

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

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This protocol describes how 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 2-d period is required. As a consequence, this is termed the double-seeded insert technique. Approximately 5–12 d after cell isolation and seeding, preparations develop electrically tight gill epithelia that can withstand freshwater on the apical cell surface. The system can be used to study freshwater gill physiology, and it is a humane alternative for toxicity testing, bioaccumulation studies and environmental water quality monitoring.

INTRODUCTION

The freshwater fish gill is a multifunctional organ, and it 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 toxicants². Whole-animal studies can use millions of fish worldwide each year, and efforts are focusing on refining, reducing and replacing (3Rs) these numbers⁵. Alternative animal-free methods used in fish toxicology include the use of immortalized 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*, and it was first described in Fletcher *et al.*⁶. We have used the DSI for studies investigating gill physiology and environmental monitoring^{7–20}.

Development of the DSI technique

Primary culture of a rainbow trout gill epithelium was pioneered by Pärt *et al.*²¹. Wood and Pärt²² subsequently developed the single-seeded insert (SSI technique), in which isolated gill cells were cultured on permeable membrane inserts for the first time. This provided a two-chamber system that allowed the cells to grow into a polarized epithelium with an apical (water-facing) and basolateral (blood- or medium-facing) surface. The preparation exhibited barrier properties that were similar to those of the gill epithelium, and it tolerated apical water exposure for limited time periods. However, the epithelium comprised only a cell population of pavement cells (PVCs), which are the respiratory cells of the gill epithelium.

This technique was further developed into the DSI technique⁶ whereby primary cells are seeded on inserts over 2 d, producing a gill epithelium of multiple cell layers and types that includes PVCs and mitochondrion-rich cells (MRCs); the latter is associated with the

transport of a number of ions and compounds across the fish gill¹. The DSI procedure produces tight epithelia with transepithelial resistance (TER) values of up to 34,000 Ω cm², which are much higher than that of SSI (typically 1,000–5,000 Ω cm²)²³. The preparation expresses tight junction (TJ) proteins such as occludin, claudins, zonula occludens^{7,8,24} and tricellulin⁹. The preparation also exhibits ion movement between the apical and basolateral compartments^{10–12}. Active Ca²⁺ transport in the cell culture system resembles that of the intact fish^{10,12}, although so far it has not been possible to produce the net uptake of Na⁺ and Cl[−] ions present in freshwater fish in cell culture.

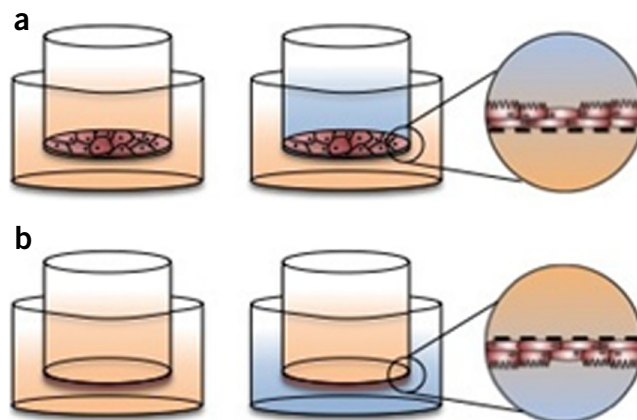
Applications of the protocol

Primary cultured gill epithelia have been used to assess how hormones and environmental conditions influence TJ protein expression and regulate epithelial permeability^{7,8}. Further applications of the gill cell culture technique include studies on cytochrome P450 activity¹⁴, lipid metabolism¹⁵, ammonia transport¹⁶, cytotoxicity²⁵ and metal-binding characteristics¹⁷. A recent study also used the DSI preparation to analyze the transport of pharmaceuticals across the gill, and found that, for some, facilitated transport processes may have a role at environmentally relevant concentrations¹⁸. It has also been used as an environmental water monitoring tool of urban streams¹³ and in metal-polluted rivers¹⁹. 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.

Generation of reliable *in vitro* cell assay results as an alternative to whole-animal tests for hydrophobic and volatile compounds has been proven to be difficult. This is because these compounds adhere to the plastic and/or are volatilized, which results in exposure concentrations in the small volumes used in *in vitro* assays that deviate substantially from the nominal concentrations. In addition, these hydrophobic compounds do not readily dissolve in medium, and to

Figure 1 | Diagram of the primary cultured rainbow trout epithelium. (a,b) Reconstructed as DSI (a) or DSII (b) preparations. The epithelium attaches to the permeable membrane supports and polarizes 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, freshwater can replace cell culture medium on the apical surface, whereas the basolateral surface is bathed in L-15 (without FBS or antibiotics).

