Title:

Closing the gap: The approach of optical and computational microscopy to uncover biomembrane organization

Authors and Affiliations:

Christian Eggeling[1], Alf Honigmann[2,*]

[1] MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, Headley Way, OX3 9DS Oxford, United Kingdom

[2] Max-Planck-Institute of Molecular Cell Biology and Genetics, Pfotenhauerstr. 108, 01307 Dresden, Germany

[*]To whom correspondence should be addressed: Alf Honigmann, honigmann@mpi-cbg.de

Abstract:

Biological membranes are complex composites of lipids, proteins and sugars, which catalyze a myriad of vital cellular reactions in a spatiotemporal tightly controlled manner. Our understanding of the organization principles of biomembranes is limited mainly by the challenge to measure distributions and interactions of lipids and proteins within the complex environment of living cells. With the recent advent of super-resolution optical microscopy (or nanoscopy) one now has approached the molecular scale regime with non-invasive live cell fluorescence observation techniques. Since in silico molecular dynamics (MD) simulation techniques are also improving to study larger and more complex systems we can now start to integrate live-cell and in silico experiments to develop a deeper understanding of biomembranes. In this review we summarize recent progress to measure lipid-protein interactions in living cells and give examples how MD simulations can complement and upgrade the experimental data.

1. Introduction:

1.1. Membrane organization

The cellular plasma membrane is the interface between the outside environment and the internal cytoplasm of the cell (Fig. 1). In this way, it can be seen as a protective barrier of the cell. However, it is more than that, since it is critically involved in many important cellular activities and a variety of cellular functions are localized at this interface zone for example signaling and sensing, selective transport of matter, cell adhesion or electrical action potential propagation. These functions result from the spatiotemporal organization of proteins and lipids into distinct complexes. The current challenge is to unravel the molecular interactions and rearrangements in the plasma membrane, and extract the underlying organization principles to understand how cellular function emerges (for example [1]–[4]). High-resolution mass spectroscopy has enabled to catalogue the composition of cellular membranes in great detail (lipidomics, proteomics and glycomics). Additionally, the list of (membrane)-protein structures solved by Xray-diffraction, Nuclear Magnet Resonance (NMR) or Cryo-Electron-Microscopy (EM) continues to grow. Despite this progress, we still have problems establishing the connection between composition, structure and biological function. For example, it is still largely unclear why cells require thousands of different lipid species. How are biological functions robustly controlled and organized in such complex composite structures as cellular membranes? To answer these questions new approaches are required that allow dissecting molecular interactions in live cell membranes and integrate the results into a simulation framework that ideally reconstitutes biological structures and functions *in silico* based on physical interactions (bottom up). This review is structured in three parts. In the first part we will introduce some of the experimental and simulation methods that have shaped our current view of membrane organization. In the second part we will explain the recent advancements in the field of optical nanoscopy, a method that allows resolving fluorescently labeled structures with near molecular resolution in an almost non-invasive manner. In the last part we will discuss some of the current frontiers of membrane research, which require a combination of experimental and simulation approaches.

1.2. Experimental Methods

Many different methods have been applied to investigate or model the organization of molecules in the plasma membrane of (living) cells. We can broadly group the approaches into three categories:

1) The 'omics' approach strives to identify and quantify the molecular components of membranes, e.g. protein-, lipid-, and sugar species. For example, the vast complexity of the eukaryotic lipidome, i.e. of the lipid composition of the cellular plasma membrane as well as the differential composition of organelle membranes, has been successfully established by high-resolution mass spectrometry [5], [6]. Additionally, mass spectrometry in combination with genetic and biological tools allows creating metabolic interaction networks as well as membrane protein interactomes [7], [8]. Mapping the lipid-protein interactome is not yet fully possible. However, the development of photo-cross-linkable lipid analogues is a promising direction towards this goal [9]. Overall, these tools have greatly advanced our knowledge about membrane composition and have the potential to build up an unbiased comprehensive interaction network of membrane components.

2) The structural biology approach tries to work out the ultra-structure of membranes and its components usually with tools that are incompatible with live-cell observations. Methods such as X-ray diffraction, NMR and Cryo-EM provide us with detailed insights into protein folding and conformations as well as lipid bilayer packing. Unfortunately, these methods are restricted to purified proteins or simple membrane reconstitutions or in the case of cryo-EM require fixation of cells (freezing). Still, these methods are indispensible for understanding membranes as they build the foundation for a molecular understanding and are the input for molecular dynamics simulations.

3) To analyze membranes in the complex environment of living cells optical far-field microscopy is usually the method of choice, since it allows observation of structures and dynamics in an almost non-invasive manner. Specificity is achieved by fluorescence labeling of the molecule of interest. Unfortunately, the full potential of far-field fluorescence microscopy is limited by constraints in spatial and temporal

resolution. The temporal resolution, i.e. the speed with which an image is recorded, determines whether the movements of membrane molecules or their spatial distribution into, for example, transient clusters may be accurately captured. Yet, the acquisition speed of the detectors, such as cameras, the scanning speed in case of raster-scanning microscopes such as the confocal device, as well as the microscopes' detection efficiency and fluorescent labels' light emission are all restricted and therefore limit the temporal resolution of any far-field fluorescence microscope to milliseconds to seconds. The spatial resolution of far-field fluorescence microscopy is on the other hand limited by diffraction to about 200 nm for visible light [10]. Consequently, many cellular features such as molecular assemblies in the plasma membrane appear blurred in the microscope image, since they are of smaller size. Additionally, molecular assemblies such as complexes or clusters can sometimes not be resolved in space and time, since the involved molecules might distribute evenly between an un-clustered and clustered state, giving no contrast between the two states in the final fluorescence image. Fortunately, over the years novel tools have been developed to tackle these limitations. Spectroscopic tools such as FCS (fluorescence correlation spectroscopy) [11], [12] enable the observation of molecular diffusion and interaction dynamics with down to below millisecond time resolution, and even over space if combined with fast beam-scanning or camera recordings [13]–[17]. Complementarily, single-particle tracking (SPT) allows disclosing those dynamics by following single isolated fluorescently-tagged molecules over space and time, with a millisecond time resolution that is only limited by the acquisition speed of the detectors (e.g. camera) (see for example [18], [19]). Enforcing its capabilities, the latter limitation has for example been tackled by interferometric scattering microscopy (iSCAT) [20]. Super-resolution fluorescence microscopy approaches such as STED (stimulated emission depletion [21]), RESOLFT (reversible switchable optical light fluorescence transition [22]–[24]), PALM (photoactivation light microscopy [25], [26]), (d)STORM ((direct) stochastic reconstruction microscopy [27], [28]) or GSDIM (ground state depletion microscopy followed by individual molecule return [29]) and resolution-improved approaches such as 4-Pi/I^{5}M [30], [31] or SIM (structured

illumination microscopy [32], [33]) have enabled live-cell imaging with spatial resolution below 200 nm, partially even down to the macromolecular scale (for a recent review see for example [34]). Obviously, the combination of both, i.e. FCS- and SPT-based super-resolution approaches such as STED-FCS [35], [36] or PALM-SPT [37] are tremendously valuable tools for investigating molecular plasma membrane organizations [38], [39].

1.3. Why we need simulations

Even though great progress has been made to measure membrane structure and dynamics with higher spatiotemporal resolution, our current understanding of the self-organization principles in biological membranes is still fragmentary. The main reasons are: 1) The best light microscopy methods are able to observe only up to three or four molecular species simultaneously and often only at sparse labeling conditions. Consequently, over 99% of the molecules are invisible in the measurement, and interactions have to be inferred indirectly from the behavior of reporter molecules. In the context of membranes there is an additional bias towards protein-protein interactions because we have no functional method to label lipids and other small molecules. 2.) Measuring dynamics in membranes is key to reveal organization mechanisms. However, in fluorescence microscopy high spatial and temporal resolution are often not achievable at the same time. 3) Like all biological systems, bio-membranes are multi-scale systems where function emerges at different hierarchical levels across space and time. Consequently, building a bottom up (from molecules to cells) model of cell membranes requires information over the complete spectrum (fast to slow and small to large) and its integration into a multi-scale model.

Overcoming all these limitations on the experimental level will require a major breakthrough in the way we infer information via light matter interactions, which is beyond our current abilities. However, the progress of *in silico* simulations methods offers a great opportunity to look beyond the current limitations of optical microscopy. In analogy to optical microscopy, simulations can be seen as

computational microscopy with practically no resolution limit in space and time [40].

In contrast to the experimental limitations molecular dynamics (MD) simulations reveal the behavior of all molecules in a system in great detail and time resolution. Unfortunately, this comes at the cost of very limited system sizes (thousands molecules) for only short time periods (microseconds) [40], [41]. In this regard the computational microscope has the inverse problem of optical microscopy, namely resolution is limited to reveal only fast and nanoscopic processes instead of slow and large processes that are accessible by optical microscopy. Figure 2 shows the current gap between experimental and simulation techniques. To close this gap on the simulation side, different methods have been developed (such as coarse grain) to extend the spatiotemporal scale of MD-simulation without scarifying too much of the molecular details [42], [43].

MD-simulations have long been successfully employed to study lipid-lipid and lipid-protein interactions [44]–[46]. The molecular details that can be visualized for processes such as formation of lipid/protein domains in complex membranes [47], [48], membrane fusion [49], ion-channel function [50] are examples that demonstrate the power of the computational microscope, whose spatiotemporal resolution is unrivaled by no other method. However, any simulation method ultimately demands adaptation with experimental data. Ideally, this adaptation should be as direct as possible, thus requiring a spatiotemporal overlap between simulation and experiment. As MD simulations are scaling up and optical microscopy is scaling down a direct comparison is becoming possible. Such comparison is the premise to bridge the scales and to gain the full understanding on how complex membrane components self-organize into functional complexes and structures.

