Mechanical induction of Osteoarthritis traits in a cartilage-on-chip model

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Abstract

The present lack of effective therapies for osteoarthritis, the most diffused musculoskeletal disease, correlates with the absence of representative *in vitro* disease models. Micro-fabrication techniques and soft lithography allow the development of Organs and Tissues on chip with increased mimicry of human pathophysiology. Exploitation of polydimethylsiloxane elasticity, furthermore, allows to incorporate finely controlled mechanical actuators which are of the utmost importance in a faithful representation of the intrinsically active environment of musculoskeletal districts, to increase our comprehension of the disease onset and to successfully predict the response to pharmacological therapies. Here we portray the fabrication and operational processes for the development of a Cartilage-on-Chip model. Additionally, we describe the methodologies to induce a phenotype reminiscent of osteoarthritis solely through hyperphysiological cyclic compression. The

techniques to assess achievement of such features through immunofluorescence and gene expression are also detailed.

Key words: Cartilage-on-Chip, Disease Modelling, Osteoarthritis, Mechanical stimulation

Running head: OA Cartilage-on-chip

1. INTRODUCTION

Osteoarthritis (OA) is the musculoskeletal disease with the highest prevalence in the world, burdening roughly 20% of women and 10% of men over 60 years of age[1].

While OA is now recognized as a whole joint failure[2], since the pathology affects also subchondral bone and synovial membrane, the majorly compromised tissue remains articular cartilage (AC). In AC the degenerative processes lead to an alteration of the anabolism-catabolism balance which brings to degradation of cartilage extracellular matrix ECM (e.g. through an alteration of collagen type II to collagen type I ratio and a decrease in the glycosaminoglycan content of the tissue), augmented production of degradative enzymes (e.g. metalloproteinases such as MMP13), and onset of tissue inflammation (e.g. increase in production of inflammatory cytokines such as IL6 and IL8) [3–5]. Moreover, chondrocytes, cartilage resident cells, upregulate the production of hypertrophic markers such as Collagen Type X eventually resulting in another OA hallmark: cartilage calcification.

OA has a multi-etiological origin, but the disease affects particularly load bearing joints (e.g. knees and hips), suggesting an intimate correlation with mechanical causes. Abnormal loading conditions such as trauma, obesity or joint misalignment, in fact, are among proven triggers of the pathology [6].

As of today, despite its diffusion, OA does not have a satisfactory treatment, and intervention options are limited to palliatives aimed at easing symptoms, or, ultimately, joint prosthetisation [5].

While promising disease modifying anti-OA drugs able to stop or revert OA's degenerative tendency are, in fact, under investigation [7, 8], their preclinical to clinical translation is still a major obstacle.

This is strictly linked to the lack of reliable and representative surrogate disease models that would allow both to faithfully test the drug response once in vivo and to help unravel OA's origin and mechanisms of progression in order to find effective therapeutic solutions[9]. Mechanical cues, for instance, are seldom adequately represented in existing OA *in vitro* models [9].

In the last decades organs on chips (OoC) solutions have been establishing themselves as more adequate disease models, increasing mimicry of the *in vivo* conditions and resemblance to the native pathophysiological cellular environment [10].

Leveraging on the soft lithography potentiality to produce sub-millimetric features, OoC devices are capable of subjecting cells in both 2D and 3D environments to physical and biochemical stimuli, including fluid induced shear stresses, mechanical cues, electrical pacing, nutrients and chemical gradients, and all of these at a cellular relevant scale [11]. The dimensional micro-scale typical of OoC, indeed, enables a level of control over the cellular environment non reachable with neither classic culture systems based on plasticware nor with macroscale bioreactors. This peculiarity makes OoC particularly suitable for a reliable replication of organ and tissue specific architectures and functions [12].

The majority of OoC devices are fabricated in polydimethylsiloxane (PDMS), which is known for its transparency , permeability to gasses and biocompatibility, thus representing an ideal candidate for cell culture applications [13]. PDMS, moreover, behaves like an hyper-elastic rubber allowing the integration of mechanical cues in OoC through accurately designed deformation states.[3, 14] This feature is particularly interesting when dealing with musculoskeletal tissues or pathologies where mechanical cues play such an important role. As an example, for instance, OA affected joints have been correlated to hyperphysiological mechanical stimuli on chondrocytes [15] and a similar stimulation should be introduced in an *in vitro* representation of OA.

In the present chapter, in particular, we describe a method to obtain an OA like cartilage-on-chip (CoC) model enabling to mimic cartilage pathophysiological mechanical environment.

To this aim, by adopting a patented technology that allows for culturing cell based 3-dimensional (3D) microconstructs and appropriately patterning the device features [16], we guarantee the application of a defined mechanical stimulus reminiscent of those highlighted as a cause of OA in patients [17, 18] (i.e. 30% confined compression, named hyperphysiological load) during the whole cell culture period, which results in induction of OA traits in a reproducible and confined way.

The CoC device was successfully adopted to differentiate human primary chondrocytes into cartilaginous like microconstructs, rich in ECM proteins characteristic of articular cartilage, such as Collagen Type II, and Aggrecan. The application of the hyperphysiological mechanical stimulation, subsequently, enabled to recapitulate in the CoC devices key hallmarks of OA. These included increased expression of degradative enzymes (i.e. MMP13), inflammatory cytokines (i.e. IL6 and IL8) and hypertrophic markers (i.e. IHH and Col10a1) and downregulation of hypertrophic brakes (i.e. Grem1, FrzB and DKK1) [15].

In the following sections, all procedures for fabrication of master molds, realization of PDMS devices and for the generation of the OA COC model are described.

