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Procedures for the preparation and culture of 'reconstructed' rainbow trout branchial epithelia

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Abstract. Techniques for the *in vitro* 'reconstruction' of freshwater rainbow trout branchial epithelia using the primary culture of gill cells on permeable polyethylene terephthalate cell culture filter supports are described. Representing models of the freshwater fish gill, epithelia grown by two separate techniques are composed of branchial pavement cells with or without the inclusion of mitochondria-rich (MR) cells. The generation of epithelia consisting of pavement cells only (via a method called single seeded inserts = SSI) involves an initial period of flask culture during which time MR cells, that appear unable to attach to the culture flask base, are excluded from the general cell populace. Alternately, the generation of a heterogeneous epithelia consisting of both pavement cells and MR cells (via a method called double seeded inserts = DSI) is facilitated by the direct seeding of cells into cell culture filter inserts. Critical to this second procedure is the repeat seeding of filter inserts over a two day period. Repeat seeding appears to allow MR cells to nest amongst the attached cell layer generated by the first day's seeding. The use of cell culture filter supports allows free access to both the apical and basolateral compartment of the epithelium and is ideal for experimental manipulation. Cells are grown under symmetrical conditions (apical media/basolateral media) and epithelium growth is measured as a function of transepithelial resistance (TER). When the epithelia exhibit a plateau in growth they can be subjected to asymmetrical conditions (freshwater apical/media basolateral) in order to assess gill cell function as *in vivo*.

Key words: Cell culture, Filter inserts, Gill, Mitochondria-rich cells, Pavement cells

Abbreviations: DSI = double seeded insert; MR = mitochondria-rich; SSI = single seeded insert; TER = transepithelial resistance

1. Introduction

The fish gill is an architecturally complex organ and like many other epithelia, it is composed of several cell types. This presents certain problems that hinder mechanistic understanding of ion transport across the branchial epithelium *in vivo*. Firstly, the structural complexity of the gill prohibits the application of key *in vivo* physiological techniques and secondly, the cellular heterogeneity of the epithelium results in difficulties associated with pinpointing specific cellular sites of ion transport. Stemming from these fundamental obstacles was the development of flat epithelial surrogate gill models, such as the opercular epithelium [14, 20] or jaw skin epithelium [25], that are now well established tools in the assessment of ion transport mechanisms across epithelia representing the seawater fish gill [27, 30]. However, despite an equally substantive research effort [4, 14, 26, 28, 29, 41], a comparable model for the freshwater fish gill still proves elusive.

The majority of gill cell types, by number and/or mass, fall into two categories: the pavement (or respiratory) cells and the mitochondria-rich (MR, or chloride) cells. It is, therefore, not surprising that both these cell types are currently accepted as 'major players' in fish ionic regulation [17, 34]. By utilizing methods for the primary culture of freshwater fish gill cells on cell culture filter supports, an entirely different approach towards the development of a surrogate freshwater fish gill model can be taken. These methods allow us to obtain a 'reconstructed' flat epithelial gill model for freshwater fish and, by virtue of our ability to culture pavement cells either alone or in conjunction with MR cells, provide the potential for considerable insight into the role that these two primary cell types play in ionic regulation.

Currently, our research is focused on using methods of primary gill cell culture to elucidate freshwater fish ion transport mechanisms that remain highly controversial [10, 21, 34] and a growing interest in fish gill cell culture would seem to indicate

that the use of such techniques is likely to gain momentum [12, 23, 36, 39]. In addition, the cultured gill epithelium has several applications in toxicology. It offers the possibility to investigate and understand toxic mechanisms at the cellular level in the gills, mechanisms that cannot be approached with general *in vivo* methods. The preparation has been used to investigate the effects of copper on the barrier properties of the gill epithelium (membrane and tight junction integrity; [19]) and has also been used to prove the presence of Phase I and Phase II drug metabolism in the gill epithelium, indicating an active role of the epithelium in the turn-over of persistent organic contaminants [6, 7, 24]. This report describes the culture systems and procedures that have been routinely used in our laboratory and have proven successful in the generation of a flat 'reconstructed' model for the freshwater fish gill [13, 15, 16, 42, 43].

2. Materials

A. Equipment

1. Laminar flow hood, No. NU-425-400 1, Nuaire.[9]
2. Centrifuge, refrigerated, model J-21C, rotor JA-20 2, Beckman.[4]
3. Dissecting equipment.
4. Portable gyrotory® shaker, model G2.[15]
5. Adjustable pipettes: Nos. P-100; P-1000; P-5000, Gilson.[12]
6. Compound microscope, No. D 35893, Leitz.[7]
7. Inverted phase contrast microscope, No. 471481, Zeiss.[5]
8. Photo-invertoscope, No. IM 35, with green filter set No. 487715, Zeiss.[5]
9. Water-jacketed incubator, No. 3158 S/N 31097–2437, Forma Scientific.[10]
10. Bright line® hemocytometer, American Optical.[1]
11. EVOM™ epithelial voltohmmeter modified to read TER up to 100,000 Ω, World Precision Instruments.[19]
12. EVOM™ 'chopstick' electrodes, No. STX-2, World Precision Instruments.[19]

