From mouth to gut: microfluidic *in vitro* simulation of human gastro-intestinal digestion and intestinal permeability

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Electronic Supplementary Information

2. Materials & Methods

2.1. Materials

Caco-2 cells (HTB-37™) were purchased from the American Type Culture Collection (ATCC®). HT29-MTX-E12 cells (ECACC 12040401) were obtained from the European Collection of Authenticated Cell Cultures. Minimum essential media (MEM) Eagle was purchased from PAN-Biotech GmbH (Aidenbach, Germany). 4', 6-diamidino-2-phenylindole, dilactate (DAPI, Invitrogen™), Sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate (Sulfo-SANPAH), Matrigel™, and rat tail type-I collagen were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Trypsin-EDTA (0.25% trypsin; 0.1% EDTA), penicillin/streptomycin 100x, foetal bovine serum (FBS), Hank's balanced salt solution (HBSS), and 1-µm pore size transwell™ inserts were bought from Merck Millipore (Burlington, MA, USA). Dimethyl sulfoxide (DMSO), paraformaldehyde (PFA), phalloidin-tetramethylrhodamine B isothiocyanate (phalloidin-TRITC), bovine serum albumin (BSA), fluoroshield™, sodium pyruvate, resazurin sodium salt, Dulbecco's Modified Eagle Medium - high glucose (DMEM), phosphate buffered saline (PBS), pepsin from porcine gastric mucosa, bovine bile, pancreatin from porcine pancreas, Lucifer Yellow, Trichloro(1H,1H,2H,2H-perfluorooctyl)silane, and sulfuric acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). TI Prime and SU-8 2025 were from Microchem Corp. (Westborough, MA, USA). Dow Corning Sylgard® 184 polydimethylsiloxane (PDMS) was purchased from Ellsworth Adhesives Ibérica (Madrid, Spain). Smooth-Cast™ 310 was purchased from Bentley Advanced Materials (Worcestershire, UK). All reagents were used as received and according to the manufacturer's recommendations.

2.2. Detailed microfabrication methods

Photolithographic chrome masks in soda lime glass were manufactured by direct write laser (DWL) lithography. Briefly, chrome and AZ400k photoresist-coated masks (Nanofilm, Westlake Village, CA, US) were patterned by UV (405 nm) using a DWL 2000 (Heidelberg Instruments, Heidelberg, Germany). The photoresist was developed for 90 seconds in OPD 262 (Fujifilm, Tokyo, Japan) followed by etching of the exposed chrome for 100 seconds using Chrome Etch 18 (Micro Resist Technology GmbH, Berlin, Germany). The remaining photoresist was stripped by immersion for 10 min in Microstrip® 3001 (Fujifilm, Tokyo, Japan) and the masks ashed in a PVA Tepla GIGAbatch 360M (PVA MPS GmbH, Wettenberg, Germany) using low power to preserve the chrome layer.

Silicon master moulds were fabricated using SU-8 2025 photoresist (MicroChem Corp., Westborough, MA, USA). Briefly, wafers were cleaned in Piranha solution (1:1 sulfuric acid:hydrogen peroxide), dehydrated overnight at 210 °C and spin-coated (3,000 rpm, 30 s) with TI Prime to promote adhesion of the photo-resist. SU-8 was spin-coated (1,000 rpm, 30 s), soft baked (65 °C, 3 min & 95 °C, 9min.), UV exposed (215 mJ·cm^{-2}) using a Suss MicroTec SE (Garching, Germany) MA/BA6 mask aligner and post baked (65 °C, 2 min. & 95 °C, 7 min). The second SU-8 layer (forming the staggered herringbone mixers) was then spin coated on top of the first (4,000 rpm, 30s) and the process repeated. The wafers were finally developed in PGMEA solvent (7 min) and hard baked at 150 °C for 20 min. The feature height was verified using a KLA-Tencor P-16^{+} surface profiler (KLA Corporation, CA, US). The silicon master moulds were pre-treated with a layer of hydrophobic 1H,1H,2H,2H-per-fluorooctyl-trichlorosilane to facilitate de-moulding. Sylgard® 184 PDMS elastomer was prepared by mixing the elastomer with curing agent (10:1 or 15:1 (for the Gut Chips), w/w), degassed and cured over the moulds at 65 °C for 1-2 h. White acetal frames (RS) machined by a FlexiCAM Viper CNC (FlexiCAM GmbH, Eibelstadt, Germany) were used to shape the PDMS stamps. Inlets/outlets were opened using biopsy punches (Kai Europe GmbH, Solingen, Germany). The PDMS stamps were oxygen plasma bonded to PDMS-coated glass-slides using a Harrick Plasma (NY, US) PDC-002-CE Plasma Cleaner.

