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Micropatterning C2C12 Myotubes for Orderly Recording of Intracellular Calcium Transients

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***Abstract*— Reconstruction of skeletal muscle myotubes *in vitro* using myogenic cell lines have been widely carried out to study functional properties and disease-related biological changes of myotubes, such as intracellular calcium dynamics. However, the analysis of biological signals in isolated single myotubes or interactions among several myotubes is quite difficult problem because of the randomness in size, morphology and orientation of differentiated myotubes cultured on a conventional tissue culture dish. In the present study, we attempted to form uniform-size myotubes and detect intracellular calcium dynamics from the fabricated myotubes. We modified surfaces of culture dishes using a poly(-dimethylsiloxane) (PDMS) stamp and a 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer solution to form line patterns for myotube formation. We could form uniform-size and –orientation C2C12 myotubes and detect intracellular calcium dynamics from it. This simple method would be a useful for studying properties in myotubes with specific sizes and morphologies.**

I. Introduction

Skeletal muscle tissue, which composed of oriented multinuclear muscle myotubes, is an abundant tissue in our body and mediate body movement by its contraction properties. Thus, the lack of functional muscle tissues caused by various diseases, such as muscular dystrophy and age-related atrophy, is a major problem for our daily life. To study functional properties and disease-related biological changes of myotubes, *in vitro* studies using skeletal muscle cell lines, such as mouse C2C12 and rat L6, have been widely carried out in research area of tissue engineering and physiology [1, 2]. The one of important biological events in skeletal muscle is intracellular calcium signal which is thought to govern various myogenic programs [3]. Spontaneous calcium dynamics in myotubes differentiated from C2C12 cell line have been investigated and discussed by now [4].

However, analyzing intracellular calcium dynamics in isolated single myotubes which have various specific size and morphology, or interactions of calcium dynamics among several myotubes are quiet difficult problems. This is because myogenic cells cultured on a conventional tissue culture dish differentiated into and formed myotubes randomly in sizes, morphologies and orientation, and differentiated myotubes layered on each other as shown in Fig. 1. Thus, it has been hard to distinguish specific single myotube and observe its isolated or interacted biological signals. To overcome these problems, some cell patterning techniques for myotubes using microfabrication techniques have been proposed [5]. However, these techniques required complex procedures and materials, thus calcium imaging or other observation of biological dynamics in the myotubes after patterning were not carried out. Here, in this research, we attempted to propose a simple method to form specific size and morphology of C2C12 myotubes using only a poly(-dimethylsiloxane) (PDMS) replica molding method [6, 7] and one chemical solution, and detect intracellular calcium dynamics from the fabricated myotubes.

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II. Materials and Methods

A. Cell Culture

C2C12 mouse myogenic cells were routinely maintained and proliferated in growth medium consisted of Dulbecco's minimum essential medium (DMEM, Invitrogen), 20% fetal bovine serum (Invitrogen) and 5-40 U/ml penicillin streptomycin (Invitrogen). Undifferentiated C2C12 cells were passaged every 2 days before reaching confluence. For induction of myotubes, C2C12 were seeded on conventional tissue culture dishes (ϕ 35 mm; Corning) or chemically-treated culture dishes for micropatterning of myotubes as described below. After 48 h, around 70 ~ 80% confluence, the medium was replaced with differentiation medium consisting of

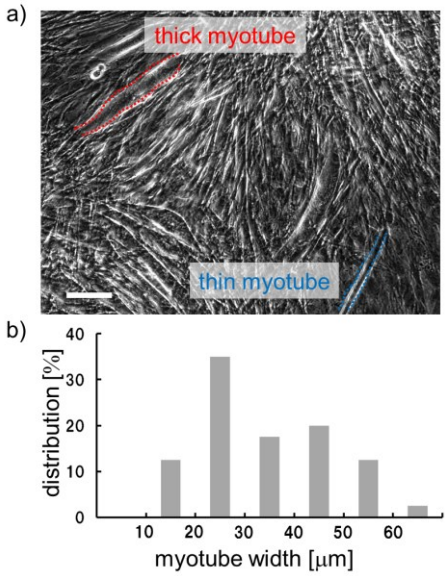

Fig. 1 (a) C2C12 myotubes cultured on a conventional tissue culture dish at 10 DIV. Scale bar: 100 μm. (b) The distribution of width of randomly constructed myotubes.

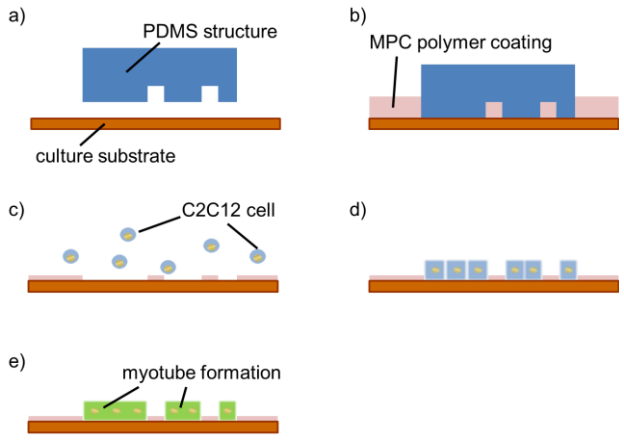

Fig. 2 Schematic depiction of the patterning processes of C2C12 myotubes. (a) PDMS stamp which had a line-pattern-indented surface was attached to a culture substrate. (b) The PDMS stamp-attached culture substrate was exposed to the MPC polymer solution to make cell-adhesive and cell-non-adhesive regions. (c) C2C12 cells were seeded onto the patterned culture surface. (d) C2C12 cells were adhered to the line patterns and differentiated into myotubes. (e) Myotubes were formed on the line patterns.

