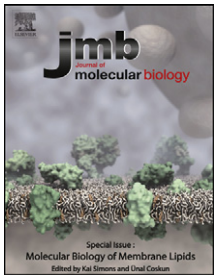

Compartmentalization of the Cell Membrane

Alf Honigmann [1,†] **and Arnd Pralle** [2,†]

*1 - **Max Planck Institute of Molecular Cell Biology and Genetics,** Pfotenhauerstr. 108 01307 Dresden, Germany*
*2 - **Department of Physics,** University at Buffalo, Buffalo, NY 14260, USA*

***Correspondence to Arnd Pralle:** apralle@buffalo.edu*
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Abstract

Many cell-membrane-associated processes require transient spatiotemporal separation of components on scales ranging from a couple of molecules to micrometers in size. Understanding these processes mechanistically involves understanding how lipids and proteins self-organize and interact with the cell cortex. Here, we review recent advances in dissecting the mechanisms of cell membrane compartmentalization. We introduce the challenges in studying cell membrane organization, the current understanding of how complex membranes self-organize to form transient domains, and the role of protein scaffolds in membrane organization. We discuss the formation of signaling domains as an important example of transient membrane compartmentalization. We conclude by pointing to the current limitations of measuring membrane organization in living cells and the steps that are required to advance the field.

Introduction: Challenges to Studies of the Cell Membrane Organization

Understanding plasma membrane organization has been a major aim in cell biology and biophysics for almost half a century. Despite knowing the majority of the molecular building blocks (lipids, proteins, and sugars) and most of the organization principles, we lack a quantitative understanding of how molecular interactions between membrane components and its coupling to the cell cortex give rise to structure and function in cell membranes. There are several fundamental reasons for this knowledge gap: first, the relevant length scales of compartmentalization in the membrane–cytoskeleton system are too small to be resolved by diffraction-limited light microscopy, and their transient nature complicates direct imaging with super-resolution microscopy [1,2]. Second, the molecular composition of the cell membrane is very complex (>500 different lipid species, >1 k sugar species, and >400 membrane proteins). In contrast to proteins, we lack methods to tag and detect most of the endogenous sugar and lipid species under live-cell conditions. Consequently, we lack molecular distribution information for many components of the cell membrane. Third, living cells are far from equilibrium, energy-driven processes constantly stir and reorganize the system. These active processes involve membrane transport and metabolism and the polymerization and motor-driven constriction of the cell cortex. How these processes couple to the composition and structure of the cell membrane is only partly understood. Lastly, molecular dynamics simulations of membranes are still far too small and short to predict bottom-up membrane behavior on a scale relevant for membrane compartmentalization, which would be at least 1 μm and 1 s [3,4]. All these facts make it very challenging to directly measure many of the relevant processes of cell membranes and to translate the knowledge gained from minimal lipid model systems or MD simulations to the membrane of intact cells.

Still, we argue that there are strong indications for the existence of compartments in the cell membrane. We present the arguments in a hierarchical order starting with the complexity of molecular interactions and the self-organization defining the “ground state” of the plasma membrane and we move up to the formation of transient signaling domains and more stable, larger membrane domains in the context of cell functions.

Self-organization of Simple and Complex Membranes

Cells are compartmentalized from the molecular to macroscopic scale to allow for the spatiotemporal control of biochemical reactions. Cellular organelles separated from the cytoplasm by dedicated lipid membranes are the most obvious form of compartmentalization. However, due to their two-dimensional (2D) and fluid nature, membranes itself are ideal structures to organize and control biochemical reactions. In fact, many important cellular processes take place at membranes, for example, signaling and sensing, energy conversion, and metabolism. The simple reduction of dimensionality upon binding to 2D membranes results in an effective increase in concentration and therefore increases the reaction rates by orders of magnitude, which is, for example, used as a switch to control some cellular signaling reactions [5]. Preferential orientation of membrane-bound molecules can additionally modulate affinities, either by blocking or exposing interaction sites [6]. In addition, Pólya's recurrence theorem states that 2D diffusion covers the entire surface [7].

