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Nanodomains in biological membranes

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Abstract

Lipid rafts are defined as cholesterol- and sphingomyelin-enriched membrane domains in the plasma membrane of cells that are highly dynamic and cannot be resolved with conventional light microscopy. Membrane proteins that are embedded in the phospholipid matrix can be grouped into raft and non-raft proteins based on their association with detergent-resistant membranes in biochemical assays. Selective lipid–protein interactions not only produce heterogeneity in the membrane, but also cause the spatial compartmentalization of membrane reactions. It has been proposed that lipid rafts function as platforms during cell signalling transduction processes such as T-cell activation (see Chapter 13 (pages 165–175)). It has been proposed that raft association co-localizes specific signalling proteins that may yield the formation of the observed signalling microclusters at the immunological synapses. However, because of the nanometre size and high dynamics of lipid rafts, direct observations have been technically challenging, leading to an ongoing discussion of the lipid raft model and its alternatives. Recent developments in fluorescence imaging techniques have provided new opportunities to investigate the organization of cell membranes with unprecedented spatial resolution. In this chapter, we describe the concept of the lipid raft and alternative models and how new imaging technologies have advanced these concepts.

Keywords:

cholesterol, cortical actin, lipid raft, high-resolution fluorescence microscopy, super-resolution fluorescence microscopy, T-cell receptor signalling.

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Introduction

Unlike pure lipid bilayers, cell membranes are not homogenous and have a heterogeneous distribution of lipids and proteins. For example, membrane lipids and proteins are asymmetrically distributed between the exoplasmic and cytoplasmic leaflet of the plasma membrane. GPI (glycosylphosphatidylinositol)-anchored proteins and the majority of sphingolipids are found in the outer leaflet of the plasma membrane. Cholesterol and glycerophospholipids such as phosphatidylserine and phosphatidylinositol are preferentially localized in the intracellular leaflet [1,2]. Within the membrane, heterogeneities in protein distribution and restricted diffusion of glycosphingolipids and membrane proteins have also been reported [3–5], suggesting a lateral membrane organization with protein clusters, lipid domains and membrane compartments.

Membrane models

Various membrane models have been proposed to account for the non-random distribution of proteins and lipids in the plasma membrane of eukaryotes that encompass homo-dimers [6], multi-molecular complexes [7], protein clusters of 5–10 nm in diameter [8,9], protein islands of approximately 70–140 nm in size [10], and confinement zones of 120–250 nm diameter [11], in which proteins are temporarily arrested.

The lipid raft hypothesis

The lipid raft hypothesis was originally formulated to explain the sorting of glycosphingolipids to the apical membrane of polarized epithelial cells [12]. Simons and van Meer [13] hypothesized that the preferential association of glycosphingolipids and cholesterol causes a lateral segregation in the lipid bilayer, which leads to the formation of membrane domains as a sorting platform for lipids. Indeed, a lipid analysis of post-Golgi vesicles in yeast revealed that glycerophospholipids are segregated from glycosphingolipids and cholesterol during trafficking to the plasma membrane [14]. The concept was later expanded to the plasma membrane and membrane proteins. In 1997, Simons and Ikonen [15] proposed that cell membranes are segregated into cholesterol- and sphingomyelin-enriched microdomains termed lipid rafts. The lipid raft model is a modification of the fluid mosaic model that was proposed by Singer and Nicolson in the 1970s [16]. In the latter, the lipid bilayer was viewed as a simple two-dimensional fluid in which membrane proteins could diffuse freely in the lateral dimension (Figure 1A).

In the lipid raft model, the phase behaviour of different lipid species is exploited to create lateral heterogeneity in the plasma membrane [15]. According to this hypothesis, the L_d (liquid disordered) phase contains mainly unsaturated phospholipids and coexists in the membrane with a L_o (liquid ordered) phase that forms from the association of saturated phospholipids, sphingolipids and cholesterol. Such phase segregation results in lipid rafts of high viscosity and membrane order that diffuse as distinct entities within the membrane. Membrane proteins with affinity for the L_o phase are thereby enriched in lipid rafts, whereas proteins with preference for L_d phases are excluded (Figure 1B). In this manner, lipid rafts can laterally sort membrane proteins.

Lipid rafts are often viewed as platforms for cell signal transduction processes. Owing to lipid modifications on proteins such as palmitoylation and GPI anchors, many signalling proteins partition thermodynamically into membranes that resemble lipid rafts biochemically and

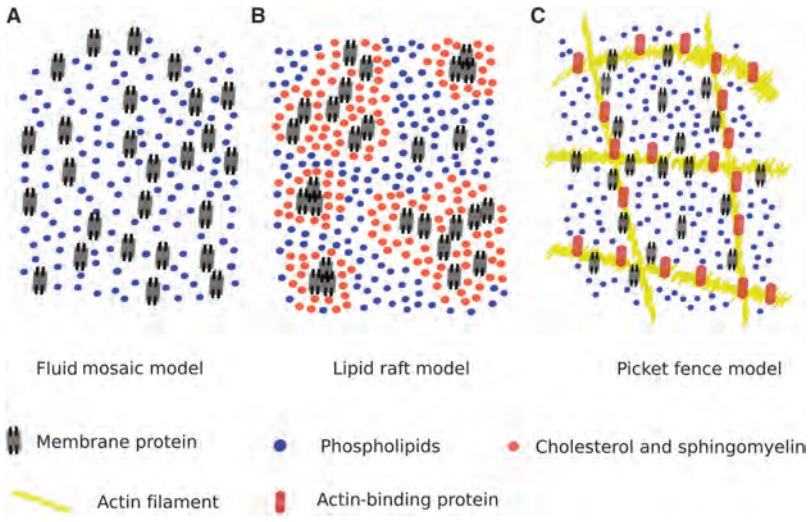

Figure 1. Models for membrane organization
(**A**) The fluid mosaic model. Membrane proteins and lipids undergo random two-dimensional diffusion within the bilayer. The membrane is not compartmentalized and no lipid or protein domains exist. (**B**) The lipid raft model. Membrane proteins partition into cholesterol- and sphingomyelin-enriched lipid domains, which are highly ordered and distinct entities in the 'sea' of fluid phospholipids. (**C**) The picket fence model. Transmembrane proteins can act as 'pickets' by binding to underlying actin filaments. The lateral diffusion of other membrane proteins is hindered by the membrane-associated actin network, which creates a fence-like barrier along the actin network.

biophysically [17,18]. Given the close proximity of proteins in lipid rafts, membrane domains and protein partitioning can physically isolate membrane reactions and thus control which signalling processes can occur. For example, the TCR (T-cell receptor) may be associated with non-raft domains when not bound to antigens and only associate with lipid rafts upon receptor ligation [19]. In contrast, the tyrosine kinase LCK that phosphorylates the TCR is constitutively associated with lipid rafts in the intracellular leaflet owing to palmitoylation and myristoylation groups. Hence, localization of the TCR in non-raft regions of the plasma membrane may prevent TCR–LCK interactions in resting cells. Upon receptor ligation, the association of both the TCR and LCK with lipid rafts would enable efficient signalling [20]. In this manner, lipid rafts also enable the transmission of an extracellular cue – the antigen – into an intracellular signal.

