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Microwave-accelerated processing of coral tissue histology

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Abstract

Background: Microwave technology has revolutionized histological processing by reducing processing time and improving tissue integrity. We describe the first microwave-accelerated tissue processing procedure for healthy and diseased tissue fragments of two species of corals: the gorgonian *Gorgonia ventalina* and the scleractinian *Acropora cervicornis*.

Methods: Fourteen tissue samples from sea fans (eleven healthy and three diseased), and one tissue sample from a healthy *A. cervicornis*, were decalcified and processed using microwaves. Histological slices were stained using hematoxylin and eosin and immunostained using an antibody against *Aspergillus*.

Results: By using microwave technology, the decalcification time, as well as the steps and time for tissue processing were significantly reduced while maintaining the integrity of the tissue.

Conclusions: This technique accelerates the chemical processing of coral tissue, while providing high quality and optimal resolution of histological sections.

Keywords: Histology, microwave, tissue processing, coral diseases

Introduction

Although the basics of tissue processing for microscopic examination remained unchanged during the early decades of the twentieth century, the advent of automation of dehydration, clearing, and infiltration processes during the 1940s added consistency to labor intensive and tedious procedures, not only simplifying the routine but also significantly reducing the time of processing from 48 or more hours to 22 to 28 hours [1-3]. First used in 1970, automated microwave processing technology has brought even greater improvement to histological procedures, and has been used across a wide spectrum of histological methods, including tissue fixation [4], decalcification and tissue processing (as shown here) [5], and staining for light and electron microscopy [6]. A major advantage of microwave energy is the reduction in turn-around time of slide completion. Processing tissue with traditional technology required 12 to 15 steps and took at least 9 hours [6]. In contrast, microwave processing reduces protocols to 4 to 5 steps that are immensely simplified and require significantly less time. Moreover, microwave processing provides vastly improved quality and resolution [7].

Microwave technology has rarely been used for histology of invertebrates. Non-variable wattage microwaves designed for cooking have been used for fixation of bivalve mollusk tissues [8], tissue dehydration of flatworms [9], tissue fixation and dehydration of tunicates [10], and for partial tissue decalcification of scleractinian corals [11]. Histologically-related studies of corals, including the field of coral diseases, have never used microwave technology for tissue processing. However, there is an urgent need to develop new tools to better describe and diagnose diseases affecting corals. Currently, most diseases of corals are diagnosed based on macroscopic characteristics [12]. This lack of concise and objective demonstration of histopathological features has fueled the debate regarding the structural features of healthy and diseased corals.

Here we present a histopathological protocol for coral using microwave technology that reduces the turn-around time of completion of microscope tissue slides, while maintaining the structural, cellular, and protein integrity of processed tissues, required to accurately describe and diagnose coral diseases. Moreover, we show that the protocol is amenable for fluorescence immunostaining by using an antibody

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against *Aspergillus*, a common fungus known to affect coral's health. Therefore, our results are remarkably relevant to the growing field of coral diseases and can be extended to many other studies where fast, efficient methodologies and the maintenance of structural properties of evaluated tissues are required.

Methods

Tissue collection and fixation

Eleven healthy and three diseased tissue samples between 10.0-49.0mm^2 and with an average thickness of 2mm were collected from specimens of the Caribbean sea fan *Gorgonia ventalina*. For this study, "healthy" is defined as sea fans that had no lesions, no purpling, and no overgrowth by fouling organisms. "Diseased" is defined as coral with dead tissue surrounded by a purpled halo. Additionally, one fragment between 4-5mm in thickness and 2.5mm in diameter was collected from a healthy scleractinian staghorn coral (*Acropora cervicornis*). The tissue samples were collected under permit (Puerto Rico Department of Natural Resources permit number 04-IC-051 issued to Carlos Toledo-Hernández). Immediately after collection, tissue samples were fixed for 1 hour in a modified Karnovsky's fixative (2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) and 2% paraformaldehyde (Sigma Aldrich, St. Louis, MO) diluted in filtered seawater) at room temperature [13]. Following fixation, tissue samples were rinsed 3 times (10 min each) in filtered seawater.

