BE-Doubleflow application notes: Gut-on-a-chip 2

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Introduction

In our previous technical note "BE-Doubleflow App. notes: Gut-on-chip 1" we explored our device BE-Doubleflow for gut-on-a-chip (GOC) models in collaboration with AINIA. In that note we compared our device with an insert platform with GOC outperforming the control system by expressing differentiation markers earlier and with increased expression of mucins and defensins. Following that work, we complemented the studies performed and deliver a detailed note with the protocols and results.

To better mimic the intestine, GOC should present adequate intestinal barrier function. Absorption studies are a way to assess the entrance and permeation of substances (such as nutrients or drugs) (Volpe, 2020).Within absorption studies, digestion blanks must be used as control samples to be compared with the digested ingredients or food of interest. The digesta contains digestive enzymes and bile salts at concentrations that although physiologically relevant are harmful to cells. For this reason, there are different strategies to detoxificate this digesta, among which is dilution with culture medium (Kondrashina et al., 2023).However, extensive dilution of the sample will difficult the posterior analysis.

This guide compares the BE-Doubleflow (BDF) device as GOC dynamic system with static culture for the set-up parameters for absorption studies using a digestion blank.

Materials

1. BE-Doubleflow device

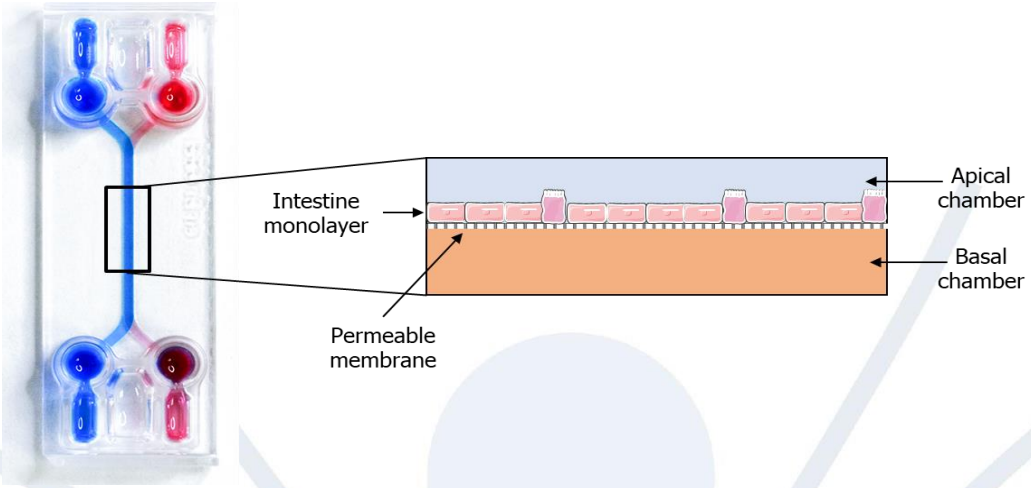


Figure 1. BE-Doubleflow device and cell culture scheme in the device channels.

BE-Doubleflow (BDF) design (Figure 1) consists of two perfusable channels connected via a PET porous membrane. This device is suitable to study the crosstalk between different 2D and 3D cultures in a biomimetic environment and control the efficiency of the interaction by selecting the optimal pore size for specific applications. It is optimal when gas control is needed, for studying the effect of circulating particles (bacteria, immune system, circulating tumour cells) and 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. Fluigent® equipment

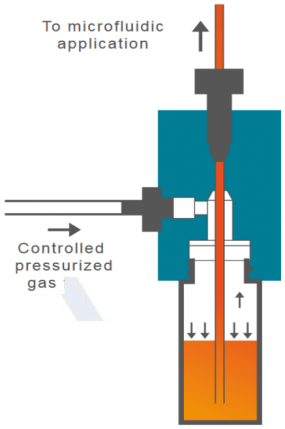


Figure 2. Positive controllers.

Fluigent® positive controllers precisely regulate the flow rate of a microfluidic system by applying small amounts of compressed air over a closed reservoir to induce the rise of a solution through an output tube (Figure 2).

