PATROLS
Advanced Tools for NanoSafety Testing

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 760813

PATROLS Standard Operating Procedures (SOP)

Triple culture of the intestine combining Caco-2, HT29-MTX-E12 and THP-1 cells

This is a SOP recommended for external use by PATROLS

Adapted from the NanoImpactNet SOP, Clift *et al* (Deliverable 5.4 under the European Commission's 7th Framework Programme, Grant Agreement 218539).

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Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	25/01/2019	Draft 1	Angela Kämpfer
2.0	25/04/2019	Amended SOP including comments from WP4 partners	Angela Kämpfer
2.1	04/01/2021	Error correction (5.7.1.1.; FBS in Caco-2 culture medium changed from 10 to 20%)	Angela Kämpfer
2.2	08/04/2021	Transfer to updated form	Angela Kämpfer

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1 Introduction:

Domain: Human Toxicology

As the importance of intestinal health for the whole organism becomes more apparent, an increasing amount of research investigates the impact of substances, e.g. particles, on the intestine in health and disease. Oral ingestion of micro- and nanoscale particles, both intentional (Peters et al., 2014, Lim et al., 2015) and accidental (von Goetz et al., 2013), has long been identified as a likely route of human exposure to particles.

In the context of the 3Rs principles – to Refine, Reduce, and ultimately Replace animal experiments – the development of novel alternative models (e.g. *in vitro* and *in silico*) is necessary. To date, the availability of relevant 3D intestinal *in vitro* co-culture models is limited but a few, mainly cell line-based, approaches have been established (Susewind et al., 2016, Georgantzopoulou et al., 2016, Leonard et al., 2010, Kämpfer et al., 2017). So far, only two models have been established solely based on human cell lines that (1) incorporate at least one immunocompetent cell type and (2) can mimic both healthy and inflamed-like conditions (Kämpfer et al., 2017, Susewind et al., 2016). Both models, however, lack the presence of mucus, which can heavily impact the fate and effect of (nano)particles in the intestine (Crater and Carrier, 2010).

1.1 Scope and limits of the protocol

Scope: Following this protocol allow one to establish a triple co-culture of human cell lines representing enterocytes (Caco-2), goblet cells (HT29-MTX-E12, hereinafter E12) and macrophages (differentiated (d)THP-1) to mimic the human small intestine in a healthy state. The model can be used to study the toxicity and inflammatory potential of particles as well as chemicals. Endpoints include, but are not limited to: barrier integrity (e.g. by Transepithelial electrical resistance (TEER), passive transport assays like Lucifer Yellow), cytotoxicity (e.g. lactate dehydrogenase (LDH) assay, nuclear staining), oxidative stress, and cytokine release.

Limitations: To induce a pro-inflammatory response, a significant stimulus might be necessary. It was previously shown that Caco-2 cells can strongly reduce or even prevent a pro-inflammatory response mediated by particles in phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 cells (Kämpfer et al., 2017). On the one hand, this

underlines the physiological relevance of the model as it more closely mimics the cellular interactions in the intestine. On the other hand, this might require focusing on more subtle changes than for instance strong induction of pro-inflammatory cytokine release to determine the pro-inflammatory potential of a test substance.

1.2 Validation state of protocol

Level of advancement towards standardization	**Level reached** (please mark only one with "X")
Stage 1: Internal laboratory method under development	
Stage 2: Validated internal laboratory method	
Stage 3: Interlaboratory tested method	X
Stage 4: Method validated by Round Robin testing	
Standardisation plans	
Is the method considered for standardisation (OECD SPSF or similar)?	No
Has the method been submitted for standardisation (to OECD, CEN, ISO,…) in its own right or as part of another standardisation project?	No
Is the method included in an existing standard (or ongoing standardisation work)	No
If yes, specify	[standard reference number, eg. EN 17199-4]

2 Terms and Definitions:

Differentiated epithelial cells

Caco-2/E12 layers cultured for 21 days on transwell filter inserts

Engineered nanomaterial

Nanomaterial designed for specific purpose or function

[SOURCE: ISO/TS 80004-1: 2016, definition 2.8]

Pre-warmed

Warmed to 37°C in a waterbath

Stable triple culture

Triple culture of differentiated epithelial cells and PMA-differentiated, non-activated THP-1 cells

3 Abbreviations:

AB	Alcian Blue
AP	Apical
BL	Basolateral
CCM	Cell culture medium
E12	HT29-MTX-E12 cell line
EtOH	Ethanol
FBS	Foetal Bovine Serum
NEAA	Non-essential amino acids
PAS reaction	Periodic acid-Schiff reaction
PBS	Phosphate Buffered Saline
Pen/Strep	Penicillin / Streptomycin
PMA	Phorbol-12-myristat-13-acetat
RT	Room temperature
TEER	Transepithelial Electrical Resistance
ZO-1	Zonula Occludens 1
LPS	Lipopolysaccharides

4 Principle of the Method:

In vitro co-cultures based on (human) cell lines representing various cell types are used to develop more physiologically relevant models. A single isolated cell type is usually not capable of adequately reflecting the heterogeneous composition of organs or the organism as a whole. More specifically, cell monocultures lack the communication

which generally regulates the interplay and homeostasis between different cell types *in vivo*. Therefore, triple cultures have been established to better reflect the complexity of an in vivo exposure situation.

The procedure can be divided into 4 parts:

1. Culture and maintenance of the 3 individual cell lines (Section 5.7.1)
2. Seeding and maintenance of the epithelial transwell co-culture (Section 5.7.2)
3. PMA-differentiation of THP-1 cells (Section 5.7.3)
4. Establishment and maintenance of the stable triple culture (Section 5.7.4)

5 Description of the Method:

5.1 Biological setting & test system used:

- set-up of a cell line-based triple culture model under sterile lab-based work conditions to mimic the intestine in a healthy state for the investigation of toxicity endpoints
- Human derived cell lines used:
    - Caco-2 (human colon adenocarcinoma): DSMZ, ACC169
    - HT29-MTX-E12: sub-clone E12 of the methotrexate-differentiated cell line HT29 (human colon adenocarcinoma), Merck/ECACC, 12040401
    - THP-1 (human monocytic leukemia): ATCC, TIB-202

