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Fabrication of Functional Gel-Microbead for Local Environment Measurement in Microchip

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Abstract— **A novel on-chip environment measurement with functional gel-microtool was developed. Environment measurement gel-microtool was fabricated by connecting the gel-microbeads impregnated with indicators in a microchip. In this paper, Bromothymol blue (BTB) and Bromocresol green (BCG) were employed as pH indicators. BTB and BCG have the different indicator range. Rhodamine B is temperature sensitive fluorescent dye and is used for temperature measurement. Gel-microbead is made by salting-out of hydrophilic photo-crosslinkable resin and is manipulated by optical tweezers. Moreover, gel-microbead is polymerized by UV illumination and connected to other gel-microbead under an electrolyte solution. The connection of gel-microbeads is performed by contact of gel-microbeads under UV illumination. Environment measurement gel-microtool with an arbitrary shape is fabricated by connection of the gel-microtool impregnated with arbitrary indicator. Multiple environments measurement gel-microtool included with several indicators is realized by assembly of the gel-microbeads impregnated with different indicators. Environment measurement is performed by detecting the color and the fluorescence intensity of each gel-microbead. We succeeded in the on-chip fabrication of the environment measurement gel-microtools such as circular pH measurement gel-microtool and wide range pH measurement gel-microtool in a microchip.**

I. Introduction

BIO industries employing the components of cells and the cell itself have been expanding in recent years. However, several unknown functions of the cell still remain to be discovered. Cell analysis by monitoring the states of a cell in specific environments is expected to be used to discover unknown properties of the cell [1, 2]. Significant research on the measurement of cellular states, for example, pH and temperature, has been performed [3, 4]. However, there are some interactions between the cell activity and the environment, such as pH and temperature that have not yet been investigated sufficiently. Therefore, the measurement of the environmental information around cells is important for detailed cell analysis.

Conventionally, the environmental conditions such as pH and temperature are measured using a probe. However, disturbances in the environment caused by the motion of the probe are a problem because the probe has to be employed in an open space such as a petri dish. Moreover, control of the probe position by the micro manipulator is also influenced by vibrations in the environment and the probe. While disturbances in the environment are a problem for measurements in an open space, measurements in a closed space, such as a microchip, are robust against such disturbances. Therefore, on-chip measurement technique has become very important in recent years.

Several techniques for measurement of the environment inside a microchip have been developed. Conventionally, fluorescence observation was the major approach for environmental measurements [5-8]. On the pH measurement, a pH sensing microscope has been developed [9]. Although the spatial resolution of this method is a few micrometers, this method can only be applied to measurements of the two-dimensional distribution of the pH value. A three-dimensional pH measurement with optically manipulated fluorescent particles has also been developed [10]. A microbead modified with a pH-sensitive fluorescent dye was prepared and positioned three-dimensionally using optical tweezers. This method enables three-dimensional, non-contact, and non-destructive measurement with high spatial resolution. Moreover, simultaneous measurement of different conditions such as O_2 and CO_2 is possible by using several fluorescence dyes with different excitation wavelength [11, 12]. However, fluorescent methods have major problems with absorption, quenching and photo-degradation. Thus, microbead modified with a pH indicator was developed [13]. Such microbead can be used for long-time measurements because of the absence of photo-degradation. However, the conventional measurement with microbead is single-point measurement, so that measurement around the cell requires the scan of the microbead. Moreover, surface modification of microbead by chemical treatment takes a long time. Although pH measurement using electrochemical reactions have been developed [14], the measurement area is limited by the sensor size and its position.

We have developed pH sensing gel-microbead impregnated with a pH indicator [15]. The pH value was measured by observing the color of the pH indicator inside the gel-microbead. The advantages of this pH sensing

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gel-microbead are the three-dimensional local measurement by non-contact manipulation of the gel-microbead such as optical tweezers [16], the *in-situ* arrangement of the pH sensing gel-microbeads arrays by positioning them on the glass surface, the low-invasive measurement around cells without cell manipulation, and the disposability of the gel-microbead. However, simultaneous measurement of multiple environmental conditions is difficult by this approach. Impregnating several indicators into single gel-microbead causes interference between indicators. In case of measurement of different conditions, measurement becomes impossible because of the interference. Even if measurement of single condition using indicators with different indicator range, measurement sensitivity becomes decrease than using single indicator.

