# Submicron-grooved culture surface extends myotube length by forming parallel and elongated motif

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During skeletal muscle development, correct cellular orientation is vital to generate desired longitudinal contraction for functional muscle fibres. In this reported study, submicron-imprint lithography was used to generate submicron-grooved surfaces on polystyrene plates to induce striated myotubes in vitro. Mouse muscle myoblast cells cultured on a submicron-grooved surface migrated faster in a directionally uniform fashion; in comparison, cells cultured on a flat surface grew and migrated slower in indiscriminate directions. Subsequent maturation of the myoblast cells formed along the submicron-groove surface resulted in a tandem of parallel myotubes that were both longer and greater in circumference than in the case of the flat surface. In a functional test, the co-culture submicron-groove-grown myotubes with neurotransmitter secreting cells further demonstrated contraction abilities, suggesting submicron-groove-guided growth served to enhance myotube formation while retaining striated motifs and physiological functionality for muscle tissue engineering.

**1. Introduction:** The maturation of skeleton muscle tissue undergoes a bundling of multinucleated fibres and packing by an extracellular matrix to generate a parallel network of contracting fibres [1]. Skeletal muscle development begins with the fusion of mononucleated myotubes to form multinucleated and maturated ones [2]. Following myotube maturation, sacromeres are formed via organisation of contractile proteins, including a highly ordered arrangement of motor proteins myosin and actin [3]. Studies have also indicated that the myoblast structure and cytoskeleton arrangement may influence the morphology and assembly of subsequent skeletal myotubes, suggesting the need for a more accurate cell model in vitro [4, 5]. To be specific, precise cellular orientation is key to generating longitudinal contraction of functional muscle fibres by neurotransmitter acetylcholine [6].

The increasing number of studies that have focused on alignment of the muscle cell may indicate the importance of spatial cell rearrangement in muscle tissue engineering. Aligned electrospun fibre combined with electrical stimulation have been shown to guide and enhance myotube formation and maturation [7, 8] and Coletti *et al.* [6] reported that static magnetic fields can improve myoblast alignment. Other than physically stimulated guidance, topographical surfaces have also been widely utilised to guide cell growth to achieve a precise spatial arrangement. Surface patterns can be fabricated by photolithography or laser holographic methods, followed by hot embossing, and chemical modification for transfer of the desired dimensional pattern to a variety of substrates such as poly(methyl methacrylate) (PMMA), polydimethylsiloxane (PDMS) or quartz [9–12]. The scientists believed that the micron on nanoscale topographical surface feature can inference cell growth [13]. However, topographical features on the micron or nanoscale also display some disparities. The larger grooves on the micronscale resulted in cells growing and imbedding, forming an absolute spatial restriction, whereas on the other hand, cells cultured on a nanogrooved surface grew on top of the grooves as a directional guide and migration was not as restricted.

In this study, we used rapidly hot embossing methods to generate the submicron-grooved surface on polystyrene (PS) substrate. We then went on to demonstrate that myoblasts cultured on the submicron-grooved surface can form multinucleated myotubes. Furthermore, myoblasts cultured on submicron-grooved topographies were able to uniformly align in direction and form elongated myotubes. Contraction of maturated myotubes was also observable under co-culture with neurotransmitter secreting cells. The submicron-features of the culture surface may be useful in mimicking the natural myoblast substratum, thus serving as a more suitable in vitro environment for healthy myotube formation.

## 2. Materials and methods

2.1. Submicron-imprinted surface: Cell culture-grade PS plates (Corning, Acton, MA) were cut into 18 × 18 mm pieces. Submicron-grooved pieces were generated by hot embossing process, using a micronpatterned Ni plate as the mould. Optimum hot embossing conditions for PS materials were as follows: the pressing temperatures for the lower mould and the upper mould were 130 and 45°C, respectively, under a load of 700 kg with press duration of 60 s. The fabricated 18 × 18 mm submicron-grooved surface was 200 nm in depth, 400 nm in width and 800 nm in space. Prior to seeding cells, submicron-grooved PS pieces were washed with 70% ethanol and dried in a laminar flow hood under UV light for 2 h. The pieces were then transferred in each well of 6-well plates (cell culture grade, Corning).