5.2 Chemicals and reagents used:

Reagent	Supplier/Catalogue number	CAS
MEM (NEAA)	Thermo Fisher Scientific, #10370-021	NA
DMEM (high glucose, L-Glutamine)	e.g. Thermo Fisher Scientific, #41965-039	NA
RPMI (HEPES, L-Glutamine)	e.g. Thermo Fisher Scientific, #52400-041	NA
PMA	Merck, P1585	16561-29-8
Penicillin / Streptomycin	e.g. Thermo Fisher Scientific, #15140122	69-57-8, 3810-74-0
L-Glutamine	e.g. Thermo Fisher Scientific, #35050038	NA
D-Glucose	e.g. Sigma, G8769	50-99-7
Sodium pyruvate	e.g. Thermo Fisher Scientific, #11360070	113-24-6
2-Mercaptoethanol (50 mM)	e.g. Thermo Fisher Scientific, #31350010	60-24-2
Trypsin	e.g. Sigma T4049	NA
FBS	THP-1, e.g. Sigma, F7524	NA
NEAA	e.g. Sigma, M7145	NA
Accutase	e.g. Merck, A6964	NA
LPS	Sigma, L4391	93572-42-0
PBS	e.g. Sigma, D1408	NA

5.3 Apparatus and equipment used:

Transwell insert and companion plates

Transwell inserts, PET, 1µm pore size[1]	Cat. N° 353103
Companion plates	Cat. N° 353503

Voltohmmeter

e.g. World Precision Instruments

Model 'EVOM' or newer with chopstick electrode STX2

Absorbance reader

e.g. Thermo Scientific, Multiskan Go

5.4 Reporting of protected elements:

This SOP does not have any associated patent restrictions, specific licenses, material transfer agreements or commercial purchase requirements required to perform the protocol described.

5.5 Health and safety precautions:

Prior to any use of this SOP a full risk assessment should be completed, considering all potential risks associated with chemicals equipment and use, in compliance with national regulation. Training of personnel should be completed before any person is working with the SOP.

5.6 Reagent preparation:

Heat inactivation of FBS

- Take a bottle of FBS from -20°C and let thaw over night

[1] *In principle, other pore sizes can be used. It is, however, possible that the cell growth and TEER development will be influenced by both the pore size and material used.*

- Place the bottle of thawed FBS in the cold waterbath and switch on; set the temperature control to 56°C
- Once the waterbath reached 56°C, keep the bottle in the waterbath for another 30 min
- In the meantime, label an adequate amount of 50 mL tubes, including your initials and the date
- After 30 min, take the bottle from the waterbath, dry and generously spray with 70% EtOh before transferring it under a laminar flow cabinet
- Carefully open the bottle and aliquot into the prepared 50 mL tubes using a Pippette Boy and serological pipettes (25 or 50 mL)
- Let the serum cool for 30 min before transferring the tubes to -20°C

Reconstitution of lyophilized PMA

- Carefully open the glass vial and add 162 µL pure ethanol to reconstitute the lyophilized powder
    - Stock 1: 10 mM
    - Store in 10 µL aliquots at -20°C
- Stock 2: add 10 µL of stock 1 to 990 µL sterile PBS
    - 100 µM
    - Prepare working aliquots of 25-50 µL each
- When handling PMA: PMA IS LIGHT AND TEMPERATURE SENSITIVE! Keep protected from light! Do not keep PMA at room temperature for more than 10 min!

5.7 Procedure:

5.7.1 Cell culture

5.7.1.1 Caco-2

Cell culture medium (CCM)

- MEM medium (500 mL)
- 20 % heat inactivated fetal bovine serum (100 mL)
- 5 mL of glutamine (Invitrogen 35050)
- 5 mL Pen/Strep

Culture / Maintenance

- Cells are seeded at $7.5x10^5$ cells in 75 cm² flasks and maintained in the above listed Caco-2 cell culture medium
- Sub-culture cells at the latest when they reach 80% confluence; generally splitting the cells on Monday and Friday is recommended
- Discard the medium and wash 1x with 5mL pre-warmed PBS
- Discard PBS, add 5 mL fresh pre-warmed PBS and place the flask at 37°C, 5% CO_2 for 5 min
- Discard the PBS and add 3 mL pre-warmed trypsin; place the flask at 37°C, 5% CO_2 for 5 min
- To de-activate the trypsin, add 7 mL pre-warmed Caco-2 cell culture medium to the flask; pipette the cell suspension up and down several times in the flask (to help detach more cells and break up cell clumps)
- Transfer cell suspension to a 15 mL tube and perform a cell count
    - The cell count can be performed on the undiluted suspension; the count should be usually be between $0.8\text{-}1.1x10^6$ cells/mL
    - Ideally cell counts should be performed using an automated cell counter. Alternatively a manual count can be performed using a haemocytometer following the protocol given in the PATROLS '3D In Vitro HepG2 Spheroid model' SOP
- Re-seed $7.5x10^5$ cells in a new flask and top up to a total volume of 15 mL with pre-warmed cell culture medium; Place flask at 37°C, 5% CO_2
- It is recommended to start a new culture after 20 passages

5.7.1.2 HT29-MTX-E12

CCM

- DMEM medium (high glucose) (500 mL)
- 10 % heat inactivated fetal bovine serum (50 mL)
- 5 mL NEAA

Culture / Maintenance

- Cells are seeded at $3x10^6$ cells in 75 cm² flasks and maintained in the above listed E12 culture medium
- Sub-culture cells before they reach 80% confluence¸ generally splitting the cells on Monday and Friday is recommended
- Discard the medium and wash 1x with 5mL pre-warmed PBS
- Discard PBS, add 5 mL fresh pre-warmed PBS and place the flask at 37°C, 5% CO_2 for 5 min
- Discard the PBS and add 3 mL pre-warmed trypsin; place the flask at 37°C, 5% CO_2 for 5 min
- To de-activate the trypsin, add 7 mL pre-warmed E12 cell culture medium to the flask; pipette the cell suspension up and down several times in the flask (to help detach more cells and break up cell clumps)
- Transfer cell suspension to a 15 mL tube and perform a cell count
    - As the cell concentration will be high (>$1.5x10^6$ cells/mL) it is advised to perform the cell count on a 1:10 diluted cell suspension
    - Ideally cell counts should be performed using an automated cell counter. Alternatively, a manual count can be performed using a haemocytometer following the protocol given in the PATROLS '3D In Vitro HepG2 Spheroid model' SOP
- Re-seed $4x10^5$ cells/cm² in a new flask (i.e. $3x10^6$ cells/flask) an top up to a total volume of 15 mL using pre-warmed E12 cell culture medium; Place flask at 37°C, 5% CO_2
- It is recommended to start a new culture after 20 passages.

