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# Micropatterning of single myotubes on a thermoresponsive culture surface using elastic stencil membranes for single-cell analysis

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**We have developed a micropatterning procedure for single myotubes and demonstrated recovery of patterned myotubes without the use of methods that might cause damage to the cells. Since skeletal muscle is a highly ordered tissue mainly composed of myotubes, analysis of single myotubes is one of the promising approaches for studying the various diseases related to skeletal muscle tissues. However, the analysis of single myotubes is quite complicated because of the difficulty in distinguishing individual myotubes differentiated on a normal cell culture surface. In the present study, thin polydimethylsiloxane (PDMS) membranes, which have rectangular holes (30, 50, 100, and 200 μm in width; 500, 750, and 1000 μm in length) through them, were fabricated by using a photolithography technique and used for single myotube micropatterning. A bovine serum albumin-coated (BSA-coated) stencil membrane was placed on a cell culture surface and C2C12 myoblasts were seeded on it. Since the cells could not attach to the surface of the stencil membrane, the cell proliferated and differentiated into myotubes in the hole areas specifically. By peeling off the membrane, a micropattern of myotubes was obtained. It was revealed that the optimum width of rectangular holes for a micropattern of single myotubes was between 30 to 50 μm. Furthermore, by placing a membrane on a thermoresponsive culture surface, recovery of the micropatterned myotubes was possible by lowering the temperature. This method involving the stencil membranes and a thermoresponsive culture surface is useful for analyzing subcellular or single myotubes.**



[**Key words:** Polydimethylsiloxane (PDMS); Orientation; Tissue engineering; Skeletal muscle; C2C12; Myoblast; Myotube; Cell patterning; Cell differentiation; Single-cell analysis]

Skeletal muscle is the most abundant tissue in our body and comprises of myotubes, which are multinuclear, elongated cells exhibiting contractile ability. Many researchers have been paid much attention to the study of skeletal muscle because it is related to various diseases, such as muscular dystrophy, diabetes, age-related atrophy and so on (1, 2). In particular, since skeletal muscle cell lines such as C2C12 and L6 have been established (3, 4), *in vitro* studies of skeletal muscle have shown significant success in wide areas such as tissue engineering, drug screening, physiology, etc. (5–10). One of the promising approaches for studying skeletal muscle is the analysis of single myotubes. However, the investigation of subcellular or single myotubes is quite difficult because it is hard to distinguish individual myotubes when the myoblasts have proliferated and differentiated into myotubes on the surface of a normal cell culture substrate (Fig. 1). The process of *in vitro* differentiation of skeletal muscle cells is illustrated in Fig. 2A; myoblasts become attached to a cell culture surface, proliferate, reach confluence, and then start to fuse to form multinucleated myotubes (11). Differentiated myotubes cultured on a normal cell culture dish are oriented randomly and layered on each other (Figs. 1 and 2A).

Recently, with the progress of microfabrication technology, investigation of subcellular or single myotubes has become possible (12–14). Griffin et al. cultured C2C12 myotubes grown as isolated strips from myoblasts on micropatterned glass, and studied the prestress and dynamic adhesive strength of single myocytes (14). Tourovskaia et al. prepared micropatterns on cell adhesive film, cultured C2C12 cells on it using a microfluidic perfusion system suitable for long-term cultures, and demonstrated membrane labeling of a region smaller than a myotube (13). Molnar et al. developed a photolithography-based method for patterning C2C12 myotubes, in which myotubes are formed exclusively on vitronectin surface patterns, and determined that the optimal line width for the formation of single myotubes (12). In these studies, the surfaces of the substrates were modified to obtain a patterned area to which the seeded cells could attach firmly. Therefore, to recover or manipulate the micropatterned myotubes, damage to the cell is inevitable due to mechanical peeling or the use of an enzymatic reaction, such as trypsin, dispase, or collagenase.

