



Method Article

The method to generate pulsatile circulatory fluid flow using microfluidics



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A B S T R A C T

Microfluidic chips provide versatile tools to mimic the biological effect of blood flow on pluripotent stem cells (PSC). This paper presents methods for the use of microfluidics to model embryonic circulation using differentiated PSC. Pulsatile circulatory flow is created with a microfluidics device with pressure-driven microvalves and ventricles. Silicone rubber devices are cast from moulds manufactured using standard and 3D laser lithography. The surface chemistry is modified to support the growth of human umbilical vein endothelial cells and pluripotent stem cells. Pulsatile circulatory fluid flow can be applied at specific stages of cell differentiation with direct observation of cellular responses by time-lapse fluorescent microscopy.

- Replicable manufacturing protocol of lab scale microfluidic device generating pulsatile fluid flow mimicry embryonic blood circulation.
- Integration of human cell lines on microfluidic chip.

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A R T I C L E I N F O

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Resource availability	

Method detail

Background

The emerging and rapid development of microfluidic technologies have led to the development of microdevices that mimic tissue microenvironments. The manufacture of devices with submicron resolution facilitates precise control of fluid flow and metabolite gradients, providing a new methodology to study the influence of a microenvironment on tissue development [1–3] with application in the study of human embryonic development using pluripotent stem cell culture [4–8].

Here we describe significant improvements in the design, manufacture and biocompatibility of a cardiac-like flow generator for long term imaging of endothelial responses [9]. The microfluidic device mimics the function of a cardiac pump connected to microcirculation for *in vitro* study of endothelial and stem cell responses to fluid dynamics. The design has the following elements: i) simulation of pulsatile circulatory flow and the phases of the cardiac cycle, ii) a closed-loop microchannel network to generate a range of shear rates, connected to the outlet and inlet of the ventricle, iii) surface chemistry to facilitate cell attachment and endothelialisation of the microcirculatory network and iv) long-term visualisation of stem cell dynamics by time-lapse microscopy. We describe several innovations that facilitate streamline manufacture and enhance biocompatibility including precision manufacture of Unger microvalve [10] moulds using 3D stereolithography, chemical bonding of polydimethylsiloxane (PDMS) to polystyrene for support of PSC lines, and robust replication of devices using Zeonor® 1060R replicas of silicon master moulds.

Method

Carry out all procedures at room temperature unless otherwise specified.

Design of microfluidic pulsatile flow generator

In this revised design, the microfluidic device (Fig. 1a) has three layers: a pneumatic layer (blue channels, top), a thin PDMS fluid layer with Unger microvalves [10] (Red, middle) connected to rectangular cross-section channels (green, middle) and a polystyrene slide that is bonded to the fluid layer (Grey, bottom). The pneumatic layer is actuated by 4 independent solenoid-driven pneumatic pressure sources (1–2 bar). Application of pneumatic pressure to heart inlet and outlet pneumatic ports results in full occlusion of inlet and outlet valves because the flow channel has an arc-shaped cross-section [10].

The ventricle is formed by 16 parallel rectangular channels (blue layer, width, 350 µm; height, 100 µm; length, 10.8 mm; total volume, 6.05 µL). These are connected to the heart inlet and outlet channels by a bifurcating network. A rectangular-shaped pneumatic channel (shown in green, width, 250 µm) connected to the ventricle control pressure port is bonded on top of the fluidic layer. Application of pressure to the overlying pneumatic channel ejects fluid from the ventricle. The cross-sectional profile of the ventricle flow layer is rectangular, so it is impossible to fully occlude this space.

