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Review

Hierarchical organization of the plasma membrane: Investigations by single-molecule tracking vs. fluorescence correlation spectroscopy

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ABSTRACT

Single-molecule tracking and fluorescence correlation spectroscopy (FCS) applied to the plasma membrane in living cells have allowed a number of unprecedented observations, thus fostering a new basic understanding of molecular diffusion, interaction, and signal transduction in the plasma membrane. It is becoming clear that the plasma membrane is a heterogeneous entity, containing diverse structures on nano-meso-scales (2–200 nm) with a variety of lifetimes, where certain membrane molecules stay together for limited durations. Molecular interactions occur in the time-dependent inhomogeneous two-dimensional liquid of the plasma membrane, which might be a key for plasma membrane functions.

1. Introduction

Just how proteins and lipids jostle around in the plasma membrane has been a source of debate for decades. Diffusion in the homogeneous membrane, a quasi two-dimensional structure immersed in water, is already a subject matter that has attracted great interest [1,2]. However, recent observations of the thermal movements of proteins and lipids in the plasma membrane, using single-molecule tracking and fluorescence correlation spectroscopy (FCS) at sufficient temporal and spatial resolutions, have revealed the unforeseen complexity of the motion. Essentially all of the molecules in the plasma membrane observed by high-speed single-molecule tracking exhibited non-Brownian diffusion. The majority of the molecules undergo suppressed diffusion: either their macroscopic (long-term) diffusion coefficients are smaller than their microscopic (short-term) diffusion coefficients or the molecules undergo temporary immobilization, making the long-term diffusion coefficients smaller than the short-term diffusion coefficients exhibited by the molecules during the mobile periods [3]. Therefore, one of the three important subject matters of the present review is to describe the technique of high-speed single-molecule tracking and its detection of the non-homogeneity of the plasma membrane.

Meanwhile, non-Brownian diffusion of membrane-incorporated molecules in non-homogeneous membranes often fails to be observed by low-speed single-molecule tracking [4,5] and FCS [6]. Therefore, the second of the three important themes of this review is to clarify why the results obtained by these techniques are different from those generated by high-speed single-molecule tracking.

Third, more concretely, we focus on two distinct membrane domains on the meso-scale (defined here as the scale between 2 and 200 nm). (1) The raft membrane domain, which is defined as dynamic, nano-sized, sterol–sphingolipid-enriched assemblies of molecules [7]. The plasma membrane consists of a non-ideal mixture of diverse molecules with differing mutual miscibilities in the fluid state, i.e., it contains dynamic meso-scale molecular complexes and domains, forming and dispersing continually within the plasma membrane on various time scales. These molecular complexes and domains range from small protein clusters with short lifetimes, such as transient dimers of rhodopsin [8], to stabilized raft domains, such as those induced by the receptors engaged in signaling, due to ligand binding and subsequent receptor clustering, including the signaling complexes/domains of T- and B-cell

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receptors [9–16], FcεR [17–19], and CD59 [3,20]. The raft domains in non-stimulated cells are likely to have properties between these two extremes.

(2) The other interesting feature of the plasma membrane, which distinguishes it from a simple two-dimensional ideal liquid, is our proposal that the plasma membrane is parceled up into apposed domains, for both proteins and lipids. Namely, the plasma membrane may be partitioned or compartmentalized for the translational diffusion of both proteins and lipids: all of the molecules incorporated in the plasma membrane undergo short-term confined diffusion within a compartment and long-term hop diffusion between the compartments. This is likely due to the filamentous actin meshwork associated with the cytoplasmic surface of the plasma membrane ("membrane skeleton" for short), and to various transmembrane proteins anchored to and aligned along the actin filaments.

In the present review, we propose how these two meso-scale structures are organized in the plasma membrane.

The research field of plasma membrane heterogeneity at various levels is presently very active, and many fine reviews have been published. Readers are directed to the following interesting reviews that generally cover this active field (limited to those published in 2007 and later), from a different viewpoint [21–29].

2. Single-molecule tracking of membrane molecules

New experimental techniques that allow researchers to track single molecules or small groups of molecules in the cell membranes of living cells are becoming important tools for investigating the dynamics, structures, and functions of the cell membrane. These techniques have given researchers the unprecedented ability to directly observe the movement, assembly, and even activation of individual single molecules in the plasma membrane of living cells in culture [30–33].

These methods are largely classified into two groups, based on the probes employed to track individual molecules. One of the methods, termed single fluorescent-molecule tracking (SFMT), employs fluorescent probes, which are usually fluorescent organic or protein molecules; each individual fluorescent molecule bound to a target molecule is visualized using fluorescence microscopy [32,34–38].

The other method, termed single particle tracking (SPT) by Sheetz et al. [39], employs colloidal gold particles of 20 or 40 nm in diameter; each individual particle bound to a single molecule (or small groups of molecules) in/on the plasma membrane is observed using Nomarski (differential interference contrast) or bright-field microscopy [30,31,39–44].

In successful SFMT, one always has to balance the spatial precision for determining the position of each single molecule, the time resolution (frame rate, e.g., how often a single molecule can be observed), and the duration of viewing a single molecule. If this balance fails, or the observation conditions are too limited by the capability of the instrument, then the information required for solving the problem will not be obtained. At typical position determination accuracies of 30–40 nm, several 10–100s of frames could be observed (at video rate, this corresponds to 1–10 s; at a 1 ms resolution, this corresponds to ~30–300 ms).

