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Research article

A histological method for marine invertebrates

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Abstract

This study aimed to increase knowledge about the structure and health assessment for evaluating histopathology as a biomarker of marine invertebrates, which must have high-quality histologic slides. Unfortunately, the accuracy of this method has been specifically modified for the marine invertebrates in Thailand; we demonstrated a modified method for permanent histological slides, including *Xestospongia* sp., *Halobates hayanus, Amphibalanus amphitrite*, and *Alpheus* sp. (n = 30 individual specimens for each species) from Libong island, Thailand, which was compared to the standard method. Optimization of tissue adapts and troubleshoots typical histological procedures, including sample collection, fixation with 10% Neutral Buffered Formalin at 2-4 °C for 24 hrs., decalcification, dehydration, clearing, embedding sectioning, and examining after staining under the modified procedures each time, which can be accomplished easily in six days. Compared to the standard method, this modified protocol showed the first report. It warranted high-quality slide scans, which are particularly ideal for the tissue, cell, and nuclei-specific features of marine invertebrates.

Keywords: Aquatic animal health, Histological slides, Histopathology, Decalcified process, Thailand

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INTRODUCTION

It is increasingly recognized that knowledge of the structure, histology, and histopathology of health assessments and disease states of aquatic animals has been proposed in various documents (Garcia and Silva, 2006; Alfaro-Montoya, 2010; Senarat et al., 2018; Senarat et al., 2019; Stewart et al., 2010; Zarella et al., 2018). Misdiagnoses and incorrect results are received when the tissue is not adequately fixed and/or processed using a standard protocol. Multiple histopathology-based prevailing research on permanent histological slides is the critical standard for evaluating tissues and cells in whole organisms under light microscopy (Frank et al., 2021; Zhang et al., 2010). It is well known that previous data showing high-quality histological slides are strongly accurate and efficient schemes for tissue and cell identification, such as in *Daphnia magna* (Ngu et al., 2022), *Danio rerio* (Nuckels and Gross, 2007; Copper et al., 2018), and other animals (Al-Sabaawy et al., 2021). Ideally, the most commonly used invertebrate histology approach has been focused on involving many regulatory steps, including sample collection, decalcification, embedding, and sectioning (Peter et al., 2005). Each of the steps mentioned must be carefully worked out (Peter et al., 2005; Barbosa et al., 2014; LaDouceur, 2021; Ngu et al., 2022).

While current protocols for marine invertebrate histology are still limited and complicated (Howard et al., 2004; LaDouceur, 2021), only one report showing the improved histological fixation of gelatinous marine invertebrates was visible (Mitchell et al., 2021), and there is no information on their featured histology, despite the efficiency of histologically assessing the structural and histopathological results. The present study, therefore, reported on a modified method specific to permanent histological slides for marine aquatic invertebrates including *Xestospongia* sp., *Halobates hayanus*, *Amphibalanus amphitrite*, and *Alpheus* sp. from Libong Island, Thailand, for comparison with the standard method.

MATERIALS AND METHODS

Photographs showing the live-collecting marine invertebrates, including *Xestospongia* sp., *Halobates hayanus*, *Amphibalanus amphitrite*, and *Alpheus* sp. (n = 30 individual specimens for each species) were collected from Libong Island, Thailand, which were then processed by modified histological methods and compared to the standard method (Table 1). The application of histopathological methods to investigate tissue structures for better histological staining and microscopic studies was done by adjusting the protocol from the standard method, including alcohol solution concentration and time steps (Table 2-3), tissue embedding, sectioning, and staining process. Table 2-3 shows the materials, equipment specifications, and chemical solutions for histological processing. The histology laboratory is located at the Department of Anatomy, Faculty of Medical Science, Naresuan University (Standard laboratory in Number 33333).

