BE-Doubleflow application notes: Gut-on-a-chip 1

Table of Contents

Introduction

The intestine is involved in key processes such as digestion and absorption, where multiple cell types are crucial. From these cell types, enterocytes are the most present, lining the entire inner surface of the organ and functioning as a biochemical, biomechanical and immunological barrier. Goblet cells are responsible for the secretion of mucins and are scattered in the intestine. Paneth cells oversee the defence of the gastrointestinal tract, secreting molecules such as lysozymes and defensins. The microbiome is also an important player in the health and disease of the intestine.

Some of these microorganisms are anaerobic, calling for low oxygen levels (Ashammakhi et al., 2020).

Gut-on-a-chip platforms are novel and versatile tools to study the physiology and pathophysiology of the intestine. The devices offered by BEOnChip are produced from Cyclic Olefin Polymer (COP), a biocompatible and transparent material. COP is also an impermeable material to gases which allows the control over the gas concentration within the devices. In case of a gut-on-a-chip, BEOnChip devices are suitable for the simulation of the low oxygen levels typical of the anaerobic environment provoked by the microbiome. Another advantage that microfluidic techniques bring is the control over the architecture of the culture model. In cases of intestinal models, it is important to assess parameters such as the transport of molecules or cells, which standard 2D well plates are not suitable. The BE-Doubleflow device is a platform designed for that type of studies, where two different cell culture channels are separated by a porous membrane, allowing the culture of multiple cell types for these transport and absorption assays. The size of the channels and the pore size of the membrane are tuneable to better suit the research need.

This guide compares the BE-Doubleflow device to an insert platform as control as *in vitro* platforms for gut models. Cell viability, layer formation and cell differentiation into intestinal cell phenotypes were evaluated in both systems.

Materials

1. BE-Doubleflow device

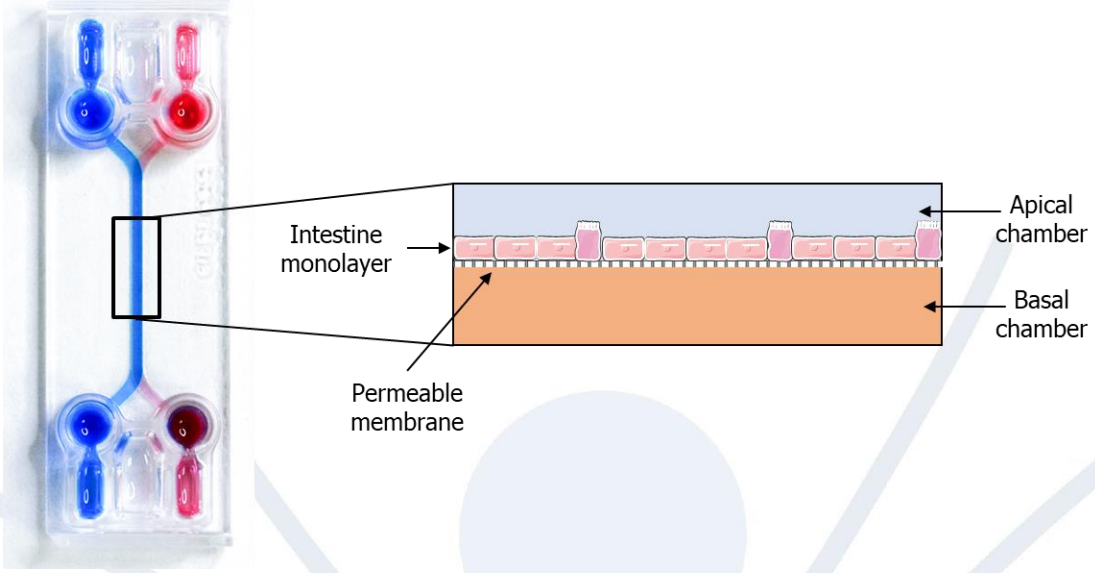

Figure 1 BE-Doubleflow device and cell culture scheme in the device channels.

BE-Doubleflow design (Figure 1) consists of two perfusable channels connected via a PET porous membrane. This device is optimal for epithelium/endothelium barrier models when flux plays a role in both sides of the co-culture. In this work, the upper/apical channel is destined for intestine lumen and the lower/basal channel as endothelial vessel.

