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Directed Collective Cell Migration Using Three-Dimensional Bioprinted Micropatterns on Thermoresponsive Surfaces for Myotube Formation

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Abstract

Directed collective cell migration governs cell orientation during tissue morphogenesis, wound healing, and tumor metastasis. Unfortunately, current methods for initiating collective cell migration, such as scratching, laser ablation, and stencils, either introduce uncontrolled cell-injury, involve multiple fabrication processes, or have utility limited to cells with strong cell–cell junctions. Using three-dimensional (3D) bioprinted gelatin methacryloyl (GelMA) micropatterns on temperature-responsive poly(*N*-isopropylacrylamide) (PNIPAm) coated interfaces, we demonstrate that directed injury-free collective cell migration could occur in parallel and perpendicular directions. After seeding cells, we created cell-free spaces between two 3D bioprinted GelMA micropatterns by lowering the temperature of PNIPAm interfaces to promote the cell detachment. Unlike conventional collective cell migration methods initiated by stencils, we observed well-organized cell migration in parallel and perpendicular to 3D bioprinted micropatterns as a function of the distance between 3D bioprinted micropatterns. We further established the utility of controlled collective cell migration for directed functional myotube formation using 3D bioprinted fingerprintlike micropatterns as well as iris musclelike concentric

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Author Contributions

W.D., S.H., and D.I.S. conceived the project, designed and executed the experiments, interpreted data, and wrote the manuscript. G.S. and M.G. performed experiments and reviewed the manuscript.

ASSOCIATED CONTENT

Supporting Information

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Parallel cells retain their orientation after being removed (Figure S1); Directed collective C2C12 migration initiated by GelMA microfiber on non-PNIPAm coated surface (Figure S2); Derivation of fusion index from primary images (Figure S3); (DOCX)

Directed collective cell migration initiated by a 3D printed line micropattern (Video S1) (AVI)

Collective cell migration after removal of GelMA micropattern (Video S2) (AVI)

Directed collective cell migration initiated by GelMA micropattern with a wide gap (Video S3) (AVI)

Parallel and perpendicular myotube formation following collective myoblast migration initiated by the GelMA micropattern with a wide gap (Video S4) and a curved boundary (Video S5) (AVI)

Myotube contraction upon the electric pulse stimulation (1–4 Hz) (Video S6) (AVI)

Notes

The authors declare no competing financial interest.

circular patterns. Our platform is unique for myoblast alignment and myotube formation because it does not require anisotropic guidance cues. Together, our findings establish how to achieve controlled collective cell migration, even at the macroscale, for tissue engineering and regeneration.

Graphical Abstract

Keywords

3D printing; temperature-responsive surface; directed collective cell migration; myotube orientation

1. INTRODUCTION

Collective or individual cell migration regulates the orientation of cells during embryo morphogenesis, tissue regeneration, and in pathological processes, such as wound healing and cancer cell invasion.^{1–3} During collective cell migration, a group of cells move together and synchronize each other's behavior. Specifically, collectively migrating cells sense and integrate external guidance cues by securing cadherin-dependent cell–cell adhesion, polarization, and mechanocoupling for cell-signaling and force transmission.^{4–8}

Collective cell migration is more efficient than individual cell migration both in the absence or the presence of guidance cues.^{9,10} In guidance free environment, the migration speed of individual cells is faster but it frequently changes the direction, whereas collective cell migration has persistent migration.¹¹ Even in the presence of environmental guidance cues, collectively migrating cells are proficient in an intercellular integration of guiding signals through long-range intercellular force transmission compared to individual cell migration.¹² Therefore, collective cell migration is the preferred method to achieve desired cell orientation.

However, the current state-of-the-art techniques in collective cell migration have practical challenges and limited utilities. Directed collective cell migration can be achieved by scratch wound-healing assay,¹³ laser ablation of cell clusters,^{14,15} and cell patterning through a PDMS stencil.^{16–18} While sudden scratching and laser ablation-induced collective cell migration create a cell-free space, it also generates an uncontrollable spatial gradient of extracellular matrix composition and cell injury at the boundaries. Conversely, PDMS stencil provides an injury-free method for initiating collective cell migration, but it has limited application. Because PDMS stencil not only requires laborious, time-consuming, and expensive clean-room facilities for photolithography of the mold, but also involves photomask fabrication, casting, curing, and peeling-off processes.

