The mystery of membrane organization: composition, regulation and physiological relevance of lipid rafts

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Abstract

Cellular plasma membranes are laterally heterogeneous, featuring a variety of distinct subcompartments that differ in their biophysical properties and composition. A large body of research has focused on understanding the basis for this heterogeneity and its physiological relevance. The membrane raft hypothesis formalized a physicochemical principle for a subtype of such lateral membrane heterogeneity, wherein the preferential associations of cholesterol and saturated lipids drives the formation of relatively packed (ordered) membrane domains that selectively recruit certain lipids and proteins. Recent years have yielded new insights into this concept and its *in vivo* relevance, primarily owing to the development of biochemical and biophysical technologies.

Introduction

Only one year after the seminal paper of Singer and Nicholson proposed the fluid-mosaic model for biomembrane organization[1], the first observations that cell membranes can be separated into detergent-labile and detergent-resistant fractions[2] sparked the idea that distinct membrane sub-compartments are present in biological membranes (for a brief history of biomembrane models, please see ref[3]). This finding was followed by a number of observations suggesting cellular membranes are laterally heterogeneous at the sub-micron level[4-9]. The membrane raft (or lipid raft) hypothesis was a specific formulation of the broad concept of lateral membrane inhomogeneity, proposing that the interactions between certain lipids (e.g., cholesterol, relatively saturated lipids, glycosylated lipids) in the plane of the membrane drive the formation of functionally important, relatively ordered membrane regions that recruit other lipids and proteins[10]. This concept was supported by observations of biomimetic model membranes, where there is clear evidence that certain lipids interact preferentially with one another, engage in collective behaviour, and generate large scale lateral domains as a consequence of liquid–liquid phase separation[11].

However, the *in vivo* presence and relevance of such ordered membrane domains was unclear, due in part to the lack of direct observations of these domains and uncertain definitions of the lipid raft concept. To address this uncertainty, a consensus operational definition of "lipid rafts" was formulated in 2006, with available evidence suggesting that rafts are heterogeneous, dynamic (in terms of both lateral mobility and association-dissociation), cholesterol and sphingolipid enriched membrane nano-domains (10-200 nm) that have the potential to form microscopic domains (>300 nm) upon clustering induced by protein–protein and protein–lipid interactions[12] (**Fig. 1**). These domains are present both in the inner and outer leaflets of an asymmetric cell membrane, are presumably coupled across leaflets[13,14], and form functional platforms for regulation of cellular functions[15]. Recently, a number of emerging biochemical and biophysical techniques have provided support for the presence of such domains in cells and suggested key roles for membrane heterogeneity in various cellular functions. The conservation of lipid rafts in the tree of life has also been demonstrated (Supplementary Box S1), providing further support for their biological significance. However, lipid rafts continue to escape direct microscopic detection, thus the presence and exact nature of rafts in live cells remains debated, particularly as different methodologies can often yield seemingly contradictory results[16].

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We agree to exchange numbering of Supplementary Boxes, first "Lipid rafts in the tree of life" and then Box S2 "Super-resolution microscopy".

Here, we define rafts as transient, relatively ordered membrane domains, whose formation is driven by lipid–lipid and lipid-protein interactions, and discuss the technological advances that have reignited the excitement around this concept and its *in vivo* relevance. In particular, we focus on the current understanding of the mechanisms of raft formation and maintenance and conclude with a discussion of the challenges remaining in this dynamic field.

Studying lipid rafts

The definition of rafts has been, in large part, influenced by the development of methodologies available for their investigation. The term "lipid rafts" has been generically applied to many distinct, though potentially related, types of membrane assemblies (**Fig. 2a**). The techniques and tools to visualize and study membrane

heterogeneity have evolved considerably since the introduction of the concept (**Fig. 2b-d**), with the recent advent of super-resolution optical microscopy (Supplementary Box S2) providing a key tool towards potentially resolving the continuing controversy.

Biochemical tools. The first evidence for a laterally heterogeneous cell membrane was the observation of differential solubilisation of membrane lipids and proteins by detergents in the 1970s[2]. The basis of the assay is that non-ionic detergents (under certain conditions, most notably cold temperatures) fractionate cellular membranes into detergent-soluble membrane fractions (DSMs) and detergent-resistant membrane fractions (DRMs) (Fig. 2b). These fractions have clearly distinct compositions, with DRMs enriched in cholesterol, sphingolipids[17,18], and glycophosphatidylinositol (GPI)-anchored proteins[5]. Although extraction of DRMs became the method of choice for probing membrane raft compositions, it quickly became clear that they cannot reflect the native composition and organization of lipid rafts in living cells. For example, the protein composition of DRMs varies widely depending on the choice of detergent used for isolation[19]. Similarly, subtle variations in temperature or detergent concentration yield different results, and considerably modify the organization of membrane proteins[20], which has led to contradicting reports of protein composition of rafts. Thus, while DRMs may inform on the propensity of some molecules to associate with specialized membrane regions[21,22], they do not faithfully reflect the native molecular or biophysical context of rafts[23] and therefore require confirmation by more robust and consistent methods, such as those discussed in the remainder of this section (for an excellent recent example, see ref [22]).

