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Dr. Vineeta Bajaj
Review Editor
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Dear Dr. Bajaj:

On behalf of our co-authors, we respectfully submit herewith the attached manuscript entitled, **A microfluidic platform for stimulating chondrocytes with dynamic compression**, which we would like to have considered for publication in *Journal of Visualized Experiments*.

Following the excellent and constructive feedback of you and the reviewers, we are submitting a revised version of our manuscript. Along with our resubmission, you will also find a document detailing our answers to the review comments. We believe that this revised version of the manuscript successfully addresses all the questions and concerns of the reviewers, and is appropriate for readers of *Journal of Visualized Experiments*.

Many thanks for your consideration.

Sincerely,

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TITLE:

A Microfluidic Platform for Stimulating Chondrocytes with Dynamic Compression

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KEYWORDS

Microfluidics, photolithography, soft lithography, growth plate chondrocytes, mechanobiology, cell mechanics, alginate hydrogel, confocal microscopy, image analysis

SUMMARY

This article provides detailed methods for fabricating and characterizing a pneumatically actuating microfluidic device for chondrocyte compression.

ABSTRACT

Mechanical stimuli are known to modulate biological functions of cells and tissues. Recent studies have suggested that compressive stress alters growth plate cartilage architecture and results in growth modulation of long bones of children. To determine the role of compressive stress in bone growth, we created a microfluidic device actuated by pneumatic pressure, to dynamically (or statically) compress growth plate chondrocytes embedded in alginate hydrogel cylinders. In this article, we describe detailed methods for fabricating and characterizing this device. The advantages of our protocol are: 1) Five different magnitudes of compressive stress can be generated on five technical replicates in a single platform, 2) It is easy to visualize cell morphology via a conventional light microscope, 3) Cells can be rapidly isolated from the device after compression to facilitate downstream assays, and 4) The platform can be applied to study mechanobiology of any cell type that can grow in hydrogels.

INTRODUCTION

Micro-engineered platforms are valuable tools for studying the molecular, cellular, and tissue level biology because they enable dynamic control of both the physical and chemical microenvironments[1-8]. Thus, multiple hypotheses can be simultaneously tested in a tightly controlled manner. In the case of growth plate cartilage, there are increasing evidences of an important role of compressive stress in modulating bone growth through action on the growth plate cartilage[9-25]. However, the mechanism of action of compressive stress – in particular, how stress guides the formation of chondrocyte columns in the growth plate – is poorly understood.

The goal of this protocol is to create a pneumatically actuating microfluidic chondrocyte compression device[26] to elucidate mechanisms of mechanobiology in growth plate chondrocytes (**Figure 1a-c**). The device consists of two parts: the pneumatic actuation unit and the alginate gel construct. The microfluidic pneumatic actuation unit is fabricated using polydimethylsiloxane (PDMS) based on the photo- and soft-lithography. This unit contains a 5 x 5 array of thin PDMS membrane balloons which can be inflated differently based on their diameters. The alginate gel construct consists of the chondrocytes embedded in a 5 $\times$ 5 array of alginate gel cylinders, and the entire alginate-chondrocyte constructs are assembled with the actuation unit. The alginate gel constructs are compressed by the pneumatically inflated PDMS balloons (**Figure 1b**). The microfluidic device can generate five different levels of compressive stress simultaneously in a single platform based on differences in the PDMS balloon diameter. Thus, a high-throughput test of chondrocyte mechanobiology under multiple compression conditions is possible.

The microfluidic device described in this protocol has many advantages over the conventional compression device such as external fixators[14,21,23] and macroscopic compression devices[16,19,27,28] for studying chondrocyte mechanobiology: 1) The microfluidic device is cost effective because it consumes smaller volume of samples than the macroscopic compression device, 2) The microfluidic device is time effective because it can test multiple compression conditions simultaneously, 3) The microfluidic device can combine mechanical and chemical stimuli by forming a concentration gradient of chemicals based on the limited mixing in microchannels, and 4) Various microscopy techniques (time-lapse microscopy and fluorescence confocal microscopy) can be applied with the microfluidic device made of transparent PDMS.

We adopted and modified the method of Moraes et al.[7,29] to create different compressive stress levels in a single device to enable high-throughput mechanobiology studies of chondrocyte compression. Our approach is appropriate for cells (e.g., chondrocytes) which need three-dimensional (3D) culture environment and for biological assays after compressing cells. Although some microfluidic cell compression devices can compress cells cultured on two-dimensional (2D) substrates[30-32], they cannot be used for chondrocytes because 2D cultured chondrocytes dedifferentiate. There are microfluidic platforms for compressing 3D cultured cells in photopolymerized hydrogels[7,33], but they are limited in isolating cells after compression experiments because isolating cells from photopolymerized hydrogel is not easy. Additionally, the effects of ultraviolet (UV) exposure and photo crosslinking initiators on cells may need to be evaluated. In contrast, our method allows rapid isolation of cells after compression experiments for post biological assays because alginate hydrogels can be depolymerized quickly by calcium chelators. The detailed device fabrication and characterization methods are described in this

protocol. A brief procedure for fabricating the microfluidic chondrocyte compression device is shown in **Figure 2**.

PROTOCOL

NOTE: Wear personal protective equipment (PPE) such as gloves and lab coat for every step in this protocol.

1 Master mold fabrication

NOTE: Perform step 1.1 - 1.3 in a fume hood.

1.1 Glass treatment

Note: Wear a face shield, gloves, and a lab coat for step 1.1.

1.1.1 Make Piranha solution (60 mL) by mixing sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2) with a volume ratio of 3:1.

CAUTION: Do not use Piranha solution and acetone in the same fume hood due to the explosion hazard.

1.1.2 Place a glass plate (50.8 mm × 76.2 mm × 1.2 mm) in Piranha solution for 30 min at 40 °C.

1.1.3 Rinse the glass plate with deionized water (diH_2O).

1.1.4 Place the glass plate in acetone at room temperature for 10 min.

1.1.5 Rinse the glass plate with isopropanol and dry it with nitrogen (N_2) gas.

1.1.6 Bake the glass plate at 200 °C for 20 min on a hot plate. All subsequent baking steps use a hot plate.

1.2 SU-8 seed layer formation on the glass plate

1.2.1 Spread SU-8 5 on the glass plate with a disposable pipette to cover around 2/3 of the plate's surface area.

1.2.2 Spin the glass plate with SU-8 5 photoresists at 500 rpm for 35 s (initial spinning cycle) and then 2,500 rpm for 40 s (final spinning cycle) using a spin coater. All subsequent spin coating steps include the same initial spinning cycle.

1.2.3 Bake the SU-8 5 coated glass plate at 65 °C for 2 min and then at 95 °C for 5 min.

1.2.4 Expose the SU-8 5 coated glass plate to UV light (60 mW/cm^2, distance between UV lamp and photomask is 20 cm, total amount of UV light energy = 60 mJ/cm^2) for 1 s.

NOTE: The UV exposure time should be adjusted according to the power of a used UV light.

1.2.5 Bake the SU-8 5 coated glass plate at 65 °C for 2 min and at 95 °C for 5 min.

1.2.6 Place the baked glass plate in the SU-8 developer for 2 min.

1.2.7 Bake the SU-8 5 coated glass at 180 °C for 20 min.

1.3 SU-8 channel pattern fabrication using photolithography (**Figure 2a Step 1-3**)

1.3.1 Pour SU-8 100 on the SU-8 5 seeded glass plate to cover around 2/3 of the plate's surface area.

1.3.2 Spin the glass plate with SU-8 100 at 3,000 rpm for 38 s (≈ 90 μm thick).

1.3.3 Bake the SU-8 100 coated glass plate at 65 °C for 10 min and then at 95 °C for 30 min. If SU-8 100 is still sticky after this procedure, bake the glass plate for a longer time at 95 °C until SU-8 100 becomes non-sticky.

1.3.4 Place a high-resolution microchannel photomask (25,400 dpi, see **Supplementary Figure 1**) on the SU-8 100 coated glass plate and expose the photomask covered glass plate to UV light for 4 s (total amount of UV light energy = 240 mJ/cm^2).

NOTE: The UV exposure time should be adjusted according to the power of a used UV light.

1.3.5 Remove the photomask from the glass plate and bake the glass plate at 65 °C for 2 min and then 95 °C for 20 min.

1.3.6 Keep the baked glass plate in a container, which is wrapped with aluminum foil to block any light, for overnight curing.

1.3.7 Develop SU-8 100 channel patterns on the glass plate in the SU-8 developer for 15 min.

1.3.8 Wash the SU-8 patterned glass plate (master mold) with isopropyl alcohol and dry it with N_2 gas. If white particles remain during this process, repeat step 7 for 5 min.

2 Pneumatic actuation unit

2.1 Microchannel layer (Layer 1)

NOTE: Perform step 2.1.1 - 2.1.2 in a fume hood.

2.1.1 Drop 200 µL of (Tridecafluoro-1, 1, 2, 2-Tetrahydrooctyl)-1-Trichlorosilane on a coverslip and place it in a vacuum chamber with the master mold.

2.1.2 Apply vacuum for 2 min in the chamber and wait for 6 h (or overnight) for silanization of the mold.

2.1.3 Mix PDMS with a weight ratio of 10:1 (prepolymer:curing agent) for 5 min.

NOTE: All subsequent PDMS casting step contains the same prepolymer and curing agent weight ratio (10:1).

2.1.4 Pour PDMS on the master mold and degas PDMS in a vacuum chamber for 30 min.

2.1.5 Sandwich PDMS with a piece of transparency film.

2.1.6 Clamp the above sandwiched assembly with a glass plate, foam pads and plexiglass plates (**Figure 2a Step 4**).

2.1.7 Cure PDMS in an oven at 80 °C for 6 h.

2.1.8 Isolate the PDMS layer (Layer 1) with the transparency film from the sandwiched structure (**Figure 2a Step 5**).

2.1.9 Activate surfaces of Layer 1 and a clean glass plate (Glass plate 1; 50.8 mm × 76.2 mm × 1.2 mm) using a plasma cleaner for 1 min.

NOTE: Plasma cleaning time may vary based on the power of a used plasma cleaner.

2.1.10 Bond Layer 1 onto Glass plate 1 and place them in the oven at 80 °C for 30 min.

2.1.11 Remove the transparency film from Layer 1.

2.2 Thin PDMS membrane (Layer 2)

2.2.1 Spin coat uncured PDMS on a transparency film at 1,000 rpm for 1 min to obtain a 60 µm thick PDMS layer.

2.2.2 Partially cure spin coated PDMS (Layer 2) in the oven at 80 °C for 20-30 min.

2.2.3 Activate Layer 1 on Glass plate 1 and Layer 2 using the plasma cleaner for 1 min.

NOTE: Plasma cleaning time may vary based on the power of a used plasma cleaner.