B. Chemicals

1. Leibovitz's L-15 medium (containing L-glutamine and phenol red), No. 11415-064, GIBCOBRL®.[8]
2. Fetal bovine serum, certified, relative growth factor > 1, No. 16000-044, GIBCOBRL®.[8]
3. Trypsin-EDTA, 0.5% trypsin, 5.3 mM EDTA.4Na, 10X, liquid, No. 15400-054, GIBCOBRL®.[8]
4. Penicillin-streptomycin, liquid, No. 15070-063, GIBCOBRL®.[8]
5. Fungizone® antimycotic, lyophilized, No. 15295-017, GIBCOBRL®.[8]
6. Gentamicin reagent solution (10 mg/ml), liquid, No. 15710-072, GIBCOBRL®.[8]
7. Sodium chloride, No. 7560-1, BDH.[2]
8. Potassium chloride, No. 645, BDH.[2]
9. di-Sodium hydrogen orthophosphate, dibasic, anhydrous, No. 807, BDH.[2]
10. Potassium dihydrogen orthophosphate, monobasic, No. 657, BDH.[2]
11. Ethylenediaminetetraacetic acid (EDTA), disodium salt, dihydrate, No. ED2SS, Sigma-Aldrich.[17]
12. Eosin, 1% w/v, No. RO3378, BDH.[2]
13. Rhodamine 123, No. R-302, Molecular Probes.[13]
14. 4-(4-(dimethylamino)styryl)-N-methyl-pyridinium iodide (4-Di-1-ASP), No. 288, Molecular Probes.[13]
15. 70% ethanol, Commercial Alcohols.[6]

C. Supplies

1. Falcon® BLUE MAX™ 50 ml polypropylene conical tubes, 30 × 115 mm style, No. [35]2070, Becton Dickinson.[4]
2. Falcon® BLUE MAX™ 15 ml polystyrene conical tubes, 17 × 120 mm style, No. [35]2095, Becton Dickinson.[4]
3. Falcon® Tissue culture flasks, 50 ml, 25 cm^2, blue plug seal cap, No. 3014, Becton Dickinson.[4]
4. Falcon® Tissue culture flasks, 250 ml, 75 cm^2, blue plug seal cap, No. 3024, Becton Dickinson.[4]
5. Cell strainers, 100 μm, No. 2360, Becton Dickinson.[4]
6. Falcon® cell culture inserts, 12 well size, 0.4 μm pore size, 1.6×10^6 pore density, transparent No. 3180, Becton Dickinson.[4]
7. Falcon® multiwell™ cell culture insert plate, 12 well size. No. 3503, Becton Dickinson.[4]
8. Disposable transfer pipettes, 3 ml, No. 60872.448, VWR.[18]
9. Single use Acrodisc® syringe filters, low protein binding, 25 mm, 0.2 μm, contains HT Tuffryn® membrane, No. 4192, Gelman.[11]
10. Cryogenic vials, 2 ml, No. 5000-0020, Nalge.[14]
11. Pipette tips, Nos. 115 (1–200 μl), 111 (101–1000 μl), 090 (1000–5000), Quality Scientific Plastic.[16]

3. Procedures

A. Preparation of solution:

1. Penicillin-streptomycin solution:
   a) Sterilize penicillin-streptomycin solution using a 0.22 μm Acrodisc® filter.
   b) Divide into 10 ml aliquots and store at –20 °C in sterile 15 ml conical centrifuge tubes.

2. Gentamicin solution:
   a) Sterilize gentamicin solution using a 0.22 μm Acrodisc® filter.
   b) Divide into 10 ml aliquots and store at room temperature (in the dark) in sterile 15 ml conical centrifuge tubes.
3. Fungizone solution:
   a) Reconstitute lyophilized fungizone® antimycotic in sterile double distilled water according to the manufacturer's instruction (should be 250 μg/ml amphotericin B and 250 μg/ml sodium desoxycholate).
   b) Divide into 0.3 ml aliquots and store at –20 °C in sterile cryopreservation tubes.
4. Phosphate buffered saline (PBS, pH 7.7):
   a) In 800 ml of Nanopure™ distilled water dissolve:
      – 8.0 g NaCl
      – 1.15 g Na_2HPO_4
      – 0.2 g KH_2PO_4
      – 0.2 g KCl
   b) Adjust to pH 7.7.
   c) Bring volume to 1 liter with Nanopure™ distilled water.
   d) Sterilize by autoclaving and store at room temperature.
5. 'WASH' solution:
   Make up the following solutions to 30 ml with PBS (pH 7.7):
   – 1.2 ml Acrodisc®-sterilized penicillin-streptomycin solution.
   – 1.2 ml Acrodisc®-sterilized gentamicin solution.
   – 0.3 ml fungizone solution.
6. 'TRYP+EDTA' solution:
   Make up the following solutions to 50 ml with PBS (pH 7.7):
   – 5 ml (0.5% trypsin, 5.3 mM EDTA.4Na) (×10).
   – 0.5 ml 2% EDTA ($2Na \cdot 2H_2O$) in PBS (pH 7.7).
7. 'STOP' solution:
   Add 2 ml fetal bovine serum to 18 ml PBS (pH 7.7).
8. 'RINSE' solution:
   Add 0.5 ml fetal bovine serum to 19.5 ml PBS (pH 7.7).
9. 'MEDIA + ANTIBIOTIC':
   Make up the following solutions to 100 ml with L-15 media:
   – 5 ml fetal bovine serum.
   – 2 ml Acrodisc®-sterilized streptomycin-penicillin solution.
   – 2 ml Acrodisc®-sterilized gentamicin solution.
10. 'MEDIA – ANTIBIOTIC':
    Add 5 ml fetal bovine serum to 95 ml L-15 media.
11. Rhodamine 123 solution:
    a) Dissolve rhodamine 123 at 1 mg/ml in PBS (pH 7.7).
    b) Sterilize using a 0.22 μm Acrodisc® filter and store at 0–4 °C in a light protected bottle.
12. DASPEI [4-(4-(dimethylamino)styryl)-N-methyl-pyridinium iodide (4-Di-1-ASP)] solution:
    a) Dissolve DASPEI at 1 mg/ml in PBS (pH 7.7)
    b) Sterilize using a 0.22 μm Acrodisc® filter and store at 0–4 °C in a light protected bottle.

