Cultivation and analysis of microphysiological systems of the human intestinal mucosa in health and disease

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1. RATIONALE/AIM

For late-stage preclinical testing of a active molecules and formulations, bioavailability and efficacy can be assessed in more complex human *in vitro* tissue models, as opposed to single cell studies, to support successful translation into clinical testing. These tissue models, also called microphysiological systems, replicate key physiological characteristics of the intestinal mucosa, e.g. different cell types, three-dimensional tissue morphology and mucus secretion, and allow the observation of pathophysiological changes as well as therapeutic effects on tissue-level. This protocol describes the cultivation and detailed characterisation of a microphysiological systems of the human intestinal mucosa in healthy and/or inflamed state using a Transwell®-based system. (Jung et al., 2024, doi: 10.1101/2024.03.08.584089).

2. EXPERIMENTAL CONDITIONS

2.1. Key Requirement:

2.1.1. Dynamic cultivation of tissue models

Transwell® inserts are seeded with enterocytes (Caco-2) and goblet cells (HT29-MTX-E12) in a ratio of 3:1 and cultured for 21 days under constant agitation at 175 rpm on an orbital shaker. The formation of an intact epithelial barrier is monitored by observing the transepithelial electrical resistance.

2.1.2. Verification of differentiated tissue models

When establishing the tissue models in a new laboratory, it is necessary to validate the differentiation of the models to ensure reproducibility and reliability of the *in vitro* models. Validation experiments include assessment of the paracellular permeability, histological features, surface morphology, mucus secretion, and expression of tight junction proteins.

2.1.3. Inflammation of tissue models

Differentiated tissue models are inflamed using a cocktail of the pro-inflammatory cytokines. The successful inflammation of the tissue is monitored by observing the transepithelial electrical resistance, paracellular permeability, as well as expression of inflammation markers.

2.1.4. Treatment and evaluation of bioavailability/therapeutic effects

Depending on the research question, healthy and/or inflamed tissue models are used to assess the bioavailability and/or therapeutic effects of small molecules/formulation. After application of the test

substance, effects may be investigated up to 24 hours using the above-mentioned readouts as well as any further assay to investigate the desired effects (ELISA, PCR, proteomics).

2.2. Key resources table:

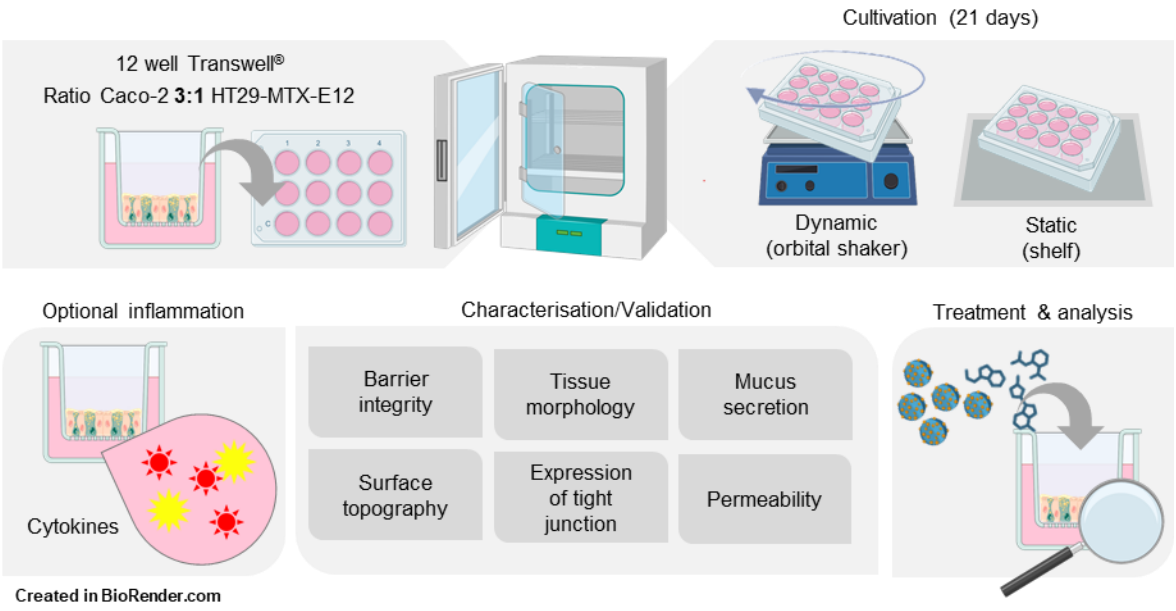
Reagent	***Supplier***	***Code***
Cell culture		
Caco-2 clone C2BBe1	American Type Culture Collection (ATCC)	CRL-2102
HT29-MTX-E12	European Collection of Authenticated Cell Cultures (ECACC)	12040401
DMEM	Gibco, Life Technologies	41966-029
Non-essential amino acids	Gibco, Life Technologies	11140050
Trypsin-EDTA	Gibco, Life Technologies	15400-054
FBS	Gibco, Life Technologies	A4766801
DBPS	Gibco, Life Technologies	14190-094
Transwell® inserts	Corning	353181
12-Well plates	Corning	353503
Histology		
DPBS	Gibco, Life Technologies	14190-094
Formaldehyde 3.7%	Sigma Aldrich/Merck	11-0705
Ethanol absolute	Merck	1.00986
Xylene histological grade	Sigma Aldrich/Merck	534056
Paraffin	Sigma Aldrich/Merck	03987
Alcian blue periodic acid/Schiff's reagent staining kit	Morphisto	11388
Embedding medium	Carl Roth	T160.1
Glass slides	Avantor/VWR	631-1553
Cover slips	Avantor/VWR	631-1575
Surface morphology assessment		
DPBS	Gibco, Life Technologies	14190-094
Glutaraldehyde	Thermo Fisher Scientific	119980250
Ethanol absolute	Merck	1.00986
Mucus quantification		
Methanol	Sigma Aldrich/Merck	M1775
Acetone	Merck	539481
Alcian blue 1%, pH 2.5	Morphisto	11388
Permeability assessment		
Hank's balanced salt solution (HBSS)	Thermo Fisher Scientific	88284
Fluoresceinisothiocyanat–Dextran (4 kDa)	Merck	46944
Black 96-well plates	Greiner, Bio-One	655076
Immunofluorescence staining		

