A versatile, simple-to-use microfluidic cell-culturing chip for long-term, high-resolution time-lapse imaging

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Supporting Information

Experimental. Detailed description of experimental methods.

Experimental

Fabrication of the microfluidic chip. The microfluidic chip consists of two layers of poly(dimethylsiloxane) (PDMS, Sylgard 184, Dow Corning Corp., USA), bonded to a 150-µm-thick cover glass (24 mm x 60 mm). Both PDMS layers were casted from the respective molds that have been fabricated by using well-established SU-8 photolithographic processes on 4-inch silicon wafers. We used a single 200-µm thick SU-8 layer for the upper vacuum layer and three layers of SU-8 for the bottom fluidic layer (**Fig. 8**). We followed the manufacturers' datasheet (Microchem Corp., USA) to structure each of the SU-8 layers. In brief, SU-8 was spin-coated at the desired thickness and soft-baked on a hotplate. The SU-8 was then exposed through a transparency mask using a UV mask aligner and baked on a hotplate for cross-linking. The first layer defines the gap between the glass and the clamping pad and was modified for the specific cell types: every cell type requires its own mold. Unexposed SU-8 was removed in the SU-8 developer. For the multi-layer mold, we used the development steps after patterning of the third layer. Both molds were coated with trichloro(1*H*,1*H*,2*H*,2*H*-perfluoro-octyl)silane (Sigma-Aldrich, Switzerland) in a vapor silanization process. Freshly mixed and degassed PDMS was poured on both molds at thicknesses of 1 mm for the fluidic layer and 3 mm for the vacuum layer. After curing on a hotplate at 80 degrees for 2 hours, both layers were activated in oxygen plasma, aligned and bonded together. Access holes were punched at inlet and outlet sites.

Cell culture. Prior to loading of the chip, *S. cerevisiae* cells were grown overnight in yeast extract peptone (YP) +2% glucose (**Fig. 6** and **7c**), YP +3% ethanol (**Fig. 7a**), and synthetic complete medium with 2% ethanol (**Fig. 7b**). *S. pombe* cells were grown overnight in yeast extract with supplements (YES) +3% glucose, and *P. putida* were grown in in Luria broth (LB). During on-chip cell cultivation, the same media were applied except when stated differently.

Human hepatic carcinoma (HepG2, DMSZ, Braunschweig, Germany) and fluorescent human colorectal carcinoma cells (HCT-116 eGFP, Sirion Biotech, Germany) were cultured in RPMI 1640 growth medium (10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin). Before starting an experiment, the cells were cultured to confluence and then digested with 0.025% trypsin/EDTA solution (in growth medium). The cell suspension was centrifuged, and the cell pellet was washed twice with PBS. The cells were re-suspended in fresh growth medium and adjusted to a concentration of $5x10^6$ cells/ml.

Loading of cells and microfluidic cell culturing. Cells were cultured using standard protocols (see supporting information) and were diluted to specified cellular concentrations. PDMS device and glass slide were rinsed with acetone, isopropanol and dried using a nitrogen gun. Both were then placed in the plasma oven and left in vacuum for 20-30 min for PDMS degassing. The oxygen plasma was then switched on for 20 seconds. Alternatively, a conventional vacuum desiccator for degassing and a handheld corona discharge device for PDMS surface treatment can be used. Right after activation, 0.45 µl of cell solution (0.8 µl for mammalian cells) was serially pipetted onto each culturing area by using a conventional pipette. The cover glass was placed on top and slightly pressed down onto the PDMS. Irreversible bonding was achieved upon first PDMS-glass contact (after approximately a few tens of minutes when using the corona discharge device). The perfusion channel was filled from the inlet and outlet with medium, and the device was then transferred to the microscope. The microfluidic device was placed on a custom-made chip holder. Prefilled tubing was used to connect a syringe pump to the outlet and a medium reservoir to the inlet. The vacuum port was connected to house vacuum. Bubbles introduced during loading were removed in less than 1 hour. After an initial settling time, the pump was switched on and adjusted to a specific pump rate.