As discussed below in the experimental evidence section, one has to consider that phase partitioning is not absolute, so that a given protein is unlikely to be completely and only associated with raft or non-raft domains. In fact, partitioning preference implies that a greater fraction of the protein is associated with the preferred lipid phase and not that the protein cannot associate with the other phase. In terms of signalling, this means that other mechanisms must exist in addition to raft partitioning to control signalling activity. For TCR signalling, such mechanisms could be the spatial segregation of the phosphatase CD45 from the ligated TCR [21] and the conformational states of Lck that regulate Lck activity and clustering [22].

The picket fence model

Although the lipid raft model proposed by Simons and Ikonen [15] has been included in many textbooks, other models have been proposed to account for membrane compartmentalization.

One such model that found widespread attention is the picket fence model. The picket fence model was proposed to resolve the long-standing dilemma of molecular dynamics in cell membranes. The diffusion of both membrane lipids and proteins is substantially slower in the plasma membrane compared with artificial membranes. The picket fence model proposed that the plasma membrane is compartmentalized by membrane proteins and cortical actin, which hinders the diffusion of membrane molecules [23].

Kusumi et al. [23] suggested that the lateral movement of membrane molecules is hindered by a 'picket fence' (Figure 1C). In this model, membrane proteins that are tightly bound to the cortical actin network constitute the pickets, whereas membrane proteins that collide with these anchored proteins or the actin network itself form permeable fences in the membrane. Lipids and proteins within these compartments can diffuse freely, but crossing the compartment boundaries is more difficult and hence less frequent. The idea of the picket fence model is based on the observation that membrane molecules display non-linear 'hop diffusion', the spontaneous crossing of boundaries, depending on their location in the cell membrane [3,4,24]. The diffusion coefficient of unrestricted membrane molecules was identical in cell and artificial membranes, whereas a fraction displayed restricted diffusion in cells [24]. Drug-induced actin depolymerization increased the size of the confinement, and decreased the frequency of hop diffusion [24].

The hopping rate of membrane proteins is substantially reduced when proteins are clustered. This reduction is severalfold higher than what would be expected from the standard diffusion law when proteins of different molecular weight are compared. Kusumi et al. [23] proposed that hop diffusion of a protein cluster is highly restricted, as it requires all the proteins in the cluster to hop across the cytoskeleton boundary at the same time. A consequence of the model is that clustering of receptors, for example upon ligand binding, renders them spatially immobilized at the activation site. This may aid the recruitment of intracellular signalling proteins to these locations because cells have sufficient time to re-polarize the trafficking machinery and deliver signalling proteins to the site of engaged receptor clusters.

The picket fence model is mainly based on single particle tracking experiments where two-dimensional trajectories of membrane components are recorded [3,4,24]. However, it has to be taken into account that the plasma membrane in intact cells is not a flat sheet, but rather has a complicated surface topology including undulations and invaginations [25,26]. Two-dimensional projections of single molecule data obtained in membranes with three-dimensional topographies can lead to an artificial increase in protein clustering [27] or immobilization. Hence, we may discover that membrane compartmentalization by the actin network may be more complicated than the picket fence model proposes.

The active actin aster model

Recently a very different model of membrane organization was proposed by Gowrishankar et al. [28]. The authors suggested that the cell membrane is organized in an ATP-dependent 'active' manner. The formation of membrane nanoclusters of GPI-anchored proteins is actively driven by association with the underlying cortical actin cytoskeleton (Figure 2A). An early study by the same researchers used high-resolution fluorescence imaging to show that GPI-anchored proteins exist as monomers and nanoclusters, where surprisingly, the fraction of nanoclusters was concentration independent [29], a result that does not fit with thermodynamic predictions. In the following investigation, Goswami et al. [9] showed that the assembly of GPI-anchored

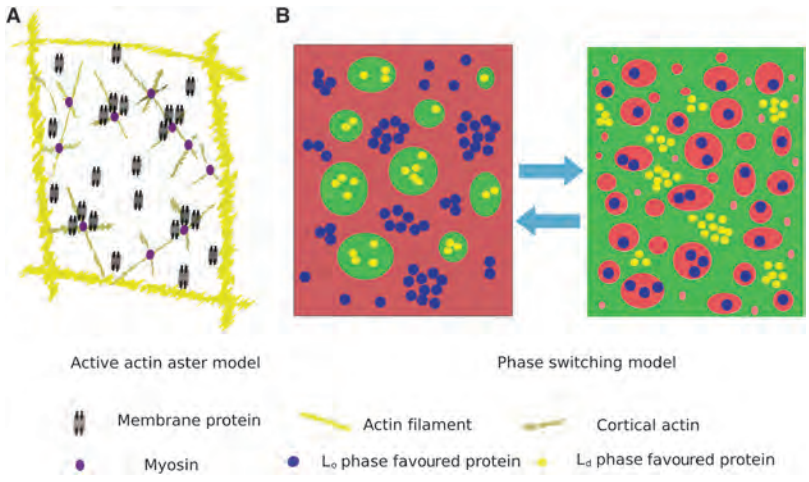

Figure 2. New models of membrane organization with limited experimental evidence
(**A**) The active actin aster model. Membrane proteins exist as monomers or small, transient clusters depending on their association with short and dynamic actin snippets that are arranged into asters by myosin motors. Active and passive linkages between actin asters and membrane proteins result in protein clustering. (**B**) The phase switching model. The plasma membrane is comprised of L_o phases (red regions) and L_d phases (green regions). Under subtle changes in physiological conditions, the L_o phase can switch from a continuous phase that surrounds the islands of the L_d phase (left panel) to a discontinuous phase with the L_d phase becoming the continuous percolating phase (right panel). Formation of lipid domains in either L_o or L_d phase induces a physical barrier for the interaction and diffusion of membrane proteins. For example, interactions between putative raft proteins (blue dots) are permitted in the continuous phase (left panel) but are largely inhibited when the L_o phases form discrete islands (right panel).

protein nanoclusters is dynamically correlated to cortical actin. Dissociation from cortical actin disassembled clusters into monomers, whereas repolymerization of actin led to the recovery of protein clusters. Theoretical modelling by Gowrishankar et al. [28] predicted that there are two populations of actin species: short and dynamic actin fibres and a longer and stable actin meshwork. Myosin motors on the short actin snippets cause the formation of asters to which membrane proteins can be actively or passively linked. This linkage can result in the dynamic clustering of proteins, including GPI-anchored proteins. However, the existence of actin asters needs to be confirmed and the nature of the linkage requires further investigations.

