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)

Culture and characterisation of mono and multi-cellular models of the gastrointestinal system

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).

Authored by:

Ali Kermanizadeh[1,2], Victor Ude[1], Helinor Johnston[1] and Vicki Stone[1]

1.Heriot Watt University, Nano Safety Research Group, School of Engineering and Physical Sciences, Edinburgh, UK

2. University of Derby, School of Human Sciences, Human Sciences Research Centre, Derby, UK

Reviewed by:

PATROLS work package 4 partners

Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	05/06/2018	Initial Document	Ali Kermanziadeh
2.0	20/07/2018	Amended Protocol	Ali Kermanizadeh
3.0	17/11/2018	Amended Protocol	Ali Kermanizadeh
4.0	15/4/2021	Amended Protocol	Ali Kermanizadeh

Table of Contents

1 Introduction:

Intestinal epithelial cell culture models, such as Caco-2 cells, are commonly used to assess absorption of drugs and transcytosis of nanomaterials (NMs) across the intestinal mucosa. However, it is known that mucus strongly impacts NM mobility and that specialized M cells are involved in particulate uptake. Thus, to get a clear understanding of how NMs interact with the intestinal mucosa, *in vitro* models are necessary that integrate the main cell types. This protocol highlifghts the step necessary for the development of a a triple culture: Caco-2 cells, mucus-secreting goblet cells and Microfold (M) cells.

1.1 Limits of the protocol:

- The set up of the test model/s is labour intensive and very time-consuming
- The quantification of M cells in the triple culture samples is currently very difficult
- The model currently uses static media without flow, and does not include membrane flexing to simulate peristalsis

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)?	Maybe

Has the method been submitted for standardisation (to OECD, CEN, ISO,…) in its own right or as part of another standardisation project?	N
Is the method included in an existing standard (or ongoing standardisation work)	N

2 Abbreviations:

Caco-2 - Human epethlial colorectal adenocarcinoma cell line

DMEM - Dulbecco's Modified Eagle's Medium

HBSS - Hank's balanced salt solution

HT29-MTX-E12 - Mature goblet cell line

M cells - Microfold cells

NMs - Nanomaterials

PBS - phosphate buffered saline

Raji cells - Lymphoblast-like cell line

RPMI - Roswell Park Memorial Institute medium

SEM - Scanning electron microscopy

TEER - Transepithelial/transendothelial electrical resistance

3 Description of the method:

3.1 Biological setting & test system used:

This SOP should be carried out under laboratory based conditions, with all work performed under sterile conditions and in a Class 2 Laminar Tissue Culture Hood.

Caco-2 - ATCC

HT29-MTX-E12 - ECACC (can be purchased from Sigma)

Raji cells - ATCC

3.2 Chemicals and reagents used:

Dulbecco's Modified Eagle's Medium (DMEM) - Gibco - 41965-039
RPMI medium - Gibco - 21875-034
Heat inactivated Fetal bovine serum (FBS) - Gibco - 10500-064
Non-essential amino acids (AA) - Sigma - M7145
Penicillin/Streptomycin - Gibco – 15070-063
Glutaraldehyde - Sigma - G6257
Sodium cacodylate - Sigma - C4945
Hexamethyldisilazane - Sigma - 379212
100% ethanol - Sigma - 51976
40% formaldehyde - Sigma - 128775
Acetic acid - Sigma - A9283
SIGMAFAST Fast Red TR/Naphthol AS-MX tablets - Sigma - N1891- S5ET
Hank's balanced salt solution (HBSS) - Sigma - H6648
FITC labelled Wheat germ agglutinin - Sigma - L4895
Hoechst - Life technologies - H1398
Trypsin-EDTA - Sigma – 14174

3.3 Apparatus and equipment used:

- 75cm^2 cell culture flask (e.g. Corning- 430641U)
- 12-well Transwell filters - Corning Incorporated - 3 µm pore size, 1.12 cm^2 surface area
- 12 well cell culture plates

- Micropipettes
- Automatic cell counter or haemocytometer
- -80°C freezer
- Liquid nitrogen
- Water bath
- Humidified Incubator (37°C and 5% CO_2)

- Epithelial voltohmmeter - i.e. EVOM2 (World precision instrument, USA)
- Inverted light microscope - i.e. Axiovert 40 C light microscope (ZEISS, Germany)

3.4 Reporting of protected elements:

This SOP does not have any associated patent restrictions, specific licenses, material transfer agreements

3.5 Health and safety precautions:

Standard health and safety precautions associated with working within a laboratory environment and performing mammalian cell culture, as described by the European Agency for Safety and Health at Work (https://osha.europa.eu/en/safety-and-health-legislation/european-guidelines), should be adopted when conducting this SOP. In addition, all health and safety precautions outlined in the MSDS data sheets associated with the specific chemicals required must also be followed.

