Supplementary Materials

Microdevice array-based identification of distinct mechanobiological response profiles in layer-specific valve interstitial cells

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Device Fabrication and Operation

The complete fabrication process is illustrated in Figure S1, and consists of preparation of a PDMS base layer and a composite PDMS-PU culture film, and bonding the two together. The PDMS base layers of the devices are fabricated by sandwich molding [1] against a multilayer master mold. Masters were fabricated on 3" × 2" glass slides, with SU-8 negative photoresist (Microchem; Newton, MA, USA), following standard parameters outlined by the manufacturer. Two-layer devices were produced using subsequent alignment and exposure steps, creating masters with a distribution channel network 150 μm thick and connecting pillars 300 μm thick (heights verified by optical surface profilometry). The circular pillars are 2 mm in diameter, and form the base for the suspended culture films after replica molding. SU-8 masters were silanized and sandwich molding was used to produce a negative replica film of the mold master on the transparency, where the SU-8 pillars form vertical through holes. This patterned PDMS layer was then bonded and transferred onto a clean glass slide using an oxygen plasma-generating corona discharge unit (BD-20A; Electrotechnic Products; Chicago, IL, USA). This procedure occasionally produces devices with a thin PDMS layer over the actuation cavities, due to inadequate pressure during sandwich mold fabrication. These films can be cleared manually using a 25G needle as was done in this work, or using higher-throughput procedures developed recently [2].

PU films were formed by dip-coating an untreated PDMS handling slab into a 0.5 wt% solution of SG-80A Tecoflex® polyurethane (Lubrizol Corporation; Wickliffe, OH, USA) in tetrahydrofuran, and allowing it to dry overnight in a covered beaker [3]. The resulting film is less than one micron in thickness, and does not adhere tightly to the PDMS slab. The exposed PU surface was treated with oxygen plasma, and uncured PDMS was spin-coated onto the slab to form a film of PDMS, 45-100 μm thick. The PDMS was partially cured in an oven at 80 °C for 20 minutes, until the PDMS layer remained sticky, but did not deform permanently. The fabricated device and PU-PDMS composite layers were then plasma treated, placed in conformal contact, and baked on a hot plate for at least 10 minutes at 80 °C to permanently bond the

substrates. The handling slab was then carefully peeled away, leaving the composite PDMS-PU film tightly adhered to the device.

Holes were then cut into a thick PDMS gasket, which was plasma bonded to the device surface to segregate groups of culture films. Devices were fabricated on 3" × 2" glass slides, and segregated into 12 groups of 9 circular suspended films each. PDMS connectors (previously described in [4]) were then used to connect the array of wells to a regulated pressure source. Pressure differentials were generated using miniature rotary vane pumps (SP-135 FZ and SP-135 FZ-LC; Schwarzer Precision, Germany), and controlled using solenoid valves (Pneumadyne; Plymouth, MN, USA), driven at 1 Hz. Relatively low strains were produced by applying a pressure of 6 kPa across a 100 μm thick film, and high strains were produced using an 11 kPa pressure source across a 45 μm thick film (parameters summarized in Table 1).

24 hours prior to experiments, devices were sterilized by immersion in 70% ethanol for 5 minutes, and subsequent exposure to a UV germicide lamp for 45 minutes in a biological safety cabinet. Device surfaces were exposed to oxygen plasma for 30 seconds each, using a hand-held corona discharge unit, and incubated with either Type I collagen (100 μg/mL in 0.02 N acetic acid), or fibronectin (100 μg/mL in PBS) overnight at 4 °C. Immediately prior to seeding, devices were washed three times in PBS, and preconditioned with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) for 2 hours at 37 °C.

