

Development of a Fiber-Optic Plate Microscope System and Visualization of Cells Stained with Fluorescent Dyes

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A new type of video-microscope system combined with a fiber-optic plate processed in the shape of a needle has been developed; the obtained fluorescence images were analyzed. The fiber-optic plate comprises thousands of optical fibers 3 μm in diameter, a sufficient size for viewing a single cell. Images at areas adjacent to the end of the fiber-optic plate are transmitted into an objective and detected with a video camera. They are then digitized for storage in an image processor. Using this system and fluorometric methods, cultured cells can be sufficiently visualized and any spatio-temporal change in the intracellular free calcium concentration can be measured. These specifications suggest that the activity and morphology of cells in internal parts of a living animal can be measured at the single-cell level by inserting a fiber-optic plate into the target organ.

Keywords Fiber-optic plate, video-microscopy, fluorescence microscopy, calcium measurement

It is quite difficult to visualize cells located in the interior of a living animal, and to measure their physiological activity. In general measurements using a whole animal, information concerning only the surface parts is acquired. In many cases, visualizing the cells of internal parts requires both evulsion and sectioning of the target organs or tissues. Since these treatments cause an interruption of communications between connected organs and tissues, it is suspected that the results obtained from these experiments are much different from those obtained from a whole animal.

A microelectrode is often used to measure the physiological activity of the internal parts of animals. Recently, an optical fiber was used to measure the intracellular calcium ion concentration in a whole tissue.¹ These tools, however, cannot provide any information concerning the morphology of the cells. Using a confocal scanning laser microscope, it is possible to obtain clear sectional images of tissues, even of internal parts, at the single-cell level by removing any blur derived from out-of-focus planes.² However, due to extinction by tissue and mechanical limitations, visualization of deep parts of an animal is almost impossible. The use of a laser microscope is therefore restricted to mainly observations of monolayer cells and tissue sections. In clinical fields, X-ray computer tomography, magnetic-resonance imaging and positron-emission tomography are often used to obtain tomographic images of a body and to determine the structure of organs as well as their

physiological parameters, such as metabolism and blood flow, by non-invasive methods.³ However, the poor spatial and temporal resolutions of the images obtained from this equipment prevent any real-time measurements at the single-cell level.

We report here on a new video-microscope system combined with a fiber-optic plate processed in the shape of a needle. The fiber-optic plate comprises thousands of optical fibers whose diameter is sufficiently small to recognize a single cell. Inserting a fiber-optic plate into an organ of a living animal and transmitting the image of an area adjacent to the end of the fiber-optic plate to a microscope installed exterior to the animal would make it possible to visualize cells located at internal parts of the body, and then to optically analyze their activity at the single-cell level. In this report, the structure of the system is presented. Cells stained with fluorescent dyes were well visualized using this system.

Materials and Methods

Fiber-optic plate microscope system

The fiber-optic plate microscope system that we report on here is basically just like a video-fluorescence microscope. The structure of this system is shown in Fig. 1. A new device, a fiber-optic plate, was set in front of the objective lens, and facing the specimen. Although in a conventional microscope the objective lens

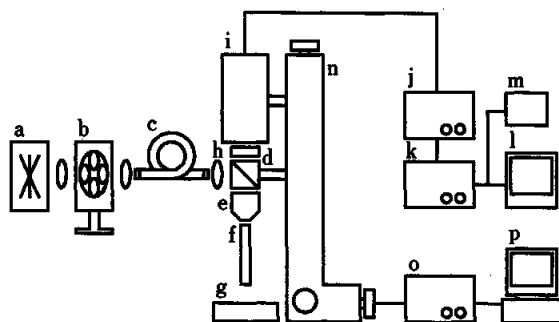


Fig. 1 Schematic drawing of the fiber-optic plate microscope system. a, xenon lamp; b, filter changer; c, bundle optical fiber; d, dichroic mirror; e, objective lens; f, fiber-optic plate; g, stage; h, emission filter; i, ICCD camera; j, camera controller; k, image processor; l, color monitor; m, optical disk unit; n, pulse stage; o, pulse stage controller; p, personal computer.

