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Development of a Micro-Scale Cell Culture System for Strawberry using Microfabricated Devices

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ABSTRACT

A microfluidic device for cell-culture systems has been studied. Micro-scale culture systems are expected to be used for a high-throughput screening system for cell selection, micro-bioreactors, environmental monitoring devices for trace hazardous compounds, etc. Selections of highly-producing cell-lines or determinations of optimal culture conditions are time- and labor-consuming processes, especially for slow-growing cell species. Recently, a number of investigations have been reported on cell cultures using microfabricated devices. However, there exist many problems related to the control of culture conditions similar to large-scale cultivation. Also, no research has been done on plant cell culture in microfluidic devices, probably because of difficulties in their cultivation at a lower cell density. In this study, microfluidic devices fabricated using polydimethylsiloxane (PDMS) were developed for the culture of plant cells. Cultured strawberry cells, which produce red pigments, anthocyanins, were introduced into a cultivation chamber in the microfluidic device. This device was composed of cultivation chambers, weir structures to retain plant cultured-cells in them, several inlets and outlets, and connecting micro-channels. The conditioned medium (CM) combined fresh medium and used-medium and was used to facilitate cell growth at a low cell density. In this micro-scale culture system, strawberry cells were successfully proliferated for more than 5 days. Moreover, the cell growth and pigment productivity using CM were compared with those using normal fresh liquid medium. Extending the applications, we examine techniques for separating specific cells in the micro-system.

Keywords: anthocyanin, heterogeneity, microfluidic device, plant cell

INTRODUCTION

Microfabrication methods enable to organize microfluidic channels for liquid supply such as reaction solution, extraction solvent, nutrition of cells, and so on. They have been developed for micro-scale analysis with quantitative liquid operations (Sato *et al*. 2003; Yamada and Seki 2004). Recently, these methods have also been applied to cell-culture systems. These micro-scale culture systems can be utilized in a high-throughput screening system for highly-producing cell selection, micro-bioreactors for producing pure valuable products without any by-products, environmental monitoring for trace hazardous compounds, such as environmental hormones, chlorine-containing chemicals and so on. Especially, in the case of slow-growing cell species, to establish a selection system of highly-producing cell-lines and to determine the optimal culture conditions are time- and labor-consuming processes when using flask cultures. The micro-bioreactor can cut costs of cellular analysis because they reduce the consumption of costly medium for animal cell culture or the release of harmful wastes in a toxicity assay. Moreover, by increasing the ratio of specific surface to volume ratio, heat transfer and mass transfer can be significantly accelerated. Therefore, the micro-bioreactor can reduce the effects of distribution of temperature or concentration in a chemical/biochemical reaction. In the case of microculture, the materials released by individual cells are not diluted by a huge amount of bulk medium and it enables a real-time microanalysis. Microculture can be carried out in experiments for individual cells which can not be achieved by traditional methods in which the cell culture is considered as a homogenous population (Inoue *et al*. 2001). Therefore, we expect to devise culture systems for every kind of cells in these microfabricated devices. These days, due to its easy handling and/or fast growth rate, cultures of animal cells, yeast and bacteria using microfabricated devices have been reported (Wakamoto *et al*. 2001; Leclerc *et al*. 2003; Wheeler *et al*. 2003). They focus on cell sorting (Fu *et al*. 2002), microchemical analysis of cell products (Schilling *et al*. 2002; Goto *et al*. 2005), investigation of cell response to a stimulus (Takayama *et al*. 2001; Tamaki *et al*. 2002), and so on.