3.6 Nanomaterials used/handling procedures:

NA

3.7 Procedure:

3.7.1 Cell culture medium:

Caco-2 and HT29-MTX: DMEM + 10% FBS + 100 IU/ml non-essential AA + 100 U/ml Penicillin/Streptomycin (termed complete DMEM)

Raji: RPMI + 10% FBS + 100 U/ml Penicillin/Streptomycin

3.7.2 Routine cell culture:

Caco-2 cells and HT29-MTX cells are sub-cultured once a week with trypsin-EDTA (0.25%, 0.53 mM) and seeded at a density of 4×10^5 per 75 cm^2 flask. The Raji B cells are sub-cultured once a week into fresh medium, restoring the cell concentration to 1×10^6. For the Caco-2 cells and HT29-MTX cells the old medium is changed every 2-3 days and replaced with pre-warmed (37°C) appropaite cell culture complete as described above.

3.7.3 Cell count:

Ideally cell counts are to be performed using an automated cell counter. Alternatively a manual count can be performed using a haemocytometer following the NanoValid protocol (Appendix A)

3.7.4 Caco-2 monoculture:

1. Seed 2 to 4×10^5 Caco-2 cells/well in 500 µl of complete DMEM (passage number 5-20) into the apical compartment of polycarbonate 12-well Transwell filters.
2. Add 1.5 ml of complete DMEM to the basal compartment.
3. Caco-2 cells are then maintained under standard incubation conditions (37°C, 5% CO_2, 95% humidity) for 21 days with medium in both the apical (500 µl) and basal compartments (1.5 ml) changed after 24 hr and then every 48 hr thereafter.
4. TEER measurements are carried out from day 13 onwards, every other day (please see below).

3.7.5 Caco-2 and HT29-MTX co-culture:

1. Mix Caco-2 and HT29-MTX cells in complete DMEM at a ratio of 9:1 and a total cell density of $8x10^5$ cells/ml.

2. Seed Caco-2 and HT29-MTX cells into the apical compartment of the Transwell filter inserts (a total of 4×10^5 cells/well in 500 µl of complete DMEM).
3. Add 1.5 ml of complete DMEM to the basal compartment.
4. The cells are then maintained under standard incubation conditions (37°C, 5% CO_2, 95% humidity) with medium on both the apical (500 µl) and basal sides (1.5 ml), changed after 24 hr and then every 48 hr thereafter to 21 days
5. TEER measurements are carried out from day 13 onwards every other day (please see below).

3.7.6 Triple culture Caco-2 and HT29-MTX and Raji cells:

1 Caco-2 and HT29-MTX cells are mixed in complete DMEM at a ratio of 9:1 and a total cell density of $8x10^5$ cells/ml.
2 Caco-2 and HT29-MTX cells are seeded on the upper side of the Transwell filter inserts (4×10^5 cells/well).
3 The cells are maintained under standard incubation conditions with medium on both the apical (500 µl) and basolateral sides (1.5 ml) changed after 24 hr and then every 48 hr thereafter.
4 At day 16, add a total of 5×10^5 (cells/well) Raji B cells, re-suspended in 1.5 ml of complete DMEM to the basolateral compartment of inserts to trigger M cell differentiation.
5 The cells are maintained for 5 days under standard incubation condition (37°C, 5% CO_2, 95% humidity) and medium changed at the apical compartment, every day and 500 µl medium also changed at basolateral compartment at day 19.
6 TEER measurements are carried out from day 13 onwards every other day (please see below).

NOTE: Antunes *et al.* 2013 suggests that normal oriented Caco-2/HT29/Raji B model may be a more physiologically relevant and reproducible *in vitro* model of the intestinal barrier to study absorption

3.7.7 Characterisation of the mono and multi-cellular models:

3.7.7.1: TEER measurements:

Trans-epithelial electrical resistance (TEER) is used to confirm cell differentiation and is conducted using an epithelial voltohmmeter. The voltohmmeter is set to resistance and placed vertically on the insert making sure that the short electrode is placed in the apical compartment while the long electrode is placed in the basolateral compartment with the cells. Maintain a constant temperature at 37°C.

The resistivity is calculated using the equation below:

$$Resistivity\ (\Omega.cm^2) = ohm2 - ohm1 \times A$$

Ohm1 = Resistance of the insert with cell culture medium only.
Ohm 2 = Resistance of the insert with cell.
A = Surface area of the insert in cm^2

Only wells with a TEER value of over 500 $\Omega.cm^2$ are utilised for subsequent experiments.

NOTE: TEER measurements are made in complete cell culture medium
NOTE 2: TEER measurements are made just prior to medium change
NOTE 3: The voltohmmeter is to sterlised with 70% ethanol and throughly washed with PBS prior to measurements

