The Continuing Mystery of Lipid Rafts

Ilya Levental[1,†] **and Sarah L. Veatch**[2,†]

1 - Department of Integrative Biology and Pharmacology, *McGovern Medical School at the University of Texas Houston, 6431 Fannin St., Houston, TX, 77030, USA*
2 - University of Michigan, *Ann Arbor, MI 48109 USA*

Correspondence to Ilya Levental and Sarah L. Veatch: *ilya.levental@uth.tmc.edu*
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Abstract

Since its initial formalization nearly 20 years ago, the concept of lipid rafts has generated a tremendous amount of attention and interest and nearly as much controversy. The controversy is perhaps surprising because the notion itself is intuitive: compartmentalization in time and space is a ubiquitous theme at all scales of biology, and therefore, the partitioning of cellular membranes into lateral subdivision should be expected. Nevertheless, the physicochemical principles responsible for compartmentalization and the molecular mechanisms by which they are functionalized remain nearly as mysterious today as they were two decades ago. Herein, we review recent literature on this topic with a specific focus on the major open questions in the field including: (1) what are the best tools to assay raft behavior in living membranes? (2) what is the function of the complex lipidome of mammalian cells with respect to membrane organization? (3) what are the mechanisms that drive raft formation and determine their properties? (4) how can rafts be modulated? (5) how is membrane compartmentalization integrated into cellular signaling? Despite decades of intensive research, this compelling field remains full of fundamental questions.

Introduction

The lipid raft model was originally proposed to explain the robust sorting of select lipid and protein cargo to the apical plasma membrane (PM) in polarized epithelial cells [1,2]. For the next 20 years, the intuitive appeal of a simple mechanism for functional compartmentalization of cellular membranes led to the application of this idea to nearly all areas of cell biology [3]. Most notable among these is signal transduction at and through the PM, with lateral domains serving as core regulators by mediating the interaction between membrane proteins [4,5]. However, despite the many cellular roles attributed to lipid rafts, the true nature of membrane organization in living cells remains unknown.

The continuing mystery of lipid rafts remains unresolved because of inherent limitations in methodology. It now seems clear that the simplistic conception of distinct, stable, and long-lived membrane domains in unperturbed PMs is inconsistent with most available evidence. Rather, cell membrane lateral organization is best conceptualized as being only somewhat heterogeneous on length scales of tens of nanometers and timescales of milliseconds. Although such length/time scales are well within range of biological relevance, few analytical techniques have the appropriate spatial and temporal resolution to accurately probe these features. Here, we review some of the major lines of inquiry into the raft phenomena from their first applications to their modern incarnations, and we highlight the key methodologies that may yield answers to the many remaining fundamental questions that continue to define the field.

A Spectrum of Membrane Models

The key physical insight behind the lipid raft hypothesis was the proposal that biomimetic model membranes could separate into coexisting liquid phases [6–9]. The early studies in this field used spectroscopic measurements of hydrated bilayers [10–13] and microscopic visualization of lipid

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monolayers formed at air–water interfaces [14] to suggest the formation of a novel liquid lipid phase in membranes composed of biological lipids. This conclusion was later confirmed by microscopic observation and characterization of coexisting liquid domains in both free and supported lipid bilayers [15,16], which were shown to have different compositions, degrees of molecular packing/order, and different single-molecule diffusivities [15,17–19]. Such observations facilitated the detailed mapping of the phase diagrams of biomimetic membrane systems, which revealed that the coexistence between a liquid-disordered (L_d) phase and a liquid-ordered (L_o) phase—distinguished by its higher molecular packing, lower diffusivity, and more extended (i.e., ordered) lipid acyl chains—was observable across a wide range of compositions and temperatures [20–24]. More recently, computational modeling has added a tool to the arsenal of probing biomimetic membranes [25], allowing the detailed molecular-level analysis of bilayer organization [26,27] and physical interactions between lipids [28] and proteins [29]. Although previously limited to relatively small and simplified membranes, advances in coarse-graining and computing technologies now allow the simulations of large and complex systems [30].

These biophysical investigations provided the necessary physicochemical underpinnings for lipid-driven lateral domains in biological membranes, but direct translation from simple models to cellular membranes was, and continues to be, hampered by their fundamental differences. Certainly important are the limited compositional complexity of model membranes and their lack, or paucity, of integral proteins, which comprise a large fraction of cell membranes (~20% by area) [31]. As detailed below, eukaryotic membranes are composed of hundreds of lipids species whose relative abundances are dynamically regulated. Furthermore, cell membranes are often asymmetric, with the two opposing leaflets containing distinct lipid compositions. In contrast, biomimetic membranes are usually constructed from less than five different lipid components and are rarely asymmetric, although recent advances have introduced technologies that should facilitate investigation into this key membrane property [32–34]. Finally, most model membranes lack key aspects that are fundamental to cell membrane functions, including but not limited to connection to a dynamic actin cortex and the transport of membrane components via both active and passive mechanisms. Recent progress has begun to address some of these issues [35–37], but the vast divide between model and cell membranes will likely remain a major challenge for bottom-up investigations for decades to come.

Liquid–liquid phase separation can also be observed in lipids extracted from biological samples, including erythrocytes [38] and luminal membranes of renal epithelia [15], lending credence to its physiological relevance. However, the vital observations validating the capacity of intact cell membranes to form coexisting liquid phases were made in PMs isolated from living cells, either as giant plasma membrane vesicles (GPMVs) [39–42] or as plasma membrane spheres (PMS) [43]. These intact PMs detach from mammalian cells by blebbing [44] and separate into coexisting liquid domains [39] of different compositions [39,45–47], orders [18,47], and diffusivities [48]. In GPMVs, this behavior is typically induced by lowering the temperature; in PMS, by crosslinking a raft component (the ganglioside GM1 using cholera toxin). Phase separation laterally sorts the endogenous components of the PM, often in agreement with previous insights into lipid raft composition. Specifically, ordered domains enrich for glycophosphatidylinositol-anchored proteins (GPI-anchored proteins) [39,45,46], glycosphingolipids [45], palmitoylated transmembrane proteins [46], and proteins with long transmembrane domains [49], and these domains exclude putative non-raft proteins such as the Transferrin receptor [43,46] and those with isoprenoid membrane anchors [50]. Altogether, these observations comprise a critical confirmation that complex biological membranes can form lipid-driven ordered domains that preferentially recruit raft components. This capability was recently confirmed in internal organelles of living yeast cells [51].

