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Investigation of Heavy Metals in Zebrafish Tissue by Total Reflection X-ray Fluorescence

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INVESTIGATION OF HEAVY METALS IN ZEBRAFISH TISSUE BY TOTAL REFLECTION X-RAY FLUORESCENCE SPECTROMETRY

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ABSTRACT

Heavy metals are present in the environment, in many consumer products and can be found in food. Some heavy metals like zinc and copper are essential for enzymatic and metabolic function while others like lead and mercury are toxic and interfere with biological pathways. Zebrafish are a very popular model organism for monitoring toxicity of heavy metals and investigating vertebrate development. In this study we explore the possibility of using total reflection X-ray fluorescence spectrometry to analyze histological sections of zebrafish embryos after exposure to nickel and lead. A methacrylate resin embedding protocol and a paraffin embedding protocol were evaluated as possible options for sample preparation for TXRF analysis of zebrafish embryos. It was found that paraffin is the superior material after an alkaline phosphatase stain was introduced. The stain made the normally transparent fish embryo embedded in paraffin clearly visible on the sample reflector material thus allowing for easy positioning and identification of the area of interest.

INTRODUCTION

Heavy metals are ubiquitous in the environment and much research has been devoted to understand their role and function in biological systems. Many heavy metals are toxic and responsible for different forms of cancer. Lead and mercury are the most studied examples and their neurotoxicity is well established (Chiba and Masironi, 1992; Grunwald and Eisen, 2002; Inuoe, 2013; Needleman, 2002; Nordberg *et al.*, 2007; Tchounwou *et al.*, 2012). However, a number of heavy metals are essential and act as co-factors in enzymes. Copper is present in a variety of enzymes including cytochrome C oxidase, which is responsible for electron transport involving iron reduction and oxidation (Nordberg *et al.*, 2007). Nickel is considered essential to bacteria, but no essentiality has been established for humans. In fact research indicates that high intake of nickel can lead to cancer (National Institute of Environmental Health, 2014; Nordberg *et al.*, 2007).

Common animal models to study toxicity of heavy metals are mice and rats. Increasingly zebrafish have been used as model organisms not only in regards to environmental toxicity, but also to understand the development of cancers, and many other diseases (Armatruda *et al.*, 2002; Dominguez *et al.*, 2010; Hill *et al.*, 2005; Spitsbergen and Kent, 2003; Vargas *et al.*, 2015).

Zebrafish have a number of advantages over other vertebrates including high fertility rates, short development periods and small size as well as easy and low cost husbandry. The transparency of the fish embryos makes it possible to observe internal organ development and the completely sequenced genome permits for disease specific studies and drug screening. Several studies have used X-ray fluorescence microtomography to image transition metals in adult zebrafish, zebrafish embryos and larvae (Bourassa *et al.*, 2014; Luan *et al.*, 2016). Other studies employed X-ray phase contrast tomography to image zebrafish muscle movement (Vargas *et al.*, 2015). In a recent manuscript by Bilo *at al.* the use of zebrafish as biomonitors in conjunction with total reflection X-ray fluorescence (TXRF) analysis was investigated. The authors developed a protocol for lead and zinc toxicity tests using single zebrafish embryos (Bilo *et al.,* 2015).

The study presented here takes a slightly different approach and explores the possibility of using total reflection X-ray fluorescence spectrometry to analyze histological sections of zebrafish embryos after exposure to the heavy metals lead and nickel. Lead was chosen based on its known toxicity to living organisms, and nickel was selected due to its presence in many consumer products and its known carcinogenicity to humans (National Institute of Environmental Health, 2014; Nordberg *et al.*, 2007). Zebrafish embryos were selected as model organisms as toxicological effects are most pronounced in early development stages and the transparency of the zebrafish embryos permits for identifying malformed and malfunctioning organs. In addition the embryos are small with only 0.5 mm by 1 mm in size at 4 days past fertilization (dpf) and an entire fish section can be analyzed with TXRF. The initial goal of the study was to develop a suitable sample preparation method for TXRF analysis of zebrafish embryos by testing different drying and embedding protocols. The ultimate goal of the project is to construct a three dimensional map of heavy metal distribution in zebrafish embryos by combining elemental analytical data obtained from TXRF measurements and high resolution microscope images of the same sections. This will be an extension of the method outlined by Rosenthal *et al.* where microscopic images of histological sections were recombined to obtain a 3D image of an organism. (Rosenthal *et al.*, 2004).