In the following we will present the current advances of super-resolution microscopy (specifically of STED microscopy) and how we combine it with fluorescence correlation spectroscopy to increase spatiotemporal resolution. Finally, we will discuss some of the current frontiers in membrane biology and give

examples how the combination of simulations and experiments has advanced knowledge in the field.

2. STED-FCS

2.1. STED microscopy

For reaching nanoscale resolution in diffraction-unlimited super-resolution microscopy, the fluorescence label has to be prepared with two states of different emission properties (such as a dark and a bright state). Separation of nearby object in an image is then realized by an optically driven transfer between these states which allows switching on and off the signal detected from those objects in space and time [51]. The first of such super-resolution fluorescence microscopes or nanoscopes was based on stimulated emission microscopy (Stimulated Emission Depletion, STED) [21], [52]. In its most preferred implementation, a laser is added to a conventional scanning (confocal) far-field microscope, which forces the fluorescent labels to their dark ground state, i.e. inhibits fluorescence emission (Fig. 3). The wavelength of this second laser is tuned to the red edge of the fluorophore's emission spectrum and induces the stimulated de-excitation of the fluorophore's excited electronic state, i.e. the transition from the fluorescent ON state (the electronically excited state) to the dark OFF state (the ground state). By detecting only the spontaneous (and not the stimulated) emission, the registered signal is efficiently decreased and completely switched off when increasing the intensity of the STED laser above a certain threshold. The introduction of a phase plate into the STED beam distorts its wave-front and, once focused by the microscope objective, creates an intensity distribution which features one or several local zeroes, such as a doughnut-shaped intensity distribution (Fig. 3). While this intensity pattern is still ruled by diffraction, large STED laser intensities force the area in which fluorescence emission is still allowed to sub-diffraction scales. An important feature of the STED microscope is that the size of its observation spot and thus its spatial resolution is tuned by the power or intensity of the STED laser thus allowing imaging with in principle unlimited spatial resolution (Fig. 3). In cellular applications, usually down to below 60 nm resolution has been achieved [53]. Remaining issues such as

potentially enhanced photobleaching or phototoxicity are currently tackled by interdisciplinary involvements: optimized fluorescent labels (chemistry and molecular biology) [54], improved laser and detection technology (physics and engineering) [55], enhanced image acquisition approaches (such as ultra-fast scanning [56] or parallelized acquisition modes [57], physics and engineering), refined data analysis (computation) [58] as well as adapting the technology from experiences in cellular applications (biology) [53].

2.2. Fluorescence Correlation Spectroscopy (FCS)

In FCS, the temporal fluctuations of the observed fluorescence signal is monitored over time as labelled molecules diffuse in and out of the observation area or volume (for example, given by the micrometer large focal laser spot of a confocal microscope), and the correlation function of these fluctuations is calculated [11], [12] (Fig. 4A,B). In the case of diffusion, the decay time of this correlation function usually renders the average transit time of the molecules through the observation area, which in turn allows calculation of a value of a diffusion coefficient. In case of free Brownian diffusion, this diffusion coefficient resembles the real diffusion constant of the molecule. However, it only resembles an apparent coefficient in the presence of hindrances or anomalies. Such anomalies usually result in a shift of the correlation curve towards larger times and the stretching of the decay. While for data analysis such curve stretching may be covered by the introduction of an anomaly coefficient, this will not give an answer on the causes of anomaly, which may stem from transient clustering, domain incorporation or other hindrances in diffusion occurring on very small spatial scales << 200 nm. Due to the diffraction limit, conventional FCS experiments will thus average over such nanoscopic hindrances. This issue has been addressed in spot-variation FCS (svFCS), where correlation data is recorded for different sizes of the observation area above the diffraction limit and the resulting dependency of the average transit time through the observation spot used to obtain more detailed information of anomalous diffusion modes [59], [60]. Unfortunately, this assignment is only realized by an extrapolation to <200 nm (sub-diffraction) sized areas, and further details of the

molecular dynamics, such as molecular interaction times or cluster areas, can only be estimated [61].

2.3. FCS with STED recordings

The STED microscope realizes unique possibilities for FCS recordings. Since the size of the observation spot can straightforwardly be tuned by the intensity of the STED laser (Fig. 4C), the principle of svFCS can be used to record and analyze FCS data for different STED intensities to determine the details of the hindrances in molecular diffusion. In contrast to the diffraction-limited svFCS data, STED-FCS can now directly study these molecular diffusion dynamics at the relevant spatial scales by plotting the apparent diffusion coefficient from diffraction-limited regions down to < 50 nm observation spot diameters [36], [38], [62]. This is highlighted in figure 4, which depicts this dependency as expected for different diffusion modes (Fig. 4D): 1) Free diffusion: The dependency remains constant, since the transit time will scale with the size of the observation area. 2) Trapped diffusion: A transient interaction with immobilized or slow moving binding partners, i.e. a transient trap or stop in the diffusion path will result in an ongoing decrease of the apparent diffusion coefficient towards small observation spot sizes, since the transit time will more and more be dominated by the trapping time. 3) Domain incorporation: Similarly to transient trapping, a transient incorporation into a domain or complex in which diffusion is still possible but slowed-down will result in a decrease of the apparent diffusion coefficient towards small observation spot sizes but with a kind of levelling off and even increase in values for spot diameters smaller than the diameter of the domains [63], [64]. A similar trend is expected for a transient interaction with an only slightly slower moving molecule, with the levelling off scaling with the difference in diffusion coefficient of the free and bound state, as well as the interaction length [39]. 4) Hop diffusion: An increase of the apparent diffusion coefficient towards smaller observation spot sizes indicates so-called hop diffusion in a meshwork-like environment [65]. While diffusion is free inside the compartments of the meshwork, crossing from one compartment to the next is perturbed (but not totally inhibited), e.g. by a fence-like structure. Such hindrance

may be evoked by the cortical actin cytoskeleton underlying the plasma membrane of living cells, with transmembrane proteins linked to the cytoskeleton establishing pickets and fences [18] or inducing strong membrane curvature [66]. As a consequence, small observation spots dominantly probe the fast diffusion inside the compartments, while a traverse through large observation spots is significantly slowed down due to the hindrances in the diffusion path. Figure 4E,F shows exemplary experimental data for fluorescent phospholipid or sphingolipid analogs, depicting the actual occurrence of such diffusion modes in the living cell. Combined with appropriate data analysis, STED-FCS then allows an accurate determination of trapping times, on- and off-rates of interactions or meshwork sizes and hopping probabilities [62], [65], [67].

Recent advances in STED-FCS have realized testing changes of these diffusion modes over time and space. While STED-FCS in combination with gated detection (STED-FLCS, fluorescence lifetime correlation spectroscopy) has allowed the recording of the dependency of the apparent diffusion coefficient within one 10-15 s measurement and thus to disclose temporal heterogeneity in diffusion modes [68], STED-FCS data recorded during fast beam scanning exposes spatial heterogeneity in diffusion [38]. Figure 5 depicts such scanning STED-FCS recordings for the fluorescent sphingolipid analog, which highlight transient trapping sites. Taking into account the whole information content from the FCS data, i.e. average transit time as well as average number of fluorescent lipids per observation spot, along with the comparison between differently labeled analogs, these measurements demonstrated that the trapping sites were not due to partitioning into highly molecular orders domains (as expected for traditional lipid “rafts”) but more probably resulted from transient molecular complexes [38]. Interestingly, this scanning STED-FCS analysis also depicted slight anomalies due to membrane curvature. Consequently, being complementary to SPT, STED-FCS has proved itself as a very versatile tool for the detailed investigation of molecular diffusion dynamics and interactions in the plasma membrane of living cells, which will further advance with optimizations such as multi-color recordings, combination with other beam-

scanning approaches (e.g. RICS or iMSD [17], [69]), and improved lipid and protein labeling technology.

3. Current frontiers in membrane research

3.1. Phospho-inositol lipid interactions

Negatively charged lipid species as phosphatidylserin (PS), phosphatidic acids (PA), phosphoinositides (PI(P)s) have important roles in signal transduction and defining membrane identity of cell organelles [70]. These lipids recruit and/or trigger conformational changes of effector proteins at membranes via ionic interaction with polybasic peptide motifs. Especially, the low abundant species of phosphorylated PI are potent signaling molecules. Their local production and turnover is key to set-up and confine signaling complexes at membranes. Additionally, on the organelle level the identity of internal membranes such as ER, golgi, endosomes and plasma membrane is in part also controlled by phosphorylation states of PIP lipids at these membranes [71]–[74]. The phosphorylation state of PI is modulated by membrane associated phosphatases and kinases [75], [76]. Obviously, a constant turnover of PIP species is required that balances the local amount of PIP species. How the network of feedback loops works, which regulates this turnover, is however not clear. Importantly, the phosphoinositol PIP (4,5)2 (or simply PIP2 throughout) has also been shown to play a structural role in sequestering trans-membrane proteins with positive juxta-membrane motifs into nanoscale domains via ionic interactions of proteins [77]–[80]. In coarse grain simulations these domains appear to be densely packed protein structures with PIP2 bridging the ionic charges of proteins, which matches with observations from super-resolution microscopy (figure 6). If this mechanism is a general organizer for membrane proteins remains to be shown. It is, however, likely that these interactions play an important role since many plasma membrane proteins contain positive juxta-membrane motifs [81]. The existence of PIP domains in live cells has not been convincingly shown [82], primarily due to difficulties in detecting transient small domains with sufficient spatiotemporal resolution. With the large number of processes that have been attributed to PIPs the question arises how specificity is achieved. Is specificity

regulated by local lipid turnover (the lipid species is only transiently and locally produced) and/or do additional factors like membrane composition, membrane potential and ion concentration fine-tune the ionic lipid protein interactions [83]? Answering these questions requires a systematic combination of simulations and experiments to understand how lipid head-group conformations are coupled to the membrane environment and how these in turn influence lipid recognition of proteins. It is also important to realize that our current spatiotemporal knowledge of lipid-protein interactions is biased towards ionic lipid protein interactions simply because these lipids can be visualized in the cellular context with available markers [84]–[87].

