Trends in Cell Biology

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Review

Lipid Rafts: Controversies Resolved, Mysteries Remain

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The lipid raft hypothesis postulates that lipid–lipid interactions can laterally organize biological membranes into domains of distinct structures, compositions, and functions. This proposal has in equal measure exhilarated and frustrated membrane research for decades. While the physicochemical principles underlying lipid-driven domains has been explored and is well understood, the existence and relevance of such domains in cells remains elusive, despite decades of research. Here, we review the conceptual underpinnings of the raft hypothesis and critically discuss the supporting and contradicting evidence in cells, focusing on why controversies about the composition, properties, and even the very existence of lipid rafts remain unresolved. Finally, we highlight several recent breakthroughs that may resolve existing controversies and suggest general approaches for moving beyond questions of the existence of rafts and towards understanding their physiological significance.

Highlights

We review the conceptual underpinnings of the raft hypothesis.

We discuss how principles of phase separation produce dynamic, nanoscopic domains.

We critically discuss the supporting and contradicting evidence for raft domains in cells.

We explain unresolved controversies about the composition, properties, and existence of lipid rafts.

We highlight several recent breakthroughs that resolve existing controversies.

We suggest general approaches towards understanding the physiological significance of rafts.

Introduction

Few topics in cell biology have spawned quite the level of spirited debate as lipid rafts have. The introduction of the raft concept [1] launched a new field and recruited to membrane biology a generation of physical chemists, cell biologists, biophysicists, optical engineers, and computational scientists [2], all to explore the seemingly trivial notion that living membranes are not laterally homogeneous. From this perspective, the raft concept has been a tremendous success, yielding insights and development across numerous disciplines. By contrast, the exciting promise of a comprehensive mechanism for regulating signaling at the plasma membrane (PM) has so far not been definitively substantiated. Worse still, despite decades of research, thousands of papers, and countless debates, the pressing question in the field remains: '…but do rafts exist?'.

These competing notions of success for the raft hypothesis are perhaps not incompatible: it is exactly the enduring mystery of the nature and function of rafts that keeps the field vital and progressive. In this review, we discuss: (i) the physicochemical principles underlying the raft concept; (ii) how cooperative regulation between proteins and lipids establish lateral structure in biomembranes; and (iii) recent developments in understanding of the raft concept and why it has proven so difficult to definitively establish the existence and relevance of lipid-driven membrane subdomains. In the final section, we highlight several recent breakthroughs across multiple fields that have overcome these complications to provide some of the most compelling evidence supporting the functional roles for raft domains in cell biology.

Order out of Chaos

Compartmentalization is a ubiquitous theme in biology: membranes originally evolved as semipermeable barriers to envelop cells and delimit compartments within the cytoplasm. Perhaps it is not surprising that compartmentalization is also possible within the plane of the membrane itself. One aspect of such compartmentalization is known as the 'raft hypothesis', which posits that preferential interactions between sterols and certain phospholipids can induce the

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formation of tightly packed membrane domains with distinct protein and lipid compositions from their surrounding membranes. The physiological implications of such organization are self-evident: with minimal energy expenditure, cells could concentrate specific reactants, exclude negative regulators, induce conformational changes, or regulate local membrane properties. Thus, rafts have been extensively implicated in signal transduction through the PM in a variety of contexts [3]. A separate functionality powered by lateral membrane partitioning, and the physiological role for which they were originally invoked, is in the sorting and trafficking of membrane components between subcellular organelles [4–6]. All organelles have unique membrane compositions and the mechanisms by which proteins and lipids traffic between them remain largely unresolved. In particular, the secretory pathway is believed to present a gradient of lipid compositions and physical properties, with endoplasmic reticulum (ER) membranes being poor in raft-forming lipids, such as cholesterol and sphingolipids, while PMs are highly enriched in these components (30–40 mol% cholesterol and 10–30% sphingolipids) [7]. The Golgi stacks are likely intermediate, possibly with progressive accumulations from *cis* to *trans* [8]. Laterally segregated raft lipid-enriched domains could provide a self-organizing explanation for such lipid distributions by formation of selective compartments that are preferentially moved from earlier to later steps in secretory pathway. A similar principle may be functional in the endosomal pathway, with the lysosome standing in for the cholesterol-poor ER and various endosomes as intermediate compartments.

Despite the longstanding debates about raft existence and confusion about their very definition, the basic physical chemistry underlying lipid-driven domains is relatively straightforward. All lipids interact with one another favorably compared with their interactions with water; this is the basis of membrane self-assembly. However, certain lipids interact more favorably with each other than with other lipids because of various chemical and geometric features [9]. With hundreds of different lipid subtypes in a given mammalian membrane [10,11], a plethora of differential interactions are a certainty and, therefore, ideal mixing should not be expected. Put another way, lateral heterogeneity is the rule rather than the exception. However, the time and length scale of these heterogeneities, which ultimately dictate their biological relevance, are highly dependent on the molecular details. Furthermore, the physical models that best describe how differential binary lipid interactions lead to lateral structures remain under active debate; some of these are discussed herein.

