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Analyzing Microdomains in Biological Membranes Using Fluorescence Techniques

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It is becoming increasingly clear that various lipids and proteins in the plasma membrane are not distributed homogeneously but are organized in compositionally and functionally variable "microdomains." Fluorescence techniques have contributed significantly to our understanding of membrane structure and function. Here we review recent studies using fluorescence methods to detect membrane domains in intact cells. We also discuss the current limitations in the available techniques and the models used to interpret experimental data.

KEY WORDS: Microdomains; plasma membrane; lipid rafts.

INTRODUCTION

Since Singer and Nicholson presented their fluid mosaic model in 1972 [1], our view of biological membranes as two-dimensional fluids in which molecules are free to diffuse laterally has been altered by the demonstration of various kinds of lateral inhomogeneities in cell membranes. Membrane domains in different cell types have been described and discussed in several earlier reviews [2–9].

One type of lateral inhomogeneities includes regions of the plasma membrane enriched in specific lipids, proteins, and cholesterol, termed lipid rafts [10]. The raft hypothesis was formulated, in part, to explain the apical sorting of certain lipids and glycosylphosphatidylinositol (GPI)-anchored proteins [10,11]. The preferential packing of sphingolipids into moving platforms was initially proposed to be responsible for their apical delivery [9,12]. The involvement of cholesterol in forming apical transport vesicles was characterized and appreciated later [10]. According to this model, glycosphingolipids (GSLs) and cholesterol segregate from bulk lipids within the trans-Golgi network, forming lipid rafts that associate with a specific set of proteins and are subsequently incorporated into apical transport vesicles. The rafts are thought to form by self-association of sphingolipids via their long saturated acyl chains, with cholesterol molecules spaced between the acyl chains, and are further stabilized by hydrogen bonding between the GSL headgroups (reviewed in Ref. 13).

The term "raft" is now adapted to refer to other membrane microdomains, including those in the plasma membrane [14]. The plasma membrane of mammalian cells contains lipids with a preference for fluid domains (such as glycerophospholipids with unsaturated tails), lipids with a preference for ordered domains (e.g., most sphingolipids), and cholesterol, an essential component having major effects on the physical properties of the membrane [15].

Lipid rafts were first isolated and now often defined operationally by their insolubility in cold, nonionic detergents [16]. Due to their high lipid content, detergent-resistant membrane (DRM) fractions float in a low-density fraction after density gradient centrifugation. There-

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fore, the most frequently used method to identify these lipid microdomains and their protein contents is to determine whether they cosediment to the low-density fraction on sucrose density gradients following extraction by certain nonionic detergents. However, isolation techniques that do not depend on detergent treatment have been developed [17,18], and this confirms that the formation of these domains does not depend on detergent treatment. Nevertheless, detergents may alter the properties of the DRMs.

Using model membranes with compositions similar to DRMs isolated from cells, Schroeder *et al.* [19] showed that detergent resistance correlates with the presence of the liquid-ordered (l_o) phase. In the l_o phase, the acyl chains of the lipid components adopt a gel phase-like ordering, yet the lipids retain substantial lateral and rotational mobility, similar to lipids in the liquid crystalline phase (reviewed in Refs. 20 and 21). DRMs are enriched in GSLs, sphingomyelin, cholesterol, and phospholipids with long, saturated acyl chains. The DRMs also contain a high content of GPI-anchored proteins, certain transmembrane proteins, nonreceptor tyrosine kinases, G-protein subunits, and several transporters [10,20,22], many of which are doubly lipidated with saturated acyl chains. Because of their properties and compositions, the lipid domains are also given names such as detergent-insoluble glycolipid-enriched membrane domains (DIGs), Triton-insoluble floating fractions (TIFFs), and GSL-enriched membranes (GEMs) [21].

The plasma membrane of cells contains several specializations, including caveolae, coated pits, microvilli, ruffles, adhesion plaques, and tight junctions. Most of these have been defined based on their protein content. These membrane specializations also may contain distinct lipid compositions. For instance, caveolae are enriched in cholesterol and sphingolipids (reviewed in Refs. 23–25). This review focuses mainly on lateral lipid domains in the plasma membrane referred to as rafts or DRMs, which include caveolae as well as membrane regions that are not caveolae. We discuss recent studies using fluorescence methods for detecting membrane domains in cells. These studies have contributed to our current understanding of how the plasma membrane is organized and how its organization regulates cellular signaling processes.