5.7.1.3 THP-1

CCM

- RPMI 1640 medium (with L-Glutamine 25 mM HEPES), 500 mL
- 3.3 mL of 0.25 g/mL of D-Glucose stock solution
- 5 mL of 100 mM sodium pyruvate
- 5 mL of 1% Penicillin-Streptomycin (100 U/100 µg/mL)
- 10 % heat inactivated fetal bovine serum (50 mL)
- 2-mercaptoethanol[2] to a final concentration of 0.05 mM (500 µL of 50 mM in 500 mL culture medium

Culture / Maintenance

- Cells are grown in suspension in 25 cm² flasks (standing upright at 37°C, 5% CO_2) at a cell density between 2 and 8x10^5 cells/mL
- To count the cells, transfer the cell suspension from the flask to a 15 mL tube; if you have multiple flasks, pool the cell suspensions in one 50 mL tube
- Take 10 µL of the THP-1 cell suspension and dilute 1:1 with Trypan Blue; ideally cell counts should be performed using an automated cell counter. Alternatively a manual count can be performed using a haemocytometer following the protocol given in the PATROLS '3D In Vitro HepG2 Spheroid model' SOP
    - If the concentration is <8x10^5 cells/mL: add 2-4 mL fresh culture medium; do not exceed a total volume of 20 mL/flask
    - If the concentration is ≥8x10^5 cells/mL: passage cells by keeping 2x10^6 cells in fresh culture medium (final volume: 10 mL); the flask can be re-used
    - Do not let the cell density exceed 1x10^6 cells/mL
- It is recommended to start a new culture after a **maximum of 15 passages**. NB: It is possible that the culture has to be replaced earlier. The PMA responsiveness, morphology and reaction to LPS should be investigated regularly.

[2] Only for cell culture, not for co-culture experiments!

5.7.2 Epithelial co-culture

Experiments should preferably be performed with biological replicates in triplicate. Two different seeding ratios are possible for the epithelial transwell co-cultures, 9:1 and 8:2 Caco-2/E12 cells, respectively. The maintenance protocol is identical for both ratios. The required cell numbers for both ratios are given in Table 5.1.

Cell seeding and maintenance

- Prepare cell suspensions of Caco-2 and one of E12 cells as described in the sub-culture instructions in Section 5.7.1.1 and 5.7.1.2 above
- Perform cell counts as described for Caco-2 and E12 cells separately
- Add 1.5 mL Caco-2 CCM to a transwell-suitable 12-well plate
- unpack the needed amount of transwell inserts and place the inserts on the medium-containing wells of the 12-well plate
- from the concentration of the Caco-2 and E12 cell suspensions calculate the required volume to obtain the following cell numbers for a 9:1 or 8:2 ratio of the two cell types

Tab. 5.1 Required cell numbers for different seeding ratios of epithelial transwell co-cultures

	9:1 ratio (cells/transwell)	8:2 ratio (cells/transwell)
Total	1.62 x10^5	1.62 x10^5
Caco-2	1.46 x10^5	1.29 x10^5
E12	0.16 x10^5	0.324 x10^5

- Top up the medium in the apical compartment to a total of 0.5 mL/transwell
- Place plate at 37°C, 5% CO_2
- Change the medium on both the apical and basolateral compartment every 2-3 days, e.g. Monday, Wednesday, Friday; follow the scheme in Table 5.2 to

gradually transition the cells basolaterally from MEM-based Caco-2 to RPMI-based THP-1 medium (without mercaptoethanol!)

- Maintain the culture for up to 21 days before using the epithelial barriers to establish the triple culture

Table 5.2 Basolateral culture medium transition from MEM-based Caco-2 to RPMI-based THP-1 culture medium

Times of medium change	Caco-2 medium (µL)	THP-1 medium (ME-free, µL)
1st	1500	0
2nd	1000	500
3rd	1000	500
4th	750	750
5th	750	750
6th	500	1000
7th	500	1000
8th	0	1500
9th	0	1500

Monitoring of barrier development

- To routinely follow barrier development, measure TEER as described in **Section 5.8.3** every 2-3 days; once the basic TEER readings stabilise, TEER measurements can be reduced, e.g. day 7 and 14 post-seeding, and 24h before the establishment of the triple culture with THP-1 cells

Day 2

- Check each flask for their response to PMA before proceeding
    - >90% of the cells should be firmly attached

- >50% of the cells should have developed a distinct macrophage-like phenotype

An example of how well-differentiated THP-1 cells should look like is given in Figure 5.1A. If the differentiation did not work well, cells will resemble the example image of Figure 5.1B.

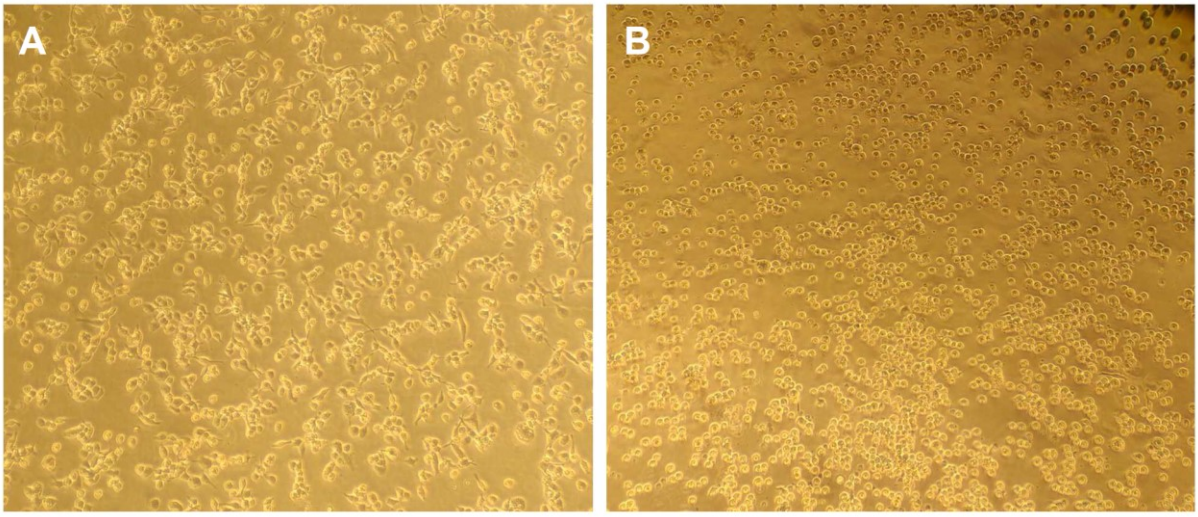

Fig. 5.1 Response of THP-1 cells to 24h PMA-exposure (A) Well-differentiated, (B) cells that did not respond well

- Discard the PMA-containing CCM and wash 2x with 5mL pre-warmed PBS
- Add 1 mL pre-warmed Accutase (1x) per flask
- Incubate for 5 min at 37°C
- Check under the light microscope for cell detachment; if many cells remain attached place back at 37°C for another 5 min
- If the cells are detached (Figure 5.2): add 3 mL fresh pre-warmed CCM to stop the Accutase activity
    - NB: The cells will not retain their amoeboid shape throughout Accutase detachment and subsequent re-attachment.
- Count cells (preferably with Trypan Blue) and re-seed $1.8x10^5$ THP-1 cells in a volume of 1.5 mL THP-1 cell culture medium containing ME onto transwell-suitable 12-well plates
- Place the cells on 12-well plates at 37°C, 5% CO_2 for 1-1.5h for re-attachment

