Supporting Information

Gradient Static-Strain Stimulation in a Microfluidic Chip for 3D Cellular Alignment

Hsin-Yi Hsieh\textsuperscript{1,2,3}, Gulden Camci-Unal\textsuperscript{2,4}, Tsu-Wei Huang\textsuperscript{5}, Ronglih Liao\textsuperscript{6}, Tsung-Ju Chen\textsuperscript{1}, Arghya Paul\textsuperscript{2,4,7}, Fan-Gang Tseng\textsuperscript{1,5,8*}, and Ali Khademhosseini\textsuperscript{2,4,7*}

\textsuperscript{1}Institute of NanoEngineering and MicroSystems (NEMS), National Tsing Hua University, No. 101, Sec. 2, Kuang-Fu Rd. Hsinchu 30013, Taiwan R.O.C.
\textsuperscript{2}Division of Biomedical Engineering, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, 65 Landsdowne Street, Cambridge, MA 02139, USA.
\textsuperscript{3}Department of Mechanical Engineering, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan R.O.C.
\textsuperscript{4}Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139, USA.
\textsuperscript{5}Department of Engineering and System, National Tsing Hua University, No. 101, Sec. 2, Kuang-Fu Rd., Hsinchu 30013, Taiwan R.O.C.
\textsuperscript{6}Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA.
\textsuperscript{7}Wyss Institute for Biologically Inspired Engineering, Harvard University, 3 Blackfan Circle, Boston, MA 02115, USA.
\textsuperscript{8}Research Center for Applied Sciences, Academia Sinica, No. 128, Sec. 2, Academia Rd., Nankang, Taipei 11529, Taiwan R.O.C.

Correspondence should be addressed to:
Ali Khademhosseini (alik@rics.bwh.harvard.edu)
Fan-Gang Tseng (fangang@ess.nthu.edu.tw)
A. Gradient Strain Chip Fabrication:

The top part of the gradient strain chip was a PDMS de-molded from a PMMA mold (Figure S-1), and the bottom part was a TMSPMA-coated slide (25 mm (W) x 37.5 mm (L) x 1 mm (H)). After punching holes on the molded PDMS and oxygen plasma treatment, the top and bottom parts were bond together to form a fluidic chip. To operate convex PDMS deformation and supply sufficient medium for cell culture, two PDMS reservoirs were also bond onto the inlet and outlet of the fluidic chip. The chip pictures can refer to the Figure S-2.

![PMMA mold](image1.png)

**Figure S-1.** The dimension of the PMMA mold for the PDMS molding. The yellow number represents the depth. (unit: mm)

![PDMS chip](image2.png)

**Figure S-2.** Pictures of the microfluidic chips. The cross-section view of PDMS chips (b) without liquid pressure on the PDMS membrane and (c) with liquid pressure for PDMS deformation, which results in the later-on gradient strain on circular hydrogels. (c) The top-view of a microfluidic chip with concentric circular hydrogel pattern.
B. Photomask Designs for Circular Hydrogels:

**Figure S-3.** Schematic illustration of photomask designs. For gradient strain chip, (a) the photomask has two fan-shape openings on the circles because uncrosslinked prepolymer cell suspension needs to be washed away in the fluidic channel after UV exposure. For uniform compressed strain, (b) the hydrogel patterns are continuously concentric circles because the uncrosslinked prepolymer cell suspension can be washed away using pipet flushing. The diameter of center circle, $c$, is fixed as 2 mm.
C. Gradient Strain Chip Calculation:

The PDMS deformation curve was calculated by assuming the curve fitted a circle with a phantom radius (r) from the phantom origin (Figure S-4). To obtain the phantom radius (r), the first equation \( r^2 = 6^2 + (r-H_0)^2 \) was based on Pythagorean theorem. Therefore, \( r = (36 + H_0^2)/(2H_0) \).

Moreover, the second equation \( x^2 + y^2 = r^2 \) was based on the phantom circle, so \( y = (r^2 - x^2)^{1/2} \).