The phase switching model

The quantification of L_o and L_d phases in live HeLa cells by Owen et al. [30] revealed that the L_o phase covered ~76% of the plasma membrane, and the disordered phase covered the remaining ~24%. The result was obtained by FLIM (fluorescent lifetime imaging microscopy) using the membrane order-sensitive dyes Laurdan and di-4-ANEPPDHQ, which have longer lifetimes in L_o than L_d phases [31,32]. By using the phasor approach to FLIM analysis [33], it was found that the lifetime distribution that originated from the cell plasma membrane was a linear combination of the pure ordered phase and pure disordered phase. The fractional contribution of each component was directly determined from the phasor plot. The data indicate the coexistence of both L_o and L_d phases in the plasma membrane and suggest that the plasma membrane is not a homogenous phase of intermediate order [30].

Considering the active role of the actin cytoskeleton in the regulation of membrane protein dynamics and membrane order [34], Owen and Gaus [35] have proposed a phase switching model (Figure 2B), where the L_o phase could switch from a continuous percolating phase to discontinuous islands under subtle physiological stimulation such as actin restructuring. Membrane proteins in the continuous percolating phase can interact and form clusters through protein–protein interactions, but switching the percolating phase to the 'island' phase introduces phase boundaries and may prevent interactions. Inversely, proteins with a strong partitioning preference for the 'island' phase would appear clustered but disperse upon phase switching. A study using GPMVs (giant plasma membrane vesicles) previously described how membranes close to the so-called critical point undergo dramatic changes in lipid phases although the variations in conditions, for example temperature, were relatively minor [36]. However, it should be noted that phase switching has not yet been directly observed in cell membranes.

Experimental evidence for lipid rafts and other membrane models

Although the lipid raft model has been exceedingly popular, it has also attracted controversy due to the difficulties in defining lipid rafts experimentally. The controversy focuses mainly on two aspects: the definition of raft markers and the size of lipid rafts. Traditionally, proteins that were found in DRMs (detergent-resistant membranes) were thought of as raft markers [37]. For example, palmitoylation of integral proteins was thought to regulate association with DRMs [38]. However, recent biophysical studies of membrane proteins showed that putative raft markers could display non-raft behaviours. For example, a fluorescent protein fused to the raft-anchoring motif of the tyrosine kinase LCK displayed remarkably different clustering and spatial confinement from full-length LCK, which indicates that forces additional to raft anchoring, probably protein–protein interactions, must be involved in LCK membrane organization [7,22]. The second aspect of confusion is the small size and highly dynamic nature of lipid rafts. In published papers, raft sizes range from less than 10 nm to over 200 nm [9,39,40], and some papers report fast assembly and disassembly rates with raft lifetimes ranging from milliseconds to seconds [40,41]. Hence it has been difficult to find a consensus for an experimental definition of lipid rafts.

Membrane proteins and lipids used to be assessed as lipid raft markers by biochemical extraction with detergents such as Triton X-100 at low temperature [15]. In these assays, DRMs are typically isolated by gradient centrifugation and often contain high levels of cholesterol and sphingomyelin and only a subset of membrane proteins, which were thus considered to be lipid raft components. This procedure was established when the hallmark study by Brown and Rose [42] described the increasing insolubility of proteins as they traffic through the Golgi apparatus to the plasma membrane in polarized epithelial cells. Early biochemical studies of T-cells using this method have identified signalling components involved in T-cell activation in the DRM fractions including LCK and LAT (linker for activation of T-cells) [19] (for an overview of T-cell activation, see Figure 3). An example of the confusion with DRMs is the palmitoylation of LAT. When these groups were removed, LAT was no longer associated with DRMs and palmitoylation-deficient LAT impaired T-cell activation [43]. However, later reports showed that palmitoylation-deficient LAT mutants did not traffic to the plasma

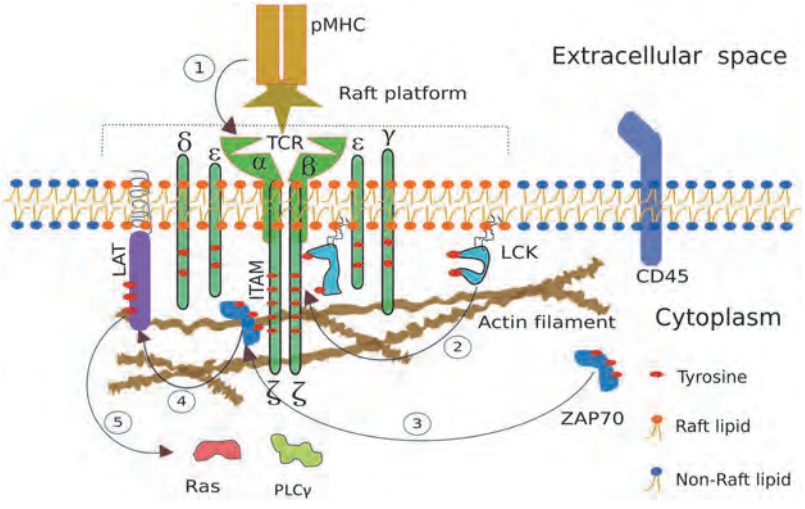

Figure 3. Sequential events involved in T-cell activation

(**1**) The T-cell signalling cascade is initiated by the physical interaction between an antigenic peptide-loaded major histocompatibility complex (pMHC) molecule and the exoplasmic α-β heterodimer of the TCR complex. (**2**) The ligand–receptor interaction causes the phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic tails of CD3ξ and CD3ζ chains that are constitutively associated with TCR α-β dimers [76]. The exact mechanism for this process is still unclear. There are two states of LCK: an open conformation that is enzymatically active and a closed conformation that is inactive. The fraction of the active form is not increased substantially during T-cell activation [77,78], suggesting that ITAM phosphorylation by LCK is regulated by the change in lipid composition upon TCR engagement [79,80] and/or by the spatial reorganization of LCK upon T-cell activation [22]. (**3**) The phosphorylated TCR complex provides docking sites for the tyrosine kinase Zap70, which becomes activated by phosphorylation. (**4**) Activated Zap70 targets LAT for phosphorylation. (**5**) LAT can recruit several downstream intracellular signalling proteins such as Ras and phospholipase Cγ (PLCγ) to the activation site that lead to the induction of key signalling pathways, including Ca^{2+} signalling, mitogen-activated protein kinase (MAPK) and nuclear factor κB [81]. These signalling processes are required for gene regulation, cytokine secretion, T-cell proliferation and differentiation.

