2017-11-01

Investigations to extend viability of a rainbow trout primary gill cell culture

Maunder, RJ

10.1007/s10646-017-1856-6
Ecotoxicology
Springer Verlag

Investigations to extend viability of a rainbow trout primary gill cell culture

Richard J. Maunder[1], Matthew G. Baron[1], Stewart F. Owen[2], Awadhesh N. Jha[1*]

[1]School of Biological and Marine Sciences, University of Plymouth, Devon, PL4 8AA, United Kingdom

[2]AstraZeneca, Alderley Park, Macclesfield, Cheshire, SK10 4TF, United Kingdom

*Correspondance: Email: a.jha@plymouth.ac.uk

Abstract

The primary culture of fish gill cells can provide functional, cell diverse, model *in vitro* platforms able to tolerate an aqueous exposure analogous to *in vivo* tissues. The utility of such models could be extended to a variety of longer term exposure scenarios if a method could be established to extend culture viability when exposed to water for longer periods. Here we report findings of a series of experiments to establish increased longevity, as monitored by culture transepithelial electrical resistance (TEER) and concurrent histological developments. Experimental cultures improved TEER during apical freshwater for a mean of twelve days, compared to previous viabilities of up to 3 days. Cultures with larger surface areas and the use of trout serum rather than foetal bovine serum (FBS) contributed to the improvement, while perfusion of the intact gill prior to cell harvest resulted in a significantly faster preparation. Detailed scanning electron microscopy analysis of cultures revealed diverse surface structures that changed with culture age. Cultures grown on membranes with an increased porosity, collagen coating or 3D structure were of no benefit compared to standard membranes. Increased culture longevity, achieved in this study and reported for the first time, is a significant breakthrough and opens up a variety of future experimentation that has previously not been possible. The extended viability facilitates exploration of *in vitro* chronic or pulse-exposure test paradigms, longer term physiological and environmental monitoring studies and the potential for interactive co-culture with other organoid micro-tissues.

Key words

In vitro; Oncorhynchus mykiss; Fish gill; ecotoxicology; Chronic exposure; Environmental risk assessment

Introduction

Rainbow trout (*Oncorhynchus mykiss)* gill cells can be extracted from primary tissue and grown on the permeable membranes of microplate inserts to form an *in vitro* gill cell culture. The initial methodology grew cells in a culture flask before transfer to the insert in a single stage (SSI; Wood and Pärt (1997)). This was later developed into directly seeding cells onto the insert, either once (SDSI; Wood et al. (2003)) or a double seeding of cells from separate fish, 24h apart (DSI; Fletcher *et al.* (2000)). The DSI method produces cultures that are polarised, electrically tight and contain pavement, mitochondria-rich (MR) and mucus cells in similar proportions to *in vivo*. (Fletcher et al. 2000; Leugen et al. 2007; Perry and Laurent 1993). Culture development through time is typically monitored using the determination of Trans-Epithelial Electrical Resistance (TEER). This is a non-destructive, quantitative measure of electrical impedance across the culture that can be used as an indicator of culture quality (Chen et al. 2015; Srinivasan et al. 2015). Culture TEER values through time reveal the formation of an extremely 'tight' culture which maintains a very high TEER for several weeks when maintained in media (Wood et al. 2002; Walker et al. 2007). Once established, the cultures are also able to tolerate an immediate switch from media to freshwater on the apical side and hence can accurately model the *in vivo* situation. TEER dynamics post water addition (PWA) can be variable and depend on the state of the culture at that time (Fletcher et al. 2000) but typically show a brief spike and return to previous levels before a collapse after 24 - 72 h (Wood et al. 2002; Walker et al. 2007). This time window when the culture has water on the apical side, culture media on the basolateral side and a stable high TEER provides a model *in vitro*

platform with the potential to provide data that can reduce the required number of *in vivo* experiments and avoid the potential animal suffering of direct exposure to contaminants (Reduction and Refinement of 3R's principles; Burden et al. 2015). These models have been proposed to have direct applications in regulatory assessments (Lillicrap et al. 2016a,b) and have already been used in the fields of physiology and toxicology (Bury et al. 2014; Stott et al. 2015; Schnell et al. 2016) and is robust enough to take to the field for use in environmental monitoring (Minghetti et al. 2014).

Despite these promising applications, a major limitation of the available model is the relatively short (24 - 72 h) culture viability PWA. It would be advantageous to be able to extend this time window to study longer term processes in a gill model without the interacting complications, costs and ethical considerations involved with *in vivo* work. Similarly, it is problematic to use short term model data to inform decisions for chemical environmental risk assessment, particularly when the standard *in vivo* tests for acute exposures have been designed to last 96 h (OECD 2012). An extension of the model viability would open up numerous avenues for future research, for example, we have also developed a trout hepatic spheroid model (Baron et al. 2012; 2017) and are working on combing these tissues from different organs together into a co-culture to obtain a holistic evaluation of potential *in vivo* conditions. Since the liver spheroids remain viable for several weeks in culture, the limiting factor for length of co-culture viability is also the viable time of gill cultures PWA. These considerations led to an experimental aim in this study to increase the time beyond the 24 - 72 h that DSI cultures remain viable after addition of apical freshwater.

Previous studies have successfully developed slight variations of the standard DSI culture to suit certain requirements, e.g. metal-sensitive cultures (Walker et al. 2007) inverted culture (Schnell et al. 2016) and cell selective seeding (Galvez et al. 2008) but the standard model and methodology remains essentially unchanged from Fletcher et al. (2000), and has been recently summarised (Schnell et al. 2016). Attempts have been made to improve the active ion transport of the culture by trialling various media additives (summarised in Bury et al. 2012) but with limited success. With these existing limitations we aimed to primarily investigate methodological protocol alterations, to seek a more fundamental change in culture physiology, or general health that might enable the cultures to tolerate a prolonged exposure to water. We decided to utilise daily TEER monitoring as an indication of culture quality and viability and report four experiments conducted to extend the longevity of the DSI gill cultures when freshwater is introduced to their apical side.

We initially made investigations into reducing the time it takes to get the cells into culture. We considered that a reduction in time that the cells are being processed might result in cells with an improved health status going into culture. One of the longest stages in the protocol is the 3 wash stages designed to remove as much blood as possible from the gills before further processing. It is a relatively simple operation to perform an *in situ* ventral aortic perfusion which clears the blood from the gill vasculature prior to excision (Perry et al. 1984). The cleared gill arches can be excised, briefly washed and put directly into the trypsin digestion, thereby reducing the processing time. The removal of blood from the tissues might also be a benefit to culture by elimination of blood cell physical interference during attachment and chemical interference during degradation. After some optimisation a viable method was

developed, so Experiment 1 was designed to test for differences in DSI culture parameters from cells produced using the standard primary and new perfused methodology.

Secondly, we thought it likely that recent developments in membrane technology might provide insert materials that are better able to support a more realistic gill culture. The existing method uses a polyethylene terephthalate membrane containing 1.6 x 10^6 0.4 µm pores/cm^2 which appears to have remained unchanged since first used in this field when became available (Wood and Pärt 1997). A 'high density' version of the same material with 1 x 10^8 0.4 µm pores/cm have also been previously used for SSI, but with no difference reported (Wood and Pärt 1997). Numerous other insert materials and coatings are available and we hypothesised that these membranes, with a higher porosity or altered surface chemistry might produce a culture with better attachment, or scope for higher transport and hence be more similar to the *in vivo* state. Experiment 2 was designed to compare culture growth on these different membranes.

We were interested in exploring the possibility of using autologous trout serum (TS; from the same donor fish) to later supplement the gill cell cultures. A literature survey revealed a related experiment had been previously carried out using fresh plasma from larger donor conspecifics to supplement *in vitro* cultures (Wood et al. 2003). No differences in TEER were observed in fresh plasma and previously frozen plasma reported as toxic to cells. In our preliminary trials, we saw some evidence of improvements in TEER when

using a heat-inactivated TS preparation (i.e. not plasma) from both fresh (unfrozen) autologous TS and frozen group-batched TS. We therefore conducted Experiment 3 to specifically test for culture TEER differences under these various serum preparations. Finally, to further explore the results of Experiment 3, we investigated if the size of culture vessel made a difference to culture TEER. This work was based on previous observations of apparently higher TEER when using larger 4.2 cm^2 inserts compared to the usual 0.9 cm^2 format. We therefore performed Experiment 4 to test culture TEER of cells from the same stock, cultured under the same conditions and seeding densities but grown in the 4.2, 0.9 or 0.3 cm^2 inserts produced varying culture TEER.

A key idea in Experiments 3 and 4 is defining what constitutes a viable culture, both before and after water addition. Viability has previously been defined for short term cultures by the maintenance of ≥50 % the TEER value pre water addition (Wood et al. 1998) and then added to by Fletcher et al. (2000) to also require a minimum 10 KΩ cm^2 starting TEER. From this, a TEER value of 5 KΩ cm^2 has been interpreted as a threshold to indicate cultures are 'tight' and suitable for experimentation (Walker et al. 2007; 2008; Minghetti et al. 2014). This value has also been shown to have significance experimentally based on observed ion fluxes (Wood et al. 1998) and mannitol permeability (Stott et al. 2015). We have used it as a working definition of viable culture here.

