Supporting Information

An adaptable stage perfusion incubator for the controlled cultivation of C_2C_{12} myoblasts

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Materials and Methods

Stage perfusion incubation system

The incubation system is comprised of a circular main body, fitted with a lid and a frame onto which the main body is fastened and that fits onto a x-y microscope stage. The frame is fabricated from polyoxymethylene, whereas the main body and lid are machined from an anodised aluminium alloy. The chip device is centrally situated within a rectangular cutout of the dimensions of a standard 24 x 40 mm glass cover slip and fixed in place by two screw clamps. For microscopy use a rectangular cutout is provided at the bottom of the main body, possessing a lid that can be clamped into place from below when not in use. Gas-tight sealing of the chip is achieved by pressing it against a flat silicon gasket fitted inside the cutout for the glass cover slip by two clamp holders also secured with silicon flat gaskets. In the centre of the incubator lid is a round cut-out window that is closed by a transparent and removable polycarbonate cap, allowing bright field microscopy. This cap and the incubator lid are placed flush against polytetrafluoroethylene (PTFE) o-rings to guarantee gas tightness. Both are fixed by screws and machined washers. To account for fluid and pressure line control of the chip device, pins are provided all around the sidewall of the main body fitting for 1/16" outer diameter (OD) tubing and finger tight nuts including ferrules. Fluid supply was enabled using fluorinated ethylene propylene (FEP) tubing (1/16" OD, 0.25 mm inner diameter (ID); BGB Analytik, Switzerland) and PTFE tubing of 1/16" OD and 0.8 mm ID (PKM S.A., Switzerland) is used for the pressure lines. All tubing is fixed into inlet ports by polyether ether ketone (PEEK) nuts (10-32 threaded) and ethylene-tetrafluoroethylene (ETFE) ferrules with stainless steel rings (both Ercatech AG, Bern, Switzerland). The pressure line tubing is connected to the chip via custom-built stainless steel connectors. The fluid supply tubing is directly inserted into the chip device. Access pins that are not in use can also be tightly sealed with blind caps (polypropylene, 10-32 treaded).

Wafer fabrication

The master molds for the double-layered chip device were prepared using 4" silicon wafers and conducted according to Kuhn *et al.*[1] Briefly, after dehydration of the silicon wafers for 10 min at 200 °C, SU-8 2050 was spin coated at 3250 rpm (fluid layer) and SU-8 2025 at 2100 rpm (control layer; both MicroChem, Newton, MA) onto the wafers to obtain 40 µm and 30 µm thick layers for the fluid and control layers, respectively. After a soft-bake at 95 °C for 6 min the layers were exposed to UV light (365 nm) with an intensity of 160 mJ cm^{-1} (SU-8 2050) and 158 mJ cm^{-1} (SU-8 2025) through a transparency photomask (Circuitgraphics, UK) on a mask-aligner (MA-6 mask aligner, Karl Süss). Following a post-exposure bake at 95 °C for 6 min, the non-exposed regions were developed in a SU-8 developer (Microchem, Newton, MA) for 5 min (SU-8 2050) and 4.5 min (SU-8 2025). Finally, both wafers were hard baked at 200 °C for 2 h followed by silanisation over night under vacuum with 1*H*,1*H*,2*H*,2*H*-perfluorodecyl-dimethylchloro-silane (ABCR, Germany).
The fabrication of the 100 µm high channel system was realised with the following adaptations to the protocol: SU-8 2050 was spin coated at 1500 rpm onto the dehydrated wafer followed by a soft bake for 21 min at 95 °C. The layer was then exposed to UV light (365 nm) at 245 mJ/cm^2 and post-exposure baked for 10 min at 95 °C prior to a 10 min development.