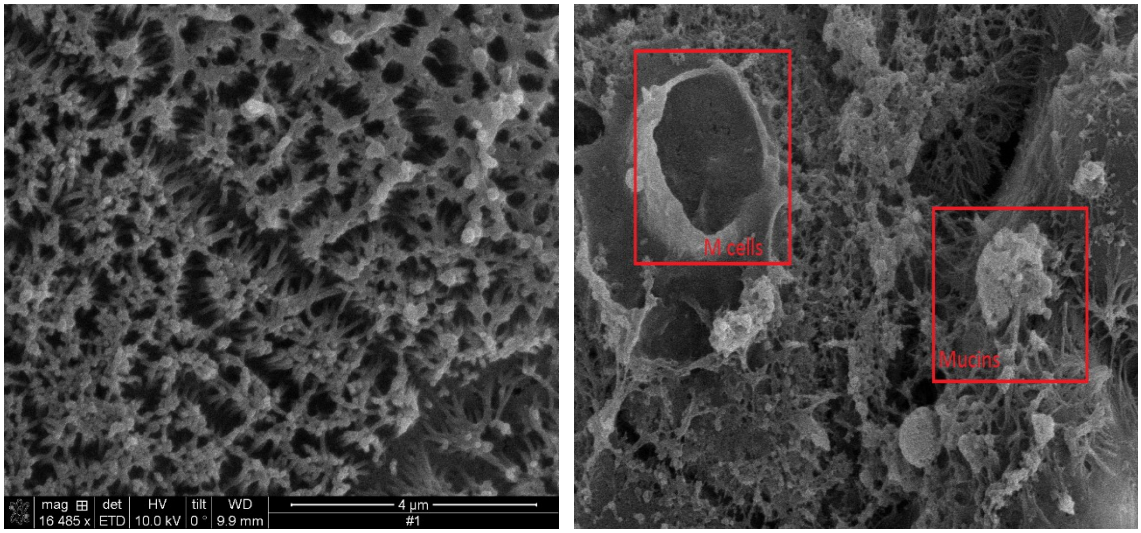
3.7.7.2 Lucifer yellow:

Lucifer Yellow to be used as additional measurement of barrier integrity - NANoREG protocol (Appendix B)

3.7.7.3 Scanning electron microscopy (SEM):

1. The different cell models are cultured for 21 days as described above.

2. The inserts containing the cells are washed with PBS twice before being fixed with 5 % glutaraldehyde (in 0.1 M sodium cacodylate) for 2 hr at 4°C.
3. The inserts are washed three times with 0.1 M sodium cacodylate in PBS.
4. The inserts are then dehydrated in graded ethanol (25, 50, 70, 80 and 90 %) for 10 min each at room temperature.
5. The inserts are further dehydrated in 100% ethanol 3x for 15 min each before being submerged in 2:1 of hexamethyldisilazane (Sigma):100% ethanol.
6. Inserts are placed in a 100% hexamethyldisilazane solution for 2 min.
7. The membranes are then removed from the inserts using a sharp scalpel and mounted on SEM specimen stubs (Aluminium, 12.5 mm diameter, 3.2 x 6 mm pin Agar Scientific UK).
8. The stubs are coated with gold (optional - the sputter coating reduces charging and thermal damage – this step is not essential but recommended) and examined using any conventional Scanning electron microscope.

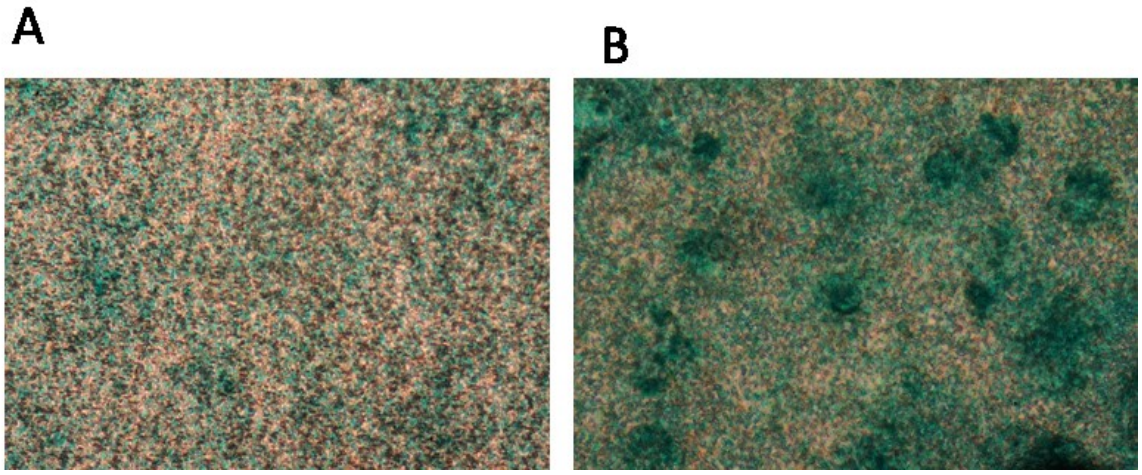


Typical SEM image of a) differentiated Caco-2 cells and b) and GIT triple cell culture model cultured for 21 days.

3.7.7.4 Alcian blue staining (used to stain mucus):

1. The cells are cultured for 21 days as described above.
2. The inserts are washed x3 with PBS.
3. The cells are fixed with 4% formaldehyde for 30 min at room temperature.

4. The inserts are placed in a 3% acetic acid (distilled water) solution for 3 mins.
5. The inserts are stained with alcian blue (10 mg/ml in 3% acetic acid) for 30 min at room temperature.
6. Rinse x2 with distilled water.
7. Optional step: Counterstain with SIGMAFAST Fast Red TR/Naphthol AS-MX tablets (each tablet dissolved in 1 ml deionized water yields a ready-to-use buffered solution) for 5 min and washed with distilled water (x3).
8. The membranes are removed from the inserts and mounted on microscopic slides before being visualised using an inverted light microscope (storage of slides is not recommended).