3.2. Hydrophobic mismatch

Hydrophobic sorting of membrane components is more elusive but it is clear that it has to happen at least in the secretory pathway because many components such as long chain fatty acids phospholipids, sphingolipids, cholesterol and long trans-membrane domain proteins become differentially enriched on the way to the plasma membrane [81]. This observation was the starting point for development of the lipid raft hypothesis, which proposes sphingolipid-cholesterol driven phase separation to be responsible for sorting [1]. Phase separation of lipids has been well characterized in model membranes [88] and was shown to fall into the universitaly class of the 2D Ising model [89]. Interestingly, the complex composition of the plasma membrane seems to be tuned closed to the transition from a homogenous state into a phase separated liquid-liquid (order/disordered) state [90]–[93]. However, under physiological conditions the plasma membrane is not large-scale phase separated [94]. To what extend and under what conditions the plasma membrane contains small phase separated domains is not fully understood, yet. It is, for example, unclear to what extend membrane proteins can drive phase separation when protein concentration in a membrane is as high as in bio-membranes [95]–[99], [47]. A number of mechanisms have been proposed how small domains can emerge in complex mixtures of lipids and proteins. The most interesting being domains based on micro-emulsion theory [100], [101] and domains based on

broadening the phase transition by a quenched disorder mechanism [94], [102]–[104]. Both theories so far lack direct experimental evidence in living cells, but it has recently been shown that interactions of ternary lipid model membranes with a simple scaffold leads to quenched disorder patterns that persist up to physiological temperatures [105]–[107] (discussed in the next section). One problem that impedes a systematic experimental approach to study phase separation in more complex membrane systems is that composition dependent phase diagrams are tedious (or impossible) to construct, simply because of the large number of compositions that have to be sampled. Already a four-component system becomes very complicated to describe [108], [109]. Additionally, nanoscopic dynamic phase separation, as is expected in complex membranes close to a phase transition, is very challenging to directly detect. These experimental shortcomings can hopefully be overcome by MD-simulations of complex membrane systems. Not only can the composition be systematically screened in MD-simulations, but also bilayer leaflet asymmetry and presence of membrane proteins may be taken into account, which is difficult in experimental model membranes [48], [110], [111]. Building a constructive feedback between simulations and experiments requires establishing ways of "translating" simulation results into data that can be directly compared to experimental data. Depending on the experiments these can be fluorescence intensity maps, single-molecule trajectories or FCS data, each with its own constrains of spatiotemporal resolution and noise. Ideally, simulations and experiments should have at least some spatiotemporal overlap that allows aligning both worlds. In this way simulations could become extensions of real microscopes that allow resolving the atomic scale.

3.3. Membrane scaffolds

Membrane scaffolds are protein structures that are associated to membranes by interaction with lipids or trans-membrane proteins. Scaffolds are ubiquitous structures on all cell membranes and serve a variety of different functions: mechanical stabilization, membrane deformations, in combination with molecular motors membrane expansion and contraction [112]–[114]. Besides these functions,

scaffolds also sort, compartmentalize and recruit components within and to membranes. For example the cortical actin cytoskeleton has been shown to confine movements of trans-membrane proteins and even lipids to sub 100nm compartments [115], [116]. These observations are summarized in the membrane fence model [18]. As discussed in the previous paragraph, scaffolds in combination with membrane phase behavior may result in local membrane domains, whose composition and location is controlled by the pinning sites connected to the scaffold (Figure 7) [105], [106], [112], [117].

We have recently used a minimal reconstitution approach to test predictions made by Ising model simulations in a controlled model membrane system [105]. We deployed biotinylated lipids as pinning sites in ternary phase separating supported lipid bilayer membranes. The pinning lipids were connected to an actin-meshwork via the adapter-protein streptavidin and phalloidin-biotin. This system allowed us to tune the pinning site density, the pinning lipid type (e.g. saturated versus unsaturated lipid) and the temperature. The main result largely confirmed the predictions of the Ising model simulations. Namely, in presence of pinning the phase transition is quenched, which results in restriction of domain coalescence at low temperatures and induction of domains at temperatures above the expected transition temperature (Figure 7A). The experiments also revealed an unexpected asymmetry towards disorder preferring pinning lipids. Quenching the phase transition by pinning disorder preferring lipids (unsaturated phospholipid DOPE) was stronger than for order preferring lipids (saturated phospholipids DSPE). This asymmetry was not predicted by the simulations and revealed that additional interactions play a role. We assumed that local curvature induced by the binding of the adapter-protein to the membrane might couple to domain formation. Introducing this scenario into the simulations reproduced the observed asymmetry between ordered and disordered pinning lipids. Additionally, the simulation showed that curvature coupling amplifies the effect of pinning, resulting in robust pattern formation already at low pinning densities (figure 7B). Overall, this work demonstrated that a tight interplay between experiments and simulations can lead to a mechanistic understanding of mechanisms of membrane patterning, which

would not be possible by intuitive interpretation of experimental results. To what extend this mechanism is at play in cell membranes is unclear. However, the results of the combined simulations and model membrane experiments give us direction where to expect strong re-organization. For example, structures with high pinning density like cell-cell junctions, focal adhesions or neuronal synapses are promising candidates [103], [107], [118].

Interestingly, membrane connected multivalent scaffolding molecules often found at signaling sites have the propensity to phase separate into protein enriched domains, which show characteristics of liquids [119]. This 3D phase separation can be triggered by membrane binding and results in local protein enriched liquid droplets [120]. It is intriguing to speculate how such a mechanism may relay and amplify signals over the plasma membrane. Unfortunately, our current understanding of scaffold membrane interactions is still very limited. The experimental data of lipid and protein mobility in live cell membranes acquired by single-molecule tracking or STED-FCS do not allow to directly inferring structural models of the underlying molecular organization. Since the mobility data are a convolute of different structural features of membranes (cortical actin meshwork, nano-domains, curvature etc.) over space and time, simulations are needed to interpret the experimental results. We have mostly relied on simple Monte Carlo random walk approaches, which explicitly simulate membrane obstacles as regions of slowed down or trapped diffusion (domains) or as semi-reflective boundaries within a Voronoi meshwork [36], [59], [65]. While these simulations reproduce some of the observed dependencies of the apparent diffusion coefficient on the observation area (diffusion law) they do not contain detailed information about molecular interactions and mechanisms (Figure 4D,F). To test, for example, the predictions made by Ising model simulations of quenched disorder in real cell membranes we require methods that allow comparing the experimental mobility data directly to simulations. As a step in this direction mobility data could be extracted from Ising model simulations [94]. This would, for example, allow comparing the signature of temperature dependence lipid diffusion in absence and presence of quenched disorder with data from real cell membranes [121]. Ultimately, to understand if our

simplified assumptions apply to real membranes (lipid/protein complexity, membrane asymmetry, diversity of pinning sites) MD-simulation that can reach the spatiotemporal scales of optical nanoscopy techniques will be required. Without doubt, understanding the organization of bio-membranes will require integrating interactions with internal and external scaffolds structures since there is a tight coupling of both.

3.4. Membrane lipid turnover

Components of bio-membranes are in constant turnover. Various exchange processes occur at different spatiotemporal scales. The best studied ones are endo- and exocytosis, which operate via vesicular transport pathways [122], [123]. Polarized uptake and delivery of vesicles is for example important in establishing apical basal membrane domains in epithelial cells [124]. Importantly, also non-vesicular transport of lipids has been discovered. Contact sites between organelles (for example endoplasmic reticulum and plasma membrane) allow localized and selective exchange of membrane lipids [125]–[127]. How and where these contact sites are established and what the turnover rates are remains to be investigated. Finally, also within an organelle membrane metabolizing enzymes constantly remodel lipids (acyl chain length and saturation, head-group identity) [128], [129]. How this remodeling is spatiotemporally controlled is largely unknown. A recent finding that highlights the importance of lipid inter-conversion is how the transition from endosomal vesicles to excocytitic vesicles in controlled. It has been shown that the inter-conversion of PI(3)P to PI(4)P by the 3-phosphatase MTM1 and the 4-kinase-2α (PI4K2α) is a key step for cargo delivery from endosome to the plasma membrane [76].

Our knowledge about general lipid turnover and flux stems mostly from pulse chase experiments analyzed by mass spectrometry. In most cases only whole cell lipid extracts are analyzed. Unfortunately, this does not allow differentiating lipid turnover in subcellular membranes (organelles). However, with the development of photo-activatable and photo-cross-linkable lipids it will become possible to target specifically the plasma membrane and visualize the turnover and inter-conversion

of lipid classes [130], [131]. This data will be essential for building non-equilibrium models of membrane organization that include lipid transport and lipid metabolism. So far the limited timescales of MD-simulations do not allow incorporating lipid transport or metabolism. However, meso-scale models have been used to investigate pattern formation in membranes with non-equilibrium processes like lipid transport and lipid-species inter-conversion [132]–[134]. These simulations indicate that localized metabolism and transport can have strong influence on membrane composition, structure and function. Reconstitution of lipid enzymes and transporters into model systems like GUVs in combination with high-resolution microscopy/spectroscopy will be required to systematically test the predictions of the meso-scale models.