	Height	**Width**	**Length**	**Total volume**
Each channel	375 µm	1,5 mm	46 mm	31,2 µL
Inlet/Outlet	7 mm	UNF 1/4" - 28		130 µL
Medium reservoir	5 mm	3,6 mm	8,8 mm	185 µL
Membrane pore size	0,4 µm			

* The volumes presented in the table are theoretical values calculated for the standard products. Changes in the device features of custom chips may modify the exact channel volume.

2. Rocker

Typically, the medium volume of the channels is limited. To promote optimal cell growth, it is essential to refresh the supply of nutrients and oxygen. Therefore, to replenish the cell culture medium (after cell attachment but before connecting any perfusion system) the rocker (orbital shaker) (Figure 2) constitutes a practical tool (https://youtu.be/EVamKBTZpkE).

Several microfluidic devices can be placed simultaneously, ensuring the renewal of the medium within the channel by tilting the devices back and ford. The rocker also permits control over the degree of inclination, speed and working time.

3. Cell types

For intestinal monolayer, two cell types are considered:

Caco-2 cell line (ATCC® HTB-37™, USA) has been used in *in vitro* intestinal models since the 90's. Culture the cells in growth medium Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotics (penicillin 100 U/mL, streptomycin 30 µg/mL) and 1% of non-essential amino acids (NEAA). Place the cells in a CO_2 incubator at 37 °C in a humidified atmosphere containing 5% CO_2.

HT-29 cell line (ATCC® HTB-38™, USA) modified in mucus-secreting cell type with methotrexate (MTX). Culture the cells in growth medium containing McCoy's 5a Medium Modified supplemented with 10% FBS and antibiotics (penicillin 100 U/mL, streptomycin 30 µg/mL). Place the cells in a CO_2 incubator at 37 °C in a humidified atmosphere containing 5% CO_2.

4. Traditional culture insert

Traditional culture inserts (0.4 µm porous size) adapted for 6-well cell culture plates are used as the control system.

Methods

1. BE-Doubleflow cell culture

Before seeding, prewarm the BE-Doubleflow device in the incubator overnight to minimise the formation of air bubbles.

1. Fill the top channel of the device with 100 µL of 0.1 mg/mL of collagen or 0.1% gelatin (diluted in PBS) and incubate at 37°C for 30 minutes. Wash the channel by smoothly

adding 100 µL of PBS into the inlet and removing it at the outlet well with the pipette. Repeat the washing step three times.

2. Aspire gently the dilution buffer completely before seeding.
3. Pipette through the pinhole of the apical channel 50 µL of culture medium with 10^6 Caco-2/HT29/MTX (9:1) cells resuspended. Cover the inlets and incubate the cells for 5-6 h).
4. After cell attachment, add 300 µL of culture medium to the medium reservoirs. Add PBS/water to the evaporation reservoirs, cover and keep the device in the incubator in the **rocker** for up to 21 days. Change cell culture medium every 2-3 days.

2. Insert culture

1. Fill the bottom of the apical side of the insert with 100 µL of 0.1 mg/mL of collagen or gelatin (diluted in PBS) and incubate at 37 °C for 30 minutes. Wash the insert with PBS three times.
2. Pipette the cell solution containing 6x10^5 cells/cm^2 (same as the BDF device).

3. Viability and proliferation assays

To assess the viability and proliferation of the cells during culture, assays such as alamarBlue (DAL1025, ThermoFisher Scientific, USA) cell viability can be performed. Resazurin, the active ingredient of amalarBlue, enters the cell membrane and is reduced to resorufin by the live cells, producing the pink fluorescence detected. Here the quantitative number of cells and viable cells was evaluated with an absorbance/fluorescence-based microplate reader.