FLUORESCENCE TECHNIQUES

Fluorescence Microscopy

Membrane rafts are not usually directly observable in cultured cells by optical microscopic techniques. Proteins and lipids expected to have a high affinity for the detergent-resistant domains typically show a uniform distribution in the plasma membranes [26,27]. However, in some circumstances clustering of some of these membrane components can lead to visible domain formation. Baird and colleagues have shown by fluorescence microscopy that the DRM markers $DiIC_{16}$ (a lipid analogue), Thy-1 (a GPI-anchored protein), and GD_{1b} (a ganglioside) coredistribute with the cross-linked immunoglobulin E (IgE) receptor (FcεRI) in intact RBL cells [28,29]. In another study, FcεRI clusters were colocalized with clustered ganglioside GM1 [30]. Similarly, when two GPI-anchored proteins were independently cross-linked with antibodies, there was a significant degree of colocalization of the small clusters of the two proteins [26]. This presumably reflects the coalescence of small rafts into larger domains that can be readily visualized, but clustering of the non-cross-linked GPI-anchored proteins could not be seen, indicating that any such clusters were below the resolution limits of optical microscopy.

Using immunofluorescence microscopy, Harder *et al.* [31] compared the patching behavior of pairs of raft markers with pairs of raft/nonraft markers cross-linked with antibodies or toxins. They observed copatching of cross-linked GPI-anchored placental alkaline phosphatase (PLAP) with influenza HA, PLAP with ganglioside GM1, and GPI-anchored Thy-1 with GM1, all of which are known to associate with rafts. On the other hand, patches of nonraft transferrin receptor were sharply separated from patched raft components. The copatching was proposed to be a consequence of the coalescence of lipid microdomains containing the cross-linked raft components [31].

Where fluorescent lipid probes with different partition preferences for distinct lipid phases are doped into the plasma membrane at low molar ratios, they provide a convenient visual marker for coexisting phases present in the plasma membrane. The diversity of lipids found in biological membranes, however, makes the coexistence of different physical states difficult to detect. Experiments in unperturbed mammalian cells have not yielded direct observation of separate domains, leading to the conclusion that domains must be small compared to optical resolution limits ($<$250 nm).

Triton X-100 (TX-100) insolubility is often a useful starting point for the biochemical analysis of raft domains. Typically, only a small fraction of membrane proteins is found in the low-density, TX-100-insoluble fractions. This indicates that most membrane proteins are not in DRMs, and this has led to a widespread impression that DRMs are a minor component of the plasma membrane. However, if one examines the lipid constituents of the

plasma membrane by fluorescence microscopy, a high fraction of the surface area of the cell is not extracted by cold TX-100. For example, when GPI-anchored folate receptors were fluorescently labeled under non-cross-linking conditions, about 70% of the cell surface area remained labeled after exposure to cold TX-100 [32]. In a recent study (M. Hao, S. Mukherjee, and F. R. Maxfield, submitted), it was similarly observed that $DiIC_{16}(3)$ (an order-preferring lipid) covered roughly 70% of the plasma membrane after cold TX-100 treatment. Observation of the same cells before and after cold TX-100 treatment shows that lipid components are rearranged during the process of extraction, and it has also been shown that the self-association of GPI-anchored proteins is somewhat perturbed by cold TX-100 treatment [32]. Furthermore, proteins from different organelles can become mixed upon detergent extraction, homogenization, and centrifugation. Nevertheless, there is a high degree of correlation between protein incorporation into DRMs and the incorporation into low-density membranes in the absence of detergent treatment [17,18], suggesting that the components left after cold TX-100 treatment are reflective of membrane organization before extraction.

One reason for the high interest in membrane domains is the importance of lateral organization in the plasma membrane for organizing complex activities such as signal transduction [33]. Domain formation plays an essential role in the initiation of high-affinity FcεRI-medicated signaling (reviewed in Refs. 34 and 35). Cholesterol depletion selectively disrupts the structural interactions between aggregated FcεRI and Lyn in intact cells [36]. These results and the fact that only the FcεRI receptor that is recruited to DRMs is phosphorylated by Lyn [37] suggest a strong link between domain formation and initiation of signal transduction. Green fluorescent protein (GFP) constructs are also widely used to look at the distribution of raft-associated signaling molecules. In double-staining experiments, GFP-tagged SH2 domains of tyrosine kinase Syk and phospholipase C (PLC-γl) localize almost exclusively to GSL-rich microdomains labeled with Cy3-cholera toxin B [30]. Similar results were obtained with GFP-Lck and GFP-tagged SH2 domains of ZAP-70, suggesting that these signaling molecules are recruited to distinct plasma membrane microdomains [38].

