19
Paradigm Shift of the Molecular Dynamics Concept in the Cell Membrane: High-Speed Single-Molecule Tracking Revealed the Partitioning of the Cell Membrane

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19.1
Introduction

The plasma membrane is the outermost membrane of the cell, surrounding the entire cell and determining the boundaries between the cell and the outside world. All of the living organisms on the earth share essentially the same fundamental plasma membrane structure. The plasma membrane has, like all of the other membranes in the cell, a quasi-two-dimensional liquid-like structure, but it is not a simple liquid. Rather, it is a nonideal mixture of various molecules with differing miscibilities in the fluid state. Therefore, the plasma membrane contains dynamic structures, like molecular complexes and domains, functioning on various time scales and space scales, and forming and dispersing continually within the plasma membrane. These molecular complexes and domains range from small protein clusters with short lifetimes, like transient dimers of rhodopsin [21], to large micron-sized stable domains, like cell-to-cell or cell-to-substrate adhesion structures (see Figure 19.1 for various membrane domains).

Another interesting feature of the plasma membrane, which makes it different from a simple two-dimensional ideal liquid, is its association with the cellular filamentous protein meshwork that consists of actin filaments, called the cytoskeleton. The actin cytoskeleton is a three-dimensional structure present throughout the cell, for the creation, modification, and maintenance of the cellular morphology. On the cytoplasmic surface of the plasma membrane, the actin filament interacts with many plasma-membrane-integrated proteins and lipids, indirectly, but specifically, via connecting molecules, and also directly, but with lower affinities (but at many sites). At the interface between the actin-based cytoskeleton and the plasma membrane, the cytoskeleton uses various protein components different from those of the bulk cytoskeleton to interact with the plasma membrane molecules, and as a result, its structure at the interface differs from the bulk structure of the cytoskeleton. Furthermore, the part of the cytoskeleton that associates with the plasma membrane is, both structurally and functionally, an integrated part of the plasma mem-

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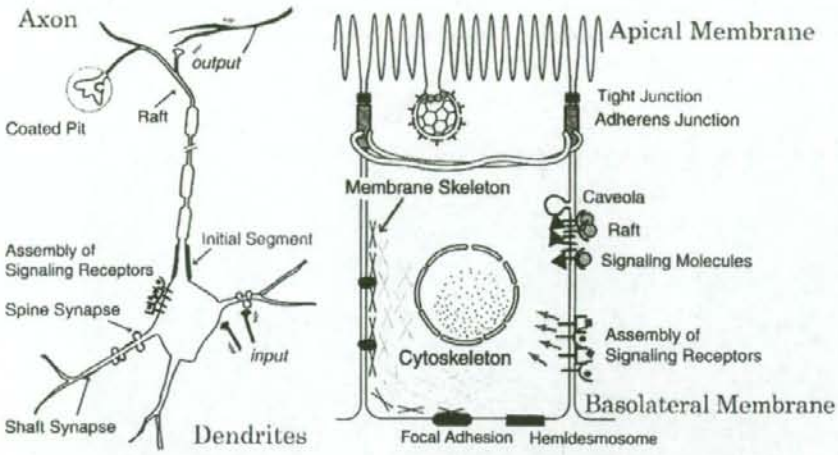

Figure 19.1 The plasma membranes are full of transient molecular complexes, domains, and compartments. Shown here are a plethora of specialized domains, key to a cell's function, which must be continually formed and dissolved during the life of a cell. Their lifetimes range between microseconds and hours. Understanding the basic mechanisms and principles for organizing these molecules is a key objective of biophysical studies of biological membranes. Schematic figures of the plasma membranes of a neuron (left) and an epithelial cell (right) are shown.

brane. Therefore, this part of the cytoskeleton is often called the membrane skeleton (Figure 19.2). Recent research has revealed that this close association of the membrane skeleton with the plasma membrane profoundly affects the dynamics and functions of membrane molecules and their interactions [22,24].

Since the plasma membrane is likely to have complex dynamic domain structures, many of which are smaller than the optical spatial resolution of 300 nm or so, unlike those in artificial membranes, the use of normal optical microscopy and electron microscopy for the studies of these complex dynamic domain structures has turned out to be difficult. The experimental results could be explained by various completely different models, and allow different interpretations, i.e., the experimental results are often unable to reveal which models better represent the dynamic membrane structure and molecular dynamics in the membrane. To clarify these complex spatiotemporal organizations and molecular dynamics, one of the best approaches appears to be high-definition single-molecule tracking, with enhanced time and spatial resolutions.

The recent advent of single-molecule techniques now allows researchers to track single molecules or small groups of molecules in the plasma membrane, even in living cells. These methods include single-fluorescent molecule tracking (SFMT) with the use of fluorescent probes, and single-particle tracking (SPT) using colloidal gold probes with a diameter of 20 or 40 nm

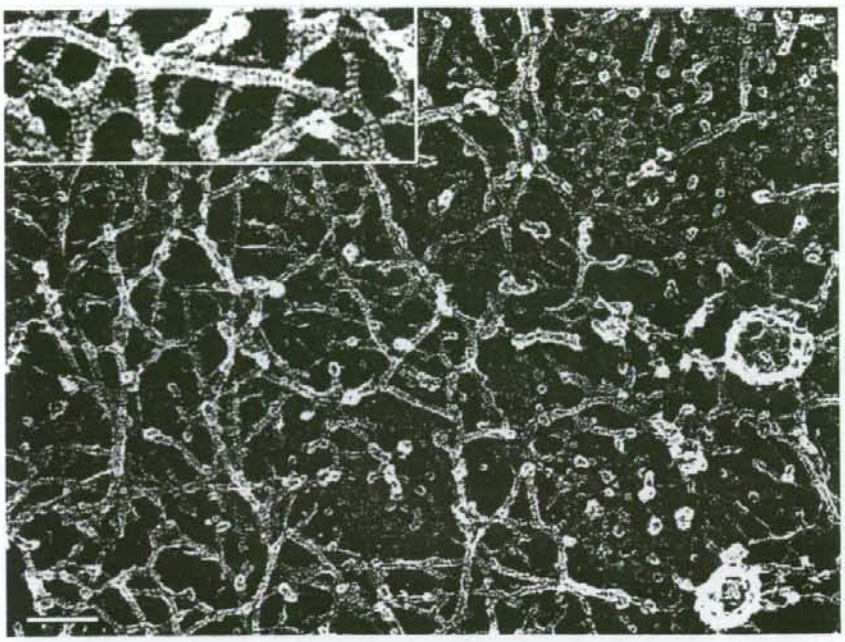

Figure 19.2 The cytoplasmic surface of the upper membrane, observed by electron microscopy. The plasma membrane specimen was rapidly frozen on a copper block precooled in liquid helium, deeply etched to remove excess ice, and platinum-carbon replicated. The presence of clathrin-coated structures (structures that look like a honeycomb or a soccer ball) shows that this image represents that of the cytoplasmic surface of the plasma membrane. The striped banding patterns with the 5.5-nm periodicity on individual filaments (see the inset) are characteristic of actin filaments. This banding pattern can be found in every filament, showing that the membrane skeleton is made of actin filaments and their associated proteins. This image also reveals the close links of the actin filaments of the membrane skeleton with the clathrin-coated structures. Bar = 100 nm (50 nm for the inset).

