

Chapter 2

DYNAMICS OF THE CELL MEMBRANE OBSERVED UNDER THE EVANESCENT WAVE MICROSCOPE AND THE CONFOCAL MICROSCOPE

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1. INTRODUCTION

The objective lens of a microscope plays an essential role for high resolution imaging. Especially, the numerical aperture (NA) of the objective lens is the determining factor for gaining the resolving power. In 1994, a novel objective lens that had a numerical aperture of 1.65 was developed by Susumu Terakawa (the author) and Katsuyuki Abe (Olympus Optical Co.). Use of this ultra high NA lens opened a new field of light microscopy beneficial for observations of living cells at a molecular level and for studies of the cell membranes in their dynamic aspects.

2. ULTRA HIGH NA OBJECTIVE LENS

Use of the ultra high NA lens (Figure 1) is quite advantageous for observation of fluorescent objects of biological specimens under the illumination of evanescent light¹. The ultra high NA lens has a large extra portion that accepts a laser light introduced from the back side of the lens, and projects it to the focal point at an angle of incidence larger than the critical angle (Figure 2). Such an extra portion lies only in the marginal zone between the line of critical angle and the edge of the lens.

The ultra high NA (NA=1.60) lens was once manufactured more than 100 years ago at Carl Zeiss, Jena. However, in fact, it disappeared from the market completely after Ernst Abbe pointed out that the extra large portion of the lens was useless for collecting the light from the objects immersed in the water.

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Figure 1. Objective lens of 1.65 NA (Olympus) developed by Terakawa and Abe in 1994.

Since then, no such lens was commercially available, and thus the ultra high NA lens has been forgotten for a long time. Aiming at an improvement of the resolution and the maximum brightness, we newly developed an objective lens that has a numerical aperture of 1.65^{1, 2}.

The lens has a high performance in three respects. 1) It is the brightest lens ever made in the history of optics. 2) The resolution reaches the highest level among all optics: the smallest resolvable distance is smaller than 150 nm. 3) It creates thinnest evanescent field (< 50 nm) easily when used for a through-the-lens (TTL)³ evanescent wave illumination. All of these properties combined together, it is the most advantageous lens for the microscopy with an evanescent wave illumination or total internal reflection fluorescence microscopy (TIRFM)¹. A disadvantage is that one has to use the lens in combination with specially selected oil and a coverslip of an optimized refractive index of 1.78.

This 1.65NA lens collects a light with its acceptance angle larger than the so called critical angle. No possible optical path exists for the light coming from the water phase in any direction illuminating the focal point O (in Figure 2). However, if the light source is smaller than the wave length of light, and if it is located exactly on the interface between the water and the coverglass i.e., the first optical element collecting the light from the source, then the light from the source radiates unevenly with a peak radiation in the direction of the critical angle, forming a distribution pattern as shown in Figure 3. A significant intensity of light is to be detected in the direction beyond the critical angle⁴. This is easily understood because the evanescent light generated in an area of 50 – 100 nm distant from the interface can travel into the glass phase in the direction beyond the critical angle. The light generated at a source located in an even shorter distance can follow a similar optical path.

