Development and adaptation of an in vitro rainbow trout gill model for use as an alternative to live fish studies



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'Double Seeded

Insert (DSI)

L-15







Introduction

In line the 3Rs, our work involves the development and validation of novel, reliable in vitro fish methods as an alternative to in vivo studies. The rainbow trout (Oncorhynchus mykiss) gill has a long history of cell

Incubate 24 h

Trout gill epithelial cell culture

Wash +

2nd seed (24 h)

L-15

L-15

and tissue models, which have developed over time from a single to a double seeding of cells in flasks and then microplate inserts²⁻⁵. One issue with the existing gill model for toxicological testing is the relatively short (~2 days) duration of viability after

1st seed (0h)

L-15

apical exposure to water. If this time could be extended, potential applications for the model would be increased e.g. to include longer term chronic testing to better represent current in vivo ecotoxicological tests. Such a model would **Reduce** the number of fish used in existing tests and **Refine** the method of exposure to test chemicals.

Epithelial cells are extracted and seeded <u>into insert</u> Waterborne

The aim of the current study was therefore to contaminants investigate the effect of different culture methods on transferred to the gill model viability and to maximise its lifespan blood via gills and functionality after apical water addition.

Incubate 24 h

O. mykiss in vivo /in vitro comparison

Measure uptake of compounds from water into gill cells and transfer to media

Measure excretion rates back across gills into water

Water L-15 culture media

Materials & Methods

Results & Discussion

dashed line =

the ~48 hours previously reported.⁷

apical water

addition

40000

35000

30000

₂₅₀₀₀

当 20000

15000

10000

5000

We have investigated various ideas to improve the time the model remains viable after addition of apical water. The methods reported here include: \(\frac{1}{1}\) Culture serum type, \(\frac{2}{2}\) Pre-perfusion of gills, and 33 Use of different membrane materials.

Blood collection from a fish before perfusion or harvesting gill cells. The serum can be used instead of FBS in later culture of cells

Cells were grown in symmetrical culture (L-15 media on both sides) with either 5% Fetal Bovine Serum (FBS) or Trout Serum (TS) before replacing the media on the apical side with artificial freshwater USEPA moderately hard water).

—TS mean ± SEM n≥6

FBS mean ± SEM n≥6

The gills were perfused in situ through the aorta with PBS via syringe to remove blood cells from filaments. The perfused cells then went straight into the trypsin stage. DSI cultures were then established using primary, perfused or both cell types, and the TEER recorded daily.

Heart is isolated Fish is Perfusion Schedule 1 tubing fed killed and into and supported clamped in upside down Blood is forced out of gills by PBS under pressure from the syringe

Whole arches dissected

without the wash steps

and processed as per

primary tissue but

Effect of seeding with primary or perfused gill preparations on subsequent culture TEER 25000 20000 E 15000 → 1st = Primary, 2nd = Primary 10000 —1st = Primary, 2nd = Perfused → 1st = Perfused, 2nd = Primary 5000 → 1st = Perfused, 2nd = Perfused (all mean ± SEM n≥4) Culture age (days)

> 2> We tested primary and/or perfused culture preparations for the two cell seeds. NSD was observed between any of the 4 combinations of cell seeding were found. Although no apparent benefit for TEER, the perfusion method does reduce the protocol time for culture preparation by limiting the wash stages and allows earlier seeding of cells.

The Trans-Epithelial Electrical Resistance (TEER) across a cell culture can be measured by a WPI EVOM2 'chopstick'

electrode. It is a standard measure of the cell membrane 'tightness' and can be interpreted as a measure of culture quality⁶

Three different plastic polymer or pore density membranes were tested against the control for TEER through time. SEM imaging was also used to assess membrane appearance and cell attachment.