Chip fabrication

For the production of the control layer PDMS oligomer and hardener were mixed at the ratio 5 : 1, degassed for 15 min and poured over the wafer bearing the control layer features. After a second degassing phase of 30 min, the polymer was pre-polymerised at 80 °C for 30 min. Single chips were then diced out of the polymer block and holes for the control layer were punched with a Biopsy puncher (1 mm diameter, Miltex).
In parallel to the control layer production, the fluid layer was fabricated based on a soft PDMS mixture (oligomer to hardener ratio of 20 : 1). The pre-polymer was degassed for 55 min and then spin-coated onto the fluid layer wafer at 1400 rpm resulting in a polymer layer height of approx. 60 µm followed by pre-curing at 80 °C for 15 min. In this manner the fluid layer elaborates both the fluid channels and a 20 µm thick flexible polymeric membrane on top. Both layer productions are timed to be finished simultaneously.
After pre-curing of the layers and preparation of the finished control layer blocks, both layers were aligned under a microscope and bonded by complete curing of the polymer mixtures at 80 °C over night. To finish the chip devices, fluid holes are punched using Biopsy punchers (1.5 mm diameter, Miltex).
For chip assembly, a 24 x 40 mm borosilicate glass cover slip (Menzel-Gläser, Germany) is sonicated for 30 min in ethanol (analytical grade, Fluka, Switzerland). The PDMS chip and the clean glass cover slip are then bonded together using a plasma cleaner (PDC-32 G, Harrick, NY, USA).
For single-layered chips, a 10 : 1 of PDMS oligomer to hardener ratio was poured over the wafer bearing the flow layer features and cured over night at 80 °C. Single chips were diced out of the PDMS slab and holes for fluid connections were punched with a 1.5 mm biopsy puncher (Miltex). Chip bonding and preparation was conducted identically to the multi-layered chip preparation protocol.

Chip characterisation

To validate the performance of the pressure control layer used for the cell traps, fluorescent imaging was conducted to visualise the deformation of the different pad geometries under defined nitrogen pressures (p). For this purpose a 10 µM fluorescein in ddH_2O solution was flushed through the channels at 1 µl/min. Starting from $p = 0$ mbar images were taken at 10 x magnification with an EMCCD camera (iXon, Andor Technologies, Ireland) and p was increased stepwise ($\Delta p = 100$ mbar) up to 900 mbar, which was the maximal stable pressure supply afforded by the Fluigent system at a maximal incoming pressure. Data analysis was done by imageJ.[2] Hereby, the intensity in the 0 mbar micrograph ($I_{0\ mbar}$) was taken equal to the maximal height of the fluid layer mold features ($h_{0\ \mathrm{mbar}}$). All other measured intensity values (I_i) were converted to the corresponding height (h_i) using the following formula:

$$h_i = I_i \cdot \frac{h_{0\ mbar}}{I_{0\ mbar}} \qquad \text{(equation S1)}$$

with i = 100 to 900 mbar and $\Delta p = 100$ mbar.
The results of the chip characterisation are depicted in figure S5.

