Comparative Biochemistry and Physiology, Part C xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Comparative analysis of fixation and embedding techniques for optimized histological preparation of zebrafish

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ARTICLE INFO

Keywords:
Atlas
Fixation
Decalcification
Embedding
Sectioning
Staining
Histology
Virtual slide

ABSTRACT

In recognition of the importance of zebrafish as a model organism for studying human disease, we have created zebrafish content for a web-based reference atlas of microanatomy for comparing histology and histopathology between model systems and with humans (http://bio-atlas.psu.edu). Fixation, decalcification, embedding, and sectioning of zebrafish were optimized to maximize section quality. A comparison of protocols involving six fixatives showed that 10% Neutral Buffered Formalin at 21 °C for 24 h yielded excellent results. Sectioning of juveniles and adults requires bone decalcification; EDTA at 0.35 M produced effective decalcification in 21-day-old juveniles through adults (≥ ~3 Months). To improve section plane consistency in sets of larvae, we have developed new array casting molds based on the outside contours of larvae derived from 3D microCT images. Tissue discontinuity in sections, a common barrier to creating quality sections of zebrafish, was minimized by processing and embedding the formalin-fixed zebrafish tissues in plasticized forms of paraffin wax, and by periodic hydration of the block surface in ice water between sets of sections. Optimal H&E (Hematoxylin and Eosin) staining was achieved through refinement of standard protocols. High quality slide scans produced from glass histology slides were digitally processed to maximize image quality, and experimental replicates posted as full slides as part of this publication. Modifications to tissue processing are still needed to eliminate the need for block surface hydration. The further addition of slide collections from other model systems and 3D tools for visualizing tissue architecture would greatly increase the utility of the digital atlas.

1. Introduction

The use of model organisms in biology is central to our ability to advance knowledge of both normal and disease states in humans. We have created an online atlas of histology and histopathology of the zebrafish to facilitate comparisons of human and model organism histology and histopathology. Zebrafish are widely used as a model for the study of development and human disorders; some 70% of human disease genes have functional homologs in zebrafish (Beckwith et al., 2000, Santoriello and Zon, 2012, Stewart et al., 2014, Zhao et al., 2015). Additionally, mutants can be readily generated, yielding histologically-apparent phenotypes (Mohideen et al., 2003; Amsterdam et al., 2004; Sadler et al., 2005; Santoriello and Zon, 2012). This also creates the opportunity to perform phenotypic comparisons between chemically-induced phenotypes (Hill et al., 2005) and mutant phenotypes. The small size of zebrafish larvae make them particularly well-suited for chemical (experimental or environmental pollutant) screening because small volumes of chemicals can be used (Görge and Nagel, 1990, Hill et al., 2005, Taylor et al., 2010, Stewart et al., 2014).

Since our website is intended to be a resource for members of the scientific community interested in human and model organism histology, consistency of quality, sectioning, staining, and histological imaging are important. To address common difficulties with various aspects of zebrafish histology, we set out to optimize protocols to produce the best possible slides and images. This paper includes answers to the large number of requests we have received for help with zebrafish histology over two decades. Our foundational work for the atlas included the testing of different protocols for tissue fixation, decalcification, and orientation in paraffin. This work addressed the most common and significant problems in zebrafish histology that makes histological sections of randomly oriented fish virtually uninterpretable: poor sectioning, twisting, and sample malorientation. Past work related to these issues includes (Tsao-Wu et al., 1998, Beckwith et al., 2000; Moore et al., 2002; Sabaliauskas et al., 2006). The work of

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https://doi.org/10.1016/j.cbpc.2017.11.003
Received 31 July 2017; Received in revised form 14 November 2017; Accepted 14 November 2017

Tsao-Wu et al. (1998) laid the foundation for agarose embedded larval tissue arrays based on a rectangular array mold. This mold was based on a four by eight agarose array in which 64 seven day old larvae can be positioned, two per well. This facilitated histologic analysis of the larvae since they could be sectioned in the same plane and visualized simultaneously. Moore et al. (2002) focused their study on the fixation and decalcification of adult zebrafish with the focus on both fixation and integrity of extracted DNA. They concluded that 10% Neutral Buffered Formalin or 4% Paraformaldehyde followed by decalcification in 0.5 M EDTA yielded satisfactory histological sections and DNA fragments useful for genotypic analysis of adult zebrafish. In subsequent years we decreased the EDTA concentration for decalcification to 0.35 M, based on the idea that this would diminish the amount of EDTA that could contaminate subsequent molecular biological reactions. Sabaliauskas et al. (2006) modified the agarose array mold to fit 50 larvae facing in the same direction. Their refined design has triangular wells that fit one larva each. This modification allowed for more consistent positioning of the larvae and easier digital imaging of individual fish. They also included descriptions of effective methods for tissue processing, staining and digital image capture. Based on the practices of other fish and comparative pathology laboratories, we have evaluated six commonly used fixatives with a focus on duration of fixation. We determined optimal decalcification durations using 0.35 M EDTA for zebrafish age 21 dpf (days post-fertilization) and older. Additionally, we developed and tested two novel agarose larval array molds designed to improve larval alignment). Finally, we refined details of both H&E staining (Hematoxylin and Eosin) and digital image capture.

