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SUBMICROSECOND IMAGING UNDER A PULSED-LASER FLUORESCENCE MICROSCOPE. ELECTROPORATION OF CELL MEMBRANE TIME- AND SPACE-RESOLVED

Kazuhiko KINOSITA, Jr.,[1]* Masahiro HIBINO,[2] Masaya SHIGEMORI,[3] Ikuo ASHIKAWA,[1]** Hiroyasu ITOH,[4] Kuniaki NAGAYAMA,[5] and Akira IKEGAMI[1]***

[1]*RIKEN (The Institute of Physical and Chemical Research), Hirosawa 2-1, Wako-shi, Saitama 351-01;* [2]*Department of Physics, Faculty of Science and Technology, Keio University, Hiyoshi 3-14-1, Kohoku-ku, Yokohama-shi, Kanagawa 223;* [3]*Department of Physics, Faculty of Science, Gakushuin University, Mejiro 1-5-1, Toshima-ku, Tokyo 171;* [4]*Tsukuba Research Laboratory, Hamamatsu Photonics K. K., Tokodai 5-9-2, Tsukuba-shi, Ibaragi 300-26;* [5]*JEOL Ltd., Musashino 3-1-2, Akishima-shi, Tokyo 196, Japan*

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Abstract. A fluorescence microscope system is described in which a pulsed dye laser is used as the excitation source of the fluorescence. The pulsed illumination allows imaging with an exposure time of less than 0.3 μs. Microsecond kinetics in a single cell can be resolved temporally and spatially.

Under this system, transmembrane potential in a sea urchin egg was visualized by staining the cell membrane with a voltage-sensitive fluorescent dye. When the egg was exposed to an external electric field, the membrane potential in the region facing the anode rose with time, with a time constant of the order of 1 μs, whereas an opposite change was observed in the region facing the anode. Both the kinetics and spatial distribution of the induced potential were in accord with theoretical predictions in which the membrane conductance was assumed to be negligible. Under an intense field, however, the membrane structure was perturbed. Once the absolute magnitude of the potential reached a critical value of about 1 V, further increase was inhibited by an abrupt increase in the ionic permeability of the membrane: Apparently, aqueous pores were introduced in those regions of the membrane which had experienced the critical potential (electroporation). The pores were formed within 1 μs after the critical potential was reached, and the total pore area amounted to 0.01–0.1 % of the membrane area. After the removal of the

Present address: *Department of Physics, Faculty of Science and Technology, Keio University, Hiyoshi 3-14-1, Kohoku-ku, Yokohama-shi, Kanagawa 223; **Tsukuba Research Laboratory, Harima-Kasei Ltd., Tokodai 5-9-3, Tsukuba-shi, Ibaragi 300-26; ***Keio University School of Medicine, Hiyoshi 4-1-1, Yokohama-shi, Kanagawa 223, Japan.

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external field the perforated membrane recovered in the time range of submillisecond.

Prospects for the improvement of the performance of the current system and the development of other types of pulsed-laser fluorescence microscopes, together with possible applications, are discussed.

INTRODUCTION

Fluorescence microscopy is now an indispensable tool of cell biology. Specific labeling with a fluorescent dye permits selective observation of a desired molecular species in the complex cellular architecture. The essentially dark background in fluorescence images, as a result of the selectivity, confers a very high sensitivity to the fluorescence detection: Observation of individual molecules is already within the reach of current technology.[1,2] Moreover, fluorescence imaging is not simply a means of showing the location of matters. Numerous fluorescent probes have been developed which respond to Ca^{2+}, pH, membrane potential, etc.[3] With these probes one can detect and follow various cellular processes as sequential images.

One problem in fluorescence micro-imaging is the difficulty in achieving a high temporal resolution. Thus, video recording normally requires 33 ms per frame, and the image lag in the ultra-sensitive camera used in fluorescence detection often limits the resolution to about 0.1 s. Even with an ideal camera a temporal resolution much better than 1 ms is impracticable under ordinary illumination, simply because the number of photons per image would be insufficient.

Pulsed illumination is the answer for overriding the resolution limit. Using a pulsed laser as the excitation source we have been able to achieve a temporal resolution of 0.3 μs in fluorescence micro-imaging.[4,5] Rapid cellular phenomena can now be detected with resolutions of a submicrosecond temporally and submicrometer spatially. Further development of the technique may well allow the imaging of rapid molecular movements under a fluorescence microscope.

