Vol. 23: 251–263, 2015
doi: 10.3354/ab00624

AQUATIC BIOLOGY
Aquat Biol

Published online April 1

Histochemistry on vibratome sections of fish tissue: a comparison of fixation and embedding methods

Bianka Grunow[1,*], Tina Kirchhoff[1], Tabea Lange[1,2], Timo Moritz[2], Steffen Harzsch[1]

[1]Cytology and Evolutionary Biology, Zoological Institute and Museum, Ernst-Moritz-Arndt-University of Greifswald, Soldmannstrasse 23, 17487 Greifswald, Germany
[2]Deutsches Meeresmuseum, Katharinenberg 14–20, 18439 Stralsund, Germany

ABSTRACT: Despite improvements in imaging techniques during recent years, for many non-model systems the fixation of tissues followed by embedding and sectioning for histochemical or immunohistochemical staining remains an important technique in vertebrate histology. The present study sets out to explore the preservation of histological sections of fish tissues using different preparation techniques. The quality of transverse vibratome sections from trunk segments of the lesser-spotted dogfish *Scyliorhinus canicula*, Atlantic sturgeon *Acipenser oxyrinchus* and zebrafish *Danio rerio* were compared using different fixatives (formaldehyde, paraformaldehyde and zinc-formaldehyde) and embedding methods (gelatine, agarose and low-temperature melting agarose). Our data show that the quality of the vibratome sections for histochemical staining is strongly dependent upon fixation and embedding media. Although paraformaldehyde fixation results in a more pronounced shrinkage of the trunk segment than the other fixatives used , the quality of the sections and the histochemical staining was best with this fixative in zebrafish and dogfish. Additionally, the embedding methods have a strong influence on the quality of the sections. In the dogfish and sturgeon samples, the preferred embedding media were agarose and low-temperature melting agarose, since gelatine often caused shrinkage of the tissues. In conclusion, for histochemical examinations, the processing protocols for vibratome sectioning need to be adapted individually to each study organism.

KEY WORDS: Histochemistry · Vibratome · Bony fish · Cartilaginous fish · Fixation · Embedding media

INTRODUCTION

Methods for studying mammalian tissue using vibratome sections have been commonly used since the early 1970s (Hökfelt & Ljungdahl 1972, Lindvall et al. 1973), and from the 1980s, publications on vibratome sections using fish tissue are also available (Funch et al. 1984, Kah et al. 1986, Yulis & Lederis 1986). This technique is well-suited to prepare tissue sections, typically between 10 and 100 µm thick, that can subsequently be processed by histochemical, immunohistochemical or electron microscopic methods. This approach has been used for studying the brain (Kálmán & Ari 2002, Lieberoth et al. 2003, Lechtreck et al. 2009, Pilaz & Silver 2014, Reichmann et al. 2015), cardiovascular system (Bryson et al. 2011, Price et al. 2014), liver (Hampton et al. 1987, Satoh et al. 2005) and retina and lenses (Freel et al. 2003, Alvarez-Viejo et al. 2004, Enski et al. 2013) in fishes and other vertebrates. Whole body transverse sections are commonly used for diagnostic work to understand the full extent of the histological lesions that are caused by fish pathogens (Bruno et al. 2013). However, to our knowledge, differences among protocols of vibratome sectioning for whole body transverse sections in fish has not yet been systematically explored.

In comparison to paraffin embedding or cryo-embedding, the advantages of vibratome sectioning are manifold (Robertson 2002). For example, there is

*Corresponding author: bianka.grunow@uni-greifswald.de

Publisher: Inter-Research · www.int-res.com

no need for dehydrating and rehydrating the tissues, which decreases loss of cell constituents. There is no need for applying high temperatures or harsh chemical treatments that may lead to antigen degradation. No expensive special blades are required, so that artefacts typically caused by paraffin embedding or freezing for cryo-sections are avoided. Furthermore, although sections of paraffin- or cryo-embedded tissues provide good internal resolution, a precise orientation of the tissue can be difficult. However, there are also disadvantages of vibratome sectioning. Sections are generally thicker than those obtained with paraffin or cryo-embedding and therefore penetration of reagents such as dyes or antibodies is more time-consuming.

In fish histology, different fixatives and embedding media are established for vibratome sectioning. Most frequently, the tissue is fixed with paraformaldehyde (Kálmán & Ari 2002, Alvarez-Viejo et al. 2004, Mahler and Driever 2007, McGrail et al. 2010) or formaldehyde (McGrail et al. 2010, Nyholm et al. 2009). For embedding, the media agarose (Kálmán & Ari 2002, Mahler & Driever 2007, Nyholm et al. 2009, Jayachandran et al. 2010, McGrail et al. 2010), gelatine (Alvarez-Viejo et al. 2004, Dietrich et al. 2010) or low-temperature melting agarose (Cheung et al. 2012) are generally used.

This study sets out to analyse in detail the effects of fixative and embedding methods on the tissue preservation of trunk segments for larvae and adult specimens of cartilaginous and bony fish species. In this report, 3 fixatives, formaldehyde, paraformaldehyde and zinc-formaldehyde, and 3 standard embedding reagents, gelatine, agarose and low-temperature melting agarose are compared. Formaldehyde fixes tissue or cells by cross-linking primary amino groups in proteins and is generally used in histology for the conservation of biological material (Rolls 2012). Paraformaldehyde is a polymerization product of Formaldehyde and the most common method for fixing animal tissue in cell biology (Kuhlmann 2009). Zink-formaldehyde is gaining importance as it has been shown to be a better fixative than paraformaldehyde for vibratome sections (especially in immunochemistry) as it enables increased penetration of antibodies (Ott 2008).

The choice of the embedding medium to stabilize tissues during the sectioning process is dependent on the tissue structure. Griffioen et al. (1992) described that a gelatine-embedding protocol prevented lesion damages in brain tissue, and Fukuda et al. (2010) used gelatine-embedded brains to create 3-dimensional histological maps and reconstruction of large-sized brain tissues. Another aspect of the embedding material is its optical properties. Blackiston et al. (2010) preferred low-temperature melting agarose because of the ease of orientation of the tissue in a fully transparent block, as is also possible with agarose.

Three fish species were used in this study: (1) larvae of a cartilaginous fish, the lesser-spotted dogfish *Scylliorhinus canicula*; (2) larvae from Atlantic sturgeon *Acipenser oxyrinchus*, representing a basal actinopterygian; and (3) the zebrafish *Danio rerio* wild-type as a representative of Teleostei and as a common model in vertebrate research.

MATERIALS AND METHODS

Animals

The lesser-spotted dogfish reproduces regularly in captivity: for animals to use in this study, 6 individuals (3 males and 3 females) in the public aquaria of the Ozeaneum (Stralsund, Germany) regularly deposited eggs on artificial plants. Parents were kept at 12 to 13°C, a salinity of 30 PSU and an oxygen supply of 92.7% in a 43500 l recirculation system. The developmental stages of the embryo and larvae described by Ballard et al. (1993) were used to determine the animals' age. Eggs were kept in a separate tank of 125 l at 15°C, 30.7 PSU and 98.5% O_2. Juveniles of 320 degree-days (DD) and newly fertilized larvae of Atlantic sturgeon were obtained from the Institute for Fisheries, State Research Center Mecklenburg-Vorpommern for Agriculture and Fishery in Born/Darss, Germany. Juveniles and eggs were kept at 20°C in a 600 l continually recirculated freshwater system. Adult zebrafish were obtained prior to fixation from the Department of Anatomy and Cell Biology, Universitätsmedizin Greifswald, Greifswald, Germany. These animals were kept at 26°C in freshwater. Prior to fixation all specimens were anaesthetised until respiratory failure using benzocaine solution (ethyl p-amino-benzoate; Sigma Aldrich) dissolved in aquarium water.