Besides these obvious differences between the reaction diffusion systems in three and two dimensions, biological membranes provide more qualities for compartmentalizing and controlling reactions. Membranes assemble via hydrophobic interactions of lipids without the need for attractive interactions between neighboring components. Depending on lipid composition, the emergent bilayer structure can be in a solid, liquid-ordered, or liquid-disordered state. In multicomponent membranes, even weak interactions between components (charge, propensity for hydrogen bonding, etc.) can lead to segregation of components and result in domains of specific composition. In addition, matching molecular characteristics of membranes components to local membrane features like thickness, curvature, and surface charge further influences the sorting of molecules in membranes [8,9]. These self-organization properties of lipid membranes have been worked out in simple model membrane systems, and all of these principles have been suggested to be important for the function of cell membranes. The cell membrane has a very complex lipid composition with a large variety of proteins, as one-third of our genes encode membrane proteins [10]. Depending on cell type and measurement method, these proteins occupy 23% to 40% of the membrane surface [11,12].

With the high cholesterol and high protein content, the overall state of the cell membrane is liquid with relatively high lipid order [13,14], and in non-polarized cells, the distribution of membrane proteins such as receptors and fluorescent lipid tracers appears to be homogenous on scales larger than 200 nm. However, intricate high spatiotemporal resolution measurements of fluorescent lipid and protein tracers in living cells have revealed anomalous diffusion characteristics or clustering on scales below 200 nm, which indicate the transient confinement of membrane components in cytoskeletal compartments and/or lipid–protein domains on the nanoscale [15–27]. The characterizations of nanoscale lipid–protein domains in intact cells come from indirect measurements because these domains are small and dynamic and their structure is easily perturbed by fixation processes required for higher resolution techniques [16,28–30]. Diffusion measurements of lipids or proteins interacting with nanoclusters require high spatiotemporal resolution, as the residence time of individual molecules in the nanodomains is likely shorter than 100 ms and the domains are smaller than 60 nm. Förster resonance energy transfer (FRET) measurements have also been applied to determine whether a protein interacts with nanodomains. As there are few proteins within each cluster, homo-FRET of the same species has provided more consistent results than dual-color FRET. The short interaction time of diffusing molecules with the nanodomains indicates binding energies not much larger than the thermal energy $k_B T$. [31]. As we can only measure the interaction of individual molecules with nanodomains, but we cannot image the domains, we currently have no information on the lifetime and shape dynamics of the domains [32].

Nanoscale domains or heterogeneities in the cell membrane can coalesce into larger domains. Cross-linking lipid or protein components by multivalent toxins or antibodies induces micrometer-scale domains in the cell membrane, which are reminiscent of liquid-ordered/liquid-disordered domains in model membranes [33]. Similar domains appear in vesicles extracted from the plasma membrane when they are cooled below room temperature [34,35]. These experiments indicate that the complex composition of the plasma membrane has evolved to be close to a transition into a state where some components spontaneously separate. The high lipid diversity found in cell membranes may be one way to be close to this point and yet avoid spontaneous demixing, because the diverse lipids reduce interface energies and act as buffer. In addition, anchoring some cell membrane components to the cytoskeleton or via adhesion to the extracellular matrix has been shown to quench segregation [36]. We will discuss this phenomenon in more detail below.

Another important structural aspect of cell membrane is their asymmetry. Most of the sphingolipids and gangliosides are located on the outer membrane leaflet, while negatively charged lipids like phosphatidylserine and phosphatidylinositol species (PIPs) are found on the inner leaflet. This lipid asymmetry is actively established and maintained by flippases and lipid transport proteins [37,38]. In the inner leaflet, protein and lipid clustering has been shown to be mediated by electrostatic interactions between divalent

cations and/or polybasic protein motifs often found close to the cytoplasmic membrane interface and negatively charged lipid head groups. [39–41]. Polyanionic PIPs serve as anchors for proteins locally clustering other lipids and proteins. This clustering mechanism together with the rapid interconversion of the phosphorylation state of PIP species by enzymes [42] offers a regulatory layer to control the formation of membrane clusters.

