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# Hele Shaw microfluidic device: A new tool for systematic investigation into the effect of the fluid shear stress for organs-on-chips



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#### ABSTRACT

This method describes a novel approach to systematically investigate the effect of the fluid shear stress (FSS) on epithelial cells thanks to a single microfluidic device based on Hele-Shaw geometry. The method was validated with intestinal Caco-2 cell monolayers and lung A549 cells. We provide guidelines to adjust the experimental parameters to apply specific ranges of FSS and to specify more accurately the area where to image the cells within the device by the performance of a computational simulation of the fluid flow. Most importantly, this simulation enables to validate the equation. This approach was successfully applied to systematically investigate Caco-2 cell monolayers-based intestine-on-chip models as reported in a companion article published in Biomaterials. This study showed that exposure to microfluidic FSS induces significant phenotypical and functional changes. A detailed understanding of the effects of the FSS will enable the realization of in vitro organs-on-chip models with well-defined characteristics tailored to a specific purpose. The Hele-Shaw approach used in this study could be readily applied to other cell types and adapted for a wide range of physiologically relevant FSS.

- Fluid shear stress is a key parameter in the differentiation of epithelial cells cultured in organ-on-chip models.
- A simple approach can be used to assess the effect of fluid shear on cellular monolayer cultured in microfluidic devices.
- Careful optimization of fluid shear stress environment is necessary for the development of better-defined organ-on-chip models.
- Computational simulation of the fluid flow gives an accurate definition of the FSS in a microfluidic channel necessary to interpret the results.

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#### Background

Organ-on-chip technology is attracting significant research interest towards the development of novel approaches for drug testing and disease modeling [1]. A specific and important feature of organ-on-chip model is that they enable the application of dynamic culture conditions that somewhat recapitulate the mechanobiology of tissues through the application of fluid shear stress (FSS). This is significant as epithelial cells experience many mechanical forces in vivo including FSS across their apical surface and these mechanical stimuli directly affect their structures and functions within tissues and organs [2]. The significant impact of the FSS on epithelial cells monolayers cultured within microfluidic environments have been demonstrated. However, most studies have typically only compared a specific FSS with static culture condition, and there is a need to precisely delineate the effects of the FSS on the structure, functions and phenotypes of organs-on-chips models. Most importantly, there is also a need for defining more accurately the actual FSS within the whole microfluidic channel to better interpret the results obtained. To this end we have recently demonstrated the utility of microfluidic devices-based on the Hele-Shaw geometry which enables to assess these effects for a range of FSS within a single experiment. This approach eliminates the experimental variations associated with the use of different devices and cell preparation [3] and therefore provides more holistic insights about the effect of the FSS. We here aimed at demonstrating the accuracy of the model by a computational simulation of the fluid flow which gives a precise overview of the FSS within the whole channel.

## Protocol

#### Design of Hele-Shaw cell culture devices

- 1. Create a script (text file) with interval points of 0.1 cm, for the two curve equations corresponding to the two channel walls of the microfluidic device. The equations correspond to the width function of the z axis shown in Fig. 1.
- 2. Enter the scripts in AutoCAD by including the curve equation (negative and positive curves) and make sure the distance  $w_1$  at the inlet corresponds to the calculated one (see Fig. 1.B). The negative and positive plines are presented in Autocad files (see Supplementary Information-1).
- 3. Close the microchamber to have an outlet and corresponding angle of 45° between the central axis and the outlet walls.
- 4. Draw the inlet and outlet holes and merge the line to create a pline. Final design is shown in Fig. 1.C.



Fig. 1. A: Equation used to draw the channel design. B: Lines drawn on AutoCad resulting from the scripts. C: Final design of the Hele-Shaw chamber adapted for Caco-2 cells.

#### Numerical simulation of the fluid flow in the Hele-Shaw device

The linear-shear Hele-Shaw microfluidic device was previously resolved following a theoretical analysis and experimentally validated by Usami et al. [3] To map the flow rate within the entire device, and also verify that the design established matched the equation of the Hele-Shaw model for the wall FSS, a computational fluid dynamics (CFD) simulation was performed using the software ANSYS. This also allows the definition of a distance *d* from the central axis on which the FSS is constant along the y axis in the defined section of the microchannel (see Fig. 1.C). In other words, at a distance inferior or equal to *d* from the central axis of the channel (*d* being perpendicular to the central axis), the FSS is not significantly different from the FSS on the central axis. It means that the cell monolayer imaged within this distance *d* from the axis can be analyzed for the FSS study one establishes. For these two types of analysis, the fluid velocity profiles and strain rates firstly were determined, along the z axis (y and x being defined), and secondly, along the y axis (z and x being defined). The axis are defined in Fig. 2A.