2. MATERIALS

2.1 Master mold fabrication equipment

1. Software for computer aided design
2. Photolithography transparency mask (e.g. grade 3 polyester PET base photomask)
3. 2 Silicon wafers (100 mm 4 inches diameter, 0-100Ωcm, 500 µm thickness)
4. Pressurized Nitrogen source
5. Stainless steel tweezers
6. Spin coater
7. Two hotplates
8. Glass Petri dishes (e.g. 120 mm diameter)
9. Chemical fume hood
10. Profiler
11. Transmitted light optical microscope

2.2 Master mold fabrication materials

1. Propanol
2. Acetone
3. TI PRIME adhesion promoter
4. SU-8 2035 negative photoresist
5. SU-8 2100 negative photoresist
6. SU-8 developer

2.3 Cartilage-on-a-chip device fabrication equipment

1. Plasma cleaner (*see* **Note 1**)
2. Oven

3. Vacuum chamber
4. Vacuum pump
5. Chemical fume hood

2.4 Cartilage-on-a-chip device fabrication materials

1. Glass Pasteur pipette
2. PDMS kit: prepolymer (Component A) and curing agent (Component B)
3. Sharpened biopsy puncher, diameter 500 µm
4. Sharpened biopsy puncher, diameter 750 µm
5. Sharpened biopsy puncher, diameter 5 mm
6. Scalpel
7. Straight razor blade
8. Stainless steel spatula
9. Cleaning tape
10. Disposable cell culture non-treated Petri dishes, 120 mm diameter
11. Stainless steel tweezers

2.5 Equipment for chondrocytes seeding, culture, and mechanical stimulation

1. Biological safety cabinet
2. Autoclave
3. Micropipette set
4. Pressurized air source
5. Pneumatic pressure regulator
6. Electropneumatic controller (*see* **Note 2**)
7. Cell culture incubator (37 °C, 5% CO_2)

2.6 Materials for chondrocytes seeding, culture and stimulation

1. Autoclave bags
2. Disposable sterile cell culture non-treated Petri dishes, 100 mm and 120 mm diameter
3. Sterile stainless-steel scissors

4. Sterile stainless-steel tweezers
5. Stainless steel couplers, 23 G, 12 mm length
6. Blunt tip syringe needles, 23 G
7. Tygon tubing, inner diameter 0.51 mm, outer diameter 1.52 mm, fitting 23 G needles
8. 1 ml syringes
9. Sterile phosphate buffered saline (PBS)
10. Serum free culture medium: Dulbecco's Modified Eagle's Medium (DMEM)
11. Chondrogenic culture medium: DMEM, 2% fetal bovine serum, 4.5 mg/ml D-glucose, 0.1 mM non-essential amino acids, 1mM sodium pyruvate, 100 mM HEPES buffer, 100 u/ml penicillin 100 µg/ml streptomycin and 0.29 mg/ml L-glutamine, supplemented with 0.1 mM ascorbic acid 2-phosphate, 10 µg/ml insulin and 10 ng/ml TGF-β3 (*see* **Note 3**)
12. 70% ethanol
13. Sterile fibrinogen solution: 100mg/mL Fibrinogen human type I, 0.9% w/v NaCl in demineralized water (*see* **Note 4**)
14. Sterile thrombin solution: 100 U/mL thrombin from human plasma, CaCl2 40 mM in demineralized water (*see* **Note 4**)
15. Sterile aminocaproic acid solution: 100 mg/ml aminocaproic acid in PBS

2.7 Materials and equipment for cartilage microtissues readouts

1. PBS
2. 4% paraformaldehyde (PFA) solution in PBS
3. Goat serum
4. Triton X-100
5. Tween-20
6. Primary antibodies (e.g. mouse anti-human collagen type I, mouse anti-human collagen type II, and rabbit anti human aggrecan to evaluate cartilage-on-a-chip constructs maturation; rabbit anti-human MMP-13 and mouse anti-human DIPEN to evaluate to assess the effect of the OA inducing mechanical compression)

7. Secondary antibodies (e.g. goat anti-mouse and goat anti-rabbit labelled with Alexa Fluor 488, Alexa Fluor 546, and Alexa Fluor 647)
8. DAPI solution
9. Proteinase K solution: 1 mg/ml proteinase K in 50 mM Tris buffer with 1 mM EDTA, 1 mM iodoacetamide and 10 μg/ml pepstatin A
10. Dimethylmethylene blue (DMMB) solution [19]
11. Chondroitin sulphate
12. DNA quantification standard kit
13. TRIzol reagent and TRIzol based total RNA extraction standard reagents
14. Complementary DNA synthesis standard reagents
15. RT-qPCR standard reagents and equipment
16. Gene assay on demand for genes of interest (e.g. *COl2A1, COL1A1, ACAN, PRG4, GDF5* to assess cartilage-on-a-chip constructs maturation and *MMP13, IL6, DKK1, FRZB, GREM1, COL10A1, IHH* to assess the effect of the OA inducing mechanical compression)
17. MMP13 detection kit

3. METHODS

3.1 Device fabrication

This section describes the fabrication of the device as depicted in Fig 1. Steps include realization of the master molds, production of device's PDMS compartments, and final device assembly.

The device is constituted by three overlying PDMS compartments, namely a top culture chamber, an un-patterned flexible membrane, and an actuation chamber. The top culture chamber, i.e. the compartment hosting the 3D cell laden hydrogel, comprises a central channel flanked by two rows of overhanging pillars which separate it from the lateral culture medium channels connected to medium reservoirs. Pillars' shape, dimension and in-between spacing were designed to confine the hydrogel upon injection, while ensuring the desired level of confined compression. Notably, the pillars height is less than the whole top culture chamber height, being present a gap that separates the pillars from the bottom of the chamber. The ratio between the pillar and the gap heights defines the compression level. The un-patterned flexible PDMS membrane separates the top culture chamber from the actuation chamber. When a positive pressure is applied to this latter compartment, the overlaying flexible membrane deflects upward until it gets stopped by the bottom surface of the pillars.

During the fabrication process PDMS is cast upon a master mold realized through a photo-curable resist (i.e. photoresist) patterned on a silicon substrate. The advancement of photolithographic techniques and the perfecting of photoresist chemistry allowed in recent years to increase the complexity of obtained features overlaying multiple photoresist layers.[3, 20]

In the present case, specifically, the master molds for the top culture chamber and for the actuation chamber are fabricated separately, while the un-patterned flexible membrane does not present features requiring a master mold produced by photolithography.

