Journal Pre-proof

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PII: S0925-4005(20)30265-3
DOI: https://doi.org/10.1016/j.snb.2020.127917
Reference: SNB 127917
To appear in: Sensors and Actuators: B. Chemical

Received Date: 24 December 2019
Revised Date: 20 February 2020
Accepted Date: 23 February 2020

Please cite this article as: Paggi CA, Venzac B, Karperien M, Leijten J, Le Gac S, Monolithic microfluidic platform for exerting gradients of compression on cell-laden hydrogels, and application to a model of the articular cartilage, Sensors and Actuators: B. Chemical (2020), doi:https://doi.org/10.1016/j.snb.2020.127917

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Monolithic microfluidic platform for exerting gradients of compression on cell-laden hydrogels, and application to a model of the articular cartilage.

Carlo Alberto Paggi, a,b,† Bastien Venzac, a,f,⁎ Marcel Karperien, b Jeroen Leijten, b and Séverine Le Gac a,⁎

†These authors contributed equally to this work

a Applied Microfluidics for BioEngineering Research, MESA+ Institute for Nanotechnology and TechMed Center, University of Twente, The Netherlands
b Developmental BioEngineering, TechMed Center, University of Twente, The Netherlands

⁎Corresponding authors: Séverine Le Gac & Bastien Venzac; s.legac@utwente.nl, b.venzac@utwente.nl

Highlights
- Monolithic device with a vertical membrane for mechanical stimulation of tissues.
- Actuation unit with 3 individual chambers to apply negative and/or positive pressure.
- Real-time tissue imaging at the single cell level: monitoring cell deformation.
- Application of healthy and hyper-physiological compressions to a cartilage model.
- Possible generation of combinations of compression and bulk shear.
Abstract

Movement is essential to our quality of life, and regulates cell behavior via mechanical stimulation. Here, we report a monolithic microfluidic platform, in which engineered tissues composed of cells in a hydrogel are exposed to gradients of mechanical compression. Mechanical stimulation is applied through the deflection of a thin polydimethylsiloxane (PDMS) vertical membrane. The device design and all actuation parameters were optimized in this work to produce physiologically relevant compression on a cartilage model (strain of 5-12%), as well as gradients of compression ranging from healthy to hyper-physiological conditions in the same device, as evidenced by the measured gradients in cell deformation. While this work focuses on mechanical compression of engineered tissues, we also demonstrated that our platform allowed creating more sophisticated multi-modal stimulation patterns. As the membrane is actuated by three independently addressed yet connected pressurized chambers, a variety of programmable deflection patterns and various cell stimulation modalities can easily be created by tuning the pressure applied in the different chambers (positive vs. negative, and amplitude). Advantageously, the fabrication of this monolithic platform is straightforward, with a single-step process. Moreover, the vertical membrane configuration allows for real-time imaging of cells encapsulated in the hydrogel matrix. The herein reported platform is highly versatile and of great interest to model other types of tissues, which also experience complex mechanical actuation patterns in vivo.

Keywords

Microfluidics; Mechanical actuation; Polydimethylsiloxane; Cartilage; Cell-laden hydrogel
1. Introduction

The microenvironment all cells experience within the body is highly dynamic. In particular, mechanical cues are essential, since they regulate a variety of biological functions and cell processes in a spatiotemporal manner, as recently reviewed [1, 2]; e.g., activation of mechanoreceptors in the cell membrane, rearrangement of the cytoskeleton, gene expression profiles, expression of cell-cell and cell-extracellular matrix (ECM) contact proteins [3], cell fate (proliferation, differentiation, phenotypic change, and apoptosis), and cell shape [4]. At the tissue and body levels, these mechanical cues have essential roles in, e.g., embryogenesis [5], tissue morphogenesis, angiogenesis [6-8], tumor progression [9], and reproductive biology [10].