To calculate the injection volume (V) for the buckle PDMS, the volume was calculated by the integration equation \( V = \int_{r-H_0}^{r} \pi y^2 \, dx \), so the correlation between the injection volume (V) and the maximum height (H_0) was \( V = \pi ((rH_0^2) - (H_0^3/3)) \). By the combination of \( r = (36 + H_0^2)/(2H_0) \) and \( V = \pi ((rH_0^2) - (H_0^3/3)) \), the maximum height (H_0) could be evaluated for 10, 20, 30, 40, 50, and 60 µL injection volume (V). Then we had H_0 for the equation of \( r = (36 + H_0^2)/(2H_0) \) to obtain the phantom radius (r). Thus, the height \( (H(x) = y(x) - (r-H_0)) \) of the buckle PDMS curve was obtained for each circular hydrogel.

\[
\begin{align*}
  r^2 &= 6^2 + (r-H_0)^2 \implies 36 - 2rH_0 + H_0^2 = 0 \\
  \therefore r &= \frac{36 + H_0^2}{2H_0} \\
  x^2 + y^2 &= r^2 \implies y = \sqrt{r^2 - x^2} \\
  V &= \int_{r-H_0}^{r} \pi y^2 \, dx = \int_{r-H_0}^{r} \pi (r^2 - x^2) \, dx = \pi (r^2 x - \frac{x^3}{3})|_{r-H_0}^{r} \\
  &= \pi \left( r^3 - \frac{r^3}{3} \right) - \pi \left( r^2 (r-H_0) - \frac{(r-H_0)^3}{3} \right) \\
  &= \pi \left( \frac{2r^3}{3} - \pi (r^3 - r^2H_0 - \frac{r^3 - H_0^3 - 3r^2H_0 + 3rH_0^2}{3}) \right) \\
  &= \pi \left( \frac{2r^3}{3} - \pi \frac{3r^3 - 3r^2H_0 - r^3 + H_0^3 + 3r^2H_0 - 3rH_0^2}{3} \right) \\
  &= \pi \left( \frac{2r^3}{3} - \pi \frac{3r^3 - 3r^2H_0 + r^3 - H_0^3 - 3r^2H_0 + 3rH_0^2}{3} \right) \\
  &= \pi \left( \frac{3rH_0^2 - H_0^3}{3} \right) = \pi (rH_0^2 - H_0^3/3) 
\end{align*}
\]

Figure S-4. Calculation of the volume, the convex PDMS curve \( (H(x)) \), and the maximum height \( (H_0) \) of deformed PDMS membrane. \( H(x) \): the height in the deformed PDMS region. \( H_0 \): the maximum height of the deformed PDMS membrane. \( r \): the radius of the deformed PDMS curve. \( V \): the volume in the pink region (not includes the volume in the bottom channel (white region)).
D. Circle Elongation of Circular Hydrogels

Figure S-5. Phase images of ripple hydrogel circles of continuous circle pattern. Although the hydrogels are immobilized onto the TMSPMA-coated glass slides and there is no free space for circle elongation, the hydrogel squeezing in circle direction causes ripple shape of hydrogel circles (if the squeezing force is too high).
E. Cell Alignment Analysis:

(1) Approach for Uniform Compressed Strain

To quickly investigate the cell behavior in the concentric circular hydrogels and select an appropriate pattern size for the gradient strain chip, we first employed 4 sizes of photomasks with the line-width/spacer of 50 μm/50 μm, 100 μm/100 μm, 200 μm/200 μm, and 300 μm /300 μm (Figure S-6 (a)). Moreover, one set of strain-free samples, the circular hydrogel facing up on glass in the dish, was for the control groups (Figure S-6 (b)). For the strain samples, the weight of the glass slide (1.2 g) applied force onto the circular hydrogels to obtain uniform elongation of ~50% (Figure S-6 (c)). The Poisson’s ratio of hydrogel usually can be assumed as 0.5,\(^1\)-\(^2\) which means the hydrogel is an incompressible material deformed elastically at small strains. In our pattern design, there was no space for the hydrogel elongation along circular direction when circular hydrogels were applied force for compressed strain. Therefore, the Poisson ratio in the radial direction (the ratio of the elongation deformation to the compressed strain) could be larger than 0.5, but smaller than 1. It also means 50% elongation represents the compressed strain is \(\geq 50\%\).

