Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

Leaf-inspired Artificial Microvascular Networks (LIAMN) for Three-dimensional Cell Culture

Rong Fan,[a] Yihang Sun,[b] and Jiandi Wan*[a]

[a] *Microsystems Engineering, Rochester Institute of Technology, Rochester, NY 14623, USA. E-mail: jdween@rit.eu*
[b] *Imaging Science, Rochester Institute of Technology, Rochester, NY 14623, USA.*

Cell maintenance: HCT116 human colon cancer cells were cultured in DMEM (Life Technologies, CA) containing 10% (v/v) fetal bovine serum (Life Technologies) and 1 % (v/v) penicillin/streptomycin (Life Technologies). The cells were maintained in a T-25 culture flask and incubated in an incubator at 37 °C with 5% CO_2. To prepare cells for culturing in agarose, cells cultured in the T-25 culture flask were washed by PBS solution and trypsinized for 5 min to detach cells from the culture flask. The cell suspension was then centrifuged and re-dispersed in a DMEM culture medium for further experimental use (1×10^8/ml). Because dead cells were not attached on the culture flask and were washed away by PBS solution, the viability of cells in the suspension is approximately the same for all experiments. To image cells under a confocal microscope, cells were stained by nucleus-blue (Life Technologies) and/or actin-green (Life Technologies) fluorescent dye following the manufacturer's protocol.

PDMS-based leaf-mimicking microfluidic device: Poly(dimethysiloxane) (PDMS) (Sylgard 184, Dow Corning) was poured to the leaf skeleton of *Hevea Brasiliensis* (Nature Bounty Supply) at the ratio of 10 : 1 for elastomer base and curing agent. After heated at 85 °C for 1 hour, the PDMS replica was peeled off from the leaf skeleton and punched with holes as inlets and outlets. The PDMS replica was then bound to a clean microscopy glass slide using oxygen plasma treatment (Fig. S1a). The inlet of the leaf-mimicking microfluidic device was always located at the petiole of the original leaf (main vein, $d \approx$ 700 μm), whereas outlets were located at either the end of the main vein, the 1st order branch ($d \approx$ 300 μm), or the networks between the 1st order branches ($d \approx$ 50 μm) (Fig. S1b).

Determination of hydraulic transport efficiency: To study the hydraulic transport efficiency of the leaf-mimicking PDMS microfluidic device, water was injected into the device by applying pressure to a water-containing syringe (Norm-Ject), which was connected to the device via a polyethylene tubing (Scientific Commodities Inc, 0.015" (0.38 mm) I.D. × 0.043" (1.09 mm) O.D.). A gas regulator with precision of 0.1 psi (Omega) was used to control the applied pressure. The pressure at which water started to flow out of the device through the outlets is recorded as the input pressure. Devices with outlets located at the main vein required the lowest input pressure, whereas devices with outlets located at the 1st order branches and networks required relatively high input pressure (Fig. S1c). On the other hand, water coverage was characterized by the ratio of the water-covered area of the device to the total area of the device. Images were took before and after the water introduction, and analyzed by using Image J. Devices that have outlets at the 1st order branch and networks had 80% area covered with water, while only 50% and 63% water coverage was obtained when outlets were located at the main vein and at the half of the 1st order branch, respectively (Fig. S1d). Each set of experiment was repeated ten times, and error bar presented as standard deviation of all trials.

Agarose-based leaf-mimicking microfluidic devices: Agarose solution was prepared by dissolving agarose powder (Sigma) in DI-water at 2 wt%, and then mixed with HCT116 cells in DMEM (Life Technologies) dispersion (1×10^8/ml). Leaf skeleton of *Hevea Brasiliensis* (Nature Bounty Supply) was used as template and sterilized by exposing to UV light for 5 min before use. Agarose-cell mixture solution was poured onto the leaf skeleton and gelled at room temperature for 1 hour. The agarose replica was then peeled off from the leaf skeleton and holes were punched at the main petiole (inlet) and the network area (outlets). The agarose matrix was placed on a glass slide and DMEM was injected into the device from a syringe (Norm-Ject) by using a syringe pump (Harvard Apparatus, PHD, Ultra). The device was then immersed in 2 ml DMEM in a petri dish and cultured at 37 °C in an incubator (Binder) for 3 days. Note that, cell growth and viability were determined by cells growing within 200 μm to the

branches, which was measured by microscope. Control experiment was conducted by using agarose matrix with simple straight microfluidic channels with the width of 800 μm for 3D cell culture. Cell viability in control experiment was measured on cells located within 200 μm to the channel and beyond 200 μm from the channel. Cell viability was determined by using Live/Dead fluorescent assay (Life Technologies) on an inverted Leica fluorescence microscope (Leica Microsystems, DMI 6000). Each set of experiment was repeated by at least three times, and error bar presented as standard deviation of all trials. Note that 2 wt% agarose is used to fabricate leaf-mimicking and leaf-inspired microfluidic devices because it provides a proper mechanical property for device fabrication.