Taken together, the thermodynamic properties of membranes allow spontaneous segregation and sorting of lipids and proteins. In cell membranes, these properties need to be spatiotemporally controlled to provide function. One interesting hypothesis is that the complex composition of cell membranes together with its coupling to the cell cortex provides an overall well-mixed fluid state, which can be locally reordered into functional clusters upon small perturbations like ligand binding [33]. Additionally, the enzymatic interconversion of polyanionic PIP species in the cytoplasmic membrane leaflet provides a robust mechanism to control cluster formation by specific electrostatic interactions [42]. Both mechanisms have been shown to be important for signaling at the plasma membrane. In fact, the coupling of both ligand-binding-induced local reorganization and local turnover of PIPs has been suggested to be necessary to transduce ligand-binding signals of nontransmembrane, (Glycosylphosphatidylinositol) GPI-bound receptors over the plasma membrane [43,44].

Role of Scaffolds on Membrane Organization

The eukaryotic plasma membrane is connected to the cell cortex via a variety of adapter proteins, which bind to membrane proteins and/or inner leaflet lipids like phosphatidylserine and phosphoinositides [45]. The anchoring type and pattern at the plasma membrane vary between cell types, and even within a single cell, the plasma membrane is often differentiated into membrane domains with a specialized membrane cortex structure. The interaction of the actomyosin cortex with the plasma membrane primarily regulates cell mechanics and determines cell morphology. One of the best-studied examples of membrane scaffolds is the erythrocyte plasma membrane, which is compartmentalized into a regular hexagonal lattice with ~100 nm compartments [46]. The lattice is composed of spectrin tetramers that are cross-linked by short actin filaments. The spectrin filaments are anchored to transmembrane proteins via the adapter protein ankyrin. A related membrane skeleton structure was recently discovered in neuronal cells using super-resolution microscopy. As in red blood cells, an actin–spectrin scaffold underlies the axonal plasma membrane. However, in contrast to red blood cells, actin forms rings around the circumference of the axonal tube. The actin rings are periodically separated about 180 nm by spectrin tetramers, which are connected to ion channels via ankyrin [47,48]. In both cases, the membrane scaffold provides mechanical robustness. In the axon, the scaffold also possibly organizes the distribution and presence of membrane proteins and lipids. At the axonal hillock, the barrier between axon and soma membrane, a similar scaffold anchors the sodium channels, which are dense enough to block the diffusion of other proteins, and clusters while letting single lipids pass [49].

In general, polarized membrane domains in most cell types are organized by special types of membrane scaffolds. Figure 1 shows a generalized structure of a membrane scaffold. The structure can be grouped into functional layers. Starting from the extracellular side, ectodomains of transmembrane receptors interact with either mobile effectors or static structures (adhesion). How the binding of a ligand is transmitted over the plasma membrane to induce adapter recruitment is not completely understood. Often, ligand binding is followed by dimerization of the receptor. On the intracellular side, adapter proteins recognize peptide motifs in the cytoplasmic tails of the receptors. Most adapter proteins contain a number of protein–protein interaction modules that lead to the sequestering of other transmembrane proteins, other scaffolders, signaling molecules, and actin filaments. Additionally, scaffolding molecules can directly interact with the membrane via lipid-binding motifs. The spatiotemporal interdependence of this interaction network including positive and negative feedbacks is thought to determine the molecular identity and the structure and function of the respective membrane domain. In particular, these integrations are modulated by lipid nanoclusters containing cholesterol, PIP2, or both [50]. Such clusters not only anchor to the cytoskeleton but attract actin polymerizing proteins and modulate the structure of the cytoskeleton [51]. Entangling these interaction networks in space and time requires a systematic combination of new experimental and modeling approaches. Examples of important membrane scaffolds are cell–matrix junctions (focal adhesions), cell–cell junctions (adherens, tight, and neuronal junctions and desmosomes), and polarized membrane protrusions (lamellapodia, axons, brush border, and cilia of epithelial cells).