While one could control migration of cells with strong cell–cell junctions, like epithelial cells,^{19–21} it is difficult to govern collective cell migration of cells with weak cell–cell junctions, such as myoblast (iris, intestine, vaginal tissue, etc.) and neuronal cells. Moreover, the current methods of collective cell migration could not achieve the orthogonal cell migration as seen with neurons.^{22,23} Together, we do not have a platform to control collective cell migration as required for physiological function of various tissues.

Here we demonstrate the parallel and perpendicular migration of cells with weak cell–cell junctions by combining 3D bioprinted GelMA hydrogel patterns with a poly(*N*-isopropylacrylamide) (PNIPAm) coated thermoresponsive surface. Our approach is fast, reproducible, involves one-step manufacturing in an ambient environment, does not require external anisotropic scaffold (micro- and nanopatterned materials) or anisotropic stimulation (electric field and mechanical strain),^{24,25} and allows directed collective cell migration at millimeter-scale. In addition, our 3D printing method provides ease-of-use over PDMS stencils because it is a direct, flexible writing technique, and does not require clean-room (soft-lithography) or photomask fabrication. Our findings further provide the proof-of-concept and a direct physiological application in the engineering of fingerprint and human iris by simultaneous control of linear and radial myotube orientation.

2. MATERIALS AND METHODS

2.1. Cell culture.

C2C12 mouse myoblast cells (ATCC no. CRL-1772) were cultured in DMEM media (ATCC) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco) in an incubator supplemented maintained at 5% CO₂ at 37 °C.

2.2. Bioink Preparation.

A 10% gelatin methacryloyl (Sigma-Aldrich & CellInk) and 0.1% photoinitiator Irgacure 2959 (Sigma-Aldrich) were dissolved in PBS at 70 °C for 1 h. The solution was transferred to a cartridge and stored in 4 °C before use. Prior to use, the cartridge was moved to a 40 °C oven for 10 min to liquify and then in the ambient environment for 10 min for cooling. Next, the cartridge was fixed on the preheated printhead for printing at 26 °C.

2.3. Cell Patterning on the Thermoresponsive Plate.

Linear and circular shaped GelMA micropatterns were printed in a poly(*N*-isopropylacrylamide) (PNIPAm) coated 6-well culture plate (ThermoFisher Scientific) using an extrusion-based 3D printer (BioX, CellInk) equipped with a 0.2 mm diameter conical nozzle. After printing, the plate was placed in a UV cross-linker (SpectroLinker) for cross-linking at the energy of 300 mJ/cm². The cross-linked GelMA micropatterns were stored at 4 °C in PBS and sterilized by exposing to UV lamp (265 nm) for at least 30 min prior to use. C2C12 mouse myoblast cells were seeded on the plate at a density of 1 × 10⁵ cells/mL. Then, the plate was placed in a 37 °C incubator for 1 h for uniform cell attachment. Next, the plate was placed in a 20 °C incubator for 30 min for cell detachment. The detached cells on the plate were gently washed away with 20 °C culture medium.

2.4. Time-Lapse Imaging of Cell Migration.

Time-lapse imaging was performed using a live cell analysis system (IncuCyte). Specifically, an automated inverted microscope (Nikon, Japan) equipped with a 10× objective was placed in an incubator with 5% CO₂ at 37 °C controlled by IncuCyte 2016 B software. The interval between two image acquisitions was 10 min for migration and 20 min for differentiation unless otherwise mentioned.

