Critical phenomena in plasma membrane organization and function

Thomas Shaw\textsuperscript{1}, Subhadip Ghosh\textsuperscript{2}, Sarah L. Veatch\textsuperscript{1,2,*}

**Keywords:** Critical Composition Fluctuations, Thermodynamics, Cell Membrane, Membrane Microdomains, Lipid Rafts

**Abstract:** Lateral organization in the plane of the plasma membrane is an important driver of biological processes. The past dozen years have seen increasing experimental support for the notion that lipid organization plays an important role in modulating this heterogeneity. Various biophysical mechanisms rooted in the concept of liquid-liquid phase separation have been proposed to explain diverse experimental observations of heterogeneity in model and cell membranes, with distinct but overlapping applicability. In this review, we focus on evidence for and consequences of the hypothesis that the plasma membrane is poised near an equilibrium miscibility critical point. Critical phenomena explain certain features of the heterogeneity observed in cells and model systems, but also go beyond heterogeneity to predict other interesting phenomena, including responses to composition perturbations.

**0. Overview**

Spatial organization of the plasma membrane on 10-100 nm length scales has been a topic of interest to biology for decades. Heterogeneity has been hypothesized to play important roles in many membrane-associated biological processes, from coordination of signal transduction machinery to endo- and exocytosis to polarization, as iconically described in the lipid raft hypothesis (1). Various biophysical notions have been marshaled to provide explanations of membrane heterogeneity, especially from equilibrium thermodynamics and phase transitions, as coexisting liquid phases are readily observed in both purified membranes and membranes isolated from plasma membranes (2, 3). One important thread of this ongoing conversation explains nanoscale membrane structure as critical phenomena. In contrast to a classical phase separation picture of plasma membrane domains, which implies stable and well-defined discrete

\textsuperscript{1}Program in Applied Physics, University of Michigan, \textsuperscript{2}Program in Biophysics, University of Michigan, *Corresponding author, contact: sveatch@umich.edu
regions of defined composition, critical phenomena are subtle, dynamic and malleable, and inhabit the relevant nanoscopic length-scales.

In this review, we conduct a brief historical survey of membrane domains in both model and biological membranes and describe the consensus that has been reached regarding the macroscopic miscibility phase behavior of model membranes as well as remaining controversies regarding the microscopic heterogeneity reported in other regions of phase space. We introduce membrane criticality and the accumulated evidence that eukaryotic plasma membranes are near-critical. We discuss where the concept of criticality fits into conventional descriptions of raft phenomenology, and describe some unique areas where criticality could plays roles in biological function that go beyond simply organizing components.

1. An early history of domains in model and cell membranes.

There is a long history of detecting heterogeneity in bilayer membranes containing cholesterol. Some of the earliest studies that interrogated purified membranes in the late 1960s and early 1970s found evidence that liquid membranes containing a single phospholipid species and cholesterol contained structure on 1-100nm length-scales accessible to the spectroscopic methods available at the time (4–9). Over the decades, evidence for these microscopic domains in binary mixtures of phospholipids and cholesterol continued to accumulate, through additional spectroscopic studies, calorimetry, the application of fluorescence techniques such as fluorescence quenching, anisotropy, and Förster resonance energy transfer (FRET), and imaging methodologies such as freeze fracture electron microscopy (10–21).
Largely in parallel to this physical chemistry characterization of membranes, cell biologists also postulated that membranes within cells could contain lipid-mediated sub-structures that could be important for cell functions. Early evidence came from the observation that cells could polarize their membrane lipid composition via differential trafficking of lipid species (22). It has also long been appreciated that cholesterol or similar sterols are vital to the proper functioning of most eukaryotic plasma membranes, as manipulation of membrane cholesterol content was shown to interfere with functions including endocytosis, several receptor mediated signaling cascades, and cell cycle control (23–26). In the early 1990s it was discovered that some detergents only partially solubilize cellular membranes, especially the plasma membrane (27, 28), and it was postulated that the insoluble fractions represented distinct membrane domains (1, 20). Researchers found that artificially clustering some membrane components individually could drive macroscopic colocalization, termed co-patching, detectable by conventional fluorescence microscopy (29). Subsequent spectroscopic and fluorescence measurements supported the hypothesis that cell membranes were heterogeneous in their lipid and protein content (30–32), and many studies correlated biological function with presumed membrane heterogeneity probed and perturbed via these and related assays (33–36). Each of these methods had well documented flaws (37), but the remarkable consistency of the conclusions drawn from different methodologies provided convincing evidence that lipid-driven heterogeneity is relevant to the plasma membrane.

2. Liquid-liquid phase separation in model membranes.

_Purified membranes:_
In 2001, the first observations of macroscopically phase separated fluid domains were reported in bilayer membranes reconstituted from cellular extracts (38, 39). These initial observations emphasized similarities between the composition of the coexisting phases in model membranes and heterogeneity measured in cells by detergent solubilization methods. Soon after, it was appreciated that at least three lipid components were required to form macroscopically phase separated domains in bilayers: a high melting temperature lipid, a low melting temperature lipid, and a sterol such as cholesterol (40). Phase diagrams describing this macroscopic phase transition have now been mapped by numerous groups using a range of methods and are in good qualitative agreement (41–49). Since three components are required to observe liquid-immiscibility, phase diagrams have been mapped by varying temperature and the molar fraction of all three components. An introduction to reading and interpreting these phase diagrams is given in Figure 1, and several experimental phase diagrams are presented in Figure 2.

A range of experimental (50–56) and simulation (57, 58) approaches using different lipid combinations have produced results consistent with phase diagrams topologically similar to those shown in Figure 1b, and their detailed characteristics have been described in several comprehensive review articles (3, 59, 60). Liquid immiscibility is most often observed at temperature below the melting temperature (Tm) of the high Tm lipid (42, 44–46), although there are exceptions (41). In the typical case, reported phase diagrams have a region of liquid-liquid coexistence, a region of three-phase coexistence (2 liquids and a solid), and two regions of liquid-solid coexistence. One of the liquid phases is called the liquid-disordered (Ld) phase, and it resembles the liquid crystalline phase of pure phospholipids (61). The second liquid phase is called liquid-ordered (Lo) (15), which was first characterized in mixtures of saturated
phospholipids and cholesterol (8, 16, 18). The solid phase is often called gel, and is most likely Lβ’ (62).

The high cholesterol edge of the three-phase triangle is sloped such that the Lo phase contains a higher cholesterol mole fraction than the Ld phase. This edge of this triangle is also the first tie-line in the liquid-liquid-coexistence region. As cholesterol is increased further, tie-lines run roughly parallel to one another, meaning that cholesterol concentration increases roughly linearly in both phases. The Lo-Ld coexistence region terminates in a miscibility critical point, where in principle tie-lines merge into a single point. In practice this region of the phase diagram is surprisingly flat, meaning that tie-lines remain long and shorten over a very small range of compositions. As temperature is lowered, the Lo-Ld immiscibility gap extends to higher concentrations of cholesterol and low Tm lipid, as does the concentration of components at the critical point (41, 45, 48). At constant temperature, the miscibility gap expands when the Tm of the high Tm component is increased or when the Tm of the low Tm lipid is decreased (41, 42, 44).

No macroscopic miscibility gap is observed for some combinations of low and high Tm lipids (42, 44, 63). A closed loop miscibility gap is found when the extremely low Tm lipid DiPhytanoyl PC is used (41), meaning that Lo-Ld coexistence occurs at temperatures above the Tm of the saturated component and there are two critical points. The phase behavior of the mixed system can depend on more than just the Tm of components. For example, sphingomyelin (SM) lipids are more effective at establishing coexisting phases as compared to glycerol-phospholipid lipids with PC headgroups (44, 58) even when the main chain transition occurs at similar temperatures for SM and PC lipids used.
Although the equilibrium thermodynamics description of the macroscopic miscibility transition is now largely accepted, questions remain regarding the thermodynamic basis of the microscopic heterogeneity also routinely observed in membranes containing cholesterol. These structures are frequently reported at temperatures and compositions where membranes remain uniform on a macroscopic scale using methods sensitive to molecular-scale organization such as FRET, fluorescence quenching, or ESR. Membranes can be tuned from a state with macroscopic phase separation to one with microscopic heterogeneity by raising temperature (64, 65), titrating in an additional component that disrupts the macroscopic phase transition (e.g. (50, 52, 66)), or by probing different ratios of the same lipid species at fixed temperature (65). Submicron structure is also reported in binary mixtures of saturated lipids and cholesterol (7, 67) and in some ternary membranes that do not exhibit macroscopic Lo-Ld immiscibility at any temperature or lipid ratio (21, 47, 63, 68). Recent experimental developments in purified membranes has begun to probe how leaflet asymmetry impacts phase separation and the presence of submicron structure in purified membranes (69, 70).