Automated time-lapse imaging. Microfluidic cell culture experiments were performed on a NIKON T*i* Eclipse inverted fluorescence microscope, placed in an environmental box to control temperature. For mammalian cell cultures, a stage-top incubator was used to additionally control humidity and CO_2 levels (5%). If not stated otherwise, bright-field (phase contrast) and fluorescence time-lapse images were acquired with a Hamamatsu ORCA Flash 4.0 camera at time intervals as indicated by using a fully automated stage positioning and hardware autofocusing (NIKON perfect focus system). The light source for bright-field images was a CoolLED pE-100 (8% power), and the light source for acquiring GFP fluorescence images was a SPECTRA X light engine using 470 nm excitation, a 495 nm beam splitter and a 525/50 emission filter. The microscope was driven either by NIKON NIS-Elements Advanced Research software or by using YouScope[1].

Measurements of medium exchange. Microfluidic chips (2.2 µm pad height, variable w_{RF}) were fabricated and bonded as described above. The chips were perfused by drawing liquid through the chip at different flow rates. Tygon tubing with a Y-shaped junction was attached at the inlet, with its ends submerged in de-ionized

H_2O and in a solution of amaranth (1 mg/ml) in de-ionized H_2O. Only one of the two solutions was allowed to flow into the chip at any time, while the flow through the other tubing was shut down by using a paper clip. To investigate the medium exchange time, the perfusion medium was switched from deionized H_2O, to the amaranth solution and pictures were taken every 15 seconds at the positions indicated in **Fig. 3a**. Imaging was performed by using an Olympus IX81 inverted microscope, equipped with an F-view camera (gain: 4 dB, exposure time: 2 ms, resolution 1376 x 1038 px). Image analysis was carried out in Matlab. In short, average intensity values were extracted from the pictures at every time and for every position. The intensity values were background corrected, and the maximum intensity values were scaled to one for every position in the chip. Scaled values were plotted over time. The time point upon reaching 50% maximum intensity was used to calculate the time delay that the medium needs to reach certain positions in the chip. The time needed for the intensity to fall from 95% to 5% was used to calculate the dispersion of the flow front at different positions in the chip.
For medium exchange measurements under the pads, microfluidic chips (2.8 µm pad height, w_{RF} = 50 µm) were fabricated and loaded with cells as described above. The cells under the pad helped to stabilize the pad height. The chips were perfused by drawing liquid through the chip at 10 µl/min. De-ionized H_2O and a solution of amaranth (100 mg/ml) in de-ionized H_2O were applied at the inlet. The complete pad was imaged every 500 ms with a 10X objective, and light intensities were analyzed at the indicated positions of the pad by using ImageJ.

Fabrication of the oxygen sensitive layer. Polystyrene (PS, average molecular weight 250,000 g/mol, Sigma-Aldrich, Switzerland) was dissolved in toluene to a final concentration of 7% (w/v) by stirring for a minimum of 1 hour. Pt(II) octa-ethylporphine ketone (PtOEPK, Livchem GmbH & Co.KG, Frankfurt, Germany) was added to the PS/toluene solution to obtain a final concentration of 1 g/l. Cover glasses (50 mm x 75 mm x 0.13-0.17 mm, Carl Roth GmbH & Co.KG, Karlsruhe, Germany) were cleaned with acetone, isopropanol, water, and subsequently blown dry by using a nitrogen gun. The slides were placed on a spin coater, and 1 ml of the PS/toluene/PtOEPK solution was added and spread using a pipette. Spin coating was performed with the following parameters (step 1: 15 s / 500 rpm / 1000 rpm/s; step 2: 30 s / 2000 rpm / 1000 rpm/s; step 3: 3 s / 0 rpm / max). The coated slides were placed into a disposable petri dish with a spacer made of scotch tape to prevent creeping of the solution underneath the slide. Slides were left to dry at room temperature for at least 10 minutes. Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning Corp., USA) was mixed in a ratio of 1:10 (w/w) and degassed under vacuum. The previously coated slide was placed into the spin coater, PDMS was added to cover 90% of the slide, and spin coating was performed using the following parameters (step 1: 15 s / 500 rpm / 1000 rpm/s; step 2: 150 s / 6000 rpm / 1000 rpm/s; step 3: 5 s / 0 rpm / max). The coated slides were placed into a disposable petri dish with a spacer made of scotch tape to prevent creeping of PDMS underneath the slide. PDMS was cured for 1 hour in a convection oven at 80 °C. The coated slides were kept in a dark place at room temperature until further use. For the experiments, chips were bonded to the slides as described earlier by using oxygen plasma activation.

