

# Hands-on 1.2

## Manipulation of zebrafish embryos and identification of embryonic stages using a bright-field stereomicroscope.

### Objective:

- Learn zebrafish husbandry.
- Manipulate zebrafish embryos.
- Identify the different stages of zebrafish embryos.

### Materials:

- Male and female adult zebrafish
- Zebrafish tanks
- Breeding tanks
- 90mm petri dishes
- Disposable pipettes
- System water
- Brightfield stereo microscope
- Methylene blue
- 2% Agarose gel plates
- Weight Balance
- Falcon tubes

### Solutions and materials preparation

- **2% Agarose gel plates.**  
Weight 2g of agarose and dissolve it in 100mL of ddH<sub>2</sub>O by heating until the agarose is completely dissolved. Pour approximately 15mL of the agarose solution into a 90mm petri dish and allow it to solidify for a few minutes.
- **Methylene blue**  
Weight 10g of methylene blue and dissolve it in 10mL of ddH<sub>2</sub>O until it is completely dissolved.

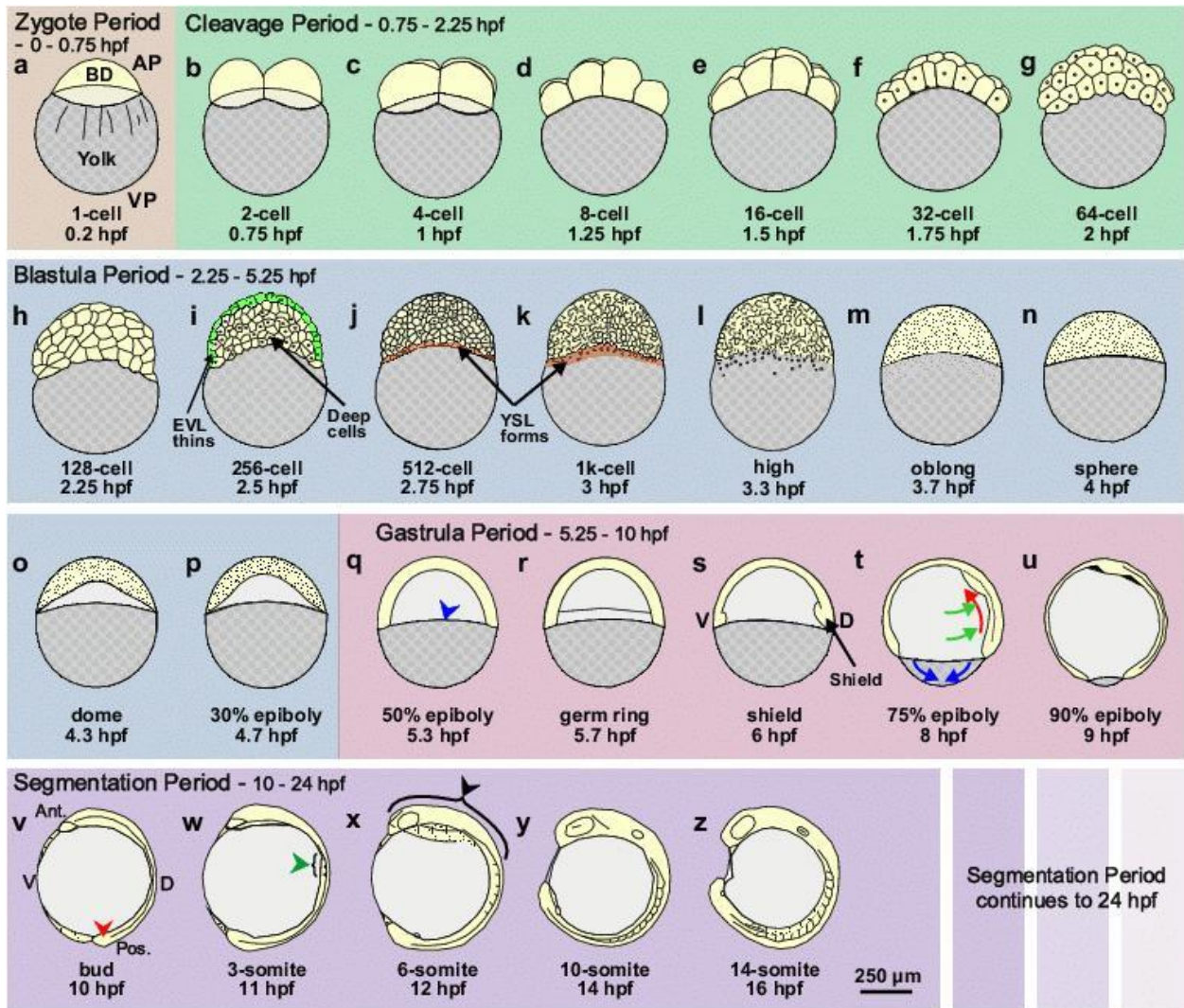
## **Methods:**

### **A) Zebrafish Coupling**

- 1) One day prior to the coupling add males and females adult zebrafish (1:1 ratio) to breeding tanks.
- 2) Separate males from females by adding a removable plastic partition, allowing zebrafish to acclimate overnight.
- 3) The next morning, remove the plastic partition to allow fish to couple and breed.
- 4) Collect the zebrafish embryos from the bottom of the tank for further manipulation.

### **B) Zebrafish embryo manipulation**

- 1) Collect the zebrafish embryos with the help of disposable pipettes and add them to a petri dish - approximately 50 embryos per 30mm petri dish.
- 2) Fill the dish with system water containing 0.1% methylene blue.
- 3) Using a brightfield stereo microscope, visualize and identify the embryonic stage of the embryos.



Adapted from Webb *et al.* CEPP (2007)

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# Hands-on 1.3

## Manipulation of zebrafish larvae and adults: accommodation in different settings/plates and exposure to experimental compounds

### Objective:

- Learn how to anesthetize, euthanize and manipulate zebrafish larvae and adult.
- Learn how to setup and perform a drug screening testing.

### Materials:

- Disposable pipette
- Buckets
- Well-plates
- 90mm petri dish
- pH meter
- Falcon tubes
- Tricaine (MS-222) (compound)
- Weight Balance
- Forceps