Materials and methods

Experimental animals

Female rainbow trout (*Oncorhynchus mykiss*) with mean bodyweight ± S.E.M. = 273 ± 9 g were obtained from a local commercial supplier and held at University of Plymouth aquarium facilities. Typical husbandry conditions were groups of 10-30 fish in 200L holding tanks on a recirculating system of aerated, dechlorinated tap water, with temperature (15 – 16 ºC), pH (6.5 - 6.8), dissolved oxygen (95 – 98 %) and photoperiod 12h: 12h. Fish were fed twice daily at 2 % body weight/day and not starved before experiments. Experiments used fish held between June 2015 and October 2016. To collect primary tissue, fish were killed by Schedule 1 procedure under UK Home Office regulations which constitutes a blow to the head and destruction of the brain. Fish were then weighed and used immediately.

Laboratory chemicals

Leibovitz's medium (L-15), FBS, Dulbecco's Phosphate-Buffered Saline (PBS), Gentamycin (10 mg/mL), Penicillin-Streptomycin (5000 U/mL) and Amphotericin B (250 µg/mL) were obtained from Life Technologies (UK). Trypsin-EDTA (0.25 %), Trypan Blue (0.4 %), and all other chemicals and reagents were obtained from Sigma-Aldrich (UK).

DSI culture preparation

Detailed methods of the standard method have been previously published (Kelly et al. 2000; Schnell et al. 2016). Briefly, all 8 gill arches were excised, filaments cut from arch and washed three times in an antibiotic (200 U/mL Penicillin-Streptomycin, 400 µg/mL Gentamycin) and antimycotic (2.5 µg/mL Amphotericin B) PBS solution. Cells were isolated by EDTA-trypsin digestion, washed, counted and seeded into media-conditioned inserts. Cultures were maintained in L-15 media supplemented with 5% serum and

antibiotics (100 U/mL Penicillin-Streptomycin, 200 µg/mL Gentamycin) in an air environment incubator at 15 ºC. After 24 ± 2 h the cultures were washed to remove cellular debris, mucus and cells prepared from a second fish were then seeded on top of the existing attached cells from the first fish. After a further 24 ± 2 h the cells were washed again and TEER monitored daily and media renewed 3 times per week. Known deviations from the published methods were the use of antibiotic solutions in media throughout culture, larger fish and 15ºC rather than 18ºC for culture incubation.

Transepithelial electrical resistance (TEER) measurements

TEER was monitored at least daily through experiments and more frequently as required for water addition experiments. TEER was measured using a STX-2 chopstick and EVOM^2 epithelial volt/ohm meter then blank corrected and resistance normalised to area (Ω cm^2) (Srinivasan et al, 2015). Blank values were generated from spare wells in plates and recorded daily for symmetrical cultures. For asymmetrical cultures the initial immediate (un-mixed) value was used for the subsequent culture. This was because the water in blank cultures quickly mixes with the underlying media (~2 hours for 4.2 cm^2 membranes) and falsely alters the blank correction value in comparison to cultured inserts where the cell layer largely blocks mixing (while facilitating some ion transport).

Water exposure

Artificial freshwater was used in all experiments for consistency. US EPA moderately hard water was prepared following standard guidelines US EPA (2002) and filter sterilised (0.22 µm PES membrane, Millex GP Millipore Ireland) before use. Basolateral media

was removed before apical media was replaced with water via pipette and repeated 4 times to ensure no residual media remained. Water was conditioned to culture temperatures before use.

Scanning Electron Microscopy (SEM)

Inserts were removed from the well with forceps, emptied and membranes removed by scalpel. Inserts were individually fixed in a 2.5 % glutaraldehyde, 0.1 M sodium cacodylate buffer (pH 7.2; 4 ºC) for 4 h. Fixed samples were rinsed in sodium cacodylate buffer, dehydrated in stepwise ethanol washes, dried in a critical point drier (Emitech K850, UK) and sputter coated with gold (Emitech K550, UK). Analysis was conducted using a JEOL JSM-5600LV (JEOL Ltd., UK) SEM using settings appropriate for the samples.

Gill pre-perfusion (Experiment 1)

Fish to be used in Experiment 1 had blood cleared from gills by a ventral aortic perfusion in a 15 ºC temperature controlled laboratory. A 50 cm length of fine bore polythene tubing was attached to a 20 mL syringe containing ambient temperature PBS. Fish were supported ventral side up and an incision made to open body cavity then held open with wound spreaders. A further incision into pericardium revealed the heart which was then gently pulled upwards using forceps around the aorta on its underside. The upper half of the heart was cut away and the syringe tubing was fed downwards through the lower half of the heart and into the aorta (~1 cm) where it was grip sealed by forceps. The syringe was then depressed by hand to push PBS through the vasculature and clear blood from the gills (see Figure 1). After perfusion the gill arches were excised and treated as for the primary preparation but with the repeated wash stages omitted. Cells were instead rinsed once in wash solution then put into the trypsin-EDTA stage. Experimental

design used the 4 combinations of culture seeding from primary and perfused cell preparations in the 4.2 cm^2 inserts; 1^{st} and 2^{nd} seed primary; 1^{st} seed primary 2^{nd} seed perfused, 1^{st} seed perfused 2^{nd} seed primary, 1^{st} and 2^{nd} seed perfused.

Effect of membrane material (Experiment 2)

Standard Falcon inserts were compared in direct trial experiments using the same seeding protocol as in Experiment 2 with corrections for growth area. The 6 well formats were typically used and comparisons made with cells of biological replicates. The types of membrane trialled were:

1. Standard: Falcon, 1.6×10^6 0.4 µm pores/cm^2, 4.19 cm^2 growth area
2. Falcon HD, 1×10^8 0.4 µm pores/cm^2, 4.19 cm^2 growth area
3. GBO Thincert, transparent, 2×10^6 0.4 µm pores/cm^2, 4.52 cm^2 growth area
4. GBO Thincert, translucent, 1×10^8 0.4 µm pores/cm^2, 4.52 cm^2 growth area
5. GBO Thincert, collagen type 1, 1×10^8 0.4 µm pores/cm^2, 4.52 cm^2 growth area
6. Reinnervate Alvetex strata, highly porous polystyrene scaffold, 3-D growth area

Supplementation of trout serum (Experiment 3)

In order to generate enough serum for culture, trout in 250 - 350 g range were selected. Fish were bled by caudal vasculature using hypodermic needles without heparin. Blood from individual fish was collected in a 15 mL tube and left overnight in the fridge (4 ºC).

Tubes were centrifuged at 750 × *g* for 10 min and serum transferred to a fresh tube. Serum compliment was heat inactivated by immersing tube in a 45 ºC water bath for 30 min then rapidly cooled on ice and filter sterilised (0.22 µm PES membrane, Millex GP Millipore Ireland). The serum from the first fish was stored in the fridge (4 ºC) and combined with the serum from the second fish before filter sterilisation to reduce losses in the filters. A separate, larger batch of TS (~100 mL) was prepared by combining together the blood collected from 20 trout from the same cohort into a single batch and processed as above but stored frozen in aliquots until required.

Since autologous TS was not ready for use in culture until Day 2, in order to maintain cells until this time we needed to use FBS media (5 %) for insert conditioning, cell processing and culture until Day 2. After this wash the apical compartment was either maintained with FBS or autologous TS media (5 %) depending on the treatment. Due to volume limitations the basolateral compartment was maintained with FBS media for both treatments. Cultures were maintained under this regime with 3 media changes in the week leading up to apical water addition. During set up of the asymmetrical phase the basolateral compartment was replaced with either fresh TS or FBS media. The osmolality of media containing FBS and TS were both measured using an Osmomat 030 (Gonotec) to confirm equal values (data not shown). No media or water renewals were performed during the asymmetrical phase.

Effect of membrane area (Experiment 4)

Falcon inserts and companion plates (BD Biosciences, USA) were purchased in 4.2, 0.9 and 0.3 cm^2 formats corresponding to 6, 12 and 24-well, respectively. Working volumes for apical and basolateral compartments were chosen from the recommended ranges suggested by suppliers. For the basolateral compartment a volume in the middle of recommended range was chosen of 3.0, 1.8 and 0.8 mL for 4.2, 0.9 and 0.3 cm^2 inserts, respectively. The upper limit of suggested working volumes were chosen for the apical compartment to both maximise nutrients and ensure a positive hydrostatic gradient was maintained during culture (2.5, 1 and 0.35 mL for 4.2, 0.9 and 0.3 cm^2, respectively; the apical media/water level was higher than the basolateral level). In order to seed cells at the same density for each insert size, a single stock was diluted to contain a minimum 4.19 million cells/mL in media, and then 1000, 208 and 76 µL seeded into 4.2, 0.9 and 0.3 cm^2 inserts, respectively. This seeded a minimum of 1 million cells/cm^2 in each well. The range of cell concentrations used was 1 - 2 million cells/cm^2, but always in proportion within a given experiment.

Data analysis

All data are expressed as means ± S.E.M. The number of biological (separate pairs of donor fish) and experimental (separate repeat runs) replicates used during each experiment is detailed in the results. Data normality and variance were assessed and group differences, through time where appropriate were assessed by comparing data using standard or repeated measures ANOVA. If required, post hoc testing was performed to find specific differences. The level of statistical significance for all analyses was $P < 0.05$. Analyses were performed using R software (v3.1.3; www.r-project.org).

Results

Gill pre-perfusion (Experiment 1)

The perfusion experiment consisted of 5 separate runs between July 2015 and March 2016, using a total of 11 biological replicates, each cultured in 3 - 6 wells. Mean ± S.E.M. wet weight of the 22 fish was 269 ± 13 g. An example gill arch after primary and perfused preparation is shown in Figure 1. Occasionally the perfusion would not work satisfactorily, as judged by visual inspection of the gills, in which case those gills were not used in this experiment. The TEER values for the 4 combinations of perfused or primary (standard) preparation techniques for the double seeding technique are shown in Figure 2. No significant differences in TEER between the treatments through time were observed. The 4 treatments follow a typical pattern of slow TEER increase until Day 5, then a linear increase until Day 11 then a plateau at around 20 KΩ cm^2. This data is from symmetrical cultures only and does not include a water addition element.

