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Single-cell Trapping Using Microwell Arrays Fabricated from Self-assembled Particle Monolayers

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This paper proposes a self-assembly fabrication method of microwells and investigates the effect of surface treatment for the cell trapping. The target cell is unicellular cyanobacterium Synechocystis *sp. strain PCC 6803, which has been widely investigated as a model organism for photosynthesis. The self-assembly monolayer of polystyrene particles (3.0 μm diameter) etched by reactive-ion etching was used as a template of polydimethylsiloxane molds. The well diameter in the mold could be controlled by the etching time. The cell-trapping efficiency was discussed with the viewpoint of hydrophilically treatment and poly-L-lysine treatment on the well arrays.*

Keywords Single-cell trapping; microwell; self-assembled; polystyrene particle; monolayers

Introduction

In the biological, pharmaceutical and medical fields, pharmacokinetics and cellular responses to various environmental stimulants have been investigated. The major techniques in current use collect and analyse a large quantity of cells. These techniques only provide the population average and cannot clarify the dynamics of individual cells. The resolution of cell behaviour is needed to accurately evaluate the individual cell response. Single-cell analyses have been developed as one such method to obtain accurate cellular dynamics [1].

Diverse methods for the isolation and analysis of large quantities of individual cells have been developed. The microfluidic platform for cell isolation has enabled easy measurement of the activation of the voltage-sensitive potassium channel [2]. In another study,

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microfluidic single-cell isolation arrays enabled the determination of the quantity of intracellular enzyme [3]. Among such isolation arrays, the microwell, which consists of a substrate with innumerable wells arrayed at the micro scale, is a remarkable device [4, 5]. Large quantities of cells dropped onto the array were spontaneously divided into individual cells and arrayed in the individual wells. Moreover, the microwell enables the simultaneous analysis of large quantity of cells, such as image analysis [6–8]. One disadvantage of such microwells is that an expensive and large system is needed to prepare the microwell and its template [9, 10].

This paper proposes a simple microwell-fabrication method using a self-assembly system. In this self-assembly system, spherical particles are assembled due to the lateral capillary force and formed a two-dimensional (2D) particle array using relatively simple and energy efficient fabrication [11–13]. In the present study, polystyrene particles were arranged in monolayers using an evaporation-driven self-assembly system. When a meniscus is formed between the suspension and the substrate, a liquid thin film is also formed at the edge of the meniscus [14]. In the liquid film, which is thinner than the particle diameter, particles with the same wet character were spontaneously assembled owing to the lateral capillary forces.

Tatsuma et al. [15] have prepared microwells by introducing a TiO_2 solution into the particle array following the calcination of this particle template. Protoplast was separated by size in the Tatsuma's microwell. However, the TiO_2 microwell was not adequate for the analysis of live cells because TiO_2 exhibits photocatalytic activity. In this paper, we propose the use of polydimethylsiloxane (PDMS); PDMS does not affect living cells when used as a microwell material. The target cells of this investigation are unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803, which has been widely investigated as a model organism for photosynthesis, and its genomic sequence has been determined [16–19]. *Synechocystis* sp. PCC 6803 has a spherical or snowman-like shape. Whether the snowman-shaped *Synechocystis* sp. PCC 6803 cells exist during division or not has not been determined. Therefore, more precise experiments on and analyses of this *Synechocystis* sp. PCC 6803—the model organism of photosynthesis—are required. Thus, we attempted to fabricate a microwell that would trap cyanobactrial cells in a single cell to achieve further biological clarification.

Materials and Methods

Cell Culture and Growth

The target organism of this research is the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 with spherical cells. The negative strain of negative phototaxis behaviour was used in this study. This strain was grown in liquid BG-11 under 15 μmE/m^{2}s of continuous fluorescent light with slow bubbling in air at 30°C for one week.

Preparation of Template and Microwell

Polystyrene particles (Pst; Duke Scientific) were arranged in a monolayer on a glass slide (Matsunami, non-fluorescent, width of 26 mm), according to previously reported methods (Fig. 1) [20, 21]. The particle size was 3.0 μm, and the concentration was 1.0 wt%. The translation speed of the stage was fixed at 4.0 μm/s. This experiment was performed at room temperature (24.8–25.6°C) and at a relative humidity of 29.8–59.8%.

