

The mystery of membrane organization: composition, regulation and roles 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 number of studies have 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, in which the preferential associations between cholesterol and saturated lipids drive the formation of relatively packed (or ordered) membrane domains that selectively recruit certain lipids and proteins. Recent studies have yielded new insights into this mechanism and its relevance *in vivo*, owing primarily to the development of improved biochemical and biophysical technologies.

Liquid–liquid phase separation

The coexistence of two phases with distinct compositions and biophysical properties. The components of both phases can diffuse and rearrange rapidly.

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Only a year after the seminal paper of Singer and Nicolson in which the fluid mosaic model for biomembrane organization was proposed¹, the first observations that cell membranes can be separated into detergent-labile and detergent-resistant fractions² sparked the idea that distinct membrane subcompartments are present in biological membranes (for a brief history of biomembrane models, see REF. 3). This finding was followed by a number of observations that suggested that cellular membranes are laterally heterogeneous at the submicrometre scale^{4–9}. The membrane raft (or lipid raft) hypothesis emerged as a way of explaining this lateral membrane inhomogeneity: it proposed that the interactions between specific lipids (for example, cholesterol, relatively saturated lipids and glycosylated lipids) in the plane of the membrane drive the formation of functionally important, relatively ordered membrane regions that recruit other lipids and proteins¹⁰. This concept was supported by observations of biomimetic model membranes, which provide 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 separation¹¹.

However, the presence and relevance of such ordered membrane domains *in vivo* were unclear, owing 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 the available evidence suggesting that rafts

are heterogeneous, dynamic (in terms of both lateral mobility and association–dissociation), cholesterol- and sphingolipid-enriched membrane nanodomains (10–200 nm) that have the potential to form microscopic domains (>300 nm) upon clustering induced by protein–protein and protein–lipid interactions¹² (FIG. 1). These domains are present in both the inner and the outer leaflets of an asymmetric cell membrane, are presumably coupled across leaflets^{13,14} and form functional platforms for the regulation of cellular processes¹⁵. Recently, several emerging biochemical and biophysical techniques have provided support for the presence of these domains in cells and suggested key roles for membrane heterogeneity in various cellular functions. The conservation of lipid rafts throughout the tree of life has also been demonstrated (Supplementary information S1 (box)), which has provided further support for their biological significance. However, lipid rafts continue to elude direct microscopic detection; thus, the presence and exact nature of rafts in live cells remain the subject of debate, particularly as different methodologies can often yield seemingly contradictory results¹⁶.

Here, we define rafts as transient, relatively ordered membrane domains, the formation of which is driven by lipid–lipid and lipid–protein interactions, and we discuss the technological advances that have reignited 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 that remain in this dynamic field.

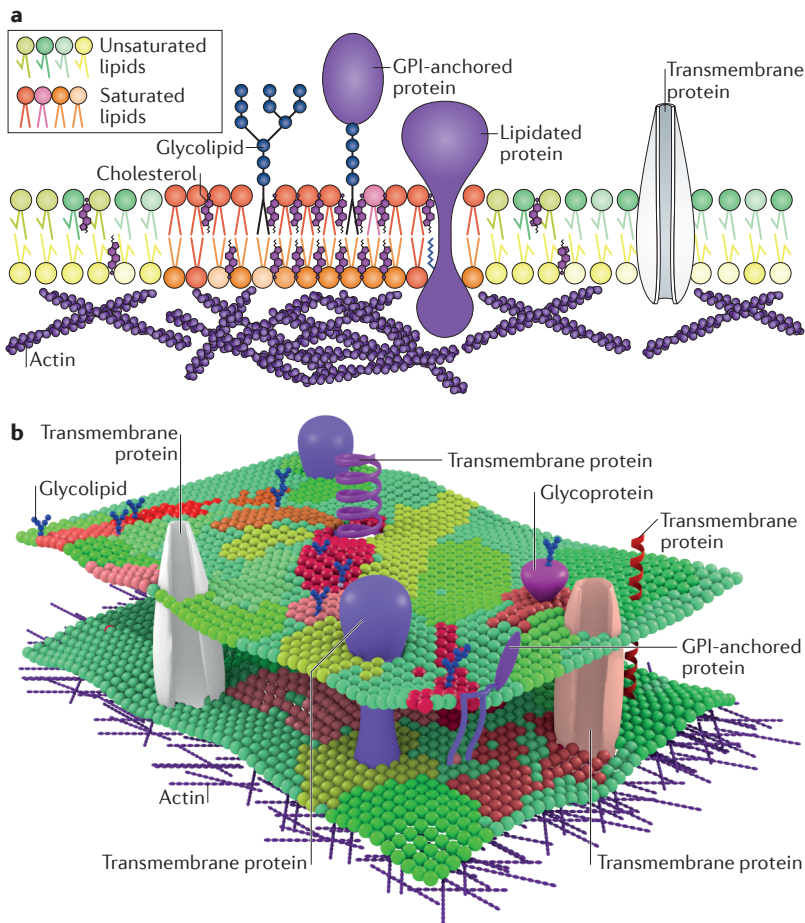


Figure 1 | General overview of lateral heterogeneity in the plasma membrane.
a | Lipid raft domains are usually defined as small, highly dynamic and transient plasma membrane entities that are enriched in saturated phospholipids, sphingolipids, glycolipids, cholesterol, lipidated proteins and glycosylphosphatidylinositol (GPI)-anchored proteins. Enrichment of these hydrophobic components endows these lipid domains with distinct physical properties; these include increased lipid packing and order, and decreased fluidity. In addition to membrane components, cortical actin plays an active part in domain maintenance and remodelling. Furthermore, membrane lipids are asymmetrically distributed in the inner and outer leaflets, which may further affect membrane organization. **b** | It is likely that membrane organization is not binary (that is, highly distinct raft and non-raft regions), but instead membranes consist of various raft-like and non-raft domains with distinct compositions and properties.

Studying lipid rafts

The definition of rafts has been influenced, in large part, by the development of methodologies available for their investigation. The term ‘lipid rafts’ has been applied generically to many distinct, although potentially related, types of membrane assemblies (FIG. 2a). The techniques and tools used to visualize and study membrane heterogeneity have evolved considerably since the introduction of the concept (FIG. 2b–d), and with the recent advent of super-resolution optical microscopy ([Supplementary information S2 \(box\)](#)) we may now have a key tool for resolving the continuing controversy.

Biochemical tools. The first evidence for a laterally heterogeneous cell membrane came from the observation of differential solubilization of membrane lipids and proteins by detergents in the 1970s². The basis of

the assay is that cellular membranes can be separated into distinct fractions — containing detergent-soluble membranes (DSMs) or detergent-resistant membranes (DRMs) — following extraction with non-ionic detergents under specific conditions (most notably, cold temperatures) (FIG. 2b). These fractions have clearly distinct compositions, with DRMs enriched in cholesterol, sphingolipids^{17,18} and glycosylphosphatidylinositol (GPI)-anchored proteins⁵. Although extraction of DRMs became the method of choice for probing membrane raft composition, it quickly became clear that DRMs do not 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 isolation¹⁹. Similarly, subtle variations in temperature or detergent concentration yield different results and considerably modify the organization of membrane proteins²⁰, which has led to contradictory reports about the protein composition of rafts. Thus, although DRM assays may provide information about the propensity of some molecules to associate with specialized membrane regions^{21,22}, they do not faithfully reflect the native molecular or biophysical composition and organization of rafts²³; therefore, the findings from these assays require confirmation by more robust and consistent methods such as those discussed below (for an excellent recent example, see REF. 22).

Biophysical tools. In parallel with studies of DRMs isolated from cells, artificial model membranes have been developed and used to study the liquid–liquid phase separation that is believed to underlie the physical principle behind lipid raft formation²⁴ (FIG. 2b). Across various experimental set-ups, membranes that consist 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, ordered phase enriched in saturated lipid species and cholesterol²⁵ (termed the liquid-ordered (L_o) phase), and a more fluid, disordered phase comprising mainly the unsaturated lipids^{26,27} (termed the liquid-disordered (L_d) phase). Owing to its tight molecular packing and enrichment of sterol and saturated lipids, the L_o phase is considered to be the model for lipid rafts. Biomimetic monolayers²⁸, supported lipid bilayers²⁹, nanoscopic bilayer vesicles³⁰ and giant unilamellar vesicles (GUVs)²⁶ have all been used to elucidate the molecular details of this phase separation^{31,32}; however, despite their important role in revealing the physical principles of L_o domain formation, a number of caveats and limitations prevent direct translation of findings from these model membranes to biological ones. First, most of these 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, inefficient and very rarely result in high protein/lipid ratios. This is in contrast to biological membranes, in which proteins are estimated to constitute up to 25% of the cross-sectional area of the membrane³⁵. Second, perhaps because of the scarcity (or even a complete lack)

Sphingolipid
 A class of lipids that comprise a long-chain sphingosine base coupled to a fatty acid chain and often a large polar head group.

Glycosylphosphatidylinositol (GPI)-anchored proteins
 Cell surface proteins that are post-translationally modified to carry a GPI moiety as an anchor to the membrane.

Cholera toxin

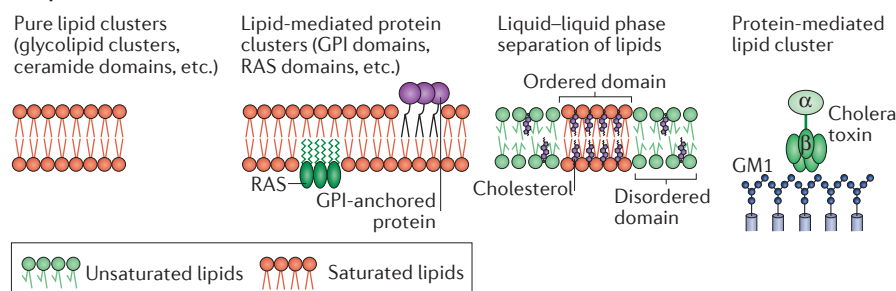
Proteinaceous toxin secreted by *Vibrio cholerae* that binds to glycolipids on the cell surface and is responsible for the symptoms of cholera infection.

of proteins, some features of domains established in synthetic membranes may not be representative of *in vivo* domains. 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 lipid³⁹ and protein⁴⁰ diversity of cellular membranes, although it is notable that they lack an assembled cortical actin cytoskeleton, phosphorylated lipids⁴¹ and strict lipid asymmetry between separate leaflets of the membrane bilayer (see REF. 42 for a detailed discussion of the advantages, caveats and applications of GPMVs). In these biological membranes, the disparity in molecular order between coexisting ordered and disordered domains is much smaller than in synthetic GUVs, for example. These differences in molecular order

between the two phases may account for the fact that the ordered phase of GUVs excludes almost 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} (BOX 1).