How membrane scaffolds can affect the compartmentalization of membrane proteins and lipids on the nanoscale has been shown by seminal studies of Kusumi's group. High-speed single-particle tracking of transmembrane proteins and outer leaflet lipid analogs revealed a confined diffusion pattern on spatial scales well below the diffraction limit of light microscopy. Membrane proteins and lipids move fast locally, below ~60–200 nm, but are confined for up to milliseconds in compartments before “hopping” to a neighboring compartment [52,53]. Aki Kusumi termed this diffusion

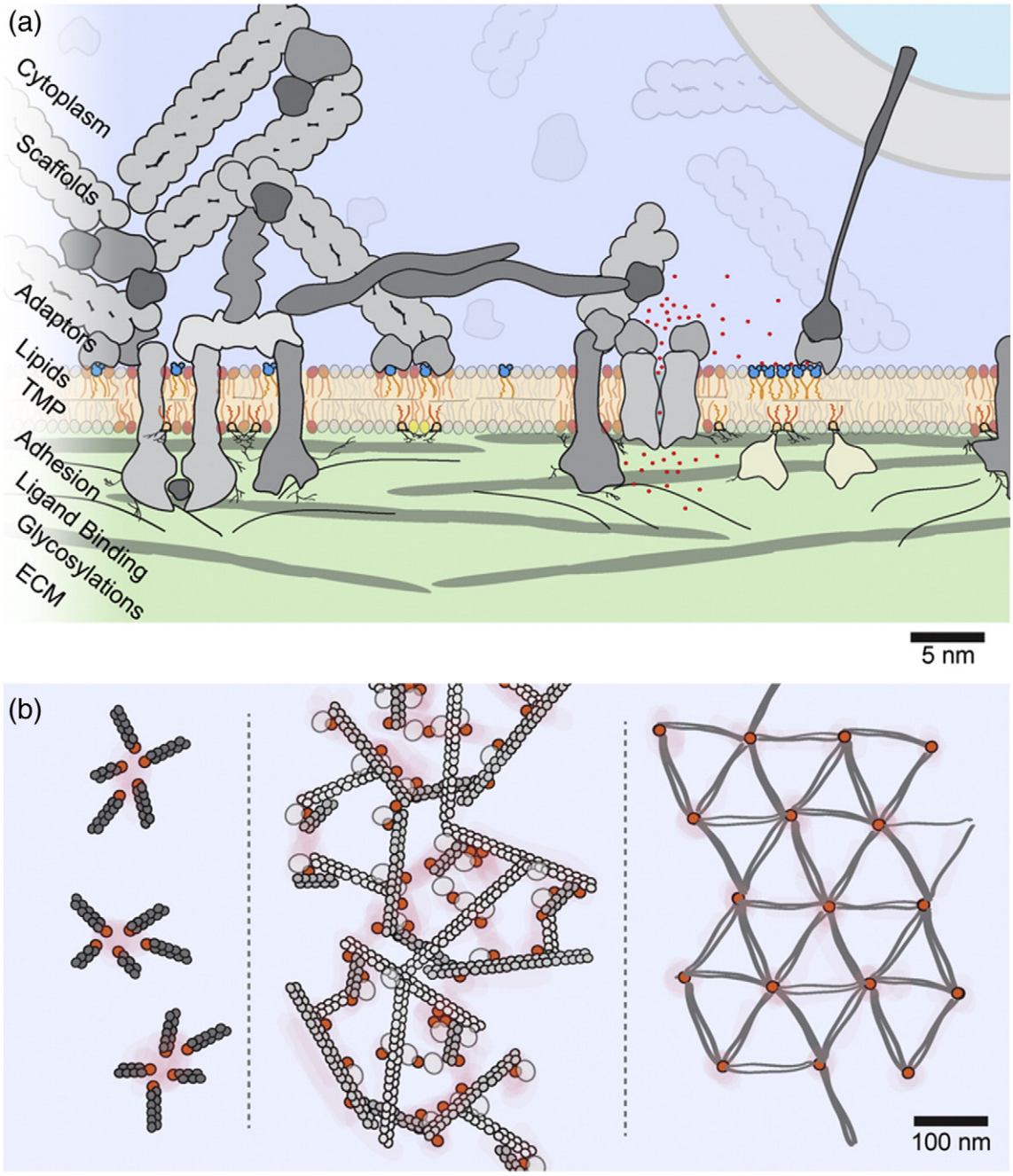

Fig. 1. Cartoon showing some of the main features of plasma membrane organization. (a) Cross-section of the plasma membrane. Green represents the extracellular matrix that is separated from the cytoplasm (blue) by the lipid bilayer. Proteins are shown in saturated gray tones. Lipids are shown in light gray or, if involved in membrane domain formation, in red tones. The outer leaflet of the bilayer contains glycosylated lipids and ectodomains of transmembrane proteins or GPI-anchored proteins (yellowish). The inner leaflet contains anionic lipids, which can interact with transmembrane and cytosolic proteins or with divalent cations (red). Transmembrane proteins and lipids can be bound by adapter proteins on the inner leaflet of the membrane. The adapters often interact with cytoskeletal scaffolding proteins such as actin or spectrin. Coupling of extracellular ligand binding or adhesion to transient internal scaffold assembly via clustering of transmembrane domains and lipids is shown on the left side. Electrostatically driven domain formation via calcium signaling is shown on the right side (docking of an exocytosis vesicle). (b) Top view on the plasma membrane from the cytoplasm showing three types of membrane scaffold structures. Left shows active actin fiber polymerizing binding to the membrane constituents and driving clustering via aster formation [67]. Middle shows actin fence formation and binding to transmembrane proteins and lipids via adapter proteins [1]. Right shows regular spectrin/actin/ankyrin-based membrane scaffold as found in red blood cells and neurons [46,47].