membrane [44]. Hence the reason for the impairment of T-cell activation was not the lack of raft targeting but the low expression level at the plasma membrane. The validity of the detergent extraction method has since been questioned because it was found that different detergents and detergent concentrations could substantially alter the distribution of proteins in the DRMs and soluble fractions, which suggests the possibility that lipid and protein re-association occurred during the extraction process [45,46].

Recent MS data showed enrichment of raft lipids at the T-cell activation site [47]. Depletion of cholesterol is often used to test whether lipid rafts are involved in a given cellular process. However, in T-cells, cholesterol depletion has been reported to disrupt or aggregate lipid rafts and impair or induce T-cell activation, respectively, depending on the level of depletion [48,49]. It should also be considered that cholesterol depletion with methyl-β-cyclodextrin can cause side effects on cells such as impaired actin cytoskeleton organization, clathrin-mediated endocytosis [50] and inhibition of the lateral movement of membrane proteins [51].

The main support for the existence of lipid rafts is derived from observations of phase transitions in model membrane systems [52]. Liposomes of phospholipids undergo phase

transitions as the temperature is raised above the transition point. The transition usually occurs from a gel-like solid phase to a liquid-like disordered phase. In the gel phase, because the acyl chains of the lipids are tightly packed together, both the lateral translocations of lipids and molecular vibration of the acyl chain are highly restricted. In contrast, in the L_d phase, the acyl chains are conformationally relaxed, and the translational diffusion of lipids is unrestricted in this phase. Interestingly, in the presence of cholesterol, a second liquid phase can separate from the L_d phase [52], known as the L_o phase. The addition of cholesterol to the L_d phase leads to the formation of a structurally more ordered phase due to a higher rigidity of the acyl chains, but the lipid mobility is similar to that in the L_d phase. Because the lateral translocation of lipid molecules is permitted, the L_o phase is regarded as a fluid phase.

In a lipid mixture that contains a certain proportion of unsaturated phospholipids, sphingomyelin and cholesterol, the L_o and L_d phases can coexist [53]. It is believed that cell membranes behave in a similar fashion, where lipid rafts are equivalent to the L_o phase and surrounded by an L_d phase composed of unsaturated phospholipids [54]. In cells, membrane order and the fraction of the L_o phase can be efficiently reduced with the oxysterol 7KC (7-ketocholesterol) [30,55]. In 7KC, the additional ketone group protrudes perpendicular from the planar cholesterol rings. Fluid membranes can accommodate the ketone groups, but the lipid packing in highly ordered membranes such as L_o phases is disrupted. Rentero et al. [55] showed that membrane condensation at the immunological synapse was inhibited by the substitution of cholesterol with 7KC, which also impaired T-cell activation.

A key part of the lipid raft model is the segregation of membrane proteins into distinct microdomains according to their affinity for raft lipids [15]. Such spatial compartmentalization of membrane proteins is crucial for the high sensitivity and fidelity of cell signalling processes. Studies examining lipid and protein partitioning in model membranes and GPMVs have supported the lipid raft hypothesis [56–58]. It was shown that lipid/lipid unmixing during phase separation could segregate GPI-anchored and palmitoylated proteins into distinctive domains. The diffusion kinetics of the putative raft proteins was substantially slower than their non-raft counterparts [56]. Similar observations were made in cell membranes, revealing the clustering [59] and confined diffusion of the putative raft proteins [60]. The latter study showed that GPI-anchored proteins had the highest viscous drag compared with transmembrane and non-raft proteins. Although the lipid composition is much more complex in cell membranes, it is generally assumed that the same lipid biochemistry and thermodynamics operate in model and cell membranes.

Owing to the difficulties associated with identifying lipid rafts biochemically and translating measurements from model to cell membranes, the actual existence of lipid rafts in cell membranes is still debated. One promising avenue of investigation is the development of lipid-phase-sensitive fluorescent dyes [61], particularly in combination with single molecule imaging. Compared with the traditional biochemical analysis, fluorescence microscopy is minimally invasive, as it does not cause substantial disturbance to the cell membrane under investigation. However, the intense laser power and prolonged illumination that are often required for high-resolution techniques can lead to oxidative stress, which could damage lipids and cause a reorganization of the membrane. Because advanced imaging techniques can detect a small number of molecules, they can probe the underlying heterogeneity of membrane organization that is inaccessible by conventional imaging techniques. With the continuing advances in spatial and temporal resolution of imaging techniques, more details on diffusion kinetics and lipid domain assembly and disassembly will emerge.

The future direction for studying membrane organization

The recently developed far-field super-resolution imaging techniques, such as PALM (photo-activated localization microscopy) [62], STORM (stochastic optical reconstruction microscopy) [63] and STED (stimulated emission depletion) microscopy [64], have truly shifted the resolution limit of fluorescence imaging techniques and allow quantitative imaging of molecular structures that are 10–100 nm in size. The details of these techniques have been reviewed previously [65,66] and are summarized in Figure 4.

