Optical pH Regulation Using Functional Nanotool Impregnating with Photo-Responsive Chemical for Intracellular Measurement

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Abstract:
We developed Optical pH regulation using functional nanotool impregnated with photo-responsive chemical for selective cell injection of nanotool. The nanotool was modified by fluorescent dye for intracellular measurement. The nanotool was included in the fusogenic liposome. Membrane fusion of the liposome to the cell membrane was used for invasive cell injection of the nanotool. The liposome fuses to the cell in weak acidic condition. Local pH regulation inside the liposome was developed using photochromic chemical for selective cell injection of the nanotool. The nanotool was modified by Leuco crystal violet (LCV). LCV emits the proton by ultraviolet (UV) illumination. The emitted proton decreases the pH value in the liposome. This pH regulation is reversible by UV/VIS illumination. The liposome was manipulated by optical tweezers. After contact of the liposome to the cell, the liposome was adhered to the cell by UV induced membrane fusion. Injected nanotool was manipulated by optical tweezers. Intracellular temperature was detected by measuring the fluorescence intensity of the nanosensor. We demonstrated optical pH regulation, selective cell injection of the nanotool, and manipulation of the nanotool in the cell.

1. INTRODUCTION

Bio industries employing components of cells and the cell itself have been expanded in recent years. However, many cell functions remain to be discovered [1]. Cell analysis by direct monitoring of cells in the specific environments is expected to reveal unknown cell properties [2]. Measurements of cellular states such as pH, oxygen and temperature were one of the most important issues [3-5]. Moreover, interactions between cell activities and the environmental conditions have not been sufficiently investigated. Investigations of such interactions require measurement of not only ambient conditions of the cells but also intracellular conditions such as distribution of temperature.

Conventionally, cell measurements were conducted by contact manipulation using micro manipulators with micropipette and micro sensors [6]. Many types of microprobe sensor were developed and these sensors were expected to achieve high sensitivity and precise measurement [7, 8]. Although these methods could measure extracellular conditions, intracellular measurement was difficult by using micro manipulators [9]. Although pH measurements using electrochemical reactions have been developed [10], the measurement area of the electrochemical pH sensor is limited by the sensor size and its position. Non-contact manipulation and measurement were necessary to achieve the intracellular measurement [11, 12].

Cell injection of the sensor was also important issue for the intracellular measurement. Cell injection methods such as endocytosis and lipofection have been developed. Endocytosis was one of the most major methods for cell injection. However, direct measurement of the intracellular conditions is difficult because the injected sensor may be included in the endosome. Lipofection was also major as the drag delivery system (DDS). This method could inject the sensor into not a specific cell but the specific cell group. However, selective injection of the artificial nano-object to the individual cell was still difficult.

In this paper, we developed selective cell injection of nanosensor into a specific cell using optical pH regulation for intracellular measurement. We used pH-responsive membrane fusion of the liposome for invasive cell injection of the nanosensor. The nanosensor included in the liposome was modified by photochromic material for pH regulation and fluorescent dye for environment measurement. We used Leuco Crystal Violet (LCV), which is a photo responsive chemical, to induce membrane fusion of the liposome. While LCV emits a proton by UV illumination, emitted proton is absorbed to LCV by VIS illumination. UV/VIS illumination switched the fusion-capability of the liposome was controlled using pH regulation induced by UV illumination. We used optical tweezers to manipulate the liposome and nanosensor. The liposome fused to the cell membrane by UV illumination and the nanosensor was injected into the cell. Injected nanotool was manipulated by optical tweezers.

We demonstrated selective cell injection of the nanotool by optical pH regulation, manipulation of the nanotool using optical tweezers and direct detection of the intracellular temperature using fluorescent observation.