2.2.4 Bond the Layer 2 onto Layer 1 and place them in the oven at 80 °C overnight.

2.3 Tubing block

2.3.1 Place metal tubes vertically on a Petri dish.

2.3.2 Gently pour PDMS in the dish to submerge about 3/4 of the metal tubes.

2.3.3 Cure PDMS in the oven at 60 °C for 6 h (or overnight).

2.3.4 Cut a piece of PDMS block containing one metal tube.

2.3.5. Punch a hole on Layer 2 for the inlet.

2.3.6 Activate the PDMS block and Layer 2 using the plasma cleaner for 1 min.

2.3.7 Attach the PDMS block onto the inlet part of Layer 2 and place the entire actuation unit in the oven at 80 °C for overnight.

3 Alginate-chondrocyte (or bead) constructs

3.1 Amino-silanized glass plate

3.1.1 Cut a glass plate (50.8 mm × 76.2 mm × 1.2 mm) into two half-sized glass plates (50.8 mm × 38.1 mm × 1.2 mm) using a diamond scriber.

3.1.2 Place the glass plates in 0.2 M hydrogen chloride (HCl) and gently shake (e.g., 55 rpm) them overnight.

3.1.3 Rinse the glass plates with dH_2O.

3.1.4 Shake the glass plates in 0.1 M sodium hydroxide (NaOH) for 1 h at 55 rpm and rinse them with diH_2O.

3.1.5 Shake the glass plates in 1% (v/v) 3-aminopropyltrimethoxysilane (APTES) for 1 h at 55 rpm and rinse them with diH_2O.

3.1.6 Dry the amino-silanized glass plates in a fume hood overnight.

3.2 Agarose gel mold for alginate gel constructs

3.2.1 Mix 5% (w/v) agarose and 200 mM calcium chloride ($CaCl_2$) in diH_2O.

3.2.2 Boil the agarose gel solution with a microwave oven (or a hot plate). Boiling time varies

based on the volume of the agarose gel solution and the power of the microwave oven (or the temperature of the hot plate).

3.2.3 Pour the boiled agarose gel solution onto an aluminum mold (see **Supplementary Figure S2**), and sandwich it with a glass plate.

3.2.4 Wait for 5 min and unmold the solidified agarose gel from the aluminum mold.

3.3 Growth plate chondrocyte harvest

3.3.1 Isolate growth plates from the hind limbs of neonatal mice.

3.3.2 Place the growth plates in 1 mL of 0.25% collagenase for 3 h in an incubator at 37 °C and 8% carbon dioxide (CO_2), to remove extracellular matrix (ECM).

3.3.3 Centrifuge the digested sample for 5 min at 125 *x g* to make a chondrocyte pellet and remove supernatant from the sample. Here, 1 *x g* is the acceleration of gravity.

3.3.4 Resuspend the chondrocyte pellet in 1 mL of Dulbecco's modified Eagle's medium (DMEM).

3.3.5 Count the number of chondrocytes in DMEM using a cell counter.

3.3.6 Centrifuge the chondrocytes in DMEM for 5 min at 125 *x g* to make a chondrocyte pellet again and remove supernatant from the sample.

3.3.7 Optionally, resuspend the chondrocyte pellet in the chondrocyte culture media (CCM)[34] containing 2 μM calcein AM, and incubate the sample at 37 °C for 30 min. Repeat step 3.3.6 and move to step 3.3.8.

3.3.8 Resuspend the chondrocyte pellet in a desired volume of CCM and keep them in the incubator at 37 °C and 8% CO_2 before use.

3.4 Alginate-chondrocyte (or bead) constructs (**Figure 2c**)

3.4.1 Mix 1.5% (w/v) alginate in ph4osphate-buffered saline (PBS) with 5 mg/mL of sulfo-NHS, 10 mg/mL of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC).

3.4.2 Add 8×10^6 chondrocytes into the 1 mL of alginate gel solution [or Add 3 μL of 1 μm-diameter fluorescent beads (542/612 nm) in 1 mL of alginate gel solution (0.3% (v/v))].

3.4.3 Place 150 μL of the alginate-chondrocyte (or bead) solution on the amino-silanized glass plate fabricated in Step 3.1.

3.4.4 Cover the alginate gel solution with the agarose gel mold for 3 min.

3.4.5 Remove excessive alginate gel solution overflowing from the agarose gel mold with a razor blade and remove the agarose gel mold. Then, cylindrical alginate-chondrocyte (or bead) constructs are obtained on the amino-silanized glass plate.

3.4.6 Place the alginate-chondrocyte constructs in cross-linking solution (50 mM $CaCl_2$ / 140 mM NaCl in diH_2O) for 1 min for further polymerization.

4 Device assembly (Figure 2d)

Note: PDMS spacers and 3D printed clamps need to be prepared separately.

4.1 Locate four 1 mm thick PDMS spacers on the four corners of the Layer 2 of the actuation unit.

4.2 Place 700 µL of CCM to cover the air chambers of Layer 2.

4.3 Place the alginate-chondrocyte (or bead) constructs on Layer 2 while carefully aligning the constructs with the air chambers.

4.4 Clamp the device with 3D printed clamps (**Figure 1 c**).

5 Actuation of the device

5.1 Connect the outlet of an air pump (see **Table of Materials**) with the inlet of a solenoid valve with a silicon tubing.

5.2 Connect the outlet of the solenoid valve with the inlet of the assembled device with a silicon tubing.

5.3 Connect the solenoid valve with a function generator.

5.4 Manipulate the solenoid valve with a square wave (e.g., 1 Hz) generated by the function generator.

5.5 Turn on the air pump to actuate the device pneumatically.

6 Imaging of chondrocytes in the device

NOTE: To obtain a good image quality, image chondrocytes (or fluorescent beads) in alginate gel through Glass plate 2 because expanded PDMS balloons and air chambers can distort optical images. If an inverted microscope is used for imaging, the device needs to be setup so that Glass plate 2 faces downward.

6.1 Prepare the device with chondrocytes (or fluorescent beads) as shown in previous sections.

6.2 Take *z*-stack images of chondrocytes (or fluorescent beads) with a confocal microscope before and under compression, respectively. Choose a *z*-step size based on the optical thickness of a used confocal imaging system.

6.3 The height of chondrocytes (or an alginate-bead construct) can be measured with automatic image processing method shown in previous literatures[26,35].

REPRESENTATIVE RESULTS

This article shows detailed steps of the microfluidic chondrocyte compression device fabrication (**Figure 2**). The device contains a 5 × 5 arrays of cylindrical alginate-chondrocyte constructs, and these constructs can be compressed with five different magnitudes of compression (**Figure 1, 3 and 4**). The height of the pneumatic microchannel is around 90 μm, and the PDMS balloon diameters are 1.2, 1.4, 1.6, 1.8 and 2.0 mm, respectively. The performance of the device was quantified with confocal microscopy with static compression conditions and image processing. Static compression was employed for the microscopic imaging because the *z*-stack imaging with the confocal microscopy takes a few minutes, so it is too slow for quantitative imaging during the dynamic compression.

Figure 3a shows the 5 × 5 arrays of alginate hydrogel columns (diameter: ~0.8 mm, height: ~1 mm) cast on Glass plate 2. These gel constructs were imaged by adding fluorescent beads in the gel. **Figure 3b** shows an example case that the gel column was compressed by 33.8% in height by the largest PDMS balloon. The resultant compressive strain of the gel constructs increased by approximately 5% per 0.2 mm increment in the PDMS balloon diameter as shown in Figure 3c.

Compressive deformation of chondrocytes was determined by imaging the cells in a 613 μm × 613 μm × 40–55 μm (*x* × *y* × *z*) volume near the gel construct center as shown in **Figure 4a**. **Figure 1d** shows an example image of a chondrocyte that was compressed by 16% by the largest PDMS balloon. **Figure 4b** shows the distribution of the measured cell compression strain values, and overall cells were compressed more by larger PDMS balloons. Therefore, the amount of alginate gel and chondrocyte compression were controlled by the diameter of PDMS balloons (**Figure 3 and Figure 4**) with a constant pressure of 14 kPa.

FIGURE AND TABLE LEGENDS

Figure 1. Microfluidic chondrocyte compression device. (**a**) Schematic of the assembled device. A 5 × 5 array of alginate–chondrocyte constructs are aligned on PDMS balloons with 5 different diameters (*D* = 1.2, 1.4, 1.6, 1.8 and 2.0 mm), where *D* is the diameter of PDMS balloon (or air chamber). (**b**) Schematic of the device operation. The device is actuated by pneumatic pressure which expands PDMS balloons. (**c**) Image of an actual device (coin diameter = 19 mm). (**d**) Vertical cross-sections of a chondrocyte before (left) and under (right) compression on the largest PDMS balloon (*D* = 2.0 mm) (cell compressive strain, ε_{cell} = |cell height change/initial cell height| × 100 = 16%). This figure is reproduced from [26].

Figure 2. Detailed steps of microfluidic chondrocyte compression device fabrication. (**a**) Photolithography for generating a SU-8 master mold and following soft lithography for creating PDMS layer with pneumatic microchannels (Layer 1). (**b**) Thin PDMS membrane (Layer 2) on a transparency film generated by spin coating. (**c**) Cylindrical alginate gel casting method on glass (Glass plate 2). (**d**) Assembly of the microfluidic chondrocyte compression device. This figure is reproduced from [26].

Figure 3. Measurement of alginate gel deformation under static compression. (**a**) 5 × 5 arrays of cylindrical alginate gel constructs (diameter: ~800 μm, height: ~1 mm). (**b**) Alginate gel compressed by the largest PDMS balloon (D = 2.0 mm). The compressive strain of an alginate gel is 33.8%. (**c**) Compressive strain of alginate gel (ε_{gel}) increases around 5% per 0.2 mm increment of PDMS balloon diameter (D). Error bar: standard deviation. Red line: linear fitting line This figure is reproduced from [26].

Figure 4. Measurement of chondrocyte deformation under static compression. (**a**) A z-stack image [613 μm × 613 μm × 40–55 μm ($x \times y \times z$)] was obtained in the middle of the gel construct, 300–400 μm from the gel bottom. (**b**) Different magnitudes of chondrocyte compressive strain (ε_{cell}) resulted as a function of the PDMS balloon diameter (D). ★: mean values. ◆: each data points. Top (or bottom) and middle lines of the box are the standard deviation and median value, respectively. This figure is reproduced from [26].

Figure S1. Microchannel photomask design for step 1.3.4 (unit = mm).

Figure S2. Aluminum mold design for step 3.2.3 (unit = mm).

Figure S3. Permanent deformation of the alginate gel (1.5%, w/v) under 1 h-long dynamic (1 Hz) and static compression. This figure is reproduced from [26].

DISCUSSION

To test the effects of compressive stress on growth plate chondrocytes, we developed the microfluidic chondrocyte compression device (**Figure 1**) to apply various levels of compressive stress to the chondrocytes in the alginate hydrogel scaffold for 3D culture in high throughput ways. To assist other researchers to adopt our device or to develop similar devices, we provided details of the device fabrication steps in this protocol article.

The crucial steps in this protocol are 1) fabricating PDMS layer with pneumatic microchannels (Layer 1) without any air bubbles since air bubbles in Layer 1 may damage pneumatic microchannels while the backing transparency film is peeled off, 2) maintaining a constant temperature (e.g., 80 °C) for curing PDMS balloons (Layer 2) because the elasticity of PDMS is known to depend on the curing temperature[36], 3) aligning the alginate gel constructs with the PDMS balloons, and 4) using fresh amino-silanized glass plate (Glass plate 2) for bonding the alginate gel columns on Glass plate 2 within two days after the salinization treatment.