Using a pair correlation analysis on PALM data, Sengupta et al. [67] observed distinct clusters of different membrane proteins. The authors showed that GPI-anchored proteins were clustered in domains of <60 nm in size with an average of three proteins per cluster. In contrast,

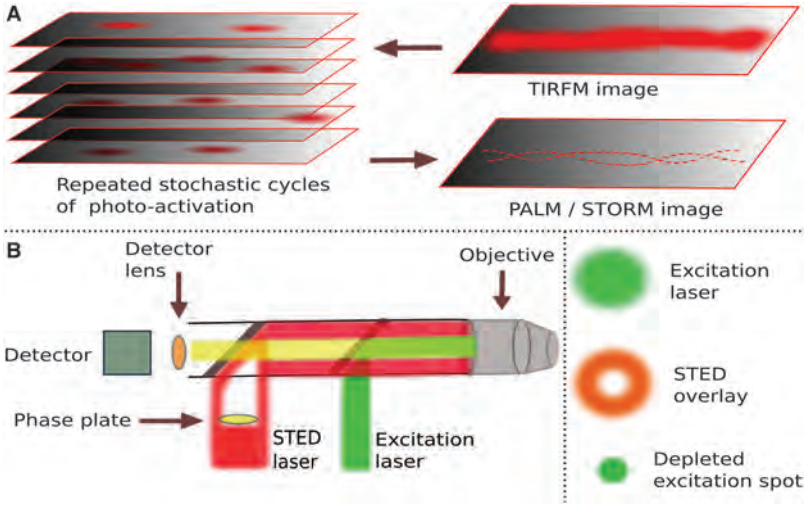

Figure 4. Principles of PALM/STORM and STED microscopy

(**A**) In PALM and STORM, the sample is labelled with photo-activatable or photo-convertible fluorophores at high density. In contrast with the traditional imaging regime, where all of the fluorophores are excited at the same time, only a sparse subset of molecules are activated in a stochastic manner in PALM and STORM. The intensity of the activation laser is tuned so that in each diffraction-limited spot there is less than one emitter on average. The spatial co-ordinates of each molecule are retrieved from the intensity profile of each emitter. During the imaging process, fluorophores are photobleached and a new set of fluorophores are converted, imaged and bleached. After repeating this cycle thousands of times, the molecular co-ordinates of all emitters are combined. This provides diffraction-unrestricted information about the distribution of proteins with nanometre precision. (**B**) STED microscopy improves conventional confocal laser-scanning microscopy, where the sample is scanned with an excitation laser. STED introduces a second fluorescence emission depletion laser that reduces the excitation spot of the conventional excitation laser. The spectrally red-shifted depletion laser returns the excited fluorophores back into the ground state without emission of fluorescence. Because of the insertion of a phase plate, the depletion laser is doughnut shaped. It switches off the fluorophores in the peripheral region only, not in the centre. As a consequence, only the fluorescence emitted from fluorophores in the geometric centre of the excitation laser is registered in each pixel. Because STED is built on laser-scanning microscopes, it can be combined with point measurement techniques such as FCS. TIRFM, total internal reflection fluorescence microscopy.

clusters of LAT and the inner leaflet, lipid-anchored protein Lyn varied in sizes and densities. Gudheti et al. [68] examined the clustering mechanisms of the transmembrane protein HA (haemagglutinin) using PALM. Clustering of HA positively correlated with the distribution of the underlying cortical actin filament, and disrupting actin altered the clustering of HA. On the other hand, the actin-binding protein cofilin was spatially excluded from the protein cluster of HA, which indicated that clustering of HA influenced actin organization. Apart from revealing the spatial distributions of molecules of interest, two-colour PALM has been employed to study the stoichiometry of membrane receptors at the single molecule level [69]. By labelling the receptor subunit with spectrally different photo-activatable dyes, the number of each subunit was retrieved by sequential activation of fluorophores in separate channels. The relative abundance of the fluorophores provides stoichiometric information of the receptor complex.

With the accessibility of the spatial distribution of molecules at nanometer resolution scales, Rossy et al. [22] showed that the clustering of LCK was determined by its conformational states and not by its association with raft lipids or protein networks. Similarly, PALM and STORM analysis of LAT revealed that there was an increased density of LAT clusters at the T-cell activation sites. Surprisingly, newly formed LAT clusters were not constructed from the lateral recruitment of LAT monomers, but instead assembled by LAT subsynaptic vesicle docking to the activation sites [70]. Another report using super-resolution imaging to examine LAT clustering showed that clustering was regulated by both protein–protein interactions and protein–lipid interactions [71].

In addition to the increase in spatial resolution, STED microscopy has the unique advantage of reducing the excitation spot, which opens the possibility of probing fluorescence in high-density samples. This enables STED to be coupled with dynamic live cell imaging methods such as FCS (fluorescence correlation spectroscopy). FCS is a fluctuation-based method that is widely used to study the diffusive behaviour of molecules in live cells. In particular, FCS can extract the molecular concentration and diffusion constants of a population of molecules from the fluctuations in a detected fluorescence signal that arise from molecules passing through the excitation spot. The recent combination of STED and FCS [72,73] thus allows the quantification of highly dynamic membrane processes such as the trapping of lipids in domains. A seminal STED-FCS study by Eggeling et al. [40] revealed the hindered diffusion of sphingomyelin and GPI-anchored proteins that could not be resolved with conventional confocal FCS. The authors showed that these putative raft markers were transiently trapped in 20-nm large domains with dwell times of approximately 10–20 ms. In contrast, the phosphoglycerolipids such as DOPE (dioleoylphosphtidylethanolamine) displayed a uniform diffusion constant of approximately 0.5 $\mu m^2 \cdot s^{-1}$. A recent STED-FCS study of artificial lipid bilayers with coexisting lipid phase reported the size of the L_o phase ranging from 40 nm to 300 nm [74]. This novel approach thus confirms previous studies that probed the coexistence of L_o and L_d phases with FRET measurements in model membranes [75]. As FRET cannot visualize L_o domains *per se*, STED imaging may resolve the long-standing question of lipid raft size in cell membranes.

Conclusion

Over the past decade, many researchers have examined the organization of cell and model membranes using a range of biochemical and biophysical techniques. However, there is still

no definite picture of the structure and dynamics of cell membranes. Despite the differences between the various models that have been proposed, it is generally accepted that the cell membrane is highly dynamic and contains heterogeneities at the nanometre scale. Model membranes have taught us about phase transition and the coexistence of L_o and L_d phases. However, cellular membranes are more complex and the underlying actin cytoskeleton may actively or passively organize lipids and proteins in the membrane. Given the nanometre size and highly dynamic nature of membrane domains in cell membranes, sophisticated new imaging approaches with high spatiotemporal resolution may unravel the contribution of protein–lipid, protein–protein and lipid–lipid interactions in the organization of cell membranes.

Summary

- Cell membranes are organized in a non-homogeneous and highly dynamic manner. Different membrane models have been proposed to explain the existence of lipid domains, protein clusters and membrane compartmentalization.
- Biochemical and biophysical approaches have been used to provide experimental evidence for the various membrane models, often producing conflicting data. A long-standing issue is the inability to directly visualize lipid domains in cell membranes.
- High- and super-resolution microscopy overcome the diffraction limit and promise new insights into membrane biology. It is anticipated that these imaging methods will reveal new and unprecedented details about the spatial and temporal heterogeneity of cell membranes.