The prominent advantages of SPT are that it yields a much better signal-to-noise ratio than that of SFMT (because the contrast originates from the incident light scattered by the particle) and photobleaching or blinking is not a concern. Using Nomarski or bright-field microscopy, colloidal gold particles could be localized with position determination precisions of ~2 nm at video rate [42,44], and ~17 nm even at 40,000 frames/s (25-μs resolution [45]). However, the large size of the colloidal-gold particles (typically 20 or 40 nm) can cause crosslinking of the target molecules or steric hindrance effects, and prevents their application to intracellular molecules.

3. Observation of dynamics of membrane molecules and membrane heterogeneity using FCS and STED-FCS

FCS also has single-molecule sensitivity, and can detect single molecules in the focal area [46]. However, FCS will not allow the tracking of single molecules, and its raw data generally become useful only after many molecules are observed. Therefore, such observations are usually made under the conditions where several 10–100s of molecules simultaneously stay within the focal area, for better signal-to-noise ratios. The signal intensity in the focal area is observed as a function of time and is auto-correlated, and from the auto-correlation function (spectral density), the average number density and the residency time of the observed molecule in the focal area are determined. For further reviews of the general method, see Chen et al. [47] and Chiantia et al. [28].

The spatial resolution of FCS is basically the same as the focal area size of the excitation laser beam, which is usually given by the optical diffraction limit of ~240 nm. Recent efforts to limit the focal area size, to improve the spatial resolution, have generated important successes, yielding illumination area sizes of ~150 nmϕ [48] and ~30 nm [49–51]. The best spatial resolution of FCS accomplished thus far has been the 30-nm focal area, attained by stimulation-induced emission depletion (STED)-FCS. However, even at this spatial resolution, membrane heterogeneity on scales smaller than 30 nm will not be directly observed.

How does FCS or STED-FCS provide information on dynamics and structures occurring on scales less than ~30 nm (or ~240 nm for normal FCS)? The information must be obtained indirectly: educated guesses must be made of the models for molecular dynamics and substructures that might be occurring in the focal area, and then they must be translated to the information obtained in FCS measurements. For quantitative analysis, a Monte-Carlo simulation of molecular movements based on the model is performed, which gives a prediction of the FCS data, and then from the FCS data, based on the model, one obtains the parameters describing the model, such as the molecular residency time within the meso-domain, the time fraction of residency within a meso-domain vs. the bulk domain, the diffusion coefficient in the bulk domain, and the size of the meso-domain [6,52]. The accuracy of the assumed model can be tested, for example, by performing FCS experiments at various focal area sizes [6,51,53]. Therefore, creating suitable models and then translating them to analysis software for FCS data are critically important for using FCS to study the membrane structure and molecular dynamics occurring in the space scales smaller than the focal area.

4. High-speed single-molecule tracking revealed hop diffusion of membrane proteins and lipids in the partitioned plasma membrane

Fig. 1a and b shows representative trajectories of an unsaturated phospholipid (a typical non-raft phospholipid probe), L-α-dioleoylphosphatidylethanolamine (DOPE) in the plasma membrane of NRK cells, recorded at time resolutions of 33 ms and 25 μs (frame rates of 30 and 40,000 Hz), respectively, using SPT (due to the probe tagging in the headgroup, it no longer exhibits the characteristics of PE). A general statistical method for analyzing these trajectories has been developed, to classify them into simple-Brownian, suppressed, and directed diffusion modes ([44]; also see Fig. 4 and its related text in Suzuki et al. [54] for the statistical analysis of trajectories and hop-confined diffusion). Note that this classification is independent of the diffusion model (many scientists

