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Regulation from within: the cytoskeleton in transmembrane signaling

Khuloud Jaqaman¹ and Sergio Grinstein²

¹Department of Systems Biology, Harvard Medical School, Boston

²Cell Biology Program, Hospital for Sick Children, and Keenan Research Centre of the Li Ka Shing Knowledge Institute, St. Michael's Hospital, Toronto

Abstract

There is mounting evidence that the plasma membrane is highly dynamic and organized in a complex manner. The cortical cytoskeleton is proving to be a particularly important regulator of plasmalemmal organization, modulating the mobility of proteins and lipids in the membrane, facilitating their segregation and influencing their clustering. This organization plays a critical role in receptor-mediated signaling, especially in the case of immunoreceptors, which require lateral clustering for their activation. Based on recent developments, we discuss the structures and mechanisms whereby the cortical cytoskeleton regulates membrane dynamics and organization, and how the non-uniform distribution of immunoreceptors and their self-association may affect activation and signaling.

Keywords

membrane domain; receptor clustering; immunoreceptor signaling; membrane skeleton; rafts; cytoskeleton

The plasma membrane: highly dynamic, yet organized

Cells continuously interact with their environment. This is necessary for cell survival and for the development and functional coordination of multicellular organisms. To this end, myriad signaling cascades are initiated at the plasma membrane upon the interaction of extracellular signals with plasmalemmal receptors.

The proteins and lipids that constitute the membrane are neither static, nor homogeneously distributed along or across the membrane bilayer. Intermolecular interactions between membrane lipids and proteins generate inhomogeneities of varying size and stability. These can vary from dimers to multi-component domains – referred to hereafter as ‘nanodomains’, because their size is generally in the tens to hundreds of nanometers – and can last from microseconds to hours. Through interactions with various membrane components, the cortical cytoskeleton can restrict the diffusion of proteins and lipids, aid in their transport, and assist in the formation, segregation or transport of nanodomains. As such, the

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Corresponding author: Grinstein, S. (sergio.grinstein@sickkids.ca).

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cytoskeleton can potentially modulate signal transduction, coordinate events in distant parts of the cell, and couple mechanical signals to biochemical responses.

Here we examine the critical role of the cytoskeleton in regulating the spatiotemporal organization of the plasma membrane, highlighting new studies and technological advances. We discuss in depth the case of immunoreceptors, which often undergo long-range translocation to become clustered and activated, and are therefore uniquely susceptible to cytoskeletal modulation.

The plasma membrane is more complex than a fluid mosaic

The ‘fluid-mosaic’ model, proposed by Singer and Nicolson forty years ago, postulated that lipids form a bilayer that is effectively a two-dimensional fluid in which proteins are embedded, forming a lipid-protein mosaic [1]. While the model captures many features of biological membranes, it makes two predictions that are at odds with experimental observations: first, it predicts that proteins and lipids undergo unrestricted diffusion in the membrane (Figure 1a); second, as a result of this unrestricted diffusion, membrane proteins and lipids are anticipated to distribute randomly and homogeneously.

Evidence in conflict with the first prediction emerged shortly after the introduction of the Singer-Nicolson model, when several studies reported that the diffusion of membrane components is rather restricted [2, 3]. The mobility of proteins in erythrocyte ghosts was found to be at least 20-fold slower than expected for a fluid lipid-protein mosaic, a reduction attributed to the presence of a cortical cytoskeleton meshwork (the ‘membrane skeleton’) [2]. Restricted diffusion of proteins and lipids has been since observed in the plasma membrane of many cell types, using a variety of techniques that include fluorescence recovery after photobleaching, single-particle tracking (SPT) and fluorescence correlation spectroscopy (FCS) (Table 1). These studies have found the reduction in molecular mobility to be caused not only by the cortical cytoskeleton [4, 5], but also by membrane ‘crowding’ with proteins [6], interactions between membrane components [7], and lateral inhomogeneity in membrane composition and state [8].

The realization that membrane composition is laterally inhomogeneous is the second inconsistency between predictions of the Singer-Nicolson model and experimental data. Convincing evidence to this end has stemmed from multiple approaches, including immunoelectron microscopy (immuno-EM) [9], FCS and its variants [10, 11], atomic force microscopy [12], and the more recently developed super-resolution microscopy techniques [13–15] (Table 1). One reason for this inhomogeneity is the tendency of varying lipid species to segregate into cognate sub-domains [16]. Though their size and lifetime are still debatable, it is generally agreed that cholesterol-enriched nanodomains – so-called ‘lipid rafts’ – exist in the plasma membrane of mammalian cells. Rafts are thought to be liquid-ordered regions that also contain sphingolipids and glycosylphosphatidylinositol (GPI)-anchored proteins. Most phospholipids, in contrast, reside in a liquid-disordered phase outside of rafts. Note that protein-lipid interactions can also generate nanodomains distinct from rafts, as recently observed for syntaxin [17].

