Supplementary Data

Methods and Materials

Biological Characterization of the Model

Probe Staining. All probe staining was performed according to manufacturer's protocols. **Table 1** details concentration and incubation times for each probe. When staining with Fluobile and MitoTracker rinsing were performed with William's E medium without red phenol. When preforming P450 Glo CYP3A4 Assay, CYP3A4 substrate (Luciferin-IPA) is after incubation time, rinsed with Detection Reagent and incubated with the same for additional 20 minutes.

Table 1| *Concentration and incubation time details for live probe staining.*

	Fluobile	MitoTracker	CYP3A4 substrate
Concentration [µM]	0.1	0.1	3
Incubation time [min]	45	10	60

Immunofluorescent Staining. A syringe pump (Standard Infusion Pump 11 Elite Syringe Pump, Harvard apparatus) was used to perform immunostaining on chips. All solutions were perfused through both side channels simultaneously. **Table 2** details duration of each perfusion step. Primary and secondary antibody production company, reference, host and dilution details are given in the **Table 3**.

Table 2| *Sequence of solutions used in immunofluorescent staining.*

Perused solutions	Perfusion period [min]
PBS washing	30
PFA fixation	45
PBS washing	30
Permeabilization	45
PBS washing	30
BSA blocking	60
Primary antibodies	180
PBS/Tween washing	45
Secondary antibodies	120
PBS/Tween washing	45
PBS washing	30

Table 3| *Table of primary and secondary antibodies used in immunostaining.*

	Antibody	Host/ Isotype	Company	Catalog Nb.	Dilution
Primary antibody	CD31	Mouse	Abcam	AB9498	1/200
	ALB	Goat	Bethyl	A80-229A	1/200
	HNF4α	Rabbit	Atlas Antibodies	HPA004712	1/200
	BSEP	Rabbit	Atlas Antibodies	HPA019035	1/200
Secondary antibody	DAPI	/	Sigma	D9542	1/10000
	Alexa488	DaM IgG	Fischer	A21202	1/1000
	Alexa 488	DaR IgG	Fischer	A21206	1/1000
	Alexa568	DaG IgG	Fischer	A11057	1/1000
	Alexa647	DaR IgG	Fischer	A31573	1/1000

Image Analysis and Quantification

Image Analysis. ImageJ (Fiji) 1.54f software (Schindelin et al., 2012) was used to adjust the brightness and contrast of each fluorescence channel of the images. The different optimized images were then merged. To deconvolve stacks of images taken with a confocal microscope, a theoretical point spread function image was generated using the Diffraction PSF 3D plugin. Then, the DeconvolutionLab2 plugin19 with 10 iterations of the Richardson-Lucy algorithm was used. The image background was subtracted with Fiji "Substract Background" tool (10-50 pixels).

Image Quantification. Cell viability was determined using fluorescent images captured in the green, FDA, (all cells) and red, PI, (dead cells) channels. Fixed intensity threshold was applied on images for both channels. Particle analysis was then performed to quantify the number of particles (cells). For Fluobile™, MitoTracker and CYP3A4 fluorescent image quantification, a fixed intensity threshold was also applied to all analyzed images. Particle analysis was performed to quantify number of particles (number of bile canaliculi) by procedure Fiji> Analyze> Analyze Particles> Count. Total fluorescent area (live mitochondria and cytochrome expression) was assessed by Fiji> Analyze> Analyze Particles> Total Area.

Finite Element Analysis

A 3D COMSOL Multiphysics model (COMSOL AB, Stockholm, Sweden) was used to estimate the distributions of velocity and shear stress of water within the device. 2D model of a chip without hydrogel was used for simulations and the applied physics model was Laminar Flow. Water properties were set as following: density 993.3 kg/m^3, dynamic viscosity 0.691 × 10^{-3} Pa.s. Pillars and walls of channels were simulated in PDMS with the following properties: density 970 kg/m^3, dynamic viscosity 1 × 10^{-3} Pa.s, Young's modulus 750 kPa, Poisson's ratio 0.49. Temperature was set on 37°C to mimic incubator environment. A flow rate of 1 µL/min (7.7 Pa) was imposed at the top inlet of the left side channel. Pressure was set at 0 Pa for the remaining outlets.