In the unperturbed cell, separation of lipid phases is not typically observed by optical microscopy. However, various perturbations such as activation of signal transduction cascades by receptor cross-linking and initiation of phagocytosis can lead to the formation of micron-scale domains that are easily observable. We have also observed that cholesterol depletion can lead to the formation of micron-scale domains in several cell types (M. Hao, S. Mukherjee, and E. R. Maxfield, submitted). These results suggest that in the resting state the plasma membrane lipids are poised at a composition such that relatively small changes can lead to large changes in lipid properties.

Near-Field Scanning Optical Microscopy

The inability to observe membrane microdomains directly under most conditions suggests that they are smaller than the resolution limit of conventional optical microscopy. Thus, techniques that provide a higher spatial resolution should be useful in analyzing such domains. Several high-resolution scanning techniques have been successfully implemented on model membranes to observe submicron domain structures directly. These include methods such as atomic force microscopy (AFM) (reviewed in Refs. 39–41) and near-field scanning optical microscopy (NSOM) [42,43]. However, the complex surface topography encountered in living cells presents difficulties for AFM and NSOM measurements.

NSOM uses the near-field interaction of light from a sharp fiber-optic probe with a sample of interest to image cell surfaces [44,45]. A feedback mechanism implemented to control the tip–sample distance during imaging generates a simultaneous force image of the surface topography [44]. As a result, one major advantage of NSOM is the simultaneous collection of fluorescence and topographic information. Images of fibroblasts labeled with a fluorescent lipid analogue showed a patchy distribution in the plasma membrane of fixed, dried cells [46]. The sizes of these patches (tens to hundreds of nanometers) are consistent with those calculated from fluorescence recovery after photobleaching experiments [46].

Fluorescence Recovery After Photobleaching

Fluorescence recovery after photobleaching (FRAP) has been used extensively to measure the lateral mobility of fluorescently labeled membrane constituents. This technique has the advantage of being noninvasive and applicable to individual living cells [47]. In this method, a short pulse of intense laser light photobleaches the fluorophores in a micrometer-sized spot [48]. The fluorophores from the surrounding area move into the irradiated spot by diffusion or flow. Two parameters are obtained: D, the lateral diffusion coefficient, and M, the mobile fraction of the diffusing species [48]. Partial fluorescence recovery ($M < 100\%$) indicates the existence of regions

at the plasma membrane where lipid and protein molecules are immobile.

FRAP experiments showed that several membrane proteins, including the GPI-anchored protein Thy-1 [49], the class I major histocompatibility complex molecules H-2D^b and Qa-2 [50], and neural cell adhesion molecules [51], exhibited lateral diffusion coefficients (D) similar to those of lipids (10^{-9}–10^{-8} cm^2/s). However, a significant fraction (up to 50%) of the lipid-linked proteins was immobile [49,50,52]. The data suggest that the basis for these membrane domains lies in the inner leaflet of the bilayer or in the cell cytoplasm. Meshes or patches formed by the cytoskeleton could organize the cell surfaces into domains [50].

Single-Particle Tracking

Unlike FRAP experiments in which information is obtained from averaging the movement of hundreds of molecules, the trajectory of a membrane lipid or protein can be followed with nanometer precision using the single-particle tracking (SPT) method [53–55]. Membrane components are labeled with either fluorescent or gold particles and imaged by low-light-level fluorescence microscopy or differential interference contrast microscopy, respectively. Comparison with FRAP performed under identical experimental conditions is possible using fluorescence SPT [56].

To explore the cause for the substantial immobile fractions observed in the FRAP measurements for GPI-anchored proteins, SPT was used to follow the movements of Thy-1, a GPI-anchored protein, GM1, and ganglioside, and fluorescein phosphatidylethanolamine (fl-PE), a phospholipid analogue, on cell surfaces of fibroblasts [57]. A significant fraction (35–40%) of Thy-1 and GM1 was found to be transiently confined, for 7–9 s, to lateral domains that are 260–370 nm in diameter. In contrast, a smaller fraction (16%) of fl-PE was confined for 6 s to much smaller regions (225 nm), which was suggested to be due to interactions between the saturated acyl chains of fl-PE and other domain components. A reduced fraction of Thy-1 experienced smaller confining domains in GSL-depleted cells, and detergent extraction left the confining regions essentially unchanged. These results are consistent with preferential association of GPI-anchored proteins with GSL-enriched domains within DRMs.

