A 3D Microfabricated Scaffold System for Unidirectional Cell Migration

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The present study demonstrates unidirectional cell migration using a novel 3D microfabricated scaffold, as revealed by the uneven sorting of cells into an area of 1 mm × 1 mm. To induce unidirectional cell migration, it is important to determine the optimal arrangement of 3D edges, and thus, the anisotropic periodic structures of micropatterns are adjusted appropriately. The cells put forth protrusions directionally along the sharp edges of these micropatterns, and migrated in the protruding direction. There are three advantages to this novel system. First, the range of applications is wide, because this system effectively induces unidirectional migration as long as 3D shapes of the scaffolds are maintained. Second, this system can contribute to the field of cell biology as a novel taxis assay. Third, this system is highly applicable to the development of medical devices. In the present report, unique 3D microfabricated scaffolds that provoked unidirectional migration of NIH3T3 cells are described. The 3D scaffolds could provoke cells to accumulate in a single target location, or could provoke a dissipated cell distribution. Because the shapes are very simple, they could be applied to the surfaces of various medical devices. Their utilization as a cell separation technology is also anticipated.

1. Introduction

Cell migration is an indispensable process in histogenesis,[1] homeostasis,[2] recovery of injured tissue,[3] and immune function.[4] The direction of cell migration is ordinarily determined by the direction from which external stimuli are received. The stimulus direction is a concentration gradient of cell attractants such as nutrients, cytokines, or extracellular matrix components.

In cell culture migration systems, cells extend protrusions and migrate toward the injection point of a cell attractant, which is injected in the immediate vicinity of the cell.[5,6] This process induces a cellular response to the concentration gradient of the cell attractant, in which the cell extends protrusions toward areas in which the attractant concentration is higher. However, in order for a cell to extend protrusions in a single direction, the attractant concentration gradient must be clear enough for the cell to perceive changes in concentration gradient originating from a single direction. Moreover, the concentration gradients that cells can perceive are limited. Considering these facts, the distance of directional cell migration is restricted, and it is difficult to precisely control cell migration direction over a long range using conventional methods.[7]

Therefore, we sought to develop a 3D microfabricated scaffold capable of precisely controlling cell migration direction over a long distance. Unidirectional cell migration directed by the 3D shape of a microfabricated scaffold confers three major advantages. First, this technology can control cell migration direction over a long distance, as the pattern shape is effective as long as the pattern can be maintained. Second, this technology allows for the development of new cellular taxis assays. Cellular taxis assays evaluate directional cell migration induced by specific stimuli, including chemotaxis[6,8] by chemical stimuli or durotaxis[9] by changes in the surface stiffness. 3D shape-induced unidirectional cell migration has not been reported before now, although a prior study reported 2D and 3D shapes that promote directional migration.[10] If directional cell migration induced by the shape of a 3D scaffold is accepted as a new taxis assay, this will make significant contributions to the field of cell biology. Third, micropatterns with 3D shapes are highly applicable to the development of medical devices. Studies that apply micropatterns with 3D shapes as medical devices are progressing quickly.[11] If unidirectional cell migration can be provoked by the 3D shape of micropatterns, this technology can be applied to regenerative medical treatments. For example, it could be applied to artificial organs, such as artificial hearts, to prevent thrombus formation by collecting endothelial cells from the circumference and promoting surface endothelialization, or to stents to remove cells from strictured segments. Moreover,
it could be used to develop simple cell separation technologies without the need for reagents or antibodies. This technology will therefore have significant impacts in highly diverse fields.

To develop a 3D shape that could induce unidirectional cell migration, we focused on the tendency of cells to adhere to 3D sharp edges,[23–14] in which cells extend protrusions along the edges of micropatterns.[13,15,16] Cells are known to migrate in the direction of protrusion extension.[17,18] We therefore hypothesized that developing a sharp edge configuration in which the direction of cell protrusions is regulated would be able to direct cell migration. We defined a sharp edge as a sharply projecting side between the planes of a micropattern. Specifically, edges with a projecting angle of 90° or less are referred to as sharp edges.