Figure 1 shows the pulsed-laser fluorescence microscope that allows imaging with an exposure time of only 0.3 μs.[4,5] A pulsed dye laser delivers a light pulse of duration 0.3 μs into an inverted epifluorescence microscope. A fluorescent sample on the specimen stage receives this light pulse and emits fluorescence. If one looks at the sample through the eye piece, the flashing fluorescence image lasting only 0.3 μs can be seen by eye as an afterimage.

For quantitative measurement the image is captured by a highly sensitive video camera (type SIT, silicone intensified target). In the camera the incoming light, the flashing image, is converted into, and held in the form of, a charge distribution on a target plate. The electrical image is then read, and at the same time erased, by raster scanning over the plate at the video rate of 33 ms per frame. The output signal from the camera is digitized and recorded in a frame memory in an image processor.

The microscope system thus operates basically as a single-shot strobe camera. Its time resolution is determined by the duration of the excitation light pulse. The use of the pulsed source eliminates the need for a high-speed shutter: The instantaneous image can be recorded on a relatively slow detector such as the eye or video camera.

The pulsed-laser fluorescence microscope enables one to visualize a transient

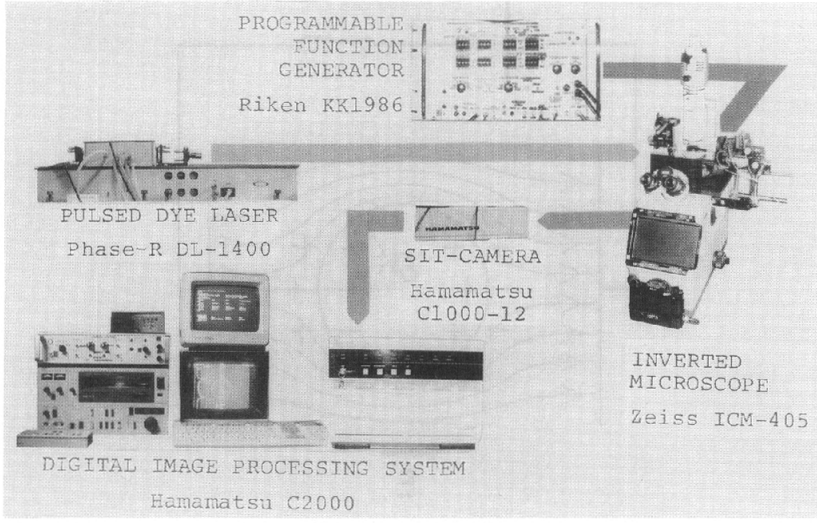

Fig. 1. Pulsed-laser fluorescence microscope. The function generator at the top is for electrical stimulation of the sample.

phenomenon that occurs for a very short period of time, or to time-resolve a fast process. Since it is a single-shot system, however, only one image is acquired in a fast event. (The video camera records at most 30 images per second, and the laser operates at about 1 Hz.) To resolve the event into a sequence of images, the event has to be repeated and the laser triggered at a different moment in each cycle. The current system is thus suited for the analysis of reproducible phenomena. A system for sequential imaging of a single transient is discussed in PROSPECT below.

MEASUREMENT OF TRANSIENT TRANSMEMBRANE POTENTIAL

As an example of application we report on the measurement of transmembrane potential using a voltage-sensitive fluorescent dye.[4,5] The transmembrane potential that we discuss here is the one induced in a cell by an externally applied electric field. Unlike the physiological transmembrane potential which is uniform all over the cell surface, the field-induced potential changes its magnitude and sign along the cell surface (see below). The induction takes place in a time of the order of 1 μs. Thus, visualization of the induction process requires a high resolution both spatially and temporally.

