

# Single-molecule tracking of membrane molecules: plasma membrane compartmentalization and dynamic assembly of raft-philic signaling molecules

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## Abstract

Tracking *single molecules* in the plasma membrane *in live cells* is becoming a useful technique for studying the spatial-temporal control of membrane molecular processes, such as signal transduction and the formation of large molecular complexes. In this review, three topics largely based on recent single-molecule observations are described, with a special emphasis on the results that are considered to be difficult to obtain using conventional methods monitoring the ensemble-averaged behavior of molecules. First, we describe the high-speed single-molecule tracking data, mostly obtained by our group that necessitated the paradigm shift of the plasma membrane structure, from the two-dimensional continuum fluid model to the compartmentalized fluid model. Second, we try to present a synthetic view of the cell membrane, which contains raft and other microdomains as well as being partitioned into small compartments. Furthermore, we present our working hypothesis, based on the literature, how large, stabilized rafts may be formed, after ligation or crosslinking, from small/unstable “reserve” rafts present in the steady-state cells. Finally, we explain our initial application of single-molecule fluorescence imaging for studies of the creation of T-cell receptor signaling complexes (immunological synapses or SMACS), by observing the recruitment of single Lck molecules as an initial approach. This revealed that the assembly of Lck at the T-cell receptor cluster site, observed by conventional fluorescence microscopy, actually represents dynamic concentrations of Lck molecules, entering and exiting the cluster domain rapidly, with the aid of the raft domains.

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## 1. Introduction

Techniques that allow researchers to track and manipulate single molecules or small groups of molecules in the cell membranes of living cells are now becoming widely available. These methods include single fluorescent molecule video imaging (SFVI) using fluorescent probes, single-particle tracking (SPT) using colloidal-gold probes, and optical trapping, which allows researchers to move the gold-particle-conjugated molecules in the living cell membrane at will [1–16]. These techniques have given researchers the unprecedented ability to observe directly the movement, assembly, and localization of individual single molecules in the

plasma membrane of living cells in culture. Furthermore, it was recently shown that not only the movement and location of single molecules can be tracked in the cell membrane, but also, under optimal conditions, the molecular activation of single molecules could be imaged [17]. New approaches to cell biological problems in the biomedical field, such as how lipid rafts might coordinate signal transduction, will become possible, by applying these single-molecule techniques and also by developing the single-molecule methods to make them more amenable for the studies of living cells.

These methods that enable researchers to see and grab single molecules in living cells will greatly advance our understanding of how the cell functions, e.g., how signaling molecules move around in the cell, how they transmit the signal to the downstream effector molecules, and how raft-philic

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molecules form rafts or enter/exit from the raft. Although such studies have only been initiated recently, they have already started to yield new significant insights that could not have been obtained without observations at the level of each individual molecule. In the present review, we describe some of these results, including those about raft domains, mostly obtained in our laboratory. We hope this article will provide readers a glimpse of the new world of the membrane organization revealed by single-molecule methods and encourage them to become involved in investigations conducted at the level of single molecules.

## 2. Why observe at the level of single molecules?

First, we would like to show the trajectories of a phospholipid, L- $\alpha$ -dioleoylphosphatidylethanolamine (DOPE), in the plasma membrane of NRK cells in culture. DOPE is an unsaturated phospholipid, and thus, it is thought to be one of the most resistant molecular species to various mechanisms for reducing the diffusion rate, such as the presence of integral membrane proteins and cholesterol, due to its bent structure at the C9–C10 *cis* double bond [18]. To observe DOPE movement, it was tagged with a fluorescent dye, Cy3, or a colloidal-gold particle with a 40 nm diameter. In our video-rate observations (33-ms resolution, and in a time window of about 100 ms), both gave about the same diffusion coefficients, which justifies the use of such a large colloidal-gold particle as a probe. While we will address the technical details later in this article, here, we first introduce the readers to the trajectories of DOPE tagged with a colloidal-gold particle (Fig. 1). One might think that this would be boring, because single-molecule observations of DOPE would simply reiterate the abundant data obtained by fluorescence recovery after photobleaching (FRAP). In fact, this was the case observed at slow observation rates, such as a video rate (33 ms/frame), in which the usual simple Brownian diffusion was observed (Fig. 1, top).

However, when the movement of gold-tagged DOPE was observed at an enhanced time resolution of 25  $\mu$ s (yes, it is 25  $\mu$ s and not 25 ms), virtually all of the gold-tagged DOPE molecules on the cell surface exhibited amazing trajectories: they showed short-term confined diffusion within a compartment and long-term hop diffusion between the compartments (Fig. 1, bottom) [19]. In these trajectories, different colors indicate various plausible compartments, which were detected by computer software. These results indicate that the plasma membrane is partitioned into submicron-sized compartments with regard to the translational diffusion of lipid molecules (*the model of plasma membrane compartmentalization*, Fig. 2A). One should note that these observations have to be substantiated by a statistical analysis (in fact, the success of much single-molecule research depends on the development of good statistical methods to analyze the data). Kusumi et al. [6] and Fujiwara et al. [19] indeed developed a statistical method to evaluate the likelihood that such tra-

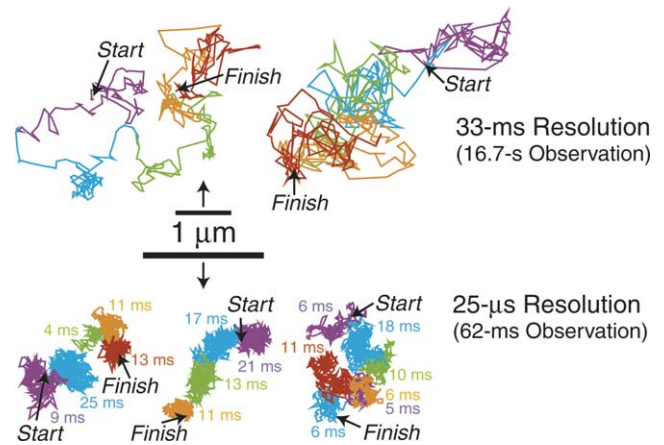


Fig. 1. Representative trajectories of single or small groups of DOPE molecules recorded at time resolutions of 33 ms and 25  $\mu$ s. See the text for details. The different colors of the trajectories obtained at a 33-ms resolution simply represent a time sequence of every 3.3 s. The different colors in the bottom trajectories obtained at a 25- $\mu$ s resolution represent various 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). These results show that the simple Brownian nature of diffusion observed at a video rate (33 ms/frame) is only superficial, and that it is due to the low time resolution of the observation: the confined + hop movement of each DOPE molecule is totally smeared out at a video rate. To resolve such movement, the time resolution must be considerably shorter than the average residency time within a compartment.

jectories could be induced by simple thermal diffusion, and found that more than 85% of the DOPE molecules were undergoing confinement + hop movement, which was termed “hop diffusion” (Fig. 2A). Such a finding would have been impossible if methods that involved ensemble averaging (like FRAP, where the hop movement of one molecule from a compartment to an adjacent one will be smeared out by averaging over all of the molecules under observation) or longer time averaging (averaging due to the camera’s frame time) had been used.

In the present review, we describe the single-molecule methods and recent results on the membrane organization (including raft domains) mainly obtained by these methods. More specifically, we will explain (1) the methods for SFVI and SPT, (2) the plasma membrane compartmentalization and hop diffusion (here, we explain what may cause the hop diffusion of lipid molecules described above), (3) the oligomerization-induced trapping of membrane molecules, one of the most significant consequences of the plasma membrane compartmentalization, (4) the possibility that the rafts in the resting-state cell may be small/short-lived and/or their constituent molecules may rapidly exchange with those in the bulk domain, (5) how clustering of raft-philic receptor molecules may induce “receptor-cluster rafts”, (6) the dynamic concentration of Lck at the place where T-cell receptor (TCR) molecules are assembled (i.e., transient recruitment of each individual Lck molecule), and (7) the formation of a macroscopic diffusion barrier in the initial segment mem-

