Deliverable 5.1
Standard operating procedures (SOPs) for new cellular pre-screening tools

Version 1

WP 5
Deliverable 5.1
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Contents

1. Objective

This Deliverable report (D5.1) is related to Task 5.3 and it describes Standard Operating Procedures (SOPs) for complex *in vitro* primary co-culture systems, immortal and targeted mutant cell lines, an *ex vivo* eye lens model and first development of organoids for cyprinid, salmonid and perciform fish species. The *use* of these new cellular pre-screening tools for studying nutrition and health will be described in a follow-up Deliverable report (D5.4) foreseen for the end of the project period.

2. Background

The global objective of WP5 is to develop innovative biotechnologies that will overcome the difficulties encountered in the production and long term maintenance of *in vivo* and *in vitro* biological models relevant for the acquisition of basic and applied knowledge in different fields of aquaculture research (reproduction, growth, nutrition, immunity, product quality for human consumption). In addition, WP5 will contribute to reduce or even replace the use of fish in experiments. Specifically, the aim for the research described here is to develop *in vitro* cellular tools to provide pre-screening tools for e.g. uptake of nutritional factors and investigate complex biological functions.

Task 5.3 "New cellular tools for in vitro pre-screening of phenotypes" (M4-M58) (Task Leader WU, Contributors: INRAE, IMR, DTU, NTNU) was formulated to address the establishment of innovative approaches such as organoids, complex primary coculture systems and targeted mutant cell lines to predict the effects of nutrients, xenobiotics, and environmental factors on fish nutrition and health in salmonids, cyprinids or perciform fish species. Task 5.3 includes several subtasks:

- Subtask 5.3.1 addressed the physiology of nutrient uptake. Hepatocyte and/or enterocyte cell lines will be used to determine (i) the metabolic role of free amino acids (AA) and AA transporters involved in growth of rainbow trout and (ii) the role of autophagy on glucose utilization (INRAE). Primary hepatocytes of isogenic Atlantic salmon will be used for evaluation of (anti-) nutritional effects. Genome-edited enterocytes will be generated to study the potential effects of new feed resources used in the formulation of future salmon feeds (NTNU). Organoids from fish intestine will be developed and grown in specific three-dimensional environments and used to study nutrient uptake and nutritional impact on intestinal integrity (INRAE, WU, IMR).
- Subtask 5.3.2 addressed fish health by studying gill function with a specific focus on immune function. Complex primary cultures of fish gill epithelium will be carried out with mucous cells and water on the apical (upper) side and gill leukocytes on the basal (lower) side. These tools will be used to study permeability, barrier function, mucus production and immune stimulation in perciforms (WU, INRAE).
- Subtask 5.3.3 should have addressed farmed fish health by studying causal factors of cataracts but has been postponed to a later phase of the project. Newly developed ex vivo models of eye lenses will be used to investigate direct effects of high glucose and establish a model to predict potential nutritional and environmental causes of cataract development in farmed fish (INRAE, WU, IMR).
- Subtask 5.3.4 addressed existing and newly developed salmonid cell lines, genome edited with CRISPR/Cas9 for specific genes related to the anti-viral interferon pathway, which will be used to generate a panel of immortal cell lines to link genomic loss of function with specific phenotypes involved in signaling processes or regulatory functions linked to fish health (INRAE).

3. Methodology

3.1 Physiology of nutrient uptake

Subtask 5.3.1 addressed the physiology of nutrient uptake. Hepatocyte and/or enterocyte cell lines were used to determine (i) the metabolic role of free amino acids (AA) and AA transporters involved in growth of rainbow trout and (ii) the role of autophagy on glucose utilization (INRAE). Primary hepatocytes of isogenic Atlantic salmon were used for evaluation of (anti-) nutritional effects. Genome-edited enterocytes were generated to study the potential effects of new feed resources used in the formulation of future salmon feeds (NTNU). Organoids from fish intestine were developed and grown in specific three-dimensional environments and used to study nutrient uptake and nutritional impact on intestinal integrity (INRAE, WU, IMR).

3.1.1. Immortal cell lines from the rainbow trout liver (INRAE)

Experiments were performed with a rainbow trout hepatic cell lines RTH-149 (ATCC® CRL-1710, LGC Standards, Molsheim, France) and RTL-W1 (from the laboratory of K. Schirmer from the EAWAG institute). Both cell lines were maintained at 18 °C in incubator with free gas exchange with air.

3.1.2. Hepatocytes from all-male salmon (IMR)

Atlantic salmon from the clonal all-male Atlantic salmon line (developed at the Institute of Marine Research (IMR), Bergen, Norway (Fjelldal et al., 2020)) was used for the isolation of the primary liver cells.

3.1.3 Intestinal organoids from common carp (WU)

Organoids have revolutionized the approach to *in vitro* studies allowing to move away from traditional *in vitro* cultures of immortalized cell lines to *in vitro* cultures of complex cell systems in which mini organs can be generated that recapitulate, partly or entirely, the function of the organ of origin. Despite their great success in the mammalian field, in which intestinal, liver, lung, retinal, mammary glands, reproductive system organoids have been generated, to mention a few, only two types of organoids have been generated thus far for cold-blooded animals: medaka and zebrafish retinal organoids and snake venom gland organoids.

3.2. Complex primary cultures of gill cells

Subtask 5.3.2 addressed fish health by studying gill function with a focus on their immune function. Complex primary cultures of fish gill epithelium were carried out with mucous cells and water on the apical (upper) side and gill leukocytes on the basal (lower) side. These tools were used to study permeability, barrier function, mucus production and immune stimulation in sea bass and Nile tilapia (WU, INRAE).

3.2.1. Complex cell culture from gills of sea bass (INRAE)

Fish gills have several functions including maintenance of gases, ions and water homeostasis. Due to their direct exposure to the external environment, fish gills also play an important role in fish immunity. This organ provides a barrier to pathogen entry and contains mucous cells responsible for local immune responses. Due to the complexity of the gills, primary cultures on permeable supports make it possible to study several functions such as transepithelial transport and barrier function. The objective was to transfer the knowledge of *in vitro* cultures of gill cells from rainbow trout (Leguen et al. 2007) to *in vitro* cultures of gill cells from seabass and Nile tilapia. For seabass, several parameters related to culture conditions were tested to increase cell viability, adhesion and confluence of gill cells on a permeable membrane (pH, osmolarity, serum, temperature, cell seeding).

3.2.2. Complex cell cultures from gills of tilapia (WU)

To some extent, primary cell cultures from gills and in vitro cell lines of gill cells can all be used to study gill physiology but with limitations. Although they are relatively easy to obtain and can be cultured for extended periods without too much effort, primary cell cultures and cell lines over-simplify and are not fully representative of the complexity of the gills. A more complex and therefore possibly more realistic *in vitro* model has recently been described for gills of rainbow trout (Nature Protocols (Schnell et al., 2016)). This model is based on the reconstruction and culture of rainbow trout gill epithelium on flat permeable membrane supports within cell culture inserts. The protocol can result in a complex build-up of heterogeneous gill epithelium with pavement cells (PVCs), goblet cells (GCs), and mitochondrion-rich cells (MRCs), including the formation of tight junctions (TJs) that can withstand freshwater on the apical cell surface. The current task focused on transferring the aforementioned knowledge from trout to a warm-water fish species, Nile tilapia, and establishment of a gill epithelium cell culture system.

3.3. Eye lens model to study cataract ex vivo

Subtask 5.3.3 addressed fish health by studying causes of cataract development in aquaculture. Newly developed *ex vivo* models of eye lenses will be used to investigate direct effects of high glucose and predict potential nutritional and environmental causes for cataract development in farmed fish (IMR).