### Solutions and materials preparation

- **Tricaine - MS222**  
Weight 400 mg of tricaine powder (Ethyl 3-aminobenzoate methanesulfonate) in 97.9 ml H<sub>2</sub>O. Add ~2.1 ml 1 M Tris (pH 9) and adjust pH to 7. To use tricaine as an anesthetic combine the following in a 250 ml beaker: 4.2 ml tricaine solution in 100 ml of fish water.

## Methods:

### **A) Zebrafish Anesthetic**

- 1) To manipulate zebrafish larvae and adults, prepare an anesthetic solution by adding 4.2 mL of tricaine solution to system water, making a final volume of 100 mL.
- 2) Using a net, remove the zebrafish from the tank system and place them in the prepared tricaine solution.
- 3) Wait for a few minutes until the fish is completely immobilized. Note: Prolonged exposure to tricaine can cause death.
- 4) Use a net or forceps to remove the fish from the tricaine solution for further manipulation or observation. **Note:** The anesthetic effect should last approximately 5 to 10 minutes.
- 5) After manipulation, transfer the fish to a recovery bucket with system water for monitoring for a few hours.
- 6) Return the zebrafish to the system tank.

### **B) Larvae exposure to compounds**

- 1) Using disposable pipettes or a net, collect the desired number of zebrafish larvae and place them in the desired plate. Note: We typically use 15-50 larvae per well in a 6-well plate.
- 2) Remove excess water from the plate and add the testing compound diluted in system water, ensuring that it covers the plate. Note: For a 6-well plate, a total volume of approximately 7 mL is used.
- 3) **(Optional)** Renew the treatment by aspirating the solution using a disposable pipette and adding a new solution. Note: It is recommended to change the solution daily or every 2 days to ensure the well-being of the zebrafish.
- 4) At the end of the exposure period, anesthetize or euthanize the larvae for further analysis. Note: Fish can be euthanized by increasing the concentration and exposure time to tricaine.

# Hands-on 2.1

Alizarin red staining of zebrafish larvae and visualization of calcified structures and warfarin induced vascular calcification

## Objective:

- Learn how to use water-soluble dyes to detect calcified tissues
- Use ectopic calcification-inducing drugs to induce ectopic mineralization in zebrafish
- Visualization and identification of ectopic mineralized tissues.