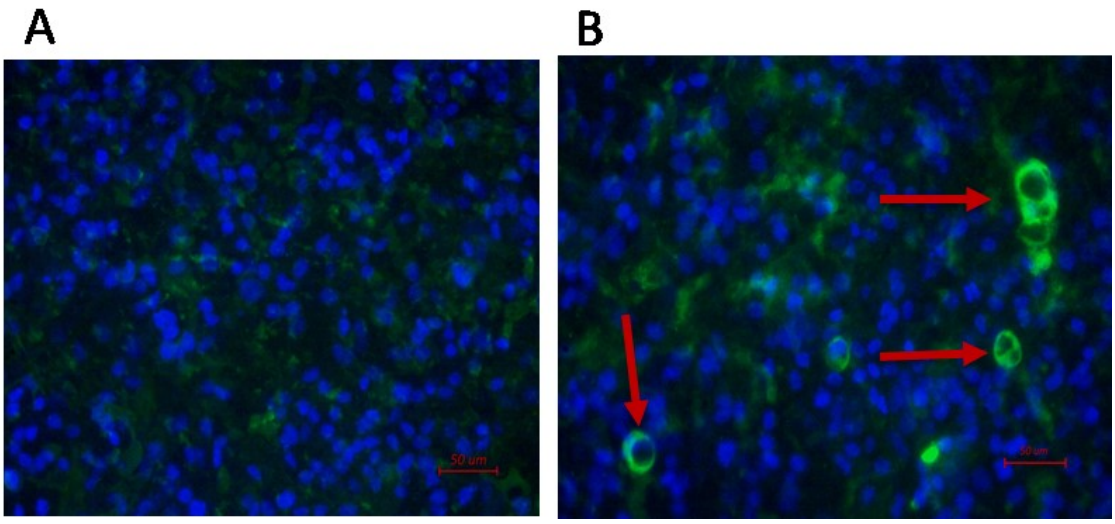


Typical Alcian blue staining image of a) differentiated Caco-2 cells and b) and GIT triple cell culture model cultured for 21 days.

3.7.7.5 Wheat germ agglutinin (WGA) conjugates staining (high affinity to sialic acid and N-acetylglucosamine on M cells):

1. The inserts containing the cells are washed x2 with warm (37°C) HBSS.
2. The cells on the inserts are fixed with 4% formaldehyde for 30 min at room temperature.
3. The inserts are washed x3 with HBSS.
4. Inserts are co-stained with 500 µl of WGA FITC (5 µg/ml) and Hoechst (1 µg/ml) (both diluted in HBSS) for 10 min at 37°C in the dark.
5. Wash the inserts x2 with HBSS.

6. Cut out membranes from the inserts and place on slides suitable for microscopy and cover with coverslips.
7. The slides are ready for fluorescent microscopic analysis (storage of slides is not recommended).



Typical Wheat Germ Agglutinin image of A) differentiated Caco-2 cells and B) and GIT triple cell culture model cultured for 21 days.

4 Suggested frequency of the use of characterisation end-points:

Routine and regular TEER measurements (every experiment)

WGA, Alcian blue, Lucifer Yellow and SEM only to be used for initial characterisation of the single/multi-cellular model

5 Data Analysis and Reporting of Data:

NOTE: Example data will be added as part of Task 4.2.

