Molecular Biology of the Cell
Vol. 10, 2475–2479, August 1999

Video Essay

Compartmentalization of the Erythrocyte Membrane by the Membrane Skeleton: Intercompartmental Hop Diffusion of Band 3[V]

Michio Tomishige* and Akihiro Kusumi*†‡

*Department of Biological Science, Graduate School of Science, Nagoya University, Nagoya 464-8602, Japan; and †Kusumi Membrane Organizer Project, Exploratory Research on Advanced Technology Organization, Japan Science and Technology Corporation, Nagoya 460-0012, Japan

Submitted March 18, 1999; Accepted June 7, 1999
Monitoring Editor: W. James Nelson

INTRODUCTION

Recent developments in microscopic instrumentation and probes have allowed the observation and manipulation of the movement of membrane proteins and lipids in the plasma membrane at the level of single molecules. These experiments are performed by tracking small colloidal gold particles attached to specific membrane proteins and lipids (single particle tracking) (Jacobson *et al.*, 1995; Sheets *et al.*, 1995; Kusumi and Sako, 1996; Saxton and Jacobson, 1997) or by dragging particle–protein complexes along the surface of the plasma membrane using laser tweezers (also termed an "optical trap" or OT) (Edidin *et al.*, 1991; Kusumi and Sako, 1996; Kusumi *et al.*, 1998). In optimal cases, a single protein molecule is attached to a 40-nm-diameter colloidal gold probe (Tomishige *et al.*, 1998). These single molecule experiments have demonstrated that most membrane-spanning proteins do not undergo simple diffusion, but that a significant number are locally slowed or stopped, some are temporarily confined, and others are transported unidirectionally (Kusumi *et al.*, 1993; Sheets *et al.*, 1997; Sako *et al.*, 1998; Simson *et al.*, 1998). Previously, these processes had not been observed, because the methods used, e.g., fluorescence recovery after photobleaching, recorded the ensemble-averaged behavior of a large number of molecules.

The underlying cellular mechanisms that induce nonrandom diffusion have remained largely unknown; however, we and others found that properties of the membrane skeleton network fundamentally affect the movement and distribution of certain membrane proteins, such as transferrin receptor, α_2-macroglobulin receptor, MHC class I molecules, E-cadherin, and band 3, through corralling and binding effects imposed by the membrane skeleton network (Edidin *et al.*, 1991; Luna and Hitt, 1992; Kusumi *et al.*, 1993; Sako and Kusumi, 1994, 1995; Sako *et al.*, 1998; Tomishige *et al.*, 1998). The results are particularly clear with band 3 in human erythrocyte ghost membranes, where the specimen consists of a lipid bilayer and its underlying membrane skeleton, which has been biochemically and biophysically well characterized (Golan, 1989; Bennett, 1990; Bennett and Gilligan, 1993; Mohandas and Evans, 1994).

The results obtained by these studies support a "membrane skeleton fence model" in which the cytoplasmic portion of a membrane protein collides with the membrane skeletal "meshwork" because of steric hindrance, thus resulting in the temporal confinement of the protein within the mesh (compartment, Figure 1A). Membrane proteins escape from these compartments and hop to adjacent ones because of the dynamic properties of the membrane skeleton. We predict that time-dependent fluctuations in the distance between the membrane and the skeleton are large or the membrane–skeleton network connections form and break continuously, as expected in a chemical equilibrium, providing membrane proteins with the opportunity to pass through the mesh barrier. In the case of band 3, macroscopic diffusion within the erythrocyte membrane occurs as the result of a series of such intercompartmental hops.

The scientific content of this work has been published previously (Tomishige *et al.*, 1998), and the purpose of the present essay is to give the reader a glimpse of the raw data from this study, as recorded by both normal and high-speed video microscopy. We hope the video sequences attached to this article will help the viewing audience develop a feel for how band 3 interacts with the membrane skeleton and undergoes intercompartmental hop diffusion within the erythrocyte ghost membrane.