In this paper, we propose an on-chip fabrication of environment measurement gel-microtool. Arbitrary shape gel-microtool is fabricated by connecting the gel-microbead impregnated with indicators. Fabricated gel-microtool enables two-dimensional measurement such as line measurement and planar measurement and is manipulated in a microchip by optical tweezers. This concept also achieves the multiple environments measurement using several indicators. Interference between indicators is prevented by connecting gel-microbeads impregnated with different indicators. When there is no interference between indicators, different indicators can be introduced into the same gel-microbead. We used BTB and BCG as pH indicators. We used Rhodamine B for the temperature measurement. The pH value is measured from the color of the gel-microbead using calibrated color information. Temperature is measured from the fluorescence intensity. On-chip fabrication of the circular pH measurement gel-microtool and local pH measurement around a yeast cell were demonstrated. Multiple measurements such as a wide range pH measurement and the simultaneous measurement of the pH and temperature were also demonstrated.

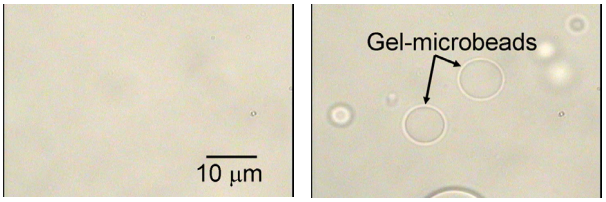
II. Material and Methods

A. Functional gel-microbead for environment sensing

An environment measurement gel-microbead is composed of the gel-microbead and an indicator. Measurement is performed by detecting the color of the indicator inside the microbead. The gel-microbead was made by salting-out of a photo-crosslinkable resin (ENT–3400, Kansai Paint, Japan), which was used for immobilizing cells and enzymes [17]. This resin consists primarily of polyethyleneglycol (PEG) prepolymer and is hydrophilic. This gel-microbead is manipulated by optical tweezers in an aqueous solution because the relative refractive index of PEG (1.4) is higher than that of water (1.3). The resin can be polymerized by irradiation of near-ultraviolet rays around 366 nm. The gel-microbead is used as a carrier of the indicator.

Figure 1 shows photographs of the gel-microbead. The concentrations for salting-out depend on the Hofmeister series [18]. Concentrations for generating gel-microbeads were confirmed visually in four different electrolytes. All electrolytes exhibited the expected concentration according to the Hofmaister series as shown in Table 1. When the electrolyte concentrations were below the limit concentration for salting-out, the un-polymerized gel-microbead melted into the solution. However, the polymerized gel-microbead swelled at a lower concentration than the limit concentration for salting-out. 0.5 M phosphate dipotassium salt required the lowest concentration for making the gel-microbeads.

In this paper, BTB (Wako Pure Chemical Industries, Ltd, Japan) and BCG (Wako Pure Chemical Industries, Ltd, Japan) were used as pH indicators. BTB and BCG have different indicator range. BTB is expressed as yellow in an acidic solution (pH < 6), green in a neutral solution, and blue in an alkaline solution (pH > 8). BCG is expressed as yellow in an acidic solution (pH < 4) and blue in a neutral solution (pH < 6). For temperature measurement, Rhodamine B (0.5mg/l) was used because Rhodamine B is a temperature sensitive fluorescence dye. The fluorescence intensity of Rhodamine B decreases according to increase of temperature.



(a) (b)

Fig. 1 Photograph of the salting-out gel-microbeads. (a) 0 M salt solution (b) 0.5 M phosphate dipotassium salt solution.