2.3. Assessment of enzymatic activity and Bile Salts concentration

2.3.1. Trypsin activity in pancreatin

The trypsin activity was determined following the protocol by Brodkorb et *al.* p-toluene-sulfonyl-L-arginine methyl ester (TAME) was used as the reaction substrate and its hydrolysis was spectrophotometrically (absorbance 247 nm) followed for 10 min at 25 °C and pH 8.1 using 46 mM Tris/HCl buffer, containing 11.5 mM $CaCl_2$, as assay solution. TAME was prepared (10 mM) in distilled water

and pancreatin (0.1, 0.5 or 1.0 mg.mL^{-1}) in 1 mM HCl. 2.6 mL of assay solution and 0.3 mL of the substrate (10 mM TAME) were mixed in a quartz cuvette, incubated in the spectrophotometer for 3-4 minutes until reaching 25 °C and 100 µL of the test enzyme concentration were added. Absorbance (247 nm) was measured in continuously for 10 min. As a blank, the same protocol was followed but replacing the enzyme with the solution assay. The slope of the initial linear section of each curve (blank and different enzyme concentrations) was determined. The trypsin activity was calculated according to equation 1, where the difference between the slope of the initial linear section of the test enzyme and the blank assay is used, 540 represents the molar extinction coefficient (L/mol×cm) of TAME at 247 nm, 3 is the volume (mL) of reaction mix and X represents the quantity (mg) of enzyme in the final reaction mixture.

$$\frac{Units}{mg}(trypsin) = \frac{[\Delta A247\ Test - \Delta A247\ Blank]\times 1000\times 3}{(540\times X)} \qquad \text{Equation 1}$$

2.3.2. Pepsin activity

The pepsin activity was determined following the protocol recommended by Brodkorb et *al*. Haemoglobin was used as the reaction substrate and its hydrolysis was spectrophotometrically (absorbance 280 nm) determined. Haemoglobin was prepared (2% w/v) in distilled water and the pH adjusted to 2.0 using 0.3M HCl. A stock solution of pepsin (1 mg.mL^{-1}) in 10 mM Tris buffer, 150 mM NaCl at pH 6.5 was prepared. The stock solution was diluted in 10 mM HCl to 5, 10, 15, 20, 25, 30 µg.mL^{-1}. 500 µL of haemoglobin were incubated at 37 °C for 3-4 min and 100 µL of pepsin at each test concentration were added and incubated for 10 min. To stop the reaction, 1 mL of 5% w/v trichloroacetic Acid (TCA) were added. The reactionubes were centrifuged at 6,000 × g for 30 min, the pellet removed and the absorbance of the supernatant read in quartz cuvettes at 280 nm. For the blank assay, the same procedure was followed but pepsin was only added after the addition of TCA. The pepsin activity was calculated according to equation 2, where Δt is 10 min and X is the amount of pepsin (µg) in 1mL of the assay solution.

$$\frac{Units}{mg}(pepsin) = \frac{[A280\ Test - A280\ Blank]\times 1000}{\Delta t\times X\times 0.001} \qquad \text{Equation 2}$$

