

Membrane Lipid Domains: Techniques for Visualization and Characterization

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Abstract: Early studies in to lipids that comprised cellular membranes noted an asymmetrical distribution of lipids between different membranes which contrasted with the contemporary concept of the plasma membrane as a homogeneous mixture of lipid in which both lipids and membrane-associated proteins exhibit unfettered mobility. The ability of lipids to form inhomogeneities capable of restricting lateral mobility has since been demonstrated in both model and cell membranes. It has further been shown that lipids possess the ability to preferentially associate and co-exist in domains rich in particular classes of lipid, most notably cholesterol, sphingolipids, and glycolipids. In cells, 'rafts' further appear to be enriched in raft-targeted proteins. These observations have led to the hypothesis that phase-separated domains may act as signaling platforms capable of recruiting proteins thus facilitating their interaction for transduction of cellular signals.

Experimental evidence largely based upon detergent isolation of raft domains has since been provided implicating laterally organized lipid domains in a litany of both physiological and pathophysiological processes including T-cell activation, B-cell antigen signaling, thromboregulation, Alzheimers disease and atherosclerosis. However, the field is often criticized for interpretations which may lack physiological relevance due to artifact arising from isolation procedures. Thus, investigators are increasingly looking to spectroscopic techniques to obtain information about phase-separated domains. This review seeks to summarize the current knowledge and understanding of raft structure, and provide a first foray in to the fundamentals and application of spectroscopic and microscopic techniques to characterize attributes and dynamics of cellular lipid microdomains.

Keywords: Lipid raft, Phase separation, Cell signaling, Cellular membrane, Spectroscopic techniques, Lipid probe.

1. INTRODUCTION

Early conceptualization imagined the plasma membrane as a homogeneously mixed bilayer of lipids [1], in which lipids and proteins associated with the bilayer were thought to exhibit unfettered lateral mobility.

Subsequent studies conducted in model membranes observed the co-existence of lipid domains of different compositions and raised the possibility that the cellular lipid bilayer could also possess regions of microheterogeneity. This theoretical framework has since been expanded to incorporate the notion of preferential lipid packing and resultant membrane domains enriched in particular lipid classes which specifically associate with lipid-modified or lipid-binding proteins, thus restricting their lateral mobility and allowing both spatial and temporal organization of functional signaling complexes.

Model systems have proven useful in the study of phase-separated domains and yielded a wealth of information regarding forces that drive lipid segregation [2], the physical differences between raft / non-raft regions [3] and insights into ambiguity surrounding cellular lipid domains [4]. Although there still exists significant ambiguity regarding the nature of co-existing liquid-ordered and disordered domains in cellular systems, it is well accepted that ternary lipid mix-

tures exhibit complex phase behaviour dependent upon the specific lipid composition in model systems.

Inquiry into the nature and role of cellular membrane domains has been driven by the belief that membrane lipid composition exerts influence over the transduction of cellular signals and conversely that cellular cues can alter the association of signaling molecules with membrane domains [5]. A framework for investigation of the role of raft domains in signaling can thus be based upon changes in the affinity of a signaling molecule for a region of the membrane whether through 1) changes in the lipid composition of the domain or 2) through changes in the chemical composition of the signaling molecule which alters its affinity for the domain. Implicit in this statement is the notion that phase separated regions of the membrane are dynamic in structure, composition and molecular associations.

It is exactly these characteristics which have hampered advances in the field and contributed to variation in experimental conclusions. Much of the early evidence regarding cellular lipid rafts has been derived from methods of isolation based upon detergent resistance. Membrane isolates have yielded micron sized domains enriched in cholesterol, saturated phospho- and sphingolipids and a variety of 'raft-associated' proteins, hence the term microdomain. However, isolation techniques are fraught with potential artifact and may not represent equilibrium conditions of the intact cellular membrane. It has been noted that the low temperature conditions and detergents employed in isolation of detergent-

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resistant microdomains may induce phase transition-driven coalescence of lipids [6]. It is also difficult to exclude contamination derived from intracellular membranes, and maintain the properties of biological samples during extractions and isolation due to lipid oxidation and dehydration. Further, detergents may also solubilize some raft proteins, thus underestimating the lipid raft content. These various factors likely account for detergent and cell type differences observed in detergent-resistant membrane preparations.

Instrumental methods have thus become increasingly attractive for characterization of phase separation in lipid membranes and spurred the development of a number of spectroscopic and microscopic methods that have facilitated study of the properties of intact cellular membranes. This review seeks to provide the novice with a basic understanding of the forces which are believed to govern the structure of lipid domains and their resultant properties. It further provides a survey of instrumental techniques available to study these domains in cellular systems, along with their strengths and weaknesses and a summary of germane findings.

2. PHASE SEPARATION OF LIPIDS IN MODEL SYSTEMS

A major aspect of the raft hypothesis is the belief that lipid interactions are sufficient to drive the lateral associations that give rise to phase separation and formation of raft domains. Indeed numerous papers have since demonstrated the formation of phase-separated domains in model systems [7, 8, 9, 10, 11], and provided a basis for understanding the forces that contribute to their formation.

The mixture most commonly accepted to crudely mimic phase separation of a cellular membrane (sometimes referred to as the 'canonical' raft mixture) is generally defined to be an equimolar mixture of cholesterol, a low melting point lipid (such as an unsaturated or short chain phospholipid) and a high melting point lipid (such as a long chain, saturated phospholipid, or sphingolipid) [6, 12, 13].

Domain formation, as evidenced from similarly composed model systems, has been found to be induced by forces which include 1) sterol-acyl chain interactions [10] which can promote mutual immiscibility and lead to domain separation and 2) lipid head group interactions [14]. Resultant domains thus exhibit altered mobility, dynamics and permeability reflective of either liquid-ordered or liquid-disordered domains, rather than homogeneously dispersed lipid mixtures.

Sterol-Acyl Chain Interactions

Cholesterol is critical to the structural integrity of raft domains and there have been numerous reports of the disruption of raft structure by agents which selectively deplete cholesterol from the plasma membrane [15, 16, 17]. However, cholesterol was initially a confounding factor in initial acceptance of the raft hypothesis, as its presence in mixtures of high melting point lipids such as dipalmitoylphosphatidylcholine (DPPC) and sphingomyelin (SM) induced the transition of both gel and liquid-disordered domains to a highly ordered structure that however maintained high lipid mobil-

ity eventually termed the liquid-ordered phase. Nevertheless, the ability of lipids bearing long, saturated acyl chains to form tightly-packed, lipid-lipid complexes with sterols in the presence of unsaturated lipids thus promoting raft formation is now widely held.