The aims of the present study are to establish a method for the patterning of single myotubes and to recover them without the use of procedures that may cause damage to the cells. We employed thin elastic stencil membranes with holes through them and thermoresponsive cell culture surfaces. These stencil membranes have previously been used for the micropatterning of cells (15–19). In

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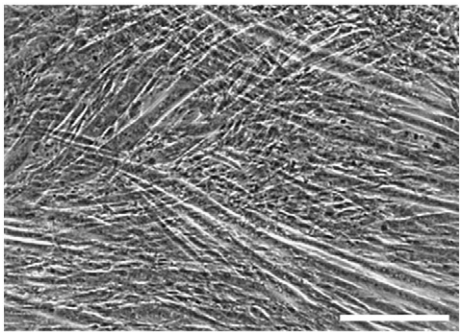

FIG. 1. Bright field picture of C2C12 myotubes differentiated in DM for 6 days on a normal cell culture surface. The myotubes were oriented randomly and crossed each other. Scale bar: 200 μm.

the process, the membranes become attached to the surface of interest and sealed the surface immediately. Cells seeded onto the membranes cannot become attached to the surface except in the hole areas, because the stencil membranes were made of cell-nonadherent materials. After seeding of the cells and peeling off of the membrane, the cells attached to the substrate remain in a pattern the same as that of the holes on the substrates. One of the biggest advantages of this method is the possibility of application to a wide range of cell culture substrates which we are interested in (15). Thermoresponsive cell culture surfaces have the unique property in that the cells cultured on them can be detached from the surface by lowering the temperature (20–22). There is no need to use an enzymatic reaction to recover the cultured cells, and thus there is no damage.

In the present study, we investigated whether the micropatterning of single myotubes was possible by using an elastic stencil membrane and whether the recovery of the micropatterned myotubes was possible by lowering the temperature using a thermoresponsive cell culture surface. Here, as illustrated in Fig. 2B, we fabricated a polydimethylsiloxane (PDMS) stencil membrane with oblong-rectangular holes of various widths (30, 50, 100, and 200 μm), coated it with bovine serum albumin (BSA) solution, placed it onto the cell culture surface, and seeded C2C12 myoblasts onto it. We observed that the myoblasts attached to the substrate specifically in the hole areas, proliferated, and differentiated into myotubes (but not on the BSA-coated PDMS membrane). We found that the number of myotubes could be controlled by changing the width of the rectangular holes and determined the optimal width for the micropatterning of single myotubes. Moreover, we applied the PDMS stencil membranes to a thermoresponsive culture surface and demonstrated the recovery of the micropatterned myotubes upon lowering of the temperature.

## MATERIALS AND METHODS

**Cell and cell culture** Murine skeletal muscle cells, C2C12, were obtained from the RIKEN Bioresource Center (Ibaraki, Japan) and cultured as described previously (5). Briefly, C2C12 cells were cultured in growth medium (GM) consisting of Dulbecco's modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; ICN Biomedicals, Inc., Aurora, OH), 100 U/ml potassium penicillin G, and 100 μg/ml streptomycin sulfate (Invitrogen) for proliferation, and in differentiation medium (DM) consisting of DMEM containing 2% horse serum (HS, JRH Biosciences, Inc., Lenexa, KS), 100 U/ml potassium penicillin G, and 100 μg/ml streptomycin sulfate for differentiation. A medium change was performed every day.

**Fabrication of PDMS stencil membranes and their use for micropatterning** Thin elastic stencil membranes were fabricated as described previously with some modifications (18). The schema is shown in Fig. 3; (i–ii) SU-8 3050 resist (SU-8; MicroChem, Corp., Newton, MA) was spin-coated onto a 4 in. Si wafer at 2100 rpm for 30 s, followed by baking at 95 °C for 30 min. (iii) After exposure to UV light through a mask, the wafer was baked at 95 °C for 4 min and the features were developed in SU-8 developer (MicroChem, Corp.). (iv–v) About 5 ml of pre-cured polydimethylsiloxan (PDMS, Sylgard184; Dow Corning, Midland, MI) was spin-coated onto the wafer at 3000 rpm for 100 s (1H-D7, Mikasa, Inc., Tokyo, Japan). (vi) After baking the wafer at 50 °C for 4 h, the thin PDMS membrane was peeled off from the wafer by means of tweezers.