2.3.3. Bile Acids concentration

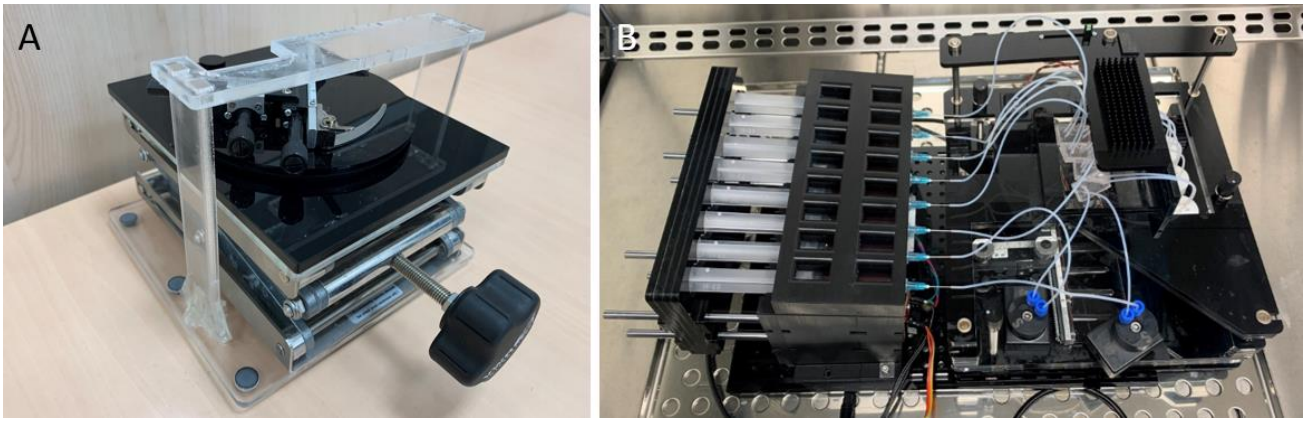
The bile acids concentration was determined using a commercial kit (Bile Acid Assay Kit, reference MAK309 from Sigma Aldrich) following the suppliers' instructions.

ESI Table 1 - Concentrations (in mM) of electrolytes in the stock (1.25x concentrated) simulated digestion fluids used for digestion. Noting that PDMS is a good solid solvent[2], both static and on-chip digestions were considered to be run in an open system. Thus, following the recommendation from Brodkorb *et al.*[1], sodium bicarbonate was replaced in all simulated fluids by NaCl at the same molar ratio to maintain the ionic strength of the electrolyte solutions and avoid unwanted pH drift. Note: The concentrations of $CaCl_2(H_2O)_2$ and bile salts indicated are that of the final digestion mixtures.

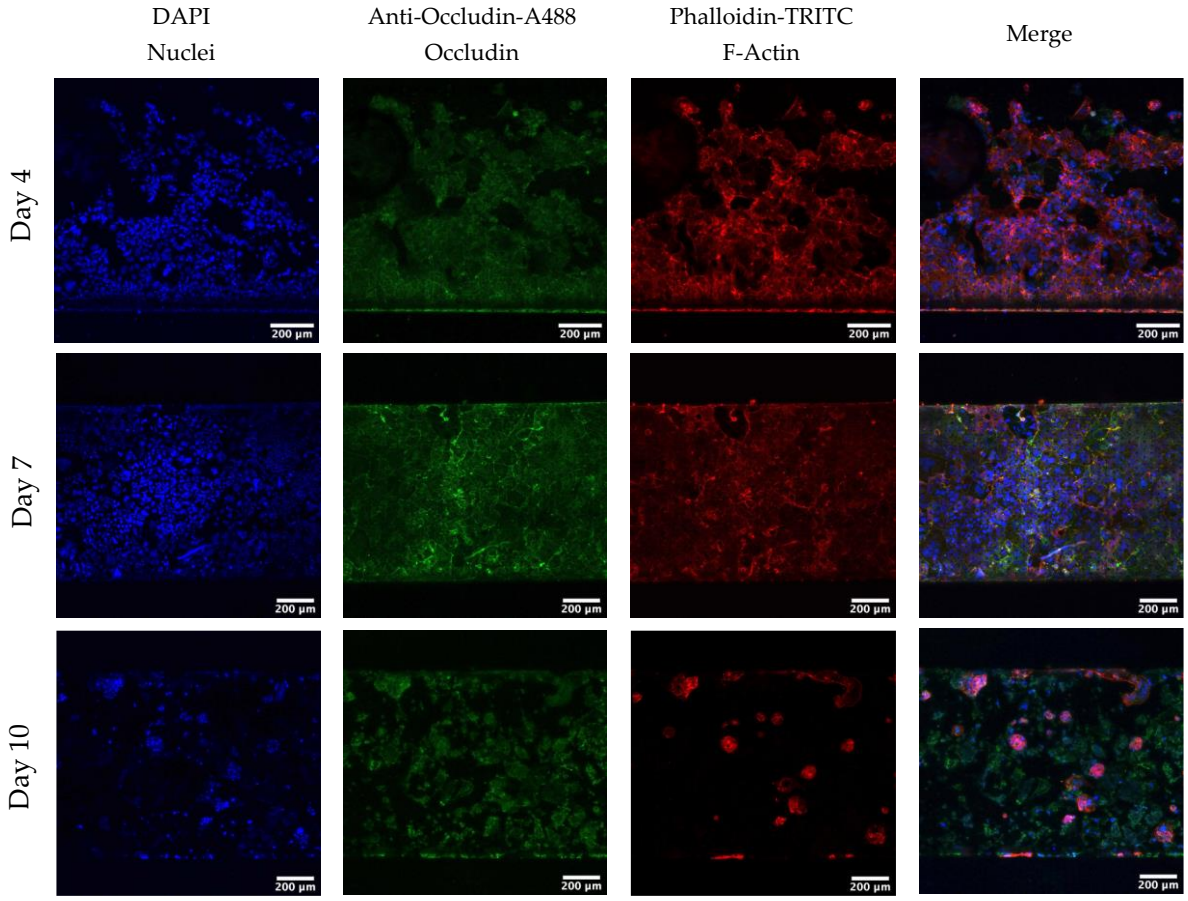
	Simulated salivary fluid (SSF)	Simulated Gastric Fluid (SGF)	Simulated Intestinal Fluid (SIF)
KCl	15.1	6.9	6.8
KH_2PO_4	3.7	0.9	0.8
$NaHCO_3$	-	-	-
NaCl	13.6	72.2	123.4
$MgCl_2(H_2O)_6$	0.15	0.1	0.33
$(NH_4)_2CO_3$	0.06	0.5	-
$CaCl_2(H_2O)_2$	0.75	0.075	0.3
HCl	1.1	15.6	8.4
Pepsin	-	2,000 U·mL^{-1}	-
Pancreatin	-	-	100 U·mL^{-1}
Bile salts	-	10	-