The major limitation of this protocol is that it is relatively labor intensive to fabricate the device

because the process involves photolithography and multiple steps of soft lithography. Additionally, the performance of microfluidic cell compression devices fabricated based on our protocol needs to be evaluated whenever different types of hydrogels and cells are used. This is because any differences in the mechanical properties of hydrogels and cells will affect device performance.

Although our microfluidic cell compression device is for applying dynamic compression to chondrocytes, its performance was evaluated by imaging statically compressed alginate gels and cells. This is because it was hard to image gels and cells under dynamic compression with the conventional confocal microscopy. We compared static (14 kPa, 1 h) and dynamic compression (14 kPa, 1 Hz, 1 h) in terms of the permanent deformation of alginate gel and found that the permanent deformation of the gel under the dynamic compression was negligible compared to the static compression (see **Supplementary Figure S3**).

One advantage of our method is that it can be used for other cell types which need 3D culture environment. The resultant compression of the device can be modulated depending on applications by changing the diameter and thickness of the PDMS balloons and/or the pressure in the balloon. It is also possible to modify the elasticity of the PDMS balloon by adjusting the mixing ratio between the prepolymer and the curing agent. Cells in this device can be imaged in real time using light/fluorescence microscopy, and the device can be rapidly disassembled for cell harvest to enable downstream analysis. Another advantage is the ability to generate five distinct mechanical stress levels with five technical replicates per each stress level using a single device. Combining replication and a dose-response analysis ensures a high degree of rigor and reproducibility in the results.

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DISCLOSURES

The authors have nothing to disclose.

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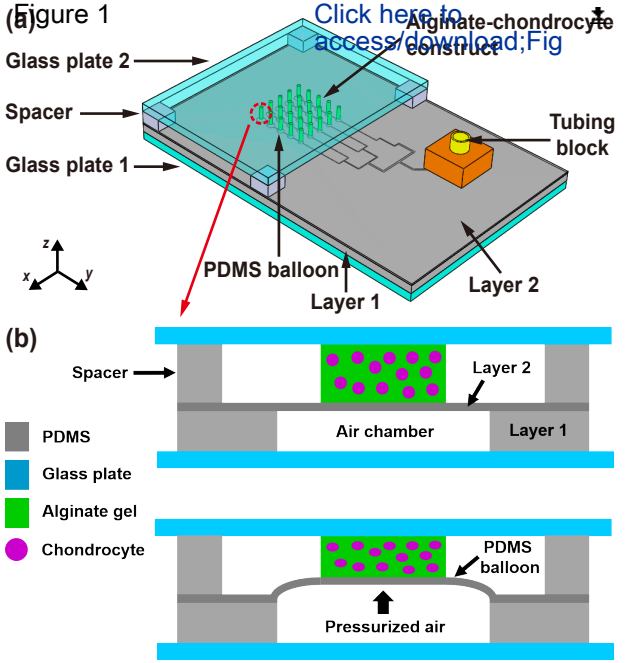
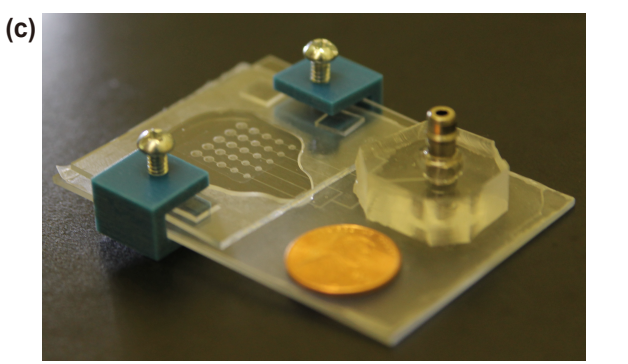
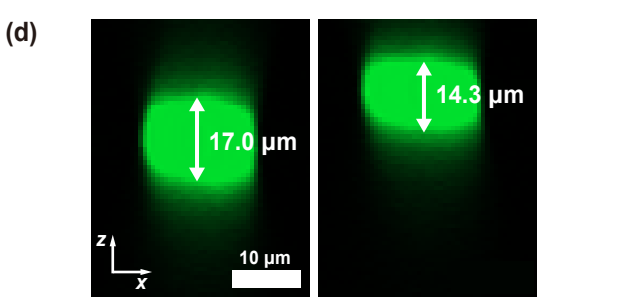
Figure 1







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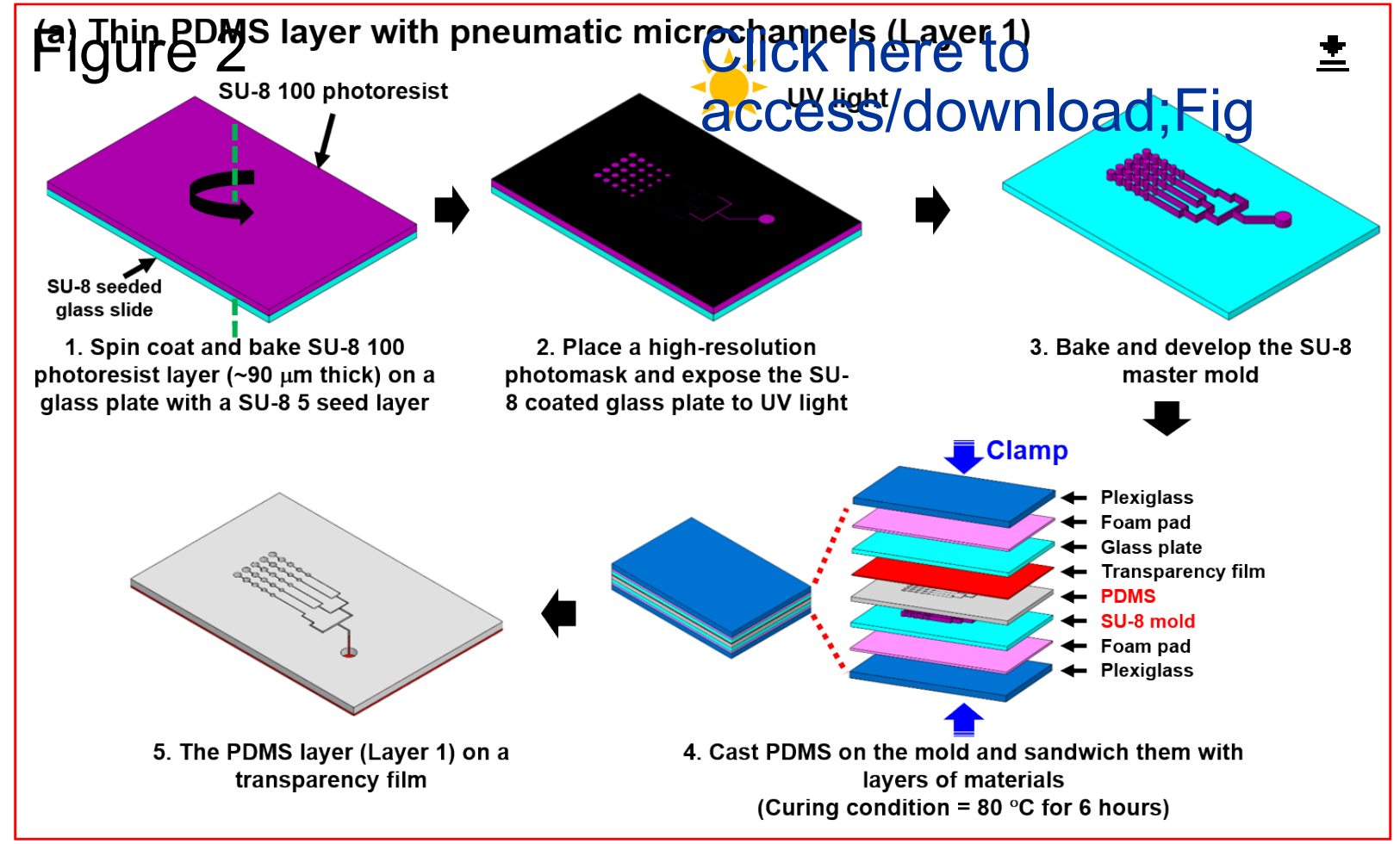
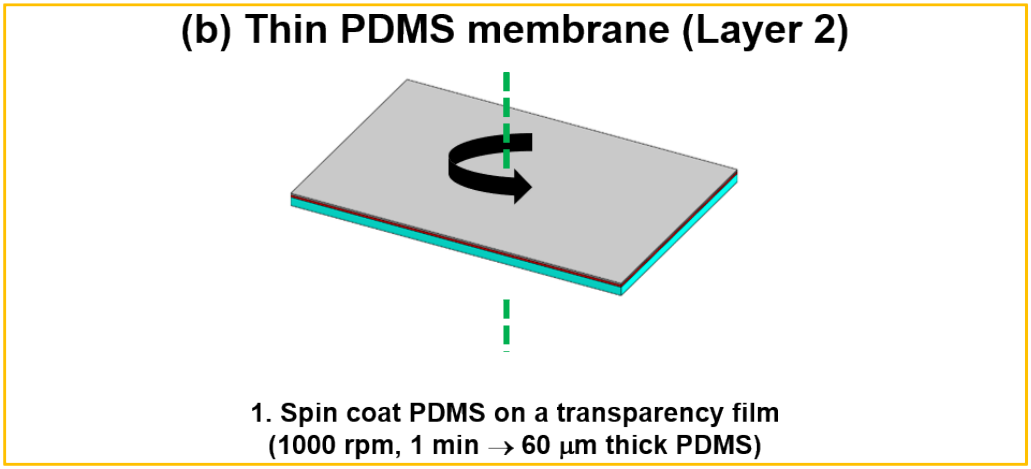
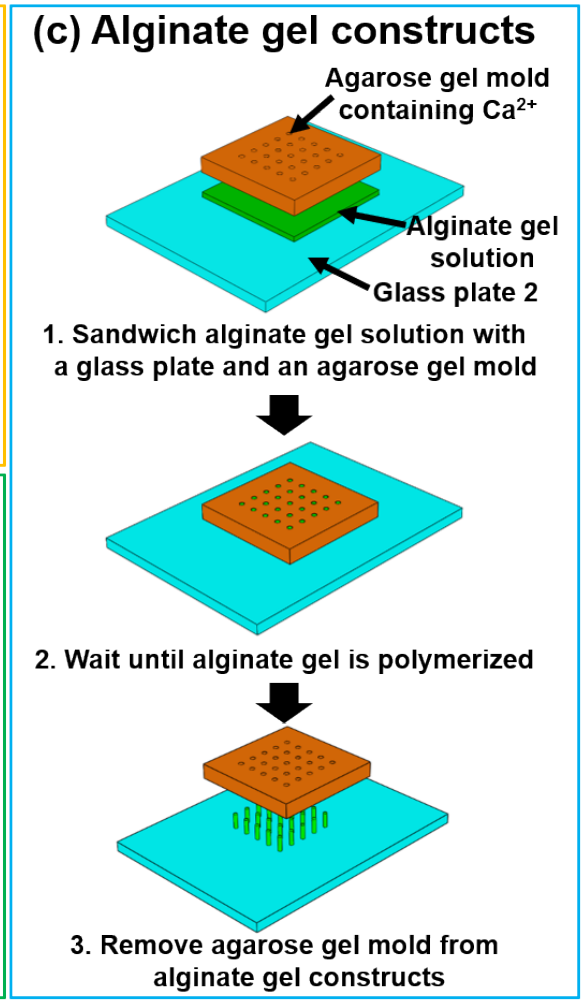