2. Materials and methods

2.1. Staging and preparation of zebrafish prior to fixation

Wild-type Connor and Ekkwill strains were used for both the development and production of the zebrafish atlas website (www.zfatlas.psu.edu), which is now renamed http://bio-atlas.psu.edu to better engage its use across model systems. Fish originally obtained from Indonesian wild caught sources were acquired through the commercial distributors Connor and Ekkwill. Fish were reared at an average temperature of 28 °C in a recirculating system with a 14:10 h light to dark cycle. Fish were fed three times a day a diet consisting of brine shrimp and flake food. All fish were staged according to the zebrafish developmental staging series of Kimmel et al. (1995; http://onlinelibrary.wiley.com/doi/10.1002/aja.1002030302/pdf). Euthanasia was performed with 160 mg/L Tricaine-S pH 7.0 (tricaine methanesulfonate, Syndel, USA) in system water. It should be noted that the relative merits of Tricaine-S and/or hypothermal shock through immersion in ice water (2–4 °C) have been recently debated (Wilson et al., 2009; Matthews and Varga, 2012). While each method has value, current data suggests that hypothermal shock results in a more rapid and humane euthanasia in small tropical species such as zebrafish. In keeping with this finding, our laboratory currently combines both methods (cold Tricaine) to produce a rapid euthanasia in our fish, ensuring that the fish do not react to subsequent immersion in fixatives.

Manipulations of the fish before and after euthanasia were kept to a minimum and were performed gently to avoid damage to the fish. Pasteur pipettes used to handle larvae were flamed to round sharp edges that can otherwise damage the fish. Fixation was performed immediately after euthanasia to prevent autolysis and degradation of cell morphology. To enhance the penetration of fixative to internal organs, adult zebrafish were incised ventrally midline from the anal pore to the base of the pectoral fin. Fish were held gently using gloved fingers rather than forceps during this process to minimize damage to the skin, external structures, and internal organs.

Table 1
Fixatives tested.

Fixative	Formulation
Zenker's	Stock solution Potassium dicromate 2.5% Mercuric chloride 5.0% Sodium sulfate 1.0% Distilled water 92% Working solution Zenker stock 95 ml Glacial acetic acid 5 ml
Zamboni's	Paraformaldehyde 2% Picric acid, saturated aqueous 15% (final 0.195%) Sodium phosphate di-basic 3.37% Sodium phosphate monobasic 0.28% Distilled water 79%
Zinc-formalin	Formalin 3.7% Zinc sulfate 1% Distilled water 97%
Bouin's	Picric acid, saturated aqueous 75% (final 0.975%) Formaldehyde 1% Acetic acid 5% Distilled Water 19%
Formaldehyde/glutaraldehyde	Formaldehyde 2% Glutaraldehyde 1% Calcium acetate 2% Distilled water 95%
10% neutral buffered formalin	Formaldehyde 4% (10% of ~40% stock solution) Sodium phosphate monobasic 0.4% Sodium phosphate di-basic 0.65% Methanol 1.5% Distilled water 93%

2.2. Fixation and decalcification

Immediately following euthanasia, fish were placed in two rinses of fixative and then incubated in at least 20 × fish volume of fixative. Samples were fixed in flat bottom glass vials to ensure that the fish were fixed in a straight orientation; fixation in a vertical position tends to result in bending of the fish. Several fixatives (Table 1) and fixation parameters such as time and temperature (Table 2) were tested as described. The merits of movement of the fixation solution by stir bar during fixation were tested with the standard fixative used in human pathology, 10% NBF (Neutral Buffered Formalin, Fisher Scientific Cat# SF100-4). Juvenile (57 dpf WT Ekkwill) zebrafish used for this comparison were placed inside embedding cassettes. Up to six cassettes were placed on top of a tray in a glass staining dish and submerged in

Table 2
Fixation times.

Fixative	Fixation time and temperature	Picric acid time and temperature
Zenker's	24 h 4 °C	None
Zamboni's	24 h 4 °C	None
Zinc-formalin	6 h 4 °C	None
Bouin's	24 h 4 °C	None
2% formaldehyde/1% glutaraldehyde	48 h 21 °C	None
2% formaldehyde/1% glutaraldehyde; 0.65% picric acid (separate step)	48 h 21 °C	48 h 21 °C
10% neutral buffered formalin	24 h 4 °C	None
10% neutral buffered formalin	48 h 4 °C	None
10% neutral buffered formalin	8 day 4 °C	None
10% neutral buffered formalin	24 h 21 °C	None
10% neutral buffered formalin	48 h 21 °C	None
10% neutral buffered formalin	8 day 21 °C	None