Biophysical tools. In parallel with studies of DRMs isolated from cells, artificial membrane models have been developed and used to study the liquid–liquid phase separation believed to underlie the physical principle behind lipid raft formation[24] (Fig. 2b). Across various experimental set-ups, membranes consisting of relatively saturated lipids with a high melting temperature, unsaturated phospholipid species with a low melting temperature and cholesterol can separate into two distinct liquid phases: a relatively packed and ordered phase enriched in saturated lipid species and cholesterol[25] (termed the liquid-ordered, or L_o), and a more fluid, disordered phase comprising mainly the unsaturated lipids[26,27] (termed the liquid-disordered, or L_d). Owing to its high molecular packing and enrichment of sterol and saturated lipids, the L_o phase is considered as the model for lipid rafts. Biomimetic monolayers[28], supported lipid bilayers[29], nanoscopic bilayers vesicles[30], and giant unilamellar vesicles (GUVs)[26] have all been used to elucidate the molecular details behind this phase separation[31,32]; however, despite their important role in revealing the physical principles of liquid-ordered domain formation, a number of caveats and limitations prevent direct translation of findings from model membranes to biological ones. First, most such experiments are performed in lipid-only systems, and although there are methods for incorporating integral membrane proteins into artificial systems[33,34], they are complex and inefficient, and very rarely result in high protein-to-lipid ratios. This is in contrast to biological membranes, in which proteins are estimated to constitute up to 25% of the membrane's cross-sectional area[35]. Perhaps because of the dearth of proteins, some features of domains established in synthetic membranes may not be representative of biology. For example, ordered domains in synthetic membranes have extremely high molecular order and tight packing, whereas the other extreme is observed in the disordered domains[27,36]. These caveats can be avoided by studying more natural systems such as giant plasma membrane vesicles (GPMVs)[37,38]. GPMVs are cell-derived, intact plasma membrane vesicles that maintain the lipid[39] and protein[40] diversity of the cellular membrane, with the notable exceptions of an assembled cortical actin cytoskeleton, phosphorylated lipids[41] and strict lipid asymmetry between separate leaflets of the membrane bilayer (we refer readers to ref[42] for a detailed discussion of the advantages, caveats, and applications of GPMVs). In these biological membranes, the contrast in molecular order between coexisting ordered and disordered domains is much smaller than, for example, in synthetic GUVs. These differences in molecular order between the two phases may account for the fact that GUV ordered phases exclude nearly all transmembrane proteins and most fluorescent lipid probes (see below), whereas the same molecules are sometimes enriched in the ordered phase in GPMVs (as would be expected for lipid rafts *in vivo*)[36,43] (see also **BOX 1**).

Analytical tools. In cells, rafts are believed to be nanoscopic (<200 nm)[7,8], and they are therefore unresolvable using conventional optical microscopy with its ≈250 nm resolution limit set by diffraction (see Supplementary Box S2). Although confocal microscopy studies have reported co-localization of certain molecules with

putative lipid raft markers (such as cholera toxin) as evidence of their raft association[44], in general the resolution of such systems is insufficient to directly assay raft domain structure and composition. To overcome this limitation, several optical tools have recently been developed[45,46] and applied to investigate nano-scale structures and dynamics in cells. For instance, super-resolution optical microscopy approaches such as photoactivated localization microscopy (PALM), stimulated emission depletion (STED) microscopy (Supplementary Box S2), and near-field scanning optical microscopy (NSOM) have been used to visualize lipid-mediated protein clustering[47-50].

For more dynamic measurements, single-molecule based techniques such as single particle tracking (SPT) have been used to evaluate the diffusion of membrane molecules and relate them to concepts of heterogeneous organization of the membrane[51]. Such studies can reveal oligomerization[52], transient arrest, domain incorporation, and/or confined and hop (compartmentalised) diffusion[53] of tracked molecules (Fig. 2b). Recently interferometric scattering (iSCAT) microscopy has further increased the sensitivity of SPT[54], and shown great potential for assessing membrane heterogeneity. For example, iSCAT was used to show that lipids can transiently stall and incorporate into sub-20nm domains within model membranes[55,56]. Complementary to SPT, fluorescence correlation spectroscopy (FCS) has been applied in combination with spot variation (svFCS[57]) or a STED microscope (STED-FCS[58]) to probe lateral diffusion of membrane components over various length scales. Particularly in STED-FCS the size of the observation spot can be reduced to ~20-40nm, revealing underlying nanoscopic features of the plasma membrane[57-59]. Finally, Förster resonance energy transfer (FRET, Fig. 2b) is a key tool for investigating membrane raft structure and composition[60,61]. The spatial regime probed by this technique makes it ideal for studying nanoscopic domains, and it has been applied to both model membranes[62] and live cells[63] not only to probe the existence of domains, but also to define their size[62,64] by using fluorescent probes with different FRET efficiencies. For a detailed review on these techniques and their caveats, see ref[45].

Most of the above-mentioned methodologies rely on fluorescent labels. This is a particular issue in investigation of membranes because the behaviour of lipids is inherently dependent on their amphiphilic properties and molecular packing, both of which are potentially affected by tags such as fluorophores, which are often nearly the size of the lipid molecules. Thus, the native behaviour of lipids is often greatly altered by the reporter[36]. To address this concern, a number of label-free techniques have been developed. Mass spectroscopy (Fig. 2b), for instance, is one the most accurate tools to address the lipid and protein composition of membranes without the necessity of external labelling[65], and has been used for label-free determination of membrane domain composition in model and cell-derived membranes[66-71]. iSCAT microscopy has also facilitated label-free observation of the dynamics of ordered domains in model membranes[72]. Raman spectroscopy is another label-free technique that has been successfully applied to monitor membrane domain composition[73]. Likewise, small-angle neutron scattering has also been used to detect raft-like domains[74] and their size[75]. Finally, electron microscopy has the necessary resolution for obtaining a snapshot of molecular arrangements at the cell surface, and a number of studies of outer and inner leaflet lipid tethered proteins, including GPI-anchored proteins, glycolipids and Ras proteins, have revealed the nanoscopic organization of proteins in rafts[76]. One potential caveat of these methods is that they usually require cell fixation and staining, which are notoriously problematic in visualizing lipid molecules. Therefore, fluorescence microscopy remains a preferred technique for direct live imaging of the putative lipid raft components, and this necessitates continued optimization of fluorescent labels for membrane components.

Probes selective to membrane domains. Non-perturbing, specific labelling of raft or non-raft domains in cells has been, and remains, one of the foremost challenges of the field. Several fluorescent markers have been used to distinguish between different membrane compartments, including cyanine dyes (e.g., DiO, DiI, DiD)[77], polycyclic aromatic hydrocarbons (e.g. naphtopyrene)[78], and fluorescently-labelled lipids[36,79] (Fig. 2c). As mentioned above, the reliability of these fluorescent lipid analogues depends strongly on the choice of both the native lipid and the fluorescent moiety[36]. The least perturbing fluorescent lipids are the intrinsically fluorescent cholesterol analogues such as dehydroergosterol[80] and cholestatrienol[81]; however, their poor photophysical characteristics in comparison with artificially tagged lipids have prevented their wide application. In the case of phospholipids, it is often challenging to preserve the natural physicochemical behaviour of the lipid after attaching a fluorophore[82,83]. In general, the least perturbing strategy is labelling the

headgroup rather than the acyl chain, and adding a hydrophilic linker to ensure that the fluorophores (which are often membrane active) do not perturb the headgroups of the surrounding lipids[84].