For lipid rafts, the most relevant interactions are between saturated lipids, sphingolipids, and sterols, which are preferred relative to their interactions with highly unsaturated lipids (Figure 1A). Individually, the magnitudes of these relative preferences are small. However, membranes are formed by a multitude of lipids, the inherent collectivity of which can multiply these small differences into macroscopic behaviors (Figure 1B). The physicochemical basis for the raft hypothesis grew out of extensive model membrane studies that established that collective interactions between sterols and saturated lipids can form a unique state of matter called the 'liquid ordered (Lo) phase' [12–14]. This Lo phase is fluid, similar to the more common disordered lipid (Ld) phase, allowing molecular motion and flexibility. However, the molecular arrangement and various physical properties (lipid packing, rigidity, and permeability) are distinct from the Ld phase, allowing Lo and Ld phases to coexist throughout a broad space of compositional and physical parameters [12]. It is this coexistence of ordered and disordered lipid phases that is believed to underlie raft assembly in living cells.

In the biological world, this simplistic version of phase coexistence is convoluted by the inherent complexity of living systems. In contrast to model membranes, biomembranes contain hundreds of different lipids and a vast array of transmembrane proteins, which comprise up to 25% of the

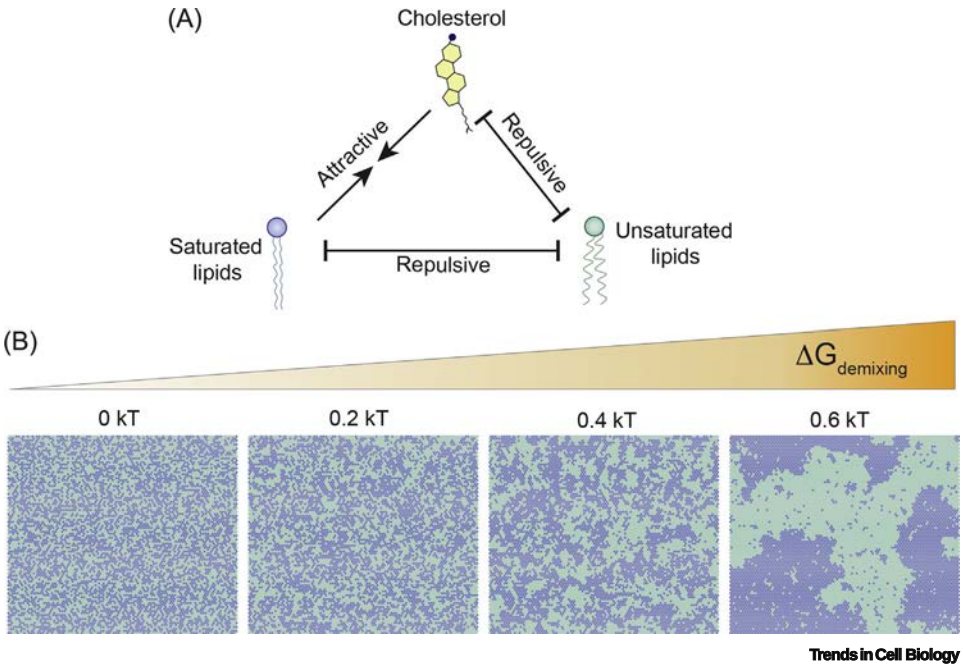

Figure 1. Differential Lipid Interactions Drive Lateral Organization. (A) Saturated lipids and cholesterol interact more favorably with each other than with unsaturated lipids. (B) Collectively, these differential interactions oppose the entropic tendency towards random mixing. When the various interactions sufficiently outweigh the demixing cost, stable lateral domains are formed.

cross-sectional area of the PM [15]. However, strong evidence supporting the biological relevance of Lo-Ld phase coexistence comes from studies in giant PM vesicles (GPMVs) [16,17], which are intact, isolated PM blebs that retain the lipid diversity and protein content of living membranes [16–18]. These vesicles macroscopically phase separate into coexisting ordered and disordered phases [19] that sort both lipid and protein components in concordance with the precepts of the raft hypothesis. Namely, saturated lipids, sterols, glycolipids, and certain proteins are co-recruited to more ordered phases, away from unsaturated lipids and most other proteins [16,20]. This spontaneous formation of coexisting fluid domains demonstrates the self-organizing capacity of mammalian PMs and the potential for such organization to laterally sort membrane components. Further research has illuminated the structural determinants of protein partitioning to the more ordered domains, suggesting that post-translational lipid modifications and transmembrane domain (TMD) features enable raft affinity [21,22]. A persistent observation from these studies, and analogous ones of synthetic membranes, is that ordered domains tend to exclude most transmembrane proteins [19,23], and even those proteins that are included are rarely enriched. This finding suggests that rafts may be relatively lipid-rich and protein-poor membrane regions, the major role of which is exclusion of most components, rather than the concentration of some. However, such measurements have only been made in isolated or synthetic systems, which may not faithfully represent some essential aspects of living cell membranes. Nevertheless, protein-depleted membrane domains in cells have been directly observed by super-resolved microscopy [24], although their underlying structure and mechanisms have not been resolved.

