Lipid rafts: at a crossroad between cell biology and physics

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Membrane lateral heterogeneity is accepted as a requirement for the function of biological membranes and the notion of lipid rafts gives specificity to this broad concept. However, the lipid raft field is now at a technical impasse because the physical tools to study biological membranes as a liquid that is ordered in space and time are still being developed. This has lead to a disconnection between the concept of lipid rafts as derived from biochemical and biophysical assays and their existence in the cell. Here, we compare the concept of lipid rafts as it has emerged from the study of synthetic membranes with the reality of lateral heterogeneity in biological membranes. Further application of existing tools and the development of new tools are needed to understand the dynamic heterogeneity of biological membranes.

The origin of the raft hypothesis[1] can be traced to the perplexing discovery that glycosphingolipids cluster in the Golgi apparatus before being sorted to the apical surface of polarized epithelial cells[2]. Subsequent studies established that glycosphingolipid clusters tend to be insoluble in Triton X-100 at 4 °C, forming detergent-resistant membranes (DRM), have a light buoyant density on sucrose gradients and are rich in both cholesterol and glycosylphosphatidyl inositol (GPI)-anchored proteins[3]. The size of the raft was proposed, on the basis of biophysical experiments, to be in the order of 50 nm[4]. The raft hypothesis was bolstered by the observation that synthetic membranes composed of glycosphingolipids and cholesterol recapitulate the detergent-resistant characteristics of the glycosphingolipid clusters[5,6], suggesting that lipid lateral heterogeneity occurs spontaneously as a function of the lipid composition of the membrane.

Two lines of inquiry emerged from these early studies: one exploring lateral heterogeneity in synthetic membranes; and the other addressing the functionality of glycosphingolipid–cholesterol rich domains in biological membranes. Few would doubt that lateral heterogeneity in biological membranes must have a major role in cell and developmental biology, but characterizing this heterogeneity in a compelling way, biochemically or biophysically, has proven to be frustratingly difficult. Indeed, the fact that a continuum of partially tenable models exists[7] — from lipid 'shells'[8] to the idea that the membrane is a collection of contiguous rafts with fluid inclusions[9] — reflects the difficulty in structurally characterizing the cell membrane[10]. A provisional contemporary definition of rafts that begins to embody these complexities emerged at the 2006 Keystone Symposium[11]: "Membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein–protein and protein–lipid interactions." It is our contention that the lipid-raft field is at a technical impasse, largely because the tools to study biological membranes as liquids structured in space and time are rudimentary. As a consequence, contemporary notions of lipid rafts have to be reconciled with the physical realities of biological membranes. This review will discuss the constraints that make biological membranes unusual liquid-like structures, the hierarchical organization of lipid–protein composites in biological membranes and the physical methods available for measuring such dynamically heterogeneous structures. We believe that new technologies and paradigms will be required to move the field forward. Other aspects of raft biology and biophysics have been covered in recent reviews on lipid rafts[7,12–20] and at an interdisciplinary conference [http:/www.biophysics.org/discussions].

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Biological membranes are different from synthetic membranes

Almost to a fault, the tenets of the lipid raft hypothesis are easily demonstrated in model membrane systems, where domains can range in size from nanoscale[21–23] to microscale[5,6] depending on the lipid mixture. These results beg the question why are rafts so 'elusive or illusive' in cell membranes[17]. The situation in biological membranes is very different from that in artificial bilayer membranes. The biological membrane is a liquid-like structure with some long-range order and short-range order in various lengths and time scales. Short-range order is manifest in a myriad of domains whose composition, size, lifetime and functionality require characterization. For the purposes of discussion, it is useful to divide the length scales characterizing membrane-dynamic structure into shells, complexes and clusters, nanodomains and microdomains (Fig. 1a). Small dynamic structures (such as shells and clusters) and the larger nanodomains may have properties different than more stable