Table 1 The comparative protocol between the standard method and modified method for tissue processing

No.	Standard method		Modified method	
	Process	Time (min)	Process	Time (min)
1	70% Ethanol	30	70% Alcohol	30
2	80% Ethanol	30	80% Alcohol	30
3	85% Ethanol	30	90% Alcohol	30
4	90% Ethanol	30	95% Alcohol I	90
5	95% Ethanol	30	95% Alcohol II	120
6	95% Ethanol	45	100% Alcohol I	120
7	Absolute Ethanol I	45	100% Alcohol II	120
8	Absolute Ethanol II	60	100% Alcohol III	120
9	Xylene I	60	Xylene I	90
10	Xylene II	60	Xylene II	120
11	Paraplast I	60	Xylene : Paraplast (1:1)	60
12	Paraplast II	90	Paraplast I	90

*Gray color indicated the different sub-protocols

Table 2 Chemical solutions for histological processing

Chemical reagents	Band	Lot	Corporation	Country
Formaldehyde 35-40%	Lab-scan	AR1072M-G2.5L	RCI Labscan Limited	THAILAND
Sodium di-hydrogen phosphate	KemAus™	1809200592	Kemans	AUSTRALIA
Di-sodium hydrogen phosphate dibasic anhydrous	KemAus™	2005206183	Kemans	AUSTRALIA
Absolute ethanol	Lab-scan	AR1069-P4L	RCI Labscan Limited	THAILAND
Xylene	Lab-scan	AR123-G4L	RCI Labscan Limited	THAILAND
Paraplast plus	Leica	2106046	LeicaBiosystems	USA
Modified hematoxylin solution	PATH. 1	NOV. 2021	C.V. Laboratories Co., Ltd	THAILAND
Eosin solution	PATH. 2	NOV. 2021	C.V. Laboratories Co., Ltd	THAILAND
Weigert's iron hematoxylin	-	JJ2RL-LP	Tokyo chemical industry Co., LTD	JAPAN
Ferric chloride anydrous	-	A334122102	Loba Chemie Pvt. Ltd	INDIA
Hydrochloric acid 37%	-	AR1107-G2.5L	RCI Labscan Limited	THAILAND
Beibrich scarlet	-	479735	HiMedia Laboratories Pvt. Ltd. (India)	INDIA
Acid fuchsin	-	448477	HiMedia Laboratories Pvt. Ltd. (India)	INDIA
Acetic Acid Glacial	-	AR1002-2.5L	RCI Labscan Limited	THAILAND
Phosphomolybdic acid	-	438517	HiMedia Laboratories Pvt. Ltd. (India)	INDIA
Phosphotungstic acid		46071	HiMedia Laboratories Pvt. Ltd. (India)	INDIA
Light green SF yellowish		FN1476341 103	Sigma-Aldrich (Merck, Germany)	Germany
Suripath Decalcifier II	Leica	70220	LeicaBiosystems	USA
Permount		202283	Fisher Scientific	USA

Table 3 The materials and equipment for histological processing

Materials / Equipment	Model	Manufacturer	Country
Automatic tissue processor	Thermo scientific STP1020	Leica Microsystem Nussloch GmbH	GERMANY
Embedding Center	Thermo Histostar Embedding Center	Thermo Shandon Limited	UK
Water Bath	WB 2800	Histo-Line Laboratories	ITALY
Cryo console	TEC 2900-2	Histo-Line Laboratories	ITALY
Microtome	HM325 Manual Microtome	Histo-Line Laboratories	ITALY
Tissue cassettes	-	PorLab Scientitic Co., Ltd	CHAINA
Low profile Microtome blades	Leica	Leica Biosystems	GERMANY
Filter papers (size. 125 mm)	Whatman TM	GE Healthcare companies, UK	CHAINA
Coverslip (size 24x40 cm)	-	HAD	INDIA
Lens Clearing Tissue (size 10x5 cm)	Newstar	Hangzhou Special Paper Industry Co., Ltd	
Latex powdered-free examination gloves	-	Sritrang glover	Thailand
Adhesion microscope slides (size 25x75 cm)	-	Citotest Labware Manufacturing Co Ltd	CHAINA
Microscope slide (size 25.5x76.2 mm)	-	HAD	INDIA

RESULTS

Fixation and Decalcification

All samples were randomly considered before used specimens were immediately euthanized. In keeping with this finding, the size of specimens was small (2-4 cm in diameter); we field-placed and fixed their whole specimens into 10% neutral buffered formalin (Optimal proportion shown in Table 2) in a plastic specimen jar of 24 hrs. at 2-4 °C in an ice box to increase the penetrating fixation. After fixation, the samples were kept in 70% ETOH three times (Figure 1), each time being used for 2 hrs. to avoid the toxic carcinogens from formaldehyde throughout the progressive tissue damage and impaired staining. Finally, the samples were placed in a 70% ethanol solution at ambient temperature (25 ºC ± 3 ºC) for long-term storage.