On the other hand, no research has been reported on plant cell culture in microfluidic devices. The reason seems to be because of difficulties in their cultivation at a lower cell density and their slow-growth. That is, a minimum effective cell-density is necessary to promote plant cell growth. It is known that conditioned medium (CM) composed of fresh medium and used medium after cultivation facilitates cell growth (Matsubayashi and Sakagami 1996) or production of secondary metabolites (Mori and Sakurai 1998). The CM is thought to contain certain growth factors (peptides) such as phytosulfokines, different from phytohormones. Another conventional method to overcome the difficulties of cultivation is the feeder-layer culture (Weber and Lark 1979). In this method, the target cells are supported by continuously feeding the growth factor derived from the high density of surrounding homogeneous or heterogeneous cells. For an enrichment of CM or feeder cells, these methods require a large amount of used medium or feeder cells for a small amount of target cells. When the selected medium or feeder cells are optimized for growth of the target cell, the system becomes complicated because of space-consuming cultivation. In the microfluidic device, the proliferation of plant cells can be easily accomplished by just a few mL of CM and the observations of individual

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cells are carried out on a light microscope.

Plant cell culture has been applied to the production of valuable secondary metabolites, such as pharmaceuticals, flavors, colorants, etc. (Tabata 2006; Roberts 2007). This method is cost-effective, and has the added advantage of producing substances that are difficult to synthesize by alternative chemical methods. Anthocyanin, one of the secondary metabolites of plant cells, is well known as a natural pigment for food colorants with low toxicity. Anthocyanins are produced by various plant-cell cultures, including wild carrot (Dougall and Weyrach 1980), sweet potato (Nozue *et al*. 1987), grape (Yamakawa *et al*. 1983; Hirasuna *et al*. 1991) and strawberry (Mori *et al*. 1993) cultures. In general, it is well known that cultured plant cells exhibit heterogeneity in secondary metabolite production (Miyanaga *et al*. 2000a, 2000b; Naill *et al*. 2005). The heterogeneity of anthocyanin production in cultured strawberry cells was investigated by image analysis (Miyanaga *et al*. 2000a). This heterogeneity of secondary metabolite production leads to lower productivity in cultured plant cells compared to that in the relatively homogeneous microbial cells. The determination factor of this heterogeneity has not been clear, although it is thought to be caused by environmental conditions or inherited difference. Strawberry was used as a model plant since the production of intracellular secondary metabolites, anthocyanins, can be colorimetrically visualized and non-destructively quantified by image analysis.

In this study, microfluidic devices fabricated using polydimethylsiloxane (PDMS) were developed for the culture of plant cells by using fresh medium and CM. Moreover, to clarify the heterogeneity of cultured plant cells, the characteristics of individual cell growth and secondary metabolite, anthocyanin, production were continuously investigated by image analysis in the micro-scale cell culture system.

MATERIALS AND METHODS

Fabrication of the microdevices

Three-dimensional microfluidic devices were fabricated using a silicone elastomer, polydimethylsiloxane (PDMS) (Hong *et al*. 2001). The fabrication procedure is illustrated in **Fig. 1**. After ultra-thick photoresist (SU-8; Microlithography Chemicals Co., MA, USA) was formed on a silicon wafer with 100 μm thickness as a master plate, PDMS prepolymer (SYLGARD 184; Dow Corning Co., MI, USA) was poured and cured on the master. The structure of the device is shown in **Fig. 2**. The device was composed of one inlet port (Port A), two drain ports (Ports B and C), eight outlet ports (Ports D) and eight cultivation chambers (**Fig. 2-1**). The cultivation chamber was built by bonding two PDMS plates with different channel depth (**Fig. 2-2**). A multilayer integration can fabricate an intricate structure. Plant cells, which are about 50 μm in diameter, have no adhesive property, while some bacteria or animal cells can attach to the surface of the substratum. To observe an individual plant cell, it is necessary to hold the cell in one place. The chamber contains a weir and a shallow ditch (ca. 30 μm) which can retain the cells and stream the medium. The width and depth of the channel were 400 μm and 100 μm, respectively.

