Advancements in instrumentation

Air-pressure-driven separable microdevice to control anisotropic curvature of cell culture surface

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Abstract

We report a novel microdevice to tune the curvature of cell-adhering surface by controlling air-pressure and micro-slit. Human aortic smooth muscle cells were cultured on demi-cylindrical concaves formed on the microdevice and their shape-adapting behaviour could be tracked when the groove direction was changed to the orthogonal direction. This microdevice demonstrated live observation of cells responding to dynamic change of anisotropic curvature of the adhering surface and could serve as a new platform to pursue the mechanobiology on curved surface.

Keywords: Mechanobiology, Curvature, Cell adhesion, Smooth muscle cell, Orientation.
Introduction

The mechanism how cells sense the surrounding mechanical environment is one of the core interests in the field so-called mechanobiology. While molecular mechanisms of cellular mechanosensing have been revealed by observing cells confined in various 2D geometry, there have been increasing number of studies investigating how cells interact with artificial 3D environment to mimic the structural complexity in vivo, in pursuit of new medical therapeutics in the future. Among these studies, effect of curvature of cell-adhering surface i.e. concave and convex structures, has been neglected in the field for a long time, however, emerging studies reported that such curvature has a strong influence on cellular behaviours including migration, microtissue morphology, and even differentiation, even though the scale of these curved structures is orders of magnitude larger than that of single cells. These studies have clearly shown that the curvature of cell-adhering plane is a significant input for cells governing their behaviours in single-cell or continuous-tissue scales, however, little is known about the mechanism how cells sense such a structural cue much larger than single cells.

To unveil the molecular mechanism, live imaging of cells exposed to dynamic change of curvature of the adhering plane is desired. However, several technical complexities have prevented the researchers from investigating cellular behaviours on micro-curvatures. First, preparation of smoothly-curved surface with sub-millimetre curvature radius is still not feasible, while former studies have utilized sophisticated fabrication principle, such as surface tension of viscous fluid, mass-transport-limited chemical etching, stereolithography or elastic substrate bent by air pressure to prepare micro-curvatures. Especially, flexible modulation of micro-curvature pattern, which would be a strong approach to reveal the adaptation mechanism of cells, remains a challenging issue. Second, a practical limitation in observation, where objective lens is usually limited to those with long working distance with typically low
magnification due to the thickness of the substrates, often limits live cell imaging. There have been promising microdevices capable of smooth modulation of curvature of cell-adhering surface by air pressure control, however, the lateral geometry of the curving structure is fixed due to the covalently-bound structure of the cell culture surface and the pressure-controlling structure. To realise flexible control of curved pattern and live imaging during cell culture, a microdevice design accompanying non-covalent assembly of cell-culture surface and air pressure-controlling system with optimised thin design for microscopy need to be developed. Establishing such a new cell-culture system would be valuable to pursue the mechanobiology on curved surfaces.

To meet the increasing demand for cell culture platform with curvature, we hereby propose a new separable microdevice for live cell monitoring, where the curvature of the cell-adhering plane can be temporarily modulated. The microdevice consists of three components; a cell culture chamber with a thin elastic silicone bottom, a micro-slit with penetrating holes and a thin vacuum chamber. The assembled microdevice modulates curvature of the cell culture surface by air-pressure control. The separable concept allowing us to reversibly separate and re-assemble cell culture chamber and air-pressure controlling system realises flexible change of curvature pattern during cell culture. As a proof-of-concept study, we demonstrate a time-lapse observation of human aortic smooth muscle cells (SMCs) adhering on a demi-cylindrical concave surface and their responses are tracked when the cylindrical direction is turned orthogonally by rotating the cell-culture chamber on the microdevice. This is, to our best knowledge, the first demonstration to observe cells responding to dynamically changing anisotropic curvature around millimetre scale and the microdevice would be a powerful tool to pursue the mechanism of cellular curvature sensing.
Experimental