Table 1 Low limit concentrations of electrolytes for salting-out of the photo-crosslinkable resin[a]

	mol/l
sodium chloride	5.0
potassium acetate	1.9
sodium acetate	1.5
phosphate dipotassium salt	0.5

[a] All value was determined under 10% photo-crosslinkable resin in each electrolyte solution

B. In-situ fabrication of functional gel-microbead

The gel-microbead formed in this study has useful characteristic for fabricating environment measurement gel-microtool. The gel-microbead is connected to other gel-microbead under an electrolyte solution. In purified water, this gel-microbead does not adhere to other objects non-specifically because PEG is an uncharged polymer. However, the gel-microbead adheres to other gel-microbead in the electrolyte solution shown in Table 1. This adhesion is weak because the gel-microbead adheres only by contact. The adhered gel-microbeads often separate in the purified water. Moreover, undesired gel-microbeads may adhere to target gel-microbead because the adhesion arises by contact of the gel-microbeads. To solve problem, we achieved firm connection of the desired gel-microbeads by using UV

illumination. The process of connection of the gel-microbeads is shown in Fig. 2. First, the gel-microbeads are manipulated and contacted to each other in an electrolyte solution of concentration lower than the concentration required for adhesion of gel-microbead. After contact of the gel-microbead to target gel-microbead, they are connected by UV illumination. The unilluminated gel-microbeads are not connected. Connection of four gel-microbeads was performed as shown in Fig. 3. These gel-microbeads were manipulated by optical tweezers and connected by UV illumination less than 1 second. The concentrations for connection of the gel-microbeads with UV illumination were confirmed in four electrolytes as shown in Table 2. The 0.1 M phosphate dipotassium salt required the lowest concentration for immobilization.

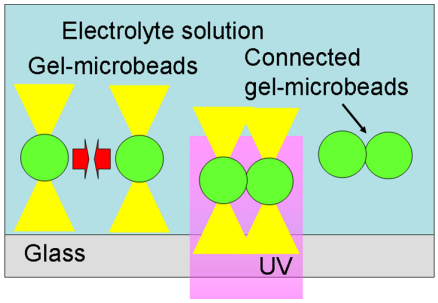
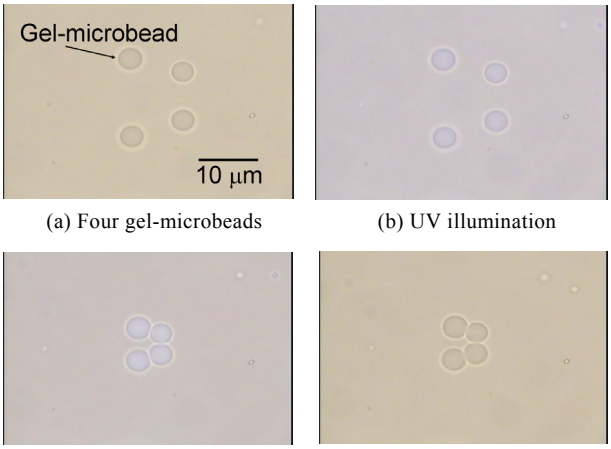


Fig. 2 A schematic of connection process of gel-microbeads.



(a) Four gel-microbeads (b) UV illumination

(c) Contact of gel-microbeads (d) Fabricated gel-microbeads

Fig. 3 *In-situ* fabrication of gel-microbeads.

Table 2 Low limit concentrations of electrolytes for fabrication of the gel-microbead using UV-ray illumination.[b]

	mol/l
sodium chloride	1.9
potassium acetate	0.5
sodium acetate	0.3
phosphate dipotassium salt	0.1

[b] All value was determined under 10% photo-crosslinkable resin in each electrolyte solution

C. *Fabrication process of functional gel-microbead*

A schematic of the fabrication process of the environment measurement gel-microtool is shown in Fig. 4. Gel-microbead impregnated with an indicator was generated by stirring the mixture of 0.9 g ENT-3400, 0.3 g indicator, and 2.4 g electrolyte solution. Then the gel-microbead was polymerized by UV illumination and injected into the microchip. The gel-microtool was fabricated by connecting gel-microbead impregnated with desired indicators. The connection of gel-microbeads is curried out by contact of the gel-microbeads manipulated with optical tweezers. When there is no interference between indicators, different indicators can be introduced into the same gel-microbead as shown in Fig. 5. This fabrication process is simple and takes much less time than the chemical surface-modification of the microbeads with several indicators.