3.1.1 Master molds fabrication

1. Through an appropriate CAD software, design the layouts of the two layers required for the fabrication of the top chamber through multi-layer photolithography, namely the gap layer and the pillar layer. (Fig 1a.) The first layout contains the outline of the whole top culture chamber, the

second one contains, in addition, the two rows of pillars constituting the central channel. Include Alignment signs to superimpose layers (*see* **Note 5**). Through the subsequent realization of the gap and the pillar layers it is possible to obtain overhanging pillars separated from the bottom of the culture chamber by a gap whose height is given by the height of the first SU-8 layer. (Fig 1b.)

2. Design the layout of the actuation layer through an appropriate CAD software (*see* **Note 6**).
3. Print a High-resolution photolithography mask (resolution should be higher than 20000 dpi) of each of the CAD designed layers (deep black for unexposed regions).
4. Perform the following steps (5-23) under clean room operations standards (*see* **Note 7**).
5. Clean two silicon wafers sequentially spraying acetone and isopropanol on the surface. Dry the wafers with a nitrogen gun and then bake them at 180 °C for 20 min on a hotplate to assure complete dehydration.
6. Place one wafer on the chuck of a spin coater making sure the wafer is centered and apply vacuum. Add approximately 1 mL of TI PRIME adhesion primer per inch of the wafer diameter on the center of the wafer (*see* **Note 8**).
7. Start the spin coater and spin the wafer using the following parameters. Ramp to 500 rpm at 100 rpm/s acceleration, hold for 10s, ramp up to 4000 rpm at 300 rpm/s acceleration, hold for 30s and ramp down.
8. Bake the substrate at 120 °C for two minutes on a hotplate and let it cool down.
9. Repeat steps 5-7 for the second wafer (Fig 1c.)
10. To fabricate the cell culture chamber, first spin the gap layer. Place one of the wafers on the spin coater chuck. Add approximately 1 ml of SU-8 2035 per inch of wafer diameter. Spin coat to obtain a thickness of 43 μm (*see* **Note 9**).
11. Bake the wafer through a three-step (two-hotplate) procedure (Soft bake). Move the coated wafer with tweezers on a 65°C pre-heated hotplate. After 3 minutes, move rapidly the wafer on a 95°C pre-heated hotplate. Bake it for 6 minutes and move again on a 65°C pre-heated hotplate for other 3 minutes. Let the wafer cool down completely before proceeding.
12. Expose the first layer. Insert the high transparency mask in the mask positioner of the mask aligner. Be carefool to place the mask with the emulsion layer (i.e. the ink used to print the mask) facing

down. Align the wafer with the mask maintaining a gap of 25 µm between the wafer and the mask. Bring mask and wafer in conformal contact and expose to 155 mJ/cm^2 of collimated UV light (wavelength 365 nm) using hard contact lithographic mode.

13. Bake the wafer through a three-step (two-hotplate) procedure (Post exposure bake) in a way similar to the soft-bake described in step 11: 1 minute at 65°C, 6 minutes at 95°C and 1 minute at 65°C. Let the wafer cool down completely before proceeding.
14. Proceed to spin the pillars layer. Place the wafer with the first SU-8 layer on the spincoater chuck. Add approximately 1 ml of SU-8 2100 per inch of wafer diameter at the center of the wafer. Spin coat to obtain a thickness of 100 µm (*see* **Note 10**).
15. Bake the wafer through a three-step (two-hotplate) procedure (Soft bake). Move the coated wafer with tweezers on a 65°C pre-heated hotplate. After 5 minutes, move rapidly the wafer on a 95°C pre-heated hotplate. Bake it for 20 minutes and move again on a 65°C pre-heated hotplate for other 5 minutes. Let the wafer cool down completely before proceeding.
16. Expose the second layer. Insert the high transparency mask in the mask positioner of the mask aligner. Be carefool to face the mask with the emulsion layer (i.e. the ink used to print the mask) facing down). Align the first layer with the mask using the alignment signs included in both layers maintaining a gap of 25 µm between the wafer and the mask. Bring mask and wafer in conformal contact and expose to 240 mJ/cm^2 of collimated UV light (wavelength 365 nm) using hard contact lithographic mode.
17. Bake the wafer through a three-step (two-hotplate) procedure (Post exposure bake) in a way similar to the soft-bake described in step x: 5 minutes at 65°C, 10 minutes at 95°C and 5 minutes at 65°C. Let the wafer cool down completely before proceeding.
18. Fill a glass Petri dish with a thin layer of SU-8 Developer, sufficient to cover the wafer completely (*see* **Note 11**). With stainless steel tweezers put the wafer in the petri with the SU-8 developer and gently stir for approximately 10 minutes.
19. Holding the wafer with stainless steel tweezers spray it with isopropanol to wash off the developer (*see* **Note 12**).
20. Dry the wafer with the nitrogen gun.

21. Inspect the features under a transmitted light optical microscope to check for imperfections and measure the layer thickness using a profiler.
22. Repeat steps 10-13 to obtain the actuation layer. Add approximately 1 ml of SU-8 2035 per inch of wafer diameter at the center of the wafer. Spin coat to obtain a thickness of 50 µm (*see* **Note 13**).
23. Repeat steps 18-20 to complete development of the actuation layer. Development of the actuation layer should take around 5 minutes.

3.1.2 Culture chamber and actuation compartments fabrication

1. Pour the PDMS kit components in a plastic cup (*see* **Note 14**) in a 10:1 ratio (w/w), prepolymer to curing agent (*see* **Note 15**).
2. Mix vigorously with a stainless-steel spatula until a uniform suspension is achieved. At this point the solution should appear white as a result of air bubbles incorporation.
3. Cover the PDMS solution and put it in a vacuum chamber connected to a vacuum pump to degas for approximately 20 minutes.
4. Tape the master mold with the desired SU-8 features facing up to the bottom of a disposable non treated Petri dish (120 mm diameter if using a 4-in wafer) (Fig 1 c. (i))
5. Whit a glass Pasteur pipet place 2-3 droplets of chlorosilane (*see* **Note 16**) on a glass cover slip and cover the glass with the Petri dish containing the master mold placed upside down. Wait for 15-20 minutes for the chlorosilane to evaporate (*see* **Note 17**).
6. Pour the degassed PDMS on top of the wafer. 50 g are enough to obtain roughly a 2.5 mm thickness in a 120 mm diameter Petri dish. (Fig 1 c. (ii))
7. Place the Mold in a vacuum chamber until all bubbles are removed
8. Cure in an oven at 65°C for 2.5 hours.