SAMPLE PREPARATION AND ANALYSIS METHODS

Zebrafish Exposure and Embedding

Zebrafish exposures were performed in 12-well culture plates. Each well had a total volume of 3 mL and 5 zebrafish embryos per well. The embryos were exposed one day post fertilization (dpf) to either 20 μg/L lead or 5 mg/L nickel, respectively. These concentrations were chosen based on the known higher toxicity for lead as opposed to nickel and in consideration of the substantially lower concentrations of the elements in the samples after sectioning. For the next three days the fish were monitored for mortality until the fish reached 4 dpf. Dead fish were removed from the wells on a daily basis and discarded. At 4 dpf, the remaining zebrafish were euthanized by

placing the well-plate in ice for 15 minutes. The fish were then fixed and embedded using either method 1 or method 2 as described below. For method 1 the fish were fixed in two subsequent steps: 1) Fish were removed from the wells and soaked in a solution containing 1% paraformaldehyde (PFA), 3% sucrose, and 2.5% glutaraldehyde in 0.2M phosphate buffer (pH 7.4) for 4-6 hours, and 2) The fixation process was then completed by washing the fish with phosphate buffered solution (PBS) several times and immersing them in 1% osmium tetroxide solution for 2-3 hours. The fish were washed again with PBS to remove any excess osmium tetroxide, dehydrated with ethanol and propylene oxide and finally embedded in methacrylate resin following the protocol described by Nuckels and Gross. (Nuckels and Gross, 2007; Sullivan Brown *et al.*, 2007).

For the second method, the fish were fixed by adding 4% paraformaldehyde (PFA) and 50% Methanol/phosphate buffered solution (PBS) to the well solution. The fixed fish were then placed in 100% methanol and stored in a -80°C freezer until further use. When ready to use, the fish were embedded in paraffin following the procedure outlined by Chuang and Zhang. (Chuang and Zhang, 2001).

The first method yielded a transparent methacrylate resin block with the fish embryo clearly visible and the second method yielded an opaque white paraffin block. Figure 1 shows the cutting blocks obtained from the resin embedding material (1a, left) and the paraffin embedding material (1b, right).

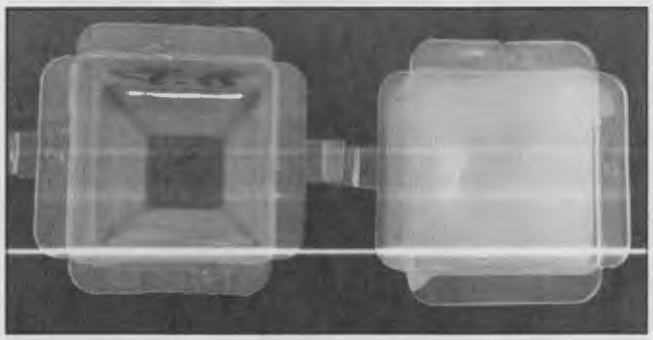

Figure 1: Microtome cutting blocks with zebrafish embryo embedded. The left block (a) shows the resin as the embedding material and the right block (b) uses paraffin as embedding material. The embryo is clearly visible in the resin block, but obscured in the paraffin block.