3.3.1. Eye lens model for Atlantic salmon (IMR)

Cataracts are a production-related disorder that can be caused by nutrition, environment and genetic factors. Cataracts are characterized by the presence of opacities in the eye lens, which can be caused by changes in the epithelial tissues surrounding the lens fibres, or the composition and structure of the fibres, resulting in reduced vision. In the last decades cataracts have been especially associated with sub-optimal dietary histidine levels, particularly after sea water transfer when a higher dietary level of histidine is needed to minimize cataract development (Remø et al., 2014). The global metabolic profile in lenses from salmon exposed to high temperatures showed changes in lens osmoregulation, redox homeostasis and carbohydrate metabolism indicating a complex aetiology behind the increased risk of cataracts at high water temperatures (Remø et al. 2017). At high water temperatures, a higher level of lens sorbitol was seen in salmon, which may have resulted in osmotic stress and consequently contributed to cataract formation, as seen in hyperglycaemic animals and humans (Remø et al., 2017). Due to the multifactorial aetiology of cataracts, developing standardized tools that can be used to test factors contributing to their formation in different strains of fish or fish species would be beneficial to elucidate the underlying factors influencing susceptibility to cataracts. The eye lens model work therefore focused on developing a SOP to refine, develop and standardize models to study cataract development in fish *ex vivo.*

3.4. Mutant cell lines for viral studies

Subtask 5.3.4 was formulated to address existing and newly developed salmonid cell lines, genome edited with CRISPR/Cas9 for specific genes related to the anti-viral interferon pathway, which will be used to generate a panel of immortal cell lines to link genomic loss of function with specific phenotypes involved in signaling processes or regulatory functions linked to fish health (INRAE).

3.4.1. Mutant cell lines from salmonids (INRAE)

In vivo gene invalidation using targeted mutagenesis (genome editing) is a powerful approach to investigate specific gene contributions to animal traits and to generate new animal models for basic research. Recent innovations allowed the development of highly efficient targeted mutagenesis, which has been widely applied in model fish species (zebrafish, medaka). However, due to the phylogenetic distance between tropical fish models and aquaculture species, understanding the genetic basis of traits of commercial importance is much more relevant in target aquaculture species. Today, the CRISPR/Cas9 system is the most convenient system to generate point mutations (knock-

out) or insert exogenous DNA at specific points of the genome. Gene edited fish cell lines, immortal cell lines, but also primary *in vitro* as well as *ex vivo* cultures of cells or structures, has several advantages over studying complex mechanisms in whole animals, including simplified measurements of function under controlled conditions as well as increased repeatability on cells from the same fish, reducing the number of animals required, or even replacing them. Our approach was to develop CRISPR/Cas9 genome editing techniques and apply this to cell lines from aquaculture species to establish *in vitro* models expressing genetic and epigenetic changes that will benefit the aquaculture research community. The cellular models, including gene edited cell lines, will provide alternatives to fish experiments for *in vitro* phenotypic screening.

4. Results and Discussion

4.1. Standard Operating Procedures (SOP) for studying physiology of nutrient uptake

4.1.1. Immortal cell lines from the rainbow trout liver (INRAE)

4.1.1.1. Chemicals

Chemicals	**art.no.**	**Suppliers**
Cell culture		
Antibiotic Antimycotic	14065-056	Gibco
Minimum Essential Medium (MEM)	61100	Gibco
Sodium Pyruvate (NaPyr)	11360-070	Gibco
Non-Essential Amino Acid (NEAA) solution	11140-50	Gibco
Fetal Bovine Serum (FBS)	10270-106	Gibco
HEPES	BP299-1	Fisher Bioreagents
DMEM w/o glutamine, w/o Cystine (CYS), w/o Methionine (MET), w/o glucose, w/o Sodium pyruvate (DMEM Minimal)	C4030	Genaxxon Bioscience
L-glutamine	61100-053	Gibco
Leibovitz's L-15 medium	21083027	Thermofisher
Insulin	10516	Sigma-Aldrich
DL-methionine (DL-MET)		EVONIK
Methionine Hydroxy Analog (MHA)	55875	Sigma-Aldrich
PBS	BP2944	ThermoFisher Scientific
Leibovitz's L-15 medium w/o Methionine, w/o Cystine (L15 Minimal)	C4063	Genaxxon Bioscience
Geneticin antibiotic	11,811	Gibco
D-(+)-Glucose	G7021	Sigma-Aldrich
Western blot		
RIPA buffer	89901	Thermo Scientific
Halt protease and phosphatase inhibitor cocktail	78442	Thermo Scientific
Polyvinylidene fluoride (PVDF) membranes	IPFL00010	Merk Millipore
Anti-ribosomal protein S6	2217	Cell Signaling Technologies
Anti-phospho-S6	4856	Cell Signaling Technologies
Anti-4EBP1	9452	Cell Signaling Technologies

Anti-phospho-4EBP1	9459	Cell Signaling Technologies
Anti-β-tubulin	2146	Cell Signaling Technologies
IRDye secondary antibody	926-68071	LI-COR
HRP-labeled goat anti-rabbit IgG secondary antibody	31460	Thermo Fisher scientific
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	34580	Thermo Fisher scientific
Gene expression analyses (RT-qPCR)		
RNeasy mini kit	74104	Qiagen
Superscript III RNAseH – reverse transcriptase kit	18080-093	Invitrogen
Random hexamers	C1101	Promega
CMA Reporter		
PA-mCherry1-N1 vector	54507	Addgene
Nucleofector Kit T	VCA-1002	Lonza
4-well cell culture slides	354114	Falcon
Microscope Cover Glasses	0107222	Marienfeld
4% PFA	22023	Biotium
Antifade mounting medium + DAPI	H-2000	Vector Laboratories

4.1.1.2. Solutions and standards

MEM Complete medium (5 mM glucose)	- MEM - 10% FBS - 10% NEAA - 2 mM NaPyr - 1% Antibiotic Antimycotic - 25-mM HEPES
CYS- and MET-free DMEM	- DMEM Minimal - 1 g/L glucose - 25-mM HEPES - 2-mM L-glutamine - 2-mM NaPyr - 4-nM insulin - 10% FBS
+MET DMEM Mediums	- CYS- and MET-free DMEM - DL-MET (2, 20 or 200 µM)
+MHA DMEM Mediums	- CYS- and MET-free DMEM - MHA (2, 20 or 200 µM)
L15 complete medium	- L-15 - 10% FBS - 1% Antibiotic Antimycotic - 25-mM HEPES
CYS- and MET-free L15	- L15 Minimal - 8.5-mM NaCl - 1-mM CYS - 10% FBS.
+MET L15 Medium	- CYS- and MET-free L15 - 500 µM DL-MET

+MHA L15 Medium	- CYS- and MET-free L15 - 500 µM MHA
High-Glucose medium	- MEM - 10% FBS - 10% NEAA - 2 mM NaPyr - 1% Antibiotic Antimycotic - 25-mM HEPES - 4.5 g/L glucose

4.1.1.3. Instruments and equipments

Cell culture

- Laminar Flow Bench
- Microscope
- Cell incubator without additional O_2/CO_2
- Pipets
- Assorted Flasks
- Sterile collection container
- Pipetting device
- 15 mL and 50 mL centrifuge tubes
- Decon ahol (70% IPA)
- Water Bath.
- 2 to 8°C Refrigerator.
- -10 to -30°C Freezer.
- Automated cell counter

Western blot

- Polyacrylamide gel electrophoresis system
- Imaging System

RT-qPCR

- Spectrophotometer
- Thermocycler

CMA Reporter

- Nucleofector 2b Device
- Light-emitting diode (LED) at 405 nm wavelength
- Fluorescent Microscope

4.1.1.4. Procedures

Initiation and maintenance of RTH-149 and RTL-W1 Cell Cultures

Initiating Cells from Frozen Stocks

- Prepare the work area in the laminar flow bench before thawing the cells.
- Withdraw the cells from storage.
- Prepare a centrifuge tube with 9 mL of complete medium. The volume of media may be adjusted depending on the density of cells in the vial.

- Thaw the vial(s) quickly by immersion in water bath.
- Transfer the vial to the laminar flow bench and rinse the outside with Decon ahol.
- Remove the contents with a pipette and add to the centrifuge tube and medium. The vial may be rinsed with medium to collect all the cells. A sample can be taken at this point or after centrifugation to count and check the viability of the cells.
- Centrifuge the cells at 500 *g* for 5 minutes (at RT)
- When the centrifugation is complete, quickly decant off the supernatant into a sterile container.
- Gently resuspend the cell pellet with complete medium and place the cell suspension in a labeled flask. Label the top or side of flask with the cell line name, date, and lot number (if applicable).
- Place the flask in the incubator at 18°C.
- Disinfect and log out of the laminar flow bench.