Testing of oxygen limitation. Saccharomyces cerevisiae (BY4741) cells were loaded as described earlier, and the chip was perfused with YP, supplemented with 2% ethanol (v/v), at a rate of 20 µl/min. The chip was incubated for 48 hours to allow the culture pads to fill completely with cells before imaging was started. Imaging was performed on a Nikon Ti Eclipse, equipped with an Orca Flash 4.0 CMOS camera, by using a Plan Apo λ 10X objective. Bright-field images were acquired using a CoolLED pE-100 light source (10% power) for bright-field illumination and a filter cube (beam splitter: HC BS 562; emission filter: 593/40) with an exposure time of 3 ms. Fluorescence images of the PtOEPK oxygen sensitive dye were taken by using 390 nm excitation through a SPECTRA X light engine (10% power), an emission filter (692/20), and an exposure time of 100 ms. All images were taken with 2 x 2 binning at 1024 x 1024 pixel resolution. Imaging of a whole-cell culture chamber as a tiled picture (20% overlap) was performed every 15 minutes. Directly after onset of imaging, the flow rate was reduced to 0.5 µl/min to stop removal of newly proliferated cells. This test was performed to prove the capability of the oxygen-sensitive layer to indicate hypoxic conditions.

pHluorin experiments. Saccharomyces cerevisiae (BY4741) cells with a plasmid encoding pHluorin, driven by an Act1 promoter and integrated at the URA3 locus and tagged at the endogenous locus of Msn2 (mKOkappa, HIS3MX) and VMA5 (mKate2, KANMX), were loaded as described earlier, and the chip was perfused with YP supplemented with 2% glucose (w/v) at a pump rate of 20 µl/min. Growth was monitored for 13 hours. At that time, the input was manually switched to YP without glucose. After an additional 2 h, the input was switched back to YP supplemented with 2% glucose. Images were acquired every 5 minutes with a 40X, 1.3 NA Plan Apo oil objective and a CoolLED pE-2 epifluorescence light source. pHluorin was imaged by using a 395/11 and 470/24 excitation filter, a 505 bandpass and a 530/11 emission filter. Msn2

was imaged by using a 540/22 excitation filter, a 565 bandpass and a 577/25 emission filter. For quantification purposes, a bright-field image, slightly out of focus, was acquired with the pHluorin emission settings.

COMSOL simulations. Computational fluid-dynamic simulations were performed with the COMSOL Multiphysics software version 3.5a using steady-state analysis in the incompressible Navier-Stokes module. Results were consolidated by varying the element size of the mesh. Simulations of the cell culturing area were executed in 3D. Simulations of the complete microfluidic device were executed in 2D by replacing the cell culturing area with a straight channel of equivalent resistance.

Image analysis. For oxygen depletion experiments tiled pictures were exported from NIS elements software as TIFF images. Tiled pictures were imported into Adobe Photoshop CS6, and overview pictures were assembled using the "move" function. Overlapping tiles were blended into one another using the mask and linear gradient tool of Adobe Photoshop CS6. Fluorescence line profiles were generated by using the plot profile function of ImageJ v1.42q. For pHluorin and Msn2 localization, the images were analyzed using CellX[2]. In short, segmentation was performed on a slightly out-of-focus bright-field image. The resulting mask was applied to the flat-field-corrected fluorescence images. For pHluorin, the median cell intensity was extracted. As the nucleus/cytoplasm ratio is independent of cell size, we extracted the highest intensity value for a disc with the radius corresponding to 7% volume of each cell for Msn2 measurements. This measurement is a good approximation for the nuclear localization[3]. The resulting values were analyzed in R and plotted using the ggplot2 package.

