Title

Procedures for the reconstruction, primary culture and experimental use of rainbow trout gill epithelia

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Abstract/Summary

This procedure documents a standardised method to reconstruct and culture the freshwater rainbow trout gill epithelium on flat permeable membrane supports within cell culture inserts. The protocol describes gill cell isolation, cultured gill epithelium formation, maintenance, monitoring and preparation for use in experimental procedures. To produce a heterogeneous gill epithelium, as seen *in vivo*, seeding of isolated gill cells twice over a two day period is required. This is termed the double seeded insert (DSI) technique. Approximately 8-12 days after cell isolation and seeding, preparations develop electrically tight gill epithelia that can withstand fresh water on the apical cell surface. The system can be used to study freshwater gill physiology, as well as a humane alternative for toxicity testing, bioaccumulation studies and environmental water quality monitoring.

Keywords: Alternative test methods, fish, gill epithelium, toxicity, *in vitro*

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INTRODUCTION

The freshwater fish gill is a multifunctional organ and is the site of gas exchange, osmoregulation, trace metal transport, nitrogenous waste excretion and xenobiotic uptake[1-4]. Fish are commonly used as indicator species to identify environmental hazards and, as their gills are constantly in contact with water, the gill epithelium is a focal point for countless studies that seek to understand deleterious effects of environmental toxicants. Whole animal studies can use millions of fish worldwide each year, and efforts are focusing on refining, reducing and replacing (3Rs) these numbers[5]. Alternative animal-free methods used in fish toxicology include the use of immortalised cell lines, as well as primary cultures of cells from fish organs. In the present article, we describe a protocol for the primary culture of a freshwater rainbow trout gill epithelium on a flat permeable membrane, via a repeated gill cell seeding protocol. This double seeded insert (DSI) technique produces a heterogeneous epithelium similar to that found in vivo. A new double seeded inverted insert (DSII) technique is also described. The DSII system can be used for solvent-free dosing, similar to that described by Kramer et al[6] for immortalised cell lines; the reduction in volume of the receiving compartment increases the chemical analytical power necessary for assessing xenobiotic biotransformation. The key features of both DSI and DSII are that they produce electrically tight gill epithelia containing the different cell types present in the *in vivo* gill, and that the preparations can tolerate fresh water, thereby physiologically resembling the intact freshwater fish gill.

This primary culture of a rainbow trout gill epithelium was pioneered by Pärt et al.[7] and later developed by Wood & Pärt[8], to the single seeded insert (SSI technique) when isolated gill cells were for the first time cultured on permeable membrane inserts. This provided a two-chamber system allowing the cells to grow into a polarised epithelium with an apical (water-facing) and basolateral (blood/media-facing) surface. The preparation exhibited barrier properties similar to that of the gill epithelium, as well as a developed apical glycocalyx and abundant rough endoplasmic reticulum. But, the epithelium comprised only of a cell population of pavement cells (PVC), the respiratory cell of the gill epithelium.

This technique was further developed into the double seeded (DSI) technique[9] whereby primary cells are seeded on inserts over two days, producing a gill epithelium of multiple cell layers and types that includes PVC and mitochondrion rich cells (MRC), the latter being associated with the transport of a number of ions and compounds across the fish gill. The DSI procedure produces tight epithelia with transepithelial resistance (TER) values of up to 34000 Ω cm^2, much higher than that of SSI (typically 1000-5000 Ω cm^2)[10]. The preparation expresses tight junction (TJ) proteins such as occludin, claudins, zonula occludens[11] and tricellulin[12] and primary cultured gill epithelia have been used to assess how hormones and environmental conditions influence TJ protein expression and regulate epithelial permeability[13-15]. The preparation also exhibits ion movement between the apical and basolateral compartments[11,16]. Active Ca^{2+} transport in the cell culture system resembles that of the intact fish[11,17], but the net uptake of Na^+ and Cl^- present in freshwater fish has so far not been possible to reproduce in cell culture. Further applications of the gill cell culture technique include studies on cytochrome P450 activity[18], lipid metabolism[18], ammonia transport[18], cytotoxicity[18] and metal binding characteristics[19]. A recent study also used the DSI preparation to analyse the transport of pharmaceuticals across the gill, and found that for some, facilitated transport processes may play a role at environmentally relevant

concentrations[20]. It has also been employed as an environmental water monitoring tool of urban streams[21] and in metal polluted rivers[22]. However, these monitoring studies are restricted to a limited time frame, because the cultures can only withstand water exposure of up to 48 h. Thus, there is scope for future development of the cell culture technique to improve both active ion transport characteristics and extended water tolerance.

The gill cells from two (~80 g) fish can provide up to 72 individual culture inserts, and thus this technique has the potential to reduce the number of organisms used in experiments. Therefore on a practical level, in order to allow the comparison of toxicological data from different aquatic ecotoxicology and physiological studies, a standardised procedure for DSI and DSII production is required. Here, we present the most current and readily available standardised DSI technique (Fig. 1A), as well as the development of a double seeded inverted insert (DSII; Fig 1B) technique.

MATERIALS

REAGENTS

- Juvenile rainbow trout (<100 g) should be acclimatised for at least 2 weeks and maintained according to national and institutional guidelines at 13-14°C in good quality water with constant 14 h light and 10 h dark photoperiod. CRITICAL STEP The fish must be kept in optimum conditions. The water must be well aerated and of good quality, and fish should eat well and not show signs of stress.
- Leibovitz's L-15 Medium, no phenol red, with L-glutamine (Invitrogen, cat. no. 21083-027)
- Fetal bovine serum (FBS) (Sigma, cat. no. F7524)
- Penicillin-streptomycin (5000 units ml^{-1} penicillin; 5 mg ml^{-1} streptomycin; Invitrogen, cat. no. 15070063)
- Gentamicin solution (10 mg ml^{-1}; Invitrogen, cat. no. 15710049)
- Fungizone (100 mg; Sigma, cat. no. A9528)
- 0.5% trypsin-EDTA x10 (Invitrogen, cat. no. 15400054)
- Phosphate buffered saline (PBS: 137 mM Sodium chloride, Sigma, cat. no. 746398; 2.7 mM Potassium chloride, Sigma, cat. no. P9333; 10 mM Disodium hydrogen phosphate, Sigma, cat. no. 255793; 1.8 mM Potassium dihydrogen phosphate, Sigma, cat. no. P0662)
- Ethanol (VWR, cat. no. 20821.321)
- Calcium chloride dihydrate (Sigma, cat. no. 223506)
- Magnesium sulphate heptahydrate (Sigma, cat. no. 230391)
- Sodium bicarbonate (Sigma, cat. no. S6014)
- Potassium chloride (Sigma, cat. no. P9333)