Gorgonian and scleractinian coral tissue sample decalcification

Gorgonian and scleractinian coral tissue samples were transferred to a decalcifying solution (100g ethylenediamine tetraacetic acid [EDTA] (Fisher Scientific, Waltham, MA), 10g NaOH/L filtered seawater, pH 7.4) and placed in a LabPulse™ Microwave Tissue Processor (H 2850, Energy Beam Sciences, CT). Sea fan fragments between 10 to 20mm^2 were decalcified for 20 min, while larger fans fragments (e.g., <20mm^2) and the *A. cervicornis* fragment were decalcified for 40 min (**Table 1**). Decalcification was determined to be completed when no sclerites (calcium carbonate spines) were observed on the gorgonian sample surface, and the calcium carbonate skeleton was dissolved from the scleractinian sample. Preliminary experiments were done using different power levels of the microwave and reagent volumes to determine the optimal microwave settings for coral tissue (data not shown). For the results presented in this report, tissues were processed at 40°C for 20-40 min at a working power of 80% (720 watts). Note that the microwave power level for decalcification and tissue processing was set significantly higher (720 watts) than the power level that normally has been suggested (250-500 watts) [14]. The overall results suggested that at constant temperature, low volume and a high power level (720 watts), the decalcification process and tissue processing were accelerated without detrimentally affecting the integrity of the tissues. Therefore, the temperature is controlled by the volume of the reagent, and independent of the power level setting of the microwave.

Table 1. Steps, with volumes, temperatures, and times required for histological processing using microwave technology.

Histological processing using microwave technology				
Step	**Solutions**	**Vol.(ml)**	**Temp(°c)**	**Time(min)**
1	Decal-solution	300	40	20-40
2	Ethanol 95%	500	40	4
3	Ethanol 95%/100% 2-Propanol	500	40	4
4	100% 2-Propanol	500	40	4
5	2-Propanol/Paraffin	500	80	10
6	Paraffin vacuum	--	60	30

Gorgonian and scleractinian coral tissue sample processing

Following decalcification, gorgonian and scleractinian coral tissue samples were washed in filtered seawater twice (10 min each). Then, tissues were placed in the microwave for dehydration in a sequence of 95% ethanol (Fisher Scientific, Waltham, MA, 95% ethanol: 100% isopropanol (2-propanol hereafter) [Fisher Scientific, Waltham, MA], and cleared in 100% 2-propanol. Each step lasted 4 min and temperature was set to 40°C. Next, tissue samples were kept in the microwave for infiltration in a 1:1 solution of melted paraffin and 2-propanol under vacuum for 10 min at 60°C (**Table 1**). A second round of infiltration was performed with 100% melted paraffin under vacuum for 30 min at 60°C. During the infiltration process, a sheet of PolarHeat™ (Energy Beam Sciences, CT) was used to convert microwave energy to heat, resulting in an increase in diffusivity of the paraffin through the tissue [14].

Sectioning and hematoxylin and eosin (H&E) staining

Thin sections (4-5µm) from gorgonian and scleractinian coral tissue were obtained using a rotary microtome (model 820; American Optical, Buffalo, NY). Sections were affixed to subbed-glass slides (chrome alum-gelatin coating solution: 50.0 g gelatin (Mallinckrodt, St. Louis, MO) and 5.0 g chromium potassium sulfate (Mallinckrodt, St. Louis, MO) in 1 L distilled deionized water.

Sections were deparaffinized with Histo-Clear twice, (30 s each, National Diagnostics, Charlotte, NC), re-hydrated through a descending series of ethanols (95%-70% ethanol each for 30 s), washed in distilled deionized water (2X each 30 s), stained in Harris hematoxylin (C.I. #75290, Sigma Aldrich, St. Louis, MO) solution for 1 min, washed in running tap water for 1 min, differentiated in Scott solution (10g sodium bicarbonate; 2g magnesium sulfate in 1 L distilled deionized water; [15]) for 30 s, and washed in running tap water again for 1 min. Bluing was done using 0.2% ammonia water for 30 s to 1 min.