Passing and maintaining the Cells

- Turn on the water bath or incubator and allow it to reach 37°C ± 2°C.
- Warm the complete medium to RT (18°C) and Trypsin to 37°C ± 2°C.
- Prepare the work area in the laminar flow bench before working with the cells.
- Aspirate or decant the supernatant from the flask into a sterile container.
- Add the selected Trypsin solution to the flask (suggested volumes: 25 cm^2 - 5 mL, 75 cm^2 - 10 mL, 150 cm^2 - 20 mL).
- Incubate the cells at 37°C for about 2-5 minutes or longer until they release from the plastic. In some cases, it may help to tap the side of the flask to dislodge the cells. Resuspend the cells in fresh medium.
- Aspirate and reshuffle the suspension until uniform and free of clumps. Count the cells.
- Transfer the required cell volume to a new flask(s). Adherent cell splits are either stated in ratios of flask surface area or cells/cm^2. Example: at a 1:6 split ratio, a 25 cm^2 confluent flask can make a 150 cm^2 flask. To split adherent cells based on cells/cm^2 (surface area), use the formulation below:
  Viable number of cells/cm^2 wanted in initial seed x Total cm^2 in new flask = Total viable cells needed
  Total Viable Cells Needed = Total Volume of cells needed from suspension
  Viable cells/mL in suspension
- Add fresh medium and gently mix cells (working volume - 25 cm^2 flask – 10 mL, 75 cm^2 - 20 mL, 150 cm^2 - 100 mL). The volume of media in the flask for adherent cells is not critical. Label the top or side of the flask with the cell line name, date, and lot number (if applicable).
- Place the flask in the incubator.
- Medium is replaced twice a week and cells are passaged at 80-90 % of confluence.

Determining the role of amino acids (Met and MHA) in the control of the expression of metabolic genes (by RT-qPCR) and the activation of growth-related signaling pathways (by Western blot) in RTH-149 and RTL-W1 Cell Cultures.

- Count cells using a Cellometer and plate them in 6-cm dishes (400,000 and 500,000 cells/dish) for RNA extraction and protein collection, respectively.
- Incubate cells at 18°C during 2 days prior treatments and wash them twice with PBS before the exposure to the appropriate treatment.
- For gene expression analyses (by RT-qPCR):

- Treat the cells with the MET-depleted medium supplemented or not with MET and MHA during 24h for RTH-149 cells and 16h for RTL-W1.
- Wash the cells with PBS.
- Extract total RNA using RNeasy mini kit following manufacturer's instructions.
- Monitor RNA concentration and purity using a spectrophotometer.
- Synthetize cDNAs from 1 µg of RNA from RTH-149 cells and 500 ng of RNA from RTL-W1 using Superscript III RNAseH – reverse transcriptase kit with random hexamers by using a thermocycler.
- Perform, the real-time quantitative PCR reactions in triplicate. For the reaction, use 3 µL of LightCycler® 480 SYBR Green I Master for 2 µL of 1/40 diluted cDNA with 0.24 µL of each gene-specific primer (at 10 µM) and 0.52 µL of nuclease-free water.
- The reaction is carried out with a Light Cycler.

- For the analysis of growth-related signalling pathways (by Western blot)
  - For both cell lines, treat the cells with the MET-depleted medium supplemented or not with MET and MHA during 5 hours.Wash the cells with ice-cold PBS
  - Treat the cells with RIPA buffer containing Halt protease and phosphatase inhibitor cocktail.
  - After 30 min of incubation on ice, centrifuge samples at 12,000 g at 4°C during 10 min.
  - Collect the supernatant and measure protein concentrations using the bicinchoninic acid method.
  - Mix equal amounts of protein with Laemmli buffer
  - Perform a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation.
  - Transfer the proteins onto a polyvinylidene fluoride (PVDF) membrane.
  - Incubate the membrane with the primary antibody targeting the protein studied.
  - Following several washes with PBS, incubate the membrane with a secondary antibody.
  - For samples from RTH-149, IRDye secondary antibody is used and signal acquisition is performed by infrared fluorescence with the appropriate Imaging System.
  - For samples from RTL-W1, membranes are exposed to HRP-labeled goat anti-rabbit IgG secondary antibody and incubated in SuperSignal™ West Pico PLUS Chemiluminescent Substrate.
  - Chemiluminescence acquisition is performed with an appropriate imager.
  - For both cell line, quantification of protein expression is performed using ImageJ software (NIH, Be-thesda, MD, USA) using β-tubulin as a loading control for phosphorylation signal normalizations.

Determining the role of Chaperone-Mediated autophagy (CMA) on glucose utilization

Monitoring the effect of High-Glucose (HG, 25 mM) treatment on CMA activity in RTH-149 cells

CMA activity can be measured in cells in culture using the photoactivable (PA) artificial substrates: KFERQ-PA-mCherry, initially developed for mammalian cells [Koga et al., 2011]. The functionality of this construct has recently been validated in fish cells [Lescat et al., 2020]. This construct is made of the N-terminal 21 amino acids of bovine RNASE1/RNase A containing its KFERQ CMA-targeting motif fused to a photoactivable-mCherry1 (PA-mCherry1) protein.

- Electropore cells with 1 to 5 µg of the KFERQ-PA-mCherry CMA reporter and plate at a density of 60,000 cells/well onto 4-well culture slides.

- Twenty-four hour after electroporation, the cells can be photoactivated by exposure to a 3.5 mA (current constant) and 90 V light-emitting diode (LED: Norlux, 405 nm) for 10 min.
- After photoactivation, expose the cells to the desired treatments: D-glucose 5 mM (Control medium) or 25 mM (HG medium).
- Fix cells at different times or at a single end time point (16 h after treatment) with 4% paraformaldehyde in PBS for 15-20 min, wash with PBS and mounting of the slides using a mounting medium including DAPI nuclear staining.
- Capture images using a fluorescent microscope. CMA can be quantified as the number of fluorescent puncta per cell.

Monitoring the role of CMA

The role of CMA is investigated by studying the consequences of inhibiting this function on the expression of metabolic genes and/or proteins.

- Electropore cells with morpholino oligos (5 µM) or siRNAs (1 µM) directed against LAMP2A (a protein essential and limiting for CMA activity) and plate them at a density of 60,000 cells/well onto 4-well culture slides for microscope imaging experiments, and in 6-cm dishes (400,000 and 500,000 cells/dish) for RNA extraction and protein collection, respectively.
- At the desired times, cells can be fixed as explained above for imaging experiments, or RNA or protein collected for RT-qPCR or Western blot analyses as described above.

4.1.2. Hepatocytes from all-male salmon (IMR)

4.1.2.1. Chemicals

Chemicals	**art.no.**	**Suppliers**
Antibiotic Antimycotic	A5955	Merck
$CaCl_2$	C5670	Merck
EDTA	ED2SS	Merck
FBS	F7524	Merck
Hepes sodium salt	H3784	Merck
KCl	P5405	Merck
collagenase	C2139	Merck
L-15 Gibco	21083027	Thermofisher
Metacain	A5040	Merck
NaCl	106404	Merck
PBS	437117K	VWR
Trypan blue dye, 0.4 %	1450021	Biorad

Laminin	L2020-1MG	Merck

4.1.2.2. Solutions and standards

Metacain stock solution	100 g is dissolved in 1 l MQ water
1M $CaCl_2$	7.35 g is dissolved in 50ml MQ water
Perfusion buffer stock solution, pH= 7.4	- 8.38 g NaCl - 0.5 g KCL - 2.4 g Hepes - Dissolved in 100ml MQ water. - Autoclave and store in refrigerator.
Perfusion buffer with EDTA, pH= 7.4	- 20 ml perfusion buffer stock solution - 1.11 g EDTA dinatriumsalt - Dissolved in 200ml MQ water. - Autoclave and store in refrigerator.
Perfusion buffer with collagenase, pH= 7.4	- 10ml perfusion buffer stock solution dissolved in 100ml MQ water. After pH adjustment, add 100µl 1M $CaCl_2$. Autoclave and store in refrigerator. - 100mg collagenase is added just before the perfusion.
L15 complete medium 10% FBS 1% Antibiotic Antimycotic	- 50 ml (1 fisk/isolation) - 44,5 ml L-15 - 5 ml FBS - 0,5 ml Antibiotic Antimycotic (10x)
Laminin coating stock solution 10 ug/ml	1 mg laminin dissolved in 100 ml PBS and the wells is coated with 2 µg/cm^2 for > 2 hours and dried before stored sterile in the refrigerator.