- **Air pressure source.** This can be a compressed air intake or external compressor (Figure 3). The use of the external pressure compressor will facilitate the assembly of systems with cell culture in sterile conditions working with a biosafety cabinet. Once the system is assembled and transported to the incubator, it can be connected to a fixed compressed air intake.
- **Controller**. Regulates pressure input to FlowEZ controllers. For a FlowEZ of 1000 mbar the maximum working pressure is 1100 mbar.
- **FlowEZ pressure controller**. The pressure controller can regulate the air inlet to the liquid reservoir to displace it (Figure 4).
- **Link/Power supply module**. The link module (linked to the FlowEZ pressure controller) allows the connection to a PC for software control.
- **CAPs**. Caps for the hermetic closure of reservoirs (Figure 5).
- **FlowUnit.** Linking a flow sensor to the pressure controller allows direct control overflow regardless of pressure, which can vary as the reservoir empties (Figure 6). The flow sensor will be previous to the microfluidic device.

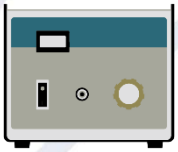


Figure 3 Air pressure compressor.

Figure 4 FlowEZ pressure controller and LineUp unit.

Figure 5. CAP.

Figure 6. FlowUnit.

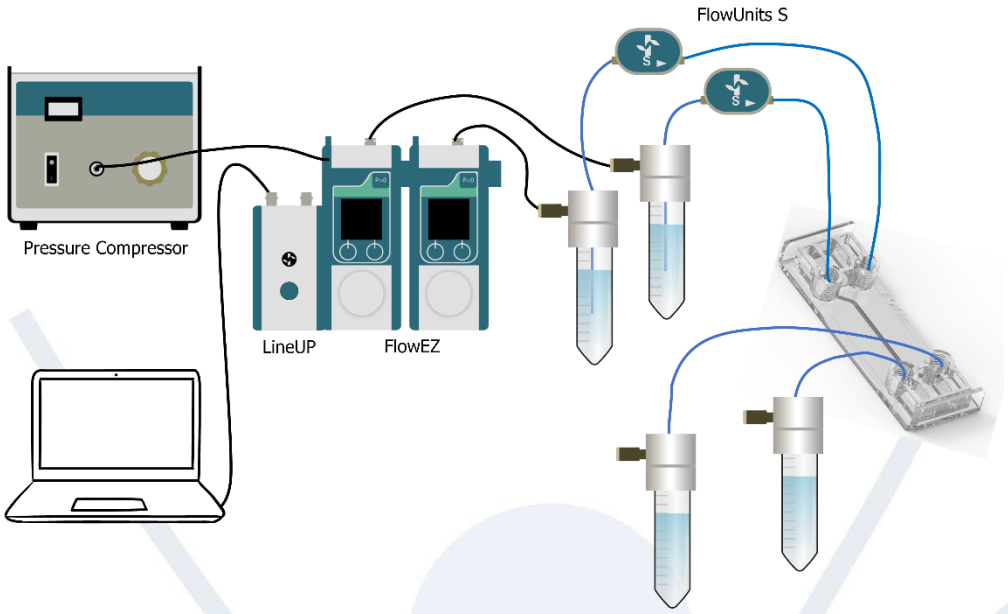


Figure 7. Schematic of the experiment set up of GOC model with BDF device and Fluigent® microfluidic system for the intestinal absorption studies.

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. Static intestinal model (control system)

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

Methods

1. Dynamic gastrointestinal digestion

The simulation of human gastrointestinal digestion is carried out using the *in vitro* Dynamic Digester developed by AINIA. This system is computer-controlled and consists in interconnected compartments that simulate the gastric and intestinal digestions. The procedure is conducted at 37 °C, in darkness and under anaerobic conditions to better mimic the physiological conditions.

