

DETECTING MICRODOMAINS IN INTACT CELL MEMBRANES

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■ **Abstract** Current models for cellular plasma membranes focus on spatial heterogeneity and how this heterogeneity relates to cell function. In particular, putative lipid raft membrane domains have been postulated to exist based in large part on the results that a significant fraction of the membrane is detergent insoluble and that molecules facilitating key membrane processes like signal transduction are often found in the detergent-resistant membrane fraction. Yet, the *in vivo* existence of lipid rafts remains extremely controversial because, despite being sought for more than a decade, evidence for their presence in intact cell membranes is inconclusive. In this review, a variety of experimental techniques that have been or might be used to look for lipid microdomains in intact cell membranes are described. Experimental results are highlighted and the strengths and limitations of different techniques for microdomain identification and characterization are assessed.

1. INTRODUCTION

Current models for cellular plasma membranes focus on the spatial and temporal heterogeneity of the outer and inner membrane leaflets and how this heterogeneity relates to cell function. It is well documented that protein-stabilized membrane domains such as caveolae and clathrin-coated pits exist in cell membranes. The existence of putative lipid-stabilized lipid raft membrane domains *in vivo* remains much more controversial (1–6) despite successful reconstitution experiments *in vitro* (7, 8).

The hypothesized existence of lipid rafts in cells has garnered much interest in part because of the finding that many signaling molecules are enriched

in the detergent resistant membrane (DRM) fraction that has become the working definition of lipid rafts *in vitro* (9). This result suggests that lipid rafts may function as cell signaling platforms (10). In addition, lipid rafts are thought of as a plausible and attractive explanation for observed heterogeneities in lipid trafficking and distribution, such as the preferential placement of glycosylphosphatidylinositol-anchored proteins (GPI-APs) in the apical membranes of epithelial cells (11). An ever increasing number of papers report on lipid raft involvement in a variety of membrane-mediated events including platelet activation (12), immune cell signaling (13), viral entry (14), and prion pathogenesis (15).

Much of the controversy surrounding lipid rafts relates to their *in vitro* definition as membrane domains that are resistant to extraction in cold 1% Triton X-100 and that float in the upper half of a 5–30% sucrose density gradient (9). Similar to the well-documented caveolae membrane domains, lipid rafts are thought to be enriched in phospholipids with saturated fatty acids, cholesterol, sphingomyelin, and glycosphingolipids. However, in stark contrast to caveolae—which are detected by their distinct 50–100 nm diameter, flask-shaped invaginations in the cell membrane—putative lipid rafts are devoid of the caveolae stabilizing protein caveolin and also lack clear morphological features, hence necessitating the use of a variety of markers for their detection *in vivo*.

There are several major caveats to the definition of lipid rafts solely as DRMs. (a) Classification of lipid raft marker proteins is detergent, temperature, and cell-type dependent (16, 17). (b) Caveolae are also detergent resistant and copurify with putative lipid rafts making it difficult to distinguish the two membrane fractions by detergent extraction alone (18, 19). (c) Detergent extraction can isolate not only molecules from plasma membranes but also from intracellular membranes (16, 20). (d) Considerable rearrangement of intermolecular associations may occur during treatment with detergent (6, 21). (e) Indeed, detergent extraction alone can induce formation of membrane domains up to several microns in diameter (21). Lipid raft involvement has also been investigated by determining effects of changes in the cellular content of cholesterol and glycosphingolipids (10). However, the use of these methods is in general limited by their global cellular effects as opposed to their local effects on membrane domains thought to be lipid rafts (1, 4, 6).

Given the controversy associated with lipid rafts, much effort has been devoted toward identifying them *in vivo*; as of yet, there are inconclusive results (18). In this review, we focus on assessing experimental methods that have been or that might be used to identify lipid microdomains in intact cell membranes (Table 1). We discuss experimental results while also critically examining limitations and consequences thereof for what can be learned about the *in vivo* correlate of DRMs. Probing membrane microdomains was also the topic of the 2004 Biophysical Discussions (www.biophysics.org/discussion).

2. RAFT MARKERS

Although a large number of diverse experimental methods have been explored for identifying lipid rafts *in vivo*, a common feature is the need for specific markers.

TABLE 1 Methods for detecting and characterizing membrane microdomains

Method	Primary observable(s)	Comments
FIIM, fluorescence intensity imaging microscopy	Spatial dependence of fluorophore density	Highly probe and topography specific; single color or multiple colors
FLIM, fluorescence lifetime imaging microscopy	Spatial dependence of fluorophore lifetime	Relatively independent of probe density; useful for FRET
TIRFM, total internal reflection fluorescence microscopy	Evanescently excited fluorophores	Useful for substrate-proximal membranes; kinetic maps and membrane topology
FRAP, fluorescence recovery after photobleaching	Fluorophore translational mobility	Well-established, ensemble-average method; useful combined with other techniques
FCS, fluorescence correlation spectroscopy	Fluorophore translational mobility & clustering	Highly sensitive to clustering; single and multiple colors
ICS, image correlation spectroscopy	Spatial dependence of fluorophore density	Image analysis method; characterizes lateral inhomogeneities
FRET, fluorescence resonance energy transfer	Donor-acceptor proximity	Cluster identification at nm scale; variety of detection methods
SPT, single particle tracking	Translational path of probe particle	Single particle sensitivity; detects domains as confinement zones
SPFT, single particle fluorescence tracking	Translational path of fluorescent particle	Single particle sensitivity; detects domains as confinement zones
SMFT, single molecule fluorescence tracking	Translational path of fluorophore	Single molecule sensitivity; detects domains as confinement zones
AFM, atomic force microscopy	Membrane topography	Primarily for domains in model systems; small height differences readily detected
NSOM, near-field scanning optical microscopy	Spatial dependence of fluorophore density	Subdiffraction limited resolution; low time resolution
OT, optical tweezers	Probe resistance to movement in membrane	Single particle sensitivity; maps membrane barriers
TEM, transmission electron microscopy	Spatial dependence of probe density	Spatial resolution well below optical; not applicable to live cells

Conventional biochemical studies as well as mass spectrometry proteomics have identified a large set of lipids and proteins that are preferentially found in DRMs (9, 20, 22, 23). Some of the more common lipid raft markers are given in Table 2.

Raft markers have been used with mixed results. For example, NBD-cholesterol and BODIPY-GM1 have been shown to be excluded from lipid raft domains in model membranes probably because of the bulkiness of the NBD and BODIPY groups (50, 51). Most phospholipids derivatized at the headgroup with fluorescent dyes are also excluded from lipid raft domains in model systems; one exception is NBD-DPPE (7). In contrast, the water soluble and membrane impermeable probe Fl-PEG cholesterol, which has been derivatized so as to minimize perturbations in the hydrophobic interior of the plasma membrane, was found to preferentially distribute to cholesterol-rich domains in model membranes and intact cells (28). Similar distributions were also obtained with filipin and dehydroergosterol (DHE), which differs from cholesterol only by the addition of three double bonds and a methyl group. The use of these two probes, however, is limited by low quantum yields, and absorption and emission in the UV and blue regions of the spectra. In the case of DHE, specialized microscope optics and cameras are required (29). In the case of filipin, cellular toxicity and probe-induced domain formation reduce its use to fixed specimens (29, 52). Many studies have used secondary labels, most commonly antibodies or toxins specific for proteins or lipids found in DRMs.