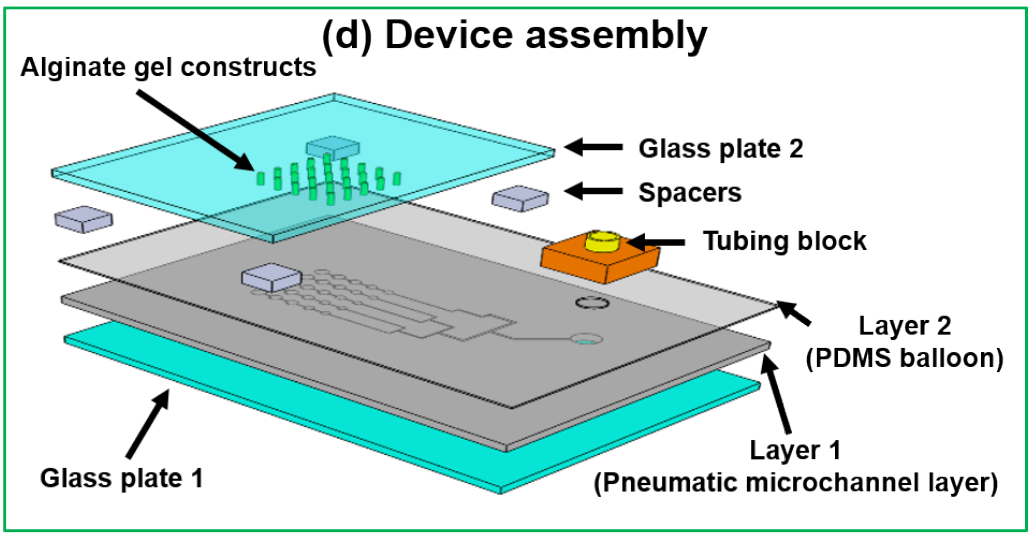
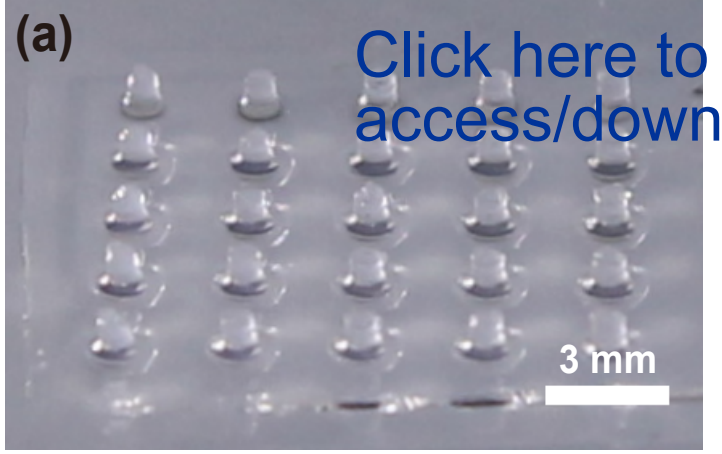
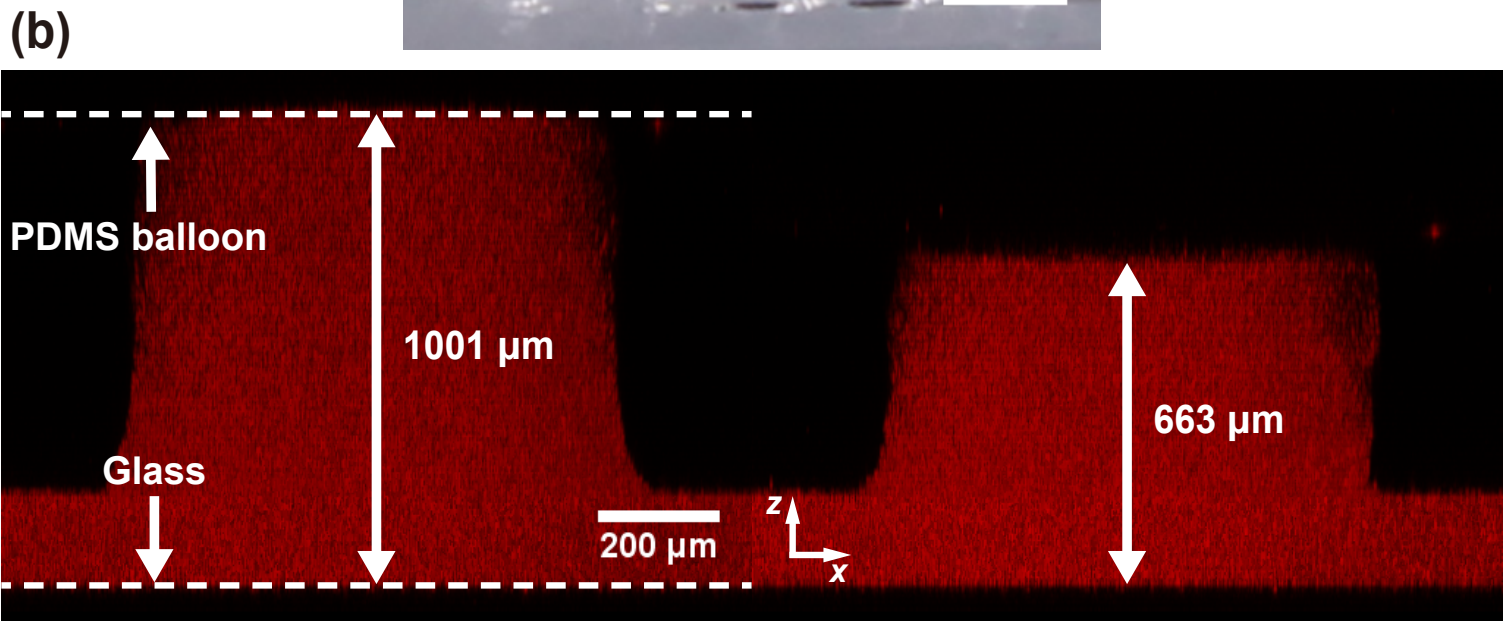


Figure 3





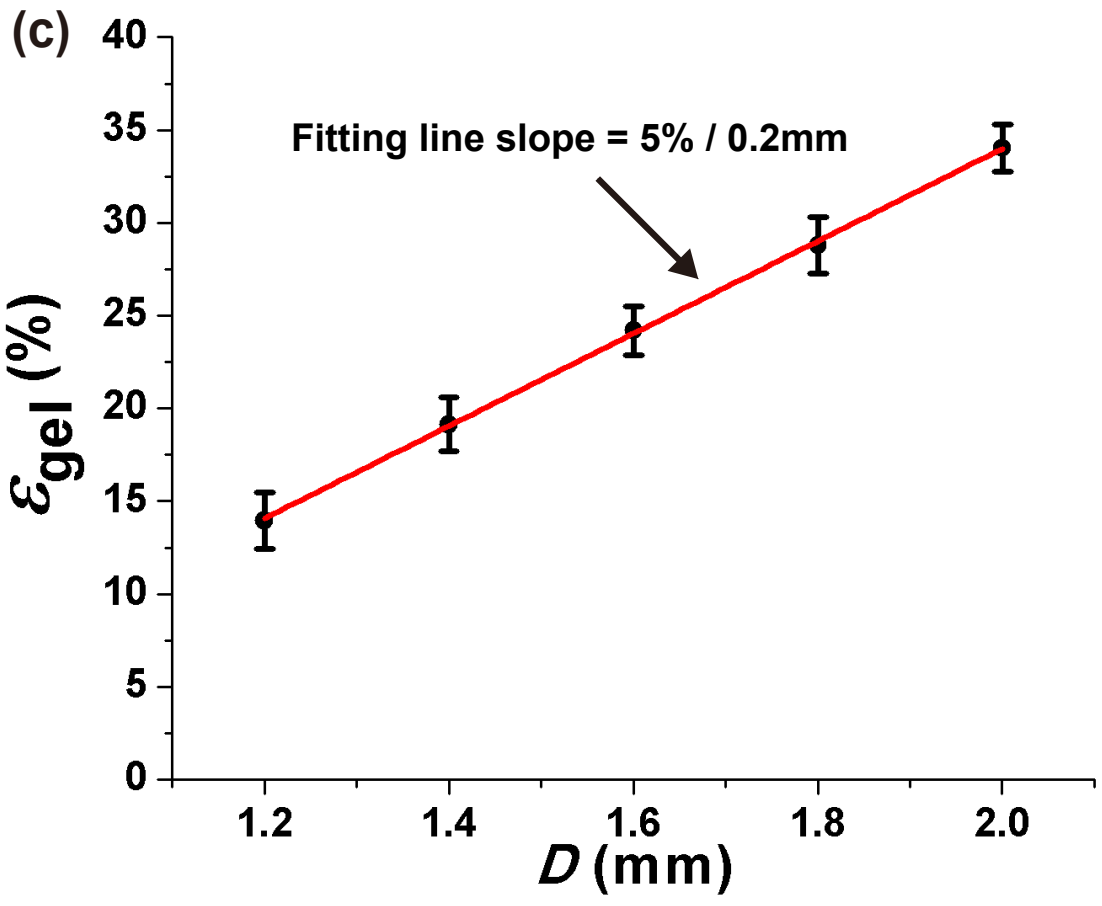
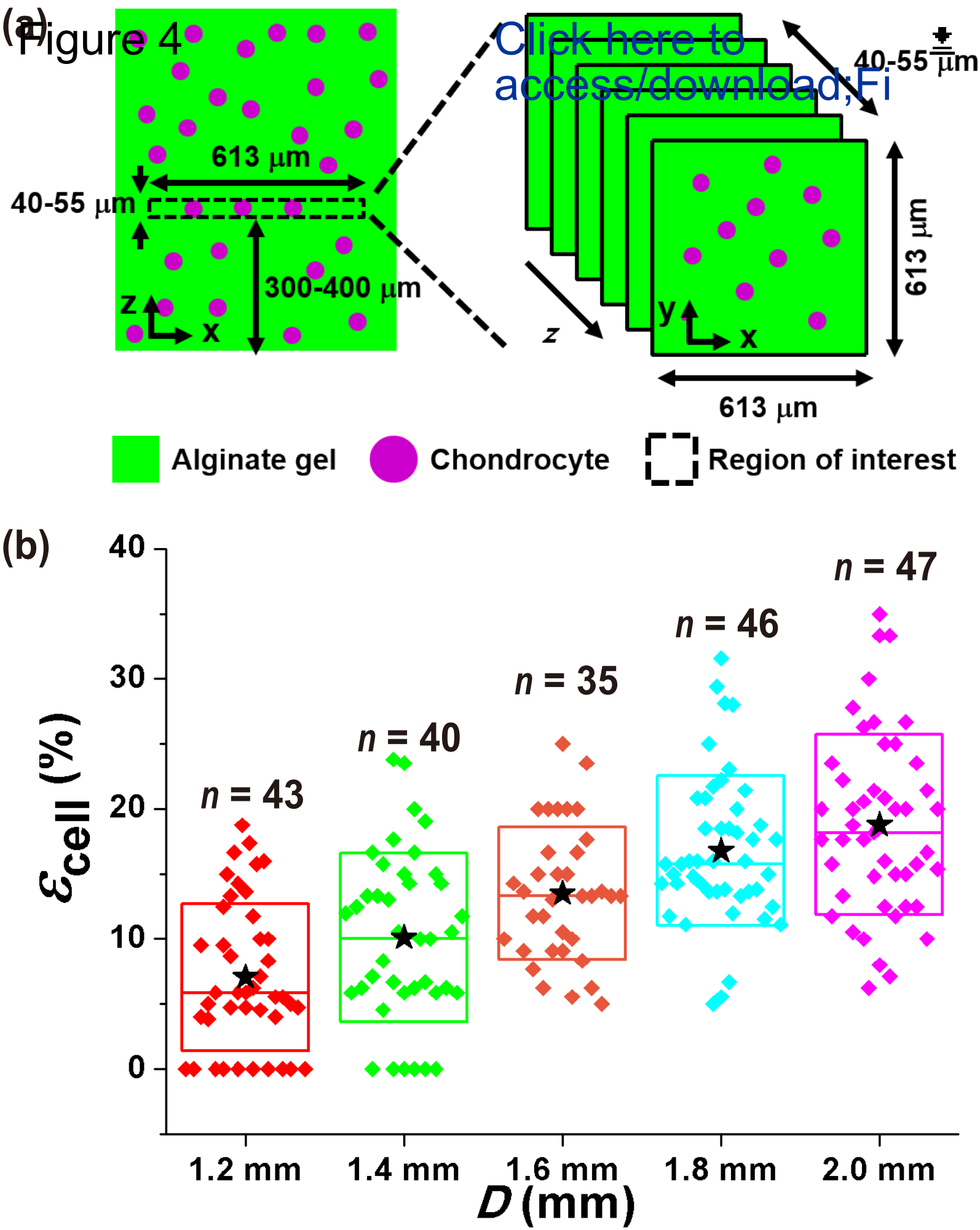