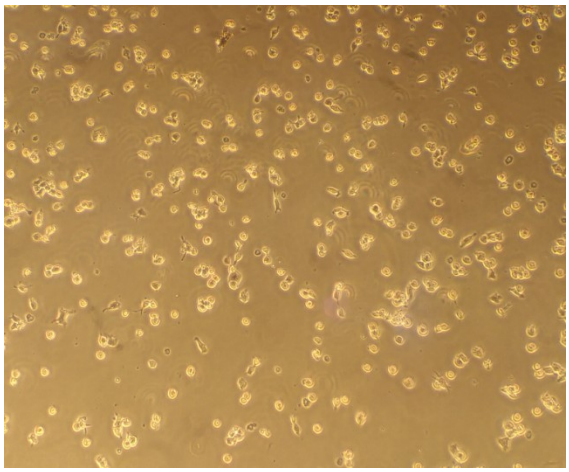

Fig. 5.2 Re-attached PMA-differentiated THP-1 cells

5.7.4 Triple culture establishment

- Once the THP-1 cells are re-attached in the 12-well plate, carefully discard the medium and replace it with 1.5 mL fresh, pre-warmed THP-1 medium (without mercaptoethanol)
- Take a transwell plate with differentiated Caco-2/E12 cells out of the incubator and measure the TEER of each transwell as described below (Section 5.9.3)
- Arrange the transwell inserts to triplicates based on the TEER readings as exemplified in Figure 5.3, so that the differences between the conditions are as low as possible

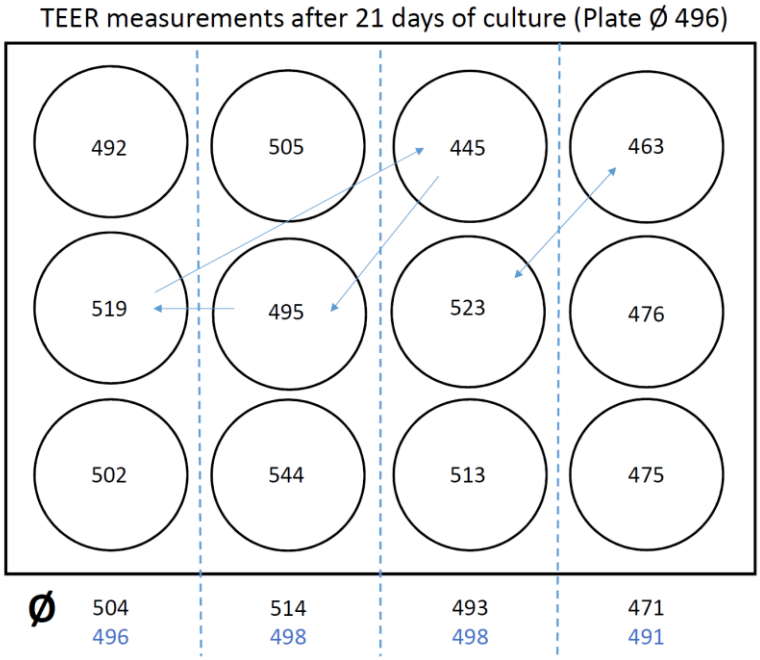

Figure 5.3 Example: How to arrange transwell inserts based on TEER measures (Ø black numbers = initial average of three transwells in a column, blue numbers = average of three transwells in a column after re-arrangement)

5.7.3 PMA-differentiation of THP-1 cells

Day 1

- Count THP-1 cells as described above (with Trypan Blue in 1:1 dilution)
- Seed 3x10^6 THP-1 cells in 5 mL THP-1 CCM (with ME) in a 25 cm² flask
- Take an aliquot of PMA (100 µM PMA stock solution) and add to the flask to obtain a final concentration of 100 nM PMA/flask
- **NB: PMA IS LIGHT AND TEMPERATURE SENSITIVE! Keep protected from light! Do not keep PMA at room temperature for more than 10 min!**
- Place the flask horizontally at 37°C, 5 % CO_2 for 24h
- NB: Prepare as many flasks as necessary; if possible, do not rely on one flask, even if few cells are needed
- Carefully discard the apical culture medium and replenish with 0.5 mL fresh pre-warmed MEM-based Caco-2 medium
- Carefully place the transwell inserts using sterile forceps in the wells containing re-attached PMA-differentiated THP-1 cells

- The plate(s) can be used immediately for experimentation, e.g. exposure to substances
- If plate(s) are not immediately used, place the plate(s) at 37°C, 5% CO_2 until further use / the first time point [e.g. in some circumstances it might be preferable to let the co-culture establish and equilibrate itself for 24h before use]

- To follow up on the triple culture, measure TEER after 4, 24, and 48h (optionally also at 18 and 42h) of culture with THP-1 cells

The epithelial co-culture and triple culture set-up is schematically summarized in Figure 5.4.