Indeed, early observations noted the non-ideal behaviour of lipid mixtures comprised of cholesterol and phospholipids [18] and the observed 'condensing effect' or the ability of cholesterol to decrease the molecular volume of phospholipid and sphingolipid acyl chains, continues to be explored and refined to this day [19]. It is believed that the condensing effect is promoted by extensive van der Waals interactions of the sterol skeleton along the length of the non-polar acyl chain, which increase with the saturation and length of the chain. This effect may confer detergent-resistance and selective protein targeting to membrane microdomains and is believed to be a 'driving force' behind the formation of raft structures [20], an assertion that finds experimental support [21, 22].

The structural features of a sterol also appear to influence its ability to promote formation of liquid-ordered domains, observing that minor modifications to cholesterol structure were capable of suppressing domain formation without complete disruption, whereas others had little to no effect [10, 23].

The ability of polyunsaturated lipids to promote phase separation in model systems has also been probed using a variety of techniques including atomic force microscopy (AFM), differential scanning calorimetry, nuclear magnetic resonance (NMR) spectroscopy and detergent extraction [24]. Results appear to indicate that phospholipids bearing polyunsaturated fatty acids such as docosahexaenoic acid may promote phase separation to a greater degree than their monounsaturated, fatty acid-bearing counterpart both in model and perhaps in cellular systems.

Head Group Interactions

Lipid head group interactions also play a significant role in promoting phase separation and determining localization of lipids to liquid-ordered domains, however given the heterogeneity of natural lipids, inquiry into this area is complex. Partitioning studies designed to measure the cholesterol partition coefficient and partition free energy in large unilamellar vesicles suggest that cholesterol favours interaction with SM > phosphatidylserine > phosphatidylcholine > phosphatidylethanolamine [21], thus underscoring the role of headgroup interactions in preferential association of lipids in agreement with earlier studies [25, 26]. Interestingly, NMR studies probing the order parameters of various phospholipids in multilamellar systems in the absence of cholesterol have concluded that the lateral packing of phospholipids is determined more by the methylation state of the headgroup than the acyl chain length [27].

Headgroup interactions may also facilitate localization of glycosphingolipids (GSLs) to ordered domains by specific hydrogen bonding [28, 14], although the nature of these interactions is still being debated. It has been asserted that interactions between headgroup sugar moieties of GSLs are weak [29, 30] however, in the absence of a strong interaction

with cholesterol [31], what forces compel localization of GSLs to liquid-ordered domains appears to remain open to question.

Together these interactions are likely a primary source of segregation within the membrane and may constitute the basis for detergent-resistance due to the hampered ability of detergents to interact with and solubilize lipids. For further review of the fundamentals of raft formation in model systems and the analysis which informs our understanding of cellular lipid rafts, one is directed to recent reviews from the group of Maxwell [32, 33].

3. AGENTS USED IN LIPID RAFT IMAGING AND ANALYSIS

There are a variety of agents used in imaging and characterization of cellular lipid microdomain properties. These agents have been largely directed towards what are believed to be the major lipid components of rafts, including cholesterol, SM and glycolipids. Investigators have also employed specific antibody- [34] and fluorescent protein-labeling [35] of putative raft proteins and coupling of fluorescent proteins to glycolipid anchors [36, 37] and lipidation consensus sequences [38], however we will restrict our discussion specifically to the three main classes of lipid-binding probes.

3A. Cholesterol-Directed Agents

Filipin

Filipin is a polyene macrolide with low antibiotic activity isolated from cultures of *S. filipinensis* [39, 40]. Discovery of the ability of filipin to promote leakage of cellular contents led investigators to hypothesize the formation of a sterol:antibiotic complex, observe cholesterol binding to a filipin complex [41, 40] and subsequently employ it in staining erythrocyte membranes [42, 43].

It has since been used extensively to visualize cellular cholesterol [44, 45, 46, 47, 46] but investigators have noted the ability of filipin to disrupt raft structure [49], cause lesion formation [50, 51], induce significant cellular damage in unfixed cells [52] and provide false-negative cytochemical results [53]. Alternately, authors have noted that 1) lesions formed appear to vary with the cholesterol content of the membrane [54] and 2) prior fixation of cholesterol-containing cells or osmotic balancing can prevent membrane damage and lysis [43].

Viable studies employing filipin investigated the distribution of cholesterol by electron microscopy in cellular membranes of primary smooth muscle cells (SMCs) as they de-differentiated from a contractile to synthetic phenotype [55]. Fixation of cells with glutaraldehyde in differentiated and de-differentiated cells revealed distinct differences in the distribution of plasma membrane filipin-sterol complexes visualized as membrane protrusions / distortions. Synthetic cells exhibited a random distribution of filipin-induced protrusions throughout the plasma membrane and a diminished content of caveolae. In contrast, contractile SMCs exhibited both a higher quantity of caveolae as well as higher concentration of membrane deformations within caveolar invaginations. Both types of cells were also treated with various agents to assess the effects of cholesterol modulation on

membrane distribution of caveolae and caveolar and non-caveolar filipin-sterol complexes. Treatment of contractile cells with LDL increased the number of non-caveolar complexes, but had little effect on the quantity of both caveolae and caveolar complexes. Treatment with mevinolin (a HMG-CoA reductase inhibitor) had little effect on the number of caveolae in either contractile or synthetic SMCs but diminished the number of caveolar filipin complexes. Alternately, treatment with β -cyclodextrin caused a near to complete loss of both caveolae and filipin disruptions.

In light of the various reports of the cytotoxic effects of filipins and the need to fix cells in order to circumvent damage, this probe appears now to be used more a means of implicating lipid rafts in cellular processes by ablation [56, 57, 58] rather than a means of assisting visualization.

Perfringolysin O

Perfringolysin O (PFO / θ -toxin) is an example of a thiol-dependent cytolysin whose cholesterol-binding activity has been well characterized [59, 60] and exploited to selectively label and visualize cholesterol-enriched rafts in cellular membranes. Early studies employed a proteolytically-nicked, biotinylated, non-cytolytic derivative of PFO (BC θ) to visualize cholesterol-rich microdomains in cellular membranes by immunoelectron microscopy [61], and detect cholesterol by fluorescence cell sorting [62] and fluorescence microscopy [63]. Subsequent efforts to characterize the cholesterol-binding activity of PFO have demonstrated that the C-terminal domain four (D4) of PFO, comprised of 110 amino acids, retains the ability to bind free cholesterol (but not esterified cholesterol or phospholipids) with the same affinity as the full length toxin by surface plasmon resonance [59]. Further, it was shown that the D4 fragment binds to live cells in a cholesterol-dependent manner and could be co-isolated in the detergent-insoluble fraction of MOLT-4 cells with several signalling proteins believed to be localized to lipid rafts [64].

