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Review

In vivo plasma membrane organization: results of biophysical approaches

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Abstract

In the last two decades, various biophysical techniques have been used to investigate the organization of the plasma membrane in live cells. This review describes some of the most important experimental findings and summarizes the characteristics and limitations of a few frequently used biophysical techniques. In addition, the current knowledge about three membrane organizational elements: the membrane-associated cytoskeleton, caveolae and lipid microdomains, is described in detail. Unresolved issues, experimental contradictions and future directions to integrate the variety of experimental data into a revised model of the plasma membrane of eukaryotic cells are discussed in the last section.

Keywords: Membrane domain; Caveolae; Cytoskeleton; Lipid raft; Single-particle tracking; Single-molecule microscopy

1. The plasma membrane, inhomogeneities and in vivo studies

Until recently the Singer and Nicolson fluid mosaic model [1] has been the textbook description of the organization and dynamic behavior of biological membranes. In this model, the lipid bilayer is represented as a neutral two-dimensional solvent in which integral membrane proteins are embedded and free to move. This view of the biological membrane is challenged by experimental results obtained in the last two decades. The current understanding is that the plasma membrane of eukaryotic cells is not homogeneous, but contains a variety of inhomogeneities [2–6]. Proteins and lipids can be temporarily confined to domains, which in turn are thought to modulate the biological functions of the trapped molecules. In the next section, three types of membrane inhomogeneities will be discussed in more detail.

One of the first structures found in plasma membrane of eukaryotic cells were caveolae. These small (~ 60 nm) flask-shaped membrane invaginations consist mainly of caveolin protein, which binds cholesterol. Caveolae have been implicated in a range of cellular functions, such as cholesterol transport, endocytosis and signal transduction [7]. So-called lipid rafts are a second class of membrane inhomogeneities, which was discovered more recently. They are enriched in cholesterol and lipids with saturated acyl chains, such as sphingolipids. Lipid raft sizes measured in vivo range from several tens of nanometers to almost a micrometer [8–11]. A third cause of membrane inhomogeneities and confined diffusion is a network of membrane-associated actin [12]. This membrane skeleton imposes effective barriers on the diffusion of membrane constituents. The size of the diffusional domains is determined by the mesh diameter of the membrane skeleton network and was shown to be about 300–600 nm.

One of the characteristics of all of the membrane inhomogeneities listed is their small size of 50–700 nm. This small size, close to or below the diffraction limit, makes them almost impossible to investigate in any detail by conventional microscopy. Novel microscopy techniques, largely based on the observation of movements of individual molecules, were developed in the last two decades to overcome this limitation. Single-particle tracking, photonic force microscopy and fluorescence-based single-molecule microscopy allow the study of the dynamical behavior of individual lipids or proteins with positional accuracies of 7–

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40 nm. All of these techniques enable accurate in vivo studies of small membrane domains and will be discussed in the following paragraphs.

In single-particle tracking [13–15] a particle, usually a small (~ 40 nm) gold bead, is coated with a low density of an antibody directed against the membrane protein of interest. Once such a bead binds to the target molecule in the cell membrane, the motion of the bead reveals the motion of the target molecule. A camera, coupled to a differential interference contrast (DIC)-equipped microscope, can record this motion. The single-particle tracking technique offers an extremely high temporal (down to tens of microseconds [16]) and positional (typically 7–40 nm) accuracy, as well as the possibility of recording long trajectories (up to 10 min).

The concept of using an antibody-coated bead is also used in techniques where a bead is trapped in a highly focused laser beam, also known as a laser optical trap or optical tweezers [17]. In this way barriers in the cell membrane were detected by dragging a trapped bead, which was attached to one or more membrane proteins, along the cell surface [18,19]. Laser trap and single-particle tracking techniques were also combined: a laser trap was used to confine the motion of a bead in a shallow potential and its local diffusion was measured by high resolution particle tracking [9]. This combination, which has been called 'photonic force microscopy', makes it possible to detect the thermal position fluctuations of the trapped bead with nanometer and sub-second (0.3 s) resolution.

Some of the potential disadvantages of both the single-particle tracking and laser trap techniques are: particle attachment might lead to an alteration of the target molecule behavior and the biochemical uncertainty of a one-to-one labeling ratio. In single-particle tracking this biochemical uncertainty can principally not be eliminated, but significantly reduced by the use of small fluorescent particles (e.g. phycobiliproteins), in combination with Fab fragments and careful preparation procedures. Indeed, single-particle fluorescence imaging studies were able to track individual receptors and detect their aggregation status [14,20–23]. To minimize the potential labeling artifacts and enable the tracking of target molecules that are not accessible form the outside of the cell, techniques were developed to label a target molecule by a single fluorescent molecule, like a fluorescent protein. The recent availability of sensitive CCD cameras has enabled detection and tracking of individual fluorescent molecules, even in live cells [24–26,31]. Excitation of the fluorescent groups or molecules in live cells can be achieved by ordinary wide-field laser illumination, offering the possibility to image both the basal and apical membrane of an adherent cell. However, the large excitation volume in wide-field illumination can result in considerable cellular autofluorescence. One way to reduce this cellular background is to use total-internal reflection (TIR) excitation [25,27]. For cellular applications objective-type TIR is commonly used. In objective-type TIR the laser beam is totally internally reflected in the objective, resulting in an evanescent field of ~ 150 nm in which fluorescent molecules can be excited. In this way the excited volume of a cell is significantly reduced and hence less cellular background is observed. A disadvantage of TIR is the strong dependence of the excitation intensity on vertical position. As membrane topology in cells has a variance of ~ 150 nm [28], quantitative analysis of signal amplitudes in TIR is difficult.

Both the wide-field and TIR techniques have high temporal- (down to 5 ms) and positional (typically ~ 35 nm) accuracies. Especially the application of fusions between autofluorescent proteins, like the Green Fluorescent Protein (GFP), and the protein of interest offers an attractive possibility to label a variety of membrane proteins anchored in the cytoplasmic leaflet at a precisely defined one-to-one ratio [29,30]. Consequently, the single-molecule microscopy technique enables the measurement of the aggregation status of labeled proteins [26,31]. However, due to blinking and bleaching of the fluorescent groups or molecules, the observation time is largely limited to a time scale of tens of seconds.