Many types of micropatterns with the described sharp edges were produced, and the adhesion, migration, and localization of cells cultured on the micropatterns were evaluated. Pattern shapes were incrementally improved based on cell localization, as described by the trial and error process (Figures S1 and S2, Supporting Information). Some of these shapes are illustrated in Figure 1A. To induce unidirectional cell migration using a micropattern 3D shape, we predicted that an edge structure in which cells put forth protrusions and an anisotropic periodic structure with a size comparable to cell size or subcellular size would be important.[13–16] We also predicted that a device that restricts the direction of cell migration to one dimension would also be important. To restrict the direction of cell migration to one dimension, we decided to employ a stripe-like pattern, which was previously reported to restrict the direction of cell migration to one dimension.[15] First, we combined a scale-like pattern[15,16] (Figure 1A1), corresponding to a micropattern with an anisotropic periodic structure, with a stripe-like pattern[15] (Figure 1A2) into a unique and uniform pattern (Figure 1A3) to restrict the direction of cell migration to one dimension. Rows of the unique pattern on a triangular prism were arranged in a stripe-like pattern. Next, to induce cells to migrate smoothly in only one direction, we performed extensive optimization; for example, we optimized the periodic structure shape and groove shape (Figure 1A4, A5). Over a thousand substrates were produced to perform the process (Figure 1A6). We experimentally evaluated 507 types of micropatterns, determining that patterns with columns of standing isosceles triangular prisms in a continuous row were most effective in inducing unidirectional cell migration.

2. Results and Discussion

2.1. Fabricated Micropattern Shapes

We expected that it would be important to determine the optimal arrangement of 3D edges, and the anisotropic periodic structures were adjusted appropriately in a micropattern (Figure 1A1, A3–A5). At first, anisotropic migration was not present, and cells migrated only vertically and horizontally along the pattern shapes. However, we repeatedly optimized the pattern shape through the trial and error process (Figures S1 and S2, Supporting Information), and after examining 507 micropatterns, developed a micropattern shape (Figure 1B) that induced highly anisotropic migration. The structure of this finalized micropattern resembled arranged edges of a double-edged saw on the same line in parallel with multiple rows, and the structure formed a uniform triangular prism. These triangular prisms had the upper surface of an isosceles triangle, overlapping for 10% of the length of the perpendicular bisector of the isosceles triangular base, and arranged straightly. The micropattern consisted of parallel prism rows. The base positions of the isosceles triangles of adjacent rows shifted with respect to each other for 50% of the length of the perpendicular bisector. Twenty-five micropatterns were fabricated by changing the parameters of the isosceles triangle on top and the interval of the rows, while unifying the basic designs described above. The 25 patterns were simultaneously fabricated on a single silicon wafer, and each patterned area was nearly the same size at \( \approx 1000 \, \mu m \times 1000 \, \mu m \) (Figure 1C). Field emission-type scanning electron microscopy (FE-SEM) images of these micropatterns are shown in Figure 1D.

This figure demonstrates that 25 micropatterns were fabricated, which were different with respect to the shape parameters of an isosceles triangle on top and row interval. Among these 25 micropatterns, the height and shape parameters on the upper surface of the triangular prism were evaluated. Although the heights of the triangular prisms that constituted these micropatterns were highly similar at 21.0 ± 3.8 \( \mu m \) (average ± SD), the shape parameters (Figure 1E) of the isosceles triangles of the upper surfaces of the triangular prisms were completely different. The reason why we fabricated grooves with a depth of 21 \( \mu m \) was to prevent the depth (or the bottom) of the grooves from influencing cell migration. As reported previously,[15,16] cells adhere and extend on the upper sides of micropatterns when the pore ratio is about 50% of the total area or less and the groove width or pore diameter is smaller than cell size. Previously, we demonstrated that NIH3T3 cells reached a depth of only 16 \( \mu m \) at most when the pore depth was 18 \( \mu m \). We considered that the micropatterns would prevent depth from influencing cell migration, because the pore ratios of the micropatterns were about 50% (pore ratio; Figure S3D, Supporting Information), the groove widths were smaller than cell size, and groove depths were about 21 \( \mu m \). The measured shape parameters of each micropattern are shown in Figure S3, Supporting Information.

However, micropatterns 21–25 were susceptible to partial breakage, possibly because their structures are fragile and easily broken during micropattern production, especially during the step of anisotropic plasma etching to the silicon substrate. Thus, we do not report the results obtained using these micropatterns in this study. However, with the aim of using more robust versions of these micropatterns in the future, we show the results of topographical analysis of undamaged micropatterns 21–25 (Figure S3, Supporting Information). Moreover, to demonstrate the influence of the characteristic sharp edges of micropatterns 21–25 on cellular morphology, we examined the morphologies of cells on micropatterns 21–23 (Figure S8, Supporting Information). The results showed that the cells tended to extend protrusions along the sharp edges.

2.2. Uneven Cell Distribution on Each Pattern

After culturing NIH3T3 cells for 76 h, the three left-end areas (A\(_L\), B\(_L\), C\(_L\)) and the three right-end areas (A\(_R\), B\(_R\), C\(_R\)) in
each micropattern area of 1000 µm × 1000 µm were imaged, as shown in Figure 2A, and cell numbers in these areas were counted (Figure S4, Supporting Information). Additionally, total cell number in each end area and the percentages of cells in left-end and right-end areas were calculated in each micropattern (Figure 2B). We observed that the cells were distributed toward the right side in the images of micropattern 12 (Figure 1D), which corresponded to the middle in all the micropatterns. When conducting uneven distribution analysis, it is important that cells do not migrate from the inside of a pattern area to the outside, or from the outside of a pattern area to the inside. We used a groove that was more than 10 µm in width and more than 20 µm in depth between the inside and outside of the entire pattern. Generally, cells found it difficult to migrate beyond this groove. Migration between the inside and outside of each pattern area was suppressed in this system.

