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The fence and picket structure of the plasma membrane of live cells as revealed by single molecule techniques (Review)

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Summary

Models of the organization of the plasma membrane of live cells as discovered through diffusion measurements of integral membrane molecules (transmembrane and GPI-anchored proteins, and lipid) at the single molecule level are discussed. Diffusion of transmembrane protein and, indeed, even lipid is anomalous in that the molecules tend to diffuse freely in limited size compartments, with infrequent intercompartment transitions. This average residency time in a compartment is dependent on the diffusing species and on its state of oligomerization, becoming completely confined to a single compartment upon sufficient oligomerization. This will be of great importance in determining cellular mechanisms for controlling the random diffusive motion of membrane molecules and in understanding signalling processes.

Keywords: Single molecule; membrane structure; compartmentalization; hop diffusion.

Introduction

The classic model of membrane organization is the fluid mosaic model of Singer and Nicholson (1972). They presented a model in which the lipid bilayer, being in a fluid state in almost all membranes, acts as a two-dimensional solvent for the integral membrane proteins. These proteins are free to diffuse through the membrane, presumably undergoing simple, random Brownian motion. Today, through direct experimental evidence, we know this model overly simplifies the complexity of the membrane and cannot explain many of its important functions, like the formation of specialized domains and localization of signalling events.

Figure 1 shows the complexity of the plasma membrane in living cells. The membrane is not just a fluid of lipids filled with transmembrane and lipid-anchored proteins. One must also take into the description the membrane cytoskeleton meshwork formed by actin and actin-binding proteins such as spectrin (fodrin) and gelsolin that come into close proximity

with the lipid bilayer. Interactions between the membrane proteins and cytoskeleton can modulate the diffusive characteristics of the proteins and even the lipids surrounding the proteins. The most dramatic instance of this is through direct binding of a protein to a member of the subsurface network that causes immobilization of the protein (beyond small fluctuations in position due to thermal fluctuations in the network).

This review will discuss three important models to explain the characteristics of diffusion of mobile proteins, lipids and protein/lipid complexes, and their inferences for membrane functions. These models have been developed to understand the important effects of the membrane skeletal meshwork and its associated proteins on the diffusion of membrane-bound molecules. They describe fundamental interactions and molecular processes in the membrane that are found in almost all eukaryotic cells, and therefore almost all molecular processes occurring in the cell's membrane will be under the influence of the membrane skeleton meshwork.

Protein diffusion: the membrane skeleton fence model

Studies of protein diffusion in the plasma membrane of live cells showed that the long-time diffusion rate of protein was slower than predicted, even when crowding effects owing to the presence of other proteins in the membrane were taken into account (Bussell *et al.* 1995a,b). Direct observation of the motion of a single/few protein(s) using single particle tracking (SPT) has revealed new features of the movement of membrane molecules that were hidden by ensemble averaging over all molecules in bulk observation techniques and which played critical roles in elucidating the membrane skeleton-based compartmentalization of the plasma membrane (De Brabander *et al.* 1985, Gelles *et al.* 1988, Kucik *et al.* 1989, Sheetz *et al.* 1989, Kusumi *et al.* 1993). SPT is a technique where a membrane protein is tagged with a small particle (a 200-nm latex particle or, to reduce the effect of cross-linking, a 40-nm diameter colloidal gold particle, which will be discussed here) and the protein/colloidal probe complex is directly observed through contrast enhanced (Nomarski or bright-field) video microscopy. Figure 2 shows a typical trajectory of a gold-labelled transmembrane CD44 molecule in the plasma membrane of a live normal rat kidney cell. The trajectory has been colour coded to reflect the passage of time, with the colour change points chosen along the trajectory, through a computer program to determine the point of escape from a compartment, to enhance the features. The trajectory does not seem a simple Brownian random walk. It appears to reveal a fine structure to the membrane. The protein undergoes random motion in a finite-sized domain, with infrequent transitions to neighbouring compartments. This motion has been termed 'hop diffusion'

