

## Research Article

**Micropatterning-Induced Alignment and Differentiation of Primary Skeletal Myoblasts on Plasma-Modified Substrates**Habibur Rahman<sup>1,\*</sup> and Alok Shrivastava<sup>2</sup><sup>1-2</sup> Dr. Preeti Global University, Shivpuri - 473665, M.P. India

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**Abstract:** Reproducing the highly aligned architecture of native skeletal muscle remains a major challenge in skeletal muscle tissue engineering. In this study, a plasma-assisted microchannel-flowed plasma ( $\mu$ CFP) micropatterning technique was used to fabricate alternating hydrophilic and hydrophobic regions for guiding the alignment and differentiation of primary skeletal myoblasts derived from rat hind limb muscles. Micropatterns with widths of 20  $\mu$ m, 200  $\mu$ m, and 1000  $\mu$ m were generated using octadecyl trichlorosilane (OTS) and 3-aminopropyltriethoxysilane (APTES) surface modification. Primary myoblasts isolated from rat skeletal muscle were cultured on these patterned substrates and evaluated for alignment, differentiation, fusion index, maturation index, myotube diameter, actin cytoskeleton organization, and spontaneous twitching activity. Micropatterned substrates significantly enhanced myoblast alignment and promoted the formation of elongated multinucleated myotubes compared with non-patterned controls. Orientation order parameter and two-dimensional fast Fourier transform (2D FFT) analyses confirmed improved directional organization of myotubes and actin cytoskeleton. The findings demonstrate that plasma-assisted micropatterning provides effective contact-guidance cues for regulating skeletal muscle cell organization and differentiation in vitro.

**Keywords:** Skeletal muscle tissue engineering, Micropatterning, Myoblast alignment, Plasma processing, Myotube differentiation, Contact guidance.

## 1. Introduction

Skeletal muscle exhibits a highly organized anisotropic architecture composed of aligned multinucleated myofibers responsible for generating contractile force [1,2]. Replicating this structural organization remains one of the major challenges in skeletal muscle tissue engineering [3]. Conventional two-dimensional culture systems fail to reproduce the aligned microenvironment of native muscle tissue, resulting in randomly oriented myotubes with limited maturation and functionality [4].

Micropatterning approaches have emerged as powerful strategies for controlling cellular organization through contact-guidance mechanisms [5]. Surface topography, geometry, and physicochemical properties can strongly influence myoblast alignment, elongation, fusion, and differentiation [6]. Previous studies have demonstrated that microgroove and chemically patterned substrates can significantly improve myogenic differentiation and myotube maturation [7–9].

Plasma-assisted surface modification techniques provide versatile approaches for generating well-defined micropatterned surfaces with alternating hydrophilic and hydrophobic regions [10]. Such patterned substrates can direct cell adhesion and regulate cytoskeletal organization.

Satellite cells are the primary stem cells responsible for skeletal muscle regeneration [11]. When isolated and cultured in vitro, these cells proliferate and differentiate into myoblasts that



fuse to form multinucleated myotubes [12]. Because primary myoblasts better represent physiological muscle behavior compared with immortalized cell lines, they are valuable for studying skeletal muscle regeneration and tissue engineering.

In the present study, a microchannel-flowed plasma ( $\mu$ CFP) process was used to fabricate micropatterned substrates for guiding the alignment and differentiation of primary skeletal myoblasts isolated from rat hind limb muscles. The effects of micropattern width on myotube organization, differentiation, and functional behavior were systematically evaluated.

## **2. Materials and Methods**

Primary skeletal myoblasts were isolated from tibialis anterior and gastrocnemius muscles of rat hind limbs under sterile conditions. Excised muscle tissues were washed with phosphate-buffered saline (PBS), finely minced, and subjected to enzymatic digestion followed by mechanical trituration to obtain mononuclear cells. The isolated cells were filtered and cultured on gelatin-coated culture dishes using Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (FBS) and penicillin–streptomycin under humidified conditions at 37 °C with 5% CO<sub>2</sub>. Progressive morphological changes associated with myogenic differentiation were monitored using optical microscopy. Immunofluorescence staining of Pax7 was performed to confirm the presence of satellite cell-derived myoblast populations.