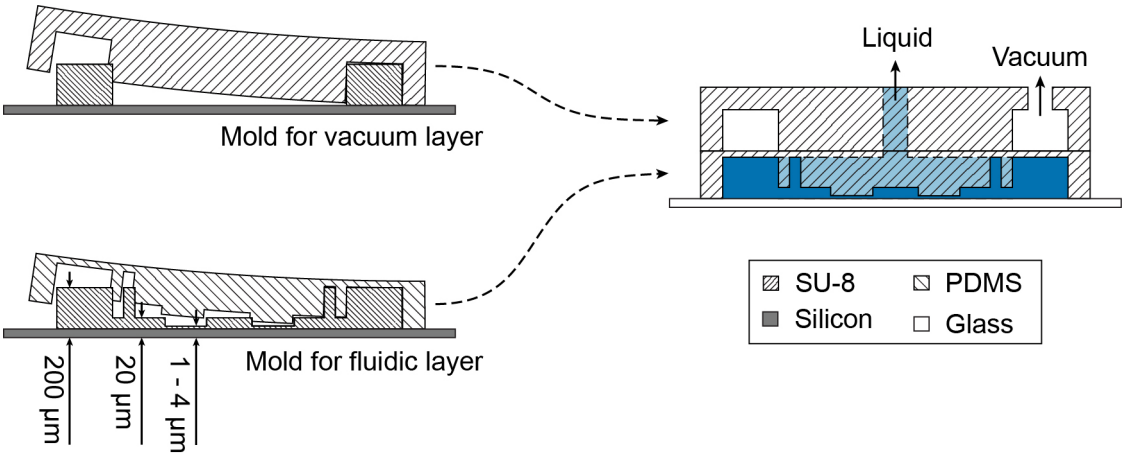

Figure S-1. PDMS device fabrication. The microfluidic chip consists of two layers of PDMS casted from two different SU-8 molds. The two layers are bonded together using oxygen plasma activation and build the ready-to-use PDMS device. Before cell loading, the PDMS device is again plasma activated and bonded on a cover glass. (see cell loading procedure).

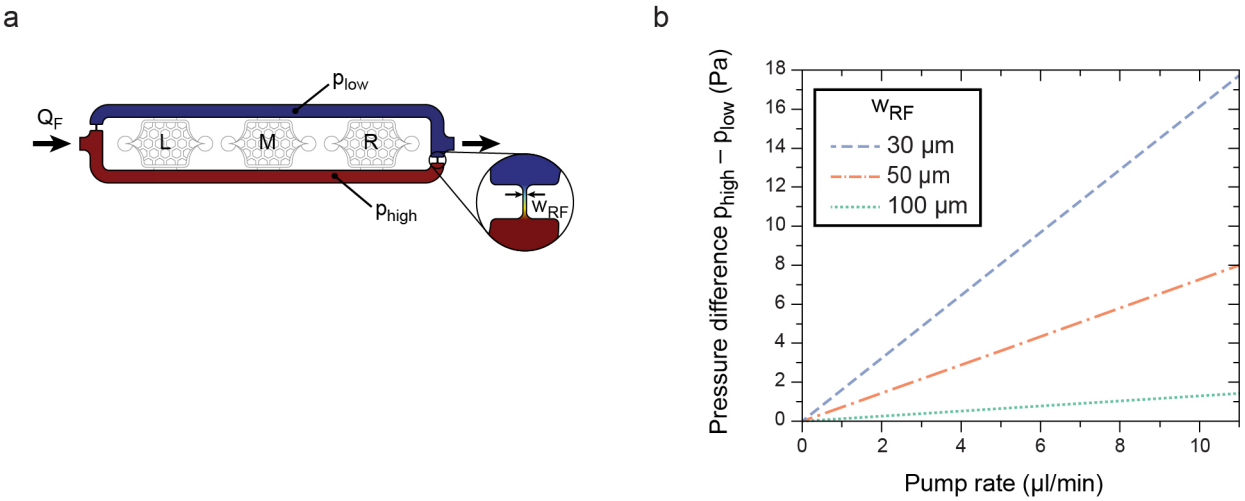

Figure S-2. Hydrodynamic pressure distribution in the microfluidic chip. (a) The two flow resistors R_F induce a specific pressure difference between upper and lower branch in dependence of the their width, w_{RF} , and the externally applied pump rate. (b) Simulated pressure difference in dependence of three different flow resistor designs and of the externally applied pump rate.