In general, the particles were hexagonally close-packed (hcp) by the evaporation-driven self-assembly system (Fig. 1). The thus-prepared two-dimensional particle arrays

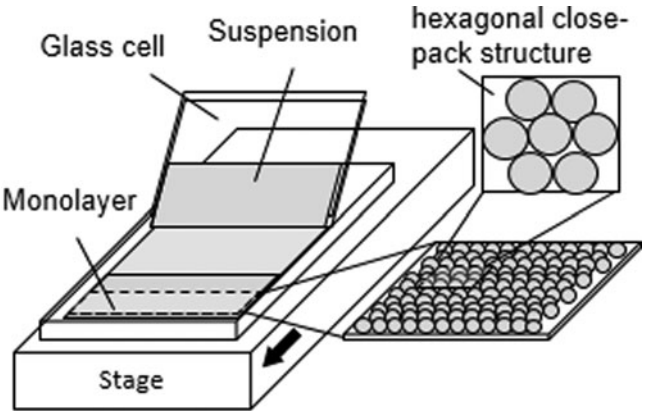

Figure 1. Schematic image of the evaporation-driven self-assembly system to prepare 2D arrays.

were etched by reactive-ion etching (RIE, EIS-700, ELIONIX) to fabricate spaces between the particles. The power of inductively coupled plasma was fixed at 200 W. The RF bias was 50 W, and the oxygen gas flow was 20 cm^3/min Two etching times, 170 and 190 s, were examined.

PDMS (Sylgard 184 Silicone Elastomer, Dow Corning Torey) was dropped onto the etched particle arrays. The PDMS base was mixed with a cross-linker in the proportion of 10 parts to 1. The PDMS was cured under room temperature for 48 h. The cured PDMS was manually peeled from the template. The particles that remained in the PDMS were dissolved in acetone. The fabricated microwell arrays were observed using a scanning electron microscope without the use of a metal coating on the samples (SEM; VE-9800SP, KEYENCE).

Surface Treatment

The microwell was immersed in NaOH (0.1 mol/L) for 30 min and subsequently washed with distilled water to be hydrophilic. The hydrophilically treated microwell was also treated with poly-L-lysine (0.1% or 0.01%) for 1 min. As a result, three types of samples were fabricated: untreated, hydrophilic-treated and poly-L-lysine treated.

Cell Trapping

The 20 μL of the culture solution (2.1×10^8 cells/mL) was dropped on the microwells and kept for 1 hour. The top view images were captured by an optical microscopy system (Olympus). All data were processed using a public domain image analysis software (ImageJ 1.41o, http://rsb.info.nih.gov/ij/).

Results and Discussion

Retting et al. [9] have seeded fibroblasts and rat basophilic leukaemia cells in microwells prepared via photolithography, and determined the optimal conditions and well sizes for the trapping of single cells in the microwells. They indicated that the microwell is required to be sufficiently wide for a cell but not sufficiently wide to fit multiple cells. On the basis of the report of Retting et al. [9], the well sizes in our study for trapping the single cells of

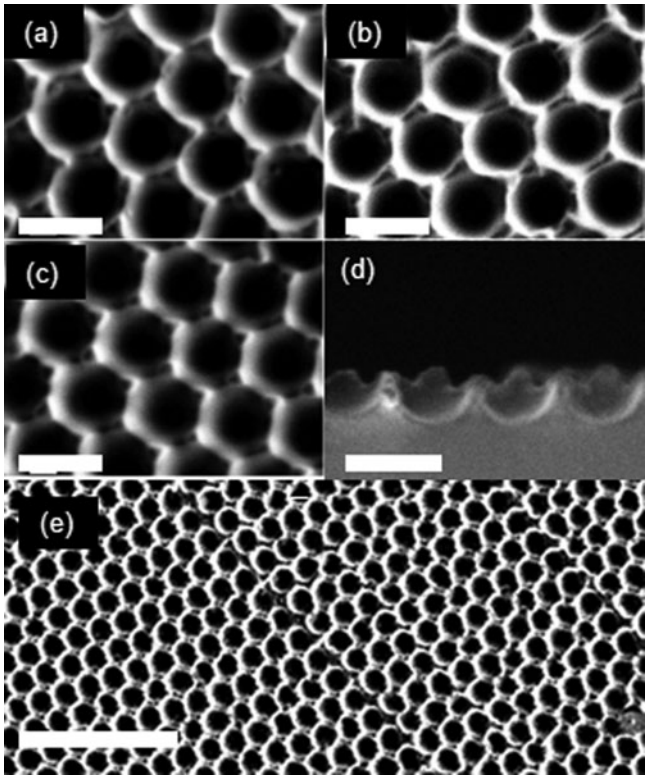

Figure 2. SEM images of microwell arrays: (a) microwells prepared from a template of non-etched monolayer, (b) and (c) microwells prepared from templates etched for 170 s and 190 s, respectively, (d) cross section of Fig. 2a and (e) an overall view of Fig. 2a; the scale bar is 20 μm. Scale bar for (a)–(d) is 3 μm.

Synechocystis sp. PCC 6803 were estimated to be 1.9–3.0 μm. Thus, we used Pst particles with a diameter of 3.0 μm for self-assembly.