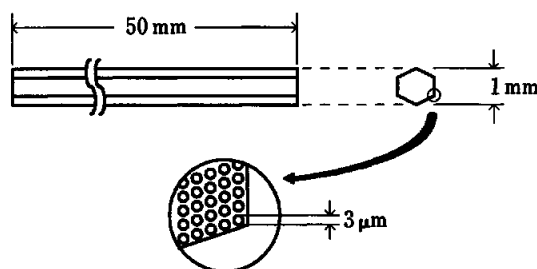


Fig. 2 Drawing of the fiber-optic plate used in the fiber-optic plate microscope system. The fiber-optic plate was processed in the shape of a needle 50 mm in length and 1 mm in diameter. This fiber-optic plate comprises thousands of optical fibers 3 μ m in diameter.

directly faces the specimen, in the fiber-optic plate microscope, the fiber-optic plate transmits an image of a specimen to the objective lens. The fiber-optic plate adopted here was made of glass and processed in the shape of a needle 50 mm in length and 1 mm in diameter. This fiber-optic plate comprises approximately 100000 optical fibers. Each optical fiber has a diameter of 3 μ m (Fig. 2).

A 75 W xenon lamp (AH2-RX, Olympus Optical Co., Ltd., Tokyo, Japan) was used as a light source for excitation. Up to four interference filters were installed in a filter changer (C4312, Hamamatsu Photonics K. K., Hamamatsu, Japan). One of them was selected to determine the excitation wavelength and was put in the light path. The excitation light was guided to a dichroic mirror with a bundle optical fiber made of quartz (2 mm in diameter). A dichroic mirror and an emission filter suitable for the fluorescent properties of a specimen were set up in the microscope. Images of fluorescence detected by an intensified charge-coupled device (ICCD) camera (C2400-87, Hamamatsu Photonics) were fed to frame memories (512 \times 483 pixels, 16 bit) in a digital

image processor (ARGUS-50, Hamamatsu Photonics). The image processor has various functions to improve images (contrast enhancement and noise reduction), to display images (a pseudocolor display and superimposition), and to analyze the intensity of the fluorescence and morphology of the cells quantitatively. The output from the image processor was displayed on a color video monitor and stored in an optical-disk unit. Parts of the microscope, including a fiber-optic plate, an objective lens, a dichroic mirror, an emission filter and an ICCD camera were installed on a three-dimensional pulse stage (Sigma Koki Co., Ltd., Saitama, Japan) in order to move them together and to easily adjust the focus. This stage was controlled so as to move them three-dimensionally every 5 μ m by a stepping motor controller controlled by a personal computer.

Materials

Fluorescent beads (Fluoresbrite) were purchased from Polysciences Inc. (PA, USA). Acridine orange, tetra-rhodamine isothiocyanate (TRITC)-labeled phalloidin, Dulbecco's modified Eagle's medium (DMEM) and dibutyryl adenosine 3',5'-cyclic monophosphate (dibutyryl cAMP) were purchased from Sigma Chemical Co. (MO, USA). Newborn fetal calf serum was purchased from Mitsubishi Kasei Co. (Tokyo, Japan). Fluo-3 acetoxymethyl ester and *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) were purchased from Dojindo Laboratories (Kumamoto, Japan). Other chemicals were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan).

Fluorescent beads

Three types of fluorescent beads were observed in order to estimate the spatial resolution of the system and the transparency of the fiber-optic plate at ultraviolet wavelengths. Two of them with mean diameters of 3.41 μ m and 9.4 μ m, respectively, were coated with a fluorescent dye for blue light excitation (excitation maximum, 458 nm). They were illuminated at 450 nm (bandwidth, 6.5 nm), and fluorescence of wavelength longer than 520 nm was observed. The other type (mean diameter of 9.33 μ m) was used for ultraviolet light excitation (excitation maximum, 365 nm). It was illuminated at 340, 360 and 380 nm (bandwidth, 10 nm), and fluorescence at 510 nm (bandwidth, 40 nm) was then observed. A 40 \times objective lens (DPlanApo UV40, numerical aperture, 0.85, Olympus) was used for observations of all the fluorescent beads. All of the fluorescence images were obtained by integrating 64 consecutive frames followed by subtraction of dark noise of the ICCD camera.