Mechanical cues, which can be exerted in a continuous, temporary, cyclic, or pulsatile manner in the body, can be classified as, e.g., surface strain, fluid-based shear, substrate topography, matrix stiffness, stretching, bulk shear stress and compression. For instance, the epithelium and endothelium are exposed to fluid flows, which enhances their barrier function and induces changes in the cell configuration and cytoskeleton [11]. Epithelial cells undergo periodic stretching, for instance due to breathing in lung and the process of digestion in the gut, [12], which significantly influences effects of drugs and is primordial for the formation of a columnar epithelium. Compressive forces in the form of mechanical loads are experienced by bones and joints; changes in the load distribution in these tissues can impact their physiological response and possibly lead to impairment and/or fracture [13]. Furthermore, cartilage in the knee joint slowly degrades during long immobilization periods, due to the absence of mechanical cues, which regulate the ECM formation [14].
Organ-on-a-chip technology [15] has become a game-changer in the field of biomechanics: it provides exquisite control on a cell microenvironment and allows studying in situ the impact of mechanical stimulations on the cell and organ function, as reviewed by the Kamm group [16]. Using a microfluidic format, the influence of fluid-induced shear stress has been examined on both the endothelium and epithelium, grown as monolayers on planar substrates [17, 18], porous membranes [19], hydrogels [20] or in tubular geometries [21]. Exposure to physiological flow levels led to endothelial cell alignment [22], cytoskeleton rearrangement, and epithelial cell polarization and differentiation [23]. Stretching can be implemented by deforming elastomeric membranes prepared from polydimethylsiloxane (PDMS) through application of positive or negative pressures, to expose cells on these membranes to well-defined surface strains [24, 25]. Noteworthy, such strain and fluid flow mechanical cues have been combined, to create more biomimetic environments, and to study the synergistic effect of various stimuli [22, 26-28]. Microfluidic platforms also lend themselves well to 3D cell cultures in hydrogel matrices [29]. By taking advantage of the deformability of these soft matrices, compressive forces have been exerted on 3D cell cultures using an elastomeric membrane: e.g., to expose cartilage cells to compressive mechanical cues [30, 31], fibroblasts to a gradient of compressive forces [32], and mesenchymal stem cells to cyclic compression [33]. However, in these previous reports, horizontal membranes were employed, which presents two main limitations. First, the device fabrication can become tedious when multiple layers must be aligned and assembled together. Secondly, imaging of both the membrane and cells, notably to examine their response to the mechanical stimulation, implies using confocal microscopy. In short, real-time imaging of the engineered tissues is limited, or even precluded.
In this paper, we present a monolithic PDMS-based platform for exerting mechanical stimulation on a 3D engineered tissue using a thin vertical membrane located next to the cell culture chamber, a configuration which was inspired by a module developed for microbial pressurization [34]. We first thoroughly examined the influence of various parameters on the membrane deflection level. Next, we mapped the deformation generated in an agarose matrix, upon application of compressive forces of different amplitudes. Following this, agarose supplemented with chondrocytes was exposed to physiological gradients of strains, and cell deformation and viability were characterized using fluorescence microscopy, as a function of their position in the hydrogel matrix. Finally, we explored the applicability of our platform to generate more sophisticated mechanical stimulation patterns allying compression and bulk shear forces, as found in articular cartilage during movement, through the sequential pressurization of the three actuation chambers. The herein reported platform opens new avenues for replicating complex physiological mechanical stimulation patterns on engineered tissues in a microfluidic format.
2. Experimental

2.1. Platform design

The device incorporates a rectangular culture chamber (1260-µm length and 4000-µm width), separated from a perfusion channel (4400-µm length and 500-µm width) by an array of pillars (100 µm x 100 µm cross-section; inter-pillar distance 100 µm) (Fig. 1). Three actuation chambers are placed on the other side of the culture chamber, a thin vertical membrane (50 or 100-µm thick) physically separating the culture from the actuation chambers. Each actuation chamber has one inlet (1-mm diameter), and a 50-µm gap along the membrane links the 3 individual actuation chambers. All microfluidic structures are 200 or 220 µm in height.