Animal measurement and fixation

Table 1 provides an overview of the animals used in this study. In total, 46 dogfishes of 5 developmental stages (700 DD, 900 DD, 1200 DD, 1500 DD, and 2000 DD), 47 sturgeons of 3 different ages (80 DD, 320 DD and 620 DD) and 41 adult zebrafish were used. Specimens of all 3 species were fixed in each

Table 1. Overview of studied material. Age (degree-days, DD), length (mean ± SE) and number of animals for the different fixation methods. FOR: 4% formaldehyde; PFA: 4% paraformaldehyde; ZnFA: 340 mM or 800 mM zinc-formaldehyde

Species Age (DD)	Length (mm)	No. of animals Total	FOR	PFA	ZnFA
Lesser-spotted dogfish					
700	26.06 ± 1.24	8	3	3	2
900	31.89 ± 0.40	10	3	4	3
1200	42.78 ± 1.59	8	3	3	2
1500	61.96 ± 3.47	10	4	3	3
2000	89.71 ± 5.13	10	3	4	3
Atlantic sturgeon					
80	8.21 ± 0.27	15	5	5	5
320	15.80 ± 0.15	18	6	6	6
620	18.59 ± 0.46	14	4	5	5
Zebrafish					
Adult	32.39 ± 0.30	41	13	14	14

of 3 chemicals: 4% formaldehyde in filtered aquarium water (FOR; prepared from a 37% formaldehyde stock solution stabilized with methanol; Berlin-Brandenburger Lager- und Distributionsgesellschaft mbH); 4% paraformaldehyde prepared following manufacturer's instructions (PFA, pH 7.8; ~1500 mOsmol kg^{-1}; Carl Roth) in 0.1 M phosphate-buffered saline (PBS); and a zinc-formaldehyde solution (ZnFA) (for details see Ott 2008). Two ZnFA solutions were prepared and applied to the samples, adjusted according to their osmolarity. Dogfishes were fixed using 800 mM ZnFA, and for the freshwater species (sturgeon and zebrafish) a 340 mM ZnFA solution was used. Before fixation, the trunk parts posterior to the pectoral fin and posterior to the dorsal fin area were cut off to ensure proper saturation of the trunk segment (Fig. 1). Size measurements of the animals and the tissue samples were taken using a digital sliding caliper (CD-20DCX absolute digimatic; Mitutoyu).

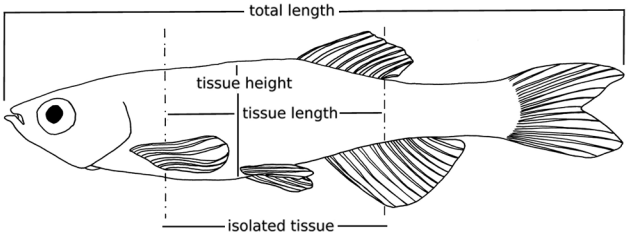

Fig. 1. Illustration of animal measurements. Total length of the animal was measured from tip of rostrum to end of tail fin. The height of the isolated trunk part was measured at the level of pelvic fin and the length was measured from one end to the other end of the trunk

These measurements included animal length (from the tip of the rostrum to the end of the tail fin), and length and height of the trunk segments (height of the tissue at the level of the pelvic fin insertion) prior and after fixation. A camera tripod was used to ensure the same angle for analyzing the size of the samples.

All samples of the trunk segments were incubated in the fixatives for 4 h at a constant temperature of 20°C followed by five 20 min washing steps in phosphate buffered saline. FOR- and PFA-fixed samples were washed in PBS whereas ZnFA-fixed samples were washed in 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer; Serva) according to the protocol of Ott (2008). All samples were kept in fresh PBS at 4°C until embedding.

Embedding and sectioning

The vibratome sections were performed from whole isolated and fixed trunk segments from dogfish (2000 DD), sturgeon (320 DD) and adult zebrafish. Specimens from each species were embedded in each of the 3 investigated embedding media prior to caudal sectioning. The embedding media were: (1) 4% porcine skin gelatine/20% chicken egg white albumin (Sigma) dissolved in distilled water (GEL) (Loesel et al. 2002), (2) 4% agarose (Serva) dissolved in distilled water (AGA), and (3) 4% low-temperature melting agarose (melting temperature ≤65°C; Sigma) dissolved in distilled water (LMA). The samples kept in gelatine blocks were post-fixed with 10% formaldehyde (diluted from 37% formaldehyde, Carl Roth) and 0.1 M PBS at 4°C overnight. All embedding blocks (1.5 × 1.5 × 1.0 cm) were stored at 4°C in PBS until sectioning. Transverse sections (50 µm thick) were prepared with a vibratome Hyrax V50 (Carl Zeiss MicroImaging), adjusting frequency, amplitude, and speed of the razor blade (Isana men; Rossmann) for each sample (Table 2). At least five 50 µm sections were prepared per sample for quality comparisons. Sections were cut by a single person to avoid variances in section quality due to operator changes. Analysis of the section quality was performed by 2 persons in a blinded manner. All sections were analyzed and evaluated as arithmetic mean. Sections of best quality (+++) showed no damage or deformation, whereas sections of good quality (++) had a little damage to the epidermis or the muscle tissue, and sections of poor quality (+) were highly damaged or showed deformation. Experiments of samples with poor quality sections were performed twice to exclude mistakes during processing.

Table 2. Overview of vibratome adjustment. Frequency, amplitude and speed for sectioning 50 µm thick fish trunk segments fixed in 4% formaldehyde (FOR), 4% paraformaldehyde (PFA), or 340 mM or 800 mM zinc-formaldehyde (ZnFA), and embedded in 4% gelatine/20% albumin (GEL), 4% agarose (AGA) or 4% low-temperature melting agarose (LMA)

	FOR			PFA			ZnFA		
	GEL	AGA	LMA	GEL	AGA	LMA	GEL	AGA	LMA
Lesser-spotted dogfish									
Frequency	50	45–60	60	40–70	50	40–70	50	50	50
Amplitude	0.7	0.7–1.2	0.7	0.7–1.2	1.0	0.9	0.9	0.9	0.9–1.0
Speed	1–3	1	1	1	4–5	1	1	3–4	3–4
Atlantic sturgeon									
Frequency	50	60–65	30	50	40	30	50	30	30
Amplitude	0.5–0.7	1.2	1.0	0.9	1.1	0.3	0.9	0.9–1.0	1.0
Speed	1	1	1	10	1	1	2	1	1
Zebrafish									
Frequency	30	50	30	30	30	60	30–75	30–75	50
Amplitude	0.7	1.2	1.0–1.2	0.3	1.1	0.8	0.3–1.2	0.3–1.2	1.2
Speed	1	1	1	1	1	1	1–4	1	1

Histochemical staining of vibratome sections

To determine the optimal fixation and embedding method for fluorescence labelling, the sections which were of very good quality were stained with the actin-binding probe phalloidin conjugated to a fluorescent dye and Hoechst, a dye for staining nuclei. The sections were washed 6 times in PBS-BSA-TX (5% BSA, 0.5% Triton X-100, 0.05% Na-acid; Sigma) for 90 min to remove the fixative. Specimens fixed in ZnFA were washed in HEPES buffered saline for the same time. Afterwards, sections were preincubated in PBS-BSA-TX for 2 h at room temperature. The tissues were then incubated in Phalloidin-TRITC 546 (1:1000; Sigma) and 0.5 µl ml^{-1} Hoechst H 33258 (1:2000; Sigma) diluted in PBS-BSA-TX for 15 min at room temperature. Thereafter, sections were rinsed in 6 changes of PBS-BSA-TX for 2 h and twice in PBS for 20 min to remove unbound remnants of the reagents. Finally, the sections were mounted in MOWIOL 4-88 (Carl Roth). Previously, different incubation times (5 min, 10 min, 15 min, 30 min) and concentrations (Phalloidin: 1:100, 1:500, 1:1000; 1:2000; Hoechst: 1:1000; 1:2000; 1:5000) of the fluorescence dyes were tested to obtain the best signal-to-noise ratio. Negative controls showed no specific labelling. The grading of the staining intensity was evaluated by assessing staining signal-to-noise ratio and required camera exposure time for photomicrographs. Samples graded with (+++) showed a very clear staining and had good signal-to-noise ratio (low background noise and visible structures of actin and DNA in high magnifications) as well as a short exposure time (<500 ms, to reduce bleaching). In comparison, samples with very long exposure times were graded with (+). Completely destroyed samples were graded with (–) as a further examination of the tissue was not possible.