Cyclodextrins

β -cyclodextrins are torus-shaped, heptameric oligosaccharides, the hydroxyl groups of which orient toward the outer circumference imparting water solubility. Conversely, the interior of the torus is inhabited by carbohydrate carbons and hydrogens and is largely hydrophobic. The structural features of the β -cyclodextrins thus enable the solubilization of normally insoluble lipid species [65] have been shown to exhibit high affinity, in particular for sterols [66, 67]. Although not used directly in imaging of lipid rafts, cyclodextrins and particularly methyl- β -cyclodextrin (M β CD), have been perhaps the most widely used agents for modulating raft structure by both depletion [68, 69] and enrichment [70, 71] of cholesterol during attempts to visualize raft characteristics.

However, recent studies suggest that despite the widespread use of M β CD to modulate membrane cholesterol, it may induce cellular effects independent of its cholesterol-extracting ability [72] and that its sterol specificity may also be questionable [73]. In light of the potential for misinterpretation of results, investigators typically corroborate observations made following cyclodextrin treatment with experi-

ments that metabolically deplete cells of cholesterol with HMG-CoA reductase inhibitors.

3B. Glycosphingolipid-Directed Agents

The non-toxic cholera toxin B (CTB) subunit binds to the glycolipid – ganglioside M1 (GM1) (Gal β 1,3GalNAc β 1,4(NeuAc α 2,3)Gal β 1,4GlcCer) with high affinity [74] and has been used extensively in the imaging and characterization of lipid rafts [75, 76, 77, 78]. GM1 is a well-accepted raft marker that has been shown to form GM1 domains in model membranes comprised of a raft mixture [79] and labelled with CTB for determination of diffusion properties in cholesterol- and SM-enriched liquid-ordered domains [75].

Interestingly, imaging studies in which SM- and GSL-enriched domains were simultaneously labeled with lysenin and CTB respectively, revealed no colocalization of the probes, suggesting that these are distinct regions of the membrane [80]. Further reports are now coming to light which challenge the utility of CTB as a probe for GM1 suggesting that there is limited correlation between binding and levels of GM1 in the membrane [81]. Alternately, use of the GSL synthesis inhibitor, D-threo-1-phenyl-2-decanoyl-amino-3-morpholino-1-propanol (D-PDMP) [82] may assist in distinguishing between non-specific and GM1-specific labelling in intact cells.

3C. Sphingomyelin-Directed Agents

Cholesterol-rich raft domains are also believed to be enriched in SM. As an abundant and important component of the cell membrane, its detection and analysis can assist in characterization of microdomains and their dynamics. Lysenin is a hemolytic and cytotoxic 41 kDa protein isolated from the coelomic fluid of *Eisania foetida*, demonstrated to specifically bind clustered SM triggering oligomerization and inducing formation of 3 – 5 nm diameter pores [83, 84]. It has thus been used a lipid- and organization-specific probe in similar fashion to the cholesterol-dependent cytolitics, in which a truncated version bearing the C-terminal SM-binding domain but lacking the N-terminal cytolytic domain has been shown to be non-toxic but able to bind membrane SM [80, 85].

4. INSTRUMENTAL TECHNIQUES

Although inquiry concerning the nature of isolated, detergent-resistant membrane domains has been the dominant form of inquiry in to the field of lipid rafts, efforts now appear to be focused on determining their cellular correlate. Spectroscopic methods cause little to no perturbation and thus provide a promising means of accessing raft properties of the intact cellular membrane. Conventional fluorescence microscopy has been used in attempts to visualize cellular raft domains via the imaging of putative raft proteins as suggested by detergent resistance. However, these images generally reveal diffuse distribution on the cell surface [86] and belie any significant order. Alternately, treatment of cells to induce clustering or cross-linking of these species can result in punctate staining patterns observable at the resolution of conventional optical microscopy (personal observations). Thus it has been suggested that lipid rafts and associated

marker proteins may exist in un-stimulated cells in ordered structures with dimensions on the order of nanometres and prompted investigation with optical methods possessing resolution capable of imaging these structures and probing their properties. The following sections will discuss these techniques, providing a brief discussion of their principles and a survey of results obtained in support of the existence of submicron, ordered domains in intact cellular membranes.

4A. Atomic Force Microscopy

Atomic force microscopy (AFM) is a powerful tool for imaging the topology of semi-fluid surfaces with both high horizontal (~1 nm) and vertical (~0.1 nm) resolution [79], an inherent strength in characterizing in-plane lateral microdomains of lipid bilayers. This technique is amenable to the study of semi-fluid surfaces bathed in solutions of physiological relevance and has yielded novel insights into structural features of bilayer lipid domains in model systems and factors which influence their formation [85, 6, 86, 89, 90, 91, 92].

To date, AFM has been directed primarily to the study of supported model lipid bilayers and examples of its utility in the study of cellular lipid domains remain few, due to the many obstacles one encounters when attempting high-resolution imaging of a complex surface, such as the exofacial membrane of the cell. However, AFM provides flexibility in obtaining images by allowing operation in several different modes, allowing one to circumvent obstacles encountered in application of this technique to live cells [93].

These modes depend largely on the nature of the interaction between the cantilever tip and the underlying substrate. Contact (static) mode is conducted such that the force between the surface of interest and the tip is maintained constant by feedback from a piezoelectric sensor and deflection of the cantilever from a point of origin is measured by reflection of a laser off the top of the cantilever. As the probe tip moves laterally across the sample, vertical deflection can be measured with high resolution.

Fluid tapping mode is a variation of AFM that allows the visualization of delicate or loosely adherent structures by overcoming technical obstacles that arise from friction, electrostatic drag and adhesion. It does so by oscillating the cantilever in a manner in which the tip oscillates vertically at a rate of 50 – 500 thousand s^{-1} with amplitude of ~20 nm, alternately touching and then being removed from contact with the surface of interest. Surface contact necessarily alters the energy / amplitude of the oscillation, which is then correlated with topographical features. It is noteworthy that application of tapping mode AFM can also be done in liquid media [94] (as opposed to air or vacuum), however generally at a lower frequency 4 - 50 thousand s^{-1} or using softer cantilevers.