As shown in Figure 2B, the red column graphs indicating the right end were higher than in the left end in all patterns evaluated, which suggested uneven distribution of the cells toward the right ends. There were clear differences in the uneven distributions of the cells toward the right end in each pattern, suggesting that this shape effectively induced cell migration in one direction. The fundamental shape parameters of the patterns 1–20, in which directional migration was observed, were 18.3–23.9 µm long on the equilateral side and 19.7–44.9 µm long on the base side. The intervals of the triangles overlapping one another were 7.2–15.7 µm, the angles between the equilateral side and the base side were 19.0–60.8°, and the intervals of the tangential lines between adjacent triangle rows were 3.2–9.3 µm. This suggested that even if the shapes and intervals of the patterns changed, the bias of cell migration to the right sides was reliable as long as the fundamental shape illustrated in Figure 1B was maintained. As mentioned below, we also expected that the ratio of the length of the base to that of the equilateral side of the isosceles triangle would need to be one or more to induce unidirectional cell migration. In addition, we were able to confirm that the reproducibility of the uneven distributions of cells cultured on these micropatterns toward the right end was sufficient using another micropatterned substrate produced independently with the same micropattern shapes (Figure S5, Supporting Information). 3D shape-induced unidirectional cell migration that allows uneven cell sorting within a defined area has not been reported previously.

2.3. Migration Directions and Preferential Placement of Cultured Cells on the Micropatterns

We next sought to determine why such shapes induced cells to migrate in one direction. Time lapse imaging of NIH3T3 cell migration on each micropattern was performed for 72 h to investigate migration motions (Movie S1, Supporting Information). On micropatterns 1–20, we observed many cells migrating toward the right and many cells in the grooves of the micropatterns. The frequencies of the cell migration in all directions, calculated using Figure 3A, were used as a standard for judging cell migration directions in time lapse movies. We judged that a cell moved horizontally when the position of its nucleus was larger than the interval of the connected isosceles triangles in each micropattern (Figure 3Ai), while we judged that a cell moved vertically when the change in the position of its nucleus was larger than the interval of the rows of the connected triangular prisms in a line (Figure 3Av). Because iii and v of each micropattern were almost the minimum cell migration distances for all directions in each micropattern in time lapse movies, iii and v were used as standards for judging cell migration directions. The cell migration frequencies toward the right were higher than those toward the left on all micropatterns (Figure 3B). Even if these results were combined for all evaluated micropatterns, the cell migration frequency toward the right was higher than that toward the left (Figure 3C). Next, the location of migrating cells on each micropattern relative to the inside or outside of the groove was evaluated using Figure 4A as a standard. The locations of migrating cells are summarized in Figure 4B. The frequencies of cells located in the grooves were higher than those of cells located outside of the grooves in all patterns from 1 to 20. In patterns 1–20, 94% of cells were in grooves, suggesting that almost all cells cultured on these micropatterns were positioned in the grooves (Figure 4C).

2.4. Cell Adhesion Morphologies

We investigated cell adhesion morphologies on the micropatterns in detail, as cells located in the grooves exhibited unusual adhesion morphologies that are not observed on flat surfaces. The direction of cell protrusions affects cell migration direction more significantly than cell morphology.[17,18] We therefore observed the directions of extended protrusions, because we expected that this approach would be effective in elucidating the mechanism of unidirectional cell migration. Protruding directions were analyzed by determining the longer protruding direction, which was categorized as right, left, or indistinguishable (Figure 5A). The lengths of each protrusion from the nucleus center were measured and compared using magnified single cell images. The indistinguishable category was defined as being round with no protrusions, or if the protrusion tip could not be recognized, such as by the protrusion contacting another cell, or if the lengths of both right and left were less than 60% of total right/left length. These analyses revealed that protrusions toward the right side were greater than those toward the left side in all patterns from 1 to 20 (Figure 5B). This suggested that the cultured cells on the micropatterns from...
Figure 2. Uneven cell distribution on the micropattern. A) Superimposed images of cellular nuclei, actin filaments, and membrane fluorescence images, and a micropattern reflected image, which were observed in six areas, in which each micropattern had dimensions of 317.3 µm × 317.3 µm. B) The percentages of cell numbers in the right (A_R, B_R, C_R) and left (A_L, B_L, C_L) areas on each micropattern. The total cell number used for counting on each micropattern is shown above the graph.
Figure 3. Frequencies of cell migration directions. A) Migration directions estimated from time lapse movies (Movie S1, Supporting Information). The cells migrated up, down, right, or left on the micropattern. The doublet lines in this figure indicate cells, and the yellow parts indicate cell nuclei. A cell was considered to have migrated in a specific direction when a point that could be regarded as the center of a cell nucleus moved to the right or left side more significantly than in iii for 1 h. A cell was considered to have migrated in one direction when the point moved up-and-down more significantly than in v for 1 h. When the point moved aslant, the cell was considered to have migrated in the direction to which the point moved for the longest distance among the four directions. B) The plotted frequencies of each migrating direction on each micropattern (1–20). The total numbers of movements used for counting on micropatterns 1 to 20 were 496, 345, 434, 573, 479, 441, 591, 513, 256, 361, 706, 498, 497, 433, 436, 533, 834, 499, 571, and 473, respectively. C) The plotted frequencies of all cell migration directions on all micropatterns (1–20).

