Invited Paper

Manipulation of Single Cell for Separation and Investigation

Fumihito Arai, Akihiko Ichikawa, Hisataka Maruyama, Kouhei Motoo, and Toshio Fukuda

Abstract: Recently, high throughput screening for microorganisms with desired characteristics from a large heterogeneous population has become possible. Single cell separation has taken on increasing significance in recent years, and several different methods have been proposed so far. In this paper, we introduce several cell manipulation methods aiming at single cell separation and investigation. At first, methods for the separation of microorganisms are classified. Then, we introduce two different approaches, that is, (1) indirect manipulation using laser trapped microtools and (2) thermal gelation.

Keywords: Bio-chip, cell, laser tweezers, micromanipulation, separation, μ-TAS.

1. INTRODUCTION

Most microorganisms visible under a microscope are viable but do not grow to form colonies visible on plates. Of such microorganisms, some are cells of known species for which the cultivation conditions utilized happen not to be suitable. Others are cells that have entered an unculturable state, while yet others are of unknown species that have never been cultured before using suitable methods [1]. Species of Bacteria and Archaea that have been cultured are only a small fraction of the ones that exist. Recently, high throughput screening for microorganisms with desired characteristics from a large heterogeneous population has become possible [2]. Single cell separation has become increasingly important in recent years, and several different methods have been proposed so far. In this paper, we introduce several cell manipulation methods aiming at single cell separation and investigation.

2. METHODS FOR CELL SEPARATION

2.1. Background and classification of methods

High-throughput separation of viable cells with desired characteristics from a large heterogeneous population is needed before conditions for the strains or for unidentified microorganisms can be found. Single-cell separation with a mechanical micromanipulator is difficult if the sample cells are suspended in a liquid, because the motion of the micromanipulator disturbs the liquid, and the cells move easily, so that the target cell is easily lost. In addition, impurities may adhere to the surface of the pipette tip. As such, a new technology is needed by which we can manipulate and separate target micro-organisms at high speed and with high purity. Figure 1 shows the map of separation methods [3]. The methods are classified into (1) line up type and (2) lay out type. Each method has merits and demerits as shown in Fig. 1.

2.2. Line up and separation

Flow cytometry is widely used for this purpose, and fluorescence-activated cell sorters were developed for high-speed separation of cells [4]. However, most conventional flow cytometers are large. Reports on the miniaturization of cell sorters and on separation chips with sequential flow have appeared [5-8]. Microorganisms flowing in a microchannel can be separated by mechanical forces, hydrodynamic flow, ultrasound, magnetic forces, laser tweezers, and electric forces [6]. However, most conventional separation processes are sequential, so it is impossible to compare multiple objects simultaneously before separation, and positional information is lost during separation.

2.3. Lay out and separation

To solve this problem, single-cell sorting of microorganisms by laser scanning cytometry was proposed [2]. A review offers an excellent introduction to the principles and applications of this method [9]. A large heterogeneous population can be handled under a microscope, and individual cells can

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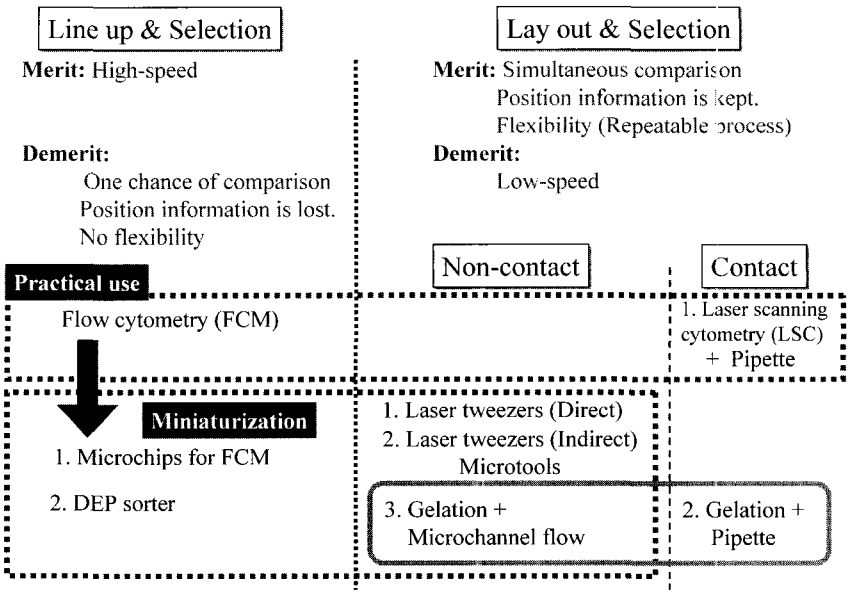


