

**RESEARCH
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**BRUSSELS 2017
SETAC EUROPE**

Primary culture of rainbow trout liver & gill

- 10th May 2017 SETAC Europe 27th Annual Meeting, Brussels
- Workshop:
- Current trends in fish *in vitro* toxicology: Applications of 3Rs principles
- Dr Richard Maunder, School of Biological and Marine Science, Plymouth University

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Liver spheroid production

Methods of Dr Matt Baron;

see Baron *et al.* (2012) *Ecotoxicology* 21, 2149-2429.

Ecotoxicology (2012) 21:2419–2429
DOI 10.1007/s10646-012-0965-5

TECHNICAL NOTE

Towards a more representative in vitro method for fish ecotoxicology: morphological and biochemical characterisation of three-dimensional spheroidal hepatocytes

Matthew G. Baron • Wendy M. Purcell •
Simon K. Jackson • Stewart F. Owen •
Awadhesh N. Jha

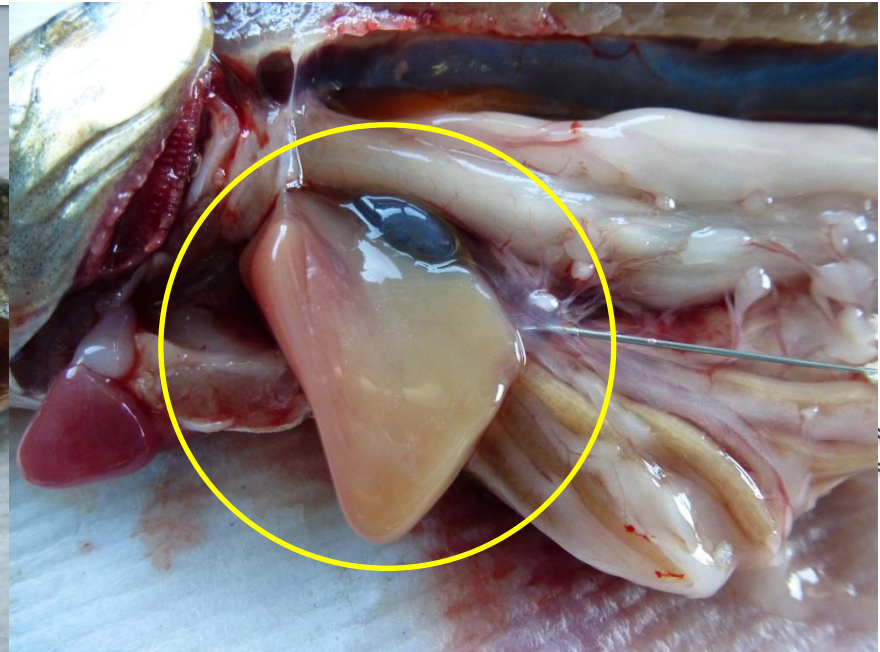
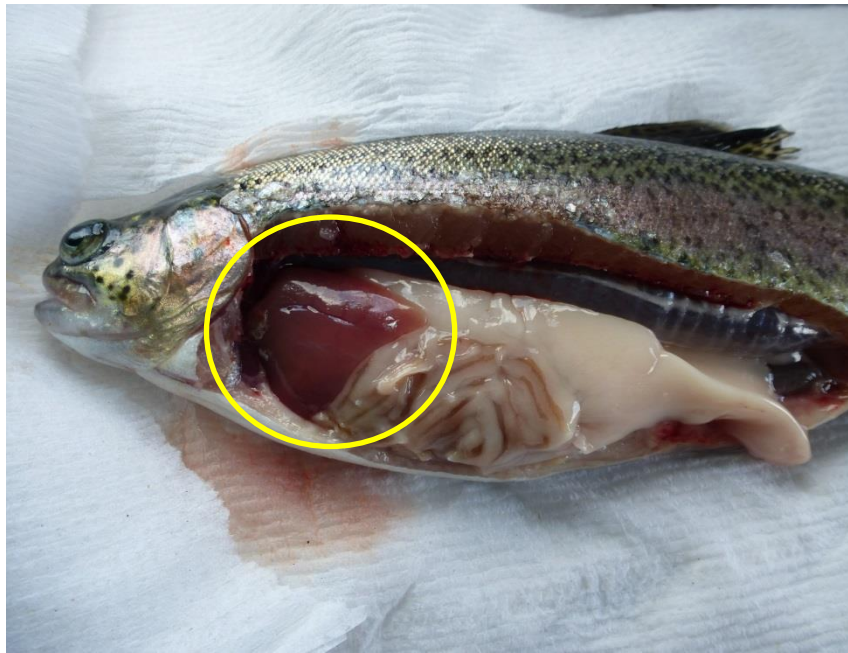
Accepted: 8 June 2012 / Published online: 26 June 2012
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SoP is available on request

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Liver spheroid production

In situ perfusion



HBSS blood clearance

Collagenase digestion

Culture media wash

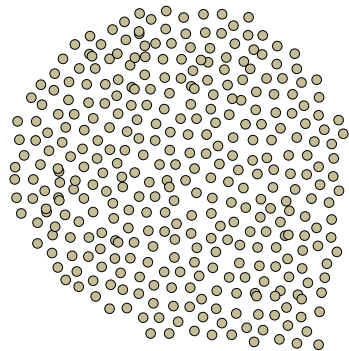


Peristaltic pump

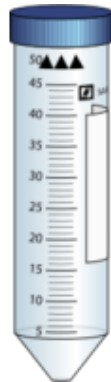
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Liver spheroid production 2

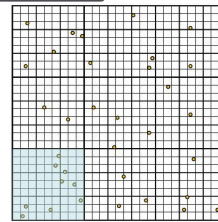
wash, count, plate



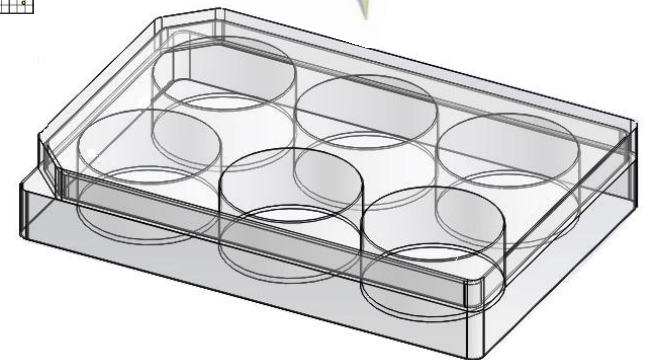
Wash hepatocytes
to purify



Haemocytometer
count of cells



Seed at 1 million
cells per mL



3 mL L-15 media +10% FBS per well
(6 well plate pre-treated with Poly(2-
hydroxyethylmethacrylate))