In addition to lipid analogues that can reveal general organization of the membrane into subdomains of variable composition, reporters that selectively bind core raft components can potentially be employed to visualize domains. These include cholesterol binding agents such as filippin[85] and Perfringolysin O[86], sphingolipid reporters such as ostreolysin A[87], lysenin[88] and pleurotolysin[89] as well as ganglioside lipid ligands such as cholera toxin[90]. The major caveats for these probes are (a) their potential perturbation of native membrane organization, for example by inducing clustering of their binding partners, as is the case for cholera toxin; and (b) their reduced specificity in the cellular context where they can potentially bind off-target species, thereby lowering their specificity for raft domains.

Probes sensitive to membrane environments. Coexisting lipid domains inherently have different physico-chemical properties. A particular property that defines lipid rafts is their tight lipid packing owing to the condensing interactions between relatively saturated lipids and cholesterol[91]. Notably, there is not a specific, unique molecular packing that would be common to the plasma membranes and their domains in different cells and contexts[92]. The diversity in membrane compositions and physical properties across cell types, and within cell types during physiological events like secretory granule secretion[92] or cell cycle progression[93], implies that a wide spectrum of different packing states exists in living cells. This lipid packing can be quantified using probes such as Laurdan that sense the level of hydration in the bilayer[94] in combination with two-photon[32] or conventional confocal microscopy[95]. These probes shift their emission spectra depending on the polarity (i.e. aqueous content or hydration) of the environment[96] (Fig. 2c), providing a ratiometric, concentration-independent quantification of the local environment, which for membranes is determined largely by lipid packing[97] (i.e. more tightly packed membrane exclude water more efficiently). Imaging of membrane packing using these probes has been applied to investigate membrane heterogeneity in live cells[47,98]. More recently, in addition to spectral shift, the lifetime[99] and energy-transfer[100] properties of these probes have been used to further investigate lipid packing in living membranes, expanding the toolbox and sensitivity of their potential applications. An important future development will be to enable efficient use of these probes in super-resolution microscopy.

Raft targeting drugs. A common paradigm to study the physiological roles of lipid rafts has been to use drugs or enzymes that impair the structure and function of these domains (Fig. 2d). Since cholesterol is usually thought to be enriched in rafts, the most common raft disrupting agent is methyl-beta cyclodextrin (MBCD), which selectively and efficiently removes cholesterol from membranes[101]. However, it needs to be considered that MBCD-mediated cholesterol removal has a broad pleitropic effects beyond raft disruption. For example, it increases membrane permeability to ions and thereby disrupts membrane potential[102], and potentially is cytotoxic[103]. Moreover, this reagent appears to preferentially remove cholesterol from liquid-disordered (non-raft) domains in model membranes[104], which can induce unexpected and inconsistent[21,67] effects on lipid packing in more complex membranes. Drugs targeting cholesterol synthesis (statins[105] and zaragozic acid[106]) or cholesterol-modifying enzymes (e.g. cholesterol oxidase[107]) have the potential to replace MBCD for raft disruption, but their specificity and effectiveness remain to be conclusively demonstrated. Other core components of rafts in cells are sphingolipids, and a number of reagents can interfere with their synthesis (e.g. fumonisin[108] or myriocin[109]) or stability (e.g. sphingomyelinase[110]), though these suffer from potential off-target effects, such as the impact on general sphingolipid metabolism and the generation of ceramides, which then alter membrane properties in a different way.

Molecular dynamics simulations. One of the biggest remaining challenges in understanding biomembranes is how the myriad of interactions between membrane molecules determines membrane organization. Overcoming this challenge requires a combination of complementary experimental approaches, as well as *in silico* techniques that integrate results from experiments (e.g. data about the structure and energetics of the system) into a simulation framework that ideally reconstitutes the natural behaviour *in silico* based solely on physical interactions[111]. An inherent advantage of such *in silico* approaches is that they model a multitude of molecules simultaneously with high spatial (atomistic level) and temporal (ns-µs) resolution, without relying on exogenous probes or labels. Thus, *in silico* molecular dynamics simulations can be regarded as a

computational microscope[112], capable of visualizing molecular behaviour with unprecedented precision. Currently, such computational microscopes have the inverse limitations of optical microscopes, in that they reveal only fast (microseconds) and nanoscopic (thousands of molecules) processes, as opposed to the slow and large scales accessible by optical microscopy[112,113]. To close the gap between computational and experimental approaches, methods such as coarse graining have been developed to extend the spatiotemporal scale of molecular dynamics simulations without sacrificing the molecular details[114]. Such simulations have been successfully employed to study lipid–lipid and lipid–protein interactions[115,116] and lipid domains in complex membranes[14,117,118]. It is important to note that such *in silico* observations are inherently model-driven and must be ultimately verified by experiments. Unfortunately, in the case of membrane domains, the spatiotemporal gap between direct observables of simulations and experiments is still too large to allow direct comparisons[111]. However, efforts from both directions are aiming to bridge this divide towards a molecular understanding of how complex membrane components self-organize into functional substructures.

Nature and composition of lipid rafts

Dissecting the physical properties – lifetime, size, and area coverage – of lipid rafts in the cellular environment remains one of most vexing issues in the field. Computational models confirm the intuitive assumption that both temporal and spatial compartmentalization of membranes into domains is crucial for membrane function[119]. Unfortunately, both the small size and short lifetime of putative raft domains *in vivo* complicate direct measurement of their properties in living cells. Furthermore, the complexity of plasma membranes suggests that a spectrum of raft-like domains, with varying sizes and lifetimes can be established *in vivo*[92,98,120], further complicating interpretations of experimental measurements. The original model of lipid rafts suggested the existence of a liquid disordered (non-raft) membrane punctuated by more ordered (raft) domains of minimal coverage[121]. However recent data indicate a much greater contribution of ordered raft-like regions, suggesting that ordered membrane domains might be in fact dominating, possibly covering the majority of the plasma membrane, with interspersed less-ordered (non-raft) domains[47,59] (**Fig. 3a**). Both the area fraction as well as the size and lifetime of membrane domains may be further tuned by cellular processes such as signalling and membrane trafficking (**Fig. 3b**) which makes it even more challenging to draw conclusions regarding these membrane domains.