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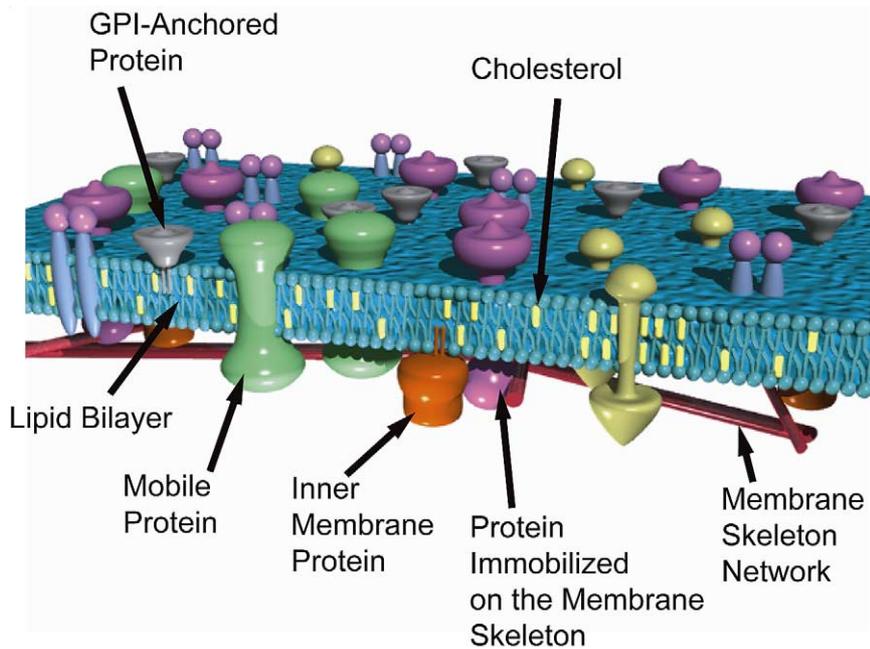


Figure 1. Schematic representation of the complexity of the membrane. The plasma membrane consists of a lipid bilayer with embedded cholesterol and proteins. The proteins are either free to move (mobile) or bound to the underlying actin-based cytoskeletal network (immobile). Even those proteins that are free may still interact sterically with the actin filaments in close proximity to the lipid bilayer. Proteins can be transmembrane, GPI-anchored or may be associated with the inner leaflet of the bilayer.

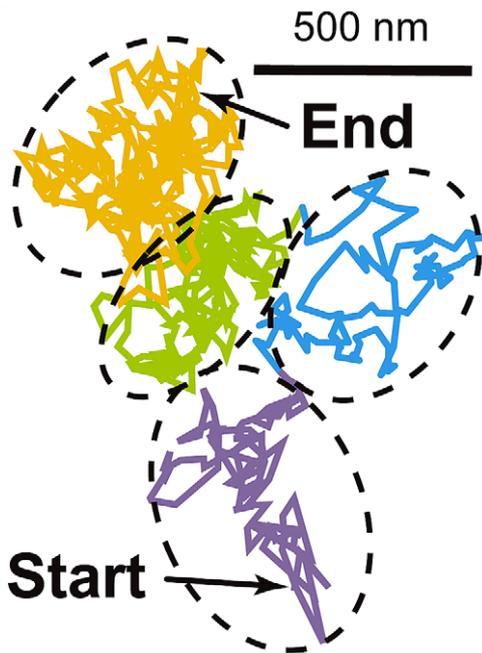


Figure 2. Hop diffusion of a transmembrane protein. The trajectory of a typical freely diffusing colloidal gold-tagged CD44 molecule in the plasma membrane of a live NRK cell is shown. The trajectory was observed at a time resolution of 2 ms per frame. The progression of time is purple–blue–green–yellow (on-line journal). In the printed journal, the progression is from dark to light shading. The dashed ovals show the plausible compartment boundaries that confine the diffusion of the protein. A fraction of CD44 shows confined or stationary motion with no long-range diffusion, presumably due to binding to the underlying actin skeleton or association with larger structures in the membrane. Reproduced with permission from Ritchie and Kusumi (2002).

(Kusumi *et al.* 1993, Sako and Kusumi 1994, Saxton 1995). (CD44 has fractions associated with detergent-resistant domains and with actin filaments through ezrin–radixin–moesin proteins, and some molecules exhibit more complex motion and some are stationary. The description above is only applicable to the fraction of CD44 that exhibits very high mobility.)