## Materials:

- Warfarin sodium (compound)
- Alizarin red S (compound)
- Tricaine solution
- Bucket
- Fluorescent stereo microscope
- Pipettes
- pH meter
- Fluorescent microscope
- 15 mL falcon tubes
- Tricaine (MS-222)

## Solutions and materials preparation

- **Warfarin (25mg/L)**  
Prepare a stock solution of Warfarin Sodium (25g/L) and dilute it in ddH<sub>2</sub>O. Add 1mL of Warfarin Sodium per 1L of system water.
- **Alizarin red S (2.5mg/mL)**  
Prepare a stock solution of 10mg/mL, diluted in ddH<sub>2</sub>O and adjust solution to pH 7.4. Dilute ¼ in system water for a working concentration of 2.5mg/mL.

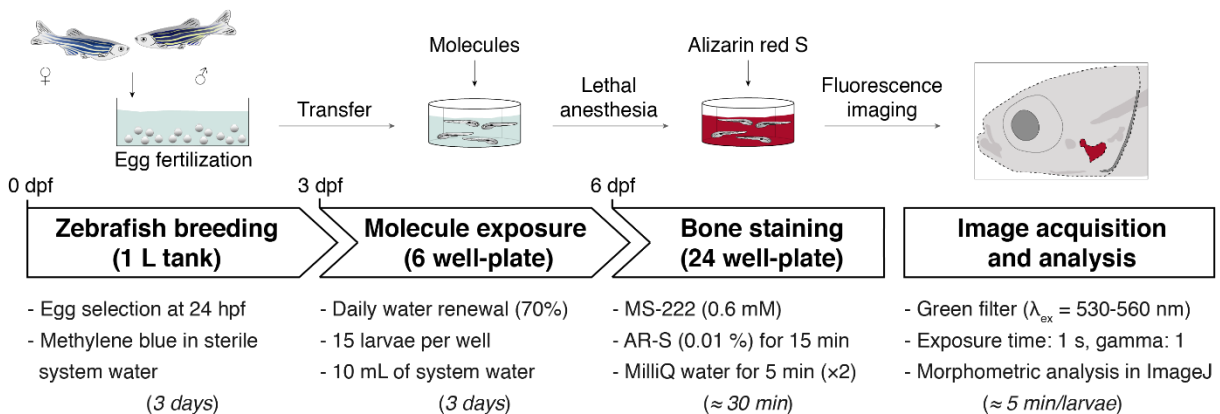
## Methods:

### A) Warfarin-inducing ectopic mineralization

- 1) Prepare fish tanks or individual cups specifically for Warfarin usage. Note: Sodium Warfarin can be toxic and should be handled with special care.
- 2) Fill the container with system water containing sodium Warfarin (25 mg/L) and add the zebrafish.
- 3) Change the solution daily for a period of 15 days.
- 4) Monitor the well-being and condition of the fish daily and euthanize any potentially sick fish. Note: Increased mortality in fish treated with Warfarin is normal.
- 5) At the end of the experiment, euthanize the fish and collect them for further analysis.

### B) In-vivo analysis of mineralized structures

- 1) Prepare a container with alizarin red S solution (2.5 mg/mL) and place the fish in it for a period of 15 minutes. Note: The incubation period can be adjusted depending on the fish's age.
- 2) Prepare another container with system water and transfer the stained fish to it for a 5-minute wash to remove excessive alizarin red S staining.
- 3) Repeat the washing process two more times.
- 4) Anesthetize or euthanize the fish using Tricaine solution.
- 5) Use a fluorescence stereo microscope to visualize calcified structures. Note: Alizarin Red S exhibits an intense fluorescence signal when exposed to light at approximately 650 nm, resulting in absorption at 420 nm.



# Hands-on 2.2

**Imaging of the opercular bone using a fluorescence stereomicroscope. Visualize transgenic fish expressing fluorescent markers of different bone proteins**

## **Objective:**

- Identify potential mineralogenic compounds by assessing the mineralization of the opercular bone.
- Manipulate and analyze transgenic fish expressing fluorescent markers of interest to assess mineralogenesis.

## **Materials:**

- Fluorescence stereomicroscope
- Alizarin red S (compound)
- Tricaine solution
- Bucket
- Pipettes
- pH meter
- Fluorescent microscope
- Falcon tubes
- Forceps