Let's Work Together: Cooperative Regulations between Membrane Proteins and Lipid Domains

Recent studies utilizing computations, synthetic membranes of increasing complexity, and cell-derived GPMVs have moved beyond simple models to begin to understand the determinants

of lateral structure in living cells. The major insights can be summarized into three broad themes: (i) lipid context is essential for lateral structure; (ii) proteins have an essential role in regulating (clustering, localizing, and templating) the lipid template (Figure 2); and (iii) the principles of macroscopic phase separation can be co-opted to produce nanodomains.

Lipid Context in Lateral Structure

The original model membrane studies that provided the crucial physical foundation of the raft hypothesis typically relied on a composition inspired by the apical PM of epithelial cells [13,25]: an approximately equimolar mixture of cholesterol, a saturated lipid-like sphingomyelin (SM), and an unsaturated phosphatidylcholine usually bearing two unsaturated acyl chains (e.g., dioleoyl phosphatidylcholine; DOPC). Since then, it has become clear that this mixture is a rather poor model of most mammalian PMs. First, this combination of headgroups is a reasonable mimic of the PM outer leaflet, whereas the inner leaflet contains abundant charged and polyunsaturated lipids [26,27]. The possible consequences of asymmetry on membrane structure are discussed later. More subtly, lipids with two unsaturated acyl chains are relatively scarce in mammalian membranes, which comprise largely mixed chain ('hybrid') lipids with one saturated and one unsaturated acyl chain (e.g., palmitoyl-oleoyl PC; POPC). This subtle distinction turns out to be of fundamental significance, because membranes with hybrid lipids as the major unsaturated components typically do not macroscopically phase separate, but rather form nanoscopic ordered domains (discussed in detail later). These studies highlight an important theme: the composition, structure, and stability of ordered domains depends not only on the abundance of order-preferring lipids (sterols and sphingolipids), but also on the type and abundance of disordered lipids. A potentially generalizable framework is that the 'contrast' between order- and disorder-preferring lipids determines the ultimate structure: disordered phases with highly polyunsaturated or very short lipids will enhance the contrast to a thick, tightly packed ordered phase and thereby stabilize phase separation. This principle has been demonstrated in simulations [28], model membranes [29–35], and isolated PMs [10,36–39]. An important conclusion from this line of evidence is that the detailed properties (size, lifetime, and stability) and compositions of domains are intrinsically context dependent and reflect the specifics of the membrane in which they arise. Thus, investigations aimed at defining the size or lifetime of such structures in cells may be doomed by fundamental heterogeneity.

Cooperative Protein and Lipid Organization

Much of the discussion of rafts in biophysics and cell biology presents a paradox: while the central purpose of membrane domains is to sort and organize membrane proteins (Figure 2A), little attention

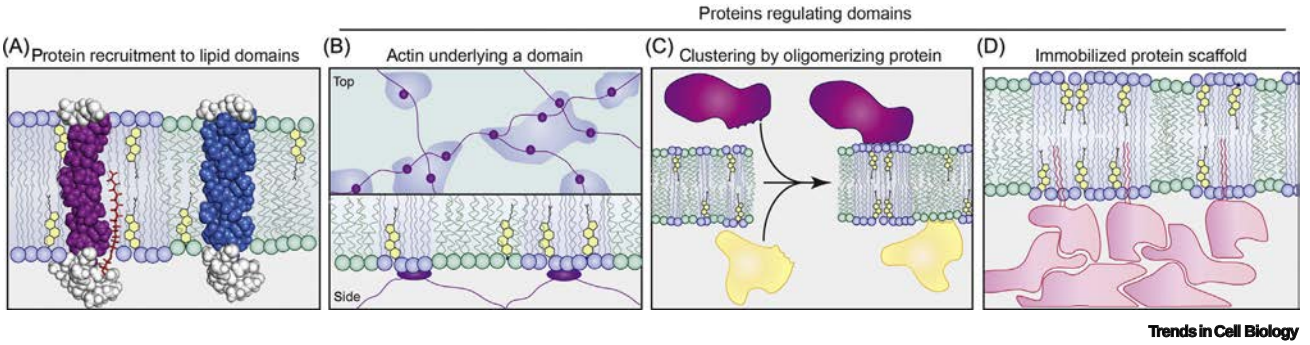

Figure 2. Cooperative Regulation between Membrane Proteins and Lateral Domains. (A) Freely diffusing proteins can be recruited to membrane domains by post-translational lipid modifications (e.g., palmitoylations) and features such as long and thin transmembrane domains. (B–D) Proteins can also template and regulate membrane domains. (B) Cytoskeletal protein networks can template membrane domains by tethering specific lipids or proteins. (C) Oligomerization of raft-associated proteins can cluster and stabilize raft domains. (D) Immobilized, membrane-bound scaffolds (e.g., postsynaptic density) may recruit specific membrane domains based on the raft affinity of their membrane-anchoring proteins.