Fig. 1. Map of separation methods.

be examined repeatedly, measured automatically, and later observed microscopically by the operator. Single-cell sorting can be carried out manually with a fluorescence microscope and micromanipulator. Cells on a microscope slide are isolated with a micromanipulator and then cultured. However, automation of target-cell separation is still impractical because of difficulty in the isolation of target cells, appropriate positioning of the pipette tip, and various other problems.

2.4. Separation by laser tweezers

For finding unidentified microorganisms, we studied the selective separation of microorganisms suspended in a liquid. We aimed at the extraction of a single selected target directly from a huge number of objects on a chip after measurement under a fluorescence microscope. The process of separation is completely different from that in flow cytometry. We integrated laser tweezers and a microfluidic controller for manipulation of the target cell, and used dielectrophoresis for exclusion of the other objects around the target by evoking a potential field in the microchannel [10-14]. An arbitrarily selected single cell can be isolated speedily in a microchannel, even though there are a large number of cells in suspension. Then the target is transported along the proper transportation trajectory by the laser tweezers. The target is released close to a split in the microchannel flow, at which point it is removed by high-speed flow (a few centimeters per second). To decrease separation time, we must reduce the distance for which the target is transported by the laser tweezers. Consequently, we improved a separation chip having a microchannel structure with stagnation point control by precise pressure adjustment [13-14]. The central part of the chip is shown in Fig. 2. The pressure balance was adjusted and the stagnation point in the microchannel was controlled to promote higher speed in extraction of the target. The transportation trajectory is shown in Fig. 3. Separation time was shortened and we could obtain one target cell in as little as 10 seconds. However, the method based on the laser trap of the target may cause the problem of direct irradiation of the high power laser.

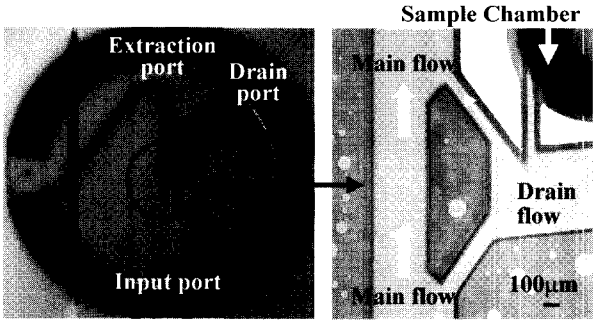


Fig. 2. Separation chip (size: 40 mmϕ).

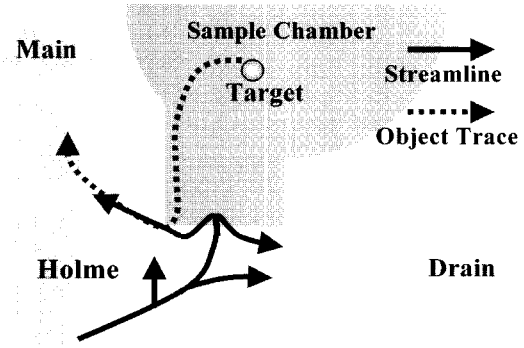


Fig. 3. Transportation trajectory of the target.

