FINAL SCRIPT: APPROVED FOR FILMING

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Title: Isolation, Fixation and Characterization of Juvenile Gilthead Seabream Head Kidney Leukocytes by Flow Cytometry

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No.**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

3. **Filming location:** Will the filming need to take place in multiple locations? **No.**

Current Protocol Length
Number of Steps: 19
Number of Shots: 39

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Isa Marmelo:** This protocol standardizes leukocyte isolation, fixation, and viability assessment from gilthead seabream head kidney via flow cytometry, enabling high-throughput sample processing without compromising quality.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What technologies are currently used to advance research in your field?

1.2. **Ana Luísa Maulvault:** Traditional techniques, like manual cell counting with hemocytometers and stained blood smears, are common in fish immunology. Flow cytometry is becoming popular, but its application in fish studies remains limited.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:4.9*

What are the current experimental challenges?

1.3. **Ana Luísa Maulvault:** Traditional fish immunology methods are labor-intensive and prone to errors. Juvenile studies are limited by challenges in obtaining pure leukocytes and the need for immediate viability assessment, restricting sample throughput.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What advantage does your protocol offer compared to other techniques?

1.4. **Zélia Silva:** This protocol enables detailed immune cell analysis by identifying key leukocyte populations and distinguishing live/dead cells. Fixation preserves viability for a month, allowing scalable, flexible, and efficient flow cytometry workflows.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

How will your findings advance research in your field?

1.5. **Daniel Bolotas:** Our findings advance fish immunology by revealing immune mechanisms and environmental impacts on cell viability, aiding the development of improved disease management strategies in aquaculture.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions :

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Isa Marmelo:** Publishing with JoVE is an innovative approach that will change the way we communicate science. Instead of getting lost in text, our methods come to life on the screen - easier to understand, harder to ignore, and more likely to inspire collaboration and engagement.

1.6.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

1.7 **Daniel Bolotas or Zélia Silva:** Once published on JoVE, we hope to make life easier for researchers trying to replicate our protocol - because, let's be honest, we've all struggled with some methods! By publishing our protocol on video, we're not just helping science; we're boosting collaborations, increasing citations, and everyone wins!

1.7.1 INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

AUTHORS: Please deliver the testimonials in both English and Portuguese

Videographer: Please capture the testimonials in both English and Portuguese

Ethics Title Card

This research has been approved by the Animal Welfare and Ethics Body at IPMA

Protocol

2. Dissection and Isolation of Head Kidney from Juvenile Gilthead Seabream

Demonstrator: Daniel Bolotas and Ana Luísa Maulvault

2.1. To begin, acclimate juvenile gilthead seabream *Sparus aurata*, in Recirculating Aquaculture Systems where water is continuously filtered and recirculated to maintain a stable environment **[1]**. Ensure that the system includes mechanical and biological filtration, aeration, and temperature control to maintain optimal abiotic parameters **[2]**. Adjust environmental factors such as photoperiod, temperature, salinity, pH, and dissolved oxygen levels according to the specific needs of the study **[3]**.

2.1.1. WIDE: Talent transferring juvenile gilthead seabream into Recirculating Aquaculture Systems.

Videographer: If possible please capture a shot of the tanks with the juvenile gilthead seabream swimming inside

2.1.2. Talent inspecting system components including mechanical and biological filters, aeration devices, and temperature controls.

2.1.3. Talent adjusting system settings for photoperiod, temperature, salinity, pH, and dissolved oxygen levels.

2.2. Use a net to gently transfer juvenile gilthead seabream fish into a temporary holding container filled with tank water **[1]**. After euthanizing the fish, place one fish on its side, on a sterile dissection tray **[2].**

2.2.1. Talent transferring the fish using a net into a container of tank water.

2.2.2. Shot of a euthanized fish being placed on its side, on a sterile dissection tray.

2.3. With a scalpel, make a careful incision along the ventral midline of the fish from the vent towards the gills **[1]**. Use dissection scissors to extend the incision and expose the internal organs **[2]**.