Effect of membrane material (Experiment 2)

When compared to the standard membrane (1), cells seeded onto Falcon HD inserts (2), GBO translucent (3), transparent (4) and collagen type 1 (5) inserts all failed to establish TEER above background after 10 days of growth (data not shown). Phase contrast microscopy of *in situ* cultures and scanning electron microscopy (SEM) of fixed membranes revealed that cell layers were incomplete or that few cells had attached. These trials were repeated for 3 - 6 wells at least 3 separate times. Cultures from the same cells seeded on standard inserts produced cultures with a typical TEER profile. Cells seeded onto Reinnervate Alvetex strata membranes

could not be accurately assessed by TEER as they were not fully compatible with the chopstick electrodes, but the data that could be collected suggested little or no difference in TEER from background. SEM imaging of the membrane surfaces are shown in Figure 3.

Supplementation of trout serum (Experiment 3)

We compared autologous fresh TS against FBS in a series of 6 separate runs between February and September 2016, using a total of 13 biological replicates, each cultured in 3 - 6 wells. Mean ± S.E.M. wet weight of the 26 fish was 303 ± 19 g. The TEER data is displayed in Figure 4. Cultures receiving the TS media showed a significant increase in TEER compared to those receiving FBS media from Day 5 to 7. Both culture types reached a similar TEER by Day 9 when the apical water addition occurred. Immediately PWA, the mean TEER of both culture types rapidly increased and then declined fairly consistently over the following 2 weeks. Interestingly, the FBS cultures maintain a significantly higher TEER than the TS on Days 10 - 12. The remaining time points do not show any significant differences in mean TEER, but the TS is above the FBS value from Day 14 onwards.

If using a TEER of >5 KΩ cm^2 threshold to define a viable culture PWA, then both culture types extend far beyond the ~24 - 72 h viability previously reported, with mean TEER values for both treatments remaining >5 KΩ cm^2 until Day 21 (= Day 12 PWA; Figure 4). The variation in duration of viability is however, high, with the TEER of individual cultures responding very differently to water addition between experimental runs.

We also conducted a smaller scale experiment (single run) to compare the TEER of the two types of trout serum (fresh autologous serum (Autologous TS) and frozen batch-prepared serum (General TS)). Two biological replicates were set up, each of which had 6 wells for each treatment. Mean ± S.E.M. wet weight of the 4 fish was 291 ± 15 g. The data is displayed in Figure 6. There was no significant difference in TEER between the serum types through symmetrical culturing or during water addition on Day 8. There was some evidence of Autologous TS treated cultures having a higher TEER than General TS during later stages (Days 15 and 16 significantly different ($p<0.05$)). No evidence of toxicity was observed in cultures treated with either serum type, as has been previously reported.

Typical culture development from initial seeding, through confluence, apical water addition and eventual degradation and typical cell surface structures is shown by SEM imaging in Figure 5. Initially, the cells quickly attach and form a monolayer after initial seeding and a confluent layer after the second seeding (Figure 5a - b). The microridge surface morphology takes a few days to appear and fully differentiate and remain present post water addition (Figure 5b - d). Culture surface morphology appears to diversify with culture age to include suspected MR cells with different microridge surface morphologies (Figure 5g - i). After a longer duration water exposure the microridges appear reduced and another, larger scale surface pattern emerges (Figure 5e) before the culture layer loses integrity (Figure 5f).

The maintenance of high TEER (>5 KΩ cm^2) for a mean of 12 days PWA (Figure 4) was encouraging, if unanticipated. Since we had primarily been using the larger 4.2 cm^2 inserts rather than the standard 0.9 cm^2 we wondered if the larger 4.2 cm^2 insert size was contributing to the apparent improved performance, so tested this hypothesis in Experiment 4.

Effect of membrane area (Experiment 4)

Experiment 4 consisted of 4 separate runs between July and November 2016, using a total of 10 biological replicates, each replicated in 3 - 6 wells. Mean ± S.E.M. wet weight of the 20 fish was 235 ± 7 g. Experiment 4 data collection was inhibited by the maximum range of the TEER instrument being 20 KΩ. A raw TEER measurement has a blank value subtracted and is then multiplied by the membrane area in cm^2 to get by the Ω cm^2 units (Srinivasan et al. 2015). For the 0.9 and 0.3 cm^2 inserts, the 20 KΩ limits the maximum recordable range of these inserts to approximately 18 and 6 KΩ cm^2, respectively (e.g. instrument measures its maximum 20KΩ in a 12 well 0.9 cm^2 insert, minus blank value of 300Ω = 19700Ω × 0.9 cm^2 = 17730 Ω cm^2). With all TEER data above this range excluded, the 0.3 cm^2 format range is Days 2-6 and 25-27 and the 0.9 cm^2 format range is Days 2-5 and 23-27. The whole 4.2 cm^2 inserts data set is available, but we show Days 2-6 and 23-27 for comparison in Figure 7. In the symmetrical phase, the 4.2 cm^2 and 0.9 cm^2 formats have similar TEER values, and both are significantly higher than the 0.3 cm^2 format on Days 4, 5 (and Day 6 for 4.2 cm^2 inserts). PWA there were no significant differences between the formats.

Since the complete TEER profile is not available, another way to analyse this data is to compare the number of days that individual cultures remained viable (TEER >5 KΩ cm^2) PWA. This data is displayed in Figure 8. All three formats produced cultures that lasted for at least 12 days, but there was a significant increase in mean duration for the 4.2 cm^2 above the 0.9 cm^2 and the 0.9 cm^2 above the 0.3 cm^2 formats.

Discussion

Gill pre-perfusion (Experiment 1)

No significant differences were found between the TEER of the four combinations of perfused and primary prepared cells throughout an extended symmetrical culture. Despite this we do consider the perfusion method to add value to this model in terms of reduced preparation time and reduced materials cost. We have not compared the primary and perfusion methods following a water addition stage, but based on the similarity of the methods under symmetrical culture, we think it is unlikely to be the cause of the large scale increases in asymmetrical culture viability reported in Experiments 3 and 4. We recommend the use of the gill perfusion method in DSI gill preparations and encourage other users to consider this methodology. The method was successfully used in the subsequent experiments.

Effect of membrane material (Experiment 2)

Despite the wide range of use of these membrane types in cell culture with other species, the use for gill cells in this application was not a success. Standard and HD membranes yield a theoretical total porous area of ~0.2% and 13% respectively and visually resemble a flat surface with occasional, or numerous pores (see Figure 3). Since trout gill cells readily attach and grow on the solid surface of a culture flask (Wood and Pärt 1997), though possibly not as well as a standard membrane (Leguen et al. 2007), it must be the higher density of pores, or a consequence of, that have caused the lack of suitable cell attachment in this case. Given the relatively large pavement cell size (~10 µm; Wilson et al. 2002; Figure 5a) in comparison to the pores (0.4 µm diameter) it is surprising that they show such an acute sensitivity to an increase in pore density. Previous studies have reported similar difficulty in growing a SSI culture on the HD pore inserts and report no benefit when a successful culture was achieved (Wood and Pärt 1997). Whatever this problem is for attachment, it seems likely that it would be increased when cells are seeded onto the Reinnervate Alvetex strata membrane given its highly porous nature and lack of flat solid substrate for attachment. The use of a collagen coating to the membrane surface has also been successfully used in other applications, but the GBO membrane used here with a collagen type 1 coating also failed to establish a viable culture. This is perhaps not surprising given the same membrane without collagen also failed. It would be interesting to test a collagen-coated standard (Falcon) insert if one became available, given the favourable growth observed using this coating in other cell types.

Supplementation of trout serum (Experiment 3)

The TS supplemented cultures in this experiment attained a higher TEER than the FBS cultures during culture establishment (Figure 4, Days 5 - 7). This finding might be of value to future research as these cultures attained the 5 KΩ cm^2 threshold a day or so sooner than those cultured with FBS. Due to costs, ethics and possible health issues using bovine serum, fish serums and those from other animals have been investigated as suitable alternatives to FBS. A variety of fish (Kocal et al. 1988; Hashimoto et al. 1997; Rathore et al. 2001; Rosa et al. 2010) and mammalian cell types (Fujiwara et al. 2010) have benefited by being grown using fish serum, or a mixture, rather than FBS. Fish serum is also now available commercially (El-Dakhly et al. 2015), can be repeat-harvested from large stock fish (Wood et al. 2003), and has even been successfully used after recovery from the waste stream of a fish processing factory (Zakaria-Runkat et al. 2006). The cost-benefit analysis of use of fish serum would depend on individual experimental aims, but there is clearly potential for use in more applications in the future. For example, we plan to utilise autologous TS for future proteomic investigations to eliminate the variation observed in proteome between individual fish. Using a mixture of TS and FBS might be a way to gain the same benefits using smaller total volumes of serum.