EQUIPMENT

- Laminar flow hood, Model no. M51424/2, Microflow Biological Safety Cabinet
- Centrifuge, refrigerated, 5810R, rotor A-4-62, Eppendorf
- Vortex, F20220176, VELP Scientifica
- Dissecting equipment; large sharp knife, forceps (SLS, cat. no. INS4291; 00:SA), scissors (VWR, cat. no. INS4808; INS4854), scalpels (Swann-Morton, cat. no. 0501)
- Portable gyratory shaker, model IKA-VIBRAX-VXR
- Adjustable pipettes: P-20 (cat. no. FA10003M); P-200 (cat. no. FA10005M); P-1000 (cat. no. F123602); Gilson
- Pipette tips: 20 µl (cat. no. S1120-1810), 200 µl (cat. no. S1120-8810) and 1000 µl (cat. no. S1122-1830), Starlab
- Tissue culture pipettes: 5 ml (cat. no. CORN4051), 10 ml (cat. no. CORN4101), 25 ml (cat. no. CORN4250); Corning
- Inverted phase contrast microscope, TE200, Nikon Eclipse
- Cell culture cooling incubator, MIR-1554, Sanyo, 18°C, no CO_2 atmosphere required
- Hemocytometer, Neubauer

- EVOM™ epithelial voltohmmeter modified to read TER up to 100,000 Ω cm^2, World Precision Instruments
- EVOM™ 'chopstick' electrodes, No. STX-2, World Precision Instruments
- 12 well inserts 0.4 µm pore, 0.9 cm^2 effective growth area, polyethylene terephthalate (PET) membrane (BD Falcon, cat. no. 353180)
- Companion cell culture plates, 12 well with low evaporation lid, notched (BD Falcon, cat. no. 353503)
- Sterile tip box with damp cotton wool and glass slides inside
- 0.2 µm sterile syringe filter (VWR, cat. no. 194-2520)
- Cell strainers, 100 µm, nylon (BD Falcon, cat. no. 352360)
- Falcon 50 ml conical tubes (BD Falcon, cat. no. 352070)
- Sterile Petri dishes (VWR, cat. no. 25382-166)

REAGENTS SETUP

PBS is prepared and autoclaved.
70% solution of ethanol is prepared in dH_20.
Fungizone is prepared as a 10 mg ml^{-1} solution and stored as 1 ml aliquots at -20°C until further use.

Cell isolation solutions Should be prepared at room temperature in sterile conditions in a tissue culture hood on the day of cell isolation.
Pre-wash solution 20 ml PBS
Wash-solution Add 1.2 ml penicillin-streptomycin, 1.2 ml gentamicin and 90 µl fungizone solution to a final volume of 30 ml PBS.
Trypsin solution Dilute 0.5% trypsin-EDTA to 0.05% by pipetting 650 µl into a conical centrifuge tube and bringing the volume up to 6.5 ml with PBS.
Stop solution Pipette 2 ml FBS into a conical centrifuge tube with 18 ml PBS.
Rinse solution Add 0.5 ml FBS made to 19.5 ml with PBS.

Cell culture medium with antibiotics and FBS (A) Pipette 27 ml FBS, 11 ml penicillin-streptomycin and 11 ml gentamicin, all sterile filtered to 500 ml of L-15 medium. Can be made up and stored at 4°C for up to 1 month.
Cell culture medium with FBS (B) Pipette 27 ml of FBS sterile filtered to 500 ml of L-15 medium. This can be made up and stored at 4°C for up to 1 month.

Exposure cell culture medium
L-15 without any supplementation
Artificial fresh water Fresh water is defined as water prepared to the standard used for maintaining fish such as the Organisation for Economic Co-operation and Development (OECD) and International Organization for Standardization (ISO) recommended[23,24].

OECD freshwater Prepare the following four solutions (40x stocks): Solution 1: Dissolve 11.76 g of $CaCl_2.2H_2O$ in dH_2O, make up to 1 L with dH_2O (0.08 M). Solution 2: Dissolve 4.93 g of $MgSO_4.7H_2O$ in dH_2O; make up to 1 L with dH_2O (0.02 M). Solution 3: Dissolve 2.59 g of $NaHCO_3$ in dH_2O, make up to 1 L with dH_2O (0.03 M) Solution 4: Dissolve 0.23 g of KCl in dH_2O; make up to 1 L (0.003 M). Once all solutions are prepared, mix 25 ml of each solution and the total volume is made up to 1 L with dH_2O. Aerate the dilution water until oxygen saturation is achieved, then store it for about 2 days with further aeration before use[23].

Natural freshwater Natural freshwater samples are filtered using a 0.2 µm sterile syringe filter prior to exposure of the cell culture.

EQUIPMENT SET UP

All equipment is sterilised using 70% ethanol or is autoclaved.

PROCEDURE

The protocol for DSI and DSII are summarised in a flow diagram of procedures (Fig. 2).

Preparation of solutions and culture inserts TIMING 1 h

1 Prepare the two types of cell culture media (see Reagents Setup). Take a 50 ml aliquot and keep on ice.
2 Prepare the cell isolation solutions and keep on ice.
3 In the cell culture hood, remove the permeable membrane inserts and companion cell culture plates from their packaging. For the DSI technique, place inserts in the wells of cell culture plates and place lid on top.
CRITICAL STEP the flanges of the inserts rest between the notches of the well to minimise media wicking and assure the proper fitting of the plate with the lid.
For DSII, turn the inserts upside down in a humidified chamber. To generate the humidified chamber, we use a sterile pipette tip box with damp sterile cotton wool.
CRITICAL STEP Microscope slides can be placed on top of the cotton wool in the tip box to create a stable and flat surface on which to place the upside down inserts . This prevents media spilling over the edge of the inverted insert.
4 Condition permeable membrane inserts by applying cell culture medium with FBS and antibiotics (A) to the surface of the insert on which the cells will be seeded.
CRITICAL STEP For DSI preparations add 200 µl cell culture medium with FBS and antibiotics (A) in the upper compartment. For DSII, add 200 µl cell culture medium with FBS and antibiotics (A) onto the top of the upside down insert.
Close the systems and keep at 18°C in a cell culture incubator.
TIMING The cell culture inserts should be conditioned with media for at least 1.5 h.