2.2. Fabrication

Designs were drawn with Clewin5 software (WieWeb software, The Netherlands). Microfluidic devices were produced from PDMS using soft-lithography [35] on a SU-8 mould. SU-8 100 photoresist (MicroChem, Westborough, MA, USA) was spin-coated on a <100> silicon wafer (Okmetic, Finland) to yield a 200 or 220-µm thick layer, exposed to UV light through a chromium-coated quartz mask, baked and developed according to the manufacturer’s specifications. The resulting mould was treated in the vapour phase with trichloro(1H,1H,2H,2H-perfluorooctyl)silane (PFOTS, Sigma-Aldrich, Zwijndrecht, The Netherlands) during 20 min after plasma activation. A mixture of PDMS pre-polymer and curing agent (Sylgard 184, Dow Corning, USA) was prepared with a weight ratio of 20:1, thoroughly degassed, poured on the mould and once more degassed, before being placed in an oven for curing at 60°C for 24 h. Thereafter, inlets and outlets were created using 1-mm and 2-mm diameter biopsy punchers. The PDMS was subsequently cleaned with 70% ethanol and dried using pressurised air.
Two types of lids were considered: microscopy glass slides (1-mm thick) with or without a ca. 4-mm thick PDMS layer. To prepare the latter, uncured 20:1 weight ratio PDMS mixture was poured in a Petri dish, degassed, and cured as described above. This PDMS lid substrate was plasma-bonded (Cute, Femto Science, South Korea) to a glass slide. Both the lid (PDMS-coated or non-coated glass) and the microfluidic structures were activated using plasma treatment, before assembly. The resulting microfluidic devices were placed in an oven at 60°C for 24 h before use.

2.3. Microfluidic set-up

For compressive stimulation, the three actuation chambers were connected to a positive pressure controller (MFCS-EZ, pressure output from 0 to 2000 mbar, Fluigent, France). For multi-modal actuation, the actuation chambers were connected to both the positive pressure controller and a negative pressure source (-350 mbar), via 2-way valves (2-switch, Fluigent) allowing rapid pressure switching for each actuation chamber using an automated script (Fluigent). The microfluidic platform was placed on the stage of an inverted microscope (IX51, Olympus, Japan) equipped with a camera (ORCA-flash 4.0 LT, Hamamatsu Photonics, Japan) and directly connected to a computer. A schematic representation of the entire set-up is provided in Supplementary information S1.

2.4. Device characterization

The membrane deflection was characterized for homogeneous compression (all three actuation chambers exposed to the same positive pressure). In each experiment, the membrane deflection was measured at four different locations along the device with ImageJ software (NIH, Bethesda, Maryland, USA), and at least three devices were characterized separately. Values obtained from each device were averaged, and a standard deviation was calculated. All
characterization steps (membrane deflection, hydrogel deformation and cell behavior) were performed in the center of the culture chamber.

To quantify the hydrogel deformation upon deflection of the membrane, agarose 2% w/w was supplemented with polystyrene microbeads (15-µm diameter, amino-coated, Kisker, Germany) at a final concentration of 61 µg/ml. Specifically, a 2% w/w agarose solution was prepared by dissolving 0.5 g agarose powder (UltraPure agarose, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) in 25 ml PBS after heating at 60°C. For each experiment, 30 µl of this solution was injected in the culture chamber. After 1 min gelation, culture medium at room temperature was added to the perfusion channel. It should be noted that the agarose hydrogel was not covalently attached to the PDMS. For homogeneous compression, the bead displacement normal to the membrane was measured as a function of their distance from the membrane. For the multi-modal stimulation, heat maps were generated for both the normal (compressive) and bulk shear strains; for this, the considered part of the culture chamber was divided in zones of 122 µm x 122 µm, and an average microbead displacement determined in each zone. All details regarding data analysis and normal and shear strain calculations are provided in the Supplementary information (S2 and S3).

2.5. Chondrocyte isolation and culture

The collection and use of human cartilage from patients undergoing total knee replacement was approved by a medical ethical committee (METC) of Zorggroep Twente, The Netherlands. Written informed consent was received from all patients. Chondrocytes were isolated from macroscopically and histologically healthy looking cartilage and expanded in T175 flasks (Cellstar®, Greiner bio-one, Germany) containing 20 ml of chondrocyte proliferation medium (DMEM with 10% FBS (Foetal Bovine Serum), 0.2 mM ascorbic acid
2-phosphate, 0.1 mM non-essential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin, 4 mM proline) [36, 37]. Medium was refreshed every three days. When chondrocytes reached 80% confluence, they were passaged using a trypsin-EDTA (1X) solution (0.25%/0.1 mM, Invitrogen) in PBS in a new flask at a concentration of 500,000 cells per 175 cm². Here, experiments were conducted with chondrocytes between passages 1 and 4.