Since our sample size was typically less than 3 mm, samples were decalcified in a Surgipath Decalcifier I (SDI) solution for tissue decalcification in approximately 2 hrs. (Figure 1). Our laboratory completed the process of dehydrating, clearing, embedding, sectioning, and staining. If the continuous process method is not preferred, these samples could be stored in 70% ETOH.

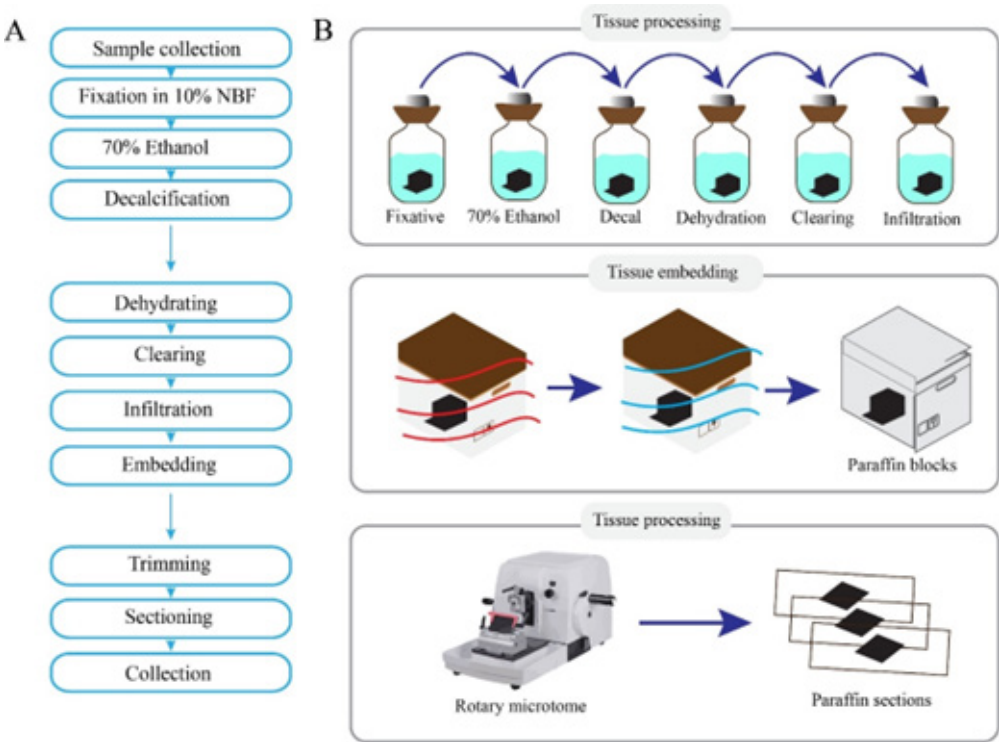

Figure 1 Recommended processing for photographic series. (A) Flowchart for modified histological protocol and (B) Illustrated procedure for tissue processing, embedding and sectioning methods for the marine invertebrates

Dehydrating, clearing, and embedding

Our histology laboratory followed a standard method (Humason, 1979; Ahmed, 2015) and a modified method, which differed in the steps performed using an automatic tissue processor (Thermo scientific STP1020, Leica Microsystem Nussloch GmbH, Figures 1-2). A complete consideration of this process is shown in Table 1. Dehydrating is a step used to remove water from tissue. Samples were dehydrated sequentially and made up using an ethyl alcohol series including 70% and 80% for thirty minutes each, 95% I and 95% II two times for ninety and one hundred twenty minutes each throughout 100% (absolute ethyl alcohol) three times for one hundred twenty minutes each. They were performed continuously under a clearing method, which was used with the clearing agent twice. Each time used ninety and one hundred twenty minutes before introducing xylene: paraplast (ratio 1:1) at 60°C. It should be noted that the Paraffin wax was melted in an oven at a constant temperature of 65 °C. The initially infiltrated process was used to maintain the hardness of the tissues. All samples were then processed with new paraplast at 60°C at least three times. After tissue processing, tissue from the standard method was still soft, while the tissue from the modified method was hard. Notably, the base mold should be heat resistant and made of aluminum alloy. Next, the specimen was embedded in paraffin wax at 60°C without the production of bubbles and affixed to an embedding ring, in which the samples were solidified (Figures 3A-3G).