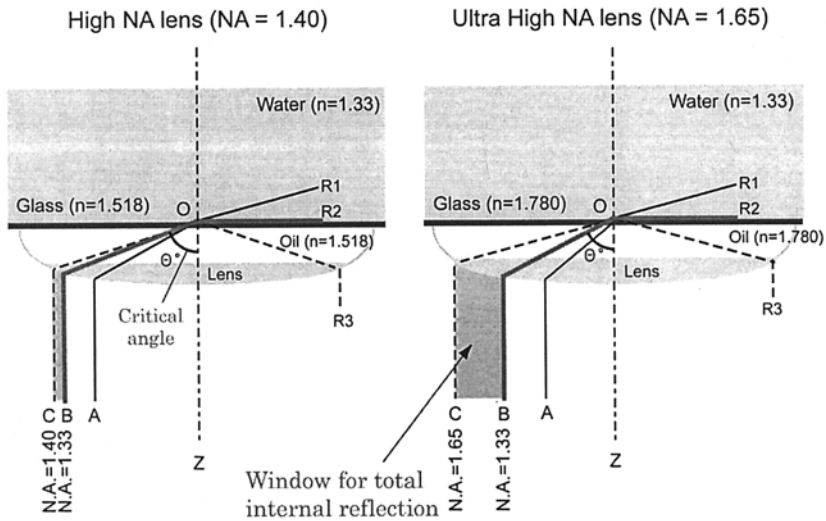


Figure 2. Ultra high NA lens focusing on a small object on the interface between the water (top half) and the glass (bottom half). A 1.40 NA lens (left) and a 1.65 NA lens (right) are compared schematically. An ordinary optical path that starts from a point (R1) in the water phase forms a line indicated by A. An optical path with the critical angle at the interface is indicated by B, and with a largest possible angle by C.

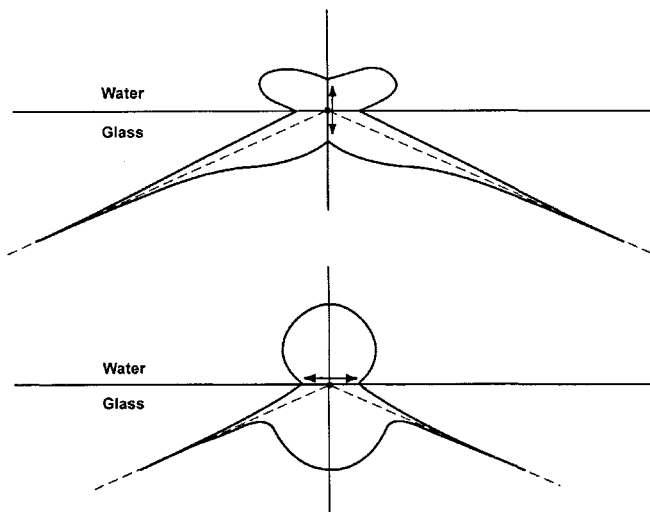


Figure 3. Radiation of light from a point source on the interface between water and glass. The dipole (arrows) lies perpendicular (top) and parallel (bottom) to the interface. The dashed line indicates the critical angle. (Hellen and Axelrod, 1987)⁴.

3. OBSERVATIONS UNDER EVANESCENT WAVE ILLUMINATION

The evanescent field formed by the TTL method is useful for visualizing the molecular image of fluorescent dyes as well as the molecular dynamics of the cell membranes.

3.1. Calcium Indicator Dye

Figure 4 shows images of a calcium sensitive dye, Rhod-2, stuck on the surface of glass. When the Ca^{2+} was chelated from the medium by addition of EGTA, no significant fluorescence was detected. However, when the Ca^{2+} concentration was raised to a level as high as 130 nM, discrete spots of fluorescence were clearly observed. A calcium dependent increase in fluorescence can be observed at the molecular level. Loading this dye into neurons made it possible to monitor Ca^{2+} signals near the plasma membrane or in the vicinity of the Ca^{2+} channels. Imaging the Ca^{2+} transients exclusively near the plasma membrane with a CCD camera of the fast rate (5 ms) was sensitive enough to detect some clustered responses.