With the exception of intrinsically fluorescent probes [e.g., DHE, filipin, and green fluorescent protein (GFP)], fluorescence microscopy additionally requires extrinsic labels (e.g., fluorescent dyes, fluorescent beads, or quantum dots). Of these, fluorescent dyes have the advantages of being small and easily conjugatable directly to possible lipid raft markers; however, their detection is limited by signal brightness and photobleaching, which usually restricts their use in single-molecule time-lapse imaging applications to ~ 100 frames. Increased fluorescence and photostability are obtainable by use of either fluorescent beads or quantum dots.

Probe multivalency is a concern when using fluorescent beads or quantum dots, as well as colloidal gold particles or dielectric spheres. The problem is presumably much less severe for the smaller gold particles (2–10 nm) used in transmission electron microscopy (TEM) and quantum dots (10–20 nm), than for the 40-nm gold particles used in single particle tracking (SPT) or the 200–500 nm spheres typically used with optical tweezers (OT). The problem of probe valency in the case of lipid raft detection is particularly relevant as it is thought that lipid rafts might be physiologically induced or stabilized by cross-linking (10, 53, 54). The problem of controlling probe valency is further complicated by the fact that several of the common lipid raft markers are intrinsically multivalent including antibodies (although monovalent Fab fragments are easily prepared) and cholera toxin subunit B (CTB) (which is pentavalent). It is possible, with careful calibration, to prepare quasi-monovalent particles, which when used for SPT give membrane diffusion coefficients similar to those of raft markers conjugated to fluorescent dyes (55). The use of larger probes also increases the likelihood of steric interaction with the extracellular matrix and ligand-induced transmembrane interaction with membrane-associated cytoskeletal components.

TABLE 2 Common raft markers

Probe	Specificity	Description	References
Cy5-DMPE ^a	Membrane interior	Phospholipid analog	(24)
NBD ^b -DPPE ^c		Phospholipid analog	(7)
Lipophilic dyes		Lipid analog	(25, 26)
Laurdan		Phase-sensitive probe	(7, 27)
FI ^d -PEG ^e -cholesterol		Cholesterol analog	(28)
DHE ^f		Cholesterol analog	(29)
Filipin	Cholesterol	Polyene antibiotic	(30)
NAP-22		Neuronal protein	(31)
Antibody		Binds cholesterol domains	(32)
Peptide		LWYIK and related sequences	(32a)
Perfringolysin O		Toxin	(12)
Lysenin	Sphingomyelin	Toxin	(33)
CTB ^g	GM1	Toxin	(34–37)
Antibody	GM3	Binds GM3 domains	(38)
Antibody	GPI-APs ^h	Binds GPI-APs	(34, 35, 37, 39–41)
Aerolysin		Toxin	(42)
GPI-GFP ⁱ		GPI-AP analog	(40, 43–46)
Antibody	TM ^j proteins	Binds TM proteins	(40)
TM-GFP ^k		TM proteins	(43)
MHC peptide		Binds MHC receptors	(45)
Acylated-GFP	Membrane inner leaflet	Fatty acylated proteins	(36, 43, 47–49)

^a1,2-dimyristoyl-sn-glycero-phosphatidylethanolamine^b7-nitro-2-1,3-benzoxadiazol-4-yl^c1,2-dipalmitoyl-sn-glycero-phosphatidylethanolamine^dfluorescein^epolyethylene glycol^fdehydroergosterol^gcholera toxin subunit B^hglycosylphosphatidylinositol-anchored proteinsⁱglycosylphosphatidylinositol-anchored green fluorescent protein^jtransmembrane^ktransmembrane green fluorescent protein

Quantum dots are an increasingly interesting possibility for labels detectable by fluorescence microscopy (56, 119). Quantum dots are very bright, possessing both large quantum yields and high absorptivities, have narrow fluorescence emission bands, and are resistant to photobleaching, although intermittency in emission (blinking) can complicate their use. Furthermore, multiple color quantum dots are excitable over a wide and overlapping range of wavelengths allowing for the possibility of long duration (> 100 frames) simultaneous single molecule tracking of multiple lipid raft markers, which is not readily possible with either gold particles or other fluorescent markers. Indeed, simultaneous tracking of different raft markers could provide novel information about raft sizes and dynamics and report on coresidency of different lipid raft markers in the same membrane domains.

3. FLUORESCENCE INTENSITY IMAGING MICROSCOPY (FIIM)

The most straightforward method of probing for microdomains in cell membranes is to label them with a fluorescent marker and then use fluorescence intensity imaging microscopy (FIIM) to look for nonuniformities in the spatial distribution of the measured fluorescence intensity. Spatial nonuniformities of different membrane probes on different cell types have been observed in a large number of studies with various markers and on different cell types. However, most putative raft markers in nonstimulated cells do not exhibit nonuniformities intense or sparse enough in their spatial distributions for ready detection and analysis (1, 3). FIIM methods are in general restricted by the diffraction-limited optical resolution of a light-microscope (approximately $\lambda/2$). This restriction makes it difficult to detect microdomains smaller than about 250 nm.

There are some cases in which straightforward FIIM has been successfully used for detecting putative raft microdomains in membranes. For example, Malinska et al. found that two raft proteins (the arginine/ H^+ symporter Can1p and the plasma membrane ATPase Pma1p) in live *Saccharomyces cerevisiae* cells were stably and simultaneously localized in nonoverlapping microdomains for periods greater than 30 min, whereas a nonraft protein (the hexose transport facilitator Hxt1p) was randomly distributed (57). In another example, using extremely low probe densities, Schütz et al. found that fluorescent analogs of saturated, but not monounsaturated, phospholipids in coronary artery smooth muscle (HASM) cells were predominantly localized in domains with an average size of 700 nm (24).

Several more sophisticated fluorescence intensity imaging methods have been adapted for the specific purpose of addressing the lipid raft hypothesis. Laurdan is a lipophilic probe that undergoes significant spectral shifts in different lipid phase environments. By calculating a "generalized polarization" function, which is derived from images observed at different visible wavelengths, it is possible to map micron-sized lipid phases in model and, recently, in macrophage cell membranes (27). Dual-color imaging can address the question of whether putative raft

markers are colocalized. This approach has been used, for example, to demonstrate that stimulation of RBL-2H3 cells by crosslinking IgE-loaded Fc ϵ RI with antigen induces intracellular SH2 domains (conjugated to GFP) and extracellular glycosphingolipids (labeled with CTB) to translocate to identical punctate membrane microdomains that apparently span both the inner and outer membrane leaflets (36). In a similar work, T cell signaling stimulated the formation of membrane regions in which various putative raft markers were colocalized (39).

4. FLUORESCENCE LIFETIME IMAGING MICROSCOPY (FLIM)

Fluorescence lifetime imaging microscopy (FLIM) is a method in which the lifetimes of fluorophores at different spatial positions are determined (58–60). A major advantage of lifetime, as compared to intensity, imaging is that the data depend only on local environmental factors affecting the fluorescence quantum efficiency. Therefore the images are predicted to depend on the local fluorophore density only to the extent that fluorescence resonance energy transfer (FRET) to nearby molecules may occur. One particularly powerful application of FLIM is its use to monitor hetero-FRET via the decrease in donor lifetime (61, 62). In addition, polarized FLIM can monitor local time-resolved anisotropy decays, enabling separation of depolarization components arising from rotational mobility and homo-FRET (63, 64). Versions of these approaches have recently been used to provide evidence for small (<5 nm) cholesterol-dependent clusters of GPI-APs in cell membranes (44).