## 1. Assumptions

The medium was considered as isothermal liquid, viscous, incompressible and Newtonian fluids. Physical properties of the fluids are constant and unaffected by the channel geometry. The flow regime is laminar, and the gravity effects on the flow field are negligible. To capture all the involved phenomena in the device scale, the 3D configuration of the chip was considered for a 3D simulation. Note that the effect of the cell surface was not simulated but that the fluid shear stress experienced by the cells is therefore only an approximation (named " $\tau_{cell}$ ", cf. point 6 further below).

## 2. Geometry and boundary conditions

The geometry used in this simulation is shown in Fig. 1C. A uniform mass flow rate was set at the inlet boundary condition, and atmospheric pressure was set at the outlet boundary condition. In computational geometry, a no-slip boundary condition was applied at the walls, where the velocity is zero, and a Cartesian coordinate system was employed.



**Fig. 2.** A: Lines defined to study the velocity and strain rates along the z axis at five points in the channel (x = 0.5; 1.5; 2.5; 3.5; 4.5 cm) on the central axis (y= width/2). B&D: Strain rate profiles along the z axis at these points in the channel on the central axis with a flow rate of 0.22 µL/min (B) or 1 µL/min (D). C&E: Wall fluid shear stress and fluid shear stress at  $z = 20 \ \mu$ m (-height of the cell monolayer) calculated based on the simulation along the x central axis for 0.22 µL/min (C) or 1 µL/min (E).

## 3. Computational domain discretisation

The tetrahedron structured computational mesh was generated in this domain using the preprocessor ANSYS Meshing software. Different mesh sizes were used to make sure that the solution of equations is mesh-independent and repeatable. No significant difference was observed in the simulations when the mesh was refined, and the solution sensitivity with the meshes will be addressed in results and discussion. The maximum mesh size used in this work was 5  $\mu$ m, and it is selected based on a proper mesh independency analysis. Different mesh sizes were used to make sure that the solution of equations is mesh-independent and repeatable. No significant difference was observed in the simulations when the mesh was refined. The solution sensitivity of the results was measured with the mesh independency study, which is 1.4%.

#### 4. Model and governing equations

The continuum assumption of the fluid was verified by Knudsen number calculation. To perform this simulation, Fluid Flow (FLUENT) package of ANSYS was used (ANSYS Inc., 2019). For the isothermal flow of a Newtonian fluid of constant density, the governing equations were written as incompressible Navier–Stokes equations, including momentum equation, and continuity equation. The SIMPLE (Semi-Implicit Method for Pressure-Linked Equations) was used for pressure velocity coupling, which reduces the internal iteration per time step. The PRESTO (Pressure Staggering Option) for the pressure interpolation, second-order upwind differencing was used for solving transport equations, and the Least Square Cell-Based gradient method for the spatial discretisation. The time step for the integration used is 10–7 s. In each time step, maximum of 30 iterations were found to be sufficient for convergence and to ensure that the residuals were lower than the absolute convergence criterion, which was 10–5. The unsteady-state flow condition was considered for simulation to monitor the flow within the microfluidic device over time and find the flow development time. The further analysis, however, on the velocity profile and strain rate were performed when the flow gets to steady condition.

The numerical simulations were carried out on a DELL Precision Tower having two processors of "Intel Xeon CPU E5–2699 V4", including 20 physical cores with the maximum speed of 2.6 GHz for an individual core and 256 GB memory.

Note: this simulation was based on a continuous fluid flow within the device. The values entered for the analysis, after meshing: Viscosity of liquid medium: 0.94 cP = 9.40E-08 N/s.cm<sup>2</sup>; Flow rate:  $Q = 3.7 \times 10^{-6}$  cm<sup>3</sup>/s (for 0.22 µl/min) or 1.67  $\times 10^{-5}$  cm<sup>3</sup>/s (for 1 µL/min) or 0.002 cm<sup>3</sup>/s (for 120 µL/min); Height of the channel: 150 µm; Width of the channel at the inlet: 0.1 cm; Volumetric mass of medium: 0.9818 g/ml and density=0.9818.

5. Verify the velocity profiles along the z axis at different x positions (corresponding to the middle of each of the five 'FSS sections' defined previously [4]; or x = 0.5; 1.5; 2.5; 3.5; 4.5 cm-middle of the predefined sections) in the channel on the central axis (y=width/2) (see Figures S1 for 0.22 µL/min, S3 for 1 µL/min). From the velocity profiles in the z direction, the strain rate -gradient of velocity- profiles along the z axis can be obtained and allow the calculation of the wall FSS and the FSS at  $z = 20 \ \mu m$  (~height of the cell monolayer) at the five different x positions (Figs. 2.B for 0.22 µL/min and 2.C for 1 µL/min). Two flow rates were tested: 0.22 µL/min (validated with Caco-2 cells) and 1 µL/min (validated with A549 cells).

