# Application of NBD-Labeled Lipids in Membrane and Cell Biology

**Sourav Haldar and Amitabha Chattopadhyay**

**Abstract** The fluorescent NBD group has come a long way in terms of biological applications since its discovery a few decades back. Although the field of fluorescently labeled lipids has grown over the years with the introduction of new fluorescent labels, NBD-labeled lipids continue to be a popular choice in membrane and cell biological studies due to desirable fluorescence characteristics of the NBD group. In this chapter, we discuss the application of NBD-labeled lipids in membrane and cell biology taking representative examples with specific focus on the biophysical basis underlying such applications.

**Keywords** FRAP · Looping up · Membrane probes · REES

## Contents

1 Introduction ..... 38
2 NBD Group Senses Slow Solvent Relaxation in Membranes ..... 40
3 Membrane Phase Dependence of Probe Looping in Acyl Chain-Labeled NBD Lipids ... 42
4 Application of NBD Fluorescence Sensitivity in Cell Biology ..... 44
5 Transbilayer Organization of Cholesterol Monitored Using NBD Fluorescence ..... 44
6 Conclusion and Future Perspectives ..... 47
References ..... 47

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## Abbreviations

| | |
|---|---|
| 6-NBD-PC | 1-Palmitoyl-2-(6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]hexanoyl)-*sn*-glycero-3-phosphocholine |
| 12-NBD-PC | 1-Palmitoyl-2-(12-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-yl) amino]dodecanoyl)-*sn*-glycero-3-phosphocholine |
| 6-NBD-CM | 6-([*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl) sphingosine |
| 6-NBD-SM | 6-([*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl) sphingosylphosphocholine |
| 25-NBD-cholesterol | 25-[*N*-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl] amino]-27-norcholesterol |
| DOPC | Dioleoyl-*sn*-glycero-3-phosphocholine |
| DPPC | 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine |
| FRAP | Fluorescence recovery after photobleaching |
| NBD | 7-Nitrobenz-2-oxa-1,3-diazol-4-yl |
| NBD-PE | *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine |
| NBD-PS | 1,2-Dioleoyl-*sn*-glycero-3-phospho-l-serine-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) |
| POPC | 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine |
| REES | Red edge excitation shift |

## 1 Introduction

Cellular membranes represent two-dimensional, non-covalent, anisotropic, and cooperative assemblies consisting of lipids and proteins. Membranes allow cellular compartmentalization and act as the interface through which cells sense the environment and communicate with each other. They confer an identity to cells (and their organelles) and represent an appropriate milieu for the proper function of membrane proteins. In addition, membranes constitute the site of important cellular functions such as signal transduction [1] and pathogen entry [2, 3]. It has been estimated that ~50% of all biological processes occur at the cell membrane [4].

The mammalian cell is made up of a large variety of lipids [5] which orchestrate diverse cellular functions with the help of membrane proteins. Tracking individual lipids in a crowded cellular milieu poses considerable challenge. It is in this context that lipid probes assume significance (see [6] for a comprehensive account of lipid probes). Various lipid probes have proved to be useful in membrane and cell biology due to their ability to monitor lipid molecules by a variety of physicochemical approaches at increasing spatiotemporal resolution [7]. Spectroscopic and microscopic techniques using fluorescent lipid analogs represent a powerful set of approaches for monitoring membrane organization and dynamics due to their high sensitivity, suitable time resolution, and multiplicity of measurable parameters.