2.5. Separation by laser trapped microtools

To avoid direct irradiation of the focused laser to the microorganism, we proposed to transport a microorganism with microtools trapped by the laser (indirect laser micromanipulation) [11]. To realize the indirect manipulation of the microorganism, we studied an injection method of microtools at the desired location in a microchip [15]. For secure transportation of the microorganism by the microtools, configuration and surface modification of the microtool proved effective. Moreover, we proposed Synchronized Laser Micromanipulation (SLM) for the trajectory control of the multiple targets by a single laser [16]. We used the laser scanning system for high-speed transportation of multiple objects. The condition of secure transportation was derived and verified through the experiment. Using the SLM, we can manipulate multiple microbeads with each designed trajectory. We assembled a band of 4 adhesive microbeads. Both ends were trapped, and the band was manipulated by SLM. It is possible to transport the microorganism by indirect manipulation of the assembled microstructure using SLM.

2.6. Separation by thermal gelation

In those studies, we aimed at isolation and transportation of a target. Following isolation, the target was transported to another area. Another way to speed up separation could be to increase the transportation speed. The transportation speed with laser tweezers is limited by the power of the laser. As in laser scanning cytometry, multiple targets are dispersed in a wide area on a chip, so changes in transportation speed strongly affect the total separation time. Channel flow might be increased, but might make the transportation of the target to the desired location more difficult. Unlike in the previous approach, we removed obstacles surrounding the target rather than transporting the target [17]. We employed a novel method to isolate the target by thermal gelation [17-20]. The targets are fixed in the gel and obstacles are washed out by a cleansing flow. By on-chip separation and monitoring, the place of isolation is used for several purposes. This concept is very different from the conventional approach.

The thermal gelation method is a simple way of fixing and releasing a target. Once we fix or trap the target somewhere by heating, and remove the obstacles around it, we can easily access the area surrounding the target by mechanical micromanipulator with the pipette. The fixed target is released by naturally cooling the temperature. Then the target is collected by the pipette [21].

In this paper, we explain some of the experimental results of single cell manipulation.

3. SEPRATION BY LASER TRAP MICROTOOLS

3.1. Indirect manipulation by microtools

Laser tweezers have been used for manipulation of particles in closed spaces [22-25]. They are utilized for manipulation of biological objects such as viruses and bacteria [23]. It has been reported that direct irradiation of the focused laser may somewhat damage the trapped object [26]. This phenomenon depends on the target, wave length, irradiation time, and power of the laser. To avoid this problem, we proposed indirect manipulation of the target by laser trapped microtools [11]. As a microtool, we can consider the following two cases.

(1) Natural object (Ex: microbe such as bucillus)

(2) Artificial object (Ex: microstructure or microbead)

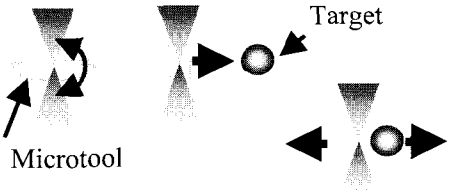
The method of how to use the microtool is classified as follows [10].

(a) Pushing or pulling by the microtool(s) one point contact multiple point contact plane contact (ex: by the micro basket) etc.

(b) Encapsulation in the micro capsule (ex: liposome)

Conceptual figures to explain those strategies are shown in Fig. 4.

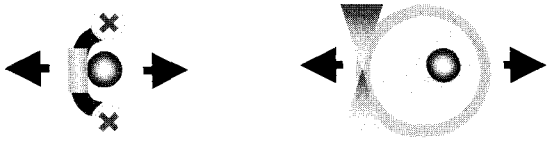
In the previous works, we used polystyrene beads as micro chopsticks. It was possible to transport the target with the microtools, however, secure transportation is not easy without maintaining orienta-



(a) Microtool: side view (One point contact).

(b) Microtools: top view (Multiple point contact by multiple beams).

(c) Microtools: top view (Multiple point contact by scanned beams).



(d) Micro basket: top view (plane contact).