Monolayers were prepared in a large area (26 mm $\times$ 3.5 mm) by the evaporation-driven self-assembly system. The arrays were hexagonally close-packed structures. The particle size in 2D arrays can be partially controlled by oxygen plasma etching [23, 24]. When the etching times were 170 s and 190 s, the particle diameters became 2.4 and 2.2 μm, respectively. The Pst particles did not maintain their spherical shape after heavy etching. These phenomena have been confirmed previously [23]. The particles of these templates were slightly larger than the target cell (1.8 μm) and were considered to have the appropriate size to trap an individual cell. The PDMS solution may penetrates deeper into the etched arrays than into the packed structure; thus, the preparation of deeper microwells was expected with our procedure. We successfully prepared microwells by dropping PDMS onto the template. The SEM images of the microwells are shown in Fig. 2. When the etching times were 0, 170 and 190 s, the well diameters of PDMS moulds were 2.6, 2.2 and 2.5 μm, respectively, and the depths were 1.17, 0.80 and 1.47 μm, respectively. The well diameters became smaller than the original particle diameter in each template when the etching times were 0 and 170 s. This result suggests that the viscous PDMS solution did not sufficiently penetrate into the particle spaces. In the case of the sampled etched for 190 s, the PDMS well

diameter of 2.5 μm was slightly larger than the etched particle diameter in the template. We considered that the microwell prepared from the template etched for 190 s was stretched during the PDMS penetration or the peel-off process because of the large spaces between particles. A precise investigation of the peel-off method and the type of organic solvent is required for the fabrication of homogeneous microwells.

Almost all cells were unsettled in the untreated microwells but were rather clumped around the edge of the cell-culture droplet. This is presumably attributed to the hydrophobicity of the PDMS or the small well size.

Non-penetration of the culture solution into small wells has been investigated using several physical methods, including degassing in a vacuum chamber and sonication under vacuum [25, 26]. The well size of 2.2–2.6 μm in this study is smaller than that of previously developed microwells. To protect the fine microstructures, chemical treatment was employed to improve the solution influx. As a result, when our microwell array was hydrophilically treated, the organisms were trapped in the wells (Fig. 3). This easy observation leads us to consider that our microwell is suitable for the analysis using such optical microscopy. Additionally, it seems that a single cell is stably positioned in a rounded microwell because of the large contact area between the cell and the microwell.

We measured the cell occupancy as the percentage of well numbers filled with cyanobacteria. The hydrophilically treated microwell with a diameter of 2.2 μm showed

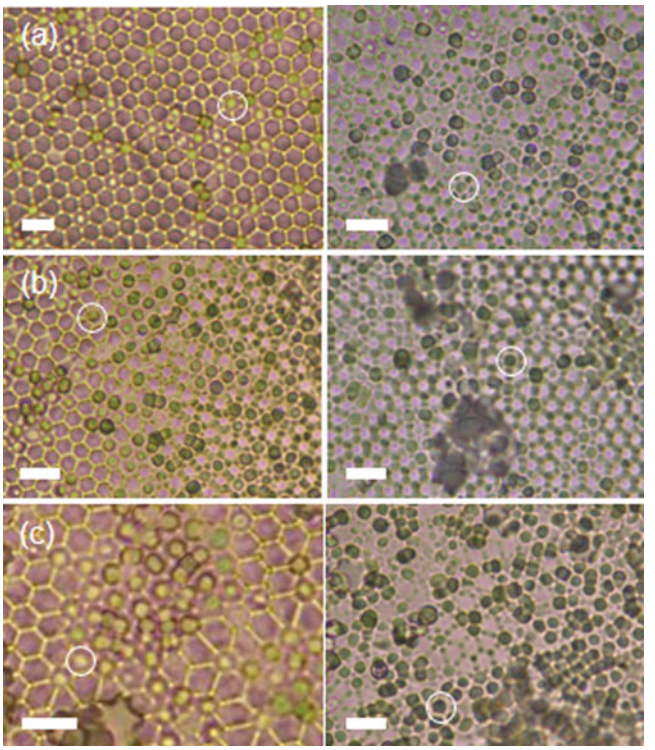

Figure 3. Single cells in microwell arrays: (a) 2.6 μm diameter microwells, (b) 2.2 μm diameter microwells and (c) 2.5 μm diameter microwells. The images on the left side are of non-treated microwells; those on the right are of microwells treated with 0.1% lysine. White circles indicate represented cells trapped in microwells. The scale bar is 5 μm.