B. Procedures for the preparation and culture of 'reconstructed' SSI epithelia (comprising pavement cells only)
   1. Preparation of fish and gills
      a) Rainbow trout (optimal size range 80–150 g) are stunned by a blow to the head and then decapitated with a sharp knife. All manipulations from this point onwards are conducted under strictly sterile conditions and ideally at a temperature lower than 20 °C. The fish head is placed on a paper towel and relocated to a laminar flow hood. Using dissecting equipment that has been swabbed with 70% ethanol, the opercula are removed and all the gill arches cut out. The gill arches are placed in a small petri dish containing PBS (pH 7.7). The remaining fish parts are removed from the laminar flow hood and the working area swabbed thoroughly with 70% ethanol. Gill arches are blotted to remove excess mucus and the filaments are cut parallel to the axis of the cartilaginous arch.
      b) First wash; the gill filaments are placed in 10 ml 'WASH' solution for 10 min.
      c) Second wash; the gill filaments are cut perpendicular to the prior axis of removal so as to obtain 3–5 filaments per cut. Cut filaments are placed in a 50 ml conical centrifuge tube and 10 ml fresh 'WASH' solution added. The filaments are 'washed' for another 10 min with frequent manual agitation to ensure thorough mixing.
      d) Third wash; aspirate wash solution from conical centrifuge tube and add remaining 10 ml of fresh 'WASH' solution. The filaments are 'washed' for another 10 min with frequent manual agitation to ensure thorough mixing. Aspirate 'wash' solution and the filaments are now ready for tryptic digestion.
   2. Tryptic digestion of the gill filaments
      a) First tryptic digestion; Rinse the filaments with a few drops of 'TRYP+EDTA' solution. Aspirate and add 5 ml of 'TRYP+EDTA'

solution. Shake cells on rotator (300 rpm) for 20 min. After shaking cells, mechanically agitate with a plastic transfer pipette ≈ 50 times. Using transfer pipette, remove tryptic digest from 50 ml conical flask and place in a 100 μm cell strainer mounted over a separate 50 ml conical flask containing 20 ml 'STOP' solution. Allow tryptic digest to strain into 'STOP' solution. Remove gill filaments from cell strainer and place back in original 50 ml conical flask.

b) Second tryptic digestion; Add 5 ml 'TRYP+EDTA' to filaments and shake on a rotator for a further 20 min. After shaking cells, repeat procedures conducted in the first tryptic digestion with a fresh cell strainer mounted above original 'STOP' solution containing the cells from the first tryptic digestion. If a lot of mucus is present and the gill filaments still appear to contain cellular material, a third tryptic digestion can be conducted. If most of the cells have been removed from the gill filaments, the resulting 'digested' filaments should take on a translucent appearance. Centrifuge the 'STOP' solution (containing cells) for 10 min (500 ×*g* at 0–4 °C). After centrifugation, aspirate 'STOP' solution, leaving a cell pellet in the bottom of the 50 ml conical tube. Add pre-chilled (0–4 °C) "RINSE" solution and resuspend cell pellet using mechanical agitation. Centrifuge for 10 min (500 ×*g* at 0–4 °C). Aspirate 'RINSE' solution and add 10 ml pre-chilled (0–4 °C) 'MEDIA + ANTIBIOTIC'. Resuspend the cell pellet by mechanical agitation.

3. Loading of culture flasks and flask culture of gill cells

a) Using cells suspended in 'MEDIA + ANTIBIOTIC', determine cell numbers with a hemocytometer. Culture flasks can then be loaded with the appropriate volume of cell suspension. Load 13 million cells/ 25 cm^2 culture flask or 50 million cells/ 75 cm^2 culture flask. Make up the volume of solution in each culture flask using 'MEDIA + ANTIBIOTIC'. In 25 cm^2 and 75 cm^2 culture flasks, final volumes of 3 ml or 6 ml should be employed respectively. Screw flask stoppers on loosely and use a rocking motion to obtain an even covering of cells across the bottom of each flask. Incubate flasks for 24 h at 18 °C in an air atmosphere.

b) After 24 h incubation at 18 °C, aspirate media containing all unattached cells. Most of the unattached cell mass consists of erythrocytes, cellular debris, mucus and MR cells. Add 6 ml or 20 ml of fresh 'MEDIA + ANTIBIOTIC' to either 25 cm^2 or 75 cm^2 culture flasks respectively. Screw flask stoppers on loosely and incubate for a further 72 h at 18 °C in an air atmosphere. Prior to incubation, cells can be observed for attachment (Figure 1a).

c) After incubating flasks for a further 72 h, aspirate and change media as previously outlined. However, replacement media should now be antibiotic-free ('MEDIA – ANTIBIOTIC' solution). Incubate flasks for a further 24–48 h at 18 °C in an air atmosphere. Cells prior to harvest can be observed in Figure 1b.