DPBS	Gibco, Life Technologies	14190-094
Formaldehyde 3.7%	Sigma Aldrich/Merck	11-0705
Bovine serum albumin	Sigma Aldrich/Merck	A8531
Goat serum	Thermo Fisher Scientific	16210064
Anti zonula occludens-1 (ZO-1) antibody	Invitrogen	61-7300
Goat-anti-rabbit Alexa Fluor™ 488 antibody	Invitrogen	A-11008
Phalloidin Alexa Fluor™ 546	Invitrogen	A-22283
4',6 Diamidin 2 phenylindol, Dihydrochlorid (DAPI)	Invitrogen	D1306
Glass slides	Avantor/VWR	631-1553
High precision cover slips	Carl Roth	LH26.2
Embedding medium	Millipore/Merck	345789

Equipment	***Supplier***	***Model***
Cell culture		
Incubator	Binder	CBF 260
Orbital Shaker	IKA	MTS 2/4
Transepithelial electrical resistance measurement		
Volt-ohmmeter	Millipore/Merck	Millicell ERS-2
Histology		
Microtome	Slee	CUT5062
Drying cabinet	Binder	ED 56
Surface morphology assessment		
Critical point dryer	Leica	CPD300
Sputter coater	Quorum Technologies	SC7620
Scanning electron microscope	Zeiss	EVO10
Optional: Confocal Raman microscope	WITec/Oxford Instruments	Alpha 300R+
Mucus quantification		
Plate reader	Tecan	Spark.
Permeability assessment		
Plate reader	Tecan	Spark.
Immunofluorescence staining		
Orbital shaker	IKA	MTS 2/4
Confocal laser scanning microscope	Zeiss	LSM900

3. PROTOCOL

3.1. Workflow



3.2. Protocol

The cultivation and characterisation of microphysiological models of the heathy intestinal mucosa is performed as described in (Jung et al., 2024, doi: 10.1101/2024.03.08.584089), where exemplary micrographs and data are included. A more detailed description is given in this document, including the procedure to induce and assess inflammation in the *in vitro* tissue model.