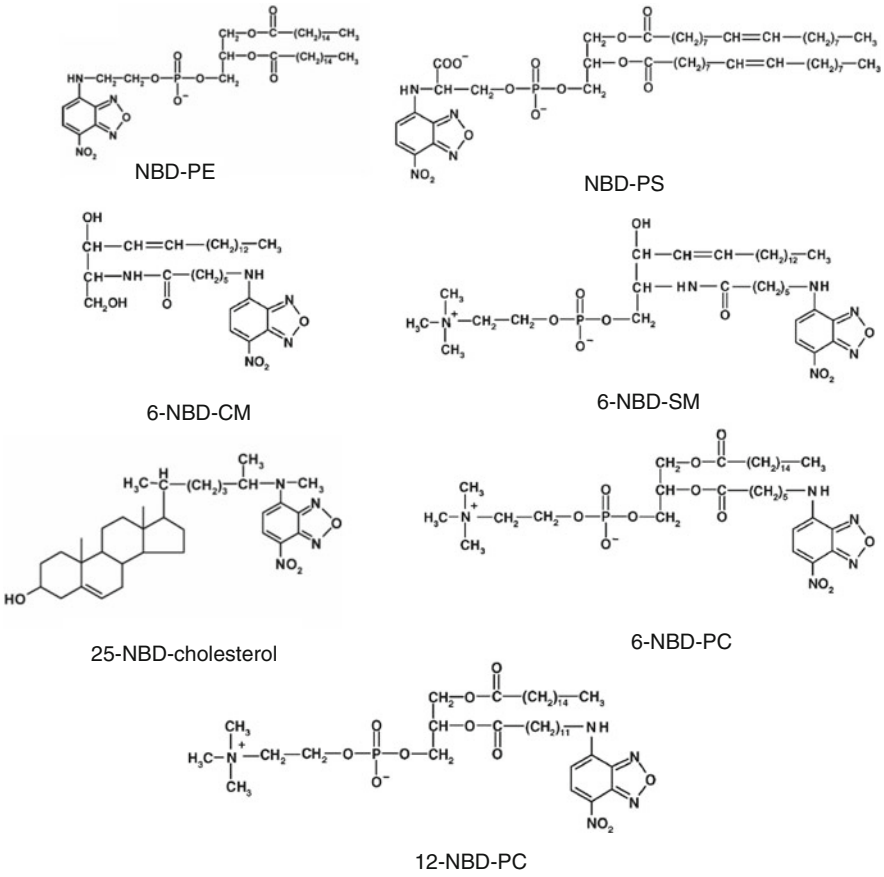


**Fig. 1** Chemical structures of representative NBD-labeled lipids. NBD-labeled lipids are extensively used as fluorescent analogs of natural lipids in membrane and cell biological studies. Depending on the specific lipid type, the NBD group could be covalently attached to the polar lipid headgroup (as in NBD-PE and NBD-PS) or to the *sn*-2 fatty acyl chain of the lipid (as in NBD-SM, NBD-CM, and NBD-PC). In case of 25-NBD-cholesterol, the NBD moiety is attached to the flexible acyl chain of the sterol

Lipids covalently linked to extrinsic fluorophores are commonly used for such studies. The advantage with this approach is that one has a choice of the fluorescent label to be used, and therefore, specific probes with appropriate characteristics can be designed for specific applications.

A widely used fluorophore in biophysical, biochemical, and cell biological studies of membranes is the NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl) group (for an earlier review on NBD-labeled lipids, see [8]). NBD-labeled lipids are extensively used as fluorescent analogs of native lipids in biological and model membranes to monitor a variety of processes (see Fig. 1). This is due to the fact that the NBD group possesses some of the most desirable properties to serve as an excellent probe for both

spectroscopic and microscopic applications [9]. For example, the NBD group is very weakly fluorescent in water. Yet, it fluoresces brightly in the visible range and exhibits a high degree of environmental sensitivity upon transfer to a hydrophobic medium [9–13]. Fluorescence lifetime of the NBD group exhibits sensitivity to environmental polarity [12, 14]. Lipids labeled with the NBD group have been shown to mimic endogenous lipids in a number of studies [15–18] although this appears to be not always true [19, 20].

In this chapter, we will focus on the application of NBD-labeled lipids in membrane and cell biology with representative examples. This chapter is not meant to be an exhaustive account of the literature on NBD-labeled lipids. Rather, we intend to provide the biophysical basis underlying specific applications. The reader is referred to a previous review [8] for earlier references and applications.