[1,6–8,14,16,18,25,29,34,36,50,59,61,65,67–69]. SFMT is advantageous in that the researcher can be sure that s/he is following a single molecule, whereas SPT gives better image contrast, allowing her/him to observe at faster frame rates or higher spatial precisions. By using both of these techniques for the same target molecule, the problems of each method can be compensated for each other. Therefore, they together have given researchers the unprecedented ability to directly observe the movement, assembly, and localization of individual, single molecules in the plasma membrane of living cells in culture [24, 57, 61, 68, 69]. Furthermore, not only the movement of single molecules, but also the *activation* of cellular signaling molecules, including the small GTP binding proteins (G proteins) H- and K-Ras, has been tracked at the level of single molecules in the living cell membrane [36].

The next important step in the development of single-molecule techniques has been the great improvement of the frame rate in SFMT and SPT. In particular, with SPT, a frame rate of 40 000 frames per second (fps), or a time resolution of 25 μs, has been achieved in the tracking of single phospholipid molecules in the plasma membrane of living cells, with a relatively small loss of the spatial precision in the determination of the positions of gold particles in the two-dimensional plane (24 nm in two-dimensional space [12]).

The ability to track single molecules at high frame rates with sufficient spatial accuracies has fostered a new fundamental understanding of molecular diffusion in the cell membrane. High-speed SPT and SFMT are revealing that the plasma membranes of virtually all mammalian cells in culture are parceled up into apposed domains, with regard to the translational diffusion of practically all of the membrane molecules. In addition, virtually all of these molecules undergo non-Brownian diffusion in the plasma membrane, i.e., short-term confined diffusion in a compartment and long-term hop diffusion between the compartments in the cell membrane (Figure 19.3; details will be given later; Fujiwara et al. [12], Kusumi and Sako [24], Murase et al. [37]).

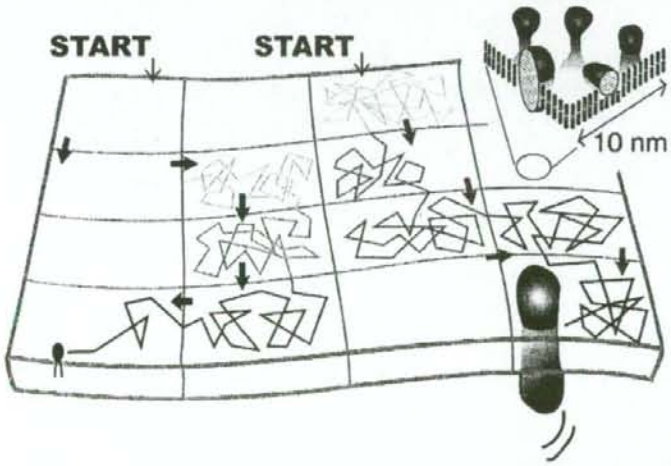

Figure 19.3 A paradigm shift for the plasma membrane concept is required from the (two-dimensional) continuum model to the compartmentalized fluid model, in which the membrane constituent molecules undergo short-term confined diffusion within a compartment and long-term hop diffusion between the compartments. The plasma membrane is partitioned into many 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 suitable if the scale is limited within 10 nm (inset), as shown in the cartoon of their original article, but it cannot be over-extended to a cell membrane structure over several 10s of nanometers.

This entails a paradigm shift of the structure of the plasma membrane from the fluid-mosaic model, proposed by Singer and Nicolson [66], and widely accepted for over 30 years: the plasma membrane in space scales greater than several 10s of nanometers should not be considered as a two-dimensional continuum fluid, but rather that partitioned into closely apposed compartments (*compartmentalized fluid model*, Figure 19.3).

In this chapter, we will first summarize the various observations that led to the concept of the partitioned plasma membrane, which induces hop diffusion of practically all of the molecules in the cell membrane. The membrane-skeleton fence model and the anchored-protein picket model will be described. We will critically compare these models with other observations and models, and evaluate them. Finally, we will discuss the important consequences of plasma membrane compartmentalization in signal transduction in the plasma membrane.

19.2 Thirty-Year Old Enigma about the Diffusion Rate of Membrane Molecules in the Plasma Membrane

For many years, physicists interested in biological membranes have struggled to understand two enigmas. First, for over 30 years, they have wondered why the diffusion coefficients for both proteins and lipids in the plasma membrane are smaller than those found in artificially reconstituted membranes and liposomes, by factors of 5 to 50, with a factor of 20 being a good round number to keep in mind (see Table 1 in Murase et al. [37] and Table 1 in Kusumi et al. [22]). Since a factor of 20 is large, this could not be explained by the crowding of membrane proteins [43] or by the presence of cholesterol [9, 31]. Note that here the diffusion coefficients are those measured at length scales greater than 300 nm, using methods like fluorescence recovery after photobleaching (FRAP) or fluorescence correlation spectroscopy (FCS), or those observed by SFMT or SPT at the normal video rate (time window of about 100 ms). In this chapter, we call these *macroscopic* diffusion coefficients. In a classical view of diffusion, one might think it strange to talk about microscopic diffusion and macroscopic diffusion, because, whatever the time scale is, the diffusion should be self-similar. However, in the case of a nonideal fluid where various membrane domains with different solubilities with the probe molecule exist, and which interacts with the associated membrane-skeleton meshwork, the diffusion depends on the time scales and the length scales over which the measurements are taken.