Micropatterned substrates were fabricated using a plasma-assisted microchannel-flowed plasma ( $\mu$ CFP) process. Octadecyl trichlorosilane (OTS) and 3-aminopropyltriethoxysilane (APTES) were used to generate alternating hydrophobic and hydrophilic surface regions. Micropatterns with widths of 20  $\mu$ m, 200  $\mu$ m, and 1000  $\mu$ m were prepared to investigate the influence of geometric confinement on skeletal muscle cell alignment and differentiation. Successful fabrication of micropatterns was confirmed using fluorescence microscopy.

Primary myoblasts were seeded onto patterned and non-patterned control substrates and cultured under growth and differentiation conditions for up to 14 days. Cellular alignment, elongation, and myotube formation were examined using fluorescence microscopy after staining the actin cytoskeleton with phalloidin and nuclei with DAPI. Quantitative analysis of cellular orientation was performed using orientation order parameter analysis and two-dimensional fast Fourier transform (2D FFT) analysis. Fusion index and maturation index were calculated to evaluate myogenic differentiation and multinucleated myotube formation. Myotube diameter and nuclear organization were analyzed from fluorescence images. Functional maturation of differentiated myotubes was further evaluated by analyzing spontaneous twitching behavior using kymograph-based methods. All experimental data were expressed as mean  $\pm$  standard deviation, and statistical significance was considered at  $p < 0.05$ .

## **3. Results and Discussion**

### **3.1 Isolation and Characterization of Primary Myoblasts**

Primary skeletal myoblasts isolated from rat hind limb skeletal muscles demonstrated progressive morphological changes characteristic of skeletal muscle differentiation. Immediately after isolation, the cells appeared heterogeneous with predominantly round or polygonal morphology. Upon continuous culture under growth conditions, adherent cells gradually proliferated and adopted elongated spindle-like morphologies typical of myoblast populations. During differentiation, neighboring myoblasts aligned with each other and fused to form elongated multinucleated myotubes resembling native skeletal muscle fibers. The differentiation process became more prominent after prolonged culture under differentiation conditions, indicating successful induction of myogenesis.

Immunofluorescence staining using Pax7 antibodies confirmed the presence of satellite cell-derived myogenic populations within the isolated cells. A significant proportion of cells exhibited positive Pax7 expression, demonstrating successful enrichment of primary skeletal muscle progenitor cells. The presence of Pax7-positive cells is important because satellite cells are responsible for skeletal muscle regeneration and contribute significantly to myotube formation *in vitro*. Optical microscopy observations additionally revealed increased cellular density and enhanced intercellular connectivity during culture progression. These findings confirmed the successful isolation, proliferation, and differentiation potential of primary skeletal myoblasts, making them suitable for subsequent micropattern-guided skeletal muscle tissue engineering studies.

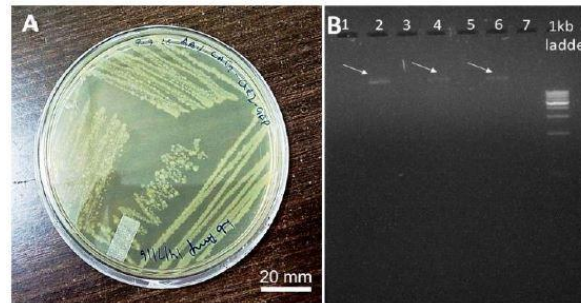


**Figure 1:** Isolation and culture of primary skeletal myoblasts from rat hind limb skeletal muscle tissues.

### 3.2 Characterization of Micropatterned Substrates

Micropatterned substrates fabricated using the microchannel-flowed plasma ( $\mu$ CFP) process exhibited well-defined and reproducible surface geometries suitable for directing skeletal muscle cell organization. The plasma-assisted surface modification strategy enabled the generation of alternating hydrophilic and hydrophobic regions through selective deposition of OTS and APTES molecules. Fluorescence microscopy analysis confirmed the successful formation of linear micropatterns with widths of 20  $\mu$ m, 200  $\mu$ m, and 1000  $\mu$ m. The patterned regions remained structurally stable throughout the experimental duration and maintained clear geometric boundaries without significant distortion.