Another reason for the inhomogeneous distribution of membrane components are protein-protein interactions, which have been detected in live cells using Förster resonance energy transfer [18], and more recently by SPT [19, 20] (Table 1). Such protein-based domains – often referred to as ‘protein islands’ – play a prominent role in the activation of lymphocytes upon interaction with antigen-presenting cells [21–23]. Protein-protein interactions come in different flavors. Within the membrane, proteins like tetraspanins associate with each other and with additional membrane proteins to form molecular complexes and nanodomains [24]. Beyond the membrane, interactions with scaffolding/cross-linking proteins inside or outside

the cell, including junctional and PDZ domain-containing protein complexes and galectins [25], can also lead to the clustering of membrane components. Another important scaffold for protein islands is the cortical cytoskeleton [9, 26]. The influence of the cytoskeleton, however, goes beyond scaffolding, as described next.

The different roles of the cytoskeleton

Membrane compartmentalization

The cytoskeleton influences the mobility and organization of molecules in the membrane in two different, yet interdependent ways. First, membrane proteins can be directly anchored or indirectly tethered to the cortical cytoskeleton [27, 28]. Proteins or lipid/protein islands attached to the cytoskeleton would be relatively immobile, with a range of movement dictated by the length and flexibility of the link (Figure 1b). Depending on the strength of the interaction, the anchorage/tethering could be transient, leading to periods of lower and higher mobility, often detected as anomalous diffusion in the membrane [29].

Second, even without specific direct or indirect interactions, the cortical cytoskeleton can generate barriers that ‘stand in the way’ of diffusing proteins and lipids. The most compelling evidence for this stems from high-speed SPT experiments, which revealed that while proteins and lipids can diffuse within biological membranes at or near the rates observed in lipid bilayers (on the order of $1\text{--}10\ \mu\text{m}^2/\text{s}$) [30, 31], such diffusion is restricted within compartments (‘corrals’) of varying size (40–250 nm). This restriction is generally transient, with residence times within a corral of 1–20 ms; molecules ‘hop’ between compartments, resulting in long-range excursions at longer timescales (Figure 1c). Consequently, when motion is sampled at intervals longer than the residence time, the apparent diffusion coefficient is 1–2 orders of magnitude smaller than that recorded in pure lipid bilayers. Such ‘hop-diffusion’ has been observed in various cell types for transmembrane proteins, including the transferrin receptor, major histocompatibility complex (MHC) molecules and G protein-coupled receptors (GPCRs), for lipids in the outer leaflet of the membrane, and for GPI-anchored proteins [32–34].

In contrast, molecules in large unilamellar vesicles and in membrane blebs, both of which lack a cortical cytoskeleton, diffuse relatively freely with diffusion coefficients close to those observed in lipid bilayers [32]. Disassembly of actin filaments using latrunculin or cytochalasin also generally increases the fraction of molecules undergoing simple Brownian motion in cellular membranes. Furthermore, electron tomography images revealed that the cortical cytoskeleton, within 10 nm from the plasma membrane, is a meshwork that delimits compartments of size similar to the corrals predicted from analyses of molecular diffusion [35]. These data strongly implicate the cortical actin cytoskeleton in compartmentalizing the plasma membrane. The ‘hop-diffusion’ model proposes that the cytoplasmic tails of transmembrane proteins bump into cytoskeletal filaments, as a result of which their motion is deterred. Furthermore, transmembrane proteins that are anchored/tethered to the cytoskeleton form ‘pickets’ that stand in the paths of other proteins and lipids, even those in the outer leaflet.

Hop-diffusion is an appealing idea that reconciles the discrepancies between molecular mobility in cell membranes and mobility in artificial bilayers. However, it should be noted that hop-diffusion at the millisecond timescale has been observed so far only using comparatively large gold particles to label membrane components. Nevertheless, the barrier effect of the cortical cytoskeleton on membrane protein mobility has been detected at longer timescales using other probes and/or techniques. For example, dragging gold particle-labeled class I MHC molecules using an optical trap (Table 1) revealed that these molecules encounter cytoskeletal barriers to their diffusion [36]. More recently, SPT experiments

monitoring the mobility of Fcε [37] or B cell receptors [38] at the single-molecule level while simultaneously imaging actin revealed that the receptors primarily move within membrane areas of lower actin density. Such long-term confinement (Figure 1d), documented for other receptors as well [20], is in the 1–10 s scale.

What allows molecules to hop from one corral to the next? First, both hop-diffusion (with transient confinement in the millisecond scale) and longer-term confinement (in the 1–10 s scale) are the result of interactions between the membrane and the cortical cytoskeleton. Second, cortical actin filament rearrangement is estimated to occur in the 10 s timescale [37–40]. Therefore, at the millisecond scale, the cortical cytoskeleton is expected to be relatively stationary, unable to account for the rapid transitions inherent to hop-diffusion. Rather, rapid hopping between corrals is most likely due to transient detachment of picket proteins from the cytoskeleton, the perchance opening of a gap between two picket proteins due to their limited diffusion while anchored/tethered to the cytoskeleton, or to transient changes in the linkages between actin filaments that generate the submembranous meshwork. On the other hand, changes in the mobility of membrane components in the 1–10 s scale can be directly due to cytoskeletal remodeling. The mechanisms by which the cortical cytoskeleton modulates molecular confinement and mobility in the membrane have not been fully resolved. A better understanding will require further studies not only of membrane lipids and proteins, but also of the cortical cytoskeleton in the relevant time and space scales.