Plant cells and medium

Whitish cultured strawberry cells (octoploid *Fragaria ananassa* cv. 'Shikinari' [white]; FAW) were proliferated on Linsmaier-Skoog (LS) medium (Linsmaier and Skoog 1965) supplemented with 30 g sucrose, 1 mg 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.1 mg benzylaminopurine (BAP) per liter of medium for more than 10 years. They had been dedifferentiated from sterilized strawberry plant leaves (Mori *et al.* 1993). Suspension cultures of FAW were established by transferring the cells from solid LS medium to liquid LS medium. The medium pH was adjusted to 5.7 before autoclaving. The cells were subcultured every week by inoculating 100 mL of fresh medium with 2 g-fresh cell weight of inoculum

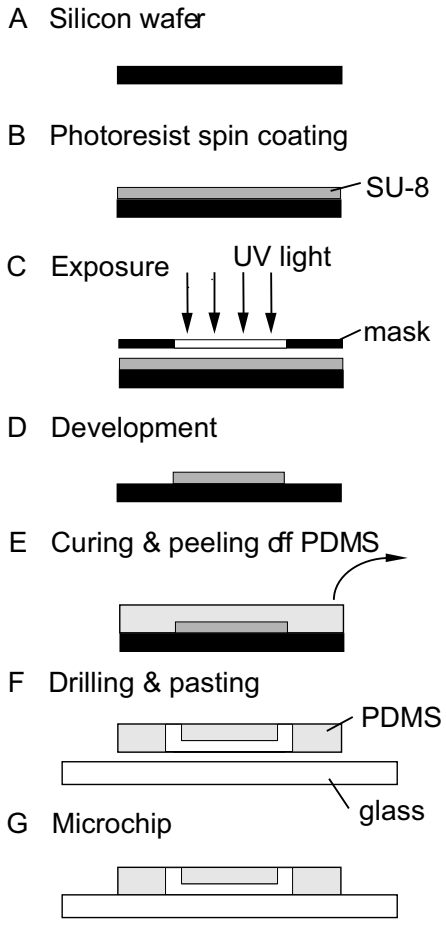

Fig. 1 Fabrication of polydimethylsiloxane (PDMS) microbioreactor.

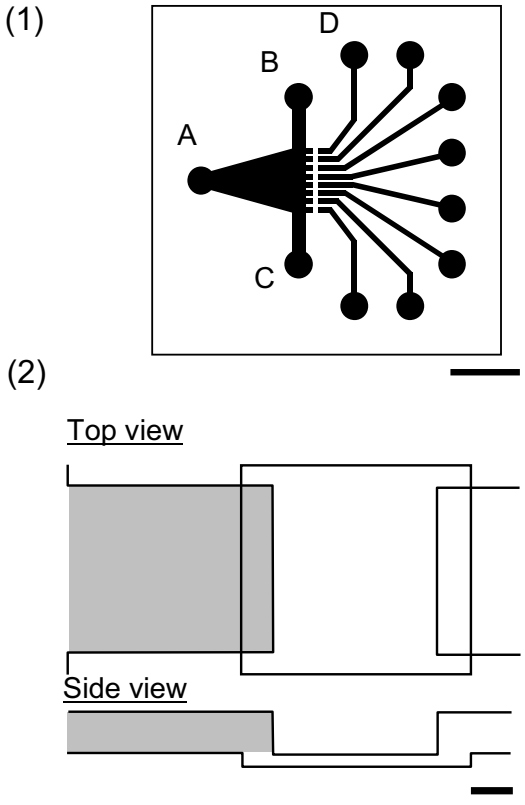

Fig. 2 Structure of the microfluidic device. (**1**) Design of the device; A: Inlet port, B, C: Side port, D: Outlet port; (**2**) top and side view of cultivation chamber. Gray area is a cultivation chamber. The volume of the chamber is 20 nl. Each scale bar indicates (1) 5 mm and (2) 100 μm, respectively.

