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Using Alizarin Red Staining to Detect Chemically Induced Bone Loss in Zebrafish Larvae

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TITLE:

Using Alizarin Red Staining to Detect Chemically Induced Bone Loss in Zebrafish Larvae

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SUMMARY:

Here, we have used alizarin red staining to show that lead acetate exposure causes a bone mass change in zebrafish larvae. This staining method can be adapted to the investigation of bone loss in zebrafish larvae loss induced by other hazardous toxicants.

ABSTRACT:

Chemically induced bone loss due to lead (Pb) exposure could trigger an array of adverse impacts on both human and animal skeletal systems. However, the specific effects and mechanisms in zebrafish remain unclear. Alizarin red has a high affinity for calcium ions and can help visualize the bone and illustrate skeletal mineral mass. In this study, we aimed to detect lead acetate (PbAc)-induced bone loss in zebrafish larvae by using alizarin red staining. Zebrafish embryos were treated with a series of PbAc concentrations (0, 5, 10, 20 mg/L) between 2 and 120 h post fertilization. Whole-mount skeletal staining was conducted on larvae at 9 days post fertilization, and the total stained area was quantified using ImageJ software. The results indicated that the mineralized tissues were stained in red, and the stained area

decreased significantly in the PbAc-exposure group, with a dose-dependent change in bone mineralization. This paper presents a staining protocol for investigating skeletal changes in PbAc-induced bone defects. The method can also be used in zebrafish larvae for the detection of bone loss induced by other chemicals.

INTRODUCTION:

Recent studies have confirmed that osteoporosis due to glucocorticoids, aromatase inhibitors, and excessive alcohol consumption is common[1,2]. Lead (Pb) is a toxic metal found in plants, soil, and aquatic environments[3]. Although the adverse effects of Pb on the human body have attracted much attention, its irreversible impact on bone needs to be investigated further. Lead intoxication causes a diverse array of pathological changes in both the developing and adult skeleton, affecting normal life activities. Studies have found an association between chronic Pb exposure and bone damage[4], including impaired bone structures[5,6], reduced bone mineral density, and even increased risk of osteoporosis[7].

Mineralized tissue is of great importance to bone strength[8], and bone mineralization matrix deposition is a critical index of bone formation[9]. Alizarin red has a high affinity for calcium ions, and alizarin red staining is a standard procedure for assessing bone formation[10]. According to this method, mineralized tissue is stained red, while all other tissue remains transparent. The stained area is then quantified by digital image analysis[11].

Zebrafish is an important model organism widely used in drug discovery and disease models. Genetic studies in zebrafish and humans have demonstrated similarities in the underlying mechanisms of skeletal morphogenesis at the molecular level[12]. Moreover, high-throughput drug or biomolecule screening is more feasible in large clutches of zebrafish than murine models, facilitating the mechanistic study of proosteogenic or osteotoxic molecules[13]. Differential staining of the skeleton *in toto*[10] is frequently used in studying skeletal dysplasia in small vertebrates and mammalian fetuses. Alizarin red staining was performed to investigate the bone developmental toxicity of chemicals in zebrafish larvae. Herein, we used lead as an example to describe a protocol for detecting lead acetate-induced bone defects in zebrafish larvae.

PROTOCOL:

All animal procedures outlined here have been reviewed and approved by the Animal Care Institute of The Ethics Committee of Soochow University.

1. Fish husbandry and embryo collection[14]

1.1. Feed fish three times every day; ensure the zebrafish are maintained at 28.5 ± 0.5 °C with a 14:10 h light/dark cycle.

1.2. Separate the male and female adult fish by isolation boards in spawning tanks at a 2:1 male to female ratio in the evening.

1.3. The next morning, remove the isolation boards at 9:00 AM and collect the embryos 2 h later.

1.4. Place the embryos in a biochemical incubator maintained at 28.5 °C before the experiment.

2. Chemical exposure

2.1. Prepare the mother stock: weigh lead acetate trihydrate (5 g), and dissolve it in ultrapure water (50 mL) with stirring.

CAUTION: PbAc is toxic. Wear suitable dust masks, protective clothing, and gloves. Perform the experiment under a fume hood.

2.2. Select and randomly distribute zebrafish embryos into clean 6-well plates (30 embryos per well in 3 mL of zebrafish breeding water; see **Table 1** for its composition). Treat the embryos with PbAc (0, 5, 10, 20 mg/L) from 2 to 120 h post fertilization (hpf).

NOTE: The concentrations of PbAc were chosen according to the dose-range-finding experiments. The results showed that the LC_{50} of lead acetate on zebrafish embryos was 41.044 mg/L at 120 hpf. Hence, the highest concentration was set to half of the LC_{50} and a series of solutions prepared by 2-fold dilution.

2.3. Feed the larvae twice a day from 5 days post fertilization (dpf). Maintain the zebrafish embryos at 28.5 ± 0.5 °C with a 14:10 h light/dark cycle.