The barriers to molecular diffusion imposed by the cytoskeleton can in turn lead to molecular clustering and nanodomain formation. A recent study of class I MHC demonstrated that the lifetime of MHC clusters depends on the stability of the cortical actin cytoskeleton [41]. Co-confinement within cytoskeleton-mediated compartments for periods of seconds also increases the chance of receptor encounter, thus enhancing epidermal growth factor receptor (EGFR) dimerization [19] and CD36 clustering [20]. This effect of compartmentalization is expected to be critical for regulating immunoreceptor activation and signaling, as discussed below.

The molecular players

Early studies of erythrocytes implicated spectrin and actin in the formation of the meshwork that lines the cytoplasmic side of the membrane, with ankyrin and band 4.1 linking actin/spectrin to membrane proteins [42, 43]. While the spectrin-ankyrin system is most prominent in erythrocytes, these proteins are also implicated in linking CD45 to the actin meshwork [44] and in confining class I MHC molecules [45].

In most other cells, actin is thought to be the key cytoskeletal element forming the cortical meshwork. A recent paper provided evidence for septins contributing to the meshwork as well [46]. Proteins of the same superfamily as band 4.1, namely ezrin, radixin and moesin (ERM proteins) link the membrane to the cortical actin meshwork [47, 48]. The ERM proteins associate with phosphatidylinositol 4,5-*bis*phosphate (PtdIns(4,5)P₂) in the membrane through their N-terminal FERM domain, and with the actin cytoskeleton through their C-terminus. A related protein, merlin, is also thought to provide a membrane-cytoskeleton bridge, although its mechanism is unclear, as it lacks the actin-binding C-terminus of ERM proteins. The ERM proteins and merlin interact with many membrane proteins, in some cases directly and in others indirectly through scaffolding proteins such as ERM-binding phosphoprotein 50 (EBP50). EBP50 itself interacts with some membrane proteins directly, and with others through yet another scaffolding protein, PDZ domain-containing protein 1 (PDZK1). Thus ERM proteins can link the membrane to the cytoskeleton and can anchor and scaffold multiple membrane proteins. They can also contribute to remodeling the actin cytoskeleton through their interactions with Rho

GTPases, upstream of actin polymerization. Membrane proteins recently found to be linked to the cytoskeleton via ERM proteins include the B cell receptor [38], the GPI-anchored protein Thy-1 [28], and the cystic fibrosis transmembrane-conductance regulator [27], among others [47, 48].

Other molecules implicated in linking the actin cortical meshwork to the membrane are filamin, talin, α -actinin and tensin. Filamin bundles actin, thus contributing to the formation of the meshwork, but also binds many cell surface receptors [49, 50]. Talin, α -actinin and tensin mostly bind to integrins at focal adhesions [50], yet can potentially interact with other membrane proteins, as reported recently for the Fce receptor [51].

It should be emphasized that the mechanisms that link most membrane proteins to the cytoskeleton are still unclear. In most studies, the interaction of membrane components with the cytoskeleton was deduced by pharmacologically perturbing actin filament integrity and measuring the effect on molecular mobility. Needless to say, a full understanding of membrane organization will require definition of the molecular mechanisms that link membrane components to the cortical cytoskeleton.

Cytoskeleton-raft crosstalk

Several recent studies suggest that the cortical cytoskeleton influences lipid raft formation and mobility, whereas cholesterol in the membrane, in turn, affects cortical cytoskeleton dynamics. FCS studies have revealed that markers of the liquid-ordered phase, such as a sphingolipid-binding domain or cholera toxin subunit B, are generally less mobile than markers of the liquid-disordered phase [52, 53]. The mobility of such raft markers tends to increase upon actin depolymerization, indicating that the cortical cytoskeleton forms barriers that curtail the movement of raft constituents, presumably via interactions between raft-associated proteins and the cytoskeleton. Furthermore, an immuno-EM study found that raft nanoclusters do not form if the cytoskeleton is depolymerized [54], implying that the cytoskeleton is necessary for lipid raft formation and/or stabilization. Conversely, there is evidence that cholesterol depletion alters the dynamics of the cortical actin meshwork, most likely via the sequestration or redistribution of $\text{PtdIns}(4,5)\text{P}_2$, found in cholesterol-enriched nanodomains [55]. These observations highlight the strong interdependence between the cytoskeleton and the membrane, and the necessity to consider both components when interpreting the results of pharmacological perturbations.

