acids [23,24]. This also suggests behavior the deep population of BODIPY fatty acids is not perturbed by the BODIPY group.

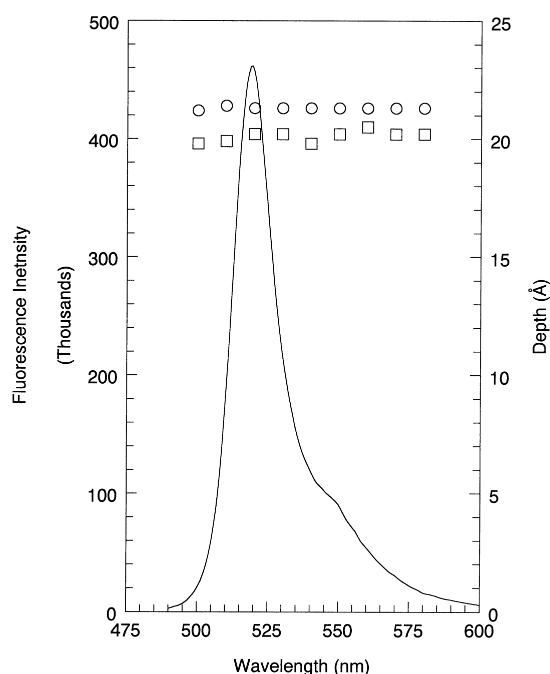


Fig. 4. The dependence of $z_{cf}$ on emission wavelength. $z_{cf}$ was measured as a function of wavelength for: (○) C10-BODIPY-C3-COOH; and (□) C8-BODIPY-C5-COOH at pH 10. (——) emission spectrum of C8-BODIPY-C5-COOH at pH 10. Emission spectra of the different BODIPY derivatives were very similar.

### 3.6. Effect of cholesterol and emission wavelength on $z_{cf}$

We also examined the effect of cholesterol on the membrane location of the BODIPY probes. As shown by a comparison of Tables 2 and 4 to Tables 1 and 3 there was little significant difference between BODIPY depth in membranes lacking or containing 33% cholesterol.

Previous studies showed that the apparent depth of some, but not all, fluorescent probes is dependent on the measured emission wavelength [5,20,24]. To determine whether this is a feature of BODIPY probes, we investigated the apparent depth dependence of two BODIPY fatty acids on emission wavelength. As shown in Fig. 4 there was no significant dependence of apparent depth on emission wavelength.

## 4. Discussion

### 4.1. BODIPY acts like a polar membrane probe, but is not as polar as NBD or dansyl probes

This study has provided a relatively direct measurement of the location of the BODIPY probes in membranes. This is important because it will allow more exact interpretation of the behavior of these widely used probes, and will help in developing rules for predicting the relationship of chemical structure to membrane depth [5,6,20].

We find that the BODIPY group has a clear tendency to locate in the polar headgroup region of the bilayer. The main population of BODIPY probes locate at a depth similar to that seen previously for such polar fluorophores as NBD [2,3] and dansyl [20] derivatives. This does not mean that the BODIPY group is as polar as NBD or dansyl, because a high degree of polarity is not necessary for a group to have a tendency to locate shallowly. For example, carbazole only has three fused rings containing one nitrogen atom, yet has a significant tendency to localize near the depth of the polar/hydrocarbon boundary [6,25].

In fact, BODIPY has a lesser tendency to locate within the polar headgroup region than NBD and dansyl. This is shown by the observation that, unlike both NBD and dansyl fatty acid derivatives, there was a deep population of BODIPY groups when BODIPY was attached to the end of a fatty acyl chain. Although the presence of a predominating shallow population makes it difficult to determine the precise depth of the deeper population, the upper limit for the depth of the deeper BODIPY population ($< 7.5$ Å) suggests the conformation of the acyl chain is near normal when the BODIPY locates deeply. Furthermore, the location of this deeper population responds to ionization of the fatty acid carboxyl group the same way (by moving to a few Å shallower location) as has been found for other hydrophobic fatty acid derivatives [23,24,26]. Therefore, we conclude BODIPY fatty acyl derivatives should be superior analogs of natural fatty acids than the corresponding NBD and dansyl derivatives.

### 4.2. The behavior of BODIPY probes is consistent with accessibility to iodide quenching

On the basis of the reduced accessibility of BODIPY derivative to quenching by KI when membrane bound relative to that when dissolved in solution, Johnson et al. [14] proposed that the BODIPY group was deeply buried in membranes. However, this result was not definitive for two reasons. First, is that a strong reduction in accessibility to quenchers can occur even when a fluorophore is bound to the polar headgroup region. We found that for carbazole quenching by the aqueous quencher acrylamide there is reduction in accessibility of a fluorophore to a quencher even upon binding to a shallow location in membranes [6]. Furthermore, the reduction of accessibility to quenching upon binding near the surface was much larger than the difference in accessibility upon shifting from a shallow to a deep location within the bilayer [6].

Second, the dependence of $F_o/F$ upon [KI] data in Johnson et al showed a slope that deviated from linearity with a negative curvature. This is characteristic of quenching behavior when there are two populations with different accessibilities to quenching [27]. In such cases, the deep population dominates the slope of the $F_o/F$ vs. [quencher] curve at high quencher concentrations. Therefore, the KI data supports our conclusion that multiple populations of BODIPY probes exist at different depths in membranes.

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