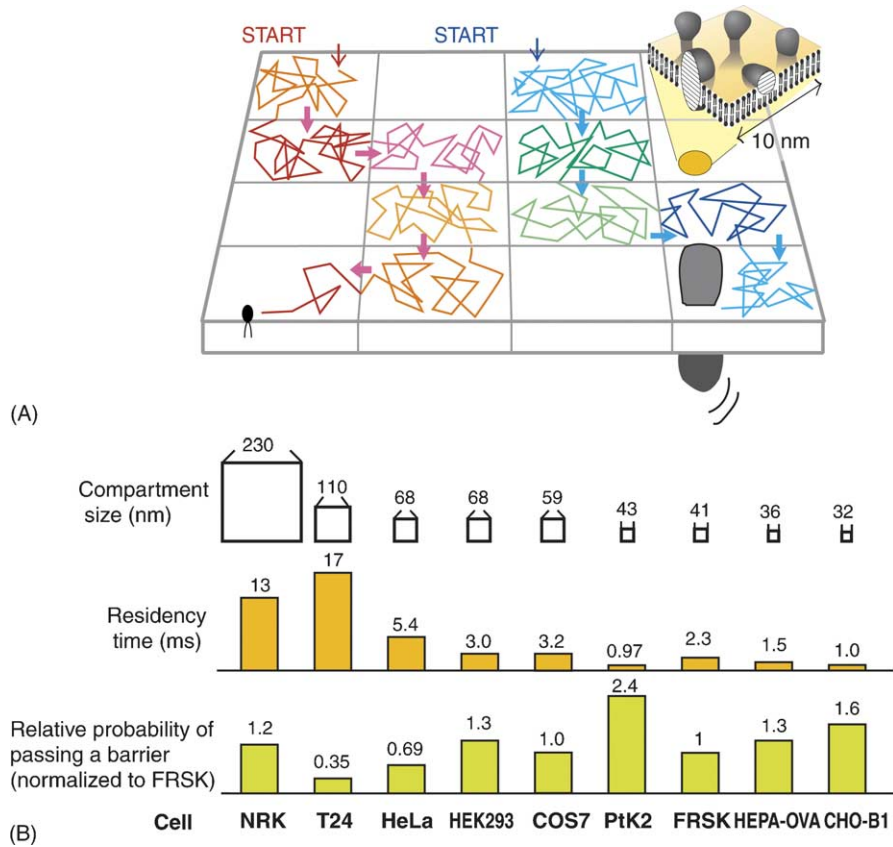


Fig. 2. A paradigm shift for the plasma membrane concept is required from the (two-dimensional) continuum fluid model to the compartmentalized fluid model [19], in which the membrane constituent molecules undergo short-term confined diffusion within a compartment and long-term hop diffusion between the compartments. (A) The plasma membrane is partitioned into many submicron-sized compartments with regard to the translational diffusion of membrane-incorporated molecules, and practically all of the molecules undergo macroscopic diffusion by repeating their confinement within a compartment and hopping to an adjacent one. The two-dimensional fluid model of Singer and Nicolson is perfectly all right if the size is limited within 10 nm (inset), as shown in their original cartoon, but it cannot be over-extended to a cell membrane structure over several tens of nanometers. (B) The average compartment size, the average residency time within a compartment, and the relative probability of passing a barrier (normalized to FRSK) for DOPE in various cell types. All of the mammalian cells in culture examined thus far had compartmentalized plasma membranes.

brane of neuronal cells, which separates the neuronal plasma membrane into the somatodendritic and axonal domains.

### 3. SFVI and SPT methods to track the movement of single molecules in the plasma membrane of living cells

For direct observations of the movement, localization, and assembly of single membrane molecules, including both lipids and proteins in the plasma membrane, the most common methods are SFVI [13–15] and SPT [7,20–22] (Fig. 3). In SPT, colloidal-gold particles of 20 or 40 nm in diameter are conjugated with small numbers of the ligand or the Fab fragment of the antibody IgG, and after binding to the target molecules on the cell surface, their movements are visualized by transmission optical microscopy. The images are electronically enhanced using both analogue and digital circuits, and the locations of individual gold particles in each frame are automatically determined [21]. In SFVI, organic

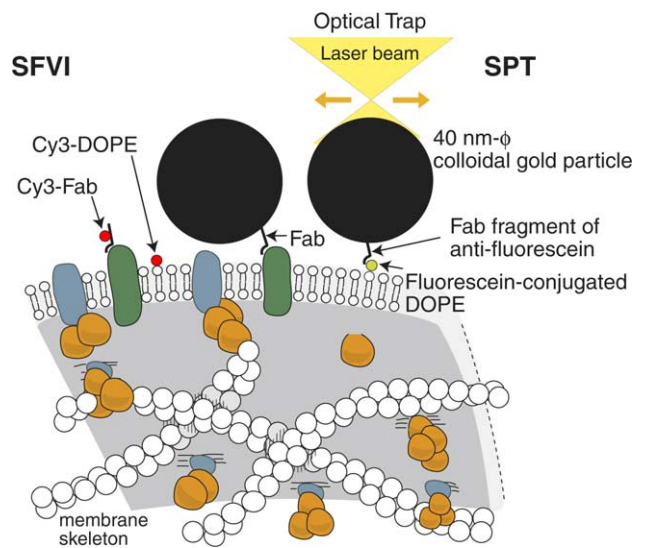


Fig. 3. Schematic diagram showing the labeling of proteins and phospholipids (DOPE) for single-molecule tracking using SPT and SFVI. See the text for details.

fluorescent dyes or the mutants of green fluorescent protein (mGFPs) are used as probes. To observe molecules located in/on the plasma membrane, fluorescence excitation using total internal reflection or oblique-angle illumination is recommended, to avoid the background signal due to autofluorescence from the cytoplasm or the probe molecules in the cytoplasm although wide-field illumination can be successfully employed [9,11,14,16]. Since fluorescence microscopes equipped with these illumination accessories are now commercially available, biomedically oriented researchers can start single-molecule observations without many initial practical and psychological barriers.

The choice of SPT or SFVI, therefore, depends on the aim of the study [19,23]. However, even when gold probes are the choice for a particular study, one has to examine if the gold probes non-specifically interact with membrane molecules, or they induce clustering of the target molecules, by comparing the diffusion behavior observed by SPT with that by SFVI [19,23]. Therefore, it is always good to wisely combine SPT and SFVI. In our laboratory, we tend to start with SFVI observations, since they are generally easier than SPT, which requires the preparation of well-behaved gold probes, and then we proceed with the development of gold probes as the necessity for SPT arises.

#### 4. Two fundamental problems of the membrane molecular dynamics that cannot be explained by the Singer–Nicolson model or the two-dimensional continuum fluid model

There have been two major long-standing problems regarding the two-dimensional continuum fluid model of the plasma membrane [24–26]. First, for 30 years, membrane biologists wondered why the diffusion coefficients for both proteins and lipids in the plasma membrane are smaller than those found in artificially reconstituted membranes or liposomes, by factors of 5 to 50 [27–46].

Second, when membrane molecules, including receptor molecules and other signaling molecules in the membrane, form oligomers or molecular complexes, their diffusion rates drop dramatically or they may be temporarily immobilized [46–48]. The macroscopic (over several 100 nm) diffusion coefficient of the transmembrane protein E-cadherin was decreased by a factor of 40 upon oligomerization (including various sizes of clusters ranging from dimers up to perhaps complexes of several tens of E-cadherin molecules) in the plasma membrane [15]. Even an unsaturated phospholipid, L- $\alpha$ -dioleoylphosphatidylethanolamine, upon artificial crosslinking, exhibited a macroscopic diffusion rate (over several 100 nm) slowed by a factor of 5 [46]. [Here, “macroscopic” generally refers to the space scales greater than several hundred nanometers, corresponding to the typical photobleaching area size in the fluorescence redistribution after photobleaching (FRAP) experiments (generally 300 nm or greater in diameter) or to the compartment size in the com-

partmentalized (or partitioned) membrane model (which will be explained later, but the compartment size varies between 30 and 230 nm, depending on the cell type).] These results are in total contrast with the general view of membrane biologists: if one believes in the two-dimensional continuum fluid model for the plasma membrane on the macroscopic scale (over several tens of nanometers), then what the diffusion theory teaches us is that oligomerization or the formation of molecular complexes hardly reduces the diffusion rate [25]. Tetramer formation from monomers (an increase in radius by a factor of 2) will decrease the diffusion rate only by a factor of 1.1, and even 100mers (an increase in radius by a factor of 10) will have a diffusion rate reduced only by a factor of 1.4 from that of monomers, assuming that the monomer radius of the membrane-spanning domain is 0.5 nm. Therefore, the great reduction of the diffusion coefficient, upon oligomerization or molecular complex formation, as described above, clearly indicates that the plasma membrane cannot be considered as a two-dimensional continuum fluid.

#### 5. Both membrane proteins and lipids undergo hop diffusion in the compartmentalized plasma membrane

The key to understanding the underlying mechanism for these two issues, the reduced diffusion rates in the plasma membrane and the suppression of diffusion upon molecular complex formation, was brought about by single-molecule observations [19,46]. High-speed SPT of DOPE molecules (Fig. 1, bottom) indicated that the plasma membrane is partitioned into submicron-sized compartments with regard to the translational diffusion of lipid molecules (*the model of plasma membrane compartmentalization*, Fig. 2A). Statistical and quantitative analyses [6,19] of DOPE trajectories, such as those displayed in Fig. 1(bottom), showed that the average compartment size was 230 nm, and the average residency time within each 230-nm compartment was 11 ms, in the case of NRK cells (these values vary greatly, depending on the cell type). No wonder one cannot detect hop movement at video rate, with a time resolution of only 33 ms. In fact, all of these trajectories shown in Fig. 1(bottom) are 62-ms long, and if Fujiwara et al. [19] had used video rate observations, there would have been only two or three points in the whole trajectory, and there would have been no way of detecting the hop diffusion of DOPE molecules.