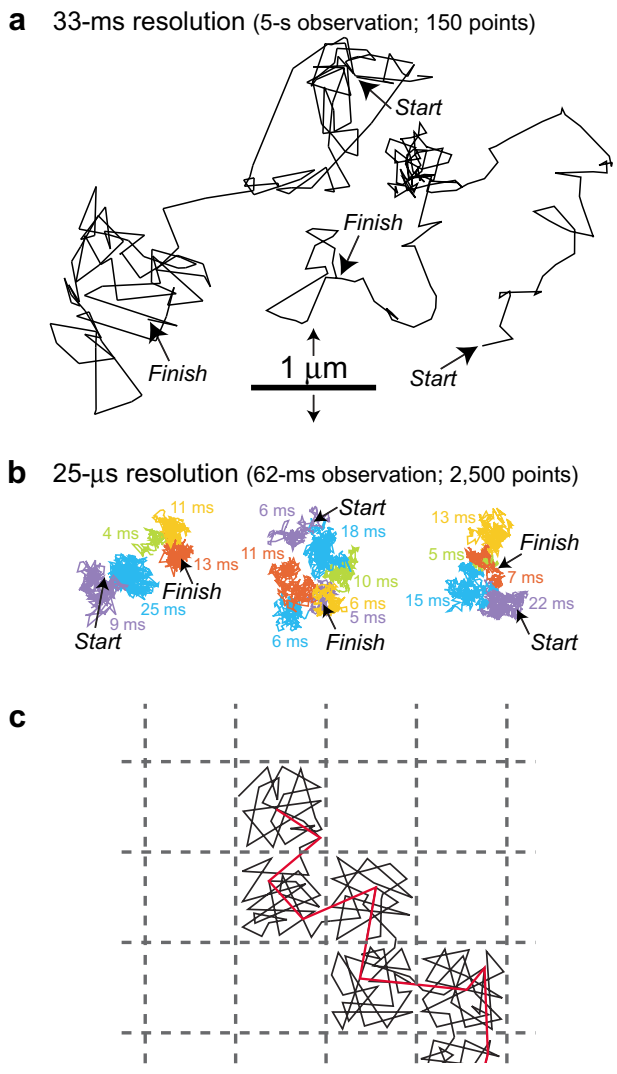

Fig. 1. Representative trajectories of single DOPE molecules recorded at time resolutions of 33 ms (a) and 25 μs (b), and a schematic figure (c) showing how different observation time-resolutions lead to simple-Brownian and suppressed diffusion modes. In b, different colors indicate different plausible compartments, detected by computer software developed in our laboratory (in a time sequence of purple, blue, green, orange, and red for both time resolutions), which finds sudden, brief increases in the local diffusion coefficient, and was used only for trajectories statistically classified as exhibiting suppressed diffusion. In this classification software and the software to find pulse-like changes of the local diffusion coefficient, no diffusion model, such as hop/confined diffusion, was assumed. The residency time within each compartment is indicated.

misunderstood [and still do] this point). It simply determines whether the given trajectory has a <5% probability of being classified into the simple-Brownian diffusion mode, and if this is true, then it is either classed into the suppressed or directed diffusion mode.

At a 33-ms resolution (normal video rate; Fig. 1a), practically all of the DOPE trajectories were classified into the simple-Brownian diffusion mode. However, when the frame rate was enhanced to 40,000 frames/s (25-μs resolution; Fig. 1b), it became clear that the simple-Brownian nature found at the 33-ms resolution is only an apparent one (Fig. 1c): the non-Brownian nature (suppressed-type diffusion) was statistically confirmed.

Then, for those trajectories classified into suppressed diffusion, their mean-square displacement (MSD)-Δt plots were fitted by the equations that assumed confined or hop diffusion, in order to obtain the average compartment size and the residency time for the single trajectory (see [44,45]). In addition, the trajectories statistically classified into suppressed diffusion were analyzed with software developed in our laboratory, which finds the instances when the local diffusion coefficient is increased suddenly and for a short time period. This is how the trajectories, such as those shown in Fig. 1b, were color-coded. Again, note that this software, and thus the color-coding itself, does not assume any diffusion model. It simply finds the instances when the local diffusion coefficient is suddenly and briefly increased. If we adopt the hop diffusion model, then the instances at which the local diffusion coefficient briefly increases might represent those when hop movements from one compartment to an adjacent one take place, but such an assumption is not required for using this program. The only assumption here is that the examined trajectory was statistically classified into the suppressed diffusion mode. This point has often been missed, partly because, in the original papers, the steps of classifying the trajectories into the suppressed diffusion mode and then fitting their MSD-Δt plots with equations describing confined/hop diffusion were combined into one step, when describing the analysis of single-molecule trajectories [44,45,54].

After performing all of these analytic procedures, the obtained results, particularly those of the phospholipid movements, amazed many membrane researchers, including us ([45,54–56]; reviewed by [30,57,58]). Virtually all of the phospholipid (DOPE and DPPE) and transmembrane protein molecules (transferrin receptor, α2-macroglobulin receptor, E-cadherin, band 3, and μ-opioid receptor) we examined exhibited short-term confined diffusion within a compartment and long-term hop movement to an adjacent one, with average compartment sizes of 30–200 nm and average residency times of several to several 100 ms (cell-type dependent). Such movements were termed hop diffusion. Namely, the intuitive impressions of the high-speed single-molecule trajectories one might obtain (Fig. 1b) are supported by the rigorous statistical and quantitative analyses.

Furthermore, these molecules undergo fast simple-Brownian diffusion in actin-filament-depleted, blebbed membranes (the lipid molecules and transmembrane molecules in these membranes diffused as quickly as those in giant liposomal membranes, with diffusion coefficients of ~9 and 4–6 $\mu m^2/s$, respectively). Very mild treatments with actin-depolymerizing drugs increased the compartment size (briefly, and then the cells tended to recover the original compartment size), whereas a mild treatment with the actin-stabilizing drug jasplakinolide prolonged the residency time within a compartment. (Note that the protein content in the blebbed membrane remained similar to that in the intact membrane, although after bleb formation, diffusion of transmembrane proteins in the neck region may be limited due to actin concentration [53]. For the intricacy of drug treatments for partial actin depolymerization and stabilization, see [45,53–55,78].)