Analytical tools. In cells, rafts are believed to be nanoscopic domains (<200 nm)^{7,8}, and therefore they cannot be resolved by conventional optical microscopy, which has an approximately 250 nm resolution limit that is set by diffraction (see [Supplementary information S2](#) (box)). Although the colocalization of certain molecules with putative lipid raft markers (such as the multivalent cholera toxin) detected by confocal microscopy has been used as evidence of their association with rafts⁴⁴, in general the resolution of confocal microscopy is insufficient to directly assay raft domain structure and composition. To overcome this limitation, several optical tools have

a Lipid-associated membrane domains



b Tools to study membrane domains

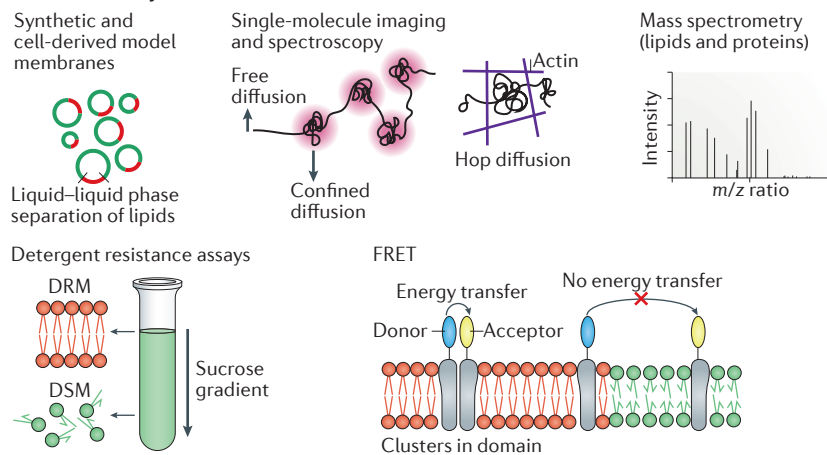
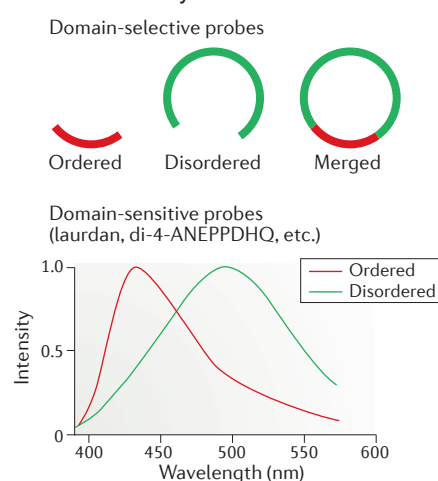
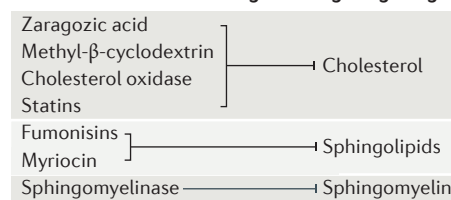


Figure 2 | Tools to study membrane domain organization, composition and function. **a** | In principle, membrane domains can be pure lipid clusters, but in most physiologically relevant cases they also contain proteins, which include clusters of, for example, glycosylphosphatidylinositol (GPI)-anchored proteins or RAS proteins. Domains can be purely lipid-driven entities, such as domains that are established by liquid-liquid phase separation in model membranes. They can also be induced by proteinaceous clustering agents such as cholera toxin, which binds to monosialotetrahexosylganglioside (GM1), or by antibodies that recognize surface receptors (not shown). **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, in which raft-like membrane regions partition

c Probes to study raft domains



d Functional studies using raft-targeting drugs



into detergent-resistant membrane (DRM) fractions, whereas non-raft components are fully solubilized and are found in detergent-soluble membrane (DSM) fractions; single-molecule microscopy to evaluate the diffusion of membrane molecules (the track of an individual molecule is depicted); and fluorescence spectroscopy methods such as Förster resonance energy transfer (FRET) and mass spectrometry. **c** | Various probes can be used to study raft domains. Domain-selective probes partition into one of the domains, whereas domain-sensitive probes partition to both domains and change their photophysical behaviour (for example, 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 disrupt rafts in cells and can shed light on the cellular functions of these domains.

Single-particle tracking

(SPT). A single-molecule technique in which the motion of individual molecules is tracked with high temporal resolution over relatively long timescales (seconds); these tracks can be used to determine the diffusion properties of a molecule.

Confined diffusion

A mode of diffusion in which the motion of the molecule is transiently arrested by molecular obstacles such as immobile clusters. It is also known as trapped diffusion.

Hop diffusion

A mode of diffusion in which molecules diffuse freely in the membrane except when they encounter a barrier (such as a structure (or structures) associated with actin filaments), the crossing of which hinders diffusion.

Interferometric scattering microscopy

(iSCAT). A microscopy technique to enhance contrast by using the interference from coherent light scattering in the focal plane and of the microscope cover glass.

Fluorescence correlation spectroscopy

(FCS). A single-molecule-based technique in which fluorescence intensity fluctuations from a microscopic observation spot are used to obtain information about molecular diffusion.

been developed recently^{45,46} and have been applied to investigate nanoscale structures and dynamics in cells. For example, super-resolution optical microscopy approaches such as photoactivated localization microscopy (PALM), stimulated emission depletion (STED) microscopy (Supplementary information S2 (box)) 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 it to models of heterogeneous organization of the membrane⁵¹. Such studies can reveal

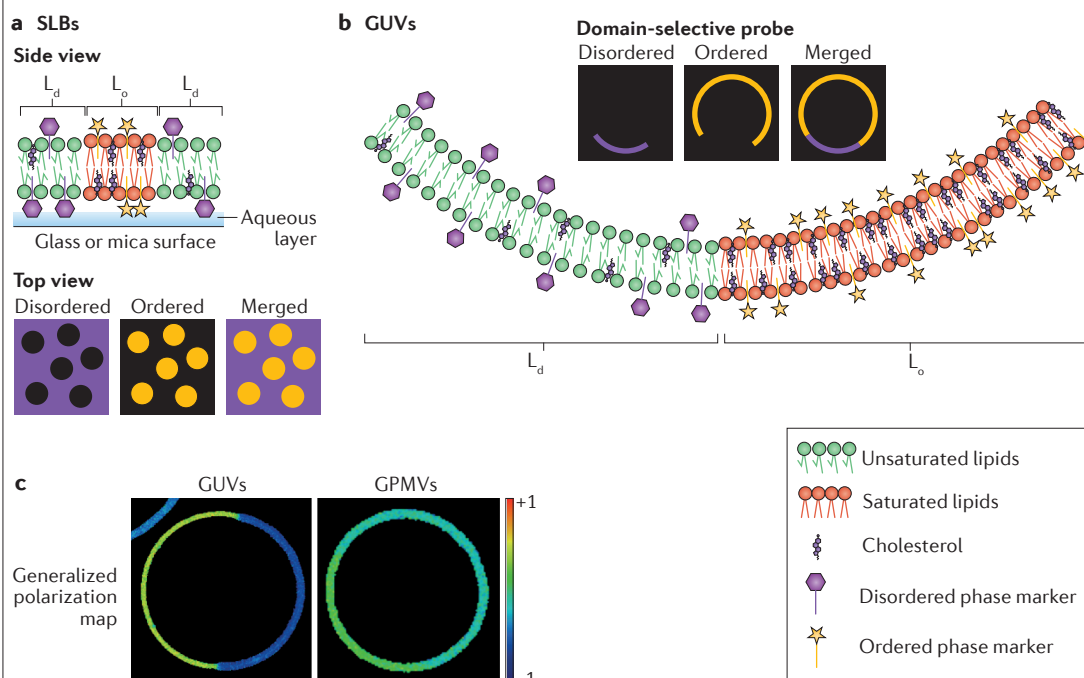
oligomerization⁵², transient arrest, domain incorporation, and/or confined diffusion and hop diffusion (also known as compartmentalized diffusion)⁵³ of tracked molecules (FIG. 2b). Recently, interferometric scattering microscopy (iSCAT) has further increased the sensitivity of SPT⁵⁴ and has shown great potential for assessing membrane heterogeneity. For example, iSCAT was used to show that lipids can transiently stall and become incorporated into sub-20 nm domains within model membranes^{55,56}. A technique complementary to SPT, fluorescence correlation spectroscopy (FCS), has been applied in combination with spot variation (svFCS⁵⁷) or a STED microscope (STED-FCS⁵⁸) to probe the

Box 1 | Model membranes for the study of the 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 (which are still liquid in nature)¹¹. One of these phases (the liquid-ordered (L_o) phase) is more viscous than the other (the liquid-disordered (L_d) phase) owing to the tighter packing and higher molecular order of its constituent lipids⁹¹. This L_o phase is believed to represent a potential physical model of lipid rafts in cellular membranes.

Supported lipid bilayers (SLBs; see the figure, part a) are planar bilayers formed on glass or mica surfaces²⁹. As these membranes are planar, they are highly amenable for microscopic imaging, either by light microscopy or by atomic force microscopy, which allows observations of the topology of nanodomains that are not resolvable by diffraction-limited optical microscopy (Supplementary information S2 (box)). The artefacts caused by the solid support in SLBs are avoided by the use of free-standing membranes such as giant unilamellar vesicles (GUVs) (see the figure, part b), which have been used frequently to investigate domain dynamics and morphologies¹⁸⁹. The limitation of synthetic model systems is their simple composition, which does not fully recapitulate the composition of the cell membrane.

Giant plasma membrane vesicles (GPMVs) are obtained from cell membranes³⁷. Similarly to GUVs, these form micrometre-scale lateral liquid domains (which confirms 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 (in GPMVs) of abundant transmembrane proteins^{37,38}, which are technically challenging to incorporate into SLBs and GUVs. The biophysical properties of GPMVs are somewhat distinct from those of artificial membranes^{37,92}. For example, the difference in packing density between L_o and L_d domains in GPMVs is much smaller than in GUVs (see the figure, part c; generalized polarization is a relative index of lipid packing, in which +1 represents maximally ordered membranes and –1 represents maximally disordered membranes), which may explain why transmembrane proteins can associate with the L_o phase in GPMVs⁴³ but not in GUVs¹⁹⁰. Despite these differences, most of the core features of the coexisting L_o and L_d domains in these model systems are fundamentally similar²⁴.



lateral diffusion of membrane components over various length scales. Particularly in STED-FCS, the size of the observation spot can be reduced to approximately 20–40 nm, which reveals underlying nanoscopic features of the plasma membrane^{58,59}. 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 membranes⁶² and live cells⁶³, 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 of these techniques and their caveats, see REF. 45.

Most of the aforementioned methodologies rely on fluorescent labels. This is a particular issue in the 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 almost the size of the lipid molecules. Thus, the native behaviour of lipids is often altered considerably by the reporter³⁶. To address this concern, a number of label-free techniques have been developed. Mass spectroscopy (FIG. 2b), for example, is one of the most accurate tools for probing the lipid and protein composition of membranes without the necessity of external labelling⁶⁵, and it has been used for label-free determination of membrane domain composition in model membranes and cell-derived membranes^{66–71}. iSCAT has also facilitated label-free observation of the dynamics of ordered domains in model membranes⁷². Raman spectroscopy is another label-free technique that has been applied successfully to monitor membrane domain composition⁷³. Likewise, small-angle neutron scattering has also been used to detect raft-like domains⁷⁴ and determine their size⁷⁵. Finally, electron microscopy has the necessary resolution to obtain 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 rafts⁷⁶. One potential caveat of these methods is that they usually require cell fixation and staining, which are notoriously problematic for visualizing lipid molecules. Therefore, fluorescence microscopy remains the preferred technique for direct live imaging of putative lipid raft components, and this necessitates the continued optimization of fluorescent labels for membrane components.

Probes selective for membrane domains. Non-perturbing, specific labelling of raft or non-raft domains in cells has been, and remains, one of the foremost challenges in the field. Several fluorescent markers — including cyanine dyes (for example, DiO, DiI and DiD)⁷⁷, polycyclic aromatic hydrocarbons (for example, naphthopyrene)⁷⁸ and fluorescently labelled lipids^{36,79} — have been used to distinguish between different membrane compartments (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 moiety³⁶. The fluorescent lipids that are free from artefacts linked to fluorescent labelling are intrinsically fluorescent cholesterol analogues such as dehydroergosterol⁸⁰ and cholestatrienol⁸¹; however, their poor photophysical characteristics compared with artificially tagged lipids have prevented their widespread 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 strategy that causes least disruption to lipid behaviour is to label the head group instead of the acyl chain and to add a hydrophilic linker to ensure that the fluorophores do not affect the head groups of the surrounding lipids⁸⁴.

In addition to lipid analogues that can reveal the general organization of the membrane into subdomains of variable composition, reporters that selectively bind to core raft components can potentially be used to visualize domains. These include cholesterol-binding agents such as filipin⁸⁵ and perfringolysin O⁸⁶, sphingolipid reporters such as ostreolysin A⁸⁷, lysenin⁸⁸ and pleurotolysin⁸⁹, as well as ganglioside lipid ligands such as cholera toxin⁹⁰. The major caveats for these probes are: first, their potential perturbation of native membrane organization by, for example, inducing the clustering of their binding partners, as is the case for cholera toxin; and, second, their reduced specificity in the cellular context, in which they can potentially exhibit off-target binding that thereby lowers their specificity for raft domains.