Analysis

Both the resin and paraffin blocks were sectioned using a manual microtome fitted with a stainless steel blade (Spencer 820 rotary microtome, American Optical). The resin block was cut into 3 μm sections and the paraffin block into 10 μm thick sections. The resin permitted for

thinner sections due to the more rigid structure of the block as opposed to the waxy paraffin, where thinner sections fractured and did not form a uniform layer on the polycarbonate sample reflector. The microtome cuts were placed directly on 2 mm thick highly polished polycarbonate slides serving as TXRF sample reflectors and cut to size to fit in the TXRF sample holder. This follows a similar procedure as for biological imaging where microtome sections are cut directly on microscope slides. To ensure complete analysis of the embryo, each cut was positioned in such way that the embryo was centered in the middle of the reflector. The size of the sectioned embryo was less than 1 mm in each direction thus enabling complete measurement of each section with an area of analysis of 2mm by 5mm. The prepared specimens were analyzed using a S2PicoFox TXRF spectrometer from Bruker Nano, Berlin. The instrument has a Mo anode x-ray tube and a X-Flash detector. The tube was operated at 50 kV and 0.6 mA. The measurement time was 2000 seconds. No quantification was attempted at this point of time.

RESULTS

Figure 2 shows a histological section of a zebrafish embryo embedded in methacrylate resin. The embryo is clearly visible within the cut section (2a, red circle) and 5 times magnification with a microscope (Zeiss Stemi, Carl Zeiss, Jena, Germany) shows head and tail in more detail (2b). Figure 3 displays a histological section of a zebrafish embryo embedded in paraffin (3a). Here, the embryo is difficult to distinguish from the background and a 20 times magnification with the microscope was needed to locate it on the sample reflector (3b). The blue circle in figure 3a marks the location of the embryo on the sample reflector to aid in positioning the embryo at the center of the analysis area.

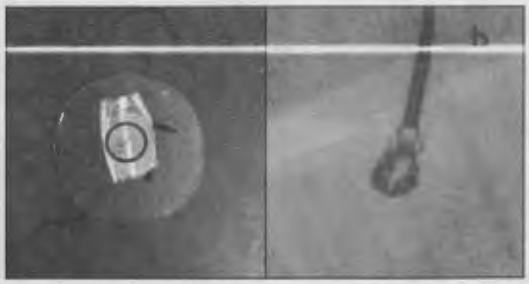

Figure 2: a) Methacrylate resin embedded zebrafish embryo exposed to 20 µg/L lead. The microtome section was directly placed on the polycarbonate sample reflector; b) microscopic image of the same embryo using a 5x magnification.

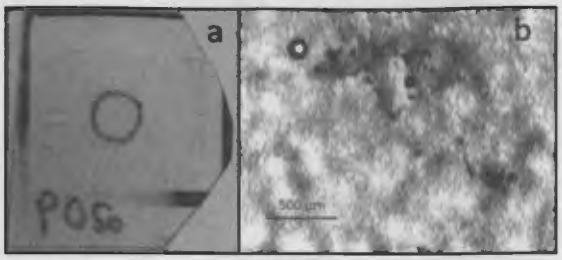

Figure 3: a) Paraffin embedded zebrafish embryo exposed to 5 mg/L nickel. The microtome section was directly placed on the polycarbonate sample reflector and the location of the embryo is marked by the blue circle; b) microscopic image of the same embryo using a 20x magnification.

Figures 4 and 5 display TXRF spectra obtained from zebrafish embryo sections exposed to either 20 µg/L Pb or 5 mg/L Ni (red) and spectra of a non-exposed zebrafish (blue). The control embryos were cut to the same thickness as the exposed ones for each embedding material. All zebrafish embryo sections feature iron and bromine as prominent peaks; with iron most likely coming from the stainless steel blade of the microtome knife and bromine as a common element present in biological tissues. Figure 4 shows a distinct set of osmium peaks for the fish sections obtained from the resin blocks (method 1) in both the lead exposed fish and to a lesser extend the non-lead exposed fish. Osmium in the form of osmium tetroxide was used as a desiccant in the drying and embedding process and must have been taken up by the biological tissue. Whereas it appears that the uptake of osmium was not uniform throughout the embryo, the osmium peaks nevertheless overlap with lead, the element of interest thus making it impossible to determine lead in this sample. In contrast, the spectrum obtained from the paraffin embedded embryo (method 2) shows a clear Ni peak at 7.5 keV indicating the presence of Ni in the tissue after exposure to this element. The control spectrum of fish not exposed to nickel did not show a Ni peak eliminating the possibility that the Ni originates from the cutting blade. It should be noted that the nickel exposure concentration was rather high at 5 mg/L. This high concentration was chosen to ensure that it would be well above any Ni present in the fish naturally.