(e) Micro capsule: side view (encapsulation).

Fig. 4. Classification of indirect manipulation.

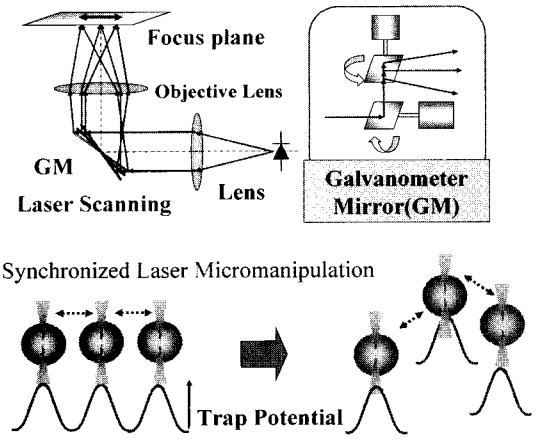


Fig. 5. Laser scanning for Synchronized Laser Micromanipulation.

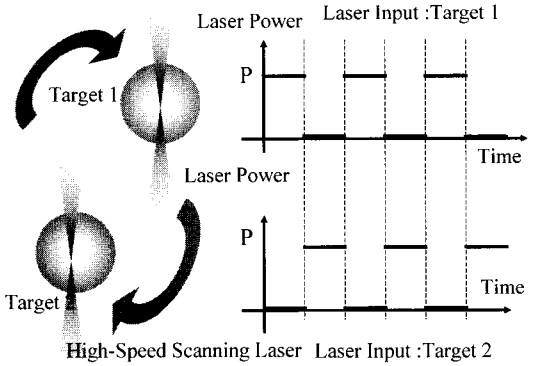


Fig. 6. Laser scanning pattern for multiple targets.

tion of the trapped target [15].

3.2. Trajectory control of multiple objects by laser manipulation

For secure transportation of the target, orientation of the laser trapped object is quite important. However, not much attention has been paid to this problem. To align the laser trapped object, we can utilize the laser scanning of single or multiple beams [16,27-32] or holographic optical tweezer arrays [33]. The laser scanning approach is suitable for orientation control and trajectory control of the object, since it is easy to create and change multiple laser trap potentials as shown in Fig. 5. In ref. [30], a time-sharing approach where the beam is repetitively scanned in the sample by rapidly moving a piezoelectric mirror was proposed. This method is known as Scanning Laser Optical Trapping (SLOT). Reports have been made on the pattern generation of trapped objects by the high speed scanning laser system [27-31,33]. However, little attention has been paid to the independent trajectory control of each trapped object.

The position of each laser trap can be controlled in the 2D or 3D space by changing the discrete laser scanning pattern. We can manipulate multiple targets with specific trajectories, if the laser irradiation time and power is appropriate to drag each object to the instantaneous focus point. We refer to this multiple trajectory control method as Synchronized Laser Micromanipulation (SLM) [16,32]. Fig. 6 shows an example of the laser scanning pattern. We need a single laser with a high speed laser scanning system. The laser irradiation time is discrete, however, if the trapping time is appropriate and trap stiffness is strong enough to realize the given trajectory, each target will move along each of the given trajectories [16].

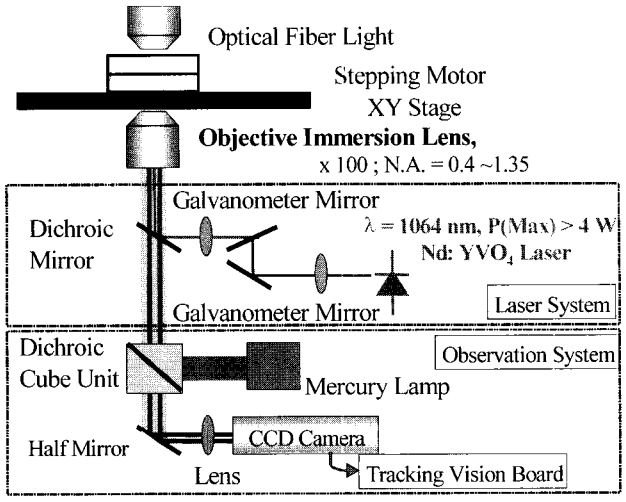


Fig. 7. Schematic diagram of the experimental system.