The induction of transmembrane potential by an external electric field has been treated theoretically.[6] When a spherical cell of radius a is suddenly exposed to a uniform electric field E, the ions inside and outside the cell flow along the field, positively and negatively charged ions in opposite directions (Fig. 2). The flow, however, is arrested by the cell membrane which poorly conducts ionic current, and ions accumulate on the surfaces of the membrane until the electric field inside the cell becomes zero and the intracellular current ceases. The profile of electrical

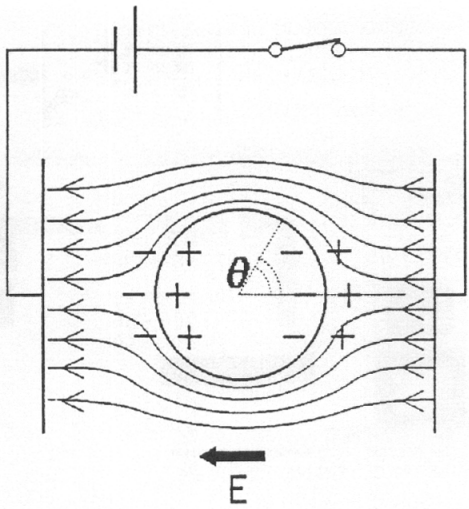

Fig. 2. Induction of transmembrane potential.

potential at the final steady-state is shown in Fig. 3, left: The potential is flat inside the cell, whereas it decreases from a high level at the positive electrode to a low level at the negative electrode as the ionic current continues to flow around the cell. The result is the formation of a large potential difference $\Delta\Psi \equiv \Psi_e - \Psi_i$ across the membrane. The development of the transmembrane potential $\Delta\Psi$, or the accumulation of ions on the membrane surfaces, requires a time τ which depends on the electrical capacitance of the membrane. The theory predicts

$$\Delta\Psi = 1.5aE \cos\theta[1 - \exp(-t/\tau)] \tag{1}$$

$$\tau = aC(r_i + r_e/2) \tag{2}$$

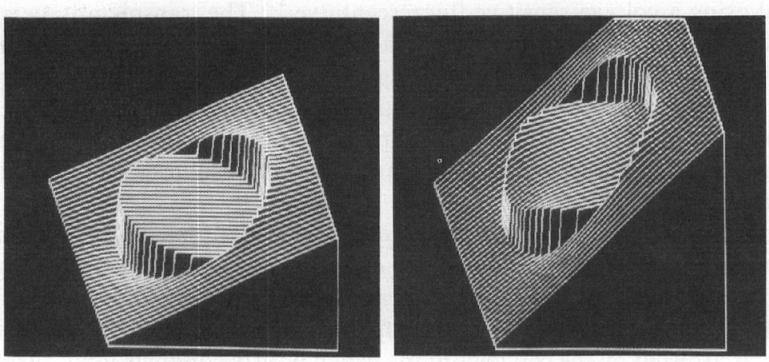

Fig. 3. Potential profiles around a cell exposed to an electric field. Left, zero membrane conductance; right, after electroporation.

where θ is the angle defined in Fig. 2, C is the membrane capacitance per unit area, and r_i and r_e are specific resistances of the intra- and extracellular media.

To measure $\Delta\Psi$ in Eq. (1) we stained the extracellular surface of the cell membrane of a sea urchin egg with the voltage-sensitive fluorescent dye RH292. This dye increases its fluorescence intensity for positive $\Delta\Psi$ and decreases the intensity for negative $\Delta\Psi$.[7] In the configuration shown in Fig. 2, therefore, we expect a stronger fluorescence on the cell surface opposing the positive electrode and a weaker fluorescence on the negative side.

The results are shown in Fig. 4, where four fluorescence images of the dye stained egg membrane at indicated times after the application of an electric field are seen as bright circles. The fluorescence on the positive electrode side rose with time while it fell on the negative side as expected. The rise time τ calculated from Eq. (2) is about 5 μs (a=0.05 mm, C=1 μ F/cm^2, r_i=200 Ω·cm, r_e=1.6 kΩ·cm for the low-salt medium), which agrees with the observed data. The rise time in sea water was about 1 μs both theoretically and experimentally. This last observation also implies that the response time of the dye RH292 was less than 1 μs.

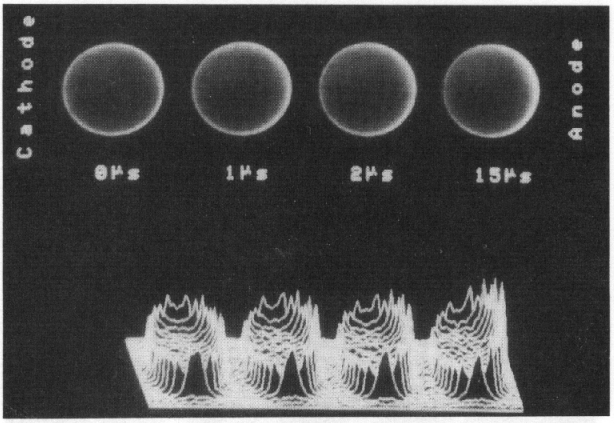

Fig. 4. Time course of the induction of transmembrane potential at 100 V/cm.