increase solubility they are administered with co-solvents that may also exert a cellular response in the assay system. Kramer *et al.*²⁶ described a solvent-free dosing system for hydrophobic compounds in which the immortalized rainbow trout gill cell line RTgill-W1 was grown on the underside of the membrane of an insert. This allows a polydimethylsiloxane membrane preloaded with test compound to be placed on the bottom of the wells in close proximity to but not touching the cells, which in turn allows for a constant dosing concentration²⁶. This technique is termed the double-seeded inverted insert (DSII) technique, and it can therefore be used in the development of a partition-controlled dosing system for hydrophobic compounds. We describe a modification to the method used by Kramer *et al.*²⁶ in which primary gill cells are cultured on an inverted insert. Characterization of the resulting primary gill epithelium growing in the DSII configuration is shown in **Supplementary Figure 1** and was carried out as described in the **Supplementary Methods**. The advantage of the DSII system is that the cultured epithelia resembles that of the intact gill (**Supplementary Fig. 1**),



and it can be used in a similar way to the RTgill-W1 cell line when grown on inverted inserts for solvent-free dosing of hydrophobic compounds. However, given that we have only obtained preliminary data to date using the primary cultured epithelium in the DSII configuration, this method may require further optimization to ensure that it is optimally set up as required for specific experiments.

Comparison with other techniques

Bols *et al.*²⁷ described the isolation of the RTgill-W1 rainbow trout gill cell line. The main difference between using the cell line and the primary gill cell culture is that when using the primary gill cell culture, both the DSI and DSII contain the different cell types present in the *in vivo* gill. The epithelium is polarized and electrically tight, with TER values of $>20 \text{ k}\Omega \text{ cm}^2$ compared with TER values in the range of $\sim 200 \Omega \text{ cm}^2$ when RTgill W1 cells are used. Importantly, the DSI and DSII tolerate the application of freshwater on the apical surface, which means that these primary cell cultures are better suited for gill physiology and functional studies than the cell line. However, the RTgill-W1 cell line viability²⁸ and cell growth²⁹ have been shown to correlate, in most cases, to both whole fish mortality and growth on exposure to pollutants. Thus, the RTgill-W1 cell line shows promise as a replacement for fish in toxicity testing.

In a number of countries, fish are used in whole effluent toxicity testing as part of environmental monitoring programs. The DSI can be used to assist in environmental monitoring because in addition to tolerating the application of freshwater in the laboratory, the DSI also tolerates the application of river water, as shown in two studies^{13,19}. In one of these studies¹⁹, we used the knowledge that the DSI responds to metals (assessed via the