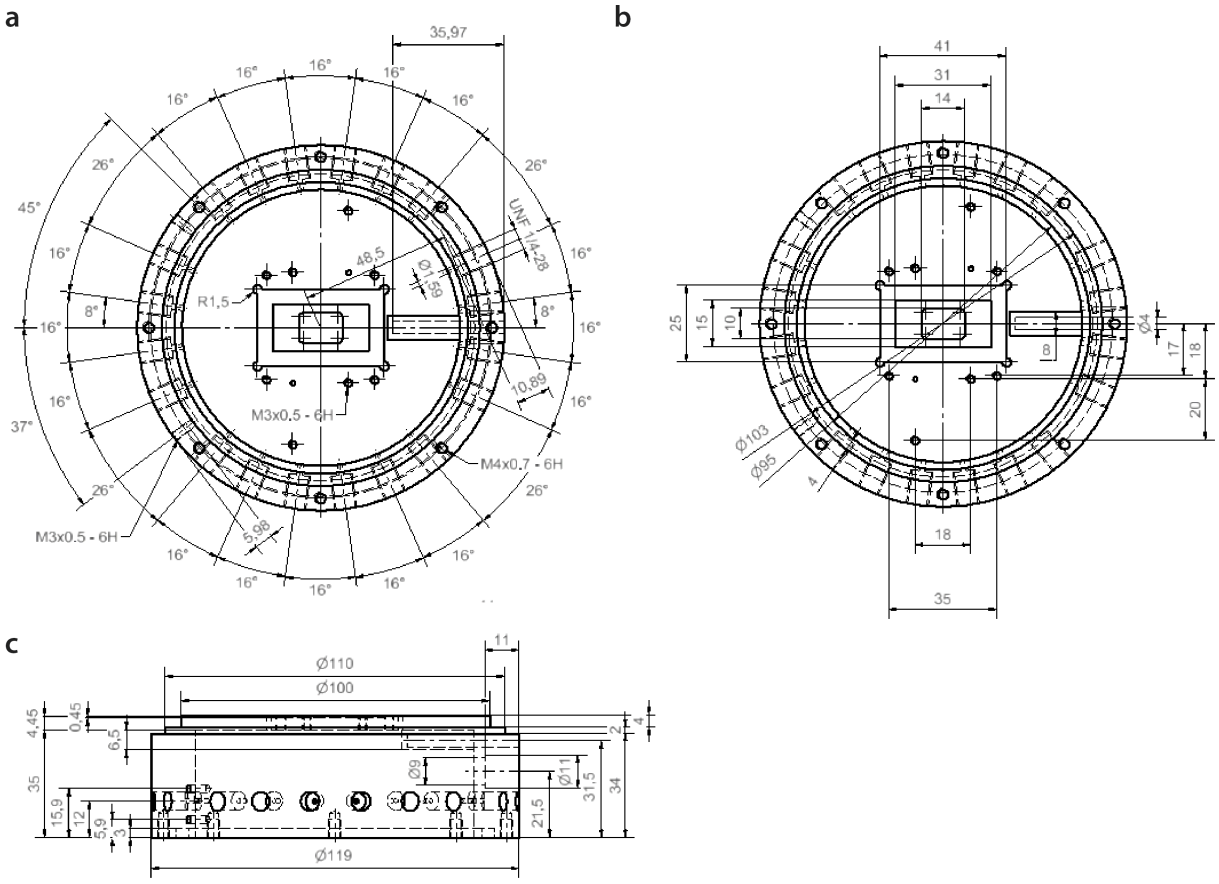

Fig. S1: Technical drawing of the main body of the stage perfusion incubation chamber: top views **(a, b)** and side view **(c)** with specific labelling.

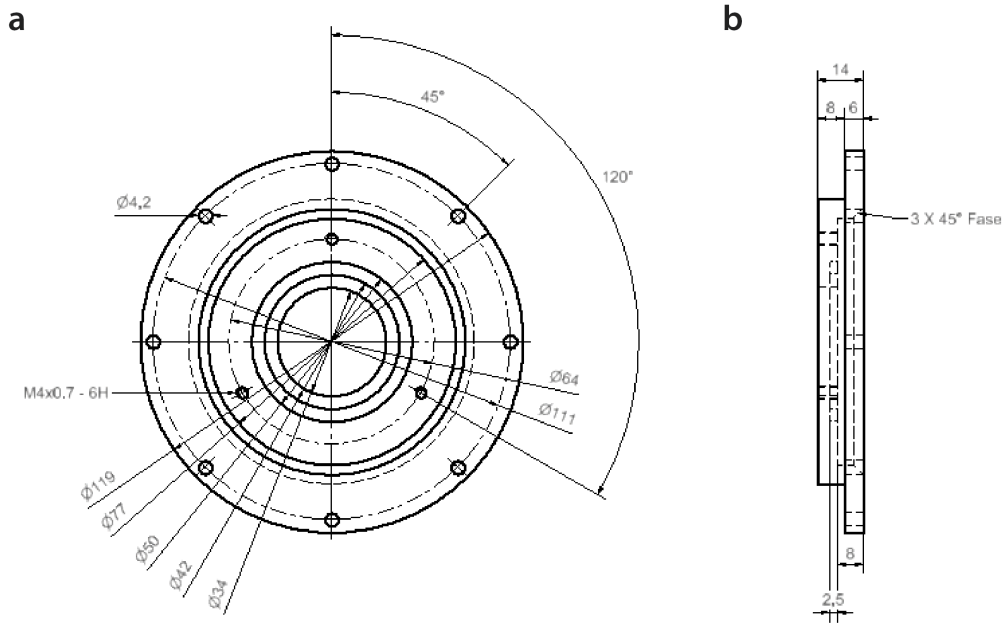

Fig. S2: Technical drawing of the metal lid of the stage perfusion incubation chamber: **(a)** top view and **(b)** side view including specific labelling.