How did it become possible to discover the hop diffusion of membrane molecules (particularly phospholipids) in the plasma membrane, which had gone undetected for 30 years after the publication of the Singer–Nicolson model [59] (although it was suggested previously [60–64]). Two factors were critical. The first was the single-molecule tracking: if a method to observe the average motion of more than one molecule, let alone thousands of molecules, was employed, then individual hop events could not be detected, due to averaging over all of the molecules under observation. The second was the vast improvement of the temporal resolution (frame rate): since the residency time in each compartment was between 1 and 1000 ms (depending on the cell type and molecule), then for detecting the compartment, there must be at least 40 points or so on average in a compartment, which necessitates the frame rate of 40 kHz (once every 25 μs) [45,55,65,66].

5. The mechanism for suppressed diffusion of transmembrane proteins: membrane-skeleton fence model

Using electron tomography, the three-dimensional structure of the membrane skeleton on the cytoplasmic surface of the plasma membrane was obtained [67]. They found that virtually the entire cytoplasmic surface is covered by the meshwork of the actin-based membrane skeleton, and that the membrane skeleton is closely associated with the cytoplasmic surface of the plasma membrane (within 0.83 nm). Since transmembrane proteins protrude into the cytoplasm, their cytoplasmic domains must collide with the membrane-associated actin filaments, and in the membrane-skeleton fence model (Fig. 2), this collision is assumed to induce temporary confinement of transmembrane proteins within the membrane-skeleton mesh (this is consistent with the dependence of protein hop diffusion on intact actin filaments, described in the previous section). Transmembrane proteins are assumed to hop between meshes, when there is space between the membrane and the actin filament due to the structural fluctuation of the membrane and/or when the actin filament that forms the compartment boundary temporarily dissociates.

Hop diffusion of transmembrane proteins has been confirmed by FCS [6], and it appears that the membrane-skeleton fence model, which is applicable to transmembrane proteins and some lipid-anchored cytoplasmic molecules, has been largely accepted in the literature [68–74], with some exceptions [75]. For a summary of the observations related to the membrane skeleton fence model, see Box 7 in Supplementary material of Kusumi et al. [57].

6. The mechanism for suppressed diffusion of phospholipids: anchored-protein picket model

Many reports have described the presence and absence of suppressed diffusion of phospholipids in the plasma membrane. Here, we start with recently published papers, supporting the hop/confined diffusion of phospholipids and GPI-anchored molecules [76–79], as well as cytoplasmic lipid-anchored signaling molecules [80].

To explain the hop diffusion of phospholipids and GPI-anchored proteins located in the outer leaflet of the plasma membrane, the "anchored-protein picket model" (Fig. 2) was proposed. In this model, various transmembrane proteins anchored to and aligned along the membrane skeleton (fence) effectively act as rows of pickets against the free diffusion of phospholipids, due to steric hindrance as well as the hydrodynamic-friction-like effects of immobilized anchored membrane protein pickets. The latter effect, first proposed by Hammer's group [81,82], propagates over about several nanometers, and is prominent in the membrane because the membrane viscosity is much greater than that of water, by a factor of ≈100, and is particularly marked when immobile pickets are aligned along the membrane-skeleton fence [45].

The concept of the anchored-protein picket effect was developed for the following reason. Fujiwara et al. [45] and Murase et al. [55] examined the involvement of the membrane skeleton, as well as the effects of the extracellular matrices, the extracellular domains of membrane proteins, and the cholesterol-rich raft domains, in suppressing phospholipid diffusion. They found that