The diffusion rate within the 230-nm compartment, which is  $5.4 \mu\text{m}^2/\text{s}$  on average, is interesting. It is almost as large as that of DOPE molecules observed in artificial membranes, such as giant liposomes.

Therefore, it is concluded that lipid diffusion in the cell membrane is slow, not because the diffusion per se is slow, but because (1) the plasma membrane is compartmentalized with regard to the translational diffusion of phospholipids, (2) the lipid molecules undergo hop diffusion over these compartments, and (3) *it takes time to hop from a compartment*

to an adjacent one (Fig. 2A), which slows the diffusion of membrane molecules in the plasma membrane as compared to those in liposomes or reconstituted membranes. Further, it was shown that smaller compartments within the smaller 230-nm compartment are not likely to exist, because the diffusion coefficient within the 230-nm compartment is already as fast as that observed in liposomes, and (5) (as explained later) oligomerization induces large hop rate reductions, leading to large decreases of the macroscopic diffusion coefficients for oligomers and molecular complexes compared with those for monomers.

How universal is this plasma membrane compartmentalization? Using the unsaturated phospholipid DOPE, Murase et al. ([46], and more recent unpublished observations) found such plasma membrane compartmentalization in all of the nine mammalian cell types examined thus far. However, the compartment size varies greatly, from 30 nm up to 230 nm, and also the residency time of DOPE ranges between 1 and 17 ms (Fig. 2B).

This was not the first time that the hop diffusion of membrane molecules was observed. Using single particle tracking [1,2,6,20,49–52], Sako and Kusumi [53] were the first to directly observe the “hop diffusion” of membrane molecules: transferrin receptor and  $\alpha$ 2-macroglobulin receptor, both transmembrane proteins, are temporarily confined within a compartment, and then these molecules hop to an adjacent apposed compartment, where they again become trapped temporarily. By repeating such confinement and hop movements between the compartments, the receptor molecules cover macroscopic areas (Fig. 2A). Since virtually all of the examined transferrin receptor molecules and  $\alpha$ 2-macroglobulin receptor molecules were found to undergo hop diffusion, it was proposed that the entire plasma membrane is basically partitioned into small compartments (except for specialized membrane domains, such as clathrin-coated pits, cell–cell and cell–substrate junctions, and microvilli).

Typical trajectories of transferrin receptor molecules in the plasma membrane of NRK cells, monitored at a time resolution of 25  $\mu$ s, are shown in Fig. 4. NRK cells have a plasma membrane with nested double compartments with sizes of 230 and 710 nm [19], but here, due to the short duration [250 ms] of the total observation time (yet each trajectory reflects the position determinations of 10,000 images), only the smaller compartment of the NRK cell membrane can be detected. The correct values of the transferrin receptor molecule hop rates for the smaller and greater compartments in NRK cells were recently found to be an average of every 55 and 570 ms, respectively, using both high-speed SPT and SFVI (direct SPT measurements gave 55 and 1800 ms, respectively, but the latter value needed to be corrected with a macroscopic diffusion coefficient determined by SFVI [a time window of 3 s, giving 0.22  $\mu$ m<sup>2</sup>/s] and the compartment size determined by SPT [710 nm]) [19]. Furthermore, all of the transmembrane proteins examined thus far, including E-cadherin [54], transferrin receptor [53],  $\alpha$ 2-macroglobulin receptor [53], CD44 (Ritchie and Kusumi, unpublished observations),

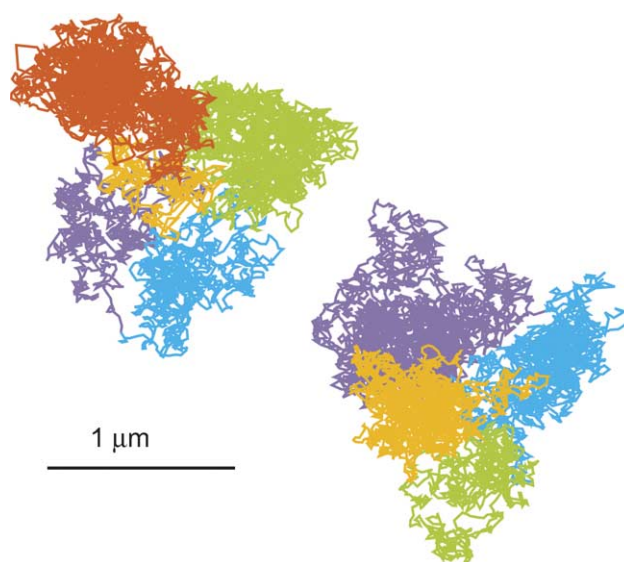


Fig. 4. Typical trajectories of 40-nm gold probes bound to transferrin receptor, recorded at a time resolution of 25  $\mu$ s for a duration of 250 ms (10,000 frames). Different colors represent various plausible compartments, detected by computer software developed in our laboratory (in a time sequence of purple, blue, green, yellow, and red). These trajectories suggest that transferrin receptor molecules undergo short-term confined diffusion in a compartment and long-term hop diffusion between the compartments. This was confirmed by the statistical analysis method developed by Fujiwara et al. [19].

band 3 [22], stem cell factor receptor (Kobayashi, Murakami, and Kusumi, unpublished observations), and various GPCRs (Kasai, Prossnitz, Suzuki, and Kusumi, unpublished observations) undergo hop diffusion. The macroscopic diffusion coefficients for these molecules, determined by SPT (reflecting the hop diffusion rate over many compartments), are basically consistent with the single fluorescent molecule video imaging (SFVI) data and the FRAP data (due to crosslinking effects, the SPT diffusion coefficients may be smaller by a factor of 1–3, but this was corrected using the SFVI data [1,2,6,19,46]).

## 6. The membrane-skeleton “fence” model for temporal corralling of transmembrane proteins

What makes the boundaries between these compartments for transmembrane proteins? Based on the single-molecule observation data and the optical trapping results using specimens with a modulated cytoskeleton or the modulated cytoplasmic domain of transmembrane proteins [6,19,20,22,53,54], as well as on the FRAP and rotational diffusion data before the single-molecule era [28–30,32,34,35,53–56], the “membrane-skeleton fence” or “membrane-skeleton corralling” model was proposed (Fig. 5, left). Transmembrane proteins protrude into the cytoplasm, and, in this model, their cytoplasmic domains collide with the membrane skeleton, inducing temporal confinement or corralling of the transmembrane proteins within the membrane-skeleton mesh. After a while, the transmembrane proteins

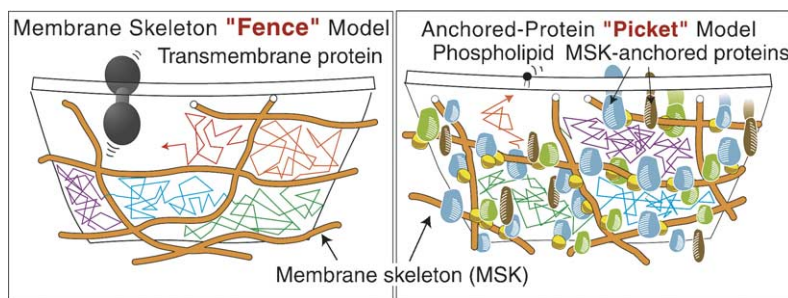


Fig. 5. The effects of the membrane-skeleton “fence” (left) and the anchored-protein “pickets” (right) that together partition the entire plasma membrane into small compartments. See the text for further details. The hydrodynamic-friction-like effect was first described by Bussell et al. [60,61].

hop to an adjacent compartment. Transmembrane proteins may hop between the compartments when a space that allows the passage of the cytoplasmic domain of the transmembrane protein is formed between the membrane and the membrane skeleton. This space is formed as a consequence of the thermal fluctuation of these structures, when the actin filament that forms the compartment boundary temporarily dissociates, and when the transmembrane protein incidentally has sufficient kinetic energy to overcome the confining potential energy of the compartment barrier when it is in the boundary region. Monte Carlo simulation results [38,40], as well as a comparison with the membrane-skeleton structure on the cytoplasmic surface of the plasma membrane observed by atomic force microscopy [57] and a three-dimensional reconstruction using electron microscope computed tomography (Morone, Usukura, Yuasa, and Kusumi, unpublished observations), supported this model.