Probes sensitive to membrane environments. Coexisting lipid domains inherently have different physicochemical properties. A defining property of lipid rafts is their tight lipid packing, which is due to the condensing interactions between relatively saturated lipids and cholesterol⁹¹. Of note, there is no specific, unique type of molecular packing that is common to the plasma membrane and its domains in different cells and contexts⁹². The diversity of membrane compositions and physical properties across cell types, and within cell types during physiological events such as secretory granule release⁹² or cell cycle progression⁹³, implies that a wide range 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 bilayer⁹⁴ in combination with two-photon³² or conventional confocal⁹⁵ microscopy. The emission spectra of these probes shift depending on the polarity (that is, the aqueous content or hydration) of the environment⁹⁶ (FIG. 2c). This shift provides a ratiometric, concentration-independent quantification of the local environment, which, for membranes, is determined largely by lipid packing⁹⁷ (that is, more tightly packed membranes 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 lifetime⁹⁹ and energy transfer¹⁰⁰ properties of similar probes have been used to further investigate lipid packing in living membranes, which has expanded the scope and sensitivity of their potential applications. Enabling the efficient use of these probes in super-resolution microscopy will be an important future development.

Förster resonance energy transfer

(FRET). A fluorescence spectroscopy and imaging technique that is based on the distance-dependent transfer of the excited state energy of a fluorescent donor molecule to a fluorescent acceptor molecule; efficient and widely used to measure intermolecular distances in the range of 1–10 nm.

Amphiphilic properties

Displaying both hydrophilic and hydrophobic character, such as for lipids with hydrophobic acyl chains and hydrophilic head groups.

Raman spectroscopy

A spectroscopy technique whereby vibrational energy of the molecules is used as their 'fingerprint'.

Ganglioside lipid

A class of glycosphingolipids with sialic acid moieties on the head group.

Raft-targeting drugs. A common paradigm in the study of the physiological roles of lipid rafts has been the use of drugs or enzymes to impair the structure and function of these domains (FIG. 2d). As cholesterol is thought to be enriched in rafts, the most common raft-disrupting agent in use is methyl- β -cyclodextrin (M β CD), which selectively and efficiently extracts cholesterol from membranes¹⁰¹. However, it is important to consider that M β CD-mediated cholesterol removal has broad pleiotropic effects that extend beyond raft disruption. For example, it increases membrane permeability to ions and thereby disrupts membrane potential¹⁰², and it is potentially cytotoxic¹⁰³. Moreover, this reagent appears to preferentially deplete cholesterol from L_d (non-raft) domains in model membranes¹⁰⁴, which can produce unexpected and inconsistent^{21,67} effects on lipid packing in more complex membranes. Drugs that target cholesterol synthesis (statins¹⁰⁵ and zaragozic acid¹⁰⁶), or cholesterol-modifying enzymes (for example, cholesterol oxidase¹⁰⁷), have the potential to replace the use of M β CD to disrupt rafts, but their specificity and effectiveness remain to be demonstrated conclusively. Sphingolipids are another core component of rafts in cells, and a number of reagents can interfere with their synthesis (for example, fumonisin B1 (REF. 108) and myriocin¹⁰⁹) or stability (for example, sphingomyelinases¹¹⁰). However, these reagents suffer from potential off-target effects on processes such as general sphingolipid metabolism and the generation of ceramides, which can then alter membrane properties in other ways.

Molecular dynamics simulations. One of the biggest challenges remaining in our understanding of 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 experimental observations (for example, data about the structure and energetics of the system) into a simulation framework that ideally can reconstitute the natural behaviour *in silico* solely on the basis of physical interactions¹¹¹. An inherent advantage of such *in silico* approaches is that they simultaneously model a multitude of molecules at a high spatial (atomic level) and temporal (nanosecond–microsecond) resolution without relying on exogenous probes or labels. Thus, *in silico* molecular dynamics simulations can be regarded as a ‘computational microscope’ (REF. 112) that is capable of visualizing molecular behaviour with unprecedented precision. Currently, such computational microscopes have the opposite limitations to optical microscopes, in that they reveal only short processes (over microseconds) at a nanoscopic scale (thousands of molecules), as opposed to processes that occur over longer timescales and at lower resolution that are accessible by optical microscopy^{112,113}. To close the gap between computational and experimental approaches, methods such as coarse-grained simulations have been developed to extend the spatiotemporal scale of molecular dynamics

simulations without sacrificing the molecular details¹¹⁴. Such simulations have been used successfully 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 ultimately be verified by experiments. Unfortunately, in the case of membrane domains, the spatiotemporal gap between the simulated and experimental observables is still too large to allow direct comparisons¹¹¹. However, efforts to bridge this divide will ensure progress towards a molecular understanding of how complex membrane components self-organize into functional substructures.

Nature and composition of lipid rafts

Dissecting the physical properties — the lifetime, size, and coverage area — of lipid rafts in the cellular environment remains one of most vexing issues in the field. Computational models have confirmed the intuitive assumption that both the temporal and spatial compartmentalization of membrane molecules into domains is crucial for membrane function¹¹⁹. 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 range 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 L_d (non-raft) membrane punctuated by more-ordered (raft) domains with minimal coverage¹²¹. However, recent data indicate a much greater extent of ordered raft-like regions in membranes (which suggests that ordered membrane domains might in fact predominate and possibly cover the majority of the plasma membrane) with interspersed less-ordered (non-raft) domains^{47,59} (FIG. 3a). The relative area 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 of the lipid raft model, raft formation was based on preferential interactions between sphingolipids and cholesterol¹⁰. Consistent with this notion, sphingomyelin has been identified as a core component of DRMs² and ordered lipid phases¹²², owing in part to strong hydrogen bonding of lipids with cholesterol^{123,124} (FIG. 4a). However, the partitioning of cholesterol between more- and less-ordered domains is less clear: experimental³⁰ and computational¹²⁵ studies suggest that it is abundant in both ordered (raft-like) and disordered (non-raft) phases, although it is enriched in more-ordered domains. Ganglioside lipids were also found to interact with cholesterol, which results in the formation of cholesterol-rich domains in model membranes⁷⁰, and these lipids have been detected consistently in the ordered domains of model membranes⁹⁰. In addition, other lipids such as relatively saturated phospholipids have often been associated with raft-like environments, especially in model membranes.

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 between a hydrogen covalently bound to an electronegative atom (as in the -NH group of sphingolipids) and another electronegative atom (such as the oxygen in the -OH group of cholesterol).

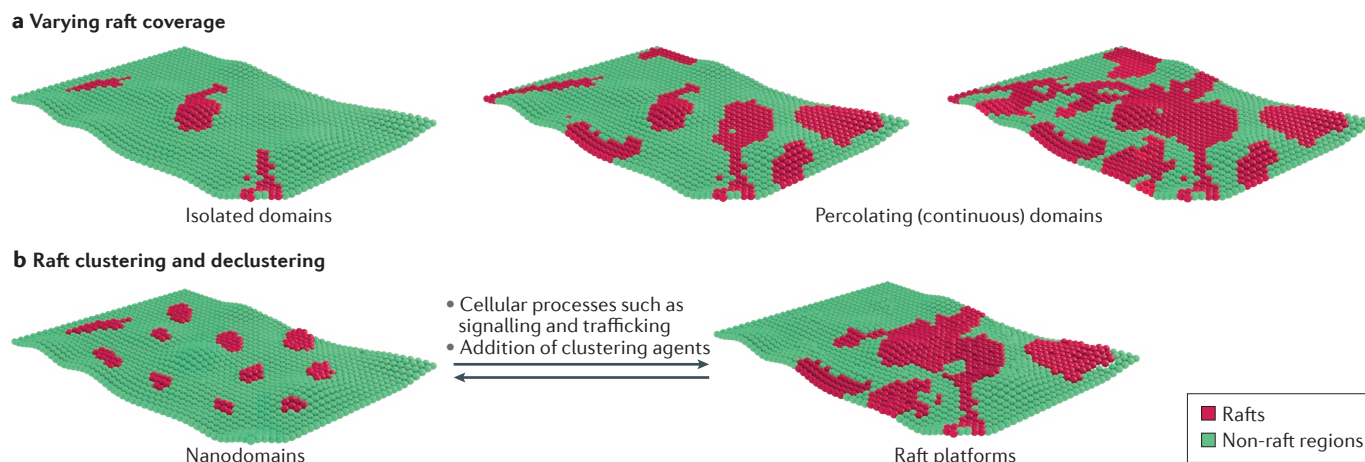


Figure 3 | Area coverage of membrane domains and domain size. a | Models of membranes with varying raft coverage. Total raft coverage in a given membrane may vary broadly, ranging from small isolated domains to percolating (continuous) raft phases of increasing size. The specific organizational state depends on a variety of factors, which include cell type, specific cellular conditions (for example, cell cycle phase) and/or the identity of the membrane (for example, 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 endocytosis and exocytosis, lipid metabolism, and so on. In addition, the binding of clustering agents (antibodies and toxins) to their receptors can promote the formation of large membrane domains.

Whereas the biophysical basis for the lipid composition of rafts can be explained by these simple principles, the basis for the selective incorporation of proteins into more-ordered (raft-like) domains largely remains a mystery. In general, proteins that interact 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, whereas branched or unsaturated anchors such as prenyl groups prefer disordered (non-raft) regions¹²⁶. In fact, GPI-anchored proteins were some of the first proteins to be identified in DRMs⁵ and later in the ordered domains of model membranes^{33,127}. Lateral GPI-anchored protein domains have been extensively characterized by single-molecule approaches^{128,129}. Although their relationship to membrane rafts remains unresolved¹³⁰, the 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 only protein species that associate with raft-like domains. In fact, in a recent experiment, 35% of all plasma membrane proteins were found in ordered domains in GPMVs⁴³. These ‘raftophilic’ proteins included GPI-anchored proteins and palmitoylated proteins, as expected (each constituting approximately one-third of the identified proteins)⁴³. However, the remaining one-third of raftophilic proteins contained neither a GPI nor a palmitoyl anchor, and the mechanism of association of many of these proteins to raft-like domains is currently unclear. Some proteins are known to become more raftophilic upon oligomerization, which may modulate their activity¹³¹. Recently, a database of putative raftophilic proteins identified in mass spectrometry studies of isolated DRMs has been established (RaftProt)¹³², although it is important to emphasize that these studies 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 raftophilic behaviour of transmembrane proteins are available¹³³. 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 — longer TMDs preferentially target the protein to the thicker, ordered domains¹³⁴.

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 membranes⁴²), the manifestation of these phenomena is macroscopic phase separation, which can be regulated by temperature¹³⁵, lipid composition^{21,26,67} or specific interactions that enhance the inherent connectivity of particular components and thus lead to enhanced clustering¹³⁶. However, cell membranes *in vivo* are not closed systems in chemical and thermodynamic equilibrium, and many potential modes of regulation contribute to the ultimate output of the inherent self-organizing capacity of biological lipids and their separation into distinct domains (FIG. 4).

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 sphingolipids¹²⁴ and possibly other lipids such as gangliosides⁶⁹ (FIG. 4a). However, an inherent limitation of studying the factors that regulate raft domain properties is the difficulty of measuring these properties *in situ*.

Epithelial–mesenchymal transition

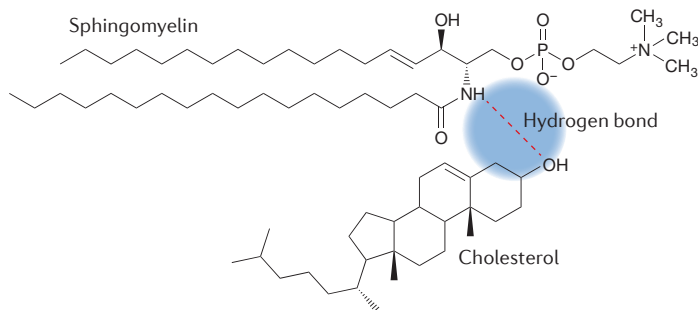
A developmental transcriptional programme that imparts mesenchymal characteristics (for example, motility) to epithelial cells.