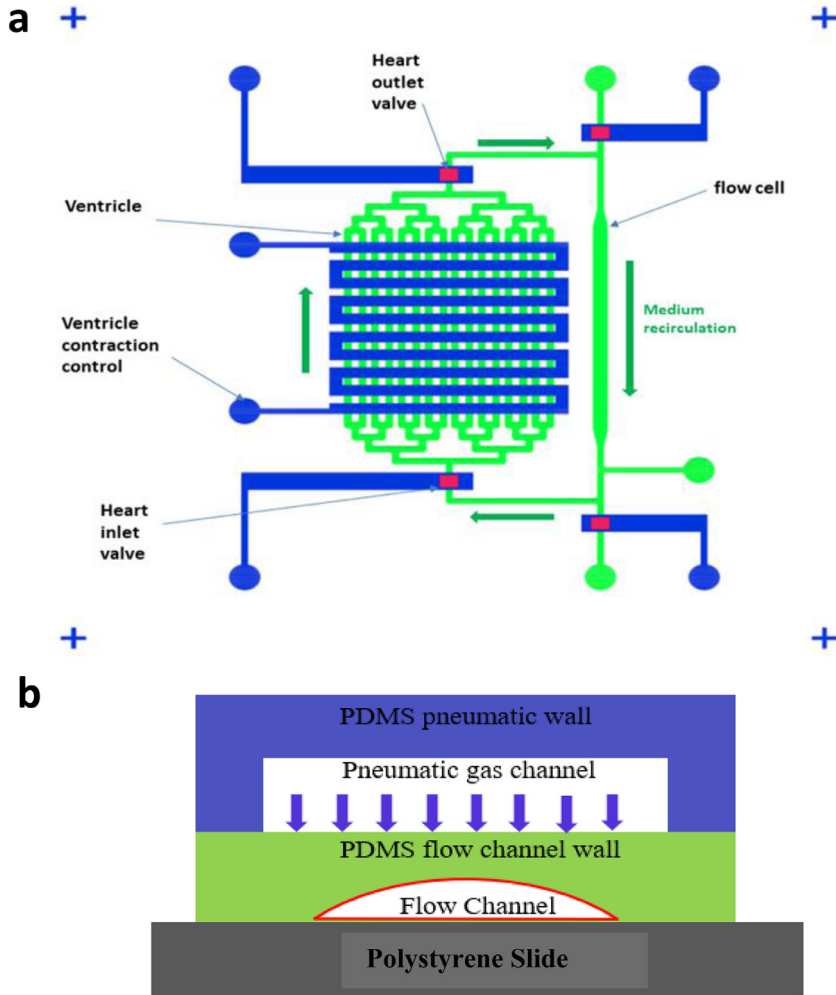


Fig. 1. Design of the cardiac device shown in the XY plane (a) and cross-section of the valve in the XZ plane (b). a. Blue channels are housed in the top layer, labelled as the hydraulic-pneumatic channels; Green channels are in the second (middle) layer denoting the fluid channel and cell seeding chambers; Red rectangles are the semicircular valves; b. Cross sectional view of the three layered cardiac device design. Pneumatic channel wall is in blue. Fluid layer channel wall is in green. The valve is outlined in red. Polystyrene slide is in grey.

A custom-built pneumatic control system [9] is employed to sequentially drive the ventricle and heart valves to mimic the cardiac cycle with working air pressure range from 1.2 bar to 2 bar. The artificial cardiac cycle consists of a) opening of the heart outlet valve and closure of the heart inlet valve, b) contraction and release of the ventricle by applying a pneumatic pressure pulse via the ventricle control, c) closure of the heart outlet valve and opening of the heart inlet valve.

The flow cell (width, 700 μm ; height, 100 μm ; length 10.8 mm) is connected to the ventricle via a 200 \times 100 μm rectangular cross-section channel. The valve channel shown in red (width, 500 μm ; maximum height, 100 μm ; length, 500 μm) has arc-shaped cross-section (Fig. 1b). The microcirculatory system is connected via the head pressure port to a manometer pressure set at around 15 cm H₂O (1470 Pa). This simulates downstream venous pressure and allows the volume of the circulatory system to vary without changes in downstream pressure. The cells and reagents are