2.5. Cell Orientation.

Phase contrast images were preprocessed by Fast Fourier Transform (FFT) band filter to enhance cell boundaries. Cell orientation was then calculated based on the Gaussian gradient of the preprocessed images through a custom-written MATLAB (The MathWorks) code.²⁶ Specifically, the algorithm could determine intensity gradient with a Gaussian differential filter. In each subregion of the image, the dominant local direction is perpendicular to the direction of the intensity gradient. Here, we set the subregion size to 25 pixels × 25 pixels (i.e., 20 μm × 20 μm). Cells that orient perpendicular to the micropattern boundary are defined to possess 0° orientation angle unless otherwise mentioned. The orientation index (OI) of cell orientation is defined as the average value of the second-order Legendre polynomial calculated using the cell orientation angle (θ):²⁷

$$\theta = \left\langle \frac{3\cos^2\theta - 1}{2} \right\rangle$$

OI will be 1 if the cell orientation angle is zero (perpendicular). OI will be 0 if there is a random alignment. OI will be −0.5 if the cell orientation angle is 90° or −90° (parallel).

2.6. Image Processing and Particle Image Velocimetry (PIV).

Background subtraction was performed in ImageJ to reduce noise. Contrast-limited adaptive histogram equalization was implemented followed by wiener2 denoise filter. The PIV analysis was performed using a PIVlab toolbox.²⁸ To yield a high vector resolution, a high signal-to-noise ratio, and a high dynamic velocity range, we performed PIV using three passes with interrogation window sizes of 128 × 128, 64 × 64, and 32 × 32 (pixels) with 50% overlap, respectively. Velocity magnitude and direction were further analyzed using the custom-written code in MATLAB.

2.7. Removal of GelMA Micropattern and Parallel Migrating Cells.

To explore the influence of GelMA stripes on parallel or perpendicular migrating cells, we performed three independent loss-of-function experiments by removing: GelMA or parallel migrating cells or both. The GelMA micropatterns were peeled off using forceps carefully. The parallel migrating cells were scratched away using a custom-made capillary needle under a microscope. Cells were placed back into IncuCyte after treatment, and their migration patterns were observed over time. Subsequently, the OI of perpendicular cells, far from the scratching sites, was calculated.

2.8. Characterization of Myotube Formation and Contractile Function.

Cell growth medium was replaced with differentiation medium (DMEM supplemented with 2% horse serum (Sigma-Aldrich) and 1% streptomycin/penicillin) for myogenic differentiation after cells reached their confluence. Cells cultured on the flat uncoated polystyrene culture dish were used as control. The differentiation medium was refreshed every 2 days until immunostaining at day 4. For myotube staining, cells were fixed with 4% paraformaldehyde in PBS, permeated with 0.5% Triton X-100 (Sigma-Aldrich) solution, and blocked with 3% BSA (Sigma-Aldrich) in PBS. Myotubes were stained with MY-32 antifast myosin skeletal heavy chain (MHC) antibody (Sigma-Aldrich) or N-Cadherin (Invitrogen) at room temperature for 1 h in the dark. Then, Alexa Fluor 555 or DyLight 488 secondary antibody (Invitrogen) was added to cells at room temperature for 1 h in the dark. Cell nuclei were stained with 300 μ M DAPI (Sigma-Aldrich) for 10 min. The samples were then mounted with prolong gold antifade mountant (Life Technology) overnight and stored in the dark at room temperature before imaging. The fusion index was calculated as the ratio of the number of nuclei in myotubes and the total nuclei. Nuclear and myotube orientations were calculated as the angle between the long axes of the best-fitted ellipses and micropattern boundary unless otherwise stated. Fluorescent images were taken by AxioScope and Zeiss 710 confocal microscope (Carl Zeiss).

After 6 days of culture, myotubes were stimulated using electric pulse stimulation. Two carbon electrodes were separated 2 cm apart with 10 V voltage. Pulse waves of 1, 2, 3, and 4 Hz with 2 ms duration were used for simulation. Videos were taken by a camera mounted onto a microscope in the phase contrast mode. Myotube contraction was analyzed using TrackMate plugin in Fiji/ImageJ. Contraction frequency was analyzed using power spectrum analysis in Origin (Origin Lab).