To address this limitation, several recent studies^{93,137} have focused on factors that regulate the temperature at which macroscopic raft-like 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 that occurs near a compositional ‘critical point’ and involves large-scale fluctuations at temperatures close to the phase transition¹³⁵. Such ‘critical fluctuations’ are present in all systems that exhibit critical behaviour, which suggests that there are scaling laws that allow extrapolation of domain size and stability to living cells^{135,139}. 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 more-ordered domains in GPMVs is affected by dietary fatty acids. In particular, exogenously supplied polyunsaturated fatty acids such as the fish oil component docosahexaenoic acid are robustly

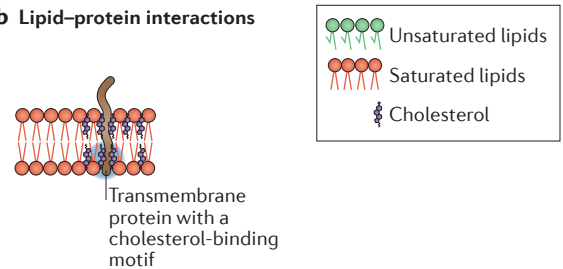
incorporated into cellular membranes, in which they induce extensive changes in lipid composition and biophysical properties, including increasing the stability of raft-like domains⁶⁷. A study relating these effects to cell behaviour showed that incorporation of docosahexaenoic acid into membranes, and the concomitant increase in the stability of raft-like domains, can repress the stem cell properties and motility of breast cancer cells by interfering with the plasma membrane remodelling that is necessary for the epithelial–mesenchymal transition¹⁴⁰.

Although variations in lipid composition are certainly key drivers of lipid membrane heterogeneity, protein–lipid interactions also have important roles in raft regulation. For example, some proteins, including the HIV glycoprotein gp41 (REF. 141), have cholesterol-binding motifs that regulate their membrane distribution (FIG. 4b). Other proteins specifically bind glycosphingolipids¹³⁸ or sphingomyelin¹⁴², which potentially mediates their recruitment to raft-like membrane domains. Furthermore, a variation on the role of palmitoylation in

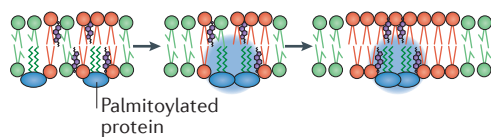
a Lipid–lipid interactions



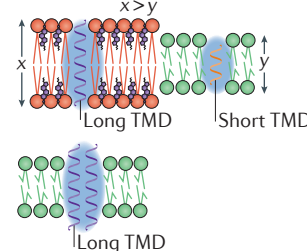
b Lipid–protein interactions



c Lipidated proteins as domain organizers



d Hydrophobic match or mismatch



e Transbilayer coupling

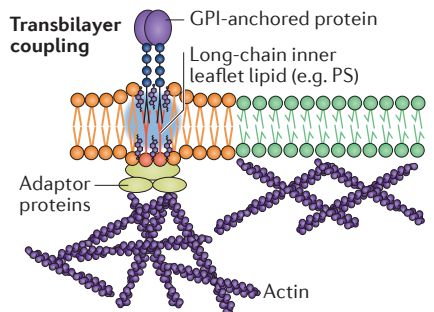


Figure 4 | Regulation of membrane domains. **a** | Lipid–lipid interactions — in particular, interactions between cholesterol and sphingolipids (but also between other relatively saturated lipids) — are the defining feature of lipid-driven ordered domain formation. The preferential interaction between sphingolipids and sterols is due to the saturation of sphingolipid hydrophobic tails, but also to 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, in which the relative paucity of water increases the stability of these bonds. **b** | Some proteins contain lipid-binding domains through which they interact with cholesterol or sphingolipids, and these lipid–protein interactions may determine the affinity of proteins for ordered lipid domains. **c** | Lipidated proteins, which are modified by the attachment of a saturated acyl chain (such as a palmitoyl moiety), are recruited to raft-like domains, but they 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 that differ in the length of their

transmembrane domains (TMDs) segregate into different lipid environments, which ensures that their hydrophobic TMDs are protected from exposure to the aqueous surroundings. For example, proteins with long TMDs associate with domains enriched in long-chain saturated lipids (top panel). 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, which leads to a local increase in protein concentration (bottom panel). **e** | Inner leaflet lipids containing long saturated acyl chains are immobilized by actin clusters, which are formed owing to the interactions between actin and membrane lipids (for example, phosphatidylserine (PS)) or adaptor proteins (such as those that possess PS-binding domains and actin-binding domains). This immobilization results in the engagement of long acyl chain-containing lipid-anchored proteins (such as glycosylphosphatidylinositol (GPI)-anchored proteins) located in the outer leaflet, in the presence of cholesterol. This results in the formation of locally ordered transbilayer domains, which are dynamic owing to the dynamics of actin clusters and may form even under conditions that do not favour liquid–liquid phase separation of lipids or other supporting interactions.

regulation of raft-like domains was recently proposed for postsynaptic density protein 95 (PSD95; also known as DLG4). Comprehensive lipidomic analysis of neuronal synapses suggested that raft-like domains are specifically recruited to synaptic sites, which was proposed to be mediated by the integration of palmitoylated PSD95 into the postsynaptic density protein scaffold¹⁴³. In this scenario, instead of raft-like domains recruiting palmitoylated proteins, it is the immobilized palmitoylated proteins that recruit saturated lipids and thus nucleate ordered domains at specific cellular sites¹⁴³ (FIG. 4c). This hypothetical mechanism and its applicability to other cellular contexts remain to be confirmed. However, the evidence that another palmitoylated protein, membrane palmitoylated protein 1 (MPP1; also known as p55), nucleates raft-like environments in erythroid cells^{144,145} suggests the existence of a more general mechanism whereby proteins dictate, or at least considerably influence, the localization and stability of organized domains.

Hydrophobic match or mismatch. Mammalian membrane lipids can contain hydrocarbon acyl chains that are 12–24 carbons in length; thus, there is the potential to yield 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, which can potentially introduce 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 the coexisting domains, such that large mismatches give rise to large domains, and vice versa¹⁴⁶. Similarly, the TMDs of nearly all eukaryotic integral membrane proteins consist of α -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 environments¹⁴⁷ (FIG. 4d). In the case of a significant length mismatch between TMDs and the surrounding lipids, lateral protein-rich aggregates can potentially be induced¹⁴⁸. However, the role of hydrophobic mismatch in membrane domain dynamics in the plasma membrane of living cells needs to be demonstrated unambiguously.

Cortical actin cytoskeleton. The cortical actin cytoskeleton is undoubtedly one of the most important factors that influence membrane organization¹⁴⁹ and mechanics¹⁵⁰. The actin scaffold has been shown to determine molecular diffusion dynamics (for example, trapped and hop diffusion) and supramolecular arrangements in the membrane^{129,151–154}. In *in vitro* cholesterol-containing membrane systems that are capable of large-scale phase separation, actin can directly stabilize or abrogate this separation depending on the nature of the lipid species that are coupled to actin^{153,155–157}. If actin filaments are coupled to, for example, saturated acyl chain-containing lipid species, they tend to stabilize L_o domains, but prevent large-scale phase separation¹⁵³. In a living cell, it is likely that the dynamics of actin

filaments will influence the organization of the membrane components that are 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 emerged from studies of the actomyosin-dependent clustering behaviour of GPI-anchored proteins in the outer leaflet of the plasma membrane. It was proposed that such clustering is the result of dynamic self-organization of actomyosin into nanoscopic contractile assemblies termed asters^{158,159}. These assemblies bind to and transiently immobilize the charged lipid phosphatidylserine in the inner membrane leaflet, possibly via specific interactions between actin and membrane adaptor proteins. This lipid species contains long saturated acyl chains that engage in cholesterol-mediated transbilayer interactions with long acyl chain-containing GPI-anchored proteins located in the outer leaflet, which results in the formation of local raft-like domains¹⁴ (FIG. 4e). Thus, an actin-driven clustering mechanism may be responsible for the formation of ordered domains in membranes of living cells, even under conditions (for example, temperature and/or lipid composition) that are not normally conducive for phase separation. A proof-of-principle for this mechanism has been demonstrated recently *in vitro*¹⁶⁰ by showing that dynamically remodelling actomyosin networks can organize and segregate associated lipids in a synthetic supported membrane bilayer system. In addition, recent live-cell work has shown that self-organizing cortical actin patterns such as asters generate more-ordered membrane environments in the immediate plasma membrane areas¹⁵⁹. As an addition 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 that are formed by several lipid or protein species. These include GPI-anchored proteins, glycolipids in the outer leaflet and RAS proteins in the inner leaflet of live-cell membranes¹⁶¹. The molecular machinery that generates these actin-based nanoclusters has not been identified, and further work is necessary to understand how these small actin-based nanoclusters may give rise to larger-scale ordered membrane domains with functional significance¹⁶¹.

Physiological functions of rafts

The most apparent function of raft-like domains (or heterogeneity in membrane lipid order in general) is to segregate specific elements in order to regulate their interactions with other membrane components and hence their activity. In addition, interactions with raftophilic lipids (cholesterol or glycosphingolipids), or with the distinct biophysical environment of rafts, may change the conformation of a raft-resident protein and thus its activity^{162,163} (FIG. 5a). These general modes of regulation may be broadly employed in cellular physiology, and a few examples are described here. However, it should be emphasized that the direct mechanistic effects of lipid rafts on cell function and dysfunction are unclear owing to the inherent difficulties in defining raft composition and properties and in achieving specificity when perturbing their function.

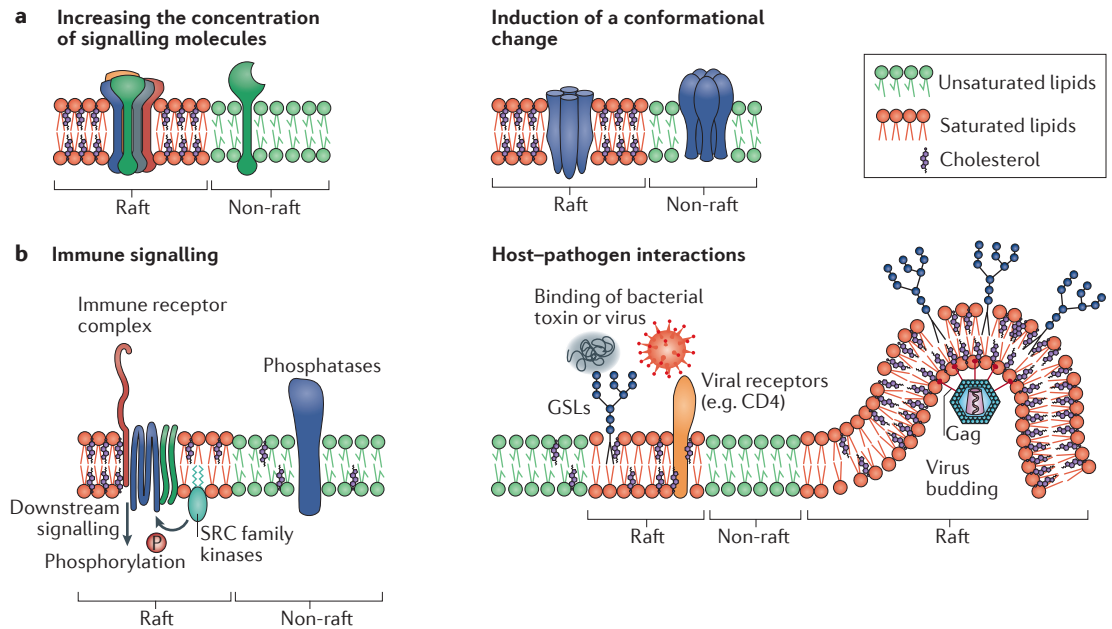


Figure 5 | Cellular functions of lipid rafts. **a** | Mechanisms by which membrane domains can potentially regulate the activity of their associated components. Raft-like domains can facilitate an increase in the concentration of certain molecules, which results in the establishment of functional catalytic platforms. For example, enzymes and substrates can be brought together to increase their encounter probability and thereby trigger reactions (for example, signal transduction). A related possibility is that distinct physicochemical environments provided by lipid rafts directly affect protein conformation, and thereby regulate protein activity. **b** | Examples of physiological functions of membrane domains. Kinases of the SRC family are enriched in raft-like domains owing to their palmitoylation, whereas transmembrane phosphatases are generally excluded from them. This segregation has been found to be important for immune signalling, in which raft-associated SRC kinases are involved in regulating the phosphorylation state, and hence the signal transduction activity, of various immune receptors that include the T cell receptor and the high-affinity immunoglobulin E receptor (FcεRI). Many pathogens and their products (such as bacterial toxins) selectively bind to membrane rafts owing to the presence of their specific receptors, such as glycosphingolipids (GSLs; for cholera toxin) or CD4 (for HIV), in these domains, and thereby gain access to 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 the Gag protein of HIV are believed to be sensitive to membrane fluidity and to associate with cholesterol-enriched domains.