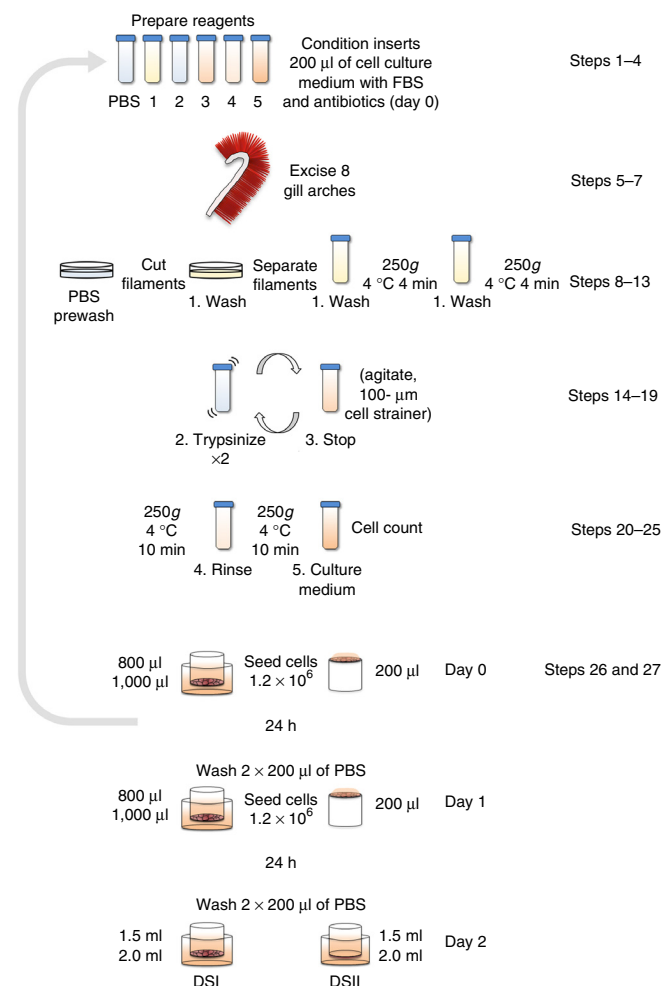


Figure 2 | Summarized flow diagram of rainbow trout gill cell isolation and seeding procedures. The reagents are prepared (see Reagent Setup) before excision of the gills; the filaments are teased apart and washed in PBS (PROCEDURE Steps 1–10). The filaments are centrifuged and washed before being treated with a trypsin solution to isolate cells (PROCEDURE Steps 11–16). The isolated cells are passed through a 100- μm strainer into a stop solution (see Reagent Setup; PROCEDURE Steps 17–21). The cell suspension is centrifuged and resuspended in cell culture medium containing FBS and antibiotics, and the cell density is determined (PROCEDURE Steps 18–25). The cells are seeded onto inserts for the double-seeded insert protocol (PROCEDURE Step 26A(i)) or onto inverted inserts for the double-seeded inverted insert protocol (PROCEDURE Step 26B(i)); this is termed day 0. The gill cell isolation procedure is repeated on day 1, and the inserts from the first seeding are washed with PBS before a second seed is placed on top (PROCEDURE Step 26A(ii–viii), 26B(ii–vii)). On day 2, the cells are washed and for the DSII protocol the insert is placed in the companion wells.

measurement of an increase in expression of the metal-responsive gene encoding metallothionein), mimics the physiological effect of metals on whole organisms in the laboratory²⁰ and conducted a trial whereby the DSI were exposed to natural metal contaminated water in the field¹⁹. The field trial study indicated that the DSI responded to metals in this complex matrix (river water), thereby showing that it is able to detect bioreactive metals. However, further experiments are required to assess the response of the DSI to other contaminants in more complex contaminant mixtures¹³ and to determine its versatility for environmental monitoring.

Experimental design

The gill cells from two (~80 g) fish provide ~40 individual culture inserts, but this can increase to 72 depending on the success of the cell isolation, and thus this technique has the potential to reduce the number of organisms used in experiments. Therefore, at a practical level, in order to allow the comparison of toxicological data from different aquatic ecotoxicology and physiological

studies, a standardized procedure for DSI and DSII production is required. Here we present the most current and readily available standardized DSI technique (**Fig. 1a**), as well as the development of a DSII (**Fig. 1b**) technique. The protocols for the DSI and DSII techniques are summarized in a flow diagram (**Fig. 2**), and typical characteristics of the cultured epithelia are described in the ANTICIPATED RESULTS section. The controls for an experiment using DSI and DSII will depend on their intended use. For a typical experiment in which the cells are to be exposed to a compound in water^{13,18–20}, a set of control inserts that are exposed to either medium or water without the test compound should be set up. By comparing results, such as expression of a gene of interest, between the test and controls, it is possible to determine whether the response is due to the effect of exposure to water, or to water and the test compound. To perform experiments that provide statistical rigor, it is recommended that the experiment include inserts derived from several different preparations—i.e., biological replicates.

MATERIALS

REAGENTS

- Juvenile rainbow trout (<100 g) should be acclimatized for at least 2 weeks and maintained according to relevant governmental and institutional guidelines at 13–14 °C in good-quality water with constant 14-h light and 10-h dark photoperiods **▲ CRITICAL** 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. **! CAUTION** Experiments must conform to relevant institutional and governmental regulations.
- Leibovitz's L-15 medium, no phenol red, with L-glutamine (Invitrogen, cat. no. 21083-027) **▲ CRITICAL** We recommend using Leibovitz's L-15 medium without phenol red, because there has been reports that phenol red may contain estrogenic activity that may influence physiological or toxicological responses³⁰.
- FBS (Sigma-Aldrich, cat. no. F7524)
- Penicillin-streptomycin (5,000 units ml⁻¹ penicillin and 5 mg ml⁻¹ streptomycin; Invitrogen, cat. no. 15070063)
- Gentamicin solution (10 mg ml⁻¹; Invitrogen, cat. no. 15710049)
- Fungizone (100 mg; Sigma-Aldrich, cat. no. A9528)
- Trypsin-EDTA, 0.5% (wt/vol), ×10 (Invitrogen, cat. no. 15400054)
- PBS (used as prewash solution)
- Ethanol (VWR International, cat. no. 20821.321)
- Calcium chloride dihydrate (Sigma-Aldrich, cat. no. 223506)
- Magnesium sulfate heptahydrate (Sigma-Aldrich, cat. no. 230391)
- Sodium bicarbonate (Sigma-Aldrich, cat. no. S6014)
- Potassium chloride (Sigma-Aldrich, 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 International, cat. no. INS4808; INS4854) and scalpels (Swann-Morton, cat. no. 0501) **▲ CRITICAL** All equipment must be autoclaved or sterilized using 70% (vol/vol) ethanol.
- Portable gyratory shaker, model IKA-VIBRAX-VXR
- Adjustable pipettes: P-20 (Gilson, cat. no. FA10003M), P-200 (Gilson, cat. no. FA10005M) and P-1000 (Gilson, cat. no. F123602)
- Pipette tips: 20 µl (Starlab, cat. no. S1120-1810), 200 µl (Starlab, cat. no. S1120-8810) and 1,000 µl (Starlab, cat. no. S1122-1830)
- Tissue culture pipettes: 5 ml (Corning, cat. no. CORN4051), 10 ml (Corning, cat. no. CORN4101) and 25 ml (Corning, cat. no. CORN4250)
- Inverted phase-contrast microscope, TE200, Nikon Eclipse
- Cell culture cooling incubator, MIR-1554, Sanyo, 18 °C, no CO₂ atmosphere required
- Hemocytometer (Neubauer)