Microscopy and image processing

Pictures were taken of the whole animal and the trunk segment isolated from head and tail prior and after fixation in order to document changes caused by the different chemicals. Images were taken using a Canon EOS 50D camera with different lenses, Canon EF-S 18-55 mm and Sigma DG Macro 105 mm or with a binocular (MZ75, Leica) equipped with a camera (DFC 425, Leica), depending on the animals' size. Embedded trunk segments were imaged with a Nikon SMZ800 Zoom stereomicroscope equipped with a digital microscope camera 'Digital Sight DS-2Mv' (Nikon). A Nikon Eclipse 90i fluorescence microscope equipped with a digital microscope camera 'Digital Sight DS-2 MBWc' (Nikon) was used for imaging sections and fluorescence samples. Autofluorescence tested at an excitation of 470 nm was negative. Unlabelled sections were documented with a cold light source (Schott KL200, Lighting and Imaging Schott) under polarized reflected light to reduce reflections.

Images were optimized with Adobe Photoshop CS 4 by adjusting tonal range as well as brightness and global contrast and some pictures were sharpened with the tool 'unsharp masking'. Some specimens were digitized as composite images in *z*-axis and *x*–*y* plane. Those images were fused using the

NIS-Elements Advanced Research Imaging software (v.3.22.15.738, Nikon). Greyscale fluorescence images were black-white inverted. Single-channel images were combined using the 'maximum intensity' or 'average intensity' tool of the freely available software ImageJ v.1.43m) to yield the merged fluorescence images. To evaluate the signal-to-noise ratio, raw images were used.

Statistical analysis

The mean ± SE was calculated from the total body length of every species for the same developmental stage (Table 1) and of the trunk segment before and after fixation for every developmental stage and fixation group as well as of the fixation group independently from the age of the specimen. Statistical analysis was performed using SPSS v.22 (IBM). Data were tested for significance via Wilcoxon rank-sum test for comparing the length and the height of tissue after fixation in the different developmental groups but also between the different fixation methods. $p < 0.05$ showed significant differences and $p < 0.001$ showed highly significant differences.

RESULTS

Influence of fixation conditions on colouration and gross morphology

Fig. 2 shows (A–C) dogfish, (D–F) sturgeon and (G–I) zebrafish trunk segments treated with FOR, PFA or ZnFA. The pigmentation of dogfish tissue did not change obviously after fixation (Fig. 2A–C``). In sturgeon tissues, fixation using FOR or ZnFA had a pronounced influence on the transparency of the fin fold, i.e. the tissue changed from transparent to white (Fig. 2D`,D``,F`,F``). In zebrafish, a change in tissue pigmentation was distinctive in all fixation groups (Fig. 2G``, I``; see '*'). The typical blue coloration in zebrafish was lost completely in FOR (Fig. 2G–G``) and nearly completely in PFA and ZnFA (Fig. 2H–I``). Furthermore, after FOR and ZnFA fixation the trunk segment showed signs of lacerations (Fig. 2G``,H``, see '#').

Influence of different parameters on tissue size

The influence of the fixation chemicals on the trunk segment was analyzed via size measurements before and after fixation. In dogfish, this analysis revealed no size differences in the fixation groups (FOR, PFA and ZnFA) in relation to the age of the larvae (Fig. 3A–C). In the FOR group, the tissue decreased in length around 3–7% and in height 4–14% after fixation (Fig. 3A). In the PFA group, a decrease of 3–13% in length and 10–32% in tissue height could be found, but no significant difference existed between these groups (Fig. 3B). In the ZnFA fixation group the influence on the tissue due to the chemicals followed a similar trend: in all age groups, we measured a decrease of 2–4% in length and 1–7% in height (Fig. 3C).

The examination of the trunk segments in sturgeon revealed no significant difference in the FOR and the ZnFA group in relation to the age of the animals (Fig. 3D,F). In the FOR and ZnFA groups, the highest decrease in tissue length was measured in the 80 DD group: the tissues shrank around 16.1 ± 2.3% (FOR) and 18.5 ± 5.4% (ZnFA). In comparison, concerning tissue height, the 320 DD FOR group showed an average decrease of 11.8 ± 3.1% and in the ZnFA group the 620 DD old larvae had a decrease of their trunk segment of around 15.4 ± 1.8%. The PFA-fixed trunk segments of sturgeon revealed a significant difference ($p < 0.05$) between the tissue shrinkage of the 80 DD (21.5 ± 3.4%) and the 620 DD group (12.1 ± 1.3%) (Fig. 3E). No significant tissue shrinkage was found for height among the 3 developmental stages tested.

The comparison of the fixation methods independent of the age of the animal is shown in Fig. 4. In all 3 species, a significant difference was calculated in the PFA groups. In dogfish, the tissue dimensions were highly significantly different ($p < 0.001$) compared to the ZnFA-fixed tissue (Fig. 4). A decrease of 7.9 ± 1.5% (length) and 14.3 ± 2.3% (height) after PFA fixation and 3.2 ± 0.6% (length) and 5.1 ± 1.2% (height) after ZnFA fixation was measured. In addition, the FOR group had 9.0 ± 1.9% less tissue height shrinkage than the PFA group ($p < 0.05$). Furthermore, there was a significant ($p < 0.001$) difference between the FOR and the PFA group in tissue height for sturgeon (Fig. 4). The tissue after PFA fixation decreased by 18.1 ± 2.9%, and after FOR fixation by 7.6 ± 1.6%. Similar to the dogfish results, in zebrafish there was a significant difference between the ZnFA and the PFA group, well as between the FOR and the PFA group (both $p < 0.05$; Fig. 4). The tissue height decreased by 5.5 ± 0.9% in FOR, 4.8 ± 0.9% in PFA and 3.1 ± 0.4% in ZnFA.