Experiments performed using the optical laser trap give further description of the obstacles a membrane protein encounters. Trapped by a near-infrared laser beam, submicrometer beads attached to the molecules of interest can be dragged across the plasma membrane until they encounter a barrier which causes the beads to escape the trap [58,59]. SPT experiments coupled to the laser trap provided evidence that gave rise to the membrane skeleton fence model [60]. In another experiment using the laser trap, lipid rafts were estimated to be complexes 26 $\pm$ 13 nm in size diffusing as small entities in the plasma membrane [61].

Fluorescence Resonance Energy Transfer

Fluorescence resonance energy transfer (FRET) has been used to detect molecular clusters in the plasma membrane [62]. FRET is good for mapping molecular proximity and interactions at the cell surface because the rate of energy transfer is inversely proportional to the sixth power of the distance between the donor and the acceptor. In a system with two different fluorophores, the molecule with higher energy absorption (shorter wavelength) is defined as the donor and the one with lower energy (longer wavelength) absorption as the acceptor [62]. Because energy is transferred from the excited donor to the acceptor, the lifetime, quantum efficiency, or fluorescence intensity of the donor decreases when the acceptor is present. As a result, the fluorescence intensity of the acceptor increases if the donor is present. Experimental techniques used to measure FRET include quenching of the donor fluorescence, sensitized acceptor emission, reduced donor lifetime, and decreased rate of donor photobleaching (reviewed in Refs. 63–65). FRET can be measured in a spectrofluorometer, a flow cytometer, or a fluorescence microscope. Since FRET occurs over distance of 1–10 nm, it measures molecular associations at a length scale that is more than adequate to detect rafts [66].

Imaging FRET combines digital fluorescence microscopy with FRET, thus increasing the resolution of conventional fluorescence microscopy to the molecular scale [67]. Imaging FRET is performed on a cell-by-cell basis, making it substantially advanced over the conventional FRET, which measures the average FRET for a cell population [67,68]. With this method, distribution of a GPI-anchored protein was examined at a resolution of $<$100 Å. Consistent with the results from conventional FRET [68] and fluorescence microscopy [26], most molecules of a GPI-anchored protein show a random distribution at the cell surface [67].

FRET Measured by Fluorescence Anisotropy

Fluorescence anisotropy measurements offer a sensitive assay for FRET between like fluorophores. The method depends on the overlap between the excitation

and the emission profiles for a single fluorophore. The nonradiative energy transfer between molecules reduces the fluorescence anisotropy compared to individual, isolated molecules. In an application of this method, the extent of energy transfer was measured in terms of the dependence of fluorescence polarization on fluorophore densities in membranes [69]. It was hypothesized that if proteins are clustered in submicron-sized "rafts" at the cell surface, the distance between fluorophores might be defined by the properties of a raft and would be somewhat independent of the fluorophore density. In this case, anisotropy values of pixels differing in fluorescence intensity would be independent of fluorophore density. On the other hand, if there were a random distribution of fluorophores, this would result in decreased anisotropy values for pixels containing a higher fluorescence intensity because the distance between individual labeled molecules would increase as the fluorophore density decreased.

Varma and Mayor [69] labeled GPI-anchored proteins with non-cross-linking fluorescein labels, and they observed a relatively constant anisotropy value over a range of fluorescence intensities. This suggested that the fluorophores are in small clusters rather than a random distribution. It was proposed that these domains are smaller than 70 nm and contain fewer than 50 molecules of the GPI-anchored protein. This inference was made based on the maximum distance between individual fluorophores ($\leq$10 nm)and the fact that the extent of depolarization in a pixel (1 μm) is independent of fluorescence intensity over a range (200-fold) of values. Consistent with a disruption of domain organization, cholesterol depletion led to an increase in anisotropy that suggested an increase in the average distance between molecules. The addition of cholesterol to depleted cells restored the organization of the GPI-anchored protein in domains.