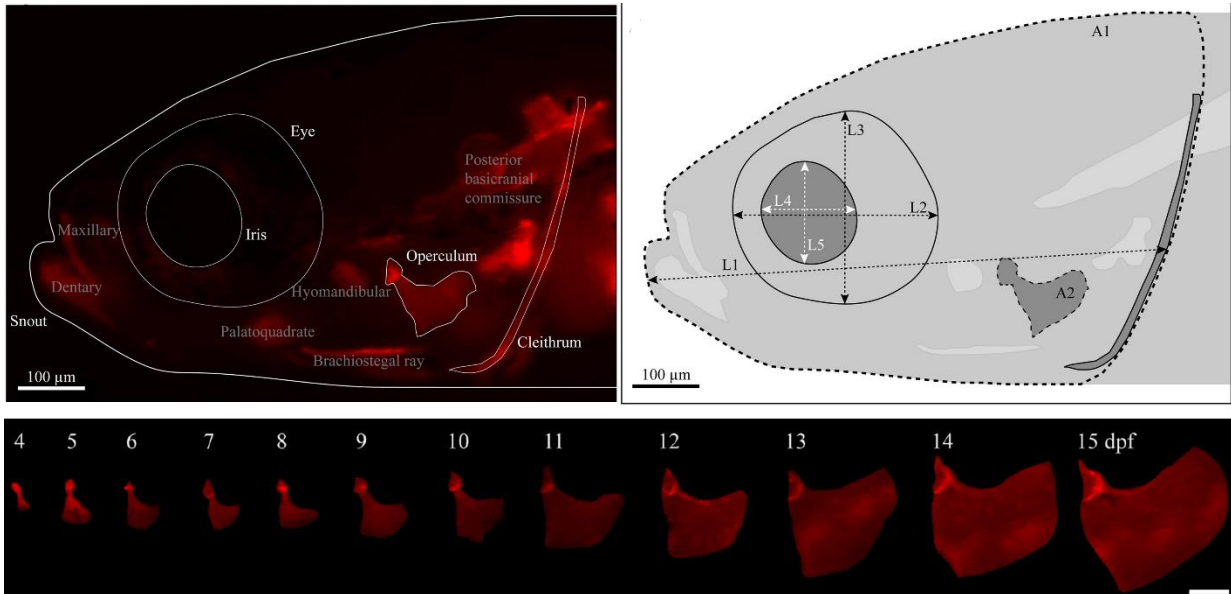
## **Methods:**

### **A) Imaging of the opercular bone**

- 1) Prepare a container with alizarin red S solution (100 mg/L) and place zebrafish larvae in it for a period of 15 minutes.
- 2) Prepare another container with system water and transfer the stained fish to it for a 5-minute wash to remove excessive alizarin red S staining.
- 3) Repeat the washing process two more times.



- 4) Anesthetize the fish using Tricaine solution.
- 5) Use a fluorescence stereo microscope to visualize the opercular bone, utilizing the fluorescence capabilities of the microscope.



Adapted from Tarasco *et al. Comp Biochem Physiol C Toxicol Pharmacol* (2017)

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## **B) Visualize transgenic fish expressing fluorescent markers of different bone proteins**

- 1) Anesthetize zebrafish larvae and adult using tricaine solution.
- 2) Use a fluorescence stereo microscope to visualize transgenic fish.

# Hands on 2.3

## Image analysis and morphometric assessment of zebrafish structures

### Objective:

- Learn how to use imageJ software and ZBONE macro.
- Investigate the mineralization effect of osteogenic compounds.

### Materials:

- Computer
- FIJI (or similar software)
- ZFBONE toolset

### A) Morphometric analysis of the operculum using FIJI

- 1) Download and install Fiji image processing package (<https://fiji.sc/>)
- 2) Download the ZFBONE FIJI-based toolset (<https://github.com/MarcoTarasco/ZFBONE>)
- 3) Open FIJI, go to the "More tools" menu and click on ">>" in the toolbar then select the option "ZFBONE". ZFBONE toolbar will appear in the main FIJI toolbar.
- 4) In the case you have an RGB image (Fiji ▶ Image ▶ Color ▶ Split Channels) and work on the red channel. (If you save the images in mono-channel there is no need to split channels).
- 5) In order to enhance the visibility of cranial bones (Fiji ▶ Image ▶ Adjust ▶ Brightness/Contrast...). Apply the same parameters to all the images.
- 6) Measure the head area (H) and the operculum area (O) with polygon and freehand selection tools, respectively. To measure each selection (Fiji ▶ Analyze ▶ Measure) or press ctrl+M.

# Hands-on 3.1

## Exposure of regenerating caudal fin to experimental compounds and alizarin red S staining

### Objective:

- Learn how to perform the amputation of the caudal fin.
- Investigate the effect of compounds in caudal fin regeneration.