Beyond membrane compartmentalization

Beyond compartmentalization, membrane proteins that associate with the cytoskeleton can get transported across long distances, sometimes while simultaneously altering the organization and dynamics of the cytoskeleton. For example, ligated EGFR dimers get transported with actin retrograde flow from the tips of filopodia to their bases before endocytosis [56]. Conversely, the actin-based motor myosin X transports receptors such as integrins to the tips of filopodia [57]. Actin retrograde flow is also involved in transporting ephrin receptor clusters from the cell periphery to the cell center [58], and is required for the formation of the immunological synapse, where small peripheral receptor and co-receptor aggregates get differentially transported centripetally to form large, concentric supramolecular clusters [59–61]. Notably, in most of these cases, the molecules that bridge the receptors to actin are unknown.

In some cases, mainly in neuronal cells, microtubules also transport surface receptors. GABA receptors activated by an external stimulus attach to the plus-ends of microtubules and get transported anterogradely to the leading edge of the cell, generating an asymmetric calcium signal [62]. SPT studies in neuronal growth cones and fibroblast lamellipodia also

reported that glutamate receptors exhibit directed retrograde movement by associating, probably indirectly, with microtubules, which in turn get transported via actin retrograde flow [63].

Immunoreceptors: an archetype of activation by clustering

Many receptors become activated primarily as a result of transmembrane conformational changes induced by ligand binding. Others, especially immunoreceptors, signal in response to clustering [64–66]: T cell, B cell, Fc ϵ and Fc γ receptors, among others, undergo extensive lateral clustering when exposed to multivalent targets (i.e. particles, such as pathogens, that exhibit on their surface multiple copies of one or more ligands), yielding robust and sustained responses. The effectiveness and specificity of the stimulation are often buttressed by the co-clustering of co-receptors and additional, independent types of receptors. Such extensive, synergistic co-clustering is best exemplified by the immunological synapse between lymphoid and antigen-presenting cells [59–61] and by the phagocytic cup [67].

The need for multimer formation for activation is most acute in cases like that of Fc γ receptors. In unstimulated circulating cells like neutrophils or monocytes, Fc γ receptors must remain quiescent, despite being continuously exposed to high concentrations of monomeric ligands (circulating IgG). Quiescence is a consequence of the low affinity of the receptors for monomeric ligands. However, the receptors' high avidity for multivalent targets, such as IgG-opsonized particles, enables Fc γ receptors to effectively bind to the target, cluster and get activated [65, 66].

For many years, the formation of receptor oligomers was envisaged to occur in a spontaneous and random fashion wherever multivalent targets collide with the cell. This notion, partly derived from the Singer-Nicolson model, entailed several assumptions: i) receptors are homogeneously distributed as monomers, ii) they diffuse freely in the plane of the membrane and iii) they have little inherent tendency to associate with each other in the absence of ligands. These premises have become increasingly questionable, with emerging evidence that several types of immunoreceptors are confined within membrane corrals anchored to the underlying cytoskeleton [37, 38].

Different modes of confinement are expected to have different effects on receptor activation and signaling. In the case of solitary confinement or anchorage of individual receptors, if the period of confinement/anchorage exceeds the time of receptor binding to the multivalent target, formation of receptor clusters is practically impeded (Figure 2a). If the half-life of confinement is, however, comparatively short, this enables receptors to hop-diffuse along the membrane and form clusters, albeit with moderate efficiency (Figure 2b). The success of cluster formation will depend on the rate of hop-diffusion, vis-à-vis the time of residence of the multivalent target in the immediate vicinity of the cell.

It is important to note that receptors need not be confined individually [37]. Co-confinement of multiple receptors within the same nanodomain can have a positive effect on receptor clustering (Figure 2c), as it enhances the likelihood of multiple receptors associating with the target if it collides with a receptor-rich confinement area (Box 1). The scavenger receptor CD36 represents a unique case of co-confinement, where receptor molecules are trapped within actin- and microtubule-delimited domains that are almost unidimensional [20].

The notion that receptors exist as monomeric entities prior to ligand exposure is also being eroded. Experimental and analytical approaches that permit the analysis of receptor interactions *in situ* in live cells indicate that receptors associate with each other in the absence of ligand [5, 19, 20]. Support for the intrinsic tendency of immunoreceptors to

associate with one another also stems from reports of the spontaneous (ligand-independent) formation of B cell receptor oligomers – and the consequent signaling – when their collision ability is enhanced by removal of cytoskeleton-dependent diffusional barriers [38].

A most interesting situation arises when the inherent affinity of receptors to associate is combined with the possibility of co-confinement (Figure 2d). In this scenario, frequent spontaneous collisions between receptors within the same confinement zone would favor the formation of a number of oligomers in the unstimulated resting state [68]. This is predicted to translate into a degree of ‘tonic’ stimulation, which has in fact been reported in several instances [38, 69, 70]. Such tonic stimulation is very modest, because oligomers formed spontaneously are rare, small and short-lived, due to the limited inter-receptor affinity. However, spontaneously formed oligomers increase the efficiency of binding to multivalent targets, translating into a highly effective means of trapping and responding to bona fide stimuli [20, 37]. Clearly, these considerations apply not only to homotypic interactions, but also to interactions between different receptors and co-receptors when their ligands are presented simultaneously on the same target.