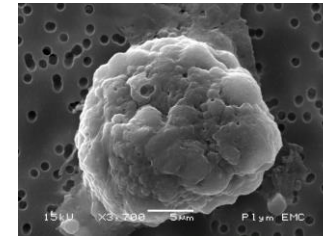
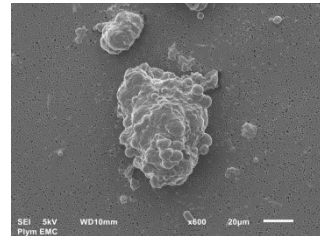
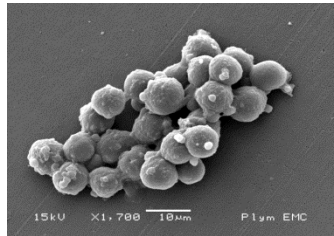
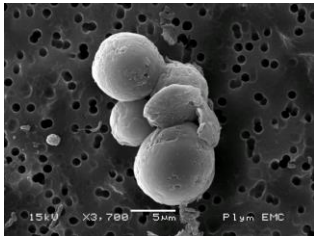
Liver spheroid production 3

rotate, renew, clean



Clean culture
and renew
1.5 mL
media every
other day

Day 1



Day 7

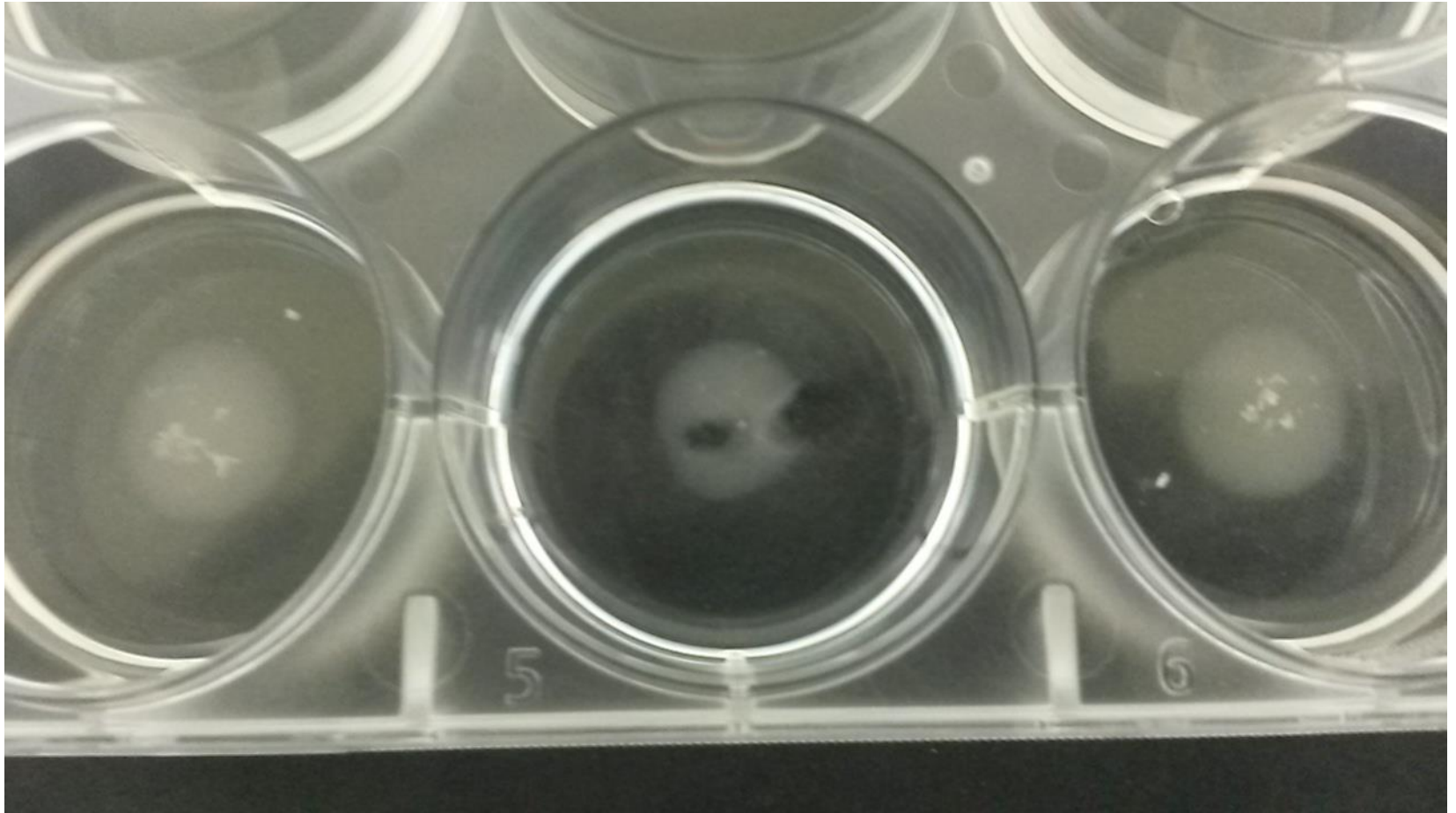


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Liver spheroid production 3

rotate, renew, clean



Spheroid metabolism assay



see Baron *et al.* (2017) PLoS ONE 12(1): e0168837



RESEARCH ARTICLE

Pharmaceutical Metabolism in Fish: Using a 3-D Hepatic *In Vitro* Model to Assess Clearance

Matthew G. Baron^{1,2}, Kate S. Mintram^{1,2}, Stewart F. Owen^{2*}, Malcolm J. Hetheridge², A. John Moody¹, Wendy M. Purcell³, Simon K. Jackson³, Awadhesh N. Jha^{1*}

1 School of Biological Science, Plymouth University, Devon, United Kingdom, 2 AstraZeneca, Alderley Park, Macclesfield, Cheshire, United Kingdom, 3 School of Biomedical & Healthcare Science, Plymouth University, Devon, United Kingdom

* Stewart.Owen@AstraZeneca.com (SFO); a.jha@plymouth.ac.uk (ANJ)



Abstract

At high internal doses, pharmaceuticals have the potential for inducing biological/pharmacological effects in fish. One particular concern for the environment is their potential to bioaccu-

Spheroid characterisation

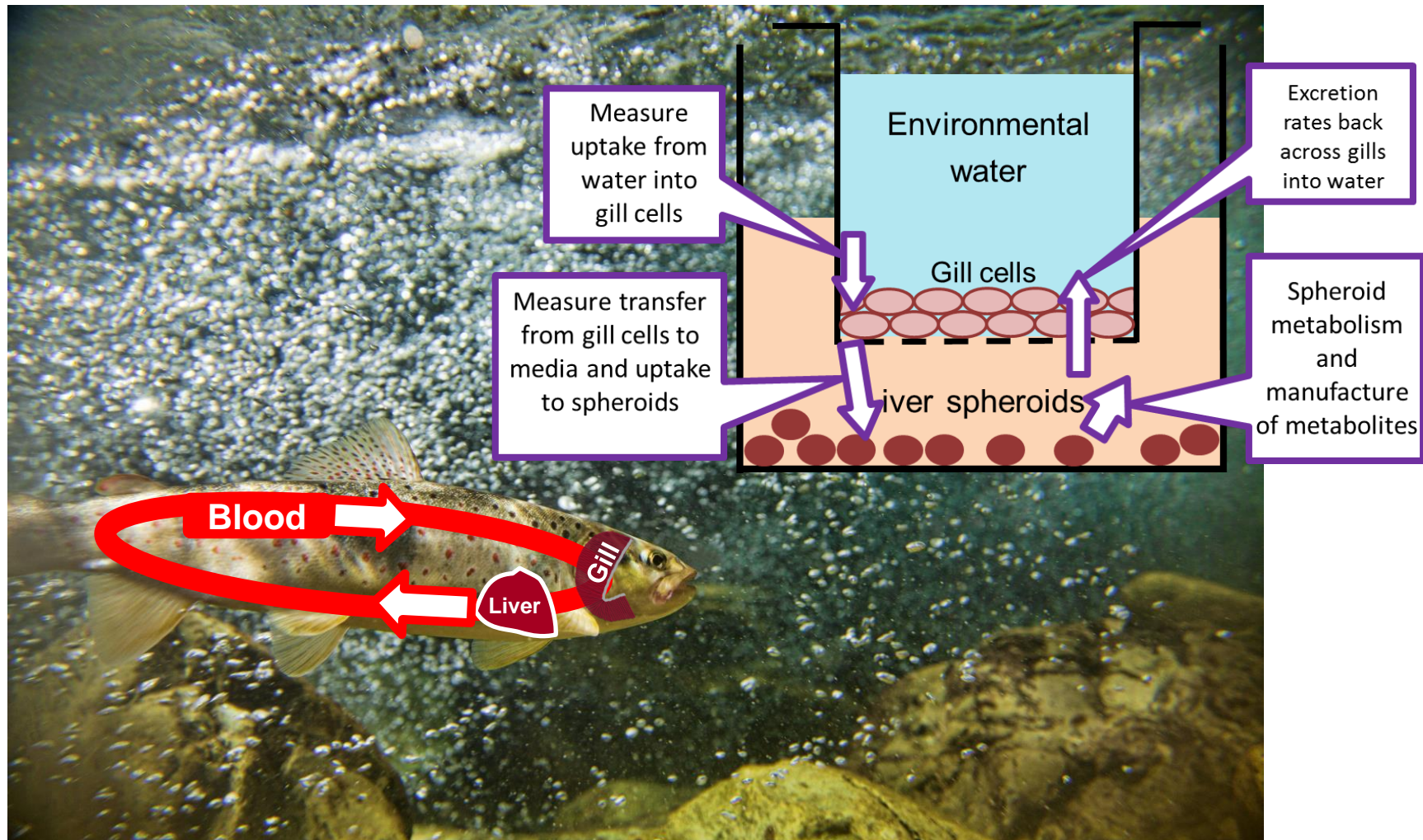
see Langan *et al.* (2015) PLoS ONE 11(2): e0149492