2.3.1. Talent making the initial incision with a scalpel.

2.3.2. Talent using dissection scissors to widen the incision and reveal internal organs.

2.4. Locate the head kidney, which is situated just behind the gills near the anterior dorsal region of the body cavity and extends along the top side beneath the vertebral column **[1]**. With a pair of fine-tipped forceps and scissors, carefully clear away surrounding tissues to better visualize the head kidney **[2]**. Then lift the head kidney and make precise cuts around it to free it from the surrounding tissues **[3]**.

2.4.1. Talent pointing to the head kidney with forceps while showing its anatomical position clearly.

2.4.2. Talent removing surrounding tissues with precision tools.

2.4.3. Talent excising the head kidney using forceps and scissors.

2.5. Immediately place the excised head kidney into a cell strainer positioned within a sterile Petri dish **[1]**.

2.5.1. Talent placing the freshly excised organ into the cell strainer inside a sterile Petri dish.

3. Isolation of Leukocytes from the Head Kidney of Juvenile Gilthead Seabream

Demonstrator: Isa Marmelo

3.1. Pipette 2 milliliters of Hanks' Balanced Salt Solution or HBSS *(H-B-S-S)* in a sterile Petri dish **[1]**. Macerate the excised head kidney on the cell strainer using the plunger of a syringe to release the cells into the solution **[2]**.

3.1.1. Talent pipetting HBSS into a sterile Petri dish.

3.1.2. Talent using a syringe plunger to gently press and macerate the head kidney tissue on the cell strainer.

3.2. Next, prepare a density gradient medium solution **[1-TXT]**. Pipette 600 microliters of the density gradient medium solution into 5-milliliter polystyrene round-bottom tubes **[2]**.

3.2.1. Talent preparing and mixing the components of the density gradient medium in a sterile container. **TXT: Density: 1.077 g/mL; Osmolarity: 353 mOsm/kg; pH: 7.4; Adjust osmolarity as per the species**

3.2.2. Talent pipetting 600 microliters of prepared density gradient medium into multiple polystyrene tubes.

3.3. Slowly transfer 2 milliliters of cell suspension from the Petri dish into each tube **[1-TXT]**. Centrifuge at 400 *g* for 45 minutes at 4 degrees Celsius with the brake turned off **[2]**.

3.3.1. Shot of the cell suspension being pipetted into the gradient tube. **TXT: Add the first drop gently to avoid destabilizing the gradient**

3.3.2. Talent placing the prepared tubes into the centrifuge and setting parameters.

3.4. After centrifugation, remove the tube and observe the leukocyte ring **[1].** Then use a sterile Pasteur pipette to gently transfer approximately 100 microliters of the leukocyte ring ~~**[1] and transfer it**~~ into a 2-milliliter microcentrifuge tube **[2]**.

NOTE: VO edited to match the shots

3.4.1. FILE: 3.4.1.mp4. 00:00-00:15

3.4.2. FILE : 3.4.2.mp4. 00:00-00:23

3.5. Now, make up the volume to 2 milliliters, with HBSS *(H-B-S-S)* **[1]** and gently resuspend the cells **[2]**. Centrifuge the sample at 400 *g* for 10 minutes at 4 degrees Celsius **[3]**.

3.5.1. Talent topping up the tube with HBSS.

3.5.2. Talent gently pipetting the suspension to resuspend.
AUTHOR'S NOTE: Shots 3.5.1-3.5.2 might have been recorded together
3.5.3. Talent placing the tube in centrifuge with appropriate settings.

3.6. After centrifugation, carefully discard the supernatant without disturbing the pellet at the bottom of the tube **[1-TXT]**. Then, resuspend the purified pellet in 1 milliliter of HBSS until the final cell concentration is between 10000 to 1 million cells per milliliter **[2]**.
3.6.1. Talent discard the supernatant carefully to leave the pellet intact. **TXT: Repeat HBSS wash until pellet is pure**
3.6.2. Talent adding HBSS and gently pipetting to resuspend the cell pellet.

