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Injection of a Fluorescent Microsensor into a Specific Cell by Laser Manipulation and Heating with Multiple Wavelengths of Light

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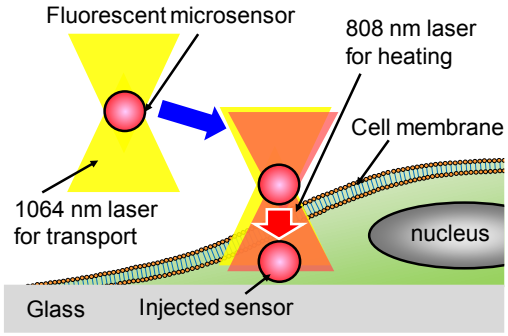
***Abstract*— In this study, we propose the manipulation and cell injection of a fluorescent microsensor using multiple wavelengths of light. The fluorescent microsensor is made of a 1-µm polystyrene particle containing infrared (IR: 808 nm) absorbing dye and Rhodamine B. The polystyrene particle can be manipulated in water using a 1064-nm laser because the refractive index of the polystyrene is 1.6 (refractive index of water: 1.3). The IR absorbing dye absorbs 808-nm light but does not absorb the 1064-nm laser. Rhodamine B is a temperature-sensitive fluorescent dye (excitation wavelength: 488 nm, emission wavelength: 560 nm). The functions of manipulation, heating for injection, and temperature measurement are achieved by different wavelengths of 1064 nm, 808 nm, and 488 nm, respectively. The temperature increase of fluorescent microsensor with 808-nm (40 mW, 10 s) laser was approximately 15°C, and enough for injection of fluorescent microsensor. We demonstrated manipulation and injection of the microsensor into Madin-Darby canine kidney cell using 1064-nm and 808-nm lasers. These results confirmed the effectiveness of our proposed cell injection of a fluorescent microsensor using multiple wavelengths of light.**

I. Introduction

Recent advances in micromanipulation and micro-sensing techniques have enabled the analysis of intracellular environmental and mechanical parameters and activity changes due to external stimuli through manipulation and measurement within specific cells [1]. For example, with the infection of a cell with an influenza virus, the growth of the virus in the nucleus leads to (1) an increase in temperature with the consumption of ATP [2], and (2) a decrease in intracellular pH [3]. Techniques for direct measurement of intracellular environmental parameters are needed to analyze the changes in activity of cells in detail [4]. These techniques can be used to develop potential therapeutic approaches by decoding cell function, inducing cell fate, and reprogramming cell behavior. There are several studies on the measurement of environmental and mechanical parameters inside cells [5]. However, direct measurements of intracellular environmental and mechanical properties are still challenging issues.



manipulation of a fluorescent microsensor and injection of the sensor into a specific cell by local heating using multiple wavelengths of light.

Direct measurement of intracellular environmental parameters requires the injection of microsensors into a specific cell and the subsequent measurement of environmental parameters [6]. Intracellular environmental measurements using microsensors can be classified into tethered and non-tethered approaches. A tethered approach involves inserting sharp micropipettes or cantilevers that have an electrode or optode probe, just a few micrometers in size integrated at the tip, into cells [7]. However, tethered approaches must maintain intracellular and extracellular microsensor connections and are therefore not suitable for long-term and multi-point measurements [8]. Moreover, the microsensor insertion involved in these approaches introduces a risk of cell damage.

Non-tethered approaches involve the injection of microsensors capable of measuring environmental parameters in a non-contact manner, such as magnetic and optical techniques. An oxygen concentration magnetic microsensor has been developed via electron paramagnetic resonance using lithium phthalocyanine microparticles [9]. Although low invasive measurements can be achieved by using magnetic particles with low cytotoxicity, there are limitations such as the need for several tens of seconds for a single measurement and the influence of other environmental parameters [9]. An optical microsensor method has been developed for producing microparticles doped with an environment-sensitive fluorescent dye and measuring changes in environmental parameters such as temperature, pH, oxygen concentration, and ion concentration from the change in fluorescence intensity [10]. Fluorescent dyes that are sensitive to a wide variety of environments have been developed and doping the

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dye into microparticles of low cytotoxicity polymers and hydrogels allows measurement of multiple parameters. The time required for a single measurement of fluorescence intensity is from several hundred milliseconds to a few seconds. We have studied the measurement of environmental parameters of cells using fluorescent microsensors and found that cells infected with influenza virus increased in temperature by 4.2°C and showed a pH reduction of 0.6 4 h after infection on the surface, using a 1 µm fluorescent microsensor [2, 3]. To achieve direct measurement of intracellular environmental parameters using fluorescent microsensors, injection of the microsensor is required.