Conclusion

As we approach bio-membranes from the bottom up of isolated molecular interactions (~10nm) to assemblies of lipid-protein complexes (~100nm) over the meso-scale level (~1um) to the macro-scale (cells) the important question arises how molecular interactions lead to the robust structure/functions that we observe on the macro-scale? How the multitude of processes occurring simultaneously spatiotemporally is controlled? To answer these questions we have to understand the organization principles that control sorting and compartmentalization of lipids and proteins in bio-membranes. Many membrane organization mechanisms and models have been proposed both from experimentalists and theorists. The ongoing challenge is to experimentally reconcile the models to come to an integrative understanding and clear up the view for new discoveries. To achieve this, we need to improve the experimental approaches but at the same time we will require system biology approaches that combine the experimental results with simulations into multi-scale models of cellular membranes.

Acknowledgement

CE thanks the the Wellcome Trust (grant ref 104924/14/Z/14), the Medical Research Council (grant number MC_UU_12010/unit programmes G0902418 and

MC_UU_12025), BBSRC and ESPRC (grant number MR/K01577X/1), the DFG research unit 1905 “Structure and function of the peroxisomal translocon” and institutional funding from the University of Oxford for financial support. CE and AH acknowledge long-years support by Stefan Hell (MPI biophysical Chemistry, Goettingen, Germany).

References

[1] K. Simons and E. Ikonen, “Functional rafts in cell membranes.,” *Nature*, vol. 387, no. 6633, pp. 569–72, Jun. 1997.

[2] E. Klotzsch and G. J. Schütz, “A critical survey of methods to detect plasma membrane rafts.,” *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, vol. 368, no. 1611, p. 20120033, Feb. 2013.

[3] J. M. Kwiatek, E. Hinde, and K. Gaus, “Microscopy approaches to investigate protein dynamics and lipid organization.,” *Mol. Membr. Biol.*, vol. 31, no. 5, pp. 141–51, Aug. 2014.

[4] G. L. Nicolson, “The Fluid-Mosaic Model of Membrane Structure: still relevant to understanding the structure, function and dynamics of biological membranes after more than 40 years.,” *Biochim. Biophys. Acta*, vol. 1838, no. 6, pp. 1451–66, Jun. 2014.

[5] P. T. Ivanova, S. B. Milne, D. S. Myers, and H. A. Brown, “Lipidomics: a mass spectrometry based systems level analysis of cellular lipids.,” *Curr. Opin. Chem. Biol.*, vol. 13, no. 5–6, pp. 526–31, Dec. 2009.

[6] C. Klose, M. A. Surma, and K. Simons, “Organellar lipidomics--background and perspectives.,” *Curr. Opin. Cell Biol.*, vol. 25, no. 4, pp. 406–13, Aug. 2013.

[7] U. Loizides-Mangold, “On the future of mass-spectrometry-based lipidomics.,” *FEBS J.*, vol. 280, no. 12, pp. 2817–29, Jun. 2013.

[8] Z. Guo, L. J. Neilson, H. Zhong, P. S. Murray, S. Zanivan, and R. Zaidel-Bar, “E-cadherin interactome complexity and robustness resolved by quantitative proteomics.,” *Sci. Signal.*, vol. 7, no. 354, p. rs7, Dec. 2014.

[9] P. Haberkant and J. C. M. Holthuis, “Fat & fabulous: bifunctional lipids in the spotlight.,” *Biochim. Biophys. Acta*, vol. 1841, no. 8, pp. 1022–30, Aug. 2014.

[10] E. Abbe, “Beiträge zur Theorie des Mikroskops und der mikroskopischen Wahrnehmung,” *Arch. für Mikroskopische Anat.*, vol. 9, no. 1, pp. 413–418, Dec. 1873.

[11] D. Magde, E. Elson, and W. W. Webb, “Thermodynamic Fluctuations in a Reacting System—Measurement by Fluorescence Correlation Spectroscopy,” *Phys. Rev. Lett.*, vol. 29, no. 11, pp. 705–708, Sep. 1972.

[12] M. Ehrenberg and R. Rigler, “Rotational brownian motion and fluorescence intensify fluctuations,” *Chem. Phys.*, vol. 4, no. 3, pp. 390–401, Jun. 1974.

[13] Z. Petrášek, S. Derenko, and P. Schwille, “Circular scanning fluorescence correlation spectroscopy on membranes.,” *Opt. Express*, vol. 19, no. 25, pp. 25006–21, Dec. 2011.

[14] E. Hermann, J. Ries, and A. J. García-Sáez, “Scanning fluorescence correlation spectroscopy on biomembranes.,” *Methods Mol. Biol.*, vol. 1232, pp. 181–97, Jan. 2015.

[15] M. A. Digman, M. Stakic, and E. Gratton, “Raster image correlation spectroscopy and number and brightness analysis.,” *Methods Enzymol.*, vol. 518, pp. 121–44, Jan. 2013.

[16] S. Coppola, D. Pozzi, S. C. De Sanctis, M. A. Digman, E. Gratton, and G. Caracciolo, “Quantitative measurement of intracellular transport of nanocarriers by spatio-temporal image correlation spectroscopy.,” *Methods Appl. Fluoresc.*, vol. 1, no. 1, Jan. 2013.

[17] C. Di Rienzo, E. Gratton, F. Beltram, and F. Cardarelli, “Fast spatiotemporal correlation spectroscopy to determine protein lateral diffusion laws in live cell membranes.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 110, no. 30, pp. 12307–12, Jul. 2013.

[18] A. Kusumi, C. Nakada, K. Ritchie, K. Murase, K. Suzuki, H. Murakoshi, R. S. Kasai, J. Kondo, and T. Fujiwara, “Paradigm shift of the plasma membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-speed single-molecule tracking of membrane molecules.,” *Annu. Rev. Biophys. Biomol. Struct.*, vol. 34, pp. 351–78, Jan. 2005.

[19] T. Schmidt, G. J. Schutz, W. Baumgartner, H. J. Gruber, and H. Schindler, “Imaging of single molecule diffusion.,” *Proc. Natl. Acad. Sci.*, vol. 93, no. 7, pp. 2926–2929, Apr. 1996.

[20] G. de Wit, J. S. H. Danial, P. Kukura, and M. I. Wallace, “Dynamic label-free imaging of lipid nanodomains,” *Proc. Natl. Acad. Sci.*, vol. 112, no. 40, p. 201508483, Sep. 2015.

[21] S. W. Hell and J. Wichmann, “Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy,” *Opt. Lett.*, vol. 19, no. 11, p. 780, Jun. 1994.

[22] S. W. Hell, “Strategy for far-field optical imaging and writing without diffraction limit,” *Phys. Lett. A*, vol. 326, no. 1–2, pp. 140–145, May 2004.

[23] M. Hofmann, C. Eggeling, S. Jakobs, and S. W. Hell, “Breaking the diffraction barrier in fluorescence microscopy at low light intensities by using reversibly photoswitchable proteins.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, no. 49, pp. 17565–9, Dec. 2005.

[24] T. Grotjohann, I. Testa, M. Leutenegger, H. Bock, N. T. Urban, F. Lavoie-Cardinal, K. I. Willig, C. Eggeling, S. Jakobs, and S. W. Hell, “Diffraction-unlimited all-optical imaging and writing with a photochromic GFP.,” *Nature*, vol. 478, no. 7368, pp. 204–8, Oct. 2011.

[25] E. Betzig, G. H. Patterson, R. Sougrat, O. W. Lindwasser, S. Olenych, J. S. Bonifacino, M. W. Davidson, J. Lippincott-Schwartz, and H. F. Hess, “Imaging intracellular fluorescent proteins at nanometer resolution.,” *Science (80-.).*, vol. 313, no. 5793, pp. 1642–5, Sep. 2006.

[26] S. T. Hess, T. P. K. Girirajan, and M. D. Mason, “Ultra-high resolution imaging

by fluorescence photoactivation localization microscopy.," *Biophys. J.*, vol. 91, no. 11, pp. 4258–72, Dec. 2006.

[27] S. van de Linde, R. Kasper, M. Heilemann, and M. Sauer, "Photoswitching microscopy with standard fluorophores," *Appl. Phys. B*, vol. 93, no. 4, pp. 725–731, Oct. 2008.

[28] M. J. Rust, M. Bates, and X. Zhuang, "Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM).," *Nat. Methods*, vol. 3, no. 10, pp. 793–5, Oct. 2006.

[29] J. Fölling, M. Bossi, H. Bock, R. Medda, C. A. Wurm, B. Hein, S. Jakobs, C. Eggeling, and S. W. Hell, "Fluorescence nanoscopy by ground-state depletion and single-molecule return.," *Nat. Methods*, vol. 5, no. 11, pp. 943–5, Nov. 2008.

[30] S. Hell and E. H. K. Stelzer, "Properties of a 4Pi confocal fluorescence microscope," *J. Opt. Soc. Am. A*, vol. 9, no. 12, p. 2159, Dec. 1992.

[31] M. G. L. Gustafsson, D. A. Agard, and J. W. Sedat, "<title>Sevenfold improvement of axial resolution in 3D wide-field microscopy using two objective lenses</title>," in *IS&T/SPIE's Symposium on Electronic Imaging: Science & Technology*, 1995, pp. 147–156.

[32] M. G. Gustafsson, "Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy.," *J. Microsc.*, vol. 198, no. Pt 2, pp. 82–7, May 2000.

[33] L. Schermelleh, P. M. Carlton, S. Haase, L. Shao, L. Winoto, P. Kner, B. Burke, M. C. Cardoso, D. A. Agard, M. G. L. Gustafsson, H. Leonhardt, and J. W. Sedat, "Subdiffraction multicolor imaging of the nuclear periphery with 3D structured illumination microscopy.," *Science*, vol. 320, no. 5881, pp. 1332–6, Jun. 2008.

[34] C. Eggeling, K. I. Willig, S. J. Sahl, and S. W. Hell, "Lens-based fluorescence nanoscopy.," *Q. Rev. Biophys.*, vol. 48, no. 2, pp. 178–243, May 2015.