Analytical model relating surface strain to applied deformation

Analytical models characterizing deformation of a clamped circular plate have been previously developed [5], and the following equations were used to relate peak vertical deflection of the membranes with radial and circumferential strains generated across the surface:

$$\varepsilon_r(r) = \frac{1}{E}(\sigma_r - \nu\sigma_\theta) \qquad \text{Eqn 1}$$

$$\varepsilon_\theta(r) = \frac{1}{E}(\sigma_\theta - \nu\sigma_r) \qquad \text{Eqn 2}$$

where ε_r and ε_θ are the radial and circumferential strains respectively, and are functions of the radial distance r. E is the Young's modulus and ν is the Poisson's ratio of the deforming material. For the composite material diaphragms developed in this work, a 'rule of mixtures' approach was used to estimate the homogenized E based on a ratio between the thicknesses of the constituent materials and their individual moduli. Since PDMS is considered to be incompressible, and forms the bulk of the diaphragms, a value of 0.5 was used for ν [6].

The radial and circumferential stresses can be determined using the following equations:

$$\sigma_r = -\frac{6D}{h^2}\left(\frac{d^2w}{dr^2} + \frac{\nu}{r}\frac{dw}{dr}\right) + \frac{1}{r}\frac{d\phi}{dr} \qquad \text{Eqn 3}$$

$$\sigma_\theta = -\frac{6D}{h^2}\left(\frac{1}{r}\frac{dw}{dr} + \nu\frac{d^2w}{dr^2}\right) + \frac{d^2\phi}{dr^2} \qquad \text{Eqn 4}$$

where D is the flexural rigidity of the circular film and is defined by

$$D = \frac{Eh^3}{12\left(1-\nu^2\right)} \qquad \text{Eqn 5}$$

Here h is the thickness of the diaphragm and $w(r)$ and $\Phi(r)$ are defined as:

$$w(r) = f\left[1-\left(\frac{r}{a}\right)^2\right]^2 \qquad \text{Eqn 6}$$

$$\phi(r) = \frac{f^2 E}{12}\left[\left(\frac{5-3\nu}{1-\nu}\right)\left(\frac{r}{a}\right)^2 - \frac{1}{4}\left(\frac{r}{a}\right)^4 + \frac{1}{9}\left(\frac{r}{a}\right)^6 - \frac{1}{48}\left(\frac{r}{a}\right)^8\right] \qquad \text{Eqn 7}$$

and f and a are the measured deflection and radius of the film, respectively.

Dynamic characterization of culture film response times

Video microscopy was used to determine the time constants required for actuation and relaxation of the bulging diaphragm. Peak deflection was tracked for each video frame, and time constants were calculated as the average time required for 95% actuation or relaxation of the diaphragm. The actuation and relaxation time constants of the culture film were defined as the time required to undergo 95% of the final deformation in response to a step application of pressure, and were found to be 0.21 and 0.42 seconds respectively and complete actuation and relaxation of the culture film was observed under load with a 1 Hz square pressure waveform (Figure S2).

Isolation of side-specific VICs

The described isolation procedure is similar to that used in a recent study in our lab [7]. Briefly, hearts were obtained from 8-month-old pigs from a local abattoir (Quality Meat Packers, Toronto, ON). At this stage, the percentage of cells expressing SMA was negligible, as previously demonstrated via histology[8]. Aortic valve leaflets were excised and rinsed in phosphate buffered saline (PBS) containing 1% penicillin and streptomycin and 0.5% amphotericin B. Rinsed leaflets were incubated in collagenase (type I, 150 Units/mL) in N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer with 0.36 mmol/L calcium chloride (pH = 7.4) for 20 minutes at 37 °C to digest the leaflet matrix. Leaflets were vortexed and lightly scraped on both surfaces to remove endothelial cells. Each leaflet was then pinned and secured (ventricular surface up) to a dissecting dish filled with black wax, using four needles (25 gauge) passing through only the fibrosa. A micro-knife (Fine Science Tools Inc; North Vancouver, BC, Canada) was used to gently cut into the spongiosa layer to remove the fibrous connections between the ventricularis and the fibrosa, while the ventricularis was gently grasped and pulled upward with fine dissecting forceps. Only the area below the nodulus of Arantius, where the tissue structure is distinctly trilayered, was separated and taken for cell isolation. Separated fibrosa and ventricularis sides of the selected region were incubated in hyaluronidase (type I-S, 2 mg/mL) in PBS for 1 hour at 37 °C. This step cleanly digested the spongiosa layer partially attached on both fibrosa and ventricularis sides. Cells from the spongiosa were released into the hyaluronidase, and subsequently discarded. The cleaned fibrosa and ventricularis layers