This paper presents a unique laser injection method that manipulates fluorescent microsensors using optical tweezers and injects the fluorescent microsensor into the cytoplasm by laser heating as shown in Fig. 1. By incorporating a dye with wavelength-selective absorption into a fluorescence microsensor, the two functions of manipulation and cell injection are controlled by switching the wavelength of the input laser. The manipulation of the fluorescent microsensor is conducted using optical tweezers with a 1064 nm laser. The fluorescent microsensors are then injected into the target cell using laser heating with a 808 nm laser. The environmental parameters are measured using fluorescence measurements at wavelengths from ultraviolet to visible light. Fabrication and evaluation of the fluorescent microsensors was conducted and injection of a single microsensor was demonstrated.

II. Related Work

A. *Manipulation method of micro object in water.*

Intracellular measurements with fluorescent microsensors using a non-tethered approach require manipulation and cell injection of fluorescent microsensor as shown in Fig. 2. Micromanipulators [11], micropipette aspiration [12,13], dielectrophoretic (DEP) tweezers [14], magnetic tweezers [15, 16], and optical tweezers [17, 18] have been developed as manipulation techniques for micro objects. While micromanipulators have advantages such as high power and multi-dimensional and high-resolution manipulation; manipulation of the order of 1 µm requires advanced skill. Manipulation of a single fluorescent microsensor by fixing to the tip of a micropipette by aspiration is also challenging for a single microsensor of 1 µm. A microsensor made of dielectric materials can be captured and manipulated with DEP tweezers, however the capture force is as low as 50 pN [14]. Magnetic tweezers achieve precise three-dimensional manipulation of a single microsensor. However, a complex magnetic field circuit must be constructed around the sample on the microscope [16]. Optical tweezers are capable of three-dimensional manipulation of a single microsensor in water to achieve a strong operating force of 100 pN or more. We applied optical tweezers with a 1064 nm laser to manipulate fluorescent microsensors [19].

B. *Injection method of fluorescent microsensors.*

For injection of fluorescent microsensors into a specific cell, high success rates, low invasiveness, and short induction

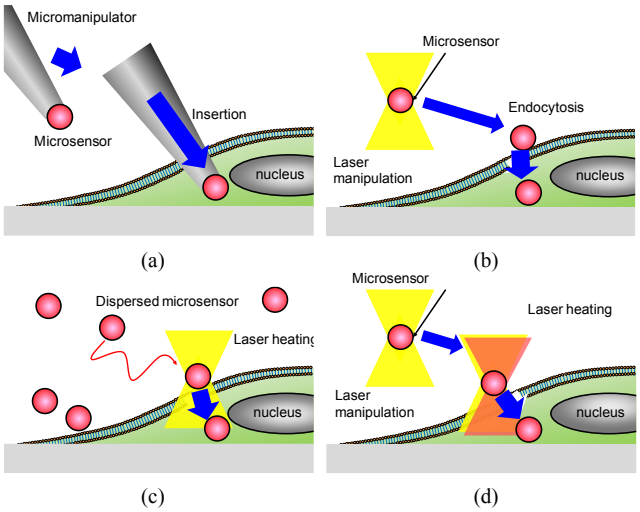

Figure 2. Classification of microsensor injection into a specific cell. (a) Micromanipulation and insertion of the microsensor by a mechanical micromanipulator, (b) Manipulation of the microsensor using optical tweezers and injection by endocytosis, (c) Dispersion of the microsensor and insertion by local laser heating, (d) Manipulation and injection of the microsensor using multiple wavelengths of light.

times are required [20]. Cell membranes naturally resist the penetration of external molecules such as drugs, organic materials, and micro/nanoparticles into the cytoplasm. Thus, successful injection of microsensors requires passage through this barrier by either biochemical or physical methods [21].