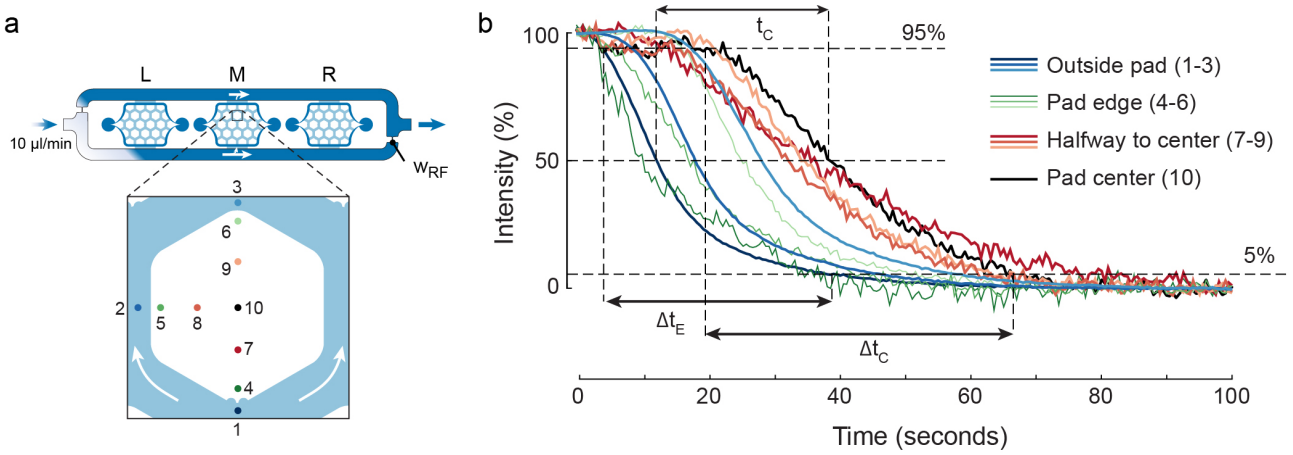

Figure S-3. Medium exchange under the pads. (a) Medium exchange under one of the central pads (2.8 µm pad height), loaded with ~20 cells, was measured by switching from deionized water to colored liquid. The colored liquid absorbs light and, therefore, reduces the grey scale values recorded by our CCD sensor (pump rate was 10 µl/min and w_{RF} = 50 µm). (b) Normalized intensity values are plotted versus time for ten marked positions outside of and underneath the pad. Curves 1-3 show the changes outside the pad as a result of the medium flow. Similar changes are observed under the pad along the edges (green curves 4-6). Towards the center (red curves 7-9) and at the center of the pad (black curve 10) the medium exchange is slightly delayed (t_C = 26.4 s). For the given chip layout, a complete turnover of the liquid volume in the center is achieved in approx. 12 seconds, taking into account the temporal dispersion at the inflow (Δt_C (47 s) – Δt_E (35 s)).