Studies point to the role that both contact force and tip composition play in the ability of AFM to image a surface without either penetrating the membrane or probing primarily submembranous structures. Deformation of a cell under the tip forces required for stable imaging of the cell surface (1- 30 nN) [95] is deemed to be significant but also a limiting factor in obtaining high lateral resolution required for lipid domain imaging. Imaging of soft biological samples

then becomes a delicate balancing act between applying sufficient pressure to obtain high resolution while avoiding sample distortion. Tip composition also plays a significant role in forces between the sample and tip and changes in force that are ostensibly topological may be due to a change in force interaction arising from the chemical makeup of the tip. Alternately, an interesting variation on AFM employs surface-modified cantilever tips in the measurement of force constants across a membrane [96, 97]. This provides exciting opportunity to probe lipid dynamics using specific lipid or raft-protein interacting probes.

Attempts made to study the surface topology of renal cells have made apparent some of the obstacles presented to characterization of cellular lipid domains [98, 99]. Indeed, surface protuberances, such as microvilli in close proximity to one another, and membrane proteins, whether transmembrane or peripheral, may hamper close proximity analysis of the membrane lipid domains.

Later work published using a low-force variation of AFM [100] showed that imaging of the CV-1 kidney cell surface can be obtained at low (20 – 50 pN) tip forces and reveals substantial heterogeneity of the membrane surface at as high a resolution of less than 10 nm. These forces are 1/100th to 1/1000th the magnitude of those commonly used in AFM imaging of cells [99] and although the problem of topographical deformation is not wholly circumvented, image distortion is certainly minimized. This variation further has the advantage that tip penetration in to the cell was deemed to be less than 10 nm, thus minimizing potential damage to the cell and contribution from intracellular structures. Despite the low forces employed in these studies, images routinely revealed submembrane, filamentous structures resembling intracellular cytoskeletal architecture in coexistence with smoother zones. Authors suggested that the regional variation in coupling between submembranous structures and the plasma membrane could account for these observations. Authors were able to obtain better lateral resolution by restricting the scan size and resulted in images in which the cell surface revealed granulation likely due to protein or protein-lipid complexes. Authors further noted their inability to image 3T3 fibroblasts and attributed this to marked variation in cell exterior that may render low force imaging impossible.

Recent efforts to image cellular lipid domains have coupled fluorescence imaging and AFM to probe the 3D topology of membrane stripped from fixed tumour mast cells [101]. Studies revealed raised regions that labeled positively for immunoglobulin E receptors (FcεRI), GM1 and clathrin and could be diminished in size by treatment with MβCD.

Interpretation of data obtained from a complex surface such as the exofacial membrane will undoubtedly provide challenge, yet these studies represent viable attempts to correlate features and behaviour of model, extracted and fixed cellular lipid domains with microdomains of live cells.

4B. Fluorescence (Förster) Resonance Energy Transfer (FRET)

The inherent ability of FRET to detect very short range interactions between fluorophores (1-10nm) [102] by non-radiative resonance energy transfer from an excited donor to

a ground-state acceptor species has yielded considerable insight into the dynamics and heterogeneous distribution of 'raft-associated proteins' of the plasma membrane. Dependence of resonant energy transfer efficiency (ϵ) upon the distance between donor and acceptor is defined by $\epsilon = 1/(1+(R/R_0)^6)$, where R_0 is the Förster distance. Non-radiative transfer of resonance energy requires significant overlap of the donor emission and acceptor excitation wavelengths, but further is influenced by the orientation of fluorophore dipoles [103] and density of donor and acceptor species in the cell membrane [104].

In a novel demonstration of raft association, FRET was used to demonstrate the enrichment and homodimerization of the ErbB2 transmembrane receptor tyrosine kinase in membrane subdomains, as well as its raft exclusion upon cross-linking of raft GM1 with fluorescently-labeled cholera toxin. Further, exclusion of ErbB2 diminished its heterodimerization with ErbB3 in a manner that reduced tyrosine phosphorylation [105]. In this study, the extent of energy transfer for the ErbB2 donor-couple was found to be density independent, an observation interpreted as denoting specific localization to sub-pixel sized domains in the membrane of the living cell. That this paper avoided use of raft depleting/disrupting agents illustrates a novel approach to associate the activity of a signaling molecule with segregated lipid domains. In light of recent reports indicating the influence of signaling molecules such as nitric oxide over the structure / molecular composition of raft domains [106], it would be of interest to determine if such physiological signals can functionally alter ErbB2 and its homodimerization.

FRET has also been used to demonstrate the organization of GPI-linked proteins in cellular membranes to regions believed to be < 70 nm in diameter [37]. Investigators measured the distance between both a GPI-linked and transmembrane chimera of the folate receptor by measuring changes in anisotropy to determine whether they were randomly or non-randomly localized. Transfer of resonant energy between a donor and acceptor fluorophore occurs with loss of polarization of net fluorescence emission. Thus, for a randomly distributed molecule, the angular dependence of fluorescence will increase as the density of the fluorophore decreases. In contrast, spatially confined molecules will exhibit a density-independence of anisotropy, indicative of nanometer scale association. Interestingly, this study employed a fluorescent folate analog (N^α-pteroyl-N^ε-(4'-fluoresceinthiocarbamoyl)-L-lysine) (PLF) to label folate receptor in both cases, thus basing measurements on homo-resonance energy transfer (donor and acceptor are the same fluorophore) rather than conventional donor-acceptor couples in which there is overlap of respective emission and excitation. This study demonstrated an inverse relationship between density and anisotropy of the fluorescently-tagged, transmembrane folate receptor over a range of fluorophore densities. In contrast, its GPI-linked counterpart, exhibited density-independence of anisotropy indicating the importance of the GPI moiety in targeting the folate receptor to submicron sized domains in the cellular membrane. The importance of cholesterol in maintaining spatial confinement was revealed by the observation of an increase in anisotropy for the GPI-linked receptor upon removal of cellular cholesterol by either MβCD, saponin or compactin. GPI-linked proteins imaged by con-

ventional microscopy often exhibit diffuse immunostaining patterns on the cell surface, thus belying domain partitioning. This finding served to underscore the limitations of conventional fluorescence microscopy in the study of submicron domain structure.