To date, biochemical methods such as endocytosis and lipofection and physical methods such as microinjection [20], electroporation [22, 23], and laser heating [24, 25] have been developed. Among the physical techniques, microinjection is a simple approach as shown in Fig. 2(a) [20]. However, the mechanical injection can easily damage cells. Electroporation injects nanoparticles from holes generated by applying a pulse current to the cell membrane, however, there are associated challenges such as difficulty in selective injection of a microsensor and risk of cell damage.

Biochemical techniques such as endocytosis and lipofection are minimally invasive to cells, however, the injection of a 1 µm object takes more than 3 hours. We have been able to reduce the injection time to 30 minutes by local vibration stimulation on the cell membrane using optical tweezers as shown in Fig. 2(b) [19]. However, this is still a long time relative to other methods. Laser heating of metal nanoparticles is accomplished within a few seconds for rapid injection [24]; however, this method has the limitation of low injected cell viability. We have used local laser heating to demonstrate rapid and low invasive injection of magnetic fluorescent microsensors made of one polystyrene microparticle containing 20 nm iron oxide nanoparticles as shown in Fig. 2(c) [26, 27]. By appropriate adjustment of the laser power, injection of a 750-nm fluorescent microsensor was achieved within 2 s with 100% injection success rate and 90% viability [26]. However, metallic fluorescent microsensors cannot be manipulated using optical tweezers because metal materials absorb laser irradiation. However, manipulation of a fluorescent microsensor using optical

tweezers could not be applied since the iron oxide nanoparticles absorb 1064 nm laser irradiation and caused a large temperature to increase during manipulation. Although we also succeeded in the manipulation and cell immobilization of the metallic fluorescent microsensor using a micromanipulator, picking up the metallic fluorescent microsensor and immobilizing the microsensor on the cell membrane take time [27]. Therefore, combination use of manipulation by optical tweezers and injection by laser heating, as shown in Fig. 2(d) is considered suitable for injection of a fluorescent microsensor into a specific cell.

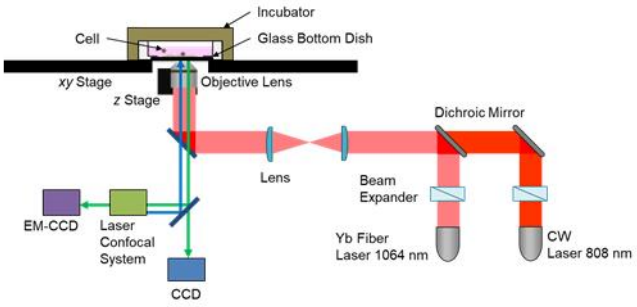

Figure 3. Experimental setup for laser manipulation and heating using multiple wavelengths of light.Figure 1. Schematic diagram of laser

III. EXPERIMENT

A. *Principle of injection of a fluorescent microsensor into a specific cell by laser manipulation and heating with multiple wavelengths of light*

In this study, we propose a unique method for injecting a single fluorescent microsensor into a specific cell using optical manipulation and heating with multiple wavelengths of light, as shown in Figs. 1. The fluorescence microsensor is made of a 1-μm polystyrene microparticle containing Rhodamine B and IR (808 nm) absorbing dye. The polystyrene microparticle can be manipulated in water using a 1064-nm laser as the refractive index of the polystyrene is 1.6 (refractive index of water: 1.3). Rhodamine B is a temperature-sensitive fluorescent dye (excitation wavelength: 488 nm, emission wavelength: 560 nm). The IR absorbing dye absorbs 808 nm light but does not absorb 1064-nm laser light, therefore the wavelengths chosen for each function do not interfere with each other. First, a fluorescent microsensor is manipulated to the target cell membrane using 1064-nm-laser optical tweezers. Then, the fluorescent microsensor is injected into the cytoplasm by heating with an 808-nm laser on the cell membrane.

This newly proposed method for the injection of a fluorescent microsensor into a specific cell using optical manipulation and heating can achieve selective, low invasive, and rapid injection of the fluorescence microsensor.