2.4. Refresh the Pb-free medium (zebrafish breeding water) every 24 h until 9 dpf.

NOTE: Upon completion of the experiments, all lead-containing solutions were poured into a designated liquid tank and treated by the experiment management center.

3. Alizarin red staining

NOTE: Wear suitable dust masks, protective clothing, and gloves during the entire staining process. The compositions of all solutions are shown in **Table 1.**

3.1. Specific staining steps

NOTE: The dyeing process was carried out in a 24-well plate at room temperature. After each solution was added, place the 24-well plate on the shaking table set at a low speed.

3.1.1. Remove 10 zebrafish larvae randomly from each group at 9 dpf and fix the fish in 1 mL of 2% paraformaldehyde/1x phosphate-buffered saline for 2 h.

3.1.2. Decant the solution and wash the zebrafish larvae with 100 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$ for 10 min.

3.1.3. Decant the solution and incubate the larvae in the following solutions for 5 min each for destaining: anhydrous ethanol (EtOH) solution (80% EtOH/100 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$, 50% EtOH/100 mM Tris-HCl (pH=7.5), and 25% EtOH/100mM Tris-HCl (pH=7.5).

3.1.4. Decant the solution, remove the pigment by bleaching with a solution consisting of 3% H_2O_2 and 0.5% KOH, and observe once every 10 min until the pigment is completely removed.

3.1.5. Rinse the zebrafish larvae several times with 25% glycerin/0.1% KOH for 10 min each until there are no bubbles.

NOTE: It is important to avoid bubbles; otherwise, the bubbles in the zebrafish body will appear as a black field of view under the microscope, which will affect observations and quantitative analysis.

3.1.6. Stain the larvae by soaking in 1 mL of 0.01% alizarin for 50 min.

3.1.7. Decant the solution and add 1mL of 50% glycerin/0.1% KOH for 10 min to clear the background. Store the fishes in fresh 50% glycerin/0.1% KOH for subsequent observation.

4. Image acquisition

4.1. Transfer one larva onto the glass slide each time, keeping the larva in the middle of the liquid drop.

4.2. Observe the larvae under a stereo microscope.

4.3. Turn on the camera, open the software (see the **Table of Materials**), and keep the default settings.

4.4. Click on **AE**, and choose an appropriate exposure time (60 ms in this experiment) to obtain the best image.

4.5. Capture all the images under the same settings. Save the images in .tif format for later analysis.

5. Image analysis

NOTE: See the **Supplemental file** for an example with a set of sample graphs for image analysis**.**

5.1. Double-click the **ImageJ** icon and analyze the images saved in step 4.5.

5.1.1. Click on **File | Open** to open the images saved in step 4.5.

5.1.2. Click on **Image | Type**, select **8-bit**.

5.1.3. Click on **Edit | Invert**.

5.1.4. Click on **Analyze | Calibrate**, select **Uncalibrated OD** in the popup interface, check **Global calibration** at the bottom left of the lower interface, and click **OK**.

5.1.5. Click on **Analyze | Set Scale | Click to Remove scale** in the popup interface, check **Global** below, and click **OK**.

5.1.6. Click on **Analyze | Set Measurements**, select the item **area** in the popup interface, check the **Limit to threshold** below (to measure only the selected range), and click **OK**.

5.1.7. Click on **Image | Adjust| Threshold**, slide the slider in the middle of the popup interface to select the appropriate threshold (change the threshold for each image) so that all targets to be tested in one image are selected, and click **Set**.

5.1.8. Click on **Analyze | Measure**. Record the dates of each group.

5.2. Use one-way ANOVA followed by Tukey's multiple comparison test to analyze the differences, and set the significance level at $p < 0.001$ (***).

REPRESENTATIVE RESULTS:

Alizarin red staining is a sensitive and specific method for measuring changes in bone mineralization in zebrafish larvae. In this study, we have observed that PbAc had adverse effects on zebrafish larvae, including death, malformation, decreased heart rate, and body length shortening. Moreover, the mineral skeleton areas of zebrafish larvae were evaluated to examine PbAc-induced bone loss. At 9 dpf (**Figure 1A)**, many bones of the head skeleton are mineralized and hence stained in red, such as parasphenoid (PS), opercle (OP), ceratobranchial (CB), and notochord (NC). In contrast, otoliths (OT) (non-bony structures) appear brown-black rather than red. Digital analysis was performed to quantify the total stained area in each image. Compared with the control group, lead acetate groups treated with 10 and 20 mg/L PbAc showed a significant decrease ($p < 0.001$) in the stained area (**Figure 1B**). The changes in bone mineralization showed dose-dependency.