The mechanism behind this anomalous diffusion was proposed to be a steric interaction between the cytoplasmic domain of the transmembrane protein and the membrane cytoskeleton, the actin meshwork that comes into close proximity with the membrane bilayer. This mechanism was termed the ‘fence’ model, where the actin strands in the skeleton network act as fences to corral the membrane proteins (Kusumi *et al.* 1993, Sako and Kusumi 1994). The requirement of the cytoplasmic tail was shown through cytoplasmic domain deletion mutants of E-cadherin in L cells (Sako *et al.* 1998) as well as cytoplasmic domain-cleaved band 3 in the erythrocyte membrane (where the membrane skeleton consists of mainly the soft, structural protein spectrin) (Tomishige *et al.* 1998). The requirement of the membrane skeleton was shown through very delicate use of actin depolymerization drugs (such as cytochalasin D and latrunculin A), where slight depolymerization of the skeleton tends to increase the average compartment size and through an actin-stabilizing drug (jasplakinolide) that tends to increase the average residency time in a compartment. In addition, membrane blebs where the membrane skeleton is largely lost, the majority of transmembrane proteins undergo simple Brownian diffusion with diffusion coefficients comparable with those in reconstructed membranes (Fujiwara *et al.*

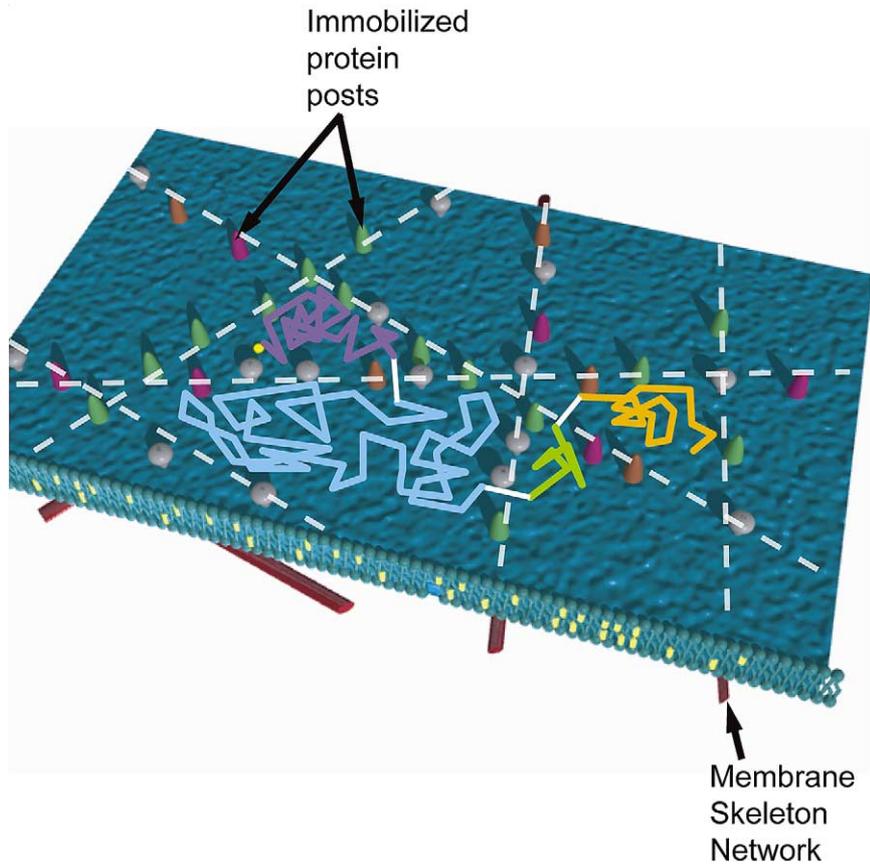


Figure 3. Anchored protein picket model. Lipid diffusion is compartmentalized, much in the same way transmembrane protein diffusion is, owing to the fraction of transmembrane protein immobilized through binding to the underlying membrane skeleton network. The hydrodynamic-like interaction predicted by the free area theory of lipid diffusion (Sperotto and Mouritsen 1991, Almeida *et al.* 1992) between the immobilized protein fence pickets acts to corral the lipids.

2002). This result again supports the membrane skeleton fence model.