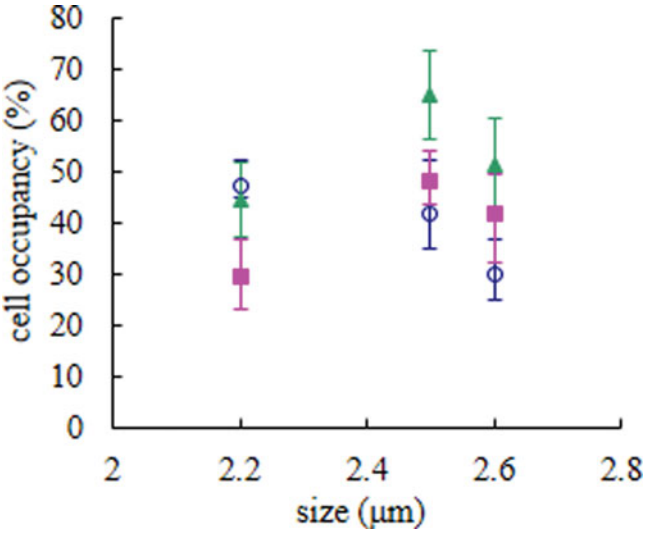

Figure 4. Effect of the each treatment on microwell arrays. Hydrophilic treatment (○), Hydrophilic and 0.01% Lysine treatment (■), Hydrophilic and 0.10% Lysine treatment (▲). The cell occupancy on of all of the microwell arrays increased with increasing concentrations of poly-L-lysine. The error bars are representative as the minimum and maximum values (n = 2∼6).

the most efficient occupancy (47.6%) in the prepared microwell arrays (Fig. 4). The occupancies of the 2.5- and 2.6-μm wells were 46.0% and 30.4%, respectively. On the basis of these results, we consider the hydrophilic treatment effective for culture influx in the wells. Actually, to maintain the viability of the bacteria in our microwells, the bacterial should be placed in culture fluid. We believe that our hydrophilic microwells are effective to maintain the culture fluid in the wells. Moreover, our cyanobacterium, that has negative photoaxis behavior, would be maintained in the traps unless the culture fluid is evaporated because the cells illuminated from the upper part in a room.

Poly-L-lysine contains numerous amino groups and is used as an adherent for the negatively charged surface of the cells. Therefore, we considered the possibility that the occupancy in the microwells could be further increased through additional poly-L-lysine treatment. In the case of treatment with 0.01% poly-L-lysine after the hydrophilic treatment, the cell occupancies of the 2.2-, 2.5- and 2.6-μm-diameter microwells were 30.1%, 48.7% and 42.5%, respectively (Fig. 4). After the treatment with 0.1% poly-L-lysine, the occupancy was 44.5% in the 2.2-μm-diameter wells, 64.9% in the 2.5-μm-diameter wells and 51.4% in the 2.6-μm-diameter wells. Cell occupancy increased with higher concentrations of poly-L-lysine on each microwell array. These results show that the treatment with poly-L-lysine after the hydrophilic treatment is effective in improving the occupancy of 2.5- and 2.6-μm wells, as expected.

However, in the case of 2.2-μm-diameter wells, the cell occupancy decreased after an additional poly-L-lysine treatment. This result was caused by the cell adherent exterior to the wells (e.g. the tops of the wells) at these shallow microwells.

In a previous investigation, Rettig and Folch showed that microwells fabricated lithographically exhibited a cell occupancy of as high as 92% [9]. However, our obtained cell occupancy was less than 70%. This discrepancy is presumably caused by the shallow depth

of 0.80–1.47 μm. This consideration is congruent with the fact that the deepest wells in our experiment—those with a diameter of 2.6 μm—showed the highest occupancy.

We expected that the further investigation of the treatment condition would reveal the enhancement of cell occupancy. Notably, the cells remained in the wells after being washed with water and methanol because of the strong binding between the cells and wells treated with 0.1% poly-L-lysine. This result suggests that reuse of the microwell may be difficult. Apparently, the excess cells, which are not trapped in the microwells, should be removed from the top surface of the microwell array with the viewpoint of practical use. Optimisation of the treatment concentration and the washing method would also be required.

In addition, TEM observations of *Synechocystis* sp. PCC 6803 (N strain) revealed the existence of flagella (unpublished results), and the presence of these flagella may decrease the occupancy in our experiments. We expect that cell occupancy can be increased through optimisation of our experimental conditions [27] and a one-step classification of spherical or snowball shapes of *Synechocystis* sp. PCC 6803 can be achieved using our template.

Conclusions

We proposes a simple microwell-fabrication method using a self-assembly system. Polystyrene particles (3.0 μm in diameter) were arranged in monolayers using an evaporation-driven self-assembly system and etched by reactive-ion etching to prepare higher microwell walls. The mould was prepared using PDMS. The well diameter was successfully controlled by the etching time. The target cells of this investigation were unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. Two types of the surface treatment, hydrophilically treatment and poly-L-lysine treatment on the microwells, were effective in improving the cell trapping efficiency. Optimisation of the treatment concentration might lead to higher occupancies. A precise investigation of the peel-off method and the type of organic solvent is required for the practical application of this method in future.

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