Rainbow trout dissection and gill cell isolation TIMING 3 h

5 Euthanize one rainbow trout

CAUTION Euthanize according to national and institutional guidelines.

CRITICAL STEP Quickly excise the head with a sharp knife and avoid haemorrhage to the gill filaments. Keep bleeding and clotting on the filaments to a minimum otherwise this will reduce the quality of the gill cells obtained.

6 With dissecting scissors and forceps, remove the operculum (Fig. 3A) and excise out the intact gill arches by cutting the dorsal and ventral cartilage of each gill arch. Place in the pre-wash solution in a Petri dish (Fig. 3B).

CRITICAL STEP Avoid damage to the filaments by holding the cartilage of the arch and not the filaments.

7 Remove mucus by careful blotting onto tissue paper and cut the filaments from the arches and place into 10 ml of wash solution in a Petri dish.

CRITICAL STEP Do not cut the filaments too close to the cartilage of the gill arch as this will contaminate the cell culture with fibroblasts.

8 Tease the filaments apart using forceps and cut with a scalpel. This should result in filaments separated in bundles of 1-5. This increases the surface areas which facilitates the action of the trypsin solution later.

9 Pour away this wash solution and add the filaments to another 10 ml aliquot of wash solution in a 50 ml conical centrifuge and replace the tube on ice. Incubate for 10 min.

CRITICAL STEP From here on, sterile culture technique is employed and the procedures take place in a laminar flow hood.

10 Centrifuge for 4 min at 250*g* at 4^{o}C to collect the filaments. Aspirate supernatant.

11 Add the last remaining 10 ml of wash solution and mix to dislodge the filaments. Incubate on ice for a further 10 min.

12 Centrifuge again for 4 min at 250*g* at 4^{o}C and aspirate the supernatant.

13 Add 500 µl of trypsin solution and agitate to remove any remaining wash solution. Centrifuge for 4 minutes at 250*g* at 4^{o}C and aspirate supernatant.

14 Add 3 ml of trypsin solution to begin tryptic digestion and shake for 12 min at 200 RPM at room temperature.

15 To mechanically agitate the cut gill filaments and improve cell yield, filaments in trypsin solution are passed repeatedly through a wide bore pipette tip immediately following the 12 min trypsinisation period. A wide bore pipette tip can be prepared by removing the tip of a 1000 µl pipette tip, so that the bore of the pipette aperture is around 2 mm. Sterilise by storing the cut pipette tip in 70% ethanol.

16 Prior to use, blot the wide bore tip on tissue to remove residual ethanol and pass the filaments/trypsin solution up and down at least 50 times.

17 Place the 100 µm cell strainer on to the 50 ml conical centrifuge tube containing 20 ml stop solution on ice. Pipette the filament/trypsin solution into the strainer and agitate to facilitate the collection of gill cells as a single cell suspension in stop solution.

CRITICAL STEP Following collection of cells, gently invert the conical centrifuge tube to mix the gill cells with the stop solution.

18 Remove the filaments from the strainer and replace back into their original conical centrifuge tube. Repeat tryptic digestion by adding the remaining 3 ml of trypsin solution and shaking for a further 12 min at 200 RPM at room temperature.

19 Place a new 100 µm cell strainer on to the same 50 ml conical centrifuge tube containing the stop solution and the previously trypsinised cells and repeat gill cell isolation as in step 13.

20 Centrifuge the cell suspension for 10 min at 250*g* at 4°C to give a red pellet (Fig. 4A). Aspirate the supernatant.

21 Re-suspend the pellet in 2 ml of rinse solution, then add the remaining 18 ml, agitate and centrifuge for 10 min at 250*g* at 4°C, resulting in another red-coloured pellet (Fig. 4B). Aspirate the supernatant.

22 Re-suspend the pellet in 10 ml cell culture medium with FBS and antibiotics (A).
CITICAL STEP First flick the tube to dislodge the pellet many times, then re-suspend in 2 ml, before adding the remaining volume, and mix well.

23 Add 10 µl of cell suspension to 90 µl Trypan blue solution in a 0.5 ml bullet centrifuge tube and mix thoroughly using a vortex.

24 Count viable cells using a haemocytometer based on the Trypan blue exclusion criterion.
CRITICAL STEP Exclude blood cells from the cell count. These appear as ellipsoidal, smaller then the gill cells.

25 Quantify the concentration of cells and adjust cell counts to required seeding density and leave on ice.
TROUBLESHOOTING

DSI cell seeding TIMING 30 min

26 Aspirate the medium conditioning the cell culture inserts before cell seeding. Seed 1.2 x 10^6 cells in 800 µl cell culture medium with FBS and antibiotics (A) per insert. Place 1 ml cell culture medium with FBS and antibiotics (A) into the basolateral compartment. Leave at 18°C overnight. This is termed 'Day 0'. CRITICAL STEP It is recommended that when establishing the protocol an insert seeding density gradient is performed to determine the optimum seeding density to produce inserts with consistently tight epithelia[11].

27 After 24 hours from the first step, begin a new gill cell preparation using another fish, by making new cell isolation solutions and repeating steps 5-25.

28 To prepare the previously seeded inserts ('day 0') for double-seeding, aspirate the lower compartment and then carefully, the upper compartment. Wash the surface of the inserts by slowly applying 200 µl of PBS down the side of the insert (not directly onto the attached cells to avoid disruption) and tap the edge of the insert gently at this wash stage to dislodge dead cells and mucus.
CRITICAL STEP When removing or changing media, always aspirate the lower compartment first, then the upper one. This maintains the hydrostatic pressure onto the cells and prevents them from lifting off.

29 Repeat another 200 µl PBS wash.
TROUBLESHOOTING

30 Carefully apply the new gill cell preparation directly on top of the previously seeded cells. Seed 1.2 x 10^6 cells in 800 µl cell culture medium with FBS and antibiotics (A) and add 1 ml in the lower compartment afterwards. Incubate at 18°C. This is termed 'Day 1'.