Alvetex 'strata' highly porous polystyrene membrane (note different scale)

(control) BD Falcon 1.6 x 10^6 0.4 μ m pores/cm² in polyethylene terephthalate (PET) membrane (GBO are similar)

BD Falcon 'High Density' 1.0 x 108 0.4 μm pores/cm² in PET membrane

35 The alternative insert membrane types generally

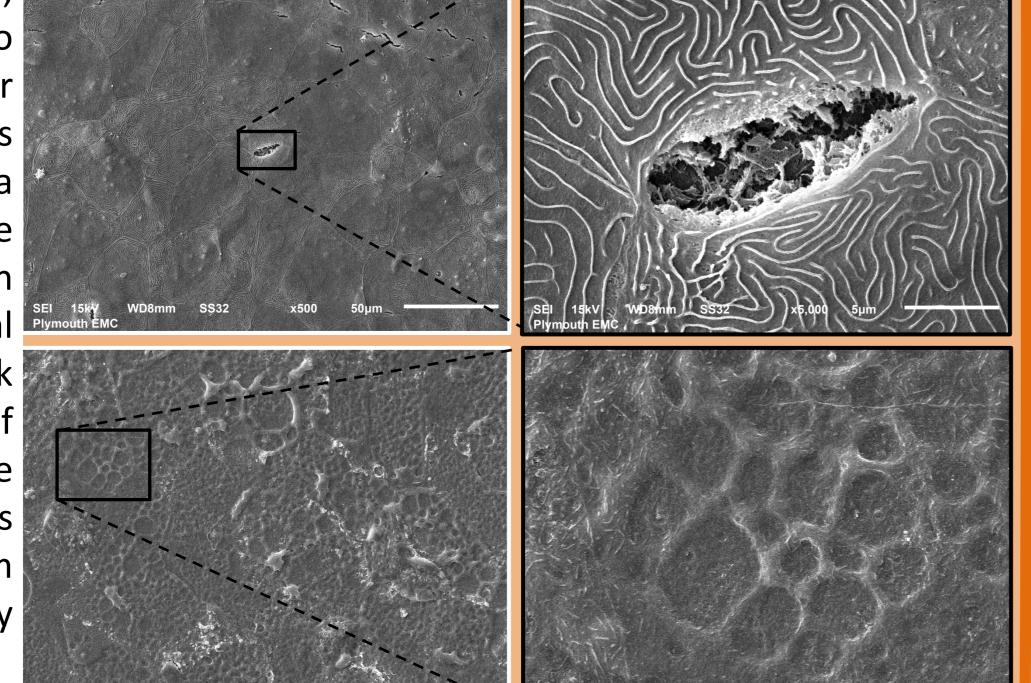
displayed low cell adhesion and low TEER. The identical PET membrane with a higher pore density did not attain TEER values above background. The Alvetex was not fully compatible with the membrane chopstick electrode, so although the TEER appeared to be low, it could not be accurately measured. Only the Transwell (polycarbonate) membrane produced similar growth to the control (data not shown). It is unclear why the cells did not attach, but the seeding densities might require optimisation for specific insert types, particularly the 3D scaffold-like Alvetex. We are also yet to try collagen-coated inserts from the major suppliers which are successfully used in mammalian in vitro cell culture.8

Culture age (days) The use of TS appears to show some improvements to TEER compared to FBS cultures. TS cultures reach $5K\Omega$ cm² (a standard threshold) a day sooner than FBS under symmetrical conditions and remain above $5K\Omega$ cm² for several days more after water addition. FBS cultures appear to reach a higher maximal TER immediately after water addition. Both of the culture types are extending far beyond

Effect of serum type on TEER in trout gill DSI cultures

SEM analysis of surface ultrastructure of a typical culture for FBS or TS at Day 5 post water addition (PWA; top) reveals microridges, tight junctions and varying cell types. After ten days PWA only TS cultures remain viable. Interestingly, TS

cultures (e.g. bottom; Day 18) appear to exhibit depressed, or coated microridges with development of a scale larger ridge structure, similar in scale to in vivo apical crypts. Further work on characterisation of this version of the model and explore its use in longer term exposures is currently being explored.



References

[**1**] Baron *et al.* (2012). *Ecotox.*, **21**, 2419-2429. **[2]** Pärt *et al.* (1993). J. Exp. Biol. 175, 219-232. [3] Fletcher et al. (2000). J. Exp. Biol. **203**, 1523-1537. **[4]** Bury et al. (2014). J. Exp. Biol. (2014) 217, 639-650. [5] Schnell, Stott et al. Nature Protocols 11, 490–498. [6] Wood et al. (2002). Bioc et Biop A 1566 72-83. [6] Walker et al. (2007) Env. Sci. Tech. **41** 6505-6513 **[7]** Takezawa *et al.* (2004). *Cell Trans* **13**, 463-473.

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