[35] L. Kastrup, H. Blom, C. Eggeling, and S. W. Hell, "Fluorescence fluctuation spectroscopy in subdiffraction focal volumes.," *Phys. Rev. Lett.*, vol. 94, no. 17, p. 178104, May 2005.

[36] C. Eggeling, C. Ringemann, R. Medda, G. Schwarzmann, K. Sandhoff, S. Polyakova, V. N. Belov, B. Hein, C. von Middendorff, A. Schönle, and S. W. Hell, "Direct observation of the nanoscale dynamics of membrane lipids in a living cell.," *Nature*, vol. 457, no. 7233, pp. 1159–62, Feb. 2009.

[37] S. Manley, J. M. Gillette, G. H. Patterson, H. Shroff, H. F. Hess, E. Betzig, and J. Lippincott-Schwartz, "High-density mapping of single-molecule trajectories with photoactivated localization microscopy.," *Nat. Methods*, vol. 5, no. 2, pp. 155–7, Feb. 2008.

[38] A. Honigmann, V. Mueller, H. Ta, A. Schoenle, E. Sezgin, S. W. Hell, and C.

Eggeling, “Scanning STED-FCS reveals spatiotemporal heterogeneity of lipid interaction in the plasma membrane of living cells.,” *Nat. Commun.*, vol. 5, p. 5412, Jan. 2014.

[39] C. Eggeling, “Super-resolution optical microscopy of lipid plasma membrane dynamics.,” *Essays Biochem.*, vol. 57, pp. 69–80, Jan. 2015.

[40] R. O. Dror, R. M. Dirks, J. P. Grossman, H. Xu, and D. E. Shaw, “Biomolecular simulation: a computational microscope for molecular biology.,” *Annu. Rev. Biophys.*, vol. 41, pp. 429–52, Jan. 2012.

[41] T. J. Lane, D. Shukla, K. A. Beauchamp, and V. S. Pande, “To milliseconds and beyond: challenges in the simulation of protein folding.,” *Curr. Opin. Struct. Biol.*, vol. 23, no. 1, pp. 58–65, Feb. 2013.

[42] M. G. Saunders and G. A. Voth, “Coarse-graining methods for computational biology.,” *Annu. Rev. Biophys.*, vol. 42, pp. 73–93, Jan. 2013.

[43] H. I. Ingolfsson, C. Arnarez, X. Periole, and S. J. Marrink, “Computational ’microscopy of cellular membranes,” *J. Cell Sci.*, vol. 129, no. 2, pp. 257–268, Jan. 2016.

[44] I. Vattulainen and T. Rog, “Lipid simulations: a perspective on lipids in action.,” *Cold Spring Harb. Perspect. Biol.*, vol. 3, no. 4, Apr. 2011.

[45] P. J. Stansfeld and M. S. P. Sansom, “Molecular simulation approaches to membrane proteins.,” *Structure*, vol. 19, no. 11, pp. 1562–72, Nov. 2011.

[46] J. Grouleff, S. J. Irudayam, K. K. Skeby, and B. Schiøtt, “The influence of cholesterol on membrane protein structure, function, and dynamics studied by molecular dynamics simulations.,” *Biochim. Biophys. Acta*, vol. 1848, no. 9, pp. 1783–95, Sep. 2015.

[47] L. V Schäfer, D. H. de Jong, A. Holt, A. J. Rzepiela, A. H. de Vries, B. Poolman, J. A. Killian, and S. J. Marrink, “Lipid packing drives the segregation of transmembrane helices into disordered lipid domains in model membranes.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 108, no. 4, pp. 1343–8, Jan. 2011.

[48] H. I. Ingólfsson, M. N. Melo, F. J. van Eerden, C. Arnarez, C. A. Lopez, T. A. Wassenaar, X. Periole, A. H. de Vries, D. P. Tieleman, and S. J. Marrink, “Lipid organization of the plasma membrane.,” *J. Am. Chem. Soc.*, vol. 136, no. 41, pp. 14554–9, Oct. 2014.

[49] H. J. Risselada, G. Bubnis, and H. Grubmüller, “Expansion of the fusion stalk and its implication for biological membrane fusion.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, no. 30, pp. 11043–8, Jul. 2014.

[50] F. Khalili-Araghi, J. Gumbart, P.-C. Wen, M. Sotomayor, E. Tajkhorshid, and K. Schulten, “Molecular dynamics simulations of membrane channels and transporters.,” *Curr. Opin. Struct. Biol.*, vol. 19, no. 2, pp. 128–37, Apr. 2009.

[51] S. W. Hell, S. Jakobs, and L. Kastrup, “Imaging and writing at the nanoscale

with focused visible light through saturable optical transitions," *Appl. Phys. A Mater. Sci. Process.*, vol. 77, no. 7, pp. 859–860, Dec. 2003.

[52] T. A. Klar, S. Jakobs, M. Dyba, A. Egner, and S. W. Hell, "Fluorescence microscopy with diffraction resolution barrier broken by stimulated emission," *Proc. Natl. Acad. Sci.*, vol. 97, no. 15, pp. 8206–8210, Jul. 2000.

[53] G. Donnert, J. Keller, R. Medda, M. A. Andrei, S. O. Rizzoli, R. Lührmann, R. Jahn, C. Eggeling, and S. W. Hell, "Macromolecular-scale resolution in biological fluorescence microscopy.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 103, no. 31, pp. 11440–5, Aug. 2006.

[54] G. Lukinavičius, K. Umezawa, N. Olivier, A. Honigmann, G. Yang, T. Plass, V. Mueller, L. Reymond, I. R. Corrêa, Z.-G. Luo, C. Schultz, E. a Lemke, P. Heppenstall, C. Eggeling, S. Manley, and K. Johnsson, "A near-infrared fluorophore for live-cell super-resolution microscopy of cellular proteins.," *Nat. Chem.*, vol. 5, no. 2, pp. 132–9, 2013.

[55] G. Vicidomini, G. Moneron, K. Y. Han, V. Westphal, H. Ta, M. Reuss, J. Engelhardt, C. Eggeling, and S. W. Hell, "Sharper low-power STED nanoscopy by time gating.," *Nat. Methods*, vol. 8, no. 7, pp. 571–3, Jul. 2011.

[56] J. Schneider, J. Zahn, M. Maglione, S. J. Sigrist, J. Marquard, J. Chojnacki, H.-G. Kräusslich, S. J. Sahl, J. Engelhardt, and S. W. Hell, "Ultrafast, temporally stochastic STED nanoscopy of millisecond dynamics.," *Nat. Methods*, vol. 12, no. 9, pp. 827–30, Sep. 2015.

[57] F. Bergermann, L. Alber, S. J. Sahl, J. Engelhardt, and S. W. Hell, "2000-fold parallelized dual-color STED fluorescence nanoscopy.," *Opt. Express*, vol. 23, no. 1, pp. 211–23, Jan. 2015.

[58] L. Lanzanò, I. Coto Hernández, M. Castello, E. Gratton, A. Diaspro, and G. Vicidomini, "Encoding and decoding spatio-temporal information for super-resolution microscopy.," *Nat. Commun.*, vol. 6, p. 6701, Jan. 2015.

[59] L. Wawrezinieck, H. Rigneault, D. Marguet, and P.-F. Lenne, "Fluorescence correlation spectroscopy diffusion laws to probe the submicron cell membrane organization.," *Biophys. J.*, vol. 89, no. 6, pp. 4029–42, Dec. 2005.

[60] P.-F. Lenne, L. Wawrezinieck, F. Conchonaud, O. Wurtz, A. Boned, X.-J. Guo, H. Rigneault, H.-T. He, and D. Marguet, "Dynamic molecular confinement in the plasma membrane by microdomains and the cytoskeleton meshwork.," *EMBO J.*, vol. 25, no. 14, pp. 3245–56, Jul. 2006.

[61] V. Ruprecht, S. Wieser, D. Marguet, and G. J. Schütz, "Spot variation fluorescence correlation spectroscopy allows for superresolution chronoscopy of confinement times in membranes.," *Biophys. J.*, vol. 100, no. 11, pp. 2839–45, Jun. 2011.

[62] V. Mueller, C. Ringemann, A. Honigmann, G. Schwarzmann, R. Medda, M. Leutenegger, S. Polyakova, V. N. Belov, S. W. Hell, and C. Eggeling, "STED

nanoscopy reveals molecular details of cholesterol- and cytoskeleton-modulated lipid interactions in living cells," *Biophys. J.*, vol. 101, no. 7, pp. 1651–1660, 2011.

[63] A. Honigmann, V. Mueller, S. W. Hell, and C. Eggeling, "STED microscopy detects and quantifies liquid phase separation in lipid membranes using a new far-red emitting fluorescent phosphoglycerolipid analogue," *Faraday Discussions*. 2013.

[64] C. Guzmán, M. Šolman, A. Ligabue, O. Blaževitš, D. M. Andrade, L. Reymond, C. Eggeling, and D. Abankwa, "The efficacy of Raf kinase recruitment to the GTPase H-ras depends on H-ras membrane conformer-specific nanoclustering.," *J. Biol. Chem.*, vol. 289, no. 14, pp. 9519–33, Apr. 2014.

[65] D. M. Andrade, M. P. Clausen, J. Keller, V. Mueller, C. Wu, J. E. Bear, S. W. Hell, B. C. Lagerholm, and C. Eggeling, "Cortical actin networks induce spatio-temporal confinement of phospholipids in the plasma membrane--a minimally invasive investigation by STED-FCS.," *Sci. Rep.*, vol. 5, p. 11454, Jan. 2015.