Single-molecule techniques are complemented by more conventional techniques to study bulk diffusion. The most frequently used is fluorescence recovery after photobleaching (FRAP) [32], which reveals the diffusion of fluorescently labeled membrane proteins or lipids from a large non-bleached area, into a small photobleached spot of typically 1–2.5 μm in diameter. After a short, high-intensity laser pulse (bleaching pulse), the recovery of the fluorescence is monitored with a fluorescence microscope. From FRAP experiments one obtains information about the diffusion coefficient of mobile molecules and, if present, the amount of molecules that do not move on the time scale of the experiment: the immobile fraction. Alternatively, FRAP data were interpreted in terms of anomalous subdiffusion [33], which has been observed in single-particle tracking experiments [16,34]. By varying the bleach spot size, additional information on diffusion barriers in the cell membrane can be obtained [35]. Barriers for lateral diffusion result in a decrease of the mobile fraction with increasing bleach spot diameter.

Fluorescence resonance energy transfer (FRET) [36], a technique commonly used to study interactions between molecules, has recently also found its applicability to study membrane organization [37,38]. In FRET an excited fluorescent dye, the donor, non-radiatively transfers energy to a second fluorescent dye with a red-shifted emission spectrum, the acceptor, which is in close proximity (0.1–10 nm). The efficiency of the transfer process is strongly dependent on distance. By measuring the dependence of the FRET efficiency on the acceptor density, one obtains information on the co-distribution of molecules in the cell membrane.

Results from the techniques discussed in the previous sections are supplemented by a plethora of results from confocal-, electron- and atomic force microscopy, fluorescence correlation spectroscopy (FCS), biochemistry, as well

as by structural techniques like electron paramagnetic resonance (EPR) and nuclear magnetic resonance (NMR). Furthermore, a wealth of important data about lipid mixtures and membrane behavior was obtained from work on model membranes [39–41]. In the following sections the current knowledge about three membrane organizational elements in live cells: the membrane-associated cytoskeleton, caveolae and lipid microdomains, will be outlined and discussed. Incorporation of all this knowledge into a revised model of the plasma membrane, unresolved issues and future directions will be discussed in the last section.

2. Membrane compartmentalization: the actin-based membrane skeleton

Work on the restriction of lateral motion of the band 3 transmembrane protein in erythrocytes indicated that the membrane associated cytoskeletal network was involved in the hindrance of free mobility [42–44]. Moreover, in FRAP experiments on class I major histocompatibility complex (MHC) molecules in K78-2 hepatoma cells, a low diffusion coefficient and bleach spot size dependence of the mobile fraction were observed [45]. To further investigate these results, class I MHC molecules (either transmembrane or glycosylphosphatidylinositol(GPI)-anchored) were labeled with antibody-coated gold particles [18]. Using a laser optical tweezer, these gold-labeled MHC class I molecules were moved across the membrane surface of murine HEPA-OVA cells until a barrier was encountered. In this way the so-called barrier-free path length (BFP) was determined. This BFP turned out to be 1.7 ± 0.2 μm for the GPI-anchored MHC class I molecules and 0.6 ± 0.1 μm for the transmembrane MHC class I molecules at 23 °C. At 34 °C the BFP increased for both molecules by about a factor of 5. From these results Edidin et al. [18] concluded that dynamic barriers on the cytoplasmic side of the plasma membrane, which were likely to contain spectrin and its membrane anchor ankyrin, impair the lateral movement of proteins.

Indeed, single-particle tracking experiments on E cadherin, a cell–cell recognition-adhesion receptor, in cultured epithelial cells showed that the membrane was compartmentalized in 300–600 nm diameter domains [19,46]. In these domains a fraction (30–64% depending on the calcium concentration) of receptor molecules was confined for 3–30 s. However, in FRAP experiments these molecules showed long-range diffusion, which can only be explained by successive movements ('hops') to adjacent compartments. These results lead to the 'membrane-skeleton-fence' model [12]. In this model the movement of membrane spanning proteins is confined, because their cytoplasmic portions sterically sense a spectrin-like cytoskeletal meshwork ('fence'), which is present close to the cytoplasmic leaflet of the cell membrane. The observed limited confinement time and intercompartimental hops were explained by thermal fluctuations of the distance between the membrane sheet and parts of the impermeable meshwork or dissociation of parts of the meshwork itself.

This interpretation was supported by experiments that showed that disruption of the actin cytoskeleton or truncation of the cytoplasmic domains of transmembrane proteins decreased the fraction of proteins exhibiting transient confinement and increased the BFP [47–49]. From the truncation experiments it was estimated that the barriers to lateral mobility were located 2–3 nm below the cytoplasmic leaflet [48]. Further support for the involvement of the cytoskeletal elements comes from experiments where the diffusion of MHC class I molecules was measured using single-particle tracking on both normal and α-spectrin-deficient MEL cells [50]. For the α-spectrin-deficient MEL cells the mean diagonal length of the confined area was larger (650 nm compared to 330 nm) and the residence time longer (45 s compared to 40 s) than in normal MEL cells, a clear indication that there were fewer barriers to lateral diffusion in the α-spectrin-deficient MEL cells.

Additional evidence for confined diffusion was provided by a study, in which single-particle tracking with temporal resolution of 25 μs and single-molecule fluorescence microscopy were used to show that the lateral diffusion of phospholipids in cell membranes of normal rat kidney (NRK) cells is even further compartmentalized [16]. The long-range diffusion of these phospholipids is mainly limited by the hop-rate across the compartment boundaries. As the boundaries were modulated by actin cytoskeleton modifications, it was concluded that the lipids sensed the actin meshwork. In fact, the single-particle tracking measurements of fluorescein-labeled 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) molecules in the membrane of NRK cells showed double compartmentalization: 230-nm compartments (average residence time of 11 ms), within larger 750-nm domains (average residence time 0.33 s). The same compartment sizes were observed when the mobility of the transferrin receptor was analyzed, indicating that the compartmentalization was also sensed by transmembrane proteins.