VIDEO SEQUENCES

Video Sequence 1: Intercompartmental Hops of Band 3 Molecules in the Erythrocyte Membrane

The movement of band 3 in human erythrocyte ghosts in a hypotonic medium was observed at the level of a single molecule or a small number of molecules. The erythrocyte ghost membrane was stably attached to a coverslip coated with poly-L-lysine. Band 3 was labeled with 40-nm colloidal gold particles conjugated with the Fab fragments of anti-band 3 antibodies (Figure 1A). We have provided evidence that most gold particles are bound to a single band 3 molecule (Tomishige *et al.*, 1998). The movement of gold particles attached to band 3 was observed by contrast-enhanced

[V] Online version of this essay contains video material for Figures 1–6. Online version available at www.molbiolcell.org.

‡ Corresponding author. E-mail address: akusumi@bio.nagoya-u.ac.jp.

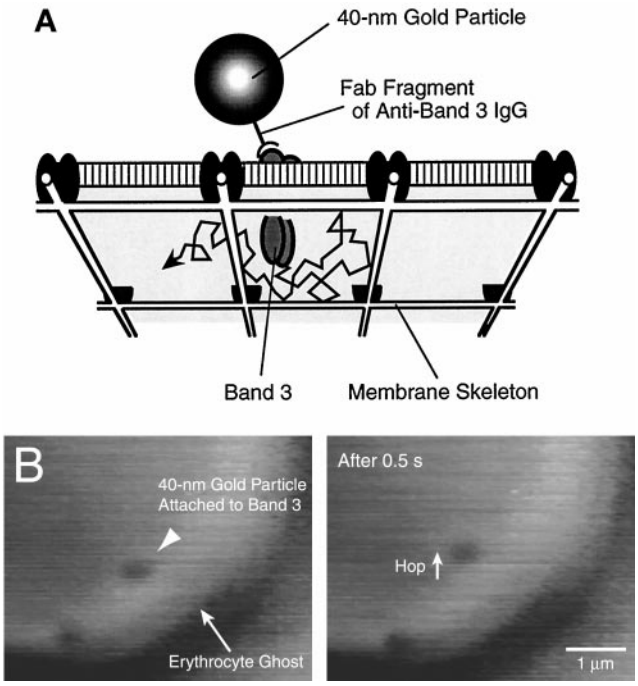

Figure 1. (A) The model to explain the confined diffusion of band 3 in the membrane (the membrane skeleton fence model). (B) Movement of gold particles attached to band 3 in the erythrocyte membrane, observed with a temporal resolution of 8 ms (4 times greater than the normal video rate).

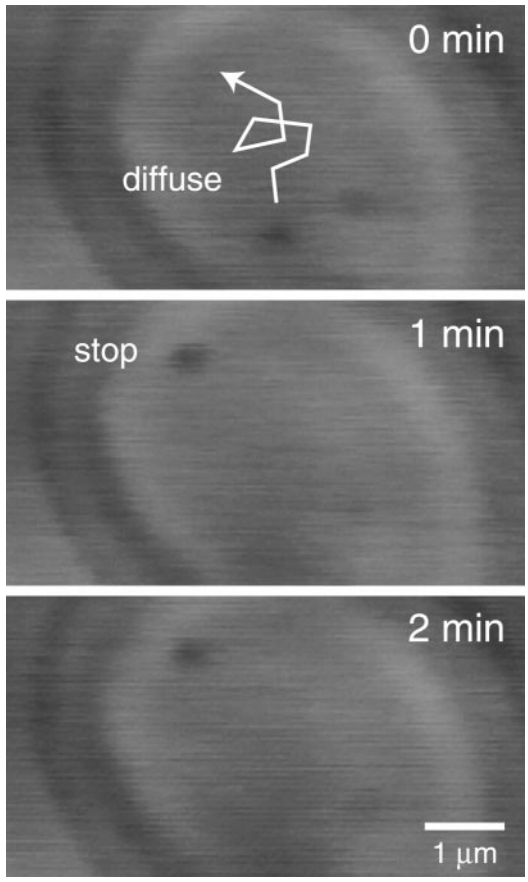

Figure 2. Long-term observations of the movement of band 3 in the erythrocyte membrane. Images recorded every 250 ms using time-lapse video microscopy.

bright-field microscopy at 37°C, with a temporal resolution of up to 0.22 ms, using a high-speed video camera.