Microdevice design

A new microdevice was designed for temporal control of the curvature of cell-adhering surface (Fig. 1A). It consists of three components; a cell culture chamber (Fig. 1B), a micro-slit (Fig. 1C) and a vacuum chamber (Fig. 1D). The supplementary material should be referred to for the detailed design and the fabrication process. Cells are cultured on an elastic silicone sheet forming the bottom part of the cell culture chamber. Applying negative air pressure from the vacuum chamber to the sheet through the micro-slit, the cell culture surface can be bent. The vacuum chamber with a glass window is designed thin enough for live microscopic observation of cultured cells. These components were assembled and set in a stage incubation system (GM-8000 and WSKMOR-GI, TOKAI HIT, Fujinomiya, Japan) installed on a microscope (Fig. 1E). The hole made in the sidewall of the vacuum chamber was connected to a vacuum pump (DAP-6D, ULVAC, Chigasaki, Japan) via a dumping chamber (FB-800-5000, ASONE, Osaka, Japan) and a pressure controller (VC900, KNF, Freiburg, Germany) (Fig. 1F).

Calibration of curvature generated by air-pressure control

Curvature of the silicone sheet generated by air pressure was calibrated using a phase-contrast microscope (Eclipse Ti, Nikon, Tokyo, Japan). The height of bottom and top parts of the deformed silicone sheet was measured. Approximating the cross-sectional shape of the deformed silicone sheet to an arc, the curvature was calculated. The differential pressure $\Delta P$ was tuned from 0 to 600 hPa using the pressure controller. Cylindrical and spherical concave surfaces were formed using the micro-slits containing rectangular (300 $\mu$m and 500 $\mu$m in width) and circular (300 $\mu$m and 500 $\mu$m in diameter) holes, respectively. The cross-sectional shape of the deformed silicone sheet was also observed via confocal microscopy.
Supplementary material should be referred to for the details of curvature measurement.

**Cell culture using microdevice**

Suspended SMCs were seeded on the bottom of the cell culture chamber covalently coated with fibronectin at $7 \times 10^4$ and $3 \times 10^4$ cells/cm$^2$ in high-density and low-density conditions, respectively, followed by incubation for 12 hours to allow cells to form stable adhesion. The supplementary material should be referred to for the experimental details. SMCs were treated with Hoechst 33342 (1:10000, H3570, Thermo Fisher, Waltham, MA, USA) in serum-free cell-culture medium for 5 min and the chamber was refilled with the cell culture medium with growth factors. The SMC-containing cell culture chamber was mounted on the micro-slit with three rectangular holes (width: 500 µm, length: 6000 µm) and the vacuum chamber installed inside the stage incubation system. The differential pressure was gradually increased to 100 hPa taking 100 min to bend the cell-adhering surface to form demi-cylindrical concave without applying direct strain on the SMCs. The SMCs were cultured for 24 hours. Following pressure release taking 100 min, the medium was exchanged. The cell culture chamber was then carefully detached from the micro-slit, rotated 90° horizontally and placed on it again so that the three rectangular holes of the micro-slit under the cell culture chamber were positioned orthogonally to the original direction. Observing the positions of tiny bubbles and dusts in the wall of the cell culture chamber under microscope, the alignment of the assembled components as well as the area of interest where demi-cylindrical concave was formed before rotating the cell culture chamber was checked carefully. The differential pressure was again increased to 100 hPa as described above, followed by incubation for additional 24 hours. Nuclei of the SMCs were observed using the fluorescence microscope with either 10x (N.A. 0.30) or 20x (N.A. 0.45) lens every 6 hours. Nuclear orientation was analysed using a custom script written in MATLAB (2016b, Mathworks, Natick, MA, USA). In brief, nuclear shape was extracted
by thresholding and binarising the fluorescence image. Each nucleus was then fit to an ellipse and the angle between the long axis of the ellipse and the axis of the demi-cylindrical structure was evaluated.
Results and Discussion

