Exploring methods to remove iodine and phosphotungstic acid stains from zoological specimens

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Aims

Micro-computed tomography (micro-CT, X-ray microtomography) has evolved into an important imaging tool for zoological research in recent years (e.g. Fernández et al. 2014, Golding and Jones 2006, Heim and Nickel 2010, Kerbl et al. 2014). The non-destructive nature of the technique makes it a powerful tool for morphological and anatomical studies, and especially so if valuable natural history museum specimens are concerned, since the deposited reference specimens may not be altered. However, zoological specimens often consist mainly of soft tissue with very low x-ray absorption and in many cases need to be treated with contrast enhancing chemicals prior to scanning. A common approach is to stain the specimen with heavy metals, which are absorbed by the tissues and thus result in a higher x-ray absorption and stronger contrast. Several effective methods are available (e.g. Metscher 2009, Pauwels et al, 2013, Paterson et al. 2014), of which iodine or phosphotungstic acid (PTA) are the most common stains for preserved specimens - mainly because they are cheap, non-toxic and easy to apply. B'oth metals tend to have a tendency to bind to specific tissues, but provide generally good overall contrast (Faulwetter et al. 2013; Metscher 2009). However, while the actual imaging through micro-CT is considered non-destructive, the contrast enhancing stains change the tissue qualities and, in case of iodine, also the colour of the specimen. Museum curators will not permit any research on reference specimens that could damage or alter the specimen, including non-reversible staining methods (Paredes et al. 2012). Methods to safely remove these contrast agents from specimen tissue are therefore of great interest for the taxonomic research community.

Concerning the removal of iodine and PTA little information is available in the literature. Both iodine and PTA staining have been reported to be stable for at least months, if not years (Metscher 2009, Paterson et al. 2014), although in some specimens the iodine stain could be removed by re-immersion into ethanol (e.g. Alba-Tercedor 2012, Paterson et al. 2014). Personal observations at the collections of the biodiversity laboratory of the Hellenic Centre for Marine Research showed, however, that in several specimens the iodine stain had not been removed even after two years of immersion in ethanol. The stability of the stain in the tissue might therefore depend on factors such as the fixation or preservation medium, the age of the specimen or the type of tissue (e.g. chitin, muscles, calcified tissues). The effectiveness of PTA to bind to tissues seems to be dependent on the pH of the solution (Quintarelli et al. 1971, 1973), but no studies exist on the removal of PTA from whole specimens stained for micro-CT imaging.

In this study, we present a first approach to removing iodine and PTA from stained specimens, using marine bristle worms as a test organism. Although the results were already promising, laboratory experiments showed that the methods still need fine-tuning, as a variety of parameters seem to affect the results.

Methods

Specimen selection

To minimize the effect of external factors on the results, we chose specimens with the same or very similar characteristics concerning their taxonomic identity, size, type of fixation and preservation medium or time since preservation. Twelve similarly sized specimens (approximately 5 mm long, 0.3 mm diameter) of *Syllis armillaris* (Polychaeta, marine bristle worms) were used (Figure 1). Specimens were collected in June 2008 from two rocky shore sites in northern Crete (Eastern Mediterranean). Details on the sampling can be found in Chatzigeorgiou et al. (2012). Specimens were fixed and preserved in 96% ethanol.

Iodine removal

Six of the twelve specimens were used for testing the effectiveness of different solutions for iodine removal. Prior to any specimen staining, all specimens were scanned to obtain a reference scan. All specimens were then stained for six days in 1% iodine in 96% ethanol, following the protocol of Metscher (2009). After staining, the specimens were removed from the 1% iodine solution, gently washed in ethanol to remove excess stain and scanned. Afterwards, three specimens were placed in 10% sodium thiosulfate ($Na_2S_2O_3$) in distilled water and three were placed in 96% ethanol. Before being transferred to the sodium thiosulfate solution, specimens were gradually rehydrated in 75%, 50% and 25% ethanol (five minutes at each concentration). To monitor the rate of stain removal, each specimen was scanned 2 h after the first transfer to the destaining solution, re-immersed into the destaining solution and scanned again after 4 h, 6 h, 24 h and 48 h. Before each scan, specimens treated with sodium thiosulfate were gently washed in distilled water to remove any chemical residue, and, since they were scanned in 96% ethanol, gradually dehydrated by reversing the above procedure. After scanning, the specimens were again rehydrated and transferred again to the sodium-thiosulfate solution. Sodium thiosulfate solutions and ethanol, respectively, were renewed after each scan. Scanning duration and dehydration/rehydration time were not included in the actual destaining time.