is paid to the structural roles of proteins in raft organization, which are often treated as passengers to lipid-driven organization. This is somewhat surprising, because some of earliest support for the raft hypothesis came from experiments that used antibodies or toxins to cluster raft-associated molecules [40,41] (Figure 2C). In cells, such clustering produces stable, microscopic structures that sort PM components. Similarly, in synthetic and cell-derived membranes, clustering of the glycolipid GM1 by cholera toxin is sufficient to induce macroscopic domains in previously uniform membranes [40], demonstrating that even subtle perturbations may have profound consequences on lipids with the capacity for phase separation. Similar behavior on the nanoscopic scale was recently reported by super-resolution microscopy in B cells, wherein clustering by B cell receptor (or cholera toxin) crosslinking produced selective raft-like nanodomains [42].

Recently, several notable examples have expanded on the principle that membrane organization involves a cooperative clustering of proteins and lipids, wherein lipid self-assembly provides the tunable template regulated by proteins (Figure 2). A striking example is the implication of the predominant structural element of the mammalian cell, the actomyosin cytoskeletal network, in membrane organization. Simulations demonstrated that filamentous supports coupled to lipids have the potential to segregate membranes into corrals and stabilize domain formation, even at relatively low connectivity to the membrane [43,44] (Figure 2B). At conditions where domains are not preferred, templating by filaments can supplement lipid connectivity to stabilize domain formation. Alternatively, when domains are present, an overlying filamentous meshwork may fragment domains into smaller subregions. Some of these predictions have been directly demonstrated in model membranes, by reconstituting an actin network linked to a bilayer capable of phase separation [43,45–47]. Actin was also demonstrated to be an essential determinant of diffusion in cell-derived membranes [48]. Importantly, a similar mechanism drives lipid domains in cells, with specific phospholipids mediating connection to an active, dynamically rearranging actomyosin cortex to effect transbilayer registration of raft-associated lipid-anchored proteins [49–51]. A conceptually similar mechanism was proposed to organize the postsynaptic PM in neurons, wherein the postsynaptic density could recruit raft-like domains via immobilization of palmitoylated proteins [52] (Figure 2D). This hypothesis presents a potentially generalizable paradigm that inverts a common conception of rafts: rather than lipid-driven domains recruiting freely diffusing proteins, immobilized order-preferring proteins could recruit a dynamic assembly of raft-forming lipids [52]. However, both models are limited, because membrane organization must inherently involve the interplay between protein and lipid components.

Raft Nanodomains

Lipid phase separation in model membranes tends to yield macroscopic domains on the orders of many micrometers, which are generally not observed in live cells, where domains remain nanoscopic [53,54]. However, clear and compelling evidence across a variety of experimental contexts has demonstrated that the principles of liquid–liquid phase separation can be coopted to produce nanoscopic domains (Figure 1). As discussed earlier, when raft-mimicking membranes are constructed using hybrid lipids such as POPC (with one saturated and one unsaturated acyl chain) as the unsaturated component, they appear microscopically uniform. However, fluorescence resonance energy transfer (FRET), electron spin resonance spectroscopy, and neutron scattering have definitively demonstrated that microscopic uniformity disguises the presence of nanoscopic ordered and disordered domains [33,55–57]. While it is still not completely clear what sets the size scale of such domains or why they transition to macroscopic upon addition of di-unsaturated lipids, a critical factor appears to be the contrast between hydrophobic thickness of the domains [33], in analogy with the ‘contrast’ arguments discussed earlier.

A distinct, although not necessarily exclusive, possibility for producing nanodomains using liquid–liquid phase separation principles are critical fluctuations. It has been convincingly demonstrated that some synthetic membrane compositions [58] and most isolated PMs [59–61] are poised near specific regions in compositional space that allow access to so-called 'critical phenomena'. At these compositions, domains do not simply melt away under unfavorable conditions (e.g., high temperatures). Rather, they begin to fragment into smaller and ever more dynamic assemblies called 'critical fluctuations'. Such criticality has been extensively explored in many nonbiological phase-separated systems [62] and shown to have some universal characteristics that are independent of the molecular details of a given system [63]. Thus, this universality allows translation of insights between systems. One such insight is how fluctuation size and lifetime should scale with temperature, which predicts that domains on the order of tens of nanometers should persist on millisecond timescales in PMs under physiological temperatures. Such submicroscopic domains have been previously reported [64]. Another universal property is the high susceptibility of critical systems to external perturbations, which has been directly demonstrated in Lo-Ld membranes [65] and may account for the susceptibility of lipid raft assembly to the protein-mediated crosslinking described earlier.