For the oral stage, the system simulates the size reduction (chewing) and enzymatic digestion of the sample. To do so, the sample is dispersed at 37 °C in a simulated salivary solution containing α-amylase with pH between 6.5-6.7. The mixture is kept under agitation for 2 min and at a pH between 6.5-6.7. For the gastric stage, a solution containing stomach residual preheated to 37 °C (oral digesta) is introduced. Next, a simulation of gastric secretion with pepsin is carried out with control over the pH curve by the addition of 1M HCl. To simulate the transit from the stomach to the intestine, the Elashoff equation is used (Blanquet et al., 2004). The subsequent gastric emptying is mixed with intestinal media. The simulated intestinal solution containing pancreatin, electrolyte solution and bile salts is gradually added and maintained at pH 6-6.5, originating intestinal digestion media. The intestinal emptying is simulated based on human *in vivo* data using Elashoff equation. The total transit time so far is 6 h. This methodology has been widely used by AINIA for evaluating the effect of bioactive compounds in food complements or ingredients (Bryszewska et al., 2019; Nieto et al., 2021; Soriano-Romaní, Nieto, & García-Benlloch, 2022; Soriano-Romaní, Nieto, Tomás-Cobos, et al., 2022).

The intestinal empties are accumulated generating the digestion blank.

2. BE-Doubleflow cell culture

Before seeding, prewarm the BDF 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/HT-29/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. Every 2-3 days the media is renewed up to day 7.

3. Microfluidic set-up

Before setting the flow up:

- Sterilize and prewarm the tubes and fluidic elements overnight at 37 °C.
- Set the system in a laminar flow cabinet.
- The channels and inlet/outlet wells should never be depleted of culture medium.
- Both inlets and outlets are designed to be able to use connectors (1/4" – 28).

To work with cells, the assembly of the circuit must be carried out under sterile conditions within a laminar flow biosafety cabinet. For this, it is advisable to autoclave the CAPs, pneumatic tubes, threaded connections, and ferrules to be used.

1. Connect all circuit components except the microfluidic device.
2. Establish a flow of ethanol (70%) for at least 15 min to sterilize the sensor and then wash with abundant sterile H_2Od. Dry by passing air through at maximum pressure. The sensor CANNOT be autoclaved.
3. Prime the tubes of the circuit of the top channel with digestive blank diluted in culture medium or the buffer and the bottom channel circuit with culture media or buffer until there are no air bubbles.
4. Remove the culture medium from the chip reservoirs (not from inlet/outlet wells) and connect the tubes to the outlet/inlet using the threaded connections and ferrules.
    a. The ferrules must be manipulated with the help of sterile forceps.
    b. Ensure that the tube is perfectly fixed.
    c. Remove the displaced medium from the reservoirs.
5. Once the system is closed, switch the flow on at 5.6 µL/min (shear stress 0.02 dyne/cm^2) for 4 h. This flow rate is calculated taking into consideration the standard measures of the BDF channels. In case the size of the channel is customized, the flow must be recalculated.
    a. Observe the system under perfusion for a few minutes to check that there are no leaks.

Consider that ethanol's sensor detection limit is higher (0±70 µL/min for S FlowUnit). For applying these values, change the liquid type to IPA (isopropanol) in the controller menu linked to the FlowUnit.

4. Dynamic intestinal absorption assay system

The "digested-like fluid" consists of digested blank diluted with HBSS or PBS without Ca^{2+}/Mg^{2+} and is inserted through the inlet of the apical reservoir. The "blood-like fluid" is the buffer used in the dilution and inserted through the inlet of the basal reservoir (Figure 8). The

outlet of the apical reservoir collects the not absorbed digested-like fluid while the basal reservoir collects the blood-like fluid with absorbed content.

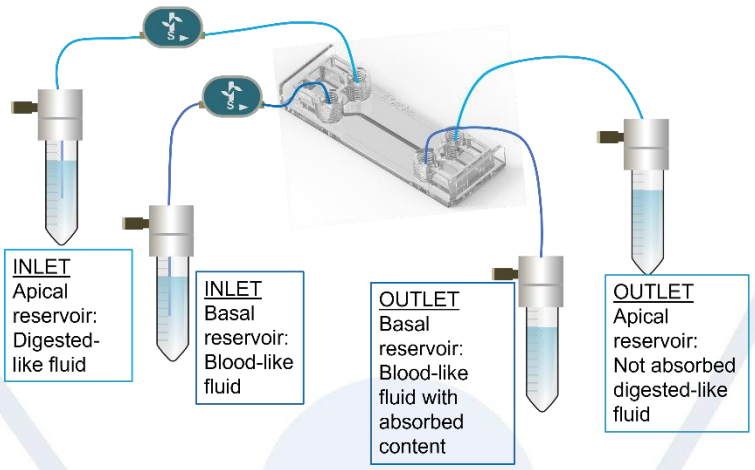


Figure 8. Inlet and outlet connections of Gut-on-a-Chip model with Fluigent® microfluidic system for simulating intestinal absorption.