The results obtained showed that the wall FSS calculated from the strain rate along the z axis decreases from inlet to outlet, from ~0.01 dyn/cm<sup>2</sup> to 0.001 dyn/cm<sup>2</sup> with a flow rate of 0.22  $\mu$ L/min (Fig. 2.C) and from 0.0447 dyn/cm<sup>2</sup> to 0.0059 dyn/cm<sup>2</sup> with a flow rate of 1  $\mu$ L/min (Fig. 2.E).

6. Compare this first data set to the theorical Hele-Shaw model.

The Hele-Shaw cell culture device is based on the initial design by Usami and colleagues, where the FSS linearly decreases along the main axis from the inlet to the outlet [3]. The mathematical Poiseuille model was applied to design a Hele-Shaw device that generates a range of FSS relevant to the physiological environment of the organ studied such as intestine-on-a-chip. This model assumed a parallel plate configuration with an infinite aspect ratio in the cross-sectional dimensions. The Hele-Shaw flow is described by the Eqs. (1) and (2) below [3]. Eq. (2) describes the wall fluid shear stress ( $\tau_{wall}$ ) [5].

$$\frac{\Delta P}{L} = -\frac{6.Q.\mu}{h^3.y} T_{\text{wall}} = -\frac{6.Q.\mu}{h^2.w_1} \left(1 - \frac{x}{L}\right)$$
(2)

To apply a specific range of FSS, both the device dimensions, *i.e.* its length (L), width at the inlet ( $w_1$ ) and height (h), as well as the flow rate can be adjusted. In a specific example used for Caco-2 cells, these parameters are selected as follow: Viscosity of the cell culture medium,  $\mu$ =



**Fig. 3.** Relationship between the wall FSS applied to (A, LEFT) Caco-2 cells and (A, RIGHT) A549 cells monolayers vs the x coordinate in the Hele-Shaw channel (or distance from inlet), obtained from the equation and the simulation. Relationship between the cell FSS applied to (B, LEFT) Caco-2 cells and (B, RIGHT) A549 cells monolayers vs the x coordinate in the Hele-Shaw channel, obtained from the equation and the simulation.

Table 1

Flow rates and FSS used for Caco-2 and A549 cell lines.

	Flow rate	FSS range experienced by cells within the device (equation)	FSS range experienced by cells within the device (CFD)
Caco-2 cells	0.22 µL/min	From 0.03 to 0.002 dyn/cm <sup>2</sup>	From ~0.03 dyn/cm <sup>2</sup> to 0.003 dyn/cm <sup>2</sup>
A549 cells	1 μL/min	From 0.135 to 0.008 dyn/cm <sup>2</sup>	From ~0.134 dyn/cm <sup>2</sup> to 0.017 dyn/cm <sup>2</sup>

 $9.4 \times 10^{-8}$  N.s/cm<sup>2</sup> (at 37 °C); Length of the first part of the channel, L = 5.3 cm; h = 0.0145 cm;  $w_1 = 0.1$  cm; Flow rate,  $Q = 3.7 \times 10^{-6}$  cm<sup>3</sup>/s (for 0.22 µl/min) or  $1.67 \times 10^{-5}$  cm<sup>3</sup>/s (for 1 µL/min). Eqs. (1) and 2 describe the Hele-Shaw model where x (cm) is a point on the central axis (or distance from the inlet).

Values obtained for the wall FSS at the five different sections in the channel show that the simulation matches the values given by the Hele-Shaw equation (see Fig. 3.A).

The relationship between the wall FSS ( $\tau_{wall}$ ) and the FSS experienced by cells adhered on the bottom wall of the microfluidic channel ( $\tau_{cell}$ ) was previously studied by Gaver and Kute [6] and accordingly, the approximation  $\tau_{cell} = 3 \times \tau_{wall}$ , was applied. Using the parameters above, the relationship between the FSS experienced by the Caco-2 cells monolayer as a function of the x axis is presented in Fig. 3.B and both the results from the equation and from the simulation are compared. A549 were exposed to a higher range of FSS by keeping the physical dimensions of the device constant and increasing the flow rate (Fig. 3.B and Table 1).

7. Finally, the simulation was used to give a distance range from the central axis which can be considered for the imaging analysis in each five sections of the microchannel. Indeed, the FSS especially on the central axis is linearly decreasing but being at a small distance *dmax* from the central axis in each section allows to get this linear trend too. Therefore, the velocity and strain rates profiles along the y axis are plotted in the middle of each 'FSS sections' (x = 0.5; 1.5; 2.5; 3.5; 4.5 cm) and at  $z = 20 \,\mu\text{m}$ . The profiles allows the calculation of the distance *d* for each section (Fig. 4; Figures S2 for 0.22  $\mu$ L/min and S4 for 1  $\mu$ L/min).