FIGURE AND TABLE LEGENDS:

Figure 1: Effects of different concentrations of PbAc on zebrafish larvae skull. (**A**) Images of the dorsal aspect of the head bone stained with alizarin red in larvae at 9 dpf. The mineralized tissue is stained in red. Scale bars = 0.5 mm. (**B**) Changes in relative mineralized area at 9 dpf; 10 larvae per group. Data are expressed as mean ± SEM. Three replicates were performed. $p <$

0.001 ***. Abbreviations: PS = parasphenoid; OP = opercle; OT = otolith; CB = ceratobranchial; NC = notochord; dpf = days post fertilization.

Table 1: Composition of solutions used in alizarin red staining protocol. Abbreviation: q.s. = quantum sufficient (as required).

Supplemental file: A set of sample graphs for image analysis.

DISCUSSION:

The zebrafish is a suitable model for studying bone metabolic disease. Compared to rodent models, zebrafish models are relatively fast to establish, and measurement of the severity of disease is easier. In wild-type zebrafish larvae, mineralization of the head skeleton occurs at 5 dpf and the axial skeleton at 7 dpf[15]. Thus, cranial bones such as PS, OP, CB, and NC are well developed at 9 dpf. After the larvae were completely destained and bleached, the soft tissues were cleared, resulting in a transparent appearance of the fish body. The alizarin red staining reagent was added to stain and visualize the mineral bones in zebrafish in red.

Image analysis is critical to obtain reliable experimental conclusions in this experiment. Photographs of zebrafish with good posture and clean background were selected for quantitative analysis. When we quantify the stained area for a single image, all mineralized bones stained in red of one fish will be calculated. Thus, we can compare the bone mass changes between the lead-exposed and the control groups. In this study, PbAc exposure caused developmental toxicity in zebrafish, and a significant reduction in the stained area of mineral bone was observed in the 10 and 20 mg/L PbAc-exposure groups at 9 dpf. Thus, early embryonic exposure to PbAc reduced the bone mass of zebrafish larvae. **Figure 1** shows PbAc-induced bone loss visualized by alizarin red staining in the zebrafish larvae.

Dyes that bind to calcified matrix are used to label the entire skeleton. Calcein is a fluorescent chromophore that can also specifically bind to calcium in live tissue and has been used to label bone structures and study bone growth[10]. Unlike calcein, alizarin red staining of fixed tissue generates a permanent record of skeletal changes that can facilitate comparisons of several specimens. Microcomputed tomography (Micro CT) can provide accurate quantitative analysis of mineralized tissue by acquiring a series of 2D X-rays. However, because of the small size of zebrafish and many of the bones of the developing zebrafish skeleton being thin, Micro CT analysis tools cannot accurately characterize these bones[16].

Fluorescent transgenic reporter lines also help visualize skeletal development in live larvae or even more mature fish in real time[17]. Similarly, alizarin red S *in vivo* staining permits the evaluation of live fish and the continuous tracking of malformations[18]. Thus, alizarin red staining is a useful and cost-effective way to analyze bone loss in zebrafish larvae. However, because of the complexity of the experimental steps and the number of solutions used, the final results of the analysis of alizarin red-stained images may be affected by the experimental operation. Further, it is difficult to use this staining method for adult zebrafish due to increased body volume and soft tissues; Micro CT analysis or transgenic lines would be a better choice for

skeletal imaging of adult zebrafish. In summary, the protocol presented here can be used to study the changes in bone mineralization in zebrafish larvae following chemical toxicant exposure. This procedure could be useful to establish a zebrafish model to study bone disease and develop new therapeutic drugs.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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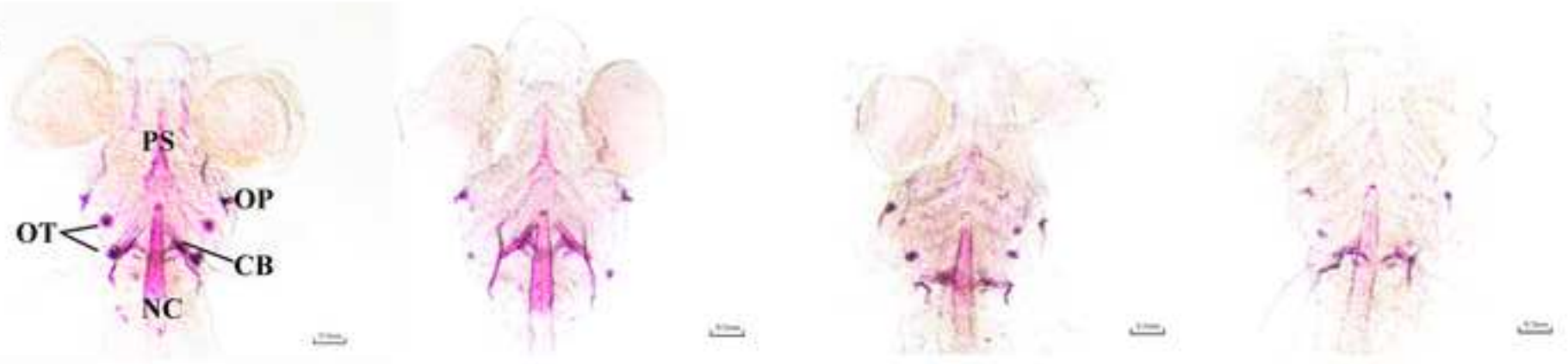
A