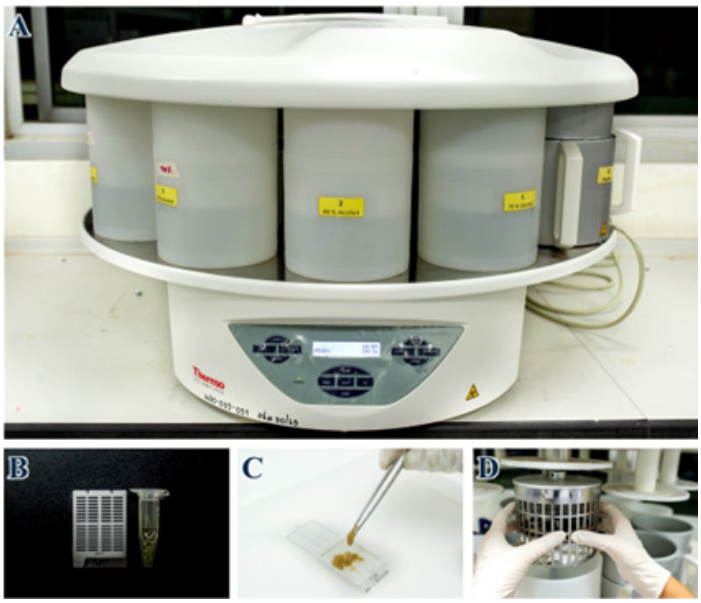

Figure 2 An automatic tissue processor for tissue processing showing Automatic tissue processor machine (A); Tissue cassettes and fixed sample in 10% neutral buffer formalin (B); Preparing tissue of *Xestospongia* sp. within cassettes (C); Tissue transportation into the cassette baskets (D)