Cell culture

NG108-15 cells⁴, a hybrid cell line between the neuroblastoma and the glioma, were cultured at 37°C in DMEM containing 5% newborn fetal calf serum under a humidified atmosphere with 5% CO₂. For experimental use, cells were plated onto glass coverslips and

cultured in a medium containing 1 mM dibutyryl cAMP for 4–6 days so as to induce neuron-like differentiation.

Staining of the cells with fluorescent dyes

Some cells were incubated for 10 min at room temperature with acridine orange of 5 $\mu\text{g}/\text{ml}$ in a non-fluorescent recording medium comprising 20 mM HEPES (pH 7.4), 115 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl_2 , 1.8 mM CaCl_2 , and 13.8 mM D-glucose. The cells were washed three times and placed on the stage of the fiber-optic plate microscope. For observations of the fluorescence images, the cells were illuminated at 490 nm (bandwidth, 7.3 nm), and fluorescence with wavelengths longer than 520 nm was detected.

Other cells were rinsed in the recording medium three times and fixed for 10 min in 3.7% formaldehyde at room temperature. After being washed three times, the cells were dehydrated in acetone for 3 min at -20°C and air-dried. Actin fibers in cells were stained with TRITC-labeled phalloidin at a concentration of 0.5 $\mu\text{g}/\text{ml}$ in the recording medium for 30 min at room temperature. The cells were then washed and examined using the fiber-optic plate microscope. For observations of the fluorescence, the cells were illuminated at 510–560 nm and fluorescence with wavelengths longer than 590 nm was detected.

All the fluorescence images were observed with the 40 \times objective and obtained by integrating 64 consecutive frames followed by subtraction of dark noise of the ICCD camera.

Imaging of $[\text{Ca}^{2+}]_i$

The cells were incubated for 60 min at 37°C with 10 μM fluo-3 acetoxymethyl ester, which is a fluorescent probe for measurements of the concentrations of intracellular free calcium ions ($[\text{Ca}^{2+}]_i$)⁵ in the recording medium. The cells were then washed three times and placed on the stage of the fiber-optic plate microscope. In order to observe the fluorescence of fluo-3, the same type of filters as those used for acridine orange were used. The fluorescence images were acquired by integrating of 64 consecutive frames. After image acquisition of the resting cells, the medium was exchanged for a solution containing a higher concentration of potassium (80 mM KCl in the recording medium), and images of the stimulated cells were obtained. The fluorescence ratio images were obtained by dividing (pixel by pixel) the images by the resting cell image after subtracting any dark noise.⁵ The ratio images were displayed in pseudocolors corresponding to the value of the fluorescence ratio.

Results

Imaging of fluorescent beads

The images of fluorescent beads for blue light excitation obtained by the fiber-optic plate microscope system are shown in Figs. 3a and b. Both beads could