2. MATERIALS AND METHODS

2.1 Optical pH regulation for selective cell injection of the nanotool for intracellular measurement

Figure 1 shows the cell injection process of the nanosensor using optical pH regulation. The nanotool was made of photo-crosslinkable resin. The nanotool was impregnated with LCV and fluorescent dye. The fusogenic liposome was consisted of Dioleoylphosphocholine (DOPC)

and Dioleoylphosphatidylethanolamine (DOPE). DOPC was used for forming the stable structure of the liposome. DOPE was used for fusion to cell membrane. DOPE expresses the fusion-capability in weak acidic condition (< pH6) [13]. DOPE fuses the cell membrane protein such as clathrin in this condition. The liposome including the nanosensor was manipulated by optical tweezers. A solution inside the liposome was adjusted to the neutral condition to avoid damage to the cell after injection. After contact of the nanosensor to the target cell, UV was illuminated to the nanosensor. The pH value inside the liposome was reduced by emission of the proton from the nanosensor. When the pH value was reduced to lower than 6, the liposome presented the fusion-capability as shown in Fig. 2. Through this process, the UV-illuminated liposome fuses to the cell membrane. On the other hand, Un-irradiated liposome doesn't present fusion-capability. Finally, the nanosensor was injected into the cell. Therefore, we can achieve the selective cell injection to the individual cell. Injected nanosensor was manipulated by optical tweezers. Intracellular environment was measured by fluorescent measurement. We could measure the conditions such as temperature from the detected fluorescence intensity of the nanotool and calibration result.

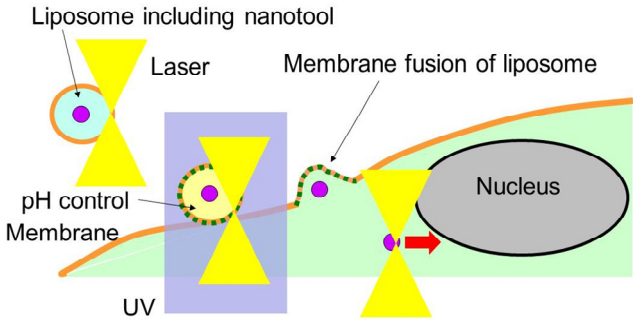


Fig. 1 A schematic of cell injection of nanotool by photo-induced pH control using photo responsive chemical.

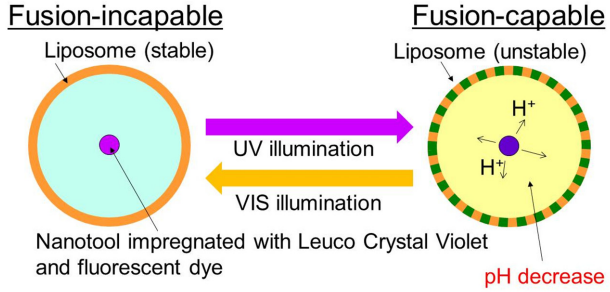


Fig. 2 A schematic of optical pH regulation in the liposome using photo-induced reaction using LCV.

2.2 Optical pH regulation in the liposome using nanotool impregnated photochromic material

We used LCV to regulate pH value inside the liposome for selective membrane fusion. LCV is a photochromic material. Molecular structure of LCV was illustrated as shown in Fig. 3. LCV of leuco type structure changes to anion type structure by UV illumination. LCV emits a proton per one molecule by this structure change. LCV of anion type changes to leuco type structure by VIS illumination. Structural change of LCV is repeatable.

pH value in the liposome was controlled by pH regulation using LCV included in the nanotool as shown in Fig 2. Amount of the maximum pH shift in the liposome was calculated by (1).

$$\Delta pH_{\max} = -Log\left\{\frac{\rho}{M_{LCV}} \times \left(\frac{d}{D}\right)^3 \times m_{lcv}/m_{gb} + 10^{-pH_{in}}\right\} \quad (1)$$

We assumed that each liposome included one nanotool. Structures of both liposome and nanotool were spherical. Where, ΔpH_{max} was amount of the maximum pH shift. M_{LCV} was the molecular weight of LCV. ρ was the density of the resin (1.11 g/ml). D was diameter of the liposome. d was diameter of the nanosensor. m_{lcv}/m_{gb} was mixing ratio of the LCV and resin. pH_{in} was initial pH inside the liposome. We can control the amount of pH shifts by mixing ratio of the resin and LCV. Fig. 4 shows the simulation results of maximum pH shift using (1).