Results

Finite Element Analysis

Finite element analysis resulted in surface and line charts of velocity, shear stress and pressure. The velocity profile in the perfusion channel can be described by Poiseuille's law for laminar flow. As can be observed from the velocity surface chart (**Fig 1A)**, the velocity is highest in the perfusion channel, and negligible in the other two channels. The maximum velocities in the middle and opposite channels correspond to 4.4% and 1.2%, respectively, of the peak velocity in the perfusion channel. The shear stress along the perfusion channel is inversely related to the velocity gradient near the walls, aligning with theoretical predictions of Poiseuille flow. Shear stress levels are highest near the inlet where pressure and velocity are maximal and progressively decrease along the channel length (**Fig 1B**). The pressure gradient shows progressive decrease of pressure from the device inlet to its outlet, ensuring steady perfusion and maintaining directional flow (**Fig 1C**).

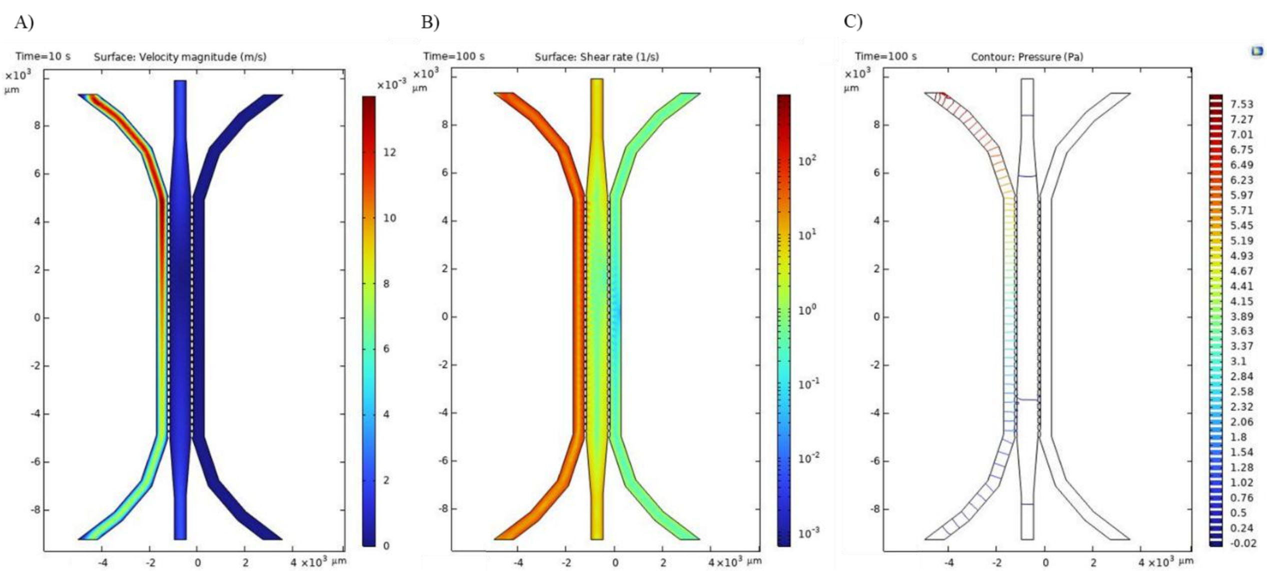

***Fig 1** Finite element simulation of water flowing through the perfusion channel. **A)** Velocity surface chart; **B)** Surface chart of shear stress; **C)** Line chart presenting pressure along the chip.*

Endothelial Tube Characterization

To evaluate the level of directionality of endothelial tissue in a microfluidic chip, LSEC cells were cultured under dynamic conditions (1 µL/min) and direct shear stress exposure for eight days. Tissue was then fixed and immunofluorescent staining of F-actin was performed. The directionality was quantified using Directionality plugin (Fiji> Analyze> Directionality). This method quantifies the orientation of cytoskeletal structures marked with the fluorescent marker by measuring the distribution of angles. **Fig 2** shows LSEC cells in the endothelial channel along with the scheme of the chip.