2.9. Statistical Analyses.

Statistical analyses were performed using t-student test for a group of two and one-way analysis of variance followed by a Tukey's posthoc test for group of three or more. All graphs are drawn by Origin.

3. RESULTS AND DISCUSSION

3.1. 3D Bioprinted Micropatterns Initiate and Guide Directions of Collective Cell Migration.

Figure 1 illustrates our strategy to use 3D bioprinted micropatterns for initiating collective migration of cells with weak cell–cell junctions in parallel and perpendicular directions. The hydrogel micro-patterns are first 3D bioprinted on thermoresponsive PNIPAm coated culture plate followed by UV cross-linking of the hydrogel at room temperature. Cells are seeded at room temperature and then incubated at 37 °C to keep them above the lower critical solution temperature (LCST) of PNIPAm (32 °C).

To create a cell-free region in between two 3D bioprinted hydrogel-based patterns without causing cell-injury, cell-laden 3D bioprinted micropatterns are incubated at 20 °C. Next, cells located between micropatterns are washed away using culture medium, resulting in

desired cell free space. To initiate directed collective cell migration from cells harbored on hydrogel micropatterns, cells are further incubated at 37 °C. Unlike conventional methods, our approach allows both parallel and perpendicular collective cell migration because of the presence of GelMA micropattern. Cells located along the micropattern boundaries migrate in the parallel direction whereas leader cells located away from the micropattern migrate in the perpendicular direction. Our 3D bioprinted micropattern-mediated directed collective cell migration lead to controlled cell orientation and polarization, which promote myoblasts differentiation to directed myotube orientation.

Our choice for GelMA was based on its ability to print, crosslink, and provide native cell adhesive ligands.²⁹ The line width of our 3D bioprinted micropattern depended on nozzle diameter, printing speed, and dispensing pressure. The line width of all of our printed micropatterns was $528.8 \pm 25.6 \mu\text{m}$ unless otherwise mentioned.

To analyze if the collective cell migration is a function of the distance from the boundary or time of their seeding, we tested the collective cell migration at three stages (Figure 2A). At the initiation stage (200 μm from the boundary; at 24 h), cells begin to migrate toward the parallel direction as indicated by the negative OI (Figure 2A–D; stage I). Specifically, cells located adjacent to the boundary of the GelMA micropattern migrated along the boundary in parallel direction (stage I). At the intermediate stage (700 μm from the boundary; at 48 h; stage II), we observed collective cell migration begin to orient in perpendicular to the micropattern as seen with their higher OI (Figure 2A–D). At the confluent stage (1100 μm from the boundary; at 72 h; stage III), we observed faster collective cell migration of leading cells in the direction perpendicular to the micropattern (Figure 2A–D, F–I, and Video S1). Therefore, the direction of collective cell migration is a function of their distance from the boundary. We also observed a reduction in the migration speed of cells in the trailing region, which may be due to their higher density as seen with cell jamming phenomenon.³⁰ We further observed that the orientation index of cells, migrating in the parallel direction, is not a function of time (Figure 2E). We have also demonstrated that cells are parallel to the boundary up to 200 μm , begin to transition from parallel to perpendicular from 200 to 700 μm , and then become perpendicular from 700 μm onward. Although number of cells migrating toward perpendicular direction exceeds the number of cells in parallel direction; here, we observed that their migration in orthogonal direction(s) coexist perhaps at varying speed (Video S1). Together, our 3D bioprinted micropattern on thermoresponsive substrate can initiate and direct collective cell migration in parallel and perpendicular directions.