Immune signalling. Compartmentalization of cellular signalling in membrane domains may be used to concentrate positive regulatory components (such as kinases¹⁶⁴), together with excluding negative regulatory elements (such as phosphatases¹⁶⁵) (FIG. 5b). Immunoglobulin E (IgE)-mediated signalling was the first signalling pathway that was shown to be associated with lipid rafts¹⁶⁶. Since then, several studies have implicated these domains in various innate and adaptive immune responses¹⁶⁷. In these contexts, the key immune receptors, including the high-affinity IgE receptor (FcεRI), the T cell receptor¹⁶⁸ and the B cell receptor⁴⁴, were found in DSMs in resting or immature cells, but these shifted to DRMs following receptor activation, which suggests that the translocation to membrane rafts is associated with active signalling through these receptors^{169–171}. This notion is supported by the co-enrichment in DRMs of the proximal signal transduction machinery that lies downstream of the immune receptors, which includes lymphocyte cell-specific protein tyrosine kinase (LCK) and a proto-oncoprotein, the tyrosine kinase FYN¹⁶⁴, as well as the signalling adaptor protein linker for activation of T cells (LAT)⁴³. Furthermore, several other immune-associated proteins are GPI-anchored (suggesting that they are

preferentially targeted to rafts) and have been found in DRMs¹⁷²; these include CD14, the receptor for bacterial lipopolysaccharides, and THY1 (also known as CD90), which is crucial for T cell activation¹⁷³.

Host–pathogen interactions. Interest in lipid rafts as modulators of host–pathogen interactions has been boosted by the recent discovery of a high level of saturated lipids (in particular, sphingolipids) and cholesterol in the viral envelope (of HIV¹⁷⁴, for example) and by finding ordered membrane domains in pathogenic microorganisms¹⁷⁵. There is now substantial evidence that viruses and bacterial products such as toxins bind preferentially to detergent-resistant highly ordered plasma membrane regions to penetrate the cell. This could be due to the enrichment of their receptors in rafts, as is the case for glycolipids¹⁷⁶ (which function as receptors for cholera toxin⁹⁰, for example) or virus receptors¹⁷⁷. Furthermore, binding of HIV Gag protein (which is necessary for virus budding and release from host cells) has been shown to occur preferentially in membrane domains with high cholesterol content¹⁷⁸, which suggests that rafts might be the preferred sites for virus budding¹⁷⁸ (FIG. 5b).

Viral envelope

The lipid membrane that covers the viral capsid and is derived from the plasma membrane of the host cell.

Cancer. A large number of proteins that are associated with malignancies have been found in DRMs: these include mucin 1 (MUC1), the overexpression of which leads to several cancer forms¹⁷⁹; urokinase plasminogen activator surface receptor (UPAR), which plays a part in tumour invasion, migration and angiogenesis in breast cancer¹⁸⁰; and RAS proteins, which show raft-dependent oncogenic activity in breast cancer¹⁸¹. The localization of oncogenic proteins to raft-like domains, together with the fact that mitogenic signalling is initiated from various cell surface receptors, suggests that rafts are potentially involved in cancer development and progression. Consistent with this idea, drugs that modulate membrane organization — including the raft-associated alkyl-phospholipids edelfosine, miltefosine and perifosine, which disrupt the raft localization of proton pumps¹⁸² — have been shown to exhibit anticancer activity¹⁸³.

Cardiovascular diseases. Atherosclerosis is a leading cause of cardiovascular disease, and it develops as a result of the uptake by macrophages of cholesterol that accumulates in the artery walls as oxidized low-density lipoprotein (oxLDL). This uptake causes a transformation of macrophages into foam cells, which accumulate in necrotic lesions in the arterial wall and can thereby clog blood vessels and lead to strokes, heart attacks and peripheral vascular diseases¹⁸⁴. Of note, this transition of macrophages into foam cells appears to be raft-dependent, as oxLDL receptors localize to raft-like domains following stimulation by oxLDL¹⁸⁵. In addition, caveolae, the 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 pits¹⁸⁶.

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, owing to the potential of membrane domains to regulate 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 a disproportionate share of both popularity and controversy. At its peak, hundreds of papers on membrane rafts were published every year; at its nadir, many refrained from using the word ‘raft’ to avoid the inevitable semantic quagmire that it conjured. The major predicament in membrane raft research has been, and continues to be, a lack of direct visualization of these domains in unperturbed living cells. However, the remarkable advances in microscopy technology over the past decade now allow direct observation of processes occurring with the spatial (nanometres) and temporal (milliseconds) regimes that are believed to be relevant for raft domains in living cells. These advances, together with improvements in *in silico* membrane modelling, suggest that direct detection of

these elusive domains in cell membranes, although still challenging, may be within reach¹⁸⁷. 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 coexisting L_o and L_d domains. Moreover, domains remarkably similar to these ordered and disordered phase-separated domains in GPMVs have been visualized directly in the subcellular organelles of budding yeast¹⁸⁸, which suggests that an investigation of internal membranes may also be a fruitful direction.

Much of the controversy about the properties of lipid rafts (such as size, lifetime and abundance) stems from attempts to make general statements about the organization of a number of different membrane components (including glycolipids, sphingomyelin, cholesterol, GPI-anchored proteins and minimal palmitoylated motifs) by using a common raft paradigm. First, it is important to note that a very specific set of physical and compositional features should not be expected for lipid rafts. Living membranes are extremely complex and varied, and thus their organization will be inherently context-dependent, and they may potentially contain 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. Second, 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 affect domain organization and dynamics. Furthermore, these bioactivities may be affected by the specific conditions of an experiment; for example, the cell type or the cell cycle phase. Altogether, to obtain reproducible results that pertain to 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 domains⁸⁴, and thus allow careful associations to be made between ordered domain affinity and other experimental read-outs²². The application of label-free methods for the detection of domains is another approach that would minimize experimental differences⁷².

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 timescales and large areas. Such data could be complemented by detailed lipidomic and proteomic analysis of nanometric regions of the cell surface⁷⁰ as well as *in silico* membrane modelling. The next step will be to integrate these observations into the framework of cellular dynamics to link membrane heterogeneity to cell biological processes. To achieve this, it will be necessary to simultaneously 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 the parallel application and development of a variety of techniques, which suggests that this field has an exciting future of interdisciplinary investigation.

Caveolae

Specialized invaginations in the plasma membrane that are enriched in caveolin, sphingolipids and cholesterol.