GPMVs can be conceptualized as an intermediate membrane model system between fully synthetic microscopic membranes [i.e., giant unilamellar vesicles (GUVs)] and living cell membranes (Fig. 1). The major disadvantage of the GPMV system compared to synthetic membranes is the lack of control over their composition and the inter-cell and inter-preparation variability inherent to biology. Conversely, in terms of lipid and protein content, GPMVs are self-evidently more suited to comparison with living membranes. In that context, comparing the features between GPMVs and GUVs can illustrate features unique to cell-derived membranes (Fig. 1). A notable example is the difference in physical properties between coexisting domains in phase-separated vesicles: while both GUVs and GPMVs separate into two phases of different orders, the relative difference in order between the phases is much lower in the cell-derived GPMVs than the synthetic membranes [18]. The disparity is largely attributable to the L_d phase, which is much more packed/ordered (and therefore more similar to the L_o phase) in GPMVs than in GUVs. This discrepancy is likely due to the compositional details of the two membranes: lipids with two unsaturated acyl chains, which disrupt packing and are commonly used components of GUVs, are not as abundant in mammalian cell membranes [52–54], where the majority of phospholipids contain a saturated and monounsaturated chain (Fig. 2). Moreover, the abundant protein content of GPMVs likely affects membrane packing [47].

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The difference in interdomain order between GUVs and GPMVs potentially resolves an important discrepancy in protein and lipid partitioning between the two systems. Namely, nearly all fluorescent lipids tested thus far show greater ordered phase affinity in GPMVs compared to GUVs [55]. The same is often true for proteins, including a GPI-anchored protein [56] and a palmitoylated protein [57], which prefer the disordered phase in synthetic membranes, in striking contrast to similar or identical proteins efficiently partitioning to the ordered phase in GPMVs [39,46]. A possible explanation is that the highly disordered L_d domain and the unnaturally packed L_o domain of GUVs combine to reduce ordered phase partitioning for nearly all probes. Finally, this interdomain order sorting has recently been shown to affect the bioactivity of membrane molecules, for example, the binding between the membrane glycolipid GM1 and its ligand cholera toxin, which was highly sensitive to membrane order and the difference between coexisting phases [55,58].

The advantage of GPMVs over live cell membranes is that they facilitate investigation into raft-associated phenomena like protein partitioning [59] and regulators of phase separation [60] in a controlled setting. However, it is important to emphasize that GPMVs are not the same as living cell membranes in many important ways including: (1) their composition is likely altered during isolation, as was shown for degradation of phosphoinositides [61], and some aspects of GPMVs are known to be affected by the chemicals used for preparation [47], highlighting potential caveats associated with the method of isolation; (2) GPMVs are not strictly asymmetric with respect to phosphatidylserine exposure [39]; (3) they are at equilibrium; (4) they are not coupled to an assembled actin cytoskeleton. These caveats do not negate the practical or conceptual value of GPMVs but are important in translating insights from this biomimetic membrane model to physiological settings.

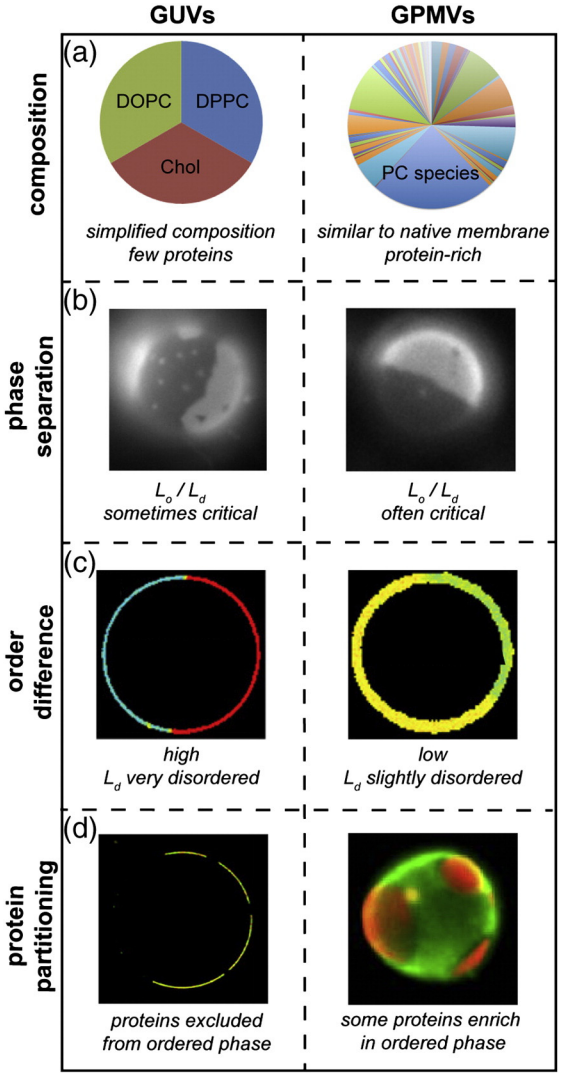

Fig. 1. Comparison between synthetic (GUVs) and biological (GPMVs) membrane model systems. (a) Synthetic and biological model membranes differ dramatically with respect to their lipid and protein composition. Synthetic model membranes like GUVs are typically composed of a small number of different lipid components and few, if any, proteins. Shown is a canonical "raft mixture" containing equimolar saturated (DPPC) and unsaturated (DOPC) phospholipid, and cholesterol (Chol). In contrast, biological membrane models like GPMVs contain hundreds of different lipids and likely thousands of proteins. Shown is a representative distribution of phosphatidylcholine species in a PM sample, which contains a few major species (e.g., POPC is ~25 mol% of all PC) and over 100 minor ones [54]. (b) Despite these differences, GUVs and GPMVs are morphologically similar, and both separate into coexisting ordered and disordered phases, visualized by staining vesicles with lipidic dyes that preferentially partition into one of the phases [93]. Notably, GPMVs often show near-critical behavior, also achievable in GUVs at specific compositions. (c) Despite these superficial similarities, these are notable difference in domain properties between GPMVs and GUVs. For example, coexisting domains in GPMVs are much more similar than in GUVs with respect to lipid packing. Images shown are pseudocolored maps of Laurdan generalized polarization, a widely used proxy for lipid packing (for experimental details, see Refs. [18]). (d) The relatively high disparity possibly results in most components being excluded from GUV ordered domains, whereas a number of lipids and proteins partition preferentially to this phase in GPMVs. Images show the partition of a peptide based on the transmembrane protein LAT. Yellow pseudocolor represents co-partitioning with a disordered phase marker in GUVs, while distinct red staining of one phase *versus* green in the other shows that the LAT-RFP protein partitions away from the disordered phase marker in GPMVs (for details, see Refs. [46,57]).