2.2. C2C12 cells and culture conditions: Murine skeletal myoblasts, C2C12 cells, were obtained from the cell bank of the Bioresource Collection and Research Center (BCRC), Taiwan. Cells were cultured in Dulbecco's modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% foetal bovine serum (FBS, Hyclone, Logan, UT) and 1% penicillin/streptomycin (Invitrogen) under a humidified atmosphere of 37°C, 5% $CO_2$ and maintained at no more than 90% confluence to avoid differentiation. To induce cell differentiation, cells were grown to confluence and shifted to DMEM supplemented with 2% heat-inactivated horse serum (HS, Hyclone) for 4–7 days. To observe cell locomotion, we used a real-time culture cell




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monitoring system (ASTEC CCM-300F, Japan) to record cell migration on both surfaces. Time-lapse images were taken every 10 min for 3 h after cell adhesion. Cell migration on both surfaces was tracked by the Image – Pro Plus software. For formation of myotubes on both surfaces, observations were made every 1 h for 2 days.

2.3. PC-12 and C2C12 co-culture conditions: Rat adrenal phaeochrompcytoma cell line PC-12, a cell type which can be induced into neuronal phenotype and secrete neurotransmitters, was obtained from BCRC. PC-12 cells were cultured in DMEM containing 10% horse serum and 5% FBS. To induce PC-12 neuronal differentiation, the medium was shifted to DMEM containing 2% heat-inactived HS and 50 ng/ml murine β-nerve growth factor (β-NGF, Peprotech, Rocky Hill, NJ) was placed. C2C12 and PC-12 cells were seeded on each side of the Culture-Insert (ibidi, Germany) on the submicron-grooved surface with their specific culture media for 2 days and then, shifted to their specific differentiation media for 4 days. On the seventh day, the Culture-Insert was removed and the medium was maintained in PC-12 differentiation medium for another 1 week for C2C12 and PC-12 myotube differentiation, maturation and migration. The contractions of C2C12 myotubes were recorded in 200X magnification under an inverted microscope (DMIRB, Leica Microsystems, German) with a digital camera (Canon EOS 5D Mark II, Japan)

2.4. Immunostaining of myotube and myoblast cytoskeleton: Myoblasts and myotubes on the submicron-grooved surface were stained by immunocytochemistry and compared with cells on the flat surface as a control. Anti-α-actinin and anti-myosin were used as biomarkers of mature C2C12 myotubes and β-tubulin III for staining PC-12 neuronal cells. Cells were fixed by 4% paraformaldehyde (Sigma, St Louis, MO) for 15 min, permeablised with 0.05% Triton-X 100 (Sigma) for 10 min and then blocked in 5% HS in phosphate buffer saline (PBS, Invitrogen) for 1 h. Samples were incubated in a 1:1000 dilution of anti-α-actinin mouse antibody (EA-53, Sigma) and 1:200 diluted anti-myosin (MY-32, Zymed, Invitrogen) and β-tubulin III (T2200, Sigma) for 2 h. After washes with PBS, cells were transferred into a 1:200 dilution of anti-mouse FITC-conjugated and anti-rabbit TRITC-conjugated second antibody. TRITC-phalloidin was used to label the F-actin filament of cytoskeleton. 4′,6-diamidino-2-2phenylindole (DAPI) was used to fluorescently label nuclei. Myotube length measurement was fixed after 7 days differentiation and measured by the Image-Pro Plus software. Thirty myotubes were counted on both the flat and submicron-grooved surfaces.

2.5. Scanning electron microscopy (SEM) observation: The cell samples were cultured on the submicron-grooved surface for 16 h and then fixed by immersion in 2% (w/v) aqueous osmium tetroxide ($OsO_4$, Sigma) for 12 h at 4°C in the dark. $OsO_4$-fixed material was washed three times in distilled water to remove the excess $OsO_4$ and then dehydrated in a 10% grade-increased ethanol series, 15 min steps from10 to 90% ethanol. The material was then washed in 95% ethanol followed by three 15 min changes of absolute ethanol. The dehydrated material was critically point dried by CPD:HCP-2 drying agent and then coated with gold (40–50 nm thickness) in a Polaron E5000 diode