31 24 hours later, wash the inserts again as in step 28-29. Now replace with maintenance volumes of culture media; 1.5 ml cell culture medium with FBS and antibiotics (A) in the upper compartment and then 2.0 ml in the lower compartment. Incubate at 18°C. This is termed 'Day 2'.

DSII cell seeding TIMING 30 min

32 Aspirate the medium conditioning the inverted cell culture inserts. Seed 1.2 x 10^6 cells in 200 µl cell culture medium with FBS and antibiotics (A) per inverted insert and leave at 18°C in the sterile tip box and leave at 18°C overnight.

33 After 24 hours, begin a new gill cell preparation using another fish, by making new cell isolation solutions and repeating steps 5-25.

34 To prepare the previously seeded inserts ('day 0') for double-seeding, wash the inverted inserts by aspirating the medium and carefully and slowly applying 200 µl PBS.

35 Repeat another 200 µl PBS wash.
TROUBLESHOOTING

36 Carefully apply the new gill cell preparation directly on top of the previously seeded cells. Seed 1.2 x 10^6 cells in 200 µl cell culture medium with FBS and antibiotics (A) and leave in the sterile pipette tip box overnight at 18°C. Termed 'Day 1'.

37 Twenty-four hours later, wash the inserts again as in step 34-35. The inserts can now be placed the right way up in companion wells. Replace with maintenance volumes of culture media; 1.5 ml cell culture medium with FBS and antibiotics (A) in the upper compartment and then 2.0 ml in the lower compartment. Incubate at 18°C. This is termed 'Day 2'.

Maintenance TIMING 5-12 d

38 Change the media on day 4 to cell culture medium with FBS (B), at 18°C, so that antibiotics are no longer present. The media should be changed every 48 h. Epithelia can be viewed under a light microscope (Fig. 5).

39 Cell culture inserts should be observed on a daily basis to check for (bacterial) contamination. Any contaminated cell culture inserts should be immediately removed and the associated plate well should be rinsed with 70% ethanol.
CRITICAL STEP Careful and thorough observation of culture preparations should take place prior to measuring transepithelial resistance (TER) (see following section) or changing media. Therefore, if one insert is found to be contaminated, it can be removed without cross contaminating other epithelia.

Monitoring TIMING 10 min

40 From day 4, a daily measurement of transepithelial resistance (TER) can be made using a custom-modified (see Equipment section) epithelial tissue voltohmmeter fitted with chopstick

electrodes. Sterilise the electrodes in 70% ethanol and rinse in PBS. After confirming no signs of contamination, the electrode is inserted over the gill cell epithelium of each insert, such that the shorter arm of the electrode is in the upper compartment, and the longer is in the lower compartment (Fig. 6).

41 The net TER is calculated by subtracting the TER of a blank insert (no cells) from the experimental value. Final resistance-area values (Ω cm^2) are obtained by multiplication of the net TER with the effective growth area (0.9 cm^2 for 12 well inserts).

42 During days 7-14, DSI and DSII inserts should show TER values of approximately 5000-30000 Ω cm^2.
TROUBLESHOOTING

Preparing for experimental procedures TIMING 30 min

43 Above a TER value of 5000 Ω cm^2 [20] freshwater can be applied to the apical surface of the cells (the upper compartment for DSI, the lower compartment for DSII).
PAUSE POINT Epithelia can be stored for later use by maintaining at 4°C for up to two weeks[25].
CRITICAL STEP Application of freshwater will cause a rise in TER (Fig. 7). After freshwater application, inserts around 5000 Ω cm^2 may rise to 30000 Ω cm^2, whilst those already around 25000-30000 Ω cm^2 may not rise by as much, to perhaps around 35000 Ω cm^2. After freshwater application, TER values should remain above 5000 Ω cm^2 for up to 48 hours.

44 Aspirate the lower compartment then the upper one.

45 To wash and remove traces of FBS, apply 800 µl PBS in the upper compartment, then 1000 µl to the lower compartment.

46 Aspirate the lower compartment and wash a further 2 times, before the final aspiration.

47 With an empty lower compartment, wash 3 x 800 µl PBS in the apical.

48 **DSI**: Apply 1.5 ml freshwater (or whatever test protocol requires) into the upper compartment, whilst leaving the lower compartment in 2.0 ml of L-15 medium. Depending on the experimental design, FBS can be removed from the medium.

49 **DSII**: Apply 1.5 ml L-15 medium without FBS into the upper compartment. 2.0 ml freshwater can now be applied to the lower compartment. The volume in the upper compartment can be reduced if required.

TIMING

Step 1-4, Preparation of solutions and cell culture inserts: 1 h
Step 5-25, Rainbow trout dissection and gill cell isolation: 3 h
Step 26-31/32-37, DSI/DSII cell seeding: 30 min
Step 38, Maintenance: 5-12 d
Step 40-42, Monitoring: 10 min (insert dependent-large sets require more time)
Step 43-49, Preparation for experimental procedures: 30 min

ANTICIPATED RESULTS

The DSI and DSII epithelia have distinct apical and basolateral surface consisting of irregularly shaped epithelial (pavement) cells with microridges and plasma membranes (Fig. 8) interspersed with mitochondria rich cells (Fig. 9B). Both preparations develop an electrically tight epithelium showing the presence of the tight junction proteins, such as zonula occludens (Fig. 10). The DSI can withstand water applied to the apical surface, but does not tolerate water when exposed via the basolateral compartment[26] and these traits are also evident in the DSII preparations (Fig. 9A and Fig. 11). Based on Rhodamine 123 staining[13] the epithelium should consist of between 10-15% MRC which is consistent with observations of the intact fish gill epithelium, however PVCs make up the bulk of the epithelium as is also seen *in vivo*. In order to perform experiments that provide statistical rigor it is recommended that experiment include inserts derived from several different preparations, i.e. biological replicates.