1. At the chosen timepoint, remove the device from the incubator and place it in the cell culture hood.
2. Remove the medium from the inlet and outlet reservoirs without emptying the cell channel.
3. Wash the channel with PBS smoothly adding 100 µL of PBS into the inlet and removing it at the outlet well with the pipette. Repeat the washing step three times.
4. Place 100 µL of the alamarBlue reagent in the inlet and wait for it to reach the outlet. If needed, slowly aspirate the liquid through the pinhole of the outlet.
5. Place the device in the incubator for 1 h at 37 °C.
6. Place the device in a plate reader or fluorescence spectrophotometer with 530/590 nm (excitation/emission) filter settings.
7. To calculate the cell viability relative to the values obtained at 7 days, use the following equation:

$$Cell\ viability\ (\%) = \frac{Fluorescence\ arbitrary\ units\ of\ sample}{Fluorescence\ arbitrary\ units\ of\ 7-day\ sample} \times 100$$

4. Microscopy monitoring

The monolayer formation can be tracked through a phase contrast microscopy on specific timepoints (days 4 and 7 for the results presented).

5. RNA isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Differentiation markers can be studied through qRT-PCR on the intended culture days (in case of the presented results it was at days 7 and 21).

1. At the chosen timepoint, remove the device from the incubator and place it in the cell culture hood.
2. Remove the medium from the inlet and outlet reservoirs without emptying the cell channel.
3. Wash the channel with PBS smoothly adding 100 µL of PBS into the inlet and removing it at the outlet well with the pipette. Repeat the washing step three times.
4. Pipette 100 µL of extraction buffer from Maxwell kit to detach the cultured cells.
5. Place the sample in MAXWELL equipment (Promega, Madison, WI, USA) for cellular RNA isolation and purification.
6. Use Nanodrop (Thermo Fisher Scientific) for quantification and quality evaluation of the extracted RNA.
7. Collect 1 µg of extracted RNA and use the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) to obtain cDNA.
8. Place the cDNA obtained with the commercial primers selected in TAQMAN™ fast advanced master mix (Thermo Fisher Scientific) for real-time PCR.

Table 1 Genes evaluated in the results presented and their reference from Thermo Fisher Scientific.

Gene	**Reference (Thermo Fisher Scientific)**
MUC2	Hs00894052_g1
MUC4	Hs04972331_g1
OCLN	Hs05465837_g1
TJP1	Hs01551871_m1
DEFB4B	Hs00823638_m1

DEFA5	Hs00360716_m1
DEFA6	Hs00427001_m1

9. Quantify the gene expression relative to a reference/housekeeping gene, demonstrating the magnitude of the physiological changes in the genes of interest. Use the formula 2-ΔΔCT.

Results obtained using the guide protocol

Cell viability and proliferation with two different coatings

Gelatin and collagen were used as coating solutions and the cell viability and proliferation were assessed after 7, 14 and 21 days of culture. Results showed that both gelatin and collagen coatings resulted in high cell viability with values compared to the control system after 7 days of culture. From that time point on, the cell viability increased in both coatings, similar to the control (Figure 3). Values above 100% of viability indicate cell proliferation, which was observed in both coatings. Between a gelatin or collagen coating, no significant differences were found. When comparing the results at day 21, gelatin coating in the BE-Doubleflow device showed a significant difference when compared with the control. Gelatin coating was chosen for the next experiments.