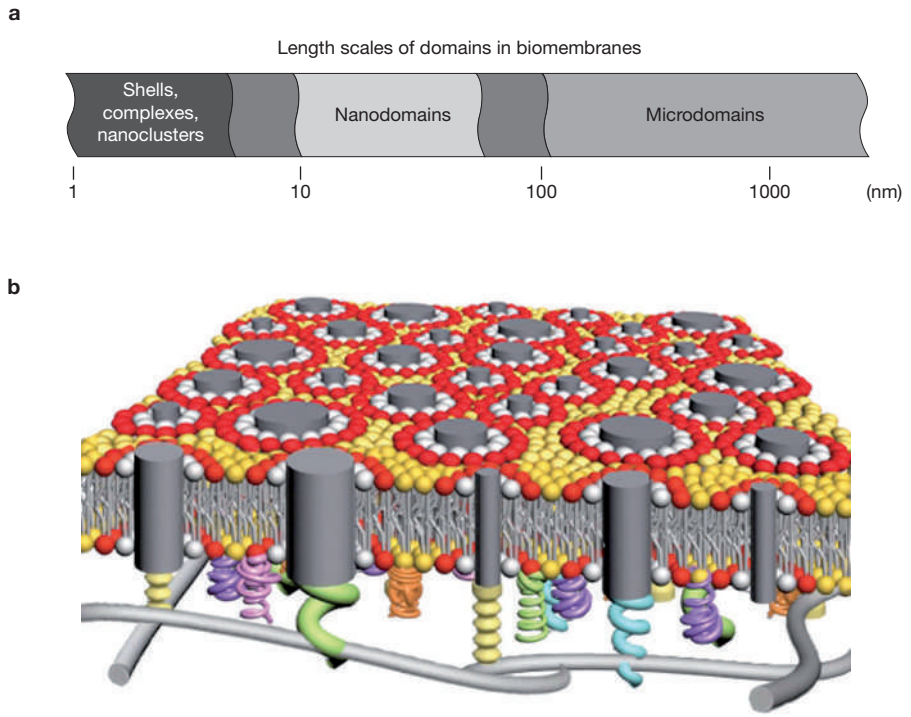

Figure 1 Domain-length scales and the biomembrane as a protein–lipid composite material. (**a**) Length scales of domains in biomembranes. Shells, complexes and nanoclusters range from 1–10 nm, whereas nanodomains such as caveolae can be as large as 100 nm. (**b**) A schematic representation of the biomembrane as a composite of lipids and proteins. Estimates of lateral protein concentration are about 30,000 per μm^2 based on rhodopsin in the rod outer segment[28,29] and transmembrane proteins in the baby hamster kidney (BHK) cell membrane[27]. Lipids were assumed to occupy a surface area of ~0.68 nm^2 (diameter ~0.93 nm) and an α-helix ~1 nm^2 (diameter ~1.1 nm). A 30 × 30 nm^2 section of membrane is depicted with 32 lipids on a side, 35 transmembrane proteins with 15 single-span, 12 tetraspan and eight heptaspan α-helical proteins, having assumed crosssectional areas in the plane of the membrane of 1 nm^2, 4.5 nm^2 and 8 nm^2, respectively. Taking into account the area excluded by the proteins, the numerical lipid : protein ratio is ~50. For a single-span helix with a diameter of ~1.1 nm, there are about seven lipids in the first boundary layer; for a tetraspan protein with a diameter of ~2.4 nm, there are about 11 lipids in the first boundary layer; for a heptaspan protein (such as rhodopsin) with a diameter of ~3.2 nm, there would be about 14 lipids in the first boundary layer. Such first-boundary layer lipids are shown in white, whereas the second layer is shown in red. All other lipids are shown in yellow. Lipid-binding proteins and adaptors linking transmembrane proteins to membrane proximate cytoskeletal filaments are also depicted as different coloured structures beneath the plane of the membrane, but ectodomains of the membrane proteins are omitted for clarity.

thermodynamic phases that, for practical purposes, have dimensions in the micrometre range (where the interfacial energy associated with the phase boundary is small compared to the free energy of the bulk phase). In addition, microemulsions may form in complex liquid mixtures that can have various characteristic sizes. They may be characterized by a lateral segregation of components, for example, in the form of two-dimensional nanoscopic 'droplets' embedded in a connected surrounding membrane. Microemulsions can be stabilized or destabilized by amphiphilic components that act as line surfactants at the boundaries of the droplet and can be changed by minute alterations in composition or environment.