PTA removal

Six specimens were used for testing the removal of PTA. Prior to any specimen preparation, all specimens were scanned to obtain a reference scan. Specimens were stained in a 0.3% PTA solution in 70% ethanol, following the protocol of Metscher (2009). After six days, the specimens were removed from the staining solution, transferred to 96% ethanol and scanned. Three specimens were placed in a 0.1M phosphate buffer with an adjusted pH of 8.8 and three specimens were placed in a water-based 0.1M sodium hydroxide (NaOH) solution with an adjusted pH of 8.9. Specimens were scanned after 2 h and 6 h, respectively, in the staining solutions (no further destaining after 6 hours was needed since the stain had already been fully removed by then). Specimens were dehydrated in ethanol before scanning and rehydrated before replacement in the respective solutions as described in the section *Iodine removal*. Solutions were renewed after each scan.

Scanning and image processing

All scans were performed with a SkyScan 1172 microtomograph at the Hellenic Center for Marine Research. Specimens were scanned in 96% ethanol in polypropylene pipette tips, which were sealed with parafilm to avoid drying out (for a setup see Metscher 2009). The settings for all scans were the same: a voltage of 60kV and a current of 167µA was chosen, a medium camera resolution of 2000x1336 pixel was used, and scans were performed for a full rotation of 360°. All scans were performed without additional random movement or averaging settings and without a filter.

The obtained projection images were reconstructed into cross sections using SkyScan's NRecon software. Using the first scans of unstained specimens, the dynamic range of the images was chosen manually with aid of the histogram window to achieve a good signal-to-noise ratio. For all scans of the same contrast agent (iodine or PTA), the same histogram values were used for every following reconstruction of projection images. To minimize file

sizes, only the region of interest (ROI) which contained the specimen was reconstructed. To assess the rate of destaining, eight cross sections were extracted from each scan at the region of the proventricle (Figure 1). This approach was preferred over an analysis of the whole animal, since individual morphological differences and specimen sizes would have resulted in a different ratio of body parts with natural density differences.

The cross section images were loaded into Skyscan's CTAnalyzer software (CTAn). Using the Binary Selection module, the specimen was isolated from the background to prevent the surrounding medium from being included in the calculation of greyscale values. Then, noise was eliminated by setting an appropriate range in the grayscale histogram (33-255). Finally, from the areas thus selected, the mean greyscale value (as a measure of relative density) was calculated for each slice. To visualize the rate of destaining, these values were averaged per specimen and treatment at each point in time (control, stained, and each destaining step) and plotted as a graph.
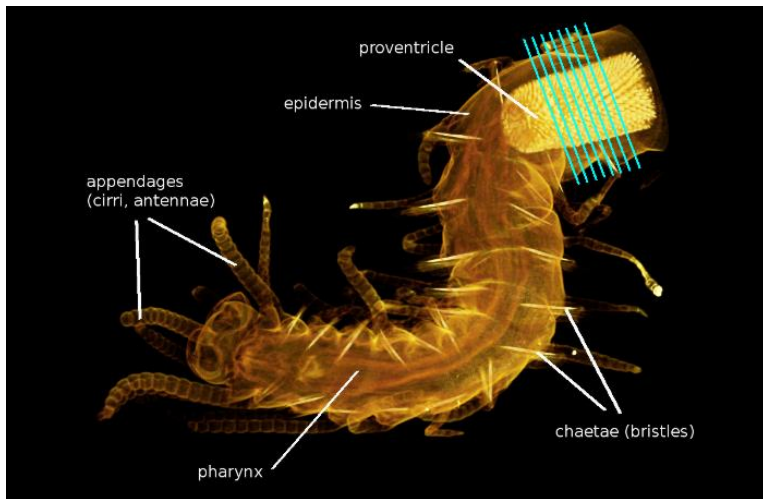

Figure 1: False-colour, semi-transparent volume rendering of anterior end of a *Syllis* species. Light blue lines indicate approximate position of cross-sections extracted for calculating average greyscale values.