Together with the 'membrane-skeleton-fence' model, these results lead to the proposition of the 'anchored-protein picket' model [16] (Fig. 1). This model assumes that close to the cytoplasmic leaflet an actin membrane skeleton meshwork is present, which has various transmembrane proteins anchored to it. The latter effectively act as rows of pickets that temporarily confine diffusing phospholipids and proteins through steric hindrance and circumferential slowing effects. The latter presumably results from the increased packing near proteins [51,52] or from increased hydrodynamic friction [53,54]. Thermal membrane undulation might play a role in facilitating hopping of proteins between adjacent compartments of the cytoskeleton meshwork [55].

Although the biological function of the membrane skeleton in relation to plasma membrane organization is not entirely clear yet, a recent study showed that accumulation

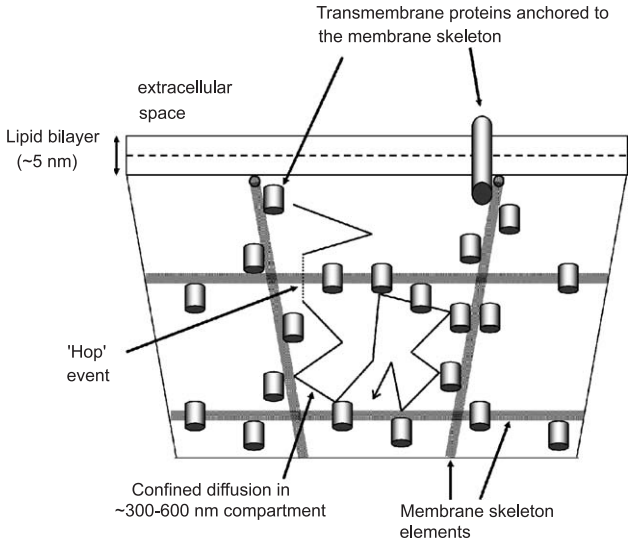

Fig. 1. Schematic drawing of the anchored membrane-protein picket model. The drawing displays a view onto the cytoplasmic leaflet of the plasma membrane. Transmembrane proteins are anchored to the membrane-associated actin cytoskeleton meshwork and effectively act as rows of pickets, which temporarily confine the movement of proteins and lipids.

of actin-anchored proteins formed membrane diffusion barriers during the neuronal polarization [56]. Moreover, cross-linked GPI-anchored proteins were shown to bind to discrete actin-associated sites in a surface scanning resistance study [57]. These sites could have an organizational role in signal transduction. Actin also seems to play an important role in the mechanism of Lck recruitment to the T-cell receptor cluster [58].

3. Membrane compartmentalization: caveolae and lipid rafts

3.1. Caveolae

Caveolae are another important class of membrane inhomogeneities [59]. They are small (~ 60 nm diameter), flask-shaped membrane invaginations containing mainly caveolin protein, a protein that binds cholesterol. The cytoplasmic coat of caveolae can occupy an area up to 150 nm in diameter [60] and EM results show partial disruption of caveolae upon cholesterol depletion [61]. Caveolar components can be isolated by treatment of cells in the cold with nonionic detergents, followed by density gradient centrifugation. As they resist detergent solubilization, they are consequently found in the low-density fraction, also called the detergent-resistant membrane (DRM) fraction.

Caveolae have been implicated in a range of cellular functions, such as cholesterol transport and endocytosis. The important role of caveolae in regulating endocytosis is illustrated by caveolin-1 knock-out mice. These mice show microvascular hyperpermeability, which could be treated by a specific nitric-oxide synthase inhibitor [62]. More recently it was discovered that caveolae are also involved in signal transduction [7]. For example, caveolin-3 knock-out mice develop a progressive cardiomyopathy and show hyperactivation of the p42/44 MAPK cascade [63].

Roy et al. [64] have shown how a dominant negative mutant of caveolin-3 selectively inhibits the small GTPase H-Ras by disrupting cholesterol-rich domains. This result was supported by flotation- and electron microscopy results on GFP-H-Ras and caveolin-1 [65], which showed that an active H-Ras mutant associates significantly less with caveolae than the wild-type H-Ras or the H-Ras membrane anchor. About 44% of the H-Ras membrane anchor was found to be associated with caveolae in the EM experiments [65].

One would expect that the association of the H-Ras membrane anchor with caveolae does result in an immobilized or confined diffusing protein fraction on short time scales. A recent study made use of the H-Ras membrane anchor which had been fused to the enhanced Yellow Fluorescent Protein (eYFP) [30]. The diffusion of individual fluorescent molecules anchored in the cytoplasmic leaflet of the cell membrane was followed on the 5–200-ms time scale. A fraction of 41 ± 7% of the H-Ras membrane anchor exhibited slow (0.29 ± 0.12 $\mu m^2/s$) diffusion which was confined in ~ 200-nm-sized domains in 3T3 cells. Cholesterol depletion and actin cytoskeleton disruption did not change the fraction of molecules in the 200-nm domains or influence the domain size; hence, they were related neither to classical lipid rafts nor to actin membrane skeleton associated domains. As 44% of the H-Ras membrane anchor has been localized in caveolar structures in an EM study [65] and 3T3 cells contain caveolae [66], it is possible that the 200-nm domains are caveolar structures. However, it must be noted that the size of caveolae (~ 60 nm) is significantly smaller than the size of the observed domains (~ 200 nm), although the cytoplasmic coat of caveolae can occupy an area up to 150 nm in diameter [60]. Furthermore, the observed cholesterol insensitivity of the 200-nm domains contradicts to a certain extent EM results that show partial disruption of caveolae upon cholesterol depletion [61].