2.6. Cell culture in the device and mechanical stimulation

Chondrocytes were cultured in agarose in the device. To that end, 1 ml of the 4% w/w agarose solution at 50°C was mixed with 1 ml of proliferation media at room temperature containing 4x10⁶ chondrocytes. Noteworthy, the final temperature of this mixture is around 37-40°C, which is not lethal for the cells. As before, 30 µl of this mixture was injected in the device, and after about 1 minute, medium was injected in the perfusion channel. Uniform compression (800 and 1000 mbar, frequency of 1 Hz) was applied on the PDMS membrane, just after introduction of the cell-laden agarose. Images of 24 individual cells (at different heights and positions in the chamber) were acquired with a 40x objective, during the 15 first minutes of stimulation and their projected surface area measured at rest and under compression, and compared to examine their response to the mechanical stimulation, as detailed in Supplementary information S4.

2.7. Cell viability

Chondrocytes were cultured in agarose in the platform for up to three days, with or without mechanical stimulation (800 or 1000 mbar for 1 h per day, 1 Hz frequency). Medium was refreshed every day by pipetting manually twice 70 µl of medium in the perfusion channel. After three days, cell viability was assessed using NucBlue® Live and NucGreen® Dead fluorescent probes (ReadyProbes™ Cell Viability Imaging Kit, Blue/Green, Life technology,
Thermo Fisher Scientific). Briefly, the perfusion channel was washed with PBS, and 30 µl of fresh culture medium supplemented with the two probes (1 ml of medium with two drops of each component) was injected twice in the system. After the second injection, devices were incubated for 30 min at 37°C before imaging using fluorescence microscopy (EVOS FL, Thermo Fisher Scientific). One-way ANOVA was performed, with Tukey’s test, to determine statistical significance on the viability of the cells in static (no pressure) and dynamic (800 mbar & 1000 mbar) conditions.
3. Results and discussion

3.1. Microfluidic platform for the mechanical stimulation of an engineered articular cartilage model

The goal of this study was to develop a microfluidic platform able to generate gradients of compressive mechanical stimulation and multi-modal actuation patterns on an engineered tissue, consisting of a cell-laden agarose matrix. This tissue model was confined in a rectangular culture chamber flanked on one side by a series of pillars, and on the other side by a thin PDMS membrane, acting as a mechanical actuator (Fig. 1). The pillars confined the hydrogel in the culture chamber by capillary action, while supporting the nutrient delivery from the perfusion channel into the cell-laden hydrogel. As a proof of concept, we demonstrate the applicability of our platform to create a cartilage model. Cartilage is a water-rich, relatively soft, elastic tissue, mainly composed of collagen (type II) fibers and proteoglycans, with a low percentage of specialized cells, called chondrocytes, in this extracellular matrix. *In vitro* cartilage models have been proposed using various synthetic or natural hydrogels such as collagen [38], alginate [39], and agarose [40, Buschmann, 1992 #74, 41-44], as used in this work.

3.2. Characterization of the device: parameters influencing the membrane deflection

First, we thoroughly characterized the PDMS membrane deflection to identify optimal parameters to emulate the compression level found in articular cartilage. For this, a positive pressure ranging from 0 to 1200 mbar was applied in all three actuation chambers concomitantly, and the influence of the PDMS formulation, the membrane thickness and height, and the nature of the lid substrate (glass vs. PDMS on glass) on the membrane deflection was quantified.
PDMS is an elastomeric material whose properties can be tuned by varying its composition and/or curing parameters [45]. More specifically, the lower the amount of cross-linker, the longer the PDMS chains between two cross-linking points, and the lower the material Young Modulus [46]. Here, we used a 20:1 prepolymer:curing agent weight ratio, which gave rise to larger deflection than a more traditional 10:1 ratio (Supplementary information S5).

Noteworthy, while further lowering the amount of curing agent would further decrease the PDMS Young modulus, it would be at the risk of having free PDMS oligomers leaching into the cell culture [47].

Similarly, a membrane deflection significantly depends on its dimensions. Here, two membranes with nominal thicknesses of 50 and 100 µm (measured thicknesses of 42.0±3.0 and 93.3±4.6 µm on 3 devices each) were compared. The deflection was almost twice higher for thinner membranes, at all applied pressures (Fig. 2a, green and red curves, respectively, for 100-µm and 50-µm thick membranes). The membrane height had less influence on its deflection. Membrane rupture was observed for much higher applied pressures of 1600-1700 mbar for the 50-µm thick membranes, and their 100-µm thick counterparts could resist 2000 mbar (maximum value generated by our pressure controller). All further experiments reported in this paper were performed using 50-µm thick and 200-µm high membranes (nominal values).