Figure 4. Frequencies of cell positioning in grooves. A) Schematic for distinguishing whether cells were inside or outside grooves using time lapse movies (Movie S1, Supporting Information). It is determined whether the cells adhered to the inside or the outside by judging the nucleus position, which was considered as the center of the largest-volume portion of PKH26-stained cells. B) Plotted frequencies of number of cells present in the grooves of each micropattern (1–20). The number of counts of cell positions on micropatterns 1–20 are 688, 385, 463, 576, 491, 636, 626, 527, 257, 361, 739, 502, 498, 435, 444, 547, 842, 499, 591, and 478, respectively. C) The plotted frequencies of cell numbers inside and outside grooves on all the micropatterns (1–20).
Figure 5. Frequencies of cell protrusion directions toward either the right or left. A) Schematic for judging the direction of cell protrusions toward the right or left using laser scanning confocal microscopy images of cells cultured on the micropatterns (Figure S8, Supporting Information). L, R, and N indicate that the cell protruded toward the left side, right side, or neither side, respectively. For N, the length of the longer protrusion is 60% or less of the whole. B) Frequencies of cell protrusion directions toward either the right or left of each micropattern (1–20). The cell numbers used for counting cell protrusion directions on micropatterns 1–20 were 19, 19, 14, 18, 25, 22, 13, 12, 22, 20, 17, 22, 24, 20, 24, 16, 25, 12, and 17, respectively. C) The frequencies of cell protrusion directions toward either the right or left for all cells on all micropatterns (1–20).

1 to 20 were prone to extend protrusions toward the right side, as 77% of cells in which the protrusion direction could be determined extended protrusions toward the right side (Figure 5C).

Next, adhesion morphologies were compared between the cells cultured on micropatterns that did or did not elicit unidirectional migration. Most cells on the micropatterns capable of eliciting unidirectional cell migration tended to assume a unique adhesion morphology, adhering to the tips of three or more edges of the double-edged saw in the groove of the micropatterns. On micropatterns capable of eliciting unidirectional migration, cells on the outside of grooves were very few, but tended to extend protrusions along the sharp edges of the isosceles triangles and extend perpendicular to the grooves (Figure 6G). On the other hand, cells on micropatterns incapable of eliciting unidirectional migration assumed an adhesion morphology extending along both the perpendicular bisector of the base side and the equilateral sides of the isosceles triangle of the upper surface of the pattern, and they adhered less to the tips of the edges (Figure 6H). This suggested that patterns in which the isosceles triangle on the upper surface of the pattern had a large basal:equilateral side length ratio were optimal for allowing easy entry of cells into the grooves, which was advantageous for unidirectional cell migration. On the other hand, if the basal:equilateral side length ratio was small, the frequency at which cells did not enter into a groove but rather extended along the surface was higher. Based on the results of a huge number of trials and error processes (Figure 6A–H), we concluded that the basal:equilateral side length ratio of the isosceles triangle should be about one or more to induce unidirectional cell migration.

Time lapse imaging confirmed that on micropatterns incapable of eliciting unidirectional migration, the cells exhibited a strong tendency to migrate both rightward and leftward on the upper surface, and cells were less able to enter the grooves (Movie S2, Supporting Information). These results suggested that positioning of the cells in the grooves and their adherence to the tips of the edges in the groove were important for the induction of unidirectional migration.