5. Static intestinal system

1. Seed into an insert culture system Caco-2 and HT-29/MTX cells in 9:1 ration as seeding in BE-Doubleflow device.
2. Use HBSS or PBS without Ca^{2+}/Mg^{2+} as buffer for the digestion blank, same to the dynamic system.
3. Incubate the solution with the cells for 4h before performing the cell viability assays.

6. Viability assay

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.

a) At the chosen timepoint, remove the device from the incubator and place it in the cell culture hood.
b) Remove the medium from the inlet and outlet reservoirs without emptying the cell channel.
c) 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.
d) 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.
e) Place the device in the incubator for 1 h at 37 °C.

f) Place the device in a plate reader or fluorescence spectrophotometer with 530/590 nm (excitation/emission) filter settings.

g) To calculate the cell viability relative to the values obtained at 7 days, use the following equation:

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

7. Microscopy monitoring

The monolayer formation can be tracked through a phase contrast microscopy on specific timepoints (4 hours after incubation with digestion blank for the results presented in figure 11).

8. Total protein quantification

At the desired timepoint (in case of the results presented, after 4h of incubation with digestion blank), cells can be extracted from the device for the analysis of parameters such as protein and RNA quantification. To extract cells, we advise you to follow the detailed protocol of our technical note "Cell recovery protocol".

For the results presented, the quantification of protein content was performed using "CelLytic™ MT Cell Lysis Reagent" buffer (Sigma-Aldrich) to isolate the total protein content. The content extracted was then quantified using Bradford protein assay kit (Bio-Rad). The absorbance was measured and extrapolated with a standard line with a known amount of protein (2000-125 µg/mL) to calculate protein concentration.

9. Total RNA quantification and evaluation

After cell extraction the RNA was isolated and purified using equipment's such as Maxwell® RSC Instruments (Promega). The quantity and quality of the RNA isolated can be measured by Nanodrop, using A260/280 and A260/230 parameters as quality assessment. Values must be ≥1.8 to be considered free of contamination by DNA, proteins or aromatic compounds.

Results obtained using the guide protocol

Cell viability and morphology after digestion blank treatment

For the development of *in vitro* gastrointestinal digestive models, it is important to start the assays with controls such as the digestive blank (digestive fluids and enzymes without food/supplements) The composition of the digestive blank itself can damage the *in vitro* culture since the digestive enzymes present are active and therefore, can detach the cells and the bile

salts at certain concentrations can be toxic for the cells. To avoid excess of cell toxicity, the digestive blank can be diluted but the specific dilution buffer and dilution factor depend on the cell culture. Previous cell viability assays are then recommended to determine the dilution factor that has to be applied to ensure biocompatibility of the digestive blank without food/supplements added (Kondrashina et al., 2023).

a) Transwell (static)

First, the cell viability after 4 h exposure to the digestion blank was evaluated in the static model. Samples included the digestion blank diluted with HBSS 1:5; 1:10 or 1:20; digestion blank diluted with PBS 1:5; 1:10 or 1:20 and cytotoxic positive control composed of DMSO 20%. Results showed that to keep high cell viability, digestion blank needs to be diluted at 1:10 with HBSS while in PBS the dilution needed is 1:20 (Figure 9).