## 2 NBD Group Senses Slow Solvent Relaxation in Membranes

It has long been recognized that organized molecular assemblies (such as membranes) may be considered as large cooperative units with properties very different from the individual structural components that constitute them. An obvious consequence of such type of organization is the restriction imposed on the dynamics of their constituent structural components. Interestingly, this kind of restriction (confinement) results in coupling the motion of solvent molecules with the slow-moving molecules in the host assembly [21]. In this scenario, red edge excitation shift (REES) represents an interesting approach that relies on slow solvent reorientation in the excited state of a fluorophore which can be used to monitor the environment and dynamics around it in an organized molecular assembly [22–25]. A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of absorption band, is termed red edge excitation shift (REES). REES arises from relatively slow rates (relative to fluorescence lifetime) of solvent relaxation (reorientation) around an excited-state fluorophore. REES therefore depends on the environment-induced motional restriction imposed on solvent molecules in the immediate proximity of the fluorophore. It allows to assess the rotational mobility of the environment itself (which is represented by the relaxing solvent molecules) utilizing the fluorophore merely as a reporter group (the definition of solvent in this context is rather pragmatic; solvent relaxation dynamics includes dynamics of restricted solvent [water] as well as the dynamics of the host dipolar matrix such as the peptide backbone in proteins [26]).

As mentioned above, an obvious consequence of high degree of organization in supramolecular assemblies such as membranes is the restriction imposed on the mobility of the constituent structural components. The biological membrane, with its viscous interior and distinct motional gradient along its vertical axis, therefore represents an ideal system for the application of REES to explore membrane phenomena ([22, 24]; see Fig. 2). The interfacial region in membranes is

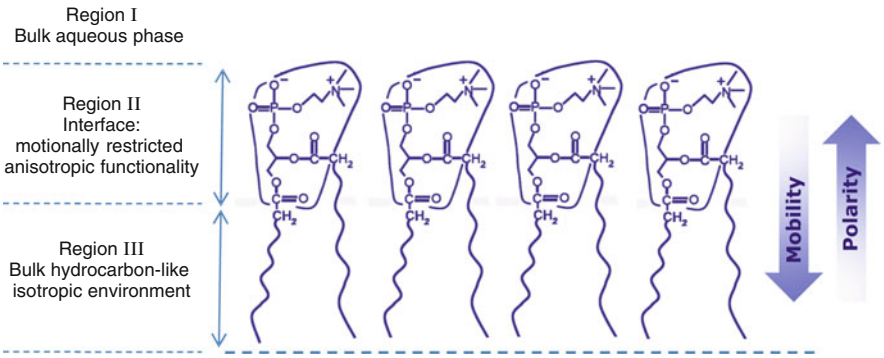


**Fig. 2** Schematic representation of half of the membrane bilayer showing the asymmetric nature of membranes in terms of anisotropy in polarity and dynamics along the monolayer. The dotted line at the bottom indicates the center of the bilayer. The membrane anisotropy along the z-axis (perpendicular to the plane of the membrane) compartmentalizes the membrane leaflet into three regions exhibiting differential dynamics. Region I comprises of bulk aqueous phase characterized by fast solvent relaxation; region II is the membrane interface, characterized by slow (restricted) solvent relaxation, and water penetration (interfacial water). This region is highly heterogeneous in chemical composition; region III represents the bulk hydrocarbon-like environment, isotropic in nature, and characterized by fast solvent relaxation. In addition, a polarity (dielectric) gradient along the z-axis is also an integral feature of membranes (see [27]). Fluorescent probes and peptides localized in the membrane interface (region II) are sensitive to REES measurements (Adapted and modified from Haldar et al. [24])

characterized by unique motional and dielectric characteristics different from the bulk aqueous phase and the more isotropic hydrocarbon-like deeper regions of the membrane. The membrane interfacial region exhibits slow rates of solvent relaxation and is therefore most likely to display the REES effect. In order to explore such effect, it is necessary to choose an appropriate probe that displays suitable properties in terms of localization, polarity, and appreciable change in dipole moment upon excitation [22, 24]. The NBD group in membrane-bound NBD-PE was found to satisfy these criteria [28]. The fluorescent NBD label is covalently attached to the headgroup of a phosphatidylethanolamine molecule in NBD-PE (see Fig. 1). The orientation and location of the NBD group in membrane-bound NBD-PE has been worked out [10, 29–33]. The NBD group in NBD-PE was found to be localized at the membrane interface characterized by unique motional and dielectric properties and therefore represents an ideal probe for monitoring REES and related effects. Interestingly, the NBD group exhibits a relatively large change in dipole moment upon excitation (~4 D; [13]), a necessary condition for a fluorophore to exhibit REES [24]. The change in emission maximum with changing excitation wavelength (REES) of NBD-PE in dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) membranes is shown in Fig. 3 [28, 32, 34]. Since the localization of the fluorescent NBD group in membrane-bound NBD-PE is interfacial [10, 29–33], these REES results imply that the interfacial region of the membrane offers considerable restriction to the reorientational motion of the solvent dipoles around the excited-state NBD group. It was later shown that