The microdevice components were assembled by mounting the cell culture chamber onto the vacuum chamber via micro-slits with rectangular penetrating holes. Sucking the air from the vacuum chamber, the silicone sheet forming the bottom of the cell culture chamber was bent to form demi-cylindrical concaves. Curvature of the silicone sheet was first characterised varying the applying pressure. The curvature linearly increased as the differential pressure was elevated from 0 to 400 hPa, and then gradually reached 2.5 mm\(^{-1}\) (Fig. 2A). The curvature could be smoothly tuned in the range of 0–2 mm\(^{-1}\), corresponding to from flat state to demi-cylindrical surface of 500 µm radius. The cross-sectional views obtained by confocal microscopy confirmed that the silicone sheet formed smooth arc without any wrinkles or buckles (Fig. S6). Using the micro-slit containing circular holes, concave pits could be also formed on the bottom of the cell culture chamber (Fig. 2B). While the curvature-differential pressure relations obtained using 300 µm and 500 µm rectangular micro-slits were similar, a considerable difference was observed between those obtained using 300 µm and 500 µm circular micro-slits. Because rectangular and circular micro-slits induce uniaxial and biaxial stretch, respectively, we assume that the difference in the stretching modes cause such different dependence on micro-slit size. After forming and releasing the demi-cylindrical concave of 1 mm\(^{-1}\) curvature, no crack, which potentially influences cellular alignment, was found on the silicone-sheet surface via scanning electron microscopy (Fig. S7). Throughout these experiments, we confirmed that this separable micro-slit device could form smoothly curved surface on the cell culture chamber.

The microdevice was next applied for cell culture. The SMCs initially oriented to all directions randomly on the flat silicone sheet, however, they preferentially aligned along the cylindrical axis 22.5 hours after forming the demi-cylindrical concave (Fig. 3A). Next, the
cell-culture chamber was rotated orthogonally to the original direction on the micro-slit to observe how SMCs react to a sudden directional change of the anisotropic curvature of the adhering surface. SMCs were exposed to a demi-cylindrical concave perpendicular to the original one and they gradually aligned along the new structure within 24 hours reproducing the same distribution profile observed prior to the chamber rotation (Fig. 3B). The representative nuclear images at each time point in high-density and low-density conditions are summarised in Fig. S8 and S9, respectively.

The SMCs on the demi-cylindrical concave in the high-density condition always adjusted their orientation parallel to the cylindrical axis (Fig. 3C), which accords well to the common knowledge in the field that cells elongate along given structural patterns when there is anisotropic chemical or physical pattern on the adhering surface. This is also supported by theoretical and numerical studies explaining the optimal angle based on minimisation of cytoskeletal strain and planer shear stress. However, the trend is less clear in the low-density condition (Fig. 3D) where SMCs showed more random orientation throughout the cell culture period, although considerable portion of SMCs oriented along the demi-cylindrical axis after turning the cylindrical axis. Comparing the orientation profiles in the both conditions, it is likely that intercellular connection help cells find a proper direction to align on curved structures in the millimetre scale.

In principle, our platform cannot separate the effects of the substrate curvature and stretch on cellular orientation. However, the stretching effect could be negligible in our case, because the circumferential strain of the silicone sheet when forming the curvature was around 1% and it was slowly operated taking 100 min, while typical stretching mode known to influence SMC orientation is 5–10% cyclic stretch of frequency above 0.5 Hz. We thus think the re-orientation behaviour of SMCs was caused by the structural change of the adhering surface. A former study conducted by Ebara and colleagues applied perpendicular transition of a stripe
surface topographic pattern in sub-micron scale to sparsely cultured fibroblasts utilising a shape memory polymer. They also reported that the cells suddenly exposed to the new topological cue adapted to the new pattern within a scale of day. Our observation accords well to their report, suggesting that a common or quite similar mechanism underlies cellular curvature sensing on curved surface and topological sensing on 2D surface. The effect of cellular density and difference in the cell types on the curvature sensing needs to be investigated to explore the mechanism in the future.

Our microdevice demonstrated that SMCs could sense subtle anisotropic curvature of 1 mm\(^{-1}\), corresponding to 1 mm of curvature radius, and adapt to directional change of the cylindrical cell-adhering surface. There is so far no general threshold of the lower limit of cellular curvature sensing in the field, and it is notable that cells sense curvature whose scale is orders of magnitude larger than that of single cell. The mechanism how connected cells sense surrounding environment as well as their curvature sensing ability has still not been explored. Our platform would serve the needs to give steric perturbations to cells, together with either static or dynamic cell-confining methods on 2D flat surfaces. Conducting the curvature-transition assay with biological inhibitors for cell-adhesion-related proteins, the mechanism how cells sense and respond to the surrounding dynamic topographic change could be revealed.