How clustering triggers signaling

Immunoreceptor activation and signaling is the ultimate paradigm of positive cooperativity (Box 1). By accumulating receptors in a tight cluster, multivalent targets trigger a highly coordinated series of phosphorylation and dephosphorylation events that are exquisitely sensitive to the local concentration of substrates, kinases and phosphatases [64, 71, 72]. The receptors themselves are the initial targets of phosphorylation at two tyrosine residues, known as the immunoreceptor tyrosine-based activation motif (ITAM). The Src-family kinases (SFKs) Lck, Fyn and Lyn are primarily responsible for ITAM phosphorylation (Figure 3a, b). The phosphorylated ITAMs then recruit downstream Syk or ZAP70 kinases through their Src Homology 2 (SH2) domains. The recruited Syk and ZAP70 in turn get phosphorylated by the SFKs, through which they get activated [71, 73] (Figure 3c).

ITAM phosphorylation at receptor clusters could be triggered by recruitment and/or activation of SFKs, by inhibition and/or displacement of phosphatases, or by exposure of latent ITAM tyrosines. These mechanisms are likely to co-operate in the initiation of signaling. As in the case of GPCRs or insulin receptors, ligand-induced transmembrane conformational changes could trigger the activation of immunoreceptors; indeed, ligand-induced exposure of a proline-rich region has been suggested to promote recruitment of the adaptor Nck via its SH3 domain [64]. However, there is no evidence that Nck fosters ITAM phosphorylation. More importantly, while monomeric ligands such as soluble antigens are unable to activate immunoreceptors, multimeric soluble or immobilized complexes are effective [64]. Thus, receptor clustering in the membrane appears to be the key factor.

SFKs, which do not associate stably with immunoreceptors prior to stimulation (Figure 3a), become an integral part of the signaling cluster upon receptor cross-linking. This has been attributed to incorporation of receptor clusters into rafts where the kinases normally reside due to their saturated acyl chains and their association with acylated co-receptors like CD4 and CD8 [64, 72] (Figure 3b). How the receptors, which at rest are largely excluded from liquid-ordered domains, migrate into rafts following cross-linking remains a mystery, but their recruitment to the vicinity of raft-localized co-receptors by cognate ligands may be a factor.

Because in unstimulated cells SFKs are largely inactive, their activation is required to trigger ITAM phosphorylation. This involves two steps: dephosphorylation of the SFKs' C-terminal inhibitory phosphotyrosine, followed by the intramolecular phosphorylation of a second tyrosine that stabilizes the active conformation (Figure 3a, b). Dephosphorylation of

the inhibitory phosphotyrosine is accomplished by various cytosolic (e.g. PTP1B, Shp1) and transmembrane (e.g. CD45, CD148, PTP α) phosphatases. Of these, CD45 is the most studied and likely the most important. CD45 partitions preferentially into liquid-disordered domains (Figure 3a), where it dephosphorylates the inhibitory phosphotyrosine of SFKs, generating a subpopulation of active kinases that are primed for recruitment into rafts [74]. However, CD45 can also dephosphorylate the activation site of SFKs, and the phosphotyrosines generated by SFKs. Thus, paradoxically, CD45 has both activating and deactivating effects. How are these prioritized? Once again, receptor clustering and lateral segregation hold the key. Because of its size, CD45 is excluded from the liquid-ordered rafts where the cross-linked receptors reside, preventing it from countering the activation of SFKs, at least initially [74, 75]. At later stages, CD45 somehow migrates into the activation zone, where it contributes to signal termination. Therefore, phosphatases play a sequential triple role in immunoreceptor activation/deactivation that is dictated by their initial exclusion and subsequent entry into the activation cluster.

This regulatory scenario is complicated further by changes in the exposure of the substrate tyrosines. There is compelling evidence that, prior to stimulation, the ITAM tyrosines of at least some immunoreceptors are buried in the bilayer, inaccessible to the SFKs [76, 77]. The tyrosines become exposed during activation, and several models have been proposed to account for this [76, 78, 79]. First, ligand binding could induce a conformational change, dislodging the tyrosines from the bilayer. Second, tight clustering of receptors may physically extrude their cytosolic tails from the membrane by displacing the annular lipids, replacing them with receptors. Third, the coalescence of receptors (and co-receptors) may result in modification of the lipid environment surrounding them. In this regard, ITAM tyrosines are often surrounded by cationic residues, which would drive the association of the motif with more negatively-charged areas of the membrane (e.g. regions rich in polyphosphoinositides and/or phosphatidylserine). These lipids may be selectively excluded from receptor complexes or modified (e.g. hydrolyzed) therein, leading to detachment of the ITAM motifs and exposure of the tyrosines.

When considered together, the various elements required to initiate signaling by immunoreceptors highlight the critical importance of their clustering. The physical redistribution of the receptors, co-receptors and associated lipids forces exposure of substrate tyrosines, attracts active SFKs to the vicinity of the receptors, brings downstream kinases like Syk and ZAP70 in contact with their activators (the SFKs), and excludes potentially inhibitory phosphatases from the active complex.