Next, we analyzed if the 3D bioprinted micropattern or the contingent of cells migrating in parallel directions could govern the speed and the direction of cells migrating in the perpendicular direction. Specifically, we performed the loss-of-function experiments. We independently removed the GelMA micropattern, parallel cells immediately adjacent to GelMA micropattern, or both (schema explained in Figure 3A, D, G) at the intermediate stage (48 h) and then followed the direction of cells previously moving in parallel or perpendicular direction. Since it is a one-step removal process, cells occupy a cell-free space (created after initial removal) and thus appear like a cell-sheet at the removal site. We first noticed that our removal of GelMA or cells adjacent to GelMA would not affect the OI (of perpendicular cells) upon the scratching as the perpendicular leader cells are far from the

boundary or the site of scratching. We found that removal of the GelMA micropattern led to migration of parallel cells toward the perpendicular direction, perhaps due to the loss of contact guidance from the GelMA micropattern necessary for cells to remain aligned in parallel direction (Figure 3B; demonstrated as lines in red). Conversely, we found that removal of GelMA did not impact leader cells already migrating in the perpendicular direction (Figure 3A–C). This suggests that the GelMA micropattern is critical for preservation of parallel migration but dispensable for maintenance of leader cells' ongoing migration in the perpendicular direction (Video S2). When we removed parallel cells, adjacent to GelMA, while keeping GelMA micropattern intact, we did not observe any impact on leader cells moving in perpendicular direction and a new set of cells got aligned in the parallel direction adjacent to the GelMA micropattern (Figure 3D–F and S1). When we removed both GelMA micropattern and parallel cells, we did not observe any impact on cells migrating in perpendicular direction (Figure 3G–I). Together, GelMA micropattern-mediated contact guidance is crucial for initiation and maintenance of parallel cells as well as initiation of perpendicular cells, but it is dispensable for the maintenance of cells migrating in perpendicular direction. Thus, contact guidance accounts for the initial parallel migration along the micropatterns. One could conceive that substrate material and stiffness affect the parallel orientation and migration of cells if we change the substrate from polystyrene to soft polymers. However, the collective cell migration in the perpendicular direction is the concept of cell–cell interaction and not cell–substrate interaction. Together, the orientation and migration of cells, between two GelMA micropatterns, are independent of substrate material and substrate properties.

Our results of 3D bioprinted line micropattern initiated collective myoblasts migration are distinct from collective myoblast migration using a PDMS stencil.³¹ Myoblasts spread in all directions when initiated by the PDMS stencil. Instead, we demonstrated a polarized directed collective myoblast migration using the printed GelMA micropattern as an initiator. To eliminate the influence of PNIPAm coating, indicating independence of collective myoblast cell migration from the substrate, we seeded myoblast cells onto GelMA micropatterns cross-linked in a hydrophobic capillary and placed the cell-seeded GelMA micropattern onto a non-PNIPAm coated culture plate for cell migration. Our results showed that we could achieve directed collective myoblast migration pattern without PNIPAm coating (Figure S2). It is pertinent to distinguish whether GelMA or physical properties of the GelMA micropattern, including stiffness, govern the directed collective cell migration in the perpendicular direction. Since the cells' contact with GelMA is before they enter the cell-free space and cells are likely to possess dose-dependent mechanical memory³² while they migrate, we suggest that the biophysical properties of the GelMA micropattern might predominantly influence how myoblast cells migrate in the cell-free space.

3.2. Distance between 3D Bioprinted Micropattern Governs the Direction of Collective Cell Migration.

We analyzed collective cell migration between 3D bioprinted micropatterns separated by narrow ($334.6 \pm 4.7 \mu\text{m}$), medium ($630 \pm 13.0 \mu\text{m}$), and wide ($952.7 \pm 20.3 \mu\text{m}$) distance. We analyzed cell orientation (Figure 4D) at 12 h after the confluence (Figure 4; right column). We found that collective cell migration is independent of time but dependent on the

distance from the boundary of 3D bioprinted micropattern. Collective cell migration in perpendicular direction is predominant in a wide gap, whereas their migration in the parallel direction is common in the narrow gap (Figure 4 and Video S3). Cells begin to transition to perpendicular alignment at 334 μm and achieve sufficient perpendicular alignment at 630 μm . In Figure 2D (x-axis), we have demonstrated that cells are parallel to the boundary up to 200 μm , begin to transition from parallel to perpendicular from 200 to 700 μm , and then become perpendicular from 700 μm onward. However, in Figure 4, we have demonstrated that cells begin to transition from parallel to perpendicular between 334 and 630 μm of gap-width. Upon comparing Figure 4 in the context of Figure 2D, the distance from the boundary for the transition from parallel to perpendicular would be approximately 200 μm and thus ~ 400 μm (>334 μm) of gap-width. This process is not time-dependent as we calculated cell orientation after they reach their confluence (Figure 4A–C; middle and right columns). Together, we demonstrate both parallel and perpendicular collective cell migration using 3D bioprinted micropatterns on thermoresponsive surfaces.

