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A Miniaturized EHT Platform for Accurate Measurements of Tissue Contractile Properties

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***Abstract*— We present a wafer-scale fabricated, PDMS-based platform for culturing miniaturized engineered heart tissues (EHTs) which allows highly accurate measurements of the contractile properties of these tissues. The design of the platform is an anisometrically downscaled version of the Heart-Dyno system, consisting of two elastic micropillars inside an elliptic microwell with volume ranging from 3 down to 1μL which supports EHT formation. Size downscaling facilitates fabrication of the platform and makes it compatible with accurate and highly reproducible batch wafer-scale processing; furthermore, downscaling reduces the cost of cell cultures and increases assay throughput. After fabrication, the devices were characterized by nanoindentation to assess the mechanical properties of the pillars and transferred to 96-well plates for cell seeding. Regardless the size of the platform, cell seeding resulted in successful formation of EHTs and all tissues were functionally active (*i.e.* showed cyclic contractions). The precise characterization of the stiffness of the micropillars enabled accurate measurements of the contractile forces exerted by the cardiac tissues through optical tracking of micropillar displacement. The miniature EHT platforms described in this paper represent a proper microenvironment for culturing and studying EHTs. [2020-0130]**

***Index Terms*— Engineered heart tissue, microfabrication, nanoindentation, pluripotent stem cells, organs-on-chip.**

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I. Introduction

CARDIOVASCULAR diseases are the most common cause of death worldwide [1]. Efficient development of effective drugs for these diseases therefore has high societal priority. In some cases, animal and *in vitro* cell culture models fail to accurately recapitulate human physiology [2] and thus contribute to the high attrition rate in the current drug development pipeline [3]. A promising alternative to these models is enabled by the emerging technology of Organ-on-Chip (OoC) populated with human cells. OoCs are dynamic engineered microenvironments which mimic the native cellular environments, provide stimuli to cell cultures and facilitate functional readouts from cells or tissue constructs [4]. In particular, engineered heart tissues (EHTs) are potentially a valuable OoC approach to model human cardiac tissue *in vitro*. These and other microphysiological models have been reported to recapitulate human heart responses more closely than 2D cell cultures [5], [6], [18] and are thus a promising tool for cardiac drug testing and disease modelling. EHTs are formed by allowing cardiac cells to self-assemble in the presence of extracellular matrix (ECM)-proteins into a beating cardiac muscle strand around flexible anchoring points. Different shapes and numbers of the anchoring points and various sizes of the tissues have been reported [7]–[10].

Even though considerable progress has been made in recent years, EHT devices still have shortcomings: in particular, they 1) may require large numbers of cells for tissue construction and 2) lack proper mechanical characterization. Some of the existing EHT platforms [14], [19], [20] use more than 500,000 cells per tissue, which could confound large scale pharmaceutical screening studies, where the cost per data point is an important consideration. Furthermore, in many papers describing existing EHT platforms [8], [21]–[23] there is little or no data regarding the mechanical characterization of the systems. Proper mechanical characterization of the EHT platforms is crucial to accurately measure tissue contractile force. Furthermore, good characterization allows inclusion of the information like tissue size, position and thickness in the force calculations. The miniaturized EHT platform we report here addresses these issues as it requires less than 50,000 cells per data point and has been mechanically characterized in great detail.

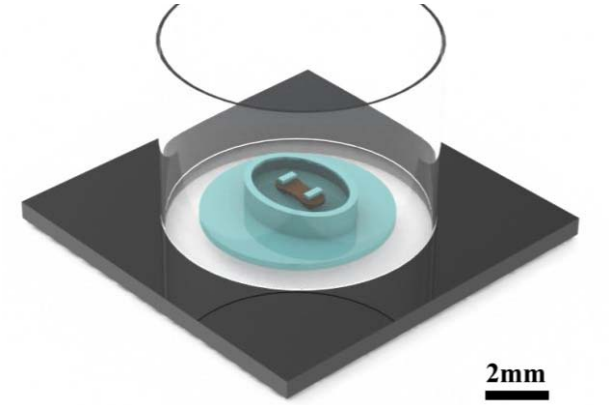

Fig. 1. A 3D model of the miniaturized EHT platform: a PDMS layer (blue) on the bottom of a cell culture well with the tissue (brown) formed around two pillars and confined by an elliptical sub-well. The transparent ring around the structure represents a single unit of a standard 96-well tissue culture plate.

