

Single-Molecule Techniques Reveal Diffusion Barrier in Neuron Membrane

by Nancy D. Lamontagne, Senior Editor

Neurons have the highly specialized task of carrying out two complicated processes: receiving and transmitting signals. These functions require two sets of proteins and two polarizations and, even more difficult, they involve the cell membrane extensively. How are the tasks separated in a continuous fluid membrane? Researchers led by Akihiro Kusumi at Nagoya University in Japan may have found the answer by dragging, tracking and imaging single molecules in living neurons.

Although proteins can be transported to different parts of the membrane to be used for a specific function, diffusion causes these proteins to gradually disperse. Scientists have thought that some sort of barrier must keep the membrane proteins from diffusing freely, but recent studies have provided contradictory results. Some have shown a barrier that even prevents diffusion of the membrane's main constituent — lipids. Because a barrier capable of stopping lipid diffusion seems difficult to explain, many questioned its existence.

However, previous studies had relied on bulk measurements of molecule diffusion, without examining the activity of single molecules. Kusumi relates this to trying to figure out if the Great Lakes are freely connected or if they are gated, by putting colored balls in one lake and observing the results from the moon. If, for example, the balls are released too far from the site of a possible gate, they will never make it to the site. An observer would conclude that a gate is present, even if it isn't.

To prevent such flawed observations when determining whether and where a diffusion barrier is present in a neuron's membrane, the researchers developed instrumentation and software especially for single-molecule live-cell imaging. With living cells, system integration for ease of operation and quick acquisition of data are key, Kusumi said. The study appeared in the July issue of *Nature Cell Biology*.

The unsaturated phospholipid dioleoylphosphatidylethanolamine (DOPE) acted as the single molecule in their experiments. The scientists labeled these

molecules with the fluorescent molecule Cy3 and introduced them to the outer leaflet of the membrane. They observed the complex's movement using a video-rate silicon-intensified target tube camera from Hamamatsu Photonics in Japan, attached to a total internal reflection fluorescence microscope that they developed based on an Olympus microscope with a 1.4-NA objective.

In an 11-day-old neuron, they found that the complex moved about very little in a portion of the neuron called the axonal initial segment but moved around more in the rest of the cell membrane. The Cy3 labeling ensured that only one DOPE molecule was attached to one Cy3 molecule, but these complexes allowed diffusion coefficients to be measured down to only $10^{-2} \mu\text{m}^2/\text{s}$ over 200 ms. Thus, they also tagged DOPE with 40-nm gold particles, allowing them to measure diffusion coefficients down to $3.3 \times 10^{-4} \mu\text{m}^2/\text{s}$ over the same time period.

They compared the DOPE trajectories they measured using gold with those using fluorescence tagging and found that the

diffusion of both was suppressed in the axonal initial segment of 10-day-old cultured neurons, but not in other parts of the cell. In 1-day-old neurons, the gold-labeled DOPE diffused rapidly throughout the cell membrane but was reduced by a factor of more than 800 after 10 days in the axonal initial segment. In other parts of the membrane, diffusion decreased only one- to threefold over the same amount of time.

They found similar results by dragging the gold-labeled molecules via optical tweezers, formed from an Nd:YVO₄ laser at 1064 nm. The consistent results from several methods showed that a diffusion barrier was present. Now they wanted to know how it could block lipid diffusion.

Immunofluorescence showed that actin and ankyrin-G — the main constituents of the membrane skeleton — become highly concentrated in the axonal initial segment during development. So the scientists plotted the diffusion coefficient of gold-labeled DOPE against the intensity of immunofluorescence of ankyrin-G for a section of the neuron and found that areas with high concentration of ankyrin-G correlated with lower diffusion coefficients of the particles. Various membrane proteins concentrate in the same spot by anchoring to the membrane skeleton.

Based on this evidence, the researchers concluded that the membrane proteins form a sort of picket fence that keeps lipids from diffusing across that area. To