3.1.3 Flexible membrane fabrication

1. Pour the PDMS kit components in a plastic cup in a 10:1 ratio (w/w), prepolymer to curing agent.

2. Mix vigorously with a stainless-steel spatula until a uniform suspension is achieved. At this point the solution should appear white as a result of air bubbles incorporation.
3. Cover the PDMS solution and put it in a vacuum chamber connected to a vacuum pump to degas for approximately 20 minutes.
4. Put the degassed PDMS into a disposable Petri dish. Use the proper quantity to achieve a thickness of 800 µm. (Fig 1 c. (ii))
5. Place the Petri dish in the vacuum chamber until all bubble s are removed.
6. Cure in an oven at 65°C for 2.5 hours. Make sure the oven shelf is on a level to avoid non uniformities in the membrane thickness (Fig 1c.)

3.1.4 Device assembly

1. With a scalpel cut the fully cured PDMS along the edge of the silicon wafer, both in the case of the top chamber and the actuation, and gently peel it off helping with tweezers if there is the need. (Fig 1 c. (iii))
2. Use a straight razor blade to shape the outer border of the PDMS slab, typically like rectangle (*see* **Note 18**).
3. With sharpened biopsy punchers bore the holes for the central chamber (750 µm diameter) and for the media reservoirs (5mm diameter) in the top culture chamber layer.
4. Cut a piece of the flexible membrane with the same dimension of the top layer.
5. Plasma bond the top layer to the flexible membrane. Clean both surfaces with a cleaning tape strip to assure removal of all dust and debris. Place the top layer and the flexible membrane in a plasma cleaner with the surfaces to be attached facing up. Turn on the cleaner vacuum pump and wait for the pressure in the chamber to reach 400 mTorr.
6. Activate the radiofrequency power switch and keep it on for 50s (*see* **Note 19**).
7. Turn off the radiofrequency and slowly ventilate the plasma cleaner chamber to re- equilibrate the pressure.

8. Rapidly put in contact the top layer and the membrane. Remove all trapped air gently pushing with a clean stainless-steel spatula. Make sure not to press directly on the features. It is essential for the overhanging pillars not to be bonded to the bottom surface for the device to function properly.
9. Place the assembled layer in an oven at 80 °C for at least 20 minutes to make plasma bonding permanent.
10. Bore the hole for the actuation chambers in the top chamber (500 μm diameter) and flexible membrane. Make sure the hole corresponds to the actuation chamber inlet preventively aligning the actuation chamber with the top chamber and signing where the access should be placed before boring the hole. (Fig 1c.)
11. Plasma bond the top culture chamber-flexible membrane assembly with the actuation chamber. Repeat steps 5-9. Notice that the flexible membrane bottom surface has to be bonded to the top surface of the actuation PDMS compartment. Assure therefore that the actuation PDMS compartment has the features facing upwards during the bonding procedure (Fig 1d.(iii)). Make sure both the chambers and the inlet are properly aligned. (Fig 1c.)
12. Cover PDMS surfaces with cleaning tape so that it remains clean during storage. Assembled device can be stored at room temperature in Petri dishes to limit dust accumulation. (Fig 1d.)

3.2 Cartilage-on-Chip maturation and OA mechanical induction

This section describes the procedures necessary to obtain cartilaginous microconstructs in the cartilage on-a-chip devices and to subsequently apply mechanical stimulation to induce OA like features. The seeding procedure, the culture conditions and the process through which mechanical stimulation can be applied are described.

3.2.1 Chondrocytes seeding and cartilage microtissues maturation

1. Remove the cleaning tape from devices surfaces and sterilize them by autoclaving (120 °C, 20 min) (*see* **Note 20**).
2. Dry sterile devices at 80 °C overnight in an oven.

3. Under a sterile cabinet hood remove the device from the sterile bags and place them in a sterile Petri dish with sterile metal tweezers.
4. Prepare chondrocytes for seeding according to supplier's instructions or following standard procedure [21]
5. Prepare a single cell suspension of human articular chondrocytes.
6. Place the thrombin, fibrinogen and aminocaproic acid solutions on ice.
7. Dilute the fibrinogen solution to a final concentration of 40 mg/ml in PBS and keep it on ice.
8. Dilute the Thrombin solution to a final concentration of 10 U/ml in serum free culture medium and keep it on ice.
9. Prepare 10 µl of cells suspension in the 10U/ml of Thrombin solution at a cell density of $100x10^6$ cells ml (i.e. 2x the desired final cellular density of $50x10^6$ cells/ml)
10. Mix 1:1 the cell suspension in the Thrombin solution with the 40 mg/ml Fibrinogen solution and immediately inject the cell laden hydrogel in the central culture chamber. The fibrin solution polymerizes rapidly. It is recommended to produce 10 µl only of fibrin solution per time, sufficient to inject approximately three cartilage-on-a-chip devices.
11. Add small PBS droplets (30 µl ca.) in the Petri dish where the device is contained and incubate the device in a cell culture incubator for 10 minutes (5% CO_2, 37 °C) to ensure complete polymerization.
12. Add chondrogenic culture medium in the side media channels and fill completely the media reservoirs. With the pipet tip aim at the inlet of the medium channel in the reservoir to properly fill it before filling the reservoir itself (*see* **Note 21**).
13. Incubate the device in a cell culture incubator and culture the constructs for 14 days to assure adequate maturation of the constructs. Change the media (i.e. chondrogenic culture medium) every other day. Used media can be stored at -80 °C for subsequent analysis.
14. At each medium change, replenish the PBS droplets in the Petri dish to avoid evaporation.