1. Singer, S. J. & Nicolson, G. L. The fluid mosaic model of the structure of cell membranes. *Science* **175**, 720–731 (1972).
2. Yu, J., Fischman, D. A. & Steck, T. L. Selective solubilization of proteins and phospholipids from red blood cell membranes by nonionic detergents. *J. Supramol. Struct.* **1**, 233–248 (1973).
3. Bagatolli, L. & Mouritsen, O. Is the fluid mosaic (and the accompanying raft hypothesis) a suitable model to describe fundamental features of biological membranes? What may be missing? *Front. Plant Sci.* **4**, 457 (2013).
4. Ahmed, S. N., Brown, D. A. & London, E. 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**, 10944–10953 (1997).
5. Brown, D. A. & Rose, J. K. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* **68**, 533–544 (1992).
6. Vanmeer, G., Stelzer, E. H. K., Wijnnaendts-van-Resandt, R. W. & Simons, K. Sorting of sphingolipids in epithelial (Madin–Darby canine kidney) cells. *J. Cell Biol.* **105**, 1623–1635 (1987).
7. Varma, R. & Mayor, S. GPI-anchored proteins are organized in submicron domains at the cell surface. *Nature* **394**, 798–801 (1998).
First evidence for nanoscopic domains of GPI-anchored proteins in living cells.
8. Pralle, A., Keller, P., Florin, E. L., Simons, K. & Horber, J. K. Sphingolipid–cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells. *J. Cell Biol.* **148**, 997–1008 (2000).
9. Friedrichson, T. & Kurzchalia, T. V. Microdomains of GPI-anchored proteins in living cells revealed by crosslinking. *Nature* **394**, 802–805 (1998).
10. Simons, K. & Ikonen, E. Functional rafts in cell membranes. *Nature* **387**, 569–572 (1997).
First work to highlight the lipid raft hypothesis and its potential functional relevance.
11. Simons, K. & Vaz, W. L. Model systems, lipid rafts, and cell membranes. *Annu. Rev. Biophys. Biomol. Struct.* **33**, 269–295 (2004).
12. Pike, L. J. Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. *J. Lipid Res.* **47**, 1597–1598 (2006).
An editorial article that summarizes the 2006 Keystone symposium on lipid rafts.
13. Kiessling, V., Wan, C. & Tamm, L. K. Domain coupling in asymmetric lipid bilayers. *Biochim. Biophys. Acta* **1788**, 64–71 (2009).
14. Raghupathy, R. *et al.* Transbilayer lipid interactions mediate nanoclustering of lipid-anchored proteins. *Cell* **161**, 581–594 (2015).
Explains the transbilayer coupling between dynamic actin organization on the intracellular side of the plasma membrane and lipid domains in the extracellular leaflet via raft-based interactions of anionic lipids.
15. Lingwood, D. & Simons, K. Lipid rafts as a membrane-organizing principle. *Science* **327**, 46–50 (2010).
16. Klotzsch, E. & Schuetz, G. J. A critical survey of methods to detect plasma membrane rafts. *Phil. Trans. R. Soc. B* **368**, 20120033 (2013).
17. Hanada, K., Nishijima, M., Akamatsu, Y. & Pagano, R. E. Both sphingolipids and cholesterol participate in the detergent insolubility of alkaline phosphatase, a glycosylphosphatidylinositol-anchored protein, in mammalian membranes. *J. Biol. Chem.* **270**, 6254–6260 (1995).
18. Schroeder, R., London, E. & Brown, D. Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. *Proc. Natl Acad. Sci. USA* **91**, 12130–12134 (1994).
19. Schuck, S., Honsho, M., Ekroos, K., Shevchenko, A. & Simons, K. Resistance of cell membranes to different detergents. *Proc. Natl Acad. Sci. USA* **100**, 5795–5800 (2003).
20. Mayor, S. & Maxfield, F. R. Insolubility and redistribution of GPI-anchored proteins at the cell surface after detergent treatment. *Mol. Biol. Cell* **6**, 929–944 (1995).
21. Levental, I. *et al.* Cholesterol-dependent phase separation in cell-derived giant plasma-membrane vesicles. *Biochem. J.* **424**, 163–167 (2009).
22. Komura, N. *et al.* Raft-based interactions of gangliosides with a GPI-anchored receptor. *Nat. Chem. Biol.* **12**, 402–410 (2016).
Combines DRM assays, SPT and GPMVs to confirm domain-mediated interactions between GPI-anchored proteins and gangliosides.
23. Lichtenberg, D., Goni, F. M. & Heerklotz, H. Detergent-resistant membranes should not be identified with membrane rafts. *Trends Biochem. Sci.* **30**, 430–436 (2005).
24. Sezgin, E. & Schwille, P. Model membrane platforms to study protein–membrane interactions. *Mol. Membr. Biol.* **29**, 144–154 (2012).
25. Ipsen, J. H., Karlstrom, G., Mouritsen, O. G., Wennerstrom, H. & Zuckermann, M. J. Phase equilibria in the phosphatidylcholine-cholesterol system. *Biochim. Biophys. Acta* **905**, 162–172 (1987).
26. Veatch, S. L. & Keller, S. L. Separation of liquid phases in giant vesicles of ternary mixtures of phospholipids and cholesterol. *Biophys. J.* **85**, 3074–3083 (2003).
27. Kaiser, H. J. *et al.* Order of lipid phases in model and plasma membranes. *Proc. Natl Acad. Sci. USA* **106**, 16645–16650 (2009).
28. McConnell, H. M., Tamm, L. K. & Weis, R. M. Periodic structures in lipid monolayer phase transitions. *Proc. Natl Acad. Sci. USA* **81**, 3249–3253 (1984).
29. Tamm, L. K. & McConnell, H. M. Supported phospholipid-bilayers. *Biophys. J.* **47**, 105–113 (1985).
30. Feigenson, G. W. & Buboltz, J. T. Ternary phase diagram of dipalmitoyl-PC/dilauroyl-PC/cholesterol: nanoscopic domain formation driven by cholesterol. *Biophys. J.* **80**, 2775–2788 (2001).
31. Koriach, J., Schwille, P., Webb, W. W. & Feigenson, G. W. Characterization of lipid bilayer phases by confocal microscopy and fluorescence correlation spectroscopy. *Proc. Natl Acad. Sci. USA* **96**, 8461–8466 (1999).
32. Bagatolli, L. A., Sanchez, S. A., Hazlett, T. & Gratton, E. Giant vesicles, Laurdan, and two-photon fluorescence microscopy: evidence of lipid lateral separation in bilayers. *Methods Enzymol.* **360**, 481–500 (2003).
33. Kahya, N., Brown, D. A. & Schwille, P. Raft partitioning and dynamic behavior of human placental alkaline phosphatase in giant unilamellar vesicles. *Biochemistry* **44**, 7479–7489 (2005).
34. Stachowiak, J. C. *et al.* Unilamellar vesicle formation and encapsulation by microfluidic jetting. *Proc. Natl Acad. Sci. USA* **105**, 4697–4702 (2008).
35. Dupuy, A. D. & Engelman, D. M. Protein area occupancy at the center of the red blood cell membrane. *Proc. Natl Acad. Sci. USA* **105**, 2848–2852 (2008).
36. Sezgin, E. *et al.* Partitioning, diffusion, and ligand binding of raft lipid analogs in model and cellular plasma membranes. *Biochim. Biophys. Acta* **1818**, 1777–1784 (2012).
37. Sezgin, E. *et al.* Elucidating membrane structure and protein behavior using giant plasma membrane vesicles. *Nat. Protoc.* **7**, 1042–1051 (2012).
38. Baumgart, T. *et al.* Large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles. *Proc. Natl Acad. Sci. USA* **104**, 3165–3170 (2007).
39. Fridriksson, E. K. *et al.* Quantitative analysis of phospholipids in functionally important membrane domains from RBL-2H3 mast cells using tandem high-resolution mass spectrometry. *Biochemistry* **38**, 8056–8063 (1999).
40. Scott, R. E., Perkins, R. G., Zschunke, M. A., Hoerl, B. J. & Maercklein, P. B. Plasma membrane vesiculation in 3T3 and SV3T3 cells. I. Morphological and biochemical characterization. *J. Cell Sci.* **35**, 229–243 (1979).
41. Keller, H., Lorizate, M. & Schwille, P. PI(4,5)P₂ degradation promotes the formation of cytoskeleton-free model membrane systems. *Chemphyschem* **10**, 2805–2812 (2009).
42. Levental, K. R. & Levental, I. Giant plasma membrane vesicles: models for understanding membrane organization. *Curr. Top. Membr.* **75**, 25–57 (2015).
43. Levental, I., Lingwood, D., Grzybek, M., Coskun, U. & Simons, K. Palmitoylation regulates raft affinity for the majority of integral raft proteins. *Proc. Natl Acad. Sci. USA* **107**, 22050–22054 (2010).
Quantitative investigation of the role of palmitoylation in protein partitioning to raft domains in GPMVs.
44. Gupta, N. & DeFranco, A. L. Visualizing lipid raft dynamics and early signaling events during antigen receptor-mediated B-lymphocyte activation. *Mol. Biol. Cell* **14**, 432–444 (2003).
45. Sezgin, E. & Schwille, P. Fluorescence techniques to study lipid dynamics. *Cold Spring Harb. Perspect. Biol.* **3**, a009803 (2011).
46. Eggeling, C. Super-resolution optical microscopy of lipid plasma membrane dynamics. *Essays Biochem.* **57**, 69–80 (2015).
47. Owen, D. M., Williamson, D. J., Magenau, A. & Gaus, K. Sub-resolution lipid domains exist in the plasma membrane and regulate protein diffusion and distribution. *Nat. Commun.* **3**, 1256 (2012).
48. Sengupta, P. *et al.* Probing protein heterogeneity in the plasma membrane using PALM and pair correlation analysis. *Nat. Methods* **8**, 969–975 (2011).
49. van Zanten, T. S. *et al.* Hotspots of GPI-anchored proteins and integrin nanoclusters function as nucleation sites for cell adhesion. *Proc. Natl Acad. Sci. USA* **106**, 18557–18562 (2009).
50. Saka, S. K. *et al.* Multi-protein assemblies underlie the mesoscale organization of the plasma membrane. *Nat. Commun.* **5**, 4509 (2014).
51. Suzuki, K. G. Single-molecule imaging of signal transduction via GPI-anchored receptors. *Methods Mol. Biol.* **1376**, 229–238 (2016).
52. Moertelmaier, M., Brameshuber, M., Linimeier, M., Schütz, G. J. & Stockinger, H. Thinning out clusters while conserving stoichiometry of labeling. *Appl. Phys. Lett.* **87**, 263903 (2005).
53. Kusumi, A. *et al.* Paradigm shift of the plasma membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-speed single-molecule tracking of membrane molecules. *Annu. Rev. Biophys. Biomol. Struct.* **34**, 351–378 (2005).
54. Ortega-Arroyo, J. & Kukura, P. Interferometric scattering microscopy (iSCAT): new frontiers in ultrafast and ultrasensitive optical microscopy. *Phys. Chem. Chem. Phys.* **14**, 15625–15636 (2012).
55. Wu, H. M., Lin, Y. H., Yen, T. C. & Hsieh, C. L. Nanoscopic substructures of raft-mimetic liquid-ordered membrane domains revealed by high-speed single-particle tracking. *Sci. Rep.* **6**, 20542 (2016).
56. Spillane, K. M. *et al.* High-speed single-particle tracking of GM1 in model membranes reveals anomalous diffusion due to interleaflet coupling and molecular pinning. *Nano Lett.* **14**, 5390–5397 (2014).
57. Wawrzyniec, L., Rigneault, H., Marguet, D. & Lenne, P. F. Fluorescence correlation spectroscopy diffusion laws to probe the submicron cell membrane organization. *Biophys. J.* **89**, 4029–4042 (2005).
58. Eggeling, C. *et al.* Direct observation of the nanoscale dynamics of membrane lipids in a living cell. *Nature* **457**, 1159–1162 (2009).
Reports differential diffusion behaviour of phospholipids and sphingolipids in the plasma membrane of live cells using super-resolution STED microscopy combined with FCS.
59. Honigsmann, A. *et al.* Scanning STED-FCS reveals spatiotemporal heterogeneity of lipid interaction in the plasma membrane of living cells. *Nat. Commun.* **5**, 5412–5412 (2014).
60. Saha, S., Raghupathy, R. & Mayor, S. Homo-FRET imaging highlights the nanoscale organization of cell surface molecules. *Methods Mol. Biol.* **1251**, 151–173 (2015).
61. Sharma, P. *et al.* Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. *Cell* **116**, 577–589 (2004).
62. Pathak, P. & London, E. The effect of membrane lipid composition on the formation of lipid ultrananodomains. *Biophys. J.* **109**, 1630–1638 (2015).
63. Engel, S. *et al.* FLIM-FRET and FRAP reveal association of influenza virus haemagglutinin with membrane rafts. *Biochem. J.* **425**, 567–573 (2010).
64. Heberle, F. A., Wu, J., Goh, S. L., Petruziello, R. S. & Feigenson, G. W. Comparison of three ternary lipid bilayer mixtures: FRET and ESR reveal nanodomains. *Biophys. J.* **99**, 3309–3318 (2010).
65. Barrera, N. P., Zhou, M. & Robinson, C. V. The role of lipids in defining membrane protein interactions: insights from mass spectrometry. *Trends Cell Biol.* **23**, 1–8 (2013).
66. Ogiso, H., Taniguchi, M. & Okazaki, T. Analysis of lipid-composition changes in plasma membrane microdomains. *J. Lipid Res.* **56**, 1594–1605 (2015).