TROUBLESHOOTING

Table 1 Troubleshooting table

Step	Problem	Possible reason	Solution
25	Low cell viability	Excessive trypsinisation	Lower trypsinisation time
		Low donor quality	Check the health of stock fish
		Slow work pace	Perform procedure faster
29, 35	Extra mucus on cells	Fish related problem	Perform an additional PBS wash
42	Low TER	Contamination	Discard the cells
		Seeding density	Perform seeding density assay
		Fish related problem	Check the health of stock fish and water quality of fish tank
		Slow development	Keep monitoring and discard if TER<5000 Ω cm^2 by day 14

Author contributions statements

SS and LCS contributed equally to the manuscript and generated the data. SS developed the DSII technique. CMW, SPK and PP developed the initial methodology. SFO, CH and NRB supported the current recent developments of the methods. CH and NRB received funding for SS and LCS that has enabled the development of the methods and expand the use of the DSI for the replacement of animals in toxicity testing and environmental monitoring. All authors contributed to the manuscript.

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Competing Interests Statement The authors declare no competing financial interests.

REFERENCES

1. Evans, D.H., Piermarini, P.M. & Choe, K.P. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev*. **85**, 97-177 (2005).

2. Wood C.M. Toxic responses of the gill. In: Target Organ Toxicity in Marine and Freshwater Teleosts, Volume **1** - Organs. (eds: Schlenk, D.W. & Benson, W.H.) 1-89 (Taylor & Francis, London, Uk) (2001).

3. Mckim, J.M. & Erickson, R.J. Environmental impacts on the physiological mechanisms controlling xenobiotic transfer across fish gills. *Physiol. Zool*. **64**, 39–67 (1991).

4. Bury, N.R., Walker, P.A. & Glover, C.N. Nutritive metal uptake in teleost fish. *J. Exp. Biol*. **206**, 11-23. (2002)

5. Scholz, S. *et al.* A European perspective on alternatives to animal testing for environmental hazard identification and risk assessment. *Regul. Toxicol. Pharm.* **67**, 506–530 (2013).

6. Kramer, N.I. *et al.* Development of a partition-controlled dosing system for cell assays. *Chem. Res. Toxicol.* **23**, 1806-1814 (2010).

7. Pärt, P., Norregren, L., Bergström, B., & Sjöberg, P. Primary culture of epithelial cells from rainbow trout gills. *J. EXP. Biol*. **175**, 219-232 (1993).

8. Wood, C.M. & Pärt, P. Cultured branchial epithelia from freshwater fish gills. *J. Exp. Biol*. **200**, 1047-1059 (1997).

9. Fletcher, M., Kelly S.P., Pärt, P., O'Donnell, M.J. & Wood, C.M. Transport properties of cultured branchial epithelia from freshwater rainbow trout: a novel preparation with mitochondria-rich cells. *J. Exp. Biol.* **203**, 1523–1537 (2000).

10. Kelly, S.P., Fletcher. M., Pärt, P. & Wood, C.M. Procedures for the preparation and culture of 'reconstructed' rainbow trout branchial epithelia. *Methods in Cell Science* **22**, 153–163 (2000).

11. Walker P.A., Bury, N.R. & Hogstrand, C. Influence of culture conditions on metal-induced responses in a cultured rainbow trout gill epithelium. *Environ. Sci. Technol*. **41**, 6505-6513 (2007).

12. Kolosov, D., Chasiotis, H. & Kelly, S.P. Tight junction protein gene expression patterns and changes in transcript abundance during development of model fish gill epithelia. *J. Exp. Biol*. **217**, 1667-1681 (2014).

13. Chasiotis, H., Wood, C.M. & Kelly, S.P. Cortisol reduces paracellular permeability and increases occludin abundance in cultured trout gill epithelia. *Mol. Cell. Endocrinol*. **323**, 232-238 (2010).

14. Sandbichler, A.M., Egg, M., Schwerte, T. & Pelster, B. Claudin 28b and F-actin are involved in rainbow trout gill pavement cell tight junction remodelling under osmotic stress. *J. Exp. Biol*. **214**, 1473-1487 (2011).

15. Kolosov, D. & Kelly, S.P. A role for tricellulin in the regulation of gill epithelium permeability. *Am. J. Physiol*. **304**, R1139-R1148 (2013).

16. Zhou, B., Kelly, S.P., Ianowski, J.P. & Wood, C.M. Effects of cortisol and prolactin on Na+ and Cl– transport in cultured branchial epithelia from FW rainbow trout. *Am. J. Physiol*. **285**, R1305-R1316 (2003).

17. Kelly, S.P. & Wood, C.M. Cortisol stimulates calcium transport across cultured gill epithelia from freshwater rainbow trout. *In Vitro Cellular & Developmental Biology – Animal*. **44**, 96-104 (2008).

18. Bury, N.R., Schnell, S. & Hogstrand, C. Gill cell culture systems as models for aquatic environmental monitoring. *J. Exp. Biol*. **217**, 639-50 (2014).

19. Zhou B., Nichols J., Playle R.C. & Wood, C.M. An in vitro biotic ligand model (BLM) for silver binding to cultured gill epithelia of freshwater rainbow trout (*Oncorhynchus mykiss*). *Toxicol. Appl. Pharmacol*. **202**, 25-37 (2005).

20. Stott, L.C., Schnell, S., Hogstrand, C., Owen, S.F. & Bury, N.R. A primary fish gill cell culture model to assess pharmaceutical uptake and efflux: evidence for passive and facilitated transport. *Aquat. Toxicol*. **159**, 127-137 (2015).

21. Schnell, S. *et al.* Environmental monitoring of urban streams using a primary fish gill cell culture system (FIGCS). *Ecotox. Environ. Safe*. **120,** 279–285 (2015).

22. Minghetti, M., Schnell, S., Chadwick, M.A., Hogstrand, C. & Bury, N.R. A primary Fish Gill Cell System (FIGCS) for environmental monitoring of river waters. *Aquat. Toxicol*. **154**, 184-192 (2014).

23. OECD Test No. 203: Fish, Acute Toxicity Test. OECD Guidelines for the Testing of Chemicals, Section 2. OECD (1992) Publishing. doi: 10.1787/9789264069961-en

24. ISO International Organization for Standardization. ISO 10229:1994 Water quality -- Determination of the prolonged toxicity of substances to freshwater fish -- Method for evaluating the effects of substances on the growth rate of rainbow trout (Oncorhynchus mykiss Walbaum (Teleostei, Salmonidae)) (1994). http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=1826

25. Wood, C.M, Eletti, B. & Pärt, P. New methods for the primary culture of gill epithelia from freshwater rainbow trout. *Fish Physiol. Biochem.* **26**, 329-344 (2002a).