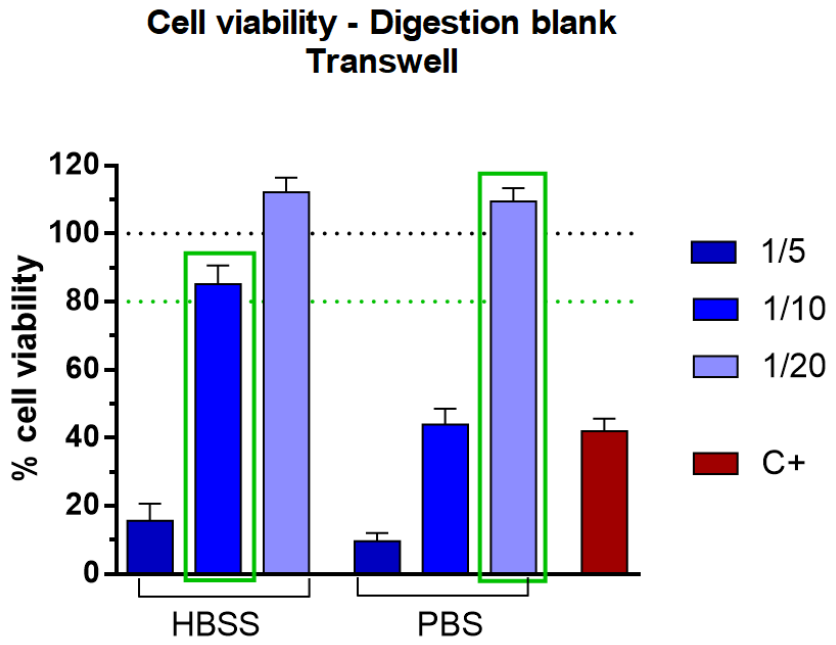


Figure 9. Cell viability of serial dilutions of digestion blank in HBSS and PBS in the Transwell (static). C+ = cytotoxic positive control (DMSO 20%).

b) BE-DoubleFlow compared to Transwell (Control)

The cell viability was assessed in a BDF device cultured for 7 days and exposed to the digestion blank for 4 h in dynamic conditions (Figure 10). The digestion blank was diluted with HBSS or PBS in a 1:5 or 1:10 dilution and compared with static model.

Results showed higher cell viability in the dynamic model in all conditions when compared with static model. In addition, the dilution necessary to keep high cell viability in HBSS was 1:5 in the dynamic model, while static model needed a dilution of 1:10. In PBS, the dilution necessary in

the dynamic model was 1:10, while in static conditions it needed to be 1:20 (as shown above in Figure 9).

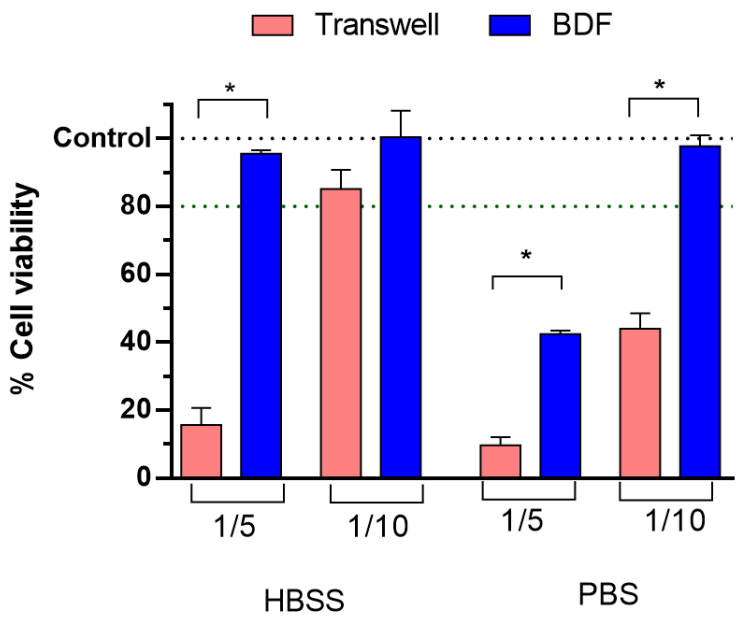


*Figure 10. Cell viability of digestion blank under same conditions in static model vs dynamic model (GOC) in HBSS or PBS buffers. *p<0.05.*

More concentrated digestion blanks equal better limits of quantification of the possible compounds of interest for uptake studies, proving that the GOC model is more advantageous as absorption model than the static control. The reason behind this improved result in the GOC system might be associated with the higher expression of mucins this model presented when compared with static culture since mucins protect the cellular epithelium (results presented in the technical note "BE-Doubleflow App. notes: Gut-on-chip 1").