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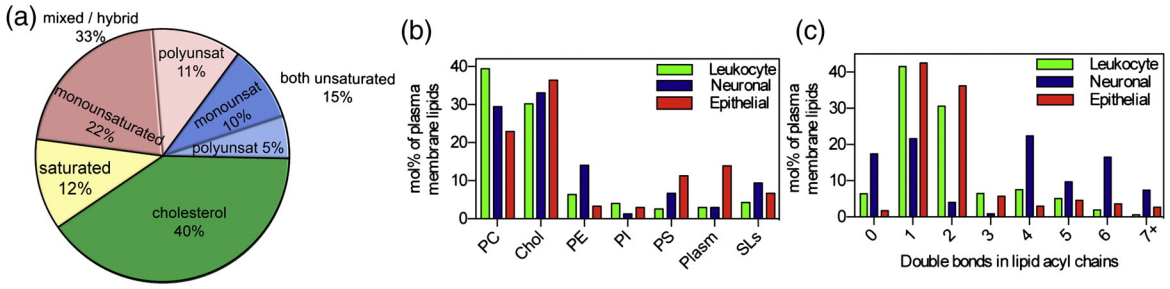

Fig. 2. Sample lipidomes from mammalian PMs. (a) The complex composition of mammalian membranes can be represented in classes that are analogous to those often used for model membrane studies. In contrast to the commonly used synthetic membrane preparations, mammalian PMs contain a high proportion of "mixed" lipid species with one saturated and one unsaturated acyl chain. Shown here is a representative lipidome of mesenchymal cell PMs from unpublished studies in the IL lab. Furthermore, it should be appreciated that most lipidomic features are cell/tissue specific. Shown are the comparisons of (b) lipid classes and (c) unsaturation among the PMs of three different cell types: leukocytes [54], epithelial cells [52], and neurons [82].

Lipidomic Complexity: the Known Unknowns

One of the initial clues suggesting the existence of raft domains in cellular membranes was the coordinated sorting of lipids and proteins in epithelial cells, which maintain a highly specialized apical membrane enriched in canonical raft components [1,62]. The isolation and characterization of specific trafficking vesicles destined for the apical *versus* the basolateral membrane [63] confirmed a membrane-mediated sorting step at the late Golgi [64]. Such apically directed cargo was resistant to solubilization by non-ionic detergents (e.g., Triton X-100), and this detergent-resistant membrane (DRM) residue was also enriched in specific lipid subsets, namely glycosphingolipids and cholesterol [2]. These observations led to the hypothesis of glycosphingolipid-enriched entities that facilitate membrane sorting in both polarized epithelia and unpolarized cells [65,66].

Early studies necessarily focused on broad classes of lipids, without much insight into the detailed compositions of the source membranes or their putative microdomains. Such insights were provided by mass spectrometric analysis of individual lipid species, which detailed the compositional enrichments in DRMs, confirming their selectivity for sphingomyelin and long, saturated phospholipids [67,68]. Comprehensive information on membrane lipid composition has only become available recently, with the implementation of shotgun lipidomics. These studies have revealed the remarkable complexity and plasticity of eukaryotic lipidomes (Fig. 2). A study in *Saccharomyces cerevisiae* identified 250 individual lipid species with significant quantitative changes in nearly all of the species induced by single-gene knockouts in biosynthetic pathways [69]. Such wholesale remodeling of the yeast lipidome appears to be a general phenomenon, as subtle variations in growth temperature, phase, and carbon source yield distinct lipidomic signatures [70]. These observations suggest deeply integrated cellular processes that sense changes in cellular metabolism and/or membrane properties and respond by tuning compositions across the spectrum of lipid subtypes. The mechanisms and purposes of these sense-and-respond modules remain generally unknown.

Comparative, lipidome-wide analyses enabled by lipidomics provide especially powerful tools to identify subtle lipid sorting signatures. A technical tour-de-force study isolated secretory vesicles leaving the Golgi apparatus by immunopurification targeting a putative marker protein for raft domains. The lipid composition of these vesicles was clearly different from their parent organelle (the *trans*-Golgi), with the vesicles being enriched in raft lipids including the major yeast sterol (ergosterol) and glycosphingolipids, providing clear evidence of lipid sorting in the late secretory pathway [71]. These vesicles were later shown to be also different from their destination organelle, the PM [72]. These observations validated the substantial literature implicating raft domains in membrane sorting processes [62].

Similar themes are observable in mammalian cells. A model epithelial cell line that develops a discrete apical PM in culture on porous filter substrates (Madin–Darby Canine Kidney) revealed the clear remodeling of the lipidome during cellular polarization, which could be largely reversed by inducing an *in vitro* model of an epithelial-to-mesenchymal transition (EMT) [53]. These distinct lipid profiles are manifested in biophysical changes, as EMT induced by several independent means dramatically reduced the stability of domain formation in isolated GPMVs [73]. Notably, these effects could be partially reversed by altering cellular lipidomes via dietary lipids [73]. EMT is thought to be involved in several cancer phenotypes, including migration and escape from apoptosis, so the observations that EMT is associated with distinctive

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lipidomic and biophysical characteristics that are tunable by dietary pertubations may have translational implications for cancer diagnosis and treatment. With respect to lipid sorting in mammalian membranes, much attention has been paid to viruses. Viral proteins (most notably influenza hemagglutinin) were some of the first to be associated with raft domains [74], leading to the hypothesis that viruses "hijack" raft domains for host cell entry, subcellular sorting, and/or assembly and budding of nascent virions [75,76]. Evidence for this hypothesis was provided by the comparison of viral lipidomes with those of their host cells. The lipid composition of the human immunodeficiency virus membrane is clearly differentiated from that of the host cells [77] and crucially the host cell PM, which is the parent organelle for the budding viral particles [78]. Viral membranes are enriched in cholesterol and sphingolipids, convincingly implicating raft-mediated sorting during viral propagation. These observations were confirmed in studies of the influenza virus [52]. Finally, lipidomic analysis has proven to be a powerful tool for diagnosing disease. For example, brain tissue from Alzheimer's disease patients and rodent models suggested compositional signatures associated with disease [79]. Most remarkably, this signature could be identified in peripheral blood, where a biomarker study found a small set of phospholipids in blood plasma that was 90% predictive for symptomatic disease onset [80].

Although these are still early days of lipidomic investigations, the data gathered have revealed an unexpected complexity and diversity of lipid compositions in cellular membranes. Figure 2a shows a sample lipidome from primary human mesenchymal stem cells grouped into broad categories based on their acyl chains. These categories contain dozens, sometimes hundreds, of different species, distinguished by their hydrophilic headgroups but also by the lengths and unsaturations of their constituent fatty acids. These features can vary dramatically between PMs of various cell types (Fig. 2b–c). Moreover, these lipidomic characteristics are not constant but are rather affected by both endogenous processes (e.g., cell cycle [81]) and exogenous cues, including dietary fats [54,73]. A dramatic, recent example is the remodeling of the lipid composition of neuronal synapses during postnatal development in the rat [82]. Over the first few weeks of life, synaptic membranes gradually become highly enriched in cholesterol and sphingolipids, in addition to the continuous accumulation of lipids with ω-3 polyunsaturated fatty acids. These changes lead to a stabilization of membrane domains in reconstituted membranes, suggesting a mechanism for localized recruitment of raft domains to the neuronal synapse [82].