Spheroids:

Metabolic activity > S9 fractions / hepatocytes

Viable for several weeks

Gill layer production



Gill layer production

Existing, well developed method exists;

See Schnell *et al.* (2016) Nature Protocols 11(3) 490-498

<http://www.burylabs.co.uk/figcs/>

PROTOCOL

Procedures for and experimen

Sabine Schnell^{1,6}, Lucy C. Stott^{1,6}
Stewart F Owen⁵ & Nic R Bury¹

¹Division of Diabetes and Nutritional Sciences, ²Zoology, University of British Columbia, Vancouver Joint Research Centre, Institute of Environment, Cheshire, UK. ³These authors contributed equally ac.uk) or N.R.B. (nic.bury@kcl.ac.uk).

Published online 11 February 2016; doi:10.1038

This protocol describes how to reconstru supports within cell culture inserts. T monitoring and preparation for use in seeding of isolated gill cells twice ov technique. Approximately 5–12 d after withstand freshwater on the apical ce alternative for toxicity testing, bloac

INTRODUCTION
The freshwater fish gill is a multifun

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FiGCS – Fish Gill Cell Culture System

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The Fish Gill Cell Culture System (FiGCS) is a surrogate of the fish gill and been used as an in vitro tool to assess the uptake of chemicals from the water into the fish (Stott et al 2015). It may thus be of interest to industrial, pharmaceutical and governmental entities as a screen to assess the potential for chemicals to be taken up by fish as part of an environmental risk assessment.

The Benefits of the Fish Gill Cell Culture System include:

- Alternative, cheap and effective way of carrying out aquatic toxicology, physiology and endocrinology
- Fulfils obligations of the NC3Rs – replace, reduce, refine the use of animals for scientific purposes
- Eliminates need to keep large stocks of live fish for testing
- Gill Cell Cultures from the gills of 2 fish effectively replaces 40-72 live fish using traditional testing methods
- Humane method – Using fish gill cell tissue means that no live fish are actually tested upon – lesser administrative burden on testing labs

This Fish Gill Cell Culture System cultures the primary gill cells of the freshwater rainbow trout on a flat permeable membrane. This procedure, known as the double seeded insert (DSI) technique, produces a heterogeneous gill epithelium



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How the Fish Gill Cell Culture System Works



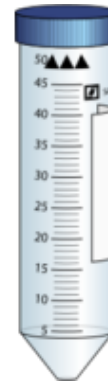
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Gill layer production

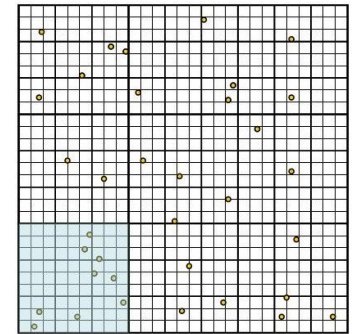
Cut away
filaments



Wash & digest
epithelial cells



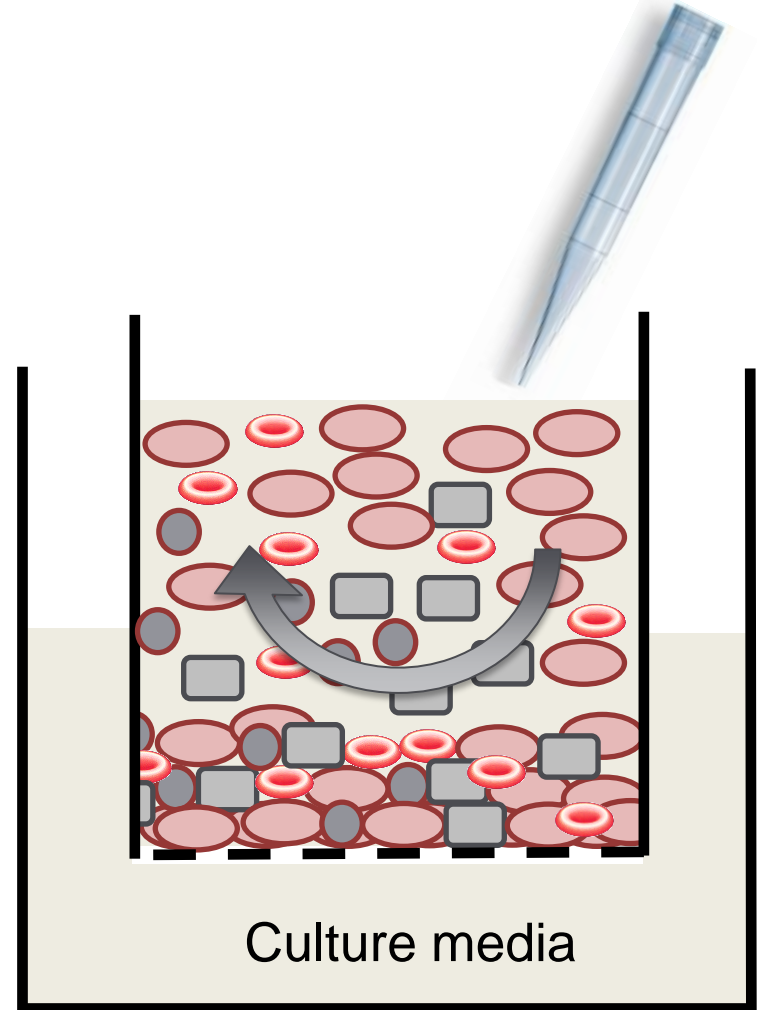
Haemocytometer
count of cells



Gill layer production

1st seed of cells

 Pavement cell



First seed

Wash

Condition
insert >2 h

Incubate 24 h

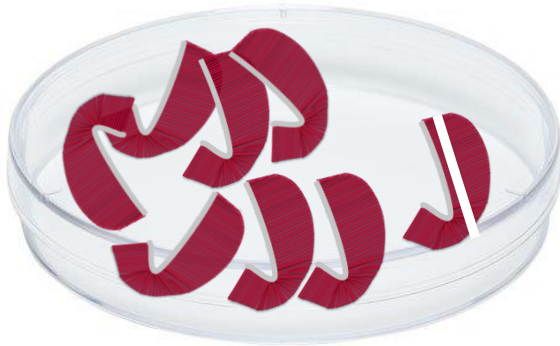
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“Single Seeded
Insert” SSI