Figure S-6. Schematic illustration of the concentric circular hydrogels on TMSPMA-coated glass for the cell behavior analysis of 3T3 cells and cardiac side population cells (CSPs) under uniform compressed strain stimulation. (a) The UV crosslinking method for concentric circular hydrogels with cell encapsulation. (b) Strain-free sample represents the circular hydrogel on glass faces up in the petri-dish for cell culture, and (c) strain sample represents the circular hydrogel on 1.2 g glass faces down in the dish.
(2) Cell Images under Uniform Compressed Strain

For the concentric hydrogel patterns, the spacer and the line width decided the maximum hydrogel elongation. Although four of our pattern sizes had the same duty cycle, the conditions of hydrogel elongation of four hydrogel sizes were different while considering the swelling ratio of hydrogels. In addition, the ratio of cell size to hydrogel width would also affect the cell alignment angle. Therefore, we tested those four sizes of hydrogels under strain-free and uniform strain (with 1.2 g applied force) conditions to investigate the cell behaviors. The phase images and LIVE/DEAD images of 3T3 cells in GelMA under strain and strain-free conditions at day 3 were shown in Figure S-7. The results show that there was circular alignment only under strain-free sample with line-width/spacer of 50 µm/50 µm at day 3. For 200 µm/200 µm and 300 µm/300 µm samples, 3T3 cells aligned along radial direction under strain conditions, and there was random alignment under strain-free conditions. For 100 µm samples, 3T3 cells seems to have both of circular and radial alignment. For LIVE/DEAD staining in Figure S-7, strain samples show only 5-10% lower viability compared to strain-free samples. The phase contrast images at day 0, day 1, and day 5 were also shown in Figure S-8.

![Image](image_url)

**Figure S-7.** The phase and LIVE/DEAD images of NIH3T3 cells encapsulated in 5% GelMA with 0.1% photoinitiator after 3 day incubation at 37°C and 5% CO₂. Strain-free and strain represent the concentric hydrogel circles on glasses face up and face down in 6-well dishes, respectively. The force applies on the strain samples is 1.2 g, the weight of TMSPMA-coated glass. The line width and spacer of hydrogel circles is 50 µm/50 µm, 100 µm/100 µm, 200 µm/200 µm, and 300 µm/300 µm from the left to the right. (Scale: 200 µm)
**Figure S-8.** The phase images of NIH3T3 cells encapsulated in 5% gelMA with 0.1% photoinitiator at day 0, day 1, and day 5 in an incubator with 37°C and 5% CO₂. Strain-free and strain represent the concentric hydrogel circles on glasses face up and face down in 6-well dishes, respectively. The force applies on the strain samples is 1.2 g, the weight of TMSPMA-coated glass. The line width and spacer of hydrogel circles is 50 µm/50 µm, 100 µm/100 µm, 200 µm/200 µm, and 300 µm/300 µm from the left to the right. (Scale: 200 µm)
(3) Histogram of Cell Nuclei Angle and Alignment Ratio under Uniform Compressed Strain

For the cell alignment, all the nuclear angles needed to be normalized and converted to the new coordinate system: 0° or 180° was the circular alignment, and 90° was the radial alignment. Following the normalization of aligned angle, the histogram of the cell alignment at day 3 in 50 µm/50 µm, 100 µm/100 µm, 200 µm/200 µm, and 300 µm/300 µm pattern sizes were shown in Figure S-9. To obtain cell alignment ratio in the blue value in Figure S-9 (b)-(d), we assumed that smaller than 20° means cells were aligned, so the cell numbers of 70°~110° would be divided by the total cell numbers of 0°~20° and 160°~180° (Equation S-1). If the value was close to 1, there was random alignment. If the value was much larger (smaller) than 1, there was more radial (circular) alignment.

**Figure S-9.** Aligned angle of 3T3 cells encapsulated in GelMA with and without strain at day 3. All cell angles are normalized based on the circular patterns. Therefore, (a) 90° represents cell aligns along the short-axis or the radius direction of the circles, and 0° or 180° represents cell aligns along the long-axis of the circles.

\[
\text{Alignment ratio} = \frac{\text{freq.}(70°\sim110°)}{\text{freq.}(0°\sim20°) + \text{freq.}(160°\sim180°)}
\]

**Equation S-1.** The formula for the calculation of the radial-to-circular alignment ratio \(RCA_{ratio}\).
(1) Cell Viability & Alignment Ratio

NIH3T3 cell behaviors were first investigated for the cell viability, alignment ratio, viable cell density, and cell spreading, under strain and strain-free conditions from day 0 to day 5. Four different pattern sizes (line-width/spacer), 50 µm/50 µm, 100 µm/100 µm, 200 µm/200 µm, and 300 µm/300 µm, were also compared to study the size effect for the cell alignment. In results of cell viability (Figure S-10(a)), there was no significant difference only at day 0. Starting from day 1, strain samples showed 5-10% smaller cell viability. However, the overall cell viability in all conditions was higher than 80%. Although ≥50% static compressed strain on hydrogels caused more cell death for longer than 1-day incubation, the overall cell viability was still larger than 80% for cell study. Therefore, there is no viability concern when applying such high strain stimulation, about 50% compressed strain on hydrogels.