The unexpected complexity and diversity of lipid compositions in cellular membranes prompt exciting but difficult questions regarding their biological purpose. In most descriptions of membrane physiology [83], the active processes (e.g., transport, signal transduction, etc.) are carried out by proteins, with the lipids reduced to subservient roles of barriers and solvents. However, these passive functions can be accomplished by very simple membranes, composed even of single lipid species. Signaling lipids (e.g., ceramide, phosphoinositide phosphates, glycosphingolipids) could perhaps account for a few more functionally important subtypes, but it is difficult to make the teleological case for the hundreds of lipid species produced in mammalian cells. Some recent studies of species-specific lipid functions begin to shed some light. A study combining photo-crosslinking, fluorescence spectroscopy, and bioinformatics showed remarkably specific binding between a single lipid (sphingomyelin with an 18-carbon acyl chain) and a transmembrane domain of a single-pass membrane protein (P24) [84]. Other examples of species-level selectivity include preferential phosphorylation of polyunsaturated phosphoinosidites by PI3Kinase [85].

Crucially, membrane behavior is dictated not just by the behaviors of individual lipids but also by the emergent properties of lipid collectives. These include bulk physical characteristics like permeability, fluidity, spontaneous curvature, and stiffness and also lateral domains resulting from preferred interactions between some lipid species. Although there is an extensive knowledge base about the compositional determinants of physical behavior in biomimetic membranes [86], most work has investigated simple membrane models. Thus, it remains an open question whether biological complexity introduces novel features to lipid collectives and how these may be regulated by compositional modulation. Our recent advances reveal that the collective behaviors of cell-derived membranes are indeed susceptible to external modulation, both by dietary lipids [54] and exogenous amphiphiles [60,87], and it remains to be addressed how these modulations affect membrane physiology.

Some Things Remain Universal

Even though cell PMs have thousands of individual components, they separate into only two microscopically discernable liquid phases [39]. In one sense, this is remarkable—the number of components dictates the number of potential degrees of freedom in the system, so in principle, it is thermodynamically possible that a complex membrane could support hundreds of liquid phases [88]. In reality, maintaining even two liquid phases in coexistence requires a delicate balance. This is because liquids are defined by relatively weak intermolecular interactions that maintain fluidity, yet relatively large intermolecular interactions are required to overcome the major entropic cost of demixing. In

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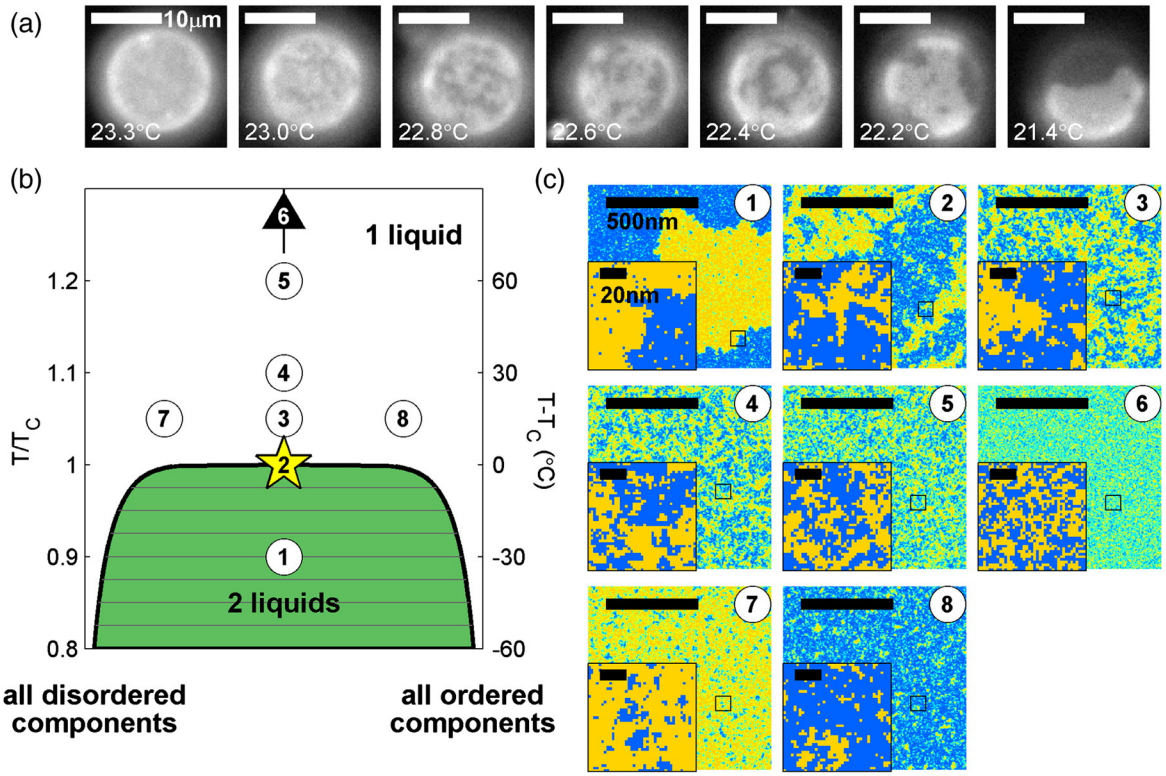

Fig. 3. Critical fluctuations in GPMVs and the 2D Ising model. (a) Membrane heterogeneity emerges over an extended temperature range in GPMVs, consistent with their passing through a critical point at the miscibility transition temperature. (b) Miscibility phase diagram for the conserved order parameter 2D Ising model or binary fluid that contains only two components, referred to here as ordered or disordered components. The left axis shows temperature in units of the critical temperature (T_C), while the right axis converts this to °C, assuming that T_C is 300 K (27 °C). (c) Simulation snapshots from an equilibrated 2D Ising model for the conditions indicated in (b). Simulations were conducted using standard methods as described previously [35]. Insets are enlarged areas of the main image in the location indicated by a black box and indicate that structure on the 20-nm scale is present even for conditions far removed from the critical point. Condition 6 is for an equal fraction of ordered and disordered components at a temperature of 2 × T_C or T-T_C = 300 °C, which is well outside of the temperature range plotted in (b).

three dimensions, most liquids are miscible with either organic or aqueous solvents, and it is rare to observe more than two phases in coexistence. While there are exceptions [89–91], they include compounds that vary dramatically in molecular weight (e.g., for the case of polymers) or atomic composition (e.g., metals, or compounds containing silicon or fluorine). In membranes, it is widely believed that cholesterol helps to manage this delicate balance [92] by preventing strong and stable interactions between acyl chains that would lead to a gel phase while also permitting the preferential interactions that drive demixing.