In the original formulation, lipid raft formation was based on preferential interactions between sphingolipids and cholesterol[10]. Consistent with this notion, sphingomyelin has been identified as a core component of detergent resistant membranes[2] and ordered lipid phases[122], in part due to strong hydrogen-bonding interactions with cholesterol[123,124] (**Fig. 4a**). However, the relative partitioning of cholesterol between more and less ordered domains is less clear, with experimental[30] and computational[125] studies suggesting that it is present in high abundance in both ordered (raft-like) and disordered (non-raft) phases, though with an enrichment in more ordered domains. Ganglioside lipids were also found to interact with cholesterol and form cholesterol rich domains in model membranes[70], and these lipids have been consistently detected in the ordered domains of model membranes[90]. In addition, other lipids such as relatively saturated phospholipids have often been associated with raft-like environments, especially in model membranes.

Whereas the biophysical basis for the lipid composition of rafts can be explained by these simple principles, the fundamental bases of selective incorporation of proteins into raft domains remains largely mysterious. In general, proteins interacting with the membrane via lipid anchors follow the rules set by the lipids: saturated lipid anchors such as GPI or palmitoyl moieties generally favour ordered membrane environments, while branched or unsaturated anchors like prenyl groups are non-raft preferring[126]. In fact, GPI-anchored proteins were some of the first proteins identified in DRMs[5] and later in the ordered domains of model membranes[33,127]. Lateral GPI-anchored proteins domains have been extensively characterized by single molecule approaches[128,129]. Although their relationship to membrane rafts remains unresolved[130], the membrane-mediated interactions between these lipid anchored proteins and lipids almost certainly regulate membrane structure and function[14,22,69].

However, lipidated proteins are certainly not the sole protein species associating with rafts. In fact, in a recent experiment, 35% of all plasma membrane proteins were found in ordered domains of GPMVs[43]. These 'raftophilic' proteins included GPI-anchored proteins and palmitoylated proteins as expected (both contributing to one-third of the identified proteins)[43]. However, the remaining one-third of raft-associated proteins contained neither GPI nor palmitoyl anchors and the mechanisms of raft association of many of these proteins are currently unclear. Some proteins are known to become more raftophilic upon oligomerization, which may modulate their activity[131]. Recently, a database of putatively raft-associated proteins has been established based on mass spectrometry studies of isolated DRMs (RaftProt)[132], though it is important to emphasize that these may be subject to the DRM-associated artefacts described above. As the actual protein content of membrane domains is uncertain, few generalizable insights into the structural determinants of raft partitioning for transmembrane proteins are available[133]. Interestingly, a recent study demonstrated that the length of the transmembrane domain (TMD) appears to be a key feature determining the raftophilic properties of a protein, with longer TMDs preferentially targeting the protein to the thicker ordered domains[134].

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Mechanisms of domain regulation

Although the raft concept and its *in vivo* relevance have been controversial, the principle of lateral membrane compartmentalization by lipids is intuitive: there are clear differences in the interaction affinities between various lipids, and these differences may be sufficient to produce a heterogeneous lipid distribution. For systems in thermodynamic equilibrium (including synthetic and biological model membranes[42]), the manifestation of these phenomena is macroscopic phase separation, which can be regulated by temperature[135], composition[21,26,67], or by specific interactions that enhance the inherent connectivity of specific components resulting in enhanced clustering[136]. However, cell membranes *in vivo* are not closed systems in chemical and thermodynamic equilibrium, and many potential regulatory modes contribute to the ultimate output of the inherent self-organizing capacity of biological lipids and their separation into distinct domains (**Fig. 4**).

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Lipid-lipid and lipid-protein interactions. In the traditional raft model, the formation of raft domains is driven mainly by the preferential binding of cholesterol to sphingolipids[124] and possibly other lipids such as gangliosides[69] (**Fig. 4a**). However, an inherent limitation of studying the factors that regulate raft domain properties is the difficulty of measuring those properties *in situ*. To address this limitation, several recent reports[93,137] have focused on factors that regulate the temperature at which macroscopic raft domains form in GPMVs, with the underlying inference that higher phase separation temperatures suggest more stable domains. This paradigm is based on observations of a specific type of phase separation in GPMVs which occurs near a compositional "critical point" and involves large scale fluctuations at temperatures close to the phase transition[141]. Such "critical fluctuations" are universal to all systems exhibiting critical behavior and suggest scaling laws that allow extrapolation of domain size and stability to living cells[135,138]. It is important to note that this hypothesis has yet to be formally evaluated; however, if validated, it will provide an important methodological tool for assaying raft properties. For example, it was recently demonstrated that the stability of raft domains in GPMVs is affected by dietary fatty acids. Specifically, exogenously supplied polyunsaturated fatty acids, e.g. the fish oil component ω-3 docosahexaenoic acid, are robustly incorporated into cellular membranes, where they induce extensive remodelling of lipid compositions and biophysical properties, including increasing the stability of raft-like domains[67]. A study relating these effects to cell behaviour showed that ω-3 docosahexaenoic acid incorporation and concomitant increase in the stability of raft-like domains can repress stem cell properties and motility of breast cancer cells, by interfering with plasma membrane remodelling necessary for the process of epithelial–mesenchymal transition[139].

While variations in lipid composition are certainly key drivers of raft behaviour, protein–lipid interactions also play key roles in raft regulation. For example, some proteins, including the HIV glycoprotein gp41[140], have cholesterol binding motifs that regulate their membrane distribution (**Fig. 4b**). Other proteins specifically bind glycosphingolipids[141] or sphingomyelin[142], potentially mediating their recruitment to membrane domains. Further, a variation on the role of palmitoylation in raft regulation was recently proposed for a post-synaptic density protein 95 (PSD-95). Comprehensive lipidomic analysis of neuronal synapses suggested specific recruitment of raft domains to synaptic sites, which was proposed to be mediated by integration of palmitoylated PSD-95 into the post-synaptic density protein scaffold[143]. In this formulation, rather than raft

domains recruiting palmitoylated proteins, it is the immobilized palmitoylated proteins that recruit saturated lipids and thus nucleate ordered domains at specific cellular sites[143] (**Fig. 4c**). This hypothetical mechanism and its applicability to other cellular contexts remain to be confirmed. However, the evidence that another palmitoylated protein (MPP1) nucleates raft-like environments in erythroid cells[144,145] suggests the possibility of a more general mechanism whereby proteins dictate, or at least considerably influence, the localization and stability of organized domains.