II. Design of the Platform

The design of the PDMS platform for formation and growth of EHTs was based on the Heart-Dyno system [12] and consists of two elastomeric posts (micropillars) surrounded by an elliptic microwell of the same height as the micropillars. The micropillars are anchors providing structural support to the tissue, while the elliptic well confines a known volume of cell/ECM mixture from which the tissue is formed. A 3D representation of the design of the platform with the EHT formed around the micropillars is depicted in Fig 1.

The Heart-Dyno system was chosen as a starting point for the design since it is a relatively small PDMS-based platform, potentially compatible with batch cleanroom processing and integration of sensors. Furthermore, it is compatible with a standard, stand-alone 96-well plate, which allows its easy adoption by pharma in existing robotic pipelines as well as by research laboratories within academia. The main shortcomings of the Heart-Dyno system are its current manufacturing method by manual PDMS moulding, use of relatively high cell numbers, and optical-only readout. By downscaling the original platform and making it compatible with batch cleanroom-based fabrication, all of these shortcomings can be addressed.

The geometry of the original Heart-Dyno system was anisometrically downscaled to reduce the number of cells required per EHT and to make the fabrication compatible with batch wafer-level processing, while retaining the original pillar stiffness. The Heart-Dyno's original volume of 3.5 μL, containing approximately 50,000 cardiac cells in the confining microwell, was scaled down respectively to 3, 2 and 1 μL in three distinct versions of the platform. The anisometric scaling ensured that the stiffness of the micropillars remained constant in all cases, so that the tissues could effectively experience the same load in all chip sizes. The dimensions of all three platforms and of the Heart-Dyno system are shown in Table I.

Prior to fabricating the devices, theoretical and numerical modelling was performed. A model of a bending cantilever was used to simulate the behavior of the elastic micropillars when the force is applied by the contracting tissue (Fig. 2). Numerical simulations were performed in COMSOL Multiphysics, using a finite element method. The force applied by

TABLE I
Design Parameters for the EHT Platforms

Symbol	Description	Heart-Dyno	3 µL platform	2 µL platform	1 µL platform
w [µm]	micropillar's width	500	487	427	352
h [µm]	micropillar's height	200	210	176	151
L [µm]	micropillar's length	700	700	560	460
d [µm]	distance between the micropillars	1	0.97	0.85	0.7
a [mm]	semi-minor axis of elliptic well	1.1	1.05	0.93	0.76
b [mm]	semi-major axis of elliptic well	1.5	1.43	1.27	1.03
t [µm]	thickness of the elliptic wall	/	200	200	200

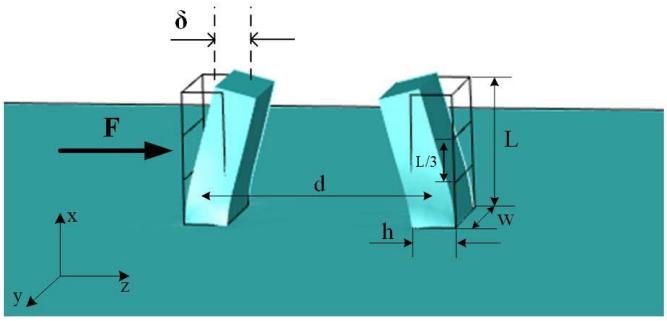

Fig. 2. Schematic of the mechanical model of bending pillars under an applied distributed load.

the tissue was modelled as a load distributed on a rectangular area in the middle of the micropillars for all three sizes. The width of this rectangular area was set to 1/3 of the micropillar's height to approximate the estimated tissue thickness. The assumption on the position of adherence of the tissue along the micropillar's height was deduced from literature [12]. Due to the applied force, the elastic micropillars bend, and by quantifying the displacement of the micropillars while knowing their mechanical properties, it is possible to estimate the applied force. Whereas bending cantilever theory assumes rigid support for the cantilever, the COMSOL Multiphysics model was implemented to more faithfully reproduce the realistic situation, where micropillars are attached to the elastic PDMS substrate.