26. Wood, C.M. *et al.* Cultured gill epithelia as models for the freshwater fish gill. *Biochim. Biophys. Acta* **1566**, 72-83 (2002b).

Figure Legends

Figure 1. Diagram of the primary cultured rainbow trout epithelium, reconstructed as (a) DSI or (b) DSII preparations. The epithelium attaches to the permeable membrane supports and polarises so as to have an apical and basolateral cell surface (right). Epithelia are bathed on both sides with cell culture medium supplemented with FBS and antibiotics during development (left). For experimental purposes, fresh water can replace cell culture medium on the apical surface, whilst the basolateral surface is bathed in L-15 (without FBS or antibiotics) (middle).

Figure 2. Summarised flow diagram of rainbow trout gill cell isolation and seeding procedures.

Figure **3.** Four gill arches are located underneath each operculum (a) on the lateral sides of the rainbow trout head. Bar = 0.5 cm. Gill arches are excised by cutting the cartilage of the arch and these are placed into 20 ml of prewash solution (PBS) (B). Bar = 1 cm.

Figure 4. Images of the gill cell pellet. The pellet is obtained after centrifugation in step 20 (a) and step 21 (b). Trapped red blood cells give the red colour. Bar = 1 cm.

Figure 5. Phase contrast micrograph of DSI gill epithelial cells grown on permeable supports. Bar = 100 μm.

Figure 6. Membrane development can be monitored by daily transepithelial electrical resistance (TER) measurements. A custom-modified (see text) epithelial tissue voltohmmeter is fitted with chopstick electrodes. The electrode arms are inserted above and below the gill cell epithelium of each insert, such that one arm of the electrode is in the upper, and the other in the lower (bottom) compartments.

Figure 7. Measurements of TER in developing double-seeded inverted insert (DSII) epithelia from 3 days. Freshwater (blue points) was added (after the dotted line) to the companion well compartment (the apical cell surface, whilst the insert contained L-15 medium) on day 7 resulting in an increase in TER still evident 24 hours later on day 8 (n = 5). Values are means ± SEM.

Figure 8. Scanning electron micrograph of the apical cell surface of DSII. The irregularly shaped epithelial cells with microridges (mr) and plasma membrane (pm) can be observed. Bar = 20 μm.

Figure 9. Transmission electron micrographs of DSII. Gill epithelia characteristics can be observed, such as (a) the tight junctions between epithelial cells (arrows) and the apical (AP) cell surface with glycocalyx and the basolateral cell surface (BL) (*bar* = 200 nm) and (b) a mitochondria rich cell (mitochondria = mc), the apical cell surface (AP), the nucleus (n) and the intercellular space (S). Bar = 500 nm.

Figure 10. Confocal microscope images of DSI epithelia. Twenty four hours before cell fixing, epithelia were exposed to (a) symmetrical conditions with L-15 medium on both sides of the epithelium or (b) asymmetrical conditions with FW in the upper compartment and L-15 medium in the companion well. Cell nuclei were stained with 5 μM Hoechst (blue) and tight junctions with zonula occludens 1 antibody (green). Bar = 50 μm.

Figure 11. The primary cultured rainbow trout gill epithelium is functionally polarised. Once the DSII epithelium has developed its signature resistance and is electrically tight (by day 10 in this case),

replacing the apical surface (the companion well compartment) from cell culture medium with FBS to fresh water (indicated by dotted line) causes a rapid reduction in TER (n = 3). Values are means ± SEM.