6 Publications:

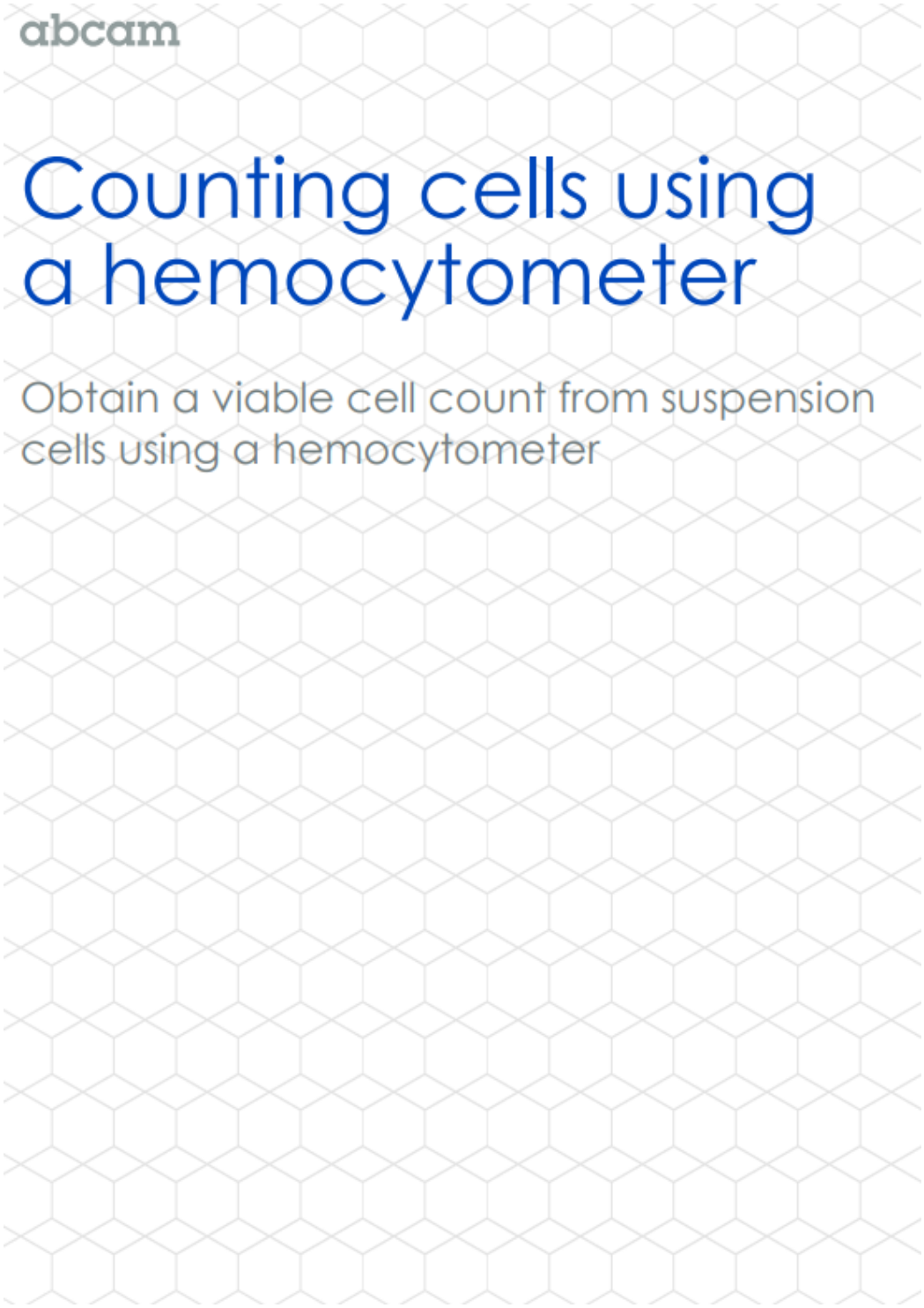
NA

7 References:

Antunes F, Andrade F, Arauio F, Ferreira D, Sarmento B. 2013. Establishment of a triple co-culture in vitro cell models to study intestinal absorption of peptide drugs. European Journal of Pharmacology and Biopharmacology 83: 427-435.

8 Appendix:

Appendix A – Abcam cell count SOP



Contents

Preparing the hemocytometer

1. If using a glass hemocytometer and coverslip, clean with alcohol before use. Moisten the coverslip with water and affix to the hemocytometer. The presence of Newton's refraction rings under the coverslip indicates proper adhesion.

2. If using a disposable hemocytometer (eg INCYTO DHC-N01), simply remove from the packet before use.

Preparing cell suspension

1. Gently swirl the flask to ensure the cells are evenly distributed.

2. Before the cells have a chance to settle, take out 0.5 mL of cell suspension using a 5 mL sterile pipette and place in an Eppendorf tube.

3. Take 100 µL of cells into a new Eppendorf tube and add 400 µL 0.4% Trypan Blue (final concentration 0.32%). Mix gently.

Counting

1. Using a pipette, take 100 µL of trypan blue-treated cell suspension and apply to the hemocytometer. If using a glass hemocytometer, very gently fill both chambers underneath the coverslip, allowing the cell suspension to be drawn out by capillary action. If using a disposable hemocytometer, pipette the cell suspension into the well of the counting chamber, allowing capillary action to draw it inside.

2. Using a microscope, focus on the grid lines of the hemocytometer with a 10X objective.

3. Using a hand tally counter, count the live, unstained cells (live cells do not take up trypan blue) in one set of 16 squares (Figure 1). When counting, employ a system whereby cells are only counted when they are within a square or on the right-hand or bottom boundary line. Following the same guidelines, dead cells stained with trypan blue can be also be counted for a viability estimate if required.

4. Move the hemocytometer to the next set of 16 corner squares and carry on counting until all four sets of 16 corner squares are counted.

Viability

To calculate number of viable cells per mL:

- Take the average cell count from each of the sets of 16 corner squares.
- Multiply by 10,000 (10^4).
- Multiply by five to correct for the 1:5 dilution from the trypan blue addition.

The final value is the number of viable cells/mL in the original cell suspension.

Example

If the cell counts for each of the 16 squares were 50, 40, 45, 52, the average cell count would be

- (50 + 40 + 45 + 52) ÷ 4 = 46.75
- 46.75 x 10,000 (10^4) = 467,500
- 467,500 x 5 = 2,337,500 live cells per mL in original cell suspension