Although the presence of compartments is seen by eye, analysis of their properties requires a more stringent methodology (Kusumi *et al.* 1993). Analysis of the hop-like trajectories follows from determining the mean-squared displacement (MSD) of the protein through its trajectory. The MSD (as a function of time) is then fit by an equation derived from the theory for diffusion through an infinite array of semipermeable barriers (Powles *et al.* 1992). The analysis gives the average compartment size, residency time in a compartment and diffusion coefficient inside of a compartment.

From such an analysis of the diffusion of transmembrane proteins in a variety of cells, compartmentalization seems to be a general feature of the plasma membrane, although there is a variance in the compartment size and residency time between cell types. The compartment size ranges from 30 nm at the smallest for CHO and FRSK cells to 200 and 700 nm for the unique, doubly compartmentalized NRK cell (Table 1 shows the variation in compartment sizes between cell types) (Murase *et al.* 2001). Interestingly, the diffusion

coefficient inside of a compartment is in the order of $5\text{--}10 \mu\text{m}^2 \text{s}^{-1}$ in all cases. This value is similar to that expected for free diffusion in pure lipid bilayers and reconstituted membranes. This suggests that the diffusion of membrane protein is not slow because it itself is slow, but rather the slowing effect is a direct result of infrequent transitions or hops between membrane compartments.

Table 1. Variation in compartment size between cell types.

Cell type	Compartment size ² (nm)
FRSK	41
CHO	32
PtK2	39
ECV304	110
NRK ¹ (outer)	750
NRK ¹ (inner)	230

¹ NRK cell is unique in that it seems to have two distinct, nested compartments (Fujiwara *et al.* 2002).

² Compartment length is determined along two orthogonal directions, (L_x , L_y). Compartment size is determined as the geometric mean of these lengths, $(L_x L_y)^{1/2}$.

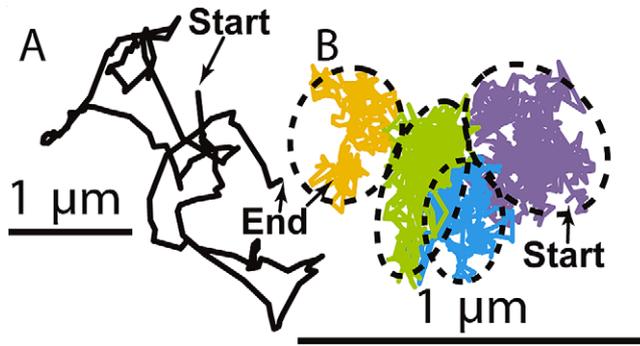


Figure 4. Hop diffusion of a DOPE lipid. The trajectory of a typical colloidal gold-tagged DOPE lipid (tagged through anti-fluorescence antibody to a fluorescein labelled DOPE lipid introduced to the cell) in the plasma membrane of a live NRK cell at (a) 33 ms and (b) 25 μ s is shown. At normal video rate, the lipid appears to undergo simple Brownian diffusion, but at much higher time resolutions, the membrane compartmentalization is seen. The progression of time is purple–blue–green–yellow (on-line journal). In the printed journal, the progression is from dark to light shading. The dashed ovals show the plausible compartment boundaries that confine the diffusion of the lipid.

Lipid diffusion: the anchored-protein picket model

SPT has also recently been applied to observe the diffusion of lipid, specifically L- α -dioleoylphosphatidylethanolamine (DOPE) in the plasma membrane of NRK cells (Fujiwara *et al.* 2002). Surprisingly, the diffusion of DOPE also reflects the compartmentalized structure of the membrane (Figure 3), although the lipid is restricted to the outer leaflet of the membrane and hence cannot directly interact with the