Fig. 7 shows the schematic diagram of the experimental system. We used the electromagnetically actuated Galvanometer mirrors. The band width of the mirror is about 950 Hz. As explained in Fig. 6, the focal point of the laser must be scanned quickly so that all targets follow the given trajectories. The number of the manipulated objects and transportation speeds are limited by the hardware property of the scanning system and laser power. To transport the targets as planned, it is necessary to allocate sufficient time to move each target in each step. Conditions for secure transportation were derived and verified through the experiment [16].

We performed several experiments to observe the effectiveness of this idea. The results are shown in Figs. 8, 9, and 10. Fig. 8 shows transportation of a yeast cell in water. It is pushed by two polystyrene microbeads (10 μmϕ) manipulated by SLM. Transportation speed is about 12 μm/s. Fig. 9 shows rotation of a yeast cell. It is rotated about $\pi/2$ in 3.5 sec. by two polystyrene microbeads (10 μmϕ) manipulated by SLM. Fig. 10 shows the dance of six microbeads by SLM. The transportation speed of each target was adjusted by properly designing the scanning pattern. The manipulation is quite stable as shown in Ref. [32].

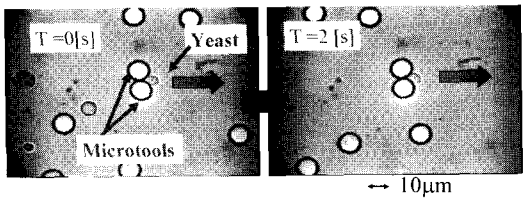


Fig. 8. Pushing a yeast cell by two microtools.

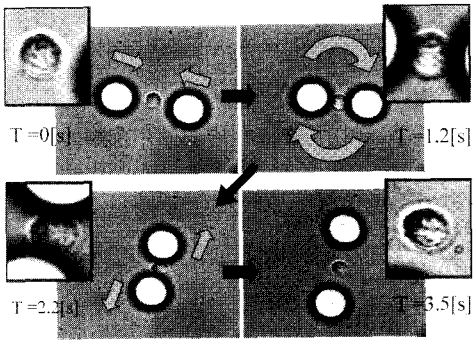


Fig. 9. Turning of a yeast cell by two microtools.

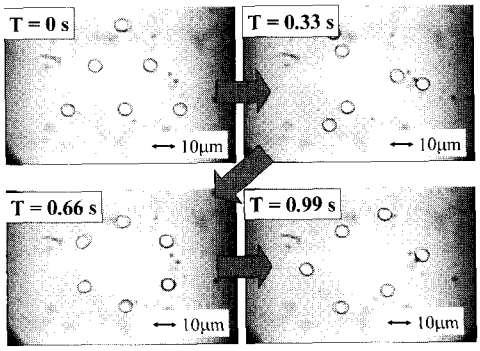


Fig. 10. Dance of six microbeads by SLM.

3.3. Assembly of microbeads and indirect manipulation of microorganisms

We employed SLM for assembly of adhesive microbeads. We constructed a band of adhesive microbeads for indirect manipulation of the microorganisms. The surface of the adhesive microbead is chemically-modified with the hydroxyl groups and the amino groups. It is possible to bind the adhesive beads to each other by means of the covalent bonds. Fig. 11 shows the process of assembly. Four adhesive microbeads (each diameter: 3μmϕ) are manipulated with the specific trajectories by SLM. We succeeded in fabrication efforts of a band of 4 microbeads as shown in Fig. 11(d). Both ends of the band are trapped and the trajectories of each ends are controlled by SLM. It was possible to control the orientation and trajectory of the band.