Similarly, Sharma *et al.* employed homo-resonance techniques based upon GPI-linked fluorescent proteins and monitored fluorescence depolarization [36]. The loss in fluorescence anisotropy provides a sensitive handle for measuring interfluorophore distances. Study of HOMO-transfer of several GPI-anchored fluorescent proteins versus their transmembrane-anchored counterparts in CHO and MDCK cells led the authors to conclude that the GPI-anchor facilitates formation of clusters of 4-5 nm for a significant fraction of these proteins in the membrane. Further, loss of the HOMO-FRET fast anisotropy decay observed upon treatment with agents that deplete/disrupt membrane cholesterol, suggest again that cluster formation is a cholesterol-dependent process.

FRET has also been used in concert with fluorescent raft and non-raft partitioning lipids to monitor the re-localization of a CXCR-1 chemokine receptor cyan-fluorescent protein adduct to raft regions upon ligation with interleukin-8 [35]. Comparisons of the resonance energy transfer between the donor CFP-CXCR-1 adduct and either DiIC16 or FastDiI were used to monitor association with raft and non-raft domains, respectively. Investigators measured FRET efficiency by monitoring the increase in donor fluorescence following photochemical destruction of the acceptor, with an increase in donor fluorescence taken as indication of colocalization. FRET efficiency was observed to increase upon stimulation with interleukin-8 when DiIC16 was used as the acceptor in contrast to an observed decrease in efficiency when FastDiI was employed. Notably, in the absence of stimulation, the CXCR-1-CFP donor showed localization in both raft and non-raft domains and greater FRET efficiency in the FastDiI regions. Results, bolstered by mobility measurements, suggest that ligation of the CXCR-1 receptor by interleukin-8 induces its relocalization to raft regions for functional association with heterotrimeric G-proteins and transduction of signals.

Collectively, these studies serve to illustrate a means of showing the functional association of putative raft-associating proteins with phase-separated domains in live cells, thus complementing or circumventing methods based upon detergent-resistance.

4C. Proximity Imaging

This relatively novel technique is based upon spectral changes induced by structural perturbations upon interaction between GFP adducts. It has been observed that upon close proximity interaction between GFP molecules that the relative fluorescence emission upon excitation at 395 vs. 475 nm changes thus providing a ratiometric means of assessing protein clustering [38]. Authors fused GFP to a GPI-anchor and expressed the construct in HeLa cells and compared the fluorescence properties to that of a GFP moiety fused to a palmitoylation signal. Results revealed distinct differences in the fluorescence profile of GPI-anchored GFP versus that of the palmitoylated fusion product and soluble GFP suggesting, in

accordance with the observations of others, that the GPI membrane anchor is capable of mediating homotypic protein interactions.

This technique has also been used to investigate the signals which induce raft re-organization in cellular lipid domains in the context of atherogenesis. Investigators validated the localization a GPI-linked thermotolerant GFP (GPI-anchored ttGFP) chimera [106]. This was an important first task in light of the noted variation in localization of GPI-anchored proteins noted by other groups [107]. The authors then sought to assess the effects of both pro- and anti-atherogenic stimuli on raft-dependent GFP interactions. Authors observed that the PRIM ratio (measure of homotypic protein interactions) increased in a dose-dependent manner upon treatment of cells with either sodium nitropruside (SNP), S-nitrosoacetylpenicillamine (SNAP) or bradykinin. In contrast, cells transfected with either wild-type ttGFP or with ttGFP tagged with palmitoylation or prenylation consensus sequences remained stable upon SNP treatment. Taken together these results suggest that the NO-dependent increase in distance between GPI-anchored ttGFP, as evidenced by the increase in PRIM, is dependent upon GPI-mediated localization of the probe. In contrast, treatment of cells with either M β CD or cholesterol oxidase resulted in substantial increase in the basal PRIM ratio suggesting the loss of ttGFP clusters. PRIM analysis thus appears to be amenable to the study of raft-localized proteins and the effects of physiological stimuli on protein clustering.

4D. Fluorescence Correlation Spectroscopy (FCS)

FCS is a well-developed instrumental technique that can be applied to the experimental study of a wide variety of physical processes including chemical and photophysical reactions [108], receptor-ligand binding [109], lipid diffusion within model membranes bearing raft-like properties [7], protein conformational dynamics [110], and the spatial localization and behaviour of reporter molecules in the membrane [111, 112, 113].

This technique is particularly amenable to the study of raft structure and dynamics, routinely monitoring the properties of single molecules and aggregates with high spatial resolution in a non-disruptive manner. FCS achieves this by the use of laser excitation, restricting the focal volume in which molecular events are observed to the femtolitre scale and computation of the autocorrelation function for emission intensity as it fluctuates around equilibrium [114]. Non-equilibrium fluctuations in the light emitted from a fluorescent molecule depend upon and allow one to probe association / dissociation processes, protein conformational changes and lateral diffusion in the membrane with a lower limit 10^{-10} cm² s⁻¹ [115]. For a more detailed consideration of the practical and theoretical principles of FCS, there exist a number of comprehensive reviews [116, 117, 110]

Lipid microheterogeneity implies that proteins and lipids localized in liquid-ordered domains will exhibit altered lateral mobility versus those in liquid-disordered domains or in homogenous mixtures. FCS displays sensitivity to phase- and composition-dependent viscosity and consequently can distinguish localization on the basis of mobility [118]. However, the dynamic range of FCS, although ideal for fast-

moving species and photophysical processes, has limited applicability in the study of more slowly moving aggregates such as multi-component aggregates at the cell surface. Additionally, an inherent short-coming of FCS encountered in the study of phase-separated lipid domains on the surface of cells is the difficulty in distinguishing between intensity fluctuations within the detection volume due to transit of fluorolabeled species within a lipid domain or due to diffusion of the entire domain itself. In light of the fact that correlation measurements and imaging are performed separately, this perhaps becomes an intractable question for investigation by this technique. However, recently developed variations in correlation spectroscopy (see ICS below) promise to overcome these difficulties via the spatial correlation of cell surface particle motion.

Nevertheless, FCS has been used in concert with confocal microscopy to visualize lipid segregation and measure diffusion coefficients in SM/dioleoylphosphatidylcholine (DOPC) giant unilamellar vesicles (GUVs). Lipid mobility was characterized as a function of cholesterol by obtaining autocorrelation curves for the lipophilic dye - 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-C18) which is excluded from SM-enriched regions and fluorescently-tagged GM1 within liquid-ordered domains. From this data, ternary phase diagrams were constructed and the authors noted the critical role that cholesterol plays both in inducing phase-separation and modulating lipid mobility in both liquid-ordered and disordered domains [7].

