Lung/liver-on-a-chip for systemic toxicity studies

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Introduction

In vitro models have limitations in mimicking complex physiological processes, which contributes significantly to drug development failure. This is, at least in part, related to the lack of interactions between cells and their natural microenvironment. The combination of 3D *in vitro* models with an engineered microenvironment, also known as "organ-on-chips", have the potential to make drug development more effective.
Recently, we created a lung–liver-on-a-chip platform for better assessing the toxicity of aerosols. The system is composed of a chip plate with 3 circuits, each composed of 2 interconnected wells. Each circuit is connected to a pump, ensuring medium circulation. We tested the characteristics of 3D organotypic bronchial epithelial cultures and HepaRG™ liver spheroids when maintained together in the platform for 28 days. The transepithelial electrical resistance (TEER) and cilia beating frequency (CBF) of the bronchial cultures were unaltered throughout the study. The liver spheroids secreted albumin and maintained their metabolic capacity over the study period. We also examined the role of the liver spheroids in modulating the toxicity of a known pulmonary toxicant, aflatoxin B1 (AFB1). The results showed that, when the bronchial cultures were exposed to AFB1 in absence of liver spheroids, TEER values and adenosine triphosphate (ATP) content decreased markedly. Conversely, in the presence of liver spheroids, bronchial cultures were unaffected by the compound for up to 48 h.
Additional modifications of the platform to include 8 wells per circuit provided the opportunity to test whether bronchial cultures could be generated faster and more optimally. For this experiment, bronchial cultures were transferred to the chip immediately after air-lift and exposed to continuous medium flow for 4 weeks. We found that the constant medium recirculation improved the homogeneity, pseudostratification, ciliation, and cell polarization of the cultures relative to those of cultures matured in standard plates.
Collectively, the results demonstrate the versatility of this chip platform for a variety of applications, including 3D tissue preparation and toxicological testing.

Methods

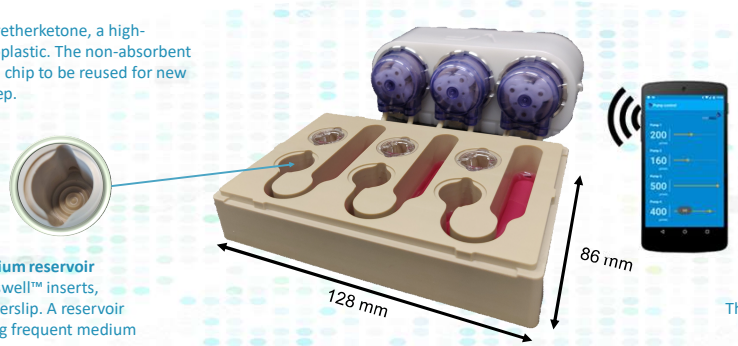
Bronchial organotypic cultures were prepared from normal human bronchial epithelial cells (Lonza, Basel, Switzerland) as previously described (Bovard et al., 2018). For Use Case 1, lung tissues were used starting from day 28 after air-lift. For Use Case 2, half of the cultures were transferred to the 32-well chip immediately after air-lift and exposed to a continuous flow of PneumaCult™-ALI medium (8 tissues per circuit; 6 mL medium per circuit; 150 µL/min), while the other half remained in a standard 24-well plate with PneumaCult™-ALI medium in the basolateral compartment (control condition). The medium was changed every day during the first week and then once a week for the three remaining weeks for cultures matured in the chip and every 2 or 3 days until week 4 for control cultures. Tissues were used from week 1 after the transfer. **Liver spheroids** were prepared from cryopreserved and differentiated HepaRG™ cells (ThermoFisher, Waltham, MA, USA) as previously described (Bovard et al., 2018). They were used once mature, at approximately 1 week after thawing. Experiments were conducted with 48 spheroids (or 600,000 liver cells).
The VitroFluid chip plate was first loaded with complete PneumaCult™-ALI medium, run without tissues to fill the tubing, and incubated at 37°C to equilibrate the medium. Organ cultures were placed in the chip, which was then returned to the incubator for 28 days. The medium was changed every 2 or 3 days. AFB1 (Sigma, St. Louis, MO, USA) was dissolved in DMSO before being diluted in the medium circulating in the chip to a working concentration of 100 µM.
The **morphology** of bronchial organotypic cultures was evaluated following fixation, paraffin embedding, sectioning, and staining with hematoxylin and eosin and Alcian blue. For **immunostaining**, the tissues were first fixed in 4% paraformaldehyde for 20 min and blocked for 1 h. Then, the tissues were stained with either an anti β-tubulin 4 antibody conjugated to Alexa 647 (Abcam, Cambridge, UK) or an anti-mucin 5AC antibody conjugated to Alexa 555 (Abcam) and incubated overnight at 4°C. Images were acquired by using the CX7 CellInsight™ platform (Thermo Fisher Scientific).
Transepithelial electrical resistance (TEER) was measured by using chopstick electrodes (WPI, Sarasota, FL, USA) connected to an EVOM-2™ epithelial voltohmmeter (WPI) in accordance with the manufacturer's instructions. **Cilia beating frequency** (CBF) was measured by using an inverted microscope (Zeiss, Oberkochen, Germany) equipped with a 4x objective, a 37°C chamber, and a high-speed camera (Basler AG, Ahrensburg, Germany). Short movies composed of 512 frames recorded at 120 images per second were captured and analyzed by using the Sisson-Ammons Video Analysis (SAVA) analysis software (Ammons Engineering, Clio, MI, USA). **ATP content** was measured by the CellTiter-Glo® 3D cell viability assay (Promega, Madison, WI, USA). The **metabolic capacity** of bronchial tissues was further confirmed by examining CYP1A1/B1 enzyme activity by a P450-Glo™ assay (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. CYP1A1/1B1 enzyme activity was induced 48 h prior to measurement with 10 nM *2,3,7,8-tetrachloro-dibenzodioxin* (TCDD; Sigma).