pattern “hop diffusion” and suggested that membrane-bound actin filaments, which are on the cytoplasmic membrane side, act as boundaries for diffusing transmembrane proteins (fence). Additionally, membrane proteins, which anchor the actin cytoskeleton to the membrane, act as obstacles within the plane of the membrane (pickets), confining the diffusion even of outer leaflet lipids. Including the finding that many proteins can switch between actin- and non-actin-bound states in a signaling-dependent manner creates a comprehensive model that explains many of the observed membrane dynamics [22].

Recent simulations and experiments indicate that membrane scaffolds can have strong effects on lipid organization that go far beyond passive diffusion obstacles. In complex membranes that are close to a phase transition, the pinning of membrane constituents by a scaffold can either induce or inhibit lipid domains, depending on the pinning pattern and temperature. Simple 2D Ising model simulations have been shown to accurately describe phase transition behavior of ternary model membranes and even complex cell-derived vesicles [34]. More advanced simulations predicted that the spatiotemporal pinning of membrane constituents results in a broadening or even a complete loss of the phase transition [36,54,55]. This mechanism is known from condensed matter and statistical physics as quenched disorder and has been studied in glass-like materials and ferromagnetic transitions. The in silico predictions for lipid membranes have been recently confirmed in a number of experimental *in vitro* approaches. Using adhesion as a source for lipid pinning in phase-separating giant unilamellar vesicles, Zhao *et al.* showed that the lipid domains are stabilized at adhesions sites at temperature far above the phase transition temperature [56]. Honigmann *et al.* used an artificial actin cortex, which was bound to a supported lipid membrane via lipid anchors. In line with the simulations, pinning by dense actin meshworks prevented large-scale phase separation at low temperature and stabilized lipid domains at high temperature [57]. Using a similar approach, Arumugam *et al.* came essentially to the same conclusions for free-standing GUVs (Giant Unilaminar Vesicle) [58].