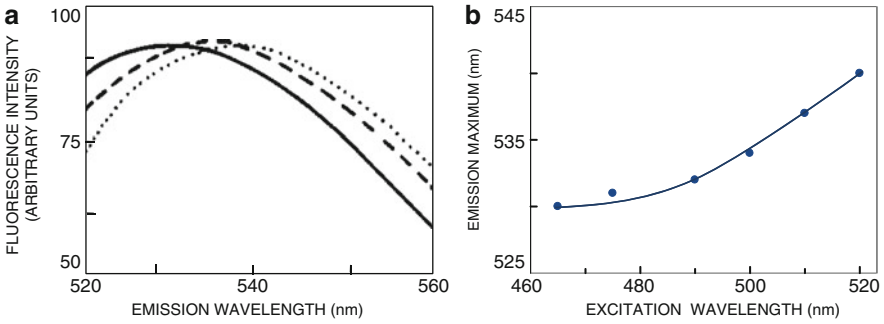


**Fig. 3** NBD-PE displays REES in membranes: (**a**) typical intensity-normalized fluorescence emission spectra of NBD-PE at increasing excitation wavelengths. Excitation wavelengths used were 465 (——), 500 (— —), and 510 (------) nm. (**b**) The effect of changing excitation wavelength on the wavelength of maximum emission (REES) of NBD-PE (Adapted and modified from Chattopadhyay and Mukherjee [28])

NBD-PE exhibits REES in membrane-mimetic assemblies such as micelles and reverse micelles [9, 14, 35]. In addition, REES exhibited by the NBD group labeled in a site-specific manner in the membrane-active peptide melittin provided novel information regarding the orientation of the peptide in the membrane [36].

## 3 Membrane Phase Dependence of Probe Looping in Acyl Chain-Labeled NBD Lipids

An important aspect of fluorescent membrane probes is their location in the membrane [37]. In the case of NBD-labeled lipids, it has been previously shown that the NBD group of acyl chain-labeled NBD lipids such as 6- and 12-NBD-PC (Fig. 1) loops up to the membrane interface in fluid-phase membranes due to the polarity of the NBD group ([10, 30, 32, 38–40]; see Table 1 and Fig. 4). This is also consistent with the observation that the NBD group in 6- and 12-NBD-PC exhibits considerable REES in fluid-phase membrane bilayers [32] and in monolayers at the air/water interface [42] since display of REES is characteristic of interfacial probe localization. The looping up of acyl chain-labeled NBD group could be due to hydrogen bonding of the NBD group at the membrane interface. The polar imino group and the oxygen atoms of the NBD group may form hydrogen bonds with the lipid carbonyls, interfacial water molecules, and the lipid headgroup. An important consequence of the looping up of the NBD group is an increase in the headgroup area. For example, it has been estimated that in POPC membranes, looping up of the NBD group results in a ~3% increase in the headgroup area [38]. It is for this reason that the looping up tendency of the NBD group in NBD-labeled lipids has been implicated in their preferred endocytic sorting [43]. The looping up of the NBD group in acyl chain-labeled NBD lipids has been utilized to monitor lipid-protein interactions in membranes [44].