0 mg/L PbAc 5 mg/L PbAc 10 mg/L PbAc 20 mg/L PbAc

B

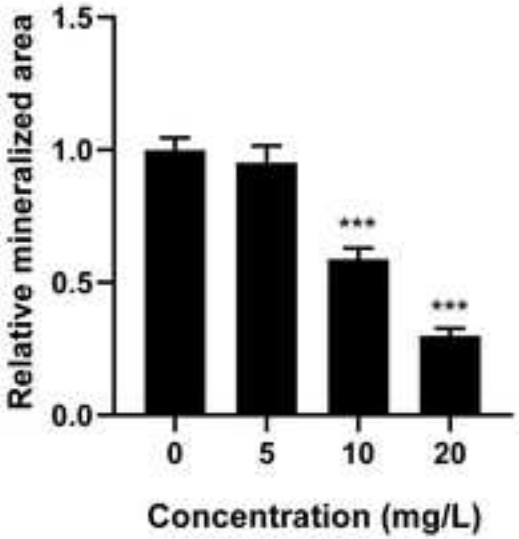


Fig. 1

Table 1

Solution
Zebrafish breeding water
Mixed solution (50 mL) of 2% paraformaldehyde and 1x PBS
Mixed solution (50 mL) of 100 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$
Mixed solution (50 mL) of 80% anhydrous ethanol, 10 mM $MgCl_2$, and 100 mM Tris-HCl (pH=7.5)
Mixed solution (50 mL) of 50% anhydrous ethanol and 100 mM Tris-HCl (pH=7.5)
Mixed solution (50 mL) of 25% anhydrous ethanol and 100 mM Tris-HCl (pH=7.5)
Mixed solution of 3% H_2O_2 solution and 0.5% KOH solution
Mixed solution (50 mL) of 25% glycerin and 0.1% KOH
0.01% Alizarin (50 mL)
Mixed solution (50 mL) of 50% glycerin and 0.1% KOH

Composition
pH 7-7.3, 27-29 °C, conductivity 450-550 µs, salinity 0.25-0.75 $\%_0$, dissolved oxygen >6 mg/L, photoperiod 14/10 h, hardness 100-200 mg/L, chlorine 0 mg/L, ammonia nitrogen concentration <0.02 mg/L, nitrite <1 mg/L, nitrate <50 mg/L, carbon dioxide <50 mg/L
25 mL of 4% paraformaldehyde, 5 mL of 10x PBS buffer, and double distilled water (ddH_2O) q.s. to 50 mL
5 mL of 1 M Tris-HCl (pH=7.5), 0.5 mL of 1 M $MgCl_2$, and ddH_2O q.s. to 50 mL
42.1 mL of 95% anhydrous ethanol, 5 mL of 1 M Tris-HCl (pH=7.5), 0.5 mL of 1 M $MgCl_2$, and ddH_2O q.s. to 50 mL
26.3 mL of 95% anhydrous ethanol, 5 mL of 1 M Tris-HCl (pH=7.5), and ddH_2O q.s. to 50 mL
13.2 mL of 95% anhydrous ethanol, 5 mL of 1 M Tris-HCl (pH=7.5), and ddH_2O q.s. to 50 mL
Equal amounts of 6% H_2O_2 and 1% KOH mixed before use
12.5 mL of 100% glycerin, 0.25 mL of 20% KOH, and ddH_2O q.s. to 50 mL
1 mL of 0.5% Alizarin, 12.5 mL of 100% glycerin, 5 mL of 1 M Tris-HCl (pH=7.5), and ddH_2O q.s. to 50 mL
25 mL of 100% glycerin, 0.25 mL of 20% KOH, and ddH_2O q.s. to 50 mL