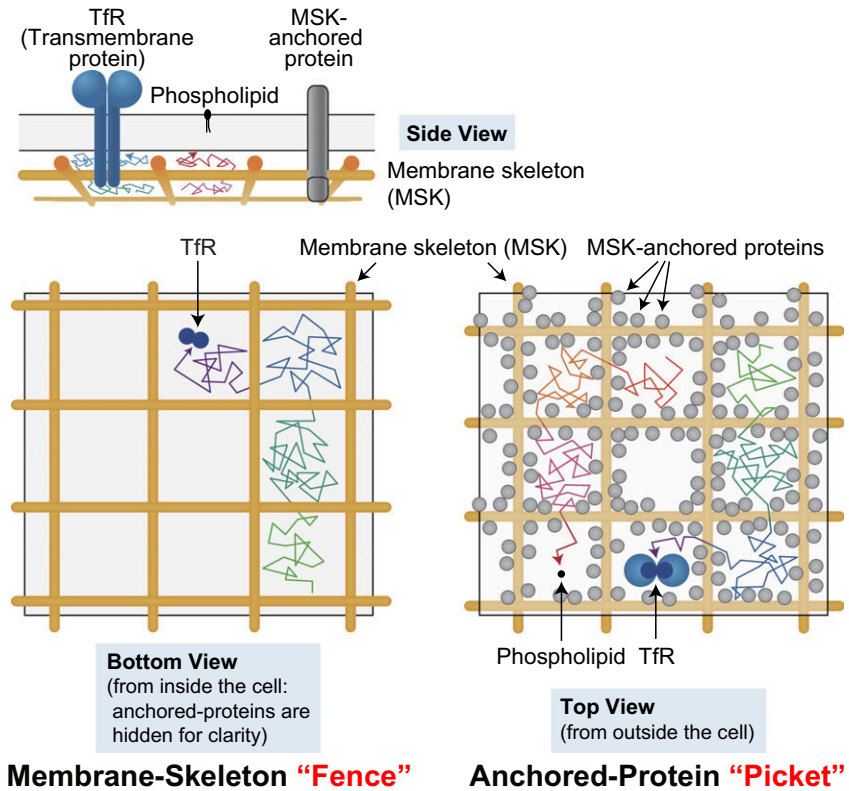

Fig. 2. The membrane-skeleton (MSK) fence model and the anchored-protein picket model. The plasma membrane may be parceled up into domains (compartments), and both transmembrane proteins and lipids undergo short-term confined diffusion within a compartment and long-term hop diffusion between these compartments. This may be due to corralling by two mechanisms: the MSK "fences" and the anchored-protein "pickets". (Side view) Schematic presentation of a transmembrane protein (here, transferrin receptor, a native dimer, is shown as an example), a phospholipid located in the outer leaflet of the plasma membrane, and an MSK-anchored protein (gray cylinder). The former two are mobile, whereas MSK-anchored proteins, a variety of transmembrane proteins that (temporarily) bind to the MSK, are immobile and act like "pickets". (Bottom view) The cytoplasmic surface of the plasma membrane, viewed from inside the cell, showing the MSK "fence" model: transmembrane proteins are confined within the mesh of the actin-based MSK, due to the collision of their cytoplasmic domains with the MSK, which induces the temporary confinement of transmembrane proteins in the MSK mesh. (Top view) The anchored-protein "picket" model: transmembrane proteins, anchored to the actin-based MSK, effectively act as rows of "pickets" along the MSK "fences", as viewed from outside the cell. Such rows of pickets suppress the free diffusion of both transmembrane proteins and lipids.

phospholipid movement was affected only when the membrane skeleton was modulated with actin drugs (or when it was observed in actin-depleted membrane blebs). All of these results point to the involvement of the membrane skeleton in both the temporal corralling and hop diffusion of phospholipids. However, this is very strange and surprising. Since the phospholipid molecules they observed were located in the extracellular leaflet of the plasma membrane (unlabeled lipids might flip to enter the cytoplasmic leaflet, but the observed molecule was tagged with a large colloidal gold particle, and thus it could not flip from the outer leaflet to the inner leaflet), whereas the membrane skeleton is located on the cytoplasmic surface of the membrane, the observed lipid molecules and the membrane skeleton cannot interact directly. Therefore, to explain this apparent discrepancy, the "anchored transmembrane-protein picket model" was proposed.

To explain the temporary confinement of phospholipids within a compartment made of aligned pickets, the inclusion of the hydrodynamic-friction-like effect of immobilized anchored transmembrane protein pickets [81,82] is absolutely required: steric hindrance of the anchored-protein pickets is not sufficient for inducing temporary confinement [45,83,84].

The dynamics of the fences and pickets has not been examined extensively thus far. When the same molecule came back to the same location in, say, 1 s, they mostly exhibited the same compartment size and shape there, although some compartment boundaries were slightly shifted or occasionally a new boundary appeared to form [54]. The hop diffusion in the human erythrocyte ghost is likely to take place as a result of temporary dissociation of spectrin tetramers to dimers [65]. Transmembrane proteins will not have to be anchored to the membrane skeleton fence longer than a millisecond to work as pickets. Assuming that the intercompartmental boundaries are about 10 nm wide, and the average diffusion coefficient of phospholipid molecules in the boundary region is 0.1 $\mu m^2/s$, it only takes 0.25 ms to pass the boundary.

7. FCS and single-molecule tracking data that do not support the hop diffusion of phospholipids and GPI-anchored proteins

In contrast to the hop/confined diffusion of transmembrane proteins and the membrane-skeleton fence model, quite a few papers have reported that either the hop/confined diffusion of phospholipids and GPI-anchored molecules or the dependence of their diffusion behaviors on filamentous actin could not be detected, which is at variance with the anchored-protein picket model. These reports mainly included two lines of observations, FCS (and FRAP) data and low-speed single-molecule tracking results, which were often coupled with the lack of an effect of actin-depolymerization drugs on the diffusion behaviors of phospholipids and GPI-anchored proteins.

The FCS results reported by Lenne et al. [6] and Wenger et al. [48] and the STED-FCS data from Eggeling et al. [51] indicated that phospholipids undergo simple-Brownian diffusion in the plasma membrane, although the diffusion coefficients were substantially smaller than those expected for free diffusion in membrane blebs and liposomes (10–20-fold). The reason for the smaller diffusion coefficients is unknown.

The focal areas employed in these studies are close to or greater than the compartment sizes. For example, the compartment size