The second enigma for membrane biophysicists is the following. When membrane molecules, including receptor molecules and other signaling molecules in the membrane, form oligomers or molecular complexes, either

their macroscopic diffusion rates drop dramatically or they may be temporarily immobilized [17,18,40]. This is completely opposite from the general view of membrane biophysicists. Based on the fluid-mosaic model of Singer and Nicolson [66], Saffman and Delbrück (1975) [47] derived an equation that relates the diffusant size to the translational diffusion coefficient in a two-dimensional continuum fluid. For a cylinder (a transmembrane protein) of radius a and height h, floating in a two-dimensional fluid of viscosity μ with a matched thickness (h) immersed in an aqueous medium of viscosity μ' (Figure 19.4), the translational and rotational diffusion coefficients, D_T and D_R, respectively, for the cylinder can be expressed as,

$$D_T = \frac{k_B T}{4\pi\mu h}\left(\log\frac{\mu h}{\mu' a} - \gamma\right) \tag{19.1}$$

$$D_R = \frac{k_B T}{4\pi\mu a^2 h}, \tag{19.2}$$

where γ is the Euler constant (≈ 0.5772). This equation predicts that translational diffusion is very *insensitive* to the diffusant size: tetramer formation from monomers (an increase in radius by a factor of 2) will decrease the diffusion rate by only a factor of 1.1, and even 100mers (an increase in radius by a factor of 10) will have their diffusion rate reduced by only a factor of 1.4 from monomers, assuming a 0.5 nm monomer radius of the membrane-spanning domain. (One should be aware that, at variance with the translational diffusion coefficient (Eq. (19.1)), the rotational diffusion rate is quite sensitive to changes in the oligomer size (Eq. (19.2)). It is inversely proportional to a^2, i.e., to the number of proteins in a complex in larger oligomers.) Furthermore, Peters and Cherry [43] found that the Saffman-Delbrück theory worked well in the reconstituted membranes of bacteriorhodopsin, which was further supported later [75]. Therefore, the considerable decreases in the diffusion coefficients of membrane receptors upon ligand binding could not be explained by ligand-induced receptor oligomerization. Alternatively, the formation of very large aggregates of thousands of receptor molecules has to be assumed to explain the reduction of the diffusion coefficient upon receptor engagement.

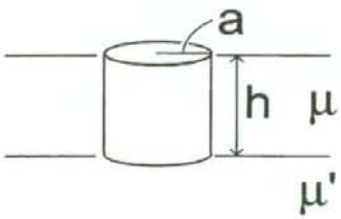

Figure 19.4 A cylinder floating in the two-dimensional fluid continuum in water.

However, the induction of such large aggregates of receptors upon liganding has never been found.

Recently, the Saffman–Delbrück theory was challenged by Gambin et al. [13]. These authors asserted that the dependence of the diffusion coefficient on the diffusant size is much stronger, based on the data by Lee and Petersen (2003) [28]. However, in their presentation, they omitted the diffusion data for the largest diffusant in the Lee and Petersen paper, which are rather consistent with the Saffman–Delbrück theory, and inconsistent with their theory. Steeper changes with an increase in the diffusant radius than those expected from the Saffman–Delbrück theory did take place around the radius of the phospholipid (the basic membrane constituent molecular species, which form the fluid membrane matrix; ~ 0.45 nm in radius) and that of an α-helix ($\sim$0.55 nm in radius), with the dependence on radius being 1/[radius] (based on the free area theory). If we adopt this model, then when a transmembrane α-helix forms a dimer, the diffusion coefficient will decrease by a factor of 1.4. The breakdown of the Saffman–Delbrück theory in this scale regime, where the diffusant size becomes comparable to the size of the phospholipid, which forms the membrane matrix, has been expected because the Saffman–Delbrück theory assumes the diffusion in a two-dimensional continuum fluid [76]. This was also implied in 1982, when the collision rates of molecular oxygen with lipid membrane probes were measured [26].

Therefore, a convenient rule of thumb with regard to the relationship between the size of the transmembrane α-helix of an integral membrane protein and its diffusion coefficient may be that the diffusion coefficient would decrease by a factor of about 1.4, following the 1/[radius] law, when the protein forms a dimer, and then any oligomers greater than dimers may follow the Saffman–Delbrück relationship, i.e., very slight dependence of the diffusion coefficient on protein oligomerization.

19.3
Macroscopic Diffusion Coefficients for Transmembrane Proteins are Suppressed by the Presence of the Membrane Skeleton

We will deal with the first enigma, i.e., why the macroscopic diffusion coefficients for both proteins and lipids in the plasma membrane are smaller than those found in artificially reconstituted membranes and liposomes, by a factor of ≈ 20. To clarify the idea, we will initially limit our argument to the diffusional motion of transmembrane proteins.

Even before the single-molecule era, there were quite a few FRAP reports indicating that the reduction of the macroscopic diffusion coefficient in the plasma membrane from that found in artificial membranes may be caused by

the actin-based membrane skeleton [42, 52, 63, 64, 71, 73, 74, 81]. Sheetz and colleagues found that the transmembrane protein band 3 (the majority of the ConA receptor observed in this study is known to be band 3) diffuses about 10 times faster [64] in spectrin-deficient mutant mouse erythrocytes than in normal cells. In mammalian red blood cells, the spectrin meshwork, instead of the f-actin network, forms the membrane skeleton. Furthermore, a number of reports have shown that the lateral diffusion coefficients of transmembrane proteins were increased in blebbed membranes or after partial depolymerization of actin filaments (for example, see Paller [42], Tank et al. [71], Wu et al. [81]).

Tsuji and Ohnishi [74] and Tsuji et al. [73] carried out *both translational and rotational diffusion measurements* for the transmembrane protein band 3 in human red blood cell ghost membranes, and showed that the translational diffusion coefficient of band 3 was increased (decreased) when the spectrin network was stabilized (destabilized, i.e., the tetramer–dimer equilibrium of spectrin was shifted toward the tetramer [dimer]), whereas the rotational diffusion coefficient of band 3 was unaffected. These results clearly indicate that (1) the spectrin meshwork partitions the membrane into small compartments, (2) the nonspecific collision of band 3 with the spectrin tetramer, which forms the compartment boundary, is responsible for the reduction of the translational diffusion coefficient in the erythrocyte membrane, and (3) the spectrin tetramer is the effective barrier, and when it temporarily dissociates into dimers, band 3 molecules can cross the compartment boundary. Based on these observations, Tsuji et al. [73] proposed a "spectrin dimer–tetramer equilibrium" gate model (SPEQ gate model).