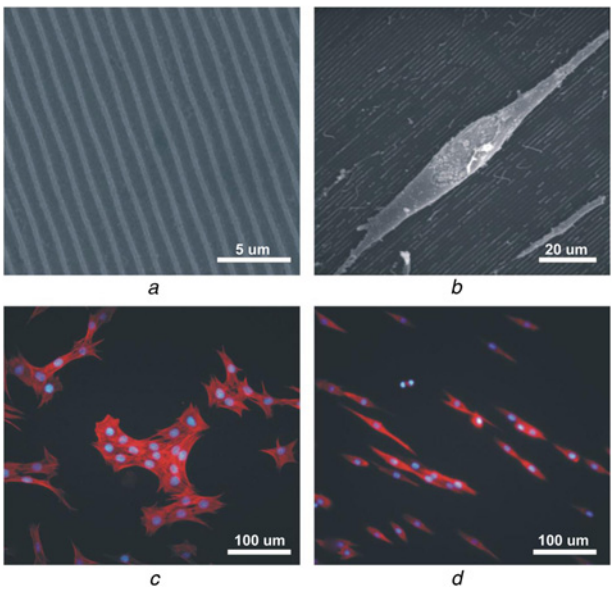


**Figure 1** *Scanning electron surface micrograph of polystyrene submicron-grooved by submicron-imprinted surface (Fig. 1a); morphology of C2C12 cultured on submicron-grooved surface (Fig. 1b); c and d immunofluorescent staining of F-actin-stained C2C12 on plane surface (Fig. 1c) and submicron-grooved surface (Fig. 1d)*

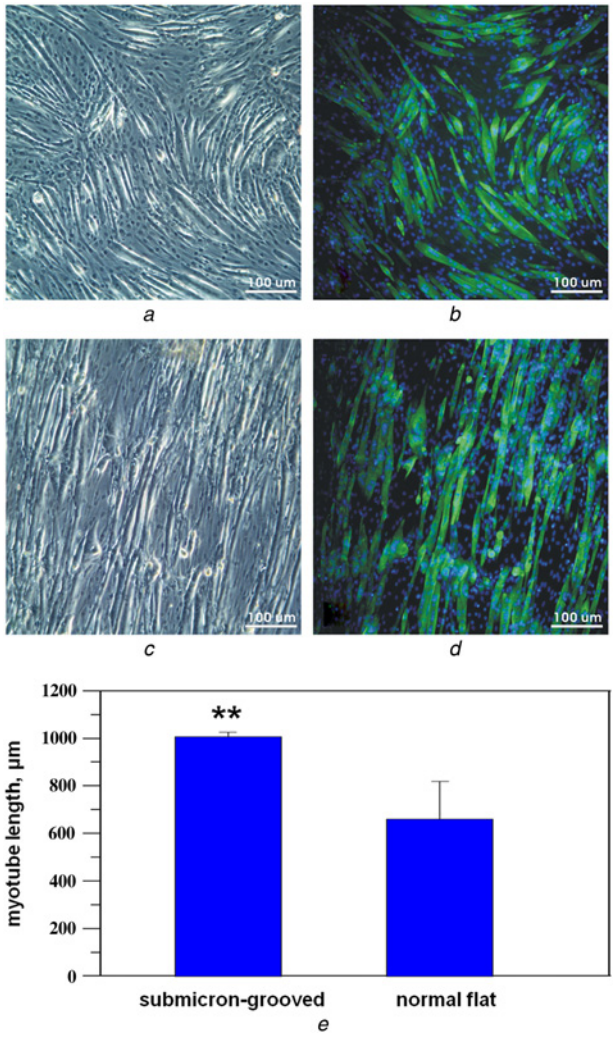


**Figure 2** *Myotubes differentiated on flat and submicron-grooved surface*
The culture was fixed and immunostained for α-actinin
*a* Light phase of myotubes
*b* Immunostained of myotubes on flat surface at fourth day
*c* Light phase of myotubes
*d* Immunostained of myotubes on submicron-grooved surface at fourth day
*e* Quantification of myotube length after seven days on flat and submicron-grooved surface

sputter coater. The sample was examined in a Hitachi S-450 SEM (SEM, LEO Elektronenmikroskopie GmbH, Germany) at 20 kV.