The experimental observation of only two liquid phases in both model and biological membranes provides an opportunity to greatly simplify some aspects of even compositionally complex membranes. The phases themselves remain compositionally complex, but many of their bulk physical properties are both robust and simply described. For example, at any given set of equilibrium conditions, phases have well-defined average composition and acyl chain order, and individual components have strictly defined partition coefficients. Furthermore, even complex membranes have discrete miscibility transition temperatures, often referred to as T_{mix} or T_{misc}. Above this transition temperature, membranes contain only a single liquid phase; while below it, two coexisting liquid phases are observed. The value of the transition temperature sets an important energy scale in the system—it is the temperature above which entropic interactions overwhelm enthalpic ones on macroscopic length-scales.

Beyond simply having a miscibility transition, GPMVs appear to have compositions that reside near a miscibility critical point [93]. A critical point is a special composition and temperature in the thermodynamic phase diagram where the free energy cost of assembling/disassembling a macroscopic domain

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becomes small compared to the thermal energy [94]. As a result, systems near critical points exhibit large and dynamic fluctuations in composition. Micron-sized composition fluctuations are observed in GPMVs close to their miscibility transition temperature [93] (Fig. 3a). Above this temperature, called the critical temperature or T_C, vesicles retain structure at and below a characteristic length, called the correlation length, which decreases as temperature rises. Below T_C, GPMVs contain coexisting liquid domains that have dynamic fluctuating boundaries that become more stable as temperature is decreased. Together, fluctuations on either side of T_C mean that GPMVs transition gradually between a single liquid phase and two coexisting liquid phases when temperature is varied slowly (e.g., at intervals of 0.2 °C; Fig. 3a). Similar behavior is also observed in synthetic GUVs with compositions carefully chosen to pass through a critical point at the miscibility transition temperature [95,96]. This is in contrast to model membranes with compositions that pass through the miscibility transition far from a critical point, which transition sharply from being uniform to containing circular domains [21,92].

The experimental observation that GPMVs are close to a miscibility critical point provides the means to make even more broadly sweeping simplifications regarding the heterogeneity of these membranes. This is because many features of critical systems are universal, implying that they do not depend on the molecular details that determine the conditions of the critical point but only on the relative proximity to this point and their dimensionality [94]. This universality allows quantitative description of fluctuating domains (e.g., characteristic size and lifetime) by defining only a few parameters: namely, the distance of the membrane from the critical point, both in composition and temperature. Crucially, both of these quantities can be derived from the macroscopic behavior of membranes. They can then be used to map the GPMV phase diagram onto a much simpler system that also exhibits critical behavior, namely the two-dimensional (2D) Ising model [35] (Fig. 3b). In the simplest case, temperature is mapped by expressing in units of T_C (in Kelvin). For example, if T_C is 22 °C or 295 K, then 37 °C is expressed as $1.05 \times T_C$. Composition is mapped onto the 2D Ising model by measuring the surface fraction of phases at a known temperature below the transition [97]. This value can be converted into composition using the lever rule and the known position of the 2D Ising model phase boundary. The surface fraction of phases is equal at the critical composition. Under this condition, the characteristic size of compositional heterogeneities varies as the inverse temperature difference from the critical temperature [94] (Fig. 3c). This reasoning was used to predict roughly 20-nm domains at physiological temperature in GPMVs isolated from mammalian cells [93]. In principle, this approach can be used to generate predictions regarding the structure in complex biomembranes even if they are not poised exactly at a critical point, as long as they are "close enough".

How close is close enough? It seems that the answer may be: not that close. As stated above, the temperature scale is set by the critical temperature itself, which is typically between 275 K (2 °C) and 300 K (27 °C) for GPMVs isolated from mammalian cells, depending on the preparation method and cell type [47,48,60,93,98]. Thus, the physiological temperature (37 °C) is between 10 °C and 35 °C above the critical temperature or $1.03 \times T_C$ and $1.13 \times T_C$, respectively. Even at the high end, this difference is within 15% of T_c, well within the range where 2D Ising model scaling laws apply. Experiments using atomic force microscopy AFM to probe supported membranes have shown the applicability of 2D Ising model predictions to nearly 10 °C from T_C by directly measuring the correlation lengths of detected domains in supported membranes [99]. Experiments in both synthetic GUVs and isolated GPMVs also showed consistency with 2D Ising model predictions over a broader temperature range (up to >30 °C above T_C), where an extended phase-like domain was stabilized through adhesion to a supported membrane [97]. This same study also found that Ising model predictions applied when synthetic GUV membranes were prepared with compositions far from a critical composition. This is likely because the shape of the high temperature edge of the 2D Ising model phase diagram is quite flat, meaning that a broad range of compositions reside close to this high temperature boundary at their transition (Fig. 3b). In the three-dimensional (3D) Ising model, as with 3D systems in general, the phase boundary has a different shape near the critical point, making fluctuations more dependent on composition than in 2D systems like membranes [94]. Finally, even vesicles that do not undergo a macroscopic phase transition can exhibit critical behavior, as long as their compositions are close to a phase boundary at some temperature [97].

A system does need to be close to the critical point to observe the micron-sized critical composition fluctuations necessary for detection by conventional fluorescence microscopy (Fig. 3a). These microscopic fluctuations are 3 orders of magnitude larger than the characteristic length scale of the system, roughly the size of a typical lipid (~1 nm). To obtain such large structures, the system has to be within 0.1% of the critical temperature (roughly 0.3 °C) and similarly close in membrane composition. However, such large fluctuations are not required for functional significance. Indeed, structures on length scales that are relevant to interactions between proteins (~10 nm) require much less stringency. Heterogeneity on this length scale is observed even 20% (60 °C) above T_c and over a large swath of compositional

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space (Fig. 3c). Therefore, since a miscibility transition has been observed in isolated PMs, it would be surprising if cell PMs did not exhibit composition fluctuations on physiologically relevant scales.

Several recent theoretical approaches have suggested alternate mechanisms to produce submicron phase-like domains in membranes. Some of these require the presence of asymmetry in the membrane [100,101] or through the incorporation of hybrid components that stabilize small domains while preventing their coalescence into larger structures [102]. These mechanisms produce so-called modulated phases that resemble emulsions such as milk but are constrained to two dimensions. These processes could potentially work in concert with composition fluctuations to make structures more robust [100,103,104]. Much work remains to be done to uncover the exact physical means by which cells organize their PMs, but a range of plausible and exciting possibilities exist.

Tuning Domains

An outstanding question is how membrane domains are regulated and how such regulation may contribute to cell physiology. The primary means for functionalization of domains is thought to be preferential recruitment of protein and lipid components to generate the compartments of specific composition. An additional control parameter is the domains themselves, namely their physical properties like size and/or lifetime. Variation of the spatial and temporal scales of membrane domains could be key for tuning the output of raft-associated signaling processes [105–107]. Unfortunately, there remains a dearth of reliable techniques for probing domains *in situ* and for controllably modulating their properties.