As such, data showing the involvement of the membrane skeleton in the reduction of the translational diffusion rate were abundant in the era before single molecule observations, particularly in human red blood cells, but direct observations of molecules undergoing short-term confined diffusion within a compartment (made of the membrane skeleton) and long-term hop diffusion between the compartments had to wait until the single-molecule technologies became available. The percolation threshold idea advanced by Saxton played an important role in these studies [53].

19.4
Single-Molecule Tracking Revealed That Transmembrane Proteins Undergo Hop Diffusion

SPT was developed in the late 1980s [6–8, 14, 20, 25, 60, 65]. Using SPT, Sako and Kusumi [48] were the first to directly observe the "hop diffusion" of membrane molecules: transferrin receptor, a transmembrane protein, is temporarily confined in a compartment of about 700 nm in diameter in the membrane,

and then it hops to an adjacent apposed compartment, where it again becomes trapped temporarily (we later found that NRK cells have nested compartments of 230 nm within 700 nm compartments. However, in earlier techniques, only the larger compartments of 700 nm could be detected). By repeating such confinement and hop movements between the compartments, a phenomenon termed hop diffusion, the receptor covers macroscopic areas (Figure 19.5). Since virtually all of the examined transferrin receptor molecules and α2-macroglobulin receptor molecules were found to undergo hop diffusion, it was proposed that the entire plasma membrane is parceled up into small, apposed domains (except for specialized membrane domains, such as clathrin-coated pits, cell–cell and cell–substrate junctions, and microvilli).

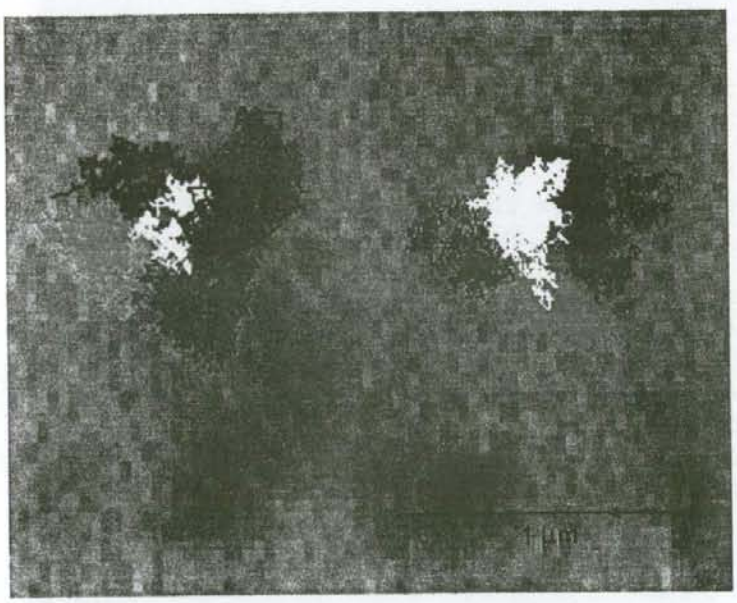

Figure 19.5 Typical trajectories of 40-nm gold probes bound to transferrin receptor, recorded at a time resolution of 25 μs for a duration of 250 ms (10 000 frames). The background is the actual image of a single frame. Different colors represent various plausible compartments, detected by computer software 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. [12].

The detection of such hop diffusion or temporary confinement within a compartment requires statistical analysis, because such trajectories might occur as a consequence of thermal diffusion [12, 25]. Here, we briefly explain how such an analysis is carried out.

For each molecule's trajectory, the mean-square displacement (*MSD*), $\langle \Delta r(\Delta t))^2 \rangle$, for every time interval is calculated according to the formula [25, 45, 65]:

$$\begin{aligned} MSD\,(\Delta t) &= MSD\,(n\delta t) \\ &= \frac{1}{N-1-n} \sum_{j=1}^{N-1-n} \left\{ \left[x\,(j\delta t + n\delta t) - x\,(j\delta t) \right]^2 \right. \\ &\qquad \left. + \left[y\,(j\delta t + n\delta t) - y\,(j\delta t) \right]^2 \right\}, \end{aligned} \tag{19.3}$$

where δt is the frame time and $(x(j\delta t + n\delta t), y(j\delta t + n\delta t))$ describes the molecule's position following a time interval $\Delta t = n\delta t$ after starting at position $(x(j\delta t), y(j\delta t))$, N is the total number of frames in a recording sequence, n and j are positive integers, and n determines the time increment.

We have developed a statistical method to classify each trajectory into the following three modes of motion (Figure 19.6): (1) simple Brownian diffusion mode, in which $MSD\,(\Delta t) = 4D\Delta t$, (2) directed diffusion mode, in which a molecule moves in a direction at a constant drift velocity (v_x, v_y), with superimposed random diffusion, $MSD\,(\Delta t) = 4D\Delta t + v^2(\Delta t)^2$, where $v^2 = v_x^2 + v_y^2$, and (3) confined diffusion mode, in which a molecule undergoes Brownian diffusion while totally confined within a limited area (compartment; $0 \le x \le L_x$, $0 \le y \le L_y$) during the observation period. The $MSD(\Delta t)$ plot levels off and asymptotically approaches a constant value, as expressed by

$$\begin{aligned} MSD_x\,(\Delta t) &= \frac{L_x^2}{6} - \frac{16L_x^2}{\pi^4} \sum_{n=1(odd)}^{\infty} \frac{1}{n^4} \exp\left\{ -\frac{1}{2} \left(\frac{n\pi\sigma_x}{L_x} \right)^2 \Delta t \right\} \\ MSD_y\,(\Delta t) &= \frac{L_y^2}{6} - \frac{16L_y^2}{\pi^4} \sum_{n=1(odd)}^{\infty} \frac{1}{n^4} \exp\left\{ -\frac{1}{2} \left(\frac{n\pi\sigma_y}{L_y} \right)^2 \Delta t \right\} \\ \sigma_x^2 &= 2D_x, \quad \sigma_y^2 = 2D_y, \quad 4D = 2D_x + 2D_y \\ L_r^2 &= L_x^2 + L_y^2. \end{aligned} \tag{19.4}$$

To describe a molecule undergoing intercompartmental jumps during the observation period, an equation for the $MSD(\Delta t) - \Delta t$ plot has been derived by Powles et al. [44], based on the model in which a Brownian particle (diffusion coefficient in the absence of barriers, D_{micro}) is placed in an infinite array of evenly spaced (L), semipermeable (with a permeability, P) barriers. At long times, relative to the average residency time in a compartment, the transitions over many compartments will look like simple Brownian diffusion with a constant diffusion coefficient, defined as D_{MACRO}, which has the relationship,

$D_{\mathrm{MACRO}}/D_{\mathrm{micro}} = [1 + (PL)^{-1}]^{-1}$. The average residency time within each compartment, τ, is often a useful parameter, and can be determined through the average compartment size and the average long-term diffusion coefficient as $\tau = L^2/4D_{\mathrm{MACRO}}$ (see Kusumi et al. [25], Sako and Kusumi [48], Fujiwara et al. [12] and Suzuki et al. [67] for the details).