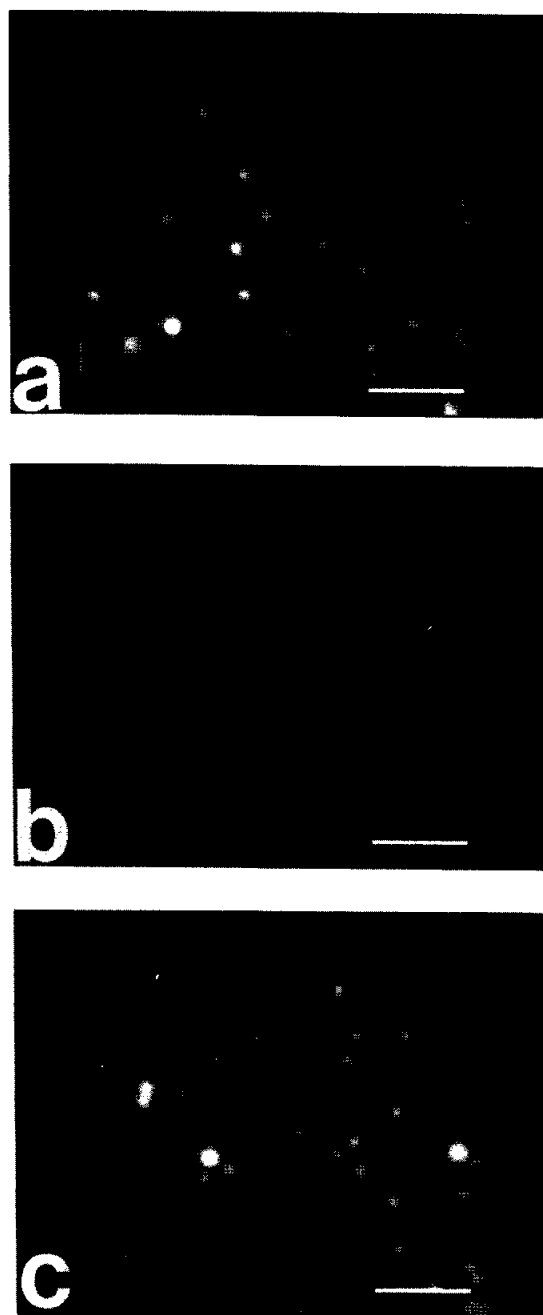


Fig. 3 Images of fluorescent beads obtained with the fiber-optic plate microscope system. a, 9.4 μm beads for blue light excitation; b, 3.41 μm beads for blue light excitation; c, 9.33 μm beads for ultraviolet excitation. Each image was obtained by integrating 64 consecutive frames followed by dark-noise subtraction. Bar, 50 μm .

be imaged clearly with high contrast. Those beads which adhered particularly to the end of the fiber-optic plate were observed as being clearly in focus. Grid-like patterns overlapping the images of the fluorescent beads show partitions of the optical fibers in the fiber-optic plate. The partitions substantially corresponded to the elements to form an image. Beads as small as 10 μm therefore looked like polygons having various shapes,

instead of having their original globular shape. Some of the 3 μm beads were observed as having different shapes and sizes compared to their original ones, since the fluorescence emitted from the beads located on the partitions was shared among a few of the neighboring optical fibers.

The beads for ultraviolet light excitation were also imaged with high contrast when they were illuminated at 380 nm (Fig. 3c). During excitation with light of wavelength shorter than 380 nm, however, the fluorescence of the beads could not be observed clearly (data not shown). It was thought that this was caused by limitations in the transparency of the fiber-optic plate for ultraviolet wavelengths.

Imaging of cells stained with fluorescent dyes

Those cells stained with acridine orange are shown in Fig. 4. From observations obtained with an ordinary fluorescence microscope it was found that the cells were stained with acridine orange very well. In brief, green



Fig. 4 Image of NG108-15 cells stained with acridine orange. The image was obtained by integrating 64 consecutive frames, followed by dark-noise subtraction. Bar, 50 μm .

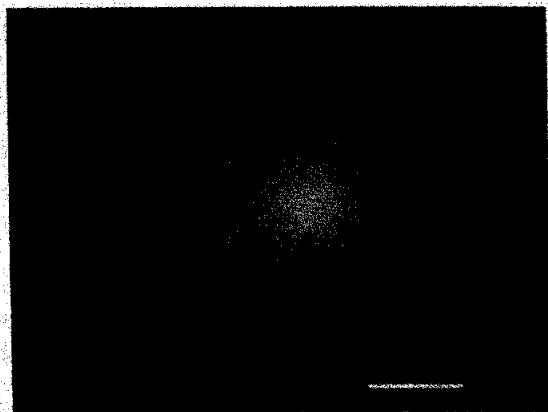


Fig. 5 Image of NG108-15 cells stained with TRITC-labeled phalloidin. The image was obtained by integrating 64 consecutive frames, followed by dark-noise subtraction. Bar, 50 μm .

fluorescence emitted from the nucleus and cytoplasm was observed. Some of vesicles distributed in the cytoplasm and in the nerve fibers emitted red fluorescence. Using the fiber-optic plate microscope, fine structures in the cell, such as vesicles, could not be recognized, although the shape of an entire cell could be sufficiently recognized.