4. Fluorescent Staining and Flow Cytometric Analysis of Leukocyte Viability in Gilthead Seabream

Demonstrator: Isa Marmelo and Zélia Silva

4.1. Add 50 microliters of dimethyl sulfoxide to a vial containing the reactive dye **[1]**. After adjusting the cell density to 1 million cells per milliliter, transfer 1 milliliter of the cell suspension into 2-milliliter microcentrifuge tubes **[2]**.
4.1.1. Talent pipetting dimethyl sulfoxide into the vial.
4.1.2. Talent transferring the suspension into labelled microcentrifuge tubes.
4.2. Prepare viability control samples as given **[1].** Induce cell death in Tubes 3 and 4 by placing them in a water bath at 70 degrees Celsius for 7 minutes **[2]**.
4.2.1. TEXT ON PLAIN BACKGROUND:
Tube 1: Live cells – unstained
Tube 2: Live cells – stained
Tube 3: Dead cells – unstained
Tube 4: Dead cells – stained
AND
Added shot: Shot of the labelled tubes.
Video editor: Please play both shots side by side
4.2.2. Talent labelling the tubes and transferring Tubes 3 and 4 into a water bath for heat-induced cell death.
AUTHOR'S NOTE: 2 videos were recorded here. 1 of labelled and the other of the tubes being placed in water bath

4.3. To stain the cells, pipette 1 microliter of the reconstituted fluorescent dye to Tubes 2 and 4 containing 1 milliliter of cell suspension and mix well **[1]**. Incubate the stained tubes at room temperature for 30 minutes, protected from light **[2]**.
4.3.1. Talent pipetting dye into the correct tubes and mixing thoroughly.
4.3.2. Shot of Tubes being kept on a benchtop, covered with aluminum foil. .

4.4. Next, wash the cells twice with 1 milliliter of HBSS **[1]** and resuspend the final pellet in 900 microliters of the same buffer **[2]**.
  4.4.1. Talent adding 1 mL HBSS to the cell pellet.
  4.4.2. Shot of the pellet being resuspended in 900 µL of buffer.

4.5. Then pipette 100 microliters of 37% formaldehyde to fix the cells and incubate at room temperature for 15 minutes **[1-TXT]**. After washing the cells twice with 1 milliliter of HBSS containing 1% BSA, resuspend in 1 milliliter of the same solution **[2]**.
  4.5.1. Talent carefully adding formaldehyde to the tube. **TXT: Incubation: RT, 15 min**
  4.5.2. Talent resuspending the fixed cells in HBSS with bovine serum albumin.

4.6. Store the samples at 4 degrees Celsius in a refrigerator for up to 1 month **[1]**.
  4.6.1. Talent placing the labelled tubes in a refrigerator.

4.7. Place the fixed sample tube in the flow cytometer sample port for analysis **[1]**. Record a minimum of 10,000 events for each sample within the singlets gate **[2]**. Save all data and back it up on external drives or cloud storage **[3]**.
  4.7.1. FILE: 67978_shot_4.7.1.mp4 00:00-00:17
  4.7.2. SCREEN: 67978_screenshot_4.7.2_part1.mp4 00:04-00:15 and 67978_screenshot_4.7.2_part2.mp4 00:03-00:15
  4.7.3. SCREEN: 67978_screenshot_4.7.3.mp4 00:02-00:18

4.8. Visualize the flow cytometry data by plotting forward scatter area versus side scatter area to assess cell size and granularity **[1]**. Perform singlet cell gating and exclude multiplets by plotting forward scatter area versus forward scatter height **[2]**.
  4.8.1. SCREEN: 67978_screenshot_4.8.1.mp4 00:02-00:24
  4.8.2. SCREEN: 67978_screenshot_4.8.2.mp4 00:00-00:43, 00:52