Fluorescence Excitation Ratio Imaging

In fluorescence excitation ratio imaging, the sample is sequentially excited by two wavelengths and fluorescence intensities are collected. The quantification of fluorescence as a ratio has the advantage of isolating the variable of interest by providing an internal control for many of the extraneous parameters. For example, BCECF fluorescence is used to measure cytosolic pH because while the fluorescence of BCECF excited by both 490-nm and 450-nm illumination is sensitive to all of the parameters in a given microscopic system, only the fluorescence excited at 490-nm illumination is sensitive to pH. By calculating the ratio of one to the other, one can obtain information that is sensitive to pH but largely insensitive to other aspects of the system [65,70].

Proximity imaging (PRIM) provides a means for imaging proximity relationships between different green fluorescent protein (GFP)-tagged proteins. PRIM exploits the fact that changes in the ratio of fluorescence emitted when excited at two wavelengths ($R_{395/475}$) take place if two GFP molecules come into physical contact [71]. PRIM complements FRET. Changes in $R_{395/475}$ upon dimerization are caused by direct but distinct structural perturbations occurring when different interfaces of two GFP molecules are brought into proximity. When PRIM was used on GPI-anchored GFP, a small but significant difference in $R_{395/475}$ was observed between GPI-anchored GFP and GFP expressed in the cytoplasm. The difference was then confirmed to be GPI anchored specific, suggesting GPI anchor-mediated clustering in the plasma membrane.

Fluorescence Correlation Spectroscopy

Fluorescence correlation spectroscopy (FCS) extracts molecular information from fluorescence fluctuations by measuring how long a fluctuation persists in time, which allows differentiation between fast and slow processes [72]. The normalized fluorescence fluctuation autocorrelation function corresponds to the probability for a fluorescent particle that is inside this volume at time t to be still inside it after a short interval, Δt [73]. FCS has been used to detect and characterize molecular clustering and dynamics on cell and model membranes [73–77]. Using FCS, the translational diffusion behavior of single lipid molecules in RBL cell membranes was found to be nonuniform, best described by models of diffusion with different coefficients in a possibly heterogenous molecular environment [73]. A variation of FCS was introduced for the analysis of aggregate formation and protein clustering in membranes [74]. Instead of correlation of temporal fluctuations, imaging FCS scans the sample through the exciting laser beam (or visa versa) and applies spatial autocorrelation of the pixel-to-pixel fluorescence fluctuations from a single fluorescence image. Using this method, it was shown that the nonuniform distribution of IgE was enhanced by unlabeled polycolonal anti-IgE and fluorescently labeled lipids became patchy only in the presence of IgE [74]. FCS measurements are performed in the 10^{-16}-liter volume range at nanomolar concentrations [75].

LIMITATIONS OF THE TECHNIQUES

Much of the evidence supporting the existence of microdomains is inferred from the behavior of membrane

probes that is not consistent with the properties of a continuous, homogeneous lipid bilayer [50]. All characterization and definitions of microdomains rely on the detection method used and are subject to technical limitations. Many of the results presented here are indirect and are therefore open to interpretation. Because the mechanisms of redistribution and insolubility of lipids and proteins upon detergent extraction are not yet completely understood, detergent insolubility can underestimate or overestimate raft formation [20,78]. Widely different detergent levels are often used, and this may reflect different types of domains or different degrees of association with domains [79]. DRMs do not represent the actual organization of rafts in cellular membranes, as GPI-anchored proteins were shown to redistribute to clusters upon extraction with TX-100 [32]. It is also shown by chemical cross-linking that detergent treatment substantially increases the size of raft complexes [80]. These results suggest that any interpretation of the function and organization of membrane components based on detergent extraction should be viewed with caution [32].

It is important to consider the relative temporal and spatial scales over which the lateral motion of molecules is measured in experiments such as FRAP and FRET. For example, the absence of FRET does not rule out the possibility that molecules form clusters, because, among other possibilities, the distance between the molecules in a cluster may be larger than can be detected by FRET [67,68]. Also, an immobile lipid fraction unable to be detected by FRAP due to a low temporal resolution does not necessarily mean the absence of lipid microdomains, as in the case of bacteriorhodopsin [47]. Bacteriorhodopsin is surrounded by about two layers of partly immobilized lipids in contact with freely diffusing lipids, as predicated by FRAP data. This means that exchange between perturbed and unperturbed lipids could occur within 0.5 ms, which is a very short time compared to the 10- to 15-s duration of a fluorescence recovery and the time required for exchange between perturbed and unperturbed lipids, thus explaining the absence of an immobile fraction [47]. Indeed, the definition of domains detected by many of our available methods is a rather static definition, limited by temporal resolution of the techniques.