Experimental observations have motivated numerous theories to explain the presence of microstructure at thermodynamic equilibrium. Critical phenomena provide a possible mechanism to bridge macro- and micro-scales in the form of dynamic fluctuations (45, 71). Other theories enable static, finite-sized domains by including some repulsive mechanism to oppose formation of macroscopic domains (72–74). These theories too predict domains that span macro-to micro- scales. Recent experimental work has begun to directly test some of these theories explicitly (75).
**Isolated membranes:** The first observations of macroscopic liquid immiscibility in vesicles blebbed directly from living cells came in 2007 (2), and these vesicles were named Giant Plasma Membrane Vesicles (GPMVs) due to their close resemblance to the Giant Unilamellar Vesicles (GUVs) used widely in fluorescence microscopy investigations of purified lipid mixtures. Earlier work using a similar vesicle preparation had characterized their lipid and protein content by mass spectrometry (76), and had observed heterogeneity using ESR (30), a spectroscopic method that can detect heterogeneity on the molecular scale. Baumgart et al (2) detected the sorting of fluorescent lipid analogs and fluorescently tagged proteins with respect to phases in GPMVs at temperatures well below those where cells were grown, leading the authors to conclude that this phase transition was not relevant for cells under normal growth conditions.

Soon after, other methods emerged to isolate plasma membranes from cells and all could yield coexisting liquid phases (77, 78), although the conditions needed to achieve phase separation differed between methods used. A subsequent study found correlations between biochemically defined detergent resistant membranes and the liquid-ordered phase detected in GPMVs (79), and that the surface fraction of ordered phase at low temperature was altered by acute treatments to manipulate cholesterol levels in vesicles (79, 80). In all cases, miscibility transition temperatures (Tmix) remained well below growth temperatures in isolated cells, emphasizing that such macroscopic domains were not likely to form under physiological conditions. A possible explanation came in 2008 when it was shown that freshly isolated GPMVs exhibited hallmarks of criticality, placing them close to a room temperature miscibility critical point (81). Over time, additional studies have documented how GPMV phase behavior is impacted by growth conditions in cells (82–84) and differentiation into other cell types (85, 86), and lipidomics
analysis has begun to characterize the vast compositional complexity of these membranes (82, 84, 87).

While there are many similarities between the phase behavior observed in GPMVs and purified model membranes, there are key differences (88). The coexisting phases detected in GPMVs differ in their physical properties from their purified membrane counterparts. The ‘fluidity’ of phases are more similar in GPMVs compared to GUVs, as measured through diffusion of membrane components or using order sensing fluorophores that report on local hydration within the hydrophobic region of the membrane (89). These different physical properties can be sensed by incorporated proteins. Some transmembrane proteins, particularly those with palmitoylated cysteines, are observed to partition into the Lo phase in GPMVs whereas few are reported to partition into the Lo phase in purified membranes (90).

It should be noted that GPMVs are model membranes that differ from intact plasma membranes in important ways. Plasma membranes exist in close association to the actin cytoskeletal cortex, while GPMVs are missing polymerized cytoskeletal components and tend to be depleted in proteins that associate with the actin (91). Notably, the cell plasma membrane does not macroscopically phase separate even under conditions that cause GPMVs to phase separate, or in any known conditions, even when phase separated GPMVs remain attached to an intact cell membrane (92). GPMVs are depleted of PI(4,5)P₂ (93), which typically makes up several mol% of the inner plasma membrane leaflet in intact cells (94). A recent report demonstrates that isolated GPMVs are frequently permeable to large hydrophilic markers (95), indicating that their membranes contain long-lived defects. While cell membranes are asymmetric in their lipid and protein composition, at least some of this asymmetry is lost in the GPMV generation and isolation
process (2, 93). The interpretation of GPMV experiments is also complicated by their sensitivity to methodological choices. For example, the most common method to prepare GPMVs involves incubating cells with a low concentration of formaldehyde and a reducing agent, and the choice of the reducing agent can greatly impact the transition temperature and physical properties of phases of the resulting GPMVs (78, 96). This is due, at least in part, to the ability of some reducing agents to modify membrane proteins and lipids.

3. Phase separated domains are related to but different from ‘raft’ heterogeneity in intact cells.

Phases are robust, macroscopic entities with well-defined compositions, and with the exception of the yeast vacuole (97, 98), structures resembling liquid-liquid phase separation are not observed in intact cells. Instead, the vast majority of membrane domains in cells are microscopic and require significant perturbations in order to be visualized or isolated. In line with this, several recent studies have directly concluded that there is no evidence of a miscibility phase transition in intact cells when cells are examined through the lens of several different experimental observables (99, 100). Nonetheless, a large number of experimental studies provide strong evidence that the heterogeneity reported in intact cells is closely related to the macroscopic phase separation observed in GPMVs. Two excellent recent reviews discuss many of these findings, as well as some exceptions (101, 102). We highlight several lines of evidence below.

1. In many cases, proteins that are associated with live-cell heterogeneity or with detergent-resistant membranes are also found to partition into the Lo-like phase of GPMVs. In particular, single-pass transmembrane and peripheral proteins containing
palmitoylations are more likely to be found in detergent resistant membranes and partition into the Lo phase in GPMVs, while proteins containing branched and unsaturated geranylgeranyl or prenyl groups tend to be solubilized by detergents and partition into the Ld phase in GPMVs. Recent work begins to extend to multi-pass proteins which can accommodate more complex protein/lipid interactions (103–105). It is likely that protein partitioning will be dictated by the identity of lipids that solvate transmembrane proteins in complex membranes (106, 107).

2. Cells actively tune their plasma membrane Tmix in response to changes in growth conditions and conditions that change Tmix lead to different phenotypes. For example, experiments indicate that cells in culture actively tune the miscibility phase transition temperature of their membrane to be a fixed temperature below their growth temperature (82), and that Tmix lowers in cells under conditions that inhibit cell growth (83). Other studies have documented that acute treatments with lipophilic small molecules or dietary lipids that alter Tmix correlate with changes in signaling outcomes, cellular differentiation, and even the general anesthetic response (85, 108, 109). While it is possible that these correlations with Tmix are a result of a mutual correlation with an unrelated quantity, the accumulated evidence suggest that maintaining Tmix plays important roles in cellular processes.

3. In model membranes, Tmix predicts structure observed in single phase membranes. While model membranes appear homogeneous at the macroscopic scale above Tmix, the value of Tmix can predict the presence of structure at smaller scales, or under conditions where membranes are perturbed by clustering one component. A very recent report that
documents the temperature dependence of both macroscopic phase behavior and microscopic heterogeneity in GPMVs under a range of perturbation conditions (96). This work found that the microscopic heterogeneity in GPMVs at elevated temperature was highly correlated with their macroscopic transition temperature $T_{\text{mix}}$, suggesting that the same could be true in intact cells. An older study finds that phase marking probes enrich in membrane domains stabilized through adhesion even well above $T_{\text{mix}}$ (80). These and other studies have led to the idea that the value of $T_{\text{mix}}$ is a measure of ‘raft’ stability under physiological conditions, even though cells do not appear to experience phase separation directly.

4. Theoretical arguments support that coupling to an intact cytoskeletal network will disrupt the macroscopic phase transition. It is well established that ‘quenched disorder’ abolishes first order phase transitions in two dimensional systems like membranes (110). The cortical actin cytoskeleton, which is linked to the plasma membrane through a system of adapter proteins, is likely to play the role of quenched disorder in the intact plasma membrane (92, 111). Experimental studies in model membranes support the main conclusions of this theory, that a broadly distributed cytoskeletal network disrupts macroscopic domains while stabilizing small-scale structure (112). Direct tests in intact cells have yet to be reported, but there is broad evidence for important connections between cortical actin and raft heterogeneity (113–117).

5. There is increasing evidence for phase-driven partitioning within intact cells. Recent work utilizing single molecule fluorescence methods are beginning to draw more direct connections between phases in vesicles and domains in cells (118, 119). These studies
monitor the recruitment and exclusion of probes with respect to clustered proteins in intact cells and find that probe concentration in clusters mirrors partitioning with respect to phase separated domains in model membranes, although typically with a smaller magnitude.

To explain these experimental developments, several theoretical avenues have been explored, with a goal of explaining the lack of a macroscopic phase separation in intact cells. Any complete biophysical model of membrane heterogeneity must first account for this fact, and therefore must be more complex than the simple phase separation picture. These explanations include coupling to quenched disorder in the form of the cytoskeleton (92, 111), coupling to curvature in a way that produces a microemulsion (72, 74, 120), and nonequilibrium suppression of domain growth, for example by active lipid transport or remodeling of membrane-coupled actin structures (121, 122). Many of these models, including the critical phenomena that are discussed in the next section, are closely related to the canonical phase separation picture (123).

4. Criticality and its connection to plasma membrane

Equilibrium critical phenomena are special relationships between thermodynamic properties of a system that emerge due to a diverging correlation length of the system in the vicinity of a critical point. Key physical quantities such as the heat capacity, the susceptibility, and the interfacial energy between domains also exhibit specific behaviors as the critical point is approached. These properties of critical systems are universal, meaning that the dominant behavior is governed by a small number of effective parameters, regardless of the complicated details of microscopic
interactions (Figure 3). The physics of criticality has been covered in detail in many textbooks (124–127). A useful introduction for biophysicists is given in (128).