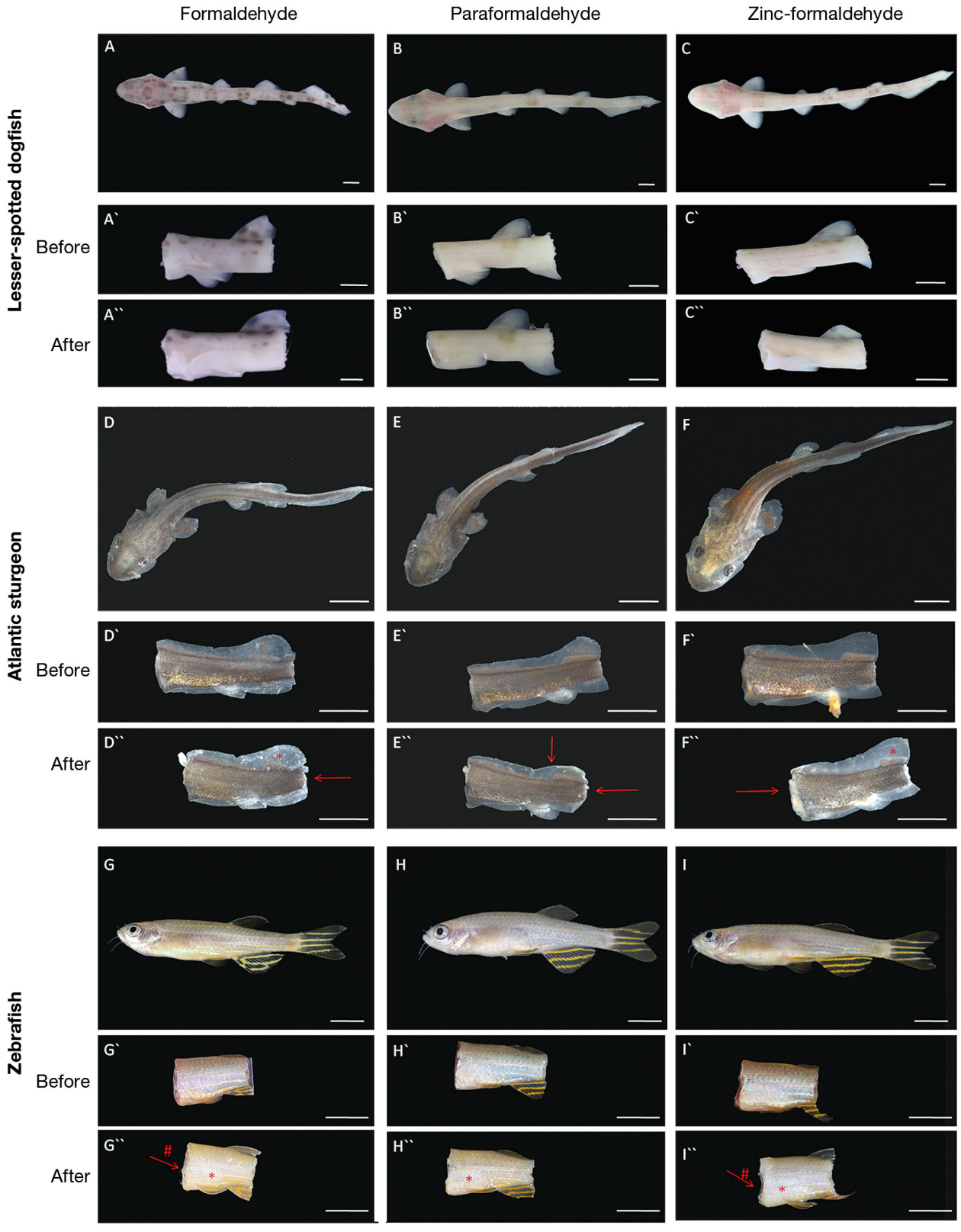

Fig. 2. Effect of different fixation methods on fish tissue. Freshly killed (A–C) lesser-spotted dogfish (2000 degree-day [DD] old; scale 5 mm), (D–F) Atlantic sturgeon (320 DD old; scale 2 mm) and (G–I) adult zebrafish (scale 5 mm). Trunk segments were documented before (`) and after (``) fixation. The pictures show the effect of (A,D,G) formaldehyde, (B,E,H) paraformaldehyde and (C,F,I) zinc-formaldehyde fixation for 4 h at room temperature (20°C) on the tissues. A loss of pigmentation (*) was present in sturgeon and zebrafish treated with (D``,G``) formaldehyde and (F``,I``) zinc-formaldehyde as well as (H``) paraformaldehyde for zebrafish. (D`–F``) The tissue from sturgeon exhibited a decrease in tissue size length (→/←) as well as height (↓). In zebrafish, after formaldehyde and zinc-formaldehyde treatment, the tissue appeared lacerated (#)

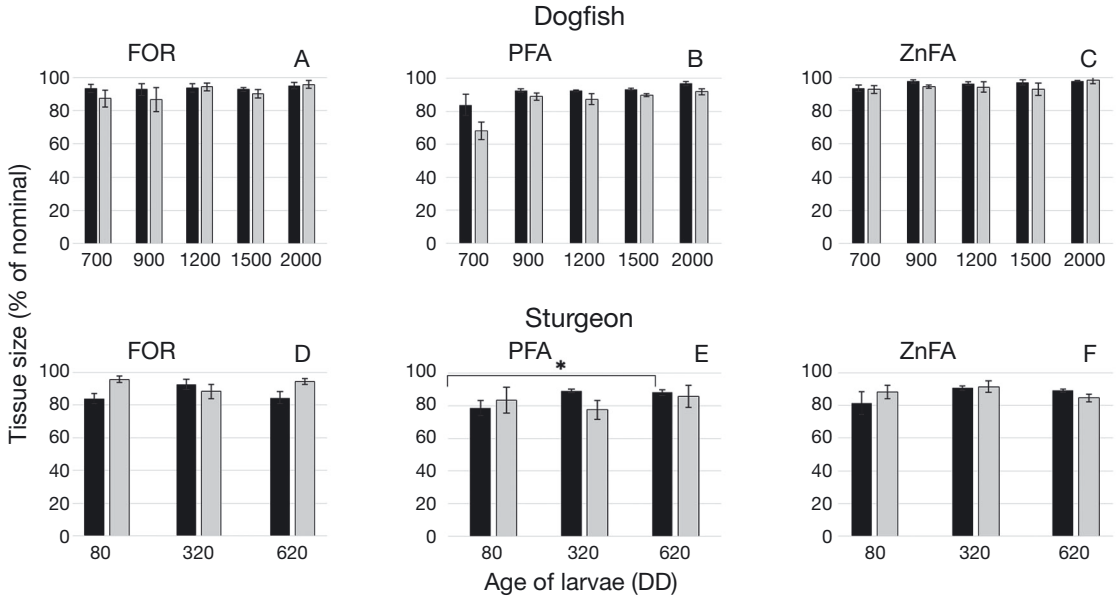

Fig. 3. Changes in tissue size after fixation in relation to age. The influence of a (A,D) 4 % formaldehyde (FOR), (B,E) 4 % paraformaldehyde (PFA), and (C,F) zinc-formaldehyde (ZnFA) fixation was analysed on body tissue length (black columns) and height (grey column) from different developmental stages for (A–C) lesser-spotted dogfish and (D–F) Atlantic sturgeon. In dogfish 700, 900, 1200, 1500 and 2000 degree-days (DD) and in Atlantic sturgeon 80, 320 and 620 DD old larvae were analysed. Between the different fixation regimes, no influence in dependence to the age of larvae in dogfish and sturgeon could be found, except in (E) PFA-fixed sturgeon of 80 and 620 DD, which shows a significant decrease in tissue length in 80 DD compared to 620 DD old larvae (*$p < 0.05$, Wilcoxon rank-sum test)

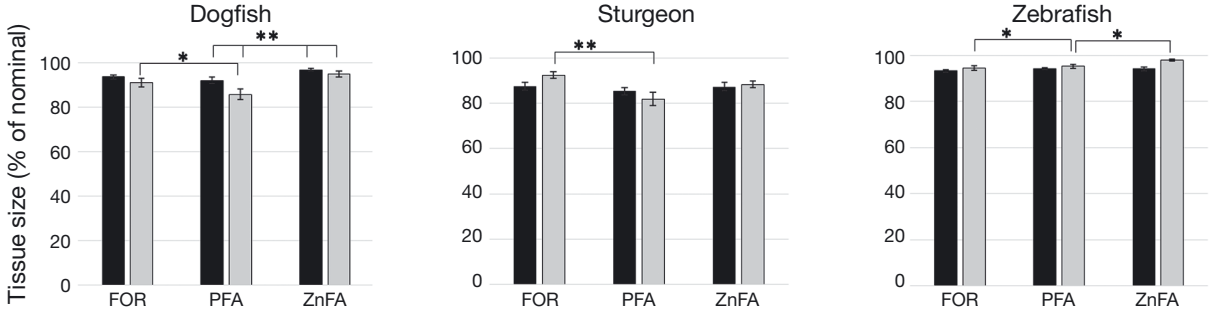

Fig. 4. Change in tissue size in relation to fixation method. The influence of 4 % formaldehyde (FOR), 4 % paraformaldehyde (PFA) and zinc-formaldehyde (ZnFA) fixation on body tissue length (black columns) and height (grey column) was analysed independently of the age of the animals in lesser-spotted dogfish, Atlantic sturgeon and adult zebrafish. In all 3 species a significant difference in tissue shrinkage was detected in the PFA groups in comparison to the FOR groups, and in zebrafish and dogfish samples in the ZnFA groups. (**$p < 0.001$, *$p < 0.05$; Wilcoxon rank-sum test)