- EVOM epithelial voltammeter modified by the manufacturer to read TER up to 100,000 Ω cm⁻² (World Precision Instruments)
- EVOM 'chopstick' electrodes, no. STX-2 (World Precision Instruments)
- Inserts, 12-well, 0.4 µm, 0.9 cm² 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
- Sterile syringe filter, 0.2 µm (VWR International, 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 International, cat. no. 25382-166)

REAGENT SETUP

PBS PBS is 137 mM sodium chloride (Sigma-Aldrich, cat. no. 746398), 2.7 mM potassium chloride (Sigma-Aldrich, cat. no. P9333), 10 mM disodium hydrogen phosphate (Sigma-Aldrich, cat. no. 255793) and 1.8 mM potassium dihydrogen phosphate (Sigma-Aldrich, cat. no. P0662). It is prepared in dH₂O and autoclaved. This can be stored for 1 month at room temperature (~20 °C). Before use, place it on ice.

Ethanol, 70% Prepare a 70% (vol/vol) solution of ethanol in dH₂O.

Fungizone Prepare a 10 mg ml⁻¹ solution and store it in 1-ml aliquots at -20 °C until further use.

Wash solution Add 1.2 ml of penicillin-streptomycin, 1.2 ml of gentamicin and 90 µl of Fungizone solution to a final volume of 30 ml of PBS. Prepare the solution at room temperature in sterile conditions in a tissue culture hood on the day of cell isolation. Before use, place it on ice.

Trypsin solution Dilute 0.5% (wt/vol) trypsin-EDTA to 0.05% by pipetting 650 µl into a conical centrifuge tube and by bringing the volume up to 6.5 ml with PBS. Prepare the solution at room temperature in sterile conditions in a tissue culture hood on the day of cell isolation.

Stop solution Pipette 2 ml of FBS into a conical centrifuge tube with 18 ml of PBS. Prepare it at room temperature in sterile conditions in a tissue culture hood on the day of cell isolation. Before use, place it on ice.

Rinse solution Add 0.5 ml of FBS in a tube, and make up the final volume to 19.5 ml with PBS. Prepare this solution at room temperature in sterile conditions in a tissue culture hood on the day of cell isolation. Before use, place it on ice.

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

Cell culture medium with FBS (medium B) Pipette 27 ml of FBS, sterile filtered, to 500 ml of L-15 medium. This can be made in advance and stored at 4 °C for up to 1 month.

Exposure cell culture medium L-15 without any supplementation can be stored at 4 °C for up to 1 month.

Artificial freshwater Freshwater is defined as water prepared to the standard used for maintaining fish, such as that recommended by the Organisation for Economic Co-operation and Development (OECD) and the International Organization for Standardization (ISO). To make up OECD freshwater, prepare the following four solutions (40× stocks). Solution 1: dissolve 11.76 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in dH_2O , and adjust the volume to 1 liter with dH_2O (0.08 M). Solution 2: dissolve 4.93 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in dH_2O ,

and adjust the volume to 1 liter with dH_2O (0.02 M). Solution 3: dissolve 2.59 g of NaHCO_3 in dH_2O , and adjust the volume to 1 liter with dH_2O (0.03 M). Solution 4: dissolve 0.23 g of KCl in dH_2O , and adjust the volume to 1 liter (0.003 M). Once all the solutions are prepared, mix 25 ml of each solution, and the total volume is made up to 1 liter with dH_2O . Aerate the dilution water until oxygen saturation is achieved, and then store it for about 2 d with further aeration before use²³.

Natural freshwater Filter natural freshwater samples using a 0.2- μm sterile syringe filter before exposure to the cell culture.

PROCEDURE

Preparation of solutions and culture inserts ● **TIMING 1 h, plus 1.5 h of incubation to condition the membrane**

▲ **CRITICAL** The recommended volumes in this section are appropriate for one cell isolation procedure.

- 1| Prepare the two types of cell culture media with antibiotics (medium A) and without antibiotics (medium B; see Reagent Setup). Take a 50-ml aliquot of each and keep them on ice.
- 2| Prepare the prewash solution (18 ml), wash solution, trypsin solution, stop solution and rinse solution, and keep them 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 the lid on top. 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** For DSI, the flanges of the inserts rest between the notches of the well to minimize medium wicking and to assure the proper fitting of the plate with the lid.

▲ **CRITICAL STEP** For DSII, 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 the medium from 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. For DSI preparations, add 200 μl of cell culture medium with FBS and antibiotics (A) in the upper compartment. For DSII, add 200 μl of cell culture medium with FBS and antibiotics (A) onto the top of the upside-down insert. Close the systems and keep them at 18 °C in a cell culture incubator for at least 1.5 h.