Note: During flask culture, routine examination of attached cells can be conducted using an inverted microscope. Any flasks that exhibit fungal or bacterial contamination should be discarded immediately. Occasionally, presumably due to rapid growth of cells, flask media may appear acidic (orange tinge to culture media) before a scheduled media change. Under such conditions media should be changed immediately and the flasks monitored for further acidification.

4. Tryptic digestion of flask cells and seeding of cell culture filter inserts.

a) Prepare 'STOP' solution in a 50 ml conical centrifuge tube according to the amount of flasks to be trypsinated and the number of source fish used. *Cells originating from different fish should always be kept separate*. Typically, 20 ml of 'STOP' solution is needed for 8 × 25 cm^2 flasks or 4 × 75 cm^2 flasks.

b) Aspirate 'MEDIA – ANTIBIOTIC' and rinse cells with a small volume (0.5 ml) of 'TRYP+EDTA'. Aspirate rinse solution immediately and add 0.7 ml or 2 ml of 'TRYP+EDTA' to 25 cm^2 or 75 cm^2 flasks respectively. Screw flask stopper on tightly and coat the bottom of the flask with the 'TRYP+EDTA' solution. Trypsination time is generally in the region of 3 min, however, careful monitoring of the flasks using an inverted microscope to determine when most of the cells detach is essential. Over-trypsinization can easily kill the cells, and waiting for the last few cells to detach is counter-productive.

c) After approximately 3 min trypsination, knock the bottom of the flasks and pour the contents of the flask into the 'STOP' solution. Rinse the flask with PBS (pH 7.7), swirl and pour into the 'STOP' solution. Using an inverted microscope,

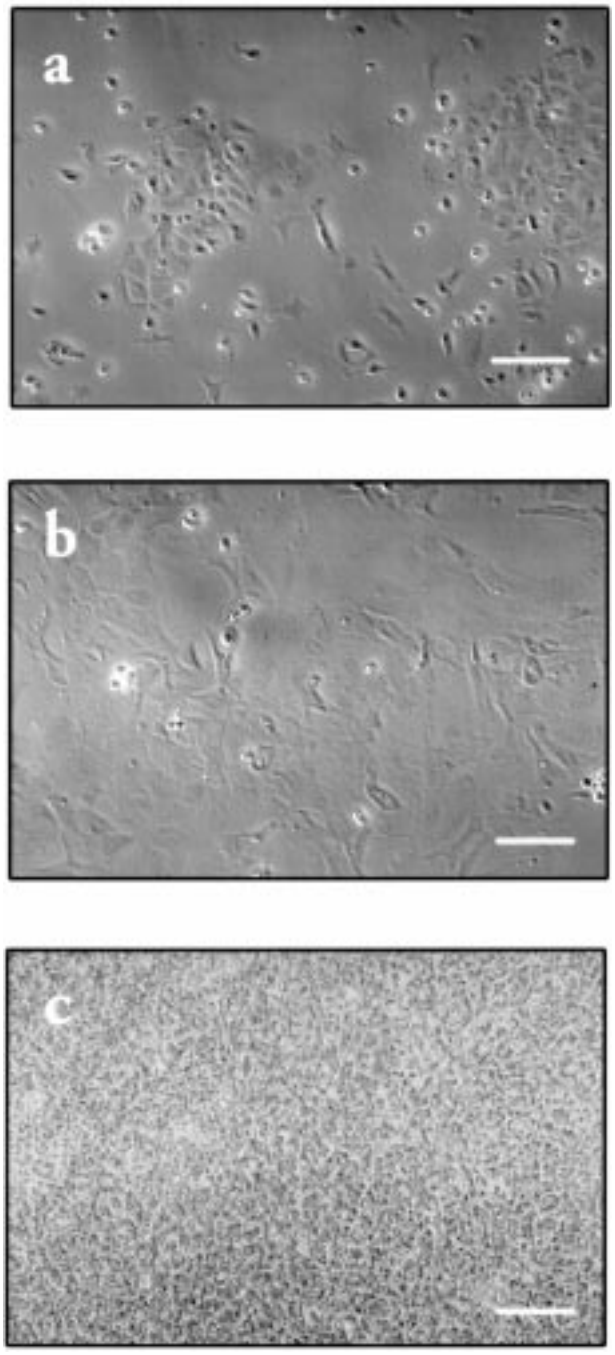

Figure 1. Phase contrast photomicrographs of cultured gill cells (a) day 1 after seeding (b) prior to harvest (day 6 in flask) and (c) on a filter insert (single seeded insert). Scale bars = 200 μm.