**Table 1** Membrane penetration depths of the NBD group in NBD-labeled lipids by the parallax method[a]

| NBD-labeled lipids | Distance from the center of the bilayer $z_{cF}$ (Å) |
|---|---|
| NBD-PE | 20.3 |
| 6-NBD-PC | 20.7 |
| 12-NBD-PC | 20.7 |
| 6-NBD-CM | 20.8 |
| 6-NBD-SM | 20.5 |
| NBD-PS (pH 7.2) | 18.8 |
| NBD-PS (pH 5.0) | 14.1 |
| 25-NBD-cholesterol[b] | 5.7 |

[a]From Mukherjee et al. [32]
[b]From Chattopadhyay and London [30]

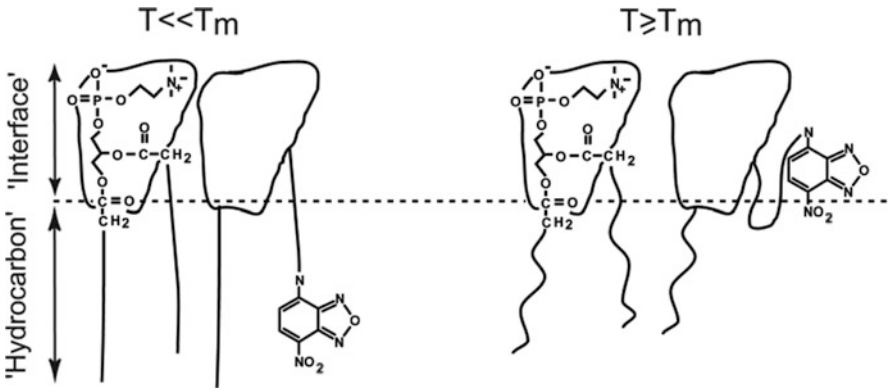


**Fig. 4** To loop up or not? A schematic representation of the acyl chain conformation in acyl chain-labeled NBD lipids below (*left*) and above (*right*) phase transition temperature of the membrane. The NBD group loops up to the membrane interface in fluid-phase ($T \geq T_m$) membranes [10, 30]. Interestingly, the looping up of the NBD group is found to be absent in gel-phase ($T << T_m$) membranes [41] (Reproduced from Raghuraman et al. [41])

Interestingly, looping up of the NBD group is critically dependent on the phase state of the membrane. In contrast to the looping up of the NBD group observed in fluid-phase (i.e., above the phase transition temperature) membranes, there appears to be a vertical distribution of the NBD group in acyl chain-labeled NBD lipids in gel (ordered) phase membranes, thereby showing that looping up of the probe is not observed under these conditions ([41]; see Fig. 4). This has been attributed to change in membrane packing induced by phase transition which could influence probe localization in the membrane.

## 4 Application of NBD Fluorescence Sensitivity in Cell Biology

An interesting feature of NBD fluorescence is its sensitivity in response to the environment in which the fluorophore is placed. The NBD group exhibits a high degree of environmental sensitivity [9–13], and fluorescence lifetime of the NBD group displays remarkable sensitivity to environmental polarity [12, 14]. For example, NBD lifetime in hydrophobic media such as membranes is high (~7 ns; [28, 32]), while NBD lifetime is considerably reduced in presence of water [9, 11, 12]. NBD lifetime reduces to ~ 1 ns in water which has been attributed to hydrogen bonding interactions between the fluorophore and the solvent [12] that is accompanied by an increase in the rate of nonradiative decay [45]. This aspect of NBD fluorescence has been effectively utilized in a number of cell biological applications. Environmental (polarity) sensitivity of NBD lifetimes was elegantly used to address the issue of movement of the signal sequence through the ribosomal tunnel during translocation of a nascent secretory protein across the endoplasmic reticulum membrane [46]. A careful analysis of lifetimes of NBD probes attached to the signal sequence of fully assembled ribosome-nascent chain-membrane complex showed that the probes displayed lifetimes corresponding to an aqueous environment (short lifetime ~ 1 ns). Based on these results, it was concluded that the signal sequence does not insert into the nonpolar core of the endoplasmic reticulum membrane. Instead, the signal sequence is localized in an aqueous environment during the early stages of the translocation process. A similar study, utilizing polarity dependence of NBD lifetimes, revealed a novel mechanism of membrane insertion for cholesterol-dependent cytolysins [47]. A rather interesting application of temperature sensitivity of NBD lifetime is the measurement of temperature in living cells as an 'optical thermometer' [48]. Another important and widely used application of NBD-labeled lipids is to monitor membrane asymmetry by chemically modifying (reducing) the NBD group with the water-soluble reducing agent dithionite [49].