TRITC-labeled phalloidin is a fluorescent probe that is used for visualizing cytoskeletal structures constructed with F-actin.⁶ From observations of stained cells with an ordinary fluorescent microscope, the axons and peripheral parts of a cell were shown to emit more intense fluorescence, and a great number of fine fibers in the cell body could be recognized. With the fiber-optic plate microscope, although the shape of the entire cell as well as the distributions of expanded nerve fibers could be well recognized, the fine fibers could not be recognized, and the cell body seemed to be stained uniformly (Fig. 5). It was thought that the difference between the images obtained with the fiber-optic plate microscope and those with an ordinary fluorescence microscope was dependent on differences in the resolving power between these microscopes.

Imaging of changes in $[\text{Ca}^{2+}]$

The fluorescence emitted from cells stained with fluo-3 could also be detected, although it was weaker than that of the fluorescent beads and other fluorescent dyes used (Fig. 6d). It was difficult to obtain in-focus images, since the fiber-optic plate was detached from the cells in order to avoid damaging them. Stimulus with a high-potassium solution intensified the fluorescence, thus increasing the fluorescence ratio (Figs. 6a – c), indicating an increase of $[\text{Ca}^{2+}]$. It seemed that elevating the external potassium ion-induced depolarization of the cell membrane and this stimulus caused Ca^{2+} entry via voltage-gated Ca^{2+} channels. Fluorescence ratioing is often used to cancel out any unevenness in the path length and illumination of the excitation light. The change in the shape of the cells during the experiment was insignificant. It was therefore thought that ratioing of the post- to the prestimulus cell image was an effective method for estimating the distribution of $[\text{Ca}^{2+}]$. This result showed that the fiber-optic plate microscope could be used to measure any change in $[\text{Ca}^{2+}]$ at the single-cell level.

Discussion

The fiber-optic plate used here comprised a great number of optical fibers (see Fig. 2). The diameter of each fiber was 3 μm , which corresponded to the limits of the spatial resolution of this system. Although this resolution is inferior to that of a conventional microscope, it is adequate for use in observations at the single-cell level. The 3 – 10 μm beads and cultured cells could, in fact, be sufficiently observed. Since the cells stained with acridine orange and TRITC could be imaged, it