One type of short-range spatial and temporal order, found in entities of the order of 10 nm or less, form because of significant fluctuations in local density and composition. This order is driven by cooperative molecular interactions between membrane constituents that are characteristic of liquids. These fluctuations are characterized by correlation times or the time that a fluctuation persists (that is, a lifetime) and correlation lengths, or the range over which membrane constituents, through direct and indirect interactions, can 'sense' each other, thereby providing a measure of domain size. A second type of order consists of more stable, disperse nanoscale structures, including shells and clusters.

In general, biomembranes have a much higher concentration of protein than most artificial membranes that have been studied in the past four decades, although our mental picture of most biological membranes, beginning with the Singer-Nicolson model[24], is one where the protein concentration is far lower than is actually found in cell membranes[25,26]. Quinn *et al.* have calculated the density of integral membrane proteins in the endoplasmic reticulum and Golgi apparatus to be ~30,000 molecules per μm[27]. The density of rhodopsin in the rod outer segment is estimated to be 30,000 per μm[28,29], whereas the density of the GPI-anchored folate receptor in caveolae following the addition of crosslinking antibodies has been calculated to be 32,000 per μm[30]. At these protein concentrations, there is only enough room in the bilayer for a few rings of lipid between individual proteins (Fig. 1b).

Biological membranes also have an associated cytoskeleton and adaptor proteins that link the cytoskeleton to specific sets of membrane proteins and lipids. The combination of membrane proximate cytoskeleton adaptors, together with regulatory proteins[31] that transiently bind to the inner leaflet surface, suggests that the region subjacent to the plasma membrane also has a very high protein concentration[32], which should be considered as part of the composite material. In addition to binding membrane proteins, cytoskeletal proteins also interact with specific lipids[33] — for example, spectrin interacts with phosphatidylserine[34] and

Table 1 Selected evidence for membrane domains

Probe/cell[1]	Method	Membrane location	Induced ?	Domain size	Cholesterol involved	Citation
Thy-1/RBL cells	TEM–immunogold	OL	No	<100 nm	Not done	50
Thy-1 & PrP/neurons	TEM–immunogold	OL	No	<100 nm	Yes	51
DC-SIGN/ dendritic cells	TEM–immunogold	TM	No	<200 nm	Yes	95
GFP–Ras targeting domains	TEM–immunogold	IL	No	H-Ras (inactive) 44 nm ---------- K-Ras ~30 nm	Yes ---------- No	96
HA/HAb2/fibroblasts	TEM & FRET (fixed)	TM	No	Non-randomness from 6–900 nm	Yes	44
Perfringolysin-O/(cholesterol) platelets	Cytochemistry	TM	Yes — by spreading on fibrinogen or by TRAP	Filopodia several μm long	Yes	94
Folate receptor and GFP–GPIAP/ CHO cells	Homo-FRET (live)	OL	No	~4–5 nm	Yes	41
MHC class I & II and other molecules/B lymphoma cells	FRET (by flow cytometry)	TM	No	Cluster detection only	Not done	97
GFP–arginine transporter/yeast	Confocal (live)	TM	No	~300 nm	No	58
GFP–GPI & other components/ Jurkat and HL60 cells	Confocal (live)	OL	Yes — by chemotractant	Several μm	Yes	98
Laurdan/macrophages	Generalized polarization (live)	TM	No	μm-sized	Yes	60
DMPE–Cy5/HASM	Single domain imaging (live)	OL	No	~700 nm (0.2–2 μm)	No	59
Various fluorescent lipid analogues	Confocal (live)	OL	Yes (by cholesterol removal)	~μm	Yes	99
Lck, LAT, CD2/Jurkat T-cells	Single-molecule tracking (live)	IL, TM	Yes, by TCR X-linking	<250 nm	No	45
CYF–YFP acylated or prenylated probes/MDCK cells	FRET (live)	IL	No	Cluster detection only	M-P/Yes ---------- G-G/No	100
EYFP-H–Ras-targeting domain/ 3T3 cells	SMT (live)	IL	No	~200 nm	No	101