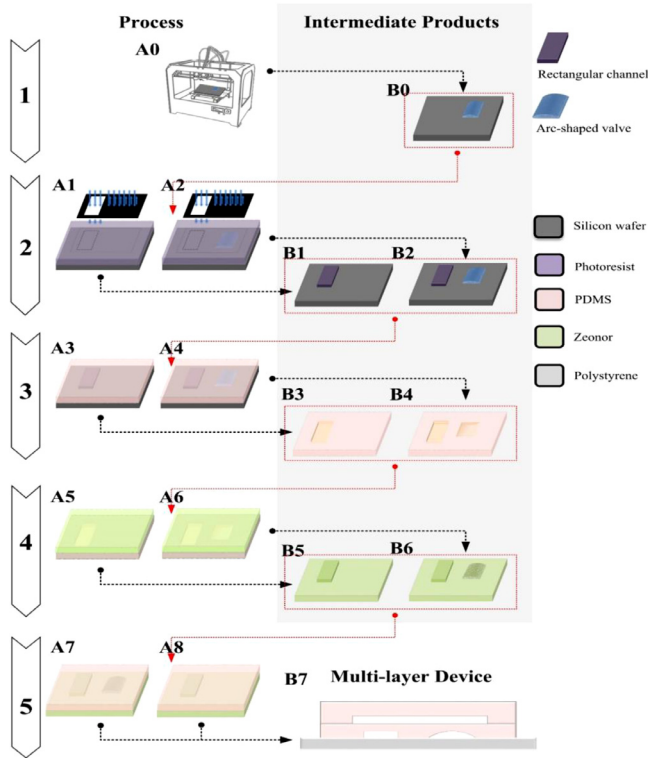


Fig. 2. Schematic drawing showing the fabrication of the pulsatile flow generator. A mould; B fabricated part (which may serve as a mould in a downstream process). Step 1: 3D stereolithographic printing. Step 2: Standard photolithography. Step 3: PDMS casting to generate PDMS negative impression. Step 4: Hot-embossing to create Zeonor® replica of device pattern. Step 5: PDMS casting off Zeonor® mould followed by bonding of PDMS layers onto polystyrene surface. A1-A7 denotes process for generating pneumatic layer of the device. A2-A8 denotes the process for generating fluid layer with semicircular-valve pattern. B1-B6 are intermittent products during fabrication, while B7 is the final assembled two-layer PDMS device.

loaded from the inlet port into the artificial circulatory system. The cell culture media circulates in the device as shown by the green arrows (Fig. 1a).

Manufacture, assembly, and operation of microfluidic cardiac flow platform

A brief fabrication protocol of the pulsatile flow generator is illustrated in Fig. 2.

Multiple layered patterns were first generated on a silicon wafer with the desired dimensions by combining standard photolithography with 3D stereolithographic laser printing (Fig. 2 Step 1&2). Semi-circular valves and alignment markers were 3D printed onto 4-inch silicon wafers (Nanoscribe GmbH, ANFF-Q, frequency-doubled, fiber laser source with a centre wavelength of 780 nm, 100 mW laser power, high scan speed 100,000 $\mu\text{m/s}$, stage scanning mode, 1.5 μm slicing, SU8 photoresist), followed by wafer cleaning with oxygen plasma etching for 10 min. Inside a fume hood, KMPR 1050 negative photoresist was spin-coated using a dedicated resist spinner on the valve-patterned wafer at 2250 rpm for 30 s starting with an angular acceleration of 300 rpm/s. After 20 min of soft baking at 100 °C, the KMPR-coated silicon wafer was aligned with the fluid channels of the chrome glass mask on an MA6 mask aligner (ANFF-NSW, UNSW) and exposed to UV light (365 nm, 20 mW/cm²) for 60.9 s followed by hard baking at 100 °C and development using propylene glycol monomethyl ether acetate (PGMEA; SU-8 developer). This procedure allowed us to fabricate fluidic channels with a height of 100 μm that were precisely aligned with the printed valves (Fig. 2). Fabrication of the silicon mould for the pneumatic layer used a similar photolithography procedure

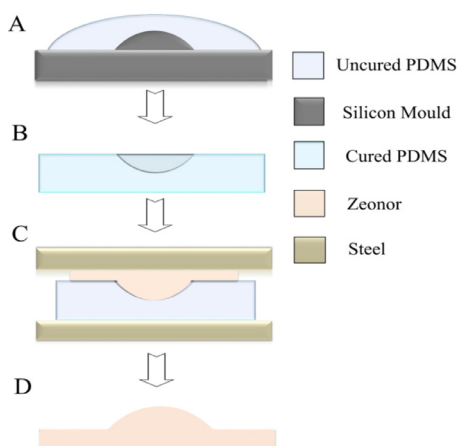


Fig. 3. Illustrations showing the soft embossing process. A. Casting of uncured PDMS on the master silicon mould; B. Cured PDMS with valve patterns peeled of a silicon waive with KMPR mould. C. Hot-embossing of thermoplastic Zeonor® with a PDMS negative mould sandwiched between two heat conductive steel plates. D. Zeonor® -replica.

but a higher spin-coating speed of KMPR 1050 at 3000 rpm and lower UV exposure of 48.7 s to create pneumatic channels with a height of 50 μm .