Several reports employing FRAP or low-speed (video rate or slower) single-molecule tracking have noted the absence of an effect of actin depolymerization on the movement of various membrane molecules [19,46,58,59]. These treatments tend to increase the compartment size, but decrease the hop rate (due to fewer number of collisions with the compartment boundaries, due to the larger compartment size), giving the opposite effects on the macroscopic diffusion coefficients measured by these techniques, and leading to minor increases in the macroscopic diffusion coefficients, by a factor of 1–2, depending on the cell type [19,46]. A clear example is found in Murase et al. ([46], cf. Table 5): after cytochalasin treatment, the average compartment size increased from 45 to 87 nm, but the hop rate decreased from an average of once every 15 ms to once every 39 ms, thus only slightly changes of the macroscopic diffusion coefficient, from 0.042 to 0.046  $\mu\text{m}^2/\text{s}$ . After ensemble-averaging over many molecules observed by FRAP, or too-much time averaging in low time-resolution single-molecule tracking (due to a slow frame rate and/or long frame time), such small changes in the motional characteristics of the membrane molecules may easily be missed (furthermore, the use of high concentrations of actin-depolymerizing drugs and/or the long incubation periods often employed in these studies complicate the results [53]), and thus high-speed

single-molecule tracking methods may be best observation choice.

### 7. The model of anchored transmembrane protein “pickets”, which work for all of the membrane molecules in the plasma membrane

The next natural question is, what makes the compartment boundaries, which even work for phospholipids located in the outer leaflet of the membrane (Figs. 1 and 2)? Fujiwara et al. [19] and Murase et al. [46] 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, on phospholipid hop diffusion, by modulating these membrane-associated molecules and structures [19,46]. They found that the phospholipid movement was affected only when they modulated the membrane skeleton, consistent with the previous FRAP observation (although how the drug treatment actually influences FRAP is complicated; see [56]). Furthermore, Fujiwara et al. observed DOPE diffusion in membrane blebs (balloon-like structures of the plasma membranes, where the membrane skeleton is largely lost [56], and Fujiwara et al. further reduced the actin-based membrane skeleton by treating cells with latrunculin) as well as in liposomes, and found that DOPE molecules undergo rapid, simple Brownian diffusion with a diffusion coefficient of  $\approx 9 \mu\text{m}^2/\text{s}$  in these membranes [19]. All of these results point to the involvement of the membrane skeleton in both the temporal corralling and hop diffusion of phospholipids.

These results are truly surprising. Since the DOPE molecules they observed were located in the extracellular leaflet of the membrane (DOPE may flip, but the large gold particle cannot get into the cytoplasm), whereas the membrane skeleton is located on the cytoplasmic surface of the membrane, DOPE and the membrane skeleton cannot interact directly. To explain this apparent contradiction, the “anchored transmembrane protein–picket model” was proposed (Fig. 5, right). In this model, various transmembrane proteins anchored to and lined up along the membrane skeleton (fence) effectively act as rows of pickets (these trans-

membrane proteins act like posts for the fence, and are thus termed 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 hydrodynamic-friction like effect, first proposed by Hammer's group [60,61], is particularly strong when exerted by immobile molecules, and it propagates over about several nanometers (this effect is prominent in the membrane because the membrane viscosity is much greater than that in water, by a factor of  $\approx 100$ ). Therefore, when these transmembrane proteins are lined up along the membrane skeleton at a density over a certain threshold (a series of Monte Carlo simulations by Fujiwara et al. indicated that 20–30% coverage of the intercompartmental boundary by these anchored transmembrane protein pickets is sufficient to reproduce the experimentally observed residency time of 11 ms in a 230-nm compartment in NRK cells), the rows of pickets on the membrane-skeleton fences become effective diffusion barriers that confine the phospholipids for some time. Note that these transmembrane picket proteins do not have to be stably bound to the membrane skeleton for a long time. Assuming that the boundary region between the compartments is 10 nm wide, it takes about  $10 \mu\text{s}$  for a molecule to traverse this region. Therefore, the zeroth approximation is that if a transmembrane protein is bound to the membrane skeleton for at least  $10 \mu\text{s}$ , then it becomes an effective picket to participate in the formation of the diffusion barrier. Note that, in this model, the transmembrane proteins anchored to the membrane skeleton are coupling the membrane skeleton, which is located on the cytoplasmic surface of the membrane, with the phospholipids that are located in the outer leaflet of the 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.

## 8. Oligomerization-induced trapping

One of the most physiologically important consequences of plasma membrane compartmentalization by the membrane skeleton “fences” and the anchored-protein “pickets” may be the suppression of diffusion of membrane molecules upon oligomerization or molecular complex formation. The partitioning of the plasma membrane into many small compartments could explain why the diffusion in the plasma membrane is very sensitive to the formation of molecular complexes (Fig. 6, right), in contrast to the prediction from the two-dimensional continuum fluid model (Fig. 6, left). Monomers of membrane molecules may hop across the picket line with relative ease, but upon molecular complex formation, the complexes as a whole, rather than single molecules, have to hop across the picket–fence line all at once, and therefore, these complexes are likely to have a much slower rate of hopping between the compartments, as found with E-cadherin-GFP [15] and oligomers of DOPE [46]. In addition, molecular complexes are, due to the avidity effect, 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” [15,20].

This oligomerization-induced trapping might be critically important in the temporary confinement of a cytoplasmic signal at the very early stages of signal transduction. When an extracellular signal is received by a receptor molecule, the receptor often forms oligomers and signaling complexes. Due to the “oligomerization-induced trapping”, these oligomeric

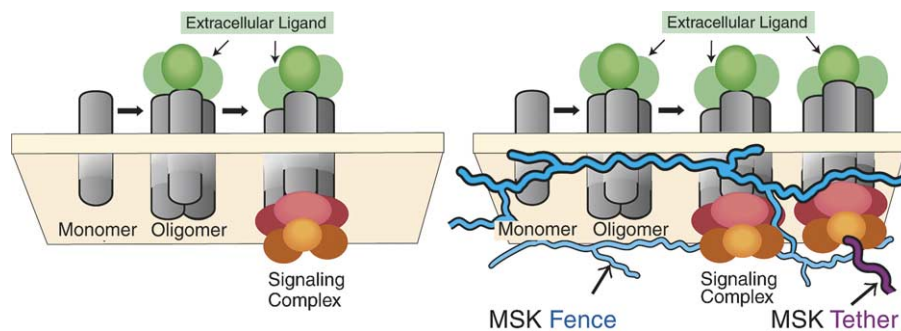


Fig. 6. Oligomerization-induced trapping model, indicating how slowing or immobilization of membrane molecules may be induced upon oligomerization or the formation of greater molecular complexes. Upon oligomerization or molecular complex formation, the hop rate across the intercompartmental barrier would be reduced greatly (right), because, in contrast to monomers, in the case of molecular complexes, all of the molecules that form the complex have to hop across the picket–fence line simultaneously. In addition, due to the avidity effect, molecular complexes are more likely to be tethered to the membrane skeleton, perhaps temporarily, which also reduces their overall diffusion rate. This enhanced confinement and the binding effects induced by oligomerization or molecular complex formation are collectively termed “oligomerization-induced trapping” [15]. This would not occur in the absence of membrane-skeleton fences and pickets (left): the diffusion theory by Saffman and Delbrück [25] predicts that the diffusion rates of the oligomeric complexes would be almost the same as those of the single receptor molecules.

complexes tend to be trapped in the same membrane-skeleton compartment as that where the extracellular signal was received. Therefore, the membrane-skeleton fence and the anchored transmembrane-protein pickets temporarily help to confine 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.

This would not occur in the absence of membrane-skeleton fences and pickets (Fig. 6, left). If there were no such structures, then even when the signaling complex is formed, the diffusion rate of such a complex would be almost the same as that of the single receptor molecules, as the diffusion theory teaches us [25].

Therefore, in the plasma membrane, oligomerization or molecular complex formation is tied to immobilization by the membrane-skeleton fence and anchored-protein pickets.

### 9. A paradigm shift of the plasma membrane structure concept is necessary: from the simple two-dimensional continuum fluid model to the compartmentalized fluid model

As described above, the membrane-skeleton “fence” and the anchored-protein “picket” together solved the two long-standing problems of molecular diffusion in the plasma membrane: (1) the oligomerization-induced temporary trapping and (2) the reduced diffusion coefficients of membrane molecules in the plasma membrane, from that found in artificial membranes, by a factor of 5–50. These results could not be explained by the two-dimensional continuum fluid model. The two-dimensional continuum fluid model works well, as long as the spatial scale is limited to the size of the original cartoon depicted by Singer and Nicolson ([24]; although at the smaller limit of the molecular scale, the continuum model would also fail), which is about 10 nm × 10 nm (Fig. 2, inset). However, in spatial scales over several tens of nanometers in the plasma membrane, simple-minded extensions of the fluid-mosaic model of Singer and Nicolson [24] and the theory by Saffman-Delbrück [25] fail. The cell appears to have developed (during evolution) means to control the long-range diffusion of membrane molecules, and to make it sensitive to the diffusant size. The long-range control of diffusion appears to be carried out by the actin-based membrane skeleton and its associated transmembrane-protein pickets, through their partitioning (corralling) and tethering effects, which are enhanced upon molecular complex formation by diffusing membrane molecules.

As such, a paradigm shift for the concept of the plasma membrane structure larger than 10 nm is required, from the two-dimensional continuum fluid to the compartmentalized fluid, in which its constituent molecules undergo hop diffusion over the compartments.