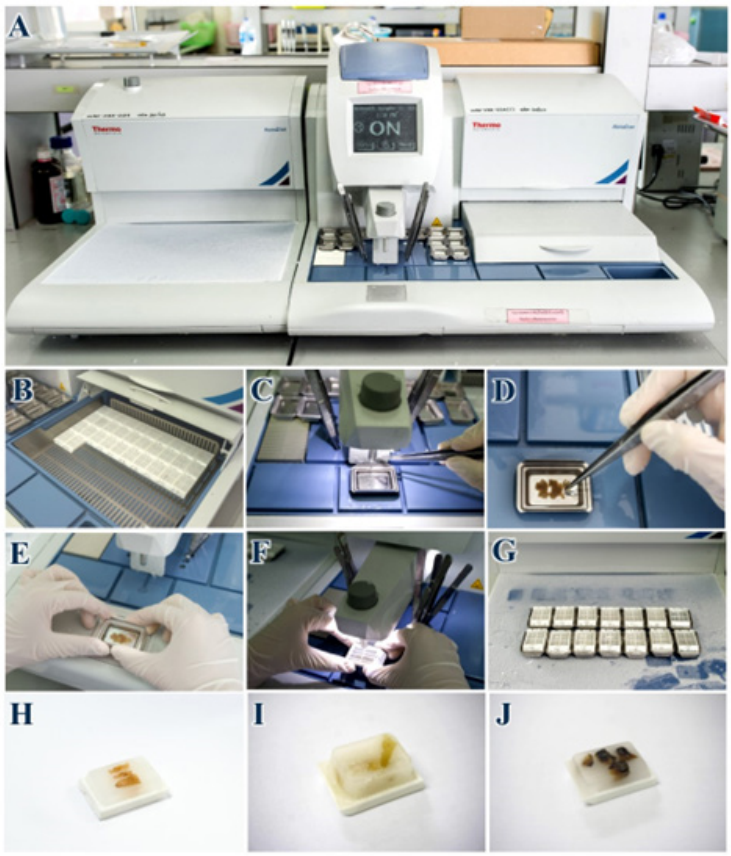

Figure 3 Photographic series for tissue embedding center (A) and an embedding process (B-H); (B) The cassettes in the paraffin bath having at 60 ºC; (C) The melted paraffin adding the base mold; (D) The sampled tissues in paraffin melt; (E-F) Moving paraffin embedding in cold place; (G) Embedding tissue into the paraffin blocks; (H) Homogenous paraffin in the paraffin block from modified method; (I-J) Heterogeneous paraffin in the paraffin blocks from the standard method.

Sectioning

After the tissue was solidified, the paraffin tissue blocks were trimmed (Figure 3G). They should be kept at -20 °C before sectioning and performed to recommend as a thinner at 4 µm using a rotary microtome (HM325 Manual Microtome, Histo-Line Laboratories. (Figures 4A-4B). Floating tissue sections were placed in a heated water bath to remove wrinkles before being affixed on glass slides. Avoiding a high temperature of more than 60 °C is recommended, which would result in the loss of the section. The unstained slides could be dried either at ambient temperature overnight or in a drying oven for a minimum of 1 h at 60°C before staining (Figures 4C-4D). It is noted that the paraffin blocks of marine invertebrate from standard tissue were a hard material and more difficult to section than the modified method. However, poor section quality, such as chatter, knife marks, folding, and wrinkling (Figure 4E) should be revised during the sectioning method.

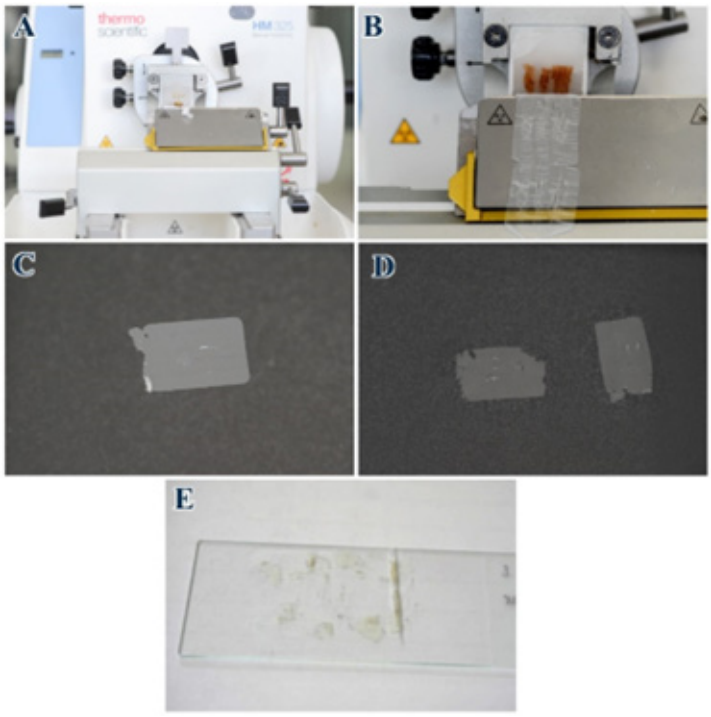

Figure 4 Photographic series in the sectioning process with the microtome shown the low quality section (A) and the high quality ribbon (B). Representative unstained-section between optimal (C) and high temperatures (D). Common artifacts from microtome sectioning (E).

Staining

A series of three stained guidelines is shown in Figure 5. After the slides were stained (Figures 6A-6B), a mounting medium was applied to the slide and covered with a glass coverslip (Figures 6C-6D).