Regardless of the mechanistic specifics, if rafts exist in cells, they are almost always nanoscopic (on the order of tens of nanometers) and dynamic, both in the sense of lateral diffusion and possibly also formation and dissolution. One obvious practical consequence of such small, dynamic domains is that they would be difficult to detect in live cells. Despite the dramatic advances in super-resolution microscopy, there is no current technology with the combined temporal and spatial resolution to directly observe such objects, were they to exist. However, the presence of nanoscopic lipid domains has been extensively reported using techniques such as FRET [66–70], fluorescence quenching [71], super-resolution diffusion [69,72,73], electron microscopy [20,74,75], and single-molecule tracking [76,77]. Moreover, lipid nanodomains have been extensively observed in fixed cell membranes and shown to sort critical signaling proteins in the PM [37,68,74,78].

Hard to Find a Raft, or Looking for Rafts in all the Wrong Places

Although coexisting Lo-Ld domains have been inferred through a variety of indirect measurements [66–73,76,77], they have rarely been directly, microscopically observed in living cells. Additionally, there have been several independent lines of evidence that have failed to find clear evidence of lipid-driven domains [79,80] or thermotropic phase transitions [81] in live cells. Thus, a healthy skepticism persists about the nature and function of lipid-driven lateral domains *in vivo*.

Seeing Is Believing

Lack of direct observation persists as the most common reason for skepticism of the raft hypothesis, raising an obvious question: if ordered domains are so easy to observe in biomimetic and isolated membranes, why are they so elusive (or illusive [82]) in live cells? One possibility was discussed earlier: there are several plausible mechanisms for generating Lo-Ld nanodomains that would be inaccessible to even modern methodology. Therefore, it may be necessary to manipulate membranes, for example by clustering raft components or releasing them from their cytoskeletal constraints, to directly observe domains in living cells.

However, the scales involved are far from the only methodological concern. Historically, a major issue was the lack of validated, domain-specific probes. This problem is conceptual as much as methodological: the Lo phase is defined by unique structural arrangements of tight, specific lipid packing. Almost by definition, tagging any raft lipid with a bulky, often hydrophilic label

reduces their affinity for raft domains. Consistently, most fluorescent lipids are excluded from ordered phases [39]. Recently, this problem was overcome by the development of novel probes that use the clever strategy of tagging lipid headgroups with the fluorophore far removed from the membrane by an inert spacer (e.g., polyethylene glycol) [83–85]. Another important tool in the arsenal for tagging rafts in cells are minimal proteins, which retain only the membrane-interacting portions of native proteins and, therefore, represent expressible probes for raft domains *in vivo*. Examples of these include GPI-anchored fluorophores [20], isolated protein TMDs [21,86], and lipidated intracellular anchors [20,74,75]. An exciting development in this field is the use of such selective anchors to target various reporters or effectors to specific membrane domains [10,87,88]. These have been used to selectively read out Akt activity and cAMP concentration in raft domains, or to sequester second messengers in specific membrane subcompartments [87,88].

You Say Yes, I Say No

Negative evidence (i.e., lack of direct visualization) is not the only reason for skepticism of the raft hypothesis. Widely used methods for raft 'isolation' have relied on cold-temperature detergent solubilization to generate detergent-resistant membranes (DRM), which have sometimes been construed as proxies for rafts in cells. These DRMs are enriched in sterols, sphingolipids, and lipidated proteins, the same components that partition to Lo phases in model and isolated PMs [89,90]. Furthermore, Lo phases in model membranes are detergent resistant, lending credence to the assumption that these DRMs represent living Lo phases [12,91]. By contrast, it is clear that residue arising from detergent dissolution of cells is unlikely to directly represent any native structure present in live cells for numerous reasons, including loss of native asymmetry, temperature-dependent lipid properties, and complex membrane–detergent interactions [82,92–96]. Thus, DRMs should not be equated with rafts and cannot provide conclusive evidence of raft composition or properties.