Using a band of 4 adhesive microbeads, we transported a yeast cell. We succeeded in the stable transportation of the cell (Fig. 12). Transportation speed in a forward direction was 21μm/s. Transportation speed in a transverse direction was 19μm/s m/s. The cell adhered to the center of the band, both ends of the band were trapped by the SLM, and it was spatially oriented. Transportation was quite successful in all directions of the focal plane without failure. This method is applicable for successful separation of the target microorganism.

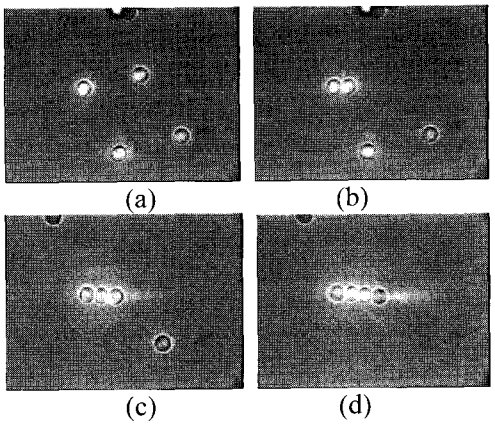
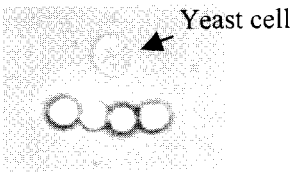
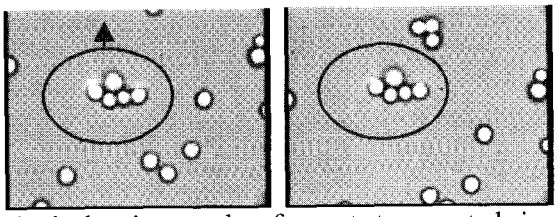


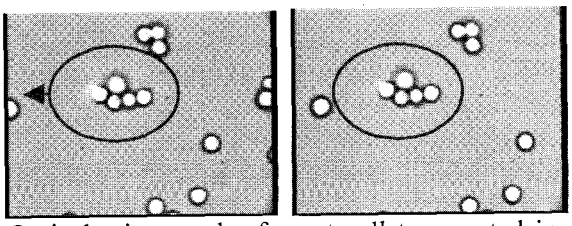
Fig. 11. Assembly of adhesive microbeads by SLM.



(a) Optical micrograph of yeast cell (4μmϕ) and a band of 4 assembled adhesive beads.



(b) Optical micrograph of yeast transported in a forward direction (Distance 7μm, time 10/30 sec).



(c) Optical micrograph of yeast cell transported in a transverse direction (Distance 5μm, time 8/30 sec).

Fig. 12. Indirect manipulation of yeast cell by a band of 4 assembled adhesive beads.

4. SEPARATION BY THERMAL GELATION

4.1. Separation based on isolation

Unlike in the previous approach, we removed

obstacles around the target rather than transporting the target [17]. We employed a novel method to isolate the target by thermal gelation [17-20]. The targets were fixed in the gel and obstacles were washed out by a cleansing flow. By on-chip separation and monitoring, the place of isolation can be used for several purposes.

4.2. Principle of fixation and isolation

Samples of interest were mixed with a poly(*N*-isopropylacrylamide)(PNIPAAm) 10% solution that was gelled at 30°C (Fig. 13) , and allowed to flow in the microchannel. To isolate a target cell, the area immediately surrounding it was heated with a microheater at the bottom of the microchannel. Fig. 14 shows a diagram of the fixation and isolation process. So that the specimen could be observed under an inverted fluorescence microscope, we fabricated a transparent microelectrode of indium tin oxide (ITO) for heating of the glass surface of the microchannel containing the microorganisms. A thin film of silicon dioxide was coated on the microelectrode for insulation. The chip with this microchannel and microelectrodes was set on the stage of an inverted fluorescence microscope. The microorganisms were allowed to flow in the microchannel with the thermosensitive hydrogel. The microelectrodes can be heated by applied voltage and when the liquid near an electrode is so heated, the target cell near it becomes immobile.