CHO, chinese hamster ovary; OL, outer leaflet; IL, inner leaflet; TM, Transmembrane or both leaflets; G-G, geranylgeranylated; M-P, myristoylated-palmitoylated; SMT, single-molecule tracking.

other lipids[35], whereas adaptors containing pleckstrin homology (PH), FYVE and PX motifs bind phosphorylated derivatives of phosphatidylinositol. Indeed, modulation of PtdIns(4,5)P_2 exposure has dramatic effects on membrane–cytoskeletal interaction[36]. Therefore, the membrane-associated cytoskeleton and its adaptors, as well as regulatory cytosolic proteins, are likely to influence the organization of membrane microdomains through both protein and lipid anchorage points on the inner leaflet. The effect of these interactions, and the high overall protein concentration in membranes, has not been fully incorporated into contemporary models of regulated lipid lateral heterogeneity in biological membranes. Thus, the membrane should be viewed as a lipid–protein composite, rather than a dilute solution of protein in a lipid solvent. Moreover, domains will rarely be isolated islands in a lipid sea, as often depicted in cartoons. The lifetimes of these domains will depend on their size and factors that may stabilize or destabilize them. These include not only lipid–lipid interactions but also protein–lipid and protein–protein interactions that occur both in the plane of the membrane, and between membrane components and elements of the cytoskeleton and pericellular matrix adjacent to the membrane[20].

A hierarchy of lipid–protein composites in biological membranes

Apart from the well-known membrane domains in differentiated cells (such as the apical–basolateral specializations and junctional complexes in epithelial cells and clathrin-coated pits), considerable evidence, both experimental and conceptual, can be marshalled for the existence of other domains of various sizes in diverse biological settings. A number of these structures exhibit lipid specificity (Table 1 and Fig. 2).

The shell model[8] proposes that specific classes of plasma-membrane proteins (such as certain transmembrane proteins and proteins anchored by lipid tails), bind to preassembled complexes of cholesterol and sphingolipid[37] or they may laterally organize specific lipids, including cholesterol, as in the case of peripheral[31,38] or transmembrane proteins[39]. Although the shell is envisioned to be stably associated with protein, component lipids can exchange with the 'bulk' bilayer lipids. The dynamics of exchange may range from the short-lived classical boundary layer lipids (~1–10 s)[40], in which shell lipids may rapidly interchange with non-shell lipids, to long-lived lipids that are tightly bound through specific lipid–protein interactions[39] and are regulated by ligands. The concept of shelled proteins, existing alone or in nanoclusters, is also supported by homo-fluorescence resonance energy transfer (FRET)[41] (between like donor and acceptor) and hetero-FRET[42] (between differenct donor and acceptor) measurements, which can be interpreted to show that DRM molecules are largely monomeric or coexist, but are not in an equilibrium, with a small fraction of 'nanoclusters' (~4–5 nm in dimension) containing four or less GPI-anchored proteins. In addition, the neurokinin-1 receptor, a G –protein-coupled receptor, was found to exist in very small (~10 nm) clusters that are cholesterol sensitive[43].