In the current study there were no significant advantages to using TS PWA (Figure 4). However, the mean data masks the variation observed in TEER profiles between runs. PWA, we observed runs where both TS and FBS significantly outperformed each other in terms of TEER scale and duration. Despite the large sample size, this variation makes it difficult to definitively recommend one type over another, though TS can be considered, on average, at least as good as FBS in terms of TEER. We speculate that the source of variation between runs could be seasonal changes in the fish leading to serum or cell differences, but our analysis cannot discern

this. Methodological inconsistencies were also considered and minimised as far as possible in experimental design. For serum, FBS from the same batch was used and TS was harvested in a standardised manner. Cortisol, or serum from stressed fish has been shown to have some small effects on this and other similar models (Zhou et al. 2003; Kelly and Wood 2001; Kelly and Wood 2002), but not to the scale of the observed variation here. The rapid collection method should also have negated the prospect of serum contamination with stress response constituents. Seasonal changes leading to variation in TEER have been previously reported (Kelly et al. 2000; Bury et al. 2014) but it is difficult to propose a satisfactory explanation when fish maintenance and culturing regimes are both under constant environmental conditions. The experimental methods were also well practiced and dependable by the time of experimentation.

When comparing the two trout serum preparations we found some evidence of a higher TEER being maintained in asymmetrical cultures receiving fresh autologous TS, rather than the frozen batch-prepared TS (Figure 6). It is therefore possible that the freezing process does inhibit certain constituents, but it is difficult to conclude if they are fish specific or if the same TEER increases would be present in 'fresh' FBS if it was available.

The frozen TS did also produce viable cultures (Figure 6) but there was no evidence of cell toxicity when using this serum type as has been previously reported for native plasma (Wood et al. 2003; Pärt et al. 1993). We suspect that the serum preparation method utilised is important and recommend both removing the coagulation factors in the clot (overnight in the fridge (4 ºC)) and using a heat

inactivation stage. As well as the heat inactivation of serum compliment, this process itself can have a beneficial effect on the resulting serum by an increase in cell growth (Fujiwara et al. 2009). The frozen serum aliquots appeared to store well at -20 ºC as were used for over 6 months with similar success.

Perhaps the key finding in this experiment was that both the culture types grown using FBS or TS maintained a mean TEER >5 KΩ cm^2 for 12 days PWA (Figure 4). Previous studies were limited to short term experimental durations of 6 – 48 h (Wood et al. 2002) due to collapse of culture integrity, recorded by TEER, after this time. One exception is the report of Walker et al. (2007, Figure 4) where cultures maintain >5 KΩ cm^2 for 96 h PWA (despite a large drop in TEER at 72h PWA). Clearly, the cultures in this experiment are lasting much longer than reported in previous works. We do not have a satisfactory explanation for why this is. The methodology has lots of scope for subtle differences in fish husbandry, cell harvest and culture, but we have first considered the known protocol deviations from the Schnell et al. (2016) published methods, which include pre perfusion of gills (see Experiment 1), media antibiotics, donor fish size and incubation temperature. Previous works using SSI concluded there was no difference in maintaining antibiotics in the media throughout culture and water addition (Gilmour et al. 1998). We therefore suggest this would have least impact on the culture duration other than reducing the chance of infection.

We have previously observed differences in primary cell culture from differing fish sizes and optimised the weight of fish for best results (Baron et al. 2012). The recommended weight of fish used to prepare the DSI cultures are 80 - 150 g (Kelly et al. 2000) or

<100 g (Schnell et al. 2016), although there are also occasional reports of fish up to 200 g being used successfully (Fletcher et al. 2000; Farkas et al. 2011). Larger fish were used throughout this study (overall mean ± S.E.M. = 273 ± 9 g). This was either by design (in order to harvest a useable volume of serum for comparison in Experiment 3), or simply due to availability. It might be worthwhile comparing similar fish at different weights, but age and seasonal differences of fish from the same stock or fish differences between different stocks could complicate interpretation of results. If we compare the TEER data from this study with the range of fish sizes used across all experiments here (155 – 510 g), there is no correlation (data not shown). Since larger fish generally yield more cells, there could be the benefit of creating more replicate cultures than if smaller individuals were used.

Incubation temperature is also a possible contributing factor. We chose to maintain cells at 15 ºC as this matched the fish holding conditions, but is 3 ºC cooler than the typically recommended temperature. While incubation temperature can be optimised in cell culture towards the experimental aim, e. g. Tollefsen (2008), and temperature changes as small as a few degrees Celsius can cause different cell responses (Pawlowski et al. 2000), it seems unlikely in this case to have caused such a profound change to the cultures. A contributing factor might be a delayed or inhibited culture degradation at the lower temperature, but similarly, the scale of the change observed here makes this explanation unlikely.

Another interesting condition within our culture system is the relationship between the fish holding water and the synthetic water added to the cultures. Due to local geology, the holding water in Plymouth is particularly soft, with previously reported mean ion

concentrations of Ca^{2+}, 0.30; K^{+} 0.02; Mg^{2+} 0.04; Na^{+} 0.39 (mmol l^{-1}; Boyle et al. 2014) which are all lower, up to 12× for Mg^{2+}, than in US EPA moderately hard water (nominal mmol l^{-1}; Ca^{2+}, 0.35; K^{+} 0.051; Mg^{2+} 0.49; Na^{+} 1.13; US EPA 2002). A theory to explain the observed culture durations here could be that the animal holding conditions are able to later influence the performance of the cell cultures. In this case that is donor fish adapting their regulatory systems to low ion conditions which later yields gill cells that are better able to cope with an ionoregulatory challenge (apical water addition) when in culture. While it is well known that fish can remodel their gill cellular structure, such as increasing MR cell density, as part of an adaptation to soft water (Perry and Laurent 1993; Perry 1997), it is not known if such changes would be passed on to subsequent primary culture. Understanding how whole animal physiology and its life history are linked to cellular function is a current topic in the study of stress and aging (Alper et al. 2015). There is evidence that individual life history can lead to alterations that are maintained in metabolic cell responses when in subsequent primary culture (Jimenez et al. 2014). This challenges the existing theory that cells grown in culture media lose the *in situ* scaling parameters provided by their position within a tissue and individual (West et al. 2003). It would be particularly interesting to further explore such adaptations in fish, both for cell type proportion and function, given the numerous demanding physiological processes the gills are tasked with.

The SEM analysis of culture surface morphology during formation and asymmetrical experimentation (Figure 5) reveals the high diversity, both in terms of surface structure and changes through time displayed by the DSI cultures. The attachment and growth and appearance of suspected MR cells observed is in line with previous descriptions (Wood et al. 2002). The microridge diversity observed

is arguably higher than previously described and shows similar morphological characteristics to *in vivo* gill cells of rainbow trout and other freshwater species (Iftikar et al. 2010; Matey et al. 2011). The culture surface changes during longer water additions have not previously been described, but include an apparent reduction in microridge height and the emergence of a larger scale ridge structure (Figure 5e). It is unclear what the significance of this larger ridge structure is but it appears to vary in scale on neighbouring cells and was present when culture TEER remained high. No quantitative assessments have been carried out on these images and they are included only to illustrate the clear diversity of cell surface morphology and changes through time.

Effect of membrane area (Experiment 4)

A working theory as to the improvements observed in culture longevity was that the larger cell numbers supported by the 4.2 cm^2 inserts created a culture that was better able to support a high TEER. This idea was investigated in Experiment 4. The experimental TEER data was impacted by a technical limitation of our TEER instrument. We do not know the peak resistance or that sustained over the experimental period. Despite this, we were able to show apparent differences in cells from the same stock but cultured on different insert sizes (Figure 7). Interestingly, all three of the insert sizes produced cultures that lasted >10 days PWA (Figure 8), but such cultures were much more common in the larger well size. This suggests some methodological variation in culture maintenance, which is in agreement with our experience in the laboratory of washing the cultures. The plates containing the 4.2 cm^2 inserts can be gently swirled on the bench a few times, causing the apical media within the inserts to rotate. This circular movement is enough to dislodge any debris and mucus on the culture, and the rotation moves it to the centre where it can be easily removed by pipette. This

is not the case for 0.9 and 0.3 cm^2 inserts where the surface tension of the media within the smaller well stops a swirl forming and the culture must be manually ‘washed’ with successive rinses of PBS via pipette. Washing by pipette is time consuming due to its ineffectiveness, increases contamination risk by having the lid off for prolonged periods, and is also inconsistent due to variation in pipetting speed. This speed variation risks disruption of the cell layer by an excessively strong jet from the pipette which would cause a delay or failure in culture confluence.

Another potential difference between the culture sizes is the variation in ratios of surface area to media volumes used within each format. These characteristics within our experimental volumes are shown in Table 1. The media replacements in symmetrical phase are designed to negate any nutrient deficits. Given the 4.2 cm^2 format have the lowest media volume per unit area of culture, the lower TEER observed in the other formats is unlikely to be limited by nutrient availability. Conversely, the 4.2 cm^2 format also have the largest surface area to volume ratio. While it is possible that oxygen concentration in the apical media or water is better maintained in this format, particularly after media changes cease PWA, experimental trials with anoxic water suggested no differences in culture TEER (data not shown). These considerations lead to a question of media renewal when in asymmetrical phase. This has not previously been an issue due to the short time periods involved (up to 72 h). However, for these longer lasting cultures (routinely 12 days), it might well be that the viable duration could be further extended, or the TEER values maintained at a higher level by replenishing the basolateral media throughout asymmetrical culture.

Overall, our study details methodological investigations which deliver a significant improvement in culture viability time from three to twelve days post water addition, as evidenced by TEER measurements and morphological developments. This is an important breakthrough and facilitates a large range of future experiments using this improved model, particularly longer term, chronic toxicity tests that are currently lacking from cell culture models. In addition, the continued use and development of this model is in line with the principles of the 3Rs in animal experimentation, as it facilitates a Reduction in the number of live fish used and offers a Refinement of the existing exposure protocols. There is scope for the continued development of similar models in other species, and this gill model could also be combined with other types of organoid cultures to provide novel co-cultures with a higher degree of relevance to the *in vivo* state.