The physics of criticality has developed over the last 200 years, beginning with the observation of a critical temperature for several liquids, above which there is no liquid-gas transition (129). The van der Waals equation of state (1873) was the first model that featured a “critical point” with this property (130). Ornstein and Zernicke, in 1918 (131) formalized the study of spatial fluctuations in liquid-gas systems, a key development. In the first half of the 20th century, precise measurements of near-critical liquid-gas systems (132) as well as magnetic systems (133) indicated that classical equations of state were inadequate to explain the near-critical region of phase space, for example that the shape of the phase liquid-gas coexistence curve is qualitatively different than predicted by van der Waals. In 1944, Lars Onsager exactly solved the 2d Ising model, which allowed for the study of critical exponents in that system (134). In 1952, Yang and Lee observed that phase transitions correspond to non-analyticities of the partition function in the thermodynamic limit (135), and Widom and others proposed power-law scaling for various quantities at critical points, and derived relationships between different critical exponents from that hypothesis (136). Real-space (Kadanoff) and momentum-space (Wilson) renormalization group methods explained the emergence of these power laws and provided avenues for computing approximate values for the critical exponents in general systems (137, 138). They also set a uniform framework for other conceptual advances. A broader class of nonequilibrium critical points can also be defined, generalizing this equilibrium concept. Biological nonequilibrium critical points have received considerable attention in recent years, see, e.g. Mora and Bialek for a review (139).
Coexistence regions generically terminate in a critical point except in special circumstances, and the Lo-Ld miscibility of purified and isolated membranes is no exception. On the phase triangle shown in Figure 1B, the critical point occurs on the high-cholesterol edge of the Lo-Ld miscibility gap. Initial evidence of critical behavior in membranes came from NMR studies of multilamellar vesicles, which detected enhanced line-broadening in the vicinity of known critical points (45). This was attributed to the diffusion mediated exchange of lipids between fluctuations with sub-micron dimensions. Later work directly visualized micron-sized critical fluctuations (above the critical temperature; Tc) and fluctuating phases (below Tc) in GUVs of purified lipids, providing evidence that membranes belong to the 2D Ising model universality class, meaning that fluctuations exhibited a temperature dependence that is universal to two dimensional systems with a one-dimensional order parameter (71). Subsequent measurements confirmed this observation in supported membranes by atomic force microscopy (48), and probed the dynamics of critical membranes (140). At this same time, it was discovered that isolated GPMVs also exhibited critical behaviors in the vicinity of a room temperature critical point (81). Again, fluctuations were consistent with the 2D Ising model universality class. These observations and their implications have been reviewed in greater detail previously (128).

One of the key features of a critical point is that its fingerprints extend well beyond the phase transition itself (Figure 3). An important parameter is t, the difference between the temperature of the system and the critical temperature (Tc) normalized by Tc in units of Kelvin. The correlation length ξ, or characteristic size of critical compositions fluctuations, is predicted to vary as \( \xi(t) = \xi_0 / t \), where \( \xi_0 \) is a parameter with dimensions close to the size of molecules in the system and in membranes was measured to be roughly 1nm. Note that as \( T \to T_c, t \to 0 \), so that the correlation
length becomes infinite. Extrapolating this relationship using a room temperature critical point (Tc = 22°C = 295K), then 20nm sized fluctuations are expected at 37°C, which corresponds to t=0.05. This prediction is in good agreement with recent experimental work probing heterogeneity in GPMVs by FRET, which detects evidence for larger than 10nm structure in GPMVs over this same temperature range (96). Similar observations have also been made in purified model membranes (141). Note that t need not be a physical temperature. Instead it is any trajectory in the phase diagram that runs perpendicular to tie-lines close to the critical point. Thus, while some Ising model images of Figure 3A are obtained by varying temperature in the model, the corresponding three-component lattice model images are obtained by varying composition at fixed temperature, as indicated in the phase diagram.

Another physical property that can extend well beyond the phase transition itself is the susceptibility (χ). The susceptibility measures how large a local composition difference arises from a local force applied to components of one of the phases, e.g. by clustering components that prefer Lo lipids. In other words, in a highly susceptible membrane, a domain of distinct local composition can be stabilized by only clustering a small subset of components, or by weakly biasing the concentration of many components that have the same order preference. In the Ising universality class, χ ∝ t^{-7/4}. This too has experimental support in vesicles, where robust domains are stabilized well above Tmix by organizing a small subset of components by an actin network or streptavidin crystal that partially decorates a vesicle surface (114, 142), or by adhesion to a supported membrane (80).

Direct theoretical predictions of critical phenomena such as the scaling of the correlation length and the magnitude of the susceptibility are quite useful for predicting consequences of
perturbations to membrane heterogeneity, but the theory quickly becomes intractable when coupled to more complex biological phenomena. Statistical mechanical lattice models based on the Ising model can be useful in this situation. These models typically only contain two components (up and down ‘spins’) positioned on a lattice, where the components at the lattice sites are either allowed to change identity (such that the composition or ‘magnetization’ can vary) or are allowed to exchange with other sites on the lattice (such that the composition remains fixed). Universality guarantees that, so long as the system is close to the critical point, the Ising model captures the relevant mesoscopic heterogeneity of the membrane, for appropriate choices of the Ising reduced temperature $t$ and magnetization $m$ (Figure 3). That is, the Ising model accurately recapitulates the thermodynamics of the effective Lo order parameter at length-scales beyond a few lipid diameters, despite the extreme simplicity of the microscopic interaction in the model – a simple nearest-neighbor interaction potential. As a result, when a biological system is coupled to the Lo order parameter, an Ising model modified to include this coupling is expected to reflect the relevant biophysical phenomena. Past work has used this approach to model the coupling of fluctuations to cortical actin (92), to explain changes in phosphorylation steady states upon clustering of a component, for various values of $t$ and $m$ (118, 143), and to predict how proximity to the critical point affects conformational state equilibria of proteins whose boundaries are sensitive to lipid order (144).

**Evidence for criticality playing a role in cells:** If intact plasma membranes exhibit similar heterogeneity to that observed in GPMVs, it could easily be relevant to the biological function of membrane proteins. An important line of evidence that criticality plays a role in biological function has come from the tuning of the GPMV critical point. For a system to be near a critical
point in the first place, two parameters must be tuned, corresponding to \( t \) (temperature) and \( m \) (composition) of the (fixed-composition) Ising model. In the extremely large space of lipid mixtures of varying composition, there are many critical points – an \( n - 2 \)-dimensional manifold in the \( n \)-dimensional space. However, there is no generic reason that tuning the concentration of any given lipid will correspond to tuning just \( t \), or just \( m \), or neither – general perturbations will affect both \( t \) and \( m \). Thus it is somewhat surprising that the cell arrives near a critical point if it constructs its membranes without explicitly or implicitly tuning to the critical point, given that lipid composition is modulated by a wide variety of perturbations. In other words, it would be surprising if plasma membrane composition is near-critical simply by coincidence. Furthermore, at least in certain cases, eukaryotic cells adapt to perturbations in ways that preserve the distance to the critical point, and corresponding physical properties. Zebrafish cells cultured at a range of temperatures from 20-32 °C produce GPMVs with correspondingly altered Tc (Burns et al 2017).

The concept of a high susceptibility near a critical point is useful in interpreting recent single molecule and super-resolution studies documenting the partitioning of phase marking probes to protein clusters in intact cells (118, 119, 143). In these studies, antibodies are used to cross-link a membrane component that prefers either the Lo or Ld phase, then the differential partitioning of probes is monitored with respect to these domains. When proteins are clustered that themselves prefer the Lo phase, then probes that also prefer Lo tend to be recruited and those that prefer Ld tend to be excluded. In contrast, when proteins that prefer the Ld phase are clustered, then probes that prefer Lo are excluded and probes that prefer Ld are recruited. Similar to experiments with vesicles adhered to supported membranes (80), the act of clustering a protein or peptide biases concentration of many components in ways that can be detected
when membranes have high susceptibility. In some cases the extent of probe partitioning approaches that observed in phase separated vesicles (143), while in others the sorting of components is much weaker (118). These differences could arise from differences in the coupling of protein clusters to membranes, or differences in the susceptibility of the membrane in different experimental systems.

5. Criticality as it relates to biological function

Since the inception of the raft hypothesis, the functional relevance of membrane domains has focused on their ability to compartmentalize protein and lipid components so that they can optimally function within biochemical networks (1). Critical phenomena are in many ways consistent with this framework. A super-critical membrane contains domains resembling ordered and disordered phases, and components that partition with the same phase will colocalize within these domains. The fluctuations are small and dynamic, consistent with evolving descriptions of rafts over the decades (145–148), but fluctuations alone are not an effective means to strongly colocalize or confine membrane components. This new reality requires us to move beyond the simple mechanisms proposed in the early raft literature to propose and test mechanisms that exploit the unique material properties of critical systems. Several proposals are highlighted in Figure 4 and described below.

**Interactions between proteins:** Composition fluctuations can mediate forces between membrane proteins, via a process termed ‘critical Casimir forces’ first described between conducting plates in vacuum (149). In essence, proteins will feel an effective attractive potential
if they partition into the same phase because their coming into close proximity allows them to share the same local lipids, as shown in Figure 4A (150, 151). In contrast, proteins that prefer different environments will feel an effective repulsion, since there is an energetic cost to mixing their local environments. These potentials are weak (on the order of the thermal energy $k_B T$) but have a range given by the correlation length and the size of the protein or protein cluster. This is long-ranged compared to other interaction modes experienced by membrane proteins such as curvature, electrostatics, and van der Waals potentials. It is notable that repulsion of components that prefer different phases has a larger magnitude than attraction between components that prefer the same phase. The Casimir force may contribute to the stability of protein assemblies, including phase separated polymer droplets that assemble on membranes. The Casimir force is also expected to alter biochemistry occurring at the membrane, by increasing or reducing the rates at which the proteins encounter one another. It is tempting to speculate that one functional role of palmitoylation, the post-translational modification that places a saturated acyl chain on proteins, is to tune the magnitude of this Casimir force for specific protein species.