The in silico and *in vitro* work clearly indicate that a quenched disorder mechanism may be important to define the physiological state of cell membranes. These indications are supported by the observation of large-scale phase separation in plasma-membrane-derived vesicles, which contain some of the membrane protein's and lipids' complexity of live cells but lack a connection to the cell cortex (reviewed in other chapters in the special edition). This implies that in intact cells, large-scale demixing is prevented by the cortex. In line with these predictions, several studies reporting on membrane heterogeneities and lipid rafts found that actin depolymerization resulted in a more homogeneous membrane at physiological temperature [17,20,59,60].

Furthermore, the cortex is constantly being remodeled by de-/polymerization of actin and microtubule fibers and motor-protein-based movements. Satyajit Mayor recently proposed a model, which designates a role of motile short actin filament for generating *trans*-leaflet lipid domains [61]. Short actin filaments that bind to the lipid phosphatidylserine containing long, saturated acyl chains via adapter proteins can induce clusters of GPI-anchored proteins on the outer leaflet of the plasma membrane.

Formation of Signaling Domains

During cell signaling, information needs to be transmitted over the cell membrane to cause a cellular response. How signals from single receptors are integrated to make global cellular decisions is still not entirely understood. Examples from well-studied signaling processes show that amplification steps are required to translate single receptor activation events into transcriptional regulation. Due to improvements in phosphoproteomics, it is becoming clear that the classical view of simple linear reaction schemes describing signaling cascades needs to be replaced by stochastic models where decisions are the result of complex interactions in large interconnected networks [62,63]. Additionally, in the recent years, it became obvious that many of the downstream processes in signaling cascades after receptor activation are actually localized directly to the site of receptor activation [64]. This is achieved by the formation of signaling clusters, which contain multiple activated receptors and scaffolding molecules that transiently recruit and therefore bring together kinases and other signaling proteins. The whole cluster is stabilized by actin-cortex remodeling and is finally inactivated by endocytosis. To understand which mechanisms actually drive the formation and termination of these signaling clusters at the cell membrane, it is helpful to refer back to the self-organization principles in membranes and its interaction with the cell cortex.

Most mechanisms of membrane organization introduced in the previous paragraphs have been suggested to be involved in the formation of signaling domains. From the wide variety of receptor types localized to the plasma membrane, some receptors may function as independent units, such as voltage-gated ion channels and some G-protein-coupled receptors. However, many receptors, including receptor tyrosine kinases, require ligand-induced dimerization for signal transduction [65]. Dimerization and the subsequent phosphorylation step have been suggested to be affected by and/or change the local membrane environment [66].

GPI-anchored receptors, which are only connected to the outer membrane leaflet, have been suggested to signal via clustering and formation of lipid raft domains, which transmit the clustering from the outer leaflet via leaflet coupling to the inner leaflet [20,30,61,67].

In general, clustering of activated receptors provides a way to optimize signaling to specific requirements: (i) cooperative signaling turns noisy receptors into a high-fidelity relay, (ii) repeated binding of a weak ligand to a cluster of receptors provides amplification and increases sensitivity, and (iii) transient clustering permits switching between local sensitive signaling and broader spatial coverage. Particular functions have evolved specific cluster size, dynamics, and organization. Modeling showed that clusters of four to eight cooperating proteins are optimally suited to transmit a signal with high fidelity across the membrane with noisy receptors requiring a limited activation energy [32,68,69]. Cooperativity, requiring all receptors to signal simultaneously, restores fidelity at the same low signaling threshold. GPI-anchored CD59 proteins cluster into three to nine GPI-anchored CD59 proteins form one cluster [70], while GTPase Ras clusters around six molecules [71–73]. Small transient clusters are ideal to increase fidelity, because quick assembly and disassembly still permit fast diffusion for broad spatial coverage.