DMEM, 2% horse serum (Invitrogen) and penicillin streptomycin. The cultures were maintained in an incubator at 37 ^{o}C and 5% CO_2 atmosphere. The culture medium was changed every 2 days.

B. Micropatterning of C2C12 Cells and Myotubes

To form uniform-size C2C12 myotubes, the surface of a tissue culture dish was modified. First, microfabricated PDMS stamps were fabricated. The master mold for the PDMS stamp was fabricated by patterning SU-8 photoresist (3005, Microchem) on a silicon wafer (ϕ 75 mm, Ferrotec Silicon). In this experiment, line patterns of SU-8 with the width of 25, 100 and 150 μm were fabricated for forming uniform-size myotubes. After fabricating the master mold, the mixture of pre-polymer and catalyst (10:1 weight ratio, Silpot 184, Dow Corning) was poured over the master, and cured on a hotplate. After curing, the PDMS stamp which had a line-pattern-indented surface was released and trimmed from the master using a surgical knife. Second, the surface of a tissue culture dish was chemically treated with the PDMS stamp. In this experiment, to make a cell-adhesive and cell-non-adhesive regions, the solution of 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer [8] (Lipidure CM5206E, NOF Corp.) was used. By coating a culture dish with the MPC polymer solution after attaching the PDMS stamp, the MPC-coated region became cell-non-adhesive and the PDMS-attached region cell-adhesive. Thus, line patterns of cell-adhesive regions were simply fabricated. Finally, undifferentiated C2C12 cells were seeded and myotubes were induced as described above. The procedure for micropatterning of C2C12 cells and myotubes was schematically outlined in Fig. 2.

C. Calcium Imaging

Spontaneous calcium transients of C2C12 myotubes formed in a culture dish and a micropatterning dish were visualized using a conventional calcium imaging method. The cultures were labeled with 5 μg/ml of calcium indicator dye fluo-4/AM (excitation peak: 491 nm, fluorescence peak: 516 nm, Molecular Probes) for 30 min, and then the culture medium was replaced with a recording solution. The composition of the recording solution was as follows: 148 mM NaCl, 2.8 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 10 mM glucose (pH 7.2). The stained samples were placed on the stage of an inverted microscope (IX71, Olympus), equipped with a temperature controller (Tokai Hit) set with 37 ^{o}C, and its fluorescence was detected with a cooled charge-coupled device (CCD) camera (ORCA-R2,

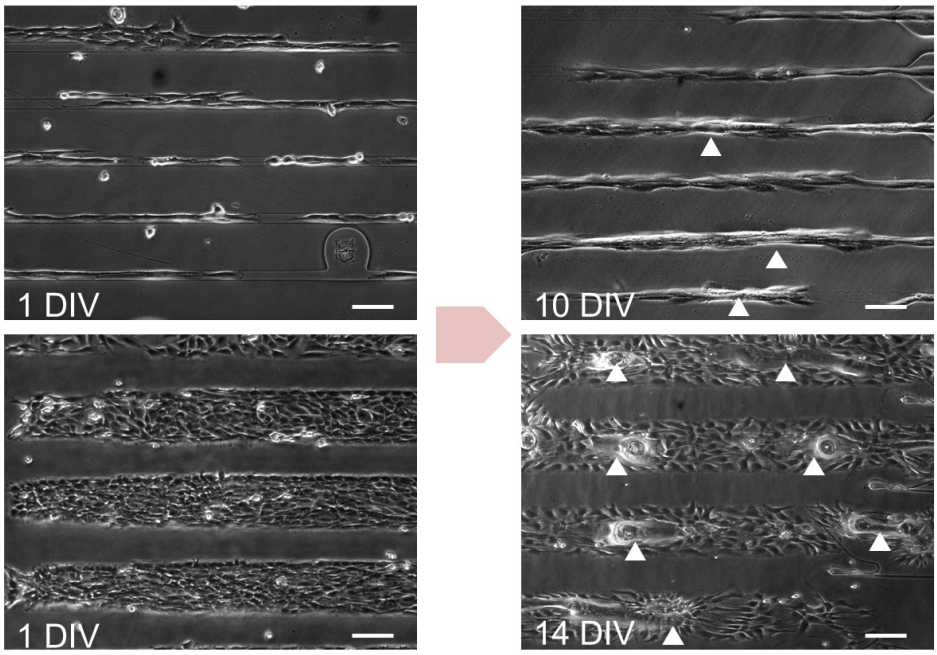

Fig. 3 Formation of uniform-size and –orientation C2C12 myotubes cultured on chemically-patterned surface. Line patterns with 25 μm width (upper) and 150 μm width (lower) are shown. Scale bar: 100 μm. White arrows indicate regions of myotube formation.