The original method for perturbing rafts and raft-related processes in cells was cholesterol depletion via cyclodextrin, a polysaccharide that extracts cholesterol with reasonable efficiency and specificity. This idea is deceptively simple—since cholesterol is required for rafts, its depletion should disrupt raft assembly and functionality. Consistent with this supposition, cholesterol depletion reduces detergent resistance of many proteins [74] and affects polarized membrane traffic, signaling [108], activation of immune cells [109], and dozens of other cellular processes. It is probably inaccurate to attribute all these effects directly to the disruption of lipid rafts, because acute cholesterol depletion is inherently pleiotropic. Cholesterol is highly abundant in the PM (up to 40 mol% [52,54]) and affects nearly all bulk membrane physical characteristics. For example, cholesterol is essential for PM barrier function [110], and its depletion almost certainly increases membrane permeability and perturbs ion polarity. Self-evidently, such perturbations are extremely disruptive and often cytotoxic [111]. Notably, even the effects of cholesterol modulation on membrane phase separation are not easily predictable. In two different cell lines, similar depletion protocols yielded opposing effects on the miscibility transition temperature, increasing in one case [48] and decreasing in another [54]. These results reflect the clearly non-monotonic behavior of phase separation with varying cholesterol concentration, consistent with simple models [21].

Recently, several approaches have taken advantage of GPMVs to introduce alternative methods of modulating raft domains. As described above, treatments that affect miscibility transition temperature in GPMVs may reflect modulators of raft properties *in situ*. One emerging theme from these studies is that exogenous amphiphiles are potentially powerful regulators of GPMV phase behavior. These include bile acids [87], menthol [112], hexadecanol [113], and synthetic detergents [54], which robustly enhance phase separation, and a broadly potent range of anesthetics [60], which all suppress phase separation. These exogenous perturbations tend to be somewhat greater in magnitude than those induced by ectopic protein binding [114] or expression [115]. One possible rationalization for these effects is that phase separation is dependent on the relative difference between coexisting domains, with treatments that increase this difference stabilizing phase separation and *vice versa*. A defining difference between L_o and L_d domains is their membrane packing/order, and it has been demonstrated that agents that increase this order difference promote phase separation in GPMVs [47,54,87] and may also affect protein–ligand interactions [58]. An attractive and related hypothesis is that some agents partition to the L_o/L_d membrane interface, thereby promoting mixing and domain dispersion [60,116].

An emerging concept takes advantage of cellular metabolism and homeostasis to tune membrane domains via physiologically relevant conditions. For example, supplementation of cultured cells with dietary fatty acids, most notably the ω-3 polyunsaturated docosahexaenoic acid, leads to their robust incorporation into membrane lipids and subsequent homeostatic lipidomic remodeling. The combined result of these effects is a notable modulation of phase separation [54]. As in the case of bile acids [87], polyunsaturated lipids appear to disorder the L_d phase, leading to enhanced interdomain order differences and stabilization of domain separation [54]. Similarly, dramatic effects are induced by modulations of cell cycle and cell density of living cells [98], with the time scales of these responses suggesting adaptation to culture conditions. Currently, neither the cellular mechanisms nor the functional consequences of these responses are understood.

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Measuring Domains

A fundamental challenge remaining for the raft field is unambiguous identification and characterization of these domains and their properties in living cells. This hurdle persists because putative raft molecules do not segregate into microscopically visible domains in live cells. This observation prompts two exclusive conclusions: either there are no structures or there is organization, but at a length scale below the resolution of optical microscopy. Evidence for the latter conclusion was produced by early studies that took advantage of antibodies to crosslink and cluster raft proteins on the surface of the cells. Such crosslinking produces large lateral patches in the PM that are selective for putative raft components, including GPI-anchored proteins and GM1 [117]. Patching could be disrupted by cholesterol depletion, supporting the conclusion that this phenomenon is related to previously unresolvable membrane rafts. Remarkably, patches induced by extracellular crosslinking of strictly outer leaflet components (e.g., GPI-anchored protein) could recruit strictly intracellular signaling molecules [118] and activate signaling [119], implying domain-mediated transmembrane communication.

Despite the tantalizing clues to underlying structure provided by these patching experiments, they do not reveal the unperturbed structure of the membrane. For this task, probes and techniques capable of resolution on the scale of tens of nanometers are required. Two such techniques are electron microscopy and fluorescence spectroscopy, in the form of Förster resonance energy transfer (FRET). An example of the former is immunogold localization microscopy of the Ras family of oncogenes in intact PM sheets, which showed that nearly identical Ras isoforms exhibited wholly distinct lateral organizations dependent on their post-translational lipid modification [120,121]. Namely, the isoform that bears two palmitate residues (H-Ras), which are known raft targeting moieties [122], segregate into separate domains from the isoform bearing a single isoprene (K-Ras), which prevents raft association. These electron microscopy observations have been validated by FRET analysis of the same constructs, and these studies were some of the first to formulate a signal transduction mechanism for such nanoscopic membrane organization [123]. Approximately coincident with this work on Ras were the investigations into the nanoscale organization of lipidated intracellular proteins [124] and GPI-APs [125,126] using FRET in live cells. These experiments convincingly demonstrated subresolution domains on the cell surface and, more recently, probed the cellular determinants of their formation and regulation. These studies implicate actively remodeled cortical actin [127,128] as an organizer of the inner leaflet via its interactions with specific inner leaflet lipids, which in turn couples this organization to the outer leaflet via interactions between lipid acyl chains [129].

Detecting Rafts through Diffusion

A large number of past studies investigating domain organization in intact cell membranes have probed the mobility of membrane components [130–133]. A common assertion is that both PM lipids and proteins show anomalous diffusion in cells, with this behavior sometimes attributed to the partitioning of the diffusing components into raft domains. Further evidence is provided by co-diffusion of distinct membrane components whose only connectivity is hypothesized to be mediated by membrane domains [134]. In many cases, the implication of raft domains in anomalous diffusion is supported by changes to confinement/diffusion behavior when membranes are perturbed, usually by reducing cholesterol but sometimes through varying other physical properties such as temperature or structure of the diffusing components. A prominent study used stimulated emission (STED)-fluorescence correlation spectroscopy (FCS) to observe the average time it takes for single molecules to diffuse through an illumination area whose size was experimentally tuned [135]. This study observed slowly diffusing components, which were present even in very small spot sizes, suggesting single molecule partitioning into very small (i.e., $<$20 nm) membrane domains. As a point of reference, even a small domain that is 20 nm in diameter would still contain ~450 lipids (assuming a mean molecular area per lipid of 0.7 nm^2), potentially large enough for collective behavior to play an important part in its organization (e.g., as in Fig. 3).