Figure 1

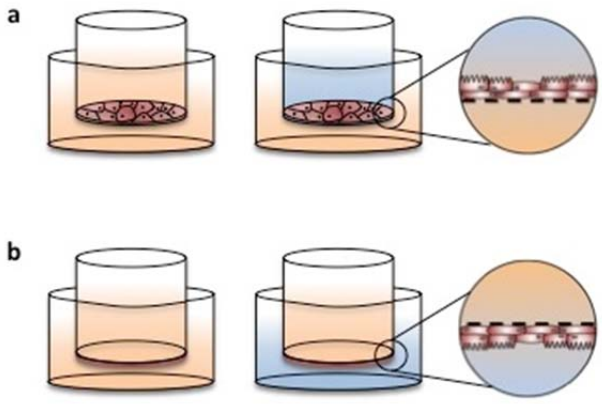


Figure 2

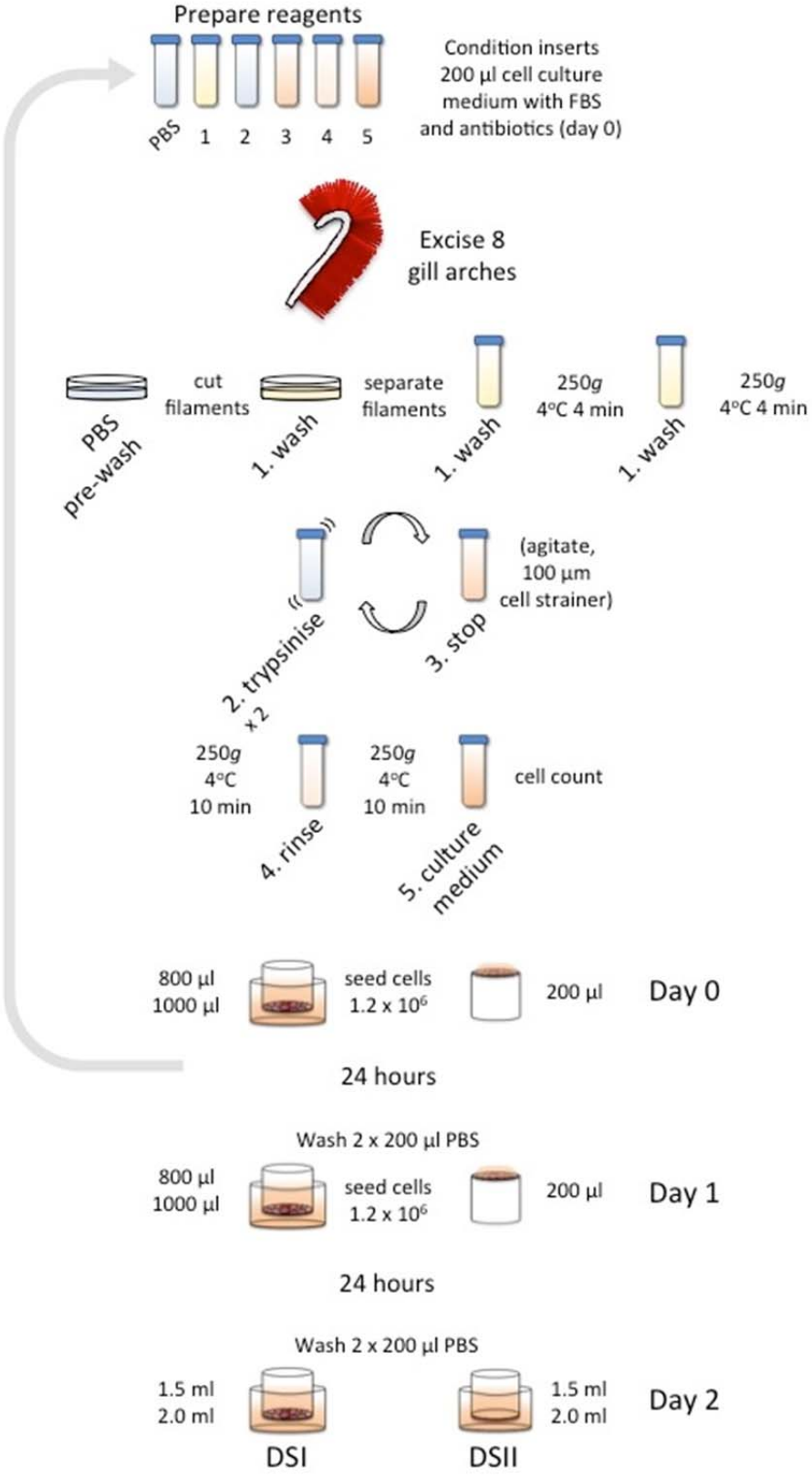

Prepare reagents
PBS
1
2
3
4
5
Condition inserts
200 µl cell culture
medium with FBS
and antibiotics (day 0)
Excise 8
gill arches
PBS
pre-wash
cut
filaments
1. wash
separate
filaments
1. wash
250g
4°C 4 min
1. wash
250g
4°C 4 min
2. trypsinise
x 2
3. stop
(agitate,
100 µm
cell strainer)
250g
4°C
10 min
4. rinse
250g
4°C
10 min
5. culture
medium
cell count
800 µl
1000 µl
seed cells
1.2 x 10^6
200 µl
Day 0
24 hours
Wash 2 x 200 µl PBS
800 µl
1000 µl
seed cells
1.2 x 10^6
200 µl
Day 1
24 hours
Wash 2 x 200 µl PBS
1.5 ml
2.0 ml
DSI
1.5 ml
2.0 ml
DSII
Day 2

Figure 3

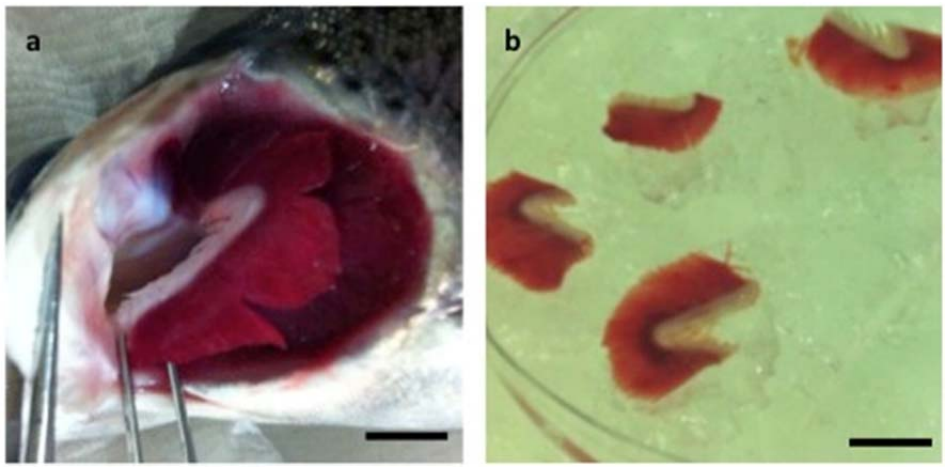


Figure 4

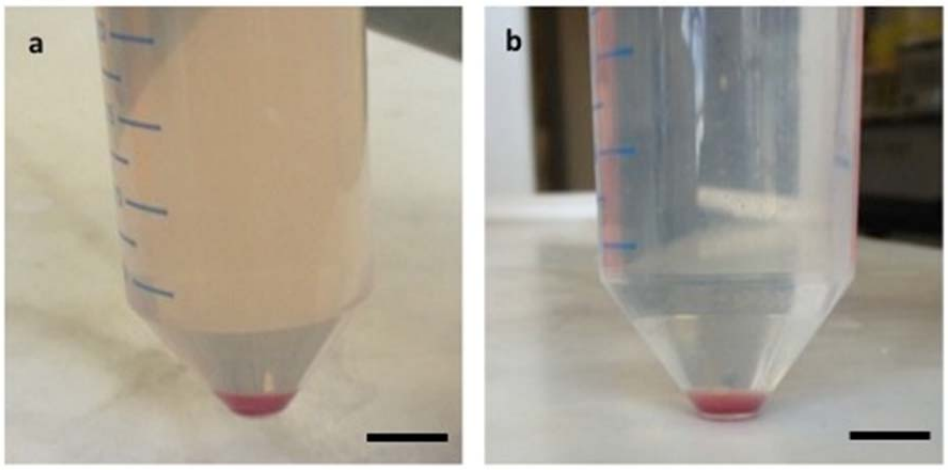
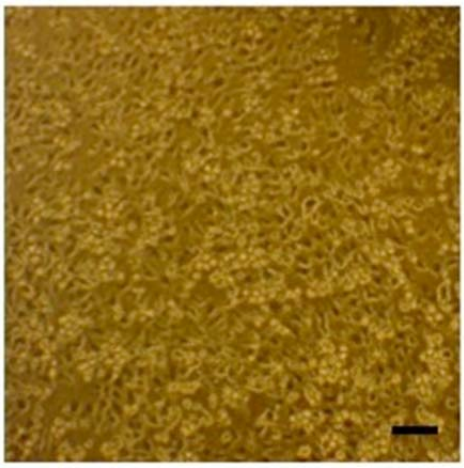