Finally, several methods have failed to directly detect rafts, despite cleverly designed approaches. One such attempt relied on mass spectrometry to scan the surface of fixed cells for chemical signatures of cholesterol and sphingolipid enrichment. While domains rich in SM [80] or putative raft proteins [97] were detected, these were not enriched in cholesterol. A separate approach attempted to immobilize GPI-anchored proteins (GPI-APs) to recruit ordered domains and did not detect enrichment of other putatively raft-preferring molecules or changes in diffusion in GPI-rich zones [79]. These are consistent with older studies of GPI-AP clustering [98]. By contrast, a study combining total internal reflection fluorescence (TIRF) microscopy with correlation analysis detected clear signatures of GPI-APs associating with membrane domains and stabilization of domains upon GPI-APs crosslinking [99]. It remains to be determined whether the above methods have the appropriate spatial and molecular resolution to definitively rule out the presence of compositionally distinct subdomains. An important concern is how different domains would need to be from each other and their surrounding bulk for these differences to be detectable. For example, model membrane experiments have reported relatively small differences in cholesterol concentration between Lo and Ld phases [100]. Moreover, lipid packing differences are smaller in natural membranes (i.e., GPMVs) than in the synthetic models often used to calibrate analytical methods [36,101]. Similarly, proteins and lipids rarely enrich by more than twofold in the ordered phase of GPMVs.

Looking for Rafts in all the Wrong Places

As discussed [5], lipid rafts may be used to organize subcellular traffic in the cell, segregating components in sorting organelles (Golgi and endosomes) to facilitate their trafficking to the PM. An extreme prediction from this hypothesis is that the PM is a 'postsorting' compartment so

distilled with raft lipids that stable domains may be hard to observe. Indeed, there is evidence that ordered domains comprise a major portion of the PM in some cells [54,66,69,102–104]. If this is the case, perhaps the search for lipid domains must be expanded to internal membranes that may have compositions more amenable to phase separation. Supporting evidence for this possibility is the direct observations of raft-like domains in endosomal compartments (vacuoles) of yeast [105]. These domains show the essential hallmarks of lipid–lipid phase separation [106] and may be the first direct demonstrations of raft domains *in vivo*. More indirect data (also discussed later) suggests that sorting of raft proteins occurs in endosomes of mammalian cells, suggesting the possibility of stable domains therein [86]. Unfortunately, the organization of internal membranes is difficult to access because they cannot be labeled form the outside and because they are generally small and not flat. However, a recent demonstration suggests that hypotonic swelling of cells allows inflation of organelles and perhaps direct visualization of membrane domains therein [107].

Break on through to the other Raft

The ultimate burden of proof for whether rafts are physiologically meaningful remains on proponents of the raft hypothesis. While a wealth of biophysical and cell biological data is supportive of selective, functional membrane domains in cells, definitive demonstrations have remained elusive. However, several recent advances have combined crossvalidated probes with biophysical and cellular insights to provide some of the most compelling evidence to date supporting relevance of liquid–-liquid phase separation in mammalian membranes.

Correlations Light the Way

A pair of studies used similar approaches to explore the detergent resistance, ordered phase preference, and *in vivo* diffusion of fluorescent analogs of raft lipids [76,77]. One group focused on SM analogs, while the other focused on a large array of analogs of glycosphingolipids. Both crossvalidated their probes, showing that only specific designs (fluorophore separated from the membrane by an inert linker) were enriched in raft domains of GPMVs and that those same analogs were detergent-resistant. These validated analogs were then evaluated via single-molecular tracking, revealing distinct behaviors for raft probes, which transiently associated with GPI-anchored proteins in nanoscopic raft-like clusters in living cells. These remarkably convergent observations from independent labs show that Lo/DRM partitioning is directly related to diffusion in live cells, providing strong evidence for the presence of submicroscopic ordered domains in live cells [76,77] (Figure 3A).

Super-Resolving Domain Assembly *in vivo*

A technical tour-de-force applied super-resolution microscopy to living B cells [108] and showed that proteins partitioning to ordered phases in GPMVs form raft-like nanoscopic clusters in activated cells [42] (Figure 3C). This behavior was shown to affect immune signaling, because clustering of the B cell receptor led to its recruitment to ordered nanodomains that sorted key regulators of cell activation. These observations were rationalized with a simple model wherein receptor clustering stabilizes pre-existing ordered fluctuations to form a signaling-competent, ‘extended ordered domain’. These results are fully consistent with classical literature in mast cells, which relied on DRMs to draw similar conclusions about the role of rafts in immune cell activation [109–113].