We next hypothesized that the lid substrate could impact the membrane deflection, and we compared glass substrates with and without a thick PDMS coating (Fig. 2a). The membrane deflection was much higher for PDMS-coated glass substrate (maximum respective deflection of 194.7±15.4 µm (PDMS coating) vs. 133.6±5.7 µm (uncoated glass) at 1200 mbar). These results collectively suggest that not only the membrane, but also the roof and the floor of the
mechanical actuation chamber were deformed upon application of the pressure. To maximize the membrane deflection, a glass substrate coated with a \textit{ca.} 4-mm thick PDMS layer was used in experiments described in this paper. Noteworthy, the use of a thick PDMS coating is not advantageous for imaging of cells in the device, and we were limited here to the use of a 40x magnification objective. Altogether, in general a compromise must be found between the amplitude of the membrane displacement and imaging possibilities, and the PDMS layer thickness chosen accordingly.

To assess the device stability, membrane deformation was examined over a period of one month. No significant variation was found at different time points (days 0, 6, 14, 26 and 34) in individual devices (Fig. 2b). Moreover, even for high deflections (> 150 µm), the membrane remained entirely elastic, and the deflection amplitude was unchanged after 27,000 cycles of stimulation (1.5 h at a frequency of 5 Hz, 800 mbar applied pressure) (data not shown). Noteworthy, implementing a post-curing step after device assembly was found to be essential to avoid large changes in the PDMS elasticity over time due to incomplete initial curing of the material.

Yet, all these characterization steps were performed in air. Therefore, we also examined the influence of the content of the culture chamber on the membrane displacement, and compared air, PBS buffer and agarose supplemented with 15-µm size microbeads at a concentration of 61 µg/ml. The nature of the material placed in the cell culture chamber had little to no influence on the membrane deflection (Fig. 2a). Consequently, the comprehensive device characterization performed in air can apply to agarose-laden culture chambers.
3.3. Creation of gradients of compressive forces as found *in vivo*

Next, we examined the agarose deformation upon application of homogeneous compression (Fig. 3a) by tracking the displacement in agarose of microbeads having a size (15 µm) similar to that of chondrocytes. Both the displacement and strain orthogonal to the membrane were quantified in front of the central actuation chamber along the full width of the culture chamber, for applied pressures of 100-1300 mbar. Overall, the applied pressure impacted the microbead displacement: the higher the applied pressure, the greater the bead displacement, and the closer the microbead to the membrane, the higher the displacement (Fig. 3b). Next to the membrane, the microbead displacement was comparable to the membrane deflection (*i.e.*, 207.2 µm vs. 201.4±3.7 µm at 1000 mbar), and regularly decreased across the chamber width to less than 25 µm close to the pillar array. Interestingly, such a behavior is reminiscent of the deformation profile found *in vivo* in the knee cartilage, in which the superficial layer of this tissue experiences much higher deformation than layers close to the bone. Here, the agarose is not covalently attached to the PDMS membrane and to the roof and bottom of the culture chamber. When the membrane is deformed, the whole hydrogel slide inside the chamber. As a consequence, while the agarose in contact with the membrane is probably less compressed close to the roof and bottom, due to the shape of the membrane, there is no difference in displacement across the entire hydrogel height already about 100 micrometers from the membrane.

The strain was evaluated and found to be almost constant across the entire culture chamber for applied pressure lower than 1000 mbar (Fig. 3c). An applied pressure of 700 mbar yielded a strain value of 5-12%, which corresponds to physiological strain in healthy knee cartilage, while applying a 1000-mbar pressure allowed emulating hyper-physiological compression (>
20% close to the membrane), as observed in diseased knee cartilage [48]. Altogether, both healthy and pathological compression profiles in knee cartilage could be reproducibly generated in our platform, with the same gradients of deformation and strain, as found in vivo.

3.4. Deformation of chondrocytes under compression

The mechanical stimulation of chondrocytes can lead to a wide range of biological responses and increased or decreased viability [48]. Here, in a first instance, we characterized the chondrocyte deformation under homogeneous compression (800 mbar applied pressure; 1 Hz frequency). Since at that short timescale, cells are incompressible [49], which has also notably been demonstrated for chondrocytes [50], here, we specifically measured changes in the cell projected area, and not in their volume, which would not be appropriate in this configuration. Changes in cell volume are typically observed after continuous and prolonged compression of cells [51], which is not the case here. The projected surface areas of a total of 24 chondrocytes across the culture chamber were measured, both at rest (before stimulation) and under compression, and the projected surface area decrease (%) plotted as a function of the chondrocyte distance to the membrane (Fig. 4). Under physiological compression (800 mbar), the chondrocyte projected surface area decreased by up to 13% at 200 µm to the membrane, and gradually decreased across the chamber from the PDMS membrane to the pillar array (2%). Altogether, cell deformation followed the same gradient as agarose displacement.