## 3. Results

3.1. Submicron-imprinted surface: Under SEM observation of surface characteristics, we observed groove dimensions of 400 nm in width and 800 nm in space (Fig. 1*a*).

3.2. C2C12 cells elongate and align along the grooved surface: Myoblast cultured on substrate patterns for 12 h were elongated and aligned along the direction of the patterns (Fig. 1*b*). Immunocytochemistry revealed F-actin fibre and nuclei was stretched along the long axis of the cell cultured on the submicron-grooved surface (Fig. 1*d*). In contrast, myoblasts cultured on flat surfaces showed neither elongation nor uniform orientation in actin filament and cell morphology (Fig. 1*c*).

3.3. Myogenic differentiation on topographical patterns: After switching to the differentiation medium, orientation of myotubes cultured on the flat surface formed random arrangements and had shorter, multinucleated morphology (Figs. 2*a* and *b*). In contrast, multinucleated myotubes arranged and elongated in the same orientation on the submicron-grooved surface (Figs. 2*c* and *d*). In addition, the size of the myotubes was more uniform on the submicron-grooved surfaces during early differentiation. Measurements demonstrated that submicron-grooved surface myotubes were significantly longer than the myotubes on the flat surface (submicron-grooved against flat surface 1008 ± 18 against 661 ± 159 μm) (Fig. 2*e*).

3.4. Myoblasts migration assay: We used a time-lapse phase microscope to record the movement of individual myoblasts on the submicron-grooved surface before differentiation. We found that movement on the submicron-grooves was generally restricted to the same direction whereas cells on the flat surface moved in random directions (Figs. 3*a* and *b*). Furthermore, the migration rate of cells on the submicron-grooved surface was faster (submicron-grooved against flat surface 0.90 ± 0.16 against 0.66 ± 0.18 μm/min) (Fig. 3*c*) and travelled a longer distance (submicron-grooved against flat surface 194 ± 36.4 against 120.4 ± 32.9 μm) than cells on the flat surface (Fig. 3*d*). We also observed during low densities that cells on the grooves surface tend to migrate in parallel to one another.

3.5. Myotubes elongate and fuse on submicron-grooved surface: When tracking the myotubes fused on the submicron-grooved surface, we observed myotube elongation in parallel to the grooves (Fig. 4*b*), whereas the direction of movement on the flat surface was sporadic (Fig. 4*a*). In addition, parallel myotubes on submicron-grooved tend to fuse laterally along the grooves, forming a single, longer myotube which would further elongate in the same orientation (Fig. 4*b*). Despite myotubes on the flat surface fusing laterally as well, evident branching after fusion resulted in two myotubes in two acute directions (Fig. 4*a*).

3.6. Myotubes co-culture with PC-12 cell for contraction: Skeletal muscle contractions can be triggered by motoneuron neurotransmitter acetylcholine, therefore to assess the functionality of the myotubes, we co-cultured the PC-12 cells with myotubes derived from C2C12 cells. After 13 days of co-culture, the skeletal myotubes started to asynchronously contract on the submicron-grooved surface. Immunostaining results showed the ordered, striated pattern of myosin protein and visible sarcomere in differentiated myotubes on both the flat and submicron-grooved surfaces after 2 weeks' induction (Fig. 5).