[66] J. Adler, A. I. Shevchuk, P. Novak, Y. E. Korchev, and I. Parmryd, "Plasma membrane topography and interpretation of single-particle tracks.," *Nat. Methods*, vol. 7, no. 3, pp. 170–1, Mar. 2010.

[67] C. Ringemann, B. Harke, C. Von Middendorff, R. Medda, A. Honigmann, R. Wagner, M. Leutenegger, A. Schönle, S. W Hell, and C. Eggeling, "New Journal of Physics," *New J. Phys.*, vol. 11, no. 10, p. 103054, 2009.

[68] G. Vicidomini, H. Ta, A. Honigmann, V. Mueller, M. P. Clausen, D. Waithe, S. Galiani, E. Sezgin, A. Diaspro, S. W. Hell, and C. Eggeling, "STED-FLCS: An Advanced Tool to Reveal Spatiotemporal Heterogeneity of Molecular Membrane Dynamics.," *Nano Lett.*, vol. 15, no. 9, pp. 5912–8, Sep. 2015.

[69] P. N. Hedde, R. M. Dörlich, R. Blomley, D. Gradl, E. Oppong, A. C. B. Cato, and G. U. Nienhaus, "Stimulated emission depletion-based raster image correlation spectroscopy reveals biomolecular dynamics in live cells.," *Nat. Commun.*, vol. 4, p. 2093, Jan. 2013.

[70] Y. Zhou and J. F. Hancock, "Ras nanoclusters: Versatile lipid-based signaling platforms.," *Biochim. Biophys. Acta*, vol. 1853, no. 4, pp. 841–9, Apr. 2015.

[71] R. Behnia and S. Munro, "Organelle identity and the signposts for membrane traffic.," *Nature*, vol. 438, no. 7068, pp. 597–604, Dec. 2005.

[72] M. Krauss and V. Haucke, "Phosphoinositide-metabolizing enzymes at the interface between membrane traffic and cell signalling.," *EMBO Rep.*, vol. 8, no. 3, pp. 241–6, Mar. 2007.

[73] R. Zoncu, R. M. Perera, D. M. Balkin, M. Pirruccello, D. Toomre, and P. De Camilli, "A phosphoinositide switch controls the maturation and signaling properties of APPL endosomes.," *Cell*, vol. 136, no. 6, pp. 1110–21, Mar. 2009.

[74] F. Nakatsu, J. M. Baskin, J. Chung, L. B. Tanner, G. Shui, S. Y. Lee, M. Pirruccello, M. Hao, N. T. Ingolia, M. R. Wenk, and P. De Camilli, "PtdIns4P synthesis by PI4KIIIα at the plasma membrane and its impact on plasma membrane identity.," *J. Cell Biol.*, vol. 199, no. 6, pp. 1003–16, Dec. 2012.

[75] S. A. Rudge and M. J. O. Wakelam, "SnapShot: Lipid kinase and phosphatase reaction pathways.," *Cell*, vol. 156, no. 1–2, pp. 376–376.e1, Jan. 2014.

[76] K. Ketel, M. Krauss, A.-S. Nicot, D. Puchkov, M. Wieffer, R. Müller, D. Subramanian, C. Schultz, J. Laporte, and V. Haucke, "A phosphoinositide conversion mechanism for exit from endosomes.," *Nature*, vol. 529, no. 7586, pp. 408–12, Jan. 2016.

[77] S. McLaughlin and D. Murray, "Plasma membrane phosphoinositide organization by protein electrostatics.," *Nature*, vol. 438, no. 7068, pp. 605–11, Dec. 2005.

[78] A. Honigmann, G. van den Bogaart, E. Iraheta, H. J. Risselada, D. Milovanovic, V. Mueller, S. Müllar, U. Diederichsen, D. Fasshauer, H. Grubmüller, S. W. Hell, C. Eggeling, K. Kühnel, and R. Jahn, "Phosphatidylinositol 4,5-bisphosphate clusters act as molecular beacons for vesicle recruitment.," *Nat. Struct. Mol. Biol.*, vol. 20, no. 6, pp. 679–86, 2013.

[79] G. van den Bogaart, K. Meyenberg, H. J. Risselada, H. Amin, K. I. Willig, B. E. Hubrich, M. Dier, S. W. Hell, H. Grubmüller, U. Diederichsen, and R. Jahn, "Membrane protein sequestering by ionic protein-lipid interactions.," *Nature*, vol. 479, no. 7374, pp. 552–5, Nov. 2011.

[80] K. B. Abd Halim, H. Koldsø, and M. S. P. Sansom, "Interactions of the EGFR juxtamembrane domain with PIP2-containing lipid bilayers: Insights from multiscale molecular dynamics simulations.," *Biochim. Biophys. Acta*, vol. 1850, no. 5, pp. 1017–25, May 2015.

[81] H. J. Sharpe, T. J. Stevens, and S. Munro, "A comprehensive comparison of transmembrane domains reveals organelle-specific properties.," *Cell*, vol. 142, no. 1, pp. 158–69, Jul. 2010.

[82] C. Ji, Y. Zhang, P. Xu, T. Xu, and X. Lou, "Nanoscale Landscape of Phosphoinositides Revealed by Specific Pleckstrin Homology (PH) Domains Using Single-molecule Superresolution Imaging in the Plasma Membrane.," *J. Biol. Chem.*, vol. 290, no. 45, pp. 26978–93, Nov. 2015.

[83] Y. Zhou, C.-O. Wong, K. Cho, D. van der Hoeven, H. Liang, D. P. Thakur, J. Luo, M. Babic, K. E. Zinsmaier, M. X. Zhu, H. Hu, K. Venkatachalam, and J. F. Hancock, "SIGNAL TRANSDUCTION. Membrane potential modulates plasma membrane phospholipid dynamics and K-Ras signaling.," *Science*, vol. 349, no. 6250, pp. 873–6, Aug. 2015.

[84] R. V Stahelin, "Lipid binding domains: more than simple lipid effectors.," *J. Lipid Res.*, vol. 50 Suppl, pp. S299–304, Apr. 2009.

[85] R. V Stahelin, J. L. Scott, and C. T. Frick, “Cellular and molecular interactions of phosphoinositides and peripheral proteins.,” *Chem. Phys. Lipids*, vol. 182, pp. 3–18, Sep. 2014.

[86] A. C. Kalli and M. S. P. Sansom, “Interactions of peripheral proteins with model membranes as viewed by molecular dynamics simulations.,” *Biochem. Soc. Trans.*, vol. 42, no. 5, pp. 1418–24, Oct. 2014.

[87] O. Idevall-Hagren and P. De Camilli, “Detection and manipulation of phosphoinositides,” *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids*, vol. 1851, no. 6, pp. 736–745, Dec. 2014.

[88] S. L. Veatch and S. L. Keller, “Seeing spots: Complex phase behavior in simple membranes,” *Biochim. Biophys. Acta - Mol. Cell Res.*, vol. 1746, no. 3, pp. 172–185, Dec. 2005.

[89] A. R. Honerkamp-Smith, P. Cicuta, M. D. Collins, S. L. Veatch, M. den Nijs, M. Schick, and S. L. Keller, “Line tensions, correlation lengths, and critical exponents in lipid membranes near critical points.,” *Biophys. J.*, vol. 95, no. 1, pp. 236–46, Jul. 2008.

[90] T. Baumgart, A. T. Hammond, P. Sengupta, S. T. Hess, D. A. Holowka, B. A. Baird, and W. W. Webb, “Large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles,” *Proc. Natl. Acad. Sci.*, vol. 104, no. 9, pp. 3165–3170, Feb. 2007.

[91] I. Levental, F. J. Byfield, P. Chowdhury, F. Gai, T. Baumgart, and P. A. Janmey, “Cholesterol-dependent phase separation in cell-derived giant plasma-membrane vesicles.,” *Biochem. J.*, vol. 424, no. 2, pp. 163–7, Dec. 2009.

[92] E. Sezgin, H.-J. Kaiser, T. Baumgart, P. Schwille, K. Simons, and I. Levental, “Elucidating membrane structure and protein behavior using giant plasma membrane vesicles.,” *Nat. Protoc.*, vol. 7, no. 6, pp. 1042–51, Jun. 2012.

[93] S. L. Veatch, P. Cicuta, P. Sengupta, A. Honerkamp-Smith, D. Holowka, and B. Baird, “Critical fluctuations in plasma membrane vesicles.,” *ACS Chem. Biol.*, vol. 3, no. 5, pp. 287–93, May 2008.

[94] B. B. Machta, S. Papanikolaou, J. P. Sethna, and S. L. Veatch, “Minimal model of plasma membrane heterogeneity requires coupling cortical actin to criticality.,” *Biophys. J.*, vol. 100, no. 7, pp. 1668–77, Apr. 2011.

[95] H.-J. Kaiser, A. Orłowski, T. Róg, T. K. M. Nyholm, W. Chai, T. Feizi, D. Lingwood, I. Vattulainen, and K. Simons, “Lateral sorting in model membranes by cholesterol-mediated hydrophobic matching.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 108, no. 40, pp. 16628–33, Oct. 2011.

[96] M. M. Sperotto, J. H. Ipsen, and O. G. Mouritsen, “Theory of protein-induced lateral phase separation in lipid membranes.,” *Cell Biophys.*, vol. 14, no. 1, pp. 79–95, Feb. 1989.

[97] S. K. Saka, A. Honigmann, C. Eggeling, S. W. Hell, T. Lang, and S. O. Rizzoli,

"Multi-protein assemblies underlie the mesoscale organization of the plasma membrane.," *Nat. Commun.*, vol. 5, no. May, p. 4509, 2014.

[98] F. Yin and J. T. Kindt, "Atomistic simulation of hydrophobic matching effects on lipid composition near a helical peptide embedded in mixed-lipid bilayers.," *J. Phys. Chem. B*, vol. 114, no. 24, pp. 8076–80, Jun. 2010.