B. *Experimental setup*

A schematic diagram of the experimental setup consisting of optical tweezers and a heating system with laser-confocal fluorescence microscopy is shown in Fig. 3. A commercial inverted optical microscope (IX71, Olympus, Tokyo, Japan) with a fluorescence observation system was used for observing the microsensor and cell. Fluorescence image was acquired by using the electron multiplying-charge coupled device (EM-CCD) (iXon Ultra, Oxford Instruments plc, UK) and confocal laser scanning system CSU-X1 (Yokogawa Co. Ltd., Japan). The diameter of the pinhole of CSU-X1 was 50 μm. The resolution of EM-CCD was 512 pixels by 512 pixels. Each pixel size was 0.8 μm/pixel. An excitation laser with a wavelength of 488 nm was used to excite the fluorescent dye in the microsensor. The power of the excitation laser was ten mW. In the manipulation of fluorescence microsensor, the frame rate of EM-CCD was 10 frames/s. In fluorescence measurement, exposure time and interval time were 500 ms and 500 ms, respectively. The optical tweezers system, which was generated using a diode pumped Nd:YAG/YVO_4 laser (IPG laser) with 1064 nm wavelength and 10 W maximum power was used for manipulation of the fluorescent microsensor [28]. The power of the 1064 nm laser was adjusted to 40 mW on the objective lens. An 808 nm continuous wave laser (OBIS, Coherent) with a maximum power of 150 mW was used for local heating. The power of the 808-nm laser was also adjusted to 40 mW on the objective lens. The diameter of the circular focus points of both lasers was approximately 1.4 μm. A piezoelectric z-stage (PI) with a high magnification objective lens (Plan Fluor 100×, Olympus, Japan) was used for acquiring 3D fluorescence images. A cell-culture chamber (ZILCOS, Tokai hit, Japan) was used to maintain the temperature of the cell environment at 37°C during the experiment.

C. *Fabrication of the fluorescent microsensor*

In this paper, we fabricated the fluorescence microsensor incorporating temperature-sensitive fluorescence dye and infrared absorbing dye as shown in Fig. 4. Fig. 5 shows the fabrication process for fluorescent microsensors made of 1-μm amino-coated polystyrene microparticles (Funakoshi, Japan), Rhodamine B (Wako pure chemical)—which is a temperature-sensitive fluorescent dye—and IR absorbing dye FDN-002 (Yamada Chemical Industry, Japan). The excitation and emission wavelengths of Rhodamine B were 488 nm and 580 nm, respectively. The IR absorbing dye FDN-002 was used for heating with the 808-nm laser.

The absorption of the 1064-nm laser by this dye was quite low. The fabrication process of the fluorescent hydrogel microsensor was as follows.

1. Injection of 100 μL of amino-coated polystyrene microparticles into a 1.5-mL microtube.
2. Preparation of a staining solution containing 1 mg/L Rhodamine B and 1 mg/mL IR absorbing dye in ethanol.
3. Staining of the amino-coated polystyrene microparticles in the staining solution for 30 min.
4. Centrifuging of the stained microsensors at 10000×g for 15 min for washing.
5. Repeat of the washing process five times.

Figure 6 shows the optical and fluorescence images of the fabricated fluorescent microsensor.

D. Cell culture

Madin-Darby canine kidney (MDCK) cells were used in the injection experiment. MDCK cells were cultured in glass bottom dishes containing 2.0 mL of Dulbecco's modified Eagle medium (DMEM) and 0.2 mL of fetal bovine serum (FBS). The culture conditions were 5% CO_2 and 95% air at 37°C. MDCK cells were cultured for 8 h prior to the injection experiment. MDCK cells were stained with Calcein-AM (excitation wavelength: 488 nm, emission wavelength: 515 nm) to investigate the cell viability after injection. Calcein-AM in the cytoplasm of alive cells is hydrolyzed by esterase activity and emits fluorescence. Therefore, alive cell can be detected by observing the fluorescence of Calcein-AM. 100 µL of 0.5 mg/mL calcein-AM solution was added to the glass bottom dish. After incubation at 37°C for 30 min, the fluorescence of calcian-AM inside the MDCK cells was observed.