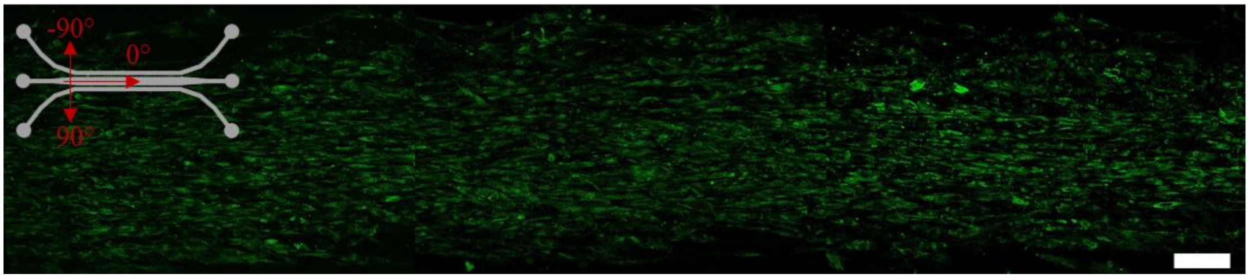

Fig 2 *Endothelial tissue on the chip after 8 days of dynamic culture marked with F-actin (phalloin- green; scale bar = 100 µm). In the top left corner, scheme of the chip design shows tissue alignment.*

Hepatic Tissue Characterization

To evaluate viability of hepatic tissue dead- live staining was carried out and fluorescent signal was analyzed as previously described (**Fig 3**).

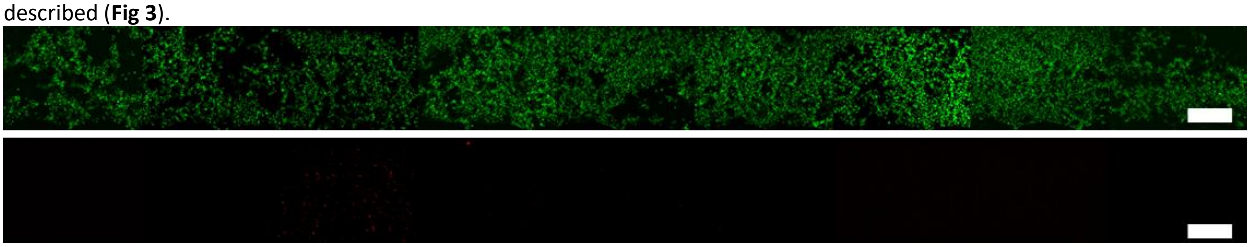

Fig 3 *Hepatocytes viability evaluation. Dead- live staining after 8 days of dynamic culture on chip: live cells- green, dead cells- red (scale bar= 200 µm).*

Higher magnification of the immunofluorescent staining of hepatic specific markers is shown in the **Fig 4**.

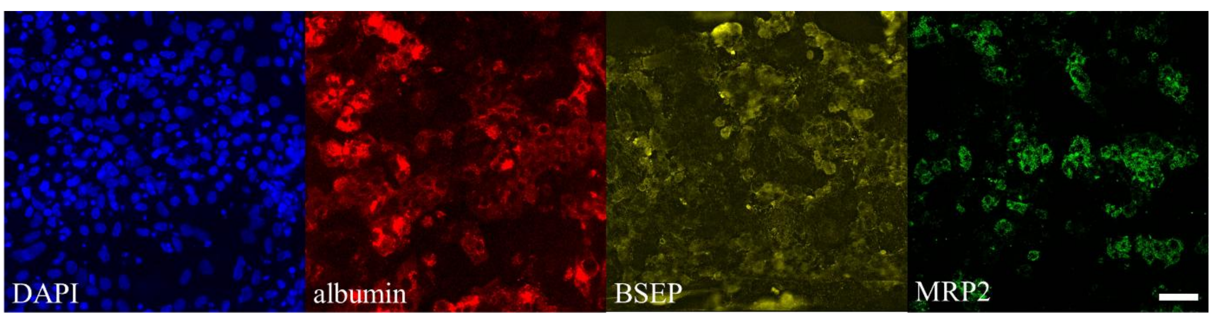

Fig 4 *Immunofluorescent staining for hepatocyte specific markers of HepaRG cells on the chip. Blue- DAPI, red- albumin, yellow- BSEP, green - MRP2 (scale bar = 50 µm).*

Impact of LSEC-HepaRG Coculture on Hepatic Expression on a Chip

Proteomic profiling of the cell culture medium was performed using the Olink Target 96 Inflammation Panel (Olink Proteomics, Uppsala, Sweden). All samples passed Olink's internal assay controls, including incubation, extension, and detection controls, ensuring consistent assay performance across plates. Data were normalized using Inter-Plate Control (IPC) normalization to correct for batch effects.