Acknowledgements

The authors would like to thank Ben Eynon, Glenn Harper, Lynne Cooper and Andrew Atfield (University of Plymouth) for technical support.

Compliance with Ethical Standards

Funding

This work was funded by a Biotechnology and Biological Sciences Research Council (BBSRC) Research Grant IPA (BB/L01016X/1), co-funded by the AstraZeneca Global Safety, Health and Environment research programme, to ANJ supporting RJM and MGB. AstraZeneca provided support in the form of salary for SFO and grant to ANJ, but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The SFO work represents an AstraZeneca contribution in kind to the Innovative Medicines Initiative (IMI) under grant agreement no.115735—iPiE: Intelligent led assessment of Pharmaceuticals in the Environment; resources of which are composed of financial contribution from the European Union's Seventh Framework Programme (FP7/2015-2018) and European Federation of Pharmaceutical Industries and Associations (EFPIA) companies' in kind contribution.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical Approval

All applicable national, and/or institutional guidelines for the care and use of animals (fish) were followed.

References

Alper SJ, Bronikowski AM, Harper JM (2015) Comparative cellular biogerontology: where do we stand? Exp Gerontol 71:109–11

Baron MG, Purcell WM, Jackson SK, Owen SF, Jha AN (2012) Towards a more representative *in vitro* method for fish ecotoxicology: morphological and biochemical characterisation of three-dimensional spheroidal hepatocytes. Ecotoxicology 21:2419–2429

Baron MG, Mintram KS, Owen SF, Hetheridge MJ, Moody AJ, Purcell WM, Jackson SK, Jha AN (2017) Pharmaceutical Metabolism in Fish: Using a 3-D Hepatic *In Vitro* Model to Assess Clearance. PLoS ONE 12(1):e0168837

Bury NR, Schnell S, Hogstrand C (2014) Gill cell culture systems as models for aquatic environmental monitoring. J Exp Biol 217:639–65

Burden N, Chapman K, Sewell F, Robinson V (2015) Pioneering Better Science through the 3Rs: An Introduction to the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs). J Am Assoc Lab Anim Sci 54(2):198-208

Boyle D, Fox JE, Akerman JM, Sloman KA, Henry TB, Handy RD (2014) Minimal effects of waterborne exposure to single-walled carbon nanotubes on behaviour and physiology of juvenile rainbow trout (Oncorhynchus mykiss). Aquat Toxicol 146:154-164

Chen S, Einspanier R, Schoen J (2015) Transepithelial electrical resistance (TEER): a functional parameter to monitor the quality of oviduct epithelial cells cultured on filter supports Histochem Cell Biol 144(5):509-515

El-Dakhly AT, Azab AES, Alrawi QK, Lashkham NM (2015) Evaluation of substitution of fetal calf serum in VERO cell cultures by fish serum. Int J Curr Res Rev 3(2):1-8

Farkas J, Christian P, Gallego-Urrea JA, Roos N, Hassellöv M, Tollefsen KE, Thomas KV (2011) Uptake and effects of manufactured silver nanoparticles in rainbow trout (*Oncorhynchus mykiss*) gill cells. Aquat Toxicol 101:117-125

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

Fujiwara M, Tsukada R, Shioya I, Takagi M (2009) Effects of heat treatment and concentration of fish serum on cell growth in adhesion culture of Chinese hamster ovary cells. Cytotechnology 59:135-141

Fujiwara M, Aizu Y, Shioya I, Takagi M (2010) Fetal-calf-serum-free suspension culture of Chinese hamster ovary cells employing fish serum. J Biosci Bioeng 109:307–309

Galvez F, Tsui T, Wood CM (2008) Cultured trout gill epithelia enriched in pavement cells or in mitochondria-rich cells provides insights into Na+ and Ca2+ transport. In Vitro Cell Dev Biol Animal 44:415-425

Gilmour KM, Pärt P, Prunet P, Pisam M, McDonald DG, Wood CM (1998) Permeability and morphology of a cultured branchial epithelium from the rainbow trout during prolonged apical exposure to freshwater. J Exp Zool 281:531-545

Hashimoto H, Toyohara H, Yokoyama Y, Sakaguchi M, Ozato K, Wakamatsu Y. (1997) Effect of carp serum on the growth of the goldfish fin cells in early passage. J Fish Biol 50:201-207

Iftikar FI, Matey V, Wood CM (2010) The Ionoregulatory Responses to Hypoxia in the Freshwater Rainbow Trout *Oncorhynchus mykiss.* Physiol Biochem Zool 83(2):343-355

Jimenez AG, Van Brocklyn J, Wortman M, Williams JB (2014) Cellular metabolic rate is influenced by life-history traits in tropical and temperate birds. PLoS ONE 9(1):e87349

Kelly SP, Fletcher M, Pärt P, Wood CM (2000) Procedures for the preparation and culture of ‘‘reconstructed’’ rainbow trout branchial epithelia. Methods Cell Sci 22:153-163

Kelly SP, Wood CM (2001) Effect of cortisol on the physiology of cultured pavement cell epithelia from freshwater trout gills. Am J Physiol 281:R811-R820

Kelly SP, Wood CM (2002) Cultured gill epithelia from freshwater tilapia (*Oreochromis niloticus*): Effect of cortisol and homologous serum supplements from stressed and unstressed fish. Membrane Biol 190:29-42

Knight E, Przyborski S (2015) Advances in 3D cell culture technologies enabling tissue-like structures to be created *in vitro*. J Anat 227(6):746-756

Kocal T, Quinn BA, Smith IR, Ferguson HW, Hayes MA (1988) Use of trout serum to prepare primary attached monolayer cultures of hepatocytes from rainbow trout (*Salmo gairdneri*). In Vitro Cell Dev Biol 24:304-308

Leguen I, Cauty C, Odjo N, Corlu A, Prunet P (2007) Trout gill cells in primary culture on solid and permeable supports. Comp Biochem Physiol A Mol Integr Physiol 148:903-912

Lillicrap A, Belanger S, Burden N, Du Pasquier D, Embry MR, Halder M, Lampi MA, Lee L, Norberg-King T, Rattner BA, Schirmer K, Thomas P (2016a) Alternative approaches to vertebrate ecotoxicity tests in the 21st century: A review of developments over the last 2 decades and current status. Environ Toxicol Chem 35(11):2637-2646

Lillicrap A, Springer T, Tyler CR (2016b) A tiered assessment strategy for more effective evaluation of bioaccumulation of chemicals in fish. Regul Toxicol Pharmacol 75:20–26

Matey V, Iftikar FI, De Boeck G, Scott GR, Sloman KA, Almeida-Val VMF, Val AL, Wood CM (2011) Gill morphology and acute hypoxia: responses of mitochondria-rich, pavement, and mucous cells in the Amazonian oscar (*Astronotus ocellatus*) and the rainbow trout (*Oncorhynchus mykiss*), two species with very different approaches to the osmo-respiratory compromise. Can J Zool 89:307-324

Minghetti M, Schnell S, Chadwick MA, Hogstrand C, Bury NR (2014a) A primary Fish Gill Cell System (FIGCS) for environmental monitoring of river waters. Aquat Toxicol 154:184-192

OECD (2012) Fish Toxicity Testing Framework. Series on testing and assessment no. 171, Organisation for Economic Cooperation and Development, Paris. ENV/JM/MONO(2012)16:171

Pandeya S, Parvez S, Ansari RA, Ali M, Kaur M, Hayat F, Ahmad F, Raisuddin S (2008) Effects of exposure to multiple trace metals on biochemical, histological and ultrastructural features of gills of a freshwater fish*, Channa punctata* Chem Biol Interact 174(3):183-92

Pawlowski S, Islinger M, Völkl A, Braunbeck T (2000) Temperature-dependent vitellogenin-mRNA expression in primary cultures of rainbow trout (*Oncorhynchus mykiss*) hepatocytes at 14 and 18 °C. Toxicol In Vitro 14(6):531-40

Pärt P, Norrgren L, Bergström E, Sjoberg E (1993) Primary cultures of epithelial cells from rainbow trout gills. J Exp Biol 175:219-232

Perry SF, Davie PS, Daxboeck C, Ellis AG, Smith DG (1984) 10 Perfusion methods for the study of gill physiology. In: Hoar WS, Randall DJ (ed) Fish Physiology Volume X: Gills, Part B: Ion and Water Transfer. Academic Press, Orlando, pp 325-388

Perry SF, Laurent P (1993) Environmental effects on fish gill structure and function. In: Rankin JC, Jensen FB (ed) Fish Ecophysiology. Chapman and Hall, London, pp 231-264

Perry SF (1997) The chloride cell: structure and function in the gills of freshwater fishes. Annu Rev Physiol 59:325-347

Rathore G, Sood N, Swaminathan R (2001) Primary cell culture from fish gills and kidney using fish serum. Indian J Exp Biol 39:936-938

Rosa J, Tiago DM, Dias J, Cancela ML, Laizé V (2010) Serum-specific stimulation of proliferation and mineralization of fish bone-derived cells. J Appl Ichthyol 26(2):251-256

Srinivasan B, Kolli AR, Esch MB, Abaci HE, Shuler ML, Hickman JJ (2015) TEER measurement techniques for *in vitro* barrier model systems. J Lab Autom. 20(2):107-126