In previous studies, it has been reported that cells tend to align along the long-axis in 3D encapsulated hydrogels with the line-width (short-axis) of 50 µm, 100 µm, and 200 µm. Furthermore, cells encapsulated in the finest line-width of 50 µm showed the most efficient alignment result, second in 100 µm, and the last in 200 µm. In our circular hydrogel design, the hydrogel elongation direction (radial direction) is perpendicular to the circular direction. Therefore, the hydrogel elongation, originated from the compressed strain, not only simply stimulates cells to align along radial direction, but also needs to complete with the effect of long-axis alignment.

In this supporting data, in addition to the line-width of 50 µm, 100 µm, and 200 µm, we also chose 300 µm as the short-axis of concentric circular hydrogels. For the cell alignment (Figure S-10(b)), cell alignment ratio gradually decreased in all the strain and strain-free samples from day 0 to day 5, which represents that cells trend to spread along the long-axis direction along time. This trend is in agreement with the previous finding. Another possibility of the reducing alignment ratio along time was the hydrogel degradation, so the elongation force on the hydrogels of the strain sample decreased along time.

**Table S-1.** The elongation \((E_i)\) and the hydrogel size after 4-hr immersing in culture medium.

<table>
<thead>
<tr>
<th></th>
<th>Spacers (µm)</th>
<th>Line-width (µm)</th>
<th>Elongation ((E_i))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mask Hydrogel (4hr)</td>
<td>Mask Hydrogel (4hr)</td>
<td>Hydrogel (4hr)</td>
</tr>
<tr>
<td>50µm/50µm</td>
<td>50 13.97 ± 3.30</td>
<td>50 86.03 ± 4.17</td>
<td>13.97 %</td>
</tr>
<tr>
<td>100µm/100µm</td>
<td>100 34.78 ± 5.98</td>
<td>100 165.22 ± 4.99</td>
<td>17.39 %</td>
</tr>
<tr>
<td>200µm/200µm</td>
<td>200 139.24 ± 6.96</td>
<td>200 260.76 ± 5.79</td>
<td>34.81 %</td>
</tr>
<tr>
<td>300µm/300µm</td>
<td>300 222.82 ± 6.01</td>
<td>300 377.18 ± 6.90</td>
<td>37.14 %</td>
</tr>
</tbody>
</table>
Four hydrogel patterns were in 50% duty cycle, so the maximum elongation was supposed to be 50%. However, the hydrogel usually swells after crosslinking and immersed in DPBS or culture medium. Therefore, the final elongation percentage of those concentric circular hydrogels was smaller than 50% after the deduction of native hydrogel swelling. The larger spacer size resulted in larger elongation percentage \((E_i)\) after considering of hydrogel swelling, as shown in Table S-1. Thus, in strain samples, the alignment ratio had the largest values in 300 µm/300 µm, and it decreased as reducing the size of line-width/spacer.

The significant difference between the strain and strain-free samples in four different hydrogel patterns all appeared at day 3 and day 5. Similarly, the strain-free samples at 100 µm/100 µm, 200 µm/200 µm, and 300 µm/300 µm had significant difference at day 3 and day 5 compared to the strain-free sample at 50 µm/50 µm. It verifies that cells need about 3 days for adjusting their orientation to the long-axis by the guidance of long-axis or to the short-axis by the hydrogel elongation stimulation.

**Figure S-10.** (a) Cell viability and (b) cell alignment ratio of strain and strain-free NIH3T3 cell encapsulation in 5% GelMA with 0.1% photoinitiator. (†) represents the strain-free data in 100, 200, or 300 µm has significant difference \((p < 0.05)\) from the strain-free data in 50 µm at respective day. (#) represents the strain-free data in 100, 200, or 300 µm has significant difference \((p < 0.05)\) from the strain-free data in 50 µm at respective day. (*), (**), and (***) indicate significant difference, \(p < 0.05\), 0.01, and 0.001, respectively.
(2) Viable Cell Density & Circularity

To understand the cell proliferation under strain condition, we calculated the viable cell density from day 0 to day 5 at different pattern sizes (Figure S-11 (a)). At day 0 to day 3, the difference between strain and strain-free samples was not significant, but the cell density in strain sample was significantly decreased at day 5. In other words, cells under 5-day strain stimulation became unhealthy, so the viable cell density dropped compared to cells in strain-free samples. For the investigation of cell spreading, the cell shape roundness was calculated following roundness \(=4\times \pi \times \text{area}/\text{perimeter}^2\) and analyzed by the imageJ. The value of roundness was assumed between 1 (circular shape) and 0 (elongated, linear morphology), and the circularity is the median value of the roundness of each sample (Figure S-11 (b)). All the circularity in strain samples was higher than those in strain-free sample. This result is fulfilled with literature that cells under strain would result in higher circularity, less cell spreading.