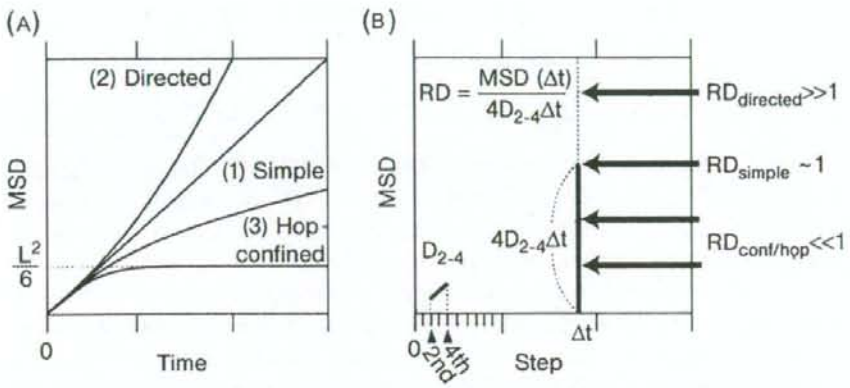

Figure 19.6 Analysis of single-molecule trajectories based on the $MSD(\Delta t) - \Delta t$ plot, and their statistical classification into the three modes of motion. (A) Theoretical $MSD(\Delta t) - \Delta t$ curves representing (1) simple Brownian diffusion, (2) directed diffusion, and (3) confined/hop diffusion. See the text for their equations. The graphs are drawn assuming that the short-term diffusion coefficients that are proportional to the slope near time 0 are identical for all of the cases. (B) The definition of the relative deviation (RD). RD is defined as the ratio of an experimental $MSD(\Delta t)$ to the fictitious MSD at time $\Delta t(4D_{2-4}\Delta t)$, assuming that the molecule undergoes simple Brownian diffusion without confinement or directed diffusion with a diffusion coefficient determined from the initial slope ($4D_{2-4}$, determined from a linear fit to the MSD values at the second, third, and fourth steps of elapsed time). The more or less that the RD deviates from 1 reflects the chances that the molecule undergoes directed or confined/hop diffusion, respectively.

In essence, the shape of the $MSD(\Delta t)$ curve is characterized based on the relative deviation (RD) from ideal Brownian diffusion (Figure 19.6). RD is defined as $MSD(\Delta t)/4D_{\mathrm{micro}}\Delta t$, where D_{micro} is the two-dimensional short-term diffusion coefficient that is proportional to the slope of the $MSD(\Delta t) - \Delta t$ plot near time 0, which is practically determined from a linear fit to the $MSD(\Delta t) - \Delta t$ plot at the second, third, and fourth frames of elapsed time (D_{2-4} as described in Kusumi et al. [25]; one has to be careful about the accuracy of these values. See Saxton [56] and Martin et al. [33]). This diffusion coefficient only reflects the viscosity properties in the space scale of tens of nanometers, and therefore can be determined independently of the motional modes (one must consider classifying molecules with very small D_{2-4} into the immobile mode, but its value has to be decided for each set of experiments, depending on the noise level and the overall distribution of the diffusion coefficient). The theoretical distribution of RD for free diffusion (which should

have an average value of 1) is obtained by a computer simulation of Brownian molecules, and RD values that give 2.5 (or 5; these are typical values and could be adjusted for the individual studies) percentile of the molecules from both ends of the distribution, referred to as RD_{min} and RD_{max}, are determined (Figure 19.7). When the trajectory of an experimental molecule shows an RD value between RD_{min} and RD_{max}, it is classified into the simple Brownian diffusion mode, and when $RD > RD_{max}$ or $RD < RD_{min}$, it is classified into the directed or confined/hop diffusion mode, respectively.

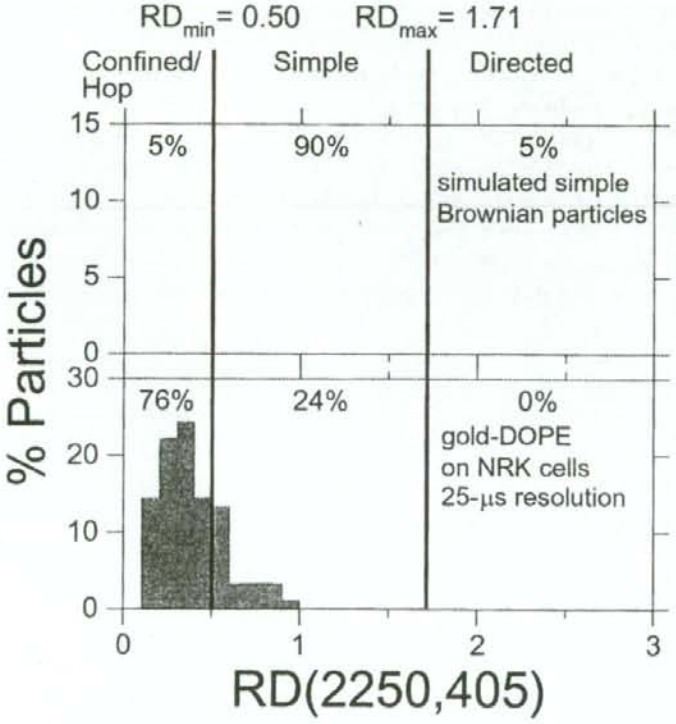

Figure 19.7 The distribution of RD for gold-tagged DOPE molecules observed in the plasma membrane of NRK cells at a 25-μs resolution (*bottom*) is quite different from that expected from simulated simple Brownian particles (*top*). In this example, the total number of frames in each trajectory is 2250 steps (56 ms) and the analysis window (Δt) is 405 steps (10 ms). For the classification of the trajectories into the three modes of motion, 1 000 simple Brownian trajectories were generated by Monte Carlo simulations, and the RD values that give 5% of the particles from both ends of the distribution were determined (RD_{min} of 0.50 and RD_{max} of 1.71, shown by bold vertical lines). When an experimental trajectory exhibited an RD value smaller than RD_{min} or greater than RD_{max}, it was classified into the confined/hop or directed diffusion mode, respectively. The majority (76%) of the DOPE trajectories ($N = 90$) were classified into the confined/hop mode at a time resolution of 25 μs.