Hamamatsu Photonics). A frame rate of 500 ms/frame was used for imaging.

III. Results and Discussion

Figure 3 showed phase-contrast images of C2C12 cells and differentiated myotubes cultured on the MPC-treated culture dishes. To obtain uniform-size myotubes, we prepared three widths of line patterns: 25 (thin), 100 (thick) and 150 μm (very thick). In this figure, C2C12 cells cultured on 25 and 150 μm line patterns after 1 day in vitro (DIV) and corresponding myotubes were shown. White arrows in the figure indicated positions of myotube formation. C2C12 cells almost localized to cell-adhesive regions and thus formed uniform-morphology and –orientation myotubes in each line-width pattern. The widths of the formed myotubes were measured as shown in Table 1. Although the widths of the myotubes were relatively thinner than the set widths in the 100 and 150 μm line patterns because C2C12 cells aggregated and fused to form myotubes, the formed myotubes in each pattern had good reproducibility in width. Furthermore, we could obtain very thick myotubes which were rarely observed under conventional culture condition (Fig. 1). Thus, this simple patterning method was useful for forming uniform-size myotubes.

TABLE 1. Widths of the fabricated patterned myotube

Set width [μm]	Actual width [μm]
25	26.2 ± 5.3
100	63.1 ± 13.1
150	104.2 ± 14.6

The actual widths of constructed myotubes cultured on patterned line were measured. (n=6 for each width pattern. mean ±SE was shown.

Then, calcium imaging was carried out to visualize intracellular calcium dynamics of myotubes as shown in Fig. 4. In a conventional culture sample, traces of intracellular calcium transients from three myotubes, which had different width and orientation, were presented. These traces had significantly different frequency of intracellular calcium dynamics. The major problem is that we can not determine the differences in intracellular calcium dynamics of the myotubes are due to properties of myotubes itself with various morphologies and widths or interactions among myotubes randomly crossed in a culture surface. On the contrary, in a patterning culture sample, we could record intracellular calcium transients from isolated myotubes with uniform width and morphology. This would be promising for studying properties in myotubes with specific sizes and morphologies, and under various culture conditions, such as related to muscular diseases and injuries [9]. Therefore, calcium imaging of patterned myotubes stimulated with electrical pulses or pharmacological treatments will be of further interest.

IV. Conclusion

We proposed the simple method to patterning C2C12 cells and myotubes using PDMS replica molding and the MPC polymer solution. We could obtain uniform-size and –orientation myotubes with high reproducibility. We also could detect intracellular calcium dynamics from the patterned myotubes. This simple method would be a useful for studying properties in myotubes with specific sizes and morphologies.

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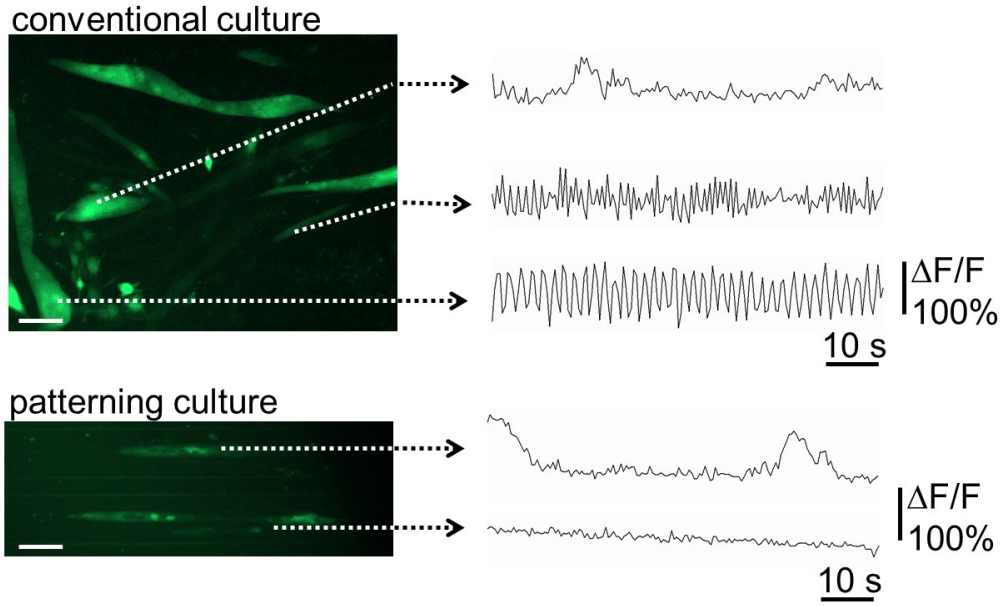

Fig. 4 Calcium imaging from randomly constructed and patterned C2C12 myotubes. Each trances show intracellular calcium transients of the myotubes. Scale bar: 100 μm.

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