Rapid Formation of Aggregates with Uniform Numbers of Cells Based on Three-dimensional Dielectrophoresis

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We applied a fabrication method for the formation of island organization of cells based on a three-dimensional (3D) device for negative dielectrophoresis (n-DEP) to produce cell aggregates with uniform numbers of cells rapidly and simply. The intersections formed by rotating the interdigitated array (IDA) with two combs of band electrodes on the upper substrate by 90° relative to the IDA with two combs on the lower substrate were prepared in the device. The AC voltage was applied to a comb on the upper substrate and a comb on the lower substrate, while AC voltage with opposite phase was applied to another comb on the upper substrate and another comb on the lower substrate. Cells dispersed randomly were directed toward the intersections with relatively lower electric fields due to n-DEP, which formed by AC voltage applied bands with the identical phase, resulting in the formation of island patterns of cells. The cells accumulated at intersections were promoted to form the cell aggregates due to the close contact together. The production of cell aggregations adhered together was easily found by the dispersion behavior after switching the applied frequency to convert the cellular pattern. When cells were accumulated at the intersections by n-DEP for 45 min, almost accumulations of cells were adhered together, and hence a formations of cell aggregations. By using the present method, we can rapidly and simply fabricate cell aggregations with a uniform number of cells.

Keywords Negative-dielectrophoresis, cell aggregates, interdigitated array electrode, intersection array, island pattern, three dimensional device

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Introduction

Dielectrophoresis (DEP) has been frequently employed to form patterns with cells and arrays of cell aggregates to apply them to cell-based biosensors,1–4 for drug development,5,6 as well as to estimate cell-cell interactions.8 The positive use of DEP force allows for a quick formation of accurate cell patterns by arranging cells to the desired positions, compared to that by arranging cells stochastically on the substrate with patterns of adhesive and non-adhesive regions. Previously, we developed a rapid and simple fabrication method of cell array by using microwell array electrodes based on the attractive force of positive DEP (p-DEP).9,10 Pairs of different types of cells in individual microwells were also formed to apply to the fabrication of hybrid cells by cell fusion. By using negative DEP (n-DEP), patterns with cells and particles can be fabricated on a substrate opposite to a substrate with an electrode pattern.11–13 The conversion of the design for cellular patterns by switching the applied frequency and/or voltage achieved the discrimination, and the separation of cells expressed specific surface antigens.14,15 Furthermore, the encapsulation of patterned cells in the layer, string and cube of a photoreactive hydrogel can be available.5,16–18 An electric field for generating DEP force was applied to accelerate the formation of cell aggregates in tissue engineering.15,20 Cells were accumulated to the convex parts of electrodes by the attractive force of p-DEP and then the regions between the convex parts were filled with cells to form the cell aggregates.21,22 However, cell aggregates could become damaged owing to the compulsory accumulation in the regions with strong electric field. The increase of the expression of genes related to the mechanical stress was investigated by applying stress with the accumulation of cells by p-DEP.23 The collection of cell aggregates formed on the substrate may be difficult to achieve through the adhesion of aggregates on the substrates.

Cellular spheroids are three-dimensional (3D) objects formed by interactions between cells with adhesive ability. The 3D cell culture model expresses similar functions to living tissues because cells in aggregates are cultured in similar environments to living tissues. For example, the ability of albumin production recovers due to the formation of multicellular spheroids with hepatocytes, while no albumin is produced by hepatocytes in the 2D culture.24 The variations of gene expressions in hepatocyte spheroids have been comprehensively estimated, resulting in the expression of large parts of genes related to liver functions.25 The common strategies for fabricating multicellular spheroids
are the prevention of cell adsorption to a solid substrate and the promotion of cell-cell interaction. Recently, the expectations have increased for the use of techniques for fabricating 3D cell structures for the reconstruction of tissues and for transplantation into a living body.\textsuperscript{26} Large numbers of spheroids can be easily and traditionally obtained by the shaking culture of cells on non-adhesive surfaces.\textsuperscript{27,28} However, it is not suitable to obtain uniform spheroids rapidly. Rotary cell culture in the rotating chamber containing cell suspension was developed for the scalable production of spheroids.\textsuperscript{29} The uniform multicellular spheroids were produced by the microwell arrays\textsuperscript{30–32} and nanoimprinted scaffolds\textsuperscript{33} fabricated by micromolding techniques. Although the basic approach of all these methods is the control of cell contact with the substrate, it is hard to collect fabricated spheroids owing to the difficulty of completely preventing cell adhesion to substrates. The hanging-drop culture was developed as an excellent method to form spheroids.\textsuperscript{34} In this method, cells were cultured in a droplet hanged from the ceiling, such as a cover of a culture dish, and spontaneously assembled to the bottom of the droplet by gravity to promote cell-cell contact. Spheroids with uniform size can be simply collected due to a lack of contact with solid substrates, while the exchange of medium is manually intensive. In these formation methods, cells were cultured for several days to obtain spheroids, and there is no technique to estimate the formation of the aggregates.