The induction of transmembrane potential by an external field is a purely physical phenomenon. At the beginning of this century when an electron microscope was not yet available, this phenomenon played an important role in predicting the presence of an insulating membrane with a thickness about 10 nm on the surface of cells.[6] At that time, however, the spatial and temporal behavior of $\Delta\Psi$ predicted in Eqs. (1) and (2) could not be measured directly. Figure 4 is the first experimental demonstration simultaneously of the spatial and temporal dependences.

In Fig. 4 the applied filed was 100 V/cm, and thus $\Delta\Psi$ at steady state (15 μs) was calculated to be +0.75 V on the positive electrode side and −0.75 V on the negative side. The intensity profile at the bottom shows that the fluorescence intensity of RH292 changed several tens of % per V. A separate experiment showed

that the intensity change was approximately proportional to $\Delta\Psi$ when $\Delta\Psi$ was below 1 V.

Under a higher field where $\Delta\Psi$ would exceed a critical value of about 1 V the cell membrane becomes permeable to small molecules as though aqueous pores are introduced.[8] This phenomenon, called electroporation, is seen in Fig. 5, where the egg was exposed to a field of 400 V/cm, 4 times higher than the field in Fig. 4. Had the membrane remained intact the induced potential should have been 4 times that in Fig. 4, or the fluorescence response should have been like the one represented by the profiles in thin line in Fig 5. The actual response (the images and the thick profiles in Fig. 5) was smaller particularly in those regions of the membrane that opposed the electrodes. There the fluorescence change, or the induced potential, saturated after 1 μs and even a slight reversal was observed. Only at the equator (the top and bottom in the images) where the magnitude of the induced potential was small did the potential continue to grow during the observed period. This behavior can be explained by the formation of pores in those regions where $\Delta\Psi$ reached the critical value of about 1 V. The pores will allow the passage of transmembrane ionic current, which will decrease $\Delta\Psi$ as seen in the theoretical potential profile in Fig. 3, right.

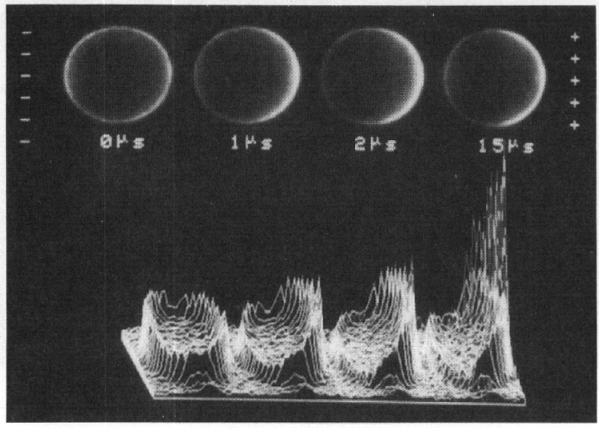

Fig. 5. Changes of transmembrane potential at 400 V/cm.

Figure 5 shows that the pores were formed within 1 μs after the potential reached the critical value. Analysis showed[4] that the electrical conductance of the porated membrane was very high, corresponding to the replacement of 0.01 to 0.1% of the membrane area by aqueous openings. This high permeability state, however, was transient: After the removal of the applied electric field the membrane conductance decreased in the submillisecond time range and became less than 1/10 of the peak value by 1 ms.

PROSPECT

The pulsed-laser fluorescence microscope can be used in many ways other than the imaging of transmembrane potential. For example, shown in Fig. 6 are polarized fluorescence images of a sea urchin egg stained with the dye RH292. This dye, being a long amphiphilic molecule, is expected to be inserted in the membrane perpendicularly to the membrane surface. Figure 6 shows that the transition moment of the dye, parallel to the major axis of the molecule, was in fact perpendicular to the membrane. These images were taken under the pulsed-laser fluorescence microscope.