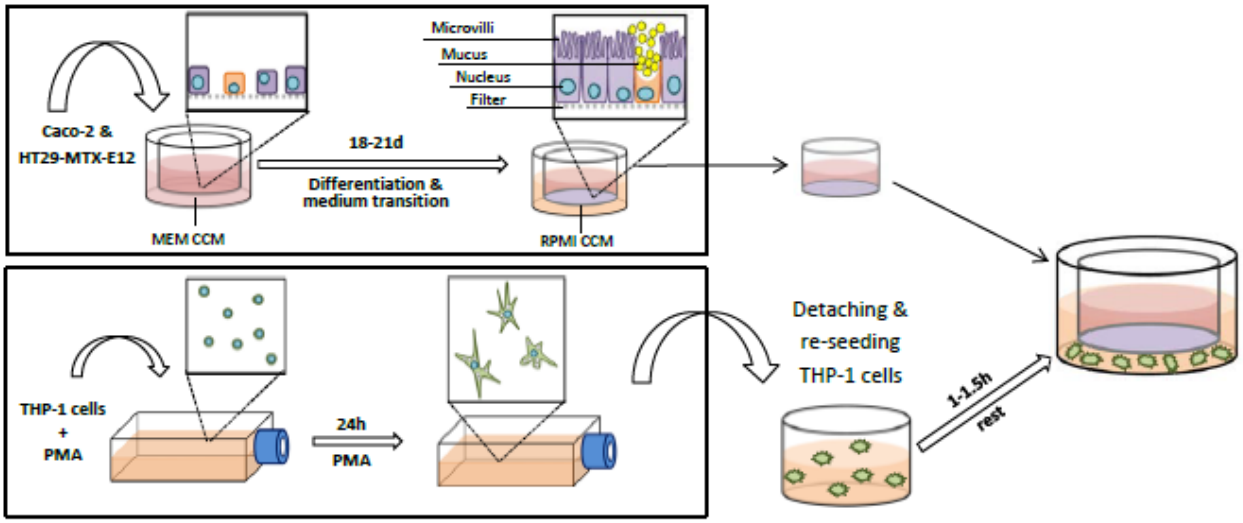

Fig. 5.4 Schematic description of the triple culture set-up

Caco-2 and E12 cells are seeded at a 9:1 ratio onto transwell filters. This epithelial co-culture is maintained for 21 days, allowing the cells to form a dense layer and differentiate into enterocyte- and goblet-like cell types (Section 5.7.2.1). While the transwell cultures are apically maintained in MEM-based Caco-2 culture medium, they are basolaterally transitioned to RPMI-based THP-1 culture medium (Table 5.1). On Day 20 of the epithelial co-cultures, THP-1 cells are differentiated to macrophage-like cells using PMA for 24h (Section 5.7.2.2). After 24h, the now adherent, amoeboid-shaped THP-1 cells are detached and re-seeded onto transwell-suitable well plates. The cells are allowed to re-attach for 1-1.5h before their medium is changed to ME-free RPMI-based culture medium and the transwells containing differentiated epithelial co-cultures are placed into the wells. This stable triple culture can be maintained for at least 5 days, if the apical culture medium is replenished every 48h.

5.8 Quality control & acceptance criteria:

5.8.1 Negative control

- with each triple culture, include an epithelial co-culture exposed to cell culture medium as a control (in triplicate) to be able to study the impact of THP-1 presence
- basolateral results (e.g. for cytokines, LDH release, …) of stable co-cultures should be compared to the levels of PMA-differentiated THP-1 monocultures (this should always be done when the cell lines, co- and triple cultures are newly established in a laboratory. Once the protocols are reproducibly established and no significant differences were seen between the basolateral results of the triple cultures and PMA-differentiated THP-1 monocultures, these controls do not have to be run routinely anymore.)

5.8.2 Positive control

- amino-modified polystyrene nanoparticles (80 µg/cm²) were tested to be cytotoxic

5.8.3 Quality control for epithelial co-cultures and stable triple cultures

1) TEER development over different passage numbers

Material / Reagent	Supplier/Catalogue number	CAS
Voltohmmeter	e.g. World Precision Instruments; model EVOM or newer	
Chopstickelectrode	e.g. World Precision Instruments, STX2	
Ethanol (dilute to 70% in H_2O)	e.g. Merck, 100983	64-17-5
PBS	e.g. Sigma	
MEM	Thermo Fisher Scientific, or comparable	

Preparation

- Fill a 50 mL tube with 30 mL 70% EtOH
- Sterilize the chopstick electrode by placing it in 70% EtOH for 15 min before use
- Prepare two 15 mL tubes: one containing 10 mL pre-warmed PBS, one containing 10 mL pre-warmed MEM-based culture medium
- Take the transwell plate from the incubator, place under the hood, take off the lid and leave for the temperature to slightly equilibrate for 1 min
- At the same point take the electrode from the EtOH to the PBS and then to the culture medium to neutralize the EtOH
- Make sure the electrode is connected to the Ohmmeter and the correct settings are chosen on the instrument (e.g. for the EVOM Voltohmmeter, make sure the instrument is switched 'On' and set to Mode 'R'); have pen and paper available to note the measurements (if no automatic recording is available)

Procedure

- Carefully place the electrode in a well, with the short chopstick in the apical compartment of the transwell and the long chopstick lightly touching the bottom of the well plate
- Hold the electrode as vertical and as still as possible
- allow the Voltohmmeter to settle on a value
- Repeat for all transwell inserts; include a blank without cells containing the same medium volumes
- Between plates or when (accidentally) touching a (potentially) non-sterile surface, wash the electrode again in 70% EtOH
- Once all measurements have been taken, place the electrode in 70% EtOH for 5-10 min and switch of the Ohmmeter

Results

- To calculate the resistance expressed as Ohm times cm^2 filter surface (i.e. $\Omega \cdot cm^2$), follow the equation

Resistivity (Ω•cm²) = (Ohm2-Ohm1)xA

Ohm1 = resistence of blank (only culture medium without cells)

Ohm2 = resistence of the filter insert with cells

A = surface area in cm² of the filter

2) Formation of a Tight Junction network (staining of ZO-1)

Material / Reagent	Supplier/Catalogue number	CAS
Zonula Occludens antibody	Thermo Fisher Scientific, 617300	NA
AlexaFluor 594	Thermo Fisher Scientific, A11037	NA
Phalloidin AlexaFluor488	Thermo Fisher Scientific, A12379	NA
DAPI	e.g. Thermo Fisher Scientific, D1306	NA
Paraformaldehyde (PFA)	e.g. Sigma, 252549	50-00-0
PBS	e.g. Sigma, D8537	NA
Bovine Serum Albumin	e.g. Sigma, A9418	9048-46-8
Triton X-100	e.g. Sigma, T8532	9002-93-1
Mounting Medium	e.g. Thermo Fisher Scientific, P36934	NA
Microscopy glass slides	e.g. Thermo Fisher Scientific, J3800AMNZ	NA
Cover slips	e.g. Marienfeld, 0101222	NA
Scalpel		
Forceps		

<u>Preparations</u>

- Prepare a 4% PFA solution in PBS
- Prepare 3% BSA in PBS by dissolving 15 g BSA in 500 mL PBS (no need for sterile PBS); unused BSA/PBS can be aliquoted and stored at -20°C
- If necessary: re-suspend the antibodies according to the manufacturer's recommendations (for phalloidin, preferably follow the DMSO-dilution)

Procedure

Fixation of cells

- Fix cells in 0.5 mL (apical and basolateral) 3.7 % Formaldehyde for 20 min
- Wash 2x with 0.5 mL apical and 1 mL basolateral of PBS
- Preferably wash a third time with H_2O if cells are meant to be used immediately
- For later use, store fixed cells in an H_2O or PBS (0.5 mL apical, 1 mL basolateral)

Permeabilisation and of cells and blocking against unspecific binding

- If cells were kept in PBS discard the buffer and wash well (~10 min) with H_2O to dissolve potentially remaining salt (that might crystalise later on)
- Permeabilise cells by adding 0.5 mL 0.1% Triton-X100 in PBS to the apical compartment and incubate for 5 min at room temperature
- Wash 3x with 0.5 mL apical and 1 mL basolateral of PBS
- Block with 3% BSA/PBS for 20 min (Tab. 5.2)
- Wash 2x with 0.5 mL apical and 1 mL basolateral of PBS

Primary ab incubation

- Prepare ZO-1 primary antibody in 1% BSA/PBS (stock = 250 µg/mL): final concentration required = 2.5 µg/mL in 200 µL per transwell
- Add 200 µL to each well and incubate for 1h at room temperature
- Wash 2x with 0.5 mL apical and 1 mL basolateral of PBS