A negative impression of the mould was fabricated by soft embossing of PDMS on silicon moulds (Fig. 2 Step 3 and Fig. 3). Master moulds were silanised with (heptadecafluoro-1,1,2,2-tetrahydrodecyl) trimethoxysilane (Sigma-Aldrich Corporation, St. Louis, Missouri, United States) for at least 20 min in a desiccator. A 'soft' negative impression of the masters was cast using silicone elastomer polydimethylsiloxane (PDMS) (SYLGARD 184 Silicone Elastomer Kit, Dow Corning, Midland, Michigan, United States) with a base to curing agent ratio of 10:1 in a petri dish. The mixture was degassed in a vacuum chamber for 60 min to suppress the formation of air bubbles during curing in a standard laboratory oven at 60 °C overnight. PDMS moulds were peeled off the master wafers. Additional alignment posts were added to four corners of the lithographic pattern of the fluid layer. This was achieved by punching 2 mm straight holes into the PDMS negative replica at the locations of the lithographic alignment markers with a biopsy punch. Now the negative moulds are ready for use in the replication of the silicon master moulds using Zeonor® 1060R.

Zeonor® thermoplastic replicas of the silicon mould by hot-embossing off the negative PDMS impression with thermoplastic material (Fig. 2 Step 4 [11]). The hot embossing process was performed on a hydraulic press with heating – Carver Hot Press (Fig. 4a). A 'soft' negative impression of the master prototype silicon mould mentioned in the last step was cast using the silicone elastomer, PDMS. The temperature of both the top and bottom platens (both 150 × 150 mm) of the Carver Hot Press were set to 160 °C and stabilised for 30 min. This is 60 °C above the glass transition temperature of the thermoplastic Zeonor® 1060R ($T_g = 100$ °C). This high moulding temperature decreases the viscosity of the molten thermoplastic so that it easily penetrates negative mould cavities. A monolayer of Zeonor® 1060R pellets were carefully placed onto the patterned PDMS mould using a tweezer, covering about 60% of the mould area and supported by a top and bottom steel plate. Once temperature was stable at 160 °C, the sample was loaded onto the Carver Hot Press by placing the sample into the bottom platen and sliding it into the centre (Fig. 4b). The moulding temperature must be kept stable during the embossing process to generate consistent and uniform replica [12]. Therefore the bottom platen and sample were then elevated using the hydraulic pump until contact was made with the top platen and then held in this position for 1 min to allow preheating of the thermoplastic from the upper platen before pressure was applied. Force was then applied to the molten thermoplastic by the mobile plate while the gap between platens decreased as the thermoplastic melted. The applied pressure between two platens was increased at 10 lb per second until 300 lb of pressure was reached and maintained for 15 min. Consequently, the mould and

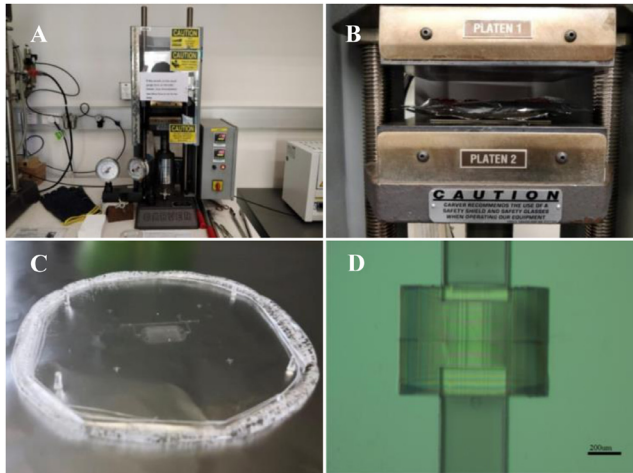


Fig. 4. Soft embossing of Zeonor® using a Carver hot press a. Carver Hot Press machine in operation; b. two steel platens loaded with PDMS negative mould and Zeonor® pellets; c. Photograph of flat Zeonor® part created by hot embossing; d. Microscopic image of the valve and fluid channels on the silicon wafer.