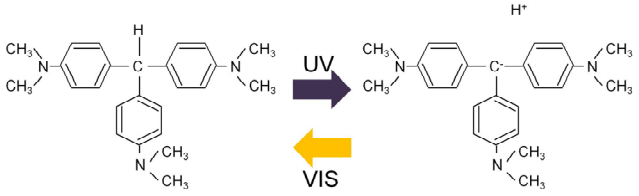


Fig. 3 A schematic of photo-induced reaction using LCV.

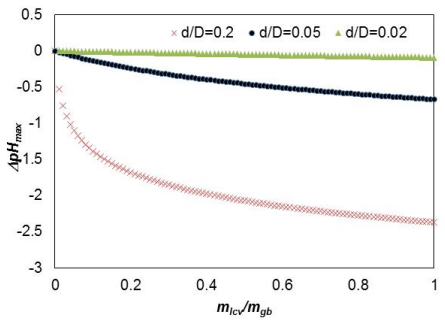
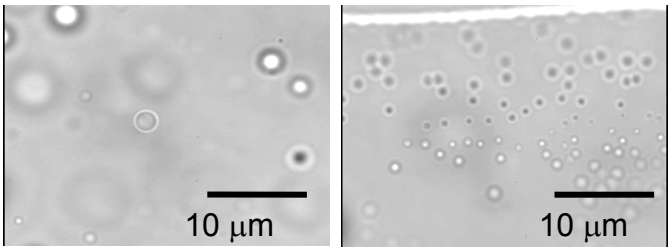


Fig. 4 A schematic of cell injection of nanotool by photo-induced pH control using photo responsive chemical.

2.3 Nanotool for optical pH regulation and intracellular measurement

The nanotool included in the liposome was injected into the cell for intracellular measurement. The nanotool was used as a career of the LCV and indicators. We used photo-crosslinkable resin. Main constitute of this resin is polyethylene glycol (PEG). PEG is biocompatible and the nanotool can be kept in the cell for long-time [14]. This resin mixed with photo-initiator was polymerized by UV illumination (around 366 nm). This resin was aggregated by introducing into a highly-concentrated electrolyte solution. The aggregated resin forms a spherical structure by surface tension in the solution. The size of the nanotool can be sorted by filtering process. Fig. 5 shows photographs of gel-tool. The nanotool could be manipulated by optical tweezers in the solution because the relative refractive index of PEG (1.4) is higher than that of water (1.3).

The nanotool can maintain functional materials inside itself. In this paper, Rhodamine B (Wako Pure Chemical Industries, Ltd, Japan) was used as the temperature indicator. The fluorescence intensity decreases according to the increase of the temperature.



(a) (b)

Fig. 5 Photographs of gel-tool. (a) Before filtration. (b) After filtration using syringe filter (pore size: 200 μm).

2.4 Preparation of liposome including nanotool

Figure 6 shows the preparation process of the liposome including the nanosensor. Materials of the nanosensor were 1g PEG-MA with photo initiator, 0.5g Rhodamine B (0.5mg/l) and 0.088g LCV. Liposome was prepared by Bangham method. Materials of the liposome were 0.05g DOPC, 0.05g DOPE. Gel-beads impregnated with Rhodamine B and LCV were generated by stirring the mixture of them in 1M K2HPO4 solution [15]. After the gel-beads were polymerized by UV illumination during 1 minute, size-classification of the gel-beads was performed by a syringe filter (mesh size: 200 nm) to collect the nanobeads.

Liposome was prepared by Bangham method [16]. The mixture of DOPC and DOPC in 2 ml diethyl ether was dried for 1hour and formed lipid membrane at the bottom of the beaker. The solution including nanotool was mixed the beaker at 50 degrees of C during 20 minutes. The liposome including the nanosensor was formed. Fig. 7 shows the fluorescent image of the nanotool and liposome including the nanotool.