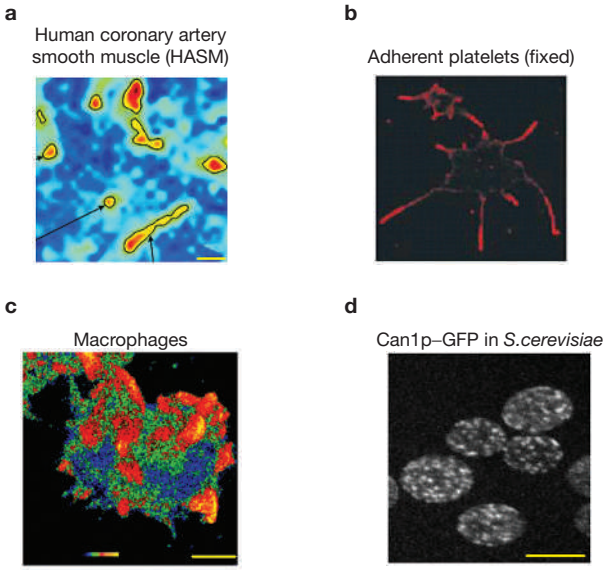

Figure 2 Examples of lipid and protein domains in cell membranes. (**a**) Single domains, enriched in the fluorescent lipid analogue DMPE-Cy5, imaged in human coronary artery smooth muscle cells[59] (reprinted with permission from the EMBO Journal). (**b**) Cholesterol-rich domains in platelet processes[94] visualized using a non-cytolytic derivative of biotinylated perfringolysin O followed by indirect immunofluorescence microscopy with an anti-biotin monoclonal antibody (reprinted with permission from the International Society on Thrombosis and Haemostasis). (**c**) Lipid domains with greater relative order than the bulk membrane, visualized in living macrophages with the fluorescent probe, Laurdan (6-Lauroyl-2-dimethylaminopahthalene), where the warmer pseudo-colours represent more ordered regions[60] (reprinted with permission of the National Academy of Sciences). (**d**) Domains formed by the proton–argenine symporter transporter (Can1p–GFP) in *Saccharomyces cerevisiae*[58] (reprinted from Molecular Biology of the Cell with permission from The American Society for Cell Biology). The scale bars represent 1 μm in **a,** and 5 μm in **c** and **d**.

In spite of criticisms[19], many of which are treated in the original postulates of the hypothesis but require proof, a virtue of the shell hypothesis is that it can accommodate seemingly disparate sets of recent data. For example, the detergent-resistant viral haemeagglutinin does not partition into lipid rafts in which it is miscible, but instead self assembles into lipid–protein complexes exhibiting order from the nanometre to micrometre scales[44]. Shelled proteins could mediate this 'self-assembly' process by enhancing the attraction between haemeagglutinin trimers. Several recent single-molecule studies also suggest that protein–protein complexes of sizes <250 nm (the resolution limit for conventional light microscopy) are fundamental units that carry out signal transduction — as opposed to these same components partitioning into lipid rafts to convey signals. Single-molecule microscopy shows that components of the immunological synapse, including Lck (a member of the Src family of protein-tyrosine kinases), linker of T-cell activation (LAT) and the coreceptor cell differentiation antigen, CD2, but not the transmembrane phosphatase CD45, exist in very small clusters that seem to be cholesterol independent[45]. In this case, phosphorylated LAT functions as a scaffold mediating downstream protein–protein interactions. In the case of IgE-triggered signalling, cross-correlation microscopy has demonstrated that activated Lyn, a tyrosine kinase, and the Fc receptor form a complex, but other putative inner-leaflet raft components do not suggest a specific association between the Fc receptor and Lyn following receptor ligation[46]. In both cases, lipid shells surrounding the protein components could help promote the formation of particular complexes.

Simons and Ikonen originally proposed that lipid "….lateral organization probably results from preferential packing of sphingolipids and cholesterol into moving platforms, or rafts, onto which specific proteins attach within the bilayer."[1] Caveolae possess many of the essential properties of the proposed lipid raft; for example, they contain clusters of glycosphingolipids, GPI-anchored proteins and a high concentration of cholesterol. The caveola is a reasonably well characterized 50–100-nm nanodomain that is detergent resistant and can be readily identified in most cells by the marker caveolin-1 (ref. 47) . Moreover, we know a lot about the function of caveolae in endocytosis and cell signalling. What is unclear, however, is whether the structure and function of caveolae depends on lateral interactions between cholesterol and sphingolipids alone, or on additional protein–lipid interactions Nevertheless, caveolae fit the original definition of a lipid raft and they may be used as a provisional standard against which other lipid rafts can be judged.