67. Levental, K. R. *et al.* Polyunsaturated lipids regulate membrane domain stability by tuning membrane order. *Biophys. J.* **110**, 1800–1810 (2016).
68. Gerl, M. J. *et al.* Quantitative analysis of the lipidomes of the influenza virus envelope and MDCK cell apical membrane. *J. Cell Biol.* **196**, 213–221 (2012). **Using lipidomics, this study shows that the influenza virus envelope is enriched in sphingolipids and cholesterol compared with the apical plasma membrane from which the virus buds, which reveals raft lipid selectivity during virus budding.**
69. Lozano, M. M., Hovis, J. S., Moss, F. R. III & Boxer, S. G. Dynamic reorganization and correlation among lipid raft components. *J. Am. Chem. Soc.* **138**, 9996–10001 (2016). **Shows that cholesterol and sphingomyelin reorganize with ganglioside, demonstrating that there is an attractive interaction between these raft constituents.**
70. Lozano, M. M. *et al.* Colocalization of the ganglioside G_{M1} and cholesterol detected by secondary ion mass spectrometry. *J. Am. Chem. Soc.* **135**, 5620–5630 (2013).
71. Frisz, J. F. *et al.* Sphingolipid domains in the plasma membranes of fibroblasts are not enriched with cholesterol. *J. Biol. Chem.* **288**, 16855–16861 (2013).
72. de Wit, G., Danial, J. S., Kukura, P. & Wallace, M. I. Dynamic label-free imaging of lipid nanodomains. *Proc. Natl Acad. Sci. USA* **112**, 12299–12303 (2015). **Presents a label-free technique to study lipid domains in model membranes.**
73. Ando, J. *et al.* Sphingomyelin distribution in lipid rafts of artificial monolayer membranes visualized by Raman microscopy. *Proc. Natl Acad. Sci. USA* **112**, 4558–4563 (2015).
74. Pencer, J., Mills, T. T., Kucerka, N., Nieh, M. P. & Katsaras, J. Small-angle neutron scattering to detect rafts and lipid domains. *Methods Mol. Biol.* **398**, 231–244 (2007).
75. Heberle, F. A. *et al.* Hybrid and nonhybrid lipids exert common effects on membrane raft size and morphology. *J. Am. Chem. Soc.* **135**, 14932–14935 (2013).
76. Prior, I. A., Muncke, C., Parton, R. G. & Hancock, J. F. Direct visualization of Ras proteins in spatially distinct cell surface microdomains. *J. Cell Biol.* **160**, 165–170 (2003).
77. Baumgart, T., Hunt, G., Farkas, E. R., Webb, W. W. & Feigensohn, G. W. Fluorescence probe partitioning between L_β/L_α phases in lipid membranes. *Biochim. Biophys. Acta* **1768**, 2182–2194 (2007).
78. Klymchenko, A. S. & Kreder, R. Fluorescent probes for lipid rafts: from model membranes to living cells. *Chem. Biol.* **21**, 97–113 (2014).
79. Juhasz, J., Davis, J. H. & Sharom, F. J. Fluorescent probe partitioning in giant unilamellar vesicles of 'lipid raft' mixtures. *Biochem. J.* **430**, 415–423 (2010).
80. Pourmousa, M. *et al.* Dehydroergosterol as an analogue for cholesterol: why it mimics cholesterol so well — or does it? *J. Phys. Chem. B* **118**, 7345–7357 (2014).
81. Robalo, J. R., Martins do Canto, A. M. T., Palace Carvalho, A. J., Prates Ramalho, J. P. & Loura, L. M. S. Behavior of fluorescent cholesterol analogues dehydroergosterol and cholestatrienol in lipid bilayers: a molecular dynamics study. *J. Phys. Chem. B* **117**, 5806–5819 (2013).
82. Sezgin, E. *et al.* A comparative study on fluorescent cholesterol analogs as versatile cellular reporters. *J. Lipid Res.* **57**, 299–309 (2016).
83. Crane, J. M. & Tamm, L. K. Role of cholesterol in the formation and nature of lipid rafts in planar and spherical model membranes. *Biophys. J.* **86**, 2965–2979 (2004).
84. Momin, N. *et al.* Designing lipids for selective partitioning into liquid ordered membrane domains. *Soft Matter* **11**, 3241–3250 (2015).
85. Lopes, S. C., Goormaghtigh, E., Cabral, B. J. & Castanho, M. A. Filipin orientation revealed by linear dichroism. Implication for a model of action. *J. Am. Chem. Soc.* **126**, 5396–5402 (2004).
86. Palmer, M. The family of thiol-activated, cholesterol-binding cytotoxins. *Toxicol.* **39**, 1681–1689 (2001).
87. Skocaj, M. *et al.* Tracking cholesterol/sphingomyelin-rich membrane domains with the osteolytic A-mCherry protein. *PLoS ONE* **9**, e92783 (2014).
88. Yamaji, A. *et al.* Lysoenin, a novel sphingomyelin-specific binding protein. *J. Biol. Chem.* **273**, 5300–5306 (1998).
89. Bhat, H. B. *et al.* Binding of a pleurotolysin ortholog from *Pleurotus erginii* to sphingomyelin and cholesterol-rich membrane domains. *J. Lipid Res.* **54**, 2933–2943 (2013).
90. Harder, T., Scheiffele, P., Verkade, P. & Simons, K. Lipid domain structure of the plasma membrane revealed by patching of membrane components. *J. Cell Biol.* **141**, 929–942 (1998).
91. Dietrich, C. *et al.* Lipid rafts reconstituted in model membranes. *Biophys. J.* **80**, 1417–1428 (2001).
92. Sezgin, E. *et al.* Adaptive lipid packing and bioactivity in membrane domains. *PLoS ONE* **10**, e0123930 (2015).
93. Gray, E. M., Diaz-Vazquez, G. & Veatch, S. L. Growth conditions and cell cycle phase modulate phase transition temperatures in RBL-2H3 derived plasma membrane vesicles. *PLoS ONE* **10**, e0137741 (2015).
94. Sezgin, E., Sadowski, T. & Simons, K. Measuring lipid packing of model and cellular membranes with environment sensitive probes. *Langmuir* **30**, 8160–8166 (2014).
95. Sezgin, E., Waithe, D., Bernardino de la Serna, J. & Eggeling, C. Spectral imaging to measure heterogeneity in membrane lipid packing. *Chemphyschem* **16**, 1387–1394 (2015).
96. Parasassi, T., Krasnowska, E. K., Bagatolli, L. & Gratton, E. Laurdan and Prodan as polarity-sensitive fluorescent membrane probes. *J. Fluoresc.* **8**, 365–373 (1998).
97. Parasassi, T., De Stasio, G., Ravagnan, G., Rusch, R. M. & Gratton, E. Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence. *Biophys. J.* **60**, 179–189 (1991).
98. Sanchez, S. A., Triccerri, M. A. & Gratton, E. Laurdan generalized polarization fluctuations measures membrane packing micro-heterogeneity *in vivo*. *Proc. Natl Acad. Sci. USA* **109**, 7314–7319 (2012). **Together with reference 27 and 92, suggests that the cell membrane contains domains with a range of properties.**
99. Golfetto, O., Hinde, E. & Gratton, E. Laurdan fluorescence lifetime discriminates cholesterol content from changes in fluidity in living cell membranes. *Biophys. J.* **104**, 1238–1247 (2013).
100. Kreder, R. *et al.* Solvatochromic Nile Red probes with FRET quencher reveal lipid order heterogeneity in living and apoptotic cells. *ACS Chem. Biol.* **10**, 1435–1442 (2015).
101. Mohammad, S. & Parmryd, I. Cholesterol depletion using methyl-beta-cyclodextrin. *Methods Mol. Biol.* **1232**, 91–102 (2015).
102. Pottosin, I. I., Valencia-Cruz, G., Bonales-Alatorre, E., Shabala, S. N. & Dobrovinskaya, O. R. Methyl-beta-cyclodextrin reversibly alters the gating of lipid rafts-associated Kv1.3 channels in Jurkat T lymphocytes. *Pflugers Arch.* **454**, 235–244 (2007).
103. Mohammad, S., Dinic, J., Adler, J. & Parmryd, I. Limited cholesterol depletion causes aggregation of plasma membrane lipid rafts inducing T cell activation. *Biochim. Biophys. Acta* **1801**, 625–634 (2010).
104. Sanchez, S. A., Gunther, G., Triccerri, M. A. & Gratton, E. Methyl-beta-cyclodextrins preferentially remove cholesterol from the liquid disordered phase in giant unilamellar vesicles. *J. Membr. Biol.* **241**, 1–10 (2011).
105. Hillyard, D. Z. *et al.* Statins inhibit NK cell cytotoxicity by membrane raft depletion rather than inhibition of isoprenylation. *Atherosclerosis* **191**, 319–325 (2007).
106. Amin, D. *et al.* RPR 107393, a potent squalene synthase inhibitor and orally effective cholesterol-lowering agent: comparison with inhibitors of HMG-CoA reductase. *J. Pharmacol. Exp. Ther.* **281**, 746–752 (1997).
107. Ahn, K. W. & Sampson, N. S. Cholesterol oxidase senses subtle changes in lipid bilayer structure. *Biochemistry* **43**, 827–836 (2004).
108. Merrill, A. H. Jr, van Echten, G., Wang, E. & Sandhoff, K. Fumonisin B1 inhibits sphingosine (sphinganine) N-acyltransferase and *de novo* sphingolipid biosynthesis in cultured neurons *in situ*. *J. Biol. Chem.* **268**, 27299–27306 (1993).
109. Zhao, Y. *et al.* ABCB4 exports phosphatidylcholine in a sphingomyelin-dependent manner. *J. Lipid Res.* **56**, 644–652 (2015).
110. Miller, H. *et al.* Lipid raft-dependent plasma membrane repair interferes with the activation of B lymphocytes. *J. Cell Biol.* **211**, 1193–1205 (2015).
111. Eggeling, C. & Honigsmann, A. Closing the gap: the approach of optical and computational microscopy to uncover biomembrane organization. *Biochim. Biophys. Acta* **1858**, 2558–2568 (2016).
112. Dror, R. O., Dirks, R. M., Grossman, J. P., Xu, H. & Shaw, D. E. Biomolecular simulation: a computational microscope for molecular biology. *Annu. Rev. Biophys.* **41**, 429–452 (2012).
113. Lane, T. J., Shukla, D., Beauchamp, K. A. & Pande, V. S. To milliseconds and beyond: challenges in the simulation of protein folding. *Curr. Opin. Struct. Biol.* **23**, 58–65 (2013).
114. Saunders, M. G. & Voth, G. A. Coarse-graining methods for computational biology. *Annu. Rev. Biophys.* **42**, 73–93 (2013).
115. Vattulainen, I. & Rog, T. Lipid simulations: a perspective on lipids in action. *Cold Spring Harb. Perspect. Biol.* **3**, a004655 (2011).
116. Stansfeld, P. J. & Sansom, M. S. Molecular simulation approaches to membrane proteins. *Structure* **19**, 1562–1572 (2011).
117. Ingolfsson, H. I. *et al.* Lipid organization of the plasma membrane. *J. Am. Chem. Soc.* **136**, 14554–14559 (2014).
118. Niemela, P. S., Ollila, S., Hyvonen, M. T., Karttunen, M. & Vattulainen, I. Assessing the nature of lipid raft membranes. *PLoS Comput. Biol.* **3**, e34 (2007).
119. Barua, D. & Goldstein, B. A mechanistic model of early FcεRI signaling: lipid rafts and the question of protection from dephosphorylation. *PLoS ONE* **7**, e51669 (2012).
120. Levental, I., Grzybek, M. & Simons, K. Raft domains of variable properties and compositions in plasma membrane vesicles. *Proc. Natl Acad. Sci. USA* **108**, 11411–11416 (2011).
121. Schutz, G. J., Kada, G., Pastushenko, V. P. & Schindler, H. Properties of lipid microdomains in a muscle cell membrane visualized by single molecule microscopy. *EMBO J.* **19**, 892–901 (2000).
122. Wang, T. Y. & Silvius, J. R. Sphingolipid partitioning into ordered domains in cholesterol-free and cholesterol-containing lipid bilayers. *Biophys. J.* **84**, 367–378 (2003).
123. Sodd, A. J., Pastor, R. W. & Lyman, E. Hexagonal substructure and hydrogen bonding in liquid-ordered phases containing palmitoyl sphingomyelin. *Biophys. J.* **109**, 948–955 (2015).
124. Ramstedt, B. & Slotte, J. P. Interaction of cholesterol with sphingomyelins and acyl-chain-matched phosphatidylcholines: a comparative study of the effect of the chain length. *Biophys. J.* **76**, 908–915 (1999).
125. Sodd, A. J., Sandar, M. L., Gawrisch, K., Pastor, R. W. & Lyman, E. The molecular structure of the liquid-ordered phase of lipid bilayers. *J. Am. Chem. Soc.* **136**, 725–732 (2014).
126. Levental, I., Grzybek, M. & Simons, K. Greasing their way: lipid modifications determine protein association with membrane rafts. *Biochemistry* **49**, 6305–6316 (2010).
127. Ozhan, G. *et al.* Lypd6 enhances Wnt/β-catenin signaling by promoting Lrp6 phosphorylation in raft plasma membrane domains. *Dev. Cell* **26**, 331–345 (2013).
128. Brameshuber, M. *et al.* Imaging of mobile long-lived nanoplateforms in the live cell plasma membrane. *J. Biol. Chem.* **285**, 41765–41771 (2010).
129. Goswami, D. *et al.* Nanoclusters of GPI-anchored proteins are formed by cortical actin-driven activity. *Cell* **135**, 1085–1097 (2008).
130. Sevsick, E. *et al.* GPI-anchored proteins do not reside in ordered domains in the live cell plasma membrane. *Nat. Commun.* **6**, 6969 (2015).
131. Shi, D. *et al.* Smoothed oligomerization/high order clustering in lipid rafts is essential for high Hedgehog activity transduction. *J. Biol. Chem.* **288**, 12605–12614 (2013).
132. Shah, A. *et al.* RaftProt: mammalian lipid raft proteome database. *Nucleic Acids Res.* **43**, D335–D338 (2015).
133. Lorent, J. H. & Levental, I. Structural determinants of protein partitioning into ordered membrane domains and lipid rafts. *Chem. Phys. Lipids* **192**, 23–32 (2015).
134. Diaz-Rohrer, B. B., Levental, K. R., Simons, K. & Levental, I. Membrane raft association is a determinant of plasma membrane localization. *Proc. Natl Acad. Sci. USA* **111**, 8500–8505 (2014). **Relates the membrane raft association of several proteins to their localization at the cell surface by showing that both properties are determined by the length of the TMD of a protein.**