Additional work by the Schwille group has shown that more cholesterol is required to induce phase segregation in ternary mixtures of saturated phospholipids, SM and cholesterol versus ternary mixtures bearing unsaturated rather than saturated phospholipids. This agrees closely with the idea that the enhanced immiscibility and compromised interaction of unsaturated acyl chains with cholesterol/SM-rich regions promotes domain formation [119]. Interestingly, the domain size observed on GUVs employed in this study was on the order of microns indicating that other factors are required for small domain formation believed to exist in cells.

FCS has also proven valuable in distinguishing between raft and non-raft markers, drawing contrast from their diffusional behaviour in both model and cell membranes [77]. Authors investigated phase-dependent lateral mobility of CTB-labeled raft GM1 and non-raft dialkylcarbocyanine dye diI in labeled rat basophilic leukemia cells (RBL)-2H3 membranes and in GUVs. CTB-labeled GM1 was virtually immobilized in cellular membranes in contrast to the significant mobility displayed by DiI. Treatment of cells with M β CD reduced the mobility of the non-raft lipid in a manner that could be reversed by cholesterol restoration and had no effect on that of the raft marker. Mobility of the CTB-labeled GM1 was however increased by cytoskeletal disruption with Latrunculin A and attributed to interactions of GM1 with other raft lipids or proteins that interact with the cytoskeleton. This explanation was further supported by the observation that CTB-labeled GM1 displays only a two-fold lesser mobility than DiI-C18 in phase-separated GUV membranes.

In addition, FCS has been used to compare the behaviour of putative raft and non-raft markers in live cells [120, 121]. Investigators probed the diffusion behaviour of a variety of

raft and non-raft localizing fluorolabeled lipids and proteins in COS-7 cells and discussed results in terms of both a lipid- and cytoskeletal-dependent lateral organization of the membrane. Results indicate that different mechanisms account for variation in diffusion behaviour observed between the raft- and non-raft partitioning species. More specifically, lipid-dependent microdomain organization determines the mobility of sphingolipid analogs and GPI-linked proteins in a manner that could be altered by treatment with either cholesterol oxidase or with sphingomyelinase and most importantly that phase-separated domains appear to exist in the membranes of live cells under equilibrium conditions. Further, results indicate that although mobility barriers for putative non-raft transmembrane proteins (transferrin receptor and dipeptidyl peptidase IV) were lowered by treatment of cells with cytoskeletal-disrupting agents (latrunculin and cytochalasin), that interestingly these proteins also appeared susceptible to lipid-mediated barriers.

The aforementioned studies serve as demonstration of the viability of FCS as a tool for both assessing the interactions which may influence formation of phase-separated domains in model membranes and evaluating the properties of raft-localized proteins in cellular membranes. FCS provides valuable opportunity for correlating and contrasting observations made in model and cellular systems, bearing numerous advantages over other instrumental methods.

To extend the capabilities of FCS, fluorescence cross-correlation spectroscopy was devised as a variation that employs labeling of distinct molecular species and monitors their correlated motion to infer interactions or colocalization. Concomitant excitation of the fluorolabeled species with superpositioned lasers generates emission signals from the detection volume with the same temporal resolution as FCS. Interacting species will exhibit synchronized temporal fluctuations in fluorescence intensity from which information regarding the interaction can be extracted by means of cross-correlation analysis. Those looking to apply this technique to questions of raft dynamics are directed to more thorough reviews of the principles and practice of FCCS [122, 118].

One of the major advantages of FCCS to the study of protein localization within phase-separated lipid domains is that, in contrast to FRET analysis, there is no spatial proximity requirement, only that molecules display correlated motion [123]. Thus, molecules which co-localize on the basis of their affinity for lipid domains may exhibit concerted motion but not interact in a manner sufficiently accessible to FRET techniques.

FCCS has been used to study diffusion processes in supported multilayers of binary lipid mixtures [124], to investigate the domain preference of SNAREs in GUVs comprised of raft-forming lipid mixtures [125] and to demonstrate to stimulated association of IgE-FC ϵ R1 with tyrosine kinase Lyn [126], which is known to require dual fatty acylation for re-distribution [127].

4E. Single Particle Tracking

This technique monitors the lateral trajectory of a protein or lipid of interest labeled with either a fluorophore or a light

scattering bead with nanometer spatial and millisecond temporal resolution. Single particle tracking (SPT) experiments are versatile and can be based upon a number of reporter systems including gold- or fluorescently-labeled antibodies and beads, quantum dots, fluorescent protein fusion proteins, and fluorophore-labeled lipids and proteins. SPT bears the advantage of not measuring the averaged motion of a composite of labeled species but instead the individual trajectory of a single molecule, thus providing a more detailed characterization of its motion and potential domain interactions.

Following the trajectory of a labeled species over a membrane area on the order of microns allows determination of whether the motion adheres to Brownian principles or is 'anomalously instructive' [102]. From this information, one can then determine whether the mode of probe diffusion is confined to a static or mobile domain or exists within unstable/dynamic domains, and the average time of residence and diffusion behaviour within a particular domain using a number of developed mathematical treatments [128].

Spatial resolution of this technique is limited by the minute physical motion of the apparatus itself over the course of data collection and is determined by following the trajectory of a stationary particle. This technique is also limited by the lack of ability to resolve motion in the z-plane. Thus, changes in the height of the membrane can appear as artifactual anomalous diffusion and mandate that measurements be confined to flat regions of the membrane. Other potential problems or caveats include the size and multivalency of membrane labels. Motion depends on the hydrodynamic radius of the species of interest and thus addition of a large label to a lipid or protein species may significantly alter the diffusion behaviour of the particle. Multivalency of membrane labels are also a potential source of artifact in that adhesive cross-linking of membrane species has been observed to induce phase-separation in model membranes [129].

With proper consideration of the pitfalls of this technique, investigators have had success in characterizing the properties of raft domains. In order to explore the preferential association of GPI-linked proteins with GSL-enriched rafts, investigators tracked the motion of 1) a putative GPI-linked raft protein – Thy-1 2) the raft-enriched lipid - GM1 and 3) the non-raft partitioning phosphatidylethanolamine (PE) [130]. From the observed trajectories, the investigators classified diffusion into a distribution of various modes, and measured the confinement times and domain sizes. The diffusion behaviour of Thy-1 was found to exhibit confined diffusion similar to that of CTB-labeled GM1, both of which displayed significantly more confinement than that of fluorescein-PE. Importantly, the diffusion properties of Thy-1 were modulated by depletion of GSLs by treatment of cells with the glucosylceramide synthase inhibitor - *D-threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) [35].