5. TOTAL INTERNAL REFLECTION FLUORESCENCE MICROSCOPY (TIRFM)

Total internal reflection fluorescence microscopy (TIRFM) uses evanescent illumination to excite fluorophores that are only very close to a planar dielectric interface (66). When used to illuminate cell membranes proximal to surfaces to which cells are adhered, TIRFM significantly reduces background from intracellular sources. In addition, polarized TIRFM has the advantage of being sensitive to the polar, rather than the usual azimuthal, angle with respect to the normal to the interface. In a recent elegant and comprehensive study, this method was used to address a variety of questions related to membrane architecture, although not specifically related to the raft hypothesis (67). When combined with evanescent illumination, fluorescence recovery after photobleaching (FRAP) can provide information about the association/dissociation kinetics of intracellular fluorescent molecules with the inner leaflet of plasma membranes in an imaging and therefore spatially resolved manner; thus, raft regions might in principle be detected by kinetics maps in the intracytoplasmic region (68). Evanescent illumination can also be combined with

fluorescence correlation spectroscopy (FCS) to provide information about the kinetics of fluorescent molecules associating with and dissociating from sites on a surface (69).

6. FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING (FRAP)

FRAP is a well established method for examining the translational mobility of fluorescent molecules in cell membranes (70). Following its initial development, this method was used extensively to characterize the lateral motions of fluorescent lipids and proteins in a variety of cell types (71–73). Indeed, the topics of lateral mobility measurement by FRAP and membrane domains have been intertwined for more than two decades (74).

FRAP has recently been revisited in the context of lipid rafts. For example, the lateral mobility of different inner leaflet Ras proteins, some of which are thought to be raft-resident and some not, were examined in Rat-1 cell membranes as a function of the expression level, the membrane cholesterol concentration, and treatment with farnesylthiosalicylic acid, which can compete with Ras for specific binding sites (47, 48). Distinct differences in the lateral mobilities of the different Ras proteins, which contained different types of membrane anchors, were observed, leading to hypotheses about the degree to which cell signaling pathways dependent on the different Ras proteins might use raft microdomains in their mechanisms. In a somewhat similar work, the lateral mobilities of influenza hemagglutinin proteins having different membrane anchors were compared in the plasma membranes of CV-1 and COS-7 cells (40). The results indicated that putative raft-associated proteins diffused more slowly, in a cholesterol-dependent manner, than those thought not to associate with rafts. FRAP has also demonstrated that the protein CD44 displays very low lateral mobility on fibroblasts unless the plasma membranes are subjected to cholesterol depletion or the cytoskeleton is disrupted (75).

Both FRAP and FCS were carried out on a variety of outer and inner leaflet green fluorescent protein-fusion proteins (GFP-FPs) expressed in RBL-2H3 cells (25). Significant differences in lateral mobility were observed for the IgE receptor Fc ϵ RI, the fluorescent lipid diI-C₁₆, a Src family kinase analog anchored to the inner leaflet by two saturated acyl chains, and a control protein anchored to the inner leaflet by an unsaturated geranylgeranyl chain with a nearby polybasic sequence. The mobilities were also dependent, in most cases, on the degree to which the receptor had been cross-linked. Taken together, the FRAP and FCS data provide preliminary information about the degree to which putative lipid raft microdomains in different membrane leaflets might be coupled during cell signaling.

Kenworthy et al. have recently presented the results of an extensive FRAP study aimed at clarifying the lipid raft hypothesis (43). The apparent diffusion coefficients and fractional mobilities were measured over rather large areas (4 μ m), designed

to be larger than raft regions, should they exist, for a variety of raft and nonraft proteins in resting COS-7 cell membranes. Measurements were also carried out as a function of temperature, cholesterol depletion, and cholesterol enhancement. The data are inconsistent with models in which a significant fraction of the cell surface is populated by stable (either mobile or immobile) raft regions. The authors conclude that, although models in which a small fraction of raft proteins are found in such domains or partition rapidly between raft and nonraft regions cannot be completely ruled out by the data, association with rafts is not the dominant factor governing lateral mobility. Instead, the apparent diffusion coefficients were much better correlated with the manner in which the proteins were anchored to the membrane rather than with their propensity to partition into DRMs.

As noted by Kenworthy et al. (43), FRAP as an isolated method is not likely to provide definitive information about the existence or nonexistence of raft microdomains. Some of the limitations are as follows. (a) Even for ideal diffusion, data interpretation depends on the geometric details of the beam shape as well as the fraction of fluorophores initially bleached (70, 76). (b) FRAP is intrinsically a macroscopic, ensemble-averaged method that may obscure details arising from putative small, dynamic, and submicron lipid rafts. (c) The lateral mobility of membrane markers is not extremely sensitive to molecular size or local viscosity. (d) A wide variety of physiological factors can contribute to the rate and shape of fluorescence recovery including transient association with relatively stable membrane components such as cytoskeletal elements, active membrane turnover, diffusional barriers, molecular crowding, hydrodynamic interactions, attractive or repulsive nonspecific protein-protein interactions in the membrane plane, local nonplanar membrane morphologies, and local number fluctuations.

Several experimental variations of FRAP have the potential to increase its efficacy for raft detection and characterization. (a) Measurements carefully carried out as a function of beam size can provide information about submicron interactions, possibly involving microdomains, that retard lateral mobility (77, 78). (b) The phenomenon of photobleaching, by itself, has been creatively combined with FRET to provide information about molecular proximity at the submicron level (see Section 8). (c) Polarized FRAP can provide direct information about rotational, as opposed to lateral, mobility, and rotational motions are usually much more sensitive to molecular (or, in the case of stable rafts, domain) size than lateral motions (79, 80).

7. FLUORESCENCE CORRELATION SPECTROSCOPY (FCS)

Among the variety of methods currently being explored for examining membrane heterogeneity are those based on signal fluctuations. Several signal fluctuation correlation methods have been developed for biological applications. These methods include, but are not limited to, FCS, image correlation spectroscopy (ICS) and cross-correlation spectroscopy (CCS) (81–85). In the first two methods, the

temporal (FCS) and spatial (ICS) autocorrelation functions of fluorescence fluctuations are calculated and interpreted. The third method (CCS) addresses the extent to which fluctuations in the fluorescence from two or more species are correlated.

Correlation functions characterize the degree to which the amplitude of a given signal influences its amplitude at some other time or place. The general definition of the normalized autocorrelation function for the observable $I(x)$ is $G(\delta) = \langle I(x)I(x + \delta) \rangle / \langle I \rangle^2$, where the brackets denote an average over the variable x (which may be either a temporal, t , or spatial, r , coordinate) and δ is the temporal (τ) or spatial (ρ) lag between signal values. $G(\delta)$ gives the probability that a change in the signal at a given time or place will be associated with a similar change at a later time or different location.