Quality of the sections

The optical characteristics of the embedding blocks were diverse. The GEL blocks were completely opaque (Fig. 5A) and therefore a precise orientation of the tissue before sectioning was time-consuming, difficult, or even impossible. Furthermore, the material was hard and bigger trimming steps resulted in breaches of the block. Multiple embedding steps of the isolated fish tissues with the medium were sometimes necessary. In comparison to GEL, AGA (Fig. 5B) and LMA (Fig. 5C) were softer and the blocks more flexible in their form, which in some cases resulted in problems cutting the embedded tissue due to high forces and pressure on the whole block. Nevertheless, tissue orientation was easier and bigger trimming steps were possible without any damage of tissue and the surrounding embedding material.

The vibratome sections of the animals differed in quality (Table 3). Sections were graded according to

Fig. 5. Optical characteristics of the embedding media: trunk segments of the lesser-spotted dogfish embedded in (A) 4% gelatine/20% albumin (trunk not visible), (B) 4% agarose (trunk visible) and (C) 4% low-temperature melting agarose (trunk highly visible)

Table 3. Quality of vibratome sections (50 µm thick) analysed via microscopic examination. Trunk segments of 2000 degree-day (DD) old lesser-spotted dogfish, 320 DD old Atlantic sturgeon and adult zebrafish were fixed with 4% formaldehyde (FOR), 4% paraformaldehyde (PFA), or 340 mM or 800 mM zinc-formaldehyde (ZnFA), and embedded in 4% gelatine/20% albumin (GEL), 4% agarose (AGA) or 4% low-temperature melting agarose (LMA). Grading: (+) poor, (++) good, (+++) best

	FOR			PFA			ZnFA		
	GEL	AGA	LMA	GEL	AGA	LMA	GEL	AGA	LMA
Lesser-spotted dogfish	++	++	++	++	+++	++[b]	++[a,b]	++	+++
Atlantic sturgeon	+[a,b]	+++[b]	++	+[a,b]	++[a]	++	+[a,b]	+	+++
Zebrafish	++[a,b]	++	++	+++[b]	+++[b]	++	+	+	++

[a]Deformation and/or extreme shrinkage of the tissue after embedding and sectioning
[b]Sections are still embedded in embedding media

aspects of the general appearance such as form and tissue completeness (no damages or deformation). For each examined fish species, the grading of the sections showed 2 very good results. For dogfish trunk segments, PFA fixation and AGA embedding (Fig. 6A) as well as ZnFA fixation and LMA embedding seem to be preferable methods (Fig. 6B). In sturgeon tissue, best results were obtained by FOR fixation and AGA embedding (Fig. 6C) and the ZnFA fixation and LMA embedding (Fig. 6D). In zebrafish, the highest quality vibratome sections were PFA-fixed tissues embedded in GEL (Fig. 6E) and AGA (Fig. 6F). The quality of all these sections was high, as the morphology of the body cross-section from the trunk segment was well recognizable and no large gap between the muscle fibres or between the muscle and the epidermis was observed. Furthermore, no deformation of the tissue was present. The deformation of the tissues was a particular problem in some GEL-embedded samples. Multiple repeated trials showed the same results. Mainly in sturgeon, all GEL-embedded trunk segments had a high ratio of deformation, restricting identification of anatomical structures (Table 2; see Fig. 6G). In comparison, ZnFA-fixed and GEL-embedded trunk segments from dogfish displayed little shrinkage but showed a curved appearance (Fig. 6H).

Quality of histochemical staining

High-quality sections were used to assess the quality of histochemical labelling with fluorescent probes. The penetration of the labelling reagents was apparently complete in the transverse sections of the trunk segment. Structures labelled with the marker for actin (phalloidin) and labelled nuclei (Hoechst stain) were present throughout the entire vibratome sections. Table 4 summarizes the morphology of the samples after staining as well as the staining intensity with background noise, resolution of details within the sections and exposure time. The staining procedure had no influence on the morphology of the samples in dogfish (Table 4, Fig. 7A–H) and zebrafish (Table 4, Fig. 7M–T). These vibratome sections were of high quality. In the category of the signal-to-noise ratio, the 2 different treatments of dogfish tissues showed no differences. In this category (signal-to-noise ratio), the samples achieved the best grading (Table 4), because the background noise was low and the structures labelled for actin, that is, the entire musculature as well as the nuclei were highly visible at higher magnifications. Furthermore, these samples shared the same short exposure time for very good fluorescence intensities. In zebrafish, the GEL-