3.5. Chondrocyte culture and mechanical stimulation in the device

To assess the impact of this gradient of hydrogel compression on cells, human chondrocytes were cultured for three days in agarose in the device, and daily stimulated by cycles of homogeneous compression (1 Hz for 1 h), using parameters chosen based on previous reports [42, 52-55]. Two pressures were included to mimic healthy (800 mbar) and hyper-
physiological (1000 mbar) compression, and static culture (no applied pressure) as a negative control. For each condition three individual devices were considered. Cell viability was evaluated at the end of the culture using NucBlue® Live reagent (Hoechst 33342), which stains all cell nuclei and NucGreen® Dead reagent that only stains the nuclei of cells, whose plasma membrane integrity is compromised. Two zones were considered, based on the cell deformation results and the presence of a gradient of strain, to evaluate cell viability (Fig. 5a). Noteworthy, chondrocytes do not proliferate in agarose [56], which was confirmed in our experiments, in which no cell aggregate was formed during this 3-day culture period, as would occur upon cell proliferation.

After three days of culture, chondrocytes were mainly NucGreen®-negative (80-90% viability) for the static (no compression) and healthy dynamic conditions (800 mbar) in the entire culture chamber, demonstrating that cell viability was not compromised in our platform (static control) and by the healthy mechanical stimulation. In contrast, using hyperphysiological stimulation (1000 mbar), high level of NucGreen®-positive cells were found close to the membrane, indicating compromised cell membrane integrity, and possibly cell death (Fig. 5a). This variation in cell fate between static, dynamic healthy, and pathophysiological conditions was found to be significantly different in zone 1 next to the membrane (P<0.05) (Fig. 5b), but not in zone 2 closer to the pillars (Fig. 5c). Noteworthy, the static control confirmed that agarose is a suitable matrix for the culture of chondrocytes, and that nutrients were successfully brought to the entire engineered cartilage via the perfusion channel, either across the agarose or via above and below this matrix, since agarose was not attached to the PDMS. Interestingly, the latter configuration would not suffer from any possible changes in the material properties (e.g., decrease in mesh size) upon repeated compression.
Only short-term changes in chondrocytes have been examined. Arguably, longer stimulation would increase the protein and matrix deposition by these cells, which would most probably translate into its stiffening of the agarose matrix, as notably reported by Bian et al. [57]. Yet, by continuously monitoring the membrane deflection, such changes could be identified, and the applied pressure increased accordingly to counterbalance those changes and ensure the same compression level is continuously applied to the engineered tissue in our microfluidic platform.

3.6. Exerting compressive and bulk shear forces on an engineered tissue

During the articulation of diarthrodial joints, mechanical stimulation comprises either intermittent compressions (standing position), or shear forces when the two bones linked by the articulation are sliding with respect to each other during movement. As a result, chondrocytes experience both compressive and bulk shear forces during movement, and both modalities induce different biological responses [58-60]. Our original mechanical actuation unit was designed to recapitulate both these compressive and more complex multi-axial stimulations, since the three actuation chambers are individually addressable, and both positive and negative pressures can be applied on-demand in any of the chamber with excellent temporal control. To illustrate this capability, different pressure sequences were applied in the three chambers. As depicted on Fig. 6a, a positive pressure was first applied to the left actuation chamber and negative pressure to the two other actuation chambers. The membrane was deflected towards the cell culture chamber only in front of the left actuation chamber. Switching from vacuum to positive pressure in the central chamber next deflected the central part of the membrane. To confirm that this actuation sequence would create both compression and bulk shear, the displacement of 15-μm microbeads was tracked in the
agarose-filled culture chamber, both orthogonally and parallel to the membrane (Fig. 6b & c). For a zone of 1220 µm x 1952 µm in the chamber (marked in Fig. 6c), vectors were plotted to represent the average direction and amplitude of the microbead displacement in this zone (Fig. 6d). Following this, heat maps were established for both the normal (in %) and bulk shear strain (in millirad) to represent the amplitude of cell deformation under compression and shear (Fig. 6e and f, respectively). Although compression remained the dominant mechanical stimulation modality, microbeads also moved along the membrane, with amplitudes amounting to 55% of their normal displacement. Compression occurred next to the central actuation chamber, and relaxation next to the left one. Normal strain was reduced in this configuration compared to a homogeneous compression modality, which can be explained by the partial deflection of the membrane in this scenario, when only one or two chambers were simultaneously pressurized. Bulk shear strain was concomitantly examined, as detailed in Supplementary information S3. The average value of the shear strain amplitude was 9.8±2.9 mrad, which is in good agreement with previously reported values for shear strain on cartilage [58-61], as summarized in Supplementary information S6. Interestingly, by further varying the actuation sequence, a great variety of stimulation patterns could be created in our device to mechanically stimulate cell-laden hydrogel matrices.