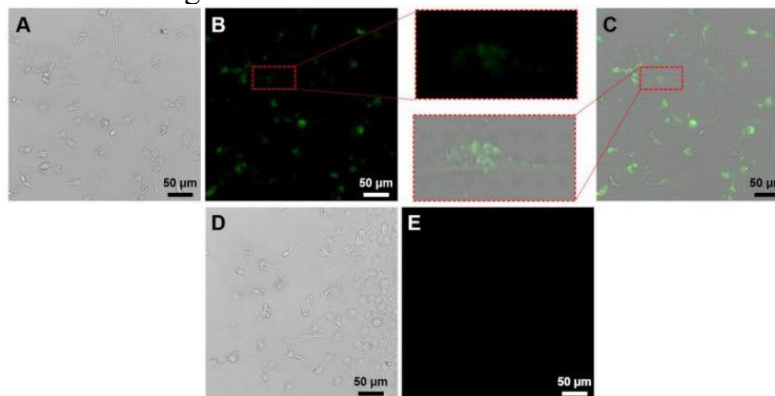


**Figure 2:** Immunofluorescence characterization of isolated primary skeletal muscle cells using Pax7 staining.

The fabricated micropatterns provided physical and chemical guidance cues that strongly influenced cell attachment and directional growth. Narrower micropatterns generated higher spatial confinement, thereby promoting anisotropic cellular orientation along the patterned direction. In contrast, wider patterns allowed relatively greater spreading of cells and reduced directional confinement. The  $\mu$ CFP process demonstrated several advantages, including

simplicity, low fabrication cost, reproducibility, and compatibility with biological substrates. Furthermore, the combination of hydrophilic and hydrophobic surface regions enhanced selective cellular adhesion and facilitated controlled organization of skeletal muscle cells. The successful fabrication of stable and biocompatible micropatterned substrates established a suitable platform for investigating the effects of geometric confinement on myoblast alignment and differentiation.

### 3.3 Alignment of Myoblasts on Micropatterned Substrates

Primary skeletal myoblasts cultured on micropatterned substrates exhibited pronounced directional alignment along the patterned regions compared with non-patterned control surfaces. Fluorescence microscopy observations demonstrated that the patterned geometries provided effective contact-guidance cues that regulated cellular orientation and elongation. Cells cultured on the 20  $\mu\text{m}$  micropatterns displayed the highest degree of alignment, forming highly organized parallel cellular arrangements. In comparison, cells on wider patterns exhibited comparatively lower alignment efficiency due to reduced geometric confinement. Cells cultured on non-patterned surfaces displayed random orientation and irregular distribution, highlighting the significance of micropattern-induced guidance.

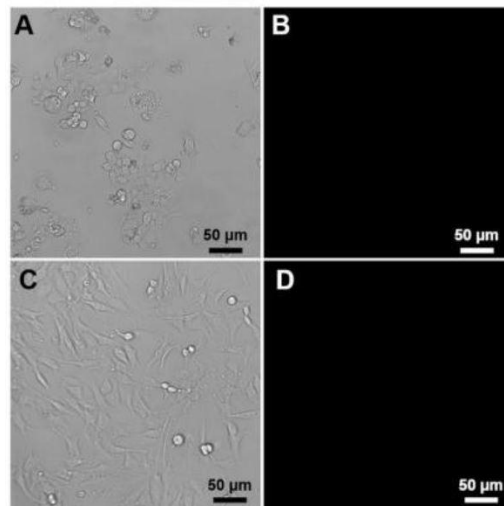


**Figure 3:** Fluorescence characterization of plasma-assisted micropatterned substrates fabricated using the microchannel-flowed plasma ( $\mu\text{CFP}$ ) process.

The enhanced cellular alignment observed on micropatterned substrates can be attributed to cytoskeletal reorganization induced by the anisotropic surface architecture. Contact guidance from the patterned edges promoted directional actin filament organization and polarized cell morphology. As the cells proliferated, alignment cues propagated collectively through neighboring cellular interactions, resulting in large-scale directional organization of myoblast populations. Such alignment is critically important in skeletal muscle tissue engineering because native muscle tissue possesses highly ordered anisotropic architecture required for efficient force generation and contractile functionality. The findings demonstrated that plasma-assisted micropatterning effectively recreates microenvironmental cues resembling native skeletal muscle organization and significantly improves cellular orientation during in vitro culture.

### 3.4 Orientation Order Parameter Analysis

Quantitative orientation order parameter analysis confirmed that micropatterned substrates significantly enhanced directional organization of skeletal myoblasts compared with non-patterned control surfaces. Cells cultured on narrow micropatterns exhibited higher

orientation order parameter values, indicating strong anisotropic alignment along the patterned direction. Among the investigated geometries, the 20  $\mu\text{m}$  patterns produced the highest alignment efficiency due to enhanced spatial confinement and stronger contact-guidance effects. Conversely, cells cultured on wider patterns or non-patterned surfaces demonstrated lower orientation values because of random cellular distribution and reduced directional constraints.