**Fig. 4.** A: Velocity contours at five points in the channel (x = 0.5; 1.5; 2.5; 3.5; 4.5 cm) in yz plans. B: Velocity contour values along the channel at  $z = 20 \ \mu\text{m}$  (-height of the cell monolayer) in the xy plan, at 0.22  $\mu$ L/min. C: Strain rate profiles along the y axis at  $z = 20 \ \mu\text{m}$  and at different x positions for 0.22  $\mu$ L/min. D: Schematic representation of the surface area within the Hele-Shaw device having constant fluid shear stress along each y axis; Table 2 summarizes the exact distance from the central axis having a constant FSS on the y axis and which can be considered for imaging at each section (same results obtained for 1  $\mu$ L/min).

Sections of shear stress in the Hele-Shaw model	Dwall (mm)	Dmax (mm)	Mean strain rate (/s)	Mean shear stress (dyn/cm <sup>2</sup> )	Shear deviation (dyn/cm <sup>2</sup> )
S1 ( $x = 0.5$ cm)	0.22	0.34	0.002	1.91E-05	1.71E-05
S2 ( $x = 1.5$ cm)	0.24	0.47	0.0021	1.96E-05	1.63E-05
S3 ( $x = 2.5$ cm)	0.18	0.82	0.0008	7.48E-06	6.63E-06
S4 ( $x = 3.5$ cm)	0.16	1.5	0.00032	3.02E-06	2.49E-06
S5 ( $x = 4.5$ cm)	0.33	3.84	0.00035	3.32E-06	2.16E-06

 Table 2

 Distance from the wall to start having constant shear stress on y axis (mm).

The profiles of the strain rates along the y axis show that the distance *d* from the central axis valid for the cell imaging is the same for all flow rates tested 0.22, 1 and additionally 120  $\mu$ L/min.

In the supplementary information, we also show the velocity and strain rate profiles on the z and y axis for a flow rate of 120  $\mu$ L/min which could be applied on endothelial cells (Supplementary Information-3. Figures S5, S6 and S7).

#### Fabrication of Hele-Shaw SU-8 mold using soft lithography

In order to prepare the Hele-Shaw microfluidic cell culture device, a SU-8 mold should first be prepared. The instructions below provide specific guidelines/examples that should be adapted to the available microfabrication facilities.

- 1. Save the selected design from Autocad as a dxf to be use in Kloe software. Contours and filling of the design are exported as a lwo file from the software, as recommended, and copied on the monitor of the mask writer.
- Spin coat a SU-8 50 (MicroChem, Newton, MA) layer of 150 μm thickness on a 6 inches silicon wafer by spinning at 300 rpm for 30 s on a Karl Suss Delta 80 spin coater (Suss MicroTec, Germany).
- 3. Bake at 65 °C for 5 min and then at 95 °C for 45 min.
- 4. Write the pattern using a Dilase 650 mask writer (Kloe, France) at 3 mm/s for contour and 10 mm/s for filling at laser dose of 30% energy modulation.
- 5. Post-exposure bake at 65 °C for 1 min and 95 °C for 15 min.
- 6. Develop the pattern in a SU-8 developer solution for 10 min, clean with isopropanol and hard bake (210 °C for 5 min).
- 7. Ensure the thickness of the SU-8 is correct using a profilometer.

#### Fabrication of Hele-Shaw devices

- 1. Cast PDMS (10:1, w/w) onto the SU-8 micropatterned master mold to obtain a 5 mm thick primary layer. Make sure there are no air bubbles in the uncured PDMS when poured in the SU-8 mold (use a desiccator and a pump).
- 2. Cure the PDMS in the SU-8 mold for at least 2 h at 70 °C.
- 3. Cut smoothly the rectangle layer on the edges of the pattern with a scalpel, peel the PDMS layer, turn it over and make incursions with biopsy punchers (Harris Core, 1.5 mm diameter for the outlet, and 3 mm diameter for the inlet) before cleaning the PDMS incursed face with isopropanol and dry with nitrogen.
- 4. Prepare a small 5 mm<sup>2</sup> piece of PDMS large enough to seal the 3 mm inlet hole bubble trap. Bond the block with oxygen plasm (treatment at medium RF intensity for 1–2 min) on the top side of the layer (the side not including the incursions from the pattern). Incubate the device for 10 min in an oven set at 65 °C.
- 5. Oxygen plasma treat the PDMS layer and bound to a glass cover slip (Proscitech, G418, No1) for 10 min to 1 h at 65 °C to produce permanent bonding. A photograph of a device is shown in Fig. 5 highlighting five different sections of FSS (arbitrarily chosen every centimeter).