Concluding remarks

We have come to realize that the plasma membrane is a complex organelle, highly organized in both space and time. Many factors contribute to its organization: from protein and lipid interactions within the plane of the membrane, to interactions between membrane components and factors inside and outside the cell. In particular, new experimental and analytical techniques are providing mounting evidence for the cortical cytoskeleton as a major regulator of membrane organization. It is critical for future studies to establish the molecular mechanisms that link the cortical cytoskeleton to the membrane and modulate the mobility and organization of membrane components.

The regulated diffusion and non-uniform distribution of receptors in the membrane most likely play a critical role in receptor signaling, especially in cases like immunoreceptors that require lateral clustering for their activation. In this regard, the cortical cytoskeleton plays at least a dual role: on the one hand, it facilitates the clustering of receptors and downstream effectors to increase the efficiency and robustness of signaling upon multivalent ligand

binding. On the other hand, it separates receptors from each other, minimizing their association and preventing signaling in the absence of ligand. What role the cortical cytoskeleton plays for individual receptors and signaling pathways is an essential question that remains to be determined. It should be borne in mind that receptor signaling itself triggers reorganization of the cytoskeleton, which in turn influences receptor mobility and signaling, a feedback of sorts!

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Box 1**Receptor clustering and signaling: insights derived from modeling**

To the best of our knowledge, to date there are no published modeling efforts investigating specifically the relationship between immunoreceptor clustering in the resting state (i.e. in absence of ligand) and the efficiency of ligand binding and signaling. However, published attempts to model other signaling pathways where receptor oligomerization is necessary for signaling, such as EGFR which dimerizes, and abstract models that investigate particular phenomena that are relevant for immunoreceptors, shed light on the effect of resting-state clustering on immunoreceptor signaling.

In terms of ligand binding, modeling studies indicate that receptor clustering decreases the effective dissociation constant of ligands – even if monovalent – because of enhanced ligand rebinding after detachment due to the increased local density of available receptors [80, 81]. While the case of multivalent ligand/target has not been explicitly investigated, these results predict that immunoreceptor clustering would increase the efficiency of multivalent target binding, as the increased local density of receptors would facilitate multiple, simultaneous receptor-ligand binding events.

Parallel to the effects of receptor clustering on ligand binding, modeling efforts suggest that receptor clustering similarly reduces the effective dissociation constant of downstream effectors, also due to enhanced rebinding [82]. Consequently, receptor clustering enhances signaling if the downstream effector needs multiple sequential modifications to get activated [83]. These results imply that receptor clustering enhances the efficiency of signal transduction whenever cooperativity between different molecular players is required, as is the case for immunoreceptors.

What about the role of the cortical cytoskeleton in clustering receptors? Overall, coupling between the membrane and the cytoskeleton has a profound effect on membrane organization, both spatially and temporally [84, 85]. Simulations suggest that diffusion within corrals might enhance receptor clustering, but only if receptors have a tendency to associate with each other, where the increased size of the receptor complexes would lead to their trapping within corrals [86]. In addition, transient binding of receptors to the cytoskeleton (i.e. anchorage/tethering), coupled to cytoskeletal remodeling, can generate receptor clusters [87]. Cytosolic scaffolds can in fact generate membrane protein clusters whose fraction and size is independent of protein density, a feature that is necessary to maintain a linear response to stimulation [88], which cannot be achieved by interactions between membrane components alone.

Figure 1.

Types of motion of membrane proteins. **a)** Free diffusion, as experienced by a protein in a lipid bilayer. Left panel: 'biophysical' view illustrating the trajectory of a molecule. Each segment of the line indicates the displacement recorded using a fast rate of image acquisition. Right panel: molecular view illustrating the lipids constituting the bilayer (beige; in all panels) and a transmembrane protein (red; in all panels). The arrows indicate the ability of the protein to diffuse in any direction. **b)** Anchorage/tethering, experienced by a protein while directly or indirectly associated with the cortical cytoskeleton. Left panel: 'biophysical' view illustrating that the mobility of a transmembrane protein is restricted to a

limited area (dotted circle) as a result of its attachment to cytoskeletal filaments (gray rods; in all panels). Right panel: molecular view illustrating attachment of the transmembrane protein to the cytoskeleton, in this illustration by association of its cytoplasmic tail to an adaptor linker molecule (blue). Shorter arrows imply restricted mobility. **c**) Hop-diffusion, experienced by a protein temporarily trapped within corrals formed by picket-fences that form on the cytoskeleton. Left panel: 'biophysical' view illustrating that the protein diffuses rapidly within the corral (red lines) but only occasionally escapes from one corral to the next, resulting in eventual long-range displacement, observable at slower rates of image acquisition (black line). Middle panel: illustrates the confinement of a transmembrane protein by a picket-fence constituted by various proteins (all non-red cylinders) that are attached directly or indirectly (via a blue adaptor) to the cytoskeleton. Right panel: transient opening of the fence – either because of detachment of pickets from the cytoskeleton or due to remodeling of the skeleton itself – enables the diffusible protein to escape the corral (gray arrow). **d**) Confined diffusion, experienced by a protein trapped within corrals formed by picket-fences that form on the cytoskeleton. Left panel: 'biophysical' view illustrating that the protein diffuses rapidly within the corral (red lines) but cannot escape (at least during the course of the recording). Right panel: confinement of a diffusible protein by a picket-fence constituted by various other proteins that are attached directly or indirectly (via blue adaptors) to the cytoskeleton. Not shown is the case where the cytoplasmic domain of the transmembrane protein itself bumps into the cortical cytoskeleton, leading to its hop-diffusion (c) or long-term confinement (d).