2.5. Correlation between Sharp Edges and Cell Migration Direction

NIH3T3 cells grown on the flat surface are spindle-shaped and extend protrusions at random angles. We next sought to determine the basis for the unusual cell adhesion morphology observed in the grooves. Adhesive cells are known to put forth protrusions and extend along both directions where sharp edges and scaffolds continue. In the scaffold used in the present study, three types of sharp edges as shown (1)–(3) in Figure 6I. Analyses of the adhesion morphologies of many cells captured by confocal laser scanning microscopy (Figure S8, Supporting Information) revealed that cells on the upper surfaces of the patterns put forth protrusions in the directions of the arrows along the two types of sharp edges on both the equilateral (1) and the base (2) of the isosceles triangle prisms (Figure 6I), with cells extending nearly in the vertical direction to the direction where the grooves continued (Figure 6C, lower left). This suggested that the cells on the upper surfaces extended into the grooves. The cells on the upper surface were not elongated parallel to the direction where the patterns or grooves continued. Furthermore, the adhesive cells in the grooves extended and remained at the middle depths of the grooves (Figure 6J). Therefore, the protruding direction on the side (3) of a triangular prism was directed to an apex in which the three sharp edges crossed (Figure 6I), facing opposite directions from (1) or (2) with the apex between. This presumption was based on the observation that the arrow on (3) was upward because no cells adhered or extended on the bottom of the grooves. If the arrow on (3) were to be pointing down, more cells would be adhering and extending on the bottom. Therefore, it is surmised that the
Figure 6. Morphologies of cells cultured on each micropattern that put forth protrusions. A–C) CLSM images of cell protrusion morphologies on micropatterns capable of inducing unidirectional migration, namely micropatterns 10, 11, and 12 of Figure 1D. D–F) CLSM images of cell protrusion morphologies on micropatterns incapable of inducing unidirectional cell migration, namely micropatterns 12, 24, and 5 in Figure S2C, Supporting Information. G) Schematic illustrations of (A–C). (i) and (ii) illustrate cells extending inside a groove and adhering to three or more projections in a
protruding directions on sides (1), (2), and (3) were facing one another across the apex. We propose that adhered NIH3T3 cells put forth protrusions and extended along these three types of sharp edges (1)–(3) in the direction of the arrow. We postulated that the cells migrated into the grooves on the micropattern, resulting in an adhesion morphology that hinged on the edges of three or more aligned projecting triangles. Additionally, the cell morphologies shown in Figure 6I strongly resembled the morphologies of cells that migrate three dimensionally in extra-cellular substrates.

It is possible that unidirectional migration on the micropatterns occurred because the mechanism involved in cell migration on the micropattern is similar to that involved in cell migration in vivo.

The cells extended into the grooves, putting forth protrusions as long as possible to ensure tight adhesion at the sharp edge (3) (Figure 6I). Thus, we postulated that the tips of the cell protrusions adhered to the sharp edge and searched for the next sharp edge, allowing them to extend to the edges of the adjoining and projecting parts on the right-hand side. The protrusions moved toward the right because the sum of the geometric vector of the extension of the protrusion on (1)–(3) in Figure 6I was expected to be rightward. Although we also expected that the width and the depth of grooves that separated the adjoining sharp edge and the peak at which the three sharp edges (1)–(3) crossed played vital roles in determining the protruding direction on (1)–(3), we cannot fully identify these roles at present. In future studies, we will perform live cell imaging at high magnification, confirming the protruding direction along the three sharp edges, its frequency, and the role of the peak at which the three sharp edges cross. It is also necessary to clarify the influence of the angle of the sharp edge on the cell protrusions and extension.

As discussed above, we postulated that unidirectional migration could be induced by devising the configuration of sharp edges in three dimensions. However, our research subjects are live cells, which are strongly influenced by various factors, such as differences in the cell cycle, different sizes, and interaction with adjacent cells. The protruding directions therefore do not fully match the arrows shown in Figure 6I in 100% of cells cultured on these micropatterns. Further, to prompt cell migration in one direction with uneven distribution, it is necessary to fine tune the micropattern shape according to the size and morphology of the cell type used.

3. Conclusion

We have developed a unidirectional cell migration system, as demonstrated by observing the uneven distribution of cells in an area of 1 mm × 1 mm, using a 3D microfabricated scaffold. The cells put forth protrusions directionally along the sharp edges of these micropatterns, and migrated in the protruding direction. The present study identified 3D shapes capable of inducing cell migration in only one direction. Because shape, rather than a cell attractant, induced unidirectional cell migration, this technology can readily be applied to many types of surfaces, such as base materials for cell culture substrates or medical devices.

This technology developed in this study is applicable under any environment, as only the shape of the material’s surface must be modified. This technology could allow development of a very simple cell separation apparatus capable of selecting a single cell type from a mixed population of cells, and potentially development of a smart artificial organ that can perform cell separation and accumulation spontaneously. It is expected that the required cell type could be collected for an extended period of time, or that unnecessary cells could be selected against. Applying simple manipulations of substrate shape in medical devices could be applied to the human body.