ESI Table 2 - Reynolds number ($Re=\rho \cdot V \cdot D/\mu$) at the range of flow rates used in the digestion-on-chip experiments. The calculations were made for the channel dimensions at the staggered herringbone regions (200 µm x 100 µm) and the hydraulic diameter (D) estimated at $2h \cdot w/h+w$. The dynamic viscosity ($\mu=1.002 \times 10^{-3}$ Pa·s) and density ($\rho=0.9982 \times 10^{3}$ kg·m^{-3}) of water at 20 °C were used.

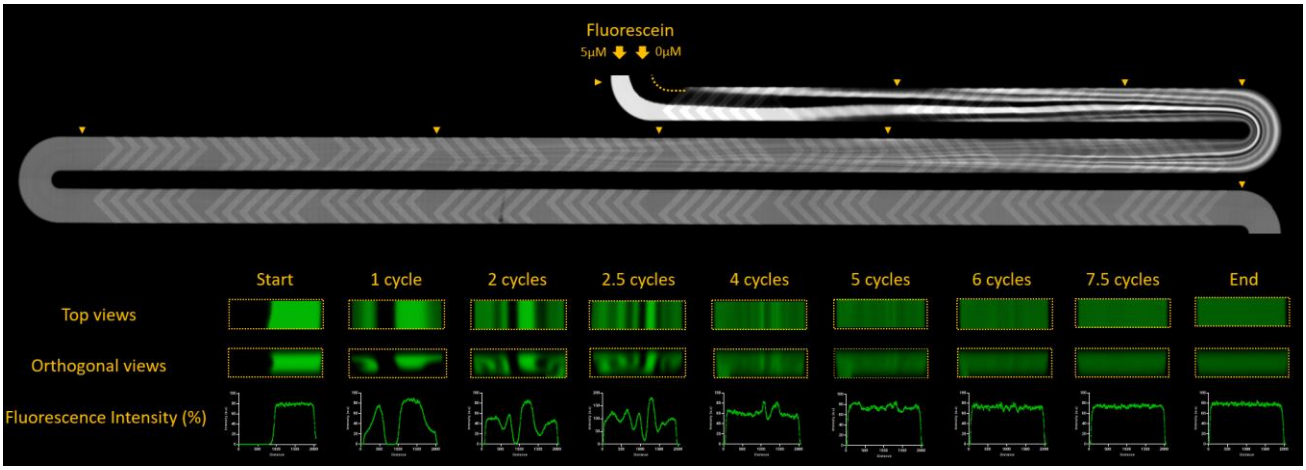
Flow rate (Q; µL·h^{-1})	Flow velocity (V; m·s^{-1})	Reynolds (Re)
24	3.3×10^{-4}	0.04
48	6.7×10^{-4}	0.09
96	1.3×10^{-3}	0.18
128	1.8×10^{-3}	0.24
192	2.7×10^{-3}	0.35
384	5.3×10^{-3}	0.71
576	8.0×10^{-3}	1.06
1152	1.6×10^{-3}	2.13
2304	3.3×10^{-3}	4.25