Video Sequence 1 shows the movement of band 3, which was recorded at a time interval of 8 ms, and is replayed at video rate (30 frames/s; see Figure 1B). Approximately two-thirds of the gold particles attached to band 3 undergo hop diffusion: as shown in Video Sequence 1, a band 3 molecule is confined within a compartment of 110 nm in diameter (on average), and on average hops to an adjacent compartment every 350 ms. This sequence contains three subsequences: the first shows a hop, the second shows two consecutive hops, and the third displays repeated hops between two domains. Band 3 molecules undergo macroscopic diffusion as the result of a series of such hops between compartments. Within a single compartment, the rate of free diffusion of band 3 molecules is only limited by the membrane viscosity, which is 5.3×10^{-9} cm^2/s. The important point of this video sequence is to show that band 3 molecules do not undergo gradual movements but rather hop from one area to an adjacent area. Movements within a compartment are very small (~3 pixels) and reflect the mesh size of the membrane-skeleton network of ~110 nm.

Video Sequence 2: Long-term Movement of Band 3: Temporal Binding of Band 3 to the Membrane Skeleton

The remaining one-third of the band 3 population exhibited random oscillatory movements within a small area of the membrane of <100 nm during the 30 s observation period. These particles do not show macroscopic diffusion and are likely to represent the band 3 molecules that are strongly bound to the membrane skeleton.

Band 3 molecules that were undergoing hop diffusion eventually traversed the entire surface of the erythrocyte membrane. During observations longer than 10 min, almost all band 3 molecules were seen to attach to and detach from the skeletal network. In Video Sequence 2, band 3 sometimes stops, remains at that spot for several minutes, and then resumes hop diffusion (Figure 2; a time-lapse recording of every 250 ms, which is replayed at video rate). Such binding may be mediated through ankyrin.

Video Sequence 3: Cleavage of the Cytoplasmic Portion of Band 3 Enhances the Intercompartmental Hop Rate

Because the erythrocyte membrane is compartmentalized with regard to the lateral diffusion of band 3 (Figure 1A), we next asked what is the molecular basis of the compartmentalized structure of the erythrocyte membrane. Our working hypothesis for the temporal confinement of (two-thirds of) band 3 is based on the steric hindrance of band 3 mobility

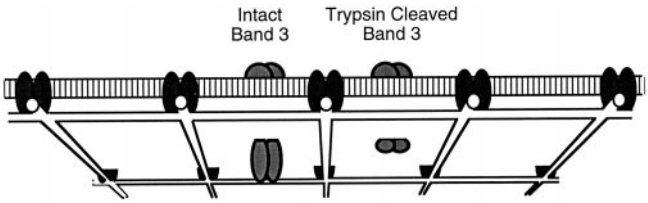

Figure 3. Movement of trypsin-cleaved band 3 was recorded with temporal resolutions of 8 and 0.89 ms.

imposed by the membrane skeleton; i.e., the cytoplasmic domain of band 3 collides with the membrane skeleton, which results in the temporal confinement of band 3 within the mesh of the membrane skeleton of ~110 nm. To test this hypothesis, the cytoplasmic portion of band 3 was cleaved off by mild trypsin treatment of erythrocyte ghosts (Figure 3). Under the conditions used, most of the cytoplasmic portions of band 3 and ankyrin were cleaved, but the spectrin network (spectrin, actin, and protein 4.1) remained basically intact.

Video Sequence 3*a* shows the movement of the cleaved band 3 recorded every 8 ms (which is the same as that for Video Sequence 1) and is replayed at a normal video rate (thus, the time is expanded by a factor of 4.1). On this time scale, the cleaved band 3 apparently undergoes simple Brownian diffusion without hopping. The frame interval was then decreased to every 0.89 ms, and the same cleaved band 3 now shows intercompartmental hops (Video Sequence 3*b*; in this video clip, the time is expanded by a factor of 38). Although trypsin treatment increased the hop rate of band 3 by a factor of 6, the diffusion coefficient within the compartment and the compartment size remained the same. These results indicate an involvement of the cytoplasmic portion of band 3 in the rate of hopping across the boundaries of compartments and are consistent with the membrane skeleton fence model (Figure 1).

