Isolation of leukocytes from seabass head kidney and *in vitro* stimulation

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- HBSS 1X - Heparin 1X – 350 mOsm/kg (cold)
- 350 mOsm/kg Percoll 1.02 g/cm^3
- 350 mOsm/kg Percoll 1.07 g/cm^3
- Sterile glass Pasteur pipettes
- 10 ml pipettes
- P 1000 WO tips
- Sterile 15 and 50 ml plastic tubes
- Sterile 100 uM cell strainers
- Sterile Syringe plunger 2.5 ml
- Sterile tweezers and scissors (two sets)
- EtOH 70%
- Poly I:C ssRNA viral mimic (Sigma catalogue number P1530)
- Vibrio extract bacterial PAMP (from INRA)
- 6-well plates (e.g. Fisher catalogue number 140675)

Washing media – 350 mOsm/kg

- 50 mL L15
- 50 µL FBS (0.1%)
- 350 µL sterile NaCl 2M

Growth media – 350 mOsm/kg

- 500 mL L15
- 10 mL FBS (2%)
- 50 µL Pen/Strep solution
- 3640 µL sterile NaCl 2M

All culture media and solutions were adjusted to 350 mOsm/kg by adding 2M NaCl.

Preparation of cells (i.e. leukocytes) from head kidney

Fish Dissection

1. Sacrifice a fish according to approved local regulations.
2. To reduce the risk of bacterial contamination, thoroughly spray the fish with 70% Ethanol before carefully opening the abdomen with scissors and knife.
3. Take a sterile 100 uM cell strainer and fit it onto a new 50 mL tube.
4. Dissect out the entire head kidney (using tweezers and scissors) and put into the sterile 100 uM cell strainer.

Procedure for cell extraction

5. Working gently, squeeze the head kidney though the 100 µm nylon mesh using the rubber end of the plunger from a 2 mL sterile syringe (few seconds) and immediately after apply 10 ml (or more) of cold HBSS 1X - Heparin 1X – seabass osmolar.
6. Make the final volume of the cell suspension to 20 mL using additional HBSS 1X - Heparin 1X – seabass osmolar.
7. Spin the tube for 10 min at 753 g in a centrifuge pre-cooled to 8°C.

Separation of leukocytes using a percoll gradient

8. In the meantime (or before) prepare 3 or 4 15ml tubes with the Percoll gradient: put 4 ml of Percoll 1,02 g/ cm^3 in the bottom of a sterile 15 ml tube, then add 4 ml of Percoll 1,07 g/ cm^3 to the bottom of the tube with a glass Pasteur pipette trying to not disturb the gradient (add Percoll 1,07 g/cm3 untill the total volume arrives to 8 ml).
9. Decant the wash medium carefully, making sure not to discard the cell pellet, inverting the tube. Add 1 ml of HBSS 1X - Heparin 1X – seabass osmolar and resuspend the pellet pipetting with a WO or edge-cutted tip, add 11,5 ml of the same buffer and resuspend by pipetting.
10. VERY gently layer 4 mL of the cell suspension from Step 9 onto the top of the percoll gradient without causing the layers to mix (touch the inner tube surface with the P1000 tip)
11. Spin the tube for 30 min at 753 g in a centrifuge pre-cooled to 8°C. NOTE: do not use a break on centrifuge! It will take approximately 1 h to stop.
12. At the end, the leucocytes will form a bit more opaque ring between the Percoll 1.02 e 1.08. In the botton, the pellet would contain mucus, eritrocytes and macrofagies, the heavier components. Use a sterile glass Pasteur pipette to carefully remove cells from the ring and put them in a new 50 ml tube.
13. Adjust the volume of the collected leukocytes to 20 mL with seabass osmolar washing media.
14. Centrifuge for 10 min at 610 g and at RT to collect the cells.
15. Decant the wash medium carefully, making sure not to discard the cell pellet. Repeat steps 13 and 14 once more to completely eliminate the percoll.
16. Decant the wash medium carefully and resuspend the pellet with 1 ml of Growth media, keeping the cells to RT.

17. Count the cells using haemocytometer and check viability with trypan blue (should be >90% viable). Dilute to 1x106mL-1 with Growth media.

Stimulation of leukocytes *in vitro*

For the stimulation *in vitro* n = 6 biological replications of each stimulation are required for analysis.

1. Add 2 mL of cells ($2x10^6$ cells) per well in 1.5 x 6-well plates (9 wells).
2. Ensure you get a min number of $18x10^6$ cells per fish for the 3 stimulations (control, Poly I:C and Vibrio extract).
3. We need minimum of $6x10^6$ cells for 3 wells of the 6-well plate per fish (see table below).
4. This can be scaled up while maintaining the same cell densities if total cell number is > $18x10^6$.

Assay:	Control 3 wells per fish	Poly I:C 3 wells per fish	Vibrio extract 3 wells per fish
RNA- and Small RNA-Seq	2 x 10^6 cells	2 x 10^6 cells	2 x 10^6 cells
ATAC-Seq	2 x 10^6 cells	2 x 10^6 cells	2 x 10^6 cells
ChIP-Seq	2 x 10^6 cells	2 x 10^6 cells	2 x 10^6 cells
reserve	2 x 10^6 cells	2 x 10^6 cells	2 x 10^6 cells

Poly I:C stimulation

5. Take an aliquot of **working stock** poly I:C (5mg/ml in PBS1X) and heat to 55°C for 15 min, cool to room temperature for 20 min.
6. Final working concentration in cells will be 50 µg mL^{-1}, which is 20 µL of stock to 2 mL of cell media). Ensure PAMP is well mixed with cells by pipetting gently.

Vibrio extract stimulation

7. Add 20 µL Vibrio extract (from INRA) to 2 mL of cells in growth media. Ensure the PAMP is well mixed with cells by pipetting gently.

Duration of stimulation

Incubate cells 12h at 20°C.