Hydrophobic (mis)match. Mammalian membrane lipids can possess hydrocarbon acyl chains from 12 to 24 carbons in length, potentially yielding drastically different hydrophobic tail lengths for individual lipids. To minimize the unfavourable exposure of hydrophobic tails to the aqueous environment, lipids segregate according to their acyl chain length, potentially introducing lateral heterogeneity. In phase-separated model membranes, this thickness mismatch between longer saturated (raft) and shorter unsaturated (non-raft) lipids appears to regulate the size of coexisting domains, with large mismatches giving rise to large domains, and *vice versa*[146]. Similarly, TMDs of nearly all eukaryote integral membrane proteins consist of alpha helices with hydrophobic amino-acid side chains, which are buried inside the hydrophobic core of the membrane. Hydrophobic matching between these TMDs and the surrounding membrane lipids minimizes the energetically unfavourable exposure of hydrophobic residues to aqueous environments[147] (**Fig. 4d**). In the case of significant length mismatch between TMDs and their solvating lipids, lateral protein-rich aggregates can potentially be induced[148]. However, the role of hydrophobic mismatch on membrane domain dynamics in the live cell membrane needs to be unambiguously demonstrated.

Cortical actin cytoskeleton. The cortical actin cytoskeleton is undoubtedly one of the most important factors influencing membrane organization[149] and mechanics[150]. The actin scaffold has been shown to determine molecular diffusion dynamics (e.g. trapped and hop diffusion)[151,152] and supramolecular arrangements in the membrane[129,153,154]. In *in vitro* cholesterol containing membrane systems capable of large scale phase segregation, actin can directly stabilize or abrogate large-scale phase separation, depending on the nature of lipid species that are coupled to actin[153,155-157]. If actin filaments are coupled to, for instance, saturated acyl chain containing lipid species they tend to stabilize L_o domains, but prevent large-scale phase separation[153]. In a living cell, it is likely that the active mechanics of actin filaments will influence the organization of membrane components associated with actin. In fact a theoretical framework for understanding the interplay between the organization of the cortical actin cytoskeleton and living asymmetric membranes has arisen from studies of the acto-myosin dependent clustering behaviour of GPI-anchored proteins on the outer leaflet of the plasma membrane. It is proposed that such clustering is the result of dynamic self-organization of acto-myosin into nanoscopic contractile assemblies termed asters[158,159]. These assemblies possibly via specific actin-membrane adaptor proteins, bind and transiently immobilize the charged lipid phosphatidylserine (PS) in the inner membrane leaflet. This lipid species contains long saturated acyl chains, which engage in cholesterol-mediated trans-bilayer interactions with the long acyl chain-containing GPI-anchored proteins located in the outer leaflet, resulting in local raft-like domains[14] (**Fig. 4e**). Thus, an actin-driven clustering mechanism may be responsible for the formation of ordered domains in live cell membranes, even under conditions (e.g. temperature and/or lipid composition) not normally conducive for phase segregation. A proof-of-principle for this mechanism has been recently demonstrated *in vitro*[160], by showing that dynamically remodelling acto-myosin networks can organize and segregate associated lipids in a synthetic supported membrane bilayer system. In addition, recent live-cell work has shown that self-organising cortical actin patterns such as asters associate more ordered membrane environments in the immediate plasma membrane areas[159]. Adding to the chemical principles of lipid–lipid interactions, this actin-driven mechanism of membrane ordering provides a consistent explanation for the dynamic properties and non-equilibrium distribution of nanoclusters formed by several lipid species. These include GPI-anchored proteins, glycolipids at the outer leaflet and Ras proteins at the inner leaflet of live-cell membranes[161]. The molecular machinery generating these actin-based nanoclusters remains as yet unidentified, and further work is necessary to understand how these small actin-based nanoclusters may give rise to larger scale ordered membrane domains, necessary for function[161].

Physiological functions of rafts

The most apparent function of raft domains is to segregate specific elements in order to regulate their interactions with other membrane components and hence their activity. Additionally, interactions with raft enriched lipids (cholesterol or glycosphingolipids), or with the distinct biophysical environment of rafts, may change protein conformation and thus bioactivity[162,163] (**Fig. 5a**). These general modes of regulation may be broadly applied in cellular physiology, and a few examples are described here. However, it should be emphasized that direct mechanistic implications of lipid rafts in cell function and dysfunction remain unclear owing to the inherent difficulties in defining raft composition and properties, and their specific perturbation.

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Immune signalling. Compartmentalization of cellular signalling in membrane domains may be used to concentrate positive regulatory components (such as kinases[164]) while excluding negative regulatory elements (such as phosphatases[165]) (**Fig. 5b**). The first signalling pathway associated with lipid rafts was IgE-mediated signalling[166]. Since then, several studies have implicated these domains in a variety of innate and adaptive immune responses[167]. In these contexts, the key immune receptors, including the IgE receptor (FceRI), T-cell receptor[168], and B-cell receptor[44], were found in detergent soluble membrane fractions in resting or immature cells, but acquired detergent resistance following receptor activation, suggesting that translocation to membrane rafts is associated with active signalling through these receptors[169-171]. This notion is supported by co-enrichment in DRMs of the proximal signal transduction machinery downstream of the immune receptors, including lymphocyte-specific protein tyrosine kinase Lck or proto-oncogene tyrosine-protein kinase Fyn[164], as well as a signalling adaptor protein LAT[43]. Further, several other immune-associated proteins are GPI-anchored (suggesting their preferential targeting to rafts) and have been found in DRMs[172]; these include the receptor for bacterial lipopolysaccharides CD14 and Thy-1 (CD90), which is crucial for T-cell activation[173].