Relying on similar methodology, several studies have questioned the existence of rafts based on diffusion measurements. A follow-up to the STED-FCS paper [136] indicated that the trapping behaviors observed in live cells were not observed in model membranes interrogated with the same molecules and instruments, prompting the inference that the anomalous diffusion observed in cells was not due to phase separation of lipids. Similarly, numerous reports suggest that raft and non-raft membrane peptides exhibit qualitatively similar confinement behaviors [130,137,138]. Another study showed that diffusion varied smoothly with temperature for a range of different probe molecules, concluding that there was no evidence for an *in situ* phase transition in live cells [139]. Yet another study found that probe localization and diffusion were not affected by the presence of macroscopic raft-like domains generated by binding raft proteins to a patterned surface [140].

An important insight from this apparent controversy is the difficulty of defining expectations in these measurements. In other words, how would the presence of

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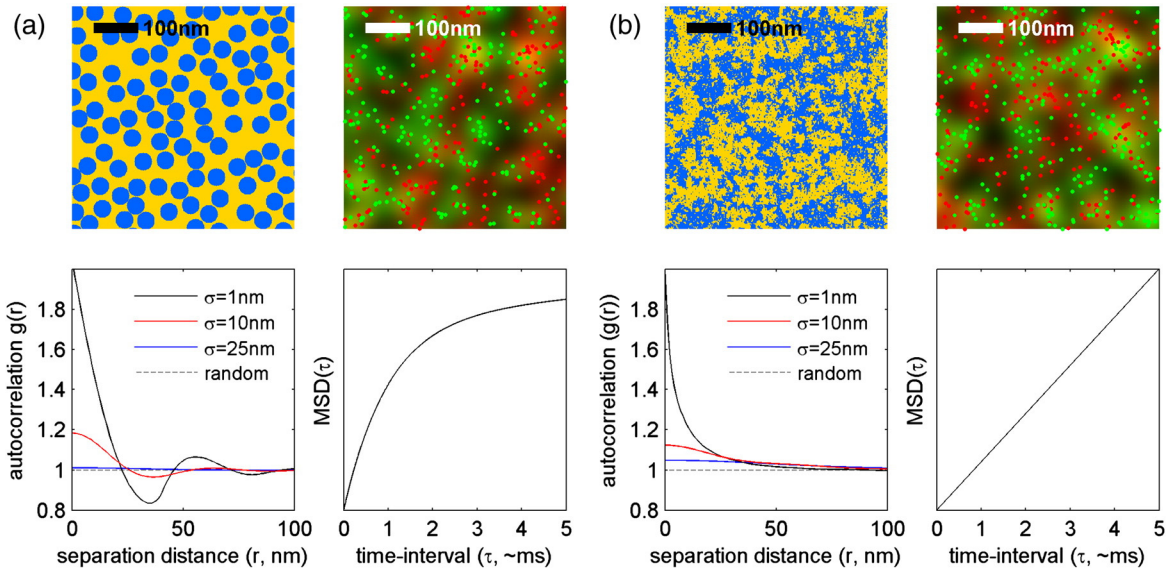

Fig. 4. Predicted experimental observations for two types of membrane heterogeneity. (a) Some models of membrane heterogeneity propose that lipids are organized into small but clearly defined structures. (b) An alternate model is that membrane domains are more akin to supercritical fluctuations. For each model, several predictions can be made. (Images at top left) Possible configurations of ordered (blue) and disordered (yellow) membrane components in the two models presented. In each case, the characteristic length of structure is 20 nm. (Images at top right) Green and red spots are a subset of components chosen randomly from the blue regions to mimic the finite concentration of a fluorescent probe. In these cases, the label is included at 0.2% per color or 2000 probes per μm^2. These spots are also blurred with a Gaussian function with standard deviation of 25 nm to represent the typical localization precision of an SR measurement as indicated in the background image. Note that both undersampling and blurring act to obscure the structures from which these images were generated, which are clearly visible in the top left panels. (Plots at bottom left) Pair of cross-correlation functions between the differently colored spots above for several localization precisions. These curves were tabulated from images like those shown at the top right as described previously [145] and indicate that differently colored points are co-clustered (correlated) when $g(r) > 1$, depleted (anti-correlated) when $g(r) < 1$, and randomly co-distributed (uncorrelated) when $g(r) = 1$. Note that finite localization precision diminishes the observations of the underlying structure even when localization precision is smaller than the characteristic size of structures. (Plots at bottom right) The predicted shape of mean squared displacement (MSD) *versus* time interval (τ) curves for both models, as discussed in the main text. Namely, single molecules that partition with the blue spots at the left will appear confined, while single molecules that partition with the blue fluctuations at the right are unconfined [136].

membrane heterogeneity impact diffusion? The effects could potentially be large if the local viscosity varies dramatically across the membrane or if the membrane contains small but well-defined domains and the energy cost for components to leave these domains is large (Fig. 4a). This type of behavior certainly occurs in model membranes with phase-separated domains that differ dramatically in composition but may not be expected in biological membranes where the contrast between domains is thought to be subtle. In the furthest extreme, it is possible to have lateral heterogeneity that does not impact single molecule mobility. This is the case for systems near a critical point, which can support large composition fluctuations that have slow relaxation times even though single-component motion is nearly Brownian (Fig. 4b) [35,141,142]. Given this range of possibilities, it is not surprising that it has been difficult to draw definitive conclusions regarding membrane heterogeneity solely from diffusion studies.

Another important caveat is that the confined motion of proposed raft components may often be convoluted by interactions with other cellular structures, including but not limited to the actin cortex (which is altered by acutely reduced cholesterol levels [143]), focal adhesions, and caveolae. Furthermore, complex membrane topology can give rise to the appearance of lateral heterogeneity, since protein mobility in cells is typically detected as a 2D projection of a 3D structure [144]. Membrane topology is also likely sensitive to cholesterol depletion, since this lipid comprises a large fraction of the PM.

Detecting Rafts using Super-Resolution (SR) Microscopy

The advent of SR microscopy brought great promise to resolving the lingering question of the existence and properties of rafts. The main justification for the inability to observe rafts in cells by diffraction-limited microscopy was that rafts are small, in the 10–100 nm range, well below the diffraction limit of visible light. Both STED

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and point-localization-based (STORM/ PALM/PAINT) SR methods provide access to these lengthscales. Nonetheless, over a decade since the invention of these methods has failed to generate a definitive result that confirms or rules out the existence of raft structures. Why is this?

First, quantitative SR measurements are difficult. An important, potential artifact is the over-counting of individual labeled molecules, which leads to apparent self-clustering when a single color is visualized [145,146]. This experimental pitfall can be overcome by imaging two distinguishable sets of molecules to measure their co-distribution [145,147–149]. This too is not without experimental complexities: fluorescent impurities and spectral overlap can give rise to misidentification and artifactual co-localization [150,151]. Although the currently available number and types of fluorophores limit these approaches, probe development is an active area of research. Second, the small size of putative raft domains still poses potential complications because the spatial resolution (practically, 15–50 nm) offered by SR methods may still not be enough to observe lipid heterogeneity directly without additional membrane perturbations to increase the size and lifetime of putative domains (Fig. 4). Finally, a key drawback of SR imaging is low temporal resolution, which is a major issue for the likely highly dynamic nature of domains *in vivo*. Indeed, most SR experiments are conducted in chemically fixed cells or membrane sheets [133,152,153], and these preparations can be prone to artifacts because lipids and even some membrane proteins are not immobilized by most fixation protocols [154]. Experiments in live cells are possible but require experimental and analytical methods that anticipate the fast dynamics expected for raft structures [155,156].