The strongest evidence for non-caveolae nanodomains (50–200 nm in dimension in the outer leaflet of the plasma membrane) comes from immunogold electron microscopy of components of, for example, the T cell receptor (TCR)[18,48] in fixed cells (Table 1), where labelled proteins are detected in clusters. The GPI-anchored protein, Thy-1, is observed in such clusters both on rat basophilic leukaemia (RBL) cells[49,50] and on neurons[51]. However, Thy-1, ganglioside 1 (GM-1), LAT or the Fc receptor show little colocalization, even though substantial amounts of these components are enriched in the Trition X-100 DRM fraction[49], indicating that detergent resistance reduces the inherent lateral complexity of the membrane to one simplified, and therefore misleading, parameter[52]. Part of the difficulty in having full confidence that such nanoscopic domains exist, however, is that measurements in living cells are more consistent with a high percentage of these proteins being monomeric. Fluoresence recovery after photobleaching (FRAP) measurements of inner- and outer-leaflet proteins give diffusion coefficients that correlate with the type of membrane anchorage (GPI, transmembrane and acylation or prenylation), not whether the proteins are detergent insoluble[53], whereas in the simple raft concept all raft molecules should have similar diffusion coefficients as they belong to similar domains. Moreover, single-molecule tracing of the transmembrane histocompability antigen is consistent with monomer diffusion[54]. Although some of the components involved in TCR signalling do exhibit very low diffusion coefficients, consistent with immobile clusters, others do not and there is no correlation with their presence in the Triton X-100 insoluble fraction[45]. In addition, FRAP measurements of inner-leaflet components show they exhibit rapid, complete recovery[53,55] after bleaching. This can be reconciled with the inner-leaflet clusters of the small GTPase, Ras, revealed by transmission electron microscopy (TEM) on fixed cells if it is assumed that activated Ras drives the formation of very transient nanoclusters from which downstream signalling ensues[56]. Single-molecule tracking and FRET studies of activated Ras are consistent with this proposal[57].

Lipid and protein reporters imaged at the light and electron microscope level by conventional and advanced means provide evidence for the existence of stable domains approaching a micrometre in dimension, or greater, in various cells (Fig. 2). Microdomains of detergent-resistant transporters were stable in growing yeast cells for more than ten minutes[58], although the exchange time of individual proteins was not determined. Microdomains in smooth muscle cells[59] and macrophages[60] were stable

Table 2 Some methods for detecting and characterizing membrane domains in live cells

Method	Primary observables	Spatial/temporal resolution	Comments	Representative citations
FCS and ICS	Fluorophore translational mobility & dynamics of lateral heterogeneity	~250 nm/ ~1 μs	Highly sensitive to clustering, single and multiple colours	46, 102–105
FRET	Donor-acceptor proximity	~5-10 nm separation detected (no size)/<1 s	Cluster identification at nm scale	41–43, 74, 106
SPT	Translational trajectory of particle (including 40 nm gold particles & quantum dots)	~250 nm (≤10 nm)[a] ≤25 μs	Single particle sensitivity detects domains as confinement zones	13, 107, 108
SMFI	Single-molecule fluorescence	~250 nm (20 nm)[a]/ ≤30 ms	Single molecule sensitivity, detects clusters by intensity & confinement	14, 45, 101, 109, 110
TOCCSL	Clusters or monomers	~250 nm/<500 ms	Detects whether molecules entering photobleached region are singles or multimers	69
Thermal noise imaging	Laser-trapped particle attached to membrane component	~10 nm/~μs	Explores positions sampled by the particle while it is trapped by laser	111

FCS, fluorescence correlation spectroscopy; ICS, image correlation spectroscopy; SPT, single-particle tracking; SMFI, single-molecule fluorescence imaging; TOCCSL, thinning out clusters while conserving stoichiometry of labelling. [a]Precision in detecting centroid of particle image.