Viruses Organize Domains

Raft domains have long been implicated in the function of viruses and other pathogens [114]. Specifically, it has been proposed that certain viral proteins can recruit a raft domain at the host PM, allowing passive recruitment of other viral proteins for ultimate assembly of the viral envelope. This process was recently directly observed by imaging of clustered matrix protein (Gag) from HIV

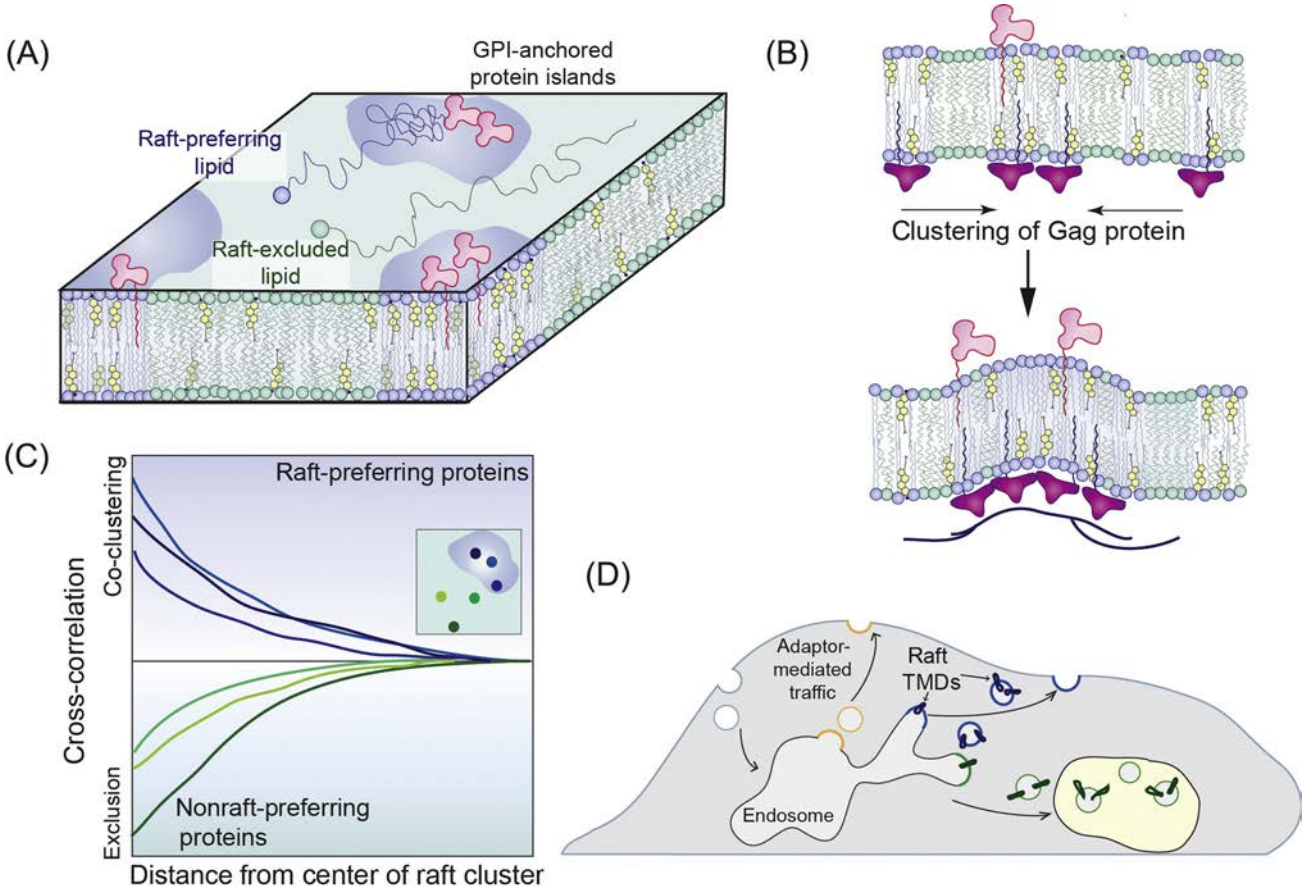

Figure 3. Accumulating Evidence for Raft Domains in Real Life. (A) A variety of crossvalidated probes have recently been developed, allowing comparison of single-molecule diffusion behaviors between raft-enriched and raft-excluded probes. Fluorescence lipid analogs that prefer ordered phases in model and bioderived membranes also tend to be detergent resistant and show distinct diffusive behaviors, statistically enriching in zones rich in glycosylphosphatidylinositol (GPI)-anchored proteins. (B) Clustering of viral Gag by matrix proteins leads to microscopically observable membrane domains that sort host proteins based on their affinity for ordered lipid phases. (C) Super-resolution microscopy in live cells reveals statistical enrichment of raft-preferring proteins near activated B cell receptors. (D) Some transmembrane proteins rely on raft affinity for their subcellular localization. For these, the transmembrane domains (TMDs) fully recapitulate the trafficking fates of the full-length protein, and their raft affinity is essential, because non-raft mutants are mis-sorted and ultimately degraded.

[115] (Figure 3B). These clusters recruited host raft proteins (raft affinity directly validated by GPMVs) as well as cholesterol and sphingolipids, all consistent with raft assembly during viral budding. A separate study with the same virus revealed that the receptor for HIV (CD4) is localized in raft domains in GPMVs, whereas the co-receptor (CCR5) enriches at the ordered-disordered boundary [116]. This configuration appears to facilitate the fusion of virus with host cell membranes, providing mechanistic insight into previous implications of rafts in viral entry [114].