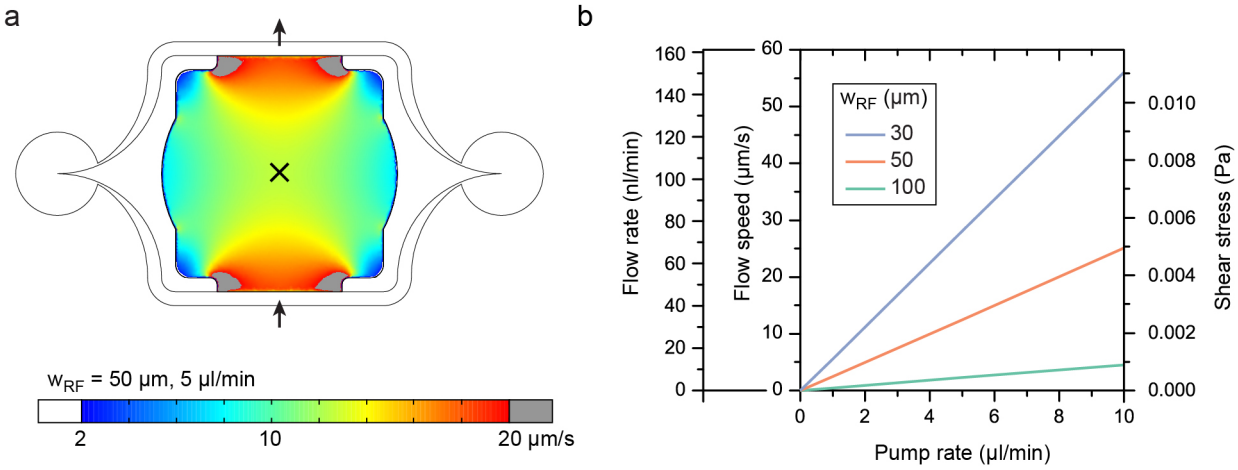

Figure S-4. Flow in the culturing chamber design for adherent cells. (a) A COMSOL CFD simulation (color scale, values higher than those represented on the scale bar, i.e. >20 µm/s, are marked in gray, values lower than those on the scale bar, i.e. <2 µm/s, are marked in white) shows a homogenous distribution of the flow speeds in a large part of the culturing area. Results are displayed for w_{RF} = 50 µm and a pump rate of 5 µl/min (b) Simulation of flow speeds and calculation of flow rates and shear stress (respective axis) showing the variability of applicable flow speeds in the center of the culturing area (black cross in (a)) for different w_{RF} values and different pump rates applied by the syringe pump.