3.2. Lipid rafts

The high cholesterol content found in caveolae is also characteristic of the most elusive class of membrane domains, the so-called lipid rafts [67,68]. Besides a high cholesterol content, lipid rafts contain fully saturated acyl chain lipids, such as sphingolipids [69]. A tight packing of these constituents is thought to result in a liquid-ordered phase, which separates the lipid rafts from the surrounding, liquid-disordered membrane. This raft concept is supported by experiments using model membranes and unilamellar vesicles which prove that liquid-disordered phases and

liquid-ordered phases can coexist [70–74] and that the latter are partially detergent-resistant [75,76].

In cellular lysates lipid raft constituents were found in the DRM fraction after cold non-ionic detergent extraction and gradient centrifugation [69]. As the DRM fraction was enriched in cholesterol and sphingolipids that form a liquid-ordered phase [77], it was hypothesized that the DRM fraction is directly corresponding to lipid rafts in vivo. Some proteins, like GPI-anchored proteins, are enriched in the DRM fraction [75,78,79]. It is thought that their saturated GPI-anchor fits well into the liquid-ordered state of lipid rafts. As caveolin is also found in the DRM fraction, caveolae are sometimes referred to as a 'special type of lipid raft'. This is confusing, as caveolae are quite distinct structures in vivo, whereas lipid rafts are still quite elusive and experimentally less well defined. However, as both lipid rafts and caveolae are dependent on cholesterol, it cannot be excluded that they are actually quite closely related and interacting in vivo.

The proposed biological functions of lipid rafts range from membrane trafficking and sorting [67,69] to a dynamic role in signal transduction [80]. Although it was recently shown that molecular characteristics of isolated DRM fractions are sensitive to the particular cell and detergent type used [81,82], DRM isolation is still generally used to identify potential 'lipid raft' components. Despite considerable effort, a more reliable in vivo method to detect lipid raft association has not yet been established. Indeed, as will be shown in the next few paragraphs, in vivo definitions and qualifications of lipid rafts differ among experimental approaches, hampering the comparison and integration of the variety of experimental results. The various experimental approaches to detect lipid rafts in the exoplasmic leaflet will be presented, followed by the current view of cytoplasmic leaflet lipid domains.

3.2.1. Detection of lipid rafts in the exoplasmic leaflet

To obtain a reliable measurement of the size of lipid rafts and their stability in vivo, various techniques, such as single-particle tracking [10], laser trapping (photonic force microscopy) [9] and single-molecule microscopy [11], have been utilized. The experiments yielded a broad range of raft diameters from $\sim$ 50 up to $\sim$ 700 nm. The smallest size was measured using a method where a laser trap was used to confine the motion of a bead bound to a raft protein to a small area ($\leq$100 nm) [9]. In this way, the local diffusion was measured by high-resolution particle tracking on the membrane of live BHK fibroblasts and PtK_2 cells. The experiments showed that when proteins are raft associated, their diffusion became independent of the type of membrane anchoring (both GPI-anchored proteins and transmembrane proteins were tested) and their diffusion constant was significantly reduced compared to the diffusion constant of non-raft proteins. Raft-associated GPI-anchored proteins were found to be stably associated with lipid rafts for up to 10 min. Cholesterol depletion resulted in an increased diffusion of raft-associated proteins to values equal to or higher than comparable non-raft proteins. Furthermore, an average raft radius of 26 $\pm$ 13 nm was reported in this study [9]. Such a size corresponds to the size of small cholesterol-dependent, non-elastic barriers (<100 nm) found in surface scanning resistance measurements of GPI-anchored proteins [57]. However, the density of these barriers was closely correlated to the density of caveolae, indicating that they actually may not be lipid rafts.

A single-molecule study employing fluorescent lipid probes revealed much larger sizes of lipid domains [11]. A saturated acyl-chain fluorescent lipid probe (1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine, DMPE-Cy5) was used to detect liquid-ordered domains in live human coronary artery smooth muscle (HASM) cells. This saturated lipid probe showed a high ($\sim$ 100-fold) partitioning into domains with an average size of 0.7 μm (0.2-2 μm). Within these domains the saturated lipid probe was diffusing fast (0.6-0.9 $\mu m^2/s$) and had a long residence time of $\sim$ 13 s. The domains covered about 13% of the membrane area. In contrast, an unsaturated lipid probe (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, DOPE-Cy5) showed free, though slightly anomalous, sub-diffusion outside the domains, proving the specificity of the saturated lipid probe. Experiments with a higher concentration of the saturated lipid probe ($\sim$ 15 per domain) indicated that the observed domains were stable on a time scale of minutes and probably linked to the cytoskeleton.

Micrometer-sized lipid domains were also observed in a another study where fluorescent lipid-analogues with preferences for liquid-disordered (dialkylindocarbocyanineC_{12}: $DiIC_{12}$ or *N*-{[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-hexanoyl}sphingosyl phosphocholine: C_6-NBD-SM) and liquid-ordered ($DiIC_{16}$) phases were used to stain the plasma membrane of several types of mammalian cells [83]. It was shown that cholesterol depletion induced the formation of micrometer-sized domains in the plasma membrane of live cells. However, these domains were enriched in fluid domain-preferring lipid analogs; an ordered domain-preferring lipid analog partitioned outside of these fluid domains. As the fluid domains covered only 20–30% of the plasma membrane area, it was concluded that the remaining 70–80% of the plasma membrane area was in the ordered state after cholesterol depletion. These results do not seem to agree with the results of Schütz et al. [11]. As different lipid analogs and cells were used and no cholesterol depletion was performed in the study of Schütz et al., additional experiments will have to be done to clarify this discrepancy. Another surprising result of the study of Hao et al. [83] was that cholesterol depletion did not visibly alter the distribution of cross-linked and non-cross-linked GPI-anchored proteins, although these proteins are often used as lipid raft markers. The difference in response to cholesterol depletion of the fluorescent lipid analogue and the GPI-anchored proteins could indicate that different types of lipid rafts are present in the plasma membrane.