## 5 Transbilayer Organization of Cholesterol Monitored Using NBD Fluorescence

Although a large body of literature exists on the organization of cholesterol in plasma membranes (with high cholesterol content, typically ~30–50 mol%), very little is known about its organization in the membrane where cholesterol content is very low (< 5 mol%). Membranes from the endoplasmic reticulum (where cholesterol is synthesized) and mitochondria are characterized with low cholesterol content. Interestingly, evidence for specific organization of cholesterol molecules in membranes at low concentrations came from studies carried out using 25-NBD-cholesterol (see Fig. 1; [50–53]). The aggregation-sensitive fluorescence of the NBD group in 25-NBD-cholesterol was elegantly utilized by Mukherjee and Chattopadhyay [51] to explore the local organization of cholesterol at low

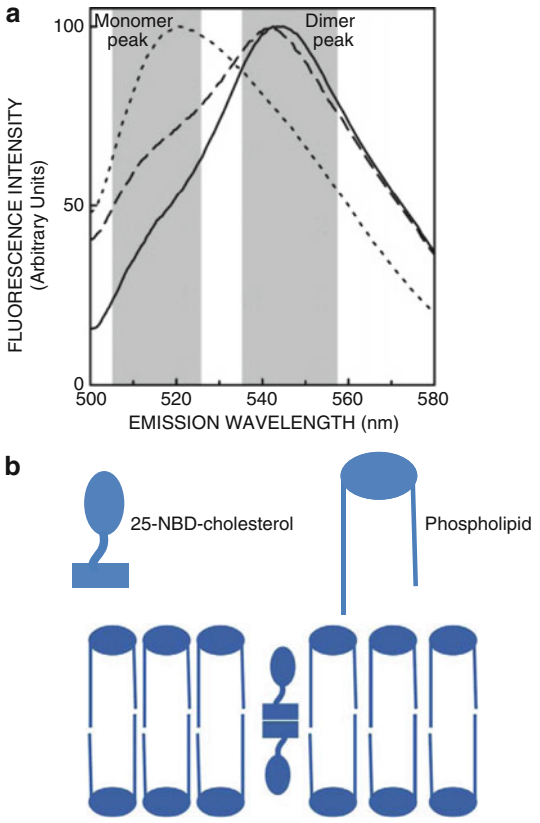


**Fig. 5** (**a**) Concentration-dependent emission spectral features of 25-NBD-cholesterol: a red shift in fluorescence emission maximum is observed with increasing concentration. Fluorescence emission spectra of 25-NBD-cholesterol in gel-phase DPPC vesicles are shown. The concentration of 25-NBD-cholesterol was 0.1 mol (------), 0.5 mol (— —), and 1 mol% (———). The shaded portions of the spectra represent the two wavelength ranges (505–526 and 537–558 nm) from which fluorescence emission of 25-NBD-cholesterol was collected for FRAP measurements described in Fig. 6. More details are in [54]. (**b**) Schematic diagram of the membrane bilayer depicting the transbilayer tail-to-tail dimers of cholesterol in membranes at low concentrations (Adapted and modified from Pucadyil et al. [54])

concentrations in membranes. By careful analysis of the emission spectral features of 25-NBD-cholesterol in DPPC membranes at the concentration range of 0.1–5 mol%, the possible presence of transbilayer tail-to-tail dimers of cholesterol in such membranes was detected both in gel- and fluid-phase membranes (see Fig. 5; [51]). It was further shown by monitoring corresponding changes in the absorption spectrum that the cholesterol dimers represented the formation of a ground-state complex (rather than an excited-state interaction). The possibility that the unique spectral feature was due to nonspecific aggregation of the NBD group was ruled out by careful control experiments. This implies that these results