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Table of Materials

Materials (4).xls

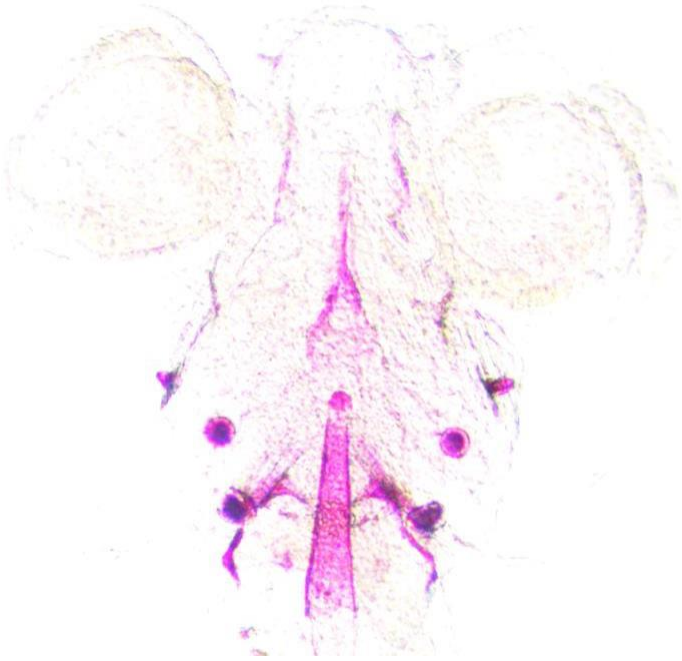
We addressed the comments in the revised manuscript.

Supplemental file:

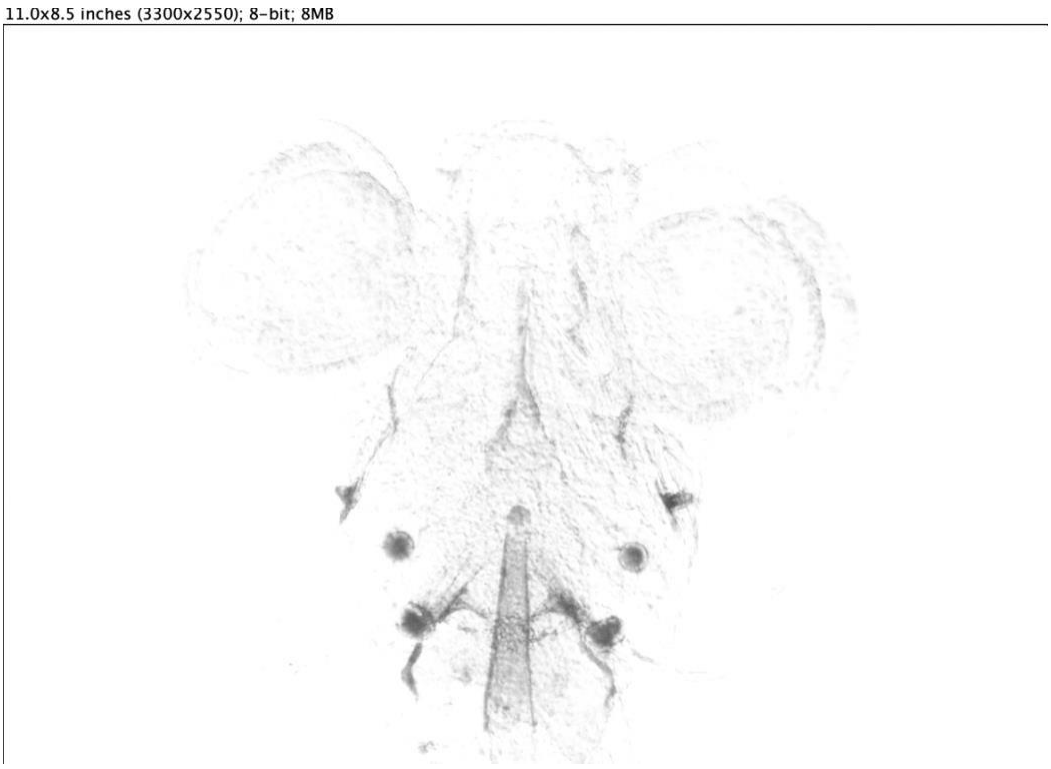
An example for ImageJ analysis, a single image represents one measurement.