molten polymer were unloaded from the carver hot press and cooled passively with a heatsink. The solidified thermoplastic cardiac device replicates were easily peeled off the PDMS elastomeric mould without fracture or defect (Fig. 4c-d). The previously punched 2 mm holes in the PDMS mould of the fluid layer were filled with molten thermoplastic then solidified generating alignment posts in the Zeonor® replica of the fluid layer.

PDMS device layer casting off of the Zeonor® moulds and layer bonding (Fig. 2 Step 5). A PDMS membrane of 150 μm was produced by spin-coating mixed but uncured PDMS at 500 rpm for 2 min or at 800 rpm for 1 min. The Zeonor® mould of the fluidic layer with alignment posts was cleaned with 4% IPA (2-propanol) and water, followed by drying with compressed air. The Zeonor® mould was then silanised with (heptadecafluoro-1,1,2,2-tetrahydrodecyl) trimethoxysilane (Sigma-Aldrich Corporation, St. Louis, Missouri, United States) for at least 20 min in a desiccator. The PDMS prepolymer and curing agent were weighed out at 10:1 ratio in a weighing cup and mixed thoroughly. The mixture was degassed in a vacuum desiccator until all bubbles dissipated (~20 min). The pre-treated Zeonor® mould was balanced on a spinner. A small amount (~10 g) of the degassed PDMS mixture was delivered on the mould with a 5 ml wide-bore pipette and allowed to spread for 30 s. The spinner was set at 500 rpm with 50 rpm/s acceleration for 2 min followed by 5 min resting to allow the spin-coated PDMS to reach mechanical equilibrium. The mould was then baked on a shelf-balanced oven at 70 $^{\circ}\text{C}$ for two hours before use. This step enables the creation of a relatively thick PDMS membrane at around 120 μm with the membrane thickness at the valve site at only 20 μm . Fluidic connection ports on PDMS were punched with 1.25 mm biopsy punch. PDMS layers were bonded together by oxygen plasma treatment (PE-250 Plasma etcher, Denton vacuum, USA) at 50 mW for one minute. After plasma etching, the PDMS pneumatic layer was immediately pressed down towards the thin PDMS fluid layer on Zeonor® mould to bond the two layers permanently, removing any air bubbles trapped in between the two layers. The two layers were weighted (~55 $\times 10^3 \text{ N/m}^2$) for 5 min and then baked in a 70 $^{\circ}\text{C}$ oven for 30 min before separation from the Zeonor® mould. Notably, the use of Zeonor® mould and alignment posts streamlined the device manufacturing process by enabling easy and precise alignment of the two patterned PDMS layers.

PDMS layer and polystyrene non-tissue culture plates were bonded by chemical gluing [13]. The surfaces of the PDMS replica and flat polystyrene slide (Cat No. L3536-100EA, Sigma Aldrich, Merck KGaA) were treated with air plasma (50 W) for 1 min to generate hydroxyl groups on the surfaces for both substrates. 1% (v/v) aqueous solutions of APTES ((3-Aminopropyl) triethoxysilane

(Cat No. 440140, Sigma-Aldrich, Merck KGaA) and GPTES ((3-Glycidioxypropyl) triethoxysilane (Cat No. 50,059, Sigma-Aldrich, Merck KGaA) were reacted with the two surfaces for 30 min to generate surface amine and epoxy functionalities, respectively [13]. After washing and drying, the silane-treated surfaces were bonded overnight with pressure ($\sim 55 \times 10^3 \text{ N/m}^2$) at room temperature to form a strong amine-epoxy bond. Notably, the growth and differentiation of human pluripotent stem cell lines depend on culturing technique and substrates. Stem cell differentiation protocols were developed on a polystyrene surface. Chemical bonding of PDMS to polystyrene allowed us to easily inoculate stem cells onto the preferred culture substrate (polystyrene) and enabled a direct comparison of the effects of fluid flow on stem cell culture with conventional static culture on a polystyrene substrate.