4.1.2.3. Instruments and equipments

- Cooling centrifuge
- Sterile bench
- Cooling incubator
- Microscope
- Bürker counting chamber
- Pipette gut
- Tweezers
- spoon
- Scissors
- Scalpel
- Weight
- Bucket

- Peristaltic pump
- Laboratory stand
- Magnet
- Artery clamp
- Measuring cylinder
- Beaker
- Blue cork bottles
- ice
- Styrofoam board
- Cell cooling incubator without additional O_2/CO_2

Disposable equipment:	**art.no.**	**Suppliers**
10ml syringe	613-3941	VWR
1ml syringe	613-2040	VWR
50ml Centrifuge tube	62.547.254	Sarstedt
cell culture plates 12 wells	83.3921	Sarstedt
celle culture plates 96 wells	83.3924	Sarstedt
Cell strains 100um	734-0004	VWR
Infusion set Intrafix® Primeline st.1.8m	BRAU4062981L	VWR
Microtube, 1.5ml	525-0226	VWR
Nitril gloves w/powder	112-3101	VWR
Petri dishes	391-0455	VWR
Pipette tips Biohit 0.1-10ul	613-0679	VWR
Pipette tips Biohit 0.5-350ul	613-5102	VWR
Pipette tips Biohit 10-1000ul	613-0687	VWR
Pipette tips Biohit 100-5000ul	613-4149	VWR
Serological pipette, 10ml	86.1254.001	Sarstedt
Serological pipette, 25ml	86.1685.001	Sarstedt
Scalpel blades no.22, sterile, Swann-Morton	233-5484	VWR
Syringe needle 18G	613-2029	VWR
Venflon	BDAM393222	VWR

4.1.2.4. Procedure

All work was carried out in agreement with the current national animal welfare act - the regulation on animal experimentation approved by the Norwegian Animal Research Authority and overseen by the Norwegian Food Safety Authority (FOTS ID, 19351).

All glassware, instruments and solutions are autoclaved prior to liver perfusion.

1. Prior the perfusion, check if the peristaltic pump delivers 4 ml/min.
2. The pump tube should be rinsed with 70% ethanol followed by autoclaved MQ water before the infusion set is connected. Fill up the pump tube/ the infusion set with perfusion buffer w/EDTA, and check that there are no air bubbles in the system.
3. The fish should be anesthetized with methacaine 5-8 ml/10 l seawater.
4. When the fish is sufficiently anesthetized, place it on a foam stand.
5. The abdomen of the fish should be cut open with a scalpel from the anus upwards (posterior – anterior direction) in such a way that the organs are not damaged.
6. To ensure free excess to the liver, 2 artery clamps were used to hold the abdominal flaps apart The venflon is inserted into the portal vein on the underside of the liver. When the venous catheter is in place, the venflon should be connected to the pump system, and the heart nicked with a scalpel. The liver should now become discoloured quite quickly.
7. One complete decolorization occurs (changes from reddish to brown), change to perfusion buffer with collagenase (approx. 40-70 ml should be enough). If perfusion occurs correctly, the buffer is injected into the portal vein and flows out through the nick made in the heart.
8. When the liver becomes swollen and glassy, it should be transferred to a petri dish on ice with ice-cold PBS (possibly L-15 without addition).
9. Before the liver is removed, the bile should be sucked up with a syringe to avoid contamination of the liver. In case of contamination, the liver must be discarded.

   ALL WORK FROM HERE SHOULD BE DONE ON ICE.

10. The cells should be gently shaken loose in PBS using two tweezers, new PBS should be added 1-2 times, until no more cells are detached from the liver.
11. The cell suspensions are filtered (100 um nylon mesh) and the cells collected by centrifugation (50 x g for 5 min).
12. The cells are filtered through a cell strainer (100 μm) into a 50 ml centrifugation tube on ice.
13. Cells are collected by centrifugation (50 x g for 5 min without brake).
14. The cells are washed twice with PBS and centrifugated at 50xg for 5 min.
15. After the last centrifugation, resuspend the cells in an appropriate volume, for example 40ml L-15 w/10% FBS, 1% Antibiotic Antimycotic.
16. To avoid cell clumping during cell counting and seeding, an 18G syringe tip can be used with a 10 ml syringe. Drag the cell medium mix up and down 5 times.
17. A dilution of the cell suspension is made before the cells are counted using a Bürker chamber.
18. The cell viability is determined with the Trypan Blue exclusion method (Biorad). Dead cells have compromised cell membranes and will be stained blue by the dye, while viable cells will remain bright since they have intact cell membranes.
19. The cell suspensions were plated on 2 μg/cm2 laminin coated culture plates and the hepatocytes were kept at 10 °C in a sterile incubator without additional O_2/CO_2.
20. The following cell concentrations are used; 7.2×10^6 cells per well in 6-well plates (in 3 ml complete L-15 medium), 2.6×10^6 cells per well in 12-well plates (in 2 ml complete L-15 medium), 0.2 × 106 cells per well in 96-well plates (in 0.2 ml complete L-15 medium).

4.1.3. Intestinal organoids from common carp (WU)

Generating (intestinal) organoids requires knowledge not only about the presence and location of adult or pluripotent stem cells but also about the growth factors required to maintain and differentiate them *in vitro* and the environment (extracellular matrix) in which mini organs can be formed. While knowledge on the presence and location of intestinal stem cells in the fish, trout, medaka, zebrafish, is available, methods on how to isolate them and grow them *in vitro* so they form intestinal organoids was not known prior to the start of this project. An important aspect that made us focus on a warm water species (i.e. common carp) was the fact that the extracellular matrix that has been thus far used for the generation of all known organoids (Matrigel) is fluid at temperatures between 4-10°C, is semi-fluid between 10-20°C, and forms a semi-solid (gel-like) matrix at temperatures above 22°C; solidification is faster at higher temperature with an optimum at 37°C. Common carp cells are generally grown at 27°C, thereby allowing solidification of Matrigel within a reasonable amount of time (45-60 min at 27°C vs 15 min at 37°C). Matrigel is required to support intestinal organoid growth.

Equipment and reagents

Fish dissection

- One polystyrene box with ice
- Anesthetic, Tricaine Methane Sulphonate
- vacutainer for blood collection and corresponding needle
- Scissors (preferably with one balled tip), small tweezers
- Marker, pen and notepad
- 70% Ethanol or isopropanol for tool sterilization during dissection
- Absorbent paper

Intestine isolation and cell isolation

- 90 mm sterile petri dish
- Disinfection buffer: referred to as PBS-PSA**B**G
- wash buffer: referred to as PBS-PSAG
- Pre-warmed 24 well plates: Keep 24 wells plate empty in incubator at 37C for a couple hours or overnight before using matrigel.
- PBS (Gibco #10010023)
- P/S Broad spectrum antibiotics (100x stock, Gibco™ 15140122)
- Gentamicin 50 mg/ml (Gibco # 15750045)
- Matrigel (Corning #356239) (Keep always at 4C to avoid solidification)
- 30mM EDTA in PBS
- TrypLE (Gibco #A1217701)
- Cold heat-inactivated foetal calf serum (FCS)
- Cold DMEM (Gibco #11965092)
- heat-inactivated, sterile, Pooled carp serum (PCS)
- Fungizone: Amphotericin B (Gibco™ 15290026)
- Betadine (buy yourself in the pharmacy or supermarket)
- cell strainers (100 µm)
- 50-15 ml tubes
- 5-10 ml strippettes

Dissociation with TrypLE and re-seeding in Matrigel

- Ice
- Ice cold PBS (+Rock inhibitor)
- Ice cold TrypLE (+Rock inhibitor)
- Ice cold complete organoid medium

- Matrigel (on ice, fluid)
- Pre-heated 24-wells plate
- Sterile 1.5mL Eppendorf tubes
- pre-warmed (27°C) Complete organoids medium

Media composition for cell isolation and organoids growth:

a. Disinfection buffer: PBS-PSA**B**G: 1x PBS + 1x P/S + Amphotericin B (500 ng/ml) + **5% Betadine** + Gentamicin 50 µg/ml

b. Wash buffer: PBS-PSAG: 1x PBS + 1x P/S + Amphotericin B (250 ng/ml) + Gentamicin 50 µg/ml

c. Organoid medium + Gentamicin (50 ug/ml) + Amphotericin B (250 ng/ml) + 10% h.i. Pooled Carp Serum (Orgmed-GAPCS)

Organoids medium

L-WRN cell culture supernatant – conditioned medium (CM)

- Prepare conditioned medium (CM) from L-WRN cells (ATCC - CRL-3276) as described on ATCC website L-WRN - CRL-3276 | ATCC
- This medium is ready to be use as base for organoids medium.