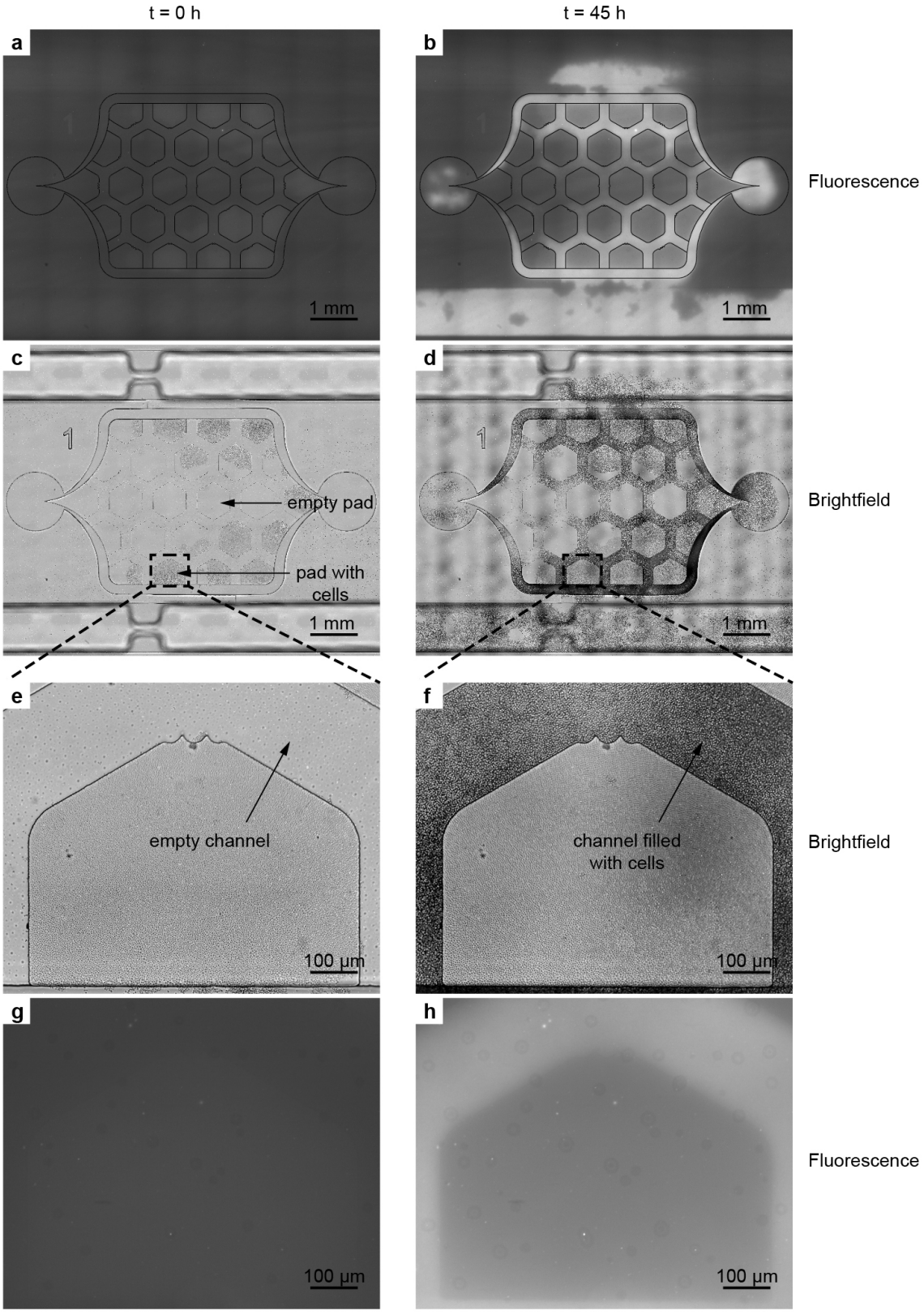

Figure S-5. Oxygen limitation study. Oxygen availability was measured by bonding the chip to a cover slip coated with the oxygen sensitive fluorescent dye Pt(II) octaethylporphine ketone (PtOEPK). *S. cerevisiae* cells were grown aerobically by using ethanol as carbon source until the culture pads were full. Flow through the chip was reduced at t = 0 h to stop removal of cells from the chip. (a) Fluorescence of oxygen sensitive dye PtOEPK at t = 0 h. PtOEPK fluorescence is quenched by high oxygen concentration in the microfluidic device. PtOEPK fluorescence is slightly increased under culture pads. (b) Fluorescence of the oxygen sensitive dye PtOEPK at t = 45 h. PtOEPK fluorescence is strong in the areas of the main channels and slightly increased under the culture pads. (c) At t = 0 h the pads are filled with *S. cerevisiae* cells, while the channels surrounding the pads do not contain any cells. (d) At t = 45 h the channels and the culture pads are filled with *S. cerevisiae* cells. (e-h) Magnified sections of panels a-d.