Figure 4



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Acrylic sheet	McMaster-Carr	8560K354
Air pump	Schwarzer Precision	SP 500 EC-LC4.5V DC
Alginate powder	FMC Corporation	Pronova UP MVG
Barb Straight Connectors (Metal tube)	Pneumadyne	EB40-250
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Dulbecco's Modified Eagle Medium (DMEM)	Gibco	11960-044
Dyed red aqueous fluorescent particles	Thermo Fisher Scientific	R0100
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Foam pad	GRAINGER	Item # 5GCE8
Function / Arbitrary Waveform Generator	Keysight Technologies	33210A
Hydrochloric acid	Fisher Chemical	A144-500
Hydrogen peroxide	Fisher BioReagents	BP2633500
Isopropyl alcohol	BDH1174-4LP	VWR
Microscope slides	Thermo Fisher Scientific	22-267-013
Plasma cleaner	Harrick Plasma	PDC-001
Polydimethylsiloxane (PDMS)	Dow Corning	184 SIL ELAST KIT 0.5KG
Power supply	Keysight Technologies	E3630A
SeaKem LE Agarose	Lonza	50004
Sodium hydroxide	Fisher Chemical	S318-1
Solenoid manifold	Pneumadyne	MSV10-1

Solenoid valve	Pneumadyne	S10MM-30-12-3
Spin coater	Laurell Technologies	WS-650Mz-23NPPB
SU8 Developer	MicroChem Corp.	Y020100 4000L1PE
SU8-100	MicroChem Corp.	Y131273 0500L1GL
SU8-5	MicroChem Corp.	Y131252 0500L1GL
Sulfo-NHS (N-hydroxysulfosuccinimide)	Thermo Fisher Scientific	24510
Sulfuric acid	EMD Millipore	MSX12445

Comments/Description

We used the model purchased in 2015. The internal design and performance of air pump (SP 500 EC-LC) changed in early 2016. Also, air pump performance has changed in the course of time. Thus, air pressure generated by an SP 500 EC-LC air pump should be calibrated before use.

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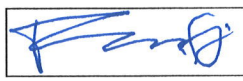
Signature:	
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We express our deep appreciation for the detailed comments and advice provided by the editor and reviewers, which have been very helpful in clarifying and improving parts of the manuscript. The manuscript has been modified in response to the remarks of both the editor and reviewers, and key revisions and new parts are marked in red in this rebuttal letter.

Response to the editorial comments

Comment 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: We double checked spelling and grammar in the manuscript.

Comment 2: Please expand the Introduction to include all of the following with citations:
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b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

Response: Following the editor's suggestion, the Introduction has been revised as follows:

"Micro-engineered platforms are valuable tools for studying the molecular, cellular, and tissue level biology because they enable dynamic control of both the physical and chemical microenvironments[1-8]. Thus, multiple hypotheses can be simultaneously tested in a tightly controlled manner. In the case of growth plate cartilage, there is increasing evidence of an important role of compressive stress in modulating bone growth through action on the growth plate cartilage[9-25]. However, the mechanism of action of compressive stress – in particular, how stress guides formation of chondrocyte columns in the growth plate – is poorly understood.

The goal of this protocol is to create a pneumatically actuating microfluidic chondrocyte compression device[26] to elucidate mechanisms of mechanobiology in growth plate chondrocytes (Figure 1 (a-c)). The device consists of two parts: the pneumatic actuation unit and the alginate gel construct. The microfluidic pneumatic actuation unit is fabricated using polydimethylsiloxane (PDMS) based on photo- and soft-lithography. This unit contains a 5 x 5 array of thin PDMS membrane balloons which can inflated differently based on their diameters. The alginate gel construct consists of the chondrocytes embedded in a 5 × 5 array of alginate gel cylinders, and the entire alginate-chondrocyte constructs are assembled with the actuation unit. The alginate gel constructs are compressed by the pneumatically inflated PDMS balloons (Fig. 1(b)). The microfluidic device can generate five different levels of compressive stress simultaneously in a single platform based on differences in the PDMS balloon diameter. Thus, a high-throughput test of chondrocyte mechanobiology under multiple compression conditions is possible.

The microfluidic device described in this protocol has many advantages over the conventional compression device such as external fixators[14,21,23] and macroscopic compression devices[16,19,27,28] for studying chondrocyte mechanobiology: 1) The microfluidic device is cost effective because it consumes smaller volume of samples than the macroscopic compression

device, 2) The microfluidic device is time effective because it can test multiple compression conditions simultaneously, 3) The microfluidic device can combine mechanical and chemical stimuli by forming a concentration gradient of chemicals based on the limited mixing in microchannels, and 4) Various microscopy techniques (time-lapse microscopy and fluorescence confocal microscopy) can be applied with the microfluidic device made of transparent PDMS.

We adopted and modified the method of Moraes *et al.*[7,29] to create different compressive stress levels in a single device to enable high-throughput mechanobiology studies of chondrocyte compression. Our approach is appropriate for cells (e.g., chondrocytes) which need three-dimensional (3D) culture environment and for biological assays after compressing cells. Although some microfluidic cell compression devices can compress cells cultured on two-dimensional (2D) substrates[30-32], they cannot be used for chondrocytes because 2D cultured chondrocytes dedifferentiate. There are microfluidic platforms for compressing 3D cultured cells in photopolymerized hydrogels[7,33], but they are limited in isolating cells after compression experiments because isolating cells from photopolymerized hydrogel is not easy. Additionally, the effects of ultraviolet (UV) exposure and photo crosslinking initiators on cells may need to be evaluated. In contrast, our method allows rapid isolation of cells after compression experiments for post biological assays because alginate hydrogels can be depolymerized quickly by calcium chelators. The detailed device fabrication and characterization methods are described in this protocol."

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For example: MicroChem Corp., Westborough, MA, WS-650MZ- Page 2 of 6 revised November 2017 89 23NPPB, Laurell Technologies Corporation, North Wales, PA, Mercury-Xenon Arc Lamp, 6295NS, 94 Newport, Irvine, CA, T2492-KG, 124 United Chemical Technologies, Bristol, PA), Sylgard 184, Dow Corning, Midland, MI, HP Transparencies for LaserJets, 133 C2934A, EB40-250, Pneumadyne, Plymouth, MN, SeaKem LE Agarose, Lonza, Basel, Switzerland, Sigma-Aldrich, St. Louis, MO, Invitrogen, 202 Waltham, MA, Pronova UP MVG, FMC Corporation, Philadelphia, PA, Thermo Fisher Scientific, 210 Waltham, MA, SP 500 EC-LC [2015 year model], Schwarzer Precision, Essen, 236 Germany, LSM 255 800, Carl Zeiss AG, Oberkochen, Germany, etc.

Response: Following the editor's suggestion, we removed all commercial language in our manuscript.

Comment 4: Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please leave a single line space between each step, substep and note in the protocol section.

Response: Following the editor's suggestion, we formatted the manuscript.

Comment 5: Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Response: Following the editor's suggestion, we formatted the manuscript.

Comment 6: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc

Response: Following the editor's suggestion, we have revised the manuscript and added the following safety procedures and use of hoods:

In page 1
"Note: Wear personal protective equipment (PPE) such as gloves and lab coat for every steps in this protocol."

In page 2
"Note: Perform step 1.1 - 1.3 in a fume hood."
"Note: Wear face shield, gloves, and lab coat for step 1.1."
"Note: Do not use Piranha solution and acetone in the same fume hood due to explosion hazard."

In page 3
"Note: Perform step 2.1.1 - 2.1.2 in a fume hood."

Comment 7: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step

Response: Based on the editor's suggestion, individual steps were checked to contain only 2-3 actions per step.

Comment 8: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Response: All unit of centrifuge speeds in our manuscript is centrifugal force. Revolutions per minutes (rpm) was used for spin coater speed only.
Comment 9: Please ensure you answer the "how" question, i.e., how is the step performed?

Response: Based on the editor's suggestion, we checked if the steps in our manuscript answer the "how" question.

Comment 10: Please ensure that the highlight is 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: Based on the editor's suggestion, we highlighted essential parts (less than 2.75 pages) of the protocol.

Comment 11: Please ensure that you discuss all figures in the Representative Results e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. However, for figures showing the experimental set-up, please reference them in the Protocol.

Response: Based on the editor's direction, we discussed all figures in the Representative Results section.