Mix 200 ml of **L-WRN CM** (without antibiotic) with:

40 ml	FBS	Ready to use		Gibco	10500-064
40 ml	PCS	In-house			
4 ml	Glutamax	100X		Gibco	35050087
4 ml	Pen/Strep	100X	1x	Thermo	10378016
800 ul	Primocin	500x stock (50 mg/ml)	100ug/ml	Invivogen	ant-pm-1
1ml	N-acetyl cysteine	400 x stock	1.25 mM	Sigma	A9165- 5G
8 ml	B-27	50x stock	1x	Gibco	17504044
4 ml	Nicotinamide	100 x stock (1M)	10 mM	Sigma	N0636-100G
400 ul	rhEGF human EGF	1000x stock (50µg/ml),	50 ng/ml	StemCell Tech	#78006.1
40 ul	Gastrin	10000x stock	7.5nM	Bio-techne	3006/1
400 ul	P38 Mapk inhibitor	1000x stock (stock: 60mM??)	10uM	Sigma	S7067-25MG
40 ul	TGF Beta type receptor inhibitor (A 83-01)	10000x stock (6mM in DMSO or 1000x stock 600µM in DMSO)	600nM	tocris	2939
	Add reagents below fresh				
400 ul	Rock Inhibitor (Y27632)	1000x stock (10 mM)	10 uM	StemCell Tech	#72304

Add DMEM/F12 up to 400 ml final volume. Filter using 0.2 µm filter, make aliquots of 40 ml and freeze at -20°C. Add Rock Inhibitor (Y27632) to medium just before seeding gut cells

Day 0 Cell isolation & seeding

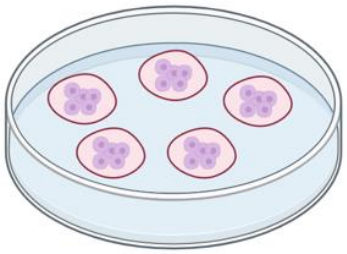
1. Sacrifice fish (carp of approx. 25 cm length, <1 year old) according to local regulations.
2. Dissect the intestine and cut a 3-4 cm piece from the 2nd segment.
3. Work on ice at all times.
4. In a sterile petri dish, flush through the lumen of the gut using a needle (23g) and syringe (10ml) and ice-cold PBS-PSABG several times until most of the gut contents is removed; clean also the outer part to remove as much fat and blood clots as possible.
6. Cut open the gut transversally by curling the entire piece of tissue over one arm of sharp scissors and then cutting.
7. Transfer the piece of tissue to a new sterile petri dish (lumen side down) containing clean PBS-PSABG and shake until no gut content is released.
8. Transfer to 15 ml tubes containing ice-cold PBS-PSABG for transport if you performed the dissection in a facility away from your lab, or proceed directly to step 9 (steps 2-8 should take you no longer than 30 min).
9. Remove the piece of tissue immersed in PBS-PSABG collecting as little liquid as possible and transfer with the inside (lumen) side up to the dry lid of a sterile petri dish.
10. Further cut the segment into tiny pieces (the smaller the better) by cutting with (curved) scissor (Just a small volume of liquid required or even cut without medium). This step can take > 1 minute!
11. Use 6 ml of ice-cols PBS-PSAG to flush the scissors and collect any tissue sticking to them. Flush the liquid directly into the lid of the petri dish. Use the liquid to collect all tissue pieces and transfer them to a 15 ml conical tube. Add some more medium to collect any remaining piece of tissue sticking to the lid. The final volume will be between 10-15 ml.
12. The tube with gut pieces is vortexed to wash. (approx. 10-15 sec).
13. Wait for the gut pieces to sink to the bottom of the tube and form a loose pellet.
14. > 2/3 of the PBS is removed by pouring it off.
15. Add approx. 6ml PBS-PSAG, wash 2 more times by repeating steps 12-14. The exact number of washes can differ per tissue or isolation. Most important is that the buffer after vortexing is clear and the tissue looks whiteish.
16. To disassociate the cells, add approx. 5 ml ice-cold PBS-PSAG with 30mM EDTA, and incubate for 15 minutes on a rotary shaker (slow speed, 14 rpm.min) at room temp.
17. Transfer the 15 ml tube to the flow hood and wait for the gut pieces to sink to the bottom of the tube and form a loose pellet; make sure no pieces are sticking to the tube or lid.
18. 2/3 of the buffer is removed by pouring from the tube.
19. Add approx. 6ml cold DMEM to stop the EDTA dissociation step.
20. Mechanical disassociation: IMPORTANT! flush the strippette with FBS to prevent cells and tissue pieces sticking. Then pipette 30-40x up and down to dislodge cells. (Test different pipettes tips to find the best for dislodging cells, if the pieces are small enough use 5 ml pipette (preferred)).
21. Filter the suspension through a 100 µm (yellow) cell strainer in a 50 ml tube.
22. Transfer the filtered cells to a 15 ml tube --> better pellet after centrifugation but if the volume is too large, keep in 50 ml tube.
23. Spin at 500 x g for 5 minutes, check if there is a pellet.
24. Discard the supernatant.
25. Add 6 ml DMEM for a second wash.
26. Spin 500 x g for 5 min. Check for a pellet again.
27. Discard the supernatant -->
28. (Optional) perform another wash if the cell pellet is large or not all the previous wash solutions could be removed. It is important to remove the EDTA.
29. Resuspend in 50-100 µl of the cells in the organoid medium. In order not to waste the Matrigel, keep the cell volume to the minimum, so centrifuge again if needed or if you want to concentrate the cell number.

30. Remove all foam bubbles.
    A. For cell seeding:
        a. Do not make bubbles (keep some volume inside the pipette tip and careful empty next to the tube wall).
        b. **Note**: Keep 24 wells plate empty in incubator at 37C for a couple hours or overnight before using the matrigel. Humidity in the incubators affects the surface tension in the wells and it makes the Matrigel drop stiffer in the wells.
        c. Matrigel is fluid when cold and solid at higher temp. (so work quickly and avoid bubbles). Matrigel is viscous so mix it well.
        d. Resuspend the intestine cells in the Matrigel kept on ice. (2:1 or 1:1 medium:Matrigel). Avoid air bubbles when emptying the pipette tip.
        e. Place small (10-20 ul) drops of matrigel+cells in 24 wells plate. Ideally 5 droplets per well for a max of 4-5 wells (to also save the medium).
        f. Cover the plate with its lid and turn the plate upside down (hanging drop). Check the matrigel consistency. It needs approx. 45 min at 27 ºC to solidify.
        g. Add pre-warmed (27°C) medium 400 - 500 µl Orgmed-GAPCS slowly, via the side of the well (add fresh ROCK inhibitor to the medium). Incubate at 27 °C with CO^2. **Note**: Small clusters of 6-8 cells are very promising as those are the ones that will turn into organoids. They should grow into nicely round shaped clusters.
        h. Change the medium every 2 days in the beginning to allow growth. If you have too many dark cells or single cells they need to be passaged.
        i. After approximately 3-4 days, remove the supernatant, put the plate on ice, add 400 ul ice cold DMEM and use a pipette tip to break the matrigel.
        j. Transfer to 15 ml tube and centrifuge 500 x g for 5 min.
        k. Remove the supernatant and leave a small amount of liquid to resuspend the cells
        l. Mix with matrigel as in step d.
        n. After approximately 1 week or when spheroids get very big, perform dissociation with TrypLE.

Dissociation with TrypLE and re-seeding in Matrigel

- Pre-cool the Eppendorf centrifuge to 4 ºC.
- Put the PBS, TrypLE, Organoid medium (if needed, Rock inhibitor, to be added 1:1000) and Matrigel on ice.
- Carefully remove the medium from the wells that you will passage, leaving just the Matrigel domes
- Add 400 ul ice cold PBS (+Rock inhibitor, 1:1000) to the well and gently pipette up and down, positioning the tip near the droplet on the well's surface helps dislodging the Matrigel. Do this until the Matrigel is dislodged from the plate and dissolved in the PBS. Matrigel becomes fluid again at 4°C.
- Transfer to a 1.5 ml Eppendorf tube
- Centrifuge for 3 minutes at 500 g, 4 ºC. If you see a streak of cells and Matrigel along the wall of the tube, instead of a pellet at the bottom, turn the tubes around 180 degrees in the centrifuge and centrifuge again.
- Carefully remove as much supernatant as possible (the organoids in the pellet can be large and will not form a firm pellet).
- Add 100 µl ice-cold TrypLE (+Rock inhibitor) and gently pipette up and down to resuspend the pellet. Incubate on ice (1-2 minutes to yield single cells + clusters, but no whole spheroids).
- Add 300 µl of ice cold complete organoid medium to dilute the TrypLE.
- Centrifuge for 5 minutes at 500 g, 4 ºC. If you see a streak of cells along the wall of the tube, instead of a pellet at the bottom, turn the tubes around 180 degrees in the centrifuge and centrifuge again.