Fig. 5. Photograph of a Hele-Shaw device highlighting five different sections of FSS calculated from Eq. (3).

#### Cell seeding and culture

### Solutions to prepare fresh

- 1. Matrigel Basement Membrane Matrix (Corning, BD Biosciences, Tewksbury, MA, USA) solution should be prepared at a final concentration of 300  $\mu$ g/mL in pre-chilled serum-free DMEM or 1x phosphate buffer saline (PBS). Keep the Matrigel solution at 4 °C or on ice to prevent unwanted premature polymerization of matrix proteins. Note: a different extracellular matrix gel type might be necessary for a specific type of cells. Matrigel was suitable for both Caco-2 and A549 cells.
- 2. Prepare the adequate cell culture medium.

In our example, human intestinal epithelial Caco-2 cells (Caco-2BBE human colorectal carcinoma line) [7] were cultured in 75 cm<sup>2</sup> tissue culture flasks in Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich, Australia) supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 1% L-glutamine (Sigma Aldrich) and 1% streptomycin/ penicillin (P/S) (Sigma Aldrich) at 37 °C with 5% CO<sub>2</sub> in a humidifying incubator.

Human lung epithelial A549 cells (ATCC<sup>®</sup> CCL-185<sup>TM</sup> human lung carcinoma line) were cultured in DMEM F12-K mixture (Gibco, ThermoFisher, Australia) supplemented with 10% FBS, 1% L-glutamine and 1% streptomycin/ penicillin as above. Cells were cultured to 70–80% confluence before being seeded inside the device.

#### Procedure

Most steps are performed within a biosafety cabinet.

- 1. Connect two Ethyl Vinyl Acetate Microbore Tubes (0.020"/ID x 0.060"/OD; John Morris Scientific Pty Ltd) to the inlet and outlet ports of a Hele-Shaw device.
- 2. Connect the inlet EVA tube to a 3-stop Tygon tube (Choice Analytical Pty Ltd) with a 3 G connector.
- 3. 3, Introduce 70% (v/v) ethanol through the tubing and the channel using a peristaltic pump (Langer Instruments, USA) for 30 min to disinfect the microfluidic system. This is done in the Biosafety cabinet with the pump inside. Ideally, one pump is used in the biosafety cabinet and another one in the cell culture incubator for the devices perfusion.

- 4. Inject PBS (Ca<sup>2+</sup> and Mg<sup>2+</sup>free, pH=7.4, Sigma Aldrich) for 20 min followed by FBS free-DMEM or PBS with 1% P/S for 1 h at room temperature (RT).
- 5. Inject and then incubate Matrigel prepared as described above at 0.3% v/v in serum-free DMEM for 2 h under static conditions at 37 °C in a humidified incubator to form a coating on the cover slip surface. 200 µL is enough to fill the entire tubing and device. Care should be taken to avoid introducing air bubble in the microfluidic chamber as these could induce defects in the Matrigel coating. To minimize air bubble, the whole device can be immersed in water in a Petri dish.
- 6. Make sure the tubing system does not have any leak before seeding the cells. Flush the channel with medium to remove any residual Matrigel solution and return the microdevice for 1 h in the incubator.
- 7. During this time, start the trypsinization of the cells from a 90% confluent T75 flask. First remove the cell culture medium from the flask, and then wash the cells with  $Ca^{2+}$  and  $Mg^{2+}$ free PBS twice. Add 2 mL of trypsin/EDTA (0.05%, Sigma Aldrich) to the flask and incubate for few minutes or until the cells detach from the bottom of the flask in the cell incubator (37 °C, 5% CO<sub>2</sub>). Count the cells with Trypan Blue (to take into account only live cells) and centrifuge the cell suspension (at 500 RCF for 5 min) and resuspend in the correct volume to obtain a final concentration of ~1.5 × 10<sup>7</sup> cells/mL. The optimal cell concentration was determined to be  $2 \times 10^5$  cells/cm<sup>2</sup> for Caco-2 cells but it might be necessary to adjust the seeding density for different cells. To calculate the concentration per volume of cells, integrate the function of the width of the channel and add the triangular part close to the outlet to find the total area of the Hele-Shaw cell culture device:

$$w = w_1., \frac{L}{(L-x)}, \text{ i.e. } \int_0^{4.5} w \, dx = 1.151 cm^2$$
 (3)

In our example, the total area of the device was 2.552 cm<sup>2</sup> and the total volume was 38.28  $\mu$ l. Cells were therefore seeded at a concentration of ~1.5  $\times$  10<sup>7</sup> cells/mL.