Secondary ab incubation and additional stainings

- Prepare staining mixture in 1% BSA/PBS in 200 µL per transwell; **KEEP PROTECTED FROM LIGHT!**
    - Secondary antibody (AlexaFluor 546): 1:300

- Phalloidin conjugated with AlexaFluor 488: if stock was prepared in PBS=1:40; if stock was prepared in DMSO=1:1500
- DAPI: 1:2000

- Add 200 µL to each transwell and incubate for 30 min at 37ºC; **KEEP PROTECTED FROM LIGHT!**
- Wash 2x with 0.5 mL apical and 1 mL basolateral of PBS

Mounting of stained filter membranes on microscopy slides

- Perform a third wash with H_2O before cutting the filters from the plastic supports using a scalpel
  - Discard the H_2O from both apical and basolateral side
  - Take out filter support and turn the insert around so that the basolateral side of the filter faces you
  - Carefully cut the filter from basolateral to apical side along the edge of the plastic support
  - Stop just before cutting the filter membrane lose
  - Take forceps to pull the filter from the support and place it, **cells facing up**, on a glass microscopy slide
- Let the filter air-dry for several minutes (observe the filter becoming **opaque** on the glass slide; if its edges start to fold up it is becoming too dry); the exact time depends on how much residual water was left on the filter when it was placed onto the glass slide
- Add a drop of mounting medium centrally on the filter and cover with a cover slip; if necessary, gently squeeze air bubbles from under the cover slip
- Keep the slides protected from light and let the mounting medium harden according to the supplier's information (e.g. at room temperature or 4°C) before analysis

3) Necrotic cell death measured by LDH release

Reagent	Supplier/Catalogue number	CAS
Lithium L-lactate	e.g. Sigma, L2250	27848-80-2
β-Nicotinamide adenine dinucleotide sodium salt (NAD)	e.g. Sigma, N0632	20111-18-6
Iodonitrotetrazolium chloride (INT)	e.g. Sigma, I8377	146-68-9
Phenazine methosulfate (PMS)	e.g. Sigma, P9625	299-11-6
DMSO	e.g. Sigma D-8779	67-68-5
Tris-HCl	e.g. Sigma T-3253	1185-53-1
Tris-base	e.g. Sigma T4661	77-86-1

Preparations

200 mM TRIS, pH 8	To prepare, add 22.2 gm of Tris-HCl and 10.6 gm of Tris-base to 1 L water.
50 mM Li L-Lactate	Add 49 mg lithium lactate into 2.5 ml of water.
NAD/PMS/INT Solution	Prepare the PMS, INT, NAD solution shortly before use. Dissolve INT in DMSO at 3.3 mg/100 µl DMSO. Phenazine Methosulfate stock: dissolve 0.9 mg in 100 µl water. Prepare NAD by adding 8.6 mg NAD to 2.3 ml water. For INT, PMS and NAD larger quantities of these solutions can be made, aliquoted, and stored at -20°C.

Procedure

Mix 100 µl PMS, 100 µl INT, and 2.3 ml NAD solution. Keep protected from light as light exposure accelerates darkening of the solution. Use as soon as possible. Samples should be run in duplicate, for each medium used a separate background sample should be included (also in duplicate). If LDH is available, three positive controls can be run or a calibration curve prepared.

The following is needed for each sample well (the required total volume can be prepared in a falcon tube and subsequently added to the well plate using a multichannel pipette):

200 mM TRIS, pH 8	50 µl
50 mM Lithium Lactate	50 µl
PMS, INT, NAD	50 µl

To the reagent mix add 50 µl sample or control. Incubate the reagent-sample mixture for 5 min at 37°C. Stop the reaction by adding 50 µL of 1 M H_2SO_4 to each well. Read the plate spectrophotometrically at 490 and 680 nm.

4) Mucus staining

Reagent	Supplier/Catalogue number	CAS
Alcian Blue (1%) (AB)	Sigma, B8438	NA
Periodic Acid	Sigma, P7875	10450-60-9
Schiff's reagent	Merck, 1.09033.0500	NA
Hydrochloric acid	e.g. Merck, 1.09057.1000	7647-01-0
Mayer's hematoxylin	Merck, 1.09249.1000	NA
Distilled water		
Acetic acid	e.g. Merck, 100063	64-19-7
Microscopy glass slides	e.g. Thermo Fisher Scientific, J3800AMNZ	NA
Mounting medium	e.g. Thermo Fisher Scientific, P36934	NA
Cover slips	e.g. Marienfeld, 0101222	NA
Scalpel		

Preparations

Periodic acid 1% (prepare fresh)

- Weigh 2 g periodic acid
- Slowly add to 200 mL MilliQ H_2O

Sulphite water (prepare fresh)

- Prepare a 10% sodium disulphite solution in MilliQ H_2O
- Add 18 mL of 10% sodium disulphite to 300 mL MilliQ H_2O and mix well
- Add 15 mL of 1N HCl

Acetic acid (3%)

- Dilute stock with MilliQ H_2O to 3% (keep at 4°C)

Procedure

- Take fixed samples out of the fridge and discard liquid
- If samples were kept in PBS, wash 1x with at least 0.5 mL of MilliQ H_2O

AB stain

- Add an adequate volume (e.g. 0.5 mL for 12-well transwell insert) of 3% acetic acid to each sample, incubate for 3 min at room temperature
- Discard acetic acid from the samples and add 0.5 mL of 1% AB in 3% acetic acid to each filter; incubate for 30 min at room temperature
- Discard AB and wash each sample 3x with at least 0.5 mL MilliQ H_2O

Periodic acid / Schiff's (PAS) reaction

- Add 0.5 mL of 1% periodic acid solution to each sample, incubate for 10 min at room temperature
- Discard the liquid and wash 3x with at least 0.5 mL MilliQ H_2O
- Add 0.5 mL of Schiff's reagent to each sample, incubate for 15 min at room temperature **protected from light**
- Discard the liquid and wash 3x2 min with at least 0.5 mL sulphite water
- Subsequently, wash 2x with at least 0.5 mL MilliQ H_2O; leave samples in a third wash of at least 0.5 mL MilliQ H_2O for 10 min at room temperature

Haematoxylin counter stain

NB: As the cell layers grow very densely, this stain might not always be helpful to distinguish the individual cells/nuclei.