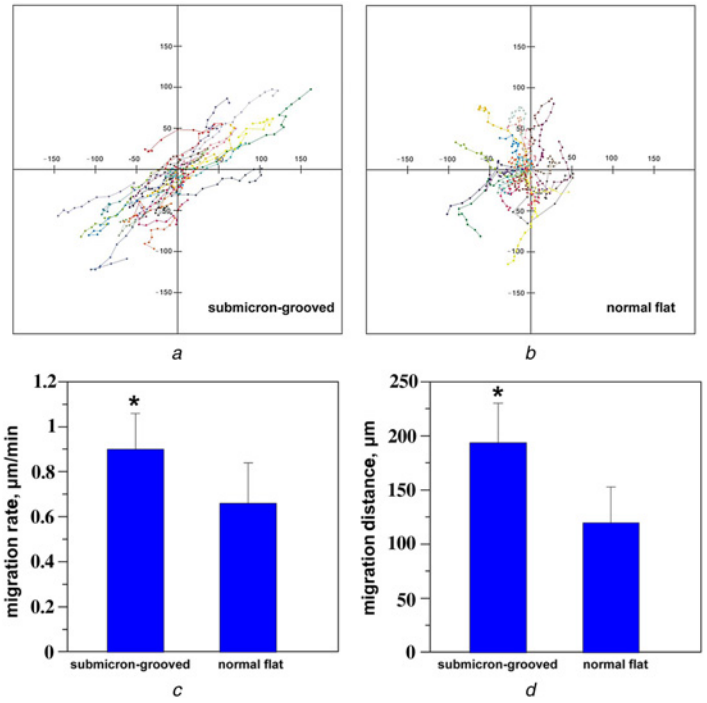


**Figure 3** *Representative diagrams of individual cell movement of 25 randomly chosen, motile cells on normal and flat surface*
*a* Most cells migrating on submicron-grooved surface were parallel moving on the grooved direction
*b* Cells migrating on flat surface were random on flat surface
*c* Migration rate of individual cells on submicron-grooved and normal flat surface. Myoblasts on submicron-grooved surface exhibited higher migration velocity than on normal surface (submicron-grooved against flat surface 0.90 ± 0.16 against 0.66 ± 0.18 μm/min)
*d* Migration distance of individual cells on submicron-grooved and normal flat surface. Myoblasts on submicron-grooved surface exhibited longer migration distance than on normal surface (submicron-grooved against flat surface 194 ± 36.4 against 120.4 ± 32.9 μm)




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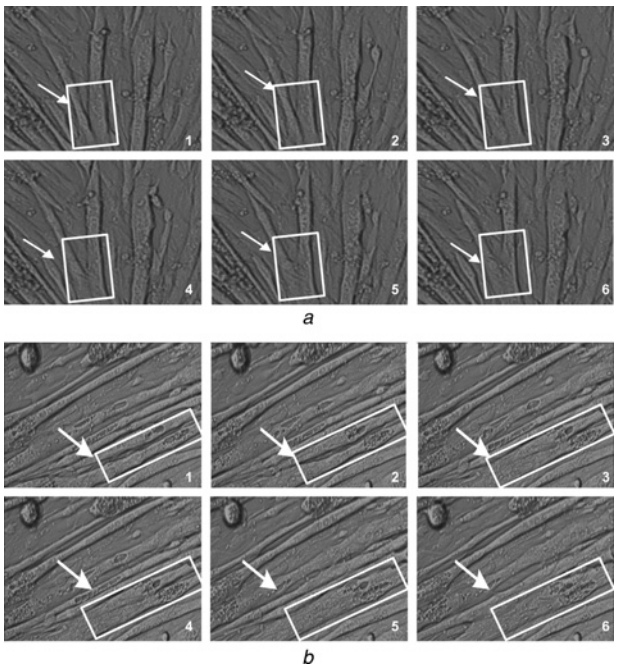


**Figure 4** *Myotubes fusion on normal flat surface (Fig. 4a), and submicron-grooved surface (Fig. 4b)*
Time-lapse microscopy of cell cultured on normal surface. White arrow indicates that two myotubes fused to form branch and more multinucleated myotubes. Time interval between each image was 1 h

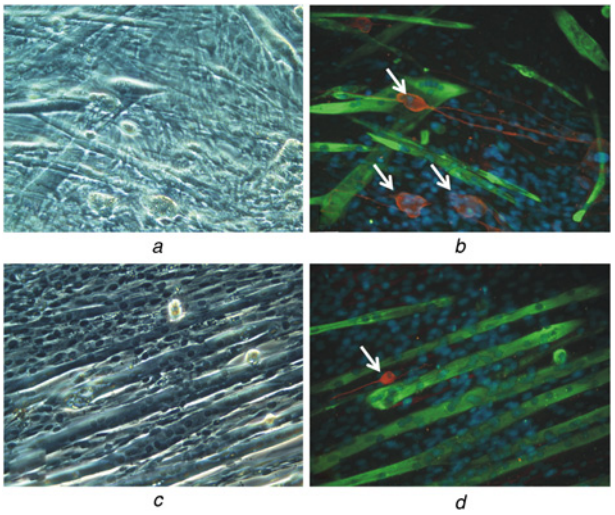


**Figure 5** *Myotubes co-culture with PC-12 cells for immunostaining*
*a* and *b* C2C12 and PC-12 co-cultured on normal flat surface
*c* and *d* Submicron-grooved surface and fixed after two weeks
*b* and *d* Stained by myosin (green fluorescence) for matured myotubes from C2C12 and β-tubulin III (red) for synapse from PC-12

Long protruding synapses from differentiated PC12 cells were also observed.