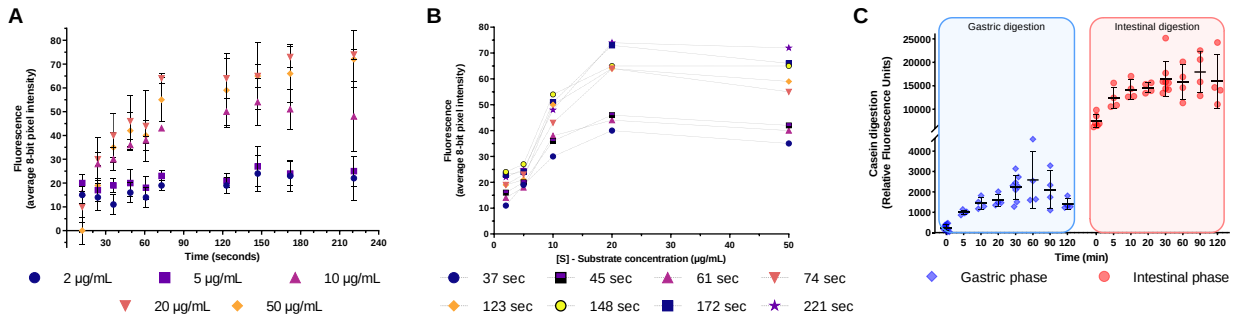
ESI Figure 1 – A) In-house built alignment rig. **B)** In-house built 8-channel syringe pump and imaging rig.



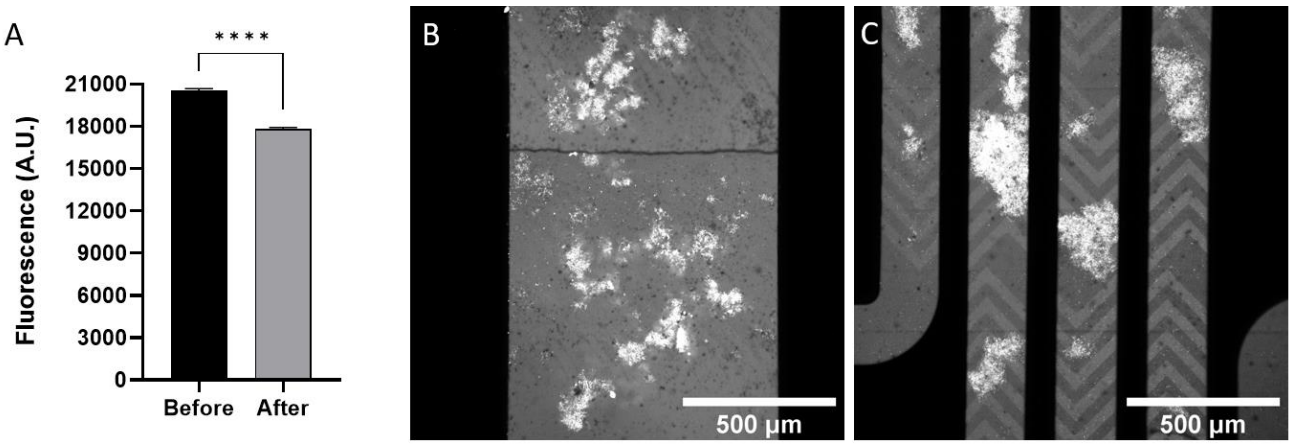
ESI Figure 2 – Confocal laser scanning microscopy images at 10× magnification of Caco-2/HT29-MTX co-cultured on-chip for 4, 7, and 10 days under continuous flow and stained for nuclei (blue), occludin (green), and F-actin (red).