This is a simplified, but practical approach to anomalous diffusion discussed throughout this book, although there may be finer or more elegant ways of describing anomalous diffusion (see the Chapters by Kimmich et al., Shlesinger, and Chechkin et al. in this book). Furthermore, the diffusion data have to be analyzed and interpreted, based on or in the way consistent with the data on the membrane-associated part of the actin cytoskeleton meshwork observed by electron tomography with subnanometer precisions [35] as well as single-molecule force measurements on membrane molecules [49, 51, 72]. Interpretations simply based on the analysis of the diffusion measurements, particularly done at low-time resolutions or for a collection of molecules, have to be re-examined based on these other important observations.

Therefore, the goal of analyzing results obtained by single-molecule tracking in the plasma membrane is to classify each experimental trajectory into one of these motional modes and to obtain the distribution or the histogram of the parameters characterizing each motional mode. Fitting the above theoretical $MSD(\Delta t) - \Delta t$ equation to the experimental $MSD(\Delta t) - \Delta t$ plot, independently in two orthogonal directions, quantitatively provides estimates for various diffusion parameters. Furthermore, for hop diffusion, individual compartments in each trajectory can be detected automatically by a computer program (see the methods section of Suzuki et al. [67] for details). Briefly, a variable size window in time is moved through the trajectory, and the local diffusivity is recorded. Intercompartmental jumps are seen as sharp increases in the diffusivity of the molecule as it extends its motion into a neighboring compartment.

The hop rates of transferrin receptor for the smaller and greater compartments in NRK cells (NRK cells have a plasma membrane with nested double compartments with sizes of 230 and 710 nm, Fujiwara et al. [12]) were recently found to be an average of every 55 and 570 ms (direct SPT measurements gave 55 and 1800 ms, respectively, but the latter value needed to be corrected for the crosslinking effect of gold probes, using a macroscopic diffusion coefficient determined by SFMT with fluorescently labeled transferrin (a time window of 3 s, giving 0.22 $\mu m^2/s$) and the compartment size determined by SPT (710 nm)) [12]. Furthermore, all of the transmembrane proteins examined thus far, including E-cadherin [51], transferrin receptor [48], α2-macroglobulin receptor [48], CD44 (Ritchie and Kusumi, unpublished observations), band 3 [72], stem cell factor receptor (Kobayashi, Murakami, and Kusumi, unpublished observations), and various GPCRs (Suzuki et al. [67], Kasai, Prossnitz, and Kusumi, unpublished observations), undergo hop diffusion. The macroscopic diffusion coefficients for these molecules, determined by SPT (reflecting hop diffusion rate over many compartments), are basically consistent with the SFMT and FRAP data, although due to the crosslinking effects of gold probes, the macroscopic diffusion coefficients may be smaller by a factor of 1–5. Since,

even in the presence of crosslinking effects, the compartment sizes determined by SPT are likely to be correct, a good strategy to evaluate the correct average hop frequency across the compartment boundaries (inverse the average residency time within a compartment) is to use the macroscopic diffusion coefficient determined by SFMT with fluorescent probes and the compartment size obtained by SPT, using the equation,

$$\text{Residency time} = \frac{(\text{Average compartment size from SPT})^2}{(4 \times D_{\text{MACRO}} \text{ from SFMT})}. \tag{19.5}$$

With this equation, one assumes a stylized model of hop diffusion (for the purpose of simple calculation), in which the molecule undergoes diffusion in the presence of an equally spaced, infinite array of barriers of the same height, and the hops occur between the central points in the compartments. Murase et al. [37] successfully employed this strategy to obtain the residency time as well as the permeability of the barriers in the plasma membranes for a phospholipid molecule, in a variety of cultured mammalian cells.

19.5
Corralling Effects of the Membrane Skeleton for Transmembrane Proteins (the Membrane-Skeleton Fence Model)

What makes the boundaries between these compartments for transmembrane proteins? We proposed the "membrane-skeleton fence" or "membrane-skeleton corralling" model (Figure 19.8, left). Transmembrane proteins protrude into the cytoplasm, and, in this model, their cytoplasmic domains collide with the membrane skeleton, which induces temporary confinement

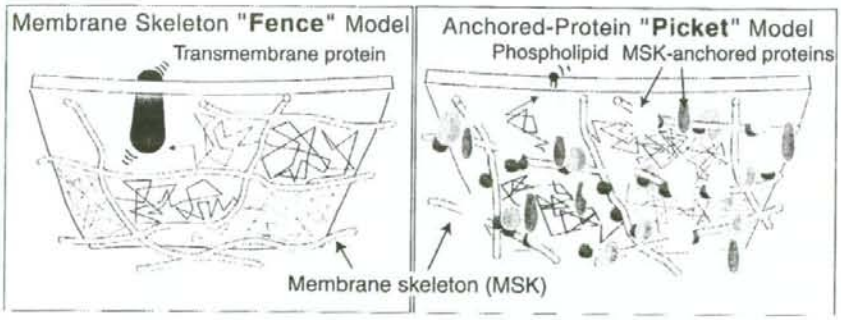

Figure 19.8 The effects of the membrane-skeleton "fence" (left) and the anchored-protein "pickets" (right) that together partition the entire plasma membrane into small compartments. See the text for further details. The hydrodynamic-friction-like effect was first described by Bussell et al. [2,3].

or corralling of the transmembrane proteins in the membrane skeleton mesh. 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 may be created as a consequence of the thermal fluctuation of these structures, when the actin filament that forms the compartment boundary temporarily dissociates, and/or 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.

Here is a summary for the evidence supporting the membrane skeleton fence model for the partitioning of the cell membrane with respect to transmembrane proteins. The problems with other models, including raft-induced compartmentalization of the plasma membrane, crowding of the extracellular surface by the extracellular domains of membrane molecules, protrusions and dips throughout the membrane, and/or a two-dimensional continuum fluid, are that they can explain one or several of these observations, but not *all* of these observations.