GPI-anchored proteins have been used extensively in particle tracking studies to observe lipid rafts by detecting confined diffusion. An early study in C3H 10T1/2 murine fibroblasts showed that 35–37% of the GPI-anchored protein Thy-1, as well as the ganglioside GM1, are transiently confined (7–9 s) in so-called 'transient confinement zones', with a diameter of 260–330 nm [84]. Decrease of cellular glycosphingolipid synthesis by 40% reduced the percentage of trajectories of Thy-1 exhibiting confined diffusion and the size of the confining domain ~ 1.5-fold, indicating that confinement was indeed partially caused by diffusion inside lipid rafts. This result was confirmed by the fact that the percentage of trajectories exhibiting confinement and the size of the domains were not affected by Triton X-100 treatment of the cells, indicting that the observed domains were detergent-resistant.

More recent single-particle tracking studies in the same cell type, again using Thy-1 and the GM1 as raft probes, proved that the abundance of transient confinement zones and their size was reduced by cholesterol depletion [10]. Furthermore, it was observed that zones can be visited again within tens of seconds after the original escape of a particle and that diffusion within the zones was reduced by a factor of 2 compared to diffusion outside of zones. The latter is consistent with the zones being in a liquid-ordered phase [52,85]. The same study showed that fluorescent lipid analogues with unsaturated acyl chains spend much less time in transient confinement zones than Thy-1, GM1 or lipid analogs with saturated acyl chains. In summary, the single-particle tracking studies showed that 200–300-nm diameter cholesterol-sensitive domains are present in the plasma membrane of various cell types. These domains temporarily trap GPI-anchored proteins and ganglioside GM1 and are stable for at least tens of seconds.

However, these particle-tracking results are not in agreement with results from a single-molecule microscopy study of the translational motion of GPI-linked and native I-E^k class II MHC membrane proteins in live CHO cells [86]. Visualization of individual I-E^k proteins was achieved by a Cy5-labeled peptide that binds to a specific extracytoplasmic site of I-E^k class II MHC. From the single-molecule fluorescence microscopy measurements, the diffusion constant of the proteins was derived on a time scale of 0.1–5 s and diffusion coefficients of ~ 0.2 $\mu m^2/s$ for both the GPI-anchored and native I-E^k proteins were reported. To distinguish between free diffusion of proteins and diffusion of proteins in small, rapidly diffusing domains, the relative diffusion of pairs of proteins was studied for intermolecular separations of 0.3–1.0 μm. Both the global diffusion analysis as well as the pair-diffusion analysis showed no deviation from Brownian motion. Hence, it was concluded that no confinement zones exists for either the native or the GPI-anchored I–E_k protein in the plasma membrane of live CHO cells. The results cannot rule out, however, the presence of domains that are larger than 0.3–4.0 μm^2 or domains with permeable barriers. Nevertheless, as described in the previous paragraph, the cholesterol-sensitive domains that temporarily trap GPI-anchored proteins in particle-tracking studies [10] have sizes (~ 0.03–0.07 μm^2) and stabilities (tens of seconds) that would allow their detection by the single-molecule technique. Cell type-related differences could offer a potential explanation for their absence in the single-molecule study.

Besides the discussed single-particle tracking methods, fluorescence resonance energy transfer (FRET) microscopy was also used to study lipid rafts. As FRET is a phenomenon that occurs when fluorophores are in very close proximity (0.1–10 nm), extremely small rafts could be detected using this technique. In a study on the occurrence of lipid rafts in the apical membrane of MDCK cells, the distribution of the GPI-anchored protein 5′ nucleotidase (5′ NT) was studied by fluorescently labeled donor and acceptor antibodies [38]. The efficiency of energy transfer correlated strongly with the surface density of the acceptor-labeled antibody. This indicated that the GPI-anchored proteins were randomly distributed, in disagreement with the expected clustering in lipid rafts. In an alternative approach, fluorescence depolarization was used to measure the extent of energy transfer between fluorescent analogues bound to a GPI-anchored folate receptor [8]. Contrary to the results on 5′ NT, the extent of energy transfer signal for this GPI-anchored folate receptor was independent of receptor density and sensitive to cholesterol removal. The authors concluded that the GPI-anchored folate receptor is clustered in cholesterol-dependent microdomains of ≤70 nm in diameter in CHO and CaCo cells.

To clarify the observed difference in distribution between the 5′ NT and GPI-anchored folate receptor, a comparative study into the organization of three endogenous GPI-anchored proteins (folate receptor, CD59 and 5′ NT) and a lipid raft marker (ganglioside GM1) in the membrane of several different cell types was performed [87]. It became evident that all the GPI-anchored proteins and the GM1 ganglioside were randomly distributed, disagreeing with the idea of a unified lipid raft type. These results make the existence of stable and large (hundreds of nanometers) rafts highly questionable. To explain their results, the authors suggest that rafts could exist as transiently stabilized structures. Alternatively, if rafts would be stable, they would be small structures that cover a minor fraction of the cell surface and contain a limited amount of GPI-anchored proteins and GM1.

This last hypothesis is supported by recent work of Sharma et al. [88]. In this study, homo-FRET (monitoring the extent of depolarization of the fluorescence emission over and above that produced by rotational diffusion of a single species of fluorophores) was used to analyze the organization of different GPI-anchored proteins expressed in various cell lines. However, the authors extended the steady-state methods of their previous study [8] by theoretically modeling the changes observed in homo-FRET efficiencies upon photobleaching of the fluorophores to obtain informa-

tion on the size of GPI-anchored protein clusters. In addition, direct measurements of the anisotropy decay rates provided information on intermolecular distances of GPI-anchored proteins in such clusters. These homo-FRET studies were supplemented by hetero-FRET experiments (monitoring the extent of energy transfer between two spectrally different fluorophore species) and theoretically explained by a model in which a minor population of GPI-anchored proteins was present in small clusters (< 5 nm). Taken together, the study showed that 20–40% of the GPI-anchored proteins in the cell membrane are organized as clusters that consist of, at most, four GPI-anchored proteins, whereas the remaining population of GPI-anchored proteins is present as monomers outside the clusters. Further experiments showed that the clusters are sensitive to cholesterol depletion and not observed when a transmembrane anchor is used instead of a GPI-anchor, supporting the hypothesis that the clusters are lipid rafts. Experiments using several species of GPI-anchored proteins showed that different species are found in the same cluster. Antibody-mediated cross-linking of a specific species segregated this cross-linked species from the pre-existing clusters and prevented its endocytosis via a GPI-anchored protein-selective pinocytic pathway. From this FRET-based study, it seems that lipid rafts are very small (< 5-nm diameter) cholesterol-dependent clusters that contain a minor fraction of the GPI-anchored proteins in the cell membrane.