IV. Results

A. Size evaluation of the fluorescent microsensor

First, the size distribution of the fluorescent microsensor was evaluated as the staining solution containing ethanol could have dissolved the polystyrene, causing changes in the sensor size and shape. In optical tweezers, the spherical structure is suitable for stable manipulation. In fluorescence temperature measurement, relative fluorescence intensity and temperature were calibrated using fluorescent microsensors with uniform size. If the size of fluorescent microsensors varies, the fluorescence intensity of microsensors varies even at the same temperature.

The mean diameter of the microsensor was evaluated using a tunable resistive pulse sensing (TRPS) nanoparticle analyzer (qNano, Izon Science Ltd., New Zealand). The mean size of the microsensor was evaluated by collecting calibration data using approximately 10000 commercial 1 µm microparticles (4010A, Thermo Scientific, USA). Fig. 7 shows the size distribution of the fluorescent microsensor. The measured mean size of the microsensor was 0.98 µm and the standard deviation was approximately 0.1 µm. These results confirmed that the fabrication process does not affect the sensor size.

B. Temperature calibration of the fluorescent microsensor

There is a proportional relationship between the relative fluorescence intensity of Rhodamine B and temperature ranging from 0°C to 100°C. Therefore, the temperature range of calibration was chosen from 30°C to 40°C to acquire the temperature sensitivity of fluorescent microsensors. Fig. 8 shows the calibration result for the relative fluorescence intensity as a function of temperature. The horizontal and vertical axes show the temperature measured by the thermopile in the chamber and the relative fluorescence intensity based on the fluorescence intensity at 30°C,

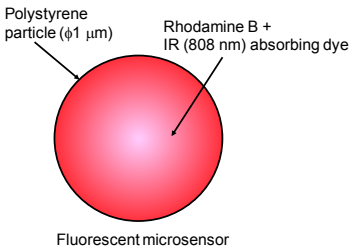

Figure 4. Schematic image of a fluorescent microsensor.

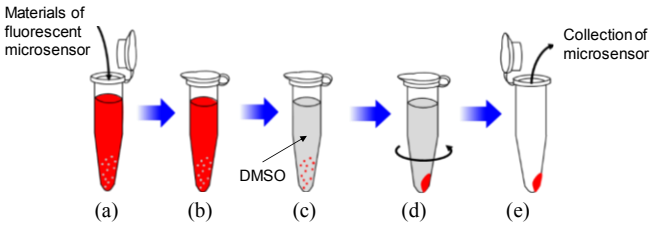

Figure 5. Fabrication of the fluorescent microsensors. (a) Mixing the materials for the fluorescent microsensor, (b) Staining, (c) Replacement of staining solution with water, (d) Washing with DI water, (e) Collection of the microsensor.

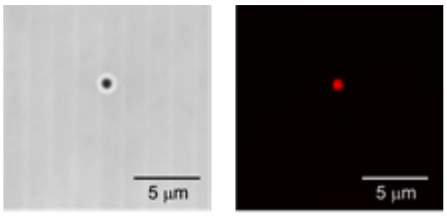

Figure 6. Optical and fluorescence images of the fluorescent microsensor. (a) Fluorescent microsensor in bright field, and (b) the fluorescence image.

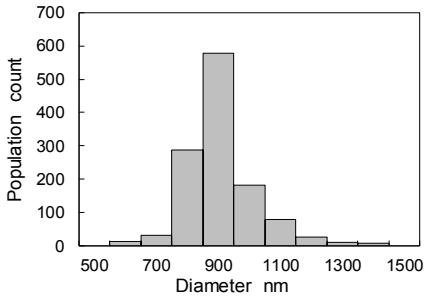

Figure 7. Size distribution of the fluorescent microsensor.

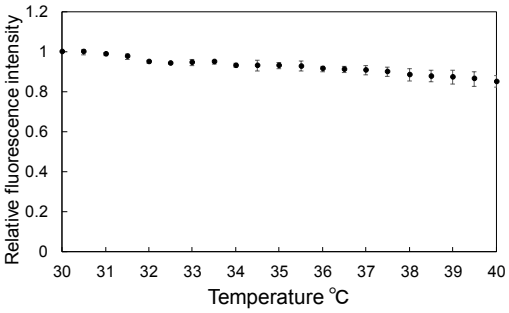

Figure 8. Calibration of the relative fluorescence intensity of the fluorescent microsensor with temperature.

respectively. The fluorescence intensity of the hydrogel fluorescence microsensor decreased monotonically with increasing temperature. The fitting equation obtained using the least squares method is shown in equation 1.

$$\frac{I}{I_0} = -0.015 \cdot Temp. + 1.5 \qquad (1)$$

From equation 1, the temperature sensitivity of the fluorescent microsensor was calculated to be −1.5 %/°C and the precision of the temperature measurement was ±0.5°C.