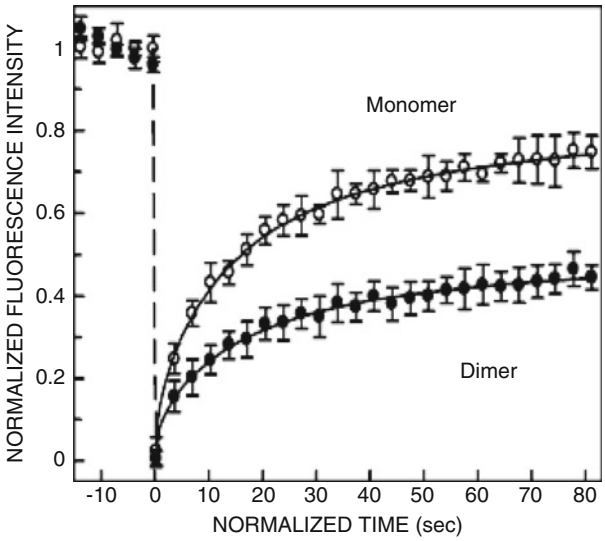


**Fig. 6** Simultaneous fluorescence recovery after photobleaching (FRAP) measurement of monomeric and dimeric populations of 25-NBD-cholesterol by the wavelength-selective FRAP approach. The figure shows the wavelength-selective FRAP of 25-NBD-cholesterol in DPPC vesicles where fluorescence emission was collected from 505 to 526 nm (○), corresponding to the monomeric population of 25-NBD-cholesterol, and 537–558 (•) nm corresponding to the dimeric 25-NBD-cholesterol population (see shaded portions in Fig. 5). Note that dimeric 25-NBD-cholesterol exhibits slow diffusion relative to monomeric 25-NBD-cholesterol. Other details are in [54] (Adapted and modified from Pucadyil et al. [54])

provide novel information about cholesterol dimerization in membranes at low concentrations, rather than providing information on NBD-NBD interactions. These results were further supported by observations from other laboratories [55]. In addition, from the distinct spectral feature of 25-NBD-cholesterol in membranes of varying curvature, it was shown that the transbilayer dimer arrangement is sensitive to membrane curvature, and dimerization is not favored in highly curved membranes [53]. The organization and dynamics of cholesterol monomers and dimers were explored by REES [52]. The environment around the cholesterol dimer appears to be rigid (relative to the monomer environment) and offer more restriction to solvent reorientation.

By the application of a novel version of fluorescence recovery after photobleaching (FRAP) measurements 'wavelength-selective FRAP', lateral diffusion coefficients of dimeric and monomeric populations of cholesterol were estimated using 25-NBD-cholesterol (see Fig. 6; [54]). In these experiments, wavelength-selective FRAP measurements were carried out in DPPC membranes containing 25-NBD-cholesterol. The diffusion characteristics of the transbilayer dimer and monomer of 25-NBD-cholesterol (evident from spectral features; see Fig. 5a) were derived by analysis of FRAP results after photoselecting a given population by use of specific wavelength-range characteristic of that population.

The results showed that the organization of 25-NBD-cholesterol in DPPC membranes is heterogeneous, with the presence of fast- and slow-diffusing species. The presence of fast- and slow-diffusing populations of 25-NBD-cholesterol was interpreted to correspond to predominant populations of cholesterol monomers and dimers.

## 6 Conclusion and Future Perspectives

The fluorescent NBD group has come a long way in terms of biological applications since its discovery a few decades back [56]. NBD-labeled lipids were first synthesized, and their sensitive fluorescence was noted in the late 1970s [57, 58]. Since then, these lipids have been used in a number of biophysical and cell biological studies to gain a variety of information. The field of fluorescently labeled lipids has grown over the years with the introduction of new fluorescent labels with desirable properties [59]. Although NBD-labeled lipids have been shown to mimic endogenous lipids in a number of studies [15–18], concerns have been raised in some cases [19, 20]. Photostability of the NBD group could also be a concern although this can be handled by using low light intensity level and other techniques [60, 61]. Nonetheless, NBD-labeled lipids continue to be widely used for various biological applications. Future exciting applications could include simultaneous attachment of the NBD group and nitroxide group in an amphiphilic molecule to explore membrane heterogeneity [62] and monitoring amyloid fibril formation utilizing NBD-labeled lipids [63].

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