[99] J. Domański, S. J. Marrink, and L. V Schäfer, "Transmembrane helices can induce domain formation in crowded model membranes.," *Biochim. Biophys. Acta*, vol. 1818, no. 4, pp. 984–94, Apr. 2012.

[100] R. Shlomovitz and M. Schick, "Model of a raft in both leaves of an asymmetric lipid bilayer.," *Biophys. J.*, vol. 105, no. 6, pp. 1406–13, Sep. 2013.

[101] G. G. Putzel and M. Schick, "Phase behavior of a model bilayer membrane with coupled leaves.," *Biophys. J.*, vol. 94, no. 3, pp. 869–77, Feb. 2008.

[102] T. Speck and R. L. C. Vink, "Random pinning limits the size of membrane adhesion domains.," *Phys. Rev. E. Stat. Nonlin. Soft Matter Phys.*, vol. 86, no. 3 Pt 1, p. 031923, Sep. 2012.

[103] T. R. Weikl, R. R. Netz, and R. Lipowsky, "Unbinding transitions and phase separation of multicomponent membranes.," *Phys. Rev. E. Stat. Phys. Plasmas. Fluids. Relat. Interdiscip. Topics*, vol. 62, no. 1 Pt A, pp. R45–8, Jul. 2000.

[104] J. Ehrig, E. P. Petrov, and P. Schwille, "Near-critical fluctuations and cytoskeleton-assisted phase separation lead to subdiffusion in cell membranes.," *Biophys. J.*, vol. 100, no. 1, pp. 80–9, Jan. 2011.

[105] A. Honigmann, S. Sadeghi, J. Keller, S. W. Hell, C. Eggeling, and R. Vink, "A lipid bound actin meshwork organizes liquid phase separation in model membranes," *Elife*, vol. 2014, no. 3, 2014.

[106] S. Arumugam, E. P. Petrov, and P. Schwille, "Cytoskeletal pinning controls phase separation in multicomponent lipid membranes.," *Biophys. J.*, vol. 108, no. 5, pp. 1104–13, Mar. 2015.

[107] J. Zhao, J. Wu, and S. L. Veatch, "Adhesion stabilizes robust lipid heterogeneity in supercritical membranes at physiological temperature.," *Biophys. J.*, vol. 104, no. 4, pp. 825–34, Feb. 2013.

[108] T. M. Konyakhina, J. Wu, J. D. Mastroianni, F. A. Heberle, and G. W. Feigenson, "Phase diagram of a 4-component lipid mixture: DSPC/DOPC/POPC/chol.," *Biochim. Biophys. Acta*, vol. 1828, no. 9, pp. 2204–14, Sep. 2013.

[109] T. M. Konyakhina and G. W. Feigenson, "Phase diagram of a polyunsaturated lipid mixture: Brain sphingomyelin/1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine/cholesterol.," *Biochim. Biophys. Acta*, vol. 1858, no. 1, pp. 153–61, Jan. 2016.

[110] D. Shorthouse, G. Hedger, H. Koldsø, and M. S. P. Sansom, "Molecular simulations of glycolipids: Towards mammalian cell membrane models.,"

Biochimie, Sep. 2015.

[111] M. Javanainen, H. Hammaren, L. Monticelli, J.-H. Jeon, M. S. Miettinen, H. Martinez-Seara, R. Metzler, and I. Vattulainen, "Anomalous and normal diffusion of proteins and lipids in crowded lipid membranes," *Faraday Discuss.*, vol. 161, pp. 397–417, Dec. 2013.

[112] B. P. Head, H. H. Patel, and P. A. Insel, "Interaction of membrane/lipid rafts with the cytoskeleton: impact on signaling and function: membrane/lipid rafts, mediators of cytoskeletal arrangement and cell signaling.," *Biochim. Biophys. Acta*, vol. 1838, no. 2, pp. 532–45, Feb. 2014.

[113] V. Astro and I. de Curtis, "Plasma membrane-associated platforms: dynamic scaffolds that organize membrane-associated events.," *Sci. Signal.*, vol. 8, no. 367, p. re1, Mar. 2015.

[114] A. Kapus and P. Janmey, "Plasma Membrane—Cortical Cytoskeleton Interactions: A Cell Biology Approach with Biophysical Considerations," in *Comprehensive Physiology*, John Wiley & Sons, Inc., 2013.

[115] Y. Sako and A. Kusumi, "Barriers for lateral diffusion of transferrin receptor in the plasma membrane as characterized by receptor dragging by laser tweezers: fence versus tether.," *J. Cell Biol.*, vol. 129, no. 6, pp. 1559–74, Jun. 1995.

[116] K. Suzuki, K. Ritchie, E. Kajikawa, T. Fujiwara, and A. Kusumi, "Rapid hop diffusion of a G-protein-coupled receptor in the plasma membrane as revealed by single-molecule techniques.," *Biophys. J.*, vol. 88, no. 5, pp. 3659–80, May 2005.

[117] R. Raghupathy, A. A. Anilkumar, A. Polley, P. P. Singh, M. Yadav, C. Johnson, S. Suryawanshi, V. Saikam, S. D. Sawant, A. Panda, Z. Guo, R. A. Vishwakarma, M. Rao, and S. Mayor, "Transbilayer Lipid Interactions Mediate Nanoclustering of Lipid-Anchored Proteins," *Cell*, vol. 161, no. 3, pp. 581–594, Apr. 2015.

[118] R. Lipowsky, T. Rouhiparkouhi, D. E. Discher, and T. R. Weikl, "Domain formation in cholesterol–phospholipid membranes exposed to adhesive surfaces or environments," *Soft Matter*, vol. 9, no. 35, p. 8438, Aug. 2013.

[119] P. Li, S. Banjade, H.-C. Cheng, S. Kim, B. Chen, L. Guo, M. Llaguno, J. V Hollingsworth, D. S. King, S. F. Banani, P. S. Russo, Q.-X. Jiang, B. T. Nixon, and M. K. Rosen, "Phase transitions in the assembly of multivalent signalling proteins.," *Nature*, vol. 483, no. 7389, pp. 336–40, Mar. 2012.

[120] S. Banjade and M. K. Rosen, "Phase transitions of multivalent proteins can promote clustering of membrane receptors.," *Elife*, vol. 3, p. e04123, Jan. 2014.

[121] I.-H. Lee, S. Saha, A. Polley, H. Huang, S. Mayor, M. Rao, and J. T. Groves, "Live cell plasma membranes do not exhibit a miscibility phase transition over a wide range of temperatures.," *J. Phys. Chem. B*, vol. 119, no. 12, pp. 4450–9, Mar. 2015.

[122] L.-G. Wu, E. Hamid, W. Shin, and H.-C. Chiang, "Exocytosis and Endocytosis: Modes, Functions, and Coupling Mechanisms*," *Annu. Rev. Physiol.*, vol. 76, no. 1, pp. 301–331, Feb. 2014.

[123] G. J. Doherty and H. T. McMahon, "Mechanisms of endocytosis.," *Annu. Rev. Biochem.*, vol. 78, pp. 857–902, Jan. 2009.

[124] K. Mostov, T. Su, and M. ter Beest, "Polarized epithelial membrane traffic: conservation and plasticity.," *Nat. Cell Biol.*, vol. 5, no. 4, pp. 287–93, Apr. 2003.

[125] W. A. Prinz, "Bridging the gap: membrane contact sites in signaling, metabolism, and organelle dynamics.," *J. Cell Biol.*, vol. 205, no. 6, pp. 759–69, Jun. 2014.

[126] M. J. Phillips and G. K. Voeltz, "Structure and function of ER membrane contact sites with other organelles.," *Nat. Rev. Mol. Cell Biol.*, vol. advance on, Dec. 2015.

[127] S. C. J. Helle, G. Kanfer, K. Kolar, A. Lang, A. H. Michel, and B. Kornmann, "Organization and function of membrane contact sites.," *Biochim. Biophys. Acta*, vol. 1833, no. 11, pp. 2526–41, Nov. 2013.

[128] J. C. M. Holthuis and A. K. Menon, "Lipid landscapes and pipelines in membrane homeostasis.," *Nature*, vol. 510, no. 7503, pp. 48–57, Jun. 2014.

[129] C. Wolf and P. Quinn, "Membrane Lipid Homeostasis," in *Membrane Dynamics and Domains SE - 10*, vol. 37, P. Quinn, Ed. Springer US, 2004, pp. 317–357.

[130] P. Haberkant, R. Raijmakers, M. Wildwater, T. Sachsenheimer, B. Brügger, K. Maeda, M. Houweling, A.-C. Gavin, C. Schultz, G. van Meer, A. J. R. Heck, and J. C. M. Holthuis, "In vivo profiling and visualization of cellular protein-lipid interactions using bifunctional fatty acids.," *Angew. Chem. Int. Ed. Engl.*, vol. 52, no. 14, pp. 4033–8, Apr. 2013.

[131] M. J. Niphakis, K. M. Lum, A. B. Cognetta, B. E. Correia, T.-A. Ichu, J. Olucha, S. J. Brown, S. Kundu, F. Piscitelli, H. Rosen, and B. F. Cravatt, "A Global Map of Lipid-Binding Proteins and Their Ligandability in Cells.," *Cell*, vol. 161, no. 7, pp. 1668–80, Jun. 2015.

[132] J. Gómez, F. Sagués, and R. Reigada, "Nonequilibrium patterns in phase-separating ternary membranes.," *Phys. Rev. E. Stat. Nonlin. Soft Matter Phys.*, vol. 80, no. 1 Pt 1, p. 011920, Jul. 2009.