cells after filtrating with nylon mesh (mean pore size: 37 μm) and kept in the dark so as not to accumulate pigment. The flasks were maintained at 27°C on a reciprocating shaker at 100 strokes per minute. Conditioned medium (CM) is generally considered to facilitate the cell growth at low cell density. Therefore, CM was used in this micro-scale culture system. CM was prepared by combining used LS medium after 7-day cultivation in the dark and fresh LS medium in the proportion of one part to four. For anthocyanin production, the cell culture was transferred to under 100 μmol $m^{-2}s^{-1}$ (*ca*. 8000 lx) light irradiation provided by fluorescent lamps (FL40SS, Toshiba Co., Japan). Besides FAW, a reddish light-independent mutant (FAR) (Nakamura *et al.* 1999) which can produce anthocyanin in the dark was also investigated.

Micro-scale cell culture

Installation of strawberry cells into the microfluidic device was carried out as follows. Firstly, the FAW suspension after 7-days cultivation was aseptically taken from the flask and filtrated by a nylon 60-mesh (mean pore size: 258 μm) to remove big aggregates. Secondly, the filtrated suspension was diluted about four hundredth by the medium. The cell suspension was injected from an inlet port (**Fig. 2-1**) with a sterile syringe under the stereomicroscope in a clean bench. The side ports were closed and eight outlet ports were opened. After installation of cells in the cultivation chambers, the outlet ports were closed. Finally, the side ports were opened and the excess cells were washed out. After closing all the channels, the microfabricated device was cultivated in the dark or under the light irradiation at 27°C. CM and fresh LS medium were used in the micro-scale cell culture and the effect of medium on the cell growth and pigment production were investigated. The medium used for micro-scale culture were filtrated by a 0.45 μm membrane filter to remove the cell debris or dust before autoclaving.

Evaluation of cell growth and anthocyanin content

Cell growth and anthocyanin content in the micro-scale culture were evaluated by image analysis. The images of growing cells were captured under an inverted microscope (IMT-2, Olympus Co.) with a digital camera (DP11, Olympus Co.). The cell growth was evaluated by calculating the cell-occupied area in the cultivation chamber. To determine the cell-occupied area, the monochromatic images of cell aggregates were digitized at an optimal threshold in image analysis software, Scion Image (Scion Co., MD, USA). The concentration of anthocyanin in the cell was estimated by a previous method (Miyanaga *et al*. 2000a). The color characteristics of anthocyanins produced by strawberry cell culture were as follows. The red component value (R) was much larger than the green (G) or blue (B) component values, and G was almost equal to B. The difference between R and G, the (R-G) value, was defined as an index for pigment accumulation in this study.

RESULTS AND DISCUSSION

Cell culture in shaking flasks or on solid medium have been the most common methods used to carry out investigations on plant cell culture. However, the evaluation of their growth or secondary metabolite production tends to be homogeneous and averages several aspects. Specifically, individual cells in the culture can not be traced. Lower density plant cell culture on solid medium has not been developed because of the difficulties in cultivation induced by the dilution of growth factors within the medium. Plant cell culture in a microfabricated device has the possibly of solving these problems. This tool has not yet been applied to plant cell culture, although there are a few studies for microbial culture (Wakamoto *et al*. 2001; Leclerc *et al*. 2003; Wheeler *et al*. 2003; Peng *et al*. 2006). To evaluate the possibility of applying a microfabricated device in plant cell culture, we investigated plant cell culture and secondary metabolite production in the microfabricated device as compared with conventional flask culture using strawberry as a model plant.

Effect of aggregate size on the cell growth

In the flask culture using LS medium, the cell growth of small aggregates (aggregate size: 37-258 μm) was compared with that of control culture whose aggregate size was not classified. The specific growth rates of small aggregates and the control were 0.38 day^{-1} and 0.37 day^{-1}, respectively. The doubling time was about 1.8-1.9 days. These results indicate that aggregate size does not affect cell growth. That is, the growth behavior of small aggregates in the micro-scale culture system will be as good as that of the control culture with various sizes of aggregates in the flask culture.