Host-pathogen interactions. Recent studies have boosted the interest in lipid rafts as modulator of host–pathogen interactions by disclosing a high content of saturated (sphingo-)lipids and cholesterol in the viral envelopes (for example of HIV)[174] or by finding ordered membrane domains in pathogenic microorganisms[175]. There is now substantial evidence showing that viruses and bacterial products (such as toxins) preferably bind to detergent-resistant and highly ordered plasma membrane regions to penetrate the cell. This could be due to the raft enrichment of receptors, such as glycolipids[176] (e.g. for cholera toxin[90]) or viral receptors[177]. Further, binding of HIV Gag protein (necessary for viral budding and release from host cells) has been shown to preferentially occur in the membrane domains with high cholesterol content[178], suggesting that rafts might be preferential sites for viral budding[178](**Fig. 5b**).

Cancer. A multitude of proteins associated with oncogenic malignancies have been found in DRMs; these include Mucin-1, overexpression of which leads to several cancer forms[179], urokinase plasminogen activator receptor, which plays a role in tumour invasion, migration and angiogenesis in breast cancer[180], and Ras proteins, which show raft-dependent oncogenic activity in breast cancer[181]. The localization of pro-oncogenic proteins to raft-like domains, along with the fact that mitogenic signalling is initiated from various cell surface receptors, suggest potential involvement of rafts in cancer development and progression. Consistently, drugs that modulate membrane organization, including the raft-associated alkyl-phospholipids[182] edelphosine, miltephosine or perifosine (for instance, by altering raft localisation of proton pumps in the membrane[182]), have been shown to exhibit anti-cancer activity[183].

Cardiovascular diseases. Atherosclerosis is a leading vascular disease, which develops as a result of the uptake of cholesterol accumulated in the artery walls (as oxidized low density lipoproteins (oxLDLs)) by macrophages. This uptake causes a transformation of macrophages into foam cells which then clog the arterial wall leading to strokes, heart attacks and peripheral vascular diseases[184]. Notably, this transition of macrophages into foam cells appears to be raft-dependent as oxLDL receptors were found to localize to raft domains upon oxLDL stimulation[185]. In addition, caveolae, formation of which has often been associated with lipid rafts, are also essential for normal cardiac functions, as various cardiac ion channels have been shown to localize to these membrane pits[186].

Conclusions and Perspective

Accumulating evidence suggests that cellular membranes are laterally heterogeneous, forming distinct, highly ordered lipid raft domains alongside less organized and more fluid regions. This heterogeneity is potentially important for various cellular functions by regulating the interactions between membrane associated components. However, the mechanisms driving and regulating lateral membrane heterogeneity remain poorly understood. For this reason, the concept of lipid rafts has received an outsized share of both popularity and controversy. At its apex, hundreds of papers per year were published on membrane rafts; at the nadir, many refrained from using the word 'rafts' to avoid the inevitable semantic quicksand that it conjured. The major predicament in membrane raft research has been, and continues to be, lack of direct visualization of these domains in unperturbed live cells. However, the remarkable advances in microscopic technology of the last decade now allow direct observation of the spatial (nanometers) and temporal (milliseconds) regimes believed to be relevant for raft domains in the living cells. These advances along with improvements in *in silico* membrane modelling suggest that direct of detection of these elusive domains in cell membranes, while remaining challenging, may be within reach[187]. Direct imaging of phase separation in isolated plasma membranes, such as GPMVs[37,38], has already provided evidence that the isolated plasma membrane bilayer is capable of generating co-existing liquid ordered and disordered domains. Moreover, domains remarkably resembling those ordered and disordered phase separated domains in GPMVs have been directly visualized in subcellular organelles of yeast[188], suggesting that investigation of internal membranes may also be a fruitful direction.

Much of the controversy about the properties (size, lifetime, abundance) of lipid rafts stems from attempts to generalize the organization of a number of different membrane components (incl. glycolipids, sphingomyelin, cholesterol, GPI-anchored proteins, and minimal palmitoylated motifs) via a common raft paradigm. First, it is important to note that a very specific set of physical and compositional features should not be expected from lipid rafts. Living membranes are extremely complex and varied, and thus their organization will inherently be context-dependent, and potentially involve many different types of coexisting domains. Such varied assemblies may have distinct organizational principles and cellular functions, which may only be apparent at specific spatial and temporal scales. Moreover, it is important to consider that most molecules that typically associate with rafts are not simply domain probes, but also possess distinct bioactivities that may impact domain organization and dynamics. Further, these bioactivities may be affected by the specifics of an experiment, e.g. cell type or cell cycle. Altogether, to obtain reproducible results regarding raft formation and their biophysical properties it may be necessary to introduce fully synthetic probes (instead of semi-native labels) that exhibit validated affinities for ordered membrane domains[84], and thus allow to careful correlations between ordered domain affinity and other experimental readouts[22]. Another approach that would minimize the experimental differences is the application of label-free detection of domains[72].

Ultimately, the controversies about the organization and dynamics of membrane domains will be resolved by direct observation of well-validated probes, with high spatial and temporal resolution, over extended time- and length-scales. Such data could be complemented by detailed lipidomic and proteomic analysis of nanometric regions of the cell surface[70] as well as *in silico* membrane modelling. The next step will be to integrate these observations into the framework of cellular dynamics, linking membrane heterogeneity to cell biological processes. For this, it will be necessary to concomitantly observe the organization, dynamics, and bioactivity of specific raft components to dissect the key principles of how domain localization modulates molecular function. Clearly, such advances will require parallel application and development of a variety of different techniques, suggesting that this field has an exciting and interdisciplinary future.

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Display items

Boxes

Box 1 | **Model membranes to study formation and organization of lateral domains**

Combining a relatively saturated lipid, an unsaturated lipid and cholesterol in a model membrane, often results in liquid–liquid phase separation and the establishment of two distinct phases (still liquid in nature)[11]. One of these phases (liquid-ordered (Lo)) is more viscous than the other (liquid disordered (Ld)) owing to the tighter packing and higher molecular order of its constituent lipids[91]. This Lo phase is believed to be analogous to lipid rafts in cellular membranes.