References

Bovard, D., and Sandoz, A. (2020). How to build your multiorgan-on-a-chip system: a case study. In Organ-on-a-Chip, (Elsevier), pp. 463–506.
Bovard, D., Sandoz, A., Luettich, K., Frentzel, S., Iskandar, A., Marescotti, D., Trivedi, K., Guedj, E., Dutertre, Q., Peitsch, M.C., et al. (2018). A lung/liver-on-a-chip platform for acute and chronic toxicity studies. Lab on a Chip 18, 3814–3829.

The VitroFluid device

Biocompatible and non-absorbent
The plates are made from polyetheretherketone, a high-performance biocompatible thermoplastic. The non-absorbent properties of this material allow the chip to be reused for new experiments after an autoclaving step.



Three-step connection and ease of use
Installation of a chip requires placement of the pump unit in the incubator, connection of a single cable to a plug (one chip = one cable), and, finally, plugging of each pump head into the pump unit. The chip is ready for use.

Versatile wells and integrated medium reservoir
Compatible wells designed for Transwell™ inserts, spheroids, or cells cultured on a coverslip. A reservoir allows for long-term culture avoiding frequent medium changes.

High-efficiency programmable pumps
The pump unit is composed of high-efficiency motors for reducing heat emission. In consequence, up to 9 VitroFluids can fit in a single incubator. The flow rates of each channel can be individually set (between 30 and 600 µL/min) by using a smartphone even when the incubator door is closed.

