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† Electronic Supplementary Information (ESI): An *in vivo*-mimetic Liver-Lobule-Chip (LLoC) for stem cell maturation and zonation of hepatocyte-like cells on chip

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S1 Cell culture medium equilibration

The cell culture medium for the HepG2 experiments was equilibrated to the gaseous environment of the incubator by over night storage in the very same incubator that were to be used during the experiment. During incubator storage, the cell culture medium was kept in ventilated T-25 or T-75 culture flasks placed horizontally in order to maximize the area of the liquid-gas interface. The syringes were then rapidly filled with the equilibrated cell culture medium, minimizing the time during which the flasks of cell culture medium were exposed to the atmosphere outside of the incubator. Cell culture medium equilibration plays a pivotal role for avoiding bubble formation in the LLoC.

S2 Antibody and dilution specifications for on-chip double-fluorescence immunostaining

The origin, manufacturer and dilution information for the used stains are stated in Table S1.

S3 Additional linear velocity profile simulations

The lower outlet of the LLoC can be sealed off to redirect the media through the artificial liver lobules and out via the central vein mimetics and the top layer. This is demonstrated in Fig. S1 for flow rates of 0.25 μL/min, 0.50 μL/min and 1.0 μL/min, respectively. Hence, the LLoC allows for flow rate customization depending of the requirements of different cells.

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S4 Z-stack of nuclear stain

The Z-stack confocal image of an example subsection of an artificial liver lobule, corresponding the maximum intensity projections presented in Fig. 3D is shown as a video on this link: https://www.youtube.com/watch?v=UXKoqM27gPA. During the duration of the movie, the view goes from the starting position at the bottom of the artificial liver lobule and up through the Z-stack. The cells were imaged seven days after seeding and nuclei of living and dead cells stained with Hoechst and ethidium homodimer-1 (EthD-1), respectively.

S5 Cell mortality under static conditions

We used a mNeonGreen-expressing HepG2 cell line and stopped the flow at 24 hours post-seeding. The cultures were maintained for seven days in parallel with LLoCs continuously perfused at 0.5 μL/min. Fig. S2 shows a comparison between the static and the perfused scenarios. HepG2 cells cultured without flow exhibited reduced proliferation and lower cell density within the lobule mimetics. Dead cell staining ethidium homodimer-1 (EthD-1) further confirmed a higher proportion of cell death in the static condition compared to the perfused control. Fluorescence micrographs of mNeonGreen-expressing HepG2 cells fixed on day seven were acquired with an LSM 710 confocal laser scanning microscope (Carl Zeiss) run in confocal mode, equipped with a water immersion Plan-Apochromat 20x/1.0 M27 objective and the Zen software. EthD-1, mNeonGreen and Hoechst were excited by Argon lasers: 514 nm and 488 nm, and laser diode 405 nm, respectively.

S6 Z-stack of cell discoid

The Z-stack confocal micrographs (1 μm interspaced confocal sections acquired with the LSM 710 as mentioned in section S5) of mNeonGreen-expressing HepG2 cells filling up an artificial liver lobule, is shown as a video on this link: https://www.youtube.com/shorts/xZY3keECV5Q. A series of orthogonal slice from this Z-stack is shown in Fig. S3. During the

Table S1 The origin, manufacturer and dilution information for the used stains. The apostrophe (') denotes the primary antibodies.

Stain	Origin	Manufacturer	Dilution
AFP'	rabbit	Daki Denmark	1:300
ALB'	mouse	R&D Systems	1:100
A1AT'	rabbit	Abcam	1:400
CK19'	mouse	InVitrogen	1:100
ARG1'	rabbit	Novus Biologicals	1:100
GS'	mouse	InVitrogen	1:500
Anti-rabbit 488	donkey	Thermo Fischer Scientific	1:300
Anti-mouse 568	donkey	Thermo Fischer Scientific	1:300
DAPI (1 mg/mL in H_2O)	—	Sigma-Aldrich	1:2000