Results

Iodine removal

Both destaining methods showed satisfactory results. Despite a certain variability between the specimens regarding the average greyscale values and noise due to the low resolution of the images, a clear trend could be observed. A first strong drop in average relative density could be seen already after 2 hours of destaining, and after 48 hours, the stain had been removed from the tissues completely (Figures 2a, 3). In specimens destained with 96% ethanol the average relative density was comparable to that of the control scans after 48 hours. Specimens destained with sodium thiosulfate showed already after two hours a lower relative density than in the unstained control scans; afterwards the relative density did not change noticeably anymore (Figures 2a, 3). Changes in tissue densities could be observed especially in the proventricle (seen as a dark round shape in Figure 3). This part of the worm's digestive tract has naturally a higher density than the surrounding tissues due to calcified granules in the muscle tissue, a characteristic which appeared to have been altered by the treatment with sodium thiosulfate (Figure 3).

PTA removal
Both methods succeed in removing PTA from the tissue, but to a different extent. The immersion in sodium hydroxide resulted in a steady decrease of relative density, until after six hours, the stain had been removed fully from two of the three specimens – except for one slightly larger specimen which was still partly stained (Figure 2b, 4). The phosphate buffer acted much more aggressively in removing the stain: density values dropped sharply already after two hours, reaching a level well below those of the control scans. In one specimen, density had decreased so much that it could not be visualized and no measurements could be taken (Figure 2b, 4).
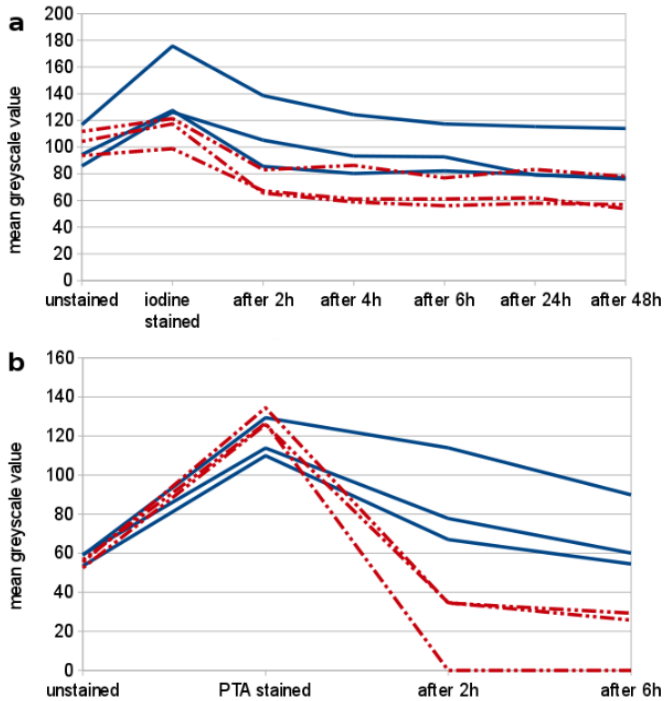