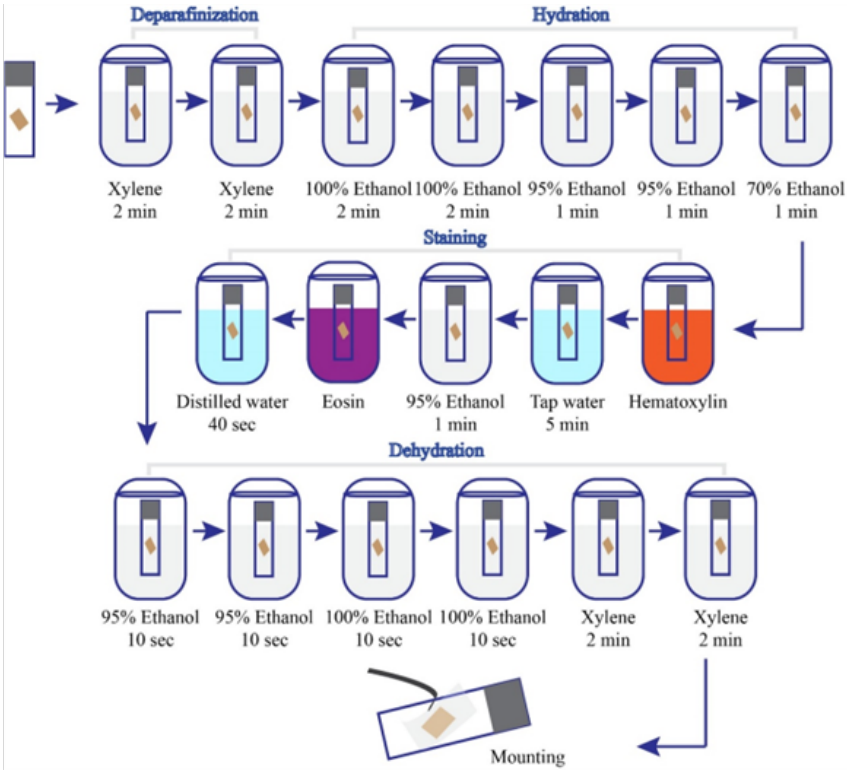

Figure 5 Recommended staining protocol throughout the mount microscope slides

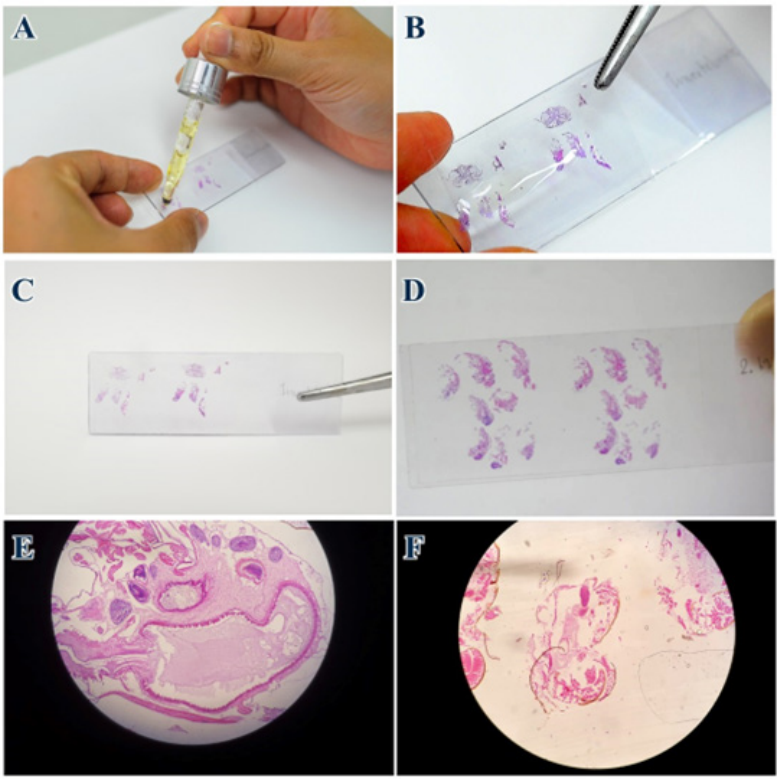

Figure 6 Photographic series on the observation of stained sections under light microscopy by adding mounting medium (A) and placing cover slips onto slides (B) throughout the complete microscope slide (C-D). Under light microscopic showed the high-quality H&E stain of *Amphibalanus amphitrite* (E), and low-quality H&E stain of *Halobates hayanus* (F).