- 8. Mix very thoroughly the cell suspension prior to injecting within the device. Load the cell suspension into a 1 mL syringe with a 23 G needle and insert the needle in the inlet EVA tube making sure not to introduce any air bubble. Then, inject the cell suspension slowly. Reconnect the Tygon and EVA tubes together, again taking care to avoid introducing air bubble (the medium can flow through the Tygon tube during this procedure).
- 9. Insert and seal the inlet Tygon tube into a reservoir with fresh complete medium (~5 mL), making sure the tip of the tube is well immersed in the medium (use 2 or 3 sheets of parafilm to seal). The whole system is illustrated in Fig. 5. Check the microchannel under a microscope to make sure cells are homogeneously distributed across the Hele-Shaw channel. If necessary, go back to the biosafety cabinet to push further the cells or reload them. Shaking horizontally or tilting the device can help spreading the cells across the width and length of the device and obtain a more homogeneous seeding density.
- 10. Leave cells to adhere to the Matrigel coated glass for at least 4 h in the cell incubator. Inspect the device to ensure good cell adhesion to the glass coverslip.
- 11. Connect the Tygon tube to the peristaltic pump in the cell incubator. Start the flow at a media flow rate set at 0.22  $\mu$ L/min for Caco-2 cells (equivalent to 0.4 rpm, after calibration with the peristaltic pump used here). A waste container is also placed at the outlet side. As noted above, the device can be immersed in water to minimize the risk of air bubbles associated to the fact that PDMS is gas permeable.
- 12. In our typical protocol, Caco-2 cells and A549 cells were grown to confluence for 5 days. Caco-2 cells formed a confluent and fully-differentiated monolayer, as previously described [4,8,9]. The cell density can be monitored daily using bright field microscopy, for example using an inverted Eclipse Ti-E Nikon microscope (Nikon, Japan). The presence of homogeneous monolayers can be better assessed by staining with Hoechst for live nuclei staining and bright field microscope (see Fig. 6.C). The cellular monolayers can also be assessed using a stereomicroscope to assess a larger area of the monolayer in the device at different culture time points (Fig. 6.A, B).



Fig. 6. Stereomicroscope images of a Caco-2 cells monolayer after 2 days (A), 3 days (B) and 5 days (C) of culture in the Hele-Shaw device.

## Morphological analysis of the cellular monolayers and dry mass calculation

1. Fix the cells with 4% (w/v) formaldehyde for 20 min at RT. Different imaging techniques could be used to analyses directly the differences between the sections of FSS. We described here the use of phase contrast microcopy, digital holographic microscopy and confocal microscopy.

Characterization of the cellular monolayer morphology and presence of vacuoles.

- 2. Capture phase contrast images with an inverted microscope (we used a Ti-E Nikon microscope equipped with an ANDOR zyla 5.5 camera, Andor Technology Ltd, Belfast, Northern Ireland) using objectives with 10 × and 20 × magnifications. Differential interference contrast (DIC) images were acquired with a Confocal Laser Scanning Microscope 710 (Zeiss, Germany) equipped with a sCMOS PCO Edge camera using an EC Plan-Neofluar 10 × with DIC objective and T-PMT (transmitted light detector).
- 3. To quantify the number and area of vacuoles in the monolayer, different methods can be used such as an automated image analysis of vacuole count and size, and monolayer height described in Kim et al. 2017 using MATLAB (MathWorks, MA, USA) or manually as described below [10]. Analyze phase contrast images using a freehand selection tool to mark vacuoles in ImageJ (NIH) as shown in the companion manuscript. At least two microscopic images for each section should be analyzed in at least three different devices to obtain statistically meaningful data.

*Calculation of the dry mass using digital holographic microscopy.* To obtain further information about the monolayer, we implemented a measurement of the "dry mass" along the x axis of the Hele-Shaw device using Digital Holographic Microscope (DHM).

Pixel number	Pixel size	Constant number	Wavelength	Readout	Dry mass (kg)	Section in the chip	Picture Number
857,472	1.404E-13	5555.556	6.67E-07	6.79	3.02908E-09	S1	18-14
857,472	1.404E-13	5555.556	6.67E-07	7.21	3.21644E-09	S1	18-19
857,472	1.404E-13	5555.556	6.67E-07	9.46	4.22019E-09	S2	18-21
857,472	1.404E-13	5555.556	6.67E-07	6.09	2.7168E-09	S2	18-24
857,472	1.404E-13	5555.556	6.67E-07	7.46	3.32797E-09	S3	18-27
857,472	1.404E-13	5555.556	6.67E-07	9.61	4.2871E-09	S3	18-29
857,472	1.404E-13	5555.556	6.67E-07	7.49	3.34135E-09	S4	18-30
857,472	1.404E-13	5555.556	6.67E-07	9.07	4.04621E-09	S4	18-32
857,472	1.404E-13	5555.556	6.67E-07	5.77	2.57405E-09	S5	18-35
857,472	1.404E-13	5555.556	6.67E-07	8.11	3.61794E-09	S5	18-44

 Table 3

 Typical data generated the DHM images.