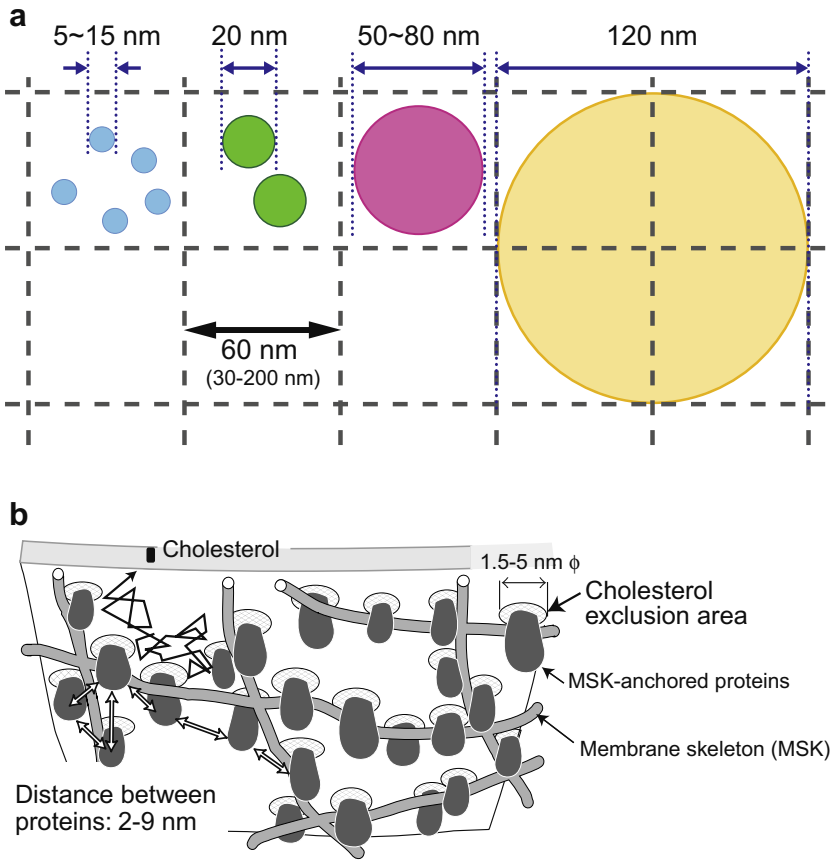

Fig. 3. Schematic displays showing (a) that raft domains with various sizes reported previously were greater or smaller than the compartment sizes (assumed to be ~60 nm, but might vary in the range between ~30 and ~200 nm), and (b) that various transmembrane picket proteins, which tend to exclude cholesterol in their annular areas, are anchored to and aligned along the membrane skeleton mesh, functioning as raft breakers. See text for details.

for the PtK2 cell plasma membrane, employed by Eggeling et al. [51], is about 45 nm on average (Fujiwara, Suzuki, and Kusumi, unpublished), whereas the smallest focal area size used there was 30 nm. Namely, the 30-nm focal area might be at the edge of detecting 45-nm compartmentalization, and such an indication is found in their Fig. 3a, around 30–40 nm on the *x*-axis (the linear line fit for τ_{D1} could be broken into two lines, connected at around 40 nm on the *x*-axis). We raise the possibility that the confinement in the membrane compartment might have been missed. The problem of detecting molecular dynamics and membrane substructures occurring on spatial scales smaller than the focal area size has been discussed in the Section 3 in this review.

Therefore, a further reduction in the focal area of FCS or STED-FCS, to the level of ~10 nm, would be extremely useful. Otherwise, as pointed out in the third section, the model-dependence would make the FCS data analysis-interpretation somewhat difficult.

High-speed single-molecule techniques are particularly powerful for obtaining information on nano-meso-scale molecular dynamics and membrane domains, because they provide a direct way to observe the molecular behaviors in real-life situations in the cell, thus leaving the least guesswork. However, the time required to gather and analyze high-speed single-molecule tracking data is considerably longer that for FCS data (perhaps 100-fold or more!). Therefore, the present strategy of our laboratory is to rely on high-speed single-molecule tracking until we obtain a suitable model for molecular dynamics and structures of interest on nano-meso-scales, and then to switch to FCS (or STED-FCS) for obtaining more voluminous data, e.g., determining various averaged kinetic parameters, and observing the effects of drugs and mutations. Namely, the complementarity of high-speed single-molecule tracking and FCS (or STED-FCS) will be a useful asset for future research.

Using single fluorescent-molecule tracking (SFMT), Wieser et al. [4,5] reported that they only found simple-Brownian diffusion of phospholipids and a GPI-anchored protein, CD59. They performed their observations at moderate rates (1000–2000 Hz, or time resolutions of 0.5–1 ms), but since the total number of observed time points was limited to only around 10 (whereas we observed several to several hundreds of thousands of time points for the same or extended durations), and given the signal-to-noise ratio and the difficulty in precisely subtracting the noise at time 0, non-Brownian diffusion would probably have been difficult to detect. In fact, their reported diffusion coefficient was 0.45 $\mu m^2/s$ [5], which falls in the middle between the microscopic diffusion coefficient representing the diffusion rate within a compartment (4–8 $\mu m^2/s$) and the macroscopic diffusion coefficient of 0.1–0.2 $\mu m^2/s$ (determined by the rate of hop movements), with more weight on the long-term diffusion rate, suggesting that, in their MSD-Δt plots, the mixture of these two processes could not be separated.

These results indicate the necessity for the further development of high-speed single-molecule techniques, particularly those usable for single fluorescent molecules. We are in the process of developing an SFMT instrument, which can operate at least 10 times faster than any reported thus far.