ESI Figure 3 – Confocal microscopy images showing mixing by chaotic advection over a staggered herringbone of a 0 µM and a 5 µM fluorescein sodium salt solution in water. Tile scan images were taken at an intermediate focal point for complete channel visualisation, and z-stacks (0.5 µm increments) were acquired after determined cycles of mixing as shown by the yellow arrows (cycles 0, 1, 2, 2.5, 3, 4, 5, 7.5, and 12.5). The tile scan image and the orthogonal view images were processed using 5-pixel and a 20-pixel median filter respectively. Fluorescence intensity profiles were extracted from ImageJ across a median line using the 'plot profile' function, and calculated as a percentage of the maximum intensity value across the same line.



ESI Figure 4 – A) On-chip fluorescence of the pepsin-catalysed digestion of a fluorescently-labelled casein derivative at different concentrations and plotted against time. **B)** On-chip fluorescence of the pepsin-catalysed digestion of a fluorescently-labelled casein derivative at different reaction times and plotted against substrate concentration. **C)** Time-resolved digestion simulations of the gastric and intestinal phases in the Digestion-Chip. Time-resolution was achieved by varying the flow rates (12-1152 µL·h-1) of the sample and simulated digestion fluids (SGFs) thus attaining different incubation times.



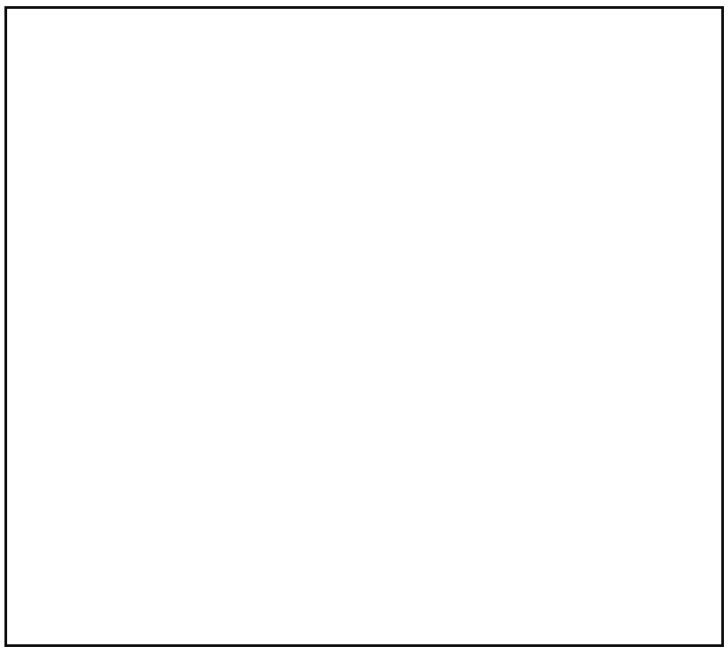
ESI Figure 5 – A) Fluorescence (arbitrary units) of digested casein before and after flowing through the PDMS Digestion-Chip. **B** and **C** show aggregates of digested casein deposited at the bottom of the Digestion-Chip.

ESI Table 3 - TEER (Ω·cm²) of Caco-2/HT29-MTX co-cultures following exposure to digesta from complete simulated digestions treated with Pefabloc® SC Plus and diluted 1:6.

	N=1					N=2				
Time (min)	**TW1**	**TW2**	**TW3**	**TW4**	**Average**	**TW1**	**TW2**	**TW3**	**TW4**	**Average**
60	1009	1020	1059	1050	**1035**	888	1010	972	976	**961**
120	1123	1093	1136	1164	**1129**	946	1065	1061	1031	**1026**
180	1130	1098	1131	1157	**1129**	1044	1187	1187	1102	**1130**
240	1116	1104	1122	1154	**1124**	1087	1186	1231	1078	**1145**

ESI Table 4 – pH of digesta following dilution (1:4, 1:6, or 1:10) in complete MEM or HBSS.

	MEM			HBSS		
	Dilution 1:10	Dilution 1:6	Dilution 1:4	Dilution 1:10	Dilution 1:6	Dilution 1:4
	7.57	7.52	7.46	7.23	7.08	6.96
pH	7.61	7.56	7.50	7.27	7.11	6.97
	7.63	7.58	7.46	7.28	7.12	6.99
Average	7.60	7.55	7.47	7.26	7.10	6.97
STDEV	0.03	0.03	0.02	0.03	0.02	0.02



ESI Video 1 – Z-stack of confocal microscopy images of occludin (green), f-actin (red) and nuclei (blue) of Caco-2/HT29-MTX co-cultures grown on the Gut-Chip.