Assembly of the microfluidic pulsatile flow generator and cell loading.

- (1) Prepare $2 \times 5 \text{ cm}$, $1 \times 50 \text{ cm}$, $4 \times 10 \text{ cm}$ Tygon tubings (0.030" \times 0.090" OD, Cat No. 1012029, John Morris Group, Australia) and 7×20 -gauge blunt needles (Cat No. 7018178, Nordson Australia) soaked in 70% ethanol.
- (2) On one side of each Tygon tubing, slip the needle end of the 20-gauge blunt needles for a snug friction fit.
- (3) Push the 5 cm long Tygon tubings into the cell inlet and outlet ports to form a friction fit between the device port and the outer diameter surface of the tubing. Push the 50 cm tubings into the pressure head ports then push the 10 cm Tygon tubings into the pneumatic/hydraulic controller ports.
- (4) Pre-load the pneumatic/hydraulic channels and connected Tygon tubings with sterile milliQ water through the pneumatic/hydraulic syringe array at 1.5 bar of pressure for 30 min.
- (5) Prime the fluid channels with 70% ethanol at 20 ml/hr for 1 hour using a syringe pump (Fusion 200-X, Chemyx Inc) to drive fluid flow from the pressure-head ports to cell inlet and outlet.
- (6) Flush the fluid channels with sterile milliQ water at 20 ml/hr for 30 min, then sterilised DPBS (Cat No. 14190144, Thermo Fisher Scientific) at 20 ml/hr for 30 min.
- (7) Fill the pressure-head reservoir with 20 ml of cell culture medium then clamp the pressure-head tubing. The Cell culture medium for HUVECs and hESCs are EGMTM-2 Endothelial Cell Growth Medium-2 BulletKitTM (Cat No. CC-3162, LONZA) and TeSRTM-E8TM (Cat No. 05990, STEMCELL Technologies)
- (8) To coat the device for HUVECs cultivation, inject 10 $\mu\text{g/ml}$ human fibronectin (Cat No. F2006, Sigma-Aldrich, Merck KGaA) into the device through the cell inlet and outlet ports using a 1 ml syringe. Incubate the device for 2 h at 37 °C. When coating for hESC cultivation, inject 1 ml of GeltrexTM (Cat No. A1413202, Thermo Fisher Scientific) (100 \times dilution in cell culture medium) into the device then incubate the device for an hour at 37 °C or overnight at 4 °C.
- (9) Rinse the coating reagent from the fluid channels using 1 ml of cell culture medium through the cell inlet and outlet ports.
- (10) Inject 100 μl of cell suspension prepared at 5 million cells per ml from the cell inlet port using a 1 ml syringe (BD Luer-LokTM, Cat No. BD3030, LIVINGSTONE). Close the on-chip valve for the cell inlet and outlet to immediately stop the flow of cell suspension inside the chip. Leave the device at 37 °C, 5% CO₂ for two hours to assure cell attachment to the coated microfluidic channel.
- (11) Refresh the microfluidic channel with 1 ml of fresh medium at 2 ml/hr. It is important to keep the on-chip-valve closed when connecting with the syringe using Luer fittings. Medium in the microfluidic channel is replaced each day with 1 ml of fresh cell culture medium.
- (12) Cell culture reaches 80% confluence after overnight incubation.
- (13) The on-chip-valve for cell inlet and outlet (V4 and V5) are kept closed. The heart inlet, outlet and ventricular contraction valve (V1, V2 and V3) are operated sequentially as stated previously [9].