3.2.2 Cartilage-on-Chip mechanical stimulation

1. After 2 weeks of static culture start the mechanical actuation to induce OA.

2. Prepare a tygon tubing for the actuation. Cut 15cm long tygon tube; assemble a 23G stainless coupler on one side and a 23G blunt needle on the other side of the tube. Wash the assembled tubing by perfusing 70% ethanol and subsequently sterile PBS. Let the tubing in an open Petri dish and expose them to an UV sterilization cycle under a safety cabinet hood
3. Fit the assembled tubing to a sterile 1 ml syringe and fill the tube with sterile PBS.
4. Fit the coupler into the inlet of the actuation chamber by using sterile tweezers.
5. Connect the Tygon tube to a pressurized air source. A 22 µm filter can be added to the needle hub to keep sterility.
6. Expose the device to a constant pressure (230 Torr) for at least 30 mins to ensure complete filling of the actuation chamber with the PBS contained in the Tygon tube.
7. Check for the complete filling of the actuation chamber under a microscope.
8. Connect the tube-needle assembly to an electropneumatic controller (*see* **Note 22**).
9. Connect the electropneumatic controller to a pressure regulator and to a pressurized air source. (Fig 2a.)
10. Use a 1 Hz square signal switching between atmospheric pressure and 610 Torr to stimulate the devices. Apply the stimulation for 2 hours, make 4 hours break, apply once again the stimulation for 2 hours, and make 16 hours pause. Repeat the stimulation pattern for 7 consecutive days. (Fig 2b.) Different stimulation patterns can be introduced according to necessity.
11. Keep the device in an incubator and refresh the media every other day (*see* **Note 23**). Used media can be stored at -80 °C for subsequent analysis.

3.3 Cartilage-on-Chip analysis and readouts

This session describes how to determine the cartilage on chip maturation and the onset of osteoarthritis like phenotype. Immunofluorescence, RT-qPCR and culture medium composition analyses are described as they have been adapted to the cartilage on chip device.

3.3.1 Cartilage-on-Chip immunofluorescence

Immunofluorescence can be performed during the cartilage maturation phase, (i.e. day 0 to day 14) to assess the cartilage-on-a-chip constructs maturation level (e.g. Staining for Collagen type-I, Collagen type-II,

Aggrecan and other ECM proteins or anabolic markers) or at the end of the OA induction period (e.g. staining for MMP13, DIPEN or other degradation or inflammation markers).

1. Remove the devices from the incubator. If necessary, detach the devices from the pressurized air source and from the electropneumatic controller.
2. Remove the culture media from the reservoirs. If necessary, collect the media and store it at -80 °C for further analysis.
3. Wash the samples with PBS. Fill the media reservoirs RV1 and RV2 (Fig 1 d.) with 100 µl PBS each, wait 1 minute then remove the PBS and fill all the reservoirs (from RV1 to RV4) with 50 µl of PBS each. Wait for 5 minutes before removing the PBS.
4. Fix the samples by filling each reservoir with 50 µl of 4% PFA solution. Store the samples in PFA at 4 °C for 6 hours.
5. Aspire the 4% PFA from all four of the reservoirs.
6. Repeat the PBS washing described in step 3.
7. Prepare a 5% Triton X-100 solution (v/v) in PBS
8. Add 50 µl of Triton X- solution in each reservoir and incubate at room temperature for 10 minutes to permeabilize cells.
9. Repeat the PBS washing described in step 3
10. Prepare a blocking solution containing 3% Goat serum (v/v) and 0.3% Tween 20 (v/v) in PBS to block unspecific antibody binding.
11. Add the blocking solution to the reservoirs, 50 µl per reservoir, and incubate at room temperature for 45 minutes.
12. Prepare the primary antibody solution in blocking solution (*see* **Note 24**).
13. Aspire the blocking solution from the devices.
14. Add 25 µl of primary antibody solution per reservoir and incubate overnight at 4 °C.
15. Gently aspirate the primary antibody solution from all four chips reservoirs.
16. Wash twice with the blocking solution pipetting it in the media reservoirs, 50 µl per reservoir, and incubating 10 minutes at room temperature each time to remove non-bound primary antibodies.

17. Prepare the secondary antibody solution in blocking solution. Keep the solution protected from light sources to avoid bleaching of the fluorophores.
18. Add 25 µl of secondary antibody solution per device and incubate at 4 °C overnight. Keep devices protected from light.
19. Wash twice with PBS. Incubate 10 minutes at room temperature each time.
20. Prepare a nuclear staining solution in PBS and add 50 µl per reservoir. Incubate 15 minutes at room temperature keeping it protected from light. DAPI was used as an example of nuclear staining, alternative dyes can be considered.
21. Rinse twice with PBS. Incubate 10 minutes at room temperature each time.
22. Remove the actuation chamber PBMS compartment from the device carefully pulling from a corner. Place the device on a glass cover slip and house it on the stage of a confocal microscope for observation (*see* **Note 25**).

3.3.2 Cartilage-on-Chip gene analysis

1. Remove the devices from the incubator. If necessary, detach the devices from the pressurized air source and from the electropneumatic controller
2. Remove the culture media from the reservoirs. If necessary, collect the media and store it at -80 °C for further analysis.
3. Wash the samples with PBS. Fill the media reservoirs with PBS (200 µl per devices), wait for 1 minute then remove the PBS and fill the reservoirs again. Wait for 5 minutes before removing the PBS.
4. Fill an Eppendorf tube with 400 µl of TRIzol reagent.
5. Open the device pulling gently apart actuation layer and flexible membrane and exposing the construct.
6. Retrieve 10 µl of TRI reagent from the Eppendorf and place it on the construct.
7. Gently scrape the construct with the tip of the pipet directly on the PDMS layer
8. Aspire the TRIzol reagent and put it back into the Eppendorf (*see* **Note 26**).
9. Perform total RNA extraction by TRIzol according to standard protocol.
10. Perform complementary DNA synthesis and RT-qPCR according to standard protocols (*see* **Note 27**).