4. Conclusion

We reported here a monolithic platform comprising a vertical PDMS membrane actuated by a series of three independent yet connected chambers, to create various mechanical stimulation patterns on engineered tissues, while providing continuous optical access in the entire culture chamber. Thorough characterization of the platform allowed identifying optimal parameters to maximize the membrane deflection under uniform compression, and to mimic both healthy and hyper-physiological mechanical stimulation, as found in articular cartilage. Chondrocytes
cultured in the device in agarose remained viable under both static and healthy dynamic conditions, while hyper-physiological stimulation led to cell membrane rupture and possibly cell death. Importantly, gradients of agarose deformation, strain and cell deformation were generated in our platform, mimicking the situation found in knee cartilage. As such, our platform is of great interest for screening the effect of various strain levels on cells in a single experiment, with real-time observation of the cell response. Finally, we exerted a combination of normal and bulk shear strains on agarose, with values very similar to those observed in the knee articulation during movement.

In future work, we will examine the influence of the multi-modal stimulation on chondrocyte behavior, in particular on their ability to create their own microenvironment and in terms of gene and protein expression patterns after retrieval of the engineered tissue from the platform after prolonged exposure to physiological compression. Moreover, it is well-known that the exact stiffness of the hydrogel material has an impact on the cell behavior and differentiation, as well as on the matrix formation [62, 63]. Other hydrogel matrices or combination of materials could be tested in our platform in combination with mechanical stimulation, to identify an optimal matrix composition to engineer cartilage tissues. Noteworthy, the herein reported easy-to-produce and cutting-edge platform is not only of great interest to study cartilage, as demonstrated here, but it also offers unprecedented options to apply unconventional mechanical stimulation in other micro-physiological organ models.

5. Author Contributions
C.A.P., B.V. and S.L.G. conceived and designed research, methodology and platform. C.A.P. performed all experimental work including cell culture, characterization of the platform, protocol optimization, and data analysis. B.V. contributes to the design and fabrication of the devices. J.L. and M.K. provided input on system needs and requirements, and offered insights
on cartilage biology. C.A.P., B.V. and S.L.G. wrote the manuscript and all authors approved its content.

CRediT author statement

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6. Conflict of Interest

There are no conflicts of interest to declare.

7. Acknowledgements

We acknowledge financial support for this project from the ReumaNetherlands grant LLP-25.
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Biographies

Carlo Alberto Paggi received his M.S. degree from University of Twente, The Netherlands, in 2018. Currently, he is currently doing his PhD at University of Twente. His research work focuses on the organ-on-chip, microfluidics, and cell biology. He is particularly focused on articular cartilage and osteoarthritis progression.

Bastien Venzac received his Ph.D. degree from the Curie Institute and Pierre et Marie Curie University, in the group of Jean-Louis Viovy and Stephanie Descroix in 2016. His post-doc research focuses on organ-on-chip and the development of simple microfluidic technology for biological applications.

Marcel Karperien obtained his PhD at the Netherlands Institute for Developmental Biology and Stem Cell Research. Currently, he is full professor and head of the Department of Developmental BioEngineering group at University of Twente. He is interested in developing new solutions for treating cartilage related disorders. His work is technology inspired and is characterized by a multidisciplinary approach integrating typical engineering disciplines with life-sciences.

Jeroen Leijten received his Ph.D. degree from Twente University in 2012. Currently, he is appointed as an associate professor at Twente University, The Netherlands. His research focuses on the development and application of microtechnological platforms including droplet generators, smart instructive micromaterials, single cell microgels, 3D cellular organoids, 3D printing, and organ-on-chips.