Figure 5



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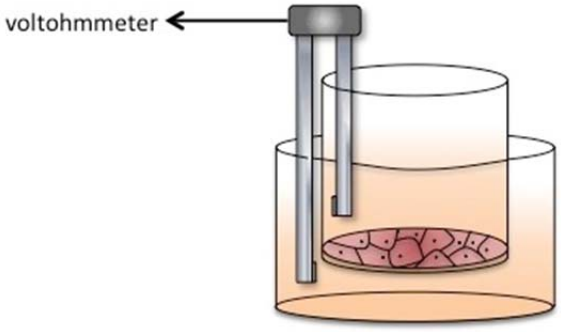

Figure 7

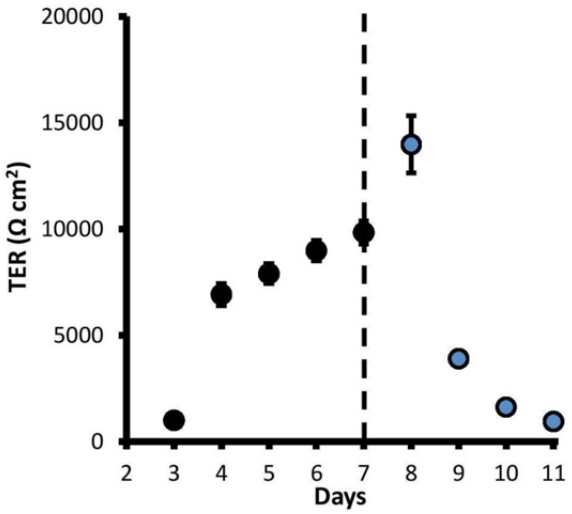

Figure 8

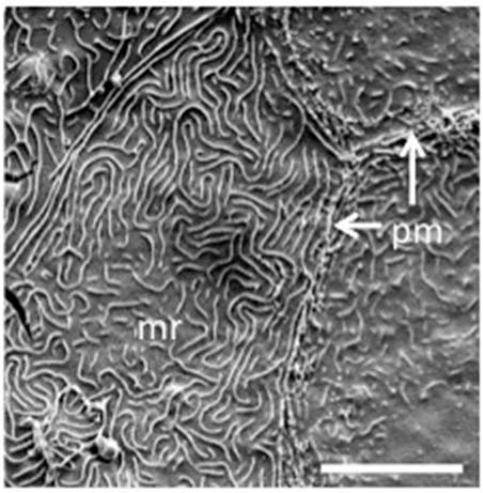

Figure 9

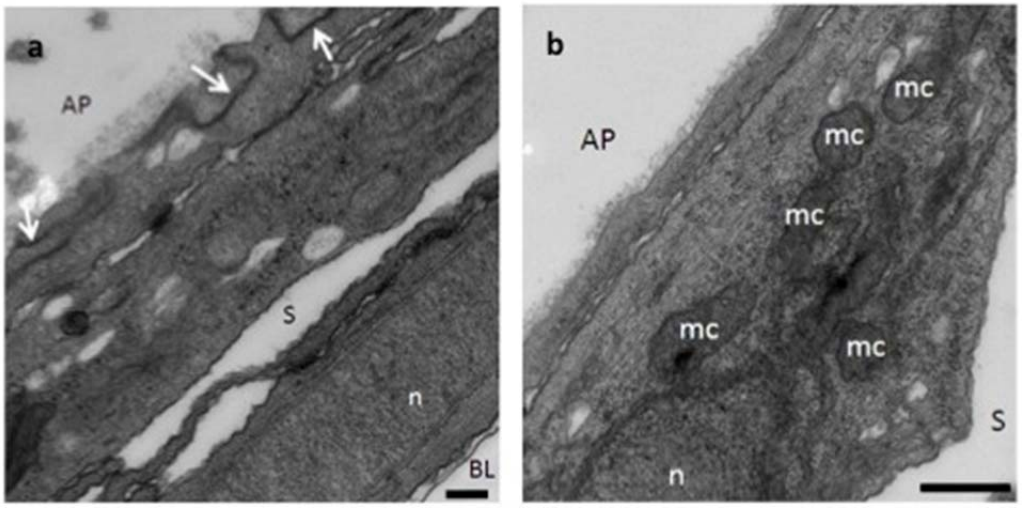

Figure 10

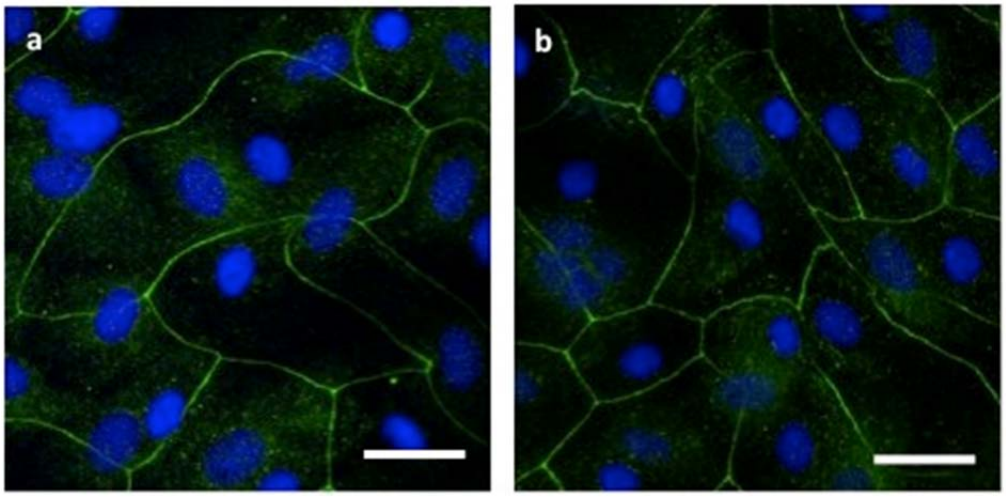

Figure 11