[133] J. Gómez, F. Sagués, and R. Reigada, "Actively maintained lipid nanodomains in biomembranes.," *Phys. Rev. E. Stat. Nonlin. Soft Matter Phys.*, vol. 77, no. 2 Pt 1, p. 021907, Feb. 2008.

[134] M. A. Lomholt, P. L. Hansen, and L. Miao, "A general theory of non-equilibrium dynamics of lipid-protein fluid membranes," *Eur. Phys. J. E*, vol. 16, no. 4, pp. 439–461, Mar. 2005.

[135] S. W. Hell, S. J. Sahl, M. Bates, X. Zhuang, R. Heintzmann, M. J. Booth, J. Bewersdorf, G. Shtengel, H. Hess, P. Tinnefeld, A. Honigmann, S. Jakobs, I. Testa, L. Cognet, B. Lounis, H. Ewers, S. J. Davis, C. Eggeling, D. Klenerman, K. I. Willig, G. Vicidomini, M. Castello, A. Diaspro, and T. Cordes, "The 2015 super-resolution microscopy roadmap," *J. Phys. D. Appl. Phys.*, vol. 48, no. 44, p. 443001, Nov. 2015.

[136] E. Rittweger, K. Y. Han, S. E. Irvine, C. Eggeling, and S. W. Hell, "STED microscopy reveals crystal colour centres with nanometric resolution," *Nat. Photonics*, vol. 3, no. 3, pp. 144–147, Feb. 2009.

[137] F. Göttfert, C. A. Wurm, V. Mueller, S. Berning, V. C. Cordes, A. Honigmann, and S. W. Hell, "Coaligned dual-channel STED nanoscopy and molecular diffusion analysis at 20 nm resolution," *Biophys. J.*, vol. 105, no. 1, 2013.

Figure captions

Figure 1: Model of plasma membrane organization. Upper left: Model of eukaryotic cell with organelle membranes. Main picture: Zoom in on the plasma membrane (PM) the extracellular matrix (ECM) and the cytosolic layer (Cytosol). Left section illustrates the crowding of proteins (~200g/l) in the cytoplasm and the PM. Right side emphasizes important structures for membrane organization (1) Cholesterol and glyco-lipid enriched rafts. (2) Cell cortex membrane interactions (picket fence). (3) Clusters of poly-anionic lipids on the inner PM-leaflet. (4) Palmitoylated proteins on the inner PM-leaflet. (5) Site of endocytosis. (6) Cell adhesion receptors. (7) GPI-anchored proteins on the outer PM-leaflet. (8) Membrane turnover by vesicular transport. (9) Extracellular matrix. Figure is modified from [135].

Figure 2: Spatial (lower axis) and temporal (upper axis) scaling of membrane processes from the bottom up (simulation, right to left) and top down (experimental, left to right). Recent developments have pushed the spatial resolution limits of optical microscopy to the near molecular range (~20 nm). Via combination with correlation spectroscopy methods like FCS temporal processes down to the microsecond range can be studied with high spatiotemporal resolution. Coming from the atomistic side MD-simulations are approaching this now experimentally accessible scale via the use of coarse graining methods and improving computational power.

Figure 3: Principle of STED nanoscopy: A) Molecular transitions during STED imaging. Fluorescent molecules are excited to the ON-state (s_1) by diffraction limited focused beam (blue, B). Spontaneous emission (green) is occurring when the molecule relaxes to the OFF-state (s_0). The red-shifted STED laser, which is focused to a donut shape having zero intensity in the middle (B), switches the molecule to the OFF-state via stimulated emission (red). B) Excitation and STED beam are co-aligned such that the zero of the STED beam is at the maximum of the excitation focus. Scanning of both beams (excitation and STED) over a point emitter results in a diffraction unlimited image of the emitter (green). C) Cross-section of the excitation (blue), the STED (red) beam and the detected emission from a scanned point emitter (green). Resolution increases (green) when the intensity overshoots the saturation threshold of the stimulated emission process of the fluorescent molecules. D) Example of increase in resolution with STED nanoscopy. A stable point emitter (nitrogen-vacancy-center in a diamond) was scanned in absence of STED yielding a diffraction limited image and in presence of STED resulting in ~30fold improvent of spatial resolution. (D) was adapted from [136].

Figure 4: Combination of STED nanoscopy with fluorescence correlation spectroscopy (FCS, STED-FCS). A) Molecular diffusion is detected by recording signal fluctuations over time stemming from fluorescence emission burst (green) of

molecules while they pass the static excitation focus (blue). The size of the detection area can be tuned by the STED beam power (red lines). B) The average molecular transit time can be estimated by auto-correlation of the fluctuation signal. The experimental correlation function (blue) can be fitted (red) to extract the concentration and the mobility of the molecules. C) Calibration measurement of a fluorescent lipid analogue diffusing in a one-component (DOPC) supported-lipid-bilayer (SLB). With increasing STED power P_{STED} the observation spot size decreases which can be detected as a shift in the correlation curve to shorter lag-times (inset, reproduced from [137]). D) Scheme of the main STED-FCS results: The dependence on the apparent diffusion coefficient D_{app} on the observation diameter holds information about anomalous sub-diffusion processes. Top show Monte Carlo simulation snapshots of 2D diffusion with transient trapping (left) and confined hop-diffusion in a meshwork (right). Both diffusion modes can be discriminated with STED-FCS (red and green lines, respectively, in the lower panel). E) Example of experimentally observed trap-diffusion of an sphingomyelin analogue in live cell membranes. (Reproduced from [62]). F) Experimentally observed hop-diffusion of a PE analogue and overlay of Monte Carlo simulations. (Reproduced from [65])

Figure 5: Scanning STED-FCS allows spatiotemporal mapping of lipid heterogeneities. A) Left: Structure of a fluorescent lipid analogue with a PEG-linker between the dye and the lipid to reduce membrane packing artifacts [63]. Top: XZ plane of a live Ptk2 cell stained with the fluorescent lipid analogue (plasma membrane staining). Lower panel XY section of the basal site of the plasma membrane. Circle indicates trajectory of the scanning STED-FCS measurement. B) Correlation carpets from two scanning trajectories (left confocal, right STED). The correlation was separated in 10s blocks (top to down). Correlation amplitude is color-coded; Y-axis depicts correlation time and X-axis position on the scanning orbit. Local mobility (average transit time through the observation spot) can be estimated by the yellow part of the decay. Interaction spots of lipid trapping are indicated with red asterisk. Note the increased resolution in the STED recording reveals more interaction sites. C) 2D histograms depicting the local mobility versus local concentration of a fluorescent sphingomyelin (SM) and phosphoethanolamine (PE) analog diffusing in the plasma membrane of the Ptk2 cells. The histogram for PE is more homogenous than the scattered histogram of SM. However, both histograms show no indication for existence of strongly ordered domains in the plasma membrane (reproduced from [38]).

Figure 6: Membrane protein sequestering by ionic protein-lipid interactions. A) Distribution of PIP2 clusters in plasma membrane of rat adrenal medulla derived cells (PC12). PIP2 was stained with PH-domain (upper panel) and an antibody (lower panel). To resolve the distribution of single clusters STED nanoscopy was used. B) The size distribution of PIP2 clusters was estimated from the STED images. C) and D) Simulations of the dynamic and amorphous PIP2-syntaxin-1A microdomains (C) Side and (D) top-view of a coarse-grained molecular dynamics simulation. 64 copies of syntaxin-1A257–288 (SxTMH) and 64 copies of PIP2 were

incorporated in a bilayer composed of a 4:1 molar ratio DOPC:DOPS. PIP2 was only present in the membrane leaflet facing the N-terminus of syntaxin-1A257–288. Simulations were performed with 150 mM NaCl. White: alkyl chains of the lipids. Cyan: DOPS headgroup. Grey: DOPC headgroup. Yellow: transmembrane region of syntaxin-1A257–288 (residues 266–288). Blue-red: polybasic linker region (residues 257–265, charges in red). Orange-blue: anionic PIP2 headgroup (charges in blue). The domains were stable over 6 μs simulation time; see . D) lower left panel: Simplified scheme of the cluster (reproduced from [79]).

Figure 7: A lipid bound actin scaffold controls lipid phase separation in model membranes. A) Top panel: Confocal image of a fluorescent lipid analog (mangenta) in a supported-lipid-bilayer (SLB) with a ternary lipid mixture that phase-separates into a liquid-ordered (Lo) and liquid-disordered (Ld) domains at low temperature (22C) and homogenous phase at high temperature (35C). The fluorescent lipid analogue stains the Ld phase. The membrane contains 1mol% of a biotinylated phospholipid (DOPE-biotin, partitioning into the Ld domain). Lower panels: Confocal and STED images (as labeled) of the fluorescent lipid analog (mangenta) and actin (green) in the same SLB at low (left) and high (right) temperature but now with an actin scaffold (green) bound to the DOPE-biotin. Phase separation is drastically changed. At high temperature domains are stabilized by actin pinning at low temperature domain coalescence is quenched. B) 2D Ising-model like simulation of phase separation in presence of pinning points distributed along a Voronoi actin-meshwork. Lipids in the Ld phase are shown as magenta, lipids in the Lo phase as black, and the actin network is shown in green as an overlay. The pinning density was 0.1/nm. (1–3) Simulation snapshots obtained without coupling to curvature for three different partitioning probabilities Lo% of the actin-pinning sites into the Lo phase: Lo% = 11 ± 2 (1), Lo% = 59 ± 5 (2), and Lo% = 78 ± 7 (3). No significant influence of the actin network is apparent. (4–6) Same as (1–3) but in the presence of curvature coupling. For snapshots (4) and (6), the lipid domains strongly correlate to the actin network, with Ld domains favoring actin in (4), and the inverse pattern in (6). The lower panels show height profiles of the images (4–6) scanned horizontally along the center of the image; the green dots indicate the positions of the actin fibers (reproduced from [105]).