- 1. Image three or more areas per section with a Transmission DHM (Lyncée Tec, Lausanne, Switzerland) using a 20x objective and a laser at 666 nm wavelength (see Table 3). Pen marks can be used to identify the FSS sections to be imaged within the device.
- 2. Reconstruct the images acquired (converted to phase image as a numeric binary file) with the Koala Acquisition software before analysis (Lyncée Tec, Switzerland).

The optical thickness of the cells (or optical path differences, OPD per pixel, corresponding to the phase shift undergone by the light emitted from two previously coherent sources when passed through the mediums, here medium with and without cells, of different refractive indices) was used for calculation of the dry mass. For reference, we use the term "optical thickness" when speaking about the cellular monolayer and "phase shift" when referring to light or optics. The binary file of each image was then input into MATLAB and the darkest area was manually segmented as background to calculate the integrated phase shift, following Eq. (4):

$$\int \varphi = \Sigma OPD_{(wholepicture)} - \Sigma OPD_{(background)}$$
(4)

In quantitative phase microscopy, the relationship between the mass and refractive index was used to estimate the cells' dry mass. Most biomolecules exhibit a linear relationship between refractive index and concentration. The slope of this relationship is the specific refractive increment, and relates the refractive index change to the increase in biomolecule mass density. Specific refractive increments for most biomolecules fall within a very narrow range, allowing estimation of a meaningful average value to be used to compute the cell mass. This average value typically equals to approximately  $1.8 \times 10^{-4} \text{ m}^3/\text{kg}$  [11,12] and can be used to convert phase shifts to dry mass using Eq. (5):

$$\mathbf{m} = \frac{1}{\alpha} \int \varphi \lambda d\mathbf{A} \tag{5}$$

where m is the cell dry mass (kg);  $\alpha$  is the specific refractive increment;  $\varphi$  is the measured phase shift as a fraction of wavelength;  $\lambda$  is the illumination wavelength, 6.67 × 10<sup>-7</sup> m; and an integration was performed across the entire cell area, with A, area of one pixel, 1.404 × 10<sup>-13</sup> m<sup>2</sup>. However, the specific value of  $\alpha$  can be typically regarded as constant [12] and practically, the calculation of the dry mass based on Eqs. (4) and (5) is done using (6):

$$m = \frac{1}{\alpha} \lambda A \left( \Sigma OPD_{(wholepicture)} - \Sigma OPD_{(background)} \right)$$
(6)

Immunofluorescence microscopy Immunofluorescence staining.

^

- 1. Permeabilize the cells with 0.1%  $(\nu/\nu)$  Triton X-100 for 15 min.
- 2. Block with 5% (w/v) bovine serum albumin (BSA) for 1 h on ice.
- 3. Wash the cells with PBS (all from Sigma Aldrich, Australia) for 5 min.



Fig. 7. Alcian blue staining of mucus at different section of the Hele-Shaw device.

- 4. Incubate the cells with primary antibodies for 2 h at RT or overnight at 4 °C using either concentrations recommended by the manufacturers or optimal ones pre-determined for the specific antibodies.
- 5. Incubate the cells with the corresponding secondary antibodies for 2 h at 4 °C.
- 6. Incubate the cells with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) or 0.1  $\mu$ g/mL Hoescht at RT (Sigma Aldrich) for nuclei staining.
- 7. Conversely, to visualize F-actin, 20  $\mu$ g/mL of FITC-Phalloidin (Sigma Aldrich) was applied at RT for 40 min to cells that were previously fixed, permeabilized, and blocked with 1% BSA. All solutions were perfused at 5  $\mu$ L/min.

*Imaging of the cellular monolayers.* Monolayers were imaged with a LSM 710 confocal microscope (Zeiss, Germany) equipped with a sCMOS PCO Edge camera using an immersion oil objective 63 x with water immersion along the z axis of the channel corresponding to the different sections of the FSS.

- 1. Capture at least 3 images of each defined section (*e.g.* every cm) in three independent devices. The FSS close to the outlet is not very well defined based on the model (see CFD simulation section) and therefore should be excluded from the analysis.
- Measure the mean fluorescence intensities of the staining or immunostaining with ImageJ (NIH). Analyze the 16-bit grey scale images with ImageJ to calculate the pixel intensity normalized per unit of area (mean pixel intensity per unit area divided by maximum intensity of the images) [13].
- 3. *Optional*: Evaluate the height of monolayer using F-actin staining and perform five measurements at random points using ImageJ in two images for each section taken from each of the three independent experiments.