Use case 1: Multi-organ-chip for toxicity testing

With the aim of creating a lung/liver model for accurate assessment of aerosol toxicity that mimics human exposure, 3D human bronchial epithelial tissues were cocultured with HepaRG™ liver spheroids for 28 days. The stability of both tissues in the chip, alone and in combination, was evaluated at the end of the experimental period. While the liver spheroid morphology remained comparable across conditions **(Figure 1A)**, the viability **(Figure 1B)** and metabolic capacity **(Figure 1C)** of spheroids cultured in the chip were slightly decreased relative to the tissues cultured in static condition. Bronchial tissue morphology remained similar to that of control tissues even after 28 days in the chip **(Figure 2A)**. While the CBF was comparable across conditions **(Figure 2B)**, TEER had increased by 50% in all tissues cultured in the chip, relative to control tissues **(Figure 2C)**. Shear stresses resulting from the medium flow beneath the inserts might explain this result.
Using this lung/liver-on-a-chip platform, we further demonstrated the role of the liver spheroids in metabolizing and inactivating a pulmonary toxicant, AFB1. In the absence of liver spheroids, TEER decreased markedly in bronchial tissues exposed to the toxin for 48 h, relative to untreated tissues **(Figure 3A)**. Interestingly, in the presence of liver spheroids, bronchial tissues were unaffected by AFB1 for the first 48 h. Similarly, the ATP content of bronchial tissues exposed to AFB1 for 72 h in coculture with liver spheroids was significantly higher than that of bronchial tissues cultured alone **(Figure 3B)**.
Together, these results show that the properties of the material used for constructing the chip (i.e., its biocompatibility and non-absorbance) combined with its ease of use, which reduces experimental errors, have made it possible to coculture bronchial and liver spheroid cultures for 28 days without an impact on either tissue's key characteristics. The advantage of the chip compared with a single culture model for assessing compound toxicity was demonstrated by using AFB1.

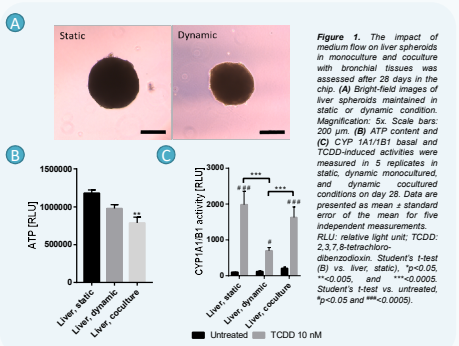


Figure 1. *The impact of medium flow on liver spheroids in monoculture and coculture with bronchial tissues was assessed after 28 days in the chip.* ***(A)*** *Bright-field images of liver spheroids maintained in static or dynamic condition. Magnification: 5x. Scale bars: 200 µm.* ***(B)*** *ATP content and* ***(C)*** *CYP 1A1/1B1 basal and TCDD-induced activities were measured in 5 replicates in static, dynamic monocultured, and dynamic cocultured conditions on day 28. Data are presented as mean ± standard error of the mean for five independent measurements. RLU: relative light unit; TCDD: 2,3,7,8-tetrachloro-dibenzodioxin. Student's t-test (B) vs. liver, static), *p<0.05, **<0.005, and ***<0.0005. Student's t-test vs. untreated, #p<0.05 and ###<0.0005).*

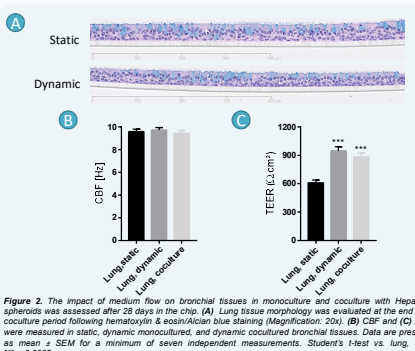


Figure 2. *The impact of medium flow on bronchial tissues in monoculture and coculture with HepaRG™ spheroids was assessed after 28 days in the chip.* ***(A)*** *Lung tissue morphology was evaluated at the end of the coculture period following hematoxylin & eosin/Alcian blue staining (Magnification: 20x).* ***(B)*** *CBF and* ***(C)*** *TEER were measured in static, dynamic monocultured, and dynamic cocultured bronchial tissues. Data are presented as mean ± SEM for a minimum of seven independent measurements. Student's t-test vs. lung, static, ***p<0.0005.*