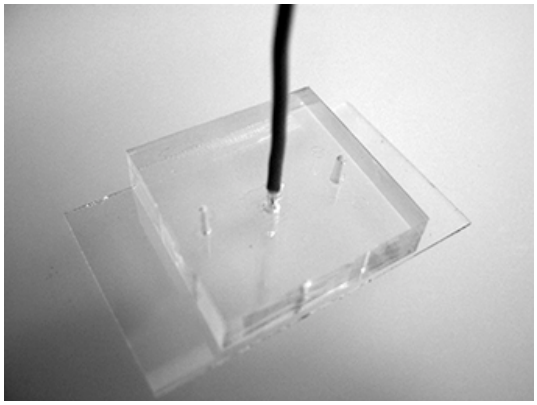

Fig. S3: Image of the setup for temperature validation of the chip device (here shown outside the incubation chamber). An additional hole (1.5 mm diameter) is punched in the middle of a regularly used PDMS chip device to measure the temperature exactly at the spot where the cell cultivation chambers are. A thermocouple is introduced into this hole so that the temperature can be validated under conditions with an open bottom lid. The PDMS block is bonded onto a 24 x 40 mm glass cover slip.

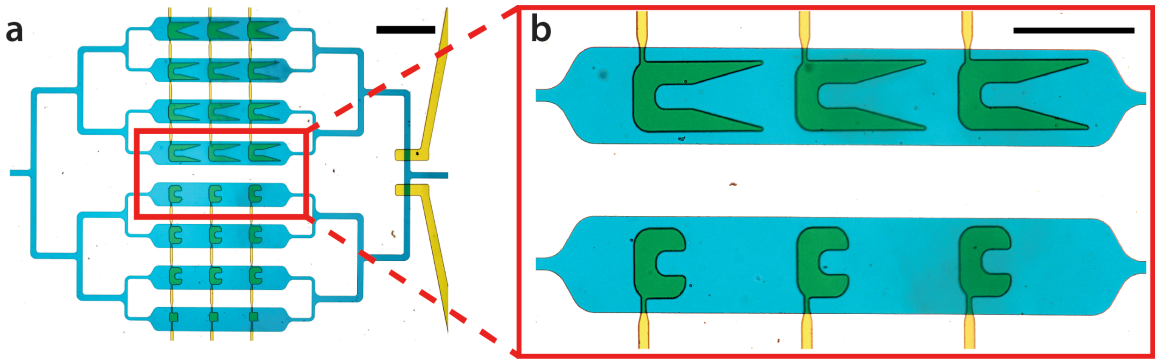

Fig. S4: Channel system and functional principle of the double-layered microfluidic device. **(a)** Micrograph of the core channel system filled with dye for visualisation. The inlet branches into 8 cell culture chambers equipped with two main types of cell catching geometries shown magnified in **(b)**. Scale bars: 1 mm (a) and 500 µm (b). For images of captured and attached cells please refer to Fig. 3 in the main article. A movie of attaching cells is provided as a supplementary source.