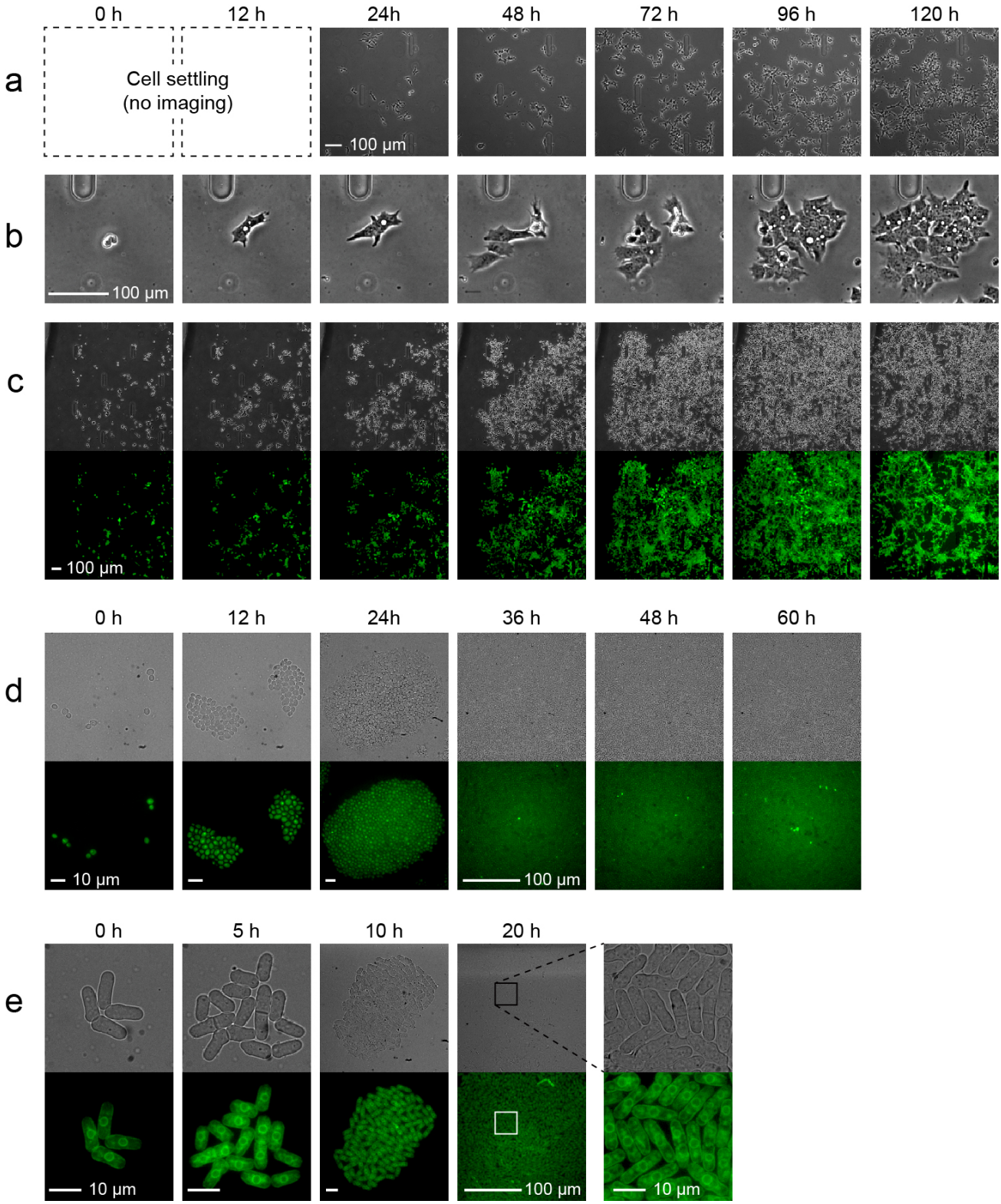

Figure S-6. Time-lapse series of different cell types. (a) Phase contrast micrographs of HEK293 cells started only after 24 hours for letting the cells adhere to glass at no flow (Plan Fluor 10X Ph1, 15 min interval, $1.2x10^6$ cells/ml loading density, see **Video S-8** for full time-lapse series). (b) Phase contrast micrographs (Plan Fluor 10X Ph1) of a single HepG2 cell and derived colony, imaged over 120 hours and grown in the chamber under continuous-perfusion conditions (see **Video S-6** for full time-lapse series). (c) Phase contrast and fluorescence micrographs (1 Plan Fluor 10X Ph1) of the HCT116 GFP cell line proliferation over 120 hours grown in the chamber under continuous-perfusion conditions (see **Video S-7** for full time-lapse series). (d) Bright field and fluorescence time-lapse micrographs (Apo 60X Oil) of *S. cerevisiae* cells bearing endogenously GFP tagged Msn2 grown in YP +3% ethanol (see **Video S-4** for full time-lapse series). (e) Bright field and fluorescence time-lapse micrographs (Apo 60X Oil) of *S. pombe* cells expressing the GFP-tagged integral endoplasmatic reticulum membrane protein ERG11. The pad height was 4 µm (see **Video S-5** for full time-lapse series).

Table S-1. Time (in seconds) needed for complete medium turnover in each chamber of the chip for different flow rates and different dimensions of w_{RF}. (e.g., turnover in chamber L is calculated by $t_{50\%,3} - t_{50\%,2}$).

Flow rate (µl/min)	Chamber	w_{RF} (µm)		
		30	50	100
5	L	128	217	501
	M	119	229	510
	R	103	223	460
10	L	62	111	270
	M	59	117	281
	R	51	115	241
20	L	32	60	145
	M	30	63	153
	R	27	62	127

Table S-2. Time (in seconds) needed for the exchange of medium at certain positions of the chip for different flow rates and different dimensions of w_{RF}. The medium switch at position 3, for example, is calculated by $\Delta t_3 - \Delta t_1$ considering the dispersion already present at the inlet (see **Fig. 3c**).

Flow rate (µl/min)	Position	w_{RF} (µm)		
		30	50	100
5	1	-	-	-
	2	8	9	57
	3	20	60	286
	4	5	11	62
	5	15	74	291
	6	0	10	54
	7	8	79	245
10	1	-	-	-
	2	3	6	12
	3	7	20	135
	4	2	8	15
	5	6	26	139
	6	0	7	12
	7	1	28	99
20	1	-	-	-
	2	3	3	4
	3	3	8	63
	4	3	5	9
	5	5	12	61
	6	2	5	4
	7	3	13	45

References

(1) Lang, M.; Rudolf, F.; Stelling, J. *Current protocols in molecular biology / edited by Frederick M. Ausubel ... [et al.]* **2012**, *Chapter 14*, Unit 14.21.1–23.

(2) Dimopoulos, S.; Mayer, C. E.; Rudolf, F.; Stelling, J. *Bioinformatics (Oxford, England)* **2014**, 1–8.

(3) Gordon, A.; Colman-Lerner, A.; Chin, T. E.; Benjamin, K. R.; Yu, R. C.; Brent, R. *Nature methods* **2007**, *4*, 175–181.