Cell growth in the micro-scale culture

As described above, it is well known than there is a critical cell density for growth in plant cell culture. This critical cell density is different depending on various kinds of plants. They have long been investigated. For example, the values of *Nicotiana tabacum* protoplast (Nagata and Takebe 1971) and *Daucus carota* (Nishi *et al*. 1974) are about 10^3 cells mL^{-1} and 2×10^4 cells mL^{-1}, respectively. In the micro-scale culture, cell growth was also affected by the initial numbers of cells. When there were a few FAW cells in each cultivation chamber (volume: *ca*. 20 nL) at the initial stage, FAW cells could not proliferate both in LS medium and in CM. However, when there were about twenty initial cells in the chamber, cell growth was observed. According to this result, in the case of strawberry cells, the critical cell density seemed to be at least ca. 10^6 cells mL^{-1} (20 cells in 20 nL). The day courses of cell growth in the dark using LS medium and CM are shown in **Fig. 3**. Considering the depth of the chamber (100 μm) and cell size (ca. 50 μm), it is thought that cells two-dimensionally proliferate with culture period. Therefore, cell proliferation was evaluated by measuring the plane area in the chamber occupied by cells. From these results, cell growth in LS medium was better than that in CM, although CM is thought to facilitate initial

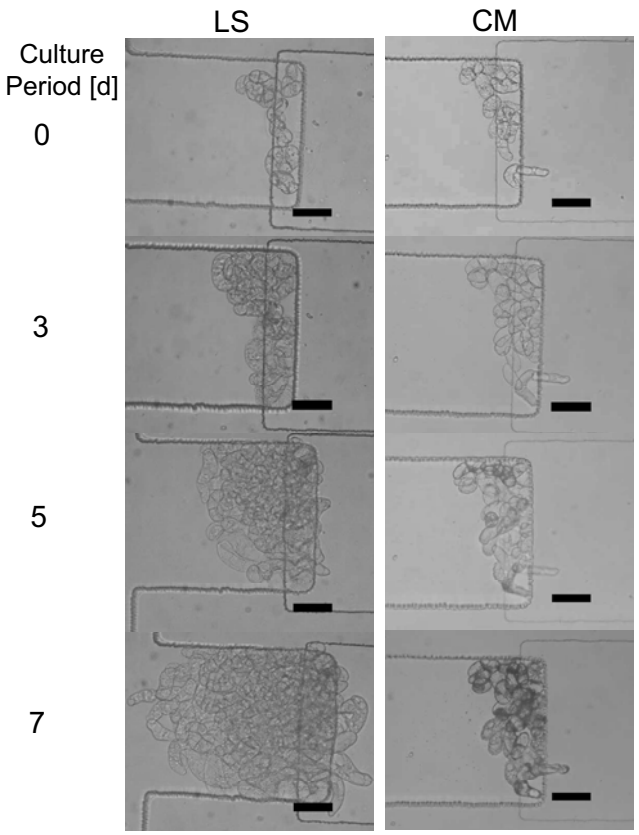

Fig. 3 Strawberry cell (FAW) culture in the microfabricated device. LS (left) and CM (right) indicate LS medium and conditioned medium, respectively. Both conditions were carried out in the dark. Each bar indicates 100 μm.