**Micropatterning of myotubes and their recovery** The stencil membranes were peeled from the wafers with tweezers and placed on the surface of 35 mm cell culture dishes (Sumitomo Bakelite, Tokyo, Japan), followed by incubation in a 1 mg/ml BSA solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 2 h to prevent undesired attachment of cells in the subsequent experiments. A BSA-coated stencil membrane was peeled from a dish, attached to another dish (Sumitomo Bakelite), and used to micropattern myotubes. C2C12 myoblasts were seeded onto the dish with a stencil membrane at the concentration of 2000 cells/cm$^2$. After 4 days cultivation in GM, the medium was switched to DM to induce differentiation into myotubes. The DM was changed every day. After 4 days culturing in DM, the PDMS stencil membrane with holes was peeled off carefully under a microscope. Thus, the micropatterning of myotubes was accomplished.

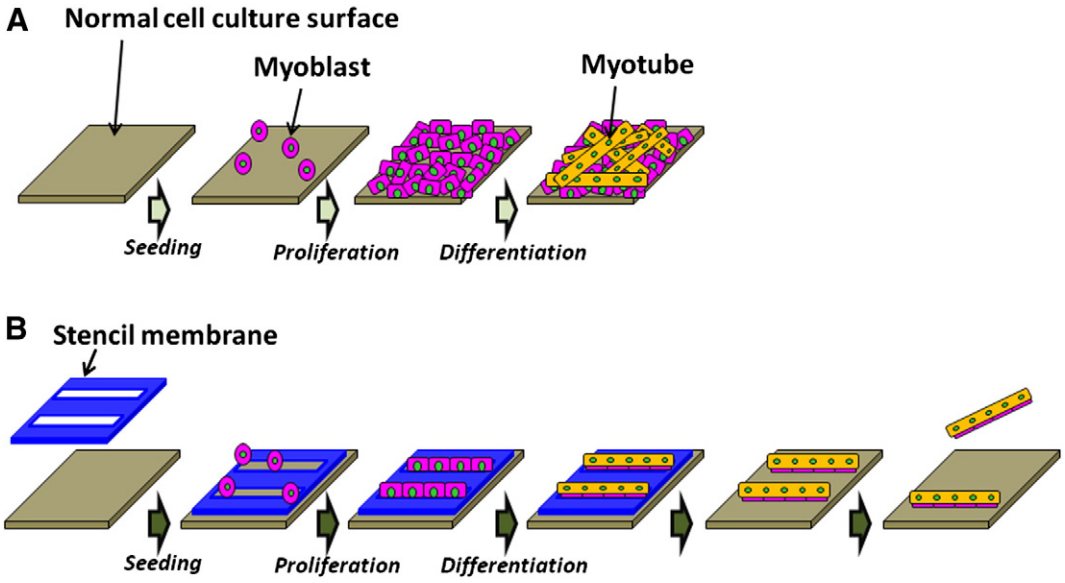


FIG. 2. Schematic illustration of myotube differentiation. A) Process of differentiation of myoblasts into myotubes on the normal cell culture surface. Seeded myoblasts become confluent and start to differentiate into myotubes. The myotubes do not align but overlap each other. B) Micropatterning of myotubes using a thin PDMS stencil membrane. After attaching BSA-coated membrane onto the surface of a cell culture dish, C2C12 myoblasts are seeded onto the membrane. After culturing in DM, the membrane is peeled off and the micropatterned myotubes can be removed.