In conventional FCS, the fluorescence signal from a small observation volume, excited by a laser beam and collected through a high aperture microscope objective, fluctuates in time owing to random, local changes in molecular concentrations driven by translational motions. For a monodisperse, homogeneous system, $G(\tau)$ contains information about the average number of molecules in the observation volume and their diffusion coefficient. In many systems, FCS gives single-molecule sensitivity with good statistical accuracy. Because $G(0)$ depends strongly on the molecular concentration (and therefore oligomerization), FCS has long been thought of as a potentially powerful method for detecting and characterizing membrane microdomains. The viability of temporal, spot-based FCS as a method for examining membrane dynamics has been confirmed by a wide variety of studies, including (as only a few examples) those of fluorescent lipids in model membranes (26), and a number of inner and outer leaflet raft markers in RBL-2H3 cells (25).

ICS was introduced as a modification to FCS for measurement of cluster sizes and concentrations. In this method, an image of a cell surface is acquired and the spatial correlation function, $G(\rho)$, is calculated. ICS is used to define the stoichiometry and density of aggregates on cell surfaces and is therefore potentially useful for the detection and characterization of membrane microdomains. ICS has been applied to images of fluorescently labeled IgE specifically bound to supported planar membranes (86) and has been used to examine clustering of a variety of membrane components on cell surfaces including influenza hemagglutinin mutants (87), PDGF receptors (88), and acetylcholine receptors (89).

More information can be obtained by using multiple fluorescent probes and cross-correlating the fluctuations in their signals; i.e., by calculating $G_{ij}(\delta) = [\langle I_i(x)I_j(x + \delta) \rangle] / [\langle I_i \rangle \langle I_j \rangle]$, where I_i and I_j denote intensities from different fluorophores emitting in different spectral regions (CCS). This method has been used in time-domain FCS for kinetic studies of DNA renaturation (90) and cleavage by endonucleases (91), and to demonstrate colocalization and oligomerization of different somatostatin receptors on cell surfaces (92). Cross-correlation methods have also been extended to ICS (93) and used to characterize colocalization of raft markers in RBL-2H3 membranes (94), influenza hemagglutinin mutants, and the clathrin-associated adaptor protein AP-2 on cell surfaces (95) as well as a

process in which Sendai virus clusters specific gangliosides upon binding to cell surfaces (96). A very powerful approach of combining temporal, spatial, and cross-correlation methods has recently been illustrated on membrane surfaces (97–99). This approach generates spatial maps of both fluorophore density and mobility.

Significantly more information about oligomerization can be extracted from fluorescence fluctuations by calculating high-order autocorrelation functions. These functions are defined as $G_{mn}(\delta) = \langle I^m(x)I^n(x + \delta) \rangle / \langle I \rangle^{m+n}$, where m and n are integers, and contain information, particularly in their magnitudes, about the concentrations and relative intensities of different molecular species when samples have multiple fluorescence components (e.g., monomers and clusters). This approach has been illustrated in both temporal (100) and spatial (101) formats.

Another method for characterizing fluorescence fluctuations is to count the photons emitted from the observed volume in sequential time elements and to use this information to generate the probability distribution for detecting a given number of photons from the observed volume and within the observation time. This method is called photon-counting histogram (PCH) analysis (102) or fluorescent intensity distribution analysis (FIDA) (103). In these methods, the intensity histogram is analyzed to obtain information about the distribution of cluster types. PCH/FIDA has recently been extended to the dual-color cross-correlation regime (104) as well as to the time domain, by generating multiple histograms arising from different sample time durations (105).

FCS and its related methods have now been established as powerful methodologies for examining cell membrane organization and dynamics. Although these methods have only begun to be applied to the study of lipid raft microdomains, this area should generate a flurry of activity in the near future. Nonetheless, these methods are not extremely simple to implement.

Worth noting are a variety of experimental complexities: (a) Methods for background correction are not straightforward. This issue is of particular concern in cellular systems where significant variability may occur in different localized spots and cannot easily be measured for each sampled region. (b) The exact spatial profile of the excitation intensity is important. In conventional, spot-based, temporal FCS, the shape of $G(\tau)$ depends critically on this intensity profile. For ICS, both long-range changes in excitation intensity (or detector sensitivity) and short-range changes in this intensity (e.g., arising from interference effects in the excitation optics) will dominate the measured autocorrelation function if not eliminated or taken into account. (c) Interference in the correlation functions from intrinsic dye photochemistry (e.g., transient “dark” states arising from long-lived triplet states) can occur. (d) In spot-based FCS on cell membranes, one can effectively bleach a “hole” in the membrane. Subsequent very small mechanical motions arising from instrument instability or cell movement can then give rise to very large fluctuations in the observed fluorescence that dominate the measured correlation function. Quantum dots have the potential for reducing problems arising from photobleaching; however, the dots exhibit intermittency in their emission, which is likely to also autocorrelate and which may be dependent on the local environment. (e) In

samples containing multiple species, $G(0) - 1$ is an average weighted by the square of the relative intensities of the different species. This property is a strength of FCS, because it means that the data are extremely sensitive to molecular clustering. However, if clustered molecules quench, then this effect must be taken into account. (f) In two-color measurements, contributions of donor emission to the acceptor channel and related factors must be dealt with quantitatively in the interpretation of the cross-correlation functions.

8. FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET)

FRET is a phenomenon in which a donor fluorophore in an excited state transfers its energy through a nonradiative mechanism to an acceptor molecule. Most importantly for raft detection, the energy transfer rate depends sharply on the distance between the donor and acceptor. For typical energy transfer pairs, the donor-acceptor distance at which one half of the excited state donors transfer their energy to acceptors is in the range of 1–10 nm. Thus, FRET can in principle detect clustering with a spatial sensitivity well below optical resolution.

A number of methods for measuring FRET on cell membranes have been used for the purpose of detecting and characterizing rafts (106). The two most conceptually straightforward methods are to probe for a decrease in donor fluorescence or an increase in acceptor fluorescence. These steady-state approaches have been augmented by examining the effect of acceptor photobleaching, which can shift the absorption spectrum of the acceptor and relieve donor quenching, thereby increasing the steady-state intensity and lifetime of the donor. Similarly, the rate of donor photobleaching decreases in the presence of energy transfer because the donor lifetime is decreased. Furthermore, FRET measurements can be characterized either as homo-transfer, in which the donor and acceptor probes have equivalent molecular structures, or hetero-transfer, in which the donor and acceptor probes are different molecules. In homo-transfer FRET measurements, the fluorescence emission is significantly depolarized and this depolarization can be detected either by steady-state or time-resolved measurements.

Energy transfer efficiencies that are independent, or dependent, on the fluorophore density have been interpreted as indicating the existence, or suggesting the nonexistence, of molecular clusters, respectively. However, the degree to which energy transfer is observed may depend on a variety of factors including not only the donor and acceptor densities, but also the relative orientations of the donor and acceptor transition dipole moments, their rotational mobilities, and their spectroscopic properties. In the presence of clusters, the cluster size, the cluster density, and the number of donor and acceptor molecules per cluster can also affect transfer efficiencies. For small clusters, the number of donor and acceptor molecules per cluster can significantly fluctuate, in theory, because of population statistics. Thus, the interpretation of observed energy transfer efficiencies can depend on considerable theoretical modeling and concomitant assumptions (44, 107, 108).