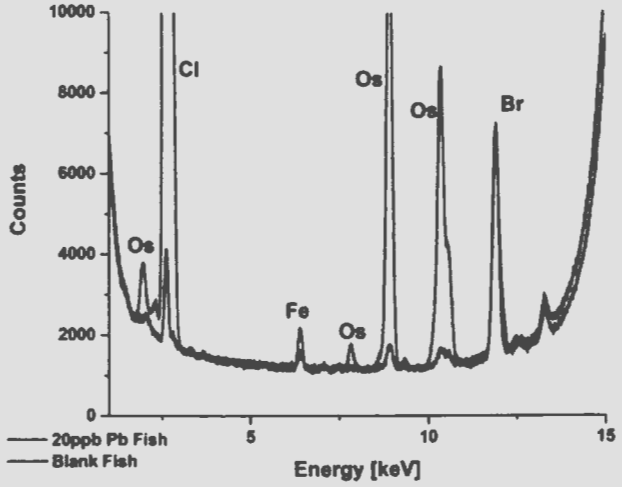

Figure 4: TXRF spectra of a methacrylate resin embedded zebrafish embryo after exposure to 20 µg/L Pb (red) and a zebrafish embryo embedded in methacrylate resin without lead exposure (blue).

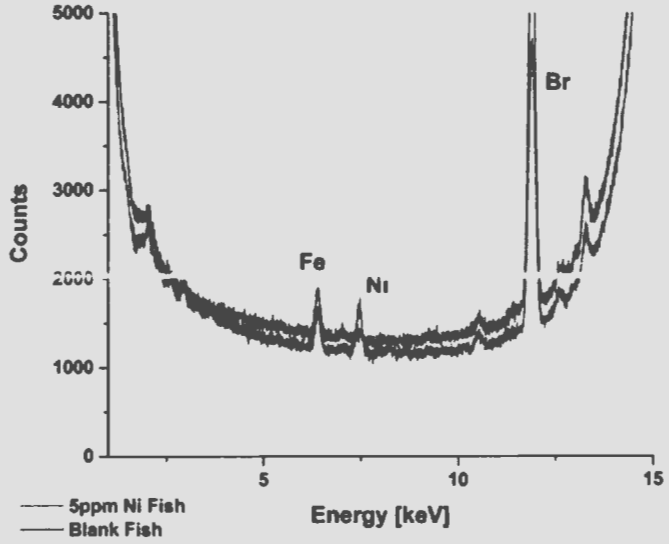

Figure 5: TXRF spectra of a zebrafish embryo stained with alkaline phosphatase and embedded in paraffin after exposure to 5 mg/L Ni (red) and a zebrafish embryo stained with alkaline phosphatase and embedded in paraffin without nickel exposure (blue).

DISCUSSION

It was found that each embedding protocol had its advantages and disadvantages. Method 1 yielded a firm transparent resin block with a good distinction between resin and embedded fish embryo. Individual parts and regions of the embryo were clearly visible, but the resin cuts were not consistent in shape and size with some crimped on the polycarbonate reflector due to non-uniform adherence to the surface. Moreover in some cases the lack of adherence resulted in complete detachment of the cut section from the reflector and the sample could not be analyzed. Also, while cutting, the resin would crack and fracture causing the fish itself to fracture and not cut smoothly. The major disadvantage of method 1, however, was the use of osmium tetroxide during the dehydration process of the zebrafish. Not only is osmium tetroxide highly toxic and extreme care is necessary to handle the compound, but also the specimen did absorb some osmium into its tissues as can be seen in figure 4. In addition the presence of osmium interferes with the detection of some elements, specifically lead, due to peak overlap. In contrast, the second method provided a much more flexible block of paraffin to cut from and the sections were much smoother and easier to handle. The disadvantage of using paraffin is that it is opaque and the fish is transparent after the dehydration process. This resulted in difficulty in distinguishing and locating the fish within each section. This was particularly cumbersome for areas where little or no pigmentation of the skin and organs is present.