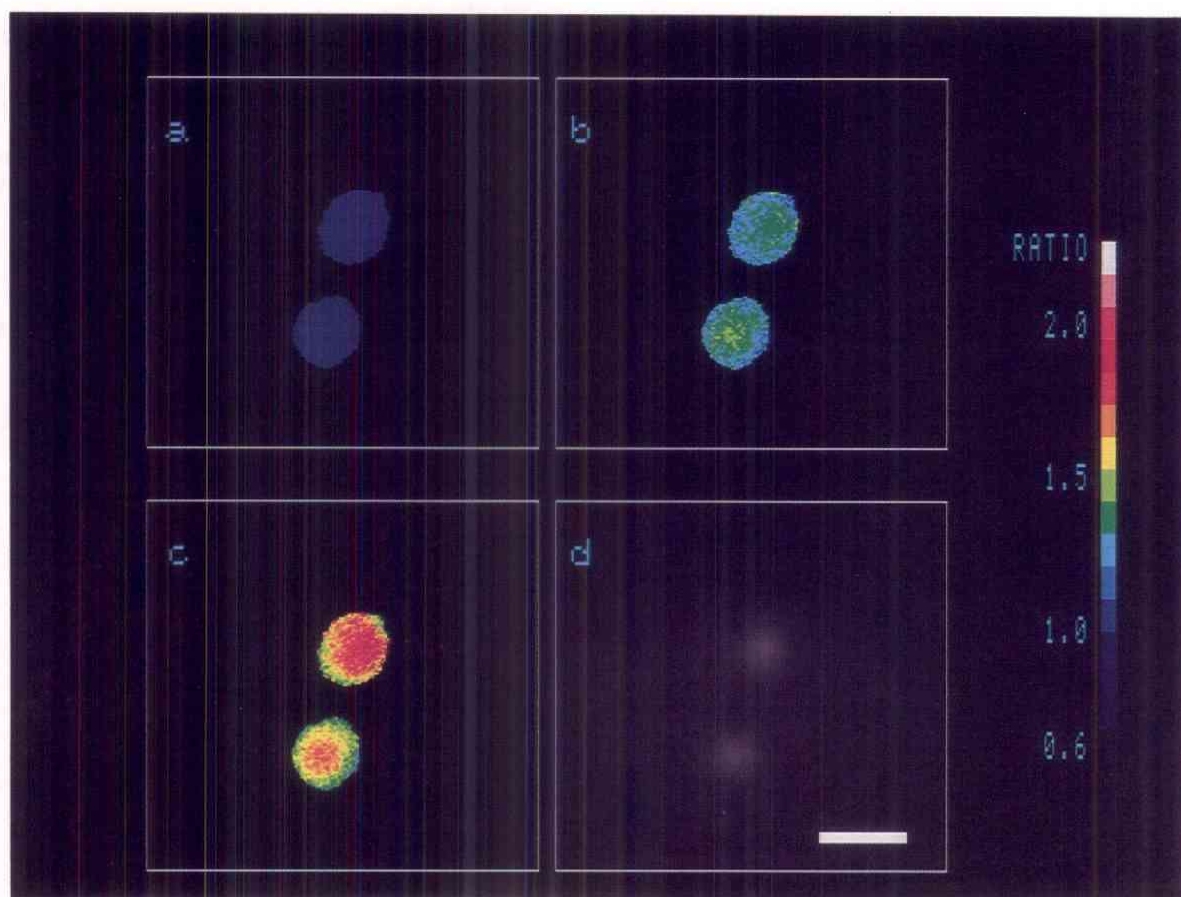


Fig. 6 Imaging of the change in the intracellular free calcium concentration. a–c, fluorescence ratio images of 0, 30 and 60 s after high potassium stimulation. The colors correspond to the value of the ratios, as shown in the scale at the right-hand side of the image; d, a fluorescence image of cells stained with fluo-3. Bar, 50 μ m.

seems that this microscope has sufficient sensitivity to detect the fluorescence of dyes generally used in cell biological fields. However, the fiber-optic plate transmits very little light of wavelengths shorter than 380 nm. When fluorescent dyes with optimum excitation wavelengths in the ultraviolet range are used, the excitation light should be directly guided to the specimen by another light path.

Many fluorescent probes which specifically label molecules in cells, such as nucleic acids, cytoskeletons and cell membranes, have been developed. They have been used not only to detect and quantify the molecules in a cell, but also to obtain much information concerning the structure and function of the cell.⁷ Hitherto, a large number of fluorescent probes for measuring several kinds of intracellular ions (e.g. Ca^{2+} , Mg^{2+} , Na^+ , K^+ , pH etc.) and the membrane potential have been developed and used for measuring the physiological activity of various kinds of cells.⁸ Video-microscopy makes it possible to analyze the dynamic process in a living cell expressed as optical, morphological and motional changes. For example, the spatio-temporal change in $[\text{Ca}^{2+}]_i$ could be measured with this system.

However, the applications of these probes have so far been limited mainly to cultured or isolated cells and tissue sections. It is suspected that the results obtained in these ways differ greatly from those obtained with a whole animal due to interruptions caused in the communications between the connected tissues and the organs. The results of this study suggest the possibility that the insertion of a fiber-optic plate could enable the physiological activity of cells in the internal parts of a living animal to be measured in real time, and that information concerning their morphology could also be obtained.

Images obtained by low-light applications tend to be insufficient regarding the signal-to-noise ratio and contrast. This gives rise to difficulties in observing details concerning the specimen. In these cases, digital image-processing techniques, such as noise reduction and contrast enhancement, are required to improve the image. It is thought that a histogram transformation of the gray level is an effective method for a contrast enhancement. A variety of mask operations are also effective to reduce random noise, enhancing details and detecting the edges of a specimen.⁹

For clinical use, it is expected that the fiber-optic plate microscope system will be utilized for both preliminary and immediate diagnosis of affected organs, instead of by preparing stained tissue sections for pathological tests. It may also be used in the visualization of tissues located in regions where it would be difficult to incise in an operation, such as in the brain.

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