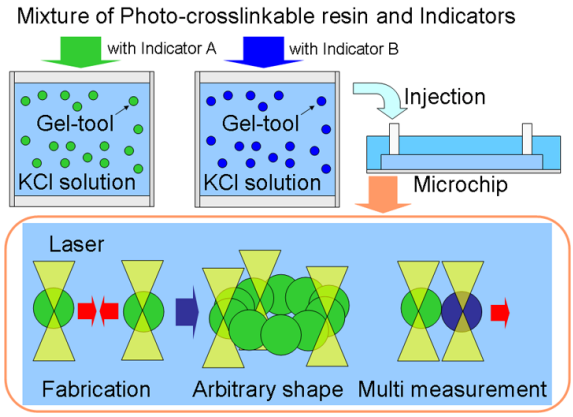


Fig. 4 A schematic of fabrication process of environment measurement gel-microtool.

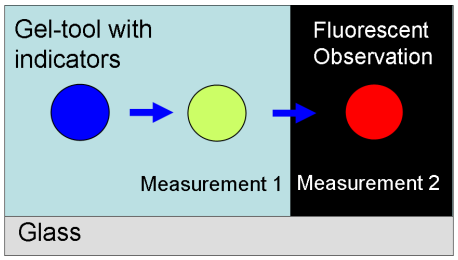


Fig. 5 A schematic of multiple environments measurement gel-microtool including different indicators.

D. *Experimental system*

The experimental system for the on-chip measurement using the environment measurement gel-microtool is shown in Fig. 6. This system was based on a modified inverted microscope (IX71, Olympus) equipped with a high numerical aperture x100 oil immersion lens (UPLSAPO 100XO, Olympus) with epi-fluorescent illumination. The NA of this lens is 1.4.

A near-infrared laser, considered to be safe for cells, was employed for the optical tweezers. The maximum power of the laser was over 5 W and its wavelength was 1064 nm. The laser beam entered through the 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. Simultaneous manipulation of multiple targets is achieved by forming multiple focal points with high speed scanning of the laser [19].

The X-Y stage of the microscope was controlled by the stepping motors. The Z axis was controlled manually by the operator. The operator controls the focal points of the laser using a joystick, and can thus manipulate the gel-microbead. Color information on the gel-microbead is acquired by a color CCD (XC-555, Sony) and is recorded using a HDD/DVD-Recorder (RDR-HX65, Sony).

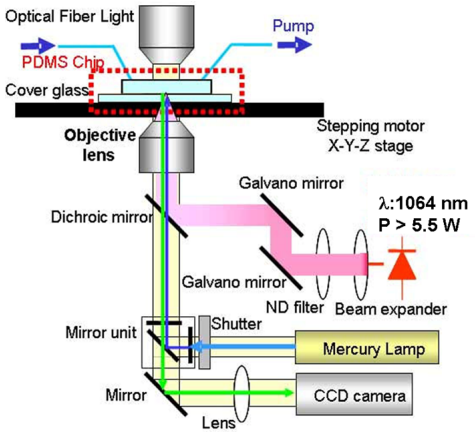


Fig. 6 A schematic of experimental system

III. Results and Dicussion

A. Calibration of functional gel-microbead

First, calibration of both pH and temperature with the color of the environment measurement gel-microtool was performed. The color of the gel-microbead is obtained as RGB information by the color CCD. RBG values are influenced by brightness which is included in each RGB value. To reduce the influence of brightness, the RGB information was converted to YCrCb information using equation 1.

$$\begin{aligned} Y &= 0.299R + 0.587\mathrm{G} + 0.114\mathrm{B} \\ Cr &= 0.5000R - 0.419G - 0.081B \\ Cb &= -0.169R - 0.419G + 0.500B \end{aligned} \quad (1)$$

The Y shows brightness, the Cr shows the color difference for red, and the Cb shows the color difference for blue. The color of both BCG and BTB changes from yellow to blue with increasing pH. Therefore, the Cr value decreases with increasing pH. However, the Cb value increases with increasing pH. In this research, the pH value was calculated using calibrated Cr value because the dispersion of Cr was smaller than that of Cb in our system. Temperature was calibrated with fluorescence intensity. $F_{Intensity}$ is represented by the fluorescence intensity based on the brightness of the brightness at 25 degrees.