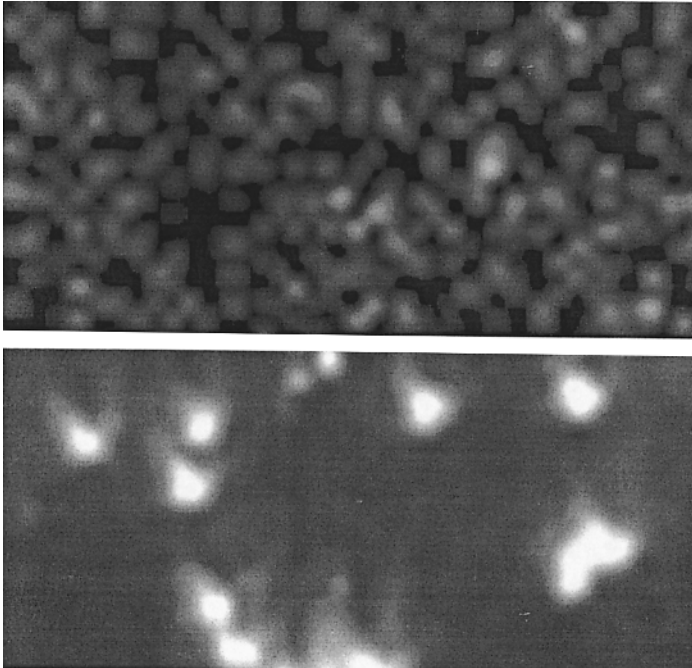


Figure 4. Fluorescence image of Rhod-2 dye molecules captured under the evanescent wave microscope equipped with a 1.65 NA lens. When $[\text{Ca}^{2+}]$ is low, almost no fluorescence was observed (top). When $[\text{Ca}^{2+}]$ is 130 nM, single molecule images appeared brightly (bottom).

3.2. Ion Channel

Labeling of ion channels with a fluorescent dye can be selectively performed by modification of the RNA code and by injection of modified RNA into a model cell like the *Xenopus* oocyte. The *Shaker* K channel was expressed in the oocyte after code modification of S351C. After the expression of proteins, the cystein residue was labeled with tetramethyl rhodamine maleimide. The cells placed under evanescent wave illumination showed many small fluorescent spots. Depolarization of the plasma membrane by 120 mV by the double electrode voltage clamp technique induced a large change in fluorescence intensity of these fluorescent spots (Figure 5). Many of them showed such a voltage-dependent fluorescence change in addition to the quantized bleaching of fluorescence, indicating that the spot was a single molecule of the K channel or at least the subunit of the channel⁵. These single channel images were rather static, and no sign of the fast mobility in lateral direction was observed.

3.3. Protein Kinase-C

The evanescent field decays exponentially with the distance from the surface of glass, with decay constant being the function of the ratio of refractive index of water to that of glass and also the function of the angle of incidence and the wavelength of the light. The fluorescent object entering the field from the outside will increase the fluorescence intensity dramatically. This property can be exploited for visualization of activated form of some enzymes. For example, protein kinase C (PK-C) is translocated to the plasma membrane from the cytoplasm upon activation by phorbol ester. When a HeLa cell expressing GFP fused with PK-C was stimulated with phorbol ester, a dramatic increase in fluorescence was observed first in a short time reversibly at 2 – 3 min, and then delayed and persistent manner after 10 min (Figure 6).

A similar response appeared clearly but in a much smaller degree under a confocal microscope.

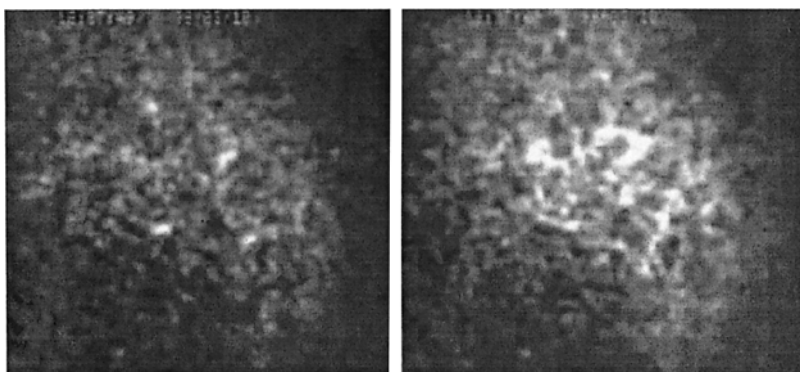


Figure 5. Tetramethyl rhodamine-labeled *Shaker* K-channel expressed in a *Xenopus* oocyte. The membrane voltage was varied from -80 mV (left) to +40 mV (right) by the voltage clamp method.