Video Sequence 4: Deformation of the Membrane Skeletal Network Using Optical Tweezers

To further observe interactions between membrane-spanning proteins and the membrane skeleton, we have developed a method to deform the membrane skeletal network using optical tweezers. Optical tweezers were used to attach a latex bead of 1 μm in diameter, coated with anti-band 3 IgG, to the center of an erythrocyte ghost membrane (Figure 4). Because such a bead can simultaneously bind to many band 3 molecules, of which ~30% are linked to the membrane skeleton, we expected that the membrane skeleton can be dragged by moving the bead by optical tweezers. Under our experimental conditions, the maximum force applied to the latex bead by our optical tweezers was ~20 pN. To visualize the deformation of the network, 40-nm colloidal gold particles coated with anti-spectrin antibodies were attached to spectrin. In these experiments, gold particles could diffuse into the intracellular aqueous space of the ghost, because the ghosts had not been resealed in the present experiment.

Video Sequence 4 shows the movement of 40-nm gold particles bound to spectrin on the internal surface of the membrane, while the latex bead bound to the membrane skeleton was dragged by the optical trap. These image data suggest that deformation/displacements of the membrane skeleton occur even when a distant part of the membrane skeleton is being dragged with an optical trap. Note that in comparison the contour of the cell was negligibly deformed, which indicates that the dragging caused deformation of the membrane skeletal network, rather than translation of the entire membrane.

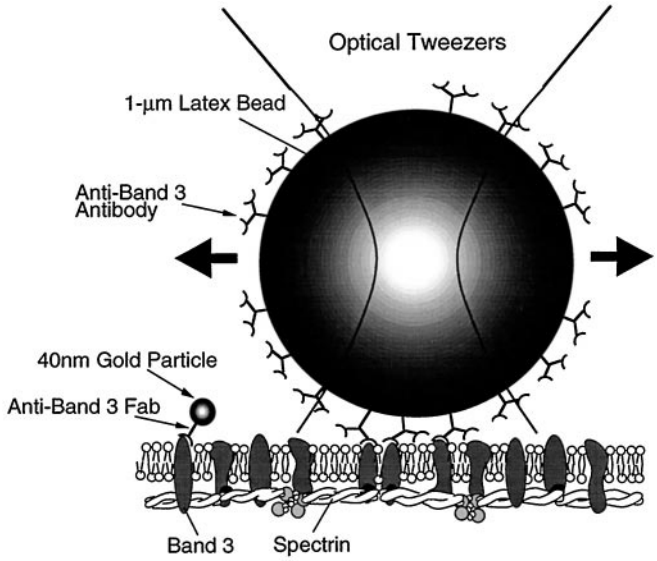

Figure 4. Deformation of the membrane skeleton using optical tweezers. A 1-μm latex bead, coated with anti-band 3 IgG, bound multiple band 3 molecules, of which ~30% are attached to the membrane skeleton. By dragging the latex bead, it was possible to deform the membrane skeleton, which can be observed by single particle tracking using gold particles specifically attached to spectrin on the cytoplasmic surface of the erythrocyte ghost membrane.

Video Sequence 5: Dragging of the Membrane Skeletal Network Forced the Displacement of Unbound Band 3 Along with the Movement of the Network

To discover whether the deformation/displacement of the membrane skeleton network causes forced movement of band 3, band 3 (in place of spectrin) was labeled with 40-nm gold particles, and the latex bead bound to the membrane skeleton was dragged using optical tweezers. We paid attention only to band 3 undergoing hop diffusion (unbound band 3). If unbound band 3 molecules collide with the membrane skeleton, they will be carried along with the movement of the membrane skeleton meshes (Figure 5A). If they do not collide with the membrane skeleton, their movement will not be affected by deformation/displacements of the membrane skeleton.