Supported lipid bilayers (SLBs, see Figure panel **a**) are planar bilayers formed on glass or mica surfaces[29]. As these membranes are planar, they are highly amenable to microscopic imaging, either by light or Atomic Force Microscopy, allowing observations of the topology of nanodomains that are not resolvable with diffraction-limited optical microscopy (Supplementary Box S2). The artefacts caused by the solid support in SLBs are avoided by the use of free-standing membranes like giant unilamellar vesicles (GUVs) (see Figure panel **b**), which have been frequently used to investigate domain dynamics and morphologies[189]. The limitation of synthetic model systems is their simple composition, which does not fully recapitulate that of the cell membrane.

Giant plasma membrane vesicles (GPMVs) are obtained from cell membranes[37]. Like GUVs, these form micron-scale lateral liquid domains (confirming the capacity for liquid–liquid phase separation in cellular membranes), but do so while maintaining the broad compositional features of the native plasma membrane. The most notable differences between GUVs and GPMVs are lipid complexity and the presence of abundant transmembrane proteins[37,38], incorporation of which into SLBs and GUVs remains technically challenging. The biophysical properties of GPMVs are somewhat distinct compared to artificial membranes[37,92]. For example, the difference in packing between Lo and Ld domains in GPMVs is much smaller than in GUVs (Figure panel **c;** generalized polarization is a relative index of lipid packing, with +1 representing maximally ordered and -1 representing maximally disordered membranes), which may explain why transmembrane proteins can associate with Lo phase in GPMVs[43] but not in GUVs[190]. Despite these differences, most of the core features of coexisting liquid-ordered and -disordered domains in these model systems are fundamentally similar[24].

Figure Legends

Figure 1 | **General overview of lateral heterogeneity in the plasma membrane. a** | Lipid raft domains are usually defined as small, highly dynamic and transient plasma membrane entities enriched in saturated phosho-, sphingo- and glycolipids, cholesterol, lipidated proteins and glycosylphosphatidylinositol (GPI)-anchored proteins. Enrichment in these hydrophobic components endows these lipid domains with distinct physical properties, including increased lipid packing and order, and decreased fluidity. In addition to membrane components, cortical actin plays an active role in domain maintenance and remodelling. Further, membrane lipids are asymmetrically distributed in the inner and outer leaflets, and this may further impact membrane organization. **b** | It is likely that membrane organization is not binary (i.e. highly specified raft and non-raft regions), but rather consists of various raft-like and non-raft domains with distinct compositions and properties.

Figure 2 | **Tools to study membrane domain organization, composition and function a** | In principle, membrane domains can be pure lipid clusters, but in most physiologically-relevant cases, also involve

proteins, including glycosylphosphatidylinositol (GPI)-anchored protein clusters or clusters of Ras proteins. These domains can be purely lipid-driven entities, such as domains established through liquid–liquid phase separation in model membranes. They can also be induced by clustering agents, such as cholera toxin which binds to monosialotetrahexosylganglioside (GM1) or by antibodies recognizing surface receptors. **b** | Tools that are commonly used to investigate membrane domains. These include various model membranes (such as synthetic giant unilamellar vesicles (GUVs) and cell-derived giant plasma membrane vesicles (GPMVs)); detergent resistance assays, wherein raft-like membrane regions persist as detergent-resistant membranes (DRMs), whereas non-raft components are fully solubilized; single molecule imaging to evaluate the diffusion of membrane molecules; fluorescence spectroscopy (such as Förster resonance energy transfer (FRET)), and mass spectrometry. **c** | Also various probes can be used to study raft domains. Domain selective probes partition selectively to one of the domains while domain sensitive probes partition to both domains and change their photophysical behaviour (for instance absorbance and emission spectra) depending on the nature of the surrounding lipid environment. **d** | Treatments that interfere with cholesterol or sphingolipid levels have been used to disturb rafts in cells and can shed light on the cellular functions of these domains.

Figure 3 | Area coverage of membrane domain and domain size. a | Models of membranes with varying raft coverage. Total raft coverage in a given membrane may vary broadly, ranging from small isolated domains to percolating raft phases of increasing size. The specific organizational state depends on a variety of factors, including cell type, specific cellular conditions (e.g. cell cycle), and/or the identity of the membrane (e.g. plasma membrane versus intracellular membranes). **b** | Another mode of modulation of membrane organization can occur without changing overall raft abundance. For example, the size and/or lifetime of individual domains may be influenced by cellular processes such as endo- and exo-cytosis, lipid metabolism, etc. In addition, binding of clustering agents (antibodies and toxins) to their receptors can promote the formation of large membrane domains.

Figure 4 | Regulation of membrane domains. a | Lipid–lipid interactions, in particular interactions between cholesterol and sphingolipids (but also between other relatively saturated lipids) are the defining feature of lipid-driven ordered domain formation. The preferential interaction between sphingolipids and sterols is due to the saturation of sphingolipid hydrophobic tails, but also hydrogen bonding between these lipid species. The amide of the sphingolipid backbone can both donate and accept a hydrogen bond, and these hydrogen bonds are within the interfacial region of the membrane, where the relative paucity of water increases the relative stability of these bonds. **b** | Some proteins harbour lipid binding domains, interacting with cholesterol or sphingolipids, and these lipid–protein interactions may determine the affinity of proteins for ordered lipid domains. **c** | Lipidated proteins, modified by the attachment of a saturated acyl chain (such as palmitoyl moieties), are recruited to raft domains, but may also nucleate and recruit membrane domains if they are integrated into a relatively static protein scaffold. **d** | Hydrophobic interactions can contribute to membrane domain organization and composition. In particular, proteins possessing transmembrane domains (TMD) of different lengths prefer different lipid environments to protect their hydrophobic TMDs from exposure to the aqueous surrounding. For example, proteins with long TMDs were found to associate with domains harbouring long chain saturated lipids (top). When there is a mismatch between the length of the TMD and the local lipid environment in which the protein resides, protein–protein interaction might be favoured instead, leading to local protein concentration (bottom). **e** | The immobilization of inner leaflet lipids containing long saturated acyl chains by actin clusters (which are formed due to the interactions between membrane lipids such as phosphatidylserine (PS) and phosphatidylinositol-4,5-bisphosphate (PIP2), adaptor proteins that possess PS/PIP2 and actin binding domains and actin) results in the engagement of long acyl chain containing lipid-anchored proteins (such as glycosylphosphatidylinositol (GPI)-anchored proteins) located in the outer leaflet, which, in the presence of cholesterol, induces their active clustering. This results in locally ordered, transbilayer nanodomains, which are dynamic owing to the dynamics of actin clusters, and may form even in conditions that do not favour liquid–liquid phase separation of lipids or other supporting interactions.