were then separately minced and incubated in collagenase (type I, 150 Units/mL, Sigma) in TES buffer for a second digestion step on a rocker at 37 °C for 2 hours. Undigested tissues were removed using a cell strainer (pore size 70 μm), and volumes of supplemented medium (Dulbecco's modified Eagle's medium; DMEM, with 10% fetal bovine serum and 1% penicillin and streptomycin) equal to the separate cell suspension volumes were added to each, and centrifuged at 284 × g. Supernatant was removed and the cells were re-suspended in supplemented medium and plated on tissue culture-treated polystyrene.

Fluorescent immunolabelling and analysis

Cells were fixed in 10% neutral buffered formalin overnight at 4 ºC, and subsequently permeabilized with 0.1% Triton X-100. Samples were blocked with 3% bovine serum albumin and incubated with monoclonal mouse anti-alpha SMA antibody (Clone 1A4, Sigma) followed by AlexaFluor 568 goat anti-mouse IgG (Invitrogen). Cell nuclei were counterstained with Hoechst 33258.

Devices maintained in a humidified environment required for immunocytochemistry tend to form condensation droplets beneath the composite diaphragms, which can hamper image collection. To obtain clear images, the underlying channel network was backfilled with deionized water in a vacuum chamber to eliminate optical distortion caused by water droplet condensation. Images were then collected using a 10× objective with a 1.6× in-line magnifier on a fluorescent microscope (Olympus 1X71, Olympus), with a CCD camera (QImaging Retiga 2000R, QImaging; Surrey, BC, Canada).

Myofibroblast differentiation was determined by counting the number of cells incorporating α-SMA into cytoskeletal stress fibers [9]. Cells containing three or more α-SMA positive stress fibers were considered as myofibroblasts, and expressed as a percentage of the total number of cells in the defined region of interest. For each condition 7-8 regions were assessed and averaged, and expressed as means ± standard deviations.