3.3.3 Cartilage-on-chip biochemical analyses: Glycosaminoglycan (GAG) quantification

1. Remove the devices from the incubator. If necessary, detach the devices from the pressurized air source and from the electropneumatic controller
2. Remove the culture media from the reservoirs. If necessary, collect the media and store it at -80 °C for further analysis.
3. Wash the samples with PBS. Fill the media reservoirs with PBS (200 µl per devices), wait for 1 minute then remove the PBS and fill the reservoirs again. Wait for 5 minutes before removing the PBS.
4. Prepare a proteinase K solution. 1 mg/ml proteinase K in 50 mM Tris buffer with 1mM EDTA, 1 mM iodoacetamide and 10 µl/ml pepstatin A.
5. Add 300 µl of proteinase K solution to an Eppendorf tube for each sample to be analyzed.
6. Gently separate the top layer from the thin membrane pulling the two layers away from a corner.
7. Add 10 µl of proteinase solution to the construct still on the PDMS layer and gently scrape the construct with the micro-pipet tip.
8. Retrieve the proteinase K solution with the construct and add it to the Eppendorf containing the rest of the solution (*see* **Note 26**).
9. Digest the samples overnight at 56 °C.
10. Prepare chondroitin sulphate standards in the range of the GAG levels in the constructs to be used for a calibration curve (*see* **Note 28**).
11. Make GAG precipitate, both in the samples and the standards through reaction with dimethyl-methylene blue solution according to standard protocol [22].
12. Measure the GAG concentration spectrophotometrically.
13. Measure the DNA content of the samples using a cells proliferation kit according to the manufacturer's instruction to be able to calculate the ratio GAG/DNA.

3.3.4 Cartilage-on-Chip culture medium analysis

1. Thaw the media samples collected during culture and stored at -80°C (*see* **Note 29**).
2. Centrifuge at 1000 x g at 4 °C for 10 min and collect the supernatant
3. Perform the analysis according to the manufacturer instruction

4. Notes

1. Both an air- and an oxygen plasma cleaner can be used. Oxygen plasma is effective in less time than air plasma treatment.
2. Either a commercial or a custom made electropneumatic controller can be used, e.g. an Arduino based controller piloting a solenoid valve (Festo Miniature Valve MH1) connected to the pressure air source.
3. It is advisable to remove TGF-β3, insulin and bovine serum from the culture medium if using the cartilage-on-a-chip to test the effect of drugs to avoid any confounding or masking effects of the compounds.
4. Fibrinogen and thrombin are used for generating a cell laden fibrin hydrogel. Alternative hydrogel formulations can be adopted. Specifically, a PEG based MMP13 cleavable hydrogel formulation was also tested in the cartilage-on-a-chip devices.[3, 23]
5. Alignment signs (e.g. squares and crosses) are necessary to be able to adequately expose the two layers in a corresponding position. Alignment sign features should have the same dimension of the lowest resolution object in the device or lower. Bigger signs to be used as orientation points are also recommended.
6. Please consider including pillars in the actuation chamber design to avoid sagging depending on its dimensions. For actuation chambers with a height to width aspect ratio lower than 1:10 in fact it is advisable to include pillars in the design to avoid for the chamber to collapse.
7. Steps 1 to 18 of section 3.1.1 should be performed in a cleanroom (Class ISO 6 or above) to prevent contamination from dust or particles. Steps involving photocurable resist before polymerization should be conducted in a yellow room to prevent specific wavelengths of the natural light to affect the curing process. Avoid introducing air bubbles when working with the photoresist and make sure all working planes are flat and on a level.
8. TI prime improve the adhesion of photoresists to the silicon wafers. It is recommended, particularly with features having low contact areas with the silicon substrates.
9. Spin coater parameters for a 43 μm height with SU-8 2035. Ramp to 500 rpm at 100 rpm/s acceleration, hold for 10s, ramp up to 2800 rpm, with a 300 rpm/s acceleration and hold for 30 s;

ramp to 3800 rpm with an acceleration of 1000 rpm/s per 1 s to remove the SU-8 buildup at the edge of the wafer and ramp down.

10. Spin coater parameters for a 100 µm height with SU-8 2100. Ramp to 500 rpm at a 100 rpm/s acceleration, hold for 10s, ramp up to 3500 rpm, with a 300 rpm/s acceleration and hold for 30 s; ramp to 4500 rpm with an acceleration of 1000 rpm/s per 1 s to remove the SU-8 buildup at the edge of the wafer and ramp down.
11. Perform all operation involving SU-8 developer under a chemical fume hood while wearing glove and face protections.
12. Underdevelopment can cause a white residue to appear in this phase. Should this happen immerse back the wafer in the SU-8 developer until the residue disappears and repeat the washing with isopropanol.
13. Spin coater parameters for a 50 µm height with SU-8 2035. Ramp to 500 rpm at 100 rpm/s acceleration, hold for 10s, ramp up to 2400 rpm, with a 300 rpm/s acceleration and hold for 30 s; ramp to 3400 rpm with an acceleration of 1000 rpm/s per 1 s to remove the SU-8 buildup at the edge of the wafer and ramp down
14. PDMS devices fabrication should be carried out under a laminar flow hood to decrease the presence of dust and debris.
15. The PDMS reticulation starts as soon as the two components (prepolymer and curing agent) are mixed and it takes from 15 min to 24h to solidify completely, depending on the temperature, from 100°C to 25°C respectively. Consider preparing fresh PDMS mixture each time.
16. All operations involving chlorosilane should be performed under a ventilated chemical fume hood wearing safety goggles, gloves and face protection
17. The salinization procedure can be performed into a vacuum chamber connected to a vacuum pump to enhance the evaporation process.
18. Optionally cut the corners with a 45 angle to facilitate the following bonding procedures.
19. Both an air plasma and an oxygen plasma can be used. In case of oxygen plasma the time can be decreased to 15-20s