Because of the above limitations, direct imaging of rafts may be beyond current technical possibilities. Instead, less intuitive image quantitation methods that rely on averaging over many domains may be required to reveal the presence of heterogeneity in intact cells. Several approaches that do not rely on image reconstruction have had some success in this area. A recent advance definitively correlated three distinct measures of raft association—DRM association, ordered phase partitioning in GPMVs, and surface patching in live cells—across more than a dozen probes, along with probing the lifetime of the small entities in unperturbed cells, using multi-color single particle tracking [156]. This study has provided some of the strongest evidence to date to link classical raft observations with novel model membranes and modern imaging techniques. It is also possible to quantify heterogeneity in mobile systems by tabulating steady-state cross-correlations between differently colored probes [155]. If raft structures are well defined but small, with strong partitioning of “raft” components, it is reasonable to expect that the improvement in resolution given by current methods will eventually lead to decisive observations of rafts in resting cells. However, the lack of positive data of this type does not exclude the presence of more subtle structures, as the ~20-nm domains predicted by the critical fluctuation hypothesis may still be below the practical detection limits of SR imaging. Most likely, any decisive measurement of raft heterogeneity will also show that the observed structures vary with perturbations in agreement with theoretical predictions. For example, it would be informative to correlate the effects of membrane perturbations (like those described above) in GPMVs to their effects in live cells. If there is indeed a correlation between the properties of isolated and in situ PMs, the former could become a reliable proxy for the latter.

From Structure to Function: How to Functionalize Membrane Domains?

The most intuitive way for lateral membrane domains to affect cell physiology is by regulating the interactions between membrane components. The simple analogy is to a reaction compartment that concentrates certain components while excluding negative regulators of a given reaction. In reality, raft and non-raft domains in biological membranes are relatively similar, so most components do not partition exclusively into either of the domains; however, this does not preclude compartments of distinct composition. An important aspect of raft functionalization is dynamic regulation of their composition. One attractive possibility for such regulation is protein modification that promotes or inhibits raft affinity. The most well-characterized instance is palmitoylation, a reversible saturated fatty acid modification believed to impart raft affinity to both transmembrane and peripheral proteins [46,122]. Another attractive possibility is protein binding to lipids with inherent raft affinity, such as glycosphingolipids [157] or cholesterol. It is also possible that the local lipid environment directly influences protein functions by setting membrane hydrophobic thickness or lateral pressure profiles or by the interactions of certain lipid headgroup or chain types with the protein surface [59].

In addition to these possibilities, the observations of critical behavior in isolated cell PMs suggest that cells tune their membrane composition to reside near a miscibility critical point in order to accomplish some biological functions. Obvious questions arise as to how cells would sense and maintain near-critical compositions and, more importantly, why? One possibility is that critical composition fluctuations can mediate weak but long-range forces between membrane proteins [158]. Two proteins that prefer the same local lipid environment will experience a long-range attraction because of their preference for the same local lipids. Conversely, proteins that prefer

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different local lipid environments will feel repulsion, because they would require unfavorable lipid interactions to approach in close proximity. Interestingly, the magnitude of the repulsive interaction is generally larger than the attractive one, suggesting that this mechanism is better suited for keeping proteins apart rather than gathering them together.

Another immediate consequence of criticality that could be exploited by cells is that critical membranes are highly susceptible, meaning they are poised to remodel in response to small inputs. For 3D systems in the vicinity of a liquid–gas critical point, susceptibility takes the form of high compressibility, meaning it takes very little work to compress or expand a supercritical fluid because it takes very little energy to convert a low density gas to a higher density liquid and vice versa. In a 2D multicomponent membrane, high susceptibility means that the membrane can easily remodel with only a slight perturbation from outside the membrane plane. This perturbation could take the form of a subtle bias for a specific membrane environment over an extended area, as in the interactions between the membrane and cortical actin [35]. Another possibility is the clustering of a small number of components that prefer a local environment, for example, via adhesion or through binding of a clustering ligand [97]. In both cases, the slight bias for a specific membrane composition in a defined location would lead to global membrane remodeling, even for components that do not directly interact with the organizing components.

Finally, a recent theoretical study describes a mechanism by which fluctuations could impact the functional states of single membrane proteins, especially large transmembrane proteins that directly interact with many boundary lipids [159]. If two internal states of a single protein have preferences for distinct local lipid environments, then the availability of these environments will impact the population of these states. An example might be a receptor that strongly partitions into a raft environment only after it becomes activated upon binding a ligand. In this case, the receptor is more likely to become activated if the membrane already contains raft domains large enough to provide the boundary lipids preferred by the active state, thereby lowering the concentration of ligand needed for activation. In principle, this mechanism could provide a tool for cells to tune the activity of a subset of membrane proteins in parallel. Furthermore, this mechanism may directly couple protein function to its localization. For example, a protein reliant on membrane domains may remain inactive within the early secretory pathway (i.e., the endoplasmic reticulum, where cholesterol concentrations are likely insufficient for domains [62]) but become activated upon transfer to the PM. While this concept still requires experimental validation, it suggests new mechanisms through which lipid rafts could impact broad cellular functions.

Conclusion

The recent years have seen a revitalization of interest in the raft field. This enthusiasm has been spurred by methodological and conceptual advances that have bolstered arguments for the existence of membrane domains and established novel principles that reconcile evidence for domains with their persistent escape from direct detection. Although it is clear that biological membranes have the capacity for lateral organization, the specific manifestations of that organization remain unknown. Domain properties such as composition, size, and lifetimes remain unclear, partly because they are likely to differ widely between various cell types, cell states, and membranes within cells. Even more nebulous are the specific mechanisms by which these domains are integrated into cellular physiology. Ultimately, to quote Winston Churchill, rafts remain a riddle, wrapped in a mystery, inside an enigma, which will likely continue to frustrate and inspire researchers for decades to come.

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†I.L. and S.V. contributed equally to this work.

Abbreviations used:
PM, plasma membrane; EMT, epithelial-to-mesenchymal transition; 2D, two-dimensional; GUVs, giant unilamellar vesicles; GPMVs, giant plasma membrane vesicles; Lo / Ld, liquid-ordered / liquid-disordered membrane phases; DRM, detergent-resistant membrane; PMS, plasma membrane spheres; GPI-AP, glycophosphatidylinositol anchored protein; SR, super-resolution; FRET, Förster resonance energy transfer; STED, stimulated emission; 3D, three-dimensional.

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