### 10. Most of the methods for observing membrane molecules’ lateral diffusion measure “effective diffusion coefficients” in space scales over 300 nm and in time scales over 100 ms

An important corollary of these results is that the rate of diffusion cannot be described by a single diffusion coefficient, and thus all of the diffusion coefficients obtained by FRAP or those estimated by single-molecule techniques at slow rates (like a video rate) must be thought of as “the *effective diffusion coefficients*”, which may be useful only when the involved time window is specified [19,20,42,44–46,62,63]. This constitutes a major difference from simple Brownian diffusion cases, where the observation time window should not matter.

Therefore, it is essential to realize the fact that membrane molecules do not undergo simple Brownian diffusion (although they may undergo “effective simple Brownian diffusion” in limited time windows), and to interpret (the changes of) the effective diffusion coefficient data based on the concept of the compartmentalized plasma membrane, which FRAP and slow single-molecule techniques cannot directly visualize. It is better to employ high-speed SPT methods to directly observe the hop diffusion of molecules, because, as described previously, all of the membrane molecules undergo hop diffusion anyway, and this technique is not particularly difficult or expensive to introduce in the lab, as compared with, e.g., confocal or total internal reflection fluorescence microscopy.

### 11. Incompatibility of raft domains and rows of anchored-protein pickets along the actin-based membrane skeleton?

Cholesterol is likely excluded from the boundary regions around transmembrane proteins in the reconstituted membranes of sarcoplasmic reticulum ATPase, phospholipids, and cholesterol [64]. Band 3 in the erythrocyte membrane is also immiscible with cholesterol in reconstituted membranes (Kusumi and Tsuji, unpublished observations). Consistent with these observations, a large majority of transmembrane proteins exhibit very low levels of partitioning into the low-temperature, detergent-resistant, low buoyant density fraction, which has been considered to be a moderately good biochemical indicator of raft-philocity of membrane molecules in the live cell membrane.

We propose that these results are due to lateral *non-conformability* of the cholesterol’s rigid, bulky sterol ring with the rough surface of the transmembrane domain of the majority of transmembrane proteins. This surface roughness is due to the amino acid side chains protruding from the transmembrane  $\alpha$ -helix. The surface of the transmembrane domain may be rough, but the alkyl chains are considered to have sufficient flexibility to accommodate the protruding amino-acid side chains. Beyond a layer or two of the alkyl

chains from the transmembrane  $\alpha$ -helix, the effects of the protruding amino acid side chains would be smoothed out, and cholesterol could be incorporated there.

The surface roughness of the transmembrane domain would probably drive the majority of transmembrane proteins away from the cholesterol-rich raft domains. Therefore, we would predict that the transmembrane receptors that take advantage of rafts for their signaling, such as the Fc $\epsilon$  receptor or the T-cell receptor, would have relatively smooth surfaces on their transmembrane  $\alpha$ -helices to facilitate their incorporation within the raft.

The results described above and the considerations given there further suggest that most transmembrane proteins may act as *raft breakers*, by driving cholesterol away from their boundary regions (see below). It follows then that the transmembrane picket proteins that are aligned along the membrane skeleton form a structure like beads on a string, the bead being the transmembrane picket protein and its annulus that excludes cholesterol, and the string being the actin-based membrane skeleton. Since the picket proteins might be 3–10 nm away from each other, and assuming that the diameter of a transmembrane domain is 0.7–4 nm [19,65], 1.5–5 nm diameter zones that exclude cholesterol are lined up 2–9 nm away from each other on the membrane skeleton (also based on the estimate that the diameter of the area that an alkyl chain occupies is 0.4–0.5 nm).

These rows of cholesterol-excluding areas might act as barriers that block the growth of the raft domains as well as the diffusion of each raft domain as a whole (if the raft size is over several nm or its lifetime or the residency time of raft-philic molecules in the raft is long). If the raft is very stable (its lifetime and the residency time of its constituent molecules are long) and has a size over several nm, one would expect that the diffusion coefficients of the raft-associating molecules would be far smaller than those for membrane molecules that do not associate with the raft domains. The results by Vrljic et al. [58] and Kenworthy et al. [66] were against this expectation. The diffusion rates are not different between these types of molecules in the majority of the cases, and even when they are different, raft partitioning was not the cause for the difference, suggesting that the hypothetical large and stable rafts actually do not exist in the cell membrane.

## 12. Rafts have not been directly observed in the plasma membranes of the resting cells

While a raft-related structure, caveolae [67–69], which may be a type of raft stabilized by the assembly of the transmembrane protein caveolin [70–72], can be imaged in intact membranes, direct observations of rafts *in resting cells* have turned out to be difficult. Close examination of the reports describing the imaging of rafts in steady-state cells using light microscopy has revealed that, in almost all of the cases, the sample preparation protocol involved procedures that could induce the clustering of raft molecules (which generally in-

duce signaling events), such as the use of crosslinkers like antibodies and fixatives (as pointed out by Mayor et al. [73]), probes that might crosslink raft molecules, such as cholera toxin, a pentavalent reagent, and latex beads conjugated with antibodies [74–79] and the employment of low temperatures (even cooling to room temperature may result in the artificial growth of domains) [14,80–83]. Therefore, *rafts have only been visualized after crosslinking or ligating raft molecules or at lower temperatures* [73,75,80], with only a few possible exceptions [14,77,84–86]. Namely, the rafts cannot be visualized by normal optical microscopy in the steady-state cells. This suggests that the raft size in the resting cell is smaller than the optical diffraction limit ( $\approx 300$  nm) and might increase after stabilization by crosslinking raft molecules or lowering the temperature [84–86]. In steady-state cells under physiological conditions, even if some raft molecules are concentrated in a raft, if the raft size is smaller than the optical diffraction limit, then their assembly in the raft would be blurred and smeared out by averaging over the area determined by optical diffraction (e.g., a  $10\times$  concentration in a 50-nm raft would appear as only  $\approx 30\%$  increase in the fluorescence signal at a diffraction limit of 300 nm).

## 13. The rafts in the plasma membrane of the resting-state cell may be small/short-lived and/or their constituent molecules may rapidly exchange with those in the bulk domain

Single-particle tracking and photonic force microscopy suggested the raft sizes ranging between 50 and 200 nm in diameter [76–78]. The problem with these methods is that since they employ gold or latex particles as probes, it is difficult to totally disregard the possibility of probe-induced crosslinking. In fact, as methods for probe preparation improve, these particle probes less often exhibit the transient confinement zone (TCZ, which is a site or a domain where raft-philic molecules temporarily stop diffusion), which may represent rafts (A. Kusumi and K. Jacobson, unpublished results).

Prior et al. [87] carried out an elegant quantitative immunoelectron microscopy study, and found 40-nm diameter raft domains that concentrate raft molecules like H-Ras (before its activation). This was further refined recently by taking the probe size into consideration, giving  $\approx 10$  nm as the diameter of the steady-state rafts [88].

FRET and pulse EPR spin-labeling experiments suggested that the rafts are even smaller and/or short-lived, and/or that the raft molecules rapidly diffuse in and out on a time scale of 100  $\mu$ s or less [89,90]. Recent FRET work on GPI-anchored proteins revealed that 20–40% of these proteins may be in clusters smaller than pentamers, with the remaining 60–80% existing as monomers [91,92]. This explained why FRET between raft-philic molecules was hard to detect using two-different dye molecules [89,93,94]. In line with these observations, McConnell has advanced the concept of the condensation complex of cholesterol and saturated-alkyl-chain

lipids, which can be formed without relevance to the liquid-ordered phase [95–101].

Using single-molecule techniques (both SPT at a 25- $\mu$ s resolution and SFVI at video rate), Suzuki et al. found that the raft that monomeric CD59, a typical raft-preferring GPI-anchored protein, is associated with may consist of only several molecules with a lifetime perhaps on the order of a millisecond or less [102–104]. Both CD59 and an unsaturated phospholipid probe, DOPE, a typical non-raft lipid, undergo short-term confined diffusion in a compartment and a long-term hop movement over many compartments with similar average hop rates of once every 1–25 ms (T24 cells, and this rate depends on the cell type). If a GPI-anchored raft protein, CD59, is always associated with large and stable rafts, the raft and CD59 would diffuse together, and one would expect that their hop rate across the compartment boundaries would be much smaller than that for DOPE. However, in reality, they both hopped at the same rate, once every 25 ms on average, consistent with the results by Vrljic et al. [58] and Kenworthy et al. [66]. Although we note that these results are not inconsistent with an extreme possibility that GPI-anchored proteins do not partition into the raft in the resting cells at all, we prefer the idea that the rafts that monomeric GPI-anchored proteins associate with in steady-state cells may be very small and/or short-lived, perhaps on the order of a millisecond or less (because both DOPE and CD59 hop across the picket line at about the same rate of once every 25 ms on average). These results are also in general agreement with the observations described above, in that the rafts in the resting cell membrane are small [89–93,95–101]. In the present review, we call these small, unstable rafts “reserve rafts”.