These results were further supported by the evaluation of the cell morphology through contrast microscopy. Figure 11 demonstrates the results obtained at 1:5 and 1:10 dilution with PBS in both dynamic and static models. In static model, both dilutions provoked gaps in the cell monolayer, especially the 1:5 dilution. In the dynamic model a confluent monolayer is visible in both conditions. In addition to the cell viability results, it can be deduced that even though the monolayer is maintained in the dynamic model, the integrity of the monolayer can be compromised in the 1:5 dilution. It can also be seen that the clear hexagonal morphology of the cells with thigh junction between cells is slightly lost after exposure with 1:5 diluted digestion blank in the dynamic model.

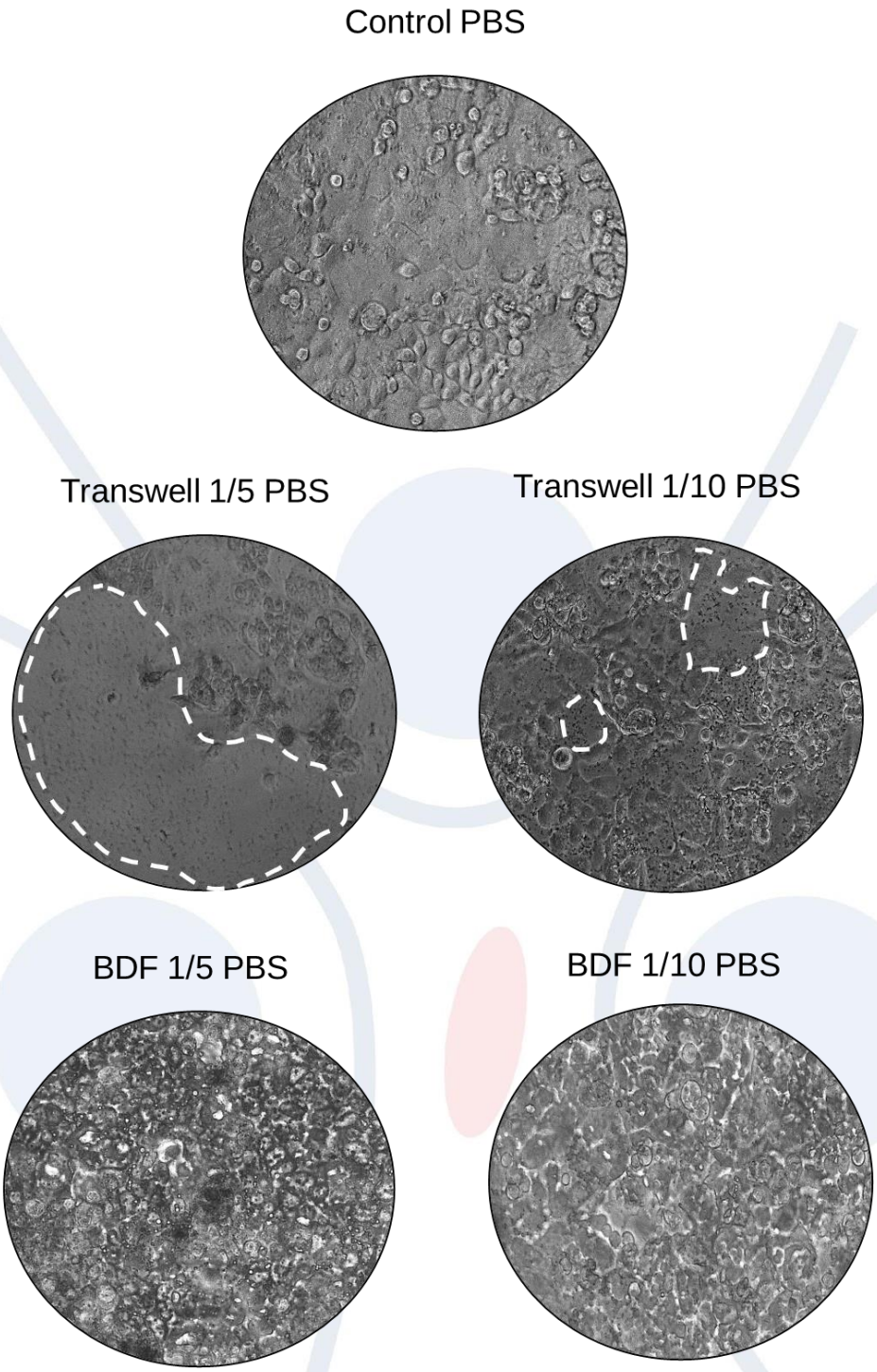


Figure 11. Phase-contrast images of cell monolayers after 4 h with digestion blank under different conditions. The dashed white line shows gaps in the intestinal cell monolayer.