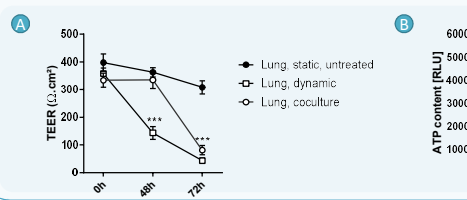


Figure 3: *AFB1 toxicity was assessed by using the lung–liver-on-a-chip.* ***(A)*** *TEER and* ***(B)*** *ATP content were measured in bronchial tissues untreated or exposed to AFB1 for 48 and 72 h. Samples exposed to AFB1 were either kept in monoculture (Lungs, dynamic) or cocultured with liver spheroids (Lungs, coculture). Data are presented as mean ± SEM for twelve independent measurements. Student's t-test vs. static untreated, **p<0.005 and ***<0.0005.*

Use case 2: Design versatility – Lung tissue maturation

Preparation of 3D organotypic bronchial tissues requires the use of primary human bronchial cells cultured at the air–liquid interface for 4 weeks with medium changes every 2 or 3 days.
With the aim of reducing the number of medium changes, accelerating tissue maturation, and improving tissue characteristics (such as thickness or pseudostratification), we connected a modified chip plate with 8 wells per circuit to the VitroFluid pump unit. Transwell™ inserts seeded with bronchial cells were then placed in the chip, and tissues were matured for 4 weeks while being exposed to continuous medium flow with weekly medium changes. The tissues were examined every week.
Comparison of tissue morphology between the conditions showed striking differences: Starting from week 2, the ciliated cells showed a more polarized morphology in tissues maintained in the chip than in tissues matured in static condition **(Figure 4)**. At weeks 2 and 3, tissues matured in the chip were also between 10 and 50% thinner. Immunostaining for cilia and goblet cells showed that tissues matured in the chip had denser ciliation **(Figure 5A)** but a 10–50% lower goblet cell population **(Figure 5B)** depending on the week of maturation.
The presence of continuous medium flow, associated with a decrease in the supply of growth factors (the regimen involving fewer medium changes), improved and accelerated ciliated cell differentiation and maturation. The thinner tissue epithelium could be attributed to better nutrient supply and, therefore, a lower tendency for the basal cells to become hyperplastic.
This case study shows that VitroFluid can be redesigned in a fast and agile manner to meet specific research needs

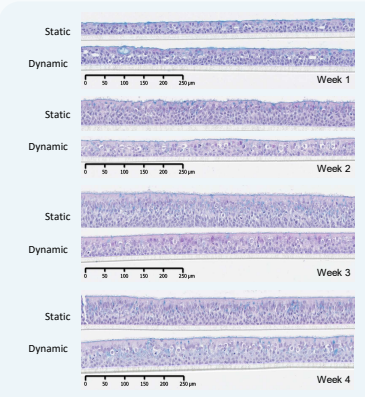


Figure 4. *Bronchial tissue morphology was evaluated every week following hematoxylin & eosin/Alcian blue staining in both conditions. Magnification: 20x.*

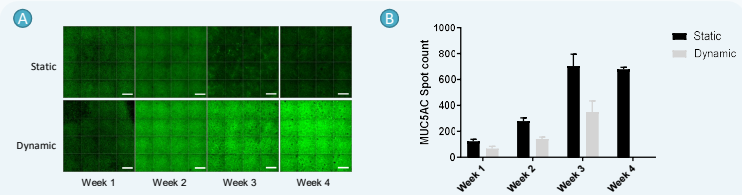


Figure 5. ***(A)*** *Tissues were stained every week with an anti-β-tubulin 4 antibody to detect cilia. The acquired images show the central region of the tissue and cover ~60% of the entire surface. Magnification: 10x. Scale bars correspond to 200 µm.* ***(B)*** *Tissues were stained every week with an anti-mucin 5AC antibody to detect goblet cells. All tissues were stained and images acquired by using the same parameters. The number of goblet cells was counted by using a CX-7 CellInsight proprietary algorithm. The spot count at week 4 for the dynamic condition could not be performed because of technical difficulties. MUC5AC = Mucin 5AC; D = Donor.*

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