Figure 2: a) Rate of iodine removal from tissue, expressed as mean greyscale value of tissue in 8 cross-sections in anterior body region. Solid blue line: 96% ethanol, dotted red line: sodium thiosulfate. The six lines correspond to the six specimens; b) Rate of PTA removal from tissue, expressed as mean greyscale value of tissue in 8 cross-sections in anterior body region. Solid blue line: sodium hydroxide, dotted red line: phosphate buffer. The six lines correspond to the six specimens.
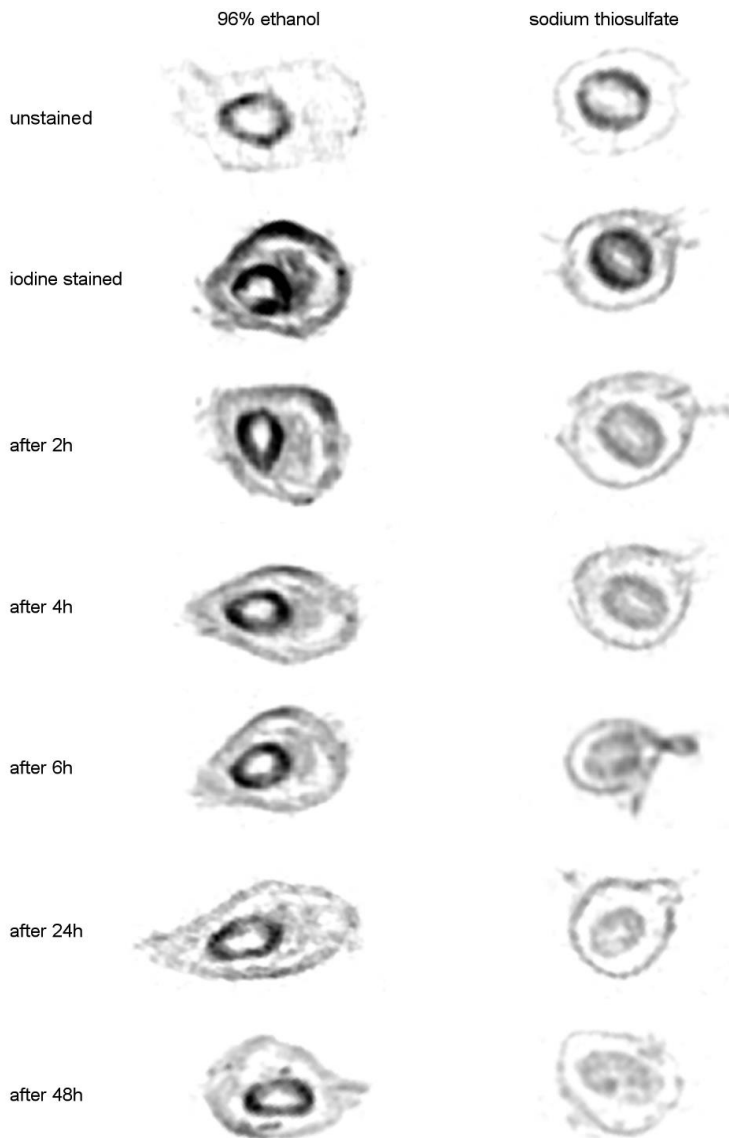
Figure 3: Examples of cross-sections from the anterior body region of one specimen of *Syllis armillaris*, at different points of the iodine staining/destaining procedure. Dark colours = higher density.
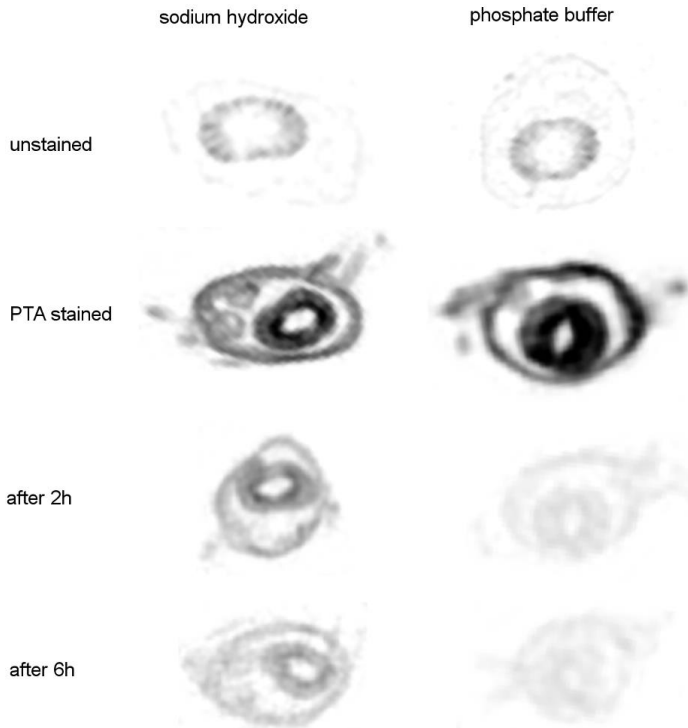
Figure 4: Examples of cross-sections from the anterior body region of one specimen of *Syllis armillaris*, at different points of the PTA staining/destaining procedure. Dark colours = higher density.