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References

1. Verkleij, A.J. and Post, J.A. (2000) Membrane phospholipid asymmetry and signal transduction. J. Membr. Biol. **178**, 1–10
2. Mondal, M., Mesmin, B., Mukherjee, S. and Maxfield, F.R. (2009) Sterols are mainly in the cytoplasmic leaflet of the plasma membrane and the endocytic recycling compartment in CHO cells. Mol. Biol. Cell **20**, 581–588
3. Murase, K., Fujiwara, T., Umemura, Y., Suzuki, K., Iino, R., Yamashita, H., Saito, M., Murakoshi, H., Ritchie, K. and Kusumi, A. (2004) Ultrafine membrane compartments for molecular diffusion as revealed by single molecule techniques. Biophys. J. **86**, 4075–4093
4. Sako, Y. and Kusumi, A. (1994) Compartmentalized structure of the plasma membrane for receptor movements as revealed by a nanometer-level motion analysis. J. Cell Biol. **125**, 1251–1264
5. Hakomori, S., Handa, K., Iwabuchi, K., Yamamura, S. and Prinetti, A. (1998) New insights in glycosphingolipid function: ‘glycosignaling domain’, a cell surface assembly of glycosphingolipids with signal transducer molecules,involved in cell adhesion coupled with signaling. Glycobiology **8**, xi–xix

6. Suzuki, K.G., Kasai, R.S., Hirosawa, K.M., Nemoto, Y.L., Ishibashi, M., Miwa, Y., Fujiwara, T.K. and Kusumi, A. (2012) Transient GPI-anchored protein homodimers are units for raft organization and function. Nat. Chem. Biol. **8**, 774–783
7. Douglass, A.D. and Vale, R.D. (2005) Single-molecule microscopy reveals plasma membrane microdomains created by protein–protein networks that exclude or trap signaling molecules in T cells. Cell **121**, 937–950
8. Plowman, S.J., Muncke, C., Parton, R.G. and Hancock, J.F. (2005) H-ras, K-ras, and inner plasma membrane raft proteins operate in nanoclusters with differential dependence on the actin cytoskeleton. Proc. Natl. Acad. Sci. U.S.A. **102**, 15500–15505
9. Goswami, D., Gowrishankar, K., Bilgrami, S., Ghosh, S., Raghupathy, R., Chadda, R., Vishwakarma, R., Rao, M. and Mayor, S. (2008) Nanoclusters of GPI-anchored proteins are formed by cortical actin-driven activity. Cell **135**, 1085–1097
10. Lillemeier, B.F., Mortelmaier, M.A., Forstner, M.B., Huppa, J.B., Groves, J.T. and Davis, M.M. (2010) TCR and Lat are expressed on separate protein islands on T cell membranes and concatenate during activation. Nat. Immunol. **11**, 90–96
11. van Zanten, T.S., Cambi, A., Koopman, M., Joosten, B., Figdor, C.G. and Garcia-Parajo, M.F. (2009) Hotspots of GPI-anchored proteins and integrin nanoclusters function as nucleation sites for cell adhesion. Proc. Natl. Acad. Sci. U.S.A. **106**, 18557–18562
12. van Meer, G., Stelzer, E.H., Wijnaendts-van-Resandt, R.W. and Simons, K. (1987) Sorting of sphingolipids in epithelial (Madin-Darby canine kidney) cells. J. Cell Biol. **105**, 1623–1635
13. Simons, K. and van Meer, G. (1988) Lipid sorting in epithelial cells. Biochemistry **27**, 6197–6202
14. Klemm, R.W., Ejsing, C.S., Surma, M.A., Kaiser, H.J., Gerl, M.J., Sampaio, J.L., de Robillard, Q., Ferguson, C., Proszynski, T.J., Shevchenko, A. and Simons, K. (2009) Segregation of sphingolipids and sterols during formation of secretory vesicles at the trans-Golgi network. J. Cell Biol. **185**, 601–612
15. Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes. Nature **387**, 569–572
16. Singer, S.J. and Nicolson, G.L. (1972) The fluid mosaic model of the structure of cell membranes. Science **175**, 720–731
17. Brown, D.A. (2006) Lipid rafts, detergent-resistant membranes, and raft targeting signals. Physiology (Bethesda) **21**, 430–439
18. Gaus, K., Chklovskaia, E., Fazekas de St Groth, B., Jessup, W. and Harder, T. (2005) Condensation of the plasma membrane at the site of T lymphocyte activation. J. Cell Biol. **171**, 121–131
19. Montixi, C., Langlet, C., Bernard, A.M., Thimonier, J., Dubois, C., Wurbel, M.A., Chauvin, J.P., Pierres, M. and He, H.T. (1998) Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. EMBO J. **17**, 5334–5348
20. Xavier, R., Brennan, T., Li, Q., McCormack, C. and Seed, B. (1998) Membrane compartmentation is required for efficient T cell activation. Immunity **8**, 723–732
21. James, J.R. and Vale, R.D. (2012) Biophysical mechanism of T-cell receptor triggering in a reconstituted system. Nature **487**, 64–69
22. Rossy, J., Owen, D.M., Williamson, D.J., Yang, Z. and Gaus, K. (2013) Conformational states of the kinase Lck regulate clustering in early T cell signaling. Nat. Immunol. **14**, 82–89
23. Kusumi, A., Ike, H., Nakada, C., Murase, K. and Fujiwara, T. (2005) Single-molecule tracking of membrane molecules: plasma membrane compartmentalization and dynamic assembly of raft-philic signaling molecules. Semin. Immunol. **17**, 3–21
24. Fujiwara, T., Ritchie, K., Murakoshi, H., Jacobson, K. and Kusumi, A. (2002) Phospholipids undergo hop diffusion in compartmentalized cell membrane. J. Cell Biol. **157**, 1071–1081
25. Adler, J., Shevchuk, A.I., Novak, P., Korchev, Y.E. and Parmryd, I. (2010) Plasma membrane topography and interpretation of single-particle tracks. Nat. Methods **7**, 170–171
26. Parmryd, I. and Onfelt, B. (2013) Consequences of membrane topography. FEBS J. **280**, 2775–2784