Figure 2.

Interaction of immunoreceptors with multivalent targets. **a)** Individually confined/anchored receptors. Left panel: 'biophysical' view of receptors with limited diffusion range, indicated by dotted circles (see Figure 1 for details of this and subsequent left panels). Right panel: because the mobility of receptors is limited, clustering by exposure to vicinal ligands (fuchsia circles) on target (blue) is ineffective. **b)** Receptors undergoing hop-diffusion. Left panel: 'biophysical' view of two hop-diffusing receptors. Right panel: hop-diffusing receptors can gradually move along the surface of the membrane, and can therefore undergo target-induced clustering, albeit slowly. **c)** Co-confined or co-anchored receptors. Left panel:

'biophysical' view of multiple receptors restricted to diffuse within individual corrals. Right panel: fast association of several receptors with multivalent target when the latter collides with region of receptor co-confinement. **d**) Co-confined or co-anchored receptors with tendency to self-associate. Left panel: 'biophysical' view of multiple receptors that have the tendency to self-associate transiently and are restricted to diffuse within individual corrals. Right panel: self-associating receptors will bind rapidly and very effectively to multivalent target when the latter collides with region of receptor co-confinement.

Figure 3.

Signal transduction by immunoreceptors. **a)** Resting state. The membrane consists of cholesterol-depleted regions (beige) and cholesterol-enriched nanodomains (rafts; brown). Cholesterol is shown in red. Src-family kinases (SFKs) are anchored to the membrane by acylation and can exist in an unphosphorylated state or be phosphorylated at an inhibitory site (yellow circle labeled P with red perimeter). CD45, a tyrosine phosphatase, can dephosphorylate SFKs, facilitating their activation. Inactivated immunoreceptors (dark blue) reside largely outside rafts. Their tyrosine residues (cyan hexagons labeled Y) constituting the ITAM motif are buried in the bilayer. Co-receptors (red), present mostly in rafts, tend to

associate with SFKs. **b)** Signal initiation. A target (light blue) bearing ligands for the immunoreceptors (green diamonds) and co-receptors (fuchsia circles) cluster immunoreceptors and co-receptors together in the context of rafts, where active SFKs can autophosphorylate (yellow circle labeled P with green perimeter) to stabilize their active state, and can phosphorylate and activate the ITAM tyrosines on immunoreceptors (also yellow circle labeled P with green perimeter). **c)** Signal propagation. Dually phosphorylated tyrosines at the ITAM motif attract tandem SH2 domains of Syk or ZAP70 (S/Z). Recruited Syk or ZAP70 can in turn be tyrosine phosphorylated and activated by SFKs and by autophosphorylation, thereby propagating the signal.

Table 1Imaging techniques that reveal protein mobility, clustering and or interactions^a.

Method	Description and measured molecular behavior	Limitations	Refs ^b
Fluorescence Recovery After Photobleaching (FRAP) (live)	<ul style="list-style-type: none"> – An area is photobleached and the recovery of fluorescence in that area is measured – In most applications, analysis of the recovery vs. time curve yields the immobile fraction of molecules and the diffusion coefficient of the mobile fraction – More sophisticated analysis of the recovery vs. time curve can yield information on binding and interactions 	<ul style="list-style-type: none"> – Measures ensemble average – Assumes that the system is stationary (i.e. molecular behavior does not change) over analysis time window – Not straightforward to apply to complex modes of motion 	[89]
Single-Particle Tracking (SPT) (live)	<ul style="list-style-type: none"> – Molecules labeled at sub-stoichiometric levels (with a fluorescent protein, organic dye, quantum dot, or gold particle) are imaged using time-lapse light microscopy – Most powerful when combined with computer algorithms that reconstruct the trajectories of the imaged molecules – Analysis of the trajectories yields their motion characteristics, potentially revealing spatial heterogeneity and temporal transients – Multi-spectral imaging and/or sophisticated image analysis algorithms can reveal interactions between molecules, such as dimerization or clustering 	<ul style="list-style-type: none"> – Visualizes only a small fraction of molecules – Needs many experiments to cover the full spectrum of molecular behaviors – Tracking can be challenging at high molecular density 	[29, 90, 91]
Förster Resonance Energy Transfer (FRET) (live)	<ul style="list-style-type: none"> – One molecular species is labeled with a 'donor' fluorophore while another molecular species is labeled with an 'acceptor' fluorophore (e.g. CFP and YFP) – Interactions between two molecules are seen as energy transfer between their fluorophores – As a variation, in homoFRET one molecular species is labeled with a fluorophore; homotypic interactions then bring fluorophore molecules together, leading to a loss of fluorescence anisotropy when illuminating with polarized light 	<ul style="list-style-type: none"> – Limited to a maximum of two molecules at a time – Depends critically on the location of the fluorophores relative to the interaction site(s) of the molecules 	[92]
Fluorescence Correlation Spectroscopy (FCS) (live)	<ul style="list-style-type: none"> – The fluctuations in fluorescence intensity (photon count) in a small volume are measured, yielding a fluorescence time series – The autocorrelation function of the fluorescence time series reflects the concentration of labeled molecules and their diffusion and/or flow characteristics – Cross-correlation between the fluorescence time series of two spectrally distinct fluorophores reflects the 	<ul style="list-style-type: none"> – Measures ensemble average – Assumes that the system is stationary over analysis time window – Probes one spatial point at a time – Not straightforward to apply to complex modes of motion 	[93]