4. Experimental Section

Fabrication and Shape Analysis of Micropatterns: Cell culture on micropatterns of various 3D shapes was conducted to determine the 3D shape that induced unidirectional cell migration, and optimized the 3D shape. The surfaces of the micropatterns were treated with oxygen plasma using reactive ion etching, which greatly enhanced the hydrophilicity of the surfaces. These micropatterns were fabricated on silicon substrate using photolithography, as has been reported previously.

The silicon substrate was a square of 10 mm × 10 mm with a thickness of 725 µm. Twenty-five types of micropatterns with different shapes were fabricated on the silicon substrate of a single sheet. Each pattern was fabricated in independent areas that were 1000 µm × 1000 µm, and were 500 µm away from one another. The 25 types of patterns consisted of uniform triangular prisms. These triangular prisms had the upper surface of an isosceles triangle, and overlapped one another for 10% of the length of the perpendicular bisector of the base of the isosceles triangles, which were in a straight row. The numbers of rows of connected lines of triangular prisms were located in parallel and constituted the micropattern. The base position of the isosceles triangle of an adjacent row shifted 50% of the length of the perpendicular bisector. A schematic illustration is shown in Figure 1B.

The photograph of whole silicon substrate produced a micropattern that was observed by a digital microscope (VHX-1000, Keyence, Japan) (Figure 1C). A top view of each micropattern produced on the same silicon substrate was observed from 60° above (tilting substrates at 30°) by a field emission-type scanning electron microscope (SU3500, Hitachi, Ltd., Japan) (Figure 1D). Evaluation of the 3D shape of each micropattern was performed using a 3D shape image acquired by color laser 3D microscopy (VK-9700, Keyence, Japan). In acquiring the 3D shape straight line, as on the edges of a saw; (iii) illustrates a cell extending perpendicularly to a groove and putting forth protrusions outside of the grooves along the base side and the two equilateral sides of the upper surface of the triangular prisms; for example, see the cell in the lower left of (C). H) Schematic illustrations of (D–F). (iv) and (v) illustrate cells extending outside the grooves along the two equilateral sides of a isosceles triangle on the upper surface of a micropattern; (vi) illustrates a cell extending along the perpendicular bisector of the base of the isosceles triangle on the upper surface of a micropattern; (vii) illustrates a cell that does not put forth protrusions inside of a groove; for example, see the cell in the third row groove at the bottom of CLSM image (F). I) Schematic illustration of the presumed protruding directions on each sharp edge (1)–(3) on the micropatterns capable of inducing unidirectional cell migration. J) Top views and side views of CLSM images of cells extending protrusions along the sharp edges of the projecting portions of the grooves on the micropatterns. Single cells in CLSM images from (A), (B), and (C) are shown left to right. Blue arrows indicate cell nuclei. To make the protrusions easily visible, the brightness and contrast of the pictures were increased.
image of each micropattern, an objective lens (CF Plan Apo 150× N.A.: 0.95, Nikon, Japan) was used. Software (VK-Analyzer, Keyence, Japan) was used to measure the 3D shape of each micropattern. Each shape parameter was measured using the 3D shape images of micropatterns 1–25 acquired by a color laser 3D microscope (VK-9700) (Figure 1E). The measured parameters were micropattern height (groove depth), i) equilateral length, ii) base length, iii) continuous isosceles triangle intervals, iv) equilateral inclination, and v) the tangent interval with the next row located in parallel. Measurement of shape parameters was conducted within the range of 70.9 µm × 94.5 µm of each pattern. Shape measurements of the 25 micropattern types were performed in the same way, and the average values and standard deviations of each shape parameter were estimated in every pattern. Six measurements (n = 6) were performed for each shape parameter. The micropatterns used for measurement of each shape parameter were then used for evaluation of cell migration. The details of the 3D shape image used for this measurement, the measurement components of each shape, and the measurement results were shown (Figure S3, Supporting Information). The measurement results of shape parameters other than (i)–(v) were also described (Figure S3, Supporting Information).

Cells, Reagents, and Laboratory Instruments: NIH3T3 cells were purchased from DS Pharma Co., Ltd. (Japan). Low-glucose DMEM (Gibco D6046) and stabilized penicillin–streptomycin solution (P4333), 0.25% Trypsin–EDTA solution (T4049), PhK26 (PKH26GL), and TWEENR 20 (PI379) were purchased from Sigma-Aldrich (USA). FBS (Gibco 26140) and Phalloidin-Alexa488 (A-12379) were purchased from Invitrogen (USA). Four percent paraformaldehyde in phosphate buffered saline was purchased from Nacalai Tesque, Inc. (Japan). DAPI (342-07431) was purchased from Nacalai Tesque, Inc. (Japan). DAPI (342-07431) was purchased from Nacalai Tesque, Inc. (Japan). Eukitt was purchased from Nacalai Tesque, Inc. (Japan). DAPI (342-07431) was purchased from Nacalai Tesque, Inc. (Japan).