Schnell S, Stott LC, Hogstrand C, Wood CM, Kelly SP, Pärt P, Owen SF, Bury NR (2016) Procedures for the reconstruction, primary culture and experimental use of rainbow trout gill epithelia. Nat Protoc 11:490-498

Stott LC, Schnell S, Hogstrand C, Owen SF, Bury NR (2015) A primary fish gill cell culture model to assess pharmaceutical uptake and efflux: Evidence for passive and facilitated transport. Aquat Toxicol 159:127-137

Tollefsen K-E, Eikvar S, Finne EF, Fogelberg O, Gregersen IK (2008) Estrogenicity of alkylphenols and alkylated non-phenolics in a rainbow trout (*Oncorhynchus mykiss*) primary hepatocyte culture. Ecotoxicol Environ Saf 71:370-383

US EPA (2002) US Environmental Protection Agency. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, 5th ed. https://www3.epa.gov/region6/water/npdes/wet/wet_methods_manuals/atx.pdf Accessed 31 January 2017

Walker PA, Bury NR, Hogstrand C (2007) Influence of culture conditions on metal-induced responses in a cultured rainbow trout gill epithelium. Environ Sci Technol 41:6505-6513

Walker PA, Kille P, Scott A, Bury NR, Hogstrand C (2008) An *in vitro* method to assess toxicity of waterborne metals to fish. Toxicol 30:67-77

Weissgerber TL, Milic NM, Winham SJ, Garovic VD (2015) Beyond bar and line graphs: time for a new data presentation paradigm. PLoS Biol 13:e1002128

West GB, Woodruff WH, Brown JH (2003) Allometric scaling of metabolic rate from molecules and mitochondria to cells and mammals. Proc Nat Acad Sci 99:2473-2478

Wilson JM, Laurent P (2002) Fish gill morphology: inside out. J Exp Zool 293:192-213

Wood CM, Pärt P (1997) Cultured branchial epithelia from freshwater fish gills. J Exp Biol 200:1047-1059

Wood CM, Gilmour KM, Pärt P (1998) Passive and active transport properties of a gill model, the cultured branchial epithelium of the freshwater rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol A 119:87–96

Wood CM, Eletti B, Pärt P (2003) New methods for the primary culture of gill epithelia from freshwater rainbow trout. Fish Physiol Biochem 26:329-344

Wood CM, Kelly SP, Zhou B, Fletcher M, O'Donnell M, Eletti B, Pärt P (2002) Cultured gill epithelia as models for the freshwater fish gill. Biochim Biophys Acta 1566:72-83

Zakaria-Runkat F, Worawattanamateekul W, Lawhavinit O (2006) Production of fish serum products as substitute for fetal bovine serum in hybridoma cell cultures from surimi industrial waste. Kasetsart J (Nat Sci) 40(Suppl.):198-205

Zhou BS, Kelly SP, Ianowski JP, Wood CM (2003) Effects of cortisol and prolactin on Na+ and Cl- transport in cultured branchial epithelia from FW rainbow trout. Am J Physiol Regul Integr Comp Physiol 285(6):R1305-R1316

Figure Legends

Figure 1. Image of perfused (top) and primary (bottom) gill arch after preparation and dissection for comparison. Note one central filament in perfused arch has not cleared.

Figure 2. comparison of mean ± S.E.M. TEER from symmetrical cultures using 4 combinations of Primary and Perfusion cell preparation techniques to supply the cells for first and second seeding. No significant differences were observed between the 4 culture seeding combinations.

Figure 3. S.E.M. imaging of different membrane surfaces for comparison. Note different scales; A = standard Falcon 1.6×10^6 0.4 µm pores/cm^2 (scale bar = 10 µm); B = Falcon HD 1×10^8 0.4 µm pores/cm^2 (scale bar = 5 µm); C = Reinnervate Alvetex strata (scale bar = 50 µm).

Figure 4. Comparison of mean ± S.E.M. TEER from DSI cultures using FBS or TS as the media serum. Water was added to apical compartment on Day 9. Significant differences between groups at individual time points are indicated by *($P < 0.05$), ***($P < 0.001$).

Figure 5. SEM photographs of typical DSI culture surface structure and morphology from cultures grown in 4.2 cm^2 inserts using 5% FBS in L-15. A - F culture development through symmetrical (A - C) and asymmetrical (D - F) conditions using apical US EPA moderately hard synthetic water. Cells attached to membrane 24 h post initial seed (A), confluent layer with smooth surface 24 h post

second seed (B), and clearly defined cell junctions and a range of surface microridge morphology at 7 days old (C). At 2 days post water addition (PWA) culture displays broad range of surface morphology and structures (D). At 10 days PWA with a high TEER maintained, cell junction definition and surface microridges are both reduced but other surface morphology has developed (E). At 18 days PWA TEER has reduced and surface layer shows clear degradation and loss of viability (F). Higher magnification of typical cell surface morphologies (G; magnified region from D) and different example (H) of suspected MR cells with finger-like apical microridge projections. An apical crypt-like structure (I; magnified from region in C). Scale bars represent 5 – 100 µm, see individual image information.

Figure 6. Comparison of mean ± S.E.M. TEER from DSI cultures using Autologous or General TS in culture media. Water was added to apical compartment on Day 8. Significant differences between groups at individual time points are indicated by *($P < 0.05$).

Figure 7. Comparison of mean ± S.E.M. TEER measurements from DSI cultures grown in the 4.2, 0.9 and 0.3 cm^2 format inserts. Only data with an uncorrected TEER of <20 KΩ shown, hence the omitted middle section. Significant differences from the 0.3 cm^2 format are denoted by *($P < 0.05$) and ***($P < 0.001$).

Figure 8. Comparison of the number of days TEER >5 KΩ cm^2 in cultures grown in the 4.2, 0.9 and 0.3 cm^2 format inserts after the addition of apical water. Whole dataset presented for transparency, with individual data points as circles and group means as

rectangle. Significant differences between the groups are denoted by *(P <0.05) and ***(P <0.001). Graph template downloaded from Weissgerber et al. (2015).

Maunder et al. Figures

Figure 1

Figure 2

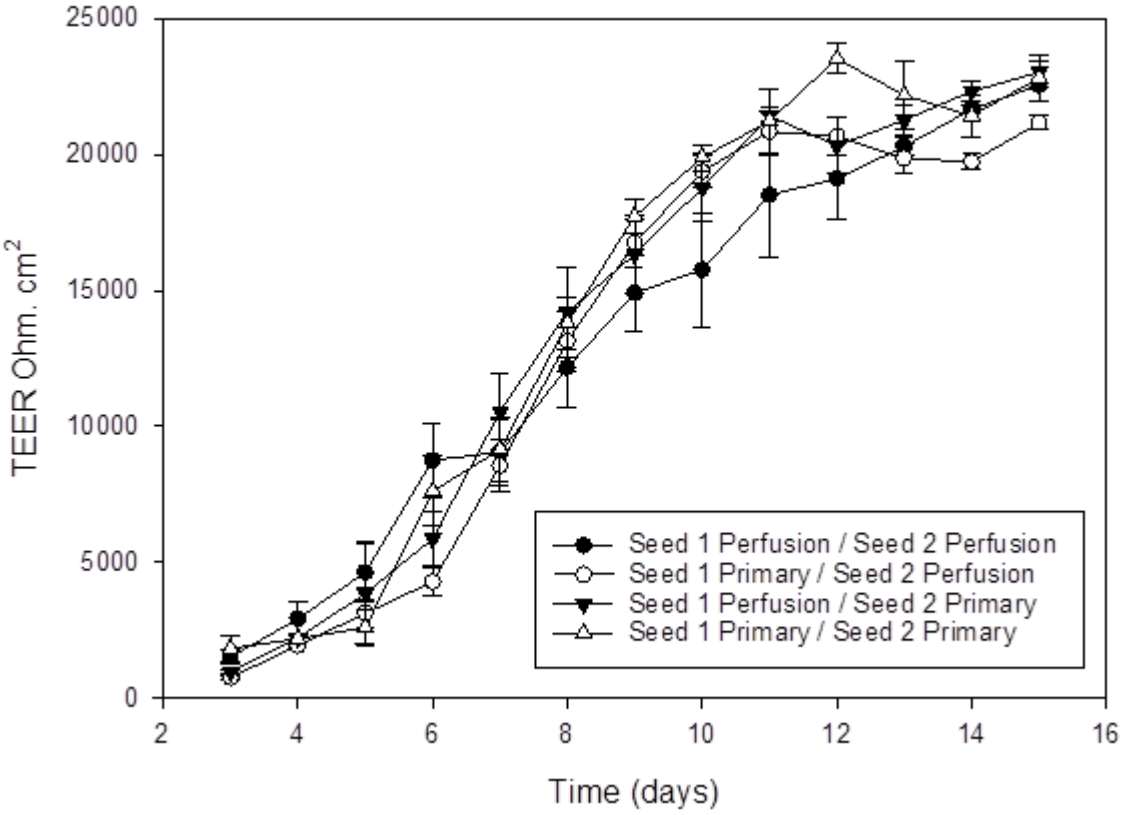
25000
20000
15000
10000
5000
0
TEER Ohm. cm2
2
4
6
8
10
12
14
16
Time (days)
Seed 1 Perfusion / Seed 2 Perfusion
Seed 1 Primary / Seed 2 Perfusion
Seed 1 Perfusion / Seed 2 Primary
Seed 1 Primary / Seed 2 Primary