Other types of experimental factors can also affect FRET measurements and their interpretation. One concern is that some fluorescent protein variants may spontaneously dimerize when confined to a membrane surface; to obviate this difficulty, monomeric mutants of GFP have been generated (109). In measurements where extensive donor or acceptor photobleaching is employed, there is considerable potential for photodamage (e.g., photoinduced cross-linking and consequent domain formation), and the time required for the photobleaching process may be long compared to putative raft lifetime or spatial stability (107, 110). Where fluorescence depolarization due to homo-transfer is observed, the fluorophore rotational mobility, which in the case of fluorescent proteins can depend on the location within the target where the protein is introduced (111), is an important consideration as well as the depolarizing effects of high numerical aperture objectives. Most if not all measurements have relied on assuming a linear relationship between fluorophore density and local fluorescence intensity; this assumption may be too simple (112).

Some experimental studies using FRET to address the possible existence of rafts on plasma membranes have suggested that at least some proteins associated with DRMs are not present in cell membranes in a detectably clustered arrangement. These measurements include steady-state studies of the dependence of hetero-transfer between different glycosylphosphatidylinositol-anchored green fluorescent proteins (GPI-GFPs) on COS-7 and Jurkat cell membranes on the donor density, as detected by acceptor emission (112), and between different GPI-APs and GM1 labeled with CTB on a variety of cell types, as detected by increased donor fluorescence after acceptor photobleaching (34, 108). Other FRET studies concluded that some proteins hypothesized to populate lipid rafts are clustered at the submicron level. These measurements include studies of the extent to which homo-transfer between GPI-AP folate receptors on CHO cells, as detected by fluorescence anisotropy, depends on the receptor density (113); of the hetero-transfer efficiency between different lipid-linked proteins present on the cytosolic leaflets of Madin Darby Canine Kidney (MDCK) plasma membranes (109); and of increased donor emission after acceptor photobleaching for GM1 labeled with cholera toxin B subunit and GPI-APs on COS-7 cells (110). Measurements based on direct calculation of energy transfer efficiency from two-color intensities measured with flow cytometry have also indicated cholesterol-dependent coclustering of interleukin 2 receptor subunits, major histocompatibility complex (MHC) proteins and a GPI-AP in human T cell lines (114).

The seemingly inconsistent results of these different measurements, some concluding that raft-resident proteins are nonrandomly distributed in tight molecular clusters and some concluding otherwise, are possibly reconciled by the increasingly accepted notion that, at least in resting cells, lipid rafts are very small and dynamic, and that only a minor fraction of classical detergent-resistant raft markers may be present in these rafts at any given time. Indeed, a recent study has examined both homo-transfer and hetero-transfer with both steady-state and time-resolved FRET methods, combined with theoretical modeling, and concluded that,

although the majority of GPI-APs on CHO cells are present as monomers, a small, detectable fraction (20–40%) does exist in small (<5 nm) cholesterol-dependent clusters each of which contains on the average less than five GPI-APs (44).

9. SINGLE PARTICLE TRACKING (SPT)

SPT is another light microscope-based method that is capable of obtaining spatial information beyond diffraction-limited optical resolution (115–117). In this technique, the translational mobilities of single or small groups of membrane proteins or lipids are inferred by tracking the movement of specifically bound extrinsic labels with a high resolution microscope to a spatial resolution of just a few to tens of nanometers.

In conventional SPT, the extrinsic label is a colloidal gold particle. These particles are readily detected by means of their intense Rayleigh light scattering. Because of signal-to-noise considerations, this approach necessitates the use of particles greater than about 30 nm in diameter (115), although an alternative method for detecting gold particles as small as 10 nm diameter on fixed cells has been described (118). This latter method, which is referred to as photothermal interference contrast, has not yet been used for conventional SPT on live cells. Limitations of conventional SPT include the large particle size and the difficulty of preparing monovalent conjugates (53, 119).

In single particle fluorescence tracking (SPFT) typical extrinsic labels are fluorescent beads (120) or quantum dots (121), whereas in single molecule fluorescence tracking (SMFT) typical extrinsic labels are phycobiliproteins (120, 122), GFP fusion proteins (49), fluorescent lipid analogs (123), or fluorescent dyes such as Cy3 and Cy5 (24, 45, 124, 125). SMFT with fluorescent proteins or dye molecules has thus far been limited by photobleaching to at best approximately 100 frames of tracking. A decrease in the signal-to-noise ratio results in a concomitant loss of spatial resolution (116). In contrast, SPFT with fluorescent beads or quantum dots, while allowing longer observation durations due to increased photostability, suffers from the same limitations as SPT (i.e., large probe size and possible multivalency), although quantum dots, which range in size from 10–20 nm, presumably suffer less from these limitations than the larger gold particles used in SPT. A major advantage of SMFT with fluorescent proteins or dyes, as compared to SPT or SPFT, is the ease with which monovalent conjugates are prepared. In fact, SMFT of lipids labeled with single fluorescent dyes is commonly used as a monovalent control for SPT and SPFT (55, 125).

Information about the heterogeneity of cell membranes by SPT, SPFT, and SMFT is obtained by characterizing the observed motion relative to that expected for Brownian motion in two dimensions (115). From such analysis, which involves assembly of particle trajectories and subsequent calculation and analysis of the mean squared displacements as a function of time, information about diffusion coefficients, particle confinement, and confinement areas and their lifetimes are

extractable by a variety of methods (115, 126, 127). Using these types of analyses, several examples of membrane domains have been documented in a variety of cell types, most of which are not fully consistent with the notion of lipid rafts.

Using a combination of SPT and SMFT to investigate the membrane mobility of the unsaturated lipid DOPE (a nonraft marker), Kusumi and coworkers have provided evidence for several types of membrane microdomains (41, 53, 55, 125). Using the unprecedented sampling frequency of 40,000 Hz (spatial resolution of 20 nm), it was found that DOPE undergoes hop-diffusion between continuous, cell-type dependent compartments of 30–230 nm every 1–17 ms. Within these domains, DOPE was found to undergo normal diffusion with coefficients similar to those in model membranes, whereas long-range apparent diffusion coefficients were significantly lower because of transiting of the barriers that produced confinement. Studies in normal rat kidney (NRK) cells also showed that similar confinement and residency times (~ 25 ms) were found for raft markers (the GPI-AP CD59 and the ganglioside GM3), whereas the transmembrane protein transferrin (a nonraft marker) was confined in nearly identically sized domains but with slightly longer residency times (41, 53). These compartments were found to be dependent on an intact actin cytoskeleton but not on cholesterol.

CD59 clustered by multivalent gold particles was found to undergo hop-diffusion between compartments of similar size but at an eightfold slower rate (~ 200 ms between hops). This slower hop-rate for clustered CD59 and the similarity of the hop-rates for DOPE and presumably monovalent CD59 indicate that if rafts (containing monomeric CD59) exist, they are either small enough where the compartment barriers do not affect the hop-rate, or they are only dynamically stable for periods less than the observed hop-rates (~ 25 ms). Clustered CD59 was further found to undergo overall Brownian motion, but with frequent visits to 90 nm domains with an average residence time of 0.7 s. Confinement to these domains was found to coincide with transient anchorage to the cytoskeleton, a subsequent rise in cytoplasmic IP_3 , and release of intracellular calcium (41, 53). These findings taken together have led Kusumi and coworkers to propose a model containing three levels of lipid rafts: (a) resting (noncross-linked) rafts consisting of at most 2–3 raft proteins, (b) cross-linked stabilized cluster rafts, and (c) transiently anchored signaling rafts (41, 53).