check all the flasks for cell detachment. If significant cell numbers appear to remain attached, repeat the trypsination procedure using a shorter trypsination time (≈ 1 min).

d) Centrifuge 'STOP' solution containing trypsinated cells for 10 min (500 ×*g* at 0–4 °C). After centrifugation, aspirate 'STOP' solution and resuspend the cell pellet in 2–3 ml (depending on pellet size) 0–4 °C equilibrated 'MEDIA – ANTIBIOTIC'. Use a hemocytometer to determine cell numbers and based on total cell counts, the number of cell inserts required can be calculated (see below). At this point cell viability can also be determined using standard techniques of eosin dye exclusion.
Note: It is desirable to continue with insert seeding immediately, however, resuspended cells can be held in a refrigerator for up to 30 min if necessary.

5. Seeding of cell culture inserts with trypsinated flask cells
   a) Place cell culture inserts into accompanying cell culture plate. Wet the inserts with a few drops of 'MEDIA – ANTIBIOTIC'. Allow inserts to sit for several minutes making sure that 'MEDIA – ANTIBIOTIC' completely covers the growth area.
   b) Typically, cells are seeded at a density of 500,000 viable cells cm^2. Aliquot the appropriate volume of cell suspension into the apical side of the cell culture insert and make the final apical volume up to 0.8 ml with additional 'MEDIA – ANTIBIOTIC'. Add 1 ml of 'MEDIA – ANTIBIOTIC' to the basolateral side and clearly label plate with seeding date. Incubate plate/s at 18 °C in an air atmosphere.
   Note: After seeding the cell culture inserts we generally assign this culture time to be day 0.
6. Culture and monitoring of cell culture inserts.
   a) After incubating culture plates for 24 h (now designated day 1 and so on), top-up apical media with 0.7 ml 'MEDIA – ANTIBIOTIC' (final apical volume of 1.5 ml). Top-up basolateral compartment with 1 ml 'MEDIA – ANTIBIOTIC' (final basolateral volume of 2 ml). Incubate for a further 24 h at 18 °C in an air atmosphere.
   b) On day 2 of the insert culture period, completely change media with fresh 'MEDIA – ANTIBIOTIC' by carefully aspirating the apical and basolateral compartment of the cell culture insert and replacing 1.5 ml and 2 ml of 'MEDIA – ANTIBIOTIC' in the apical and basolateral side respectively. At this point the first TER measurements can be made and recorded.
   c) From day 2 onwards, TER measurements are recorded every 24 h and complete media changes (all antibiotic-free) are conducted every 48 h. Inserts can be inspected on a daily basis, using an inverted microscope, for fungal or bacterial contamination. Ideally, by day 6 TER measurements should be ≥ 1000 Ω cm^2 (after correction for background resistance). Cells on inserts can be observed in Figure 1c.

Note: Observation of inserts for contamination should always be made prior to measuring TER. If this is not done, the 'chopstick' electrodes, which need to be immersed in the insert media, may cause cross-contamination of inserts. If contamination is observed, the contaminated insert/s can be removed without affecting neighboring inserts. It is also common practice to wash 'chopstick' electrodes with ethanol and rinse with PBS (pH 7.7) between plates to avoid cross-plate contamination. If a cell culture insert is removed, it is good practice to rinse out the associated (now empty) well with 70% ethanol.

C. Procedures for the preparation and culture of 'reconstructed' DSI epithelia (comprising pavement cells and mitochondria-rich cells)