Moreover, we assessed the viability of the culture before and after the 4 h incubation with the digestion blank, comparing the static with the dynamic system applying same conditions in terms of buffer and dilution. Results showed no significant differences between timepoints and conditions, which validates the non-toxicity of the treatment (Figure 12).

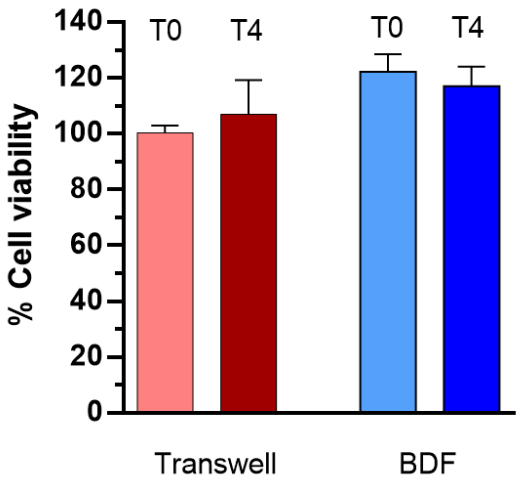
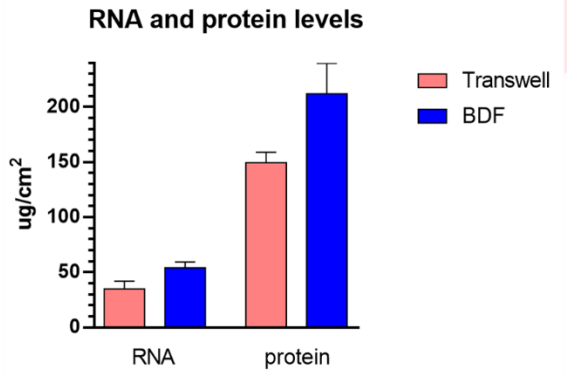


Figure 12. Cell viability of the Transwell and BE-Doubleflow device before (T=0) and after (T=4h) the 4h of intestinal absorption simulation.

Total RNA and protein content

RNA and protein content and quality of the sample is important when using both for further determinations, including omics assays (eg. transcriptomic or proteomic analysis). Comparing insert culture and BE-Doubleflow device, results showed similar RNA and protein content on both systems, with the protein levels being slightly higher ($p<0.1$) on the DE-Doubleflow device. The quality of RNA was also similar in both systems. These results corroborate the suitability of GOC systems to be used as standard intestinal models.

A



B

	A260/280	A260/230
Transwell	2,11 ± 0,04	2,2 ± 0,04
BDF	2,11 ± 0,03	2,18 ± 0,08

Figure 13. Total RNA and protein quantification and quality assessment. (A) RNA and protein levels on cells cultures in control (Transwell) and BE-Doubleflow device (GUTOnChip) after 4 h of incubation with digestive blank. (B) A260/280 and A26/230 values obtained through Nanodrop analysis as a marker for RNA quality.

Conclusions

Through this study it can be concluded that the BE-Doubleflow device is a suitable platform for a gut model and when connected with Fluigent® microfluidic pumps it is interesting to be used as dynamic intestinal absorption *in vitro* over the static control model. The developed dynamic model allowed to use a less diluted sample of the digested which implies less sample manipulation and closer to the *in vivo* situation. A less diluted sample also allows a better quantification analysis of the components present. The total protein and RNA content can be easily isolated from the BE-Doubleflow devices and the quantity and quality were similar or slightly higher than the control system.

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Related products from Beonchip/Fluigent:

- BE-Doubleflow
- Microfluidic flow control system connection kit
- FlowEz
- LineUP link
- FlowEZ supply kit
- Flow Unit S
- P-CAPs
- Pressure source FLPG+