In this paper, we developed a rapid and simple fabrication method for cell aggregates consisting of uniform numbers of cells. We used a device consisting of a 3D construction of upper and lower interdigitated array (IDA) electrodes, which was previously applied to the immunosensing,\textsuperscript{35} the investigation of cells expressing a specific surface antigen,\textsuperscript{36} the formation of 3D embryonic stem cell aggregates\textsuperscript{37} and electrorotation system.\textsuperscript{38} Cells dispersed in the device randomly were compulsively accumulated at intersections of IDA electrodes by n-DEP to form an island organization, resulting in promotion of close adhesion. The production of cell aggregates adhered together can be easily observed by the dispersion behavior after switching the applied frequency to convert the cellular pattern.

**Experimental**

**Fabrication of the device for accumulating cells**

IDA electrodes were fabricated by conventional photolithography on glass substrates deposited with indium tin oxide (ITO). Each band element was 4.0 mm long and 35 μm wide. Three types of IDA electrodes were prepared with different distances between adjacent bands, 35, 70 and 105 μm. The electrode area exposed to the solution was a 5-μm thick insulating layer of negative photoresist (SU-8 3005, MicroChem Corp. Newton, MO) fabricated on the band pattern of ITO-IDA to regulate the number of bands to 40. Figure 1(a) schematically depicts the assembly of the components for the DEP patterning device. The upper ITO-IDA electrode was mounted on the lower ITO-IDA electrode with 60 μm thick polyester films (Nitto Denko Corp., Osaka, Japan), which was used as a spacer to define the fluidic channel in the patterning device. The distance between the upper and lower electrodes was determined as 70 μm owing to the polyester film and insulating layers on both upper and lower substrate. Grid formation of electrodes was fabricated by rotating the upper template ITO-IDA by 90° relative to the lower ITO-IDA.

**Island organization with HuH7 cells and formation of cell aggregates by n-DEP**

Human hepatoma cell line (HuH7), which were obtained from the Japanese Collection of Research Bioresources, were suspended in a solution consisting of 4.8 mM phosphate buffer and 190 mM sucrose (conductivity: 50 mS m$^{-1}$) for the
manipulation of cells by n-DEP. A cell suspension (2.0 μL of 1.2 × 10^7 cells mL⁻¹) was introduced into the fluidic channel on the patterning device. The AC voltage was applied to the band electrodes to form the electric field in the device by using the function generator (7075, Hioki E.E. Co., Ueda, Japan) and the voltage amplifier (HSA4011, NF Corp, Yokohama, Japan). Cell manipulation was performed under an optical microscope (IX70, Olympus, Tokyo, Japan) equipped with a CCD camera (DP72, Olympus). Figure 1(b) shows the arrangement of four band elements for cell manipulation. To form the island organization of cells, AC voltage of typically 20 V peak-to-peak (V pp) in the n-DEP frequency region (100 kHz) was applied to the band elements, while the phase of the AC voltage applied to the upper band (A) and lower band (i) was opposite to that applied to the bands (B) and (ii).

Figures 1(c) and 1(d) show the cross-sectional illustrations of the device along bands (A) and (B), respectively. The dotted lines indicate pseudo-electric lines of flux. The strong electric fields were formed between bands (A) and (ii), and bands (ii) and (i) in Fig. 1(c), and between bands (B) and (i), and bands (i) and (ii) in Fig. 1(d) due to the application of the voltage with opposite phases. Cells that experienced n-DEP moved to the intersections (A-i) and (B-ii) with relatively low electric fields to form aggregates. After the self-adhesion of cells was prompted, the frequency applied to band (B) was switched from 100 to 80 kHz to form the electric field between bands (B) and (ii). The formation of cell aggregates was estimated by the movement of cells at the intersections (B-ii).