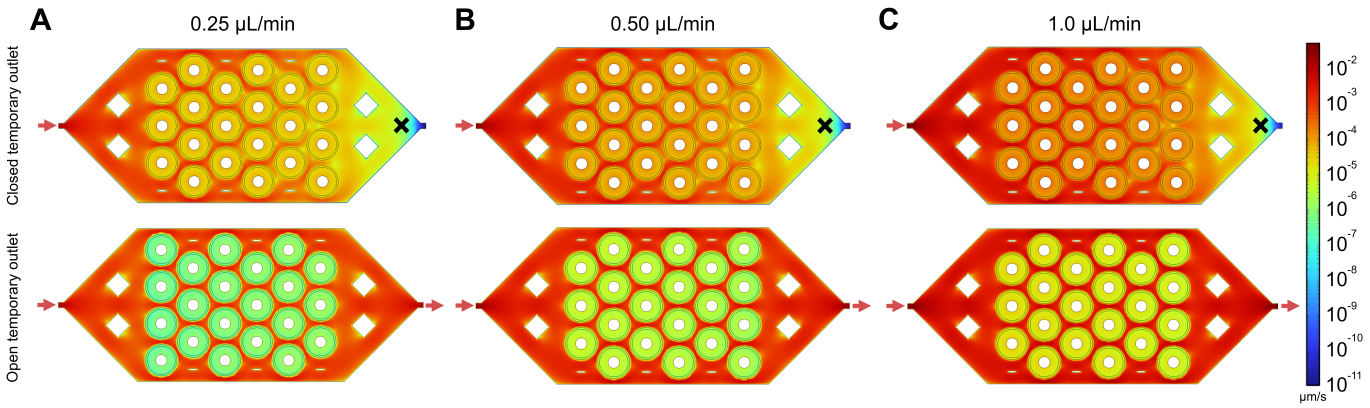

Fig. S1 Computational fluid dynamics analysis displaying linear velocity profiles at three simulated flow rates. A closed outlet scenario (upper panel) is compared with an open outlet scenario (lower panel) at a flow rate of A) 0.25 μL/min, B) 0.50 μL/min, and C) 1.0 μL/min.

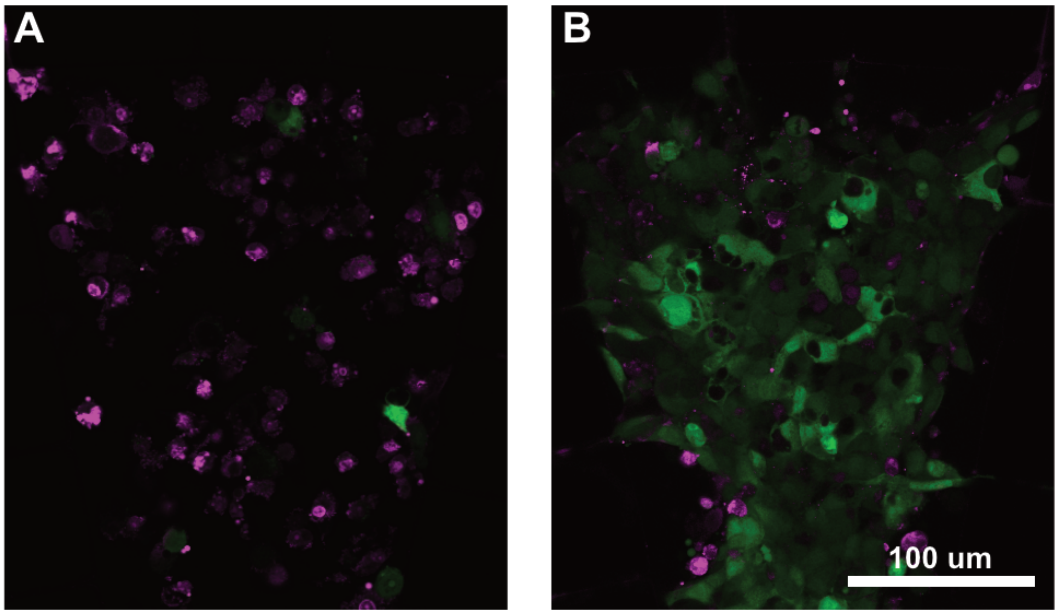

Fig. S2 Comparison of static and perfused culture conditions in LLoC devices using cytosolic mNeonGreen-expressing HepG2 cells. Cytosolic fluorescence from mNeonGreen (green) indicates viable cells, while dead cells are counterstained with Ethidium Homodimer-1 (EthD-1, magenta). Compared to the perfused condition, static cultures show reduced cell density, loss of green fluorescence, and elevated EthD-1 staining—indicative of impaired proliferation, decreased metabolic activity, and increased cell death. (A) Static culture condition, with flow halted 24 h post-seeding and maintained for 7 days without perfusion. (B) Continuous perfusion at 0.5 L/min over the same period.

duration of the movie, the view goes from the starting position at the bottom of the artificial liver lobule and up through the Z-stack, 60 μm. The cells were imaged seven days after seeding.