Anderson and Jacobson [105] proposed a model of a “lipid-shell” surrounding raft-associating molecules. The difference between the lipid shell model and our small/unstable reserve raft model is the lifetime (although the size may also differ by a factor of 3–10 in diameter or 10–100 in the number of molecules). They assume that the lipid shells have long lifetimes, and they argued that this model could explain the fast hop frequency of CD59 across the rows of pickets (compartment boundaries), because the shells are soft and can change their shape, allowing for passage between the pickets. However, such a property may not be readily compatible with the raft integrity (long lifetime), i.e., if it were very soft, then it would tend to break easily, reducing its lifetime, and thus the shell concept becomes very similar to the small/unstable reserve raft concept.

#### **14. Clustering of raft molecules by ligand binding or crosslinking induces stabilized rafts, “receptor-cluster rafts”**

In contrast to the cell in the steady-state (in the absence of extracellular stimulation), abundant evidence exists for the formation of greater, stabilized rafts upon stimulation by liganding or crosslinking raft-philic molecules, suggesting the

recruitment of raft-philic structural and signaling molecules in cholesterol-dependent manners, colocalizing with the activated receptor molecules [48,74, 83–85,106–116]. However, it is important to realize that the constituent lipid molecules of such “receptor-cluster rafts” may still be able to exchange with those in the bulk disordered-liquid domain, just like the lipids moving back and forth between the ordered- and disordered-liquid-phase domains in artificial membranes [117].

#### **15. How can raft-molecule clustering induce stabilized rafts?**

How can the clustering of GPI-anchored proteins (induced by ligand, lectins, pathogens or other reagents) induce greater, stabilized rafts? What makes the clustered raft molecules different from the monomeric raft molecules? As an example, consider the hypothetical oligomerization of four GPI-anchored receptor (GPI-AR) molecules, which induces the close assembly of 4–8 saturated alkyl chains. Note that since the protein domain is much larger (say 3–5 nm in diameter) than the two alkyl chain anchors (say 0.6 nm  $\times$  1.2 nm), there is always enough space for other lipids to stay among the anchoring chains in a GPI-AR cluster. We suggest that such a loose saturated-alkyl-chain cluster would recruit cholesterol, glycosphingolipids, and raft-preferring molecules into the cluster, due to the following interactions.

First, cholesterol mixes well with saturated chains while it tends to be excluded from the bulk, disordered liquid domains because of its immiscibility with unsaturated alkyl chains [18,118]. Therefore, if given a choice, cholesterol would move to the region where the saturated chains are concentrated.

Second, since the long alkyl chains of sphingolipids might also be excluded from the bulk, disordered-liquid domain due to mismatches in their hydrophobic lengths, particularly when the long saturated chains are in contact with cholesterol [119–121], they tend to assemble in the clusters of GPI-AR-cholesterol, where the hydrophobic chains are longer and the membrane is thicker.

Third, importantly, since the GPI-AR oligomerization prevents their saturated chains from diffusing away from the cluster and blocks the changes in their relative positions, it reduces the diffusion of other molecules in the area of the GPI-AR oligomer, thus restricting the movements of the recruited cholesterol and glycosphingolipids.

Fourth, since the cholesterol-saturated alkyl chain interaction enhances the trans conformation and thus higher orders of saturated alkyl chains [18,118], which are predominant in the GPI-AR region, this further reduces the thermal agitation in this region and prolongs the residency time of cholesterol, glycosphingolipids, and sphingomyelin in this GPI-AR cluster region.

Each of these processes would involve a tiny free energy gain, but collectively these gains may be sufficient to main-

tain high concentrations of cholesterol and sphingolipids in the GPI-AR oligomer area. Further, we again would like to stress the delicate balance between the concentration and the dispersion of these raft molecules: the oligomerization of GPI-AR only slightly suppresses thermal diffusion in the oligomer area, but the small reduction in the thermal motion could greatly enhance the ability of GPI-AR to collect other raft molecules to form large and stable rafts.

This argument is analogous to that for the effect of transmembrane protein clustering on lipid movement found in boundary lipid research. When transmembrane proteins are monomeric, the lipids at the boundary (i.e., the lipids that directly contact the transmembrane protein) exchange with the lipid in the bulk domain at a rate of  $10^7 \text{ s}^{-1}$ , or once every 0.1  $\mu\text{s}$  on average [122,123]. Note that if the transmembrane protein is anchored to the membrane skeleton, then its anchoring effect on the surrounding lipids is greatly enhanced and distantly propagates due to the hydrodynamic-friction like effects [19,124,125]. Such rapid lipid exchange is often overlooked by raft researchers. Upon transmembrane protein oligomerization, the lipids become sandwiched between two transmembrane proteins (including the lipids trapped between boundary lipids) or trapped in the protein-rich domain, and the rate of their exchanges with lipids in the bulk domain drops dramatically [121,126,127]. Both the geometric and packing effects of the clustered proteins would contribute to the prolonged residency of lipids in the clustered protein area. GPI-AR clustering would similarly suppress the movement of molecules in the cluster region, thus prolonging their stay in the cluster domain.

The essential behavior of boundary lipids that raft researchers must understand may be summarized as follows. In the instances that the alkyl chain enters the boundary region, the trans-gauche isomerization in the alkyl chain is strongly restricted (thus free volume in this area is substantially smaller than that in the bulk region) [128–131], but the chain leaves the boundary region within 0.1  $\mu\text{s}$  on average (a new lipid comes into the boundary region, and the isomerization of its alkyl chains instantaneously stops; therefore free volume there is always less than that in the bulk region). Prolonged residencies of lipids in the boundary region of a transmembrane protein only takes place when the transmembrane proteins are clustered or form protein-enriched domains. For further details of the boundary lipid behaviors, see our previous review [65]. An analogy can be made to GPI-AR: cholesterol and glycosphingolipids would not remain in the boundary region of GPI-AR unless GPI-AR clustering is induced, leading to the prolonged, but temporary, trapping of other raft lipids and cholesterol in the cluster domain.

The receptor-cluster rafts are the rafts that are directly involved in biological functions. The considerations given in this section suggest that the receptor-cluster rafts are on the verge of forming even in resting state cells, and with only small modulations of the delicate balance of molecular interactions, such as clustering of GPI-AR, receptor-cluster rafts are formed. Therefore, the small, unstable rafts found be-

fore stimulation are those ready for forming greater stabilized rafts, and this is why these molecular complexes are called “reserve rafts” in this review.

## 16. Mechanism of Lck recruitment to the T-cell receptor clustering site

T-cell receptor (TCR) signaling has fascinated many researchers, because, in addition to its immunological importance, it provides interesting paradigms for studying the basic design of how cellular signal transduction is carried out and regulated in various cell types. The multiple signaling pathways triggered by TCR involve various conceptually different and important mechanisms for the regulation of signaling working simultaneously [132,133]: some signaling pathways have to be activated continuously for a long time (over 24 h) [134,135]; the TCR signaling network should contain circuits to prevent and/or turn off faulty signals [136]; and it should amplify or reduce the signal when another signal comes from co-receptors, such as CD28 and CD4/8 [137]. T cells achieve such complex signal transduction by recruiting specific signaling molecules to the TCR clusters and their neighborhood, and also by having them escape from the stimulation zone, in the correct order and at the right time [138]. In other words, the recruitment process itself is the fundamental regulation mechanism for this complex signal transduction [139–142], and since the formation and dynamics of raft domains may play critical roles in the regulation of signaling molecule recruitment processes, the interest on how the raft domains may be involved in TCR signaling has recently drawn extensive attention [116,143].

Ike et al. [144] studied the regulation mechanism by which Lck, a Src family kinase, is recruited to the site of TCR clustering. Lck is recruited to the TCR clustering site during the very early stages after TCR engagement [145,146]. They used SFVI to observe single molecules of Lck and its N-terminal 10 amino acid sequence (N10), both fused with green fluorescent protein (GFP) (Lck- and N10-GFP, respectively, Fig. 7) and expressed in the Jurkat T cell line. Lck consists of an SH4 domain that includes N10 and the domain for the interaction with the coreceptor CD4, an SH3 domain, an SH2 domain, and a kinase domain. N10 has no site for specific interactions with other proteins, but contains all of the Lck anchoring, saturated alkyl chains (one myristoyl and two palmitoyl), which induce the partitioning of about half of Lck [147] and 80% of N10-GFP [144] into the detergent-resistant membrane (DRM) low-buoyant density fraction.

Two mechanisms have been proposed for Lck recruitment: one is that Lck is carried along with its binding protein, CD4, which is recruited to the TCR clustering site due to its affinity for MHC II in the antigen presenting cell (the target cell; MHC II binds to both TCR and CD4) [148]; and the other is that the Lck molecules associated with the (stabilized) raft are assembled when the (stabilized) rafts are carried by the actomyosin system to the site of TCR clustering [149].