3.2.1. Cell culture

Enterocytes and goblet cells are cultured separately in Dulbecco's Modified Eagle Medium (DMEM) with 10% foetal bovine serum (FBS) and 1% non-essential amino acids at 37°C and 5% CO_2 and are subcultured once a week. The cell lines are kept for a maximum of 12 weeks in culture or discarded earlier if fluctuations in cell division kinetics are observed. *In vitro* models are seeded in a Caco-2:HT29-MTX-E12 ratio of 1:3 (25%:75%) with a seeding density of $5x10^4$ cells/cm^2. Cells are seeded in 12-well Transwell® inserts with a pore size of 3.0 µm and cultivated for 21 days under dynamic conditions, achieved by placing the cell culture plates on an orbital microtiter shaker at 175 rpm 24 hours after seeding. Dynamic cultivation will induce three-dimensional tissue morphology to better replicate physiological properties of the intestinal mucosa *in vivo*. Alternatively, tissue models can be cultured statically by placing cell culture plates on the incubator shelf. The medium is replaced every 2 to 3 days.

3.2.2. Inflammation of tissue models and assessment

After 21-day cultivation, the *in vitro* tissue models can be inflamed using a mixture of the pro-inflammatory cytokines interleukin 1β (25 ng/mL), tumour necrosis factor α (50 ng/mL), and interferon γ (50 ng/mL). Cytokines are added to the basolateral compartment and tissue models are incubated for

24 hours. In addition to the test cultures, controls of untreated *in vitro* models as well as models with apically applied cytokines (should show no effect) should be cultured as reference points. For subsequent experiments (transport and exposure studies), the basolateral compartment should always contain the cytokine cocktail to uphold the inflammatory state.

3.2.3. Transepithelial electrical resistance (TEER) measurement

Over the course of the cultivation period of 21 days, the TEER of tissue models are measured every 2 – 7 days. Averaged values of empty cell culture inserts (blanks) are subtracted from the resistance measurements of samples and normalised to the area of the cultivation area (0.9 cm^2).

Quality attributes:

TEER measurements are established in the research community and can be used to obtain a quick estimation of barrier integrity of the tissue in a non-invasive manner. However, depending on the cell passage and the FBS batch, TEER values might fluctuate notably (100 – 400 Ohm*cm^2), making the TEER value an unreliable measure for the sole investigation of barrier integrity. TEER values of approx. 150 Ohm*cm^2 can be considered physiological, however, it is strongly recommended to validate a functional barrier via permeability studies using fluoresceinisothiocyanat–Dextran (see below).

Inflamed tissue models exhibit an approx. 30 – 40% decrease in TEER values after 24 hours of cytokine stimulation.

3.2.4. Histology

The distribution of Caco-2 and HT29-MTX-E12 cells within the *in vitro* models as well as the morphological organisation of tissues can be assessed using histological analyses. *In vitro* models are washed with DPBS and fixed using a 3.7% formaldehyde solution (in DPBS) for 15 min at room temperature. Again, samples are washed with DPBS and dehydrated using an ascending ethanol series (70%, 80%, 95%, 100%) for 30 min each. After incubation in xylene for 10 min, samples are incubated for 60 min in liquid paraffin at 65°C in a drying cabinet. Afterwards, samples can be embedded in paraffin and cured overnight at room temperature. Before cutting, samples are placed on ice for 15 min and then cut into 5 µm sections using a microtome. Sections are placed in water to unfold and then carefully placed on microscope slides. The samples are dried for at least 8 hours at 37°C to remove excess water. For staining, sections are rehydrated using the ethanol series mentioned above (5 min each) and stained using alcian blue and periodic acid/Schiff's reagent, following the manufacturer's protocol. Lastly, the sections are dehydrated (ethanol series, 5 min each), cleared in xylene (5 min), and mounted using a suitable embedding medium and a coverslip. The slides are then observed using a light microscope.

Quality attributes:

Using the Alcian blue/PAS staining kit, enterocytes appear pink while mucus-containing goblet cells appear blue in the histological sections. Both cell types should be visible in the samples and appear approx. in the seeding ratio of 1:3. Dynamically cultured tissue models should display a monolayer of epithelial cells on the Transwell® membrane with elongated/columnar cell morphologies and an approximate layer thickness of 25-30 µm. Three-dimensional protrusions are expected to be present at multiple spots in the

sample, spanning dimensions of 80-120 µm in height and 100 µm in width. These morphological features can also be found in microfluidic chip-based microphysiological systems (Kim et al., 2013 doi: 10.1039/c3ib40126j). Keep in mind that due to the sample preparation, protrusions might be flattened or destroyed. More detailed information can be gained from topography measurements.