3.3. Directed Myoblast Differentiation for Parallel and Perpendicular Myotube Formation.

To exploit the utility of collective cell migration in tissue engineering, we performed directed myoblast migration and myotube formation. During directed collective myoblasts migration, elongated cells are aligned in an end-to-end sequence and thus promote myoblast fusion for controlled myotube orientation (Figure 5A). The unpatterned flat surface generated randomly oriented myotubes, whereas a 3D bioprinted micropattern initiated collective cell migration facilitated formation of myotubes both parallel and perpendicular to micropattern (Figure 5B–D, Videos S4 and S5). Our fusion index analyses, a measure of differentiated myoblasts, demonstrate that myotubes induced by collective cell migration (0.48 ± 0.02 for perpendicular; 0.37 ± 0.10 for parallel) is higher than that of myotubes obtained on a unpatterned flat surface (0.27 ± 0.06) (Figures 5E; S3). Moreover, we found that the myotubes contain the sarcomere structure, a hallmark of functional, contractile myotubes (inserted in Figure 5C). To demonstrate the contractile function of the myotubes, we further performed electric pulse stimulation of myotubes (1–4 Hz). We found that myotubes contract with the external electric stimuli (Figure 5F–G; Video S6). We have also stained fused myoblasts with N-cadherin (green) and DAPI (light blue) (Figure 5H). We found that N-cadherin are mainly expressed on myotube cell membrane, indicating that cell-cell contacts formed via N-cadherin triggered signaling events that promote the commitment to myogenesis (Figure 5H).³³ Since we differentiate cells to myotubes after myoblasts reach confluence, we do not observe, as anticipated, discrimination between cell–cell junctions or fusion events.

Together, we showed that collective cell migration initiated by 3D bioprinted micropatterns facilitate functional myotube formation. Since the hydrogel–polystyrene interface defined by the GelMA micropattern facilitates both perpendicular and parallel myotubes, it could be utilized in developing orthogonal muscle fibers as seen with the wall of the intestine.³⁴

3.4. Directed Myoblasts Differentiation for Radial Muscle Fiber Regeneration.

We performed collective myoblasts migration and differentiation on 3D bioprinted fingerprint pattern. The pattern contains parallel curves with interfiber spacing varying from

240 to 1500 μm (Figure 6A, B). We found that myotube orientation depends on the distance between micropattern spacing. Myotubes were oriented along micropatterns ($17.7^\circ \pm 11.9^\circ$) when they are formed in a 240 μm wide gap. The orientation angle was higher as the micropattern spacing was wider (Figure 6C). Likewise, myotubes were formed in the perpendicular direction ($101.2^\circ \pm 19.7^\circ$) when the micropattern spacing reaches 1500 μm (Figure 6D). We also found parallel myotubes formed adjacent to micropattern boundaries. The curvature is known to influence cell orientation at micrometer scale.³⁵ However, cells in our fingerprint pattern experience the curvature at millimeter scale; negating its role on cell orientation. Therefore, we anticipate that the curvature in the fingerprint, which is lacking the context of a single cell, would not have any influence on the myotube orientation. Our analyses of the fusion index further demonstrated that wide gap offers higher fusion index than the narrow gap (Figure 6E)

To demonstrate the physiological utility of our platform, we tested if we could maneuver the formation of both parallel and perpendicular myotubes. Specifically, we embarked upon developing radial and circular muscle fibers mimicking the human iris to potentially control the aperture of the pupil in response to external stimuli (Figure 7A–C). We first 3D bioprinted a disc (6 mm diameter) surrounded by a circular ring (10 mm diameter) to mimic the real size of a human iris (Figure 7B) for initiating directed collective myoblast migration followed by developing radial muscle fibers (Figure 7D). Next, we performed myotube orientation analyses to measure the similarity of the engineered radial muscle fibers to the real iris. We found that the orientation angle distribution of the engineered radial muscle fibers and the real iris were overlapped, indicating that the engineered iris replicates the structure of real iris radial muscle (Figure 7E–F). Likewise, we demonstrated the formation of circumferential muscle fibers mimicking the circular muscle fiber of the iris.