Séverine Le Gac received her Ph.D. degree cum laude from the University of Lille, France, in 2004. She is Professor at the University of Twente, The Netherlands, leading the Applied Microfluidics for BioEngineering Research (AMBER) team. Her research focuses on the use of miniaturized devices for biological and medical applications, and in particular for cancer research and the field of assisted reproductive technologies.
9. Figure captions

Figure 1.

**Design of the microfluidic platform to mimic articular cartilage.**

*Left:* Top view of the device comprising (*from top to bottom*): a mechanical actuation section composed of 3 connected actuation chambers separated from the rest of the system by a thin vertical PDMS membrane; a 3D cell culture chamber; an array of pillars; a medium perfusion channel.

*Right:* Microscopic picture showing a section of the system (red square on the left picture) containing a chondrocyte-laden agarose matrix. *Left,* static condition; *Right,* homogeneous compression (1000 mbar). Scale bar: 500 µm

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Figure 2.

**Influence of various device parameters on the vertical membrane deflection.**

*a.* Membrane displacement in devices for a 0-1200 mbar applied pressure: with a 50-µm thick PDMS membrane and a 4-mm thick PDMS base in presence of agarose (orange) (n=3) or air
(red) (n=10) in the cell culture chamber; 50-µm thick membranes with a glass base (blue) (n=3); 100-µm thick membranes with a 4-mm PDMS base (green) (n=3). The standard deviation corresponds to measurements in n independent devices, n being specified for each condition.

b. Membrane displacement in a single device over a period of time of > 1 month.

Figure 3.

Agarose deformation under homogeneous compression.

a. Top view of a device filled with an agarose matrix supplemented with 15-micron microbeads at rest (top) or under homogeneous compression mechanical stimuli (middle 500 mbar; bottom 1000 mbar).

b. Displacement of microbeads in the zone indicated by the red rectangle, as a function of the applied pressure and distance to the membrane for homogeneous compression.

c. Associated strain (%). The light-blue zone in the strain graph indicates the physiological strain range. The dashed lines on the right indicate the position of the pillar array.
Figure 4.

Chondrocyte culture and compressive mechanical stimulation.

a. Cell surface area decrease (projected area, in %) for individual chondrocytes exposed to homogeneous compression (800 mbar, 1 Hz frequency) as a function of their distance to the membrane. The standard deviation corresponds to three manual measurements for the same cell.

b. Cell deformation under compression: comparison of their shape and projected surface area at rest and under homogeneous compression.
Figure 5.

Chondrocyte viability under healthy and hyper-physiological mechanical stimulation

a. Left, top view of the system with a subdivision in two zones, close to the vertical PDMS membrane (zone 1, black frame) and close to the pillars (zone 2, red frame); Right, live/dead assay after three days of culture using the ReadyProbes™ Cell Viability Imaging Kit in zone 1 for three culture conditions; no stimulation (top), 800 mbar (healthy) (middle), and 1000 mbar (hyper-physiological) (bottom). Quantitative chondrocyte viability in zone 1 (b) and zone 2 (c). For each condition 3 individual platforms were considered. Scale bars: 500 µm. (** indicates P<0.05, one-way ANOVA with Tukey’s test).
Figure 6.

Multi-modal deformation of the agarose in the cell culture chamber.

a (1-4). Pictures of the mechanical actuation system with the three chambers pressurized with different conformations. Red arrows indicate negative pressure (-350 mbar) and blue arrows positive pressure (+800 mbar). b. & c. Pictures of the agarose matrix supplemented with microbeads during sequential actuation of the chambers: (b) left chamber pressurized with +800 mbar, and central chamber with –350 mbar; (c) both chambers pressurized with +800 mbar. Scale bars: 250 µm. d. Vector map representing the average microbead displacement in the agarose upon sequential actuation as depicted in pictures b & c in zones of 122 µm x 122
μm; arrows have been drawn 1.5 longer than the actual measured displacement for visualization purposes. Heat maps of the normal (compressive) (e) and bulk shear strains (f) in agarose generated by the sequential actuation presented in pictures b & c, and averaged for zones of 122 μm x 244 μm. In (e) positive normal strains are oriented toward the pillars, and negative towards the PDMS membrane, and in (f) positive shear strain angles are oriented clockwise.