Quality control thresholds were applied as follows: standard deviation < 0.2 NPX within technical replicates and exclusion of outliers differing by > 0.3 NPX from replicate means. Proteins with more than 15% missing values across all samples were removed prior to analysis. Final NPX values were log_2-transformed for downstream statistical evaluation. **Table 4** shows z- normalized NPX vales of 36 different protein expressions across 13 tested samples.

Table 4| *Proteome profiling of HepaRG mono- and LSEC-HepaRG coculture: NPX values of 36 biomarkers.*

Assay	**IL8**	**VEGFA**	**GDNF**	**CDCP1**	**LAP-TGFβ1**	**uPA**	**IL6**	**TRAIL**	**CXCL1**	**SCF**	**MCP-4**	**IL-10RA**
HepaRG D1	7.74121	3.17785	-0.48692	-1.20981	-0.43313	-0.47607	1.33639	0.39391	4.93609	-0.33441	6.09027	-0.28093
HepaRG D2	6.47401	2.29040	-0.47899	-1.12549	-0.25128	-0.45202	0.70859	0.38196	4.34219	-0.45108	6.12805	-0.22211
HepaRG D3	5.87638	3.63820	-0.41065	-1.19402	-0.39902	-0.39551	0.00376	0.55634	4.00044	-0.33540	5.77329	-0.24314
HepaRG D4	5.06296	1.66787	-0.37659	-1.36136	-0.67499	-0.55448	-0.55702	0.32282	3.91256	-0.71635	6.15150	-0.25265
HepaRG D7	5.33177	3.05070	-0.25201	-1.06222	-0.37499	-0.36570	-0.17493	0.42804	3.66262	-0.31554	6.23501	-0.23911
HepaRG D8	7.36747	4.20211	-0.43260	-0.93339	0.69858	-0.31240	0.85764	0.39679	5.28643	-0.17304	6.31410	-0.25867
LSEC-HepaRG D1	10.59517	11.31077	0.02471	-0.15923	0.58668	4.95863	4.56304	1.20359	7.18391	-0.23521	5.60584	-0.27411
LSEC-HepaRG D2	8.59939	10.84570	-0.34894	-0.29919	0.31793	2.44377	3.09194	0.88360	5.37474	-0.34289	5.09784	-0.33945
LSEC-HepaRG D3	8.95657	11.13576	0.14610	0.34962	0.83618	2.25696	3.85964	0.90058	6.42269	0.06435	5.40921	-0.29190
LSEC-HepaRG D4	7.01377	10.81701	-0.33679	-0.43512	0.18649	1.35371	2.72671	0.82051	4.80419	-0.01751	5.02122	-0.33418
LSEC-HepaRG D6	6.67062	10.90266	-0.20299	-0.50016	0.34754	0.80544	2.23121	0.94830	4.56711	-0.04718	4.79035	-0.22231
LSEC-HepaRG D7	7.94140	11.27901	0.14962	0.36705	0.91458	1.12425	2.88322	0.86372	5.59573	0.16233	5.12489	-0.38098
LSEC-HepaRG D8	7.00759	11.09919	-0.08187	-0.14350	0.44598	0.59659	1.75840	0.76079	4.80231	-0.11808	4.91364	-0.35581
Assay	**FGF-5**	**MMP-1**	**LIF-R**	**FGF-21**	**IL-18R1**	**PD-L1**	**HGF**	**MMP-10**	**CCL23**	**CD5**	**Flt3L**	**4E-BP1**
HepaRG D1	-0.66981	1.60335	-0.24294	1.45689	-0.72000	0.47180	-0.21557	1.19056	-0.62285	-0.73590	-0.74648	-0.83945
HepaRG D2	-0.50112	0.84043	-0.25001	1.50130	-0.65723	-0.17660	-0.16938	0.77016	-0.47929	-0.83205	-1.22384	-0.85467
HepaRG D3	-0.49962	0.74019	-0.21030	1.43153	-0.82075	0.18260	-0.07122	0.05280	-0.38793	-0.66260	-2.48573	-1.27639
HepaRG D4	-0.56841	-0.37847	-0.29147	1.50486	-0.69312	0.08226	-0.58728	-0.01777	-0.44606	-1.02523	-1.25589	-1.47786