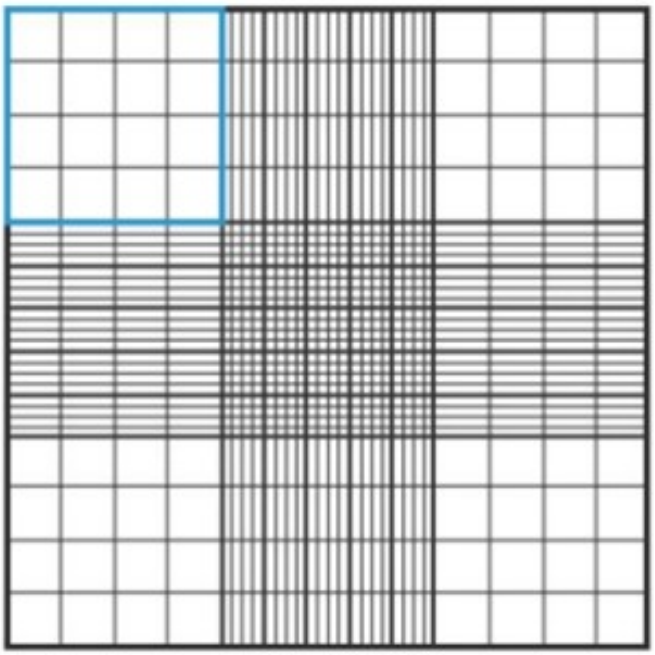


Figure 1. Hemocytometer gridlines diagram indicating one of the sets of 16 squares that should be used for counting.

Appendix B - NANoREG Lucifer Yellow SOP

7. ANALYSIS OF NPS IMPACT ON THE EPITHELIUM INTEGRITY FOLLOWING LY PASSAGE

At the end of passage experiments, the impact of NPs on epithelium integrity is evaluated by lucifer yellow (LY, Sigma # L0259) assay to determine any difference in this paracellular marker ability to cross the monolayer between NPs-treated inserts untreated inserts.

- Prepare freshly a LY stock solution at 0.4 mg/ml in pre-warmed (37°C) HBSS by resuspending 4 mg of LY in 10 ml of HBSS.

Rem: A 5 days old LY stock solution can also be used without problem if stored at 4°C in the dark.

- After collecting Ap and Bl media, carefully wash 3 times both Ap and Bl sides of inserts with pre-warmed (37°C) HBSS to remove any residual culture medium and cellular fragments. Put the inserts plate on a heating plate (37°C) during all the washing step.
- Add pre-warmed (37°C) HBSS firstly into Bl compartment (1.5 ml/well) and then in Ap compartment (0.5 ml/insert), to avoid percolation of HBSS from Ap side to Bl side especially for cell free inserts.
- Throw away the washing HBSS starting with the Ap HBSS and following with the Bl HBSS. Do not remove the Ap and Bl HBSS from all inserts at once to avoid their drying. Proceed by collecting the HBSS of the triplicate inserts of the same condition at once before continuing with the others inserts.
- After washing steps, inserts are then transferred into a new multi-well plates containing 1.5 ml of pre-warmed (37°C) HBSS and gently filled in Ap side with 0.5 mL of 0.4 mg/mL LY in HBSS.
- Inserts are incubated for 2h at 37°C in 5% CO_2 incubator.
- Prepare a LY standard curve (see Table 1 below) by serial dilutions and add 100 µl/well of each standard in duplicate in the **black** 96 well plate (follow the template in the excel file "LY permeability template"). Dilute 200 x stock solution LY at 400 µg/ml (C_0 in the P_{app} equation; see below) and then add 100 µl/well in duplicate. HBSS is used as blank; 0 µg/ml.

Table 1: Serial dilutions to prepare LY standards.

Standards	Final concentration (µg/ml)	LY standard solution (µl)	HBSS (µl)	dilution
Std. 1	4	20 µl of 400 µg/ml solution	1980	100
Std. 2	2	200 µl of std. 1	200	2
Std. 3	1	150 µl of std. 2	150	2
Std. 4	0.4	50 µl of std. 1	450	10
Std. 5	0.2	200 µl of std. 4	200	2
Std. 6	0.1	150 µl of std. 5	150	2
Std. 7	0.04	50 µl of std. 4	450	10
Std. 8	0.02	150 µl of std. 7	150	2
Blank	0	0	100 µl/well	0

- Collect 2 x 100 µl of the Bl HBSS of each insert (including cell free inserts) by pipetting 3 times, add these volumes into a **black** 96 well plate (following the layout in the excel file "LY permeability template").
- Collect 2 x 100 µl of the Bl HBSS of each cell free insert by pipetting 3 times.

- Proceed to a 50x dilution of the Bl HBSS of these cell free inserts with HBSS before adding 100 µl of diluted solution into a **black** 96 well plate (following the layout in the excel file "LY permeability template").
- Measure by fluorimetric detection (ex. 428 nm, em. 536 nm) the LY content. Use excitation and emission wavelengths as close as possible to the recommended wavelengths (for instance ex. 485 nm, em. 538 nm).
- Percentage of LY passage in Bl side after treatment is compared with percentage of LY passage in the negative control.

Calculate the crossing of LY by two methods:

1) By calculating the % of LY passage (100% x (C_{Bl} x Bl volume)/(C_0 x Ap volume) and compare with untreated control. C_{Bl} is the concentration of LY in basolateral side and C_0 is the initial concentration in the donor (apical) compartment. Bl volume = 1.5 ml and Ap volume = 0.5 ml

2) Calculate the Papp value of LY passage through the monolayer exposed to test compound (Papptc) and compare it with Papp of LY in control cells (Pappc). If Papptc ≤ Pappc the concentration of the test compound do not alter the epithelium integrity. Use the following equation:

$$Papp = ((\Delta Q/\Delta t) \times V) \times (1/AC_0)$$

where $\Delta Q/\Delta t$ is the amount of LY transported in the basolateral compartment per time unit (t), V is the basolateral volume (cm^3), A is the surface area of the filter (1.12 cm^2) and C_0 is the initial concentration in the donor (apical) compartment.