The organization of GPI-anchored proteins in the plasma membrane of live cells was also investigated in a recent paper by Glebov and Nichols [89]. However, in this study a ratio-imaging FRET analysis of fluorescently labeled GPI-anchored proteins was used. GPI-anchored proteins fused to monomeric CFP (mCFP) and monomeric Citrine fluorescent protein (mCitFP), a FRET pair, were used to determine if GPI-anchored proteins are clustered in the plasma membrane of unstimulated cells. Coexpression of GPI-anchored mCFP and GPI-anchored mCitFP resulted in a linear dependence of the FRET efficiency on the mean fluorescent donor (mCFP) intensity in COS7 and Jurkat cells, indicating a random distribution of these GPI-anchored proteins. A similar behavior was observed when the FRET efficiency between a GPI-anchored protein and a non-DRM, transmembrane protein was analyzed. Furthermore, cholesterol depletion using β-methyl cyclodextrin did not affect the random distribution of GPI-anchored proteins. To prove that the ratio-imaging FRET approach could indeed detect clustering of GPI-anchored proteins, GPI-anchored fluorescent proteins were clustered by an anti-GFP antibody. This treatment resulted in a clear change of the FRET efficiency dependence on the mean fluorescence intensity of the donor, proving that induced clustering can be detected. In summary, the ratio-imaging FRET study did not detect clustering of GPI-anchored proteins in unstimulated conditions.

These results do not seem to agree completely with the results obtained by Sharma et al. [88], who showed that a minor fraction of 20–40% of GPI-anchored proteins were localized in small (< 5 nm), cholesterol-dependent clusters, containing at most four GPI-anchored protein molecules. Glebov et al. estimate that at most 10% of the GPI-anchored proteins could be in clusters similar to those induced by anti-GFP antibody treatment without detection in their ratio-imaging assay. Hence, at first sight Glebov et al. should have been able to detect the 20–40% clustered fraction observed by Sharma et al. However, this only holds if one assumes that the antibody-induced clusters of Glebov et al. are comparable in size and make up to the clusters observed by Sharma et al., something which is not necessarily true. As the average size of the clusters of Sharma et al. is not known (four molecules per cluster is an upper limit estimate), it is hard to judge if the 20–40% clustering they observe could indeed be have been observed by the ratio-imaging method of Glebov et al. A direct comparison of the same constructs in the same cell lines with both methods would provide a more definitive answer.

In conclusion, the studies on potential lipid-raft marker molecules in the exoplasmic leaflet of the cell membrane (like GPI-anchored proteins or raft-associated lipid analogs) yield a wide range of characteristic parameters for liquid-ordered lipid microdomains in terms of size (5 nm–1 μm), stability (0.1 s–10 min) and abundance (13%–80% of the membrane surface). In Fig. 2, some of the experimental results on lipid microdomains are depicted. Of course, one has to be aware that differences in size and stability of lipid microdomains could be related to the different techniques, experimental conditions, and particular cell types used. Nevertheless, despite the substantial amount of experimental data, it is at the current state of research unrealistic to formulate a consistent model of exoplasmic leaflet organization and lipid rafts in vivo.

3.2.2. Detection of lipid rafts in the cytoplasmic leaflet

Contrary to the exoplasmic leaflet, current knowledge about the organization of the cytoplasmic leaflet of the cell membrane is very limited. Research into the in vivo existence of lipid raft-like structures and coupling between the two membrane leaflets has only started recently. Model membrane experiments indicate that due to the different lipid composition of the cytoplasmic leaflet, the spontaneous segregation of liquid-ordered domains in this leaflet is not likely [90]. Nevertheless, the organization of the cytoplasmic leaflet is of great biological interest. The transduction of cellular signals from the outside to the inside of the cell usually involves the formation of specific phospholipid species (phosphatidylinositol-2-phosphate and phosphatidylinositol-3-phosphate) in the cytoplasmic leaflet, which function as docking sites for several PH-domain-containing cytoplasmic proteins.

Moreover, a wealth of proteins that are anchored in the cytoplasmic leaflet play essential roles in signal transduction processes. Well-known examples are G-proteins, small GTPases and Src-family kinases. These proteins are anchored in the cytoplasmic leaflet of the plasma membrane

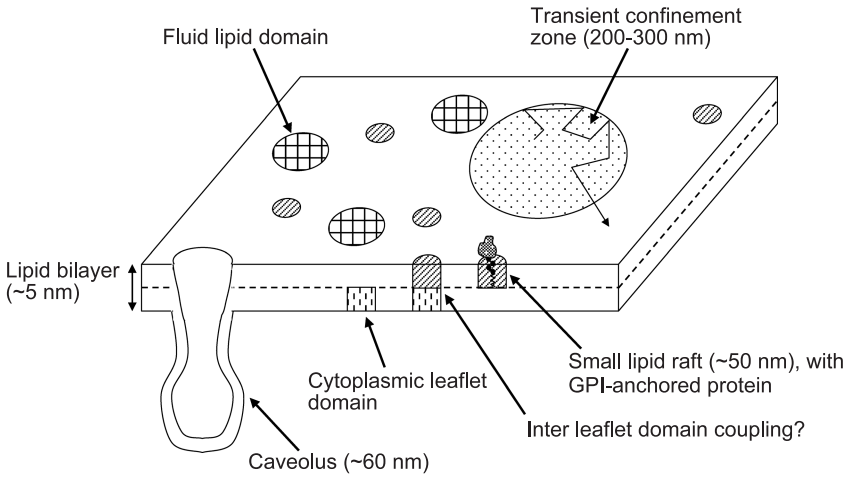

Fig. 2. The mosaic model of the plasma membrane. In this model the plasma membrane contains several types of domains, like caveolae, transient confinement zones, smaller fluid lipid domains, cholesterol-dependent liquid-ordered domains (lipid rafts) and cytoplasmic leaflet domains. For simplicity the bilayer indication is omitted in the caveolus. Structures are not all to scale; approximate sizes are indicted in drawing.

via covalently attached lipid modifications including long chain fatty acids. However, the acyl chains differ significantly between various membrane-anchored proteins. This variety of lipid modifications is thought to be involved in a differential localization of membrane-anchored proteins in putative cytoplasmic leaflet lipid-rafts, something suggested by their differential affinity for the DRM fraction [65,91–94].