- Remove as much supernatant as possible.
- Take the pre-heated 24-wells plate from the incubator.
- Carefully resuspend the pellet in ~50ul of Matrigel (usually results in ~60-70ul final volume), avoid emptying the pipet completely to prevent air bubbles.
- Transfer the MG/Cell mixture to the 24-wells plate as a Matrigel droplet:
    - Gently press down on the pipet so a small droplet forms at the pipette tip
    - Gently touch the plate with the droplet to form a Matrigel dome.
    - Repeat to form 4-5 droplets per well.



- Invert the plate (hanging drop) and place in the incubator at 27°C, allowing the Matrigel to solidify for 45-60 minutes. Pre-warm complete organoid medium to 27 ºC during this time.
- Flip the plate right side up and add 400 ul of medium to each well. Return to the incubator.

4.2. Standard Operating Procedure (SOP) for generating complex primary cell cultures

4.2.1. Complex cell culture from gills of sea bass (INRAE)

Required equipment, reagents

For fish dissection

- Forceps, scissors, scalpel
- Small glass Petri dishes
- Absorbent paper
- Balance

For cell culture

- Leibovitz's L-15 Medium (Sigma, L4386)
- 10X Hanks Balanced Salt Solution (HBSS) without calcium and magnesium (Sigma, H4641)
- EDTA (Sigma, EDS)
- HEPES (Sigma, H4034)
- Sodium bicarbonate (Sigma, S6297)
- Fetal bovine serum (FBS) (Eurobio, CVFSVF00))
- Antibiotics : Penicillin-streptomycin-amphotericin (Sigma, catalogue number A5955)
- Trypsin (Sigma, T4799)
- Falcon® Cell strainers, 100µm (Corning, 352360)
- Falcon® 50ml conical tubes (Corning, 352070)
- Pipettes/tips for 20, 200 and 1000µl
- Pipet-aid/pipettes for 10 and 25ml
- Hemocytometer, Thoma
- 24, 12 or 6 wells Transwell Polyester, Corning, pore density 4×10^6 pores/cm²; pore size 0.4 µm (Corning 3470, 3460,3450)
- EVOM^{tm} epithelial voltohmmeter (World Precision instruments)

Media recipes

- Culture medium: L15 complemented with 10mM HEPES, 2mM NaHCO3 pH 7.6, osmolarity 365mOsmol/l
- Wash-solution: add 1% of antibiotics in HBSS complemented with 10mM HEPES, 2mM NaHCO3 pH 7.6, osmolarity 365mOsmol/l

- Trypsin solution: add 0.05% tryspin powder in HBSS complemented with 10mM HEPES, 2mM NaHCO3 and 0.02% EDTA pH 7.6, osmolarity 365mOsmol/l
- Stop solution: culture medium + 10% FBS and 1% antibiotics.
- Culture media with FBS and antibiotics: culture medium +10% FBS + 0.5% antibiotics.

Preparation of cells from sea bass gills

1. Young sea bass (less than 1 year) should be killed according to local regulations.
2. Lift the opercula and excise all gills arches with scissors and forceps. Place the gill arches into 5 ml of wash solution in a small Petri dish for 5 min.
3. Remove mucus and blood by blotting each gill arch onto absorbent paper and place into a second glass Petri dish for 5 mn (5 ml of wash solution in Petri dish)
4. Place into a third 5 ml of wash solution in a glass Petri dish. Cut the filaments from the arches then the filament into smaller pieces with 2 pair of scissors.
5. Put all small filament pieces in a 50 ml conical centrifuge tube (tube 1), centrifuge for 5min at 200*g* at room temperature then discard the supernatant.
6. Add 3 ml of trypsin solution to tube 1 and shake for 10 min at 250 r.p.m. at room temperature.
7. Place a 100 µm cell strainer onto another 50 ml tube (tube 2) containing 10 ml stop solution. Pour the filament/trypsin solution in tube 1 into the strainer and use a pipette tip to facilitate the passage of isolated cells through the cell strainer. Remove the remaining filaments from the strainer and replace back into tube 1. Then rinse the cell strainer with 2 ml of stop solution.
8. Repeat the trypsin digestion (steps 6-7) twice using 2 new cell strainers. In the last trypsin digestion, rinse the third strainer with 3*2 ml of stop solution with gentle mechanical agitation with a pipette tip.
9. Centrifuge the cell suspension for 5 min at 200*g* at room temperature.
10. Rinse the pellet twice with 10 ml stop solution. For the last rinse, re-suspend the pellet in 3-5 ml culture medium with FBS and antibiotics.
11. Count viable cells (except red blood cells) using a haemocytometer based on the Trypan blue exclusion criterion (20 µl Trypan blue solution + 20 µl cell suspension)
12. Seed cells at 2 to 2.5×10^6 cm^2 on Transwell inserts.
13. Twenty-four hours after seeding, non-adherent cells were removed. As from the third day after seeding, the medium was changed every 2 days.
14. Transepithelial resistance (assessing the barrier function of the epithelium) are measured with an EVOM from day 3.

4.2.2. Complex cell cultures from gills of tilapia (WU)

<u>Equipment and reagents</u>

Fish dissection

- One polystyrene box with ice
- Anesthetic Tricaine Methane Suplhonate
- vacutainer for blood collection and corresponding needle
- Scissors, small tweezers, blade
- Marker, pen and notepad
- 70% Ethanol or isopropanol for tools and fish sterilization during dissection
- Absorbent paper

Primary culture of tilapia fish gill epithelia.

- HBSS: HBSS without calcium and magnesium (Gibco), osmolarity 280-300 mOsm/l. Add 10 mM HEPES and 2 mM $NaHCO_3$.
- Wash-solution: 1x Pen/strep (Gibco 15140122) in HBBS.
- Trypsin-EDTA solution: Gibco 25200056; 0.05 % trypsin solution + 0.25 % EDTA.

- L15 without phenol red: Gibco 21083027.
- Stop solution: 10 % FBS in L15
- Rinse solution: 2.5 % FBS in L15
- Cell culture medium: L15 without phenol red + 1x Pen/strep (Gibco 15140122) + 5% FBS + 10mM HEPES + 2 mM NaHCO3. osmolarity 280-300mOsm/l.
- 1ml pipette/tips
- 100 µm cell strainer
- 90 mm petri dishes
- surgical instruments for dissection

Nile tilapia dissection and gill isolation (approx. 1h)
Fish: Healthy, younger than 10 months, avoid stress to minimize mucus formation.

Prepare all solutions just before isolation; perform all steps at Room temperature unless stated otherwise.

1. Kill fish according to local regulations.
2. Tap the fish dry, using paper towels.
3. Spray the fish with 70% Ethanol.
4. Remove the operculum and excise the intact gill arches, with dissection scissors and forceps. Place the gill arches into 10 ml of wash solution in the lid of a Petri dish.
5. Remove clotted blood from gill arch with forceps in wash solution.
6. Cut the filaments (primary lamellae) from the arch and place in wash solution (in the deep side of a Petri dish).
7. Cut filaments in smaller pieces (bundles of 1-5) with a forceps and a scalpel.
8. Transfer the filaments to one 50 ml tube with 10 ml wash solution.

Gill cell isolation (approx. 2 h.)