The esterase activities in HuH7 cells were investigated by staining the cells with 3′,6′-di(Ο-acetyl)-4′,5′-bis[Ν,N-bis-(carboxymethyl)aminomethyl]fluorescein, tetraacetoxymethyl ester (Calcein-AM, Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Calcein-AM is hydrolyzed to calcein, and shows the yellow-green fluorescence in the living cells by the enzyme reaction of esterase presented in the cytoplasm. After the cell aggregates were fabricated, they were collected from the channel and were incubated in the cell culture medium (D-MEM) containing 10% fetal bovine serum and Calcein-AM (1000 folds dilution) for 20 min in a CO₂ incubator at 37°C. Fluorescent images of cell aggregates were obtained by fluorescent microscopy at different focal distances by stepping z-axis. Three dimensional images were constructed with Lumina Vision (Mitani Corp., Tokyo) and after obtaining xy-images, that were deconvoluted with AutoQuant X (Nippon Roper K. K., Tokyo). Hoechst33342 (1000 fold dilution, Abcam plc, Tokyo) for staining cell nucleus to blue was also used to count the number of cells in single aggregates.

Results and Discussion

Island organization with cells and aggregation of accumulated cells

An island pattern with cells was fabricated by DEP devices with a gap distance of 35 μm between adjacent bands based on n-DEP. Figures 2(a) and 2(b) show images before and after forming the island pattern with HuH7 cells, respectively. A suspension of cells was introduced into the fluidic channel. The particles dispersed randomly in the channel (Fig. 2(a)) started to move immediately after applying the voltage and were guided to the areas at intersections (A-i) and (B-ii) within 3 s (Fig. 2(b) and Movie S1, Supporting Information). After the self-adhesion of cells was prompted, the frequency applied to band (B) was switched from 100 to 80 kHz to form the electric field between bands (B) and (ii). The formation of cell aggregates was estimated by the movement of cells at the intersections (B-ii).

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frequency to 80 kHz, the cells accumulated at the intersections (B-ii) (Fig. 2(c)) started to move toward the intersections (A-i) on every side (Fig. 2(d)), while the cells at the intersections (A-i) did not move (Figs. 2(c) and 2(d)). The cells present at the intersections (B-ii) arrived at the intersections (A-i) 10 s after the frequency was switched. When the frequency for band (B) was switched to 80 kHz, the strong electric field was generated at the intersections (B-ii) due to the difference of the frequencies applied to bands (B) and (ii). Thus, the cells at the intersections (B-ii) experienced the repulsive force of n-DEP, resulting in the movement of cells at the intersections (B-ii) to the intersections (A-i) where the generated electric field was relatively low.

Figures 2(e) and 2(f) show images immediately before and 5 s after switching the frequency after the island pattern with cells was maintained for 1 min. Images (c) immediately before and (d) 5 s after switching the frequency after the island pattern with cells was maintained for 30 min. All cells at the intersections (B-ii) moved toward the intersections (A-i) in the upper-right corner of the image together. Therefore, the cells accumulated at the intersections for 30 min formed aggregates of cells due to mutual adhesion to each other. The direction of the movement of cell aggregates could be determined by the differences in the center of balance of accumulated cells at the intersections.

Next, DEP devices fabricated with ITO-IDA electrodes with a gap distance between the bands of 70 μm were used to form the cell aggregates. Figure 3 shows the images of the cells manipulated by n-DEP. The cells randomly dispersed in the fluidic channel (Fig. 3(a)) also accumulated at the intersections (A-i) and (B-ii) (Fig. 3(b)) within 10 s. The time required to form the island pattern by using the devices with 70 μm gap is more than that using devices with 35 μm gap. This is owing to an increase in the average moving distance of cells for accumulation. The number of cells accumulated at a single intersection by using the devices with 70 μm gap is numerous compared to that with 35 μm, because the area for accumulating cells at a single intersection increases as a result of increasing
the gap distance. When the frequency applied to the band (B) was switched 1 min after maintaining the island pattern, cells accumulated at the intersections (B-ii) were dispersed on every side (Figs. 3(c) and 3(d)). However, when the frequency was switched after 30 min, the cells moved together to the lower-left corner (Figs. 3(e) and 3(f)). The accumulated cells adhered to each other.

We also used DEP devices with 105 μm gap. Figures 4(a) and 4(b) show images of cells 30 s after applying AC voltages of 20 Vpp and 35 Vpp, respectively. Although cells started to move toward the intersections (A-i) and (B-ii) after applying AC voltage (20 Vpp), cells stopped moving after approximately 30 s, resulting in cells remaining at the region between the intersections (A-i) and (B-ii) (Fig. 4(a)). The increase of the gap distance gave rise to the appearance of a lower electric field region between the intersections (A-i) and (B-ii). When AC voltage of 35 Vpp was applied, almost all the cells accumulated at the intersections adhered to each other to aggregate, because accumulated cells moved together after the frequency was switched to 80 kHz.