In a recent study by Zacharias et al. [95], dimerization-suppressed mutants of the FRET-pair Cyan- and Yellow Fluorescent Protein (CFP and YFP) were coupled to different lipid anchors and caveolin. Combinations of these constructs were expressed in MDCK cells and the dependence of the FRET efficiency on the acceptor density was measured. The results showed that myristoyl-palmitoyl-, but not geranylgeranyl-modified fluorescent proteins are clustered in cholesterol-dependent aggregates and co-cluster with caveolin. As no visible aggregates and no intradomain, density-dependent FRET was seen for the myristoyl-palmitoyl-modified fluorescent proteins, the authors conclude that the observed aggregates must be fairly small. Interestingly, also the geranylgeranyl-modified fluorescent protein seemed to cluster, but did not co-cluster with either caveolin or the myristoyl-palmitoyl-modified fluorescent protein. Moreover, the clustering of the geranylgeranyl-modified fluorescent proteins was not disrupted by cholesterol depletion, indicating that these aggregates are probably not lipid rafts.

However, the biological significance of these cholesterol insensitive aggregates is questionable as the membrane-anchoring sequence of the geranylgeranyl-modified fluorescent proteins used consisted of the CAAX-motif (consensus sequence for prenylation) only. In cells prenylated proteins require a second membrane-binding motif (like a polybasic domain or palmitoylation) for correct and stable membrane binding [96]. Hence, it is not clear how the clustering results for the geranylgeranyl-modified protein in the FRET study are related to prenylated proteins in cells, such as Ras. Another point, which is not addressed in the study, is the pronounced scatter in the FRET data, which could reflect the natural heterogeneity in the observed clusters. Alternatively, the scatter could be partially due to heterogeneity in the lipid anchors themselves. Palmitoylation, for example, was shown to be reversible and dynamic in cells [97]; this could give rise to membrane anchors with a variable degree of palmitoylation.

Although the existence of cholesterol-dependent clusters on the cytoplasmic leaflet of the cell membrane was demonstrated by the abovementioned FRET experiments, no exact size and stability could be measured. For this, techniques like particle tracking or single-molecule microscopy could be used. However, as the attachment of particles to proteins anchored in the cytoplasmic leaflet is not practical, fluorescence-based single-molecule microscopy is the most logical approach for such a study. Cytoplasmic leaflet anchored proteins could be visualized by fusion to an autofluorescent protein. It has been shown that the eYFP variant is, at least up till now, the best suited for wide-field single-molecule fluorescence microscopy in cells because of its high photon yield and minimal spectral overlap with cellular autofluorescent background [29].

Therefore, eYFP fusions to the membrane anchoring sequence of the small GTPase H-Ras and the membrane anchoring sequence of the Src-kinase Lck were used in recent single-molecule studies aimed at detecting potential cytoplasmic leaflet lipid microdomains [30]. The H-Ras membrane anchor contains three cysteines to which one S-prenyl and two S-acyl groups are attached, whereas the Lck membrane anchor has two cysteine-linked S-acyl groups and one glycine-linked myristoyl group. Lck has been found in the DRM fraction [91,92]. For H-Ras the DRM association is less clear, both enrichment [65,94] and absence [93] in DRM fractions have been found.

The mobility of thousands of individual membrane-anchored fluorescent molecules was studied in live tsA201 cells on time scales between 5 and 200 ms. Analysis of the trajectory sets and the corresponding mean square displacement distributions revealed that there were two populations of diffusing molecules [30]. For the H-Ras membrane anchor, it was found that the largest population, 73 ± 5% of the molecules, showed free diffusion at 22 °C, with a diffusion coefficient of 0.53 $\mu m^2/s$. The remaining 27% of the molecules showed a much lower mobility (0.06 $\mu m^2/s$) and was confined to domains with an average size of ~ 250 nm. FRAP experiments were performed to analyze the stability of those domains on longer time scales. Indeed, it was found that an immobile fraction of the same size was present, which in turn indicated that the domains identified in the single-molecule experiments were stable for up to 10 s. Similar, though smaller, immobile fractions have been observed before in FRAP experiments where a GFP fused to the full-length H-Ras protein was used [98].

The 200-nm domains were also observed when the diffusion of the H-Ras membrane anchor and the Lck membrane anchor was studied in 3T3-A14 fibroblast cells at 37 °C. About 41% of the H-Ras membrane-anchored molecules were localized in the 200-nm domains under these conditions. Preliminary experiments showed that the fraction of Lck membrane-anchored molecules in 200-nm domains is significantly smaller (Lommerse, P.H.M. et al., unpublished data), indicating that the affinity for the 200-nm domains is dependent on the type of membrane anchor. For both the H-Ras and Lck membrane anchor, cholesterol depletion (using methyl-β-cyclodextrin) and actin cytoskeleton disruption did not change the fraction of molecules in the 200-nm domains nor did it influence the domain size, hence the domains were neither related to classical lipid rafts nor to membrane skeleton associated domains. As indicated in the caveolae section of this review, it is possible that the 200-nm domains are caveolar structures.