Inflamed tissue models exhibit no obvious alterations compared to healthy controls.

3.2.5. Surface morphology & enterocyte differentiation assessment

Using scanning electron microscopy (SEM), more detailed information about the morphology of the tissue surface can be obtained. Before imaging, tissue models are washed with DPBS and fixed using a 2.5% glutaraldehyde solution (in DPBS). Samples are washed again and dehydrated in an ethanol series with 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% and 100% for 20 min each on an orbital shaker. Subsequently, samples are dried using a critical point dryer, placed on sample holders for SEM imaging and sputter-coated with a thin layer of palladium/gold for six minutes.

Quality attributes:

SEM imaging allows for the better assessment of successful three-dimensional tissue formation. On the surface of the tissue, multiple protrusions (as described in histology) should be visible. In higher magnifications, enterocytes should display the formation of microvilli as crucial indicator for successful differentiation. Due to harsh sample preparation, there might be cracks forming in the tissue, this is not an indicator of an impaired barrier.

Inflamed tissue models exhibit no obvious alterations compared to healthy controls.

3.2.6. Topography measurement

Surface morphology of the *in vitro* tissues can further be visualised in more detail using profilometry based methods, e.g. white light reflectance. Dried and sputter-coated samples from SEM analysis can be analysed using e.g. the topography feature of an Alpha300R^{+} confocal Raman microscopy. Acquired z-values, representing the surface topography, are exported as txt-files, and analysed in MATLAB regarding the height of protrusions. Additionally, MATLAB can be used to plot the topography for visual representation. An exemplary custom script for plotting can be found below (yellow sections can be adapted to the respective measurement).

```
% Convert Table to Array and assign to Z
Zpre=table2array(YOURFILE) ;

% Define the baseline as the 10th percentile of the data plus 20
baseline = 20;

% Convert to double
Zpre = double(Zpre);

% Adjust Z-Baseline
Z = Zpre + baseline;

% Define dimensions
[X, Y] = meshgrid(1:500, 1:500); % Use meshgrid to create 2D arrays for X and Y

% Create Mesh 3D Plot
 mesh(X, Y, Z)

% Adjust the Aspect ratio
daspect([1, 1, 2])

 % Define the colormap and its range
cmin = min(0);
cmax = max(140);

% Adjust the colormap range and label
clim([cmin, cmax]);

% Adjust Labels and Title
xlabel('X');
ylabel('Y');
zlabel('Z');

% Define z-limits and add Color Scale
zlim([-20 150])
colorbar;
c = colorbar;
c.Label.String = 'Elevation (µm)';
```

Quality attributes:

The obtained data can be used to acquire more detailed information about the z dimensions of the protrusions in the tissue models, which should be between 80-120 µm.

Inflamed tissue models exhibit no obvious alterations compared to healthy controls.

3.2.7. Mucus quantification

For quantifying mucus in *in vitro* models, an *in situ* alcian blue staining can be performed. Tissue models are washed twice with DPBS and fixed using ice-cold methanol and acetone (1:1) for 15 min at -20°C. Fixative is removed, tissue sample are washed again with DPBS and incubated for 15 min with 1% alcian blue pH 2.5. Unbound dye is removed by repeated washing with ultrapure water. Samples are dried under ambient conditions. Using a plate reader, absorbance is measured at 630 nm in multiple positions within each sample (>200 measurements per sample). The amount of mucus is given as the relative amount after normalisation to the statically cultured Caco-2 monoculture in each independent experiment.

Quality attributes:

After 21 days of cultivation, the mucus layer can already be observed via visual inspection of the *in vitro* models, however the in situ Alcian blue staining can help novice experimenters in gauging the mucus secretion. As a reference point, the same experiment should be performed with a control tissue model

without goblet cells. In comparison, the dynamic cultures with enterocytes and goblet cells in a ratio of 1:3 should exhibit dark blue spots where mucus is accumulating on the tissue. The relative absorbance is expected to be approx. twice as high as the control. A more detailed analysis of mucus secretion can be performed by using qRT-PCR and assessment of MUC5Ac and MUC5b.

Inflamed tissue models exhibit no obvious alterations compared to healthy controls.