Equation 1 shows the relationship between the tip displacement of a bending micropillar δ and the applied force F, represented as the micropillar stiffness k:

$$F = k \cdot \delta \tag{1}$$

In the following equations the distributed load was approximated with a point force applied in the middle of a micropillar:

$$k = \frac{Ewh^3}{2\left(-x^3 + 3Lx^2\right)} \tag{2}$$

$$k \sim \frac{4Ewh^3}{5L^3}\left(c_1 - c_2\frac{x}{L} + c_3\frac{x^2}{L^2} - c_4\frac{x^3}{L^3}\right) \tag{3}$$

Here, E is Young's modulus, x is the position of the applied force along the micropillar length, c_i's are constant fitting

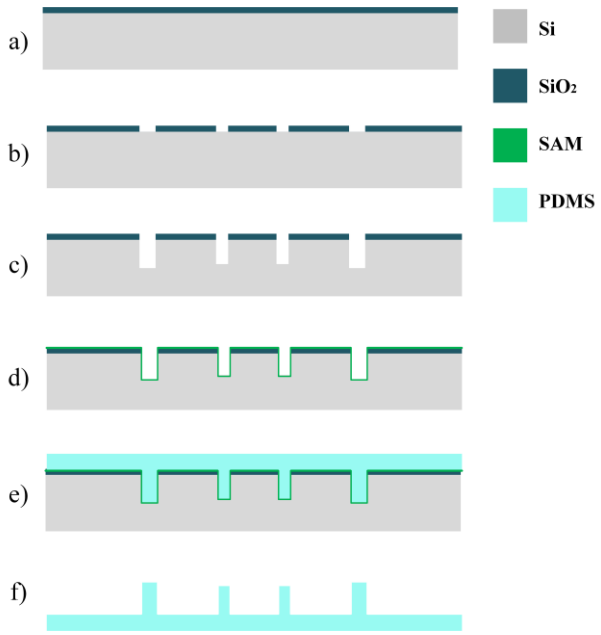

Fig. 3. Sketch of the fabrication process of the PDMS-based EHT platform. a)-b) definition of SiO_2 hard mask; c) DRIE of micropillar moulds; d) SAM deposition; e) spin-coating of PDMS; f) demoulding of the final EHT platform.

coefficients, and w, h and L are width, height and length of the micropillar, respectively. As a starting point in the design, the stiffness in the middle of the polydimethylsiloxane (PDMS) micropillars (*i.e.*, for $x = L/2$) was chosen to be 14 μN/μm, as in the original Heart-Dyno system. This value was only used in the analytic equations of the bending cantilever theory in order to guide downscaling of the micropillars. The dimensions of the micropillars and the volume of the well were scaled down, while keeping the stiffness constant for the 3, 2 and 1 μL PDMS platforms, resulting in three different chip sizes with micropillar lengths of 700, 560 and 460 μm, respectively (Table I).

III. Fabrication

The three different-sized EHT platforms were fabricated at wafer-level, using silicon-based micromachining and polymer moulding techniques (Fig. 3). The fabrication started with deep reactive ion etching (DRIE) of a 4-inch, 1 mm-thick, single side-polished Si wafer, using SiO_2 as hard mask, to accurately define deep holes for PDMS moulding of the micropillars (Fig. 3a-c). Prior to PDMS spin-coating the wafers were functionalized with a perfluorinated anti-adhesion self-assembled monolayer (SAM) to ease PDMS removal (Fig. 3d). The anti-adhesive layer was formed by evaporation of a few droplets of perfluorooctyl-trichlorosilane in a vacuum chamber for two hours. As a result, the silicon dioxide-covered surface of the Si wafer became highly hydrophobic, which was confirmed using water contact angle measurements (117°).

An uncured PDMS mixture of Sylgard 184 elastomer and its curing agent (ratio 10:1) was spin-coated using a two-step process (Fig. 3e). The first step was used to ensure the deep holes in the wafer were filled with PDMS. The second spin-coating step defined the final thickness of the PDMS substrate supporting the pillars (~300 μm). In between the two spin-coating steps, the wafers were degassed under vacuum to make sure that the PDMS reached the bottom of the holes without trapping air. The PDMS was then cured in an oven at 85°C for 30 minutes and peeled off the wafer easily (Fig. 3f). After demoulding, the platforms were manually cut out of the PDMS substrate and transferred to the bottom of the wells in a 96-well plate.

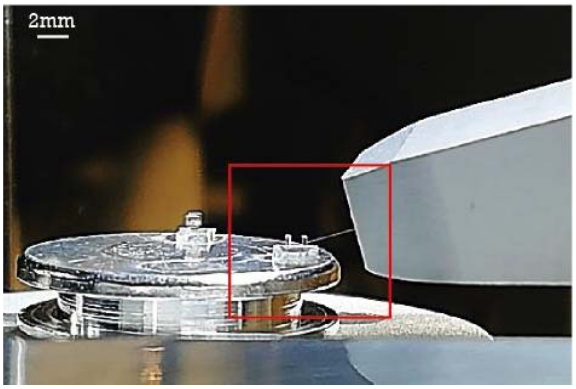

Fig. 4. Setup for the mechanical characterization of the elastic micropillars using nanoindentation. The red frame highlights a single pair of PDMS micropillars cut out from the wafer-sized PDMS substrate, as well as the sensor tip of the nanoindentation tool.