Comment 12: Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Response: The figures in the manuscript are reproduced from our previous article published in *Lab on a Chip* (Lee, D. Erickson, A. You, T., Dudley, A. T. & Ryu, S. Pneumatic microfluidic cell compression device for high-throughput study of chondrocyte mechanobiology. *Lab Chip.* 18 (14), 2077-2086, 2018). According to the policy of *Lab on a Chip*, the authors of the article do not need to obtain permission to reproduce figures if the author uses correct acknowledgement. The screen shots of the policy were attached on a separate word (.docx) document and uploaded to our Editorial Manager account.

Comment 13: As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Response: Following the editor's suggestion, we revised Discussion section as shown below:

"To test the effects of compressive stress on growth plate chondrocytes, we developed the microfluidic chondrocyte compression device (Figure 1) to apply various levels of compressive stress to the chondrocytes in the alginate hydrogel scaffold for 3D culture in high throughput ways. To assist other researchers to adopt our device or to develop similar devices, we provided details of the device fabrication steps in this protocol article.

The crucial steps in this protocol are 1) fabricating PDMS layer with pneumatic microchannels (Layer 1) without any air bubbles since air bubbles in Layer 1 may damage pneumatic microchannels while the backing transparency film is peeled off, 2) maintaining a constant temperature (e.g., 80°C) for curing PDMS balloons (Layer 2) because the elasticity of PDMS is known to depend on the curing temperature[36], 3) aligning the alginate gel constructs with the PDMS balloons, and 4) using fresh amino-silanized glass plate (Glass plate 2) for bonding the alginate gel columns on Glass plate 2 within two days after the salinization treatment.

The major limitation of this protocol is that it is relatively labor intensive to fabricate the device because the process involves photolithography and multiple steps of soft lithography. Additionally, the performance of microfluidic cell compression devices fabricated based on our protocol needs to be evaluated whenever different types of hydrogels and cells are used. This is because any differences in mechanical properties of hydrogels and cells will affect device performance.

Although our microfluidic cell compression device is for applying dynamic compression to chondrocytes, its performance was evaluated by imaging statically compressed alginate gels and cells. This is because it was hard to image gels and cells under dynamic compression with the conventional confocal microscopy. We compared static (14 kPa, 1 h) and dynamic compression (14 kPa, 1 Hz, 1 h) in terms of the permanent deformation of alginate gel, and found that the permanent deformation of the gel under the dynamic compression was negligible compared to the static compression (see Supplementary Figure S3).

One advantage of our method is that it can be used for other cell types which need 3D culture environment. The resultant compression of the device can be modulated depending on applications by changing the diameter and thickness of the PDMS balloons and/or the pressure in the balloon. It is also possible to modify the elasticity of the PDMS balloon by adjusting the mixing ratio between the prepolymer and the curing agent. Cells in this device can be imaged in real time using light/fluorescence microscopy, and the device can be rapidly disassembled for cell harvest to enable downstream analysis. Another advantage is the ability to generate five distinct mechanical stress levels with five technical replicates per each stress level using a single device. Combining replication and a "dose-response" analysis ensures a high degree of rigor and reproducibility in the results."

Comment 14: Please expand the journal title in the reference section.

Response: Based on the editor's direction, we replaced the abbreviation of journal title with full journal title in the reference section.

Response to Reviewer #1's comments

The manuscript by Lee et al. describes a microfluidic platform for compression of chondrocytes, that follows the Lab on a Chip article from the authors. There is significant interest in the mechanobiology community for microengineered tools for mechanically stimulating cells, so this is a timely contribution. While the topic is of interest, I find the introduction and discussion can be further developed. Even though this is aimed at a methodology-focused audience, the current presentation of the introduction and discuss do not meet the instructions provided by JoVE. Some clarifications are also needed in the protocol section.

Comment 1: Introduction. The introduction is rather brief and the authors lump a large number of references together without differentiating them. The authors mentioned this is modified from the work of Moraes et al., but without describing the actual device. Given the scope is to developed a microfluidic platform, I don't find enough introduction on what the device working principle is in the introduction. This would be true even for an expert in the field, let alone someone who might be new to the topic. It launches right away to fabrication with a series of steps that would make little sense if the reader does not have a big picture view of device. I suggest that the authors might want to move part of Figure 2 and use it as Figure 1 in the introduction. There should be a figure that describe the overall design of the device. The authors should consider describing the rationale behind the development/use of this method, and improve on the description of the context of the method with respect to other approaches. A quick google search on microfluidic compression shows two relevant papers (PMIDs: 30386779 and 21975691), but there may be more with similar working principle. There are also many constriction channel-based devices that many groups have worked on for compression, but the cells are traveling through the channels which may be less relevant here.

Response: Following the reviewer's advice, we revised Introduction section as shown below:

"Micro-engineered platforms are valuable tools for studying the molecular, cellular, and tissue level biology because they enable dynamic control of both the physical and chemical microenvironments[1-8]. Thus, multiple hypotheses can be simultaneously tested in a tightly controlled manner. In the case of growth plate cartilage, there is increasing evidence of an important role of compressive stress in modulating bone growth through action on the growth plate cartilage[9-25]. However, the mechanism of action of compressive stress – in particular, how stress guides formation of chondrocyte columns in the growth plate – is poorly understood.

The goal of this protocol is to create a pneumatically actuating microfluidic chondrocyte compression device[26] to elucidate mechanisms of mechanobiology in growth plate chondrocytes (Figure 1 (a-c)). The device consists of two parts: the pneumatic actuation unit and the alginate gel construct. The microfluidic pneumatic actuation unit is fabricated using polydimethylsiloxane (PDMS) based on photo- and soft-lithography. This unit contains a 5 x 5 array of thin PDMS membrane balloons which can inflated differently based on their diameters. The alginate gel construct consists of the chondrocytes embedded in a 5 × 5 array of alginate gel cylinders, and the entire alginate-chondrocyte constructs are assembled with the actuation unit. The alginate gel constructs are compressed by the pneumatically inflated PDMS balloons (Fig. 1(b)). The microfluidic device can generate five different levels of compressive stress simultaneously in a

single platform based on differences in the PDMS balloon diameter. Thus, a high-throughput test of chondrocyte mechanobiology under multiple compression conditions is possible.

The microfluidic device described in this protocol has many advantages over the conventional compression device such as external fixators[14,21,23] and macroscopic compression devices[16,19,27,28] for studying chondrocyte mechanobiology: 1) The microfluidic device is cost effective because it consumes smaller volume of samples than the macroscopic compression device, 2) The microfluidic device is time effective because it can test multiple compression conditions simultaneously, 3) The microfluidic device can combine mechanical and chemical stimuli by forming a concentration gradient of chemicals based on the limited mixing in microchannels, and 4) Various microscopy techniques (time-lapse microscopy and fluorescence confocal microscopy) can be applied with the microfluidic device made of transparent PDMS.
We adopted and modified the method of Moraes *et al.*[7,29] to create different compressive stress levels in a single device to enable high-throughput mechanobiology studies of chondrocyte compression. Our approach is appropriate for cells (e.g., chondrocytes) which need three-dimensional (3D) culture environment and for biological assays after compressing cells. Although some microfluidic cell compression devices can compress cells cultured on two-dimensional (2D) substrates[30-32], they cannot be used for chondrocytes because 2D cultured chondrocytes dedifferentiate. There are microfluidic platforms for compressing 3D cultured cells in photopolymerized hydrogels[7,33], but they are limited in isolating cells after compression experiments because isolating cells from photopolymerized hydrogel is not easy. Additionally, the effects of ultraviolet (UV) exposure and photo crosslinking initiators on cells may need to be evaluated. In contrast, our method allows rapid isolation of cells after compression experiments for post biological assays because alginate hydrogels can be depolymerized quickly by calcium chelators. The detailed device fabrication and characterization methods are described in this protocol."

Comment 2: Discussion. The discussion is also under developed. It should not be focused on discussing the experimental result, but rather describing what are the critical steps in the protocols. Are there steps that are particular tricky or require more attention? It should also be used to point out the significance of the method with respect to alternative methods (such as the two mentioned above). I like the high throughput nature and that it spans a range of strains. The authors say 'dynamic' compression several places (including the title). To me, this implies cyclic loading, but I did not see description of this. Putting (or statically) may cause confusion as well. Finally, the authors should point out any limitations to the approach.

Response: Based on the reviewer's comments, we revised Discussion section as shown below:

"To test the effects of compressive stress on growth plate chondrocytes, we developed the microfluidic chondrocyte compression device (Figure 1) to apply various levels of compressive stress to the chondrocytes in the alginate hydrogel scaffold for 3D culture in high throughput ways. To assist other researchers to adopt our device or to develop similar devices, we provided details of the device fabrication steps in this protocol article.

The crucial steps in this protocol are 1) fabricating PDMS layer with pneumatic microchannels (Layer 1) without any air bubbles since air bubbles in Layer 1 may damage pneumatic microchannels while the backing transparency film is peeled off, 2) maintaining a

constant temperature (e.g., 80°C) for curing PDMS balloons (Layer 2) because the elasticity of PDMS is known to depend on the curing temperature[36], 3) aligning the alginate gel constructs with the PDMS balloons, and 4) using fresh amino-silanized glass plate (Glass plate 2) for bonding the alginate gel columns on Glass plate 2 within two days after the salinization treatment.

The major limitation of this protocol is that it is relatively labor intensive to fabricate the device because the process involves photolithography and multiple steps of soft lithography. Additionally, the performance of microfluidic cell compression devices fabricated based on our protocol needs to be evaluated whenever different types of hydrogels and cells are used. This is because any differences in mechanical properties of hydrogels and cells will affect device performance.

Although our microfluidic cell compression device is for applying dynamic compression to chondrocytes, its performance was evaluated by imaging statically compressed alginate gels and cells. This is because it was hard to image gels and cells under dynamic compression with the conventional confocal microscopy. We compared static (14 kPa, 1 h) and dynamic compression (14 kPa, 1 Hz, 1 h) in terms of the permanent deformation of alginate gel, and found that the permanent deformation of the gel under the dynamic compression was negligible compared to the static compression (see Supplementary Figure S3).

One advantage of our method is that it can be used for other cell types which need 3D culture environment. The resultant compression of the device can be modulated depending on applications by changing the diameter and thickness of the PDMS balloons and/or the pressure in the balloon. It is also possible to modify the elasticity of the PDMS balloon by adjusting the mixing ratio between the prepolymer and the curing agent. Cells in this device can be imaged in real time using light/fluorescence microscopy, and the device can be rapidly disassembled for cell harvest to enable downstream analysis. Another advantage is the ability to generate five distinct mechanical stress levels with five technical replicates per each stress level using a single device. Combining replication and a "dose-response" analysis ensures a high degree of rigor and reproducibility in the results."

Comment 3: Minor Concerns. Protocol: The protocol is generally thorough, but I have a few suggestions and places for clarification.

Comment 3-1: 1. photomask design could be uploaded as supplemental file, if it is not already made available.

Response: We have included our photomask design as a supplementary figure 1 as shown below:

"1.3.4 Place a high-resolution microchannel photomask (25,400 dpi, see Supplementary Figure 1) …"

Comment 3-2: 1.1.1. Please include total volume used.