**Susceptibility to receptor clustering:** The high susceptibility of a critical membrane provides a means for the cell to sense a redistribution of a subset of membrane constituents by an external force (Figure 4B). Clustering a membrane protein that prefers one phase would bias the local lipid composition in proportion to the heightened susceptibility of the system. This effect can impact biochemical reactions that take place within these clusters, drastically altering the chemical steady state of the system. We have studied this effect in the context of B cell receptor (BCR) signaling (118, 143). Here, the act of clustering the BCR or another ordered membrane
component by an extracellular ligand stabilizes an ordered domain that contains a higher local concentration of kinase and a lower concentration of phosphatase than the membrane as a whole. This establishes a local environment that favors receptor phosphorylation and activation. In principle, this class of activation mechanism could contribute to a wide range of signaling pathways that are initiated by receptor clustering at the cell surface. This type of mechanism could also play a role in establishing biochemical environments in membrane regions where components are organized by processes occurring at the inner plasma membrane leaflet, such as at junctions between the ER and plasma membrane (152), or at sites where scaffolding adaptor proteins are anchored to membranes such as in neuronal synapses (153).

**Allosteric regulation of single proteins:** Beyond contributing to the organization of proteins, the functioning of single proteins can also be impacted by the size and stability of fluctuations in the membrane. One mechanism that has been proposed requires that two conformational states of the protein in question have different boundary lipid preferences for Lo or Ld lipids (144). If that is the case, then a change in Tc will differentially affect the free energies of the two conformational states. Roughly, a spatially extended lipid preference carries a free energy cost that decreases near Tc, so that a conformational state with strong order preference becomes more probable when fluctuations are large compared to the protein diameter. This model was proposed to explain striking correlations between the Tc-altering effects and anesthetic or anesthetic-reversing potencies of a wide range of treatments, including short and long-chain n-alcohols and hydrostatic pressure (108, 109).

**Tuning binding of allosteric regulators:** The chemical potential \( \mu \) of a component is the thermodynamic parameter that controls the proclivity of that component to enter or exit a
system. For example, its availability to bind in a binding pocket. Formally, the chemical potential is the increment of free energy to move one particle into the system from a particle bath. Equivalently, the chemical activity \( a \propto e^{\mu/kT} \) can be used. In an ideal gas or ideal dilute solution, the chemical potential has a simple logarithmic relationship to concentration and linear in temperature (so that activity is proportional to concentration), and insensitive to the concentrations of other components (154). However, a near-critical mixture is far from ideal – the critical point is precisely where weak cooperative interactions between the many components lead to strong effects (124). Therefore, we expect strong relationships between the chemical potentials of different components, especially when those components modulate \( T_c \).

Many transmembrane proteins have been shown to be modulated by binding to membrane components, prominently including cholesterol (154, 155) and phosphatidylinositol lipids (156), and many other signaling lipids (157). If the chemical potential of some of these components is strongly modulated by concentration changes of other components, binding site occupancy will also vary, and we expect to see changes in protein functions that depend on binding of those components. Recent work by Ayuyan and Cohen has developed sensitive methods for measuring and controlling the chemical potential of cholesterol in the plasma membrane, and found that cholesterol chemical potential varies by \( \sim 2kT \) in different physiologically relevant cellular conditions (154). That amount is certainly adequate to induce substantial changes in binding site occupancy. Work remains to be done to explore if these differences can be attributed in any way to the critical phase transition, but it is exciting to speculate that perturbations that change membrane criticality may act indirectly by impacting the activity of membrane components.
**Criticality coupled to other processes describes a broad array of raft phenomena:** The Ising universal critical phenomena are a good start for understanding membrane heterogeneity, but they are also clearly insufficient to explain all phenomena. As stated above, critical phenomena alone are not expected to give rise to regions of tight clustering or confinement of proteins and lipids, as is sometimes attributed to membrane domains. This said, it is possible that the local membrane environment can impact the conformational states sampled by membrane proteins in ways that facilitate binding through stronger protein binding sites. This type of synergistic effect could underlie a range of cholesterol dependent processes observed at the plasma membrane, including for example the transient pinning observed in studies of membrane protein and lipid dynamics (158).

Another example where there is potential for synergy between criticality and other organizing principles relates to the ‘Active Composite Model’ proposed by Mayor and coworkers (159). This model posits that the plasma membrane interacts with a heterogeneous cortical actin network, composed of both active and passive components. The passive components largely resemble quenched disorder, as described earlier in this review. The active component is composed of motor driven short actin filaments that can actively drive certain membrane proteins and lipids into close proximity, coupling across membrane leaflets. Considering this model in a critical membrane provides a simple means to correlate domain structure across leaflets without requiring strong interactions such as interdigitation, since the cooperativity inherent in a phase transition can amplify weak couplings that may be present. Moreover, the high susceptibility of a critical membrane allows it to robustly remodel when external forces are applied, including those originating from the actin cortex.
More broadly, we envision that plasma membrane criticality is only one of several organizing principles that contribute to plasma membrane functions. It could be that interactions mediated by curvature and electrostatics superimpose with those mediated via criticality to define the plasma membrane interactome. It is also possible that there is interesting cross-talk between these various interaction modes. For example, studies have shown that the sorting of lipids into curved membranes can be mediated by the binding of curvature-sensing proteins that have preferences for one membrane phase (160). Proteins and peptides can also organize lipids through electrostatics, which in turn can stabilize domains impacted by fluctuations of ordered and disordered phase lipids. The broader implications of this potential cross-talk is largely unexplored, and could give rise to qualitatively new phenomena accessible to cell membranes (161).

6. Concluding remarks

While it has long been appreciated that plasma membrane lipids are capable of intriguing, non-ideal behaviors, much of the past literature is clouded by imperfect methods and an incomplete conceptual framework to conceptualize experimental observations. This backdrop led to controversial and often unphysical descriptions of lipid rafts. The past decade or so has brought key advances, including membrane isolations that largely preserve plasma membrane protein and lipid content and super-resolution imaging methods that do not suffer from the same pitfalls that plagued early raft research. Together with this, the membrane community has begun to appreciate the rich phenomena that naturally occur near miscibility critical points, many of which
exhibit strong parallels with long-standing observations in both the membrane-biophysics and membrane biology literatures. Moving forward, the challenge will be to isolate these effects to enable a definitive measurement of the role of criticality in cell membranes, and to explore how these immiscibility-mediated interactions work alongside other physical and biochemical organizing principles to contribute to the rich array of biological functions at the cell surface.
Literature Cited:

1. Simons K, Ikonen E. 1997. Functional rafts in cell membranes. *Nature*. 387(6633):569–72

2. Baumgart T, Hammond AT, Sengupta P, Hess ST, Holowka DA, et al. 2007. Large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles. *Proc. Natl. Acad. Sci.* 104(9):3165–70

3. Veatch SL, Keller SL. 2005. Seeing spots: Complex phase behavior in simple membranes. *Biochim. Biophys. Acta BBA - Mol. Cell Res.* 1746(3):172–85

4. Oldfield E, Chapman D. 1971. Effects of cholesterol and cholesterol derivatives on hydrocarbon chain mobility in lipids. *Biochem. Biophys. Res. Commun.* 43(3):610–16

5. Oldfield E, Chapman D. 1972. Dynamics of lipids in membranes: Heterogeneity and the role of cholesterol. *FEBS Lett.* 23(3):285–97

6. Engelman DM, Rothman JE. 1972. The planar organization of lecithin-cholesterol bilayers. *J. Biol. Chem.* 247(11):3694–97

7. Shimshick EJ, McConnell HM. 1973. Lateral phase separations in binary mixtures of cholesterol and phospholipids. *Biochem. Biophys. Res. Commun.* 53(2):446–51

8. Chapman D, Penkett SA. 1966. Nuclear magnetic resonance spectroscopic studies of the interaction of phospholipids with cholesterol. *Nature*. 211(5055):1304–5

9. Ladbroke BD, Williams RM, Chapman D. 1968. Studies on lecithin-cholesterol-water interactions by differential scanning calorimetry and X-ray diffraction. *Biochim. Biophys. Acta BBA - Biomembr.* 150(3):333–40
10. Opella SJ, Yesinowski JP, Waugh JS. 1976. Nuclear magnetic resonance description of molecular motion and phase separations of cholesterol in lecithin dispersions. *Proc. Natl. Acad. Sci.* 73(11):3812–15

11. Lentz BR, Barrow DA, Hoechli M. 1980. Cholesterol-phosphatidylcholine interactions in multilamellar vesicles. *Biochemistry.* 19(9):1943–54

12. Rubenstein JLR, Owicki JC, McConnell HM. 1980. Dynamic properties of binary mixtures of phosphatidylcholines and cholesterol. *Biochemistry.* 19(3):569–73

13. Recktenwald DJ, McConnell HM. 1981. Phase equilibriums in binary mixtures of phosphatidylcholine and cholesterol. *Biochemistry.* 20(15):4505–10