4.3. Application to the on-chip culture

We proposed a continuous-culture chip with monitoring in situ of individual cells. Fig. 15 depicts the diagram of a microreactor that uses thermal gelation of a thermosensitive hydrogel solution [17]. It has at least three ports, I to III. With these, growth media, dyes, and reagents can be provided by control of the flow pattern. Contamination by impurities is prevented by dual flow control of T-shaped microchannel and continuous laminar flow. First, the thermosensitive hydrogel solution containing sample cells is pumped into the microchannel from port I. A buffer or some water is pumped through port II to avoid contamination. Port III is used as a drain. The target is selected under a fluorescence microscope, and fixed by thermal gelation of the hydrogel on the microelectrode. Anything other than the target is washed away, leaving the target isolated. Then, we supply growth media, dyes, and reagents from port II if necessary. In this case, ports I and III are used as drains.

The advantages of the proposed method are as follows.

(1) Reaction conditions, including the use of substrates, culture media, and fluorescent dyes, can be controlled.
(2) Contamination is unlikely and procedures can be sterile.
(3) Specific cells can be sorted according to their characteristics.
(4) The state of individual specimens can be monitored in real time. Each specimen is fixed and its position is recorded, so images of the target cells are easily taken.
(5) The cells can be released from the gel by cooling of the electrode to room temperature.

Fig. 16 depicts the results of continuous culture of yeast cells and monitoring. First, a 10 wt% hydrogel solution containing sample yeast cells was pumped into the microchannel through port I. Three yeast cells were fixed on the microelectrode by thermal gelation. When a buffer was provided, the isolated yeast cells did not grow. However, when growth medium (5 wt% Difco Bacto YPD broth; Nippon Becton-Dickinson Co., Ltd., Tokyo, Japan) was provided at the flow rate of 100 m/s, cells grew. Next, the cells were stained with 5 (and 6)-carboxyfluorescein diacetate with an excitation maximum at 494 nm and an emission maximum at 517 nm injected through port II. This dye is in general use as an indicator of cell viability. The results indicated that the separated cells cultured on the microelectrode were viable. Contamination by impurities was prevented in the same way as for the separation described above. Continuous culture and reaction on the chip had the advantages we expected; it was easy to isolate selected cells and keep them in a small space.

5. CONCLUSIONS

In this paper, we introduced several cell manipulation methods aiming at single cell separation and investigation. At first, methods for separation of microorganisms were classified. Then, we introduced

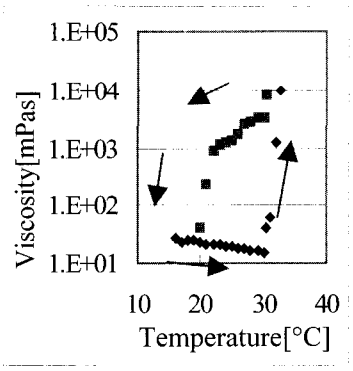


Fig. 13. Graph of the viscosity of PNIPAAm 10% solution during heating and cooling. An 8-ml volume of a 10 wt% solution was heated from 16.0°C to 32.5°C, at the rate of 0.3°C/min.

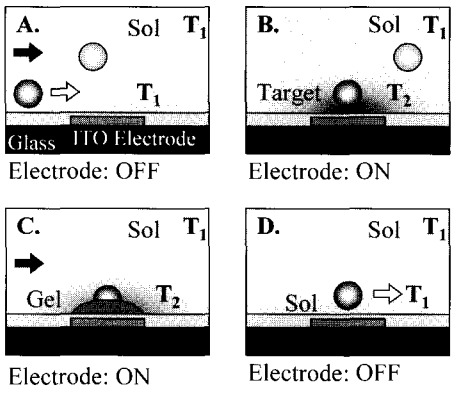


Fig. 14. Isolation process of a target cell by thermal gelation. T1 is the room temperature and T2 is the gelation temperature. (A) A target cell is selected. (B) A microelectrode is heated. (C) The cell is fixed by the gel. After the undesired cells are washed, the cell is isolated. (D) When the heater is switched off, the cell is released.