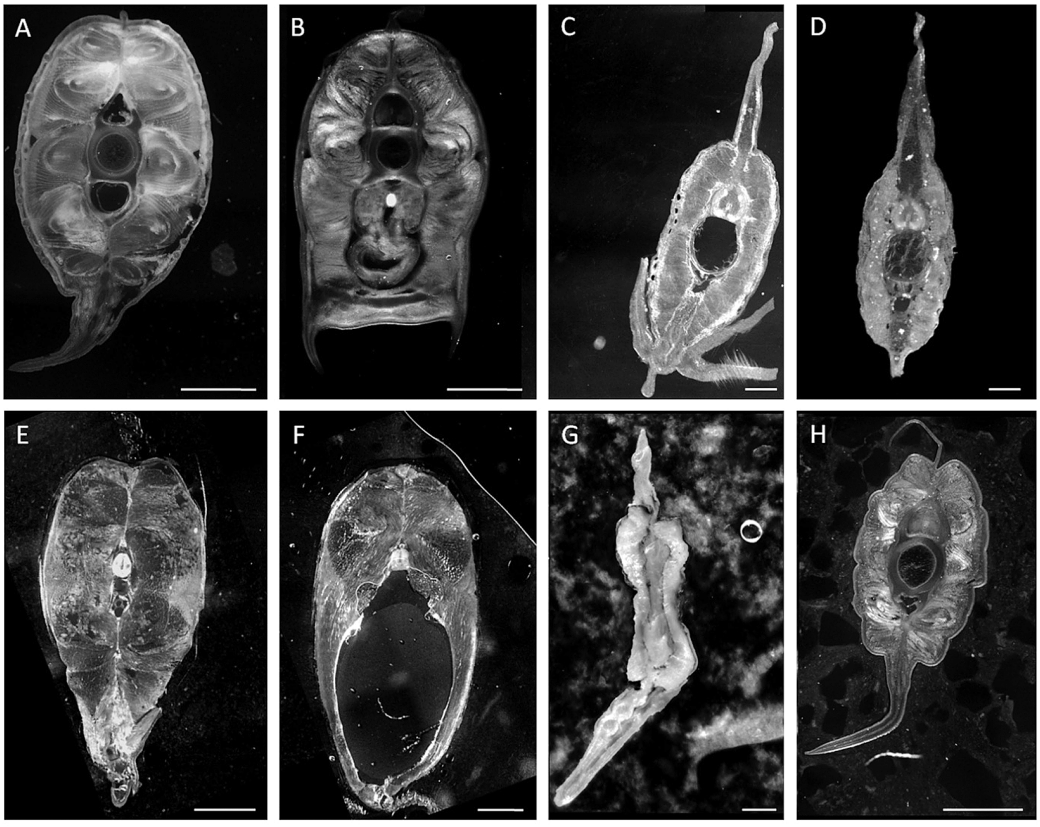

Fig. 6. Vibratome sections (50 µm thick) of trunk sections from 3 fish species with different fixation and embedding techniques which are graded as high quality (+++; A–F) and low quality (+; G, H) (see 'Material and methods: Embedding and sectioning'). Lesser-spotted dogfish: (A) PFA-fixed and embedded in AGA and (B) 800 mM ZnFA-fixed and embedded in LMA. Atlantic sturgeon: (C) FOR-fixed and embedded in AGA and (D) 340 mM ZnFA-fixed and embedded in LMA. Adult zebrafish: PFA-fixed and embedded in (E) GEL and (F) AGA. (G) Trunk segment from sturgeon fixed in FOR and embedded in GEL showed a high level of deformation. (H) Trunk segment from dogfish fixed in ZnFA and embedded in GEL showed a curved appearance due to shrinkage. See Table 3 for abbreviations. Scale bars: 1000 µm for dogfish and zebrafish, 100 µm for sturgeon

Table 4. Sample quality and staining intensity after fluorescence staining for high quality vibratome sections of trunk segments for 3 fish species analysed via microscopic examination. Grading: (–) n/a, (+) poor, (++) good, (+++) best. Fixation: FOR: 4% formaldehyde, PFA: 4% paraformaldehyde, ZnFA: 340 mM or 800 mM zinc-formaldehyde. Embedding mediums: GEL: 4% gelatine/20% albumin, AGA: 4% agarose, LMA: 4% low-temperature melting agarose

Species	Fixation	Embedding medium	Staining intensity			Morphology of sections
			Signal-to-noise ratio		Exposure time	
			Background	Detailed structures		
Lesser-spotted dogfish	PFA	AGA	+++	+++	+++	+++
	ZnFA	LMA	+++	+++	+++	+++
Atlantic sturgeon	FOR	AGA	++	+	++	+
	ZnFA	LMA	–	–	–	–
Zebrafish	PFA	GEL	++	++	+	+++
	PFA	AGA	+++	++	+++	++

embedded samples (Fig. 7M,O,P) needed up to 5 to 6 times longer exposure times to document the fluorescence signal of the phalloidin probe than in the AGA-embedded samples, indicating that the labelling was weaker (Fig. 7Q,S,T, Table 4). The resolution of details in both zebrafish samples achieved only a 'good' grading because, in comparison to the dogfish samples, the structures were not as visible (Table 4). The vibratome sections of the ZnFA-fixed and LMA-embedded trunk segment from sturgeon could not be stained because during the procedure all samples within this approach were completely destroyed (the

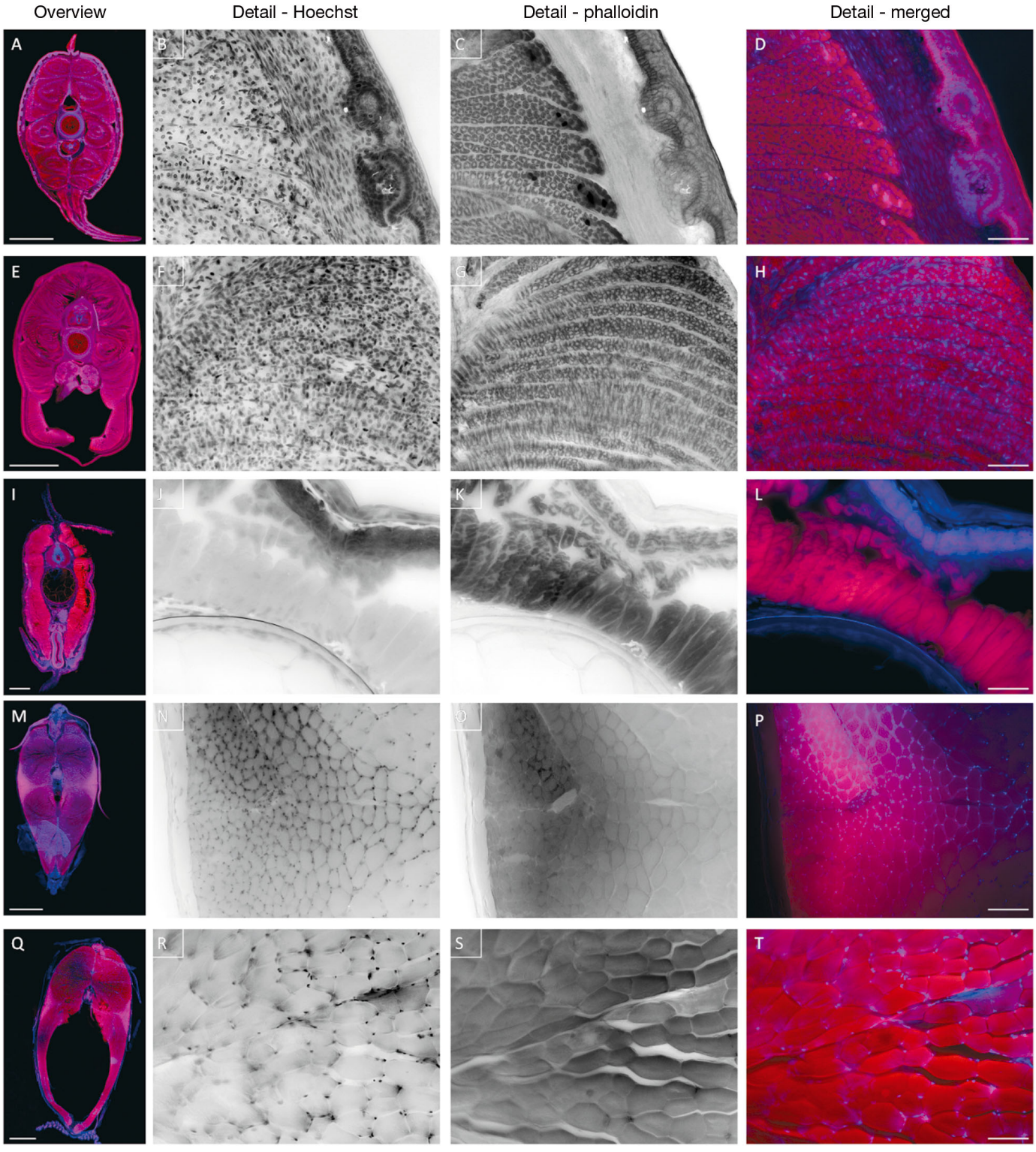

Fig. 7. Double-fluorescence staining for DNA (Hoechst H 33258) and with a probe for actin (phalloidin) in high-quality vibratome sections from 3 fish species. Lesser-spotted dogfish: (A–D) PFA-fixed and embedded in AGA, and (E–H) 800 mM Zn-FA-fixed and embedded in LMA. (I–L) Atlantic sturgeon: FOR-fixed and embedded in AGA. Adult zebrafish: PFA-fixed and embedded in (M–P) GEL and (Q–T) AGA. The first column shows the overview of the whole sections; the other 3 columns show details of the stain with Hoechst, phalloidin, and a merged image of both channels. Scale bar 1000 µm for A, E, M and Q, 100 µm for I, 50 µm for B–D, F–H, N–P and R–T, and 25 µm for J–L. See Table 4 for abbreviations

tissue disintegrated). One reason could be the long washing steps. Also, the FOR-fixed and AGA-embedded sturgeon samples showed a massive degradation in their morphology (Fig. 7I–L). In these sections, structural details were only poorly resolved (Table 4).