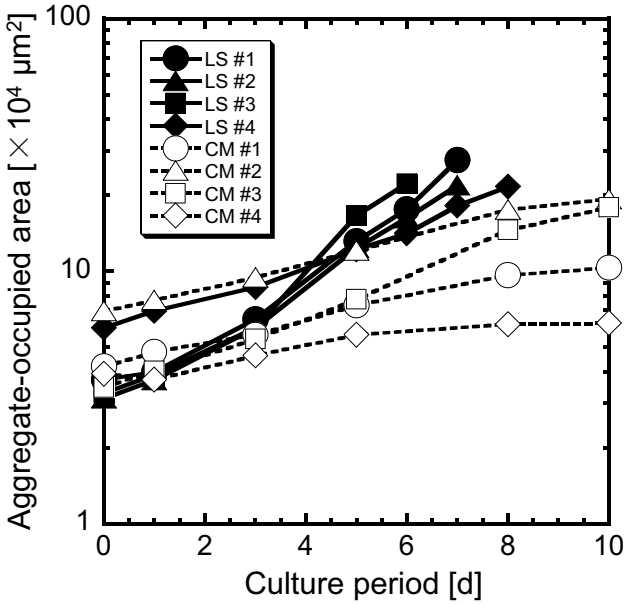

Fig. 4 Aggregate-occupied area in the micro-scale batch culture. Solid symbols and open symbols indicate LS medium and CM, respectively.

cell proliferation. The reason seems to be the accumulation of waste products in the chamber because of the batch culture using CM. To attenuate these effects, the perfusion culture where the medium was slowly supplied from the inlet port to the outlet port was tested. However, the clogging of the weir by the cells occurred during the perfusion culture (data not shown). It is necessary to modify the design of the fluid channel so as not to obstruct the medium flow.

Fig. 4 shows part of the aggregate-occupied area changes which indicate individual cell growth in the micro-scale batch culture. The growth of aggregates in each chamber (8 ports) was observed in the LS medium and in the CM. In **Fig. 4**, typical growths of four aggregates in both conditions are depicted. Significant differences between cell growth in the LS medium and that in the CM were observed, although much more data must be gathered for statistical analysis. Moreover, there were differences of cell growth tendency among individual aggregates in the same medium. Because the initial aggregate areas were not so different, these differences are probably due to the heterogeneity of cell growth. From these results, specific growth rates were determined. The specific growth rates in the LS medium and in the CM were (0.35 ± 0.12) day^{-1} and (0.14 ± 0.06) day^{-1}, respectively. The specific growth rate in LS medium in the micro chamber was greater than that in CM and similar to that in the flask culture (0.35 ± 0.05) day^{-1} using LS medium (in triplicate). In the view of cell culture, the micro-scale cell culture system can achieve a similar condition as the macro-scale cell culture system. The facilitation of oxygen transfer in the PDMS may contribute to the cell growth in the microfabricated device.

Pigment production in the micro-scale culture

For pigment production, FAW cells in the microfabricated device were cultivated under light irradiation. The behavior of pigment production is shown in **Fig. 5**. Although the well cell-growth was not observed, image analysis revealed that pigment accumulated in some cells and the concentration changed daily in individual cells. Moreover, CM was non-effective for pigment production as well as cell growth. The cells in CM became brownish on and after about the third day. The rate of turning brownish was much faster than in LS medium. Therefore, the intracellular pigment concentration when using CM was less than that when using LS medium. However, from the microscopic observation, even though in using LS medium (under light irradiation), the cell growth was suppressed compared with cultivation in the dark (data not shown). These are due to inhibitory effects on cell growth by the intracellular/extracellular accumulation of metabolic decomposition products in the CM and/or activation of secondary metabolism by light irradiation. In this article, only the batch cultures of plant cells have been investigated, because of the difficulty of keeping each cell in a given position with medium flow. In a further study, the effect of the compounds in the CM on pigment production will be investigated by using perfusion culture of the microfabricated device where fresh liquid medium is continuously supplied into each microchamber.