Figure 5 | Cellular functions of lipid rafts. a | Mechanisms by which membrane domains can potentially regulate bioactivity of their associated components. Rafts can concentrate certain molecules resulting in the establishment of functional catalytic platforms. For example, enzyme and substrates can be brought together to increase their encounter probability and thereby trigger reactions (e.g. signal transduction). A related

possibility is that distinct physicochemical environments provided by lipid rafts directly impact protein conformation, thereby regulating bioactivity. **b** | Examples of physiological functions of membrane domains. Kinases of the SRC family are enriched in raft domains owing to their palmitoylation, whereas transmembrane phosphatases are generally excluded. This segregation has been found to be important for immune signalling, where raft associated SRC kinases are involved in regulating the phosphorylation state, and hence the signal transduction activity, of various immune receptors (including the T-cell receptor and the IgE receptor) (left). Many pathogens and their products (such as bacterial toxins) selectively bind membrane rafts owing to the presence of their specific receptors, such as glycoshpingolipids (GSLs; for the cholera toxin) or CD4 (for the human immunodeficiency virus (HIV)) in these domains, thereby gaining access to their host cells. Virus budding is also thought to occur preferentially at raft-like domains. Although the mechanism behind this selective budding is not yet clear, viral proteins such as Gag proteins of HIV are believed to be sensitive to membrane fluidity, and to associate with cholesterol-enriched domains.

Glossary

liquid–liquid phase separation: coexistence of two phases with distinct compositions and biophysical properties. The components of both phases can diffuse and rearrange rapidly.
sphingolipid: a class of lipids based on a long chain sphingosine base coupled to a fatty acid chain and often a large polar headgroup
glycophosphatidylinositol-anchored proteins: cell surface proteins that are post-translationally modified to carry a glycophosphatidylinositol moiety as an anchor to the membrane.
cholera toxin: proteinaceous toxin secreted by *Vibrio cholerea* that binds glycolipids on the cell surface and is responsible for cholera infection.
single particle tracking (SPT): a single molecule technique where the motion of individual molecules is tracked with high temporal resolution over relatively long time scales (seconds); these tracks can be used to determine the molecules' diffusion properties.
confined diffusion: also known as trapped diffusion; a diffusion mode where the motion of the molecule is transiently arrested by molecular obstacles such as immobile clusters.
hop diffusion: a diffusion mode where molecules diffuse freely on the membrane except when encountering a barrier, such as structures associated with actin filaments.
interferometric scattering (iSCAT) microscopy: a microscopy technique wherein interference from coherent light scattering in the focal plane and of the microscope cover glass is used to enhance contrast.
fluorescence correlation spectroscopy: a single molecule-based technique wherein fluorescence intensity fluctuations from a microscopic observation spot are used to obtain information about molecular diffusion.
Förster resonance energy transfer (FRET): a fluorescence spectroscopy and imaging technique based on the distance-dependent transfer of the excited state energy of a donor fluorescent molecule to an acceptor fluorescent molecule; efficient and widely used to measure intermolecular distances in the range of 1-10 nm.
amphiphilic molecules: molecules showing both hydrophilic and hydrophobic character, such as lipids with hydrophobic acyl chains and hydrophilic headgroups.
Raman spectroscopy: a spectroscopy technique where vibrational energy of the molecules is used as fingerprints of the molecules.
ganglioside lipid: a class of glycosphingolipid with sialic acid moieties on the head group.
ceramides: a class of lipids composed of sphingosine and a fatty acid.
coarse-grained simulations: simulations that rely on simplified representations of the simulated components.
hydrogen -bonding: non-covalent chemical bonds formed between a hydrogen covalently bound to a electronegative atom (as in the -NH group of sphingolipids) and another electronegative atom (such as the oxygen in the -OH group of cholesterol).
epithelial–mesenchymal transition: a developmental transcriptional program that imparts mesenchymal characteristics (e.g. motility) to epithelial cells.

viral envelope: the lipid membrane that covers the virus capsid protein and is derived from the host cell plasma membrane.
caveolae: specialized invaginations in the plasma membrane enriched in caveolin, sphingolipids, and cholesterol.

Online summary

- **Cellular membranes are lateral heterogeneous, consisting of transient and dynamic domains with varying properties, prominently including ordered lipid-driven domains referred to as lipid (or membrane) rafts.**
- **Membrane domains can be induced and regulated by a variety of interactions, including specific lipid–lipid and lipid–protein interactions, bulk membrane properties, and interactions between membrane components and the underlying cytoskeleton.**
- **Advanced microscopy and biochemistry techniques facilitate the study of membrane domains, however they still elude direct *in vivo* visualization. The multiplicity of possible organizational states and their context-dependent nature most likely account for experimental inconsistencies.**
- **Membrane rafts potentially play crucial physiological roles across cell types, spanning from immune cells to cancer cells.**
- **Membrane domains are conserved throughout the domains of life, supporting their important functions in biological systems.**

Author biographies

Erdinc Sezgin has been an EMBO and a Marie Skłodowska-Curie fellow in University of Oxford since 2014. He carried out his PhD work in the group of Petra Schwille at the Technical University of Dresden and a short postdoctoral period in Kai Simons lab at Max Planck Institute of Cell Biology and Genetics in Dresden, Germany. His research is focused on the role of membrane heterogeneity in immune system.

Ilya Levental received his PhD from the University of Pennsylvania under the guidance of Dr Paul Janmey. His postdoctoral research on the mechanisms of protein recruitment to membrane domains was carried out during a Humboldt Fellowship in the laboratory of Dr Kai Simons at the MPI for Molecular Cell Biology and Genetics (MPI-CBG) in Dresden, Germany. In 2012, he became a group leader and CPRIT scholar for cancer research at the McGovern Medical School of the University of Texas Health Science Center at Houston. His research continues to focus on the physiological consequences of membrane composition and organization, with specific focus on dietary lipids.

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