20. Do not insert too many devices in the same autoclave bag, make sure they are evenly distributed. Devices which are in contact with each other during autoclavation might be difficult to separate afterwards.
21. As an alternative, media channels can be filled cutting a pipet tip so that it completely occupies the medium reservoir and creating a pressure gradient in the reservoir itself. To do so, with stainless steel sterile scissors, cut a 1000 μl pipet tip so that its outer diameter is roughly 5 mm. Aspire the culture medium with the cut tip, fit it into the 5mm media reservoir and push until the liquid appears in the downstream reservoir (e.g. from RV1 to RV4). Finish filling the downstream reservoir. Repeat then the operation on the other side of the chip (e.g. fit the cut tip in RV 1 and RV2 and have the media flow in RV4 and RV 3 respectively)
22. The tube needle assembly is to be connected to the electropneumatic pressure controller providing the stimulation pattern. When multiple devices are stimulated at the same time three-way valves can be used to connect the devices in parallel. It is advisable to have a second piece of tube placed after a filter an connecting the devices in an incubator with the controller placed outside. An example of the assembly is depicted in figure 2a
23. Mechanical actuation can increase the media evaporation. It is advisable to check for the evaporation level every day and if necessary, increase the medium refreshing frequency to once a day
24. Anti-human collagen type II, Anti-human collagen type I, Anti-human aggrecan, Anti-human MMP13, and Anti-human DIPEN antibodies have been tested into the device.
25. For observation at higher magnifications (e.g. 40x or higher) it is possible to also remove the flexible membrane exposing the constructs. Gently remove the flexible membrane pulling from a corner. Pay attention to where the construct remains, the top layer or the membrane. Put that layer in direct contact with a glass cover slide for confocal observation.
26. Constructs are barely visible, repeat this step at least twice on both the top layer and the flexible membrane to be sure to retrieve all the material.
27. Assays on demand for *COL1A1, COL2A1, ACAN, PRG4, GDF5*, and *ATX* were assessed to establish cartilage-on-a-chip microconstructs maturation level; *MMP13, IL6, IL8, COL10A1, IHH, FRZBB, DKK1* and *GREM1* were adopted to determine the assumption of OA-like traits.

28. Absolute GAG quantities in the constructs ranged from 0.5 μ g at day o to 6 μg at day 14.
29. Exhausted culture medium from cultured cartilage-on-a-chip devices can be collected upon media refreshing and stored at -80 °C until use for analysis. As an example, MMP13 and proMMP13 released upon mechanical stimulation was measured adopting the Sensolyte Plus 520 MMP-13 Assay Kit. Similarly, different ELISA kit can be used to investigate the release of different inflammatory cytokines and/or enzymes.

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Figures and captions

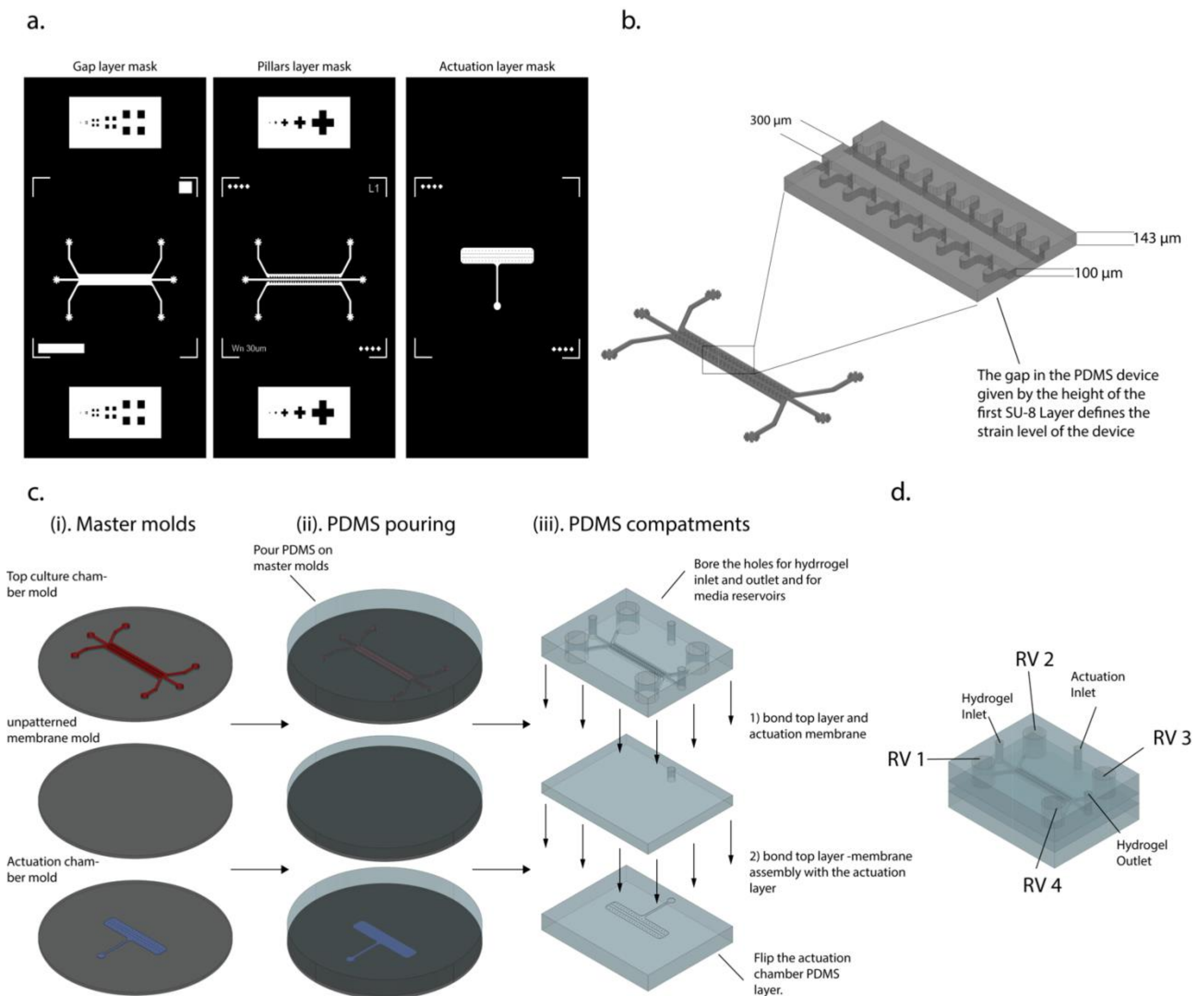

Figure 1. Device fabrication. a. Layout of the masks for the three layers. Gap layer and Pillars layer masks are used for realization of the top culture chamber through multi-layer soft lithography. The actuation mask is used for realization of the actuation chamber through classic single layer photolithography. b. Structure of the SU-8 features of the top culture chamber. The zoom in highlights the height of the gap layer and that of the pillar

layer. c. Passages for the realization of the master-molds and of the PDMS device. (i) realization of Master molds, (ii) PDMS pouring on molds, ad (ii) PDMS compartments refinement and access holes boring d. Assembled PDMS device. Inlets and outlets are highlighted, reservoirs are indicated as RV.