Response: Following the reviewer's suggestion, we include total volume of Piranha solution.
In page 2

"1.1.1 Make Piranha solution (60 ml) by mixing sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2) with 3:1 volume ratio."

Comment 3-3: 1.2.4. It might be helpful to include the power of the UV lamp and the distance it is placed by the sample

Response: Following the reviewer's suggestion, we included the UV light exposure system information. In page 2

"1.2.4 Expose the SU-8 5 coated glass plate to UV light (60 mW/cm^2, distance between UV lamp and photomask is 20 cm, total amount of UV light energy = 60 mJ/cm^2) for 1 second. The UV exposure time should be adjusted according to the power of a used UV light."

Comment 3-4: 2.1.9. Can the authors indicate the plasma etcher model used?

Response: Based on JoVE editor's directions, we moved all the product information to a separate material list file.

Comment 3-5: 3. It stated that the protocol for machining of the aluminum mold is not included. The authors should at least describe the geometry and the feature of the mold.

Response: Following the reviewer's suggestion, we included the design of the aluminium mold as a supplementary figure as shown below:

"3.2.3 Pour the boiled agarose gel solution onto an aluminum mold (see Supplementary Figure S2), and sandwich it with a glass plate."

Comment 3-6: 3.3.8. Can the authors describe the desirable cell density?

Response: We've already suggested a working cell number density value as shown in our response to the following comment.

Comment 3-7: 3.4.2. This step doesn't make sense to me. Do you mean 8 x10^6 particles/ml of chondrocytes?

Response: To prevent confusions, we revised step 3.4.2 as shown below:

"3.4.2 Add 8×10^6 chondrocytes into the 1 ml of alginate gel solution [or Add 3 µl of 1 µm-diameter fluorescent beads (542/612 nm) in 1 ml of alginate gel solution (0.3% (v/v))]."

Comment 3-8: 3.4.3. Where does this amino-silanized glass plate come from? I suppose this is from step 1.6. If so, please make it explicit.

Response: Following the reviewer's suggestion, we revised a sentence as shown below:

"3.4.3 Place 150 μl of the alginate-chondrocyte (or bead) solution on the amino-silanized glass plate fabricated in Step 3.1."

Comment 3-9: 5.4 and 5.5. These two steps are quite vague. Maybe the two steps can be combined somehow and example square wave parameters can be provided (this is where I thought the authors are doing cyclic loading, but I don't see it from the representative results).

Response: Following the reviewer's comments, we added detailed explanation why we used static compression to characterize the device. Based on editor's direction, we added example square wave parameter in step 5.4.

In page 5
"5.4 Manipulate the solenoid valve with a square wave (e.g., 1 Hz) generated by the function generator."

In page 6
"This article shows detailed steps of the microfluidic chondrocyte compression device fabrication (Figure 2). The device contains a 5 × 5 arrays of cylindrical alginate-chondrocyte constructs, and these constructs can be compressed with five different magnitudes of compression (Figure 1, 3 and 4). The height of the pneumatic microchannel is around 90 μm, and the PDMS balloon diameters are 1.2, 1.4, 1.6, 1.8 and 2.0 mm, respectively. The performance of the device was quantified with confocal microscopy with static compression conditions and image processing. Static compression was employed for the microscopic imaging because the *z*-stack imaging with the confocal microscopy takes a few minutes, so it is too slow for quantitative imaging during the dynamic compression."

Comment 3-10: Abstract: line 45: microscope instead of microscopy

Response: Following the reviewer's advice, we revised the sentence as shown below.

In the abstract
"……..2) It is easy to visualize cell morphology via a conventional light microscope,……"

Response to Reviewer #2's comments

In this paper, authors observed the change of cell with fluorescent according to the compressed pressures and pressure areas through a microfluidic chip. The chondrocytes in the chip was given the compressive stress to observe whether compressive stress could affect the cell growth. The protocol of this study is from Lab on a chip paper (Lee, D. Erickson, A. You, T., Dudley, AT & Ryu, S. Pneumatic microfluidic cell compression device for high-throughput study of chondrocyte mechanobiology. Lab Chip. 18 (14), 20177-2086, 2018).

Comment 1: There is no explanation of channel design(length/width/height) and PDMS balloon diameter to have 5 different compressive stresses. I recommend to add this information in protocol.

Response: Following the reviewer's advice, we added detailed channel dimensions in the supplementary information, and revised representative result section as shown below:

In page 6
"……The height of pneumatic channels is around 90 μm and the PDMS balloon diameters are 1.2, 1.4, 1.6, 1.8 and 2.0 mm, respectively……..."

Comment 2: At line 94&109 : please provide the total amount of the UV light energy for curing photoresist especially for SU-8 5 and SU-8 100

Response: Following the reviewer's suggestion, we revised manuscript as shown below:

In page 2
"1.2.4 Expose the SU-8 5 coated glass plate to UV light (60 mW/cm^2, distance between UV lamp and photomask is 20 cm, total amount of UV light energy = 60 mJ/cm^2) for 1 second. The UV exposure time should be adjusted according to the power of a used UV light."

"1.3.4 Place a microchannel photomask (25,400 dpi) on the SU-8 100 coated glass plate and expose the photomask covered glass plate to UV light for 4 seconds (total amount of UV light energy = 240 mJ/cm^2). The UV exposure time should be adjusted according to the power of a used UV light."

Comment 3: At line 140. The merchandise information of plasma cleaner is needed.

Response: Based on JoVE editor's directions, we moved all the product information to a separate material list file.

Comment 4: At line 142. In figure 1(a) there is not "Glass plate 1". Please correct either figure or sentence.

Response: Although Glass plate 1 is mentioned in step 2.1.10 (formerly line 142), it is not needed in the procedure shown in Figure 2(a) [figure 1(a) in previous manuscript].

Comment 5: At line 149. It seems that the punching hole step of layer 2 is missing.

Response: We agree with the reviewer's point. We added a step as shown below:

In page 4
"2.3.5. Punch a hole on Layer 2 for the inlet."

Comment 6: At line 182. The microwave oven power & time OR the hot plate temperature & time are needed.

Response: The power of our microwave is 1200 W, and we heated agarose gel powder in diH_2O for 35 seconds. However, we did not provide the specific method for making agarose gel solution because the detailed method is already provided by each agarose gel powder manufacturer. Also, depending on the specific agarose gel products, the volume of agarose gel solution, and dimension of glass ware (in the case of hot plate), the heating time varies. Thus, heating time for melting agarose powder in diH_2O needs to be determined by each lab empirically.

Comment 7: The values of PDMS balloon diameter are needed.

Response: Following the reviewer's advice, we revised representative results section as shown below:

In page 6
"… the PDMS balloon diameters are 1.2, 1.4, 1.6, 1.8 and 2.0 mm, respectively…"

Comment 8: The picture resolution should be improved.

Response: Each figure as a vector file will be uploaded on JoVE to ensure high resolution images.

Response to Reviewer #3's comments

In this manuscript, the authors used a microfluidic platform to apply dynamic mechanical compressions with five different magnitudes for stimulating chondrocytes. Specifically, they well described the detailed methods of the fabrication and characterization for the presented device. I recommend this manuscript to be published in the journal after the following revision.

Comment 1: As the authors mentioned in the paper, the device was fabricated and modified from the one of Moraes et al. and the authors published the similar work recently at the journal of Lab Chip in 2018. The authors are recommended to clearly highlight the novelty of this study in the manuscript.

Response: We agree with the reviewer's point. We revised introduction part of the manuscript to highlight the novelty of our manuscript as shown below:

In page 1
"Micro-engineered platforms are valuable tools for studying the molecular, cellular, and tissue level biology because they enable dynamic control of both the physical and chemical microenvironments[1-8]. Thus, multiple hypotheses can be simultaneously tested in a tightly controlled manner. In the case of growth plate cartilage, there is increasing evidence of an important role of compressive stress in modulating bone growth through action on the growth plate cartilage[9-25]. However, the mechanism of action of compressive stress – in particular, how stress guides formation of chondrocyte columns in the growth plate – is poorly understood.

The goal of this protocol is to create a pneumatically actuating microfluidic chondrocyte compression device[26] to elucidate mechanisms of mechanobiology in growth plate chondrocytes (Figure 1 (a-c)). The device consists of two parts: the pneumatic actuation unit and the alginate gel construct. The microfluidic pneumatic actuation unit is fabricated using polydimethylsiloxane (PDMS) based on photo- and soft-lithography. This unit contains a 5 x 5 array of thin PDMS membrane balloons which can inflated differently based on their diameters. The alginate gel construct consists of the chondrocytes embedded in a 5 × 5 array of alginate gel cylinders, and the entire alginate-chondrocyte constructs are assembled with the actuation unit. The alginate gel constructs are compressed by the pneumatically inflated PDMS balloons (Fig. 1(b)). The microfluidic device can generate five different levels of compressive stress simultaneously in a single platform based on differences in the PDMS balloon diameter. Thus, a high-throughput test of chondrocyte mechanobiology under multiple compression conditions is possible.

The microfluidic device described in this protocol has many advantages over the conventional compression device such as external fixators[14,21,23] and macroscopic compression devices[16,19,27,28] for studying chondrocyte mechanobiology: 1) The microfluidic device is cost effective because it consumes smaller volume of samples than the macroscopic compression device, 2) The microfluidic device is time effective because it can test multiple compression conditions simultaneously, 3) The microfluidic device can combine mechanical and chemical stimuli by forming a concentration gradient of chemicals based on the limited mixing in microchannels, and 4) Various microscopy techniques (time-lapse microscopy and fluorescence confocal microscopy) can be applied with the microfluidic device made of transparent PDMS.
We adopted and modified the method of Moraes *et al.*[7,29] to create different compressive stress levels in a single device to enable high-throughput mechanobiology studies of chondrocyte

compression. Our approach is appropriate for cells (e.g., chondrocytes) which need three-dimensional (3D) culture environment and for biological assays after compressing cells. Although some microfluidic cell compression devices can compress cells cultured on two-dimensional (2D) substrates[30-32], they cannot be used for chondrocytes because 2D cultured chondrocytes dedifferentiate. There are microfluidic platforms for compressing 3D cultured cells in photopolymerized hydrogels[7,33], but they are limited in isolating cells after compression experiments because isolating cells from photopolymerized hydrogel is not easy. Additionally, the effects of ultraviolet (UV) exposure and photo crosslinking initiators on cells may need to be evaluated. In contrast, our method allows rapid isolation of cells after compression experiments for post biological assays because alginate hydrogels can be depolymerized quickly by calcium chelators. The detailed device fabrication and characterization methods are described in this protocol."

Comment 2: Alginate hydrogels crosslinked with Ca ions are known to have viscoelastic properties. However, to be used in the device under dynamic compressions, gels should be more elastic so that the alginate-based constructs encapsulating cells may need to be examined whether these gels could be fully recovered under applied strains. The authors are recommended to provide any evidence to show the gels used in the study could be fully recovered under cyclic compression or describe any insight regarding this point in the manuscript.