As with all probe approaches, the validity of the fluorescence techniques relies on the ability of the probes to report faithfully on the host membrane components. Some striking differences between FRAP and SPT measurements are due to the effects of the label. Valency alters lateral diffusion because it can cross-link binding sites and affect both the cluster size and the distance between the lipids anchoring the label [81]. In the plasma membrane, the label could cause drag by interacting with the extracellular matrix or its binding could induce cytoskeleton association, thus markedly reducing diffusion [81]. Caution should also be taken when examining the distribution of proteins that are sensitive to labeling and fixation conditions. For example, a GPI-anchored folate receptor was reported to exist in clusters over caveolae [82], which were later revealed as an artifact induced by secondary antibody crosslinking [26]. Certain fixation conditions have also been suggested to disperse preexisting clusters of the folate receptor [83].

Despite the obvious usefulness of fluorescent lipid analogues, only a small number and variety are available for membrane study, thereby severely limiting our understanding of membrane structure from the lipid point of view. While the fluorescent lipid analogues are not necessarily mimics of specific lipids, they can trace the effects of different properties (length, saturation, shape, etc.). For example, lipid analogues with preferences for different phases could indicate the presence or absence of coexisting lipid phases [77], and lipid analogues with different shapes (e.g., cone, cylinder, inverted cone) could provide information about membrane curvature [84]. However, one should take caution when incorporating lipid analogue into cell membranes, since a concentration higher than a few mole percent of the natural lipids could interfere with the native membrane structure. A major difficulty that has prevented the wide application of fluorescent lipid analogues to cell membranes is the lack of methods for incorporation of highly insoluble lipids. Traditionally, fluorescent lipid analogues are first incorporated into liposomes and then delivered to the plasma membrane [85]. An alternate method uses fatty acid-free bovine serum albumin as carriers for the delivery of lipid analogues to the plasma membrane [84]. For lipids that are extremely insoluble in aqueous media, special soluble chelators have to be used as carriers. For example, dehydroergosterol, a fluorescent cholesterol analogue, is soluble when complexed with methyl-β-cyclodextrin and can be incorporated into the plasma membrane.

Other Important Aspects of Membrane Study

Comparisons of results obtained in living cells with domain studies using model membranes provide invaluable insight to domain properties and origins. The idea that domains resembling the l_o phase exist in biological membranes is well supported by comparisons with model membrane studies. Many excellent reviews are devoted to the formation of the l_o phase involving cholesterol and sphingolipids in model membranes and its relationship to the microdomains found in biological membranes

[13,20,21,33,78,86,87]. Several of these studies show that lateral phase separation can occur among different lipid molecules. For example, using two-colored fluorescent lipid analogues with differing order preferences, micron-scale domain separations have been observed directly by confocal microscopy in large unilamellar liposomes [77].

By no means are lipids the only source for domain formation. Studies show that a cytoskeletal meshwork closely placed at the cytoplasmic side of the plasma membrane may be responsible for the confinements of membrane-spanning proteins measured by FRAP, SPT, and laser trap experiments (for review see Refs. 48 and 88). Interactions between FcεRI and lipid raft components are also shown to be regulated by the actin cytoskeleton [89]. The two bases for the formation of membrane domains, namely, lipid segregation and cytoskeleton linkage, are not mutually exclusive. For instance, lipid domains created by lipid/lipid immiscibilities could result from the lateral pressure exerted by cytoskeleton proteins on the bilayer (reviewed in Ref. 88). Certain proteins, by virtue of their transmembrane domains, may preferentially associate with order- or disorder-preferring lipids [10]. At a sufficient density, these might create zones of one type of lipid phase that is poorly permeated by lipids preferring the other type of phase [86].

Vesicle traffic, responsible for the delivery and removal of membrane components, also plays a role in determining the persistence and relative concentration of a constant population of membrane patches [90]. While the average number and size of patches are maintained by vesicle traffic, individual patches are short-lived as a result of component molecules diffusing away from the site of vesicle delivery [90].