Rainbow trout dissection and gill cell isolation ● **TIMING 3 h**

- 5| Euthanize one rainbow trout.

! **CAUTION** Euthanize rainbow trout according to national and institutional guidelines. We follow UK Home Office Schedule 1 procedures in which the fish is euthanized via the destruction of the brain, which is achieved by a blow to the cranium and the use of a sharp probe.

- 6| Remove the head behind the operculum with a sharp knife.

▲ **CRITICAL STEP** It is important to perform this procedure quickly so as to reduce the extent of gill hemorrhaging and to avoid excessive bleeding and clotting on the filaments, which will reduce the quality of the gill cells obtained.
- 7| 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 the gill arches into 10 ml of prewash solution in a Petri dish (**Fig. 3b**).

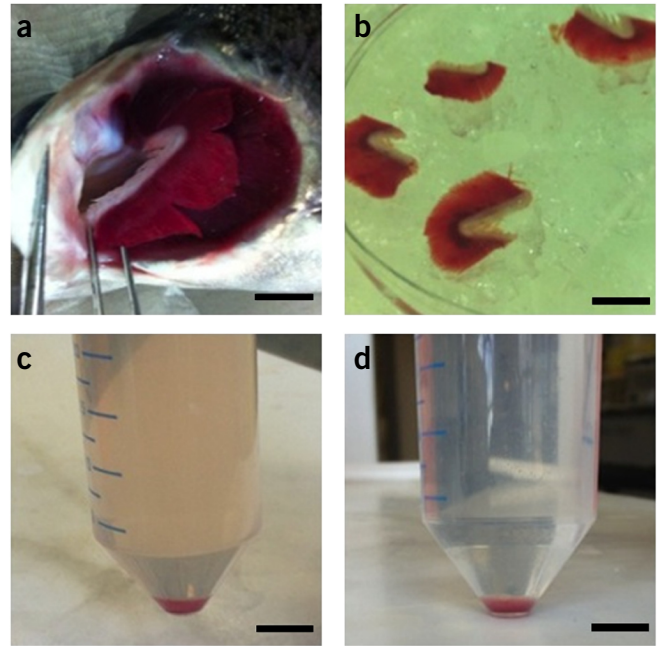
▲ **CRITICAL STEP** Avoid damage to the filaments by holding the cartilage of the arch and not the filaments.
- 8| Remove mucus by carefully blotting each gill arch onto tissue paper, and then cut the filaments from the arches and place the filaments into 10 ml of wash solution (prepared in Reagent Setup) 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.
- 9| Tease the filaments apart using forceps, and cut them 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.
- 10| Pour away this wash solution, and add the filaments to a 10-ml aliquot of wash solution in a 50-ml conical centrifuge tube, and place the tube on ice. Incubate the tube for 10 min.

▲ **CRITICAL STEP** From this step onward, sterile culture technique must be used, and the procedures should therefore take place in a laminar flow hood.

PROTOCOL

Figure 3 | Dissection of the gills and isolation of the cells. (a) Four gill arches are located underneath each operculum on the lateral sides of the rainbow trout head. Scale bar, 0.5 cm. (b) Gill arches are excised by cutting the cartilage of the arch, and these are placed into 20 ml of prewash solution (PBS). Scale bar, 1 cm. (c,d) The gill cell pellet is obtained after centrifugation in Step 21 (c) and Step 22 (d). Trapped red blood cells give the red color. Scale bars, 1 cm.



11 | Centrifuge the tube for 4 min at 250g at 4 °C to collect the filaments. Aspirate and discard the supernatant.

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

13 | Centrifuge again for 4 min at 250g at 4 °C, and then aspirate and discard the supernatant.

14 | Add 500 µl of trypsin solution to the pelleted filaments, and agitate to remove any remaining wash solution. Centrifuge the tube for 4 min at 250g at 4 °C, and then aspirate and discard the supernatant.

15 | Add 3 ml of trypsin solution to begin tryptic digestion, and shake for 12 min at 200 r.p.m. at room temperature.

16 | To mechanically agitate the cut gill filaments and to improve cell yield, pass the filaments in trypsin solution repeatedly through a wide-bore pipette tip immediately after the 12-min trypsinization period. A wide-bore pipette tip can be prepared by removing the tip of a 1,000-µl pipette tip, so that the bore of the pipette aperture is ~2 mm. Sterilize by storing the cut pipette tip in 70% (vol/vol) ethanol. Before 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 onto the 50-ml conical centrifuge tube containing 20 ml of stop solution (prepared in Reagent Setup) on ice. Pipette the filament/trypsin solution into the strainer, and agitate it to facilitate the collection of gill cells as a single-cell suspension in stop solution.