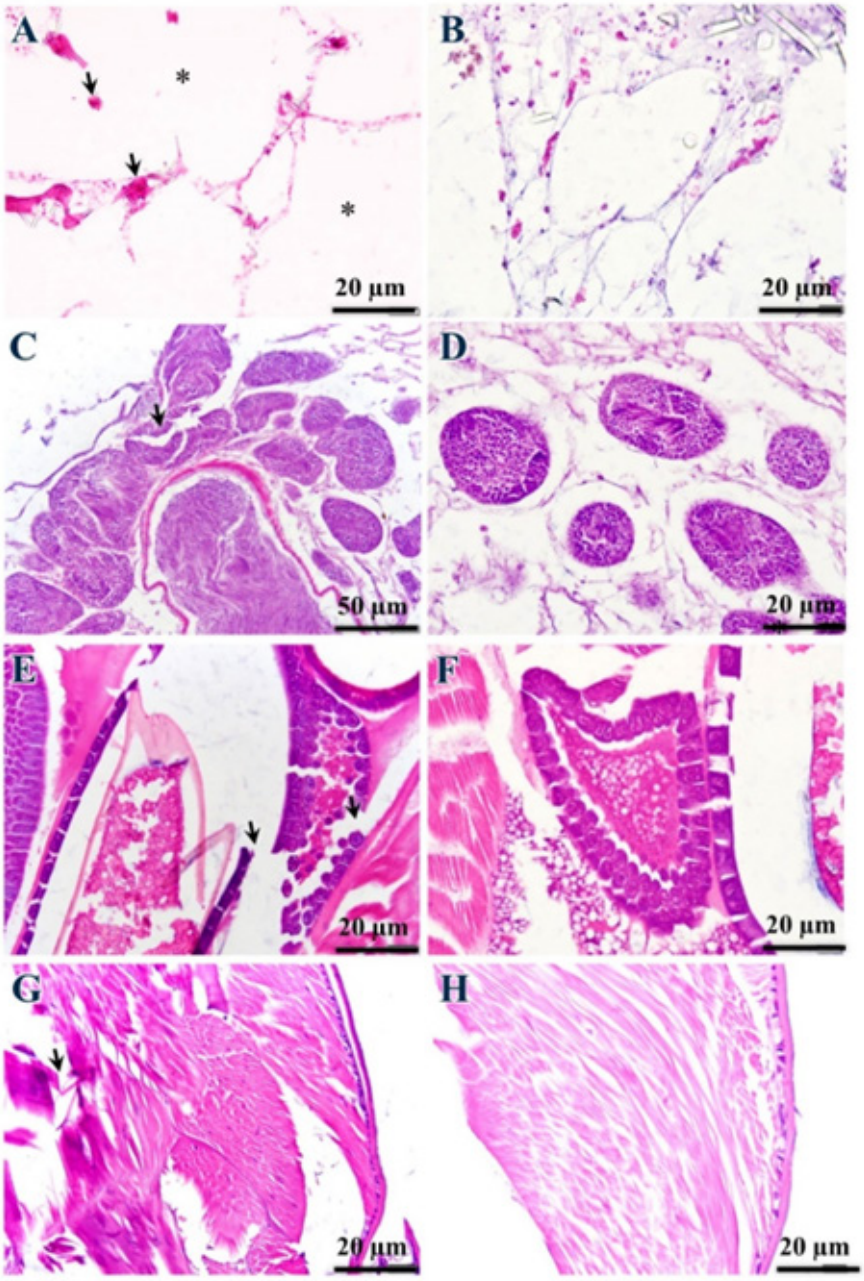

Figure 7 Comparative histological images with H&E staining method from the selected samples between the standard method (A, C, E, G) and modified method (B, D, F, H) including *Xestospongia* sp.(A-B), testicular follicles of *Amphibalanus amphitrite* (C-D); the oocyte of *Halobates hayanus* (E-F) and the muscular tissue of *Alpheus* sp (G-H). Noted: Asterisks = tissue loss, Arrows = artifacts