In Video Sequence 5*a*, the large bead in the center (attached to the membrane skeleton via band 3 molecules) was dragged toward the left, while, at the same time, the movement of band 3-gold that had previously been undergoing hop diffusion was observed. The sequence is shown in real

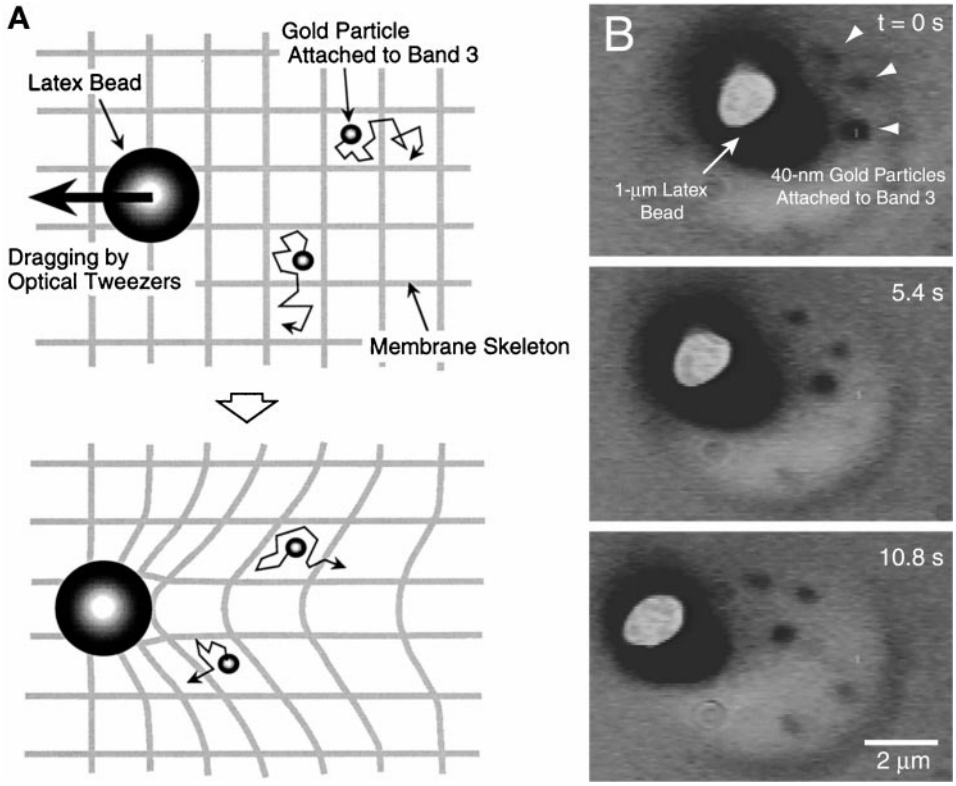

Figure 5. (A) Effect of lateral dragging of a 1-μm latex bead attached to the membrane skeleton on the movement of several band 3 molecules capable of undergoing hop diffusion. (B) The gold particles bound to band 3 undergoing hop diffusion followed the bead when it was dragged toward the left at a rate of 0.15 μm/s.

time (video rate recording). When the latex bead was dragged toward the left at a rate of 1.8 μm/s, the gold particles attached to band 3 (seen on the right) were displaced in the direction of dragging of the latex bead. When dragging of the latex bead was stopped, the same band 3 molecules continued a hop diffusion, indicating that they are not bound to the membrane skeleton. When the large bead was moved back toward its original position, the gold-bound band 3 molecules returned, but not to their original positions, because of the hop diffusion that kept occurring during this sequence. This result strongly suggests that the cytoplasmic domain of band 3 collides with the membrane skeleton.