1. Prewarm the trypsin-EDTA solution at 27 °C.
2. Filaments should sink to the bottom of the tube, otherwise centrifuge the tube for 4 min at 250g at 16 °C.
3. Pour the solution off and add 3 ml of trypsin-EDTA solution to begin digestion and shake in a tabletop shaker, for 12 min at 250 RPM at 27 °C (This step can be performed in a rotary shaker, but time and speed need to be optimized again).
4. To increase cell yield, pass filaments still in trypsin solution at least 20 times through a wide bore pipette tip, immediately following the 12 min trypsinisation period (A wide bore pipette tip can be prepared by cutting the tip of a 1000 µl pipette tip, so that the bore of the pipette aperture is around 2 mm).
5. Place a 100 µm cell strainer onto a 50 ml conical tube containing 20 ml stop solution. Pour liquid first and then the filaments onto the cell strainer, gently move the filaments around to facilitate cell loosening and passage through the mesh. Then put filaments back into the original 50 ml conical tube.
6. to properly stop trypsinization, mix the solution in the tube containing the cell suspension.
7. Add 3 ml of trypsin solution to the tube containing the filaments to start trypsin digestion again, and shake for 12 min at 250 RPM at 27 °C.
8. repeat steps 4-6
9. Centrifuge the cell suspension for 10 min at 300 g at 16 °C. A red pellet should be obtained. Discard supernatant.
10. Re-suspend the red cell pellet in 1 ml of rinse solution, then add the remaining 19 ml, flick briefly and centrifuge for 10 min at 300 g at 16 °C. Discard the supernatant.

11. Re-suspend the red pellet in the tube in 2-5 ml cell culture medium and flick the tube to dislodge the pellet and mix well.
12. Count viable cells using a Bürker counting chamber based on the Trypan blue exclusion criterion or other method of choice. Do NOT include red blood cells.
13. Seed cells at a density of 3 million/cm^2 in a 25 or 75 cm^2 flask.
14. Replace the culture medium every two days.
15. An epithelial cell monolayer will form on day 1, and tight junctions should start forming by day 7 onwards. Under these conditions goblet cells will also be present.

4.3. Standard Operating Procedures (SOP) to study cataract *ex vivo*

4.3.1. Eye lens model for Atlantic salmon (IMR)

The development of this SOP has been postponed to M48 and will be reported as part of D5.4

4.4. Standard Operating Procedure (SOP) for generating mutant cell lines for viral studies

The immune response to a viral infection in vertebrates can be broken down into several stages. Firstly, cells of different cell types detect the presence of the virus via receptors present on their surface or in the cytoplasm after entry of the pathogen. Activation of these receptors triggers a cascade of molecular reactions culminating in the production of type I interferons (IFNs), released into the intercellular space and bloodstream, and acting on a large number of cells to induce an "antiviral state", refractory to infection, by activating the expression of numerous genes, known as "ISGs" for IFN stimulated Genes. Numerous ISGs have been identified in fish, but their mode of action remains largely unknown. In the long term, activation of the IFN system and its action on ISGs reduces and delays the spread of the virus in the organism, while the adaptive response takes hold. At a cellular level, IFN increases resistance to viral infection and reduces viral replication. Partial or total inactivation of the type I IFN response makes cells (and fish) much more susceptible to viral infections, even if other antiviral mechanisms exist.

Viral diagnostics and vaccinology rely on the availability of stable cell lines, usually isolated from the host species, enabling large quantities of virus to be produced in the laboratory. Treatment of these viral particles (inactivation) or their genetic characteristics (attenuation) make it possible to obtain safe and effective vaccine preparations. On the other hand, to test the efficacy of any type of antiviral vaccine, it is absolutely necessary to set up clinical trials under controlled experimental conditions. A system for producing large quantities of field virus strains is therefore extremely important. At present, no *in vitro* propagation system is available for various piscine viruses causing important emerging diseases, such as piscine myocarditis virus (PMCV) or Piscine Reovirus (PRV). Others can be propagated/produced in some existing fish cell lines but with low yields.

As the IFN system makes a major contribution to cellular resistance to viral infection, we hypothesize that it largely conditions virus production efficiency in available cell culture systems. We have recently developed a genome-editing system in salmonid fish cell lines that enables genes involved in immune defense against viruses, and in particular ISGs, to be easily invalidated.

Mutant cell lines from salmonids (INRAE)

Required equipment, reagents

- Leibovitz's L-15 Medium (Thermofisher, 11415064)
- Trypsin-EDTA (0.25%), phenol red (Thermofisher, 25200056)
- D-PBS (Thermofisher, 14190144)
- Fetal bovine serum (FBS) (Eurobio, CVFSVF00-01)
- G418 (Invivogen, ant-gn-5)
- Hygromycin B Gold (Invivogen, ant-hg-5)
- Penicillin-Streptomycin (10,000 U/mL) (Thermofisher, 15140122)
- Sorenson™ low binding aerosol barrier tips for 10, 20, 200 and 1000µl pipets (Sigma, Z719390-960EA/Z719412-960EA/ Z719439-960EA/Z719463-1000EA)
- 2ml, 5ml, 10ml, 25ml pipettes (Sarstedt, 86.1252/1253/1254/1685)
- 25 cm2 flasks (Sarstedt, 83.3910)
- nCas9n TrueCut™ Cas9 Protein v2 (Thermofisher, A36499)
- Q5® High-Fidelity 2X Master Mix (New England Biolabs, M0492S)
- T7 Ribomax Express Large Scale RNA Production System (Promega, P1320)
- NUCLEOSPIN Gel and PCR Clean-Up kit (Macherey-Nagel, 2548462)
- NUCLEOSPIN Tissue kit (Macherey-Nagel, 2548558)
- TRIzol (Thermofisher, 15596026)
- Electroporator Neon (Thermofisher, discontinued)
- Neon™ Transfection System 10 µL Kit (Thermofisher, MPK1025)
- RNse-free water (Sigma, W4502-1L)
- PCR machine Eppendorf (Mastercycle Pro)
- 20°C cell culture incubator
- Laminar flow cell culture cabinets

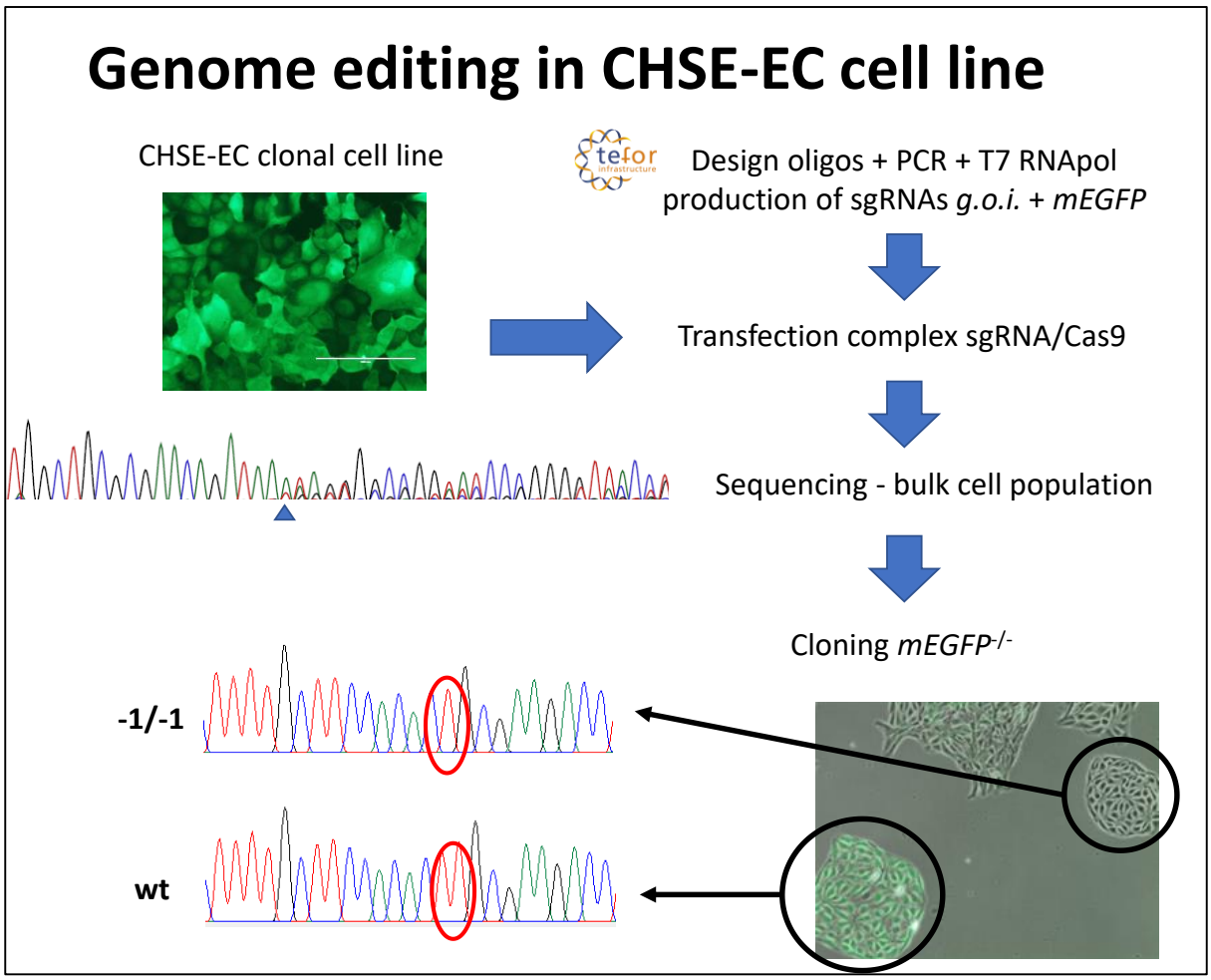
Media recipes

- Culture medium (CHSE-EC and all derived mutants): L15, 10% FBS, 1X penicillin-streptomycin, 500 µg/ml G418, 30 µg/ml Hygromycin