To remediate this problem and enhance visibility of the embryos, an alkaline phosphatase stain was introduced, staining the nucleotides and proteins within the fish purple. Alkaline phosphatase staining is commonly used for visualizing cells and the fish are treated with the alkaline phosphatase before fixing (Nuesslein-Vollhard and Dahm, 2002). The stain colored the fish purple and did not interfere with TXRF analysis as can be seen in Figure 5, where the blue spectrum shows the data for a stained, but not exposed fish embryo embedded in paraffin. Figure 6a shows a non-stained zebrafish embryo and 6b an alkaline phosphatase stained embryo. Whereas the stained entire embryo (Figure 6b) appears to have little transparency to reveal individual organs, the right picture in Figure 7 (Figure 7b) showing a magnified histological section of a stained embryo on the TXRF sample reflector indicates that individual features are clearly distinguishable once the embryo was sectioned. In addition, the embryo can be easily identified within the paraffin section thus helping to position it in the center of the analysis area.

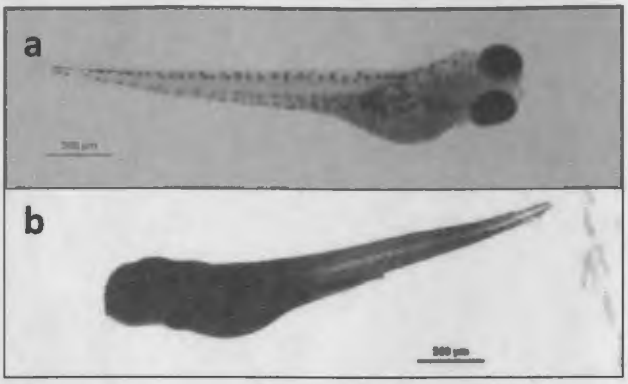

Figure 6: Comparison between a non-stained (6a) and an alkaline phosphatase stained zebrafish embryo (6b). Both images were acquired with a microscope at the same magnification before embedding the embryos in paraffin.

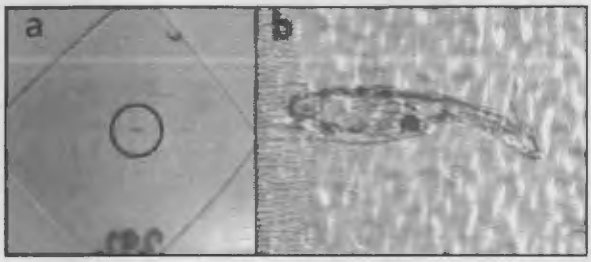

Figure 7: a) Histological section of a zebrafish embryo placed on a TXRF sample reflector after exposure to 5 mg/L nickel, staining with alkaline phosphatase and embedding in paraffin. The embryo is highlighted by the red circle and can be easily distinguished on the reflector. b) Microscopic image of the same embryo as shown in Figure 7a.

CONCLUSION

A sample preparation method for analysis of histological sections of zebrafish embryos by total reflection X-ray fluorescence was developed based on two common fixing and embedding protocols for biological samples. One protocol employed a methacrylate resin and a second one paraffin to yield cutting blocks for sectioning. It was found that the protocol using paraffin as embedding material was superior as it not only permitted for easier sectioning, but also allowed for safer handling and did not interfere with the analysis of elements of interest. The next steps in this project will include the quantification of toxic metals such as lead in individual sections and construction of an elemental map by combining quantitative elemental analysis of each subsequent embryo section by TXRF with microscopic images taken from the same sections.

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