Sizes of the sample gel-microbead ranged from 5 μmϕ to 15 μmϕ. The white balance of the CCD was adjusted manually. Illuminance was adjusted to 2000 lux. The color of the gel-microbead was obtained in the state where the focus was adjusted in the equatorial plane. The pH values of the sample buffer were measured by a commercial pH meter (pHep5, HANNA). Temperature of the solution was controlled by using thermal robo (TR-1AR, As one corp.).

The calibration results are shown in Figs. 7, 8, and 9. 6 data points were taken in each pH. In Figs. 7 and 8, the pH value decreased with increasing Cr. There were proportional relationships between the pH and Cr. Equations 2 and 3 show the linear approximation formulas for the plots in Figs. 7 and 8. Precision of the pH measurement was about 0.4 because the maximum standard deviations of Cr were about 0.4. In Fig. 9, $F_{Intensity}$ represents relative fluorescence intensity based on the intensity at 25 degrees. There was also proportional relation between temperature and $F_{Intensity}$ and the linear approximation formula was shown in equation 4.

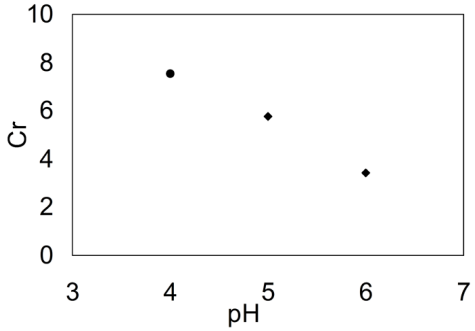


Fig. 7 Calibration result of pH with BCG

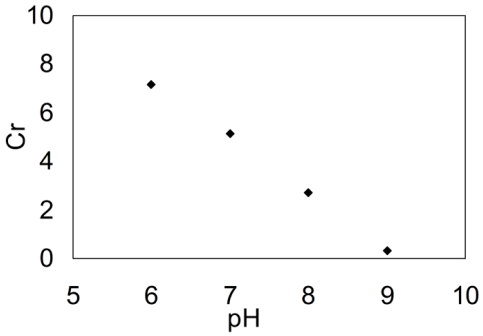


Fig. 8 Calibration result of pH with BTB

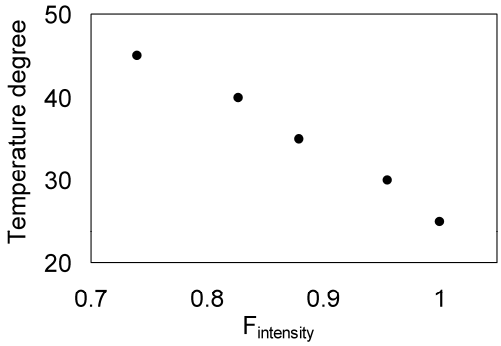


Fig. 9 Calibration result of temperature

$$pH = -4.8 \times 10^{-1} \times Cr + 7.7 \quad (2)$$

$$pH = -4.3 \times 10^{-1} \times Cr + 9.2 \quad (3)$$

$$Temperature = -1.3 \times 10^{-2} \times F_{Intensity} + 1.3 \quad (4)$$

B. Fabrication of circle gel-microtool and local pH measurement around a yeast cell

Figure 10 shows a schematic of the fabrication of circular gel-microtool impregnated with BTB and local pH measurement around a yeast cell. Gel-microbeads impregnated BTB was manipulated and connected. Fabricated gel-microtool was manipulated in the microchip and a yeast cell was set inside the circle gel-microtool. The pH value around the yeast cell is measured. Fig. 11 shows the experimental result. Six pH sensing gel-microbead was connected in alkaline solution. Yeast cell was positioned at the center of the gel-microtool. The pH value around the yeast cell was measured by detecting the color change of the gel-microtool. The shape of the gel-microtool can be selected at the measurement according to the purpose and situation.