DISCUSSION

Pre-analytical variables such as species and age of the animals and therefore structure of the epidermis, skeleton, sample size, type of fixative, fixation time and processing conditions can have a profound influence on the quality of tissue preservation in vertebrate histology (Chung et al. 2008, Verderio 2012). Samples can be under-fixed, so that tissue preservation is poor; samples can be over-fixed so that the sample becomes difficult to infiltrate with other reagents (Wu et al. 2012). Increasing the temperature of fixation will increase the rate of diffusion of the fixative into the tissue and speed up the rate of chemical reaction between the fixative and tissue elements. It can also potentially increase the rate of tissue degeneration in unfixed areas of the specimen. Rolls (2012) noted for light microscopy that the initial fixation is usually carried out at room temperature. Therefore, in this study all 3 chemicals were used for 4 h at 20°C to compare the influence of the fixation process on the trunk segments. Taking into account that PFA is a standard fixation method (e.g. Ward et al. 2008, Kuhlmann 2009), it was surprising that our analysis of the tissue revealed in all 3 examined species that PFA fixation is inferior in comparison to FOR and ZnFA, due to higher shrinkage of the tissue. Similar effects were also described by Wehrl et al. (2014), which analyzed the degree of shrinkage by measuring the volumes of mouse brains. Shrinkage was highest in paraformaldehyde-lysine-periodate and PFA. Furthermore, in Wehrl et al. (2014), a zinc-based fixative caused the smallest degree of brain shrinkage and only small deformations and consequentially was recommended for *in vivo–ex vivo* comparison studies. Especially in morphometric analysis, the shrinkage process has to be considered. In our study, we adapted the FOR and ZnFA fixatives to the sample organisms' osmolarity, and this step was crucial for achieving good tissue preservation. Therefore, in future studies, the use of PFA should be undertaken carefully because proper adaptation to the osmolarity is essential. In conclusion, our findings show that the influence of the fixative must be examined carefully for other tissues in fish, especially when studying marine species. Furthermore, the size of the tissue samples has to be considered because the time of incubation within the fixative has to be prolonged with increasing sample volume, and multiple fixatives should be tested, such as glutaraldehyde, Bouin or Hollande. Benerini Gatta et al. (2012) reported that for different human organs the Hollande fixative (containing cooper actetat, pciric acid, formalin, and acetic acid) proved to be the best for morphology and histochemistry. In comparison, the results obtained with Bouin fixation (containing picric acid, acetic acid, and formalin) was comparable to the one with formalin (Benerini Gatta et al. 2012). Enzymes such as collagenase are frequently used after fixation of whole mount preparations in order to improve the penetration of the labelling reagents. Additionally, Olson (1985) described another technique for microscopic analysis of tissue: the perfusion fixation with specific radionuclides, fluorescent tags, or polymerizable resins can provide a wealth of information on the anatomy, physiology, pharmacology, and biochemistry of fish tissues.

Wu et al. (2012) described the influence of fixatives at different incubation temperatures. Compared to fixations at room temperature (20–25°C); fixation of samples at 4°C tends to reduce extraction of cell contents, to slow down autolytic processes and to reduce tissue shrinkage. However, 4°C will also slow down the process of the fixation itself and therefore a longer incubation time is necessary at lower temperature. This finding should also be analysed in fish trunk segments in future studies.

The sectioning of the fish tissue via a vibratome revealed differences in the quality of the sections. Problems ranging from deformation to breaking of the tissue were encountered in this study. In these cases, the embedding medium was not the perfect choice for this kind of tissue due to the fixation of the tissue structure itself. Our results show that in sturgeon, the GEL embedding method is not optimal because of the deformation or shrinkage of the tissue. The reasons for the extreme shrinkage can be manifold. Kuhlmann (2009) noted that a hypertonic solution will lead to shrinkage of the tissue, caused by an inappropriate pH, buffering capacity or osmolarity. These aspects need to be addressed in future studies. The sectioning of the tissue from dogfish and sturgeon was better in the softer embedding material AGA and LMA because of the compact epidermis. In contrast, in the teleost zebrafish the GEL embedding method was very successful. In this species, the PFA–GEL combination yielded especially high quality vibratome sections. Nevertheless, the PFA fixation

caused a significant shrinkage of the tissue. Furthermore, most of the trunk segments, mainly in AGA and LMA, broke out of the embedding material after cutting (Table 3). This factor can be difficult to control for smaller samples. In the current study this played a minor role because the tissues were large enough for optical localisation and easy handling. Additional factors affecting tissue preservation need to be examined further, including different concentrations of the embedding medium or different brands and providers of the embedding medium with different melting temperatures. For example, the quality of low-temperature melting agarose in our study proved to be of prime importance for the performance of this medium.

Because of the time-consuming and expensive nature of optimizing protocols for immunostaining in a market full of different antibodies, in the subsequent next step in this study we only examined the staining properties of the sections using histochemical probes for actin (phalloidin) and DNA (Hoechst stain). The staining procedure yielded good to very good results in dogfish and zebrafish samples, but not in sturgeon. The use of fluorescent probes instead of antibodies might be also the reason for a good staining intensity in FOR-fixed samples because they are much smaller molecules than immunoglobulins. Immunochemical examinations showed poor or no penetration of many antibodies after FOR fixation (Molgaard et al. 2014) due to the extensive cross-linking of proteins and blocking of amino groups (Sternberger 1979). We conclude that for immunochemical examinations of fish tissues with different antibodies, fixation, embedding and sectioning parameters need to be adjusted again according to the specific area of interest.

CONCLUSIONS

The goal of this study was to explore the effects of different fixation and embedding protocols on the quality of vibratome sections and fluorescence histochemistry in 3 different fish species. In dogfish, a ZnFA fixation and LMA embedding medium is recommended as there was little tissue shrinkage and very good staining intensities as well as no morphological degradation of the vibratome sections. In sturgeon, we did not obtain good results with any of our methods — other protocols or methods such as cryosectioning will have to be tested. For zebrafish, and likely other teleosts, the PFA fixation combined with ARA embedding is recommend for optimal staining intensity and preservation of the samples, even if this fixation shows a significant decrease in tissue size. Overall, the success of histological examination of vibratome sections from trunk segments will depend on the fish species as well as the development and adjustment of appropriate protocols.

Acknowledgements. The Institute for Fisheries, State Research Center Mecklenburg-Vorpommern for Agriculture and Fishery in Born/Darss and the Department of Anatomy and Cell Biology, Universitätsmedizin Greifswald, Greifswald, Germany are gratefully acknowledged for supplying the Atlantic sturgeon and the zebrafish. The aquarium team of the Ozeaneum in Stralsund helped in supply and keeping of the specimens. We wish to thank all members of the Department of Cytology and Evolutionary Biology for their support during this study. This work was supported by the project starter grant of the University of Greifswald (Germany) E404 3100.