From engineering perspective, our separable microdevice is unique as a cell-culture platform to realise dynamic topographic change of cell culture environment. Various microfluidic vascular models have been widely developed in the field so-called Micro Total Analysis Systems. While biomechanical stimuli including fluid shear stress and cyclic stretch have been implemented in these microvascular models, the effect of substrate curvature that potentially affect cellular properties such as motility and phenotype has not been highlighted so far. On the other hand, topographic change of cell-adhering surface in
sub-micron scale was already realised in biomaterial field using a polymeric shape-memory effect triggered by temperature change. While such chemical change can be triggered only once and it requires temperature change that affects on cellular metabolism, our system enables the dynamic control of anisotropic curvature without changing temperature and it, in principle, does not have limitation in repeating the topographic change. There have been several cell-culture platforms controlling air pressure to apply cyclic stretch to cells, which can be potentially applied to tune the curvature of cell-adhering plane, however, the geometry of curved structure is fixed due to the irreversible bonding of the elastic membrane and the vacuum structure. On the other hand, our separable microdevice is capable of flexibly changing the design of the curved part by replacing the micro-slit. The current microdevice is limited to create concave structures because of the working principle based on negative air pressure. However, convex curvature could be potentially realised by applying reversible bonding techniques for microfluidics. Although there is a tradeoff relation between the range of tunable curvature, the tunable area and the thickness of the vacuum chamber limiting the usage of high-NA lenses, further high-resolution imaging of the cells on curved surface could be potentially realised by compromising flexibility in curvature-tuning. Enriching the toolbox currently limited to investigate cellular behaviours on curved surface, our microdevice would contribute to the mechanobiology field.
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Supporting Information

The details on microdevice fabrication and cell culture are described in a supporting material.

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.
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Figure Captions

Fig. 1  Design of microdevice.  (A) The microdevice is composed of a cell culture chamber with a thin bottom made of silicone sheet, a micro-slit made of SU-8 and a vacuum chamber with a glass bottom.  Cells are cultured on the thin silicone sheet and the curvature of the adhering plane can be temporarily controlled by tuning the air pressure inside the vacuum chamber.  The cellular response can be observed by microscope in real time.  (B) Cell culture chamber with a thin silicone bottom.  (C) SU-8 micro-slit containing three straight penetrating holes in the center (width: 500 µm, length: 6 mm).  (D) Vacuum chamber with glass window.  (E) Assembled microdevice containing cells and cell culture medium installed on the microscopic stage.  (F) Schematic of air-pressure control system.  The microdevice installed in the stage incubation system was connected to a vacuum pump via a pressure controller that allows flexible modulation of differential pressure applying to the cell culture surface.

Fig. 2  Calibration of curvature tuned by air-pressure control.  Curvature of demi-cylindrical surface (A) and demi-spherical surface (B) generated using the micro-slits with rectangular holes and the one with circular holes, respectively, was calibrated as a function of applied differential pressure.  Representative 3D structures observed in the experiment were reconstructed from the stacks of confocal images.  The error bars represent the standard deviations of three different silicone sheets.

Fig. 3  Curvature-transition assay applied for human aortic SMCs.  Transition of nuclear orientation was analysed.  (A) Nuclei of Hoechst-treated SMCs were imaged and the angle between the long axis of each nucleus and the cylindrical direction was evaluated as nuclear angle.  Each nucleus is coloured according to the nuclear angle.  (B) Cell culture surface was
bent to form demi-cylindrical surface of 1 mm$^{-1}$ of curvature in the initial 1.5 hours. The axial
direction of the demi-cylindrical concave was turned orthogonally to the initial direction 24
from starting the experiment. Nuclear alignment of SMCs were tracked for 51 hours. (C, D)
Transition of nuclear orientation of SMCs were analysed. More than 1000 and 250 nuclei in
high-density (C) and low-density (D) conditions, respectively, were analysed at each time point.
The experiments were duplicated to ensure the reproducibility.
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