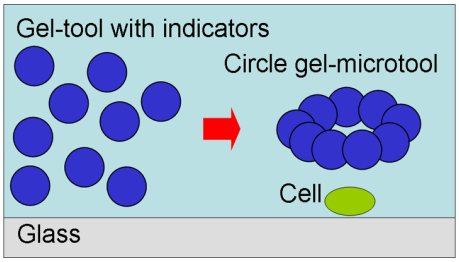
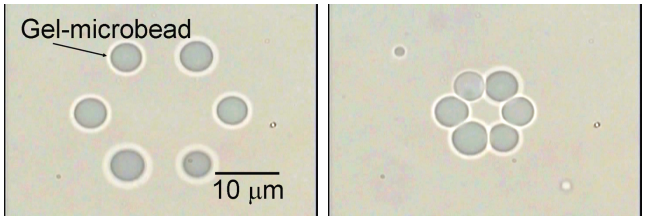
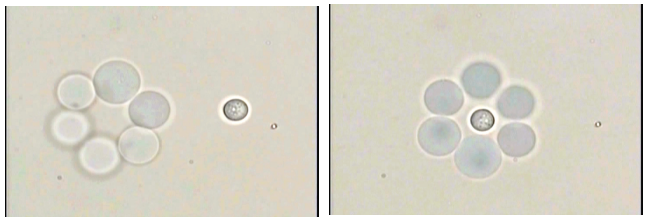


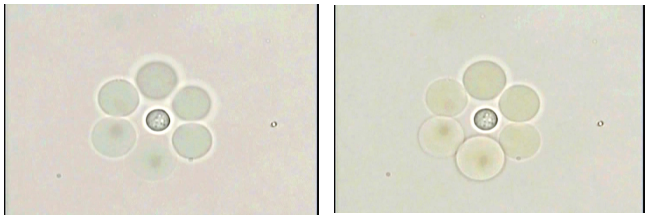
Fig. 10 A schematic of fabrication of measurement gel-microtool.



(a) Six pH sensing gel-microbeads (b) circular pH sensing gel-microtool



(a) Position of a yeast cell (b) pH8.9



(c) pH7.5 (d) pH5.0

Fig. 11 On-chip fabrication of circular pH measurement gel-microtool and local pH measutment around a yeast cell.

C. Fabrication of multiple measurement gel-microtool

Figure 12 shows a schematic of the multiple environments measurement gel-microtool. Two types of the gel-microtool were demonstrated. One is binding type gel-microtool. This gel-microtool is used for the wide range pH measurement and is composed of two gel-microbeads impregnated different pH indicators. BCG and BTB were used for this gel-microtool. Another is coexistence type gel-microtool. This gel-microtool includes the pH indicator and temperature sensitive fluorescent dye in a single gel-microbead. BTB and Rhodamine B were used.

Figure 13 shows a schematic of the wide pH measurement gel-microtool. Gel-microbead impregnated with BCG was manipulated and contacted to the gel-micorbead impregnated BTB. These gel-microbeads were connected by UV illumination. Fig. 14 shows the experimental results of the *in-situ* fabrication of the gel-microtool and wide range pH measurement. The gel-microtool was fabricated in pH 9 solution. Then we introduced pH 4 solution and observed the color change of each gel-microbead.

Figure 15 shows a schematic of the pH and temperature measurement gel-microtool. Fig. 16 shows the experimental result of the pH and temperature measurements by single gel-microbead. BCG and BTB are not excited by the wavelength for exciting Rhodamine B. 0.5 mg/l Rhodamine B does not show the color. Therefore, BCG and BTB can coexist with Rhodamine B in the same gel-microbead.