27. Owen, D.M., Williamson, D.J., Boelen, L., Magenau, A., Rossy, J. and Gaus, K. (2013) Quantitative analysis of three-dimensional fluorescence localization microscopy data. Biophys. J. **105**, L05–L07
28. Gowrishankar, K., Ghosh, S., Saha, S., C, R., Mayor, S. and Rao, M. (2012) Active remodeling of cortical actin regulates spatiotemporal organization of cell surface molecules. Cell **149**, 1353–1367
29. Sharma, P., Varma, R., Sarasij, R.C., Ira, Gousset, K., Krishnamoorthy, G., Rao, M. and Mayor, S. (2004) Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. Cell **116**, 577–589
30. Owen, D.M., Williamson, D.J., Magenau, A. and Gaus, K. (2012) Sub-resolution lipid domains exist in the plasma membrane and regulate protein diffusion and distribution. Nat. Commun. **3**, 1256
31. Parasassi, T., De Stasio, G., Ravagnan, G., Rusch, R.M. and Gratton, E. (1991) Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence. Biophys. J. **60**, 179–189
32. Jin, L., Millard, A.C., Wuskell, J.P., Dong, X., Wu, D., Clark, H.A. and Loew, L.M. (2006) Characterization and application of a new optical probe for membrane lipid domains. Biophys. J. **90**, 2563–2575
33. Digman, M.A., Caiolfa, V.R., Zamai, M. and Gratton, E. (2008) The phasor approach to fluorescence lifetime imaging analysis. Biophys. J. **94**, L14–L16
34. Dinic, J., Ashrafzadeh, P. and Parmryd, I. (2013) Actin filaments attachment at the plasma membrane in live cells cause the formation of ordered lipid domains. Biochim. Biophys. Acta **1828**, 1102–1111
35. Owen, D.M. and Gaus, K. (2013) Imaging lipid domains in cell membranes: the advent of super-resolution fluorescence microscopy. Front. Plant Sci. **4**, 503
36. Veatch, S.L., Cicuta, P., Sengupta, P., Honerkamp-Smith, A., Holowka, D. and Baird, B. (2008) Critical fluctuations in plasma membrane vesicles. ACS Chem. Biol. **3**, 287–293
37. Simons, K. and Toomre, D. (2000) Lipid rafts and signal transduction. Nat. Rev. Mol. Cell Biol. **1**, 31–39
38. Arni, S., Keilbaugh, S.A., Ostermeyer, A.G. and Brown, D.A. (1998) Association of GAP-43 with detergent-resistant membranes requires two palmitoylated cysteine residues. J. Biol. Chem. **273**, 28478–28485
39. Lillemeier, B.F., Pfeiffer, J.R., Surviladze, Z., Wilson, B.S. and Davis, M.M. (2006) Plasma membrane-associated proteins are clustered into islands attached to the cytoskeleton. Proc. Natl. Acad. Sci. U.S.A. **103**, 18992–18997
40. Eggeling, C., Ringemann, C., Medda, R., Schwarzmann, G., Sandhoff, K., Polyakova, S., Belov, V.N., Hein, B., von Middendorff, C., Schonle, A. and Hell, S.W. (2009) Direct observation of the nanoscale dynamics of membrane lipids in a living cell. Nature **457**, 1159–1162
41. Brameshuber, M., Weghuber, J., Ruprecht, V., Gombos, I., Horvath, I., Vigh, L., Eckerstorfer, P., Kiss, E., Stockinger, H. and Schutz, G.J. (2010) Imaging of mobile long-lived nanoplatforms in the live cell plasma membrane. J. Biol. Chem. **285**, 41765–41771
42. Brown, D.A. and Rose, J.K. (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell **68**, 533–544
43. Zhang, W., Trible, R.P. and Samelson, L.E. (1998) LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. Immunity **9**, 239–246
44. Hundt, M., Harada, Y., De Giorgio, L., Tanimura, N., Zhang, W. and Altman, A. (2009) Palmitoylation-dependent plasma membrane transport but lipid raft-independent signaling by linker for activation of T cells. J. Immunol. **183**, 1685–1694
45. Schuck, S., Honsho, M., Ekroos, K., Shevchenko, A. and Simons, K. (2003) Resistance of cell membranes to different detergents. Proc. Natl. Acad. Sci. U.S.A. **100**, 5795–5800

46. Magee, A.I. and Parmryd, I. (2003) Detergent-resistant membranes and the protein composition of lipid rafts. Genome Biol. **4**, 234
47. Zech, T., Ejsing, C.S., Gaus, K., de Wet, B., Shevchenko, A., Simons, K. and Harder, T. (2009) Accumulation of raft lipids in T-cell plasma membrane domains engaged in TCR signalling. EMBO J. **28**, 466–476
48. Kabouridis, P.S., Janzen, J., Magee, A.L. and Ley, S.C. (2000) Cholesterol depletion disrupts lipid rafts and modulates the activity of multiple signaling pathways in T lymphocytes. Eur. J. Immunol. **30**, 954–963
49. Mahammad, S., Dinic, J., Adler, J. and Parmryd, I. (2010) Limited cholesterol depletion causes aggregation of plasma membrane lipid rafts inducing T cell activation. Biochim. Biophys. Acta **1801**, 625–634
50. Subtil, A., Gaidarov, I., Kobylarz, K., Lampson, M.A., Keen, J.H. and McGraw, T.E. (1999) Acute cholesterol depletion inhibits clathrin-coated pit budding. Proc. Natl. Acad. Sci. U.S.A. **96**, 6775–6780
51. Goodwin, J.S., Drake, K.R., Remmert, C.L. and Kenworthy, A.K. (2005) Ras diffusion is sensitive to plasma membrane viscosity. Biophys. J. **89**, 1398–1410
52. Ipsen, J.H., Karlstrom, G., Mouritsen, O.G., Wennerstrom, H. and Zuckermann, M.J. (1987) Phase equilibria in the phosphatidylcholine-cholesterol system. Biochim. Biophys. Acta **905**, 162–172
53. de Almeida, R.F., Fedorov, A. and Prieto, M. (2003) Sphingomyelin/phosphatidylcholine/cholesterol phase diagram: boundaries and composition of lipid rafts. Biophys. J. **85**, 2406–2416
54. Edidin, M. (2003) The state of lipid rafts: from model membranes to cells. Annu. Rev. Biophys. Biomol. Struct. **32**, 257–283
55. Rentero, C., Zech, T., Quinn, C.M., Engelhardt, K., Williamson, D., Grewal, T., Jessup, W., Harder, T. and Gaus, K. (2008) Functional implications of plasma membrane condensation for T cell activation. PLoS ONE **3**, e2262
56. Kahya, N., Brown, D.A. and Schwille, P. (2005) Raft partitioning and dynamic behavior of human placental alkaline phosphatase in giant unilamellar vesicles. Biochemistry **44**, 7479–7489
57. Dietrich, C., Bagatolli, L.A., Volovyk, Z.N., Thompson, N.L., Levi, M., Jacobson, K. and Gratton, E. (2001) Lipid rafts reconstituted in model membranes. Biophys. J. **80**, 1417–1428
58. Levental, I., Lingwood, D., Grzybek, M., Coskun, U., and Simons, K. (2010) Palmitoylation regulates raft affinity for the majority of integral raft proteins. Proc. Natl. Acad. Sci. U.S.A. **107**, 22050–22054
59. Zacharias, D.A., Violin, J.D., Newton, A.C. and Tsien, R.Y. (2002) Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. Science **296**, 913–916
60. Pralle, A., Keller, P., Florin, E.L., Simons, K. and Horber, J.K. (2000) Sphingolipid-cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells. J. Cell Biol. **148**, 997–1008
61. Kwiatek, J.M., Owen, D.M., Abu-Siniyeh, A., Yan, P., Loew, L.M. and Gaus, K. (2013) Characterization of a new series of fluorescent probes for imaging membrane order. PLoS ONE **8**, e52960
62. Betzig, E., Patterson, G.H., Sougrat, R., Lindwasser, O.W., Olenych, S., Bonifacino, J.S., Davidson, M.W., Lippincott-Schwartz, J. and Hess, H.F. (2006) Imaging intracellular fluorescent proteins at nanometer resolution. Science **313**, 1642–1645
63. Rust, M.J., Bates, M. and Zhuang, X. (2006) Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). Nat. Methods **3**, 793–795
64. Hell, S.W. and Wichmann, J. (1994) Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. Opt. Lett. **19**, 780–782
65. Hell, S.W., Dyba, M. and Jakobs, S. (2004) Concepts for nanoscale resolution in fluorescence microscopy. Curr. Opin. Neurobiol. **14**, 599–609