1. The preparation of fish and gills should be performed as outlined in the procedures for the preparation and culture of epithelia comprising of pavement cells only.
2. The fist tryptic digestion of the gill filaments should be performed as outlined in the procedures for the preparation and culture of epithelia comprising of pavement cells only.
3. First direct seeding of cell culture inserts:
   Using cells suspended in 'MEDIA + ANTIBIOTIC', determine cell numbers with a hemocytometer. At this stage, cell viability can be determined using standard techniques of eosin dye exclusion. Based on cell counts, place the appropriate number of cell culture inserts into accompanying cell culture plates. Seeding density is normally in the region of $2–3 \times 10^6$ viable cells cm^2. Before adding cell suspension, wet the cell culture inserts with a few drops of 'MEDIA + ANTIBIOTIC' and allow to stand for several minutes. After aliquoting the appropriate volume of cell suspension into the apical side of the cell culture insert, make the final apical volume up to 0.8 ml with 'MEDIA + ANTIBIOTIC'. Add 1 ml of 'MEDIA + ANTIBIOTIC' to the basolateral side and clearly label each plate with seeding date. Incubate plate/s at 18 °C in an air atmosphere for 24 h.
   Note: After seeding the cell culture inserts we generally assign this culture time to be day 0.
4. After 24 h incubation a second direct seeding of the inserts should be conducted. The procedures for the preparation of fish and gills should be performed as outlined in the procedures for the preparation and culture of epithelia comprising of pavement cells only. In addition, the procedures for tryptic digestion of the gill filaments should be performed as outlined in the procedures for the preparation and culture of SSI epithelia.
5. Second direct seeding of cell culture inserts
   a) Using cells suspended in 'MEDIA + ANTIBIOTIC', determine cell numbers with a hemocytometer. Cell viability can, again, be determined using standard techniques of eosin dye exclusion. The second seeding density is also normally in the region of $2–3 \times 10^6$ viable cells cm^2. Prior to the addition of this second cell suspension, aspirate all media from the inserts and rinse any mucus away with PBS (pH 7.7). This may take several attempts, depending on how much mucus has accumulated. After aliquoting the appropriate volume of cell suspension into the apical side of the cell culture insert, make the final apical volume up to 0.8 ml with 'MEDIA + ANTIBIOTIC' and add 1 ml of 'MEDIA + ANTIBIOTIC' to the basolateral side of the insert. Incubate plate/s at 18 °C in an air atmosphere for 24 h.
   b) On the following day, aspirate all media from the cell culture inserts and rinse away mucus with PBS (pH 7.7). Again, several rinses may be required to remove all mucus. Add 1.5 ml 'MEDIA + ANTIBIOTIC' to the apical side of the cell culture insert and 2.0 ml 'MEDIA + ANTIBIOTIC' to the basolateral side. At this point, TER measurements can be made. Typically, it is encouraging to get TER readings that are above 100 Ω cm^2 (after background correction).
   Note: If initial TER readings are significantly lower than 100 Ω cm^2 (after background correction) a third direct seeding may be necessary.
   c) Measure and record TER daily and change media every 48 h. Typically, during the media change that occurs 96 h after the initial seed (day 4 of culture) we change from using 'MEDIA + ANTIBIOTIC' to using 'MEDIA – ANTIBIOTIC'. All media changes after this point are conducted using 'MEDIA – ANTIBIOTIC'. Ideally, after 6–7 days culture, the inserts should exhibit a TER > 1000 Ω cm^2.
   Note: Cell culture inserts should be observed on a daily basis to check for contamination. These observations should, again, be conducted prior to measuring TER for reasons previously outlined. Any contaminated cell culture inserts should be immediately removed and the associated plate well should be rinsed with 70% ethanol.
6. Confirming the presence of mitochondria-rich (MR) cells
   a) Add 5 µl of rhodamine stock solution or

15 µl of DASPEI stock solution to both the apical and basolateral compartments of the cell culture insert. Incubate for 30 min at 18 °C in the dark.

b) Wash epithelia with 'MEDIA – ANTIBIOTIC' for three consecutive 5 min periods.

c) Epithelia can now be observed using an epifluorescence microscope with the appropriate filter for rhodamine 123 (absorption 507 nm/emission 529 nm) or DASPEI (absorption 475 nm/emission 605 nm). Under fluorescent illumination, MR cells exhibit bright fluorescence relative to the low background staining of the pavement cells.

Note: Observation of cells with mitochondria specific dyes are procedures normally considered terminal for preparations due to compromised epithelial integrity.

4. Results and discussion

Generation of cultured epithelia

The procedures outlined above are based on methods originally outlined for the flask culture of gill cells [32] and are routinely employed in our laboratories to generate 'reconstructed' flat epithelial preparations from freshwater rainbow trout [13, 15, 16, 42, 43]. However, several factors have to be considered before adopting these procedures. Firstly, the quality of epithelia appears to vary according to season. In the warmer summer months we find that the generation of high quality epithelia is less successful than during the cooler months of the year. It has been suggested that this may relate to fish condition itself [43] however, this may also relate to high room temperatures, a phenomenon that can be offset if the procedures are carried out on ice. Secondly, the onset of warmer weather is often associated with increased contamination problems. Normally we prefer to exclude the use of antibiotics in our culture media after day 4 (flask culture for SSI epithelia) or day 5 (DSI epithelia) however, during the warmer months antibiotics may be used throughout the culture period. This does not appear to alter epithelial function [16] and will greatly reduce contamination problems.

Growth of 'reconstructed' epithelia

The TER of all epithelial preparations was sigmoidal over time and, typically, the TER of both SSI and DSI epithelia reached a stable plateau 6–9 days after first seeding (Figure 2).

The TER of SSI preparations normally plateau around 1000–5000 Ω cm^2, (Figure 2b) with occasional preparations displaying a TER as high as

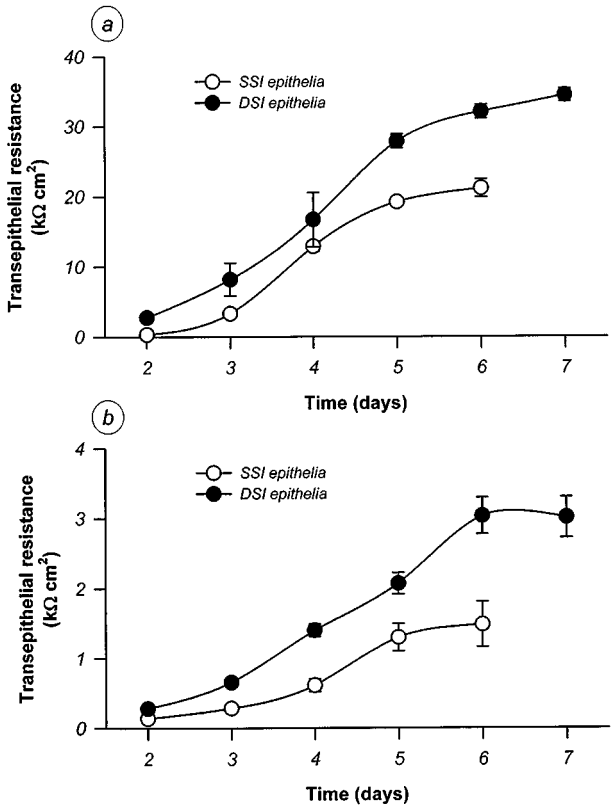

Figure 2. Growth curves of transepithelial resistance with time in culture for (a) high end and (b) low end resistance measurements in both single seeded and double seeded epithelia.