### Materials:

- 3 mL pipette
- Petri dish
- Forceps
- Scalpel
- pH meter
- 300 mL cup
- 1L cup
- Fluorescent microscope

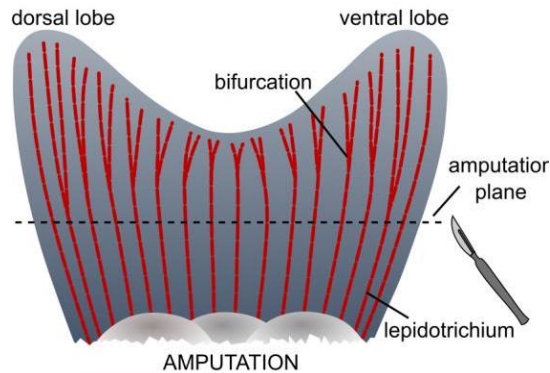
### Solutions and materials preparation:

- **Tricaine - MS222**  
Weight 400 mg of tricaine powder (Ethyl 3-aminobenzoate methanesulfonate) in 97.9 ml H<sub>2</sub>O. Add ~2.1 ml 1 M Tris (pH 9) and adjust pH to 7. To use tricaine as an anesthetic combine the following in a 250 ml beaker: 4.2 ml tricaine solution in 100 ml of fish water.
- **Alizarin red S (0.1%)**  
Dissolve 0.01 g of Alizarin red S in 100 mL of fish water and adjust solution to pH 7.4 with NH<sub>4</sub>OH.

## Methods:

### A) Caudal fin amputation and molecules exposure:

- 1) Anesthetize adult zebrafish (3-4 month) with tricaine (4.2 ml / 100 ml of fish water)
- 2) With the help of a forceps, place the fish on top of a petri dish.
- 3) Amputation should be performed between the 1st and 2nd segments below the amputation plane (consider the first bifurcating rays of both lobes), as in the scheme.



- 4) After amputation fish are allowed to recover in fish water.
- 5) Expose specimens to the given treatment (either before amputation and/or during regeneration) by waterborne exposure or intraperitoneal injection. In the case of waterborne exposure, 70% of the treatments is renewed daily.
- 6) Allow specimens to regenerate at 33 °C for 5 days. (Recommended fish density: 5 adults/L)

### B) Alizarin red *in vivo* staining:

- 1) Stain fish with alizarin red S (0.01% in system water, pH 7.4) for 15 min
- 2) Wash (20 min) and anaesthetize for imaging.

### C) Visualization and imaging of the caudal fin:

- 1) Using a glass petri dish, first place the fish with the head on the left then turn the plate and zoom on the regenerated caudal fin.
- 2) For each fin, brightfield and fluorescence images are collected sequentially to assess the regenerated fin and the mineralized area, respectively. Image are acquired with a fluorescent microscope equipped with green light filter ( $\lambda_{ex} = 530\text{--}560\text{ nm}$  and  $\lambda_{em} = 580\text{ nm}$ ). Suggested settings: exposure time 200 ms (brightfield acquisition) and 1 s (fluorescent acquisition), gamma 1.00, image format 1376 × 1032 pixels, binning 1 × 1. Save images in TIFF.

# Hands-on 3.3

## Image analysis and morphometric assessment of new bone mineralization and patterning

### Materials:

- Fluorescent and brightfield images of zebrafish adult caudal fin
- Computer with the following software installed: ImageJ/FIJI, Excel

### Methods:

#### Image analysis and morphometric assessment:

- 1) Open the brightfield image and measure STU (use straight-line selection tool then measure; (Fiji ► Analyze ► Measure) or press ctrl+M)
- 2) Measure REG (use polygon selection tool then measure), keep the selection active
- 3) Open the correspondent fluorescent image, transform to 8-bit (Fiji ► Image ► Type ► 8-bit) and restore selection (Fiji ► Edit ► Selection ► Restore Selection)
- 4) Select RMA using a threshold ((Fiji ► Image ► Adjust ► Threshold...)) then measure (ctrl+M)
- 5) Close fluorescent image and select the brightfield one
- 6) Using a straight-line selection tool, measure the width of each ray before STU
- 7) Using any spreadsheet software calculate RAY (average of the rays width before amputation) and determine (REG/STU) to evaluate effects on tissue regeneration and finally ((RMA/RAY)/(REG/STU)) to assess effects on *de novo* bone mineralization
- 8) Compare ratios of control/vehicle *versus* treatment/molecule with a statistical software