A possible explanation for the forced movement of band 3 by dragging the membrane skeleton may be a hydrodynamic coupling effect, because all membrane proteins and lipids that are bound to the membrane skeleton network will move in concert with the membrane skeleton. We examined the extent of this effect by lowering the rate of dragging of the membrane skeleton (by a factor of 12, to 0.15 μm/s; Video Sequence 5*b*). Even at this slow rate of dragging, the gold particles attached to band 3 still followed the large bead's movements (Figure 5B). These results show that the band 3 molecules undergoing hop diffusion do indeed collide with the membrane skeleton network, and this results in their temporal confinement within a compartment of the membrane skeleton.

Video Sequence 6: Dragging of the Membrane Skeleton Does Not Induce Displacement of Phospholipids Located on the Outer Surface

To further examine the possibility that the hydrodynamic coupling was responsible for the concerted movement of band 3 as the membrane skeleton was dragged, we next examined the effect of dragging the membrane skeleton on the lipids in the outer leaflet of the membrane. Direct interaction of these lipids with the membrane skeleton is not possible. As such, any effect must be caused by hydrodynamic coupling through the fluid bilayer. Fluorescein-phosphatidylethanolamine was artificially incorporated into the erythrocyte ghost and labeled with gold particles coated with anti-fluorescein Fab.

In Video Sequence 6 (Figure 6), a gold particle bound to fluorescein-phosphatidylethanolamine in the outer leaflet of the membrane is seen near the right edge of the cell. This particle is seen to undergo rapid Brownian diffusion. The membrane skeleton was then dragged toward the left at a rate of 1.8 μm/s; however, the lipid showed no sign of following the large bead and thus the membrane skeleton.

CONCLUSION

The image data shown in this video essay indicate that the erythrocyte membrane is compartmentalized with regard to

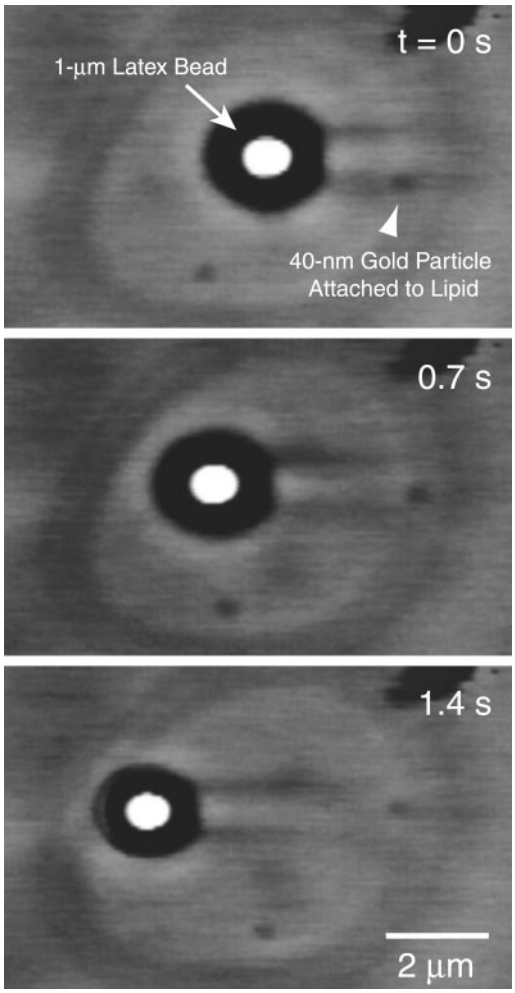

Figure 6. Effect of lateral dragging of a 1-μm latex bead attached to the membrane skeleton on the movement of a lipid that is located in the outer leaflet of the cell membrane.

the lateral diffusion of band 3 and presumably other transmembrane proteins. The confined diffusion is most likely caused by the collision of the cytoplasmic portion of band 3 with the membrane skeleton network (membrane–skeleton fence model). These conclusions could only be reached by studying the movements of single band 3 molecules using single particle tracking techniques and by direct mechanical manipulations of the membrane skeleton using optical tweezers. Because single molecule techniques can be applied to study the movements of various other proteins in the supramolecular complexes contained in living cells, they provide powerful tools for cell biologists to investigate the dynamics and underlying mechanisms for many cellular processes.