C. *Heat evaluation of the fluorescent microsensor using 1064 nm and 808 nm lasers*

To confirm the wavelength-specific heating of the fluorescent microsensor, the absorbance properties of the fluorescent microsensor were evaluated as shown in Fig. 9. First, we evaluated the absorbance properties of the FDN-002. A glass microchannel (depth: 150 μm) was fabricated and the FDN-002 ethanol solution was injected into the microchannel. 1064 nm and 808 nm lasers were applied, and the laser power was measured before and after passing through the microchannel. Fig. 9 shows that the absorbance rates of the 1064 nm and 808 nm lasers at 1 mg/mL were 1% and 85%, respectively. This result confirmed the wavelength selectivity of the laser heating of the fluorescent microsensor.

Figure 10 shows the experimental results for the temperature measurement using the fluorescent microsensor during optical heating with 1064 nm and 808 nm lasers. Both laser powers were adjusted by 40 mW. The environmental temperature was maintained at 37°C using the cell-culture chamber. Temperature measurement was conducted every 1 s. First, a single fluorescent microsensor was trapped using the 1064 nm laser to avoid heat diffusion to the glass substrate. After 22 temperature measurements, the fluorescent microsensor was irradiated with the 808 nm laser. In the case of 1064 nm laser irradiation, the temperature remained almost uniform, while the temperature dramatically increased under 808 nm laser irradiation. The approximate temperature of the fluorescent microsensor increased by 15°C following 10 s of heating. In our previous study, 50°C was required to inject a microsensor using laser heating [28]. As the environmental temperature was maintained at 37°C, the observed heating properties were considered sufficient for the injection of the fluorescent microsensor into the cytoplasm. These results confirmed the effectiveness of selective laser heating of the microsensor using an IR absorbing dye.

D. *Manipulation and injection of the fluorescent microsensor into an MDCK cell*

Figure 11 shows the optical injection of the fluorescent microsensor using optical manipulation with a 1064 nm laser and heating using a 808 nm laser. Both laser powers were adjusted to 40 mW. The environmental temperature was maintained at 37°C by a cell-culture chamber. MDCK cells were used as a sample specimen.

As shown in Fig. 11 (a), the fluorescent microsensor was successfully trapped by optical tweezers with a 1064 nm wavelength. After transport of the fluorescent microsensor to the target cell membrane, the fluorescent microsensor was heated using an 808 nm laser for 10 s. After laser heating, the focus plane of the fluorescent microsensor was moved

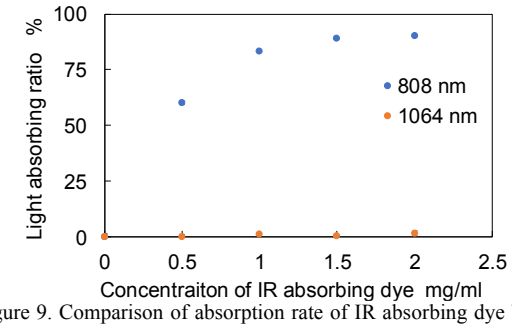

Figure 9. Comparison of absorption rate of IR absorbing dye between 808 nm and 1064 nm (Thickness of microchannel 150 μm).

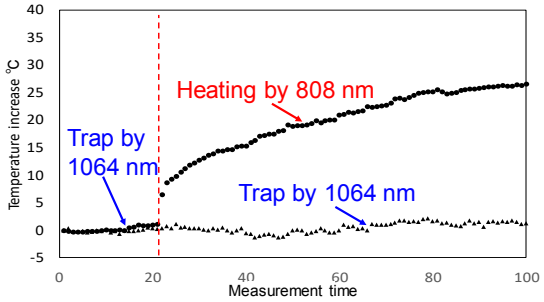

Figure 10. Measurement of temperature increase of fluorescence microsensor by irradiating 808 nm laser.