Procedure

1. Single guide (sg) RNA is designed within the first coding exon of the gene of interest using the CRISPOR software (Concordet and Haeussler, 2018).
2. A dsDNA template is then amplified by PCR using the forward primer DR274F AAAAGCACCGACTCGGTGCCAC (final concentration 500 nM), the reverse primer T7R TCCTAATACGACTCACTATA (final concentration 500 nM) and a 120 nt long oligo AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCT AGCTCTAAAACNNNNNNNNNNNNNNNNNNNNTATAGTGAGTCGTATTAGGA (template, final concentration 1 µM) and the Q5 High-Fidelity DNA Polymerase2X master mix . Cycling protocol was as follow: 98°C 30 sec, 35 cycles [98°C 5 sec, 60°C 10 sec, 72°C 10 sec], 72°C 30 sec, Hold 4°C.
3. The PCR product (120 nt; approx. 1 µg) was purified (Macherey-Nagel, PCR and Gel purification kit), used as template for T7 *in vitro* transcription () according to the manufacturer's instructions and the resulting RNA purified using 1 ml TRIzol reagent according to the manufacturer's instructions. The resulting RNA pellet was dissolved in 50 µl of RNase-free water.

4. The monomeric Enhanced Green Fluorescent Protein (mEGFP) sgRNA (Dehler et al., 2016) was produced as described above using the template oligo AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCT AGCTCTAAAACTAGGTGGCATCGCCCTCGCCTATAGTGAGTCGTATTAGGA.
5. Each sgRNA (1µg in 1µl) were mixed with 1 µg (in 1µl) of recombinant nCas9n separately, incubated at room temperature for 5 min. Nucleoprotein complexes were pooled and used in transfection as described previously by Dehler et al., 2016 (4 µl Cas9-sgRNAs mixed with 30 µl of CHSE-EC suspension in solution R at 10^7 cells/ml; 3 electroporation's cycles of 2X 20ms pulses at 1,300 volts using the Thermofisher Neon electroporation system, 10µl kit).
6. All transfected cells were mixed in 5 ml CHSE-EC culture medium in a 25cm^2 flask and incubated at 20 °C for 1-2 weeks, until the monolayer reached confluency.
7. The cells were then passaged using a surface ratio 1:3 with one third being used for genomic DNA extraction using the NucleoSpin Tissue according to the manufacturer's instructions specific to cultured cells.
8. A 500-nucleotides genomic fragment is amplified by PCR using relevant primers encompassing the sgRNA targeted site (New England Biolabs Q5 2X mastermix), purified using the PCR clean-up and Gel extraction (Macherey-Nagel), and sequenced (Sanger sequencing service, Eurofin).
9. After sequence analysis and evidence for presence of genome edited cells in the population (chromatograms showing polymorphism at the sgRNA location), the cells were passaged a second time and a small proportion was seeded at very low density (10-fold serial dilutions) on 6-well plates.
10. After 4-5 weeks, clonal cell patches were marked by a circle using a marker pen under an inverted microscope and analysed under a fluorescent microscope. Non-fluorescent "Dark" (mEGFP-deficient) clones were selected, detached mechanically by pipetting using a P200 pipet under sterile conditions and sub-cultured in separate 25cm^2 flasks pre-filled with 5 ml of complete culture medium (see figure below).



11. After 3-4 weeks of expansion at 20°C, the absence of mEGFP was confirmed in two clones under the fluorescent microscope, passaged using a surface ratio of 1:1, left to grow until confluency and passaged again using a surface ratio of 1:3.
12. One flask for each clone was sacrificed for genomic DNA purification and genotyping at the targeted locus as described above whereby the two other flask were expanded further into 75 cm^2 flasks.
13. From this stage, the cells were passage every 2 weeks at a surface ration of 1:3.
14. Regularly, a third of a confluent 75 cm^2 culture was pelleted (30 sec 13,000g), resuspended in 500µl FBS in a cryovial and mixed with 500µl complete culture medium supplemented with 20% DMSO place in a polystyrene box filled with cotton wool. The box stored at -70°C ensured a cooling rate lower than 1°C/min. After 24 hours, the cryovial was transferred in a liquid nitrogen contained (BRAND).

5. Conclusions

Subtask 5.3.1 Phsyiology of nutrient uptake

SOP on immortal hepatocyte cell lines (INRAE):
Results gathered from the two rainbow trout (*Oncorhynchus mykiss*, Salmoniformes, Salmonidae) liver-derived cell lines revealed a better absorption and metabolization of DL-MET than DL-MHA, with the activation of the mechanistic Target Of Rapamycin (mTOR) pathway for DL-MET and the activation of integrated stress response (ISR) pathway for MHA (Pinel et al., 2022). The results also demonstrated the existence of a major pathway of lysosomal proteolysis known as Chaperone-mediated autophagy, and its important protective role against high glucose-induced stress (Velez et al., 2023). This research unequivocally underscores the significance of employing these models in the realm of fish nutrition. In addition to aligning with the 3Rs principle by reducing the necessity for animal testing, these models provide a streamlined and meticulously controlled environment for experimental investigations.

SOP on salmonid all-male hepatocytes (IMR)
Individuals from a clonal all-male Atlantic salmon (*Salmo salar*, Salmoniformes, Salmonidae) line were used for the isolation of the primary liver cells. Using all-male salmon reduced the use of fish to a minimum (no females had to be discarded) thus favouring the 3R principles.

SOP on intestinal organoids (WU)
In this project we optimized intestinal cells isolation, seeding in Matrigel and compared the growth and formation of common carp (*Cyprinus carpio*, Cypriniformes, Cyprinidae) intestinal organoids in medium containing mammalian (mouse) growth factors and carp-specific growth factors. The current SOP is based on cultures using mammalian growth factors supplemented with pooled carp serum (PCS) and can profit from further optimisation studies using culture medium containing in-house carp-specific growth factors for increased reproducibility.

Subtask 5.3.2 Fish health and gill functions

SOPs on complex cell cultures from fish gills (subtask 3.2 INRAE, WU)
This project aimed to transfer the knowledge of in vitro cultures of gill cells of rainbow trout to in vitro cultures of gill cells of perciform fish. For European seabass (*Dicentrarchus labrax*, Moroniformes, Moronidae), a complex cell culture was obtained with mainly pavement cells and few mucus cells. Transepithelial resistance reached a plateau 7 to 9 days after seeding, providing a primary culture ready for use. For Nile tilapia (*Oreochromis niloticus*, Perciformes, Cichlidae) a complex gill epithelium primary cell culture also comprising Goblet cells was achieved.

Subtask 5.3.3 Fish health and causal factors for cataract

SOP on *ex vivo* eye lens model (IMR)
The effort on this SOP has been postponed to M48 and will be reported as part of D5.4

Subtask 5.3.4 Fish health and salmonid cell lines

SOP on KO mutant cell lines for viral studies (INRAE):
The method developed have been used successfully to generate >15 clonal cell lines. To date, the method can generate single, double or triple mutants. Initially developed in chinook salmon (*Oncorhynchus tshawytscha*, Salmoniformes, Salmonidae) the method has now been transposed

successfully to a cyprinid fish cell line (EPC from fathead minnow (*Pimephales promelasone*, Cypriniformes, Cyprinidae); cell line obtained with viperin antiviral gene invalidated) and is currently being tested for the rainbow trout (*Oncorhynchus mykiss*, Salmoniformes, Salmonidae) (RTG-E, a RTG2 derived fluorescent clonal cell line).

6. Appendix

7. References

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