Following work by Jacobson and coworkers, signaling rafts were further identified as transient confinement zones (TCZs), a term coined to describe regions of the plasma membrane where a protein or lipid is confined much longer than would be expected by simple Brownian motion (41, 54, 128). Much work has been devoted to the characterizations of TCZs and their relation to lipid rafts (35, 54, 128, 129). TCZs are typically on the order of 100–300 nm in diameter and have lifetimes of hundreds of milliseconds to seconds; observed sizes and confinement times are dependent on the employed sampling rate (35, 41, 54). It has been shown that putative raft markers including GPI-APs (Thy1, CD59) and gangliosides (GM1, GM3) spend more time in TCZs than do lipid analogs, whereas glycosphingolipid depletion, cholesterol extraction, actin depolymerization or Src family kinase

inhibitors all decrease the time spent in TCZs (35, 41, 54, 129). It has also been shown that there is a strong correlation between probe valency and the time spent in TCZs; probes thought to be monovalent reside minimally in TCZs as compared to known multivalent probes (41, 54).

Using SMFT, Schütz et al. found that a fluorescent analog of a saturated phospholipid in HASM cells exhibited restricted diffusion, whereas a fluorescent analog of a monounsaturated probe did not. Analysis of trajectories as well as FIIM results were consistent with the existence of 700 nm microdomains (24). Also using SMFT, Vrljic et al. studied the translational mobility, in CHO cells, of full-length I-E^k and GPI-anchored I-E^k class II MHC membrane proteins labeled with monovalent fluorescent peptides (45). In this system, no evidence for confinement was found for either full-length or GPI-anchored I-E^k. However, this system was studied at a low sampling rate (10 Hz, spatial resolution of ~60 nm) for which hop-diffusion would not be expected to be observed. The possibility of unintentional clustering of I-E^k was also likely minimized (or possibly eliminated) by the use of monovalent fluorescent peptides as the extrinsic probes. This in turn would be expected to lead to significantly less confinement of I-E^k in TCZs.

SMFT has also been used to study inner leaflet microdomains in human embryonic kidney cells and mouse fibroblasts (49). This study found that 30–40% of an acylated fluorescent protein, consisting of the H-Ras membrane targeting sequence (raft marker) fused to yellow fluorescent protein, was confined to 200 nm domains. These domains, however, were insensitive to cytoskeletal rearrangement and more importantly to extraction of 60% of the membrane cholesterol. This result suggests that these domains are inconsistent with hypothesized lipid rafts but rather represent another type of membrane microdomain.

Unlike methods such as FRAP and FCS, which report on ensemble averages of the translational mobility of extrinsically labeled lipids and proteins over membrane areas $\sim 1 \mu\text{m}^2$, SPT reports on the translational mobility of individual lipids and proteins and is particularly useful for characterizing membrane heterogeneities at spatial scales of tens of nanometers. To date, domains identified as either TCZs or signaling rafts are most consistent with the lipid raft hypothesis in live cells. These domains, however, are rarely detected in the absence of external cross-linking of putative lipid raft markers indicating that they reflect cluster-stabilized lipid rafts. The best evidence for unstimulated lipid rafts in live cells has been provided by Schütz et al. using saturated, single fluorescent dye labeled lipids in HASM cells (24). These domains, however, are much larger (average of 700 nm diameter) than domains detected in other cell-types by other methods (~ 5 –50 nm) and are thought to represent noninduced lipid rafts (37, 44, 46, 130). Unfortunately these experiments were carried out at room temperature, leaving questions about their applicability at 37°C. HASM cells are also highly differentiated, which may be associated with an unusually long-range membrane domain structure. Indeed, very stable microscopically resolved domains were observed in the membranes of yeast, perhaps another special situation (57). Kusumi and coworkers have also argued that observed similarities in hop-diffusion rates for unsaturated lipids and

GPI-APs indicate that unstimulated lipid rafts are either very small, consisting of at most a few molecules, or unstable with lifetimes less than a few milliseconds (41, 53). An equally plausible model, consistent with these data and that of Vrljic and coworkers, would be that lipid rafts in cells are only present after some form of external stabilization; alternatively, unstimulated lipid raft markers may exist in the membrane surrounded by small lipid-shells composed of cholesterol and sphingolipids, as has been hypothesized by Anderson & Jacobson (18).

10. ATOMIC FORCE MICROSCOPY (AFM)

AFM is a potentially powerful tool for studying membrane microstructures in the 1–10 nanometer range (131–135). In this method, topography maps of a sample are acquired by measurement of deflection forces (greater than nN) on a sharp needle, in close proximity to a sample surface, which is raster-scanned across the sample. No extensive sample preparation or vacuum are needed, so the method is (in some cases) amenable to *in vivo* measurements.

Many pioneering AFM studies have imaged protein structure and dynamics on lipid surfaces. Although most of these studies are not directly related to the lipid raft field, the composite results suggest possible effectiveness of the method in this area. For example, a dramatic mapping of the surface of the hexagonally packed intermediate layer of *Deinococcus radiodurans* has been produced using AFM (136). In a similarly striking report, diffusion of single sodium-driven rotors from a bacterial ATP synthase were observed in lipid membranes by using time-lapsed AFM topographs (137). Ultimately, this type of topographic information might significantly enhance our understanding of membrane microdomains, particularly if the measurements were carried out on live, intact cells.

A number of AFM studies have addressed domain structure and dynamics on model membrane surfaces (132, 138–143). Most of these works examine coexistent gel/liquid or liquid/liquid phases in substrate-supported monolayers or bilayers. Different domain types are distinguished either by their lateral height differences (which are often on the order of 1 nm) or by the presence of bound protein indicators such as CTB. These studies have primarily addressed the dependence of large domain characteristics on chemical composition, and many have used lipid mixtures containing cholesterol, sphingolipids, and/or gangliosides in an attempt to address the lipid raft hypothesis. The most novel contributions of these studies include observations of domain substructures smaller than those that are observable with optical microscopy.

The ability of AFM to map small subdomain structures suggests that this method might in principle have the capability of illuminating small microdomains on intact cells. However, AFM studies on intact cell membranes are much more difficult to execute and have much lower lateral resolutions (e.g., 20–50 nm) as compared to resolutions in imaging single, isolated molecules (~nm). The resolution is not as great for intact cells in part because of rearrangements of the plasma membrane

and/or movements of submembrane filament bundles. Furthermore, cell surfaces are covered by a pericellular matrix consisting of proteins, proteoglycans, and glycosaminoglycans, making direct access to the lipid surface difficult. Nonetheless, the application of AFM to live cells holds promise. For example, a recent study indicated a possible reduction of the number of microvilli on the surfaces of MDCK cells by inhibition of either sphingolipid or cholesterol biosynthesis (144).