Digital image capture

According to the digital image capture, we used the slide scanner technology for highly effective performance. All stained slides were scanned and photographed using a 3DHISTECH Panoramic Viewer (3DHISTECH, Hungary) at a higher resolution (40x) and digital Canon 750. The final sets of the histologically-scanned figures were saved and observed with a SlideViewer program (3DHISTECH Ltd, https://www.3dhistech.com/research/software-downloads/). They clearly showed the ability to distinguish different tissue types and well-identified nuclei. The representative organs from marine invertebrates are shown between the standard method and the modified method in Figures 7A-7H. It is noted that the standard method showed some artifact techniques as well as loss of tissue (Figure 7A) and muscular tissue (Figure 7G).

DISCUSSION

It is accepted that the samples were euthanized to prevent autolysis and degradation of body morphology with a rapidly cooling shock. This method should be recommended for hypothermal shock with immersion in ice water (2–4 °C) as well as a more rapid and reduced stressful status (Wilson et al., 2009; Matthews and Varga, 2012). Afterward, the samples were fixed in 10% neutral buffered formalin. Similarly, Berzins et al. (2021) suggested that the invertebrate samples should be kept within 24 hrs. to reduce the negative effect on the cells. It should be noted that the fixation times might vary depending on the sample size. If the samples are more than 5 cm in diameter, they should be trimmed to a smaller size to reduce tissue degeneration. This is a general histological tissue fixative that is ideally suited for cross-linking certain proteins and preventing degradation by inactivating enzymes, suggesting a standard recommended solution for formalin-fixed paraffin-embedded samples (Copper et al., 2018; Snyder et al., 2022).

Following fixation and immersion in 70% ETOH, decalcification using 7% hydrochloric acid (HCl), 5% nitric acid, and 10% ethylenediaminetetraacetic acid (EDTA) is widely used (Copper et al., 2018; Snyder et al., 2022). However, we recommend that the surgipath decalcifier solution (Surgipath Decalcifier I: SDI) be utilized to decalcify the samples' exoskeleton, mineralized tissue, and thickness-gut diets prior to routine sectioning of paraffin-embedded blocks. According to previous reports, the SDI has the best ability to fix and preserve high-quality microscopic features in short processing times (https://shop.leicabiosystems.com/us/histology-consumables/reagents-solutions/pid-decalcifier-system).

Our histology laboratory followed a standard protocol for tissue processing, dehydrating, clearing, and staining samples as typically used and previously described (Clarke et al., 2007; Sun et al., 2009; Ghanaati et al., 2011). It is accepted that the xylene solution is routine histopathology since it clears the tissue and renders it transparent (Swamy et al., 2015) while removing the alcohol (Muddana et al., 2017). The histological slides were successful when the purple nucleus and pinkness cytoplasm were accepted. The standard method showed some artifact techniques and potential coincidental structural damage to specimens. In contrast to the modified method, there are good results to support marine animal histology.

CONCLUSIONS

We developed a modified histological-assisted method for evaluating tissue and cells in marine aquatic invertebrates that can be easily accomplished in four days. This approach has several advantages, i.e. impressive efficiency for tissue and cell identification when compared to the standard method. From a practical standpoint, this is also true, and we anticipate that this modified technique could be applied as an adjunctive method, such as for cryosections and transmission electron microscopy (TEM), etc.

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AUTHOR CONTRIBUTIONS

Kitiya Kongthong: Investigation, methodology, formal analysis, manuscript preparation.
Natthawut Charoenphon: Methodology, editing and formal analysis.
Narit Thaochan: Methodology, editing and formal analysis.
Piyakorn Boonyoung: Methodology, editing and formal analysis.
Atsuo lida: Editing and formal analysis.
Archig Jeamah: Sample collection, editing and formal analysis.
Supapong Imsonpang: Editing and formal analysis.
Koraon Wongkhamhaeng: Editing and formal analysis.
Piyamat Kongtueng: Methodology, editing and formal analysis.
Sinlapachai Senarat: Conceptualization, Investigation, formal analysis and design the experiment, supervision and finalization.

CONFLICT OF INTEREST

We have no conflict of interest.

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