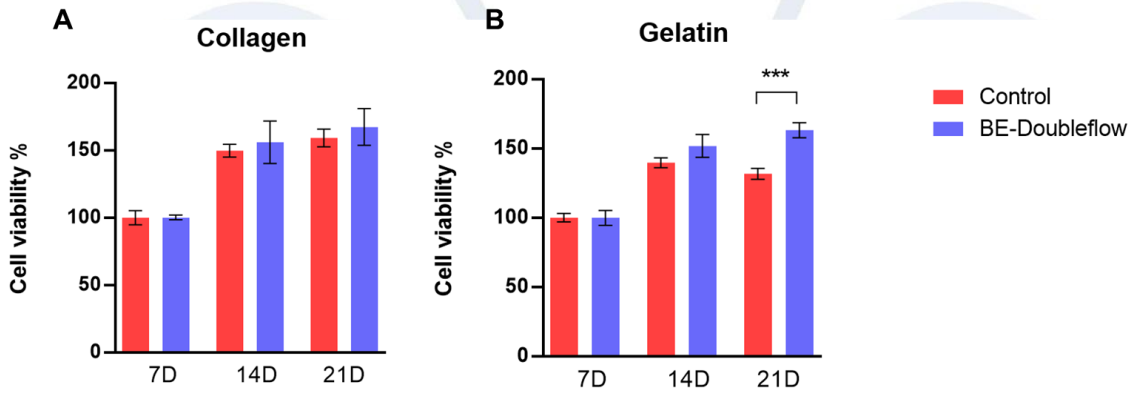

*Figure 3 Cell viability (and proliferation) in the culture on the BE-Doubleflow device and control system for 7, 14 and 21 days. (A) Cell viability with collagen coating and (B) cell viability with gelatin coating. Data are presented as the mean ± standard error of the mean. Unpaired t-test with Welch's correction statistical analysis performed: *** = p < 0.001.*

Epithelial monolayer evaluation

The morphology of the cells was observed in both systems with a gelatin coating on days 4 and 7. The cell epithelial monolayer formed in BE-Doubleflow showed no gaps, validating the integrity of the same, similar to the one formed in the control system (Figure 4).

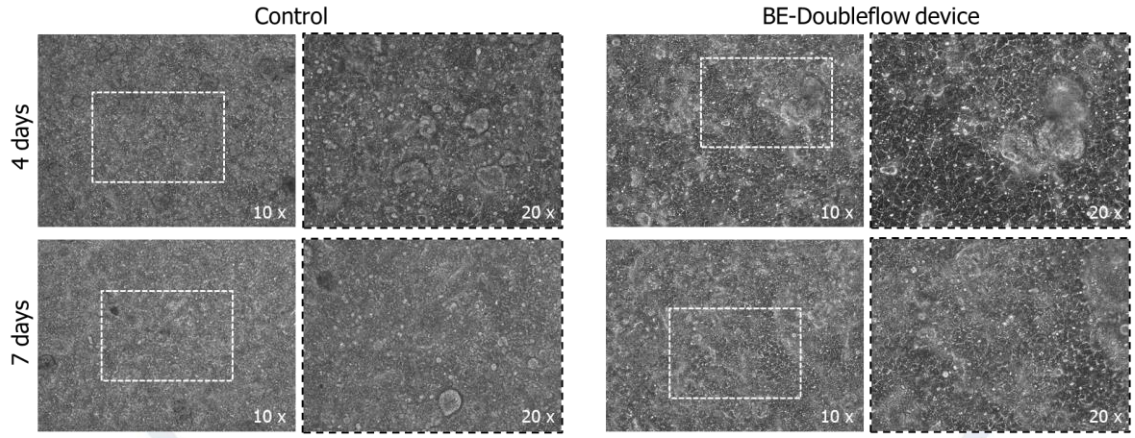

Figure 4 Phase contrast images of the cell (Caco-2) monolayer on the control system (Transwell) and BDF device (chip) at day 4 and day 7 of culture.

Cell differentiation in the model

The cell differentiation was evaluated on days 7 and 21 in BE-Doubleflow device and in the control system. Results showed that the cells cultured in BE-Doubleflow devices had a significant increase in Goblet and Paneth cell biomarkers after 21 days, compared with control, which points to a more heterogeneous cell population, more reminiscent of the native tissue (Figure 5). Between days 7 and 21, there are no significant differences, which can indicate that these biomarkers are expressed as early as the first time point.