IV. Mechanical Characterization

A FemtoTools Nanomechanical Testing System (FT-NMT03) was used for the mechanical characterization of the fabricated PDMS platforms. The nanorobotic system is designed to test mechanical properties of micro/nanostructures and is equipped with a micro-force sensing probe with silicon tip that can apply a specific force and measure the displacement of the tip. The PDMS substrate with micropillars was mounted onto a holder next to the sensing probe. The silicon tip was positioned at a predetermined height along the micropillar with nanometer precision, as shown in Fig. 4.

During the measurement, force was applied to the micropillar by the silicon tip and the displacement of the tip was continuously measured with a high resolution piezo-scanner. After a certain displacement of the probe or after the defined force limit had been reached, the micropillar started returning to the initial position, thereby pushing the silicon tip backwards. This movement of the tip was again measured with piezo-sensors and the force-displacement curve was obtained. The stiffness of the micropillar is represented by the slope of the force-displacement curve during the micropillar's return to its initial position. Measurements were performed at five distinct micropillar positions for all three different platforms.

Comparison between measured data and data from numerical simulations showed curves with very similar slope and only slightly shifted apart (Fig. 5). The same trend and very similar graphs were obtained for all three micropillar sizes, therefore only results for 700 μm-long micropillars are shown. In order to fit the simulations to the measured values, an analytical function was introduced to precisely describe the model of bending pillars on the elastic substrate, which could not be approximated using only (2). The fitting function in (3) was

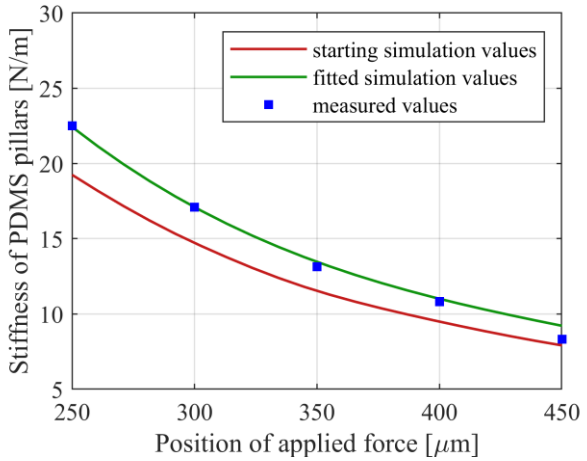

Fig. 5. Stiffness analysis for 700 μm-long PDMS pillars. Data from finite element simulations (red) compared and fitted to the measured data (blue) to estimate the Young's modulus of PDMS for the EHT platform.

generated by applying Taylor's expansion to (2) for $x = L/2$ and finding the missing coefficients c_i using the least square difference fitting tool in Matlab®. Consequently, the value of the Young's modulus of PDMS was calculated to be 1.7 MPa in all three cases. which is consistent with the use of the same PDMS mixture for all platform types. This value of E was used in the further contractile force estimation.

V. Inclusion of Cardiac Cells

Human-induced pluripotent stem cell (hiPSC)-derived cardiomyocytes and hiPSC-derived cardiac fibroblasts (4:1 ratio) [18] were mixed in a collagen/Matrigel-based ECM gel and seeded in the three different chips. As a source of differentiated cardiomyocytes and fibroblasts, the LUMC0020iCTRL-06 hiPSC-line was used. For the ECM gel mixture, we used acid solubilized collagen I and growth factor reduced Matrigel (2.6 mg/ml collagen I and 9% Matrigel). The number of cells used per chip was approximately 47000 (volume: 3 μL), 31000 (2 μL) and 16000 (1 μL). After pipetting the gel mixture into the PDMS microwells, the 96-well plate was shortly centrifuged to achieve level and homogenous distribution of the mixture throughout the well, to make sure that the tissue formed around midway along the micropillars, and to remove any trapped air. Self-assembly and compaction of the tissue around the micropillars was visible within 24 hours and spontaneous contraction of EHTs started within 72 hours. After approximately 2 hours of gel solidification in a humidified incubator, wells of the 96-well plate were filled with 200 μL formation medium, preventing the tissues from drying out. Formation medium was used during the first 3 days of cell culture and maturation medium thereafter, according to the Heart-Dyno protocol [12]. Cells were kept in a humidified incubator for the whole time and medium was refreshed every 3 to 4 days. All tissues successfully formed regardless of the chip size, demonstrating that the miniaturized design of the elastic micropillars is suitable for self-assembly of different-sized tissues. Representative images of the three different tissue sizes formed around the micropillars in the PDMS