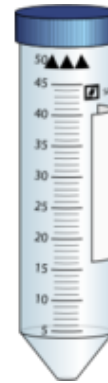
Gill layer production

Prepare a 2nd set of epithelial cells

Cut away
filaments



Wash & digest
epithelial cells







Haemocytometer
count of cells

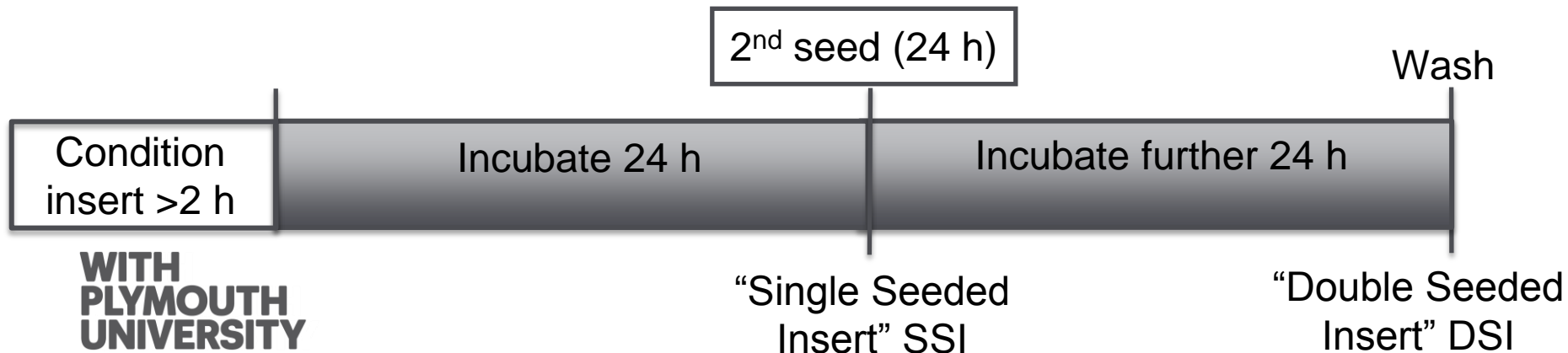
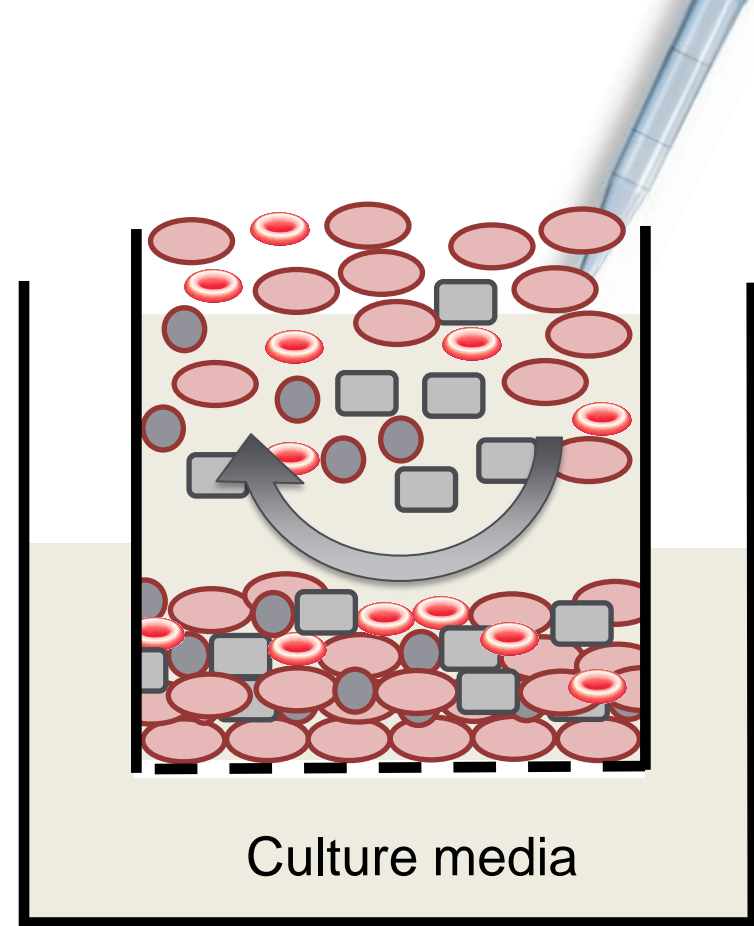


Gill layer production

2nd seed of cells

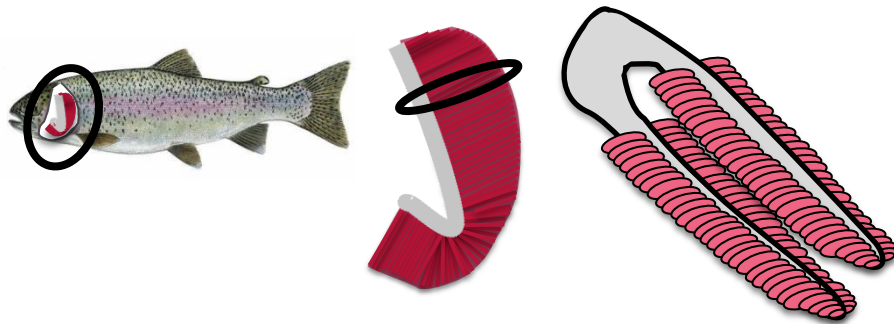
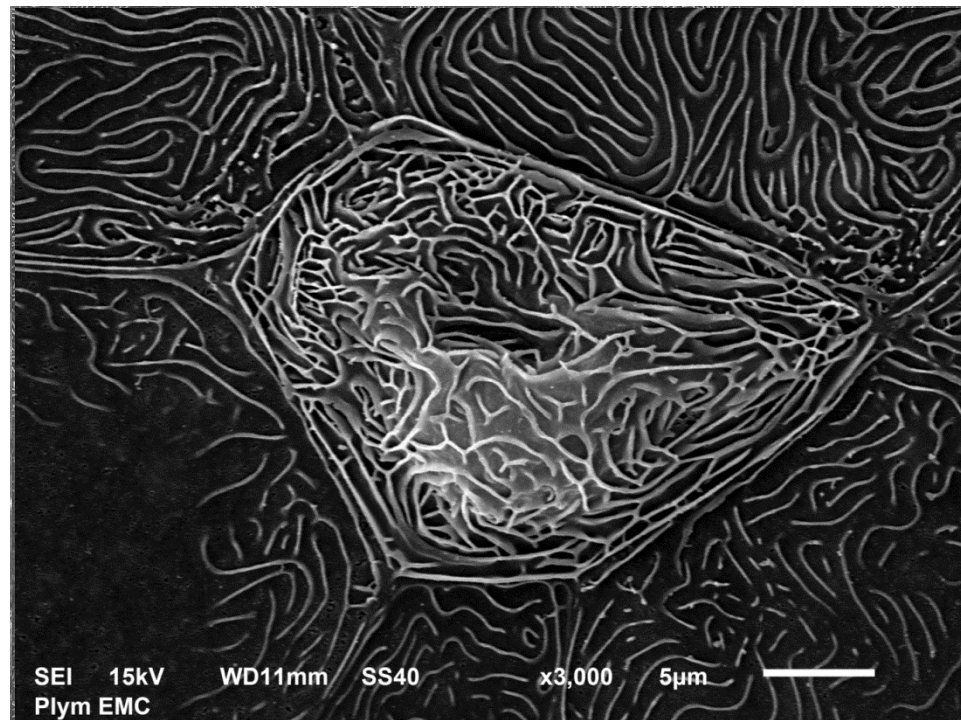
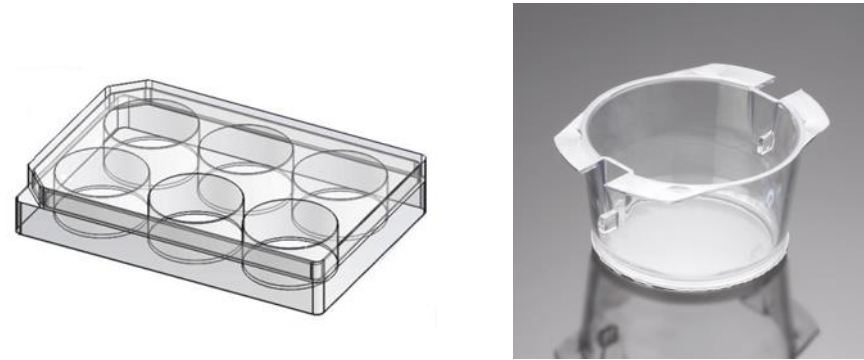
Higher diversity of cell types present in DSI