The single-molecule experiments further revealed interesting results for the remaining, supposedly freely diffusing fraction of molecules. For both the H-Ras membrane anchor and the Lck membrane anchor, the diffusion coefficient decreased by ~ 15–30% after cholesterol depletion. The interpretation put forward was that small lipid rafts into which the membrane anchors were captured aggregate after cholesterol depletion, resulting in an increased raft size and consequently in a decreased mobility. The formation of larger (micrometer-sized) domains after cholesterol depletion has been observed before [83]. The single-molecule microscopy technique used has a positional accuracy of 35 nm, which would enable one to observe domains with a diameter of more than 70 nm directly. As no domains were directly observed, even after cholesterol depletion, their size must be smaller than 70 nm. Such small domains would be more likely to be slowed down on larger (micrometer) length scales than individual membrane-anchored molecules. Indeed, a significant slowdown of the mobile fraction of the Lck membrane anchor was observed in FRAP experiments.

The estimated raft diameter of < 70 nm is in good agreement with the lipid raft sizes of < 70 nm that have been reported before [8,9]. However, these studies as well as the FRET study of Hao et al. [83] indicated that cholesterol depletion results in lipid raft dissociation. This contradicts our interpretation of cholesterol-depletion-induced raft aggregation as well as a finding that cholesterol depletion induced large domain formation in live cells [83]. Additional experiments, employing a combination of techniques (EM, FRET- and single-molecule microscopy) using several membrane-anchored proteins and methods of cholesterol depletion/repletion, will most likely provide a more definitive view on these contradictory findings.

As described, the few experiments done so far to investigate the presence of domains in the cytoplasmic leaflet of the plasma membrane indicate that different kinds of small domains exist, some of which are cholesterol-dependent, while others are not. There is still considerable uncertainty about the in vivo relationship and overlap between caveolae and potential 'pure' cytoplasmic leaflet lipid microdomains. Additional experiments will have to be performed in order to obtain a more conclusive picture of the cytoplasmic leaflet structure and its potential role in signal transduction.

3.2.3. Inter-leaflet coupling

A very interesting and important, but hardly investigated, issue is the inter-leaflet coupling of membrane domains. This inter-leaflet coupling could potentially facilitate signal transduction, which often involves interactions between transmembrane proteins and molecules anchored in the cytoplasmic leaflet. In a study to investigate whether lipid rafts are involved in the interactions between the cytoplasmic leaflet-anchored tyrosine kinase Lyn and the IgE high affinity receptor, IgE-FcεRI [99,100], Pyenta et al. [101] used cross-correlation analysis to study inter-leaflet coupling. For this purpose, a GFP linked to the membrane anchoring sequence of Lyn was constructed and its behavior compared to a GFP fused to the membrane-anchoring sequence of K-Ras, a protein with a low affinity for lipid rafts [64,65,102]. The ganglioside GD_{1b} and the GPI-anchored protein Thy-1 were used as markers for exoplasmic leaflet rafts.

When IgE-FcεRI, GD_{1b} and Thy-1 were cross-linked to form large patches in RBL-2H3 cells, the co-redistribution of the cytoplasmic leaflet anchored GFPs was quantified using cross-correlation analysis. It was found that GFP with the Lyn membrane anchor co-redistributed significantly more with exoplasmic leaflet lipid raft markers and IgE-FcεRI than the GFP with the K-Ras membrane anchor and an endogenous prenylated protein (Cdc42). These results indicate that the functionally important interaction between Lyn and cross-linked IgE-FcεRI could indeed be facilitated by their mutual co-association with lipid rafts and provides the first evidence for inter-leaflet coupling.

4. Conclusion and outlook

It is clear that the original fluid mosaic model of the plasma membrane, as formulated by Singer and Nicolson [1], is not completely satisfactory any more. In view of the types of membrane inhomogeneities described in the previous sections, one must conclude that the plasma membrane is not homogeneous, even on small length scales of tens of nanometers. The second conclusion must be that the exact properties of and relationships between the different membrane organizing components (e.g. actin membrane skeleton, caveolae, lipid rafts) are not understood well enough to propose a defined, generalized model of plasma membrane organization. Nevertheless, based on our current knowledge, the membrane can best be depicted as a heavily compartmentalized, quasi-two-dimensional structure, which is more mosaic-like than fluid (see Fig. 2), as has been proposed recently by Vereb et al. [6].

This rather crude membrane model should be tested, modified and made more quantitative in the future. More specifically, research should focus on explaining the differences found in the properties of lipid rafts reported in the experiments done so far. A consistent study, combining several techniques in one or just a few cell types, should be able to produce detailed information on putative lipid rafts on a wide range of time- and length scales. This is by no means an easy task, but would indicate if the wide variation in lipid raft properties found so far is an effect of the different experimental approaches and cell types used up till now, or an inherent property of lipid rafts.

Besides the basic questions of lipid raft size, composition and stability, there are other important aspects of membrane organization in general that need further investigation. One of these aspects is the effect of the high protein concentration present in biological membranes (molecular crowding) on diffusion and membrane domains [103]. New concepts, like the proposed lipid shells [104], which might function as a molecular address to target proteins to lipid domains, will need to be experimentally verified. A very interesting question, which has only partially been addressed, is whether membrane domains actually compartmentalize signal transduction and if so, how this is achieved. This issue is closely linked to the characterization of cytoplasmic leaflet domains, their potential association with caveolae, and the inter-leaflet coupling. In all these areas our current knowledge is limited. Related to membrane organization is the role of membrane domains in intercellular trafficking. Indeed, membrane turnover [105] due to endo- and exocytosis and its effects on membrane organization [106,107] will have to be included in any future model of the plasma membrane as well as the association between the bilayer and the membrane skeleton [16]. These unresolved issues offer a scientific challenge and will be addressed in the near future with the help of techniques like the ones used to produce the results described in this review. Moreover, recent advances in microscopy like dual-wavelength imaging of differently colored fluorescent particles [108] or molecules [109], imaging of quantum dots [110] and photothermal interference contrast imaging using 10-nm gold particles [111] will prove useful to obtain more insight in membrane organization in the near future.

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