The relationship of transient confinement zones (TCZs) to putative raft structures was further explored in C3H 10T1/2 murine fibroblasts [131]. Residence times of Thy-1 and GM1 were measured and compared to those of both saturated and unsaturated phospholipids. In agreement with the results of Sheets *et al.*, Thy-1 and GM1 displayed increased residence time in TCZs compared to phospholipids

and that the residence time of the unsaturated phospholipid was less than that of the saturated analog. To further elaborate on the relation between TCZs and raft, authors noted a major reduction in zone abundance upon depletion of cellular cholesterol.

In another study aiming to indirectly assess the attributes of lipid domains, the lateral motion of Alexa 588- and FITC-labeled polyoma virus-like particles (VLPs) bound to live 3T6 mouse fibroblasts was recorded for up to 100 sec and the motion characterized by SPT algorithm [132]. VLPs are ligands for their cognate ganglioside GD1a and GT1b receptors. Authors noted a brief period (5-10s) of free diffusion at a rate only slightly slower than the diffusion of GD1a-bound VLPs in DOPC bilayers. VLPs were then observed to subsequently inhabit transient confinement to zones of 30 – 60 nm diameters for up to 100s during which confined particles were observed to occasionally again exhibit free diffusion before being re-trapped. Interestingly, methyl- β -cyclodextrin treatment of cells resulted in the absence of free diffusion during data collection, a result mimicked by nystatin and progesterone, and restored following cholesterol repletion. Further, extended periods of confinement could only be altered by agents that alter the actin cytoskeleton, suggesting that both lipid domains and membrane-cytoskeleton contacts contribute to confinement on different temporal scales.

These examples demonstrate the feasibility of this technique in determining domain properties and contributing to important questions in the field of cellular lipid domains including - what is the size of a particular domain, how long does a particular domain remain in existence, and most importantly what are the physiological signals that trigger exodus or localization from or to a lipid domain?

4F. Near-Field Scanning Optical Microscopy

Near-field scanning optical microscopy (NSOM) is a type of microscopy in which an external light source is filtered through a tapered probe with an aperture below the wavelength of the source radiation. The probe thus acts as a waveguide and in employing a 'sub-wavelength' light source, scanning the probe at a height several nanometres above the surface of interest, high resolution images can be obtained with optical resolution beyond that of conventional microscopy, defined not by the source wavelength but by the aperture dimensions and typically as low as 50 nm [133].

Similar to AFM, the tip sample distance is controlled by a piezoelectric feedback mechanism which measures the lateral amplitude of the probe vibration. In close proximity to the sample, shear forces dampen the amplitude of vibration and the electronic controls adjust the height so that the scan is conducted at a partially dampened value [134]. The strengths of NSOM are therefore its ability to generate high resolution optical images in both bright-field and fluorescence mode, but also to provide topographical images in shear force mode. NSOM has been used primarily in the characterization of lipid phase-separation in model systems [135, 136, 137, 138]. Although technical hurdles regarding the use of NSOM on live cells still need to be overcome [139, 140], NSOM has been used to image fixed cells with some success. Successful examples include detection and characterization of ErbB2 clustering in quiescent and acti-

vated cells [141], and interactions between fluorescently labeled concanavalin A on 3T3 fibroblasts by NSOM coupled with FRET [143].

NSOM was also used to image human skin fibroblasts labelled with 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (BODIPY-PC) and fluorescently labelled antibodies to HLA-1 under buffer and in dried form [142]. In dried form, scans obtained by NSOM in both optical and shear force mode revealed a heterogeneous distribution of fluorescently labelled lipid suggestive of lipid domains that did not correlate with fluorescent domains generated by antibody labelling of the transmembrane protein HLA-1. Images resolved HLA-1 patches with radii of ~ 70 nm and ~ 600 nm in dried cells but only larger radius patches in cells imaged in buffer. Although this study did not report successful imaging of cellular lipid domains in buffer, it appears to represent one of the first attempts at scanning fixed cells by NSOM, an important first step to application to live cell imaging.

Adaptation of this technique to the study of cells under physiological conditions is the perhaps the greatest obstacle to widespread application in the study of cellular lipid domains. However, given the flexibility of coupling NSOM to fluorescent techniques [143], advances in the application to live cells [144, 145] and the promise of resolution comparable to that of electron microscopy under physiological conditions [146], NSOM is poised to make substantial contributions to the field of cellular lipid rafts.

4G. Image Correlation Spectroscopy

The basic premise of the raft hypothesis envisions the lipid raft as a triage centre within which membrane bound signaling proteins will assemble to transduce cellular signals. A valuable component of information regarding the assembly/structure of these species is the number of proteins found within an aggregate and their redistribution over time in response to cellular stimuli. Recent elaborations on the technique of FCS sought to overcome its major limitation in characterizing slow diffusion processes and in doing so make accessible the question of cluster size and dynamics.

One of the inherent limitations of FCS is the increasing difficulty encountered in characterizing the motion of relatively slowly diffusing (beyond 10^{-10} cm² s⁻¹) or immobile proteins species which require extended measurement times and thus enhance the risk of photobleaching and resultant artifactual intensity fluctuations. Whereas FCS appears to be well suited to the study of single molecules or aggregates that diffuse on a scale of 10^{-6} to 10^{-10} cm² s⁻¹, characterization of the dynamics of some proteins and protein clusters at the cell surface occurs on a scale several orders of magnitude slower [108]. The recently developed technique of image correlation spectroscopy (ICS) expands the analytical capabilities of FCS by measuring position-, instead of time-dependent fluctuations in fluorescence intensity in images [147], the theoretical principles of which have been well reviewed [148].

One of the first variants of ICS introduced, employed a confocal laser scanning microscope to obtain fluorescent intensity fluctuations from cellular images by spatial auto-

correlation [149]. Whereas FCS is suited to obtaining diffusion rates of protein and lipid species influenced by their localization to raft / non-raft regions or association with other molecular species, ICS enables determination of the average number of fluorescent clusters per unit area as well as the number of protein entities which comprise them [148, 108] via the pixel-by-pixel spatial analysis of a sequence of successive images. Notably, although ICS can potentially provide information of great utility to the study of lipid domain-dependent molecular association and requires no additional hardware external to the imaging system (in contrast to FCS), it bears the limitations of being unable to provide information regarding the nature of molecular interactions between different species and further requires additional analysis to characterize a multi-population distribution of clusters of various size distributions [150]. However, analogous to the techniques of FCS and FCCS, characterization of the co-distribution of different species has become accessible through the development of image-based cross correlation analysis (see below).