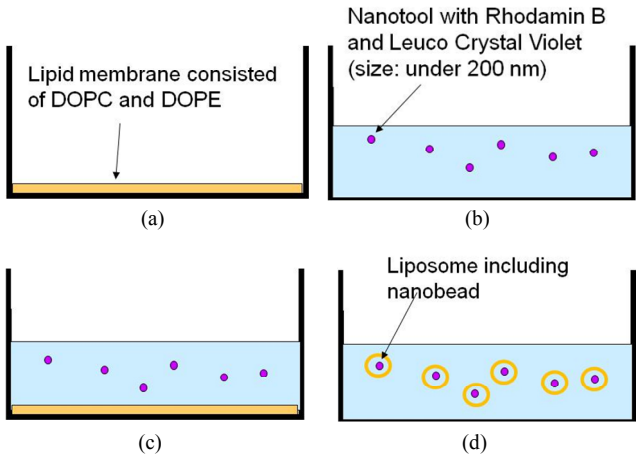
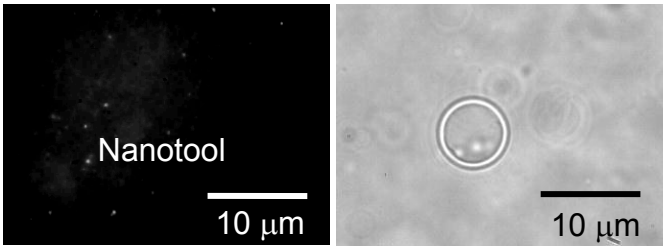


Fig. 6 Preparation process of nanotool including fusogenic liposome. (a) Form of lipid layer composed of DOPC and DOPE. (b) Generation of nanosensor impregnated with LCV and Rhodamine B by aggregation of photo-cross linkable resin in 1M K_2HPO_4 solution. (c) Mix of the nanosensor solution with lipid layer. (d) Form of the liposome including nanosensor by Bangham method (50 ^oC, 20 min).



(a) (b)

Fig. 7 Photographs of nanotool and liposome. (a) Fluorescent image of nanotool. (b) Fusogenic liposome including the nanotool.

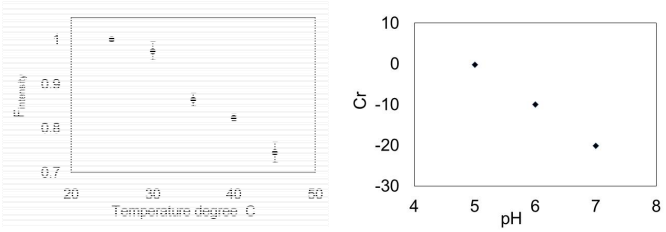
2.5 Calibrations of temperature and pH with CCD image

Calibration of temperature with the fluorescent intensity of the nanotool was performed. The fluorescence of the nanotool was obtained as RGB information by the color CCD (WAT-250D2, Watec co. td.). RGB information was converted to YCrCb information by (2) [17].

$$\begin{aligned} Y &= 0.299 \times R + 0.587 \times G + 0.114 \times B \\ Cr &= 0.500 \times R - 0.419 \times G - 0.081 \times B \\ Cb &= -0.169 \times R - 0419 \times G + 0.500 \times B \end{aligned} \quad (2)$$

The Y shows brightness, the Cr shows the color difference for red, and the Cb shows the color difference for blue. Temperature was calibrated with brightness. pH value

was calibrated by *Cr* value. We used Bromocresol Green for pH measurement and Rhodamine B for temperature measurement. Temperature was calculated by *Y* value. Fig. 8 shows the calibration results. $F_{Intensity}$ is represented by the relative fluorescence intensity based on the brightness of the brightness at 25 degrees.



(a) (b)

Fig. 8 Calibration results. (a) Calibration of temperature with relative fluorescence intensity based on 25 ºC. (b) Calibration of pH with Cr value.

2.6 Experimental setup

The experimental system for intracellular measurements using the gel-nanotool is shown in Fig. 9. This system was based on a inverted microscope (IX71, Olympus) equipped with a high numerical aperture (NA = 1.4), 100 oil immersion lens (UPLSAPO100XO, Olympus) with epi-fluorescent illumination. A near-infrared laser (maximum power: 6 W, wavelength: 1064 nm), which is considered to be safe for cells, was employed for the optical tweezers. The laser beam entered through a side port located on the mirror unit cassette of the microscope. The focus of the laser is controlled by scanning the galvano mirrors in the observation plane. The X-Y stage of the microscope was controlled by stepping motors and the Z axis was controlled manually. The operator controls the laser focal points with a joypad, and hence can manipulate the nanotool. Fluorescent information on the gel nanotool is acquired by a CCD camera (WAT-250D2, WATEC) and recorded using HDD recorder.