The cultivation of FAR cells (light-independent mutant) in the microfabricated device in the dark is also shown in **Fig. 6**. CM was fed at a low flow rate (ca. 300 μL/h) from Port B to Port C (**Fig. 2-1**). Cells could produce anthocyanin without a need for light irradiation during cultivation, although some exotic cell aggregates from a different place moved in and out of the microchamber because of a subtle fluid movement derived from silicone tube flection. Moreover, most FAR cells can highly accumulate anthocyanin and are insusceptible to the inhibitory effect of metabolite accumulation on their growth compared with wild type, FAW cells. The maximum concentrations of anthocyanin in the individual FAR cells were almost similar to each other before turning brownish. However, during the incubation period after setting the cells in each microchamber, the pigmented cells also turned brownish due to a depletion of nutrients or the accumulation of inhibitory substances for cell growth in the batch culture. In perfusion culture we can expect that continuous culture of individual cells can be achieved in the microchamber without accumulation of any inhibitory substances.

Pigment production by individual cells

At a low cell concentration, FAW cells were incubated in LS medium under light irradiation (**Fig. 7**). Anthocyanin accumulation in the individual cells was evaluated by image analysis. The changes in anthocyanin concentration in the individual cells are shown in **Fig. 8** Some cells started anthocyanin production from day 4, while others accumulated anthocyanin from day 1. They gradually accumulated more anthocyanin as culture period increased and turned brow-

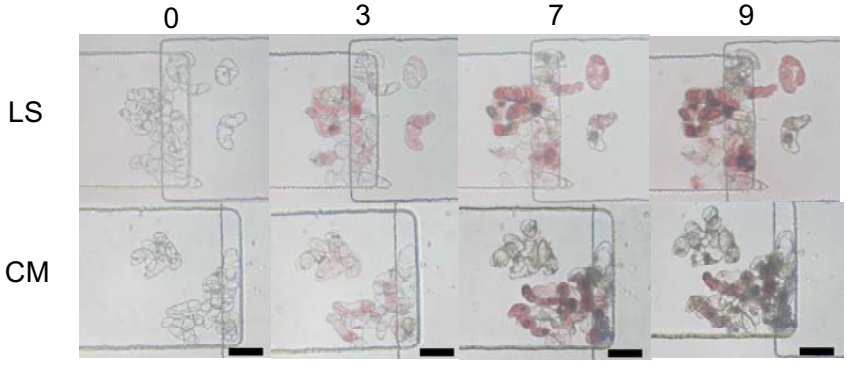

Fig. 5 Pigment production of cultured strawberry cells in LS medium and conditioned medium (CM) under light irradiation. Each number indicates culture period [unit: day]. Scale bars indicate 100 μm.

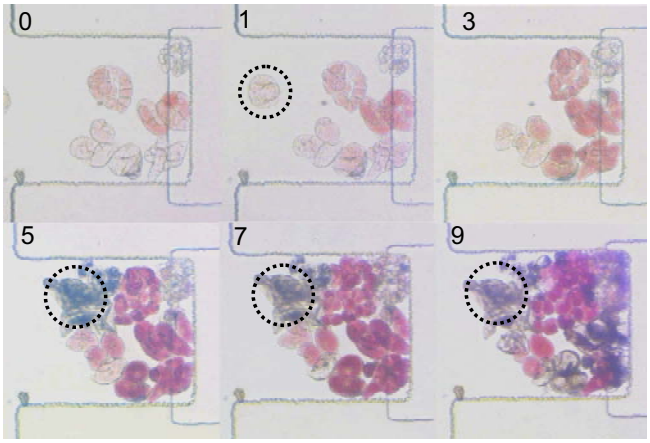

Fig. 6 Cultivation of light-independent mutant strawberry cells (FAR) in the dark. Each number indicates culture period [unit: day]. The exotic cell aggregates are circled by a dotted line.