-  Pavement cell
-  Chloride cell
-  Erythrocyte
-  Goblet cell

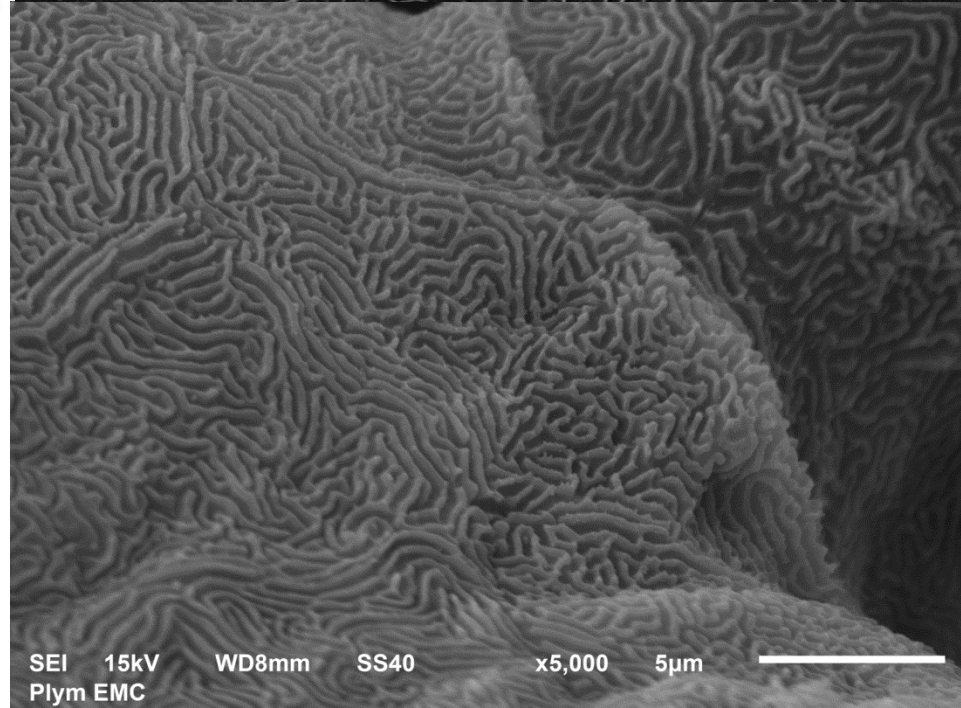


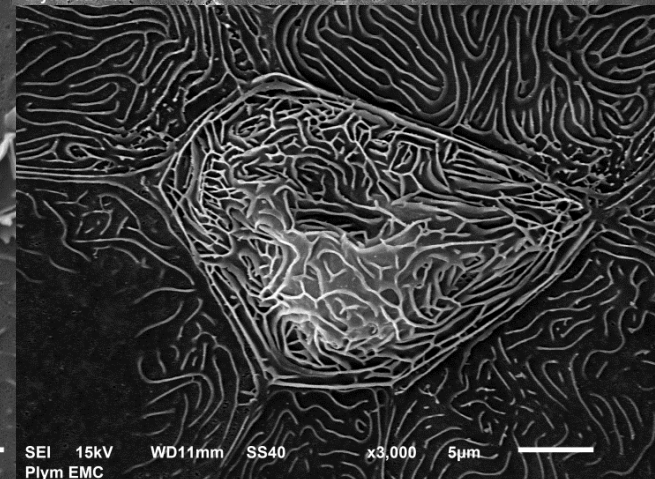
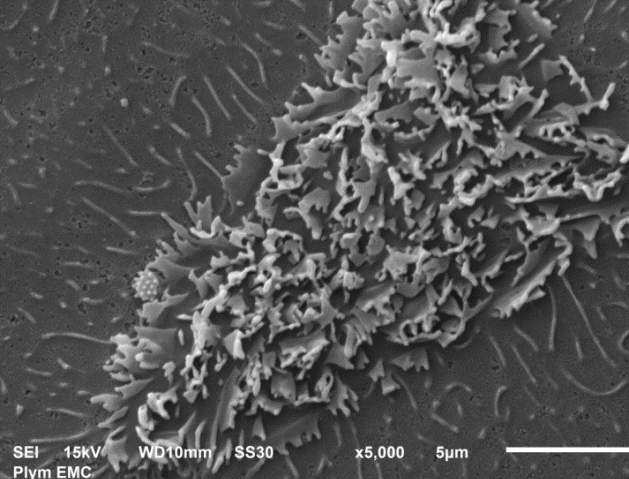
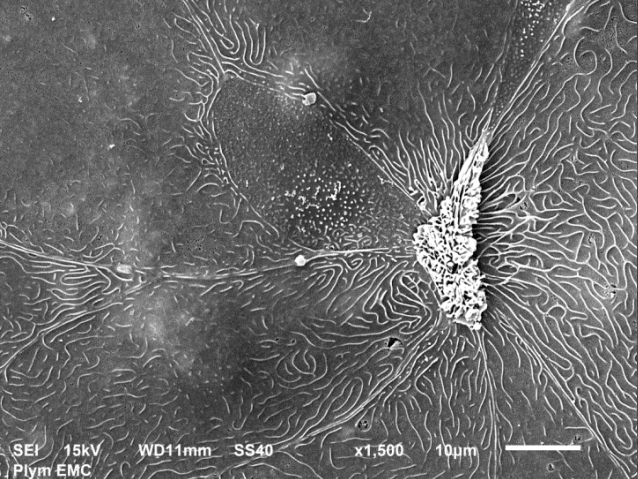
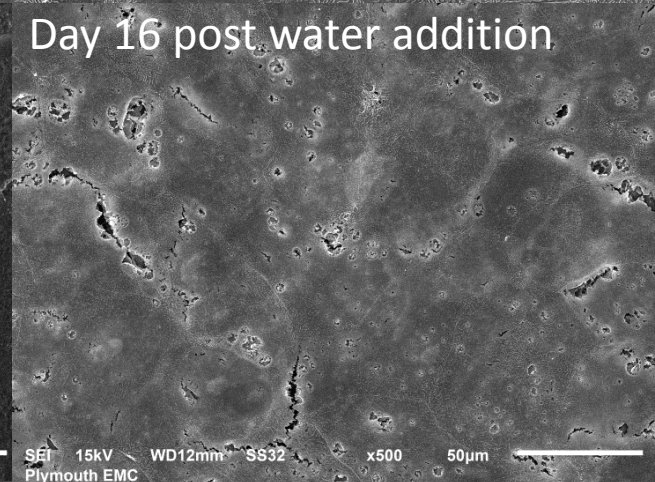
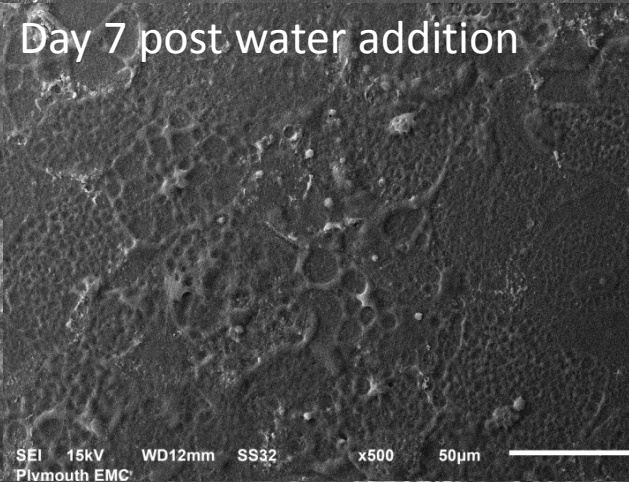
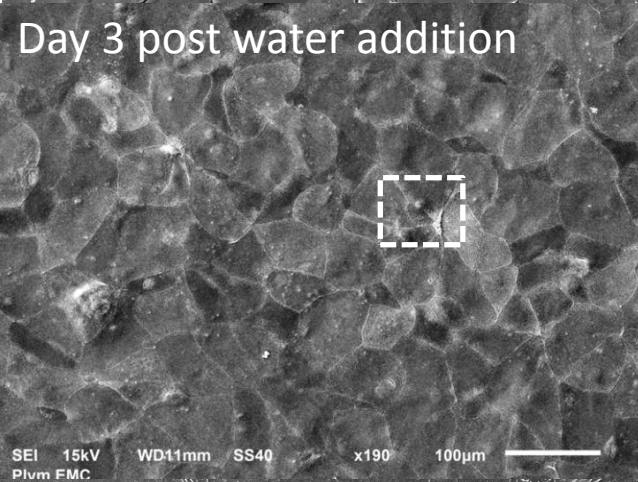
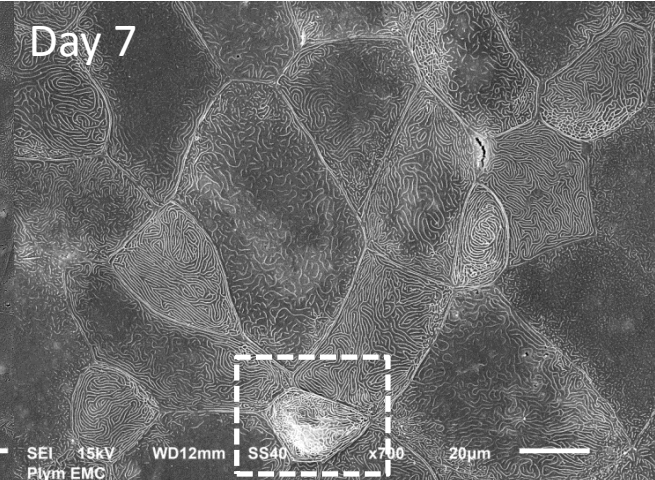
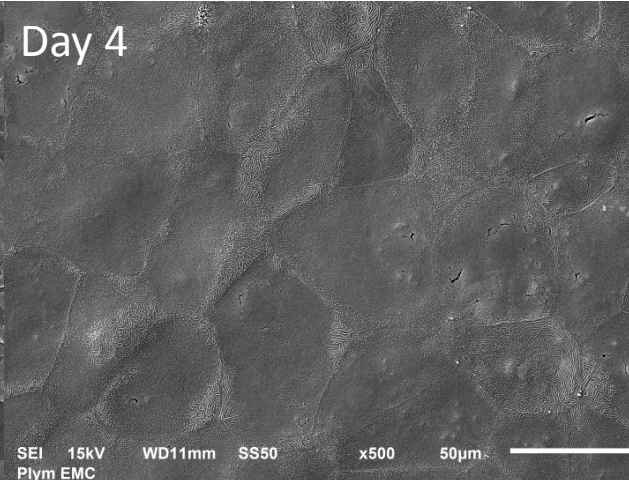
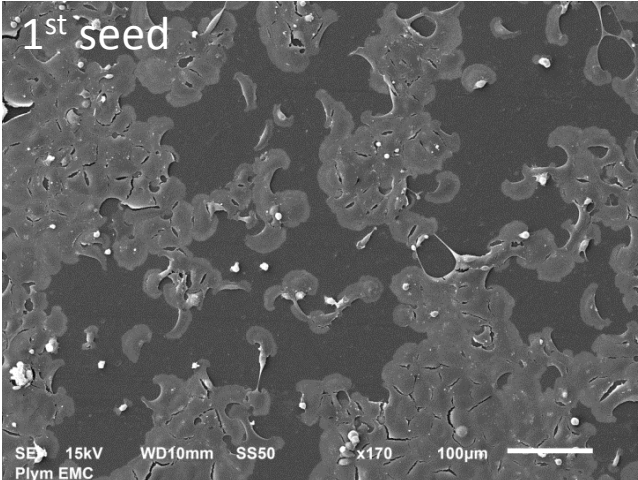
Gill layer production