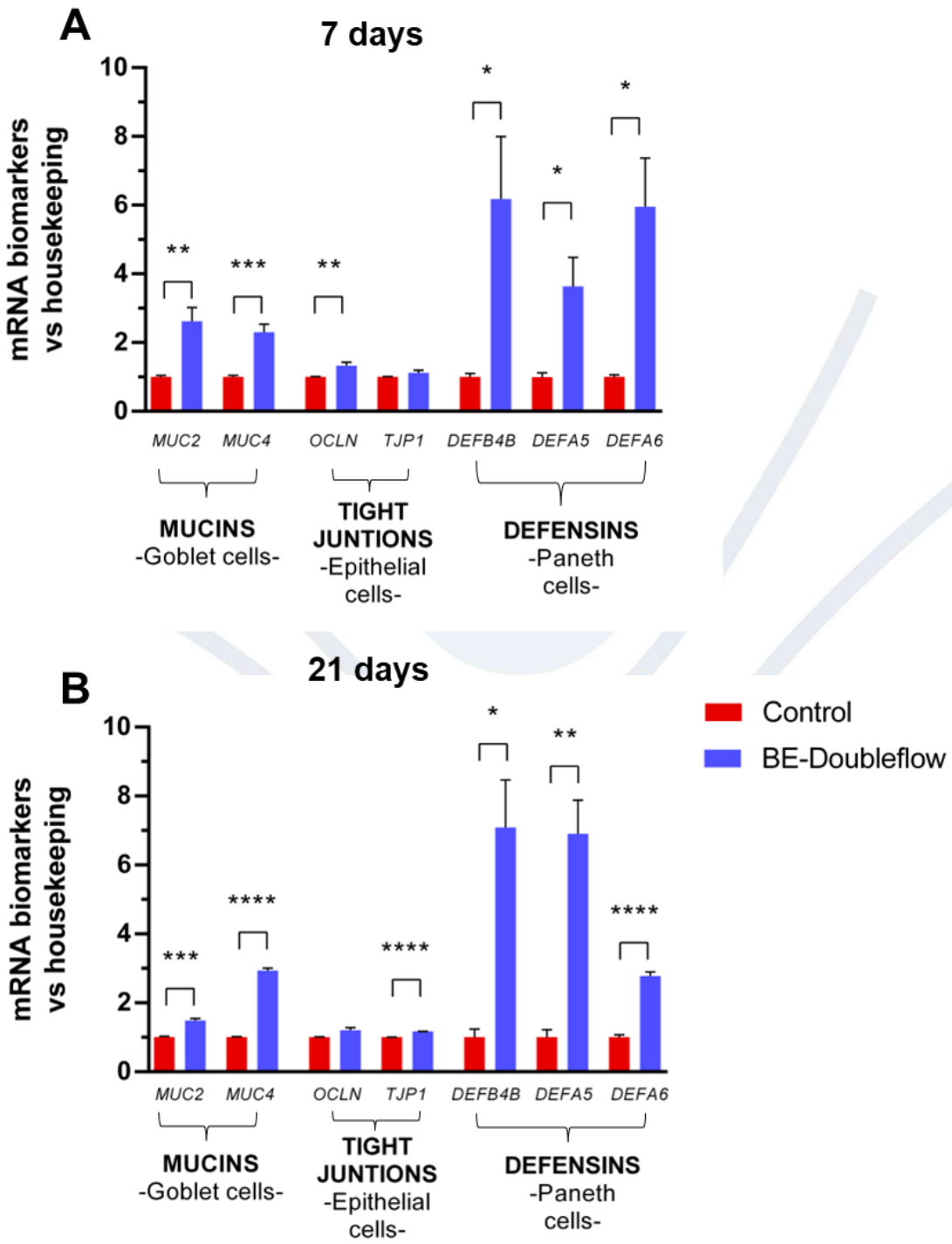

*Figure 5 Gene expression of cells cultured in control system and BE-Doubleflow device at days 7 (A) and 21 (B). Data are presented as the mean ± standard error of the mean. Unpaired t-test with Welch's correction statistical analysis performed: * = p < 0.05; ** = p < 0.01; *** = p < 0.005; **** = p < 0.001;*

Conclusions

BE-Doubleflow device is a suitable platform for intestine modelling as gut-on-a-chip. Results showed high cell viability and proliferation rates, similar to the control system. The cell differentiation was improved in the BE-Doubleflow device, with an increase in mucin- and

defensin-secreting cells present in this model. Moreover, the increase in gene expression of these cells was visible as early as day 7, which can point to a quicker differentiation in the microfluidic system, compared with the standard 21 days of culture.

References

Ashammakhi, N., Nasiri, R., Barros, N. R. de, Tebon, P., Thakor, J., Goudie, M., Shamloo, A., Martin, M. G., & Khademhosseini, A. (2020). Gut-on-a-chip: Current progress and future opportunities. *Biomaterials*, *255*, 120196.