Discussion and conclusions

Being able to remove X-ray contrast agents from tissues is crucial in many areas of research, especially when valuable specimens are concerned. We presented here new methods for reversing the two most commonly used stains in micro-CT imaging of biological specimens, iodine and phosphotungstic acid (PTA).

Iodine is soluble in ethanol, and several reports indicate that it can be removed from stains through re-immersion in ethanol (e.g. Alba-Tercedor 2012, Paterson et al. 2014). However, this method does not seem to work reliably on all specimens, as personal experiences have shown. Sodium thiosulfate reacts chemically with iodine in an aqueous solution, reducing it to iodide and thus removes the tissue bonds. Our results show that 96% ethanol did indeed reduce the amount of the stain in the tissue of *Syllis armillaris*, resulting in a lowered contrast after already a few hours to days. However, especially 3D visualizations (not shown here) revealed that the animal was not completely destained yet. Sodium thiosulfate, on the other hand, had removed the stain completely after only two hours. A yet unresolved problem is that the relative density of the tissue after destaining was even lower than in the unstained (control) specimens. This may indicate an actual alteration of the natural tissue characteristics. In particular the proventricle, which contains calcium granules in the muscle cells, had much lower contrast than in the unstained specimens. Furthermore, after having been immersed for over a day in the sodium thiosulfate solution, the animals became more fragile and soft and

had a dissolved appearance. The reason for this might be that the aqueous $Na_2S_2O_3$ solution, unlike ethanol, does not preserve tissue characteristics. Another explanation, supported by the strong destaining effect, might be that in the current experiment a too high concentration of $Na_2S_2O_3$ was used.

Phosphotungstic acid stains are known to have a pH optimum (below 5.0, depending on tissue: see Quintarelli et al. 1971, 1973), and indeed by immersing the specimens in a solution with a slightly elevated pH, the stain could effectively be removed from the tissue. Although both solutions used here had an almost identical pH (NaOH: 8.9, phosphate buffer: 8.8) their effect was very different, indicating that another yet unknown factor might play a role as well. Using sodium hydroxide, the stain was removed at a steady rate within a few hours, whereas the phosphate buffer seems to work "too well" – already after two hours the contrast was lower than in the control specimens and in some cases the tissue seemed to have the same density as the surrounding ethanol. The latter indicates an alteration of the tissue characteristics. No adverse effects could be seen using NaOH. Sodium hydroxide has the additional advantage of being soluble in both water and ethanol, the latter prevents the disintegration of tissue if longer destaining times are needed.

The approaches presented here are a very useful first step towards developing a standardised method for removing stains from biological specimens. Such methods are crucial for micro-CT research on valuable museum specimens, but might also be of use in, for example, developmental biology when different tissue types should be subsequently visualised with different stains. However, while the results presented here show a clear trend, an optimal protocol is not yet available. The current experiments showed that both the use of sodium thiosulfate and phosphate buffer resulted in possible alteration of tissue characteristics, and thus more research is needed to optimize the protocols and determine, among other parameters, the optimal concentration of sodium thiosulfate to avoid tissue damage. None of the methods is expected to affect the genetic material, but this should be tested in separate experiments. Scans in higher resolution in combination with a more refined method of density comparisons are needed to determine the exact effect of each method. The comparison of relative densities showed that the effects of staining and destaining had considerable variations among specimens and might depend on individual reactions, the identity of the specimen (taxon/tissue types), fixation and preservation methods and size of the animal. The effects of these factors are currently being investigated in a series of additional experiments.

Acknowledgements

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