Revisiting the "Raft Model"

In looking to future contributions of fluorescence to understanding membrane domains, it is helpful to review our current state of knowledge and to ask whether fluorescent techniques have the potential to provide critical information. Compared with our knowledge of protein structure and function, our understanding of membrane organization and dynamics is remarkably primitive. Even issues such as whether the plasma membrane is mostly in a phase like l_o and whether such l_o membrane domains are small minor components (rafts) remain subject to discussion. Considering the high content of cholesterol and GSLs present [21], the plasma membrane is more likely to be in a liquid ordered phase with patches of fluid domains [91]. Part of the problem with current models may be the appealing simplicity of a binary partition that would be suggested by a simple version of a raft model. However, the presence of thousands of different lipid moieties and membrane proteins could impart a much more complex structure to the plasma membrane. Increasing fractions of lipids and proteins can be incorporated into DRMs by lowering the detergent concentrations used [79], suggesting that there may be more of a continuum of different types of membrane organizations rather than a small number of well-defined phases. Understanding such a diversity of lipid organizations will require increasingly sophisticated methods for analyzing the distributions of lipids and proteins in biological membranes.

Several of the methods described in this review can be used to obtain distance distributions between pairs of molecules, and this can provide information about nanometer-scale lipid organization. FRET between lipids and proteins may also be used to probe the lipid environment around GFP-labeled proteins. SPT measurements on single lipid molecules can describe the diffusional properties of molecules over space and time, and this can further refine our understanding of the size and persistence of lipid domains. FCS can also provide information on the number of fluorophores that move in a unit and their motional characteristics.

At distances of 10–200 nm, NSOM may provide the best opportunity for direct observation of small lipid domains in living cells. This method has been successfully applied to immobilized model membranes, but significant challenges remain in applying it to living cells.

Conventional optical microscopy (wide-field or confocal) can be useful for observing the formation of larger scale domains and for measuring properties of individual cells. For example, using fluorescent lipid probes with preferences for ordered domains ($DiIC_{18}$) and fluid domains (C_6-NBD-SM), segregation of micron-scale phases could be observed after cholesterol depletion. About 70% of the plasma membrane was covered by the ordered lipids (M. Hao, S. Mukherjee, and F. R. Maxfield, submitted). This is consistent with studies of cold TX-100 resistance which indicated that most of the plasma membrane area was resistant to extraction [32].

Studies regarding the distribution of GPI-anchored proteins at the cell surface reveal seemingly contradictory results. On one hand, clusters or microdomains consisting of 15–50 GPI-anchored protein molecules exist on a nanometer scale [69,80]; SPT and FRAP experiments also find a significant fraction of GPI-anchored proteins confined to lateral domains that are hundreds of nanometers in diameter [49,57]. On the other hand, a random and uniform distribution was shown by conventional FRET [68], high-resolution imaging FRET [67,92], and fluorescence microscopy [26,67].

It is interesting to consider a scenario in which a mixture of a large fraction of uniformly distributed and a small fraction of clustered GPI-anchored protein molecules spreads randomly over the cell surface [67]. In such a model, very small microdomains (nanometer-sized) with "raft"-like properties (i.e., ordered domains) would comprise the majority of the cell surface, making the plasma membrane mainly ordered. These ordered microdomains would not be continuous; rather, they would be separated by thin lines of fluid lipids. This implies that the membrane domains are not necessarily circular in shape, a concept discussed extensively in theoretical and model membrane studies (reviewed in Ref. 93). In this case, although the fluid phase is a minority of the plasma membrane, it is a percolating (or continuous) phase. Therefore, a molecule that partitions into the fluid phase is free to diffuse through the entire membrane, whereas raft components are confined to dispersed ordered domains. This would be consistent with results obtained from FRAP and SPT. It has even been proposed that ordered domains might be smaller than 70 nm in diameter and thus too small to be detected by FRET and fluorescence microscopy methods [92]. In this case, coalescence of such components into larger complexes might occur in response to cellular signaling.

SUMMARY

Modern fluorescence techniques have provided important insights to the existence of membrane inhomogeneities. In this review, we have focused on the use of fluorescence techniques in detecting a subset of membrane domains, i.e., specialized regions in the plasma membrane that are resistant to solubilization in nonionic detergents, termed DRMs. Despite extensive studies on the structure of biological membranes during the last decade, we still do not have concrete information on the nature of membrane domains. These domains seem to be small and dynamic structures that are aggregated and stabilized by detergent extraction, antibody-induced cross-linking, or cholesterol modulation [92]. New methods are needed for a more accurate estimate of the size, composition, and dynamics of these rafts.

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