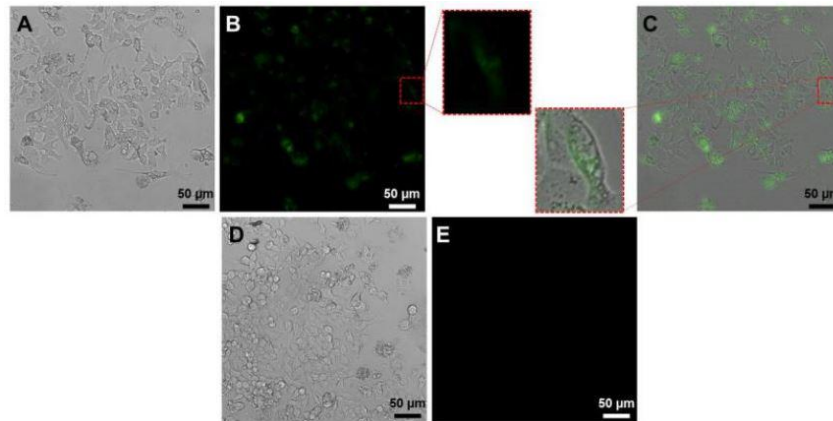
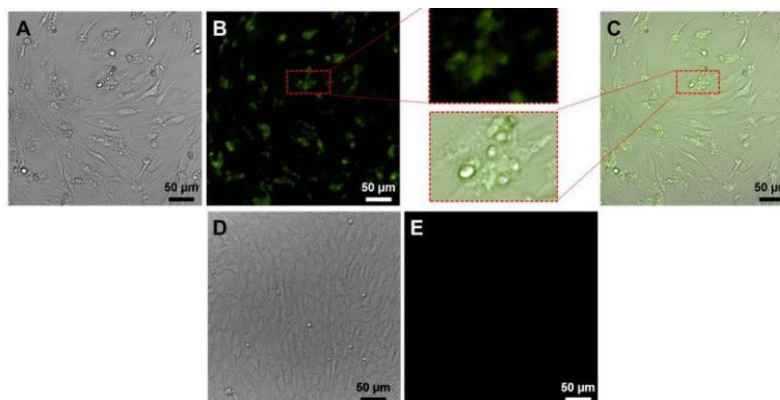


**Figure 4:** Alignment of primary skeletal myoblasts on micropatterned substrates with different pattern geometries.

Two-dimensional fast Fourier transform (2D FFT) analysis further supported the fluorescence microscopy observations by demonstrating highly concentrated orientation distributions along the principal pattern axis. The FFT profiles obtained from aligned cultures exhibited narrow directional peaks, whereas control samples showed broad isotropic distributions. These results indicate that micropattern geometry strongly influences cytoskeletal organization and collective cellular orientation. The enhanced anisotropic organization observed on micropatterned surfaces is highly relevant for skeletal muscle tissue engineering because aligned myoblast organization is essential for subsequent myotube fusion, maturation, and contractile behaviour. The quantitative analyses therefore confirmed the effectiveness of the plasma-assisted micropatterning strategy in directing skeletal muscle cell alignment and recreating physiologically relevant muscle-like architecture in vitro.

### 3.5 Myotube Differentiation and Fusion



Micropatterned substrates significantly promoted myogenic differentiation and multinucleated myotube formation compared with non-patterned controls. During differentiation culture, aligned myoblasts fused efficiently to form elongated multinucleated myotubes oriented parallel to the micropattern direction. Fluorescence microscopy images revealed dense and organized myotube networks on patterned surfaces, whereas control cultures exhibited randomly

distributed and less mature myotubes. The enhanced differentiation observed on patterned substrates suggests that geometric guidance cues strongly influence cell–cell interactions and fusion processes required for skeletal muscle maturation.