- Use the excel LY permeability template to calculate the LY Papp and % of LY passage.

8. REFERENCES

- ECVAM DB_ALM n.142

Appendix C

Reduction of ratio of Caco-2 and HT29-MTX-E12 in M cell model

In a meeting between Heriot-Watt University and IUF partners, it was agreed that the ratio of Caco-2 cell and HT29-MTX-E12 in the M cell model will be changed from 8:2 to 9:1. In the intestine, the percentage of goblet cells gradually increases from duodenum to colon with ~4 to 16%, respectively (Kim and Ho, 2010). As microfold cell (M cell) are primarily located in the small intestine, the model should be oriented towards a lower ratio of goblet cells. This is because increase in HT29-MTX-E12 do not only increase the level of mucus secretion, but also impacts on the integrity of the cell monolayer by reducing the transepithelial electrical resistance (TEER), interact with nanomaterials thereby affecting absorption and translocation properties of the intestinal M cell model (Pan et al., 2015; Calatayud et al., 2012; Hilgendorf et al., 2000). Chen et al. (2010) and Pan et al. (2015) concluded that a seeding ratio of 9:1 resulted in the most precise and reproducible outcomes regarding TEER and drug permeability. Reducing the HT29-MTX-E12 to 10% will enable our model to better align with the published literature on both Caco-2/ HT29-MTX co-culture (Chen et al., 2010; García-Rodríguez et al., 2018; Ude et al., 2019) and Caco-2/HT29-MTX/Raji B triple co- culture (Antunes et al., 2013 Lozoya-Agullo et al., 2017) models. In addition, the histological comparison of 9:1 co-cultures to murine small intestinal tissue has shown good correlation (Figure 2), which supports the reduction of the seeding ratio.

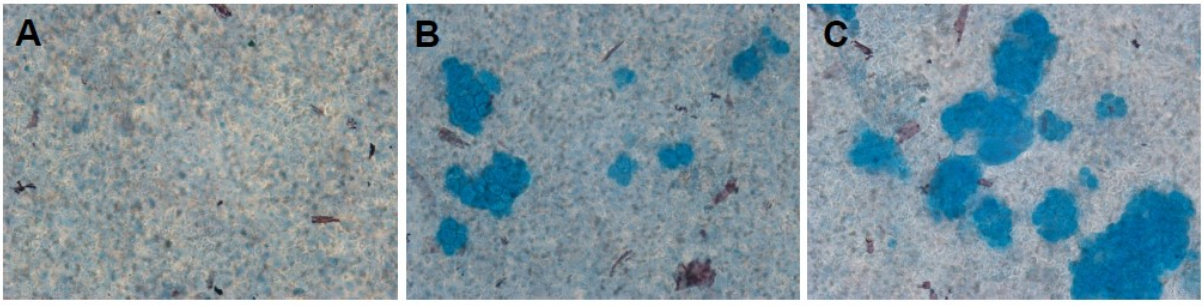


Figure 1. Alcian Blue staining for acidic mucus in (A) Caco-2 monocultures, and Caco-2/HT29-MTX-E12 co-cultures at (B) 9:1 and (C) 8:2 seeding ratio