SEM imaging:

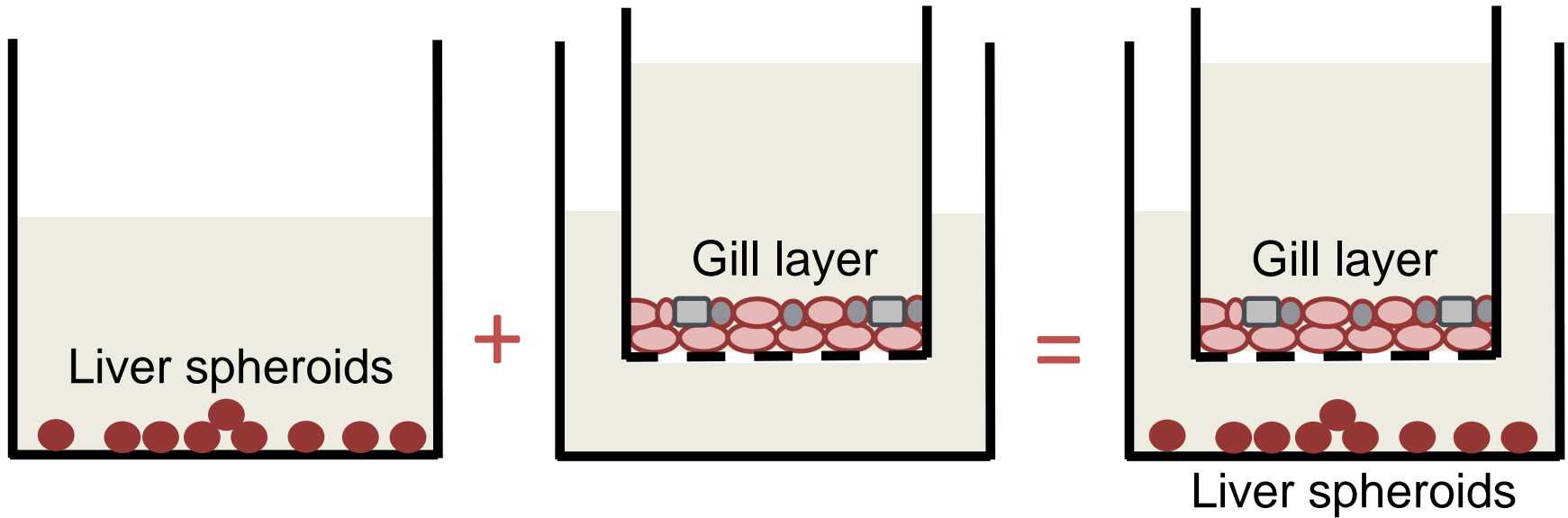


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Co culture...