Since its introduction, ICS has been used to study the localization [151] and diffusion of transferrin receptors [152], the density and localization of clathrin-associated adaptor protein (AP-1) in the membrane of CV-1 cells [153], the presence of pre-existing aggregates of platelet-derived growth factor receptors on the surface of non-transformed human fibroblasts [154], the fusion of Sendai virus to cultured cell membranes [155], the dynamic interactions of caveolin-1 isoforms with bone morphogenetic proteins on the cell surface [156], the binding of Sendai virus to the ganglioside GD1a [157], changes in distribution of cell surface receptors in response to metabolic perturbation [158], to establish the degree of aggregation, dynamics and associations of the α_5 integrin during nucleation of nascent adhesions [159] and with particular importance to the study of cellular raft domains, the partitioning of proteins into plasma membrane microdomains [160].

Recently, a pair of critically important papers published from the labs of Lui and Schroeder employed multi-photon ICS with statistical analysis to directly image and demonstrate that the naturally-occurring, fluorescent sterol – dehydroergosterol (DHE) exhibits non-random, strong clustering patterns in the plasma membranes of L-cell fibroblasts [161, 162]. These papers represent perhaps the first direct imaging of sterol-rich domains in live cells and provide the groundwork for further inroads in to their role in cellular signaling.

Image cross-correlation spectroscopy (ICCS) is an elaboration on conventional ICS measurements that seeks to characterize the co-localization of different molecular species whether through direct interaction or *via* co-distribution to discrete membrane domains. It does so via simultaneous imaging of distinct fluorolabeled species in separate detection channels and cross-correlation of their spatial intensity fluctuations. Recent effort has evaluated the accuracy and dynamic range of both ICS and ICCS providing useful practical information to investigators attempting to apply these techniques to the study of protein distribution on the cell surface [163]. This technique has revealed the distribution of inner-leaflet-anchored proteins with IgE receptors and outer leaflet raft components in RBL-2H3 cells [164]. In this study

investigators monitored the co-redistribution of inner-leaflet-targeting GFP constructs (bearing consensus sequences for either prenylation or myristoylation/palmitoylation) with each of IgE-Fc ϵ RI, GD_{1b} and GPI-linked Thy-1 upon cross-linking of these external components. This study confirms the preferential redistribution of the palmitoylated/ myristoylated-GFP with lipid raft components versus that of the prenylated-GFP and represents a viable example of the application of this technique to the study of how raft dynamics during cell stimulation may facilitate signal transduction.

4F. High Resolution Mass Spectrometry

In the field of modern biological chemistry, mass spectrometry has been the pre-eminent analytical tool for unambiguous determination of the chemical identity of species in biological systems. Mass spectrometry has the advantage of analysing for native biochemical species rather than fluorophore-labeled species that can alter the behaviour / mobility of the molecule of interest as is often the case in fluorescence-based imaging techniques.

Recent studies have demonstrated the applicability of high resolution secondary ion mass spectrometry (SIMS) to the quantitative chemical analysis of both micropatterned protein adlayers [165], and supported lipid membranes [165, 166] deposited on a SiO₂ substrate. This technique employs a tightly focused beam of Cs⁺ ions (100 nm) to fragment and ionize isotopically or atomically labelled chemical species into both negatively charged atomic and molecular secondary ions which are then separated and detected by a high resolution mass spectrometer. On heterogeneously patterned surfaces, the secondary ion species volatilized varies with the path of the ion beam as it scans across the surface.

Studies conducted on adlayers of ¹⁹F- and ¹⁵N-labeled proteins micropatterned as grids along with a distinct natural abundance fibronectin grid, show ¹⁹F⁻ and ¹⁵N⁻ signals that corresponded exclusively to those regions in which respective protein grids had been formed. Alternately, images of ¹²C⁻ and ³²S⁻ abundance corresponded to all patterned proteins, thus demonstrating both the high selectivity as well as high lateral resolution of this technique. Authors also noted the high ion yields obtained for fluorine-labeled protein, which in concert with the uniqueness of the label make it an appealing atomic label for future studies. Authors were further able to characterize supported lipid bilayers in regions within fibronectin corrals via the ¹²C⁻ and ³¹P⁻ signals showing the lipid specificity through the single phosphorous atom of phospholipids.

Subsequent results obtained from study of a supported lipid bilayer comprised of ¹³C₁₈-distearoylphosphatidylcholine (DSPC) and ¹⁵N-dilauroylphosphatidylcholine (DLPC) demonstrated the chemical heterogeneity of the bilayer by SIMS in regions on the order of 100 nm that displayed phase separation by AFM [166]. The chemical composition data was further used to construct secondary ion images that exhibited geometries that closely correlated to those obtained by AFM and suggest the utility of SIMS as a complimentary tool capable of providing quantitative chemical data regarding phase-separated domains in lipid bilayers derived from live cells.

5. CONCLUSION

Although valuable information regarding lipid microdomain structure has been gleaned from model systems, features including membrane asymmetry, lipid diversity and the contribution of intracellular and membrane proteins continue to impede a fundamental understanding of cellular raft organization and dynamics. Despite general agreement that rafts are not discernable by conventional fluorescence microscopy, there are also widely divergent reports on the size of lipid rafts ranging from low nm [169] up to 1000 nm [170,171]. Regardless, several models of membrane microheterogeneity have been suggested [70, 167, 168] and are used to guide inquiry in to the properties of cellular lipid domains.

However, even the simplest questions about membrane domain structure appear to lack consensus. Which phase (Lo or Ld) predominates in the membrane? Is there more than one class of liquid-ordered domain in the membrane? What proportion of the membrane is considered to possess 'raft' properties? What is the time scale in which rafts exist? What are the physiological signals that influence raft dynamics? Whether this is reflective of cellular differences, the techniques used to detect rafts or the definitions of raft being employed is unknown.

As is often the case, scientific inquiry requires harmonization of data from a variety of sources. Although no single technique can be expected to provide definitive conclusions, instrumental techniques such as those outlined herein will continue to make valuable contributions toward a comprehensive understanding of the properties and dynamics of lipid domains and their influence on cell function.

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