Supplementary References

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Supplementary Figures

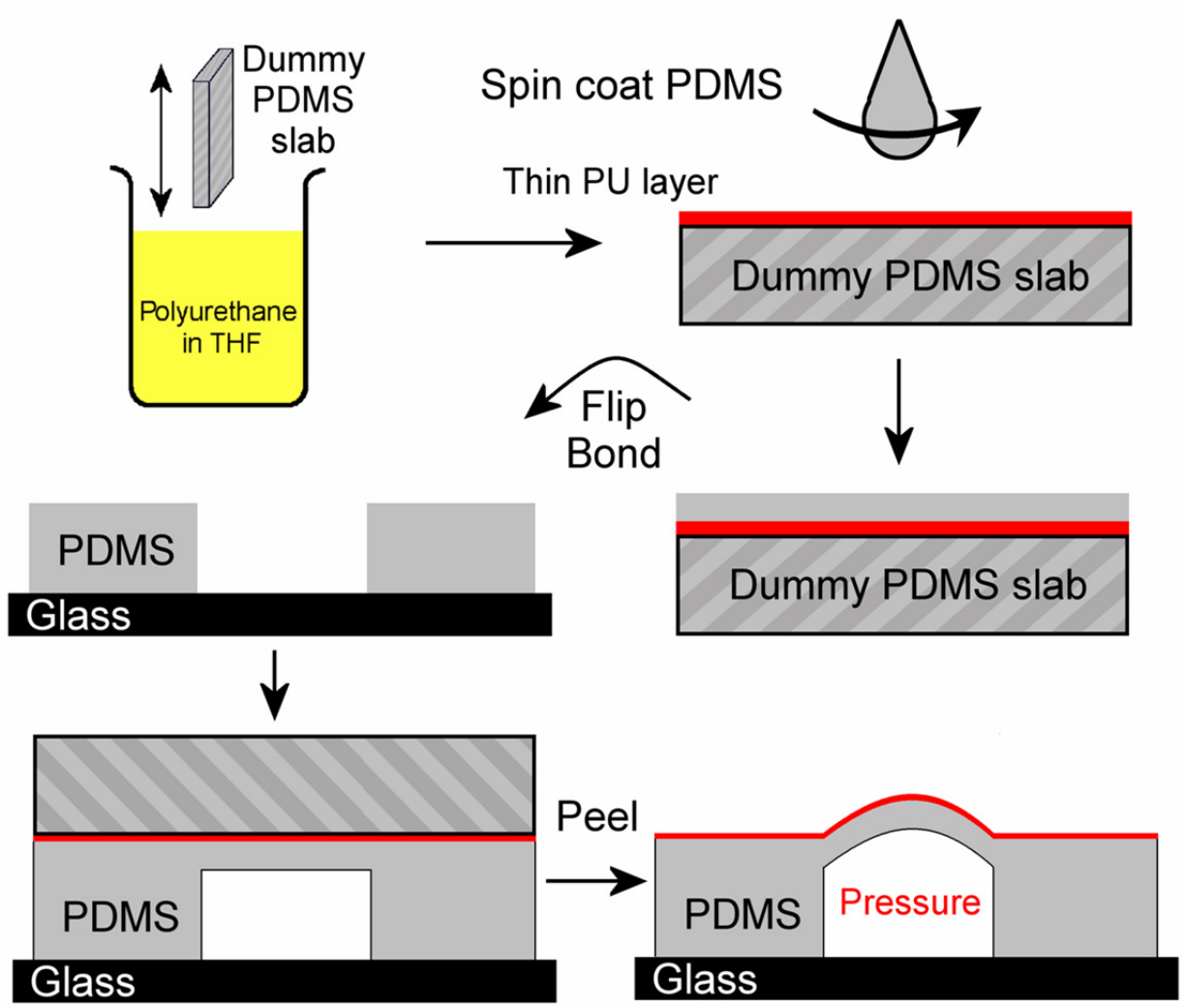

Figure S1. Fabrication process for the composite culture films and mechanical stimulation system.

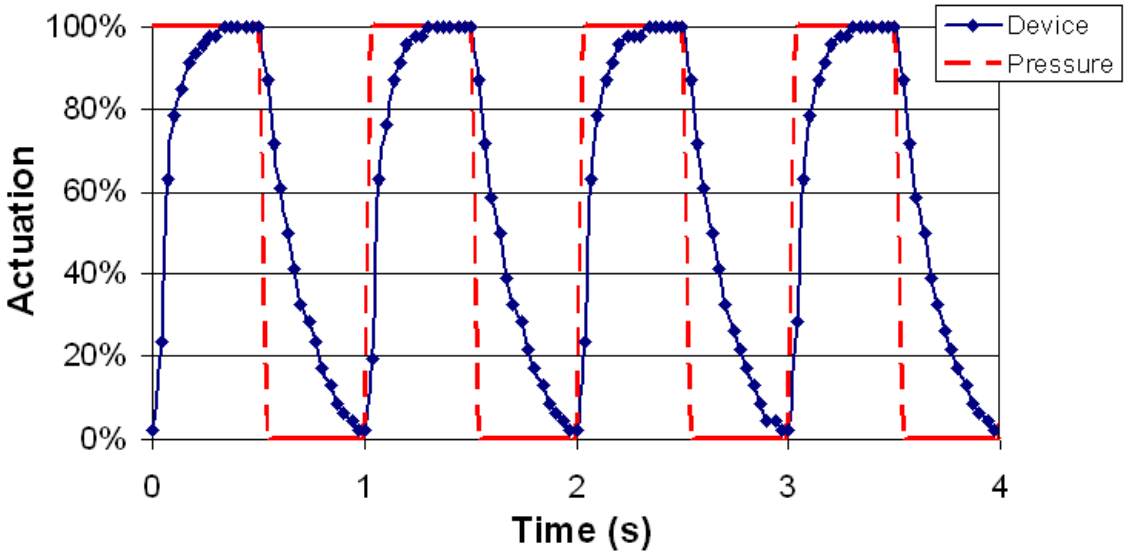

Figure S2. Dynamic characterization of device actuation in response to a 1 Hz cyclically applied square pressure waveform. Results obtained by video microscopy and subsequent image analysis of culture film deflection.