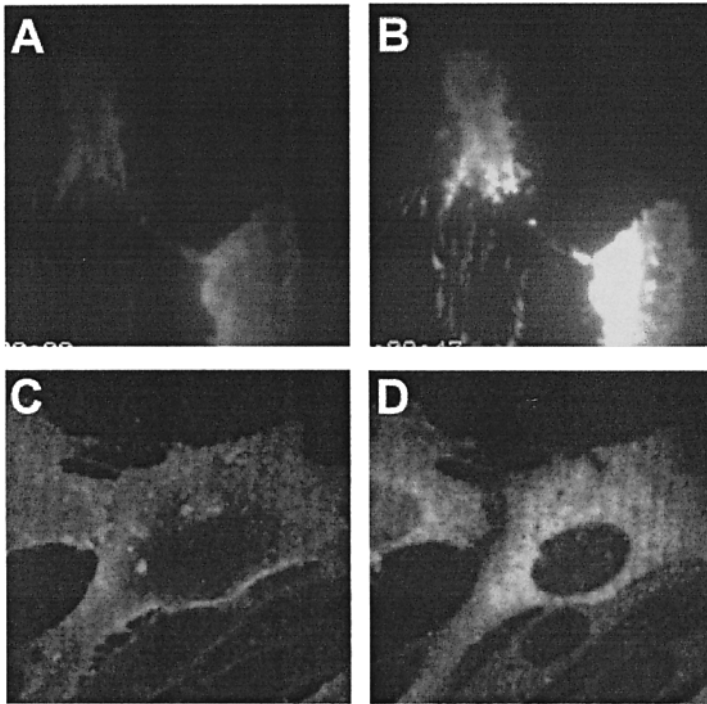


Figure 6. Fluorescence images of cells expressing PK-C/GFP observed under evanesence mode (top) and confocal mode (bottom), before (left) and 9 min after (right) stimulation with TPA.

3.4. Dynamin

The dynamin activity can also be observed in a similar manner when the protein is fused with GFP. Stimulation of a cell (PC12) with an electrical pulse induced a translocation of the protein, i.e., many clusters of the dynamin-GFP fusion protein appeared on the plasma membrane in 30 – 90 s after stimulation. These fluorescent clusters showed rather rapid Brownian movement under the plasma membrane. Some of them were lined up and moved a few micrometer as if they swept up scattered debris⁶. In rare cases, hairy clusters were assembled into a circular pattern which enlarges itself in a minute or so as shown in Figure 7. These dynamic images provide evidence that the dynamin interacts continuously from one pit (or invagination) to the other on the plasma membrane. Probably dynamin pinches off the pit membranes at the neck without its detachment from the plasma membrane. We hypothesized that the dynamin action takes place just like a sweeper cleaning on the floor in a scanning manner (sweeping model). This is in some contrast to the conventional view that dynamin binds to the neck region and detaches the plasma membrane together with a pit membrane to form an endocytic vesicle.

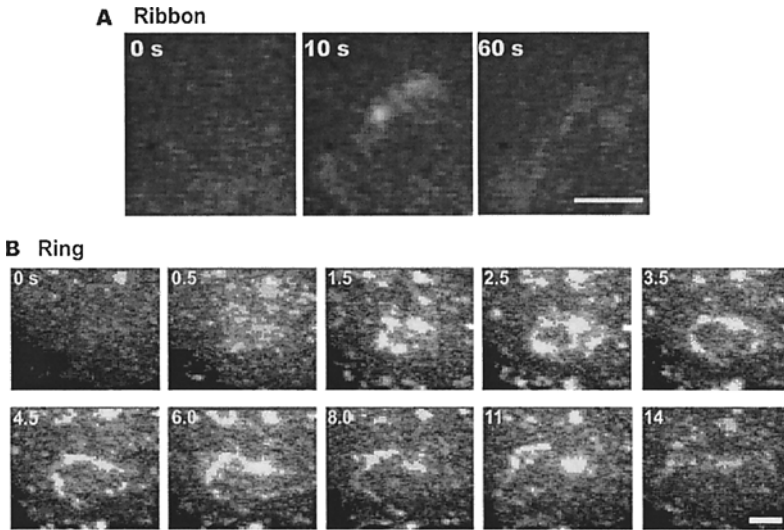


Figure 7. Super clusters of dynamin visualized in PC12 cells expressing the dynamin-GFP fusion protein⁶. The cell was stimulated with an electrical pulse of 1 ms in duration, and then video sequences were captured. The numbers indicate the time after the stimulation in second. Bars, 1 μ m.