As discussed above, small changes in intermolecular interactions easily lead to local demixing of membrane components and nanodomain formation. Interestingly, for the formation of transient signaling nanodomains, some properties of the signaling molecules can be modulated by the signaling event. For example, the tilt of transmembrane domains changes for some ion channels and receptor during activation, which means that they now prefer a different membrane thickness. Also, other activation processes, such as phosphorylation or polymerization, change the charge or hydrophobic surface of membrane proteins, thereby influencing their preference for a particular local environment [8,74–76]. These mechanisms may drive the initial formation of signaling clusters of activated receptors. In addition, active aggregation by energy-consuming polymerization and reaction kinetics has recently become the focus of several studies [77–79]. As signaling generally involves amplification steps, it is intriguing to think of positive feedbacks accumulating more signaling molecules. A collaboration between the groups of Ron Vale and Michael Rosen recently demonstrated how T-cell receptor phosphorylation is recognized by downstream proteins and is converted into a signaling cluster by the multivalent adapters and scaffolding proteins and the actin cytoskeleton. The scaffolders and adapters allow for local and transient sequestering of kinases and other signaling molecules to the site of receptor activation. Interestingly, the assembly of signaling clusters via scaffolding proteins seems to be based on liquid phase separation of the multivalent scaffolding proteins, which allows for amplification and formation of specific local reaction environments, and it is conceptually very similar to what has been proposed for phase separation of lipids and proteins within the membrane [80–82]. On top of this, motor-driven molecular flow is clearly essential for micrometer-sized signaling platforms, such as the immunological synapse [64,83], but may also occur in smaller domains.

The role of lipids for the formation of signaling clusters is best understood in the inner leaflet, where clustering is mostly caused by electrostatic interactions between divalent cations and/or polybasic protein motifs often found close to the cytoplasmic membrane interface and negatively charged lipid head groups [39–41]. It has been proposed that divalent ions, such as calcium influx during signaling, may aggregate these charged lipids, creating possible feedback loops. The different phosphorylation states of PIPs can be specifically recognized by a set of lipid-binding domains such as Pleckstrin homology domain, C2 domains [84], or PDZ domains [85], which, for example, serve as site-specific docking adapters for synaptic vesicles in neurons [40,86] or are modules of multivalent signaling scaffolders. These PIP2 nanoclusters also strengthened the connection between membrane and cytoskeleton and help relay the signal from the membrane toward the nucleus.

In intact cells, domain growth is limited by the pinning of the domains through interactions to the underlying cytoskeleton, unless the process involves active cytoskeleton remodeling. Eventually, the signaling event has to be terminated and the nanodomains have to be disassembled. The trigger for this is not known and could be a feedback from some downstream event. However, the simplest trigger may be the size of the nanodomains and may be correlated to the strength of its connection to the cortical cytoskeleton. The common route for the removal of activated signaling complex is endocytosis [87], which could be triggered by increasing actin polymerization on the growing nanodomains on the inner membrane leaflet.

Conclusions

Many cellular processes such as cell signaling, vesicle fusion and budding, cell division, cellular adhesion, and cell polarity [88–91] require transient spatiotemporal separation of the membrane and the cortical cytoskeleton components on scales ranging from a couple of molecules to micrometers in size [92]. Understanding these important processes on a mechanistic level involves understanding how complex membranes self-organize. Recent advancements in optical and spectroscopic techniques help reveal membrane organization in live cells with high

spatiotemporal resolution. These tools will allow us to test theoretical predictions and hypothesis based on model membrane experiments in live cells. However, most of the endogenous lipid species are not yet detectable by fluorescence techniques, which greatly limits our understanding of cell membrane organization. New directions and tools have to be developed to overcome this significant gap. Additionally, the number of mechanisms, processes, and molecular components involved in defining the functional structure of the cell membrane is becoming increasingly large. Therefore, development of multiscale models for cell membrane structure and function would be useful to bridge the molecular self-organization scale to the cellular and tissue scale.

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†A.H and A.P. contributed equally to this work.

Abbreviations used:
2D, two-dimensional; FRET, Förster resonance energy transfer; PIP, phosphatidylinositol species; GPI, Glycosylphosphatidylinositol.

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