for tens of seconds; moreover, in muscle they were observed as cohesive entities that rotate as units, whereas individual lipid-probe molecules had a domain residence time of ~10 ms[59]. Thus, it seems that stable, larger domains can exist in various cells but the stabilizing factors[20] are largely unknown. In some instances, proteins from the four-transmembrane domain tetraspanin superfamily[61] may organize special microdomains as in microvillae[62] and HIV may induce tetraspanin-enriched domains as export portals in infected cells[63]. Caveolae may also form micrometre sized clusters[64] in some cells. The importance of the lipid phase in developing longer-range order in the plasma membrane has been illustrated by two recent studies. Electron-spin resonance results from several intact living-cell types indicate that a large fraction of outer-leaflet phospholipids is in the liquid-ordered state at temperatures near 5 °C and decreases as temperature increases to 37 °C[65]. Although the individual ordered units have a lower size limit of ~ 2 nm, the authors suggest that the liquid-ordered state may actually be the contiguous (percolating) phase in the outer leaflet[9]. A similar view has been advanced based on interpretation of FRAP results on the apical surface of polarized epithelial cells, but not fibroblasts, at temperatures ~<25 °C; at 37 °C, the non-raft, liquid-disordered phase becomes the percolating phase[66].

Biophysical tools to study membrane domains in biological membranes

To be biologically significant, domains should have a minimum size of a few protein diameters and a minimum lifetime corresponding to a short enzyme turnover time of ~microseconds. When domains are stable and of sufficient size, detection is possible using both traditional and advanced imaging techniques (Fig. 2). But, when domains are small and transient, the biophysical challenges of measuring them are similar to those facing physicists a half-century ago when they attempted to describe the structure of liquids. Predictably, the most controversial area of membrane lateral organization is on the nanoscale level, where technology with sufficient simultaneous spatial and temporal resolution is not available. Thus, refining existing methods and developing new ones is now key to progress[67,68]. As the goal is to study dynamic membrane structure in living cells, applicable techniques, for the most part, rely on fluorescence microscopy because of the high sensitivity of these methods and their facile application to single, living cells.

In Table 2, we enumerate several techniques with the potential to most directly detect and characterize dynamic heterogeneity in living cells, together with all-important estimates of temporal and spatial resolution. It is likely that advances will be made in characterizing dynamic domain structure using both the single molecule and particle techniques, and advanced fluorescence correlation microscopy. The latter should be useful in characterizing domains that are not visible by ordinary fluorescence microscopy but can be detected using other specialized techniques[41,59,69]. Moreover, there are several emerging technologies that 'beat' the resolution limit of fluorescence microscopy[70]. There are also a number of developing techniques with great potential to provide complementary information. These include secondary ion mass spectrometry on the 100-nm scale[71,72] to quantitatively determine composition of microdomains *in situ* with submicrometre resolution, and atomic force microscopy (AFM) and near field scanning microscopy (NSOM) for membrane topography and microcomposition[73–75]. Such techniques can complement fluorescence microscopy, but are limited in that they either operate on quick-frozen specimens (nano-SIMS) or they lack the time resolution of the fluorescence techniques (AFM and NSOM). Provided that adequate contrast between structures can be obtained, the ultimate tools to determine the length scale of dynamic clusters, nanodomains and rafts in the nanometre range are scattering techniques, such as neutron and X-ray scattering[76,77]. However, because of difficulties in sample preparation, these techniques have so far only been used in model-membrane studies.

Other challenges

As current biophysical tools mature and new methods emerge, we must not lose sight of the fact that energy must constantly be supplied to biological membranes[78], both directly through the expenditure of high-energy intermediates and indirectly through intracellular and extracellular transport processes that constantly add and remove lipids and proteins[79,80]. This leads to creation of an active region[7,12] with scale-dependent organization and function that depends on mutual interactions between lipids and proteins[46,81].