**4. Discussion:** In this study, we observed that the C2C12 myoblasts cultured on a submicron-grooved surface fabricated by submicron-imprinting exhibit an elongated morphology and parallel alignment along the grooved surface. Myoblasts migrated quicker and in tandem along the grooved surface. Previous studies on nanoscale topography features have also shown the corneal epithelial cell migration rate accelerates [14]. From this, we speculate that the topography of the cell culture submicron-scale surface may play an intrinsic and vital role in cell quality, particularly where cells are known to grow in uniform, parallel motifs. Myotubes on the submicron-grooved surface also fused laterally, forming a longer and multinucleated myotube. In contrast, myotubes on the flat surface grew in random directions and fused in a branched motif. Immunostaining showed myotubes on the grooved surface were evidently longer and more uniform in size compared with myotubes on the flat surface.

The contact guidance is the phenomenon of cells which respond to the physical clue from the growing environment such as substratum orientation. Previous studies indicated that the remodelling of cytoskeleton fibres actin and microtubules played an important role to regulate cell alignment on the groove surface. The aligned and condensed actin fibres along the groove could be observed in the early stage of cell attachment [15]. In this study, we showed that the actin fibres stretched along with long axis of the submicron grooved surface. We were also able to observe the unilateral growth of myoblasts because of spatial restriction imparted by specific topographical features of the submicron-grooved surface. Cell alignment and elongation can be attributed as a cell response to a spatial signal [16]. Fusion was also made possible as the striated cells were not imbedded in grooves. This phenomenon has also been published in epithelial cells [17].

Parallel alignment of myoblasts is a crucial step for fusion into myotubes [18]. An aligned extracellular matrix could trigger elongated multinucleated myotubes and display physiological force length behaviour, further suggesting myoblast alignment promotes myotube formation [19]. Our results also support this observation, as we found myoblasts aligned in parallel and tandem on the submicron-grooved surface promoted myotube formation.

For the development of muscle in vivo, primary myotubes form first, serving as a scaffold for secondary myotubes to fuse and form multinucleated myotubes [2, 9]. Under time-lapse microscopy, of myotube fusion on the submicron-grooved surface, two parallel aligned myotubes were laterally fused to form a longer and wider myotube. Two smaller and thinner myotubes on the side can also be seen to be beginning the early stages of fusion. Compared with the branch shape of myotubes formation on the plane surface, myotubes fused on the submicron-grooved surface are more reminiscent of physiological muscle development. The parallel alignment of two myotubes utilises one myotube as a scaffold and guide for another myotube to fuse with it. The myotubes formed were also of wider diameter and less varied in size myotubes forming on the flat surface.

Furthermore, in this study, we co-cultured myoblasts with a PC12 cell, a cell which can secrete the neurotransmitter acetylcholine responsible for neural synapse, and we were able to observe myotube contraction on the submicron-grooved surface. The arrangement of cells plays an critical role to have proper function in specific tissue such as skeleton muscles and neurons. Skeleton muscle is high order tissue which consists of a unidirectional bundle of myofibres to generate the longitudinal force by contraction. Therefore controlling the cell growing direction by designed scaffolds could facilitate the forming of a proper structure of highly organised tissue.

**5. Conclusions:** We used a submicron-imprinting method to generate a submicron-grooved surface for culturing myoblasts. The submicron-scaled topographic groove features enhanced both the length and uniformity of the myotubes, which facilitated newly formed myotubes to fuse and mature into longer, functional myotubes. Sacromere fibres and contractile properties were also clearly evident upon co-culture with motoneuron cells. The similarities between myogenesis and the observed fusion behaviour of myotubes under the submicron-groove system may provide future guidance for the design of biomaterial scaffolds.

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