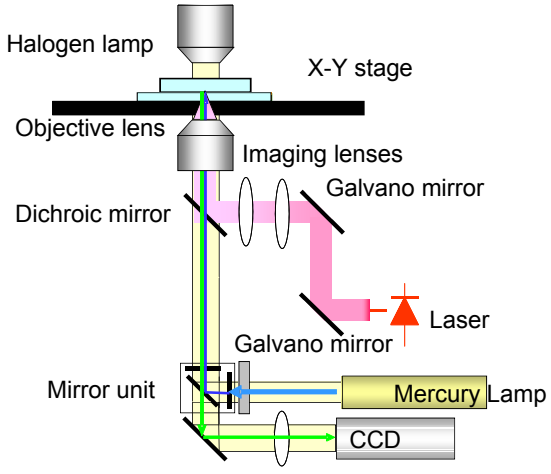
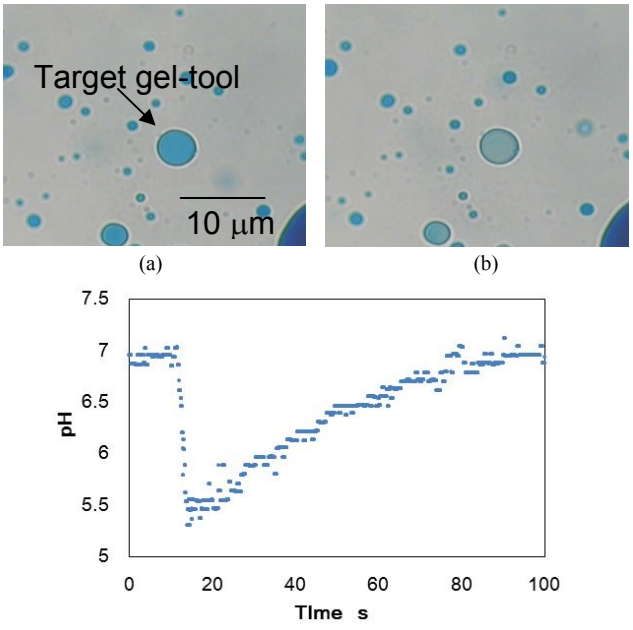


Fig. 9 Laser manipulation system.

3. Experiments

3.1 Optical pH regulation using gel-tool impregnated with Leuco Crystal Violet

Figure 10 showed the optical pH regulation using gel-tool impregnated with LCV in a neutral solution. The diameter of the gel-tool was 4 μm. Initial pH in the liposome was 7.0. Amount of the pH shift was estimated 2 from (1) (assumption: $d = D$). We measured the pH reduction in the gel-tool by color change of the gel-tool impregnated with BCG. UV-ray (54 nW/μm^2 at 366 nm) was illuminated for 4 seconds from 11 sec. Initial color of the gel-tool was blue. pH value was reduced to 5.3. pH value was recovered to 7 by VIS illumination (3.5 nW/μm^2 at 420 nm) and diffusion of proton from environment. We confirmed that optical pH regulation using LCV.



(a) (b)

(c)

Fig. 10 Time-course of pH shift inside gel-tool. (a) Before UV illumination (t=0s). (b) After UV illumination (t=18s). (c) Time course shift of pH value of the gel-tool.

3.2 Repeatable pH regulation by UV illumination control

Figure 11 showed the repeatable pH regulation using gel-tool. Initial pH in the solution was 7. Illumination time of UV-ray (7.2 nW/μm^2 at 366 nm) was controlled by electric shutter. Interval time during UV illumination was 60 seconds. We succeeded in repeatable pH regulation as shown in Fig. 11(d). Autofluorescence from the photoinitiator during UV illumination inhibited the dynamic pH measurement. Currently, our method achieved static pH control. Dynamic pH regulation avoiding autofluorescence is the future work.