Method	Description and measured molecular behavior	Limitations	Refs ^b
	<ul style="list-style-type: none"> interactions between the labeled molecules – Combining FCS with STED allows monitoring molecular behavior in smaller volumes, enhancing sensitivity 		
Image Correlation Spectroscopy (ICS) (live)	<ul style="list-style-type: none"> – The ‘imaging analog’ of FCS – Image correlation in space reveals cluster density and degree of aggregation – Image correlation in time reveals diffusion and/or flow characteristics for slowly moving molecules (where FCS fails) – Cross-correlation between two channels reveals the degree of co-clustering and interactions between the labeled molecules 	<ul style="list-style-type: none"> – Measures ensemble average – Assumes that the system is stationary over analysis time window – Insensitive to movements and interactions below the resolution limit 	[94]
Particle Image Correlation Spectroscopy (PICS) (live)	<ul style="list-style-type: none"> – A ‘combination’ of SPT and ICS: molecules are detected in each image, and their positions are then correlated in space and time to extract their mobility characteristics and degree of clustering/co-clustering 	<ul style="list-style-type: none"> – Measures ensemble average – Assumes that the system is stationary over analysis time window 	[95]
Number and Brightness (N&B) analysis (live)	<ul style="list-style-type: none"> – The intensity mean and variance in each image pixel over several frames are analyzed to extract the average number of molecules and their brightness per pixel – The intensity cross-variance between two channels yields the stoichiometry of molecules in complexes 	<ul style="list-style-type: none"> – Assumes that the system is stationary over analysis time window 	[96]
Immuno-Electron Microscopy (Immuno-EM) (fixed)	<ul style="list-style-type: none"> – Molecules in membrane sheets on an EM grid are labeled with gold-conjugated antibodies and then imaged – Spatial statistical analysis of the resulting molecule positions (e.g. using Ripley’s K-function) reveals the degree of molecular clustering (or exclusion) – Labeling and imaging two molecular species simultaneously with gold particles of different size allows the analysis of their co-clustering 	<ul style="list-style-type: none"> – Yields only spatial information – Fixation might perturb protein distribution/clustering 	[97]
Single-molecule localization microscopy (fixed/live)	<p>In fixed cells:</p> <ul style="list-style-type: none"> – Molecules are labeled with photo-activatable/convertible fluorophores which are stochastically ‘turned on’ and imaged; localization of individual fluorophores in many images allows the reconstruction of a ‘super-resolution’ compound image – The resulting molecular positions can be analyzed with spatial statistical analysis methods <hr/> <p>In live cells:</p> <ul style="list-style-type: none"> – Essentially SPT, taking advantage of fluorophore photoactivation/conversion 	<ul style="list-style-type: none"> – Fixation might perturb protein distribution/clustering – Fluorophore ‘blinking’ complicates counting number of molecules – Analysis in live cells generally assumes that the system is stationary over analysis time window 	[98, 99]

Method	Description and measured molecular behavior	Limitations	Refs ^b
	to visualize sparse molecule subsets at a time; the accumulation of molecule trajectories over many activation cycles then yields many more data points per cell than SPT with other labeling strategies		
Atomic Force Microscopy (AFM) (fixed/live)	<ul style="list-style-type: none"> – A surface is scanned using a cantilever probe and the force exerted on it is measured as a readout of surface topography, with nanometer resolution – Recent developments in high speed AFM might allow application to live cells to provide new insights into the dynamics of cell surface processes 	<ul style="list-style-type: none"> – Lacks molecular specificity – The dynamic range required for cell surface imaging limits the acquisition speed 	[100]
Optical tweezers (live)	<ul style="list-style-type: none"> – A molecule labeled with a gold particle is trapped in an optical tweezer and moved across the cell surface – Analysis of the distance moved by the molecule before escaping the trap sheds light on the barriers in its path or its anchorage/tethering 	<ul style="list-style-type: none"> – Requires labeling with relatively large gold particles – Does not reveal 'natural' mobility of molecules – Low number of data points 	[36]

^a An elegant graphical depiction of the length and time scales probed by many of these techniques can be found in [101].

^b Recent reviews, where available.