Figure 3

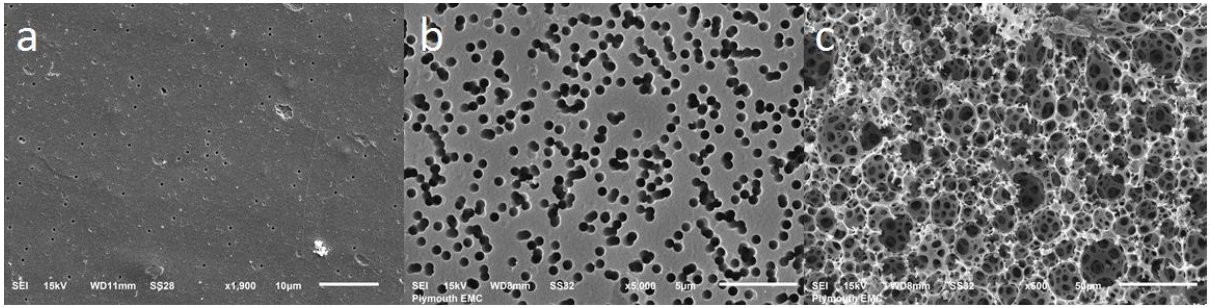
a
b
c
SEI 15kV WD11mm SS28 x1,900 10µm
SEI 15kV WD8mm SS32 x5,000 5µm
Plymouth EMC

Figure 4

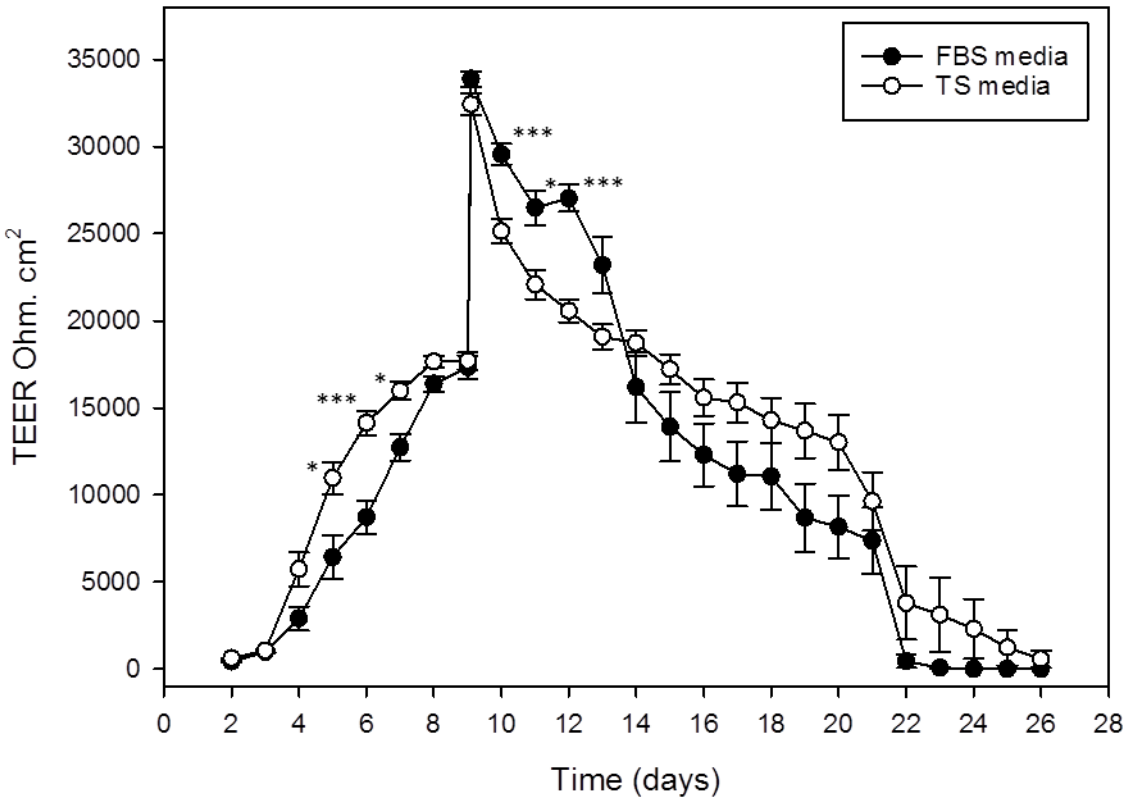
FBS media
TS media
TEER Ohm. cm2
Time (days)
0
5000
10000
15000
20000
25000
30000
35000
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
*
***
*
***
*
***

Figure 5

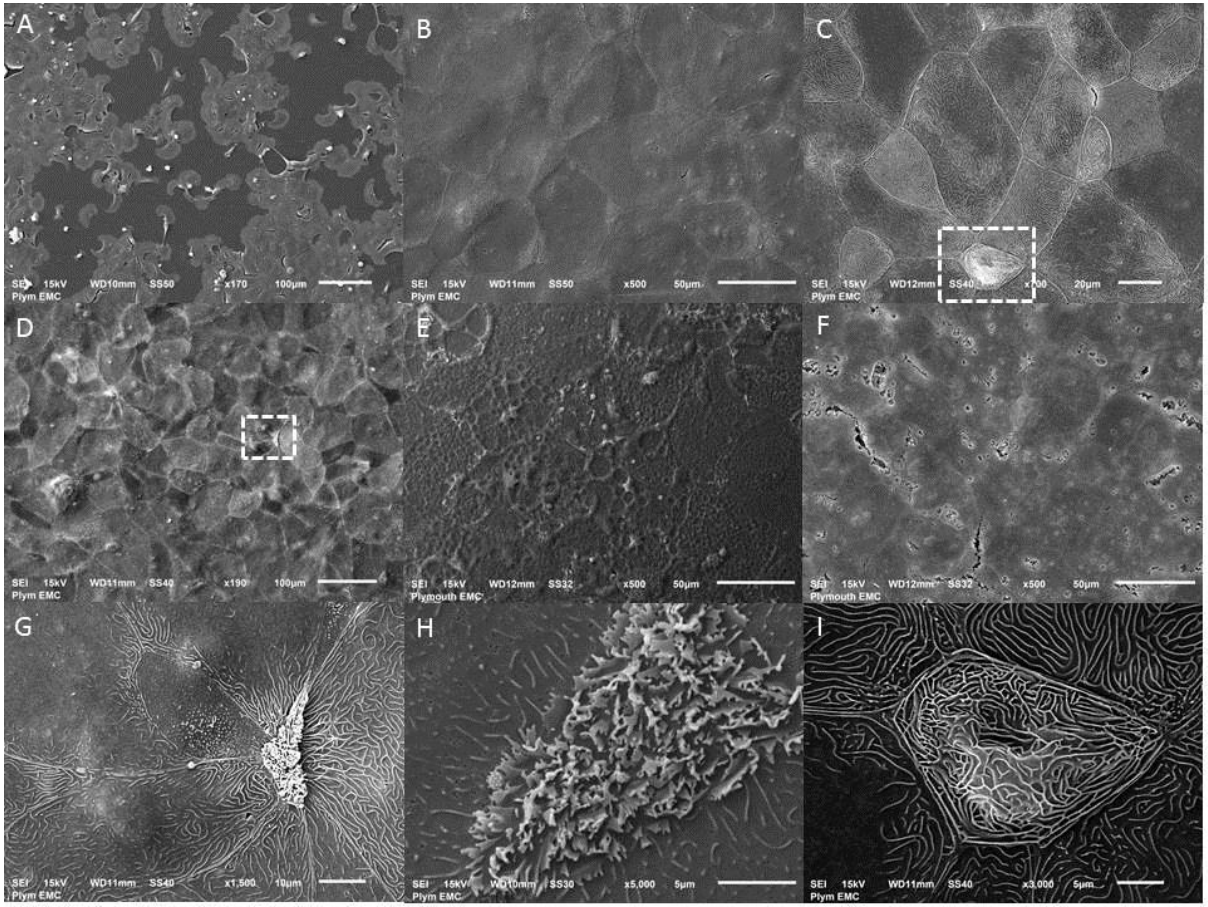
A
SEI 15kV WD10mm SS50 x170 100µm
Plym EMC
B
SEI 15kV WD11mm SS50 x500 50µm
Plym EMC
C
SEI 15kV WD12mm SS40 x500 20µm
Plym EMC
D
SEI 15kV WD11mm SS40 x190 100µm
Plym EMC
E
SEI 15kV WD12mm SS32 x500 50µm
Plymouth EMC
F
SEI 15kV WD12mm SS32 x500 50µm
Plymouth EMC
G
SEI 15kV WD11mm SS40 x1,500 10µm
Plym EMC
H
SEI 15kV WD10mm SS30 x5,000 5µm
Plym EMC
I
SEI 15kV WD11mm SS40 x3,000 5µm
Plym EMC