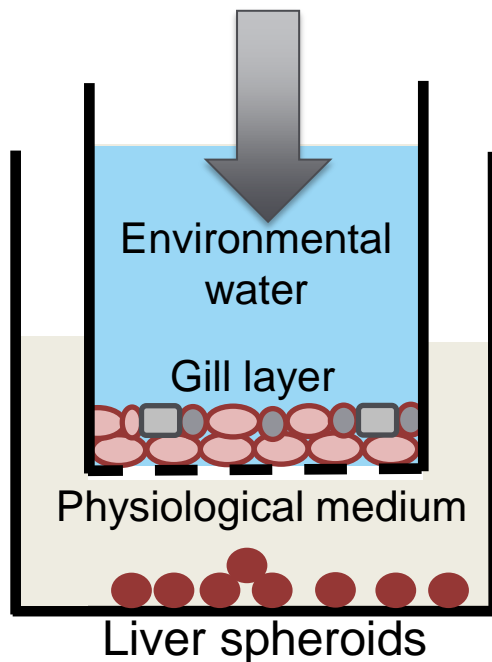


Easy..ish ...for static / short term

Co culture longer term

Challenges:

1. The swirling action is blocked by the insert.
2. Gill cultures only remain viable for ~2 days when exposed to water (to match *in vivo* situation)

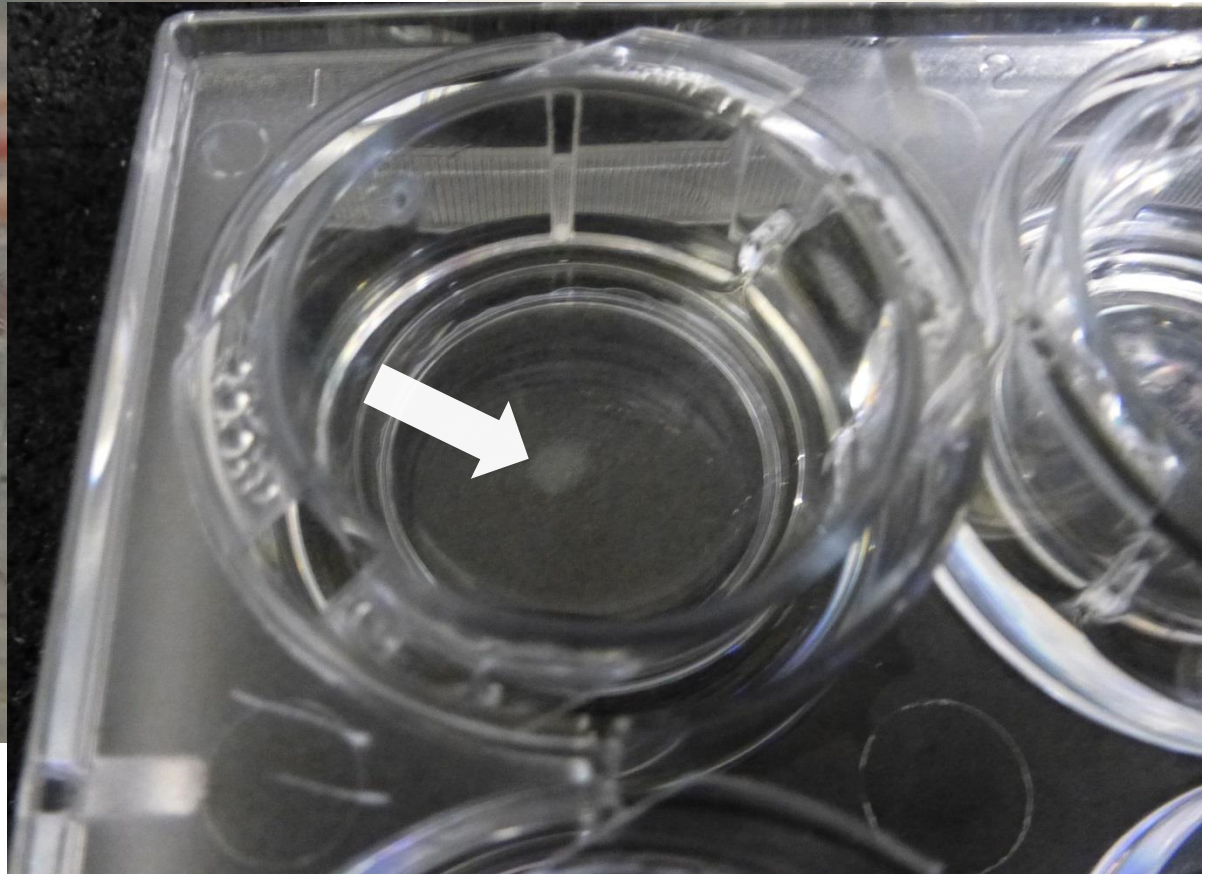


Co culture longer term

Issues:

1. The swirling action is blocked by the insert.

Solution: Upside-down culture?

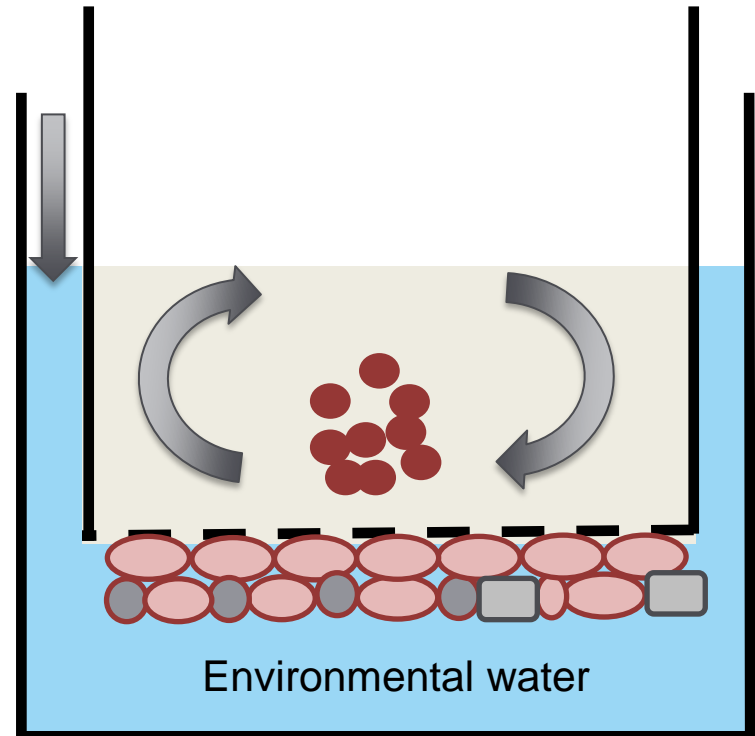
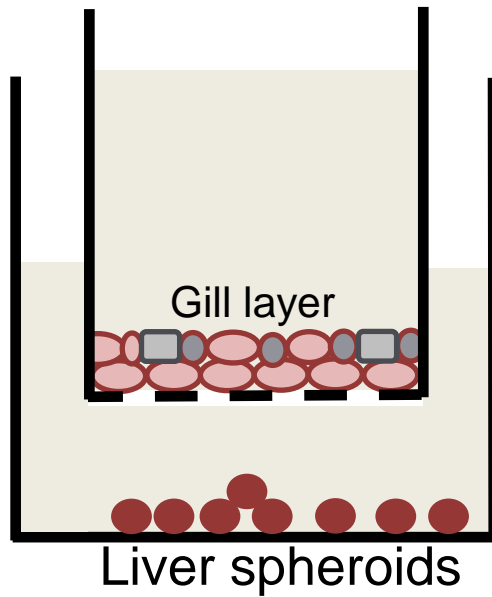


Co culture longer term

Issues:

1. The swirling action is blocked by the insert.

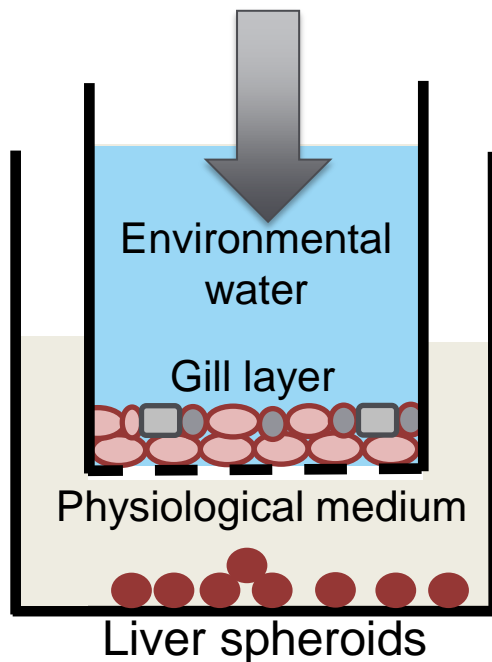
Solution: Upside-down culture?