4. CONCLUSION

Directed collective cell migration governs cell orientation and function during tissue development, repair, and regeneration. Using 3D bioprinted hydrogel micropattern on thermoresponsive surfaces, we have developed an injury-free method to regulate directed collective cell migration. Unlike conventional approaches, our method is simple and does not involve multistep photolithography-based fabrication. We demonstrate parallel and perpendicular migration of cells as a function of distance between micropatterns, their velocities, as well as their dependence on micropatterns for initiating and preserving the direction of cell migration, but not dependent on time. The myotubes generated by our method have contractility upon electrical stimulation. Next, we demonstrated the utility of our method in developing a 3D bioprinted fingerprint as well as in engineering of a human iris using radial and circular myotube formation. Thus, our finding is a stepping stone to precisely control the direction of cell migration for macroscale physiological tissue engineering and regenerative medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

GelMA	gelatin methacryloyl
PNIPAm	poly(<i>N</i> -isopropylacrylamide)
OI	orientation index

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Figure 1.

3D bioprinted hydrogel micropatterns to promote directed collective cell migration. (A) Schema (top) and a picture (bottom) of 3D bioprinted GelMA micropatterns on PNIPAm coated surface. (B) Uniform cell seeding on GelMA micropatterns and PNIPAm coated surface at 37 °C. (C) Detachment of cells to generate cell-free space by reducing temperature of thermoresponsive PNIPAm coated interface to 20 °C. (D) Initiation of directed collective cell migration by 3D bioprinted micropatterns at 37 °C. Yellow lines with arrowheads show cell migration directions parallel or perpendicular to 3D bioprinted micropatterns. (E) Directed collective myotube fibers' orientation after changing the growth media to differentiation media. Yellow lines with arrowheads show myotube orientation directions either parallel or perpendicular to 3D bioprinted micropatterns.

Figure 2. Directed collective cell migration in parallel and perpendicular directions initiated by a line micropattern as a function of distance from the boundary. (A) Time-lapse images of directed collective cell migration in 72 h. The micropattern boundary is indicated as a yellow dotted line. Cell migration perpendicular (red arrowhead) and parallel (blue line with double arrowheads) to the micropattern boundary is indicated at a different distance (red dotted line) from the GelMA boundary over time. The perpendicular direction to the micropattern boundary is defined as 0°. (B–C) Orientation maps and orientation index of collectively migrating cells in the perpendicular direction over time and at different distances. Cells located along the micropattern boundary are not calculated. (D) Orientation index of cells migrating away, in the perpendicular direction, from the micropattern boundary at different distances, suggesting cell migration direction transition from parallel to perpendicular after 200 μm which becomes perpendicular after 400 μm from the boundary. Thus, cell migration is a function of the distance from the boundary of the GelMA micropattern. (E) Orientation maps and orientation index of collectively migrating cells, located along the micropattern boundary, in the parallel direction over time, suggesting that cell migration is not a function of time. The wind rose maps show the overlapped orientation angle distribution. (F) Velocity cloud map of collective migration of cells in the perpendicular direction after reaching confluence from 72 to 80 h. (G) Mean velocity orientation map of (F). (H–I) Velocity

magnitude and orientation as a function of distance from the micropattern boundary. * $p < 0.05$.

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Figure 3.