The lack of the effect of actin filaments on the diffusion behaviors of phospholipids and GPI-anchored proteins has been reported [75,85,86]. However, observations in actin-depleted blebbed membranes have not been done, except for those by our group. Instead, actin-depolymerizing drugs have been utilized. In these experiments, the actin membrane skeleton mesh has to be slightly modified without changing the overall organization of the membrane skeleton. Otherwise, the results would be too complicated, due to the secondary effects of the drugs. However, the detection of the drug effect then becomes difficult. Even in single-molecule tracking, the only major observed change was the increase of the confined area size by ~20%. Furthermore, drug effects vary strongly, depending on the kinds of drugs employed [78,87], and also on the reaction of the cells to recover the original membrane skeleton ([54]; Fujiwara and Kusumi, unpublished).

8. Further evidence, supporting the anchored-protein picket model

Further evidence supporting the anchored-protein picket model (and membrane-skeleton fence model) is summarized.

(1) Using electron tomography of rapidly-frozen, deeply-etched specimens of plasma membranes, Morone et al. [67] determined the distribution of the mesh size of the actin-based membrane skeleton on the cytoplasmic surface of the plasma membrane (within 0.83 nm), and found that it agrees well with that for the compartment size determined from the DOPE diffusion data. Good agreements between the electron tomography data and the DOPE diffusion results were observed for two cell types, NRK and FRSK cells, which exhibited quite different compartment sizes for DOPE diffusion, 230 nm and 42 nm, respectively, further supporting the anchored-protein picket model.
(2) The phospholipid hop diffusion is not affected by removing the major fraction of the extracellular domains of transmembrane proteins and the extracellular matrix [45,55], indicating that these are not the major causes for the induction of hop diffusion.
(3) The removal of cholesterol has no major effects on the hop diffusion parameters [45,55], suggesting that lipid rafts are not the primary cause of membrane compartmentalization or hop diffusion.
(4) The compartment sizes detected by transmembrane proteins and phospholipids are the same in all of the cell types examined thus far (Fujiwara, Iwasawa, and Kusumi, unpublished observations), supporting the membrane-skeleton fence and anchored-protein picket models.
(5) Monte-Carlo simulations reproduced the experimentally observed residency times when only 20–30% of the compartment boundaries were occupied by the anchored transmembrane protein pickets [45,55]. This represents the anchoring of only about 15% of the total transmembrane proteins in the plasma membrane.

The anchored transmembrane protein pickets would be operative on any molecules incorporated in the membrane, including transmembrane proteins. Therefore, the diffusion of transmembrane proteins will be doubly suppressed in the membrane. Both the fence and picket will act on transmembrane proteins.

9. Oligomerization-induced trapping of proteins and lipids, based on the membrane-skeleton fences and pickets: possible biological functions of fences and pickets

At variance with the prediction from the two-dimensional continuum fluid model of Saffman and Delbrück [1], which was later confirmed by Peters and Cherry [88], the diffusion coefficient in the plasma membrane is measurably decreased upon the oligomerization of membrane molecules or the formation of molecular complexes. The diffusion coefficients observed in the plasma membrane (~0.2 $\mu m^2/s$) are already ~20-fold smaller than those found in artificial lipid membranes or in actin-depleted blebbed membranes, but they are further decreased after the observed molecules form dimers and oligomers.

This effect should not be confused with the diffusion coefficient reduction induced by oligomerization in lipid membranes, re-

ported by Gambin et al. [2]. The actual value for the diffusion coefficient detected by Gambin et al. was about several $\mu m^2/s$ for monomers (thus about 20-fold greater than those in the plasma membrane), and oligomerization reduces it (in lipid bilayers) to about half or one-third of this value. The effect reported by Gambin et al. is due to the similar sizes of the solute and the solvent.

The oligomerization-induced reduction of the (macroscopic) diffusion coefficient in the plasma membrane, in contrast, can be explained by the partitioning of the plasma membrane into many small compartments, due to the presence of fences and pickets. Monomers of membrane molecules may hop across the picket-fence with relative ease, but upon oligomerization or molecular complex formation, the oligomers or the complexes as a whole, rather than single molecules, have to hop across the picket-fence all at once, and therefore, a longer-lasting greater free space is required for the passage of these oligomers across the picket-fence of the intercompartmental boundary. Hence, these complexes are likely to have a much slower rate of hopping between the compartments, as found with oligomers of DOPE [55] and E-cadherin [37]. In addition, molecular complexes are more likely to be bound or tethered to the membrane skeleton, perhaps temporarily, which also induces (temporary) immobilization or trapping of molecular complexes. Such enhanced confinement and binding effects induced by oligomerization or molecular complex formation are collectively termed "oligomerization-induced trapping" [30,37].

Therefore, in the plasma membrane, oligomerization or molecular complex formation is tied to immobilization by the membrane skeleton fence and anchored-protein pickets. This effect was confirmed by Fu et al. [89] in the heterodimerization of integrin Mac-1 subunits ($\alpha_M\beta_2$). By observing FcεRI diffusion, Andrews et al. [90] reported antigen-induced (thus oligomerization-induced), actin-dependent receptor immobilization.

Oligomerization-induced trapping might play very important roles in the temporary localization of signal transduction complexes. When an extracellular signal is received by a receptor molecule, the receptor often forms oligomers and signaling complexes by recruiting cytoplasmic signaling molecules. Due to the "oligomerization-induced trapping", these oligomeric complexes tend to be trapped in the same membrane skeleton compartment as that where the extracellular signal was initially received. Therefore, the membrane skeleton fence and the anchored transmembrane-protein pickets help to temporarily localize the initiation of the cytoplasmic signal to the place where the extracellular signal was received. Such spatial confinement is particularly important for signals that induce local or polarized reorganization of the cytoskeleton or chemotactic events.