11. NEAR-FIELD SCANNING OPTICAL MICROSCOPY (NSOM)

Another scanning probe method is near-field scanning optical microscopy (NSOM) (145–149). In contrast to AFM, NSOM is a light microscopy-based technique. In the most common configuration (aperture-NSOM), a probe with a subwavelength aperture is scanned in close proximity (less than λ) to the sample surface. Under these conditions, light incident on the sample is predominantly evanescent while being minimally diffracted, and the area of illumination and the resulting spatial resolution are proportional to the aperture size. The intensity of the evanescent light decays approximately exponentially with distance from the NSOM aperture, resulting in highly specific illumination of the sample to a depth of only about 100 nm (near-field). Using this approach, optical images with a resolution of about 50 nm are typically obtainable. There are also apertureless configurations of NSOM, examples of which include the use of far-field illumination of a FRET donor-acceptor pair (150) and fluorescence quenching by a metal tip (151). Such apertureless NSOM methods are in theory capable of obtaining spatial resolutions of just a few nm (146).

For NSOM, the requirement of accurate and precise placement of the probe within a few nanometers of the sample surface, while scanning the probe, proves to be the greatest challenge, in particular for live cell imaging (145, 146). The most common methods to date are those involving surface force feedback effectively, leading to combined NSOM/AFM measurements of the sample composition and its topography. These methods have been used to image microdomains in model membranes (51) and fixed cells under dry and aqueous conditions (152, 153).

Although surface force feedback methods work well for controlling the distance of separation between the NSOM probe and the sample under dry conditions, these methods are less sensitive under aqueous conditions because of viscous dampening (146). In addition, under aqueous conditions cells typically range in thicknesses of over $1\ \mu\text{m}$ and remain very sensitive to direct contact. Hence, the method for controlling the distance between the cell membrane and the probe needs to be accurate to within a few nanometers, ideally without ever making contact with the cell membrane, while having a range of several hundred nanometers (145, 147). Much effort has been devoted to either improving force-feedback stabilized methods or alternatively finding new methods for controlling probe-to-sample distances in aqueous conditions suitable for imaging live cells at lateral resolutions

similar to those possible for fixed cells in dry conditions. Improved force-feedback stabilized methods for use under aqueous conditions have resulted in images of tobacco mosaic virus with a lateral resolution of 60 nm (154), the nuclear envelope of *Xenopus laevis* oocytes with a lateral resolution of 70 nm (155), and nanometric domains on the membrane of dendritic cells with a lateral resolution of 90 nm (155a).

As an alternative to force feedback stabilized methods, scanning ion conductance microscopy (SICM), in which an electrolyte-filled glass nanopipette is scanned along the sample surface, was proposed by Hansma et al. (156). In this noncontact approach, the distance of separation is controlled by maintaining a constant ion flow from the pipette aperture; the resolution, similar to that for aperture-NSOM, is dependent on the size of the tip aperture. Hybrid approaches of SICM and NSOM have been used for simultaneous topography and light imaging of live cardiac myocytes (157) and renal tubular cells A6 (158). In this latter approach, a topographic resolution of about 220 nm and an optical resolution of about 190 nm were reported.

12. OPTICAL TWEEZERS (OT)

The basis of the OT technique was demonstrated by Ashkin, who found that dielectric particles can be accelerated and trapped by laser-induced radiation pressure (159). In this technique, microspheres as small as 0.1 μm and as large as 100 μm can be manipulated without mechanical contact. OT have been used in a variety of biological contexts, including studies of the mechanisms of microtubule motors (160) and the mechanical properties of single DNA strands (161). An extensive overview of biological applications is given in Reference 162.

OT have also been used to examine membrane heterogeneity. In the first study, MHC class I molecules were labeled with antibody-coated gold particles and then dragged across cell surfaces (163). The MHC molecules were found to encounter barriers to lateral movement arising primarily from the cytoplasmic side of the membrane. This work was followed by a much more refined and in-depth study in which transferrin receptors were labeled with either gold or latex particles and then forced through fibroblast membranes (164). The results confirmed that transmembrane protein mobility can be significantly retarded by cytoskeletal barriers, and provided quantitative information about barrier architecture. OT have also been used to deform the cytoskeleton while observing trajectories of the membrane-spanning protein band 3 on erythrocytes by using SPT (165). The results were consistent with a model in which the mobility of band 3 is strongly correlated with structures in the cytoskeleton. The particle-dragging technique has recently been used to address the lipid raft hypothesis by scanning aggregates of GPI-APs across plasma membranes (166, 167). In this case, two types of barriers were observed. One type was correlated with the actin cytoskeleton, presumably reflecting the presence of a transmembrane linker molecule, and the other type was correlated

with membrane cholesterol. The results were interpreted as suggesting direct interactions between cross-link stabilized rafts and membrane substructures.

In a very elegant work, Pralle et al. expanded the degree to which OT can be used for investigating membrane microdomains (46). Putative raft and nonraft proteins were labeled with 0.2 μm fluorescent microspheres and optically trapped and then tracked within an area of about 100 nm. At 50 kHz, the lateral and axial resolutions were better than 1 and 5 nm, respectively. The thermal fluctuations of the beads within the trapping zones were observed and used to obtain local diffusion coefficients. Confining the particles to small regions has the possibility of avoiding complications arising from perhaps more sparsely distributed membrane barriers. The authors found that the majority of the raft proteins exhibited a large viscous drag, whereas the majority of the nonraft proteins exhibited a much smaller drag. The apparent diffusion coefficients of the nonraft proteins agreed well with those previously measured for transmembrane proteins in model membranes, whereas the apparent diffusion coefficients of the raft proteins were much smaller. After cholesterol extraction, the raft proteins had reduced drags approximately equivalent to those of the nonraft proteins. The authors postulate that the much larger drags observed for the raft proteins before cholesterol depletion reflect stable (approximately one minute) rafts that move as intact entities and have diameters of about 50 nm. This conclusion, although not entirely consistent with many recent results, has been used to suggest that the elementary raft size is 50 nm.

13. TRANSMISSION ELECTRON MICROSCOPY (TEM)

The increased spatial resolution available by TEM with immunolabeling has also been applied to the search for lipid rafts in cell membranes (37, 168, 169, 169a). In this technique, lipid raft markers are labeled with antibody-coated gold particles typically ranging in size from 2–10 nm. Use of different sized gold particles makes colocalization experiments of raft markers at nanometer scales possible (37, 130, 168), whereas standard osmium staining allows for simultaneous visualization of lipid domains rich in unsaturated lipids. Because sample observation requires high vacuum pressures, extensive sample manipulation (including fixation in formaldehyde, glutaraldehyde, and often osmium), and dehydration, obtaining dynamic information is precluded. Sample specimens must also be very thin (50–100 nm) requiring specimen embedment in a resin and subsequent sectioning, although this limitation has elegantly been circumvented by the preparation of plasma membrane sheets from the apical cell membranes of cultured cells (37, 130, 170). This method is particularly useful because both inner and outer membrane leaflets are accessible for immunostaining while membrane architectural features such as clathrin-coated pits and caveolae are preserved. It has found considerable use in identifying single putative raft markers and clusters thereof, and in mapping their location relative to membrane features. Application of spatial statistics to particle positions in images has additionally allowed for quantitative determination of domain presence and average domain size (37, 169).