Parallel collective cell migration requires the presence of micropattern whereas perpendicular collective cell migration is preserved after initiation. (A) Schema demonstrating removal of the GelMA micropattern. (B) Time-lapse images of collective migration after removal of the GelMA micropattern. Red dotted rectangles indicate the region for calculating local orientation index. The red rectangle indicates the parallel cells. Yellow dotted lines indicate the boundary of micropatterns. (C) Local orientation index as a function of culture time after removal of GelMA (left). Overall orientation index of cell monolayer as a function of time after removal of GelMA (right). (D) Schema demonstrating removal of a lining of parallel cells adjacent to the GelMA micropattern (and not GelMA micropattern). (E) Time-lapse images of collective migration after removal of a lining of parallel cells adjacent to the GelMA micropattern. (F) Local orientation index as a function of culture time after removal of a lining of parallel cells adjacent to the GelMA micropattern (left). Overall orientation index of cell monolayer as a function of time after removal of a lining of parallel cells adjacent to the GelMA micropattern (right). (G) Schema demonstrating removal of both the GelMA micropattern and a lining of parallel cells adjacent to the GelMA micropattern. (H) Time-lapse images of collective migration after removal of both the GelMA micropattern and a lining of parallel cells adjacent to the GelMA micropattern. (I) Local orientation index as a function of culture time after removal of both the GelMA micropattern and a lining of parallel cells adjacent to the GelMA micropattern (left). Monolayer orientation index of cell monolayer as a function of time after removal of both the GelMA micropattern and a lining of parallel cells adjacent to the GelMA micropattern (right). Each experiment was repeated at least three times. $*p < 0.05$.

Figure 4.

Directed collective cell migration as a function of a distance between two GelMA micropatterns. (A–C) Time-lapse images of directed collective cell migration initiated by two micropatterns separated by narrow (334 μm), medium (630 μm), and wide (952 μm) distances. (D) Cell orientation distribution for micropatterns separated by narrow (334 μm), medium (630 μm), and wide (952 μm) gaps at 12 h after confluence.

Figure 5.

Directed collective cell migration of functional myotubes in perpendicular and parallel directions at millimeter-scale. (A) Schema of directed myoblast migration followed by myotube formation. (B–C) Immunofluorescent images show myosin (MHC, red) and nuclear (DAPI, light blue) staining in unpatterned and patterned myotube aligned after directed collective cell migration. Arrowheads indicate the position of the parallel myotubes after differentiation. Inserted image shows the sarcomere structure (green) in the myotube. (D–E) Myotube and nuclear orientation as well as fusion index of myotubes emerged from

unpatterned (blue), parallel (green), and perpendicular (orange) collective cell migration. (F) Myotube contraction using 1–4 Hz electric pulse stimulation. (G) Fourier transform analysis of the myotube contraction frequency. (H) High resolution image showing N-cadherin (green) and DAPI (light blue) staining of myotubes. * $p < 0.05$.

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Figure 6.

Directed collective cell migration to establish a 3D bioprinted fingerprint with precise myotube orientation. (A–B) 3D model and G-code for fingerprint. (C) Immunofluorescent images of myotube (MHC), nuclei (DAPI), and merged in 3D bioprinted fingerprint pattern with different micropattern spacing. Blue arrows indicate the orientation of myotubes. Yellow lines indicate GelMA micropattern boundaries. (D–E) Myotube orientation angle and fusion index to demonstrate that the cell migration pattern is dependent on the distance between the GelMA micropattern. * $p < 0.05$.

Figure 7.

3D bioprinted directed collective cell migration to engineer iris radial and circumferential muscle fibers. (A) Human iris radial muscle fiber (top) was reverse-engineered in an iris-mimic structure (bottom). (B) G-code for 3D printing of an iris. (C) Schema shows relative orientation of radial and circular muscle fibers in the human iris. (D) Engineering of patterned radial and circumferential muscle fibers to mimic human iris (left). Zoomed in (2×) image shows the interface of radial and circumferential muscle fibers (right). Yellow dotted lines indicate micropattern boundaries. White and blue arrows indicate the radial and circumferential muscle fibers. (E) Zoomed in (3×) image of muscle fiber orientation in human iris as seen in A. (F) Distribution of the orientation angle of radial muscle fibers in engineered 3D bioprinted iris (red) and in human iris (blue).