▲ CRITICAL STEP After 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 them back into their original conical centrifuge tube. Repeat tryptic digestion by adding the remaining 3 ml of trypsin solution and by shaking for a further 12 min at 200 r.p.m. at room temperature.

19 | Place a new 100-µm cell strainer onto the same 50-ml conical centrifuge tube containing the stop solution and the previously trypsinized cells, and repeat the gill cell isolation as in Step 13.

20 | Centrifuge the cell suspension for 10 min at 250g at 4 °C. A red pellet should be obtained (**Fig. 3c**). Aspirate and discard the supernatant.

? TROUBLESHOOTING

21 | Resuspend the red pellet in 2 ml of rinse solution (prepared in Reagent Setup), and then add the remaining 18 ml; agitate the mixture briefly and centrifuge it for 10 min at 250g at 4 °C. This should result in another red-colored pellet (**Fig. 3d**). Aspirate and discard the supernatant.

22 | Resuspend the red pellet initially in 2 ml of cell culture medium with FBS and antibiotics (medium A), and flick the tube to dislodge the pellet many times; next, resuspend in 8 ml (for a total of 10 ml) and mix it well.

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

24 | Count the viable cells using a hemocytometer on the basis of the trypan blue exclusion criterion.

▲ CRITICAL STEP Exclude blood cells from the cell count. These appear as ellipsoidal cells, which are smaller than the gill cells.

25| Determine the concentration of cells and adjust cell counts of cell suspension from Step 21 to required seeding density detailed in the following steps.

? TROUBLESHOOTING

■ **PAUSE POINT** Cells can be left up to 2 h on ice.

Cell seeding

26| Follow option A to seed the cells for a DSI experiment, and follow option B to seed cells for a DSII experiment.

(A) DSI cell seeding ● **TIMING 30 min**

- (i) Aspirate the medium conditioning the cell culture inserts (from Step 4) before cell seeding. Seed 1.2×10^6 cells in 800 μ l of cell culture medium with FBS and antibiotics (medium A) per insert. In addition, set up three blank inserts (no cells) for TER measurements. Place 1 ml of cell culture medium with FBS and antibiotics (medium A) into the basolateral compartment. This is termed 'day 0'.
 ▲ **CRITICAL STEP** It is recommended that when establishing the protocol an insert seeding density gradient be performed to determine the optimum seeding density for producing inserts with consistently tight epithelia¹⁰.
- (ii) Incubate the cells at 18 °C overnight.
- (iii) After the overnight incubation, begin a new gill cell preparation using another fish, by repeating Steps 5–25.
- (iv) To prepare the previously seeded inserts ('day 0') for double-seeding (from Step 26A(i)), aspirate the medium first from the lower compartment and then carefully from the upper compartment. Wash the surface of the inserts by slowly applying 200 μ l 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 you are removing or changing the medium, always aspirate the lower compartment first, and then the upper one. This maintains the hydrostatic pressure onto the cells and it prevents them from lifting off.
- (v) Repeat the wash as described in Step 26A(iv), with 200 μ l of PBS.
 ? **TROUBLESHOOTING**
- (vi) Carefully apply the new gill cell preparation (obtained in Step 26A(iii)) directly on top of the previously seeded cells. Seed 1.2×10^6 cells in 800 μ l of cell culture medium with FBS and antibiotics (medium A), and add 1 ml in the lower compartment afterward. Also apply medium to your blank insert controls. This is termed 'day 1'. Incubate the cells at 18 °C.
- (vii) After 24 h, wash the inserts again, as described in Step 26A(iv,v).
- (viii) Add maintenance volumes of culture medium; 1.5-ml cell culture medium with FBS and antibiotics (medium A) in the upper compartment and then 2.0 ml in the lower compartment. This is termed 'day 2'. Incubate the cells at 18 °C.

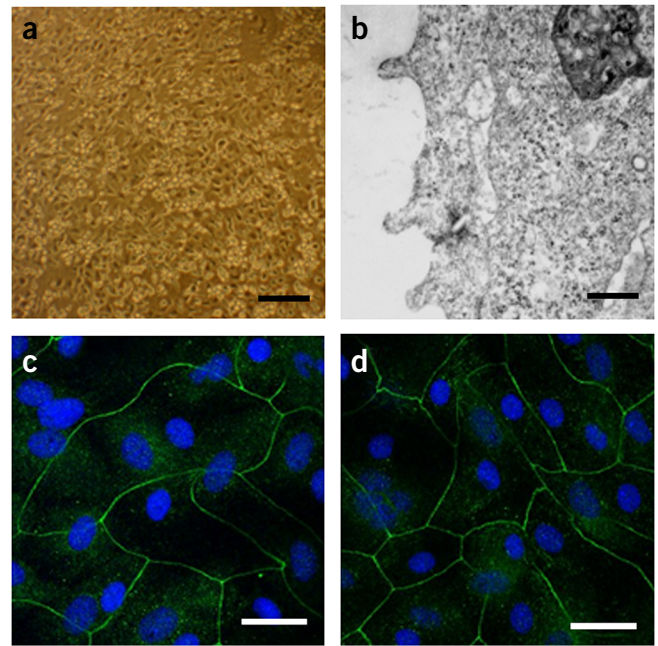
(B) DSII cell seeding ● **TIMING 30 min**