14. Alecio MR, Golan DE, Veatch WR, Rando RR. 1982. Use of a fluorescent cholesterol derivative to measure lateral mobility of cholesterol in membranes. *Proc. Natl. Acad. Sci. U. S. A.* 79(17):5171–74

15. Hjort Ipsen J, Karlström G, Mourtisen OG, Wennerström H, Zuckermann MJ. 1987. Phase equilibria in the phosphatidylcholine-cholesterol system. *Biochim. Biophys. Acta BBA - Biomembr.* 905(1):162–72

16. Vist MR, Davis JH. 1990. Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures: deuterium nuclear magnetic resonance and differential scanning calorimetry. *Biochemistry.* 29(2):451–64

17. Almeida PF, Vaz WL, Thompson TE. 1992. Lateral diffusion in the liquid phases of dimyristoylphosphatidylcholine/cholesterol lipid bilayers: a free volume analysis. *Biochemistry.* 31(29):6739–47
18. Huang TH, Lee CW, Das Gupta SK, Blume A, Griffin RG. 1993. A 13C and 2H nuclear magnetic resonance study of phosphatidylcholine/cholesterol interactions: characterization of liquid-gel phases. *Biochemistry*. 32(48):13277–87

19. McMullen TPW, McElhaney RN. 1995. New aspects of the interaction of cholesterol with dipalmitoylphosphatidylcholine bilayers as revealed by high-sensitivity differential scanning calorimetry. *Biochim. Biophys. Acta BBA - Biomembr*. 1234(1):90–98

20. Ahmed SN, Brown DA, London E. 1997. On the Origin of Sphingolipid/Cholesterol-Rich Detergent-Insoluble Cell Membranes: Physiological Concentrations of Cholesterol and Sphingolipid Induce Formation of a Detergent-Insoluble, Liquid-Ordered Lipid Phase in Model Membranes. *Biochemistry*. 36(36):10944–53

21. Feigenson GW, Buboltz JT. 2001. Ternary Phase Diagram of Dipalmitoyl-PC/Dilauroyl-PC/Cholesterol: Nanoscopic Domain Formation Driven by Cholesterol. *Biophys. J*. 80(6):2775–88

22. van Meer G, Simons K. 1982. Viruses budding from either the apical or the basolateral plasma membrane domain of MDCK cells have unique phospholipid compositions. *EMBO J*. 1(7):847–52

23. Heiniger H-J, Kandutsch AA, Chen HW. 1976. Depletion of L-cell sterol depresses endocytosis. *Nature*. 263(5577):515–17

24. Alderson JCE, Green C. 1975. Enrichment of lymphocytes with cholesterol and its effect on lymphocyte activation. *FEBS Lett*. 52(2):208–11
25. Lohr KM, Snyderman R. 1982. Amphotericin B alters the affinity and functional activity of the oligopeptide chemotactic factor receptor on human polymorphonuclear leukocytes. *J. Immunol.* 129(4):1594–99

26. Dahl C, Biemann HP, Dahl J. 1987. A protein kinase antigenically related to pp60v-src possibly involved in yeast cell cycle control: positive in vivo regulation by sterol. *Proc. Natl. Acad. Sci. U. S. A.* 84(12):4012–16

27. Brown DA, Rose JK. 1992. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell.* 68(3):533–44

28. Fiedler K, Kobayashi T, Kurzchalía TV, Simons K. 1993. Glycosphingolipid-enriched, detergent-insoluble complexes in protein sorting in epithelial cells. *Biochemistry.* 32(25):6365–73

29. Harder T, Scheiffele P, Verkade P, Simons K. 1998. Lipid Domain Structure of the Plasma Membrane Revealed by Patching of Membrane Components. *J. Cell Biol.* 141(4):929–42

30. Ge M, Gidwani A, Brown HA, Holowka D, Baird B, Freed JH. 2003. Ordered and Disordered Phases Coexist in Plasma Membrane Vesicles of RBL-2H3 Mast Cells. An ESR Study. *Biophys. J.* 85(2):1278–88

31. Gidwani A, Holowka D, Baird B. 2001. Fluorescence Anisotropy Measurements of Lipid Order in Plasma Membranes and Lipid Rafts from RBL-2H3 Mast Cells. *Biochemistry.* 40(41):12422–29

32. Kenworthy AK, Edidin M. 1998. Distribution of a Glycosylphosphatidylinositol-anchored Protein at the Apical Surface of MDCK Cells Examined at a Resolution of <100 Å Using Imaging Fluorescence Resonance Energy Transfer. *J. Cell Biol.* 142(1):69–84
33. Sheets ED, Holowka D, Baird B. 1999. Critical Role for Cholesterol in Lyn-mediated Tyrosine Phosphorylation of FcεRI and Their Association with Detergent-resistant Membranes. *J. Cell Biol.* 145(4):877–87

34. Pierce SK. 2002. Lipid rafts and B-cell activation. *Nat. Rev. Immunol.* 2(2):nri726

35. Plowman SJ, Muncke C, Parton RG, Hancock JF. 2005. H-ras, K-ras, and inner plasma membrane raft proteins operate in nanoclusters with differential dependence on the actin cytoskeleton. *Proc Natl Acad Sci U A.* 102:15500-5.

36. Brown DA, London E. 1998. Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* 14(1):111–136

37. Munro S. 2003. Lipid Rafts: Elusive or Illusive? *Cell.* 115(4):377–88

38. Dietrich C, Bagatolli LA, Volovyk ZN, Thompson NL, Levi M, et al. 2001. Lipid Rafts Reconstituted in Model Membranes. *Biophys. J.* 80(3):1417–28

39. Samsonov AV, Mihalyov I, Cohen FS. 2001. Characterization of Cholesterol-Sphingomyelin Domains and Their Dynamics in Bilayer Membranes. *Biophys. J.* 81(3):1486–1500

40. Veatch SL, Keller SL. 2002. Organization in Lipid Membranes Containing Cholesterol. *Phys. Rev. Lett.* 89(26):268101

41. Veatch SL, Gawrisch K, Keller SL. 2006. Closed-Loop Miscibility Gap and Quantitative Tie-Lines in Ternary Membranes Containing Diphytanoyl PC. *Biophys. J.* 90(12):4428–36

42. Veatch SL, Keller SL. 2003. Separation of Liquid Phases in Giant Vesicles of Ternary Mixtures of Phospholipids and Cholesterol. *Biophys. J.* 85(5):3074–83
43. Ionova IV, Livshits VA, Marsh D. 2012. Phase Diagram of Ternary Cholesterol/Palmitoylsphingomyelin/Palmitoyloleoyl-Phosphatidylcholine Mixtures: Spin-Label EPR Study of Lipid-Raft Formation. *Biophys. J.* 102(8):1856–65

44. Veatch SL, Keller SL. 2005. Miscibility Phase Diagrams of Giant Vesicles Containing Sphingomyelin. *Phys. Rev. Lett.* 94(14):148101

45. Veatch SL, Soubias O, Keller SL, Gawrisch K. 2007. Critical fluctuations in domain-forming lipid mixtures. *Proc. Natl. Acad. Sci.* 104(45):17650–55

46. Zhao J, Wu J, Heberle FA, Mills TT, Klawitter P, et al. 2007. Phase studies of model biomembranes: Complex behavior of DSPC/DOPC/Cholesterol. *Biochim. Biophys. Acta* BBA - Biomembr. 1768(11):2764–76

47. Petruzielo RS, Heberle FA, Drazba P, Katsaras J, Feigenson GW. 2013. Phase behavior and domain size in sphingomyelin-containing lipid bilayers. *Biochim. Biophys. Acta* BBA - Biomembr. 1828(4):1302–13

48. Connell SD, Heath G, Olmsted PD, Kisil A. 2013. Critical point fluctuations in supported lipid membranes. *Faraday Discuss.* 161(0):91–111

49. Khadka NK, Ho CS, Pan J. 2015. Macroscopic and Nanoscopic Heterogeneous Structures in a Three-Component Lipid Bilayer Mixtures Determined by Atomic Force Microscopy. *Langmuir.* 31(45):12417–25

50. Konyakhina TM, Wu J, Mastroianni JD, Heberle FA, Feigenson GW. 2013. Phase Diagram of a 4-Component Lipid Mixture: DSPC/DOPC/POPC/chol. *Biochim. Biophys. Acta* BBA-Biomembr.
51. Mills TT, Tristram-Nagle S, Heberle FA, Morales NF, Zhao J, et al. 2008. Liquid-Liquid Domains in Bilayers Detected by Wide Angle X-Ray Scattering. *Biophys. J.* 95(2):682–90

52. Konyakhina TM, Feigenson GW. 2016. Phase diagram of a polyunsaturated lipid mixture: Brain sphingomyelin/1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine/cholesterol. *Biochim. Biophys. Acta BBA - Biomembr.* 1858(1):153–61

53. de Almeida RFM, Borst J, Fedorov A, Prieto M, Visser AJWG. 2007. Complexity of Lipid Domains and Rafts in Giant Unilamellar Vesicles Revealed by Combining Imaging and Microscopic and Macroscopic Time-Resolved Fluorescence. *Biophys. J.* 93(2):539–53

54. Sibold J, Tewaag VE, Vagedes T, Mey I, Steinem C. 2020. Phase separation in pore-spanning membranes induced by differences in surface adhesion. *Phys. Chem. Chem. Phys.* 22(17):9308–15