1. Double-click **ImageJ** and analyze the image as follows.

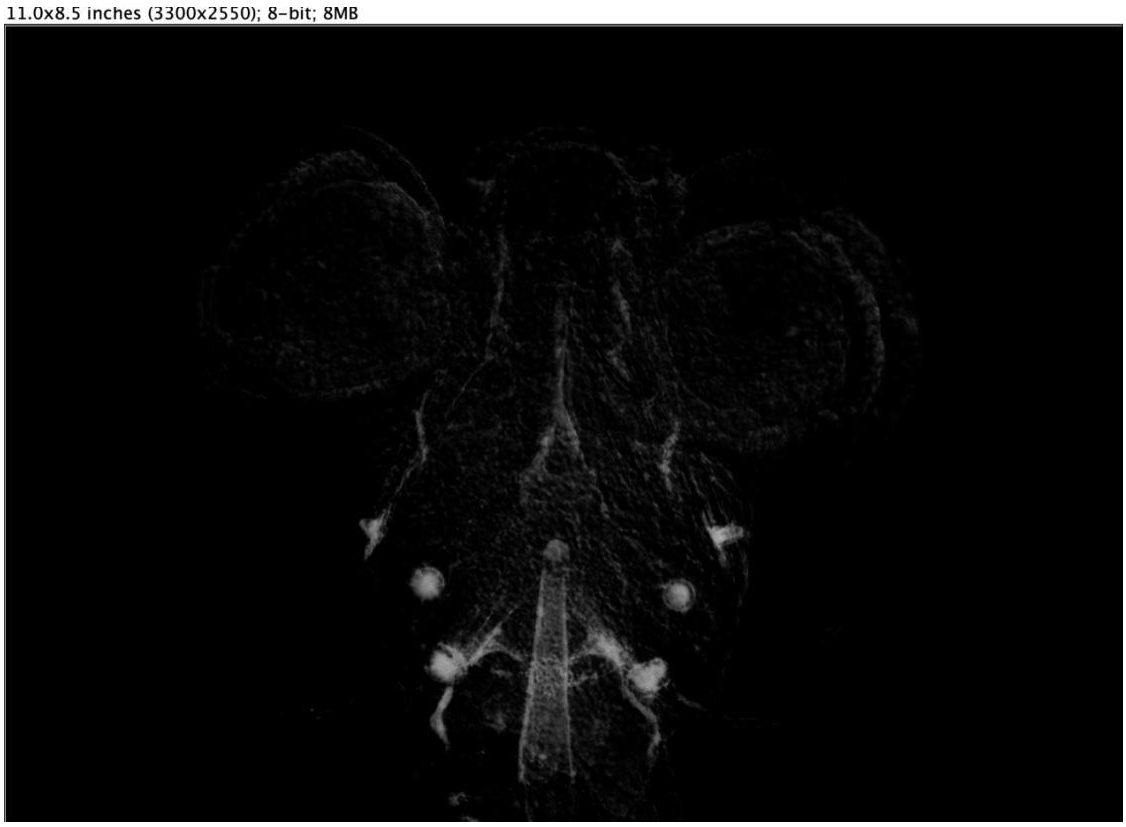
1.1. Click **File | Open** to open the images.



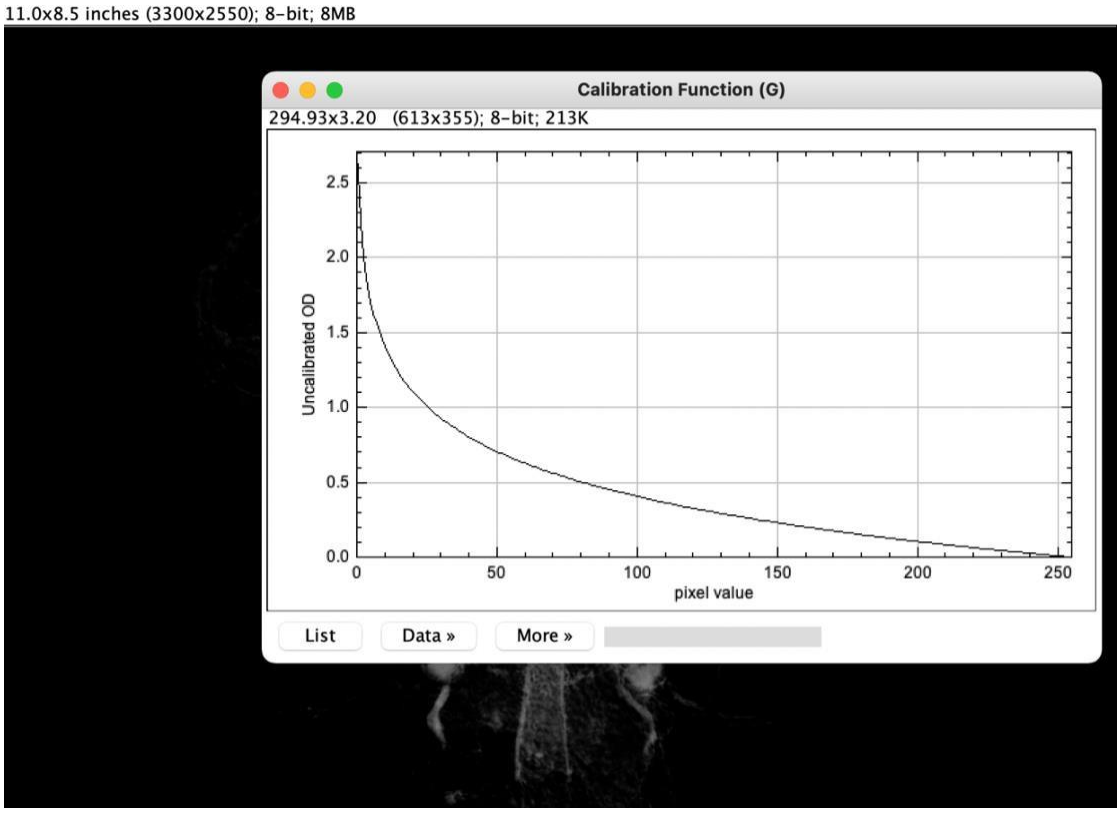
1.2. In the **Image** input box, type 8-bit.