Using plasma membrane sheets, Hancock and coworkers determined that GFP fused to a membrane targeting region from H-Ras (a raft marker) was found in cholesterol-sensitive membrane microdomains that covered 35% of the plasma membrane and had an average diameter of 44 nm (130, 169). This result is in contrast with data obtained by SMFT, which found that 30–40% of the same membrane marker, but in different cell-types, was localized in 200 nm cholesterol-insensitive domains (49). This discrepancy has not yet been resolved.

Also using plasma membrane sheets, Wilson et al. have recently reported on the distinctly different localization patterns of putative raft markers in resting mast cells (37). In the absence of external cross-linking, GPI-AP Thy-1 coclustered with the dually palmitoylated single transmembrane adapter protein LAT, the IgE receptor Fc ϵ RI was predominantly found in separate 20–50 nm diameter microdomains, and the ganglioside GM1 was uniformly distributed. Upon cross-linking, GM1 and Fc ϵ RI were found in nonoverlapping osmiophilic (indicative of unsaturated lipids) microdomains, sometimes exceeding 500 nm in diameter, whereas Thy-1, increasingly coclustered with LAT, was found in microdomains of similar size but with no apparent preference for osmiophilic microdomains. Only after simultaneous cross-linking were Fc ϵ RI and GM1 found colocalized (also in osmiophilic microdomains). Consistent with these observations, Madore et al., using more conventionally prepared specimen sections, found that the GPI-APs Thy-1 and prion protein (PrP) on neurons are predominantly organized in separate domains (171), which showed distinctive differences in detergent solubility and lipid compositions (168, 171).

These studies demonstrate the potential for using TEM to qualitatively and quantitatively probe for the existence of microdomains in both the outer and inner membrane leaflets in fixed cells. Data reported thus far are consistent with an elementary raft size of 50 nm and much larger aggregation-stabilized rafts. Intriguingly, the increased resolution available by TEM has revealed the possible existence of a large heterogeneous population of membrane domains that may correlate with DRMs (37, 168, 171). In particular, the absence of coclustering of GM1 and the GPI-AP Thy-1 is noteworthy because arguably the two most widely used raft markers to date have been CTB for GM1 and antibodies to GPI-APs such as Thy-1 (34, 43, 110). However, this observation is consistent with FRET data, which also failed to find evidence for heteroclustering between GM1 and several GPI-APs in HeLa and NRK cells (34). The absence of coclustering between the GPI-APs Thy-1 and PrP likewise indicates the existence of a large population of membrane domains that correlates with DRMs, or alternatively that a functional role, in addition to the GPI-anchor, may be required for lipid raft localization (168, 171).

14. EMERGING METHODS

A number of nascent optical imaging methods are noteworthy. There have been several recent reports on new versions of light microscope methods designed to operate beyond the diffraction limit. These methods include the use of structured

illumination (172, 173), harmonic excitation light microscopy (174), and zero-mode waveguides (175). A major effort to define the ability of small metallic structures deposited on surfaces to fine-tune the properties of nearby fluorophores is underway (176). Other optical methods under development for cell membranes include imaging signals from surface plasmon resonance (177, 178), surface-enhanced Raman scattering (179), and second-harmonic generation (180, 181).

The complexities associated with using membrane markers could in principle be circumvented by developing methods less dependent on their use by providing spatially resolved information with chemical sensitivity. A possibility within this category is mass spectrometry imaging. Although still in the early stages of development, imaging based on time-of-flight secondary-ion mass spectrometry has been used to view aspects of the lipid distribution across mating protozoans, albeit after a rather harsh freeze-fracture preparation, but with submicron resolution (182). In a related work, after freeze drying and deposition on silver substrates, phosphatidylcholine and cholesterol distributions were imaged in erythrocyte membranes (183). Another possible technique that does not rely on extrinsic labels but is capable of obtaining chemical information is infrared NSOM (184). In this method, an aperture-NSOM probe is used to collect infrared light reflected from fixed, dried or live cells with a subdiffraction limited spatial resolution of ~ 100 nm.

Creative new approaches to thermodynamic studies of lipid phase behavior in model systems have the potential for significantly increasing our understanding of the factors that drive putative protein/lipid microdomain formation, dissolution, size, and composition (2). In addition, a serious understanding of lipid/protein microdomains, because of their probable dynamic nature, may ultimately require considerable new work in the area of molecular dynamics simulations (185, 186). Because both of these areas highlight possible intermolecular and intramolecular interactions between specific chemical groups, the results have the potential for stimulating the formation of new, more chemically nuanced hypotheses about the molecular nature of microdomains in biological membranes.

15. EXPERIMENTAL CONSIDERATIONS

A variety of methods that have been or might be used to investigate the lipid raft hypothesis have been discussed. In comparing and evaluating these techniques, it is important to consider a number of factors. Some methods require extensive sample preparation including cell destruction, others require chemical fixation, and some can be carried out on live, intact cells. Ultimately, it is important to examine live cells because of the growing evidence that membrane microdomains change in size, lifetime, and chemical composition during cell function. The role of membrane remodeling (i.e., uptake, deposition, and recycling of membrane components) as well as enzyme catalyzed lipid turnover in the membrane can also be best addressed in live cells. Different methods have different spatial and temporal sensitivities, and these are also likely to be critical factors because of the

growing evidence that domains can, under certain circumstances, be very small and very dynamic. The degree to which certain membrane markers (e.g., those that are multivalent) might perturb or induce microdomains is also of both experimental and physiological importance. In addition, different techniques vary in their ability to work at subsaturation to saturation of membrane markers or microdomains. This factor is important because at subsaturation levels only tight-binding sites might be probed and at saturation levels the nature of the microdomains might be perturbed by competition effects.

16. CONCLUSIONS

Following the introduction of the functional lipid raft hypothesis, many authors have presumed the existence of lipid rafts although there is at present significant uncertainty about the issue. The existence of DRMs suggests the possibility that these fractions are derived from entities that pre-exist in cell membranes. In addition, model membrane studies suggest a physical basis for raft formation, i.e., that membrane lipids can differentiate into different coexistent liquid-like phases. However, neither of these two groups of results proves that rafts, as they were originally conceptualized, exist *in vivo*.

Most certainly, there are a variety of spatial, temporal, and compositional membrane inhomogeneities participating in processes underlying cell function. However, the possibility that microdomains of significant biological importance exist solely through lipid-lipid interactions seems at this point unlikely particularly in view of the high surface concentration of membrane proteins. Rather, it is thought that domains result from concerted lipid and protein interactions, are nonequilibrium, dynamic entities, are cell-type specific, and arise or dissolve in response to various biochemical influences.

It seems reasonable, then, to shift the attention in the lipid raft field from the search for stable, lipid-driven liquid-ordered phases (rafts) present in a surrounding environment of a liquid-disordered phase to the identification and characterization, under defined physiological conditions, of what might be better termed as membrane microdomains. Microdomain composition, size, and lifetime are still unanswered questions. Also unavailable is a categorization of the heterogeneity of microdomain types, as well as the degree to which microdomains depend on cell type, environment, and history.

We have described a variety of methods that have been or might be used to detect membrane microdomains; emphasis has been on their characterization in the plasma membranes of intact, live cells. Although no single technique can be expected to definitively solve this very important issue, nonetheless, the large variety of methods available or under development should facilitate significant contributions. At present, the best approach toward understanding membrane microdomains is to compare and contrast results obtained with the variety of existent methods, looking for consistencies between results, as well as exploring new techniques as they are developed.

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