- Add at least 0.5 mL of ready-to-use haematoxylin staining to each sample
- Incubate for 1-5 min (depending on sample, cell density and desired colour intensity) at room temperature
- Discard stain, wash 2x with at least 0.5 mL MilliQ H_2O
- leave samples in a third wash of at least 0.5 mL MilliQ H_2O for 5 min at room temperature

Mounting

- Perform a third wash with H_2O before cutting the filters from the plastic supports using a scalpel
    - Discard the H_2O from both apical and basolateral side
    - Take out filter support and turn the insert around so that the basolateral side of the filter faces you
    - Carefully cut the filter from basolateral to apical side along the edge of the plastic support
    - Stop just before cutting the filter membrane lose
    - Take forceps to pull the filter from the support and place it, **cells facing up**, on a glass microscopy slide
- Let the filter air-dry for several minutes (observe the filter becoming **opaque** on the glass slide; if its edges start to fold up it is becoming too dry); the exact time depends on how much residual water was left on the filter when it was placed onto the glass slide
- Add a drop of mounting medium centrally on the filter and cover with a cover slip; if necessary, gently squeeze air bubbles from under the cover slip

5) PMA differentiation efficiency of THP-1 cells

- PMA reactivity of THP-1 cells
    - Over a time course of 24h, >90% of THP-1 cells ($3x10^6$ cells/5 mL in a 25 cm² flask) should have attached firmly to the flask
    - In a majority of cells (> 50%) a morphological change should be visible, from spherical to amoeboid
- Reactivity of PMA-differentiated THP-1 cells towards stressors, e.g. LPS → cytokine release in response to LPS exposure (quantify release of pro-inflammatory cytokines, e.g. TNF-α, IL-8, IL-6 or IL-1β, by ELISA or comparable technique)
    - Release of pro-inflammatory cytokines in undifferentiated, unstimulated THP-1 cells should be absent

- After PMA differentiation, a low background release of IL-8 is acceptable
- Release of pro-inflammatory cytokines should be significantly higher compared to THP-1 cells not treated with PMA

5.8.4 Acceptance criteria for triple cultures

1) For Caco-2/E12 epithelial co-cultures

NB: as endpoints like TEER can heavily depend on the types of plastics and cell culture media used, the following criteria are strictly limited to the here recommended supplies and culture conditions

- TEER should be ≥250 Ω•cm² at day 21
  - The barrier integrity should gradually increase over 21 day growth period; the TEER increase should be stronger in the first 10 days after seeding. Around Day 12 to 16, the TEER increase should reach a plateau. It might slightly decrease again thereafter (Figure 5.5)
  - In case the TEER is <250 Ω•cm² at Day 21, the cell layer might not have developed properly, may be damaged or the filter of the transwell could not be intact
  - If the TEER is >1000 Ω•cm² using the here recommended materials and procedures, it should be considered to confirm the cell line's genetic integrity

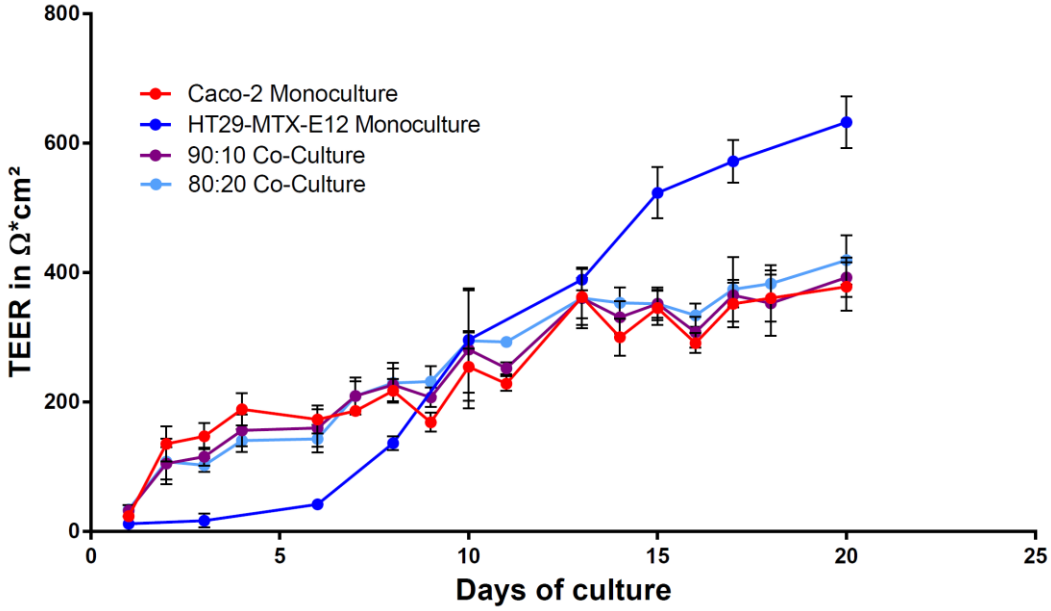

Fig. 5.5 Barrier development measured as TEER over a 20 day growth period and basolateral adaption to RPMI-based culture medium (Average N=3±SD)

- The barrier integrity, determined for instance using Lucifer Yellow or dextran blue, should be stable for at least 5 days

- Acidic mucus should be detectable on the epithelial layers; preferably it should be evenly distributed, however, a patchy occurrence associated with 'islands' of E12 cells is more likely (Figure 5.6)

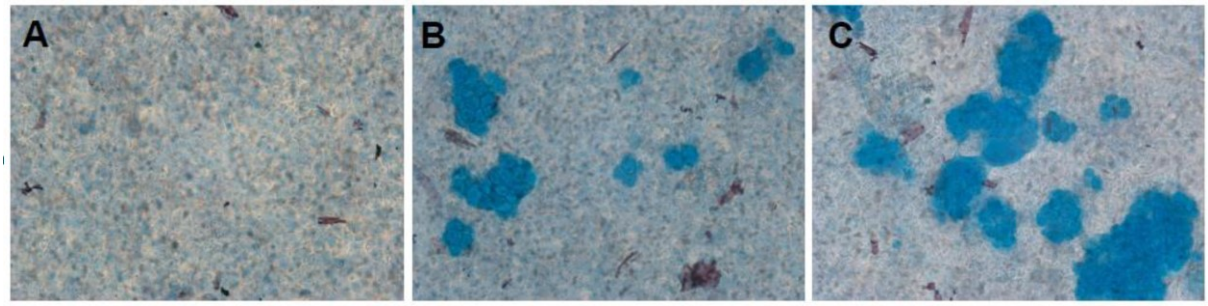

Fig. 5.6 Alcian Blue staining acidic mucus on 21-day-old transwell cultures: (A) Caco-2 monoculture, (B) Caco-2/E12 co-culture in 9:1 ratio, (C) Caco-2/E12 co-culture in 8:2 ratio

- The TEER of the triple cultures should not significantly vary from the TEER of epithelial co-cultures