(1) The hop rate of band 3 in the red-cell ghost membrane and that for E-cadherin in cultured L cells (both are transmembrane proteins) were increased with a decrease in the cytoplasmic domain size [51, 72]. This clearly indicates that the cytoplasmic domain of the transmembrane protein is involved in temporarily trapping the transmembrane protein.

(2) When each individual transmembrane protein molecule was dragged by an optical trap (transferrin receptor and E-cadherin), the compartment size estimated by the free dragging length in optical trap experiments employing very weak trapping forces agrees with that detected by single-molecule diffusion measurements [49, 51], indicating the presence of actual force barriers between the compartments.

(3) The experiments in which each individual molecule of transferrin receptor, E-cadherin, and band 3 was dragged using an optical trap revealed that the compartment boundaries are elastic, consistent with the model in which the basis for the compartment barrier is the membrane skeleton meshwork [49,51,72].

(4) When the membrane skeleton is dragged, by moving the optical trap that grabbed a polystyrene bead bound to the membrane skeleton, transmembrane proteins (band 3) that are not bound to the membrane skeleton and undergo diffusion were also moved, along with the movement of the membrane skeleton [72].

(5) Hop diffusion of transmembrane proteins depends on the integrity of the membrane skeleton [48, 67, 72]. Very mild latrunculin or cytochalasin D treatments increased the average compartment size. Note that since harsher treatments tend to induce membrane protein aggregation and overall changes in the cell shape, making the interpretation of diffusion data virtually impossible, only very mild treatments are useful. Under these conditions, one should note that the effects of these drugs are complex, depending on the treatment duration (because cells start compensating for the initial changes in the actin filaments), the cell type (the overall amounts of actin and the ratio of the amounts of actin molecules in stress fibers vs. single filaments), the action mechanisms of the drugs, and the drug concentrations. Furthermore, the effects of latrunculin or cytochalasin D are difficult to find if one measures the macroscopic diffusion coefficients by FRAP or slow-rate (like video-rate) single-molecule tracking, because, after cells are treated with these drugs, the compartment size slightly increases, whereas the hop rate tends to decrease slightly, resulting in only minor increases in the macroscopic diffusion coefficient (between a factor of 1 and 2). In fact, several reports have noted the absence of the effects of latrunculin, cytochalasin, gelsolin, and siRNA of spectrin on the movement of various membrane molecules [11, 32, 41, 58, 77], whereas Lenne et al. [30], using fluorescence correlation spectroscopy, found that the diffusion of transferrin receptor is affected by drugs that target the actin-based membrane skeleton.

Furthermore, hop diffusion could not be found in liposomes and in membrane blebs, where the membrane skeleton is essentially absent (Suzuki et al. [67]; in some cell types, considerable fractions of the actin skeleton remain in the blebbed membrane, and if this happens, the membrane has to be further treated with latrunculin or cytochalasin D to remove the remaining actin skeleton, for the elimination of hop diffusion). In these membranes, the membrane molecules undergo rapid, simple Brownian diffusion that can be characterized by a single diffusion coefficient in the range of 5–10 $\mu m^2/s$ for DOPE or 3 $\mu m^2/s$ for transmembrane proteins in all of the observation time scales (0.025 ms–1 s, i.e., by a factor of 40 000).

(6) Electron microscopy results, in particular those with rapidly frozen, deeply etched specimens of the plasma membranes [35] or those obtained with a scanning electron microscope (Morone and Kusumi, unpublished observations), showed that, except for the specific locations where internalization apparati, cell adhesion structures, microvilli, or filopodia are located, the plasma membrane exhibits a gently undulating surface, which generally lacks sharp protrusions or dips. In fact, in our previous observations of high-speed single-particle tracking, the candidate

cell lines were first examined by scanning electron microscopy, and only those without too many microvilli were selected for our observations. The presence of membrane protrusions and dips has been thought to cause apparent confinement. However, this cannot be true. First, to reduce the macroscopic diffusion coefficient by a factor of 10, the protrusions or dips must be as large as 100–300 nm for a compartment size of 200 nm, and they must be present throughout the cell membrane. This is clearly inconsistent with the electron microscope observations (possibly except for the brush-border membranes of epithelial cells) [35,46]. Furthermore, the idea of membrane protrusions and/or dips for the apparent confinement is inconsistent with the observations (1), (2), and (3) described above.

(7) The instances of hops are clearly visible and also are detectable with a computer program in the analysis of single-molecule trajectories with sufficient time resolutions [67].

(8) Oligomerization of transmembrane proteins reduces the macroscopic diffusion coefficient by decreasing the intercompartmental hop rate (without affecting the compartment size), a phenomenon termed oligomerization-induced trapping by [18]. This can be easily explained by the membrane skeleton fence model, but cannot be naturally explained by the two-dimensional continuum fluid model, the viscoelastic model, the general anomalous diffusion model, or the model of long membrane protrusions and deep dips throughout the membrane.

The other aspect of this result is that it indicates a need for control experiments for the crosslinking effect of the gold probes using SFMT or FRAP, when SPT with gold particles is employed. To circumvent such a nuisance, in our lab, we always begin our studies using SFMT with a fluorescent tag, and only when we need high-speed single molecule tracking or when we wish to carry out optical trapping experiments, we develop colloidal gold probes. The crosslinking effect could become very extensive, so that it could cause the long-term trapping of the target protein within a compartment, when the probes were attached to cells at lower temperatures for over several 10s of minutes [4,5,67].

19.6 Phospholipids Also Undergo Hop Diffusion in the Plasma Membrane

The next natural question is "What about lipids?" Fujiwara et al. [12] and Murase et al. [37] indeed addressed this question, by observing an unsaturated phospholipid, L-α-dioleoylphosphatidylethanolamine (DOPE), which is considered to be one of the most difficult molecules to immobilize in the

plasma membrane, due to its unsaturation and low-levels of headgroup interactions. To observe the movement of single DOPE molecules, the DOPE was tagged with the fluorescent dye Cy3 or with a 40-nm diameter colloidal gold particle, and was observed by SFMT and SPT, respectively. The colloidal gold probes were necessary to observe DOPE diffusion at frame rates much higher than video rate (30 Hz). Due to low sensitivity, SFMT would not accommodate observations at frame rates much faster than 200 Hz. In video-rate observations (30 Hz) and in NRK cells, both methods yielded about the same diffusion coefficients, as long as they remained in time windows shorter than 100 ms. This result justifies the use of such a large colloidal gold particle as a probe for a small molecule, like DOPE. In longer time scales, the diffusion coefficient of gold-tagged DOPE was smaller than that of Cy3-tagged DOPE by a factor of 3, due to the crosslinking effect of gold probes (Fujiwara et al. [12], which, by the way, in itself shows that the plasma membrane cannot be considered as a two-dimensional continuum fluid).