66. Huang, B., Babcock, H. and Zhuang, X. (2010) Breaking the diffraction barrier: super-resolution imaging of cells. Cell **143**, 1047–1058
67. Sengupta, P., Jovanovic-Talisman, T., Skoko, D., Renz, M., Veatch, S.L. and Lippincott-Schwartz, J. (2011) Probing protein heterogeneity in the plasma membrane using PALM and pair correlation analysis. Nat. Methods **8**, 969–975
68. Gudheti, M.V., Curthoys, N.M., Gould, T.J., Kim, D., Gunewardene, M.S., Gabor, K.A., Gosse, J.A., Kim, C.H., Zimmerberg, J. and Hess, S.T. (2013) Actin mediates the nanoscale membrane organization of the clustered membrane protein influenza hemagglutinin. Biophys. J. **104**, 2182–2192
69. Renz, M., Daniels, B.R., Vamosi, G., Arias, I.M. and Lippincott-Schwartz, J. (2012) Plasticity of the asialoglycoprotein receptor deciphered by ensemble FRET imaging and single-molecule counting PALM imaging. Proc. Natl. Acad. Sci. U.S.A. **109**, E2989–E2997
70. Williamson, D.J., Owen, D.M., Rossy, J., Magenau, A., Wehrmann, M., Gooding, J.J. and Gaus, K. (2011) Pre-existing clusters of the adaptor Lat do not participate in early T cell signaling events. Nat. Immunol. **12**, 655–662
71. Sherman, E., Barr, V., Manley, S., Patterson, G., Balagopalan, L., Akpan, I., Regan, C.K., Merrill, R.K., Sommers, C.L., Lippincott-Schwartz, J. and Samelson, L.E. (2011) Functional nanoscale organization of signaling molecules downstream of the T cell antigen receptor. Immunity **35**, 705–720
72. Kastrup, L., Blom, H., Eggeling, C. and Hell, S.W. (2005) Fluorescence fluctuation spectroscopy in subdiffraction focal volumes. Phys. Rev. Lett. **94**, 178104
73. Mueller, V., Honigmann, A., Ringemann, C., Medda, R., Schwarzmann, G. and Eggeling, C. (2013) FCS in STED microscopy: studying the nanoscale of lipid membrane dynamics. Methods Enzymol. **519**, 1–38
74. Honigmann, A., Mueller, V., Hell, S.W. and Eggeling, C. (2013) STED microscopy detects and quantifies liquid phase separation in lipid membranes using a new far-red emitting fluorescent phosphoglycerolipid analogue. Faraday Discuss. **161**, 77–89; discussion 113–150
75. Heberle, F.A., Wu, J., Goh, S.L., Petruzielo, R.S. and Feigenson, G.W. (2010) Comparison of three ternary lipid bilayer mixtures: FRET and ESR reveal nanodomains. Biophys. J. **99**, 3309–3318
76. Palacios, E.H. and Weiss, A. (2004) Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. Oncogene **23**, 7990–8000
77. Paster, W., Paar, C., Eckerstorfer, P., Jakober, A., Drbal, K., Schutz, G.J., Sonnleitner, A. and Stockinger, H. (2009) Genetically encoded Förster resonance energy transfer sensors for the conformation of the Src family kinase Lck. J. Immunol. **182**, 2160–2167
78. Nika, K., Soldani, C., Salek, M., Paster, W., Gray, A., Etzensperger, R., Fugger, L., Polzella, P., Cerundolo, V., Dushek, O. et al. (2010) Constitutively active Lck kinase in T cells drives antigen receptor signal transduction. Immunity **32**, 766–777
79. Xu, C., Gagnon, E., Call, M.E., Schnell, J.R., Schwieters, C.D., Carman, C.V., Chou, J.J. and Wucherpfennig, K.W. (2008) Regulation of T cell receptor activation by dynamic membrane binding of the CD3ε cytoplasmic tyrosine-based motif. Cell **135**, 702–713
80. Gagnon, E., Schubert, D.A., Gordo, S., Chu, H.H. and Wucherpfennig, K.W. (2012) Local changes in lipid environment of TCR microclusters regulate membrane binding by the CD3ε cytoplasmic domain. J. Exp. Med. **209**, 2423–2439
81. Balagopalan, L., Coussens, N.P., Sherman, E., Samelson, L.E. and Sommers, C.L. (2010) The LAT story: a tale of cooperativity, coordination, and choreography. Cold Spring Harb. Perspect. Biol. **2**, a005512