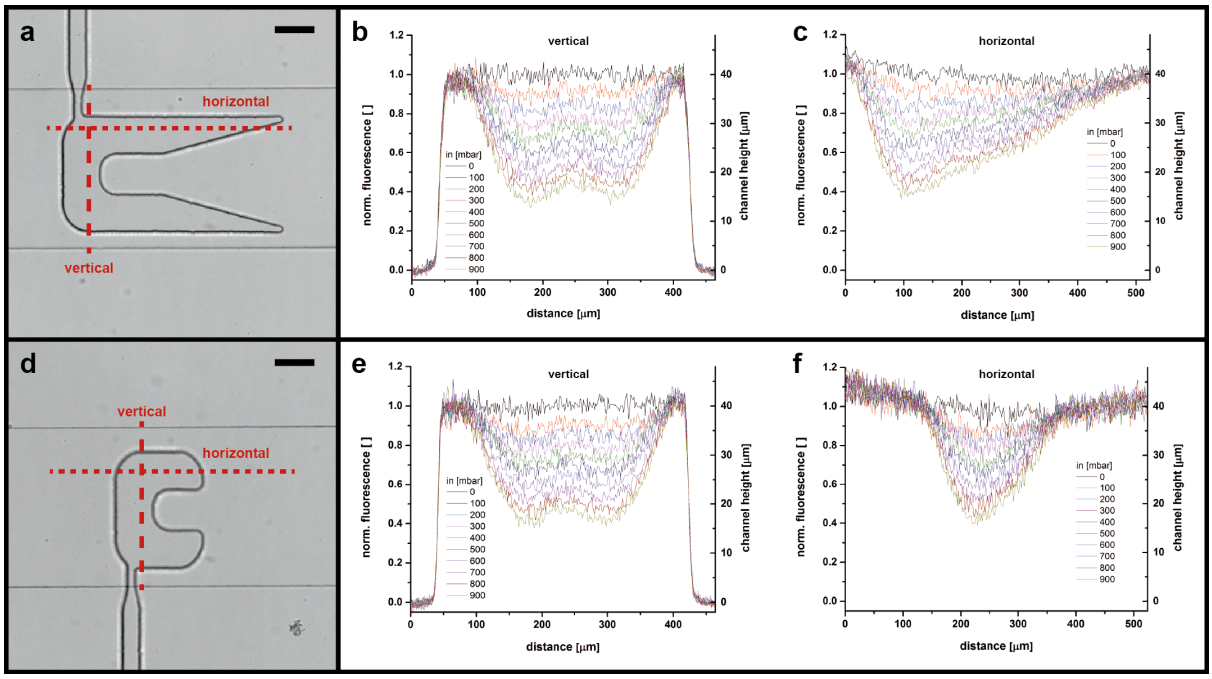

Fig. S5: Membrane deflection profiles of the two prominent cell capturing geometries as indicated in a and d. The channels were flushed with a fluorescein solution and each trap type was monitored in the range of $p = 0$ to $p = 900$ mbar with $\Delta p = 100$ mbar. **(a-c)** Profiles of the long geometry type indicate a maximal deflection of about 25 µm at 900 mbar leaving a gap of ~15 µm in between the deflected membrane and the channel bottom for fluid passage. The gap in between the actuated membrane and the channel bottom is essential, as without it, fluid flow would redirect fluid flow (and cells) around the trap and preclude cell entrapment. The lengthened design allows a higher capturing number than the short type. **(d-f)** Deflection profiles of the short capturing geometry with a maximal deflection of ~23 µm leave a gap of ~17 µm for fluid flow passage. Scale bars (a, d): 100 µm. A movie file showing cell capture is available separately.