The pneumatic system was driven by a purpose-built AVR (Atmel Corporation, San Jose, California, United States) microprocessor board. Microprocessor firmware was controlled via software written in MATLAB[®]. The microprocessor board maintained pressure set points and controlled the state and timing of micro-miniature solenoid valves (Part no.AL2106, ASCO Valve Inc, USA) during the cardiac cycle. Compressed air was regulated to the desired pressure (1.2 - 2 bar) in an air reservoir

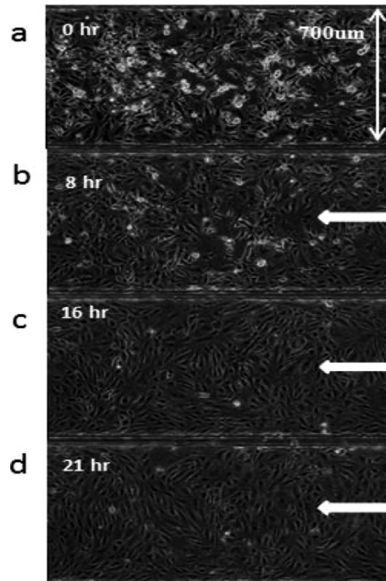


Fig. 5. Phase-contrast image (10 \times) showing the morphology of human umbilical vein endothelial cells exposed to unidirectional laminar flow ($\tau = 0.5$ Pa) in flow cell chamber (300(W) \times 50(H) μ m) for 24 h; a: immediately after flow started (0hr); b: 8 h after flow initiated, cells started to rearrange alignment and migrate; c: 16 h after flow was initiated, cells aligned along the flow direction and migrated opposite to the direction of flow; d: 24 h after flow initiated (See Supplement Video at <https://thebox.unsw.edu.au/B6CA9510-A9B3-11E8-A713F24ABF86A0E1>).

(Norgren Ltd, Camellia, New South Wales, Australia). The pressure was further adjusted by the AVR microcontroller with an accuracy of 0.25 kPa using a differential pressure transducer (24PCCFA6G, Honeywell Switch Sensing and control, Canada) and proportional feedback. The regulated air was then connected to a manifold with micro solenoid valves which were also connected to downstream microchannels in the microdevice using high-pressure 4-mm nylon tubing and push-in fittings.

Upon onset of flow, phase-contrast images of HUVEC response in the flow cell chamber can be acquired at 3-minute intervals and assembled into videos with MATLAB[®] 2017a (MATLAB 2017a, The MathWorks Inc., Natick, MA). We observed consistent migration of HUVECs with the flow direction in our cardiac flow generator (Fig. 5. Also see supplement video at <https://thebox.unsw.edu.au/B6CA9510-A9B3-11E8-A713F24ABF86A0E1>). HUVEC in pulsatile shear flow ($\tau_m = 0.36$ Pa) showed moderate morphological change by the end of 24 h and instead of aligning along the flow direction, they elongated and aligned perpendicular to the flow direction after 20 h of shear exposure (Fig. 5d). We also found that the difference between hESC maintenance culture in standard tissue culture plates and the microfluidic device in a static condition was not significant ($p > 0.05$). The doubling time for hESC H9 in both culture systems matched that reported in the literature (doubling time: 24.2 h) [14].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Additional information

Material

The products and vendors listed here have been employed in our research; however, suitable alternatives may exist for microfluidic culture platforms and syringe pumps. Custom microfluidics could easily be adapted to the system with special attention to the prevention of bubble formation.

HUVEC and hESC culture

HUVECs were cultured using Endothelial Growth Medium (EGM2, Lonza Group, Basel, Switzerland) which contains 5% foetal bovine serum (FBS) and 1% Penicillin-streptomycin (PS) with supplements (Lonza Group, Basel, Switzerland) in a humidified incubator at 37 °C with 5% CO₂. hESC H9 line were regularly tested to exclude mycoplasma contamination (MycoAlert™ Mycoplasma Detection Kit, Lonza Group, Basel, Switzerland). hESC lines were maintained by culture on 6 well plates in TeSR-E8 medium (STEMCELL Technologies Inc., Vancouver). Plates were pre-coated with GelTrex® growth factor reduced (Thermo Fisher Scientific, Waltham, Massachusetts, United States) at 1:100 in DPBS (Thermo Fisher Scientific, Waltham, Massachusetts, United States) overnight. hESC lines were passaged before 80% confluency, by splitting 1:4 every 48 h using enzymatic harvesting with TrypLE (Thermo Fisher Scientific, Waltham, Massachusetts, United States).

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