Using high-speed SPT with a time resolution of 25 μs (a frame rate of 40 kHz, about 1300-fold faster than normal video rate), Fujiwara et al. [12] and Murase et al. [37] found that an unsaturated phospholipid, DOPE, undergoes hop diffusion. Representative trajectories of DOPE in the plasma membrane of NRK cells, recorded at time resolutions of 33 ms and 25 μs, are shown in Figure 19.9. At a 33-ms resolution (normal video rate), practically all of the DOPE trajectories were classified into the simple Brownian diffusion mode. However, when the time resolution was enhanced to 25 μs, it became clear that the simple Brownian nature found at the 33-ms resolution is only an apparent one. The hop diffusion is clearly visible (individual compartments were detected by the computer program we developed), and the statistical analysis method described above indeed showed that over 85% of the DOPE trajectories were classified into the hop-confined diffusion mode, rather than the simple Brownian diffusion mode.

Quantitative analysis of the trajectories, such as those displayed in Figure 19.6, revealed that the average compartment size was 230 nm in the case of NRK cells. The average residency time within each 230-nm compartment was 11 ms. No wonder we did not see hop movement at video rate, with a time resolution of only 33 ms. In fact, all of these trajectories shown in Figure 19.9 (bottom) are 62-milliseconds long, and if we had used video rate observations, there would have been only 2 or 3 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 $\mu m^2/s$ on average, is interesting. It is almost as large as that of DOPE molecules observed in artificial membranes, such as giant liposomes. Therefore, lipid diffusion in the cell membrane is slow, not because the diffusion per se is slow,

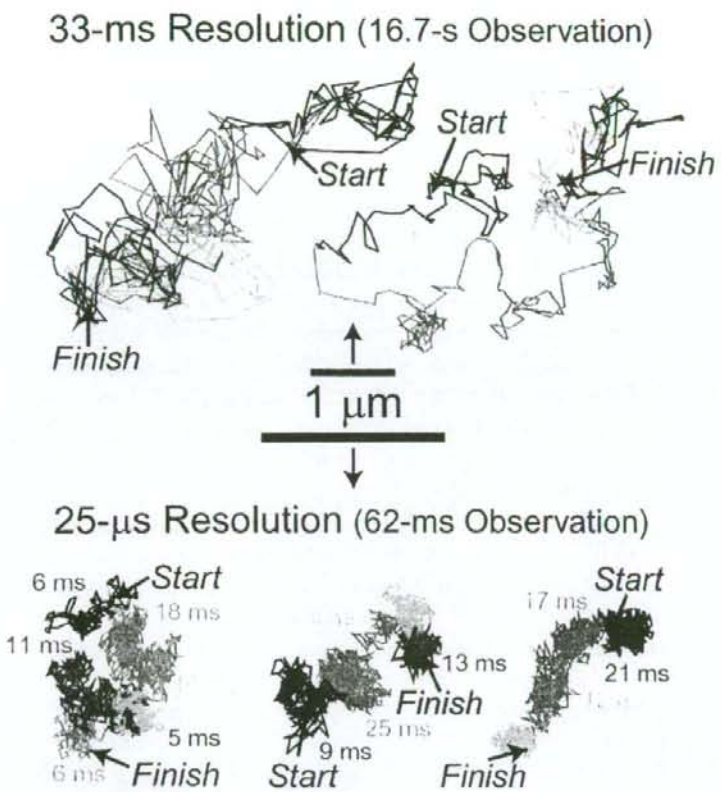

Figure 19.9 Representative trajectories of single or small groups of DOPE molecules recorded at time resolutions of 33 ms and 25 μs (see the text for details). The different colors of the trajectories obtained at a 33-ms resolution simply represent a time sequence of every 3.3 s. The different colors in the bottom trajectories obtained at a 25-μs 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 video rate. To resolve such movement, the time resolution must be considerably shorter than the average residency time within a compartment.

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 (Figure 19.3). These observations solved the 30-year old enigma in the fluid mosaic model: the mechanism underlying the reduction of the diffusion rate in the plasma membrane by a factor of ~ 20 from that found in artificial membranes.

What makes the boundaries between these compartments, which even work for phospholipids located in the outer leaflet of the membrane? Fujiwara et al. [12] observed DOPE diffusion in membrane blebs (balloon-like structures of the plasma membranes, where the membrane skeleton is largely lost, and Fujiwara et al. further reduced the actin-based membrane skeleton by treating the cells with latrunculin) as well as in liposomes, and found that DOPE molecules undergo rapid, simple Brownian diffusion with a diffusion coefficient of $\approx 9\ \mu m^2/s$ in these membranes. Furthermore, Fujiwara et al. [12] and Murase et al. [37] 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 phospholipid hop diffusion. They found that the phospholipid movement was only affected when they modulated the membrane skeleton with actin drugs. This is consistent with the previous FRAP observations, in the sense that the modulation of the membrane skeleton influences the lipid movement (although FRAP did not allow researchers to observe such detailed motion; see Paller [42]). 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 a very strange and surprising result! Since the DOPE molecules they observed were located in the extracellular leaflet of the membrane (unlabeled DOPE may flip, but the large DOPE molecule tagged with a gold particle cannot flip to enter the cytoplasmic leaflet), whereas the membrane skeleton is located on the cytoplasmic surface of the membrane, the DOPE and the membrane skeleton cannot interact directly. To explain this apparent discrepancy, the "anchored transmembrane-protein picket model" was proposed (Figure 19.8, right). In this model, various transmembrane proteins anchored to and lined up along the membrane skeleton (fence) effectively act as rows of pickets (these transmembrane 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 latter effect, first proposed by Hammer's group [2, 3], 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 $\approx$ 100, and is particularly marked when immobile pickets are aligned along the membrane-skeleton fence. When the number density of these transmembrane picket proteins exceeds a certain threshold (20–30% coverage of the intercompartmental boundary to reproduce the experimentally observed residency time of 11 ms in a 230-nm compartment in NRK cells, as determined by a series of Monte Carlo simulations by Fujiwara et al. [12]), 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