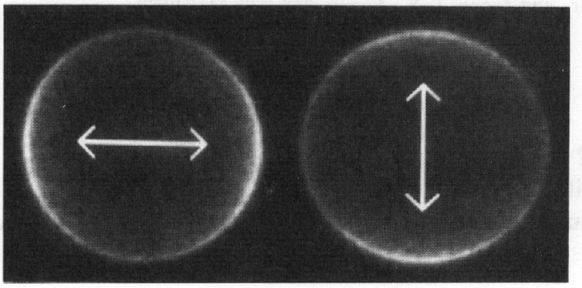

Fig. 6. Polarized fluorescence images of a sea urchin egg stained with RH292. Arrows indicate the direction of polarization.

This implies that we have been able to measure molecular orientations at a resolution of 0.3 μs. (The system that we are developing now allows the simultaneous acquisition of the two polarized images, with a single camera, with an exposure time of a few ns.) Possible disorganization of the membrane structure as a result of electroporation, for example, should be detected in such measurements. So far, however, we have not obtained a positive result: Poration appears to be a highly localized phenomenon that does not significantly affect the average membrane structure.

The pulsed-laser microscope we describe here is basically a single-shot system suitable for the analysis of reproducible phenomena. To analyze a single, irreproducible event into sequential images one needs a light source that produces a burst of light pulses as well as a detector that can record the burst of multiple images separately. We are now developing such a system. This system with a fast camera can also be used in a single-pulse excitation mode. Then we can obtain fluorescence or phosphorescence lifetime-resolved images: Sequential images of the luminescence that develop after the pulsed excitation are captured. Combined with the polarization optics this set-up should be powerful in analyzing molecular motions in a living cell.

REFERENCES

1) Taylor, D. L., Waggoner, A. S., Lanni, F., Murphy, R. F., and Birge, R. R. eds.: *Applications of Fluorescence in the Biomedical Sciences*", Alan R. Liss, Inc. (1986).
2) Harada, Y. and Yanagida, T.: *Cell Motility Cytoskeleton*, **10**, 1–6 (1988).
3) Haugland, R. P.: *Handbook of Fluorescent Probes and Research Chemicals*", Molecular Probes, Inc. (1985).
4) Kinosita, K., Jr., Ashikawa, I., Saita, N., Yoshimura, H., Itoh, H., Nagayama, K., and Ikegami, A.,: *Biophys. J.*, **53**, 1015–1019 (1988).
5) Kinosita, K., Jr., Ashikawa, I., Hibino, M., Shigemori, M., Yoshimura, H., Itoh, H., Nagayama, K., and Ikegami, A.: *Proc. O-E/LASE'88*, **909**, 271–277 (1988).
6) Cole, K. S.: "*Membrane, Ions and Impulses*", University of California Press, Berkeley (1972).
7) Grinvald, A., Hildesheim, R., Farber, I. C., and Anglister, L.: *Biophys. J.*, **39**, 301–308 (1982).
8) Kinosita, K., Jr., and Tsong, T. Y.: *Nature*, **268**, 438–441 (1977).

DISCUSSION

Q1. Is RH292 non-toxic?
Q2. Does it diffuse?
Q3. Could you use it in multicellular organisms to image the nervous system?
(Howard, C. V.)

A1. It is not toxic: for example, tetrahymena stained heavily with RH292 continued swimming at least for a few days. Stained cells, however, die under intense illumination (e.g. a few min. of full illumination from 100 W Hg-lamp through 40× objective ... observation / measurement requires a light level 1/30–1/100 of the killing level).
A2. The dye is water soluble. It will diffuse into organisms through aqueous channels if they exist. The dye also crosses cell membrane and enters the cell if you wait for a long time. If this happens, however, you lose the voltage-dependent signal.
A3. Yes. I haven't done it myself, but Grinwald's group and several others have succeeded in imaging neural activities in brain using related dyes. The major problem encountered at the organism level is that many cells (many membranes) other than the excitable ones will contribute background fluorescence; S/N is much lower compared to single cell measurements.

Q. Is there any biological damage from the sub-microsecond laser pulse?
(Brakenhoff, G. J.)

A. Not at our light level (<1mJ/pulse). Damage does occur at higher levels, but continuous illumination is also harmful (see discussion with Dr. Howard). For the same amount of total energy, pulsed illumination appears to be more harmful than continuous illumination, but the difference is rather small.