HepaRG D7	-0.48364	0.32243	-0.18679	1.51676	-0.44295	0.03508	-0.30456	0.05689	-0.36972	-0.68883	-0.73829	-1.44679
HepaRG D8	-0.44437	0.76569	-0.29158	1.64081	0.16168	0.37486	0.34295	1.20736	-0.53244	-0.82398	-0.36558	-0.23148
LSEC-HepaRG D1	-0.23143	11.65655	0.06170	1.18048	1.21999	2.72609	5.49192	8.45028	4.93157	-0.25232	1.86888	1.87524
LSEC-HepaRG D2	-0.32234	11.58464	-0.24392	1.02870	0.49585	1.33349	4.37199	7.52235	3.53568	-0.66059	-0.33041	-0.17417
LSEC-HepaRG D3	-0.23846	10.91343	-0.11567	1.24930	1.47469	2.46019	4.36248	6.36234	3.71505	-0.55074	0.16643	0.67872
LSEC-HepaRG D4	-0.46524	10.21084	-0.05820	1.08191	0.15984	0.67873	2.77029	5.18438	2.51481	-0.66412	-0.30237	-0.64936
LSEC-HepaRG D6	-0.31250	8.75854	-0.19115	1.02824	0.55589	0.75357	2.05534	3.71809	1.58087	-0.77591	-0.78532	-0.34763
LSEC-HepaRG D7	-0.35579	8.35183	-0.19396	1.66251	1.20277	1.90653	2.00520	3.45706	2.15488	-0.50909	-0.56797	1.07335
LSEC-HepaRG D8	-0.29191	7.41086	-0.19317	1.13597	0.69700	0.70242	0.92233	2.96854	1.23333	-0.75565	-0.33195	-0.52777
Assay	**DNER**	**CD40**	**IL33**	**FGF-19**	**LIF**	**CASP-8**	**CCL25**	**TNFRSF9**	**NT-3**	**IL5**	**ADA**	**CSF-1**
HepaRG D1	1.31651	4.39229	-1.07810	-0.64959	0.40496	0.73273	-1.03018	-0.41280	-0.75952	0.22002	-0.04304	1.41510
HepaRG D2	1.25457	3.29162	-0.81761	-0.73412	-0.00542	0.32118	-1.00940	-0.10851	-0.68001	0.35344	0.13321	0.84342
HepaRG D3	1.36299	2.97678	-0.78868	-0.64690	-0.01029	-0.02826	-1.04847	-0.67519	-0.68841	0.12832	-0.01666	1.06964
HepaRG D4	1.26124	2.96535	-0.91223	-0.71648	-0.24446	-0.12121	-1.17551	-0.51005	-0.65632	0.18980	-0.57639	0.60935
HepaRG D7	1.60237	3.03414	-0.80090	-0.56067	0.08196	-0.03522	-1.01746	-0.16610	-0.61657	0.46793	-0.23566	1.61569
HepaRG D8	1.42774	5.30158	-0.92653	-0.72902	0.89165	1.15305	-1.04727	-0.19885	-0.54325	0.19356	0.83542	4.14953
LSEC-HepaRG D1	1.30168	8.37095	0.85821	-0.48713	2.66556	1.29244	-0.78641	0.40753	-0.83323	0.14262	5.26777	7.32945
LSEC-HepaRG D2	1.02332	6.35394	-0.14434	-0.56804	1.42050	0.69226	-0.90265	0.05597	-0.96830	0.13975	2.27268	4.18756
LSEC-HepaRG D3	1.17021	8.08142	-0.10348	-0.55546	2.01630	1.37016	-0.96828	0.13819	-0.86744	0.09012	2.50636	5.29724
LSEC-HepaRG D4	0.97772	5.57694	-0.62652	-0.66028	1.23775	0.52009	-1.00402	-0.17655	-0.80505	0.03448	1.05059	3.41200
LSEC-HepaRG D6	1.06981	5.96520	-0.73254	-0.52654	1.74352	0.57176	-0.94029	-0.29515	-0.79388	0.06860	1.12264	3.56076
LSEC-HepaRG D7	1.04600	7.27452	-0.47954	-0.62582	2.58550	1.63916	-0.95369	0.02637	-0.85246	0.01621	2.04811	5.10736
LSEC-HepaRG D8	1.09616	5.60043	-0.63270	-0.55859	2.40803	0.53949	-0.83327	0.06034	-1.01073	-0.08347	0.89452	4.17201