Agarose-based leaf-inspired microfluidic devices: Leaf-inspired microfluidic master was fabricated using established soft lithography technique. HCT116 cell-containing agarose mixture was prepared as described above and poured onto the microfluidic master to form a single layer of agarose hydrogel replica at room temperature. The thickness of agarose replica was controlled by adjusting the volume of agarose mixture solution being used and measured under a microscope. To assemble the 3D LIAMN, three pieces of agarose replica were aligned and stacked together. The assembled device was placed under room temperature for additional 30 min so that the agarose became slightly dry and adhered better to each layer. Inlet and outlet holes were then punched throughout the three-layer construct. The inlet and outlet tubing, however, were only inserted at the top layer. The assembled device that was continuously supplied by DMEM through the embedded perfusion channels was then immersed in 5 mL DMEM and cultured in an incubator. Cell growth, viability, morphology, and distribution were measured under an inverted Leica fluorescence microscope or confocal microscope (Leica Microsystems). Each set of experiment was repeated by at least three times, and error bar presented as standard deviation of all trials.

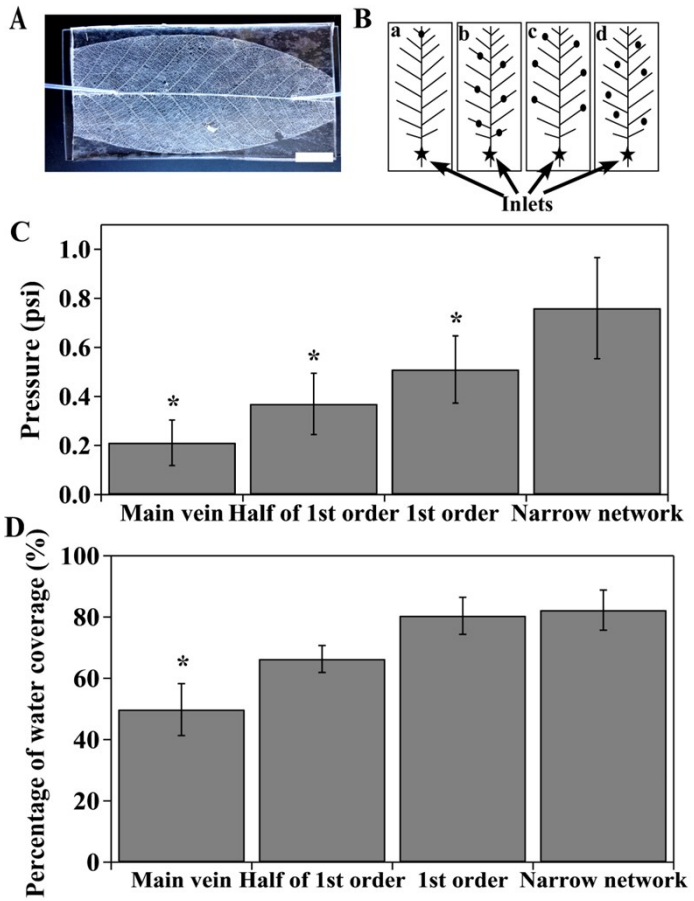

Fig. S1 Analyze the hydraulic transport efficiency of leaf-mimicking PDMS microfluidic devices. (A) Image of a typical leaf-mimicking PDMS microfluidic device. Scale bar: 2 cm. (B) Schematics of positions of inlets and outlets in the leaf-mimicking PDMS microfluidic devices. The star symbol indicates the position of inlets, which is always at the petiole of the original leaf (main vein). Solid circle indicates the position of outlets, which could be at the main vein (a), the half of the 1st order branch (b), the 1st order branch (c), or in the network (d). (C) Effect of position of outlets on the input pressure. * $P < 0.05$. (D) Dependence of fluid coverage on the position of outlets in leaf-mimicking PDMS microfluidic devices. Each set of experiment was repeated ten times, and error bar presented as standard deviation of all trials. * $P < 0.05$.

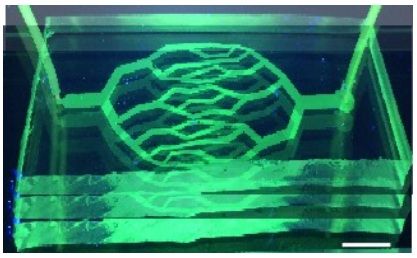

Fig. S2 Fluorescent image of a three-layer PDMS microfluidic device when fluorescein solution is flowing through the device (Scale bar: 3 mm).

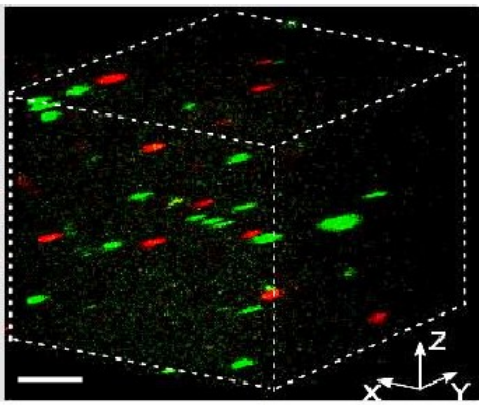

Fig. S3 3D construction of confocal images of HCT116 cells stained with cell Live/Dead assay at day 14 in the developed three-layer leaf-inspired agarose matrix.

Table S1 Calculated parameters of the leaf-inspired branching system.

Level (*n*)	Diameter (*d*, mm)	Length (*l*, mm)
0 (main vein)	2.00	5.00
1	1.41	3.97
2	1.00	3.15
3	0.79	2.50
4	0.63	1.98
5	0.50	1.57

Note that the distances between the smallest branches (5^{th} order branches) are from 400 to 500 µm.