Figure 6

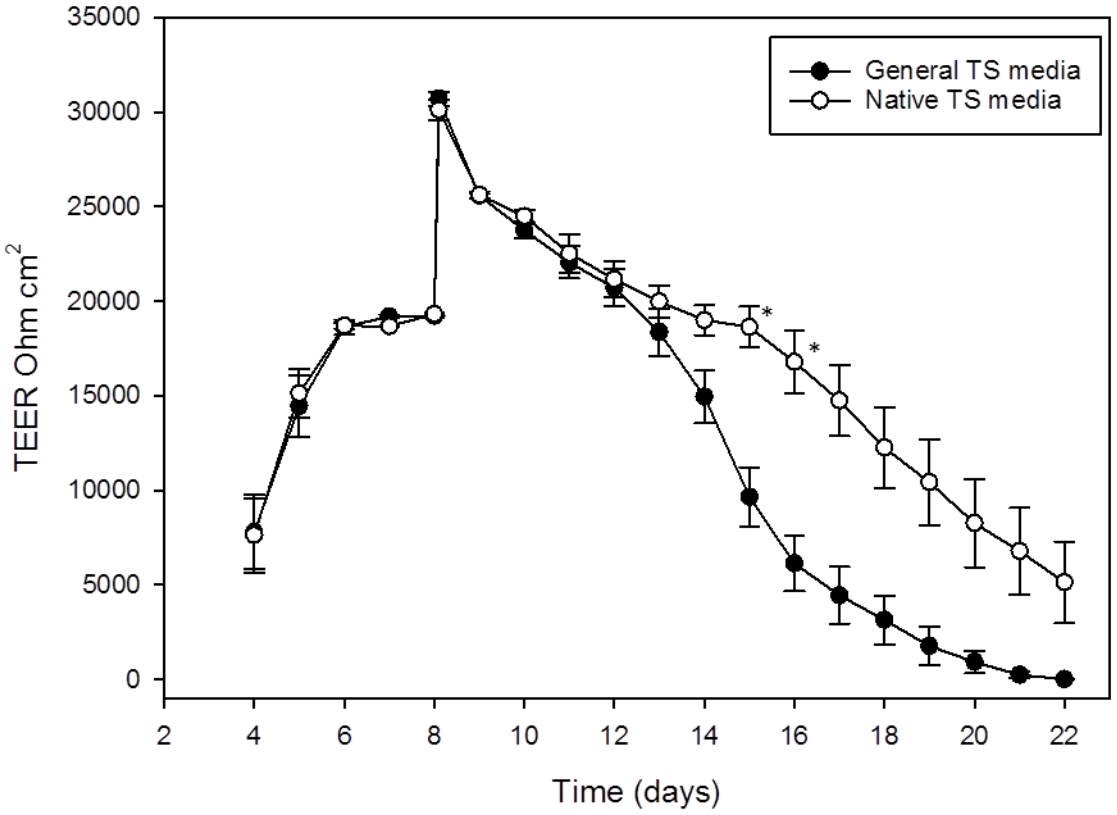
35000
30000
25000
20000
15000
10000
5000
0
TEER Ohm cm^2
2
4
6
8
10
12
14
16
18
20
22
Time (days)
General TS media
Native TS media
*
*

Figure 7

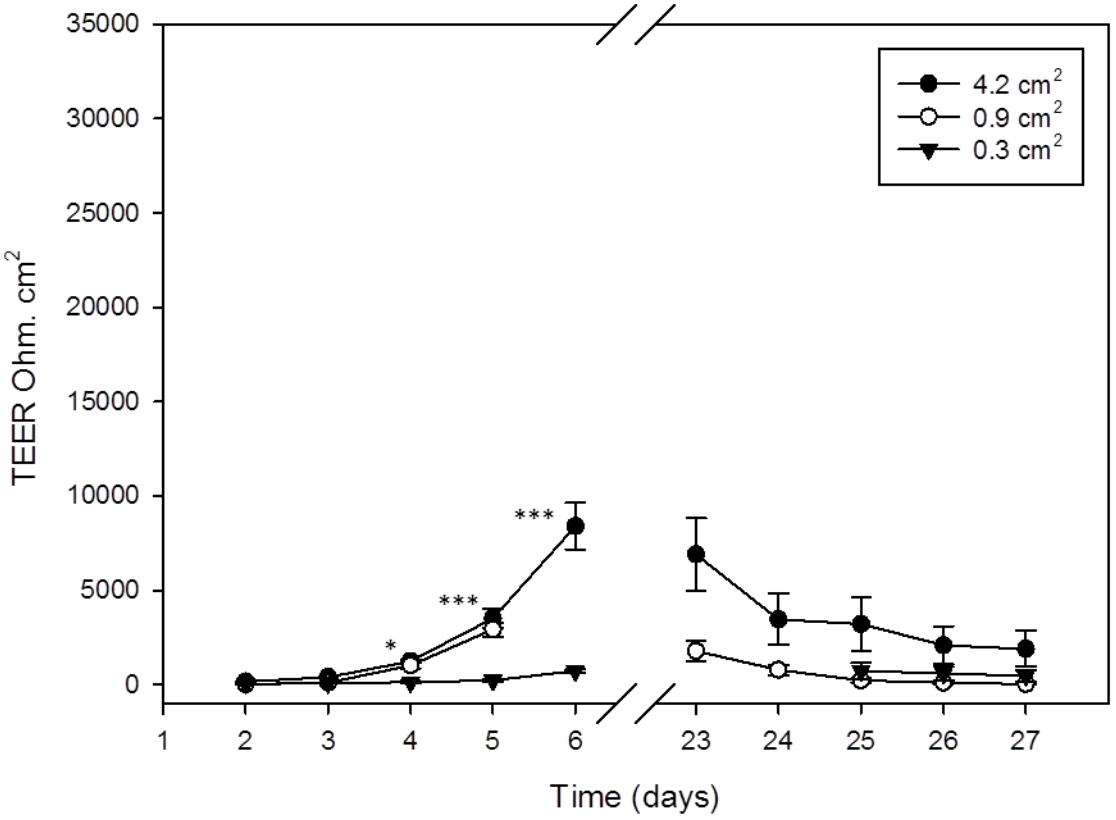
35000
30000
25000
20000
15000
10000
5000
0
TEER Ohm. cm2
4.2 cm2
0.9 cm2
0.3 cm2
*
***
***
1
2
3
4
5
6
23
24
25
26
27
Time (days)

Figure 8

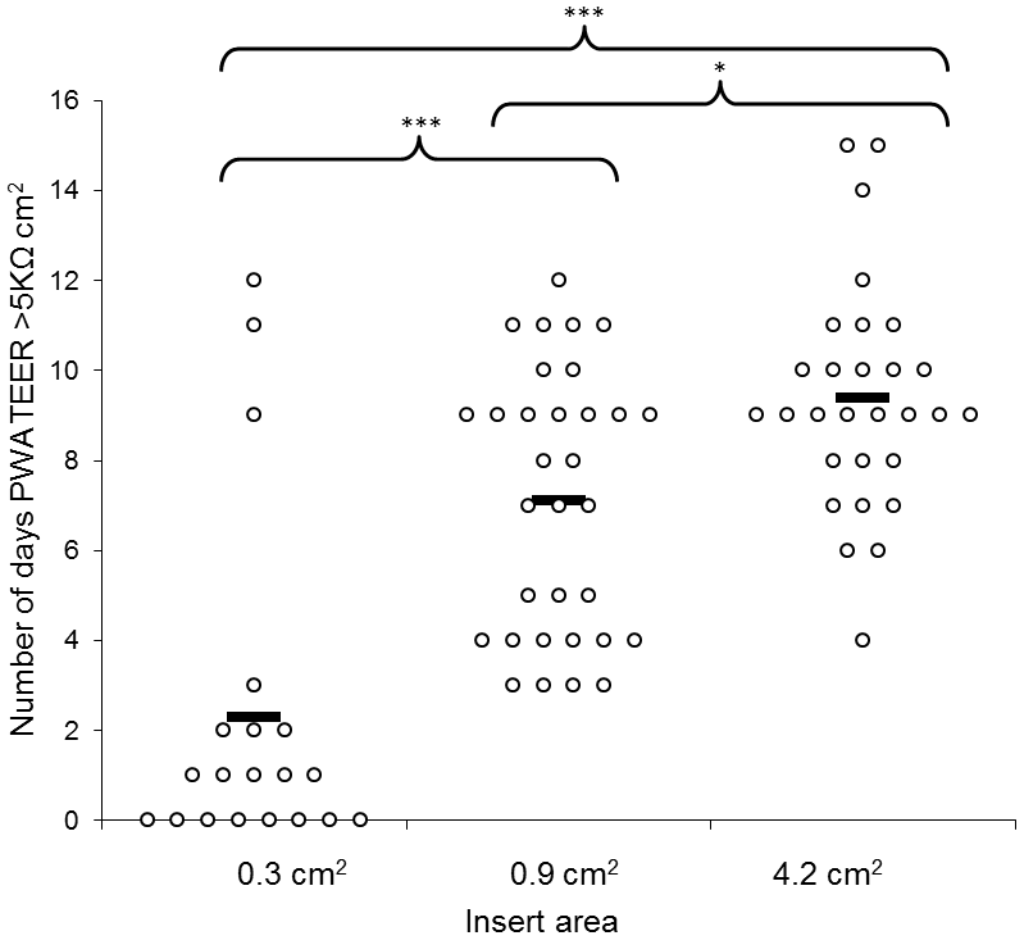
***
*
***
16
14
12
10
8
6
4
2
0
Number of days PWA TEER >5KΩ cm2
0.3 cm2
0.9 cm2
4.2 cm2
Insert area

Table 1 Characteristics, culture volumes and comparisons of three different sized microplate insert format.

Insert format	**Insert area (cm^2)**	**Seeding ratio**	**Apical media (vol. mL)**	**Basolateral media vol. (mL)**	**Apical media per cm^2 (mL)**	**Surface area: Volume (ratio of apical compartment)**
4.2 cm^2	4.19	1	2.5	3	0.24	1.7:1
0.9 cm^2	0.87	0.208	1	2	1.11	0.9:1
0.3 cm^2	0.32	0.076	0.35	0.8	2.67	0.9:1