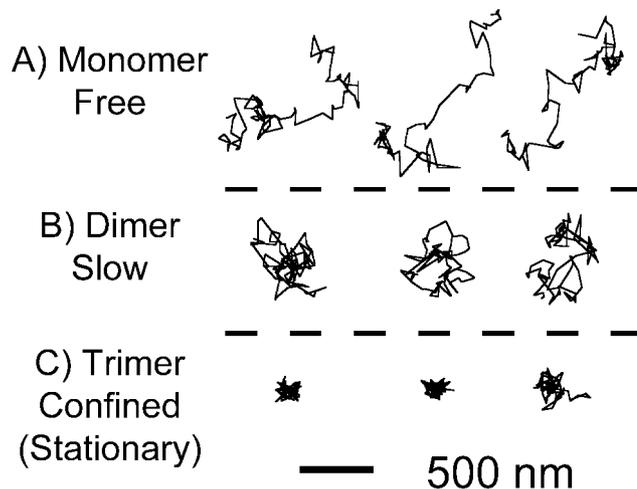


Figure 5. Trajectories of E-cadherin-GFP in L cells reveal that oligomerized molecules undergo slower long-range diffusion. E-cadherin-GFP is seen through single molecule fluorescence video imaging. The trajectories are 3 s (90 video frames) in length at 33 ms/frame. (A) Trajectories of a fluorescent spots deemed to represent a single molecule undergoing simple diffusion. Compartmentalization cannot be visualized owing to the slow frame rates required for fluorescence imaging. (B) Trajectories of fluorescent spots that were double in intensity. Their diffusion is more compact (i.e. their long-range diffusion is slowed). (C) Trajectories of fluorescent spots of triple intensity showing confined motion (i.e. no long-range diffusion).

membrane cytoskeleton. The parameters found for the hop diffusion of DOPE are very similar to those found with transmembrane protein on the same cells, except for the hop frequency (residency time within a compartment). In addition, actin stabilizing/depolymerizing drugs have the same effects on the compartmentalization of DOPE as they do on transmembrane protein. Furthermore, removal of extracellular matrix proteins and extracellular domains of membrane proteins, and partial cholesterol depletion did not affect the hop parameters. It has been proposed that the membrane skeleton interacts indirectly with even the lipid on the outer leaflet through a hydrodynamic-like interaction with the transmembrane protein that is immobilized to the skeleton. These immobilized protein ‘posts’ or ‘fence pickets’ hold the membrane skeleton ‘fences’ in close proximity to the membrane and hence reflect the structure of the underlying actin-based membrane skeleton meshwork (Figure 4). The hydrodynamic-like interaction predicted by the free area theory of lipid diffusion (Sperotto and Mouritsen 1991, Almeida *et al.* 1992) has been shown through Monte Carlo simulation to be able to reproduce the experimental results. Only 20–30% coverage of the compartment boundaries with anchored transmembrane proteins was sufficient to reproduce the experimentally observed hop frequency (Fujiwara *et al.* 2002).

Protein/lipid complex diffusion: the oligomerization-induced trapping model

The existence of the actin skeleton scaffolding and its associated immobilized proteins lends a fine structure to the fluid membrane. As such, long-range diffusion is slowed even for single lipids due to this fine structure. The implications are that an aggregate of lipid and protein (even GPI-anchored protein), as expected in the formation of lipid domains or rafts, would severely restrict the long-range motion. In fact, after growing to a certain finite size the complex would be permanently trapped in its local compartment. Such effects have been seen in complex formation of E-cadherin (labelled with green fluorescent protein, GFP) in L cells through single fluorescence video imaging (SFVI) (Iino *et al.* 2001) (Figure 5). The technique of SFVI uses a fluorophore-tagged protein or lipid that is viewed through a total internal reflection microscope equipped with an intensified video-imaging system. The advantage of this technique over SPT is the ability to tag, with certainty, a single molecule at the cost of the higher signal-to-noise ratio of fluorescence imaging that limits the observation to standard video rates. This slowing of molecular complexes may be an important mechanism for the localization of external signals (Figure 6). Without a localization mechanism, after receiving a signal through ligand binding, the spatial information of the signal would be quickly lost due to random diffusion of the receptor molecules. A freely diffusing molecule in a pure model bilayer devoid of a compartmentalized fine structure should have a diffusion coefficient in the range 5–10 $\mu\text{m}^2 \text{s}^{-1}$ (Fujiwara *et al.* 2002) and thus will cover an area of diameter 2–4 μm in 1 s. In the plasma membrane of a CHO or FRSK cell, after aggregation, the signalling complex can be restricted to a