3.5. IP_3

Some of the intracellular messengers involve translocation of signal molecules from the plasma membrane to the cytoplasm. As a result of enzymatic reactions, activation of G-protein and phospholipase C for example, IP_3 is produced on the plasma membrane and is released to further activate Ca^{2+} channels located intracellularly on the membranous structures. This process can be monitored by a GFP fusion protein capable of binding the so called PH domain. When phospholipids are in their original form, the protein binds to the PH domain on the phospholipids. After cleavage of the phospholipids, the protein loses its binding site on the plasma membrane. Under the evanescent wave illumination, a large loss of membrane fluorescence was observed in HeLa cells expressing the PH-domain binding protein fused to GFP when the cell was stimulated with histamine. Sometimes, waves of darkness propagated across the bottom part of the cells, indicating a repeated cycle of IP_3 signals in a few minutes.

3.6. Exocytosis

Exocytosis is a very dynamic process taking place on the cell membrane. When contents or the vesicle membrane is stained with fluorescent dyes, the process can be observed at a high spatio-temporal resolution. Adrenergic vesicles are easily stained with acridine orange, as it mimics the adrenaline or noradrenaline in the uptake process on the vesicle membrane. Expression of a GFP fusion protein either as a content of secretory

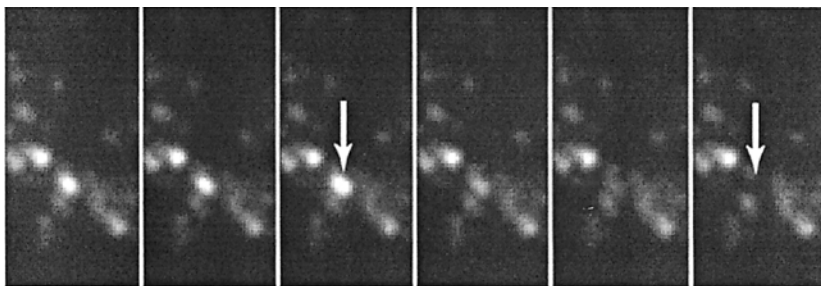


Figure 8. Exocytosis of an acridine orange-loaded secretory granule in a MIN-6 cell. The cell was stimulated by irradiation of an UV laser flash after loading with caged Ca^{2+} . A sequence of video images were recorded at a frame rate of 33 ms. The granule (left arrow) transiently increased the fluorescence and then rapidly disappeared (right arrow). Almost all granules shown in this figure responded similarly in a few second.

vesicles or as an intrinsic protein on the limiting membrane is an obvious alternative to visualize the exocytotic response under the evanescent light⁷.

The exocytotic response of fluorescent vesicles appeared in a two different manners⁸. The vesicles rapidly disappeared one by one when the cell was stimulated with agonists or by membrane depolarization. There was no optical sign before the disappearance. The fluorescence intensity decreased to zero level in 30 – 100 ms in the chromaffin cells and MIN-6 cells. In the case of response of transmitter release from the nerve terminals, the rate of decrease reached 6 ms which was the smallest time resolved by our custom made CCD camera. Another form of response was a transient flash response immediately followed by disappearance as mentioned above (Figure 8). The flash was an increase in fluorescence intensity as well as in fluorescent area. In the evanescent field, the intensity of excitation light decreases with the distance from the surface. Because of this property, fluorescent objects increase in intensity as they move closer to the glass surface. We interpret that two types of exocytotic responses found in a single cell reflect the variation in the velocity of releasing process. When the velocity is faster than the diffusion rate, the concentration of substances released would increase near the glass surface transiently before they diffuse away. This is why the flash response appears before the disappearance of vesicles. The release of substance from a vesicle by diffusion process only would appear as simple disappearance of the vesicle. The extra factor that facilitates release is a volume flow of water due to secretion from the vesicles⁸.

4. DISCUSSION

The evanescent wave microscope can be applied to molecular visualization of membrane proteins such as ion channels, receptors, and transporters. It is also useful for dynamic analyses of the transmembrane signaling, release of signal molecules, exocytosis, and endocytosis. The evanescent wave illumination provides very localized uncaging of Ca^{2+} and other caged compound. The resulting distribution of Ca^{2+} may be very physiological similar to that induced by opening of Ca^{2+} channel upon the membrane depolarization. Ca^{2+} transients near the cell membrane is also selectively monitored by

this evanescent wave illumination. As the evanescence microscope can be assembled easily even by beginners, this field of research is very promising in future.

5. ACKNOWLEDGEMENTS

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