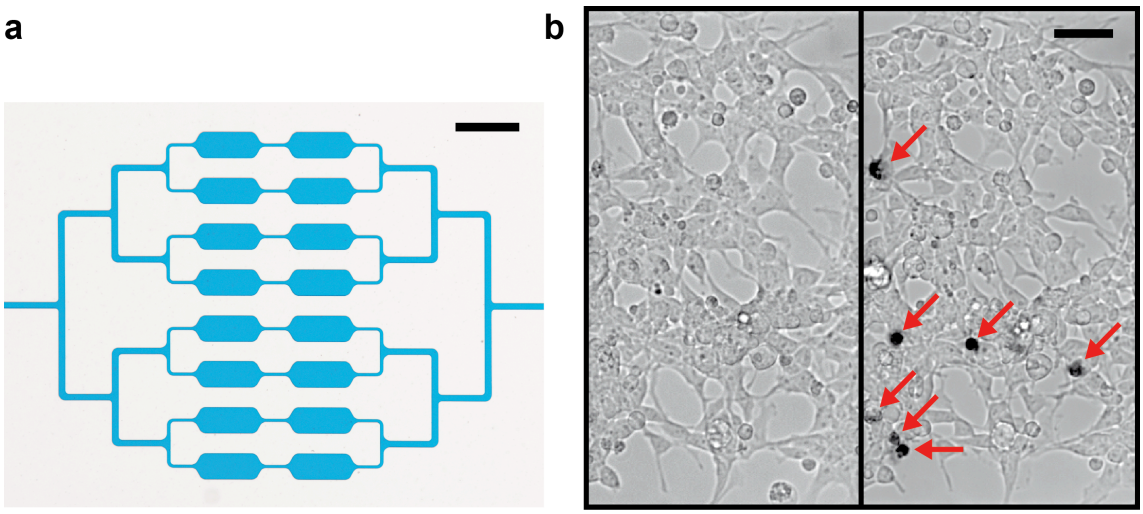

Fig. S6: (a) Chip design used for the long-term culture of HEK-293 cells. Here, the longer culture chambers of the previous design (see Fig. S4) are each replaced by two shorter culture chambers placed in series. In this manner the seeding density gradients as shown in figure S8 are avoided. The channels and chambers are 100 µm in height and filled with food dye for visualisation. **(b)** Cell viability determination using Trypan blue. Trypan blue enters necrotic cells and colours dead cells dark. The left image shows a bright field micrograph of HEK-293 cells in regular cell medium, the right image shows the identical HEK-293 cells after staining by Trypan blue. Dead cells are indicated with red arrows. Scale bars: 1 mm (a), 50 µm (b)