Co culture longer term

Issues:

- 1.
2. Gill cultures only remain viable for 2-3 days when exposed to water (to match *in vivo* situation)



See Poster tomorrow:

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

THPC09, Exhibition Hall, Thurs 0830

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



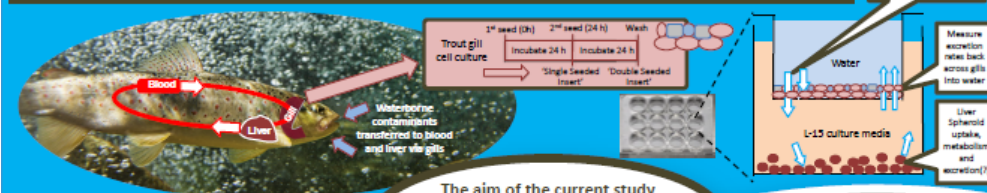
Richard Maunder^{1*}, Matthew Baron¹,
Stewart Owen², Awadhesh Jha¹
¹School of Biological Sciences, Plymouth University, Plymouth PL4 8AA
²AstraZeneca Safety, Health & Environment, Alderley Park, Macclesfield SK10 4TG
*Richard.Maunder@plymouth.ac.uk; A.Jha@plymouth.ac.uk



Investigate 4 methodological alterations to existing protocol:

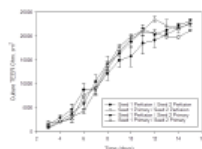
1. Membrane material
2. Gill pre-perfusion
3. Native serum supplementation
4. Larger inserts

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. Using primary tissues, we have already developed a method of creation and testing of *in vitro* hepatic spheroids¹ and aim to combine these with an existing primary gill model² to create a functional co-culture that is better able to predict *in vivo* uptake and metabolism of contaminants. Such a model would reduce the number of fish used in existing tests and refine the method of exposure to test chemicals. In modelling the *in vivo* exposure scenario, the gill culture can already tolerate the replacement of apical media with water, but currently only for ~2 days. It would be extremely useful for a variety of applications if this duration could be extended.



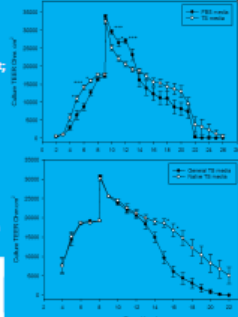
The aim of the current study was therefore to investigate the effect of different culture methods on the gill model viability and to maximise its lifespan and functionality after apical water addition

Results
1. Membrane type
When compared to the standard membranes, cells seeded onto Falcon HD inserts, GBO translucent, transparent, collagen type 1 and Reinnervate Alvetex inserts all failed to establish TEER above background after 10 days of growth. Microscopy revealed few cells had attached to these membranes.

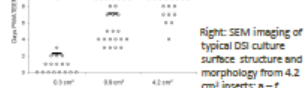


2. Gill pre-perfusion
The TEER values for the 4 combinations of preparation techniques for the double seedings are shown opposite. No sig. diff. in TEER between the treatments through time were observed. This data does not include a water addition stage.

3. Serum type
We compared autologous fresh trout serum (TS) against FBS in a series of 6 separate runs between Feb and Sept 2016, using a total of 13 biological replicates, each cultured in 3 - 6 wells. The TEER data is displayed on right; cultures receiving the TS media showed a significant increase in TEER compared to those receiving FBS media from Day 5 - 7. Immediately PWA, mean TEER of both culture types rapidly increased and then declined fairly consistently over the following 2 wks. Interestingly, the FBS cultures maintained a significantly higher TEER than the TS on Days 10 - 12. In a smaller experiment we also compared fresh autologous (native) TS to a mixed TS batch that was stored frozen until use. There appears to be a slight benefit in using fresh serum over batch-frozen serum (opposite) but both types support cell culture.



4. Membrane area We compared the effect of area on culture characteristics using up to 10 biological replicates, each replicated in 3 - 6 wells. One way to analyse this data (left) is to compare the number of days that individual cultures remained viable (TEER > 5 KΩ cm²)³ PWA. All 3 formats produced cultures that lasted for at least 12 days, but there was a significant increase in mean duration for 4.2 cm² above 0.9 cm² and 0.9 cm² above 0.3 cm² formats.



Right: SEM imaging of typical DS culture surface structure and morphology from 4.2 cm² inserts; a - f culture development through symmetrical (a - c) and asymmetrical conditions (d - f) using apical US EPA HW-24 h post 1st seed (a), confluent layer 24 h post 2nd seed (b) and clearly defined cell junctions and range of surface microridge morphology at 7 days (c). At 2 days 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 surface layer shows clear degradation and loss of viability (f). Higher magnification of surface morphology (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 c).

Methods

We investigated 4 protocol alterations from the standard methods³ that we considered might improve the subsequent culture characteristics. These were tested against control cultures by monitoring Trans epithelial Electrical resistance (TEER)

1. Membrane material; Standard/HD Falcon/Collagen/3D structure
2. Ventral aorta pre-perfusion of gill to clear blood prior to cell harvest
3. Replacement of FBS with heat inactivated autologous serum
4. Use of inserts with different membrane areas seeded at the same seeding ratio (cells/cm²)

Discussion

- (1) Despite the wide range of use of these membrane types in cell culture from other species, the use for gill cells proved unsuccessful in improving culture characteristics.
- (2) No differences in culture TEER following pre-perfusion of gills were found, but we do consider the perfusion method to add value to this model in terms of reduced preparation time and reduced materials cost.
- (3) The TS supplemented cultures attained a higher TEER than the FBS cultures during early establishment. However, the key finding in this experiment was that both culture types maintained a mean TEER > 5 KΩ cm² for 12 days PWA. This is longer than the previously reported values of up to 48 h^{4,5}. While we do not have a satisfactory explanation for why this is, the methodology does have scope for subtle differences in fish husbandry, cell harvest and culture. We have also conducted SEM investigations into culture surface structure.
- (4) A working theory as to the improvements observed in culture longevity was that the larger cell numbers supported by the 4.2 cm² inserts created a culture that was better able to support a high TEER. This was not fully supported by the data which showed all insert sizes supported cultures for up to 12 days, but that the larger inserts did so more often. It remains unclear if this is due to a methodology difference or an underlying difference in culture characteristics.

References

- [1] Baron et al. (2012). *Ecotox.*, 21, 2419-2429. [2] Fletcher et al. (2000). *J. Exp. Biol.*, 208, 1523-1537. [3] Bury et al. (2014). *J. Exp. Biol.*, 217, 639-650. [4] Wood et al. (2002). *Biochim. Biophys. Acta* 1566 72- 83. [5] Schnell et al. (2016). *Nat. Protoc.*, 11 490-498.

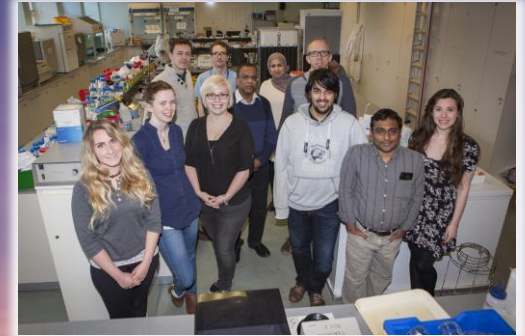
Ongoing / Future work

- Development and characterisation of single and co-culture models to suit specific exposure scenarios
 - Investigate communication between tissue types?
 - Can same fish supply multiple tissues?
- Single and co-culture exposures to various environmental contaminants (Pharmaceuticals / metals / PAHs)
- Analytical chemistry support to quantify concentrations in different compartments through time
- Development of Gut model for dietary uptake route...



Thanks

Project team and staff
at Plymouth University



Project funding:

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NERC
SCIENCE OF THE
ENVIRONMENT

 **BBSRC**
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