20,000 Ω cm^2 (Figure 2a) Similar variation in TER can be observed in DSI preparations, however, DSI preparations exhibit a propensity towards higher TER measurements (with values ranging from 1300 to 34,000 Ω cm^2). These variations appear to be natural and originate from the fish themselves as even the strictest laboratory standardization cannot eliminate them. Furthermore, it has been noted that fish cultured during the cooler months (water holding temperatures of 6–10 °C) of the year often produce higher resistance epithelia than those cultured during the warmer months (water holding temperatures of 11–16 °C) [43]. This natural variation can be exploited to examine epithelial 'tightness' and parameters that may be affected by changes in epithelial permeability (see [43]), however, the worker should be aware that individual experiments should, when possible, always be carried out on a single batch of inserts. Comparison between the current preparations and TER values from other cultured epithelia would seem to indicate that the cultured gill preparations are markedly 'tighter' than 'leaky' epithelia from tissues such as the fish kidney (23 Ω cm^2 [11]), dog kidney MDCK cells (100 Ω cm^2 [2, 8]), IEC-6 rat small intestine cells (10–15 Ω cm^2 [2]). However, the TER of gill cell preparations often fall in the range of 'tight' epithelia

derived from tissues such as rat alveolar cells (2.3 kΩ cm^2 [9]) and toad A6 kidney cells (1.6–5.0 kΩ cm^2 [5, 38]). Particularly encouraging is the similarity found between TER measurements across the cultured preparations and TER measurements found across dissected epithelial preparations that have been tested *in vitro* as possible surrogate models for the freshwater gill (11 kΩ cm^2 for the cleithral epithelium of trout [26]; 3.7 kΩ cm^2 for the opercular epithelium of freshwater-adapted tilapia [14, 31]). Similarly, cultured gill pavement cells from the marine fish (*Dicentrarchus labrax*) also exhibit a high TER [1].

Epithelium composition

It has been established [42], and can be routinely demonstrated using mitochondrial specific dyes such as rhodamine 123 or DASPEI, that the SSI epithelium is comprised solely of pavement cells. Transmission election microscopy has revealed that the SSI epithelium consists of multiple overlapping cell layers that are normally 2–4 layers thick (occasionally 5 or 6) [42]. The apical surface of the epithelium has a prominent glycocalyx and exhibits characteristic apical projections that are consistent with the development of structural polarity. Cell-to-cell contact in the form of tight junctions can also be easily observed. All of these observations are in line with observations of pavement cell structure in an intact gill epithelium [22].

The majority of cells comprising a DSI preparation are pavement cells and are structurally consistent with observations on the SSI epithelium; however, the presence of MR cells is unique to the DSI preparation. These MR cells fluoresce brightly when stained with rhodamine 123 (Figure 3) and normally constitute around 15% of the total epithelial cell number [13].

The majority of MR cells can be observed as individuals nested in the general epithelium; however, prominent MR cell 'clusters' can often be observed [13]. Transmission electron microscope observations have revealed that the cultured MR cell also exhibits numerous similarities with MR cells found in intact gill epithelia. Most notable is the abundance of mitochondria within the cell. In addition, both cell types are also open apically to the external environment (or in the case of cultured cells, to the apical culture media) and possess an anastomosing tubular system [13].

Applications of 'reconstructed' epithelia

The principal intended application for 'reconstructed' flat epithelia is to employ this system as a surrogate model for the freshwater fish gill and a considerable degree of success in this area has already been established [13, 15, 16, 42, 43]. This is particularly so with regard to the passive electrical and transport properties of the epithelium which duplicate the intact gill quite well [42, 43]. However, the active transport properties of the epithelia still require considerable development [42, 43].