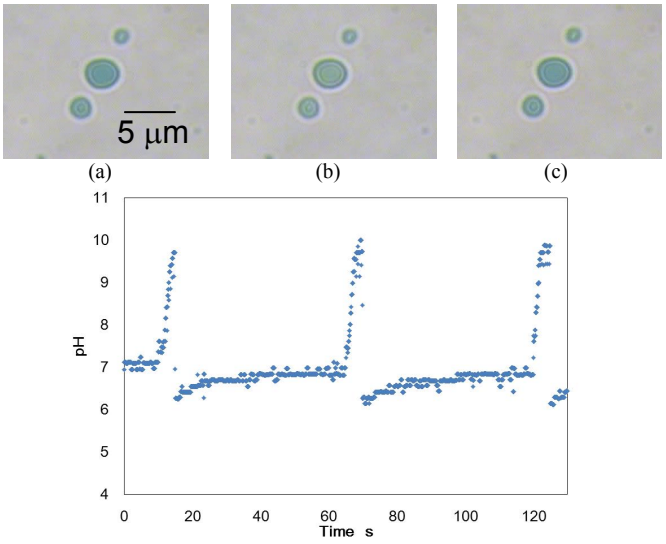
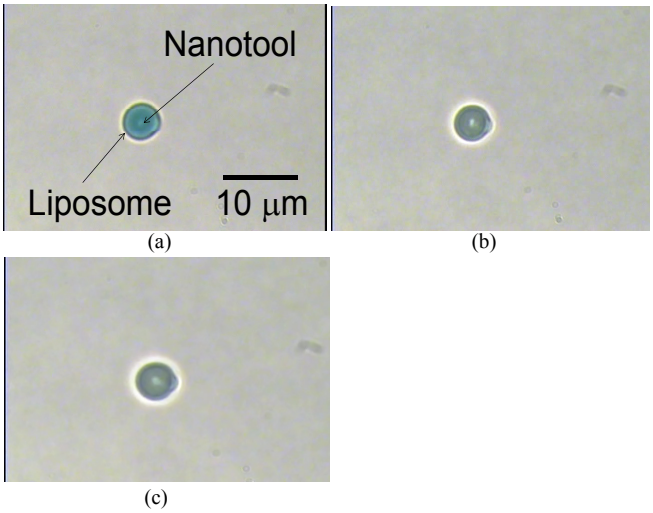


Fig. 11 Control of pH shift by adjusting UV-ray illumination (power: 7.2 nW/μm^2 at 366 nm). (a) Repeatable pH regulation of the gel-tool (t= 10s). (b) First pH reduction by UV illumination for 5 seconds (t= 15s). (c) Recover of pH (t= 75s). (d) Time-course shift of pH value.

3.3 Optical pH regulation in the liposome using nanotool

Figure 12 showed the pH regulation in the liposome using the nanosensor. The diameter of the liposome was 4 μm and that of the nanosensor was 200 nm. Initial pH in the liposome was 6.8. Maximum amount of the pH shift was estimated 2 from (1). UV-ray (54 nW/μm^2 at 366 nm) was illuminated for 8 seconds from 1 sec, for 2 second from 12 sec, for 2 seconds from 16 sec, and 3 sec from 36 sec. Rate of the pH reduce by UV illumination was calculated at 0.0023 pH/nJ. We confirmed pH inside the liposome was reduced lower than 6 and this method was applicable to selective membrane fusion.



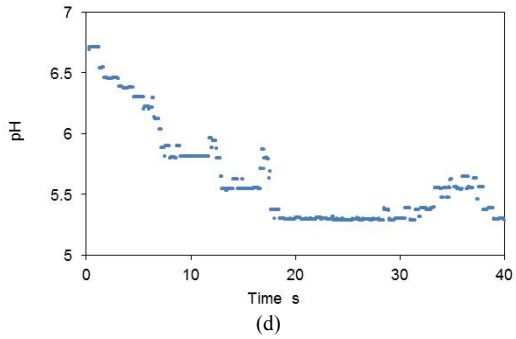


Fig. 12 Optical pH regulation in liposome. (a) Initial state (t=0s). (b) After UV illumination (t=30s). (c) After recover of pH (t=50s). (d) Time-course of pH value regulated by optical pH regulation.