Cell Culture and Time Lapse Observations on Micropatterns: A sterilized 10 mm × 10 mm silicon wafer with 25 types of micropatterns was degassed by immersion in culture medium in a 35 mm cell culture dish (−0.08 to −0.09 MPa, 1 min).[15,16] NIH3T3 cells were seeded uniformly on the degassed micropattern at a density of 1 × 10^6 cells per cm^2, and were cultured (37 °C, 5% CO_2) for 2 h. After 2 h in culture, the plasma membrane of NIH3T3 cells was stained with PhK26 to perform fluorescence live cell imaging.[15,16] Cells adhered to a micropattern substrate on the 10 mm quadrant were turned upside down on the glass base with a depth of 1.3 mm and diameter of 12 mm in a glass base dish, and were started in culture (DMEM, 10% FBS, 1% penicillin streptomycin, 37 °C, 5% CO_2).[15,16] 4 h after seeding, time lapse observation of the PKH26-labeled cells was initiated in the specified fixed points on each micropattern and flat surface. Time lapse observation was performed at intervals of 30 min for 72 h using a fluorescence microscope (Observer Z1, Zeiss, Germany) attached to a Stage Top Incubator (Inu-Zics, Tokai Hit, Japan). Using an objective lens (Plan-Apochromat 10× N.A.: 0.95 Zeiss, Germany), fluorescence images of PKH26-labeled cells and reflected images of the micropattern at the specified fixed area (871 µm × 690 µm) were captured with a charge coupled device camera. After time lapse observation was completed, the sample used for live imaging was immediately immersed in 4% paraformaldehyde-phosphate buffered saline solution and fixed at room temperature.

Fluorescent Staining and Uneven Distribution Analysis of Cells: It was determined whether the cells cultured on each micropattern would be unevenly distributed on the sides of each micropattern. When such uneven distributions of cells were observed, placement of the apex angle of the isosceles triangle constituting the triangular prisms of a micropattern faced in the left-hand direction was unified, and the base of the isosceles triangle faced in the right-hand direction. NIH3T3 cells were used for time lapse observation from 4 h until 76 h after seeding. First, the actin filaments of the cells cultured and fixed on the micropattern were stained with Phalloidin-Alexa488 (1:200, 7 h, R.T.), and the nuclei were stained with DAPI (1:1000, 20 min, R.T.). Next, fluorescence images of the cell nuclei, actin filaments, and membranes, and reflective bright-field images of cells on each micropattern from 1 to 20 were observed in six areas of 317.3 µm × 317.3 µm using a confocal laser scanning microscope (CLSM) (FV-1000 D, Olympus, Japan) (Figure 2A). Membranous fluorescence was emitted from PKH26 used for time lapse observation. In this observation, 31 images were captured every 2.0 µm until 30 µm along the Z-axis, which was enough to observe cells from the top to the bottom surface of the micropattern, using a CLSM with an Uplspao 40 × 2 objective lens (Olympus, Japan). In addition, the observed six areas of 317.3 µm × 317.3 µm were located in the three areas at the right end (A_r, B_r, C_r) and the three areas at the left end (A_l, B_l, C_l) of each micropattern. The three right-end areas (A_r, B_r, C_r) and the three left-end areas (A_l, B_l, C_l) were a pair of right-and-left every A, B, and C area, and the pairs of right-and-left corresponded to both ends of the same rows of connected triangular prisms. Cell numbers of each of the observed six areas on every micropattern were counted to calculate the percentages of cells in right-and-left ends of each micropattern. The percentages of cells on micropatterns 1–20 are shown in Figure 2B. The observed images of the right-end areas (A_r, B_r, C_r) and the left-end areas (A_l, B_l, C_l) on each micropattern used for the cell count in creating Figure 2B are shown (Figure S4, Supporting Information). The cell count results in each area and the total count number for each micropattern were also indicated in this figure.

Position Analysis and Migrating Direction Analysis of Cells to Grooves: Time lapse movies of cells migrating on each micropattern obtained by the above-mentioned time lapse observation were analyzed, determining whether cells were moved either inside or outside of the groove, and which direction the cells were migrating. These analyses were manually performed using AxioVision4.8 (Zeiss, Germany). To minimize the influence of cell–cell contact, we determined whether cells were migrating from the time lapse movie analyses, the analysis time was limited to no more than 24 h, in which cell density was comparatively low. Moreover, although time lapse observation was performed every 30 min, the migration distances were too short to distinguish the position change and the migration direction of the cell from images captured at 30 min intervals. Therefore, the position changes and migration directions of each cell at each point were judged by comparing time lapse images at 1 h intervals. First, the cell migration directions were determined, and the frequencies of each direction were calculated. The judgment of migration directions was in four directions to the right, left, up, and down on the time lapse pictures. The cell migration direction was judged by the change of the nucleus position. The central part the PKH26-stained cell volume was regarded as the nucleus position. Cells were judged to be obliquely migrating when the migrating distance was longer, vertical or horizontal. A schematic illustration for the judgment procedure is shown in Figure 3A. The total cell numbers used for counting the migration directions was 678, and the total number of movements used for counting on micropatterns 1–20 was shown (Figure S4, Supporting Information).