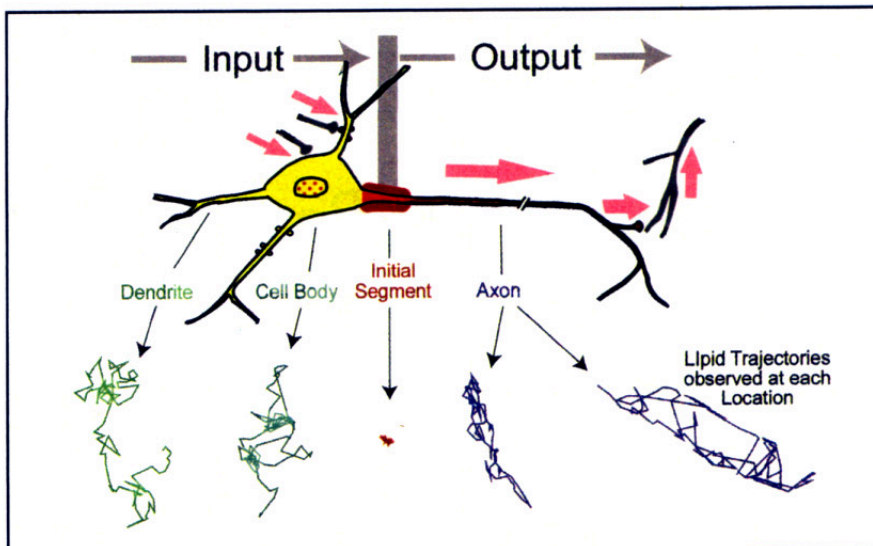
test this model, they treated the membrane with a chemical that breaks apart actin filaments. After treatment, not only was a larger percentage of Cy3-DOPE mobile, but sodium channels were also more spread out, and gold-labeled DOPE could be dragged through the cell body membrane. Treatment with a chemical that enhances actin polymerization and stabilization made Cy3-DOPE less mobile, even in the axonal initial segment membrane of younger neurons where lipids are generally more mobile. Thus, all their results were consistent with the "picket fence" model. They think that diffusion barriers found in other types of cells may be based on a similar mechanism.

To reduce background signal, the cells must be kept healthy during imaging, Kusumi said. Temperature control over the instrument and the cell is a must. The microscope becomes unstable if there is a change in temperature or temperature gradient, and the cells do strange things.

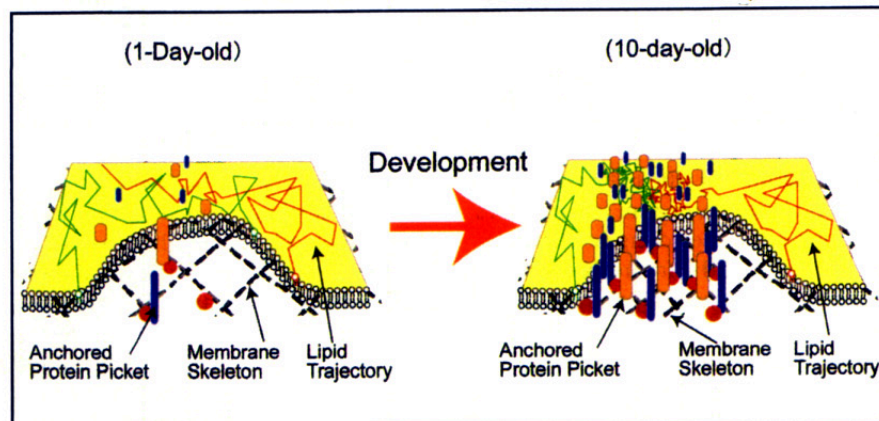
The researchers automated many steps of the signal acquisition process, and they developed software and interfaced it to the signal acquisition system to simultaneously track the movement of many probes in real time. This allowed them to clearly see the trajectories and to understand what was happening immediately, while the cells were still healthy. The trajectories imported to another home-built software program for immediate quantitative/statistical analysis.

Applying single-molecule nanobiology to the functions of the cell membrane is very important because the membrane is *not a simple liquid*, Kusumi said. "Rather, it is a nonideal liquid mixture of molecules with molecular clusters, domains and compartments (the domains made by the membrane skeleton and its associated transmembrane proteins). And since these are very dynamic structures with a variety of space-time scales, [the] single-molecule method will provide very powerful ways to understand them. ... I think that these are the keys to the understanding of the membrane functions, like signal transduction and cell-cell and cell-substrate interactions."

Now that they understand the diffusion barrier, the researchers are studying how the signals from the receptor-type kinases are processed in the cell membrane, how rafts may be involved in concentrating proteins for signal transduction and how neurons develop their contacts with each other. □



Single-molecule imaging and dragging by optical tweezers revealed that a diffusion barrier exists in the initial segment of the neuron's membrane. This segment divides the neuron into the parts that receive and output information. The trajectories for a labeled lipid as observed in each segment of the neuron are shown at the bottom.



The researchers believe that the barrier is formed gradually during development as membrane proteins become concentrated and anchored to the membrane skeleton that is also being accumulated in the initial segment membrane, forming a "picket fence" that blocks lipid diffusion.