55. Kahya N, Scherfeld D, Bacia K, Poolman B, Schwille P. 2003. Probing lipid mobility of raft-exhibiting model membranes by fluorescence correlation spectroscopy. *J. Biol. Chem.* 278(30):28109–15

56. Aufderhorst-Roberts A, Chandra U, Connell SD. 2017. Three-Phase Coexistence in Lipid Membranes. *Biophys. J.* 112(2):313–24

57. Sodt AJ, Sandar ML, Gawrisch K, Pastor RW, Lyman E. 2014. The Molecular Structure of the Liquid-Ordered Phase of Lipid Bilayers. *J. Am. Chem. Soc.* 136(2):725–32

58. Sodt AJ, Pastor RW, Lyman E. 2015. Hexagonal Substructure and Hydrogen Bonding in Liquid-Ordered Phases Containing Palmitoyl Sphingomyelin. *Biophys. J.* 109(5):948–55
59. Goñi FM, Alonso A, Bagatolli LA, Brown RE, Marsh D, et al. 2008. Phase diagrams of lipid mixtures relevant to the study of membrane rafts. *Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids.* 1781(11):665–84

60. Heberle FA, Feigenson GW. 2011. Phase Separation in Lipid Membranes. *Cold Spring Harb. Perspect. Biol.* 3(4):a004630

61. Ladbrooke BD, Chapman D. 1969. Thermal analysis of lipids, proteins and biological membranes a review and summary of some recent studies. *Chem. Phys. Lipids.* 3(4):304–56

62. Janiak MJ, Small DM, Shipley GG. 1979. Temperature and compositional dependence of the structure of hydrated dimyristoyl lecithin. *J. Biol. Chem.* 254(13):6068–78

63. Heberle FA, Wu J, Goh SL, Petruzielo RS, Feigenson GW. 2010. Comparison of Three Ternary Lipid Bilayer Mixtures: FRET and ESR Reveal Nanodomains. *Biophys. J.* 99(10):3309–18

64. Pathak P, London E. 2011. Measurement of Lipid Nanodomain (Raft) Formation and Size in Sphingomyelin/POPC/Cholesterol Vesicles Shows TX-100 and Transmembrane Helices Increase Domain Size by Coalescing Preexisting Nanodomains But Do Not Induce Domain Formation. *Biophys. J.* 101(10):2417–25

65. Pathak P, London E. 2015. The Effect of Membrane Lipid Composition on the Formation of Lipid Ultrnanodomains. *Biophys. J.* 109(8):1630–38

66. Konyakhina TM, Goh SL, Amazon J, Heberle FA, Wu J, Feigenson GW. 2011. Control of a Nanoscopic-to-Macroscopic Transition: Modulated Phases in Four-Component DSPC/DOPC/POPC/Chol Giant Unilamellar Vesicles. *Biophys. J.* 101(2):L8–10
67. Collado MI, Goñi FM, Alonso A, Marsh D. 2005. Domain formation in sphingomyelin/cholesterol mixed membranes studied by spin-label electron spin resonance spectroscopy. *Biochemistry*. 44(12):4911–18

68. de Almeida RFM, Fedorov A, Prieto M. 2003. Sphingomyelin/Phosphatidylcholine/Cholesterol Phase Diagram: Boundaries and Composition of Lipid Rafts. *Biophys. J.* 85(4):2406–16

69. Doktorova M, Heberle FA, Eicher B, Standaert RF, Katsaras J, et al. 2018. Preparation of asymmetric phospholipid vesicles for use as cell membrane models. *Nat. Protoc.* 13(9):2086–2101

70. Lin Q, London E. 2015. Ordered Raft Domains Induced by Outer Leaflet Sphingomyelin in Cholesterol-Rich Asymmetric Vesicles. *Biophys. J.* 108(9):2212–22

71. Honerkamp-Smith AR, Cicuta P, Collins MD, Veatch SL, den Nijs M, et al. 2008. Line Tensions, Correlation Lengths, and Critical Exponents in Lipid Membranes Near Critical Points. *Biophys. J.* 95(1):236–46

72. Leibler S, Andelman D. 1987. Ordered and curved meso-structures in membranes and amphiphilic films. *J. Phys.* 48(11):2013–18

73. Palmieri B, Yamamoto T, Brewster RC, Safran SA. 2014. Line active molecules promote inhomogeneous structures in membranes: Theory, simulations and experiments. *Adv. Colloid Interface Sci.* 208:58–65

74. Brodbek L, Schmid F. 2016. Interplay of curvature-induced micro- and nanodomain structures in multicomponent lipid bilayers. *Int. J. Adv. Eng. Sci. Appl. Math.* 8(2):111–20
75. Cornell CE, Skinkle AD, He S, Levental I, Levental KR, Keller SL. 2018. Tuning Length Scales of Small Domains in Cell-Derived Membranes and Synthetic Model Membranes. *Biophys. J.* 115(4):690–701

76. Fridriksson EK, Shipkova PA, Sheets ED, Holowka D, Baird B, McLafferty FW. 1999. Quantitative Analysis of Phospholipids in Functionally Important Membrane Domains from RBL-2H3 Mast Cells Using Tandem High-Resolution Mass Spectrometry. *Biochemistry.* 38(25):8056–63

77. Lingwood D, Ries J, Schwille P, Simons K. 2008. Plasma membranes are poised for activation of raft phase coalescence at physiological temperature. *Proc. Natl. Acad. Sci.* 105(29):10005–10

78. Levental I, Grzybek M, Simons K. 2011. Raft domains of variable properties and compositions in plasma membrane vesicles. *Proc. Natl. Acad. Sci.* 108(28):11411–16

79. Levental I, Byfield FJ, Chowdhury P, Gai F, Baumgart T, Janmey PA. 2009. Cholesterol-dependent phase separation in cell-derived giant plasma-membrane vesicles. *Biochem. J.* 424(2):163–67

80. Zhao J, Wu J, Veatch SL. 2013. Adhesion Stabilizes Robust Lipid Heterogeneity in Supercritical Membranes at Physiological Temperature. *Biophys. J.* 104(4):825–34

81. Veatch SL, Cicuta P, Sengupta P, Honerkamp-Smith A, Holowka D, Baird B. 2008. Critical Fluctuations in Plasma Membrane Vesicles. *ACS Chem. Biol.* 3(5):287–93

82. Burns M, Wisser K, Wu J, Levental I, Veatch SL. 2017. Miscibility Transition Temperature Scales with Growth Temperature in a Zebrafish Cell Line. *Biophys. J.* 113(6):1212–22
83. Gray EM, Díaz-Vázquez G, Veatch SL. 2015. Growth Conditions and Cell Cycle Phase Modulate Phase Transition Temperatures in RBL-2H3 Derived Plasma Membrane Vesicles. *PLOS ONE.* 10(9):e0137741

84. Levental KR, Lorent JH, Lin X, Skinkle AD, Surma MA, et al. 2016. Polyunsaturated Lipids Regulate Membrane Domain Stability by Tuning Membrane Order. *Biophys. J.* 110(8):1800–1810

85. Levental KR, Surma MA, Skinkle AD, Lorent JH, Zhou Y, et al. 2017. ω-3 polyunsaturated fatty acids direct differentiation of the membrane phenotype in mesenchymal stem cells to potentiate osteogenesis. *Sci. Adv.* 3(11):eaao1193

86. Cammarota E, Soriani C, Taub R, Morgan F, Sakai J, et al. 2020. Criticality of plasma membrane lipids reflects activation state of macrophage cells. *J. R. Soc. Interface.* 17(163):20190803

87. Symons JL, Cho K-J, Chang JT, Du G, Waxham MN, et al. 2020. Lipidomic atlas of mammalian cell membranes reveals hierarchical variation induced by culture conditions, subcellular membranes, and cell lineages. *Soft Matter*

88. Sezgin E, Levental I, Grzybek M, Schwarzmann G, Mueller V, et al. 2012. Partitioning, diffusion, and ligand binding of raft lipid analogs in model and cellular plasma membranes. *Biochim. Biophys. Acta BBA - Biomembr.* 1818(7):1777–84

89. Kaiser H-J, Lingwood D, Levental I, Sampaio JL, Kalvodova L, et al. 2009. Order of lipid phases in model and plasma membranes. *Proc. Natl. Acad. Sci.* 106(39):16645–50
90. Lorent JH, Diaz-Rohrer B, Lin X, Spring K, Gorfe AA, et al. 2017. Structural determinants and functional consequences of protein affinity for membrane rafts. Nat. Commun. 8(1):1–10

91. Menon AK, Holowka D, Webb WW, Baird B. 1986. Cross-linking of receptor-bound IgE to aggregates larger than dimers leads to rapid immobilization. J. Cell Biol. 102(2):541–50

92. Machta BB, Papanikolaou S, Sethna JP, Veatch SL. 2011. Minimal Model of Plasma Membrane Heterogeneity Requires Coupling Cortical Actin to Criticality. Biophys. J. 100(7):1668–77

93. Keller H, Lorizate M, Schwille P. 2009. PI(4,5)P2 Degradation Promotes the Formation of Cytoskeleton-Free Model Membrane Systems. ChemPhysChem. 10(16):2805–12