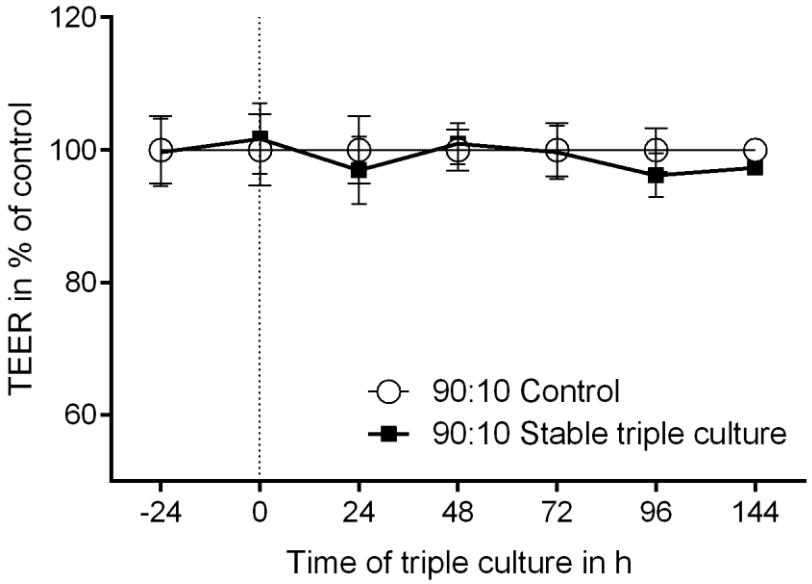

Fig. 5.7 Barrier integrity measured as TEER of stable triple cultures compared to epithelial co-culture controls (-24-72h: Average N≥3±SD, 96h: Average N=2 N≥3±SD, 144h: N=1; start of the triple culture is indicated by dotted line)

- The apical cytokine values should not significantly exceed those of epithelial co-culture control (e.g. IL-8); the basolateral cytokine values should not exceed the sum detected in epithelial co-culture controls and THP-1 monocultures (e.g. TNF-α, IL-8, IL-6 or IL-1β)
    - Especially regarding IL-8 as PMA-differentiated THP-1 cells are known to release significant amounts of IL-8 without additional activation
- The apical LDH release should not significantly exceed the LDH detected in epithelial co-culture controls

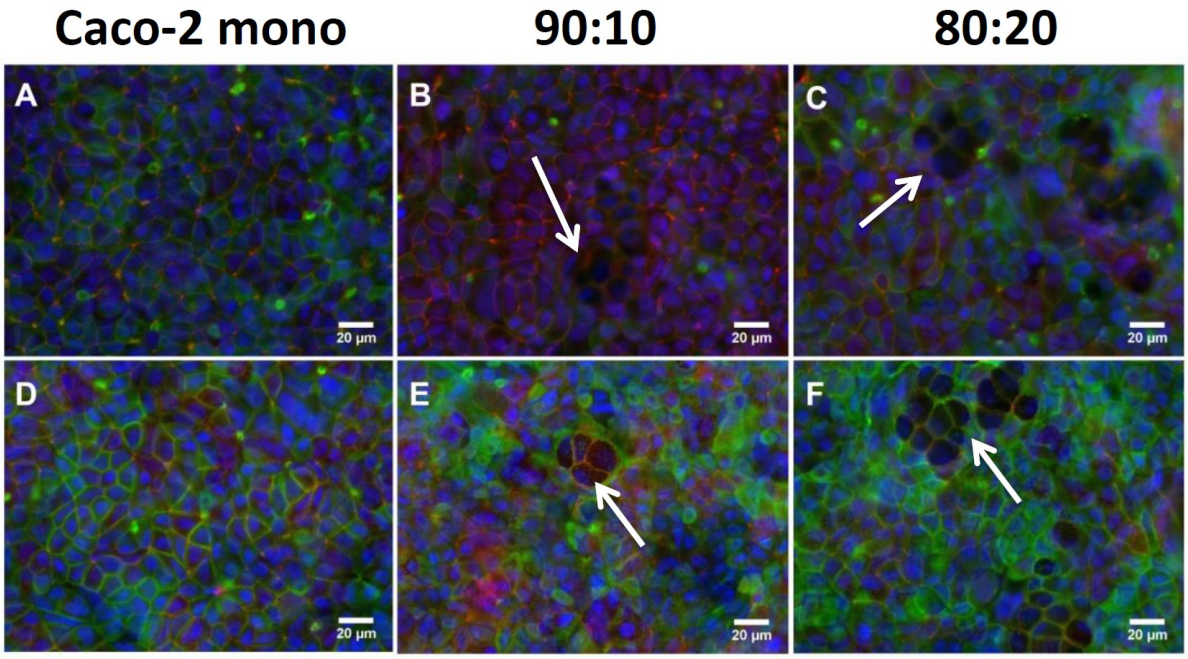

Fig. 5.8 ZO-1 (red), F-actin (green) and nuclei (blue) in (A,D) Caco-2 monoculture, (B,E) 90:10 and (C;F) 80:20 Caco-2/E12 co-cultures in absence (A,B,C) of differentiated THP-1 or after 48h triple culture (D,E,F) with differentiated THP-1 cells

Both the actin and tight junction network should be well developed and continuous. The barrier should be composed as tight monolayer. Imaging the cell layer in one focal area might be difficult as the shape of the two epithelial cell types appears to be clearly different with the E12 cells adopting a higher columnar phenotype (white arrows) compared to the Caco-2 cells.

2) Quality control for differentiated THP-1 cells

- more than 90% of the cells should be firmly attached to the flask in response to PMA treatment (Figure 5.1 A)
- if large numbers of cells remain unattached and/or spherically shaped, the flask should not be used (Figure 5.1 B)

6 Data Analysis and Reporting of Data:

6.1 TEER

- Express the TEER values as % of the negative control (i.e. the average of the epithelial co-culture triplicates)
- In case of exposure experiments, e.g. using ENM, expressing the TEER results as % of the untreated triple culture control is recommended

6.2 Cytokine release

- The standard curve should be plotted as 4-parameter logfit unless the supplier/manufacturer of supplies states otherwise
- The cytokine release can be expressed as
    - total values (usually pg/mL or ng/mL)
    - fold increase compared to the negative control
- basolateral results of stable co-cultures should initially be compared to the cytokine levels of PMA-differentiated THP-1 monocultures

6.3 LDH release

- The LDH release can be expressed as
    - absorbance value
    - total value (usually pg/mL) if a standard curve is prepared
    - fold increase compared to the negative control

7 Publications:

The basic principles of this method are described in

KÄMPFER, A. A. M., URBAN, P., GIORIA, S., KANASE, N., STONE, V. & KINSNER-OVASKAINEN, A. 2017. Development of an in vitro co-culture model to mimic the human intestine in healthy and diseased state. *Toxicol In Vitro,* 45**,** 31-43.

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Studies: Effects of Silver and Titanium Dioxide Nanomaterials in Intestinal Models.*small,* DOI 10.1002/smll.202004223.

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