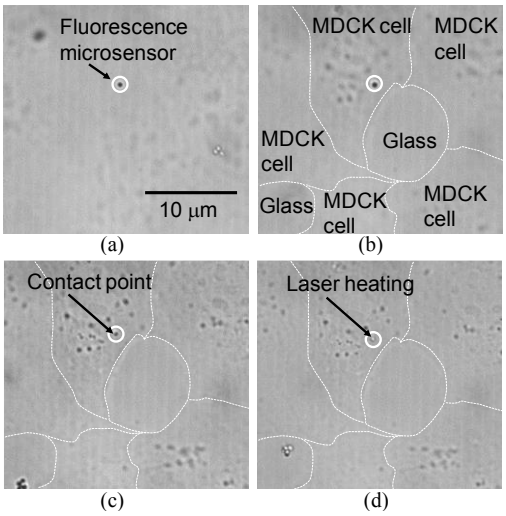

Figure 11. Manipulation and injection of fluorescence microsensor to MDCK cell. (a) Trap of the microsensor, (b) Transport to MDCK cell, (c) Contact to cell membrane, (d) 808 nm laser irradiation to the sensor.

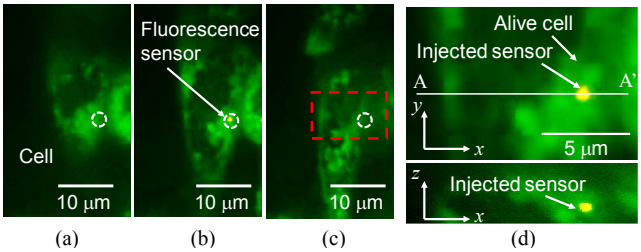

Figure 12. Fluorescence image of injected fluorescence microsensor. (a) Fluorescence image at 3 mm upper to focus plane of the microsensor, (b) Fluorescence image at focus plane of the microsensor. (c) Fluorescence image at 3 mm upper to focus plane of the microsensor. (d) Upper: zoom image of red rectangle at (c), lower: cross-sectional image at A-A' line. (red: fluorescence microsensor, green: cytoplasm of alive cell)

downward (inside the cytoplasm). The success rate of the injection was 70 % (total number of experiments was 10).

To confirm the injection of the fluorescence microsensor into MDCK cells, laser-confocal fluorescence imaging of the MDCK cell and the injected fluorescent microsensor was acquired for the whole cell (pitch of the slice image: 0.5 μm) as shown in Fig. 12. From Fig. 12 (b) we observed the presence of the fluorescent microsensor inside the cytoplasm of the MDCK cell. Moreover, the location of the fluorescent microsensor inside the MDCK cell was confirmed by the cross-section image as shown in Fig. 12(d).

From these results, the effectiveness of the proposed method as confirmed.

V. Conclusion

In this paper, we proposed and experimentally confirmed the optical injection of a single fluorescent microsensor into a specific cell using optical manipulation and heating with multiple wavelengths of light. The fluorescent microsensors stained with Rhodamine B and IR absorbing dye were successfully fabricated. The fluorescent microsensor was not heated by the 1064 nm laser, but the 808 nm laser did induce temperature changes. We therefore succeeded in the trapping and manipulation of the fluorescent microsensor with optical tweezers using a 1064-nm laser. In the current experimental setting, the direct measurement of light absorption property in each 1 μm microsensor was difficult. The direct evaluation of light absorption property is future work. In addition, we succeeded in injecting the fluorescent microsensor into a specific cell by heating with an 808 nm laser. Furthermore, we showed the presence of the injected fluorescent microsensor in the cell and demonstrated that the injected cell was still alive. These findings demonstrate a high success rate (70 %), low invasive, and rapid injection within 10 s. However, the measurement of time required for fluorescent microsensor to cross the cell membrane is difficult in the current experimental system. The measurement of crossing time for fluorescent microsensor is future work.

The presented method can control the temperature of the microsensor during the heating by measuring the temperature of the microsensor. Therefore, this method has the potential for automated injection of fluorescent microsensors to the target cells. Automated injection of a fluorescent microsensor into a specific cell is our future work.

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