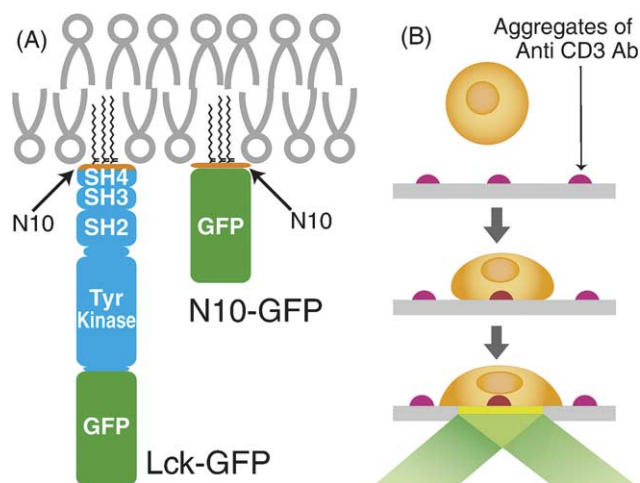


Fig. 7. Schematic structures of Lck- and N10-GFP molecules (A) and a diagram showing the method for stimulating a cell at a single site by clustering TCR, to investigate the mechanism of Lck recruitment to the TCR cluster. The movements of Lck and its N-terminal 10 amino acid sequence (N10), both fused to GFP (left), and expressed in the Jurkat T cell line were visualized at the Single-molecule level near a single TCR cluster (right).

To study the events occurring at the very early stages of TCR signaling, a simplified model system has been developed (Fig. 7) [144]. First, the TCR clustering was induced at a single site in a cell using aggregates of OKT3, an antibody against CD3 $\epsilon$ , one of the subunits of the TCR complex, and the cell was very locally stimulated. The antibody aggregate induced TCR clustering, which in turn triggered the subsequent cellular signaling, including Lck assembly. In other words, using the aggregates of the anti-CD3 $\epsilon$  antibody placed on the coverslip, antigen presentation to the T cell was mimicked to a certain degree by locally inducing TCR clustering. To do this, aggregates of OKT3 were sparsely adsorbed on a coverslip, and then the cells were centrifuged onto it. By quickly sedimenting the cells onto the coverslip, the initial time for TCR clustering is well defined. Bunnell et al. [150,151] previously coated the cover slip with an anti-CD3 $\epsilon$  antibody to induce cellular signaling from all over the surface attached to the coverslip, and the cell was highly extended on the cover slip. In the strategy by Ike et al. [144], using the antibody aggregates, very local stimulation at a single, small site per cell was achieved.

One of the problems of this approach is that, although time 0 can be specified quite accurately (which is a major advantage of this method over simply mixing antigen presenting cells and T cells in solution), observations right after the signal input cannot be done. Four minutes usually pass, from the time of centrifugation of the cells for interaction with the antibody aggregate until the initiation of single-molecule observation of Lck assembly at the TCR cluster. Therefore, using such an experimental system, the initial assembly events of Lck cannot be investigated. However, a steady-state, more robust assembly of Lck could be monitored, after the initial TCR-clustering-induced rapid rise of recruitment of various

signaling molecules and the initial reorganization of actin filaments are already well underway.

Ike et al. [144] indeed found that, upon TCR clustering at a single site in a cell, both Lck- and N10-GFP became concentrated at and around the TCR cluster in a cholesterol- and actin-dependent manner, indicating the critical roles of rafts and their associations with actin filaments, which also become concentrated near the TCR cluster. Interestingly, the assembly of Lck and N10 was dynamic, and both molecules concentrated at and around the TCR cluster were exchanging rapidly with those in the surrounding membrane region at a rate on the order of several seconds.

Single-molecule observations showed that neither Lck- nor N10-GFP undergoes directed movement toward the cluster, but that they both undergo apparently simple Brownian diffusion in the observation time scales of several seconds. However, their diffusion rates strongly depend on how far the molecules are located from the cluster (when they were measured 4 min or later after the signal input from the antibody aggregate). Their diffusion coefficients were decreased near the stimulation point, but were increased in the area far from the stimulation point, as compared to the rate without stimulation (Fig. 8). The overall variations of the diffusion coefficient over the cell surface were, by factors of  $\approx 3$  and  $\approx 10$  for Lck- and N10-GFP, respectively. Ike et al. [144] proposed a model in which the actin membrane skeleton (cortical actin filaments) becomes more (less) dense near (far from) the TCR cluster site, and hence, the movement of Lck- and N10-GFP associated with the raft is decreased (increased) due to the enhanced (reduced) corralling and tethering effects of the actin membrane-skeleton mesh and various transmembrane protein pickets (Fig. 9, cf. Fig. 5). The reduced diffusion rate near the cluster would allow Lck sufficient time to interact with the TCR cluster and the downstream signaling molecules, to trigger the subsequent signaling events in the area within and around the TCR cluster.

The assembly of N10-GFP at the TCR cluster site suggests that the binding of Lck to CD4 may not be an absolute requirement for Lck recruitment to the TCR cluster site. Perhaps Lck associations with CD4 and raft domains may facilitate both its recruitment and/or its interactions with TCR and ZAP-70. Furthermore, as the dynamic nature of the Lck concentration at the TCR cluster site suggests, the Lck interactions with CD4, raft domains, TCR, and ZAP-70 are likely to be transient. Although many publications appear to assume or conclude that long-term associations occur between Lck and CD4 [146,148], the data are rather indirect or suggest low-affinity binding ( $K_d \approx 0.4 \mu\text{M}$  [152]). Namely, Lck molecules may become concentrated at the CD4 cluster, but at the level of individual molecules, the Lck molecules at the CD4 cluster are rapidly exchanging with those in the bulk membrane.

Molecular immunology has always eagerly embraced new technologies to open new vistas in signal transduction analyses. Recent examples include the three-dimensional reconstruction of SMACs by a deconvolution analysis of fluores-

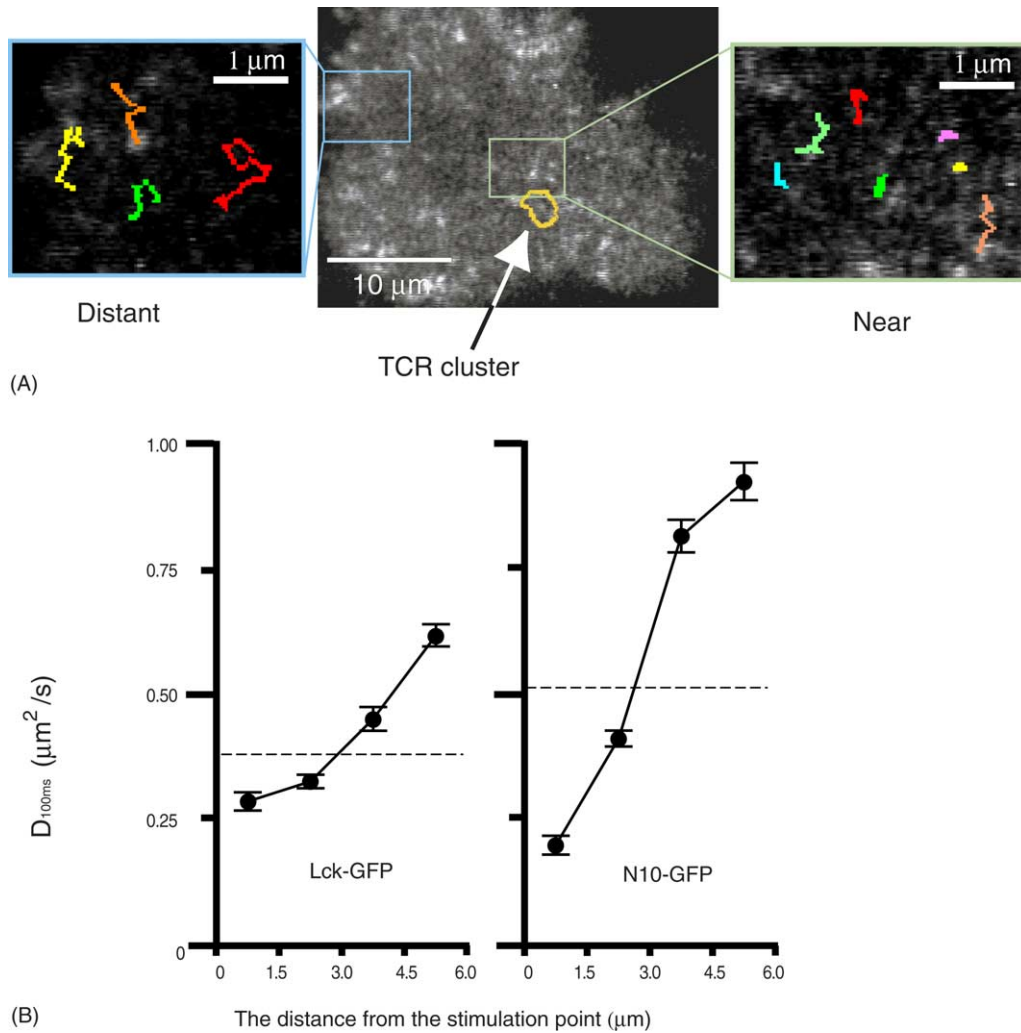


Fig. 8. Reduction in the diffusion rate of both Lck and N10 near the TCR cluster site. Although no directed motion toward the TCR cluster site was observable, a significant reduction in the diffusion rate (3- and 10-fold for Lck and N10, respectively) was found in the vicinity of the cluster. (A) The yellow contour in the central image indicates the aggregation site of the anti-CD3 $\epsilon$  antibody attached to this cell (only cells with single-site stimulation were selected). The images on the right and left show the expanded images near and far from the stimulation site, respectively, together with the trajectories of Lck molecules (different molecules are represented by different colors). A comparison of the trajectories in the left image with those in the right image shows that the molecular diffusion near the TCR cluster site is suppressed. (B) The average diffusion coefficient of Lck- and N10-GFP, plotted as a function of the distance from the TCR cluster site. Dashed lines indicate the average diffusion coefficient before stimulation.