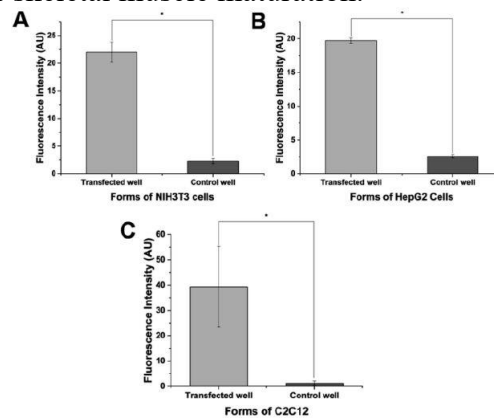


**Figure 5:** Quantitative orientation order parameter analysis of cellular alignment on micropatterned substrates.

Quantitative analysis of fusion index and maturation index further confirmed improved myogenic differentiation on micropatterned substrates. Cells cultured on narrow micropatterns demonstrated significantly higher fusion efficiency and greater numbers of multinucleated myotubes compared with wider patterns and controls. The aligned microenvironment likely facilitated efficient cytoskeletal organization and promoted membrane interactions necessary for myoblast fusion. Furthermore, the elongated morphology of aligned cells enhanced directional cellular communication, thereby supporting synchronized differentiation and maturation. The results indicate that micropattern-induced alignment not only regulates cellular organization but also directly enhances functional aspects of skeletal muscle differentiation. Such improvements in myotube formation and maturation are particularly important for developing physiologically relevant skeletal muscle tissue models for regenerative medicine, disease modelling, and drug screening applications.

### 3.6 Myotube Diameter and Nuclear Organization

Micropatterned substrates strongly influenced myotube morphology, diameter, and nuclear organization during skeletal muscle differentiation. Differentiated myotubes formed on patterned surfaces exhibited elongated and highly organized structures resembling native skeletal muscle fibres. Quantitative analysis demonstrated that micropattern-guided cultures produced more uniform myotube diameters compared with non-patterned controls. The improved structural organization observed on micropatterned substrates is likely associated with enhanced cellular alignment and efficient fusion processes during myogenesis.

Nuclear organization within the differentiated myotubes also showed significant differences between patterned and non-patterned cultures. Myotubes cultured on micropatterned surfaces displayed evenly distributed nuclei aligned along the longitudinal axis of the myotubes, whereas randomly organized cultures exhibited irregular nuclear distribution patterns. Proper nuclear positioning is an important indicator of skeletal muscle maturation and functionality because native skeletal muscle fibres possess highly ordered nuclear arrangements that contribute to efficient cellular signalling and contractile performance. The anisotropic microenvironment generated by the micropatterns likely promoted coordinated cytoskeletal tension and directional force distribution, thereby regulating nuclear positioning and myotube morphology. These findings demonstrate that plasma-assisted micropatterning effectively

enhances structural maturation of engineered skeletal muscle tissue and contributes to the development of biomimetic muscle-like constructs with improved physiological organization.

### **3.7 Actin Cytoskeleton Organization and Functional Behavior**

Phalloidin fluorescence staining demonstrated highly organized actin cytoskeleton alignment in myotubes cultured on micropatterned substrates. The actin filaments exhibited strong directional orientation parallel to the patterned regions, whereas cells cultured on non-patterned surfaces showed randomly distributed cytoskeletal organization. The aligned actin structures observed on micropatterned substrates indicate enhanced cytoskeletal maturation and efficient transmission of intracellular mechanical forces. Two-dimensional fast Fourier transform (2D FFT) analysis further confirmed the anisotropic organization of actin filaments by showing concentrated directional frequency distributions along the principal alignment axis.

Functional evaluation of differentiated myotubes revealed spontaneous twitching activity in aligned cultures, indicating successful development of contractile skeletal muscle-like tissue. Kymograph analysis demonstrated coordinated contractile motion and synchronized activity among neighboring aligned myotubes. In contrast, non-patterned cultures exhibited less organized and weaker contractile behavior. The improved functional maturation observed on micropatterned substrates can be attributed to enhanced cellular alignment, efficient myoblast fusion, and organized actin cytoskeleton formation. The development of spontaneous contractility is a key indicator of physiological maturation in engineered skeletal muscle tissues. These findings demonstrate that plasma-assisted micropatterning not only regulates structural organization but also significantly improves the functional behavior of engineered skeletal muscle constructs, thereby highlighting its potential for advanced tissue engineering and regenerative medicine applications.

## **4. Conclusion**

A plasma-assisted microchannel-flowed plasma ( $\mu$ CFP) micropatterning strategy was successfully employed to fabricate substrates capable of guiding the alignment and differentiation of primary skeletal myoblasts. Micropatterned surfaces significantly enhanced cellular alignment, myotube formation, cytoskeletal organization, and functional maturation compared with non-patterned controls. The developed approach provides a simple and effective strategy for reproducing the anisotropic organization of skeletal muscle tissue in vitro and may contribute to future developments in skeletal muscle tissue engineering.

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**Conflicts of Interest:** The authors declare no conflicts of interest to report regarding the present study.

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