LITERATURE CITED

Alvarez-Viejo M, Cernuda-Cernuda R, Alvarez-López C, García-Fernández JM (2004) Identification of extraretinal photoreceptors in the teleost *Phoxinus phoxinus.* Histol Histopathol 19:487–494

Ballard WW, Mellinger J, Lechenault H (1993) A series of normal stages for development of *Scyliorhinus canicula*, the lesser spotted dogfish (Chondrichthyes: Scyliorhinidae). J Exp Biol 267:318–336

Benerini Gatta L, Cadei M, Balzarini P, Castriciano S and others (2012) Application of alternative fixatives to formalin in diagnostic pathology. Eur J Histochem 56:e12

Blackiston D, Vandenberg LN, Levin M (2010) High-throughput *Xenopus laevis* immunohistochemistry using agarose sections. Cold Spring Harb Protoc 12:pdb.prot 5532

Bruno DW, Noguera PA, Poppe TT (2013) A colour atlas of salmonid diseases, 2nd edn. Springer, Dordrecht

Bryson JL, Coles MC, Manley NR (2011) A method for labelling vasculature in embryonic mice. J Vis Exp 56: 3267

Cheung ID, Bagnat M, Ma TP, Datta A and others (2012) Regulation of intrahepatic biliary duct morphogenesis by Claudin 15-like b. Dev Biol 361:68–78

Chung JY, Braunschweig T, Williams R, Guerrero N and others (2008) Factors in tissue handling and processing that impact RNA obtained from formalin-fixed, paraffin-embedded tissue. J Histochem Cytochem 56:1033–1042

Dietrich HW III, Westerfield M, Zon LI (2010) The zebrafish: cellular and developmental biology, Part A, 3rd edn. In: Wison L, Tran P (eds) Methods in cell biology, Vol 100. Elsevier, p 103–108

Enoki R, Koizumi A (2013) A method of horizontally sliced preparation of the retina. Methods Mol Biol 935:201–205

Freel CD, Gilliland KO, Mekeel HE, Giblin FJ, Costello MJ (2003) Ultrastructural characterization and Fourier analysis of fiber cell cytoplasm in the hyperbaric oxygen treated guinea pig lens opacification model. Exp Eye Res 76:405–415

Fukuda T, Morooka K, Miyagi Y (2010) A simple but accu-

rate method for histological reconstruction of the large-sized brain tissue of the human that is applicable to construction of digitized brain database. Neurosci Res 67:260–265

Funch PG, Wood MR, Faber DS (1984) Localization of active sites along the myelinated goldfish *Mauthner axon*: morphological and pharmacological evidence for salutatory conduction. J Neurosci 4:2397–2409

Griffioen HA, Van der Beek E, Boer GJ (1992) Gelatine embedding to preserve lesion-damaged hypothalami and intracerebroventricular grafts for vibratome slicing and immunocytochemistry. J Neurosci Methods 43:43–47

Hampton JA, Klaunig JE, Goldblatt PJ (1987) Resident sinusoidal macrophages in the liver of the brown bullhead (*Ictalurus nebulosus*): an ultrastructural, functional and cytochemical study. Anat Rec 219:338–346

Hökfelt T, Ljungdahl A (1972) Modification of the Falck-Hillarp formaldehyde fluorescence method using the vibratome: simple, rapid and sensitive localization of catecholamines in sections of unfixed or formalin fixed brain tissue. Histochemie 29:325–339

Jayachandran P, Hong E, Brewster R (2010) Labelling and imaging cells in the zebrafish hindbrain. J Vis Exp 41: 1976

Kah O, Dubourg P, Onteniente B, Geffard M, Calas A (1986) The dopaminergic innervation of the goldfish pituitary. An immunocytochemical study at the electron-microscope level using antibodies against dopamine. Cell Tissue Res 244:577–582

Kálmán M, Ari C (2002) Distribution of GFAP immunoreactive structures in the rhombencephalon of the sterlet (*Acipenser ruthenus*) and its evolutionary implication. J Exp Zool 293:395–406

Kuhlmann WD (2009) Fixation of biological specimens. www.immunologie-labor.com/cellmarker_files/IET_tissue_02.pdf (accessed 06/10/2014)

Lechtreck KF, Sanderson MJ, Witman GB (2009) High-speed digital imaging of ependymal cilia in the murine brain. Methods Cell Biol 91:255–264

Lieberoth BC, Becker CG, Becker T (2003) Double labelling of neurons by retrograde axonal tracing and non-radioactive in situ hybridization in the CNS of adult zebrafish. Methods Cell Sci 25:65–70

Lindvall O, Björklund A, Hökfelt T, Ljungdahl A (1973) Application of the glyoxylic acid method to vibratome sections for the improved visualization of central catecholamine neurons. Histochemie 35:31–38

Loesel R, Nässel DR, Strausfeld NJ (2002) Common design in a unique midline neuropil in the brains of arthropods. Arthropod Struct Dev 31:77–91

Mahler J, Driever W (2007) Expression of the zebrafish intermediate neurofilament Nestin in the developing nervous system and in neural proliferation zones at postembryonic stages. BMC Dev Biol 7:89

McGrail M, Batz L, Noack K, Pandey S, Huang Y, Gu X, Essner JJ (2010) Expression of the zebrafish *CD133/prominin1* genes in cellular proliferation zones in the embryonic central nervous system and sensory organs. Dev Dyn 239:1849–1857

Molgaard S, Ulrichsen M, Boggild S, Holm ML, Vaegter C, Nyengaard J, Glerup S (2014) Immunofluorescent visualization of mouse interneuron subtypes. Version 2. F1000 Res 3:242

Nyholm MK, Abdelilah-Seyfried S, Grinblat Y (2009) A novel genetic mechanism regulates dorsolateral hinge-point formation during zebrafish cranial neurulation. J Cell Sci 122:2137–2148

Olson KR (1985) Preparation of fish tissues for electron microscopy. J Electron Microsc Tech 2:217–228

Ott SR (2008) Confocal microscopy in large insect brains: zinc–formaldehyde fixation improves synapsin immunostaining and preservation of morphology in whole-mounts. J Neurosci Methods 172:220–230

Pilaz LJ, Silver DL (2014) Live imaging of mitosis in the developing mouse embryonic cortex. J Vis Exp 88

Price RL, Haley ST, Bullard T, Davis J, Borg TK, Terracio L (2014) Confocal microscopy of cardiac myocytes. Methods Mol Biol 1075:185–199

Reichmann F, Painsipp E, Holzer P, Kummer D, Bock E, Leitinger G (2015) A novel unbiased counting method for the quantification of synapses in the mouse brain. J Neurosci Methods 240:13–21

Robertson N (2002) Vibratome sectioning of tissues: an alternative to paraffin methods. http://www4.ncsu.edu/~rgfranks/research/protocols/immuno%20histio%20chemistry/robertson%20vbratmimuno.doc (accessed 12/09/2014)

Rolls G (2012) Fixation and fixatives (2) – factors influencing chemical fixation, formaldehyde and glutaraldehyde. www.leicabiosystems.com/pathologyleaders/fixation-and-fixatives-2-factors-influencing-chemical-fixation-formaldehyde-and-glutaraldehyde/ (accessed 24/09/2014)

Satoh K, Takahashi G, Miura T, Hayakari M, Hatayama I (2005) Enzymatic detection of precursor cell populations of preneoplastic foci positive for gamma-glutamyltranspeptidase in rat liver. Int J Cancer 115:711–716

Sternberger LA (1979) Immunocytochemistry, 2nd edn. John Wiley & Sons, New York, NY

Verderio P (2012) Assessing the clinical relevance of oncogenic pathways in neoadjuvant breast cancer. J Clin Oncol 30:1912–1915

Ward TS, Rosen GD, von Bartheld CS (2008) Optical disector counting in cryosections and vibratome sections underestimates particle numbers: effects of tissue quality. Microsc Res Tech 71:60–68

Wehrl HF, Bezrukov I, Wiehr S, Lehnhoff M and others (2014) Assessment of murine brain tissue shrinkage caused by different histological fixatives using magnetic resonance and computed tomography imaging. Histol Histopathol (in press)

Wu S, Baskin T, Gallagher KL (2012) Mechanical fixation techniques for processing and orienting delicate samples, such as the root of *Arabidopsis thaliana*, for light or electron microscopy. Nat Protoc 7:1113–1124

Yulis CR, Lederis K (1986) The distribution of 'extraurophyseal' urotensin I-immunoreactivity in the central nervous system of *Catostomus commersoni* after urophysectomy. Neurosci Lett 70:75–80

Editorial responsibility: Helmut Segner, Bern, Switzerland

Submitted: October 20, 2014; Accepted: February 3, 2015
Proofs received from author(s): March 17, 2015