135. Veatch, S. L. *et al.* Critical fluctuations in plasma membrane vesicles. *ACS Chem. Biol.* **3**, 287–293 (2008).
Shows critical fluctuation behaviour in GPMVs, which suggests that the cell membrane is near a miscibility critical point that could be modulated by temperature.
136. Lingwood, D., Ries, J., Schwillie, P. & Simons, K. Plasma membranes are poised for activation of raft phase coalescence at physiological temperature. *Proc. Natl Acad. Sci. USA* **105**, 10005–10010 (2008).
137. Zhou, Y. *et al.* Bile acids modulate signaling by functional perturbation of plasma membrane domains. *J. Biol. Chem.* **288**, 35660–35670 (2013).
138. Coskun, U., Grzybek, M., Drechsel, D. & Simons, K. Regulation of human EGF receptor by lipids. *Proc. Natl Acad. Sci. USA* **108**, 9044–9048 (2010).
139. Levental, I. & Veatch, S. L. The continuing mystery of lipid rafts. *J. Mol. Biol.* **428**, 4749–4764 (2016).
140. Tisza, M. J. *et al.* Motility and stem cell properties induced by the epithelial–mesenchymal transition require destabilization of lipid rafts. *Oncotarget* **7**, 51553–51568 (2016).
141. Schwarzer, R. *et al.* The cholesterol-binding motif of the HIV-1 glycoprotein gp41 regulates lateral sorting and oligomerization. *Cell. Microbiol.* **16**, 1565–1581 (2014).
142. Contreras, F. X. *et al.* Molecular recognition of a single sphingolipid species by a protein's transmembrane domain. *Nature* **481**, 525–529 (2012).
Shows direct specific interactions between the single-pass TMD of the coat protein I (COP1)-machinery protein p24 and a single sphingomyelin species.
143. Tulodziecka, K. *et al.* Remodeling of the postsynaptic plasma membrane during neural development. *Mol. Biol. Cell* **27**, 3480–3489 (2016).
144. Lach, A. *et al.* Palmitoylation of MPP1 (membrane-palmitoylated protein 1)/p55 is crucial for lateral membrane organization in erythroid cells. *J. Biol. Chem.* **287**, 18974–18984 (2012).
145. Podkalicka, J., Biernatowska, A., Majkowski, M., Grzybek, M. & Sikorski, A. F. MPP1 as a factor regulating phase separation in giant plasma membrane-derived vesicles. *Biophys. J.* **108**, 2201–2211 (2015).
146. Heberle, F. A. *et al.* Bilayer thickness mismatch controls domain size in model membranes. *J. Am. Chem. Soc.* **135**, 6853–6859 (2013).
147. Jensen, M. O. & Mouritsen, O. G. Lipids do influence protein function — the hydrophobic matching hypothesis revisited. *Biochim. Biophys. Acta* **1666**, 205–226 (2004).
148. Kaiser, H.-J. *et al.* Lateral sorting in model membranes by cholesterol-mediated hydrophobic matching. *Proc. Natl Acad. Sci. USA* **108**, 16628–16633 (2011).
149. Koster, D. V. & Mayor, S. Cortical actin and the plasma membrane: inextricably intertwined. *Curr. Opin. Cell Biol.* **38**, 81–89 (2016).
150. Fritzsche, M., Erlenkamper, C., Moendarbary, E., Charras, G. & Kruse, K. Actin kinetics shapes cortical network structure and mechanics. *Sci. Adv.* **2**, e1501337 (2016).
151. Fujiwara, T. K. *et al.* Confined diffusion of transmembrane proteins and lipids induced by the same actin meshwork lining the plasma membrane. *Mol. Biol. Cell* **27**, 1101–1119 (2016).
152. Saha, S. *et al.* Diffusion of GPI-anchored proteins is influenced by the activity of dynamic cortical actin. *Mol. Biol. Cell* **26**, 4033–4045 (2015).
153. Honigsmann, A. *et al.* A lipid bound actin meshwork organizes liquid phase separation in model membranes. *eLife* **3**, e01671 (2014).
154. Mueller, V. *et al.* STED nanoscopy reveals molecular details of cholesterol- and cytoskeleton-modulated lipid interactions in living cells. *Biophys. J.* **101**, 1651–1660 (2011).
155. Ehrig, J., Petrov, E. P. & Schwillie, P. Near-critical fluctuations and cytoskeleton-assisted phase separation lead to subdiffusion in cell membranes. *Biophys. J.* **100**, 80–89 (2011).
156. Machta, B. B., Papanikolaou, S., Sethna, J. P. & Veatch, S. L. Minimal model of plasma membrane heterogeneity requires coupling cortical actin to criticality. *Biophys. J.* **100**, 1668–1677 (2011).
157. Liu, A. P. & Fletcher, D. A. Actin polymerization serves as a membrane domain switch in model lipid bilayers. *Biophys. J.* **91**, 4064–4070 (2006).
158. Gowrishankar, K. *et al.* Active remodeling of cortical actin regulates spatiotemporal organization of cell surface molecules. *Cell* **149**, 1353–1367 (2012).
Provides a compelling theoretical framework to understand how actomyosin-driven activity can create non-equilibrium clusters of membrane proteins.
159. Fritzsche, M. *et al.* Self-organizing actin patterns shape membrane architecture but not cell mechanics. *Nat. Commun.* **8**, 14347 (2017).
160. Koster, D. V. *et al.* Actomyosin dynamics drive local membrane component organization in an *in vitro* active composite layer. *Proc. Natl Acad. Sci. USA* **113**, E1645–E1654 (2016).
161. Rao, M. & Mayor, S. Active organization of membrane constituents in living cells. *Curr. Opin. Cell Biol.* **29**, 126–132 (2014).
162. Lingwood, D. *et al.* Cholesterol modulates glycolipid conformation and receptor activity. *Nat. Chem. Biol.* **7**, 260–262 (2011).
163. Laganowsky, A. *et al.* Membrane proteins bind lipids selectively to modulate their structure and function. *Nature* **510**, 172–175 (2014).
164. Filipp, D., Leung, B. L., Zhang, J., Veillette, A. & Julius, M. Enrichment of Ick in lipid rafts regulates colocalized fyn activation and the initiation of proximal signals through TCR alpha beta. *J. Immunol.* **172**, 4266–4274 (2004).
165. Zhang, M. *et al.* CD45 signals outside of lipid rafts to promote ERK activation, synaptic raft clustering, and IL-2 production. *J. Immunol.* **174**, 1479–1490 (2005).
166. Field, K. A., Holowka, D. & Baird, B. Fc epsilon RI-mediated recruitment of p53/56lyn to detergent-resistant membrane domains accompanies cellular signaling. *Proc. Natl Acad. Sci. USA* **92**, 9201–9205 (1995).
167. Varshney, P., Yadav, V. & Saini, N. Lipid rafts in immune signaling: current progress and future perspective. *Immunology* **149**, 13–24 (2016).
168. Dinic, J., Riehl, A., Adler, J. & Parmryd, I. The T cell receptor resides in ordered plasma membrane nanodomains that aggregate upon patching of the receptor. *Sci. Rep.* **5**, 10082 (2015).
169. Beck-Garcia, K. *et al.* Nanoclusters of the resting T cell antigen receptor (TCR) localize to non-raft domains. *Biochim. Biophys. Acta* **1853**, 802–809 (2015).
170. Sproul, T. W., Malapati, S., Kim, J. & Pierce, S. K. Cutting edge: B cell antigen receptor signaling occurs outside lipid rafts in immature B cells. *J. Immunol.* **165**, 6020–6023 (2000).
171. Stone, M. B., Shelby, S. A., Nunez, M. F., Wissner, K. & Veatch, S. L. Protein sorting by lipid phase-like domains supports emergent signaling function in B lymphocyte plasma membranes. *eLife* **6**, e19891 (2017).
172. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J. & Mathison, J. C. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* **249**, 1431–1433 (1990).
173. Beissert, S. *et al.* Impaired cutaneous immune responses in Thy-1-deficient mice. *J. Immunol.* **161**, 5296–5302 (1998).
174. Lorizate, M. *et al.* Comparative lipidomics analysis of HIV-1 particles and their producer cell membrane in different cell lines. *Cell. Microbiol.* **15**, 292–304 (2013).
175. Farnoud, A. M., Toledo, A. M., Konopka, J. B., Del Poeta, M. & London, E. Raft-like membrane domains in pathogenic microorganisms. *Curr. Top. Membr.* **75**, 233–268 (2015).
176. Iwabuchi, K. Involvement of glycosphingolipid-enriched lipid rafts in inflammatory responses. *Front. Biosci. (Landmark Ed.)* **20**, 325–334 (2015).
177. Teissier, E. & Pecheur, E. I. Lipids as modulators of membrane fusion mediated by viral fusion proteins. *Eur. Biophys. J.* **36**, 887–899 (2007).
178. Dick, R. A., Goh, S. L., Feigensohn, G. W. & Vogt, V. M. HIV-1 Gag protein can sense the cholesterol and acyl chain environment in model membranes. *Proc. Natl Acad. Sci. USA* **109**, 18761–18766 (2012).
179. Staubach, S., Razawi, H. & Hanisch, F. G. Proteomics of MUC1-containing lipid rafts from plasma membranes and exosomes of human breast carcinoma cells MCF-7. *Proteomics* **9**, 2820–2835 (2009).
180. Raghun, H. *et al.* Localization of uPAR and MMP-9 in lipid rafts is critical for migration, invasion and angiogenesis in human breast cancer cells. *BMC Cancer* **10**, 647 (2010).
181. Larsen, J. B. *et al.* Membrane curvature enables N-Ras lipid anchor sorting to lipid-ordered membrane phases. *Nat. Chem. Biol.* **11**, 192–194 (2015).
182. Cuesta-Marban, A. *et al.* Drug uptake, lipid rafts, and vesicle trafficking modulate resistance to an anticancer lysophosphatidylcholine analogue in yeast. *J. Biol. Chem.* **288**, 8405–8418 (2013).
183. Gajate, C. & Mollinedo, F. Edelfosine and perifosine induce selective apoptosis in multiple myeloma by recruitment of death receptors and downstream signaling molecules into lipid rafts. *Blood* **109**, 711–719 (2007).
184. Shashkin, P., Dragulev, B. & Ley, K. Macrophage differentiation to foam cells. *Curr. Pharm. Des.* **11**, 3061–3072 (2005).
185. Rios, F. J. *et al.* Uptake of oxLDL and IL-10 production by macrophages requires PAFR and CD36 recruitment into the same lipid rafts. *PLoS ONE* **8**, e76893 (2013).
186. Maguy, A., Hebert, T. E. & Nattel, S. Involvement of lipid rafts and caveolae in cardiac ion channel function. *Cardiovasc. Res.* **69**, 798–807 (2006).
187. van Zanten, T. S. & Mayor, S. Current approaches to studying membrane organization. *FI000Res.* **4**, 1380 (2015).
188. Toulmay, A. & Prinz, W. A. Direct imaging reveals stable, micrometer-scale lipid domains that segregate proteins in live cells. *J. Cell Biol.* **202**, 35–44 (2013).
The first demonstration of microscopic raft-like domain formation in the vacuoles of live yeast cells.
189. Kahya, N., Scherfeld, D., Bacia, K. & Schwillie, P. Lipid domain formation and dynamics in giant unilamellar vesicles explored by fluorescence correlation spectroscopy. *J. Struct. Biol.* **147**, 77–89 (2004).
190. Shogomori, H. *et al.* Palmitoylation and intracellular domain interactions both contribute to raft targeting of linker for activation of T cells. *J. Biol. Chem.* **280**, 18931–18942 (2005).

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Competing interests statement

The authors declare no competing interests.

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