![Figure S-11.](image)

**Figure S-11.** (a) Cell numbers and (b) cell circularity of strain and strain-free NIH3T3 cell encapsulation in 5% GelMA with 0.1% photoinitiator. (†) represents the strain-free data in 100, 200, or 300 µm has significant difference (p < 0.05) from the strain-free data in 50 µm at respective day. (#) represents the strain-free data in 100, 200, or 300 µm has significant difference (p < 0.05) from the strain-free data in 50 µm at respective day. (*), (**), and (***) indicate significant difference, p < 0.05, 0.01, and 0.001, respectively.
(3) Parameters for the Gradient Strain Chip

Previous results imply that larger than 200 µm/200 µm patterns could be sufficiently generated radial stretch by the compressed strain on hydrogel compared to the strain-free samples. Moreover, 200 µm/200 µm pattern in the gradient strain chip could have 12 circles, but 300 µm/300 µm pattern could only have 8 circles. Thus, 200 µm/200 µm pattern was chosen for the experiments in the gradient strain chip with more strained hydrogels in the gradient. Previous result also shows that cells need more than 3 days for alignment, but 5-day compressed strain on cells causes cell death. Therefore, we selected 3-day incubation for the experiments for the gradient strain chip.
G. CSP Cell Behavior Analysis:

Because different cell types may have different response to the mechanical stimulation, cardiac side population (CSP) cells were employed for comparison. One obvious different between 3T3 cells and CSPs is the cell size. In Figure S-12 (a) and (b), the average size of 3T3 cells and CSPs are 52 µm and 124 µm in diameter, respectively. The CSP cell viability in strain and strain-free samples showed similar trend compared to 3T3 result. CSP viability in strain samples was 5-10% lower than strain-free samples, and the minimum viability was about 80% (Figure S-12 (c)). For the CSP alignment, the main difference from 3T3 was the CSPs in strain-free conditions significantly aligned along circular direction along time. A possible reason is the size of CSP is closer to the line-width of 200 µm circular hydrogels, so CSPs only have free space along circular direction for their proliferation. Thus, CSPs tended to align along circular direction compared to more random alignment for 3T3 cells with smaller sizes. The average size of different cell types may be an important factor to affect the alignment trend in the concentric circular hydrogels. CSPs under strain sample also seem to be more sensitive to the elongated hydrogels because of the higher alignment ratio. In Figure S-12 (e), CSPs in strain samples have equal or even more viable cell density. It suggests that CSPs can tolerate higher compressed strain, and CSPs even like strain condition, compared to 3T3 cells. For the circularity in Figure S-12 (f), the CSP circularity in strain samples was higher than that in the strain-free samples, in agreement with the circularity results of 3T3.

Figure S-12. Cell morphology of (a) NIH 3T3 cells and (b) cardiac side population (CSP) cells and the cell viability of CSP encapsulated in concentric circular hydrogel pattern with the line-width/spacer of 200 µm/200 µm from day 0 to day 5. (*), (**), and (***)) indicate significant difference, p < 0.05, 0.01, and 0.001, respectively.
**H. Diffraction Effect via Glass Gap:**

**Figure S-13.** Integrated UV Energy on the hydrogel after considering diffraction effect based on 1-mm glass gap. If the photomask closely contacts hydrogel (0 μm gap), the distribution of UV energy is an ideal square wave as shown in (a). (b) When there is a 500-μm gap, the diffraction effect results in a ripple shape of UV energy distribution. (c) When the gap increases to 1000 μm, equal to the TMSPMA-coated glass bottom of the GSS-microChip, the ripple wave of UV energy becomes broader. (d) The integrated energy in hydrogel number 1 and 12 is smaller than number 2-11 because there is less UV energy contributed from the energy via neighboring aperture (see the point of green arrows).

**REFERENCE**