Ratio of the aggregation and number of cells in single aggregates

We investigated the ratio of the aggregation of cells accumulated at the intersections by using devices fabricated by the IDA electrodes with 35 μm gap and 70 μm gap. The aggregation ratio was defined as the number of cell aggregates that moved together upon switching the frequency to the number of cell-accumulations at the intersections. Figure 5 shows the aggregation ratio as a function of the time for the cell-accumulation to form the island pattern (trial order number is five). The aggregation ratio increased with increasing time for cell-accumulation and saturated at 45 min, in both cases when electrodes with 35 μm gap and 70 μm gap were used. When the cells were accumulated for over 45 min, almost accumulated cells moved together after switching the frequency to 80 kHz, and accordingly, the accumulated cells became aggregated. Thus, the time required to form the array of cell aggregates in the present experimental condition is as short as 45 min, which is significantly rapid compared to that by methods based on the control of the cell contact. Those methods require cell cultivation for at least 1 day. The cells received n-DEP force toward the center of intersections and accumulated at the intersections by the repulsive forces generated by the strong electric fields at four neighboring intersections. Therefore, the reduction of the time to form the cell aggregations could be caused by the increase of the contact area between the reciprocal cells due to the slight flatness of cells accumulated by n-DEP force.

We also investigated the number of cells in the single aggregates that were fabricated by DEP devices for 45 min to form the island pattern. Figure 6 shows histograms for the number of cells in the single aggregates. There were approximately 7000 cells in the region between the whole band electrodes on the upper and lower substrates (0.5 μL). The average number of cells in the single aggregates was estimated and found to be 9.8 cells for the devices with 35 μm gap. The number of cells in single aggregates was estimated by the calculation of the initial concentration of cells and the volume of the region between the whole band electrodes, and found to be 8.2 cells for 35 μm gap and 70 μm gap devices, respectively. The theoretically calculated number of cells in a single aggregate coincided with the numbers.
investigated by the present experiments. Thus, the results suggest that the distance between adjacent bands allows for the fabrication of cell aggregates to regulate the number of cells in the single aggregates. Standard deviations were calculated for 35 μm gap and 70 μm gap devices and found to be 3.5 and 4.8, respectively. The dispersion of the number of cells that formed aggregates could be responsible for the bias of the initial position of the cells dispersed in the fluidic channel on the device.

The arrangement of cell nuclei and the esterase activity of the aggregated cells were investigated by staining the cells with Hoechst33342 and Calcein-AM, respectively. Figures 7(a) and 7(b) show the 3D fluorescence images of the aggregated cells stained in blue by Hoechst33342 and in green by Calcein-AM. Blue spots indicating cell nuclei were observed in the cell aggregate (Fig. 7(a)). The green signal with a deformed sphere was also observed (Fig. 7(b)). The result indicates that cells in the aggregates fabricated by n-DEP maintained the esterase activity and were alive. It is reported that cells in aggregates with a diameter less than 100 μm maintained their viability.39 The 3D image merged Figs. 7(a) and 7(b) and the rotation of the merged 3D image is shown in Fig. 7(c) and Movie S2 (Supporting Information). Blue spots enveloped in green signal spread broadly, indicating the cytoplasm. Thus, the green signal could represent the shape of the cell aggregate. The number of cells in the single aggregates can be easily counted by the number of blue spots and was found to be 10 cells in the present cell aggregate.

Conclusions

We have developed a novel platform for producing aggregates rapidly consisting of a uniform number of cells with a device of 3D construction of upper and lower IDA electrodes based on n-DEP. When the AC voltage was applied to the IDA band electrodes, the cells rapidly accumulated at the intersections formed by the bands with an identical phase due to the repulsive force of n-DEP. The incubation of accumulated cells allowed for aggregation of cells together at each intersection. The production of cell aggregates can be estimated by the conversion of accumulated position by switching the applied frequency. The number of cells in the aggregates increased with increasing the gap distance of bands; aggregates with an average of 9.8 and 18.0 cells were produced by using devices with 35 and 70 μm gap, respectively. The present method allows for the fabrication of cell aggregates to regulate the number of cells in single aggregates rapidly.

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Supporting Information

Movies S1 and S2. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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