3.2.8. Immunofluorescence staining

To assess the formation and polarisation of tight junctions, *in vitro* models are washed twice with DPBS and fixed using ice-cold acetone and methanol (1:1) for 20 min at -20°C. Afterwards, samples are rinsed with DPBS and cell culture inserts with tissues are cut from the Transwell® using a scalpel. The samples are transferred to a fresh well plate, blocked with 3% bovine serum albumin in DPBS with 1% goat serum for 20 min. Then, an anti-zonula occludens 1 (ZO-1) antibody (1:400) in the before used blocking buffer, is added to each sample, and incubated over night at 4 °C under gentle shaking. After washing with DPBS (3x 5 min), samples are incubated with a goat-anti-rabbit Alexa Fluor™ 488 antibody (1:500 in DPBS) for 1 hour at room temperature. Accessible actin filaments and nuclei are stained using Alexa Fluor™ 546 Phalloidin for 1 hour (1:400 in DPBS) and 4',6-Diamidin-2-phenylindol, Dihydrochlorid (DAPI) for 5 min (1:100 in DPBS), respectively. Samples are mounted on glass slides using an embedding medium and high precision cover slips. Tight junctions are visualised by acquiring z-stacks using a confocal laser scanning microscope.

Quality attributes:

It is highly recommended to acquire z-stacks as opposed to simple 2D micrographs to assess the correct (apical) expression of tight junctions. DAPI staining is used to differentiate neighbouring cells from another. Healthy tissue models display a continuous, straight, thin line of tight junctions between all cells. Indicators of disrupted tight junctions include accumulation of ZO-1 positive spots in the cytoplasm (internalisation of protein) or wavy lines between two cells (“ribboning”). Additionally, Phalloidin staining can give indication of the formation of microvilli on the tissue surface (appears as furry texture) if e.g. SEM can not be easily accessed.

Inflamed tissue models exhibit potentially higher internalisation and ribboning of tight junctions.

3.2.9. Permeability assessment

To assess the barrier integrity of *in vitro* models in detail, transport studies using fluorescein isothiocyanate (FITC) labelled 4 kDa dextran are conducted. TEER of *in vitro* models is measured before pre-incubation with Hank's balanced salt solution (HBSS) for 30 min in the incubator on an orbital shaker at 175 rpm. TEER is measured again and fresh HBSS is placed in the basolateral compartment while a solution of 250 µg/mL FITC-dextran is added to the apical compartment. *In vitro* models are placed on an orbital shaker again and every hour, 100 µL samples are collected for a total duration of min. 4 hours. After each sampling point, 100 µL of fresh, pre-warmed HBSS is added to the basolateral compartment. Samples are collected in a black 96 well plate and fluorescence intensities are measured immediately using a plate reader (excitation: 485 nm, emission: 535 nm, gain: 60). After the transport study is

complete, TEER is measured again to validate the barrier integrity. The apparent permeability coefficient (P_{app}) of each sample is calculated using the following equation:

$$P_{app} = (\mathrm{dQ/dt}) \times (1/\mathrm{A\ x\ C0})$$

dQ/dt = the rate of cumulative drug transport (µg/s)
A = the surface area of the cell monolayer (cm^2)
C0 = the initial concentration of the drug in the donor compartment (µg/mL)

Quality attributes:

HBSS incubation should not exert any effect on TEER values. P_{app} of intact tissue models is expected to be below 5×10^{-7}. In comparison, static enterocyte monocultures should exhibit P_{app} values of $< 5 \times 10^{-8}$.

Inflamed tissue models exhibit approx. > threefold increase in permeability compared to healthy controls.

3.2.10. Enzyme-Linked Immunosorbent Assay (ELISA)

The inflammation state of *in vitro* models can further be characterised in more detail by employing ELISA for relevant cytokines (interleukin 8). Here, the medium in the apical compartment of the models is collected before and after the cytokine stimulation, as well as after the respective treatment in exposure experiments.

Quality attributes:

After 24 hours of cytokine stimulation, the concentration of secreted interleukin 8 may reach up to 1000 pg/mL. The relative in-/decrease of interleukin 8 after treatment can be used as reliable readout for therapeutic efficacy. The cytokines used for stimulation (interleukin 1, tumour necrosis factor α, interferon γ) cannot be reliably used in ELISA studies but may be quantified using qRT-PCR for additional information.