94. McLaughlin S, Wang J, Gambhir A, Murray D. 2002. PIP2 and Proteins: Interactions, Organization, and Information Flow. Annu. Rev. Biophys. Biomol. Struct. 31(1):151–75

95. Skinkle AD, Levental KR, Levental I. 2020. Cell-Derived Plasma Membrane Vesicles Are Permeable to Hydrophilic Macromolecules. Biophys. J. 118(6):1292–1300

96. Li G, Wang Q, Kakuda S, London E. 2020. Nanodomains can persist at physiologic temperature in plasma membrane vesicles and be modulated by altering cell lipids. J. Lipid Res. jlr.RA119000565

97. Toulmay A, Prinz WA. 2013. Direct imaging reveals stable, micrometer-scale lipid domains that segregate proteins in live cells. J. Cell Biol. 202(1):35–44

98. Rayermann SP, Rayermann GE, Cornell CE, Merz AJ, Keller SL. 2017. Hallmarks of Reversible Separation of Living, Unperturbed Cell Membranes into Two Liquid Phases. Biophys. J. 113(11):2425–32
99. Lee I-H, Saha S, Polley A, Huang H, Mayor S, et al. 2015. Live Cell Plasma Membranes Do Not Exhibit a Miscibility Phase Transition over a Wide Range of Temperatures. *J. Phys. Chem. B*. 119(12):4450–59

100. Sevcsik E, Brameshuber M, Fölser M, Weghuber J, Honigmann A, Schütz GJ. 2015. GPI-anchored proteins do not reside in ordered domains in the live cell plasma membrane. *Nat. Commun.* 6(1):1–10

101. Levental I, Levental KR, Heberle FA. 2020. Lipid Rafts: Controversies Resolved, Mysteries Remain. *Trends Cell Biol.* 30(5):341–53

102. Kusumi A, Fujiwara TK, Tsunoyama TA, Kasai RS, Liu A-A, et al. 2020. Defining raft domains in the plasma membrane. *Traffic*. 21(1):106–37

103. Castello-Serrano I, Lorent JH, Ippolito R, Levental KR, Levental I. 2020. Myelin-Associated MAL and PLP Are Unusual among Multipass Transmembrane Proteins in Preferring Ordered Membrane Domains. *J. Phys. Chem. B*

104. Marinko JT, Kenworthy AK, Sanders CR. 2020. Peripheral myelin protein 22 preferentially partitions into ordered phase membrane domains. *Proc. Natl. Acad. Sci.*

105. Yang S-T, Kreutzberger AJB, Kiessling V, Ganser-Pornillos BK, White JM, Tamm LK. 2017. HIV virions sense plasma membrane heterogeneity for cell entry. *Sci. Adv.* 3(6):e1700338

106. Anderson RGW, Jacobson K. 2002. A Role for Lipid Shells in Targeting Proteins to Caveolae, Rafts, and Other Lipid Domains. *Science*. 296(5574):1821–25

107. Corradi V, Mendez-Villuendas E, Ingólfsson HI, Gu R-X, Siuda I, et al. 2018. Lipid–Protein Interactions Are Unique Fingerprints for Membrane Proteins. *ACS Cent. Sci.* 4(6):709–17
108. Gray E, Karslake J, Machta BB, Veatch SL. 2013. Liquid General Anesthetics Lower Critical Temperatures in Plasma Membrane Vesicles. *Biophys. J.* 105(12):2751–59

109. Machta BB, Gray E, Nouri M, McCarthy NLC, Gray EM, et al. 2016. Conditions that Stabilize Membrane Domains Also Antagonize n-Alcohol Anesthesia. *Biophys. J.* 111(3):537–45

110. Grinstein G, Ma S. 1982. Roughening and Lower Critical Dimension in the Random-Field Ising Model. *Phys. Rev. Lett.* 49(9):685–88

111. Yethiraj A, Weisshaar JC. 2007. Why Are Lipid Rafts Not Observed In Vivo? *Biophys. J.* 93(9):3113–19

112. Honigmann A, Sadeghi S, Keller J, Hell SW, Eggeling C, Vink R. 2014. A lipid bound actin meshwork organizes liquid phase separation in model membranes. *eLife.* 3:

113. Kwik J, Boyle S, Fooksman D, Margolis L, Sheetz MP, Edidin M. 2003. Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. *Proc. Natl. Acad. Sci.* 100(24):13964–69

114. Liu AP, Fletcher DA. 2006. Actin Polymerization Serves as a Membrane Domain Switch in Model Lipid Bilayers. *Biophys. J.* 91(11):4064–70

115. Shelby SA, Veatch SL, Holowka DA, Baird BA. 2016. Functional nanoscale coupling of Lyn kinase with IgE-FceRI is restricted by the actin cytoskeleton in early antigen-stimulated signaling. *Mol. Biol. Cell.* 27(22):3645–58

116. Gudheti MV, Curthoys NM, Gould TJ, Kim D, Gunewardene MS, et al. 2013. Actin Mediates the Nanoscale Membrane Organization of the Clustered Membrane Protein Influenza Hemagglutinin. *Biophys. J.* 104(10):2182–92
117. Chichili GR, Rodgers W. 2009. Cytoskeleton–membrane interactions in membrane raft structure. *Cell. Mol. Life Sci.* 66(14):2319–28

118. Stone MB, Shelby SA, Núñez MF, Wisser K, Veatch SL. 2017. Protein sorting by lipid phase-like domains supports emergent signaling function in B lymphocyte plasma membranes. *eLife*. 6:e19891

119. Kinoshita M, Suzuki KGN, Matsumori N, Takada M, Ano H, et al. 2017. Raft-based sphingomyelin interactions revealed by new fluorescent sphingomyelin analogs. *J. Cell Biol.* 216(4):1183–1204

120. Allender DW, Giang H, Schick M. 2020. Model Plasma Membrane Exhibits a Microemulsion in Both Leaves Providing a Foundation for “Rafts.” *Biophys. J.* 118(5):1019–31

121. Foret L. 2005. A simple mechanism of raft formation in two-component fluid membranes. *EPL Europhys. Lett.* 71(3):508

122. Gowrishankar K, Ghosh S, Saha S, C. R, Mayor S, Rao M. 2012. Active Remodeling of Cortical Actin Regulates Spatiotemporal Organization of Cell Surface Molecules. *Cell*. 149(6):1353–67

123. Schmid F. 2017. Physical mechanisms of micro- and nanodomain formation in multicomponent lipid membranes. *Biochim. Biophys. Acta BBA - Biomembr.* 1859(4):509–28

124. Goldenfeld N. 2018. *Lectures On Phase Transitions And The Renormalization Group*. CRC Press
125. Cardy J. 1996. *Scaling and Renormalization in Statistical Physics*. Cambridge: Cambridge University Press

126. Kardar M. 2007. *Statistical physics of fields*. Cambridge; New York: Cambridge University Press

127. Chaikin PM, Lubensky TC. 2010. *Principles of condensed matter physics*. Cambridge: Cambridge Univ. Press. 699 pp. 1. paperback ed. (with corr.), 5. print ed.

128. Honerkamp-Smith AR, Veatch SL, Keller SL. 2009. An introduction to critical points for biophysicists; observations of compositional heterogeneity in lipid membranes. *Biochim. Biophys. Acta BBA - Biomembr.* 1788(1):53–63

129. Cagniard de la Tour C. 1822. Exposé de quelques résultats obtenu par l’action combinée de la chaleur et de la compression sur certains liquides, tels que l’eau, l’alcool, l’éther sulfurique et l’essence de pétrole rectifiée. *Ann. Chim. Phys.* 21:127–32

130. van der Waals JD, Rowlinson JS. 2004. *On the continuity of the gaseous and liquid states*. Mineola, N.Y: Dover Publications. 297 pp.

131. Ornstein L, Zernike F. 1918. Die linearen dimensionen der dichtheschwankungen. *Phys Zeit.* 19:134–37

132. Guggenheim EA. 1945. The Principle of Corresponding States. *J. Chem. Phys.* 13(7):253–61

133. Fisher ME. 1967. The theory of equilibrium critical phenomena. *Rep. Prog. Phys.* 30(2):615

134. Onsager L. 1944. Crystal Statistics. I. A Two-Dimensional Model with an Order-Disorder Transition. *Phys. Rev.* 65(3–4):117–49
135. Lee TD, Yang CN. 1952. Statistical Theory of Equations of State and Phase Transitions. II. Lattice Gas and Ising Model. *Phys. Rev.* 87(3):410–19

136. Widom B. 1965. Equation of State in the Neighborhood of the Critical Point. *J. Chem. Phys.* 43(11):3898–3905

137. Kadanoff LP. 1966. Scaling laws for ising models near Tc. *Phys. Phys. Fiz.* 2(6):263–72

138. Wilson KG. 1971. Renormalization Group and Critical Phenomena. I. Renormalization Group and the Kadanoff Scaling Picture. *Phys. Rev. B.* 4(9):3174–83

139. Mora T, Bialek W. 2011. Are Biological Systems Poised at Criticality? *J. Stat. Phys.* 144(2):268–302

140. Honerkamp-Smith AR, Machta BB, Keller SL. 2012. Experimental Observations of Dynamic Critical Phenomena in a Lipid Membrane. *Phys. Rev. Lett.* 108(26):265702