### Staining for mucus (depending on the epithelial cells used)

Alcian blue is commonly used to stain the glycocalyx (acidic substances of the mucopolysaccharides, part of the sialylated glycocalyx within the intestinal mucus [14]).

- 1. Fix the cells with formal dehyde solution (4%, w/v) for 20–30 min.
- 2. Perfuse 0.1% (w/v) alcian blue solution prepared in 3% (v/v) acetic acid (pH 2.5; 8GX Sigma Aldrich) into the device at 1  $\mu$ L/min for 2 h.
- 3. Wash the cells with PBS for 30 min.
- 4. Acquire images with an inverted microscope equipped with a color camera. Alcian blue can also be visualized by stereomicroscopy (Fig. 7).
- 5. Convert all images (including background image for cells without staining used as control) to 8-bit images and measure color mean intensities.
- 6. Subtract the mean intensity of the background images from the respective values of the images. Normalize values against the images captured in the inlet where accumulation of mucus was observed.

## Statistics

1. Acquire and analyse three images of each section of the Hele-Shaw culture to obtain the corresponding data (mean fluorescent intensity, Alcian blue intensity values calculated as described above or height of the monolayer) and present them as a mean value for each section. Repeat each



Fig. 8. Epithelial to mesenchymal transition for A549 cells cultured under different FSS in the Hele-Shaw device over 7 days, as shown by the expression of vimentin (red).

experiment of interest three times independently (three different devices, prepared independently) so that each section is represented by a set of data of three independent mean values. Present the data as the mean  $\pm$  standard error of mean (SEM).

- 2. Perform a Kruskal-Wallis test (non-parametric one-way analysis of variance (ANOVA)). The difference between each section is then compared with the Dunn's test. All the statistical data analysis can be performed with GraphPad Prism 7.04 for Windows (GraphPad Prism Software, La Jolla, CA).
- 3. A p-value<0.05 indicates statistically significant difference between the groups. On the contrary a p-value>0.05 shows that the two sections have no significant difference.

## Method validation

The method described here has been validated for two different type of cells. A detailed report on the characterization of the effect of the FSS on Caco-2 cells based intestine-on-chip model is presented in the companion manuscript [4]. We also included here in the supplementary information the results without any FSS (no flow, static) to see the effect of flow on cells functions in the same sections as a comparison (Figure S8). We also investigated the effect of the FSS on alveolar A549 cells that are commonly used to prepare lung-on-chip models. To this end we used A549 VIM RFP cell line in which vimentin is constitutively tagged with the red fluorescent protein, thereby enabling direct monitoring with confocal microscopy of the effect of the FSS on the expression levels of this epithelial-mesenchymal transition (EMT) marker. EMT is a process in which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties [15,16]. It has been



Fig. 9. Confocal microscopy images of A549 lung cells at day 7 in a Hele-Shaw device showing vimentin (red) and Pan cytokeratin expression and DAPI. B: Mean fluorescence intensity for vimentin and cytokeratin along the FSS axis.

demonstrated that A549 cancer cells undergo EMT under specific conditions like the exposure to TGF- $\beta$ 1 [17], menthol or tobacco-flavored EC liquids or aerosols [18]. In addition, EMT can be significantly affected by the FSS [19]. A549 cells cultured in Hele-Shaw devices expressed more vimentin over time, especially when exposed to the higher range of FSS (Fig. 8). Vimentin expression decreased from section 1 to section 5. It can also be observed that the morphology of the cells is different across the sections of the Hele-Shaw channel as cells were more spindle shaped than circular at higher FSS. Upon staining with anti-Cytokeratin (CK3–6H5)-FITC antibody (Myltenyi Biotec, Australia), it is also evident that the FSS induced increased expression of vimentin correlated with decreased expression of cytokeratin by the A549 cells (Fig. 9).

This method described a novel approach to systematically investigate the effect of the FSS on cellular monolayers using a single microfluidic device based on the Hele-Shaw geometry. It provided a detailed guideline on how to adjust the experimental parameters to apply specific ranges of FSS and precisely map out the shear stress distribution within the device using of a computational simulation of the fluid flow. This approach was successfully applied to systematically investigate Caco-2 cell monolayers-based intestine-on-chip models as reported in a companion article published in Biomaterials [4]. This study showed that exposure to microfluidic FSS induces significant phenotypical and functional changes and consequently that detailed understanding of the effects of the FSS enables the realization of *in vitro* organs-on-chip models with well-defined characteristics tailored to a specific purpose. We also showed changes in the phenotype of A549 cells within the device. The methodology reported here could be readily applied to other cell types and adapted for a wide range of physiologically relevant FSS.

## **Declaration of Competing Interest**

The authors declare that there are no conflicts of interest.

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#### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi: 10.1016/j.mex.2020.100980.

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