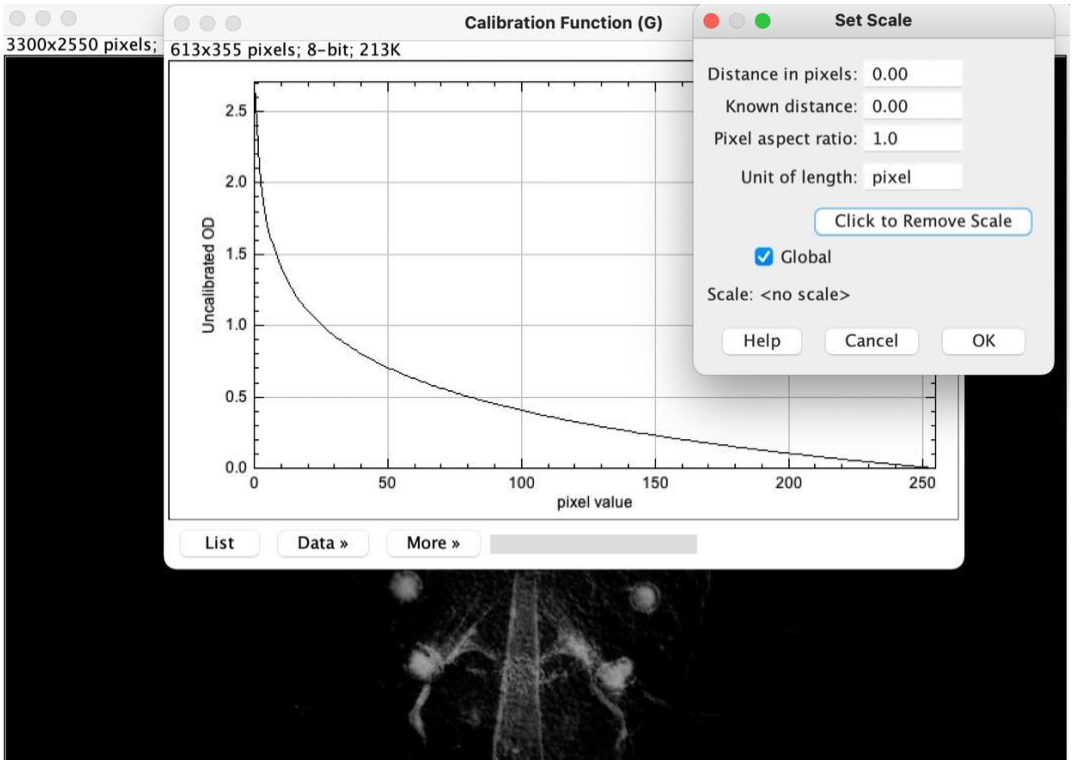
1.3. Click on **Edit | Invert**.



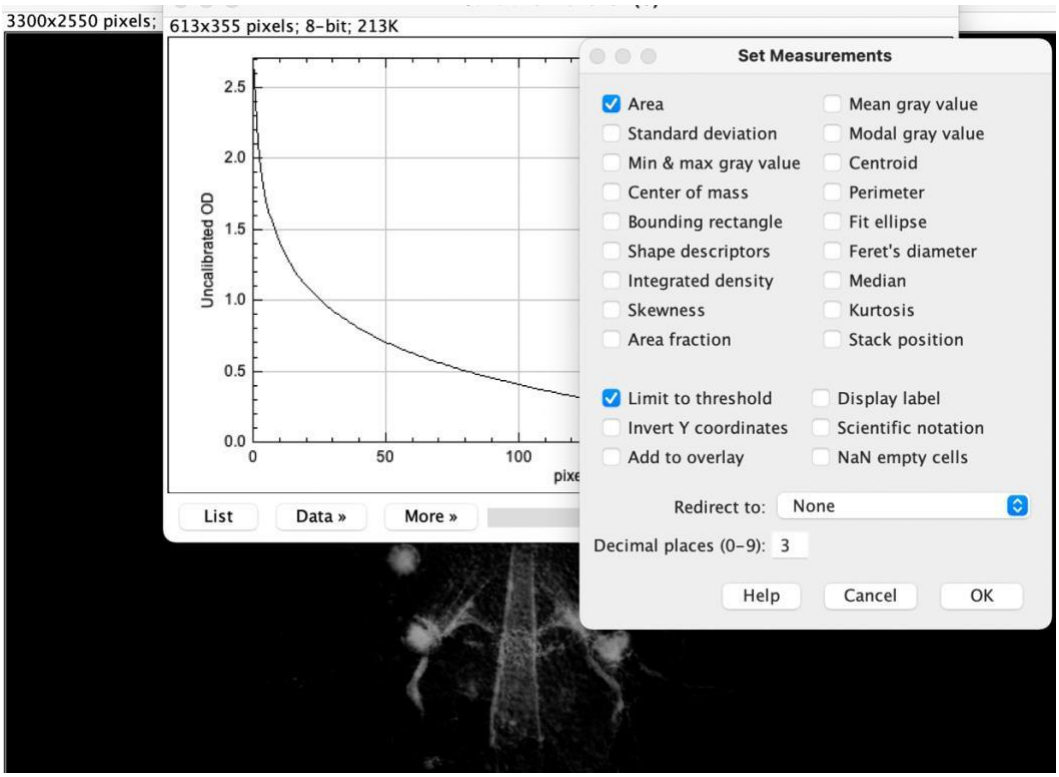
1.4. Click on **Analyze | Calibrate**, select **Uncalibrated OD** in the popup interface, check **Global calibration** at the bottom left of the lower interface, and click **OK**.