The confinement due to membrane-skeleton-based fences and pickets has been proposed to play key roles in clathrin-coated pit formation [91,92], B-cell receptor signaling [93], and EGF receptor signaling in tumor cells [94].

10. What are "correct" raft sizes?

A number of studies have evaluated the raft size, but the estimates are quite diverse. Somewhat strangely and surprisingly, the variations among different studies are greater in non-stimulated cells: one would expect that, based on the raft hypothesis [95,96], due to signal-induced coalescence of some raft domains, the variations would be greater in stimulated cells (although this might be due to the very extensive stimulation employed in many investigations). As shown in Fig. 3a, the estimated raft diameters of the nano-clusters of raft-associated molecules ranged from 5 to 15 nm ([83,97–100]; Plowman, S., Muncke, C., Parton, R., and Hancock, J.F., personal communication), 20 nm [51], 50–80 nm [101–103], 120 nm [6], and 700 nm [36].

Why such variations? Kusumi and Suzuki [104] previously pointed out the problems in immunofluorescence and immunoelectron microscopy studies: without the use of glutaraldehyde, even protein molecules tend to remain mobile after chemical fixation-crosslinking (during antibody application), leading to antibody-induced clustering of raft-associated molecules.

Here, we propose another major issue in raft-size evaluation: each method only detects possibly very small and different subfractions of the raft domains present in the plasma membrane. As emphasized in this review, since raft domains and raft-associated molecules are dynamically moving, and since the temporal and spatial spectra of the dynamics appear to be extremely broad, each method, with its unique limitations in temporal and spatial spectra, can detect only the raft domains that they are designed to detect. Another way of stating this proposal is that the raft domains have an extremely broad spectrum of lifetimes and sizes. Namely, we think that all of the numbers given above are probably correct, in the sense that in the plasma membrane, raft domains of such sizes are present. Therefore, the next issue we have to address is to determine the populations (fractions, which can be the percent occupancy in the plasma membrane plane) of raft domains of each size. Here, we also emphasize the possible importance of very small raft domains, possibly made of three to a dozen or so molecules, as the core of greater raft domains, as described in a previous review [105].

11. Raft domains may be impregnated in the actin-induced compartments: hierarchical organization of the plasma membrane

How do the raft domains fit into the view of the compartmentalized plasma membrane?

According to the anchored-protein picket model, various transmembrane proteins (pickets) are anchored to and lined up along the membrane skeleton mesh, to induce temporary confinement of phospholipids and GPI-anchored proteins. For their hydrodynamic-friction effects to work, they have to be about 3–10 nm away from each other (Fig. 3b; assuming that the diameter of a transmembrane domain is 0.7–4 nm). In addition, for the movement of raft-associated molecules as well as for the growth of raft domains, we have to consider the fact that cholesterol tends to be excluded from the boundary regions of transmembrane proteins. It follows then that, on the membrane skeleton, 1.5–5 nm diameter zones that exclude cholesterol are lined up 2–9 nm away from each other (Fig. 3b; assuming that the diameter of the area occupied by an alkyl chain is 0.4–0.5 nm). These rows of cholesterol-excluding areas would act as barriers that block the passage of raft-associated molecules and the growth of raft domains. This would limit the raft size to less than the compartment size (30–250 nm, depending on the cell type). Therefore, larger raft domains, perhaps those greater than 50 nm might exist rather rarely, in greater membrane-skeleton-mesh-induced compartments.

Both the raft sizes and compartment sizes might be determined by the interaction of raft-associated molecules and actin-regulating molecules. The interactions of certain raft domains and actin-based membrane skeleton have been reported [3,20,100]. Therefore, for the organization of the plasma membrane, the following interactions would be particularly important: (1) the formation of actin-based membrane skeleton, (2) its general association with the cytoplasmic surface of the plasma membrane and (3) its more specific interaction with raft domains, including its involvement in raft domain formation and its own new formation due to actin polymerization at the raft domain, (4) transmembrane protein pickets anchored to and aligned along the membrane skeleton, (5) the structural non-conformability of these picket proteins with

cholesterol, (6) weakly-cooperative assembly of cholesterol, (glyco)sphingolipids, and GPI-anchored proteins, (7) which is enhanced by the tendency of cholesterol to be excluded from the bulk unsaturated alkyl chain environment, and (8) induction of greater and stabilized raft domains by clustered GPI-anchored proteins [3,20,105].

Based on these interactions, the hierarchical organization of the plasma membrane may be formed: the plasma membrane is partitioned into compartments by the membrane skeleton fences-pickets, which might be associated with specific raft domains, while excluding general rafts. The membrane compartment thus contains diverse sizes of raft domains within it, which are generally smaller than the compartment size, but can grow at the raft domain that was involved in the initiation of actin polymerization. Within each membrane compartment delimited by membrane-skeleton fences and pickets, numerous small rafts may exist, rapidly and restlessly forming and dispersing as well as coalescing and disintegrating, where raft-associable molecules may enter/exit continually.

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