Response: Following the reviewer's comments, we included the following graph, which is from our previous article published in *Lab on a Chip* (Lee, D. Erickson, A. You, T., Dudley, A. T. & Ryu, S. Pneumatic microfluidic cell compression device for high-throughput study of chondrocyte mechanobiology. *Lab Chip.* 18 (14), 2077-2086, 2018), as a supplementary figure. As shown in the figure, the cyclic compression (1 Hz, 1 hour) produced 0.5-6% permanent deformation of alginate gel while static compression (1 hour) resulted in 9-30% permanent deformation. Thus, the permanent alginate gel deformation seems negligible under dynamic compression.

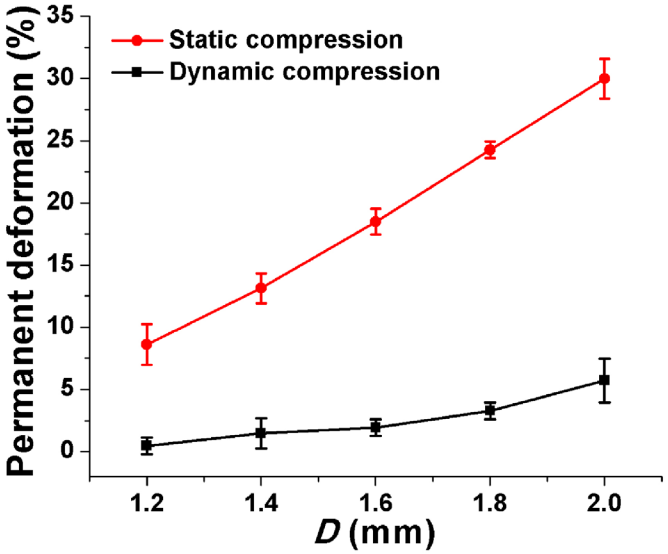


Figure S3. Permanent deformation of the alginate gel (1.5%, w/v) under 1 hour-long dynamic (1 Hz) and static compression. Reproduced from [26] with permission.

Supplementary Figures for "A microfluidic platform for stimulating chondrocytes with dynamic compression"

Figure S1

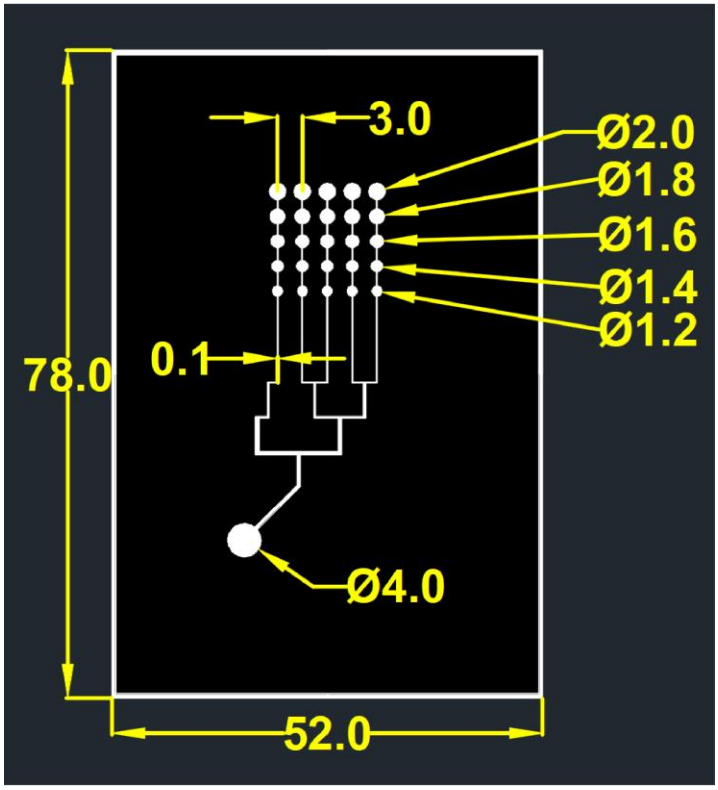


Figure S2

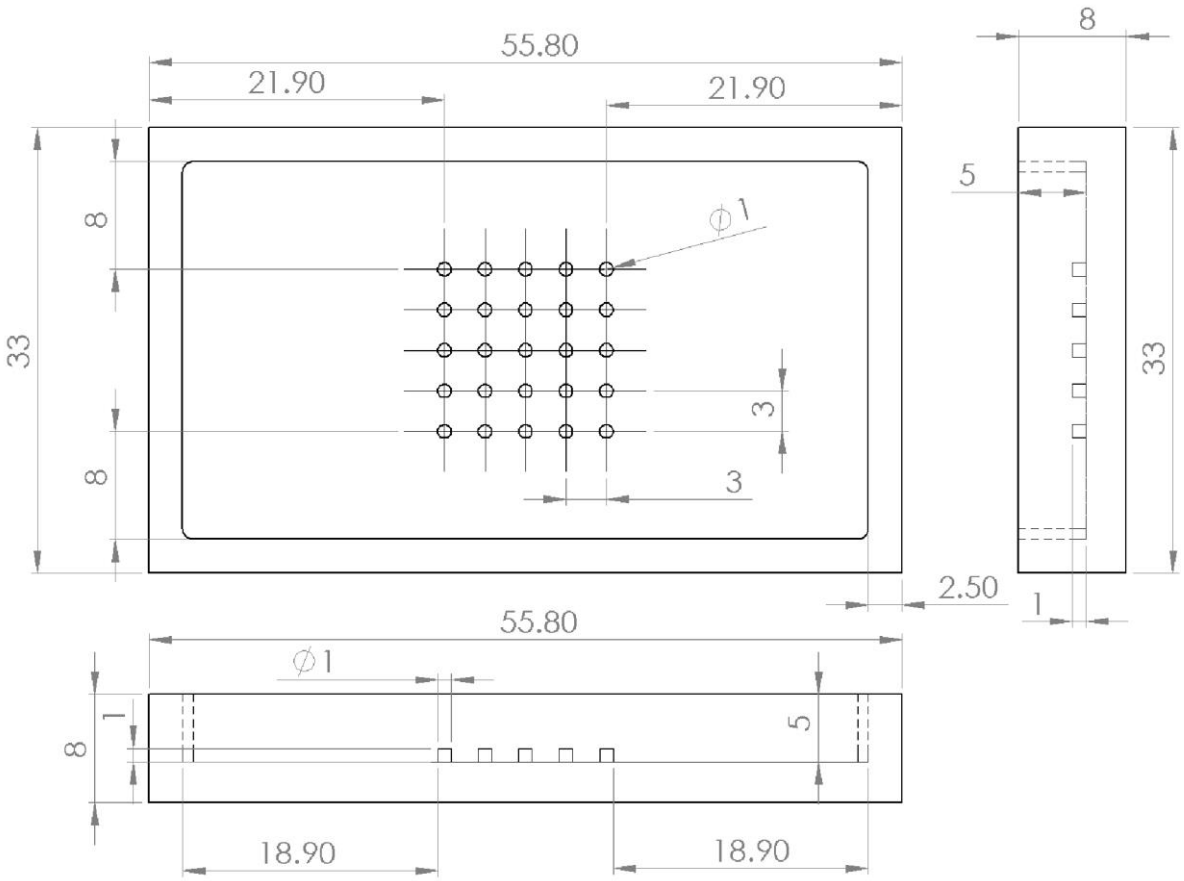
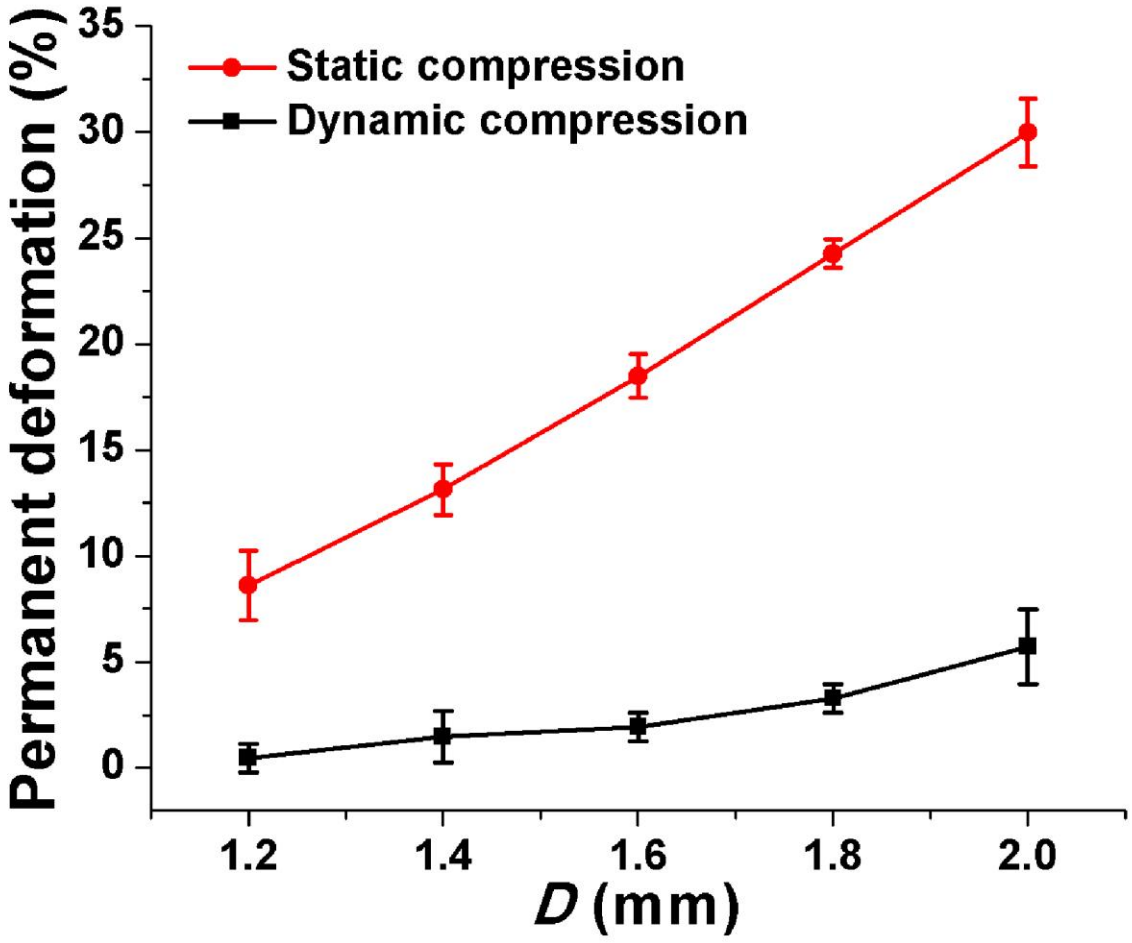


Figure S3


Permanent deformation (%)
35
30
25
20
15
10
5
0
Static compression
Dynamic compression
1.2
1.4
1.6
1.8
2.0
D (mm)

Pneumatic microfluidic cell compression device for high-throughput study of chondrocyte mechanobiology

D. Lee, A. Erickson, T. You, A. T. Dudley and S. Ryu, *Lab Chip*, 2018, **18**, 2077
DOI: 10.1039/C8LC00320C

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