141. Frazier ML, Wright JR, Pokorný A, Almeida PFF. 2007. Investigation of Domain Formation in Sphingomyelin/Cholesterol/POPC Mixtures by Fluorescence Resonance Energy Transfer and Monte Carlo Simulations. *Biophys. J.* 92(7):2422–33

142. Manley S, Horton MR, Lezczynski S, Gast AP. 2008. Sorting of Streptavidin Protein Coats on Phase-Separating Model Membranes. *Biophys. J.* 95(5):2301–7

143. Núñez MF, Wisser K, Veatch SL. 2019. Synergistic factors control kinase–phosphatase organization in B-cells engaged with supported bilayers. *Mol. Biol. Cell.* 31(7):667–82

144. Kimchi O, Veatch SL, Machta BB. 2018. Ion channels can be allosterically regulated by membrane domains near a de-mixing critical point. *J. Gen. Physiol.* 150(12):1769–77

145. Lingwood D, Simons K. 2010. Lipid rafts as a membrane-organizing principle. *Science.* 327:46–50
146. Pike LJ. 2006. Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. *J Lipid Res.* 47:1597–98

147. Levental I, Veatch SL. 2016. The Continuing Mystery of Lipid Rafts. *J. Mol. Biol.* 428(24, Part A):4749–64

148. Sezgin E, Levental I, Mayor S, Eggeling C. 2017. The mystery of membrane organization: composition, regulation and roles of lipid rafts. *Nat. Rev. Mol. Cell Biol.* 18(6):361–74

149. Casimir HBG. 1948. On the Attraction Between Two Perfectly Conducting Plates. *Indag. Math.* 10:261–63

150. Machta BB, Veatch SL, Sethna JP. 2012. Critical Casimir Forces in Cellular Membranes. *Phys. Rev. Lett.* 109(13):138101

151. Reynwar BJ, Deserno M. 2008. Membrane composition-mediated protein-protein interactions. *Biointerphases.* 3(2):FA117–24

152. Saheki Y, De Camilli P. 2017. Endoplasmic Reticulum–Plasma Membrane Contact Sites. *Annu. Rev. Biochem.* 86(1):659–84

153. Zeng M, Chen X, Guan D, Xu J, Wu H, et al. 2018. Reconstituted Postsynaptic Density as a Molecular Platform for Understanding Synapse Formation and Plasticity. *Cell.* 174(5):1172-1187.e16

154. Ayuyan AG, Cohen FS. 2018. The Chemical Potential of Plasma Membrane Cholesterol: Implications for Cell Biology. *Biophys. J.* 114(4):904–18

155. Fantini J, Epand RM, Barrantes FJ. 2019. Cholesterol-Recognition Motifs in Membrane Proteins. *Adv. Exp. Med. Biol.* 1135:3–25
156. De Craene J-O, Bertazzi DL, Bär S, Friant S. 2017. Phosphoinositides, Major Actors in Membrane Trafficking and Lipid Signaling Pathways. *Int. J. Mol. Sci.* 18(3):

157. Shimizu T. 2009. Lipid mediators in health and disease: enzymes and receptors as therapeutic targets for the regulation of immunity and inflammation. *Annu. Rev. Pharmacol. Toxicol.* 49:123–50

158. Eggeling C, Ringemann C, Medda R, Schwarzmann G, Sandhoff K, et al. 2009. Direct observation of the nanoscale dynamics of membrane lipids in a living cell. *Nature* 457(7233):1159–62

159. Rao M, Mayor S. 2014. Active organization of membrane constituents in living cells. *Curr. Opin. Cell Biol.* 29:126–32

160. Sorre B, Callan-Jones A, Manneville J-B, Nassoy P, Joanny J-F, et al. 2009. Curvature-driven lipid sorting needs proximity to a demixing point and is aided by proteins. *Proc. Natl. Acad. Sci.* 106(14):5622–26

161. Mitra ED, Whitehead SC, Holowka D, Baird B, Sethna JP. 2018. Computation of a Theoretical Membrane Phase Diagram and the Role of Phase in Lipid-Raft-Mediated Protein Organization. *J. Phys. Chem. B.* 122(13):3500–3513
**Figure Captions**

**Figure 1:** Phase diagram of lipid mixtures. (A) Phase diagrams of three component mixtures are conventionally drawn on an equilateral triangle. The three vertices are pure mixtures of each lipid component, points along the edges are binary mixtures, and points within the triangle contain all three components. Compositions can be read by measuring the perpendicular distance to each edge, which have the property of always summing to 100%. Two examples are shown. (B) Qualitative phase diagram for ternary lipid mixtures of high melting temperature (Tm) lipids, low Tm lipids and cholesterol. Thick red lines indicate boundaries of liquid-solid (So) coexistence and the thick blue line represents the boundary of liquid-disordered (Ld) liquid-ordered (Lo) coexistence. Points along this boundary also indicate the composition of coexisting phases, and the specific compositions in coexistence are indicated by tie-lines (lighter weight lines within binary coexistence regions). The green triangle represents compositions that exhibit all three phases in coexistence, whose compositions are indicated by the three vertices of the triangle. The Ld-Lo coexistence region terminates at a miscibility critical point along the high cholesterol edge, indicated by a yellow star.

**Figure 2:** Phase diagrams of ternary lipid mixtures exhibit the same overall topology. (A) DiPhytnanolPC/DPPC/Chol by fluorescence microscopy at 16°C, redrawn from (41). (B) DOPC/DPPC/Chol by deuterium NMR, from (45). (C) DOPC/DSPC/Chol by fluorescence microscopy and FRET, from (46). (D) DPC/PSM/Chol by FRET, neutron scattering, and DSC, from (47). (E) POPC/PSM/Chol by EPR, from (43). (F) DOPC/eggSM/Chol by AFM, from (48).

**Figure 3:** Critical systems exhibit universal features that extend beyond the two phase region. (A) Simulation snap-shots of two lattice models. (Top) an Ising model at various reduced temperatures $t$ and compositions $m$ and (middle) a three component model with interactions designed to reproduce ternary GUV phase diagrams, at fixed temperature and various compositions. (Bottom) Compositions of the three-component model are chosen so that the effective reduced temperature $t$ and $m$ of the model match $t$ and $m$ of the corresponding Ising model. Miscibility gaps, tie lines, and the directions of $t$ and $m$ are shown on each schematic phase diagram. (B) Measurements of the divergence of correlation length $\xi$ vs $t$ as the critical point is approached from the one-phase region. (Top) In the two models from panel A. For the three-component model, $t$ is the cholesterol (Chol) content normalized by the Chol content at the critical point. (Middle) In GUVs and GPMVs, varying temperature. In each case, the expected 2D-Ising power law $\xi \propto t^{-1}$ is observed although the proportionality constant differs, resulting in each dataset being plotted with a different y axis scale. (Bottom) Representative GUV and GPMV images near the critical point, data points replotted from (128).

**Figure 4:** Four Functional Mechanisms Primarily Driven by Lo/Ld Partitioning in a Near-Critical Membrane. Membrane proteins and other components are characterized by how they partition into Lo/Ld domains, as schematically shown. The combination of the partitioning of various
components confers to the systems particular properties that can be used to drive biological function. The four mechanisms described here correspond to those described in greater detail in section 5 of the text. (A) Critical Casimir forces yield an effective attraction between components with like order preference (blue-blue pair), and an effective repulsion between components that prefer opposite lipid order (blue-magenta pair). The strength of these interactions increases rapidly when $t$ is reduced, as $\xi$ becomes large. (B) Clustering a protein that prefers Lo lipids (green) induces a distinct Lo domain due to the high susceptibility of the membrane near the critical point. As a result, other Lo-preferring proteins (blue) are recruited to the cluster, and Ld-preferring proteins (magenta) are excluded. Similarly, an Ld domain could be stabilized by clustering an Ld-preferring component. C) The white object is a large protein with two conformations (pentagon/star). One conformation (pentagon) has no order preference and the other (star) prefers an Lo environment. The star conformation becomes more likely as $t$ is decreased because there are large patches of Lo membrane that satisfy its preferred boundary condition. (D) Changes in $t$ also result in changes in the chemical potentials of some membrane components, including components (orange oval) that can bind to a membrane protein (square) as an allosteric modulator, therefore a change in $t$ can induce differences in binding site occupancy and the resulting distribution of protein states.
Figure 1:

(a) C1 + C2 + C3 = 100%
- Blue: Equal fractions of C1, C2, and C3
- Orange: 10% C1, 50% C2, 40% C3

(b) Cholesterol
- Low Tm lipid
- High Tm lipid
Figure 2 omitted due to pending copyright requests.
Figure 3:

A

Ising Model

Three Component Model

B

Graphs showing data with markers for 2 component and 3 component models. Additionally, images of GPMV and GUJ are shown.
Figure 4:

A. Interactions between proteins
- high t/small $\xi$: negligible interactions
- low t/large $\xi$: long-range interactions

B. Susceptibility to clustering
- high t/small $\xi$: minimal sorting
- low t/large $\xi$: effective sorting

C. Allosteric regulation of single proteins
- high t/small $\xi$: state with weak partitioning
- low t/large $\xi$: state with strong partitioning

D. Tuning binding of allosteric regulators
- high t/small $\xi$: reduced regulator binding
- low t/large $\xi$: enhanced regulator binding