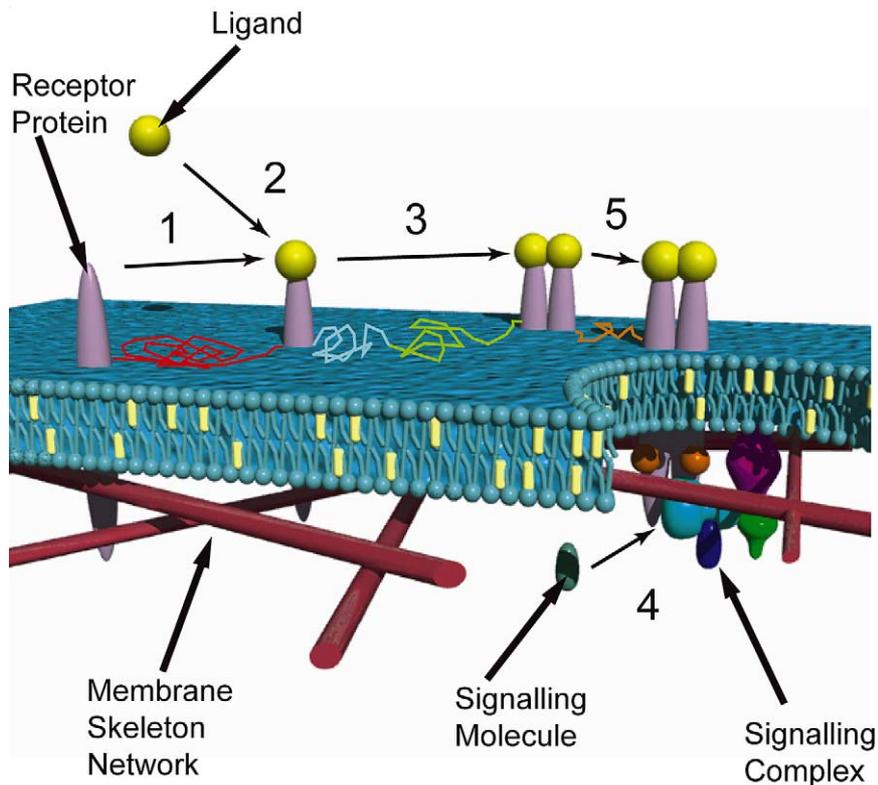


Figure 6. Oligomerization-induced trapping model. (1) A receptor protein, which is freely diffusing between compartments in the membrane, binds a ligand molecule (2). (3) The receptor with ligand diffuses until it meets a similar molecule to form a dimer, slowing its transition rate between compartments. (4) Signalling molecules, both membrane-bound and cytoplasmic, begin to collect around the receptor molecule forming a signalling complex (5). The signalling complex is now arrested in a compartment owing to its size (i.e. it can no longer escape through the fence and/or pickets).

compartment of only 30 nm in extent until dissolution of the signalling complex. Such a mechanism to restrict the diffusion of a signalling complex requires no input of energy from the cell.

Discussion

The membrane fine structure forms a compartmentalized mosaic across the plasma membrane that affects the free diffusion of the mobile constituents. The effect has far-reaching implications as to the mobility of protein, lipid, lipid domains or rafts and signalling complexes. In addition, subtle control of the membrane skeleton, through external agents such as drugs or through internal actin network restructuring, can have direct consequences on the diffusive characteristics of the membrane molecules.

Extreme cases of control of the diffusive character can be seen in the requirement to keep a polarity in many cells, such as in the epithelium and along the neuronal axon. Accumulation of immobilized protein in the membrane separating two sections of the fluid bilayer can be sufficient to restrict diffusional mixing allowing for a differential distribution of molecules in the continuous bilayer. Such an effect has been seen in the initial segment of hippocampal neurons where

accumulation of actin-based membrane skeletal proteins and anchoring of various transmembrane proteins including sodium channels at the initial segment practically blocks diffusion in that region, preventing mixing of proteins, transmembrane and GPI-anchored, between the cell body and its axon's distal regions (Nakada *et al.* 2001).

With the membrane skeleton and its associated immobilized membrane proteins interacting with and controlling the movements of all membrane molecules, the effects of the fence and picket model should be taken into account during the analysis of all membrane molecule interactions. Specifically during signalling, localization of a signal after stimulation seems intimately linked to the aggregation state of the signalling complex. This gives the cell a very efficient means to arrest the motion of a signalling complex, and hence determines with positional accuracy the direction of the signal.

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