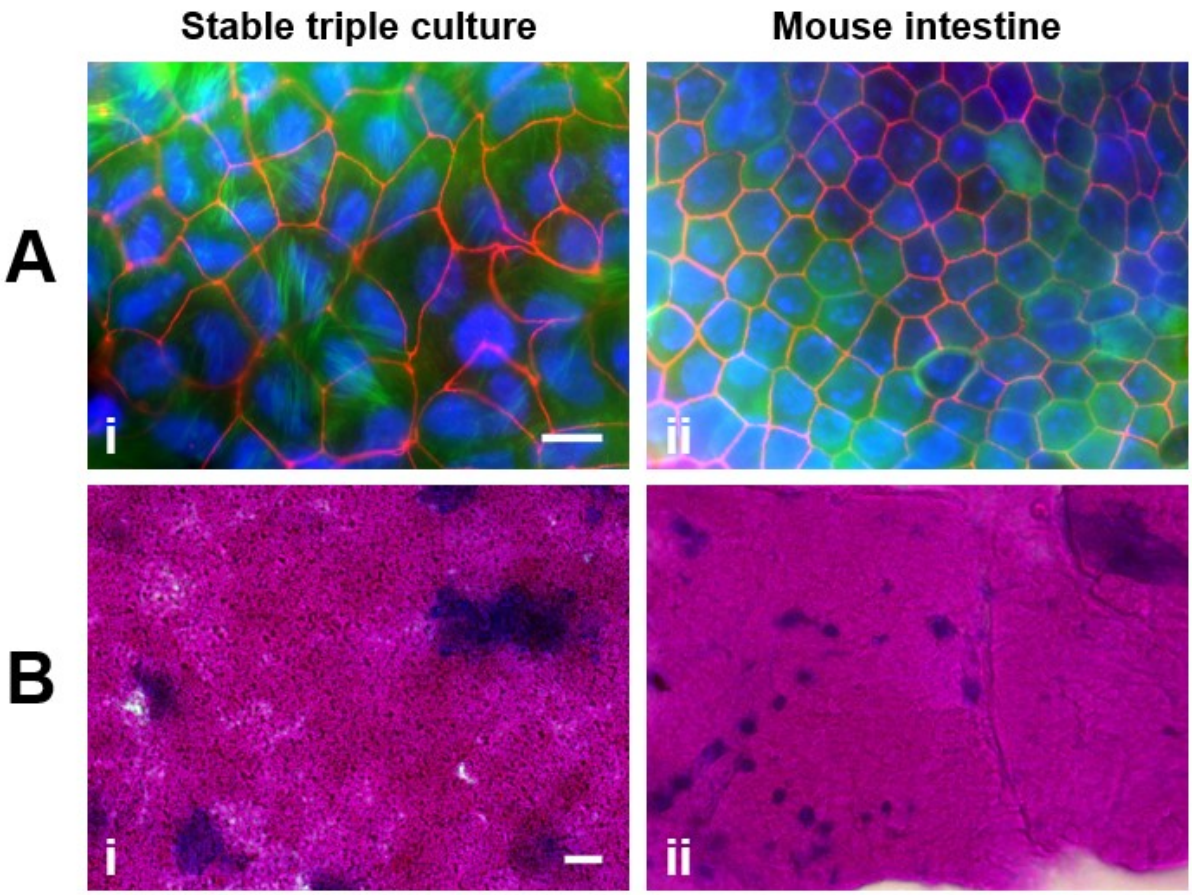


Figure 2. Comparison of untreated in vitro stable triple culture epithelial layers and control intestinal tissue. (A) Morphological comparison between epithelial transwell cell layer after 48h stable triple culture (i) and small intestinal epithelium of a healthy C57BL6/J mouse (ii) (blue: nuclei, red: ZO-1, green: F-actin; scale bar = 10 µm, 1,000x magnification). (B) Mucin staining of the epithelial layer of stable triple cultures after 48h (i) and murine small intestinal epithelium (ii) (Magenta: neutral mucus (PAS reaction), dark blue: acidic mucus (Alcian Blue); scale bar = 50 µm; 400x magnification).

References

Antunes, F., Andrade, F., Araújo, F., Ferreira, D. and Sarmento, B., 2013. Establishment of a triple co-culture in vitro cell models to study intestinal absorption of peptide drugs. European Journal of Pharmaceutics and Biopharmaceutics, 83(3), pp.427-435.

Calatayud, M., Vázquez, M., Devesa, V. and Vélez, D., 2012. In vitro study of intestinal transport of inorganic and methylated arsenic species by Caco-2/HT29-MTX cocultures. Chemical research in toxicology, 25(12), pp.2654-2662.

Chen, X.M., Elisia, I. and Kitts, D.D., 2010. Defining conditions for the co-culture of Caco-2 and HT29-MTX cells using Taguchi design. Journal of pharmacological and toxicological methods, 61(3), pp.334-342.

García-Rodríguez, A., Vila, L., Cortés, C., Hernández, A. and Marcos, R., 2018. Effects of differently shaped TiO 2 NPs (nanospheres, nanorods and nanowires) on the in vitro model (Caco-2/HT29) of the intestinal barrier. Particle and fibre toxicology, 15(1), pp.1-16.

Hilgendorf, C., Spahn-Langguth, H., Regårdh, C.G., Lipka, E., Amidon, G.L. and Langguth, P., 2000. Caco-2 versus caco-2/HT29-MTX co-cultured cell lines: permeabilities via diffusion, inside-and outside-directed carrier-mediated transport. Journal of pharmaceutical sciences, 89(1), pp.63-75.

Kim, Y.S. and Ho, S.B., 2010. Intestinal Goblet Cells and Mucins in Health and Disease: Recent Insights and Progress. *Current Gastroenterology Reports*, 12(5), pp. 319-330.

Lozoya-Agullo, I., Araujo, F., González-Álvarez, I., Merino-Sanjuán, M., Gonzalez-Alvarez, M., Bermejo, M. and Sarmento, B., 2017. Usefulness of Caco-2/HT29-MTX and Caco-2/HT29-MTX/Raji B coculture models to predict intestinal and colonic permeability compared to Caco-2 monoculture. *Molecular pharmaceutics*, *14*(4), pp.1264-1270.

Pan, F., Han, L., Zhang, Y., Yu, Y. and Liu, J., 2015. Optimization of Caco-2 and HT29 co-culture in vitro cell models for permeability studies. International journal of food sciences and nutrition, 66(6), pp.680-685.

Ude, V.C., Brown, D.M., Stone, V. and Johnston, H.J., 2019. Using 3D gastrointestinal tract in vitro models with microfold cells and mucus secreting ability to assess the hazard of copper oxide nanomaterials. Journal of nanobiotechnology, 17(1), p.70.