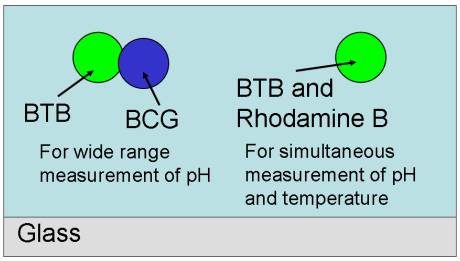


Fig. 12 Schematics of the gel-microtool for multiple measurements

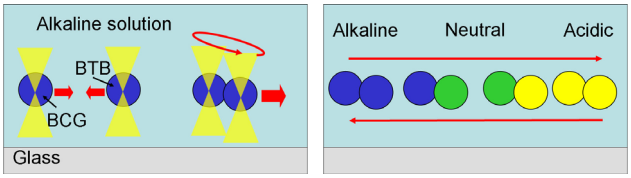
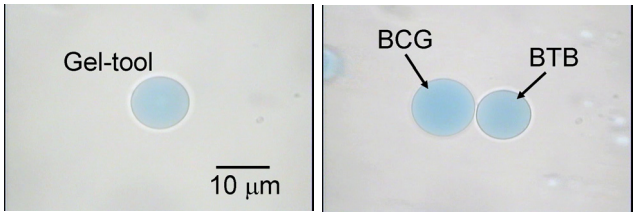
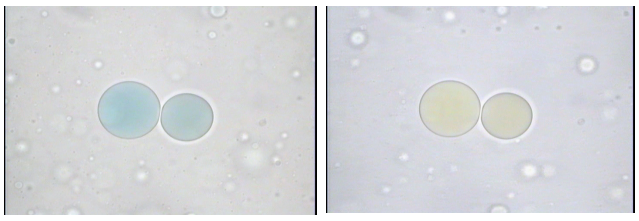


Fig. 13 Schematics of wide rage pH measurement gel-microtool



(a) Gel-microbead with BTB (b) Fabricated microtool (pH9)



(c) pH8.1 (d) pH4.6

Fig. 14 Wide range pH measurement

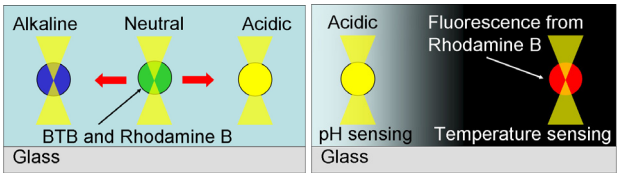
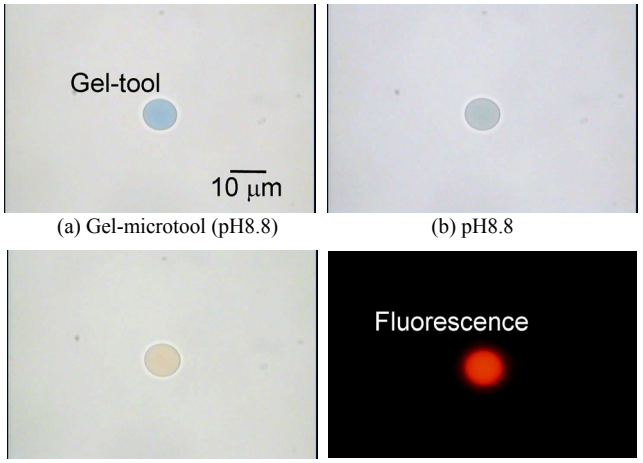


Fig. 15 Schematics of pH and temperature measurement gel-microtool



(a) Gel-microtool (pH8.8) (b) pH8.8

(c) pH4.6 (d) Temperature sensing (25 degrees)

Fig. 16 pH and temperature measurement

IV. Conclusions

We have developed an on-chip environment measurement method using environment measurement gel-microtool made by fabricating functional gel-microbead impregnated with indicators, and have demonstrated on-chip fabrication of the gel-microtool and local pH measurement around single yeast cell. Gel-microbeads created by salting-out of a hydrophilic photo-crosslinkable resin were used as a carrier of indicators. Gel-microbeads impregnated with the indicators were obtained using a simple and short process.

The gel-microbeads could be manipulated by optical tweezers. The connection of arbitrary gel-microbeads was realized by using UV illumination in the solution that adjusted the electrolytic concentration. The connection of the gel-microbeads impregnated different indicators prevents the interference between the indicators. Type of the gel-microtool can be chosen depending on a purpose such as the number of the measurement condition and the characteristics of the indicators. Moreover, we can achieve the gel-microtool, which can measure a large number of environmental conditions, by combination of these two types of gel-microtools.

This on-chip environment measurement method will make a great contribution to cell biology in the future.

Acknowledgment

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