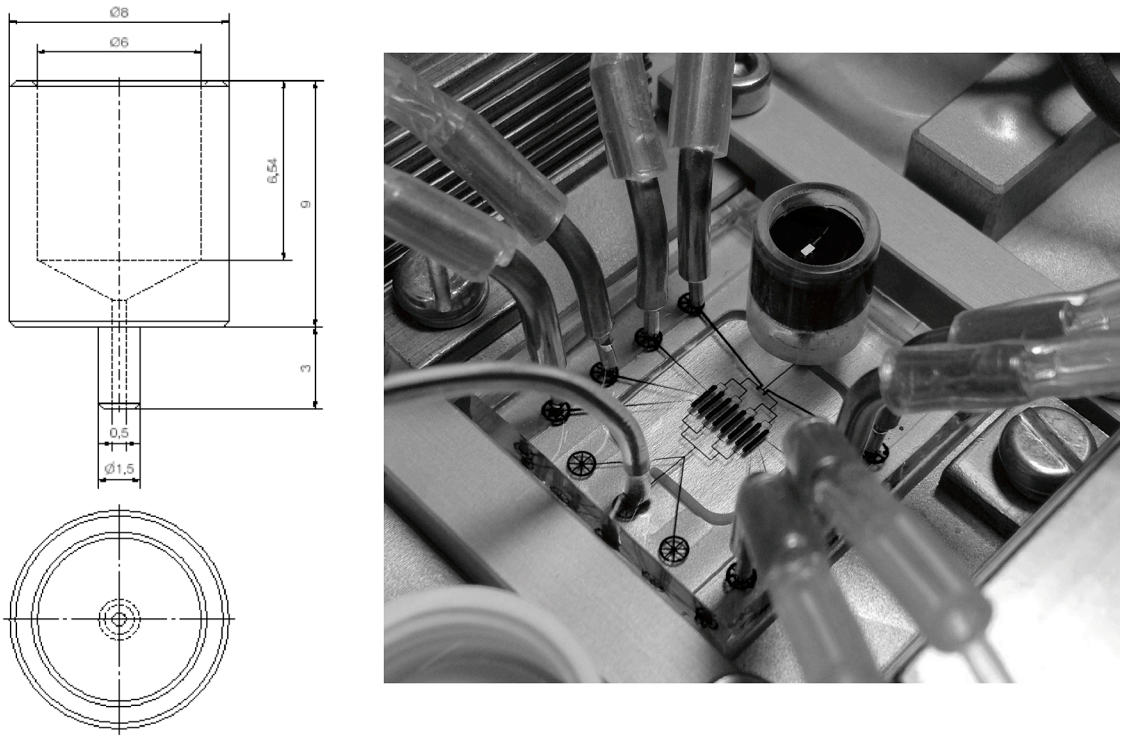

Fig. S7: Fluid supply reservoir designed for easy accessibility and solution exchange. **(left):** Technical drawing of the custom-built liquid reservoir fabricated from PMMA. Due to the conical shape at the bottom of the reservoir, cell feed to the chip is not negatively affected by cell sedimentation to the bottom of the reservoir potentially enabling long-term cell feed. **(right):** Photograph of the PDMS chip embedded in the perfusion incubation chamber including the implemented reservoir. Reservoir and channels are visualised by filling with food dye. The tubing for fluid control is inserted into the chip opposite to the reservoir. All tubing accesses connected to the chip with angled metal connectors (4 on each side of the chip) account for nitrogen pressure supply for the pressure layer.

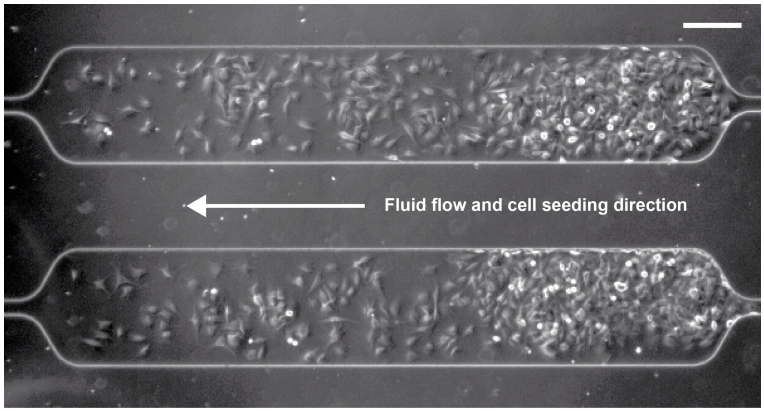

Fig. S8: Passive cell deposition. Cells were seeded in the same cultivation chambers under identical conditions, but without the application of additional trapping tools. A lower flow rate was chosen to allow cell settlement to the channel surface. The passive cell seeding leads to a steep cell density gradient within the chambers, which is unbeneficial for cell types that are strongly affected by the culture confluence. The image was taken 2 h after cell seeding. For a comparison on how cell confluence benefits from the use of the presented catching structures, please refer to Figure 3 (a, b) in the main article. Scale bar: 200 µm.

Tab. S1: List of parts of the perfusion incubation system. For more details, please refer to the CAD files in the ESI.

Number	Type	Annotation
1	chamber main body	aluminium (AL 6036), anodised
1	chamber lid	aluminium (AL 6036), anodised
1	microscope adapter plate	POM
1	removable chamber lid	PMMA
1	removable bottom lid	aluminium (AL 6036), anodised
2	chip clamp	aluminium (AL 6036), anodised, incl. silicon flat gasket
1	silicon flat gasket	
20	blind cap	PP, ≤ 20
20	access hole nut	PEEK, ≤ 20 (9 applied in presented setup)
20	access hole ferrule	ETFE and stainless steel, ≤ 20 (9 applied in presented setup)
8	metal capillary connector	stainless steel
2	coach spring	phosphorus bronze
3	threaded bolt	M4 x 16 mm, stainless steel
3	winged nut	M4, stainless steel
8	hexagon socket head cap screw	M4 x 12 mm, stainless steel
8	machined washer	M4, stainless steel
4	hexagon socket head cap screw	M3 x 6 mm, stainless steel
2	resistance heater	25 W
1	PT100 sensor	
1	temperature feedback control unit	
1	high accuracy diaphragm pressure regulator	Beswick Engineering, Part No. PRDB-3N1-0

References

1. P. Kuhn, J. Puigmarti-Luis, I. Imaz, D. Maspoch, and P. S. Dittrich, *Lab Chip*, 2011, **11**, 753–757.
2. C. A. Schneider, W. S. Rasband, and K. W. Eliceiri, *Nat Methods*, 2012, **9**, 671–675.