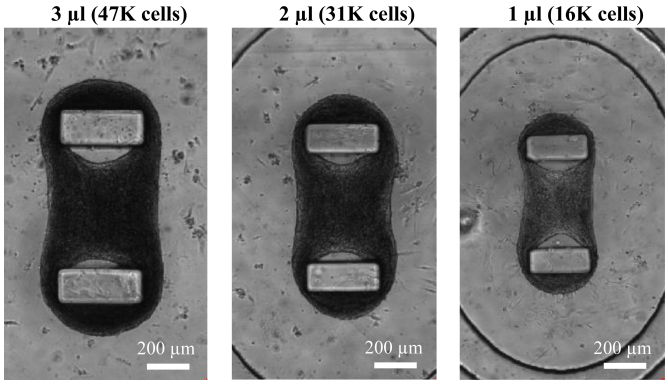

Fig. 6. Representative images of the three different sizes of hiPSC-based cardiac tissues successfully formed around PDMS micropillars in the miniaturized. EHT platforms, taken on day 4 after chip seeding.

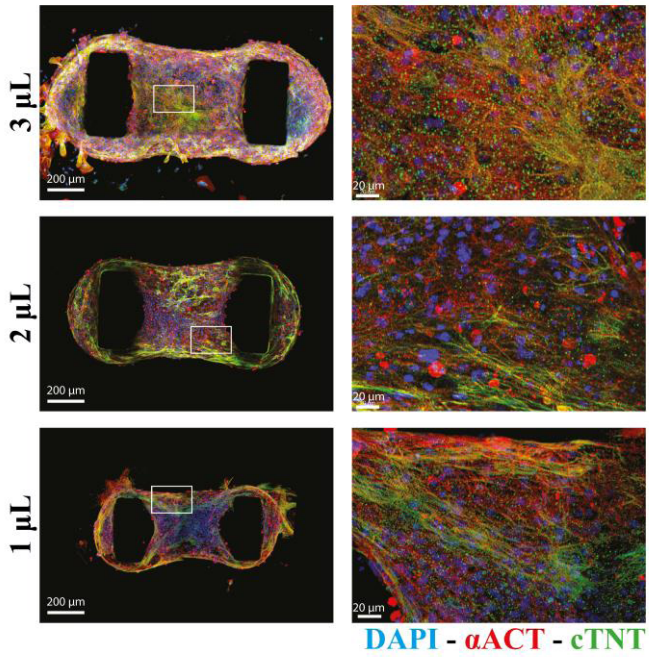

Fig. 7. Immunostaining of the three tissue sizes for cardiac specific markers alpha-actinin (αACT) and cardiac troponin T (cTNT). Nuclei were stained with DAPI.

platforms are shown in Fig. 6. Brightfield images were taken live on day 4 since the beginning of the experiment with a Nikon Eclipse Ti2.

Immunostaining of the intracellular sarcomeric structures demonstrated the cellular organization around the flexible PDMS micropillars and sarcomere orientation. The tissues were stained whole–mount and imaged with an Andor Dragonfly 500 on day 19 from the beginning of the experiment. Sarcomeres were stained using cardiac specific antibodies against alpha-actinin (ACTN2) and cardiac troponin T (TNNT); nuclei were stained with DAPI (Fig. 7).

During the 18 days following formation, contractile activity of the tissues was recorded three times per week and analyzed using custom made software for tracking movement of the PDMS micropillars. Recordings of tissues were taken on a Nikon Eclipse Ti with a custom-built environmental chamber at 37 °C and 5% CO_2 and equipped with a high-speed camera