4.9. Identify the three leukocyte populations based on forward versus side scatter profiles **[1].** Set the threshold for viability dye staining in the corresponding fluorescence channel to distinguish live and dead cells **[2]**.
  4.9.1. SCREEN: 67978_screenshot_4.9.1.mp4 00:50-00:54,01:04-01:16,01:32, 02:12-02:17
  AND
  TEXT ON PLAIN BACKGROUND:
  $FSC\text{-}A^{high}/SSC\text{-}A^{high}$: Granulocytes
  $FSC\text{-}A^{medium}/SSC\text{-}A^{medium}$: Monocytes
  $FSC\text{-}A^{low}/SSC\text{-}A^{low}$: Lymphocytes

  Video Editor: Please play both shots side by side
  4.9.2. SCREEN: 67978_screenshot_4.9.2.mp4 00:11-00:26, 00:42-00:55, 00:57-01:01,01:36-01:42
  Video Editor: Please speed up the video

Results

5. Representative Results

5.1. Under optimal conditions, lymphocytes, monocytes, and granulocytes constituted 31.0%, 38.0%, and 31.0% of the leukocyte population respectively **[1]**, while under thermal stress, lymphocytes decreased to 21.3%, monocytes increased to 45.6%, and granulocytes remained stable at 33.1% **[2]**.

5.1.1. LAB MEDIA: Figure 6 A *Video editor: Pleases sequentially Highlight the three groups of cells marked LY, MO, and GR*

5.1.2. LAB MEDIA: Figure 6 B *Video editor: Pleases sequentially Highlight the three groups of cells marked LY, MO, and GR*

5.2. The sample exposed to optimal conditions exhibited higher viability, with a predominance of live cells across all leukocyte populations **[1]**. In contrast, the thermally stressed sample showed a significant increase in cell death **[2]**.

5.2.1. LAB MEDIA: Figure 6 A.1, A.2, A.3
Video editor: Please highlight the blue dots in each panel

5.2.2. LAB MEDIA: Figure 6 B.1, B.2, B.3
Video Editor: Please highlight the red areas

Pronunciation Guide:

Leukocyte

- **Pronunciation link**: Merriam-Webster
- **IPA**: /ˈluː.kəˌsaɪt/
- **Phonetic Spelling**: loo-kuh-syte

2. Cytometry

- **Pronunciation link**: Merriam-Webster
- **IPA**: /saɪˈtɑː.mə.tri/
- **Phonetic Spelling**: sy-tah-muh-tree

3. Dimethyl Sulfoxide

- **Pronunciation link**: Merriam-Webster
- **IPA**: /ˌdaɪˈmɛθ.əl ˈsʌlfˌɒk.saɪd/
- **Phonetic Spelling**: dye-meth-uhl sul-fok-side

4. Formaldehyde

- **Pronunciation link**: Merriam-Webster
- **IPA**: /fɔːrˈmæl.dəˌhaɪd/
- **Phonetic Spelling**: for-mal-duh-hide

5. Monocyte

- **Pronunciation link**: Merriam-Webster
- **IPA**: /ˈmɒn.əˌsaɪt/
- **Phonetic Spelling**: mon-uh-syte

6. Lymphocyte

- **Pronunciation link**: Merriam-Webster
- **IPA**: /ˈlɪm.fəˌsaɪt/
- **Phonetic Spelling**: lim-fuh-syte

7. Granulocyte

- **Pronunciation link**: Merriam-Webster
- **IPA**: /ˈgræn.jə.ləˌsaɪt/

- **Phonetic Spelling**: gran-yuh-luh-syte

8. Osmolality

- **Pronunciation link**: Merriam-Webster
- **IPA**: /ˌɒz.məˈlæl.ə.ti/
- **Phonetic Spelling**: oz-muh-lal-uh-tee

9. Photoperiod

- **Pronunciation link**: Merriam-Webster
- **IPA**: /ˈfoʊ.toʊˌpɪr.i.ˌɑːd/
- **Phonetic Spelling**: foh-toh-peer-ee-od

10. Hemocytometer

- **Pronunciation link**: Merriam-Webster
- **IPA**: /ˌhiː.mə.saɪˈtɒm.ɪ.tər/
- **Phonetic Spelling**: hee-muh-sy-tom-uh-ter