- (i) Aspirate the conditioning medium from the inverted cell culture inserts (from Step 4) before seeding. Seed 1.2×10^6 cells in 200 μ l of cell culture medium with FBS and antibiotics (medium A) per inverted insert, and leave them at 18 °C in the sterile tip box. Also set up three blank inserts (no cells) for blank TER measurements. This is termed day 0.
 ▲ **CRITICAL STEP** It is recommended that when establishing the protocol an insert seeding density gradient be performed to determine the optimum seeding density to produce inserts with consistently tight epithelia¹⁰.
- (ii) Incubate the cells at 18 °C overnight.
- (iii) After the overnight incubation and 24 h after Step 26B(i), begin a new gill cell preparation using another fish, by repeating Steps 5–25.
- (iv) To prepare the previously seeded inserts ('day 0') for double-seeding, wash the inverted inserts by aspirating the medium and by carefully and slowly applying 200 μ l of PBS.
- (v) Repeat another 200- μ l PBS wash.
 ? **TROUBLESHOOTING**
- (vi) Carefully apply the new gill cell preparation (obtained in Step 26B(iii)) directly on top of the previously seeded cells. Seed 1.2×10^6 cells in 200 μ l of cell culture medium with FBS and antibiotics (medium A), and leave them in the sterile pipette tip box overnight at 18 °C. This is termed 'day 1'.
- (vii) Twenty-four hours later, wash the inserts again, as described in Step 26B(iv,v). The inserts can now be placed the right way up in companion wells. Replace it with maintenance volumes of culture medium: 1.5 ml of cell culture medium with FBS and antibiotics (medium A) in the upper compartment and then 2.0 ml in the lower compartment. Incubate the inserts at 18 °C. This is termed 'day 2'.

Maintenance ● **TIMING 5–12 d**

27| Incubate the inserts until day 4. On day 4, remove the medium and replace it with cell culture medium with FBS (medium B) without antibiotics. Continue to incubate cells at 18 °C, replacing the medium every 48 h. Monitor appropriate growth of the epithelia under a light microscope (**Fig. 4**). Cell culture inserts should be observed on a daily basis to

Figure 4 | Images of the double-seeded insert epithelium. (a) Phase-contrast micrograph of DSI gill epithelial cells. Scale bar, 100 μm . (b) Transmission electron micrograph of DSI gill epithelia showing the characteristic microridges on the apical surface. Scale bar, 200 nm. (c,d) Confocal microscope images of DSI epithelia in which 24 h before cell fixing, epithelia were exposed to symmetrical conditions with L-15 medium on both sides of the epithelium (c) or asymmetrical conditions with freshwater in the upper compartment and L-15 medium in the companion well (d), and cell nuclei were stained with 5 μM Hoechst (blue) and tight junctions with zonula occludens 1 antibody (green). Scale bars, 50 μm . (See **Supplementary Methods** for details of how we stained tight junctions using zonula occluden 1-specific antibodies.)



check for (bacterial) contamination. Any contaminated cell culture inserts should be immediately removed, and the associated plate well should be rinsed with 70% (vol/vol) ethanol. From day 4 onward, TER can be monitored daily if desired, as described in the following section.

▲ CRITICAL STEP Careful and thorough observation of culture preparations should take place before measuring TER (see following section) or changing the medium. Therefore, if one insert is found to be contaminated, it can be removed without cross-contaminating other epithelia.

Monitoring TER ● TIMING 10 min

▲ CRITICAL From day 4 onward, a daily measurement of TER can be made using a custom-modified (see Equipment) epithelial tissue voltohmmeter fitted with chopstick electrodes, as described in this section.

28| Sterilize the electrodes in 70% (vol/vol) ethanol and rinse them in PBS.

29| Confirm that the cells are not contaminated. If the cells are showing no signs of contamination, insert the electrode 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. 5a**), and measure TER.

30| Calculate the net TER by subtracting the average TER of the blank insert (no cells; Step 26A(i) for DSI and Step 26B(i) for DSII) from the experimental value. Obtain final resistance-area values ($\Omega \text{ cm}^2$) by multiplying the net TER by the effective growth area (0.9 cm^2 for 12-well inserts). During days 7–14, DSI and DSII inserts should show TER values of ~5,000–30,000 $\Omega \text{ cm}^2$ (**Fig. 5b** and **Supplementary Fig. 1a**). Once a TER value of 5,000 $\Omega \text{ cm}^2$ or above is obtained, if desired you can proceed to assaying freshwater, as described in the next section.