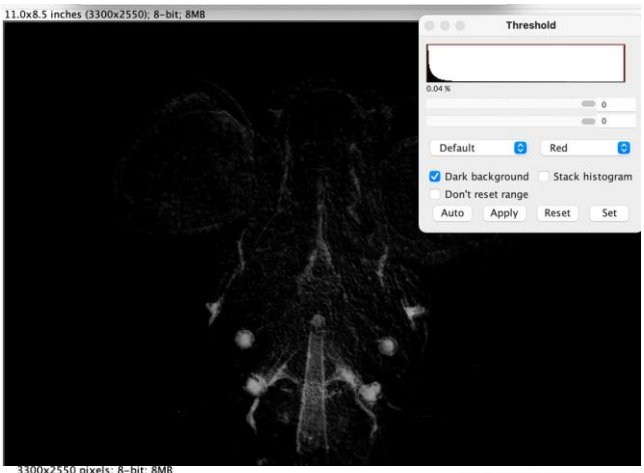
1.5. Click on **Analyze | Set Scale | Click to Remove scale** in the popup interface, check **Global** below, and click **OK**.

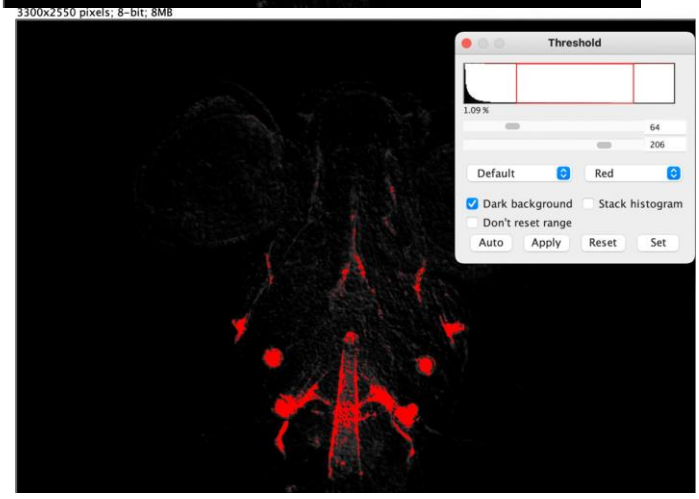


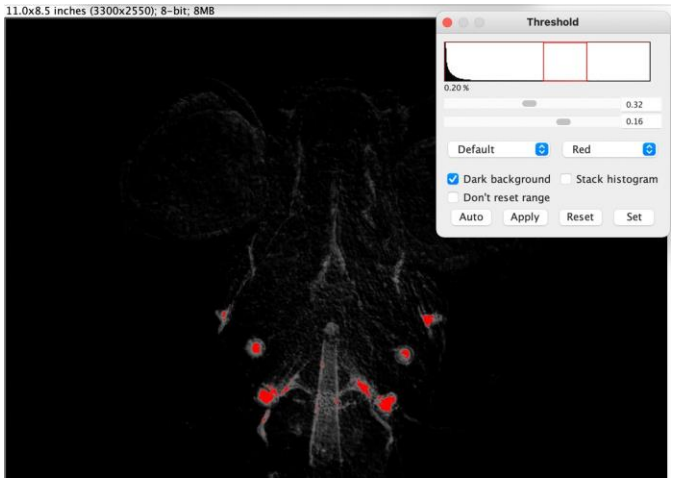
1.6. Click on **Analyze | Set Measurements**, select the item **area** in the popup interface, check the **Limit to threshold** below (to measure only the selected range), and click **OK.**



1.7. Click on **Image | Adjust| Threshold**, slide the slider in the middle of the popup interface to select the appropriate threshold for the measurement of all the targets, and click **Set**.







1.8. Click on **Analyze | Measure**.