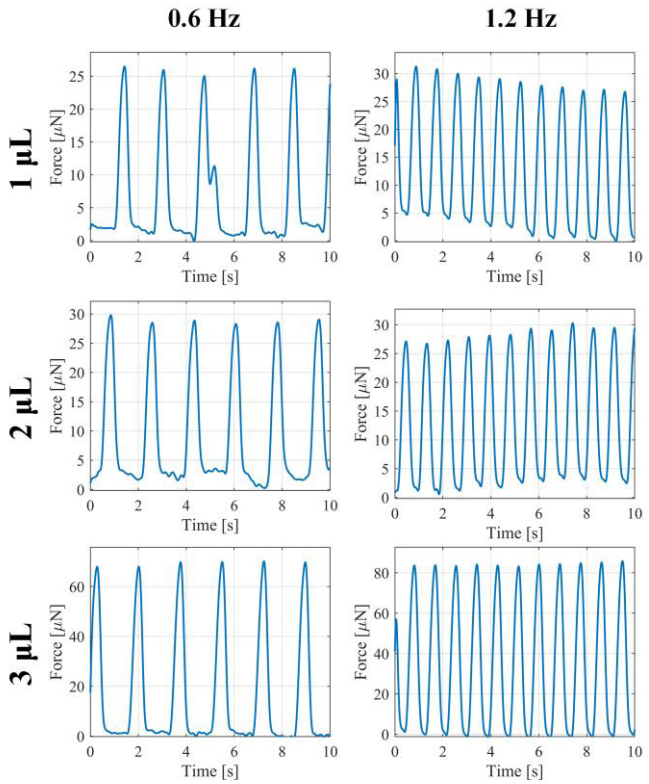

Fig. 8. Contraction force of 1, 2 and 3 μL-derived EHTs, paced with 0.6 and 1.2 Hz, estimated using software for tracking the displacement of pillars.

(Thorlabs). For each datapoint, 10 seconds of footage was saved at 100 frames per second. Using image processing techniques within the Computer Vision Toolbox in Matlab® the position of the rectangular tip of the micropillars was tracked throughout all of the frames in recorded videos. Movement of the rectangular tips of the micropillars was measured in pixels and later converted to micrometers. Displacement of the tip of the micropillars is proportional to the tissue's contractile force, which was further calculated using the stiffness of micropillars k. Using (2), and assuming that the tissues adhered to the middle of the micropillars, the contraction force of EHTs could be obtained. Measured force values are lower than in [12], in all three platforms, possibly due to smaller numbers of cells per tissue. For the detailed analysis of measured contractile force and its comparison among different sized tissues, more data from repeated experiments are required in the future.

Furthermore, to test the response of the tissues to the electrical stimulation, the EHTs were paced with external platinum electrodes. Voltage in the form of biphasic rectangular pulses (40V peak-to-peak) was applied to the tissues, with frequency ranging from 0.5 to 3 Hz. It was seen that all EHTs followed the pacing frequency up to 2.4 Hz. Output of the software for tracking micropillar motion in the case of pacing at 0.6 and 1.2 Hz on day 11, for all three chip sizes, is shown in Fig 8.

VI. Conclusion

We presented here three miniaturized and well-characterized PDMS platforms suitable for EHT formation. The design of the PDMS platform for supporting cardiac tissue formation is a downscaled version of the Heart-Dyno with three different chip volume sizes: 3, 2 and 1 μL. The PDMS platforms containing micropillars with rectangular cross-section within an elliptic microwell were fabricated by combining wafer-scale silicon- and polymer-based processing. After fabrication, chips were mechanically characterized using nanoindentation and finite element simulations, which allowed accurate measurements of the contractile force of the tissues. For cell seeding, the chips were transferred to a standard 96-well plate. Mixtures of hiPSC-derived cardiomyocytes and hiPSC-derived cardiac fibroblasts were seeded in all three chip sizes. Tissues formed successfully in all wells and were functionally active. Contractile properties of the tissues were evaluated by optically tracking the movement of PDMS pillars. The tissues were viable and functional for at least 18 days. Experiments with electrical stimulation showed that miniaturized EHTs followed the pacing frequency up to 2.4 Hz.

The main limitation of the system developed here is that the exact position of the tissues and their thickness could not be measured during the experiments. Obtaining these two parameters in the future will allow us to perform comparisons between contraction forces generated by differently sized EHTs. Future experiments will also provide data for more comprehensive analysis of the functional differences between the three sizes of the tissues.

Through this preliminary research we demonstrated that downscaling of the existing Heart-Dyno system is possible and convenient. The downscaling limit in this work was set by the smallest cell medium volume that could be accurately pipetted manually (*i.e.*, 1 μL). Since fundamental problems were not encountered while downscaling, even smaller devices might be fabricated in the future if handled by pipetting robots or other precision cell culture technologies. By showing that PDMS platforms could be made using scalable fabrication methods, we came a step closer to high-throughput cardiac drug screening and disease modelling using OoCs. Finally, cleanroom-based fabrication opens many possibilities for further upgrades, including integration of electrodes and of sensors for platform automation.

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