The caveolae nanodomain illustrates the challenge of understanding the role of energy dissapation in thermodynamically open systems[82]. In addition to whatever lateral forces are required for lipid organization in a caveola, there must also be a constant flow of cholesterol through the domain for it to function[83]. The mechanism of cholesterol supply may involve the movement of caveolin-1 from the endoplasmic reticulum[84] and lipid droplets[85,86] to caveolae, although the precise details remain unknown. Shutting off the supply of cholesterol causes caveolae to malfunction, both during endocytosis[87] and signal transduction[86]. Studies of caveolin-1-expressing cells shows that cholesterol rapidly moves from its site of synthesis in the endoplasmic reticulum across the caveolae bilayer into contiguous membrane, as well as to extracellular lipidic acceptors like high-densisty lipoprotein (HDL), with a half-time of ~6 min[84]. Even though cholesterol flux seems to be critical, biophysical calculations fail to explain why. Given the large number of animal genes devoted to moving cholesterol around in cells and between tissues, transmembrane cholesterol flux most likely is common, and therefore important for membrane organization. In fact, the energy-dependent turnover of lipids in general is probably critical for the normal function of membranes and membrane domains. The challenge is to incorporate these parameters into future models of nanodomain structure and function, determine how lipid fluxes contribute to nanodomain order and develop new methods to detect lipid fluxes in cell membranes.

Because of the dynamic composition of local membrane structures, there may not be a consensus non-caveolae nanodomain. In addition to the lipid and protein composition, the characteristics of nanodomains may be strongly dependent on available soluble and pericellular matrix-bound ligands for membrane receptors, as well as on transmembrane potential and mechanical stress. We also need to determine whether microdomains are pre-existent structures into which certain components partition[88,] or whether they are preassembled protein–lipid complexes that are actively inserted into the membrane[7,20,44] but induced to cluster into domains by specific stimuli. Indeed, the field has been moving toward the concept that lipid-shelled proteins and protein nanoclusters can be triggered to aggregate by external influences, such as ligands, to produce larger functional domains[8,13,89,90].

We need to assess the degree to which appropriate artificial membranes, at equilibrium or near equilibrium, can be used to study aspects of the biological membrane. In this regard, it will be important to ask how the principles apparent in relatively simple model lipid-membrane systems[19,88], many of which are quite intriguing[91], are reflected in the complexity of cell membranes. However, investigators seeking more relevant model membranes will need to deal with the issue of protein concentrations and the natural lipid asymmetry of biomembranes[19], as well as energetics. Model membranes with high protein concentration are used in nuclear magnetic resonance (NMR)[92], electron microscopy and diffraction investigations. Such membranes may be useful in revisiting the boundary layer and shell issue by using a more expanded set of proteins and lipids than in the original studies. Ultimately, we will need to develop novel lipid–protein model systems with functioning proteins (such as channels, multienzymes systems, etc.) that have some of the properties of biological membranes, but that are also sufficiently well-defined to study domain properties such as formation, size, lifetime and morphology. Finally, there is a clear need for the development of new computational models and algorithms (for example, based on dissipative particle dynamics and molecular dynamics) that will allow improved computer simulations of the large assemblies of molecules found in domains[93]. The complementary expertise of biophysicists and cell biologists will be required to make meaningful progress in understanding membrane lateral heterogeneity. □

ACKNOWLEDGEMENTS

K.J. thanks A G. Lee, G. Schutz and H. Stockinger for stimulating comments. We also thank A. Diehl and B. Yang for artwork and assistance in preparing the manuscript. This work was supported by the National Institutes of Health (NIH) grant GM 41402 (K.J.), the Kenan Distinguished Professorship (K.J.), NIH grant HL 20948 (R.A.), NIH grant GM 52016 (R.A.), the Perot Family Foundation (R.A.) and the Cecil H. Green Distinguished Chair in Cellular and Molecular Biology (R.A.). MEMPHYS-Center for Biomembrane Physics is supported by the Danish National Research Foundation (O.G.M.).

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