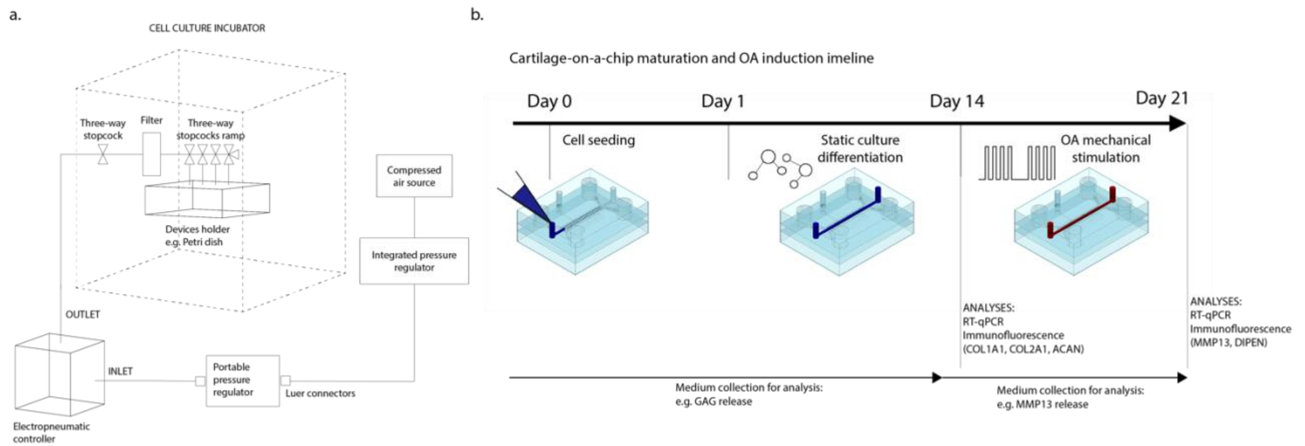

Figure 2. a. Schematic of the complete setup. A portable pressure regulator is used to define the pressure level at the inlet of the electro-pneumatic controller (e.g. Arduino based controller connected to a pneumatic electro-valve). Three-way stopcocks are used to open and close the accesses to the devices, a filter is adopted to isolate the sterile environment (e.g. the inside of a Petri dish containing the devices) from the unsterile outside. Holder and filters are placed inside a cell culture incubator while the controller remains outside. b. Temporal experimental evolution. After seeding chondrocytes within the organ on chip devices are cultured in static conditions for 14 days to achieve the necessary maturation. A subsequent period of 7 days is adopted to induce OA traits (as indicated by the red color of the construct) through hyper-physiological compression. Both endpoint and online analyses that were tested are indicated in the figure.

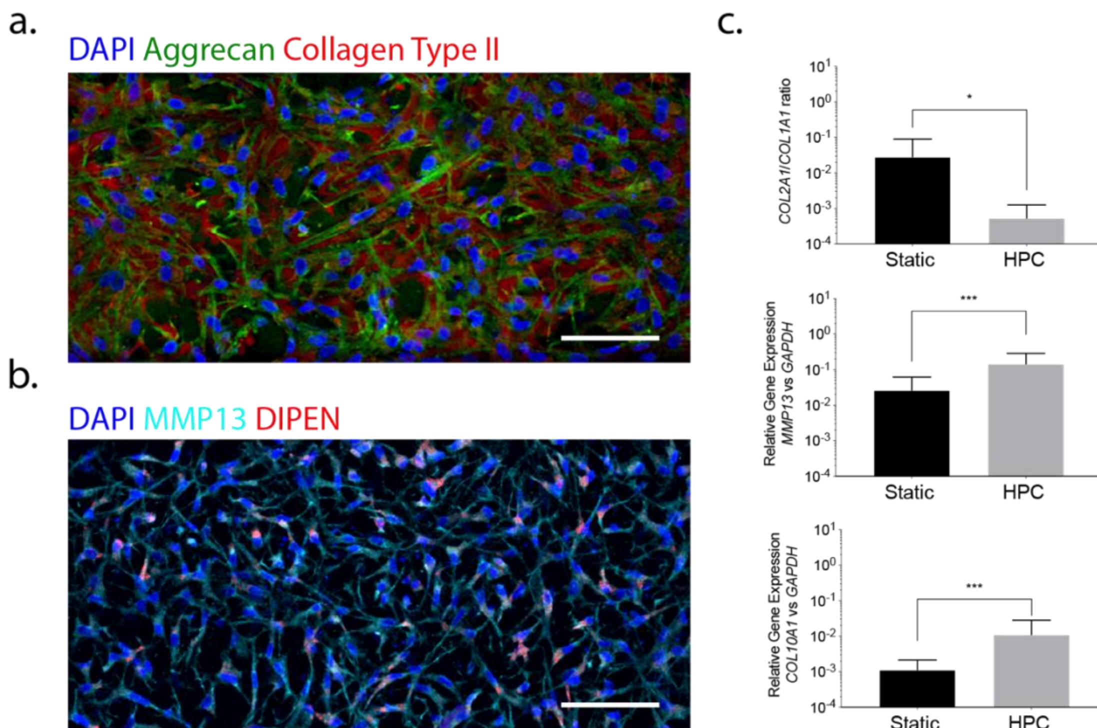

Figure 3. Examples of analyses done on the devices. a. Immunofluorescence imaging of a static construct after 14 days of maturation. DAPI is represented in blue, Aggrecan in green and Collagen Type II in red. Scale bar 100 µm. b. Immunofluorescence imaging of a construct at day 21 after 7 days of HPC. DAPI is represented in blue, MMP13 in cyan and DIPEN in red. c. RT-qPCR results of constructs at day 21. N= 3 primary donors were considered in analyses. At least n=3 biologically independent devices per donor were considered. Statistics by Mann-Whitney test for non-normal distribution.