Rafting through Traffic

As discussed earlier, raft domains are well suited to provide a platform for the accurate and robust sorting of membrane components between organelles [4,6]. Isolation of post-Golgi vesicles from yeast appears to confirm the potential for lipid-mediated sorting of proteins in the secretory pathway [117,118]. The involvement of rafts in subcellular sorting in mammalian cells was recently confirmed by analysis of the trafficking and localization of single-pass protein TMDs (Figure 3D). Such TMDs have been extensively engineered to evaluate the determinants of transmembrane

protein affinity for raft domains in GPMVs [21,86], revealing structural features, such as TMD length and surface area, as key drivers of raft affinity. Given that these raft-partitioning TMDs have no residues that can interact with known cytosolic trafficking machineries, they act as genetically encoded probes of trafficking of ordered domains in cells. Remarkably, for a broad panel of TMDs, raft association was fully sufficient for their recycling to the PM after endocytosis, establishing a quantitative and functional relationship between raft association and subcellular protein localization [86]. These observations support the conclusion that ordered membrane domains mediate the recycling of specific membrane components from the endosomal compartments to the PM. Subsequent bioinformatic analyses suggest that PM-resident proteins tend to have higher raft affinity than those of secretory organelles [21], in line with the putative role of rafts in secretory sorting. Finally, sorting of SM into specific post-Golgi vesicles destined for the PM was recently directly observed [119,120].

An overarching conclusion of the studies discussed in this section is that L_o-like domains are present in live cell membranes as nanoscopic entities that influence cellular physiology, signaling, and trafficking. Importantly, the features of live cell rafts inferred from these observations (nanometric, highly dynamic, and potentially heterogeneous) make them inherently difficult to observe in their native context. Studies such as those discussed earlier, which rely on natural membrane models (i.e., GPMVs) to directly quantify raft-associated behaviors and relate them to cellular effects, are enabling rapid progress towards resolving the raft controversy.

Concluding Remarks: Rafts Keep on Slipping, Slipping, Slipping…into the Future

The studies described in the earlier section have provided compelling evidence that strongly supports the existence of rafts *in vivo*. These advances have some common themes: they rely on bioorthogonal probes for ordered membrane environments (e.g., synthetic lipids or non-natural TMD sequences) and validate those probes using several independent methods. The behavior of these probes is then evaluated in living cells relative to analogous probes with distinct preferences for disordered membrane regions. Finally, the methodology for detection must either be extremely sensitive to nanometer and millisecond scales or rely on cellular machineries to amplify differences between probes. This general set of approaches may provide a useful guide for coherent characterization of the physiological roles of membrane domains.

One of the broadest and most important open questions in membrane biology concerns the biophysical consequences of lipid asymmetry (see Outstanding Questions). The asymmetric distribution of lipids between the two bilayer leaflets is a fundamental feature of nearly all PMs [27], but the effect of this asymmetry on biophysical properties of the membrane is largely unexplored. Part of the reason is the historical lack of well-controlled experimental model systems with stable, robust lipid asymmetry. This limitation has been recently overcome by several clever approaches [121–123], paving the way towards exploration of the coupling between lateral domains and transverse lipid organization. A fundamental open question is: what is the lateral organization of a membrane, wherein one leaflet is expected to separate into coexisting Lo/Ld phases whereas the other leaflet would not? Such is the most likely configuration of the mammalian PM, with a SM- and cholesterol-rich outer leaflet and an inner leaflet generally lacking in saturated lipids. In most experiments (both in model membranes [124,125] and in cells [67,115]), information about lateral domains appears to be transmitted across the bilayer midplane; however, the mechanism of this transbilayer coupling remains mysterious. An exciting possibility is that asymmetric membranes represent an entirely unexplored state of matter, wherein large-scale domain formation is suppressed due to a strong mismatch penalty between leaflets, which can be rapidly released by modulation of lipid asymmetry.

Outstanding Questions

What are the physicochemical principles underlying raft formation?

How are these principles manipulated by cells to produce and regulate nanoscopic, dynamic domains?

What is the evidence that rafts exist *in vivo*? What is the evidence against such existence?

How does membrane asymmetry contribute to lateral organization?

What is the interplay between lipids and membrane protein in regulating membrane structure?

Finally, continuing advances across microscopic modalities suggest that 'seeing' rafts in real life may be just a matter of time. Expansion microscopy and other forms of increasing contrast combined with continuing extension of the boundaries of temporal and spatial resolution may eventually allow direct observations of domains *in vivo*. As noted earlier, these searches should not be limited to the PM, because internal membranes may be no less likely to have observable domains. Finally, the recent developments in cryogenic electron microscopy in intact cells [126] may open doors towards directly imaging membrane properties *in situ*.

Acknowledgments

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