Next, the frequency of cells positioning in the grooves of a micropattern and cells positioning outside of the grooves was investigated. Almost all cellular positions could be distinguished between the inside and outside of grooves. Some cells, which adhered to both the inside and the outside of the grooves, were judged by nucleus position, where was considered as the center of the large-volume portion of the PKH26-stained cells. A schematic illustration for the judgment procedure is shown in Figure 4A. The total cell number used for counting on micropatterns 1–20 was 678, and the total number of counts of cell positions was 10585. The cell numbers used for counting cell positions and the number of counts of cell positions on micropatterns 1–20 were shown (Figure S5, Supporting Information). There were some errors in the center positions in the parts that were perceived as nuclei, as the center positions used for these analyses were determined by visual measure.

Cellular Morphology Observation: Cellular morphology observation was performed using the same sample that had been used for the
uneven distribution analysis. Fluorescence images of the cell nuclei, actin filaments, membranes, and reflective bright-field images of each micropattern from 1–25 were observed in three, four, or five areas of 126.7 µm x 126.7 µm using a CLSM (FV-1000 D, Olympus, Japan) with an objective lens (UPLSAPO100x oil NA: 1.40, Olympus, Japan) (Figure S8, Supporting Information). In this observation, 61 images were captured every 0.5 µm until 30 µm along the Z-axis to observe cells from the top to the bottom surface of the micropattern using a CLSM. The reflective bright-field images of the cells could clearly indicate the 3D cellular morphologies of even small projections, as the reflective bright-field images consisted of not only cellular surface reflections, but also fluorescence of PKH26-stained plasma membranes and Alexa488-stained actin filaments by configuring the irradiation and the detection wavelength. 3D side views of single cellular morphologies were created using Crop with a selected ROI of AutoQuantX3 (Media Cybernetics Inc., USA); for example, see the confocal laser scanning microscopic images (CLSM images) in Figure 6j.

Directional Analysis of Cell Protrusions: Directional analysis of cell protrusions was performed using CLSM images of cells cultured on micropatterns (Figure S8, Supporting Information). The images were taken under the conditions described in the “Cellular Morphology Observation” section. Superimposed images were prepared using CLSM images, which were fluorescence images of the cell nuclei, actin filaments, and membranes, and reflective bright-field images of each micropattern with cells to allow thorough observations of cell protrusions from right above and just beside by clipping each single cell using the Crop-Selected ROI of AutoQuantX3 above mentioned, and the expanded images were reviewed on a personal computer screen. The lengths of the cell protrusions were measured manually on a personal computer screen using Crop Selected ROI, and were used for the judgment of whether the right or left portion of the protrusion was longer. This judgment determined whether the protrusion length from the nuclear center to the projection tip of the right side or left side was longer than the other, or could not be distinguished. Morphologies in which direction could not be distinguished were defined as being round with no protrusion or with no clear projection tip such as contacting other cells, or if the protrusion length of the longer protrusion was 60% or less of the overall length. In this judgement, lumps of cells and gatherings of two or more cells that could not be distinguished, or the protrusions that were difficult to distinguish due to extension out of the observation area were excluded. However, if it was possible to distinguish between sides despite protrusions extending partially out of the observation area, these cells were included in analysis. The illustration of this judgment procedure is shown in Figure 5A. The results for each cell were indicated over the images (Figure S8, Supporting Information). The total cell number used for counting cell protrusion direction as L, R, and N was 385. The cell numbers used for counting the cell protrusion directions on micropatterns 1–20 were shown (Figure S9, Supporting Information). In addition, the nuclear center location by eye measurement of the above-mentioned CLSM images in these analyses was determined. Therefore, there were some errors due to uneven fluorescence dyeing or the resolution limitation of the microscope.

Cleaning of the Micropatterns for Reuse: The micropatterns were reusable. After cell culturing, the micropatterns were cleaned for reuse. Organic matter on the surface was removed using oxygen plasma treatment (100 sccm, 10 Pa, 100 W, 15 min) with a reactive ion etching system (RIE-10NRV, Samco, Inc., Japan), after which the micropatterns were soaked in fluoric acid to remove any remaining metals. Finally, the hydrophilicity of the micropatterns was improved by oxygen plasma treatment, which made them as good as new. Cleaning did not result in any deterioration of the micropattern surface.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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