3.4 Selective cell injection and manipulation of nanotool

Figure 13 shows UV-induced adhesion of liposome to cell. Initial pH inside the liposome was 7. Liposome was manipulated and contacted to the cell membrane by optical tweezers. The liposome did not adhere to the membrane without UV illumination. The liposome adhered to the membrane by UV illumination. Success rate of the membrane fusion using optical pH regulation was 95%. After incubation on the glass substrate in a petri dish for 3hour (Concentration of Carbon dioxide: 5.0 mg/l, temperature: 37 degrees of C), the nanotool was injected into the MDCK cell.

Figure 14 shows the intracellular manipulation of the nanotool. We succeeded in manipulation of the injected nanotool by optical tweezers. Local temperature inside the MDCK cell could be measured by detecting the fluorescent intensity of the nanotool as shown in Figs. 14 (b)-(d).

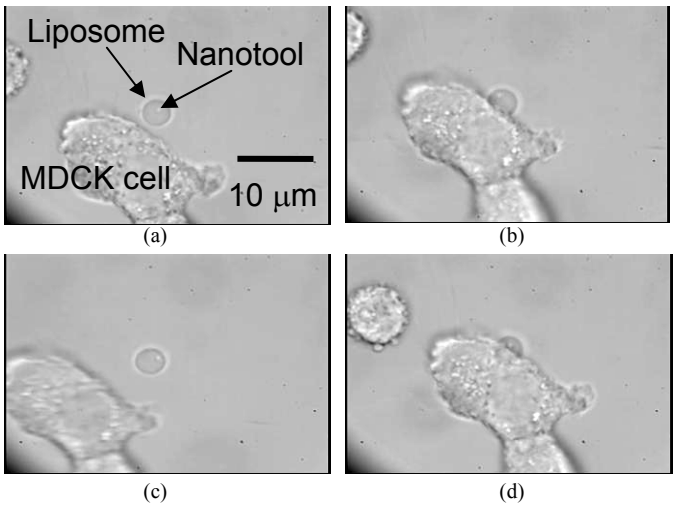


Fig. 13 Photo induced adhesion of liposome to MDCK cell. (a) Liposome including nanotool. (b) Contact without UV illumination. (c) Move of un-adhered liposome. (d) Adhesion with UV illumination

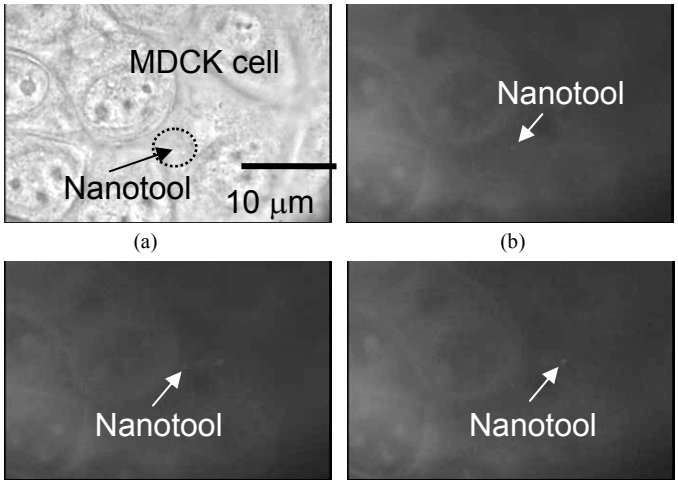


Fig. 14 Intracellular manipulation of nanotool (solution temperature: 25 °C). (a) Photo of MDCK cell. (b) Trap of nanotool (32 °C). (c) Manipulation of nanotool (31 °C). (d) Manipulation of nanotool (31 °C).

4. CONCLUSIONS

We developed optical pH regulation using nanotool for intracellular measurement in a microfluidic chip. Local pH was regulated by optical structural change of LCV. Autofluorescence during UV illumination will be solved by optimization of the mixing rate of the photo initiator and it is future work. The membrane fusion of the fusogenic liposome was controlled by pH regulation induced by UV illumination. Injected nanotool was manipulated by optical tweezers. Photo-induced membrane fusion could be used for invasive and selective injection of nanotool into a specific cell. This technique for intracellular measurement will make great contributions for cell biology.

8. ACKNOWLEDGEMENT

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