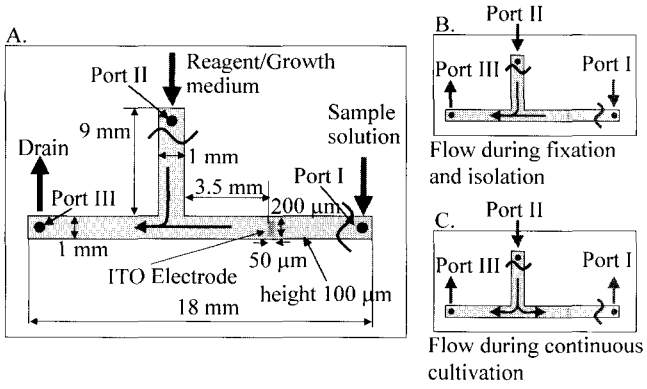


Fig. 15. Diagram of the microreactor designed for continuous culture and monitoring in situ of individual microorganisms on a chip. (A) Overview of the microreactor. (B) Flow during sample injection and fixation. (C) Flow during isolation and continuous culture.

(1) indirect manipulation using laser trapped micro-tools and (2) the thermal gelation method.

In the first case, it was possible to transport the microorganism by the indirect manipulation of the assembled microstructure. In this application, since the size of the microtool was relatively large, particle diffusion caused by Brownian motion did not create difficulty in the positioning of the target. Extensive investigation on the stable manipulation, in case the size effect is not negligible, was also studied. However, it was omitted here because of page limitation. The proposed method will be applied for separation of the target microorganism. Safety of the

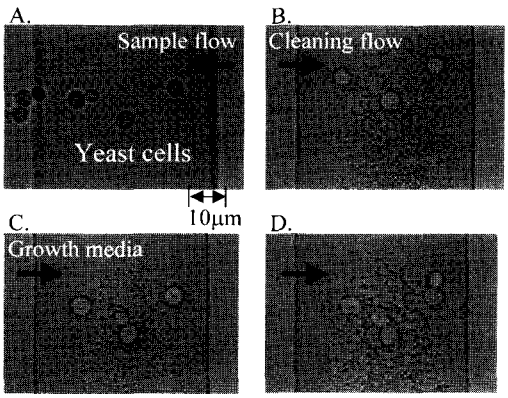


Fig. 16. In-situ monitoring of continuous culture of yeast cells on a chip. (A) Yeast cells before fixation. (B) Yeast cells fixed by gelation. (C) Cells after 1 hour of culture. The cell in the center has grown. (D) Cells after 2 hours of culture. All cells have grown.

transported object will be examined in future.

In the second case, the specimen was fixed by the thermal gelation of a PNIPAAm solution and selected cells were separated from a large population. We succeeded in the separation of yeast cells, their continuous culture on a chip, and monitoring of the culture by fluorescence staining in situ. We confirmed the viability of the separated and cultured yeast cells on the microelectrode using PNIPAAm. The result showed the effectiveness of the method when applied to live microorganisms. Our method is extremely useful in the pure cultivation of microorganisms and will be a promising method for biologists when screening for useful microorganisms.

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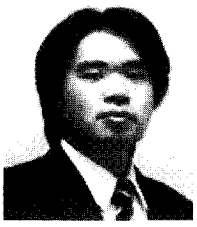

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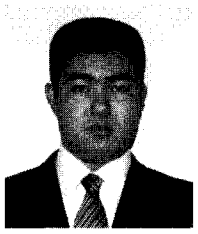

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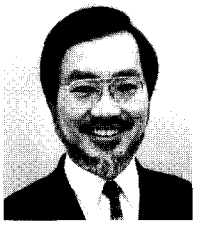

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