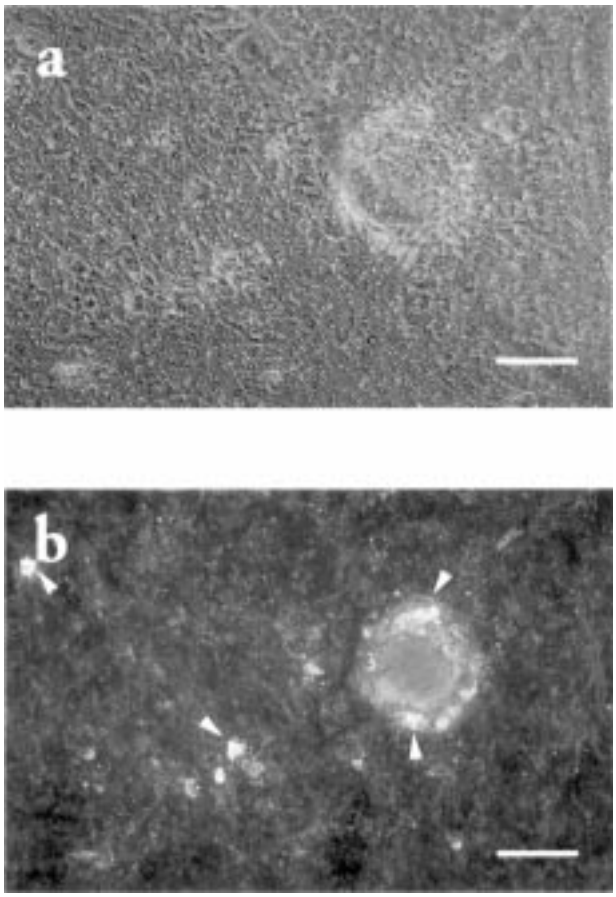

Figure 3. Photomicrographs showing rhodamine 123 stained double seeded epithelia (a) without and (b) with fluorescent illumination. Representative mitochondria-rich cells indicated by arrowheads. Scale bars = 200 μm.

Under symmetrical conditions, ion efflux rates for Na^+ and Cl^- are very similar to those found *in vivo* (Table 1). On the other hand, under asymmetrical conditions ion efflux rates (for Na^+ and Cl^- at least) are usually several fold higher than those found *in vivo* (Table 1). However, ion influx rates under asymmetrical conditions are either very low and passive, as is the case with Na^+, or very low but suggestive of active uptake, as is the case with Cl^- [42, 43]. This occurs regardless of MR cell presence and as such we currently attribute both phenomenon to the pavement cells [13]; however, the presence of MR cells does result in low active Ca^{2+} uptake across the DSI epithelium under symmetrical conditions (a development that is notably absent in the SSI preparation) [13]. To our knowledge, no theory places only Cl^- uptake on the pavement cells [17, 34] which would suggest that either additional hormonal support, nutritional supplementation or other improvements are required before the cells in the

Table 1. Comparison between *in vivo* and *in vitro* efflux rates of Na^+, Cl^- and Ca^{++} for rainbow trout branchial epithelia

		Na^+	Cl^-	Ca^{++}
In vivo efflux (μmol kg^{-1} h^{-1})	33% seawater (isosmotic)[a]	≈ 750	≈ 750	–
	Freshwater[b]	≈ 250	≈ 250	≈ 7.5
In vitro efflux (μmol kg^{-1} h^{-1})	Symmetrical[c]	≈ 500	≈ 600	≈ 1.0
	Asymmetrical[c]	≈ 900	≈ 1100	≈ 2.0

[a] *In vivo* Na^+ and Cl^- efflux data from [3].
[b] *In vivo* Na^+ and Cl^- efflux data from [40] and [17] and Ca^{2+} data from [33].
[c] *In vitro* efflux data from [13] calculated according to gill area data of [18].

epithelium function in a manner identical to those found *in vivo*. Alternatively, current theories on ion transport across the gill are flawed. In order to establish which of these possibilities is more accurate, further development of the epithelium culture regime using a mixture of the former suggestions is necessary.

The cultured epithelium as a tool for toxicity screening

The cultured gill epithelia can be developed into a screening tool in *in vitro* toxicology. The preparation has two advantages over the currently used cell-based *in vitro* methods. Firstly, to our knowledge this is the first *in vitro* method where cells can be directly exposed to water. Therefore, it offers a unique possibility to mimic the exposure situation in the aquatic environment in an *in vitro* system. Water samples (lake water, wastewater, drinking water) can be screened directly without a previous extraction procedure. Secondly, the epithelium represents a higher level of organization than single cells.

Endpoints based on epithelial integrity could therefore be expected to be more sensitive than the single cell endpoints currently used in *in vitro* toxicology (LDH leakage, neutral red assay, MTT assay, calcein assay etc). A recent demonstration of this is the study of [37]. The toxicity of a number of chemicals was tested in a standard cytotoxicity assay using gill cells in suspension. A selected group of the same chemicals were simultaneously tested on cultured gill epithelia using TER as an endpoint. The epithelial preparation was 2–1000 times more sensitive compared to the isolated cells depending on the type of chemical. *In vitro* cytotoxicity tests are generally less sensitive than *in vivo* tests. Clearly, single cell toxicity does not reflect organismal toxicity fully and one reason is probably that cell-cell interactions are the targets in the toxic response. Cultured epithelia are one approach to bring *in vitro* methods closer to the *in vivo* situation.

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Notes on suppliers

1. American Optical, Scientific Instruments Division, Buffalo NY, 14215, USA
2. BDH Inc., 350 Evans Avenue, Toronto, ON M82 1K5, Canada
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17. Sigma-Aldrich Chemical Company, P.O. Box 14508, St. Louis, MO 63178-9974, USA
18. VWR Canlab, 2360 Argentia Road, Mississauga, ON L5N 5Z7, Canada
19. World Precision Instruments Inc., International Trade Center, 175 Sarasota Center Boulevard, Sarasota, FL 34240-9258, USA

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