? TROUBLESHOOTING

■ PAUSE POINT Epithelia can be stored with medium A in both the apical and basolateral compartments for later use by maintaining them at 4 $^{\circ}\text{C}$ for up to 2 weeks³¹.

Experimental assay ● TIMING 30 min

31| Remove the medium from the lower compartment, followed by the upper compartment.

32| To wash and remove traces of FBS, apply 800 μl of PBS in the upper compartment, and then apply 1,000 μl to the lower compartment.

33| Aspirate the lower compartment and wash a further two times before the final aspiration.

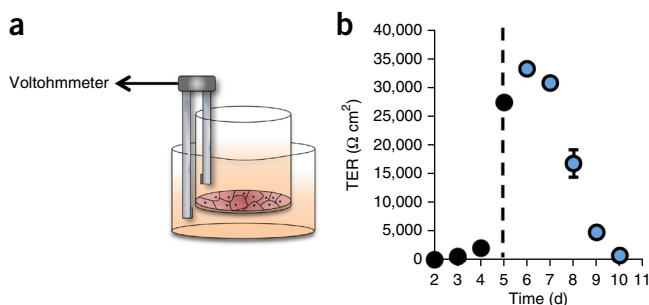


Figure 5 | Membrane development can be monitored by daily TER measurements. (a) A custom-modified epithelial tissue voltohmmeter is fitted with chopstick electrodes. The electrode arms are inserted above and below the gill cell epithelium of each insert, such that one arm of the electrode is in the upper compartment and the other arm is in the lower (bottom) compartment. (b) Measurements of TER in developing DSI. Freshwater (blue points) was added (after the dotted line) to the companion well compartment (the apical cell surface, while the insert contained L-15 medium) on day 4, resulting in an increase in TER still evident 24 h later on day 5 ($n = 6$). Values are means \pm s.e.m.

34 | Apply the solution of interest, e.g., freshwater, to the test compartment. For DSI, apply 1.5 ml of freshwater into the upper compartment, while leaving the lower compartment in 2.0 ml of L-15 medium. Depending on the experimental design, FBS can be removed from the medium. For DSII, apply 1.5 ml of L-15 medium without FBS into the upper compartment; 2.0 ml of freshwater can now be applied to the lower compartment. The volume in the upper compartment can be reduced if required. Application of freshwater should cause a rise in TER (**Supplementary Fig. 1a**). After freshwater application, inserts around 5,000 Ω cm² may rise to 30,000 Ω cm², whereas those already around 25,000–30,000 Ω cm² may not rise by as much. After freshwater application, TER values should remain above 5,000 Ω cm² for up to 48 h.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
20	Pellet is not red	Excessive trypsinization	Check the trypsin concentration and batch, and reduce the trypsinization time
		Trypsin digestion has not stopped	Check the FBS, and if it is out of date, remake the stop solution
25	Low cell viability	Excessive trypsinization	Decrease the trypsinization time
		Low donor quality	Check the health of stock fish
	Low cell number	Slow work pace	Perform the procedure faster
		Low EDTA concentrations in trypsin solution (Reagent Setup)	Increase EDTA concentration to 5 mM in the trypsin solution (Reagent Setup)
26A(v), 26B(v)	Extra mucus on cells	Fish health-related problem	Perform an additional PBS wash
30	Low TER	Contamination	Discard the cells
		Seeding density	Perform a seeding-density assay
		Fish health-related problem	Check the health of stock fish and water quality of the fish tank
		Slow development	Keep monitoring, and discard it if TER is <5,000 Ω cm ² by day 14

● TIMING

Steps 1–4, preparation of solutions and cell culture inserts: 1 h; inserts also need to be left to condition for at least 1.5 h

Steps 5–25, rainbow trout dissection and gill cell isolation: 3 h

Step 26, DSI/DSII cell seeding: 30 min

Steps 27–30, maintenance and monitoring: this will depend on the number of inserts, and it can take between 30 min and 2 h on the day of medium changes; cell preparation reaches maturity over 5–12 d

Steps 31–34, experimental assay: 30 min

ANTICIPATED RESULTS

The epithelia should have distinct apical and basolateral surface consisting of irregularly shaped epithelial (pavement) cells with microridges and plasma membranes (**Fig. 4b** and **Supplementary Fig. 1c,d**) interspersed with MRCs (**Supplementary Fig. 1e**) typically from day 5 onward while growing in both the DSI and DSII configurations. Both preparations should develop an electrically tight epithelium showing the presence of the TJ proteins, such as zonula occludens (**Fig. 4c,d** and **Supplementary Fig. 1f,g**), typically from day 5 onward. The DSI can withstand water applied to the apical surface, but it does not tolerate water when exposed via the basolateral compartment³¹, and these traits are also evident in the DSII preparations (**Supplementary Fig. 1b**). On the basis of rhodamine 123 staining⁶, the epithelium should consist of between 10 and 15% MRCs, 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*¹.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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