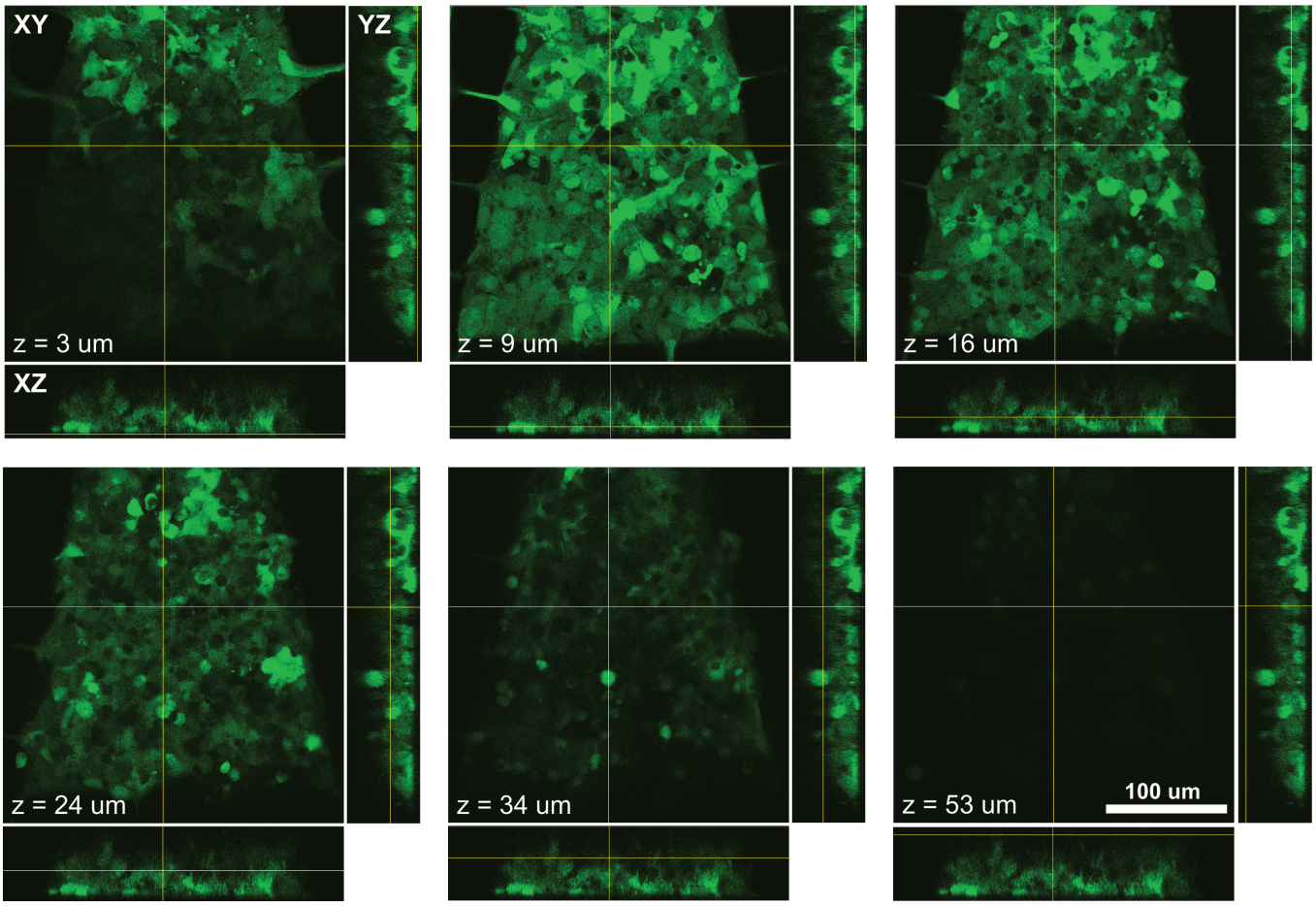

Fig. S3 Z-stack CLSM images of HepG2 cells expressing mNeonGreen cytosolic protein, within a LLoC lobule. A representative cross-sectional series of six Z-planes displayed as ortho slices (3, 9, 16, 24, 34 and 53 µm above the glass) demonstrates cellular distribution across the vertical height (∼60 µm) of the chamber. Fluorescence from the glass base to the PDMS ceiling confirms uniform cellular occupation across ∼5–6 stacked cell layers. The gradual decrease in intensity with depth is attributed to light scattering within the densely packed tissue.