cence images [138,145], the observation of lymphoid tissues using two-photon excitation fluorescence microscopy [153,154], and the use of supported membranes [155,156], in addition to the use of gene knock-out or transgenic mice. The next reasonable addition to these arsenals would be single-molecule observation and manipulation methods.

### 17. Developmental formation of a diffusion barrier in the neuronal initial segment cell membrane

One of the major functions of the “membrane-skeleton fence” and the “anchored-transmembrane protein pickets” may be to form large diffusion barriers in the cell membrane. Recently, Nakada et al. found that neuronal cells develop diffusion barriers in the plasma membrane, using the fence

and picket mechanisms [23]. The neuron has two distinct domains: one is the somatodendritic domain, which functions in the input of the electrical signal, and the other is the axonal domain, which is responsible for the output of the signal, and each domain has its characteristic membrane proteins. However, the plasma membrane is one continuous membrane, and therefore, there must be a diffusion barrier in the boundary region between the two domains, so the cell can prevent the intermixing of the membrane molecules located in each of these two domains. The domain that separates these two domains is called the initial segment (IS), an elongated domain with a length of  $\approx 30 \mu\text{m}$  in mature neurons, located at the foot of the axon. Protein and lipid transport helps to define the two separate domains, but without a barrier at the IS, proteins and lipids in the cell membrane will eventually intermix.

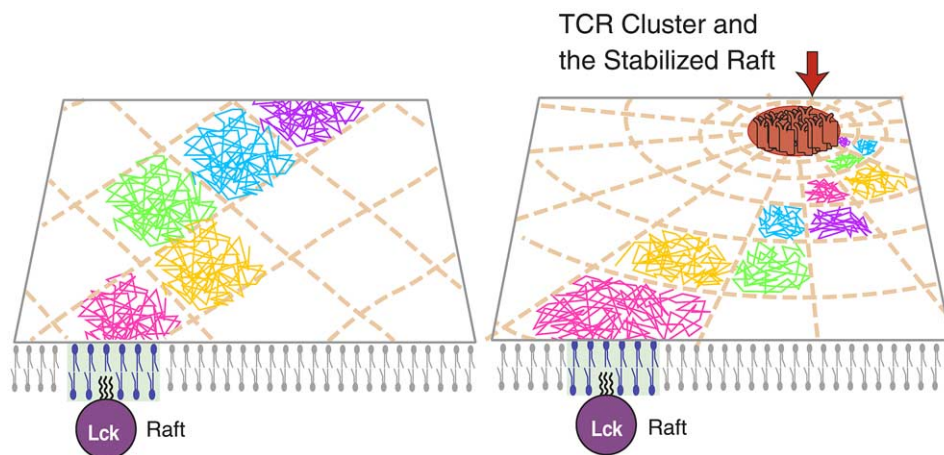


Fig. 9. Model for the recruitment of Lck to the TCR cluster site. Formation of the TCR cluster (shown in the upper right corner of the cartoon on the right) increases the density of the actin skeleton (both the “fences” and the “pickets”) in the vicinity of the cluster site, thus decreasing the macroscopic diffusion coefficient of Lck there. This slowing allows more time for Lck interactions with the TCR cluster and with various required signaling molecules.

Previous studies probing the existence of a diffusion barrier in the neuron’s IS domain produced contradicting conclusions [157–161]. The distance that the introduced dye molecules had to travel to cross the IS may be too far for the experiment period, or the latex bead used as a probe might have crosslinked the test molecules, and thus its diffusion is suppressed.

In the study by Nakada et al., the test molecule that they primarily observed was an unsaturated phospholipid, DOPE, which was used in previous studies of membrane compartmentalization and hop diffusion [23]. To avoid the ambiguity of bulk observations, they observed the diffusion of individual DOPE molecules using single-molecule techniques: SFVI, SPT, and single-molecule dragging by laser tweezers. At 1 day in vitro (DIV), DOPE diffuses rapidly everywhere in the cell membrane, including the IS membrane. In contrast, the diffusion of DOPE in 10-DIV neurons in the IS membrane was severely restricted, whereas it was still free elsewhere on the cell membrane. Furthermore, colloidal-gold probes attached to DOPE in the mature IS membrane could not be dragged laterally by optical tweezers, whereas those attached to the IS membrane or to younger neurons could be dragged freely, suggesting that a diffusion barrier to even phospholipids exists and is formed during neuronal development.

Nakada et al. examined the mechanism for the barrier formation, following the developmental stages, and found that the diffusion barrier arose coincident with the concentration of actin, ankyrin-G, and  $\text{Na}^+$  channels in the IS area. In addition, partial depolymerization of the actin filaments made DOPE and the  $\text{Na}^+$  channels mobile once again, whereas stabilization of actin filaments just before the barrier function is established greatly suppressed DOPE diffusion only in the IS membrane. Ankyrin-G is known to anchor various transmembrane proteins, including the  $\text{Na}^+$  channel, on the membrane skeleton [162–164] and the accumulation of the  $\text{Na}^+$  channel in the IS membrane was also recently reported by other groups [165,166]. These findings, based on

the anchored-protein picket model, suggest that the diffusion barrier in the IS membrane may be created by the local concentration of various transmembrane proteins [167] and membrane-skeletal proteins [168], and by their binding to each other. Namely, very dense rows of anchored-protein pickets are formed in the IS membrane, and since they are formed over the 30- $\mu\text{m}$  long domain, they can practically block the macroscopic diffusion of phospholipids in the IS domain (a rough estimate suggests that it takes over 2 weeks for DOPE to pass this domain).

In the membrane region surrounding the immunological synapses, the passage of membrane signaling molecules may be controlled, and this may be achieved by the dense rows of various transmembrane proteins, such as cell adhesion molecules, anchored to the concentrated actin filaments in the synapse region.

## 18. Conclusions

Recent advancements in single-molecule methods have allowed researchers to observe the movement, recruitment, and activation of single molecules in the plasma membrane in living cells. In particular, high-speed SPT at a frame rate of 40,000 frames/s has revealed the partitioning of the fluid plasma membrane into small compartments throughout the membrane, and the hop diffusion of virtually all of the molecules in the plasma membrane. This explains why the diffusion coefficients in the plasma membrane are considerably smaller than those in artificial membranes, and why the diffusion coefficient is reduced upon molecular complex formation (oligomerization-induced trapping).

Observations of dynamic Lck assembly after TCR engagement in immunological cells, and the formation of a large-scale diffusion barrier in the neuronal cell membrane showed that membrane-skeleton “fences” and anchored transmembrane protein “pickets” play important roles in the orga-

nization of various membrane molecules in the plasma membrane. In the studies of immunological signal transduction mechanisms, in particular the recruitment and assembly/disassembly of signaling molecules at the site of signal transduction, the involvement of these actin-based membrane-skeleton “fences” and anchored transmembrane protein “pickets” should always be considered.

Single-molecule methods could provide insights that may never be obtained by employing methods that involve the ensemble averaging of all of the molecules under observation, like the hop movement of membrane molecules (for this observation, the time averaging also had to be reduced, but the length of the trajectory must be kept sufficiently long). We hope that more researchers engaged in immunological research will start to employ single-molecule techniques, as it appears that abundant opportunities exist for very many interesting, single-molecule studies in immunology, at the levels of molecules, cells, and tissues. Specially, for the investigations of raft organization and raft-based coordination of signaling molecules, single-molecule techniques, the high-speed SPT and SFVI in particular, are likely to make important contributions. Because, for understanding how the raft domains are formed and function, the raft dynamics, structure, size, and lifetime, and the entry and exiting kinetics of raft-philic molecules in and out of raft domains have to be known, with single-molecule techniques, many of these issues can be directly addressed.

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