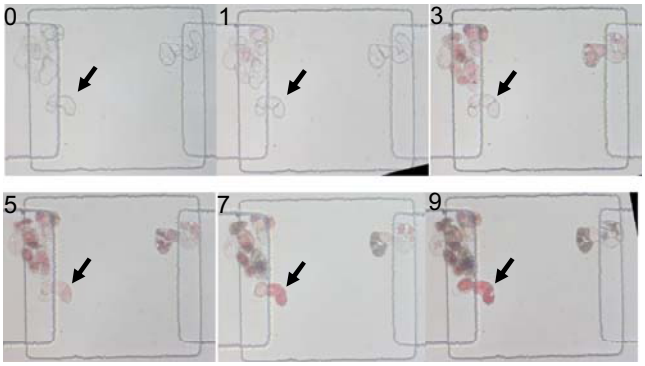

Fig. 7 Anthocyanin accumulation in individual strawberry cells in the microfabricated device at a lower cell concentration. Each number indicates culture period [day]. Arrows indicate the same cell.

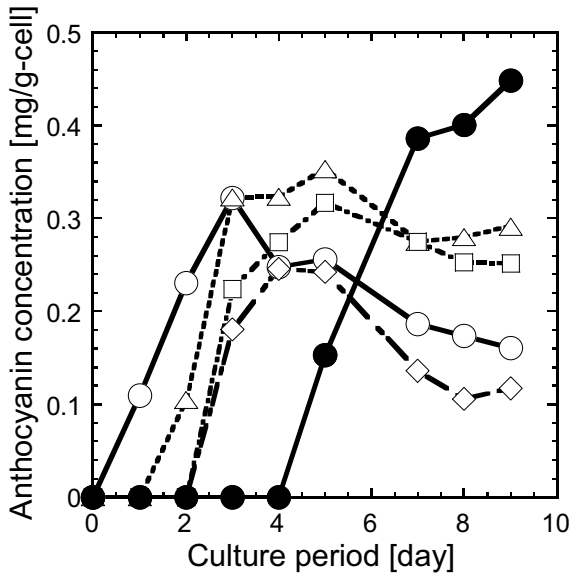

Fig. 8 Anthocyanin concentration in an individual strawberry cell in the microfabricated device. Solid symbols indicate the cell marked by an arrow in **Fig. 7**.

nish after a few days. In addition, the maximum concentrations were not so different from each other.

As described above, significant growth could not be observed because of low cell concentration in the light. However, each cell could be traced individually without an overlap. Moreover, the effects of mutual cells seemed to have almost vanished or were negligible in the chamber. Consequently, even though the cells were adjacent, their behavior of pigment accumulation differed. Considering that the volume of the cultivation chamber is small, the surrounding condition of cells is considered to be relatively uniform compared to the macro-scale cell culture. Therefore, the heterogeneity of pigment production seems not to be due to environmental conditions but rather due to other factors, such as growth cycle, inherited factor, etc. For future experiments, gene expression in secondary metabolism of each cell and the effect of synchronous culture are needed. Furthermore, for more statistical discussion, much more individual cells should be investigated in the various conditions.

CONCLUSIONS

Microfluidic devices fabricated using polydimethylsiloxane (PDMS) were developed for the culture of plant cells. The micro-scale culture of strawberry cells was carried out. The cell growth in the micro-scale culture using LS medium was similar to that in the flask culture. The cell growth and red pigment, anthocyanin, production were evaluated by image analysis of microscopic images. In the batch culture, the cell growth and pigment production in LS medium were much greater than those in conditioned medium (CM). These results seem to indicate that some compounds of CM accumulated in the cultivation chamber and inhibit cell growth or secondary metabolism. Further experiments are necessary to construct the perfusion culture of plant cells in the microfluidic devices and techniques for separating specific cells.

From the observation of individual cells in the microfabricated devices, it seemed that the heterogeneity of pigment production is dependent on some factors, such as growth cycles, inherited factors, etc. It is possible that the surrounding condition of individual cells did not affect its anthocyanin production to a great extent. Microculture in the microfabricated devices can be a powerful tool to clarify the behavior of individual cells and to investigate the heterogeneous cell population. Moreover, for the propagation of virus-free plants, microculture of vegetative cells can be applied as a first screening step. In a further study, much more data will be accumulated to suggest a statistically coherent argument.

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