ACKNOWLEDGMENTS

We thank Professor Gerard Marriott and Dr. Kenneth Ritchie for critical reading of this manuscript and Prof. Yuichi Takakuwa for the gift of antibodies.

REFERENCES

Bennett, V. (1990). Spectrin-based membrane skeleton: a multipotential adaptor between plasma membrane and cytoplasm. Physiol. Rev. *70*, 1029–1065.

Bennett, V., and Gilligan, D.M. (1993). The spectrin-based membrane skeleton and micron-scale organization of the plasma membrane. Annu. Rev. Cell Biol. *9*, 27–66.

Edidin, M., Kuo, S.C., and Sheetz, M.P. (1991). Lateral movements of membrane glycoproteins restricted by dynamic cytoplasmic barriers. Science *254*, 1379–1382.

Golan, D.E. (1989). Red blood cell membrane protein and lipid diffusion. In: Red Blood Cell Membranes, ed. P. Agre, and J.C. Parker, New York: Marcel Dekker, 367–400.

Jacobson, K., Sheets, E.D., and Simson, R. (1995). Revisiting the fluid mosaic model of membranes. Science *268*, 1441–1442.

Kusumi, A., and Sako, Y. (1996). Cell surface organization by the membrane skeleton. Curr. Opin. Cell Biol. *8*, 566–574.

Kusumi, A., Sako, Y., Fujiwara, T., and Tomishige, M. (1998). Application of laser tweezers to studies of the fences and tethers of the membrane skeleton that regulate the movements of plasma membrane proteins. Methods Cell Biol. *55*, 173–194.

Kusumi, A., Sako, Y., and Yamamoto, M. (1993). Confined lateral diffusion of membrane receptors as studied by single particle tracking (nanovid microscopy). Effects of calcium-induced differentiation in cultured epithelial cells. Biophys. J. *65*, 2021–2040.

Luna, E.J., and Hitt, A.L. (1992). Cytoskeleton-plasma membrane interactions. Science *258*, 955–964.

Mohandas, N., and Evans, E. (1994). Mechanical properties of the red cell membrane in relation to molecular structure and genetic defects. Annu. Rev. Biophys. Biomol. Struct. *23*, 787–818.

Sako, Y., and Kusumi, A. (1994). Compartmentalized structure of the plasma membrane for receptor movements as revealed by a nanometer-level motion analysis. J. Cell Biol. *125*, 1251–1264.

Sako, Y., and Kusumi, A. (1995). Barriers for lateral diffusion of transferrin receptor in the plasma membrane as characterized by receptor dragging by laser tweezers: fence versus tether. J. Cell Biol. *129*, 1559–1574.

Sako, Y., Nagafuchi, A., Tsukita, S., Takeichi, M., and Kusumi, A. (1998). Cytoplasmic regulation of the movement of E-cadherin on the free cell surface as studied by optical tweezers and single particle tracking: corralling and tethering by the membrane skeleton. J. Cell Biol. *140*, 1227–1240.

Saxton, M.J., and Jacobson, K. (1997). Single-particle tracking: applications to membrane dynamics. Annu. Rev. Biophys. Biomol. Struct. *26*, 373–399.

Sheets, E.D., Lee, G.M., Simson, R., and Jacobson, K. (1997). Transient confinement of a glycosylphosphatidylinositol-anchored protein in the plasma membrane. Biochemistry *36*, 12449–12458.

Sheets, E.D., Simson, R., and Jacobson, K. (1995). New insights into membrane dynamics from the analysis of cell surface interactions by physical methods. Curr. Opin. Cell Biol. *7*, 707–714.

Simson, R., Yang, B., Moore, S.E., Doherty, P., Walsh, F.S., and Jacobson, K.A. (1998). Structural mosaicism on the submicron scale in the plasma membrane. Biophys. J. *74*, 297–308.

Tomishige, M., Sako, Y., and Kusumi A. (1998). Regulation mechanism of the lateral diffusion of band 3 in erythrocyte membranes by the membrane skeleton. J. Cell Biol. *142*, 989–1000.