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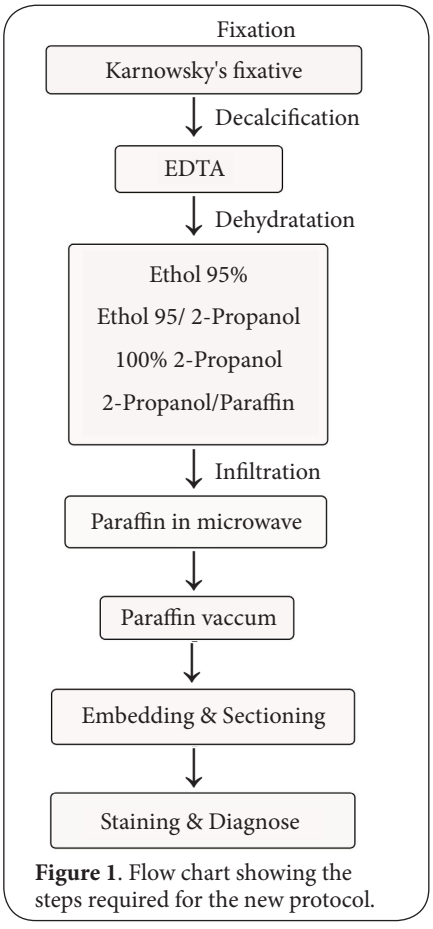

Figure 1. Flow chart showing the steps required for the new protocol.

Sections were washed in running tap water for 5 min; rinsed in 95% alcohol (10 dips); counterstained in eosin Y (C.I. #45380, National Aniline and Chemical Company, Buffalo, NY) solution for 30 sec to 1 min; dehydrated through 95% alcohol and 2 changes of absolute alcohol, 2 min each; cleared in Histo-Clear (2X each for 5 min); and coverslipped using Permount (VWR, Radnor, PA), a xylene based mounting medium.

Immunohistochemistry

For fluorescence immunohistochemistry, thin tissue sections (5µm) from healthy gorgonian coral were deparaffinized and hydrated through a descending series of ethanols (explained previously). Sections were permeabilized in 0.05% Triton X-100 (Sigma Aldrich, St. Louis, MO) for 15 min at room temperature. Then, tissue sections were rinsed in filtered seawater and incubated for 1 hour at room temperature in a blocking solution (0.05% Triton X-100; 3% of normal goat serum; Jackson ImmunoResearch, West Grove, PA) in filtered seawater to prevent non-specific binding. After blocking, the tissue sections were rinsed with filtered seawater for 5 min and then incubated with a rabbit polyclonal primary antibody (1/500 diluted in blocking solution) against *Aspergillus* (Abcam, Cambridge, MA) at 4°C for 24 hours. Next, tissue sections were rinsed with filtered seawater for 5 min and immediately after, to prevent any endogenous fluorescence, tissue sections were blocked with 0.1% sodium borohydrate for 30 mins. Tissue sections were rinsed again with filtered seawater for 5 min and incubated for 2 hours at room temperature with Alexa Fluor 546® (Invitrogen, Eugene, OR) goat anti-rabbit secondary antibody (1/200 diluted in blocking solution) (Life Technology, Carlsbad, CA). Finally, tissue sections were washed twice (30 s each) in filtered seawater and then labeled with Alexa Fluor 488 Phalloidin (Life Technology, Carlsbad, CA) for 5 min, rinsed again in filtered seawater (2X each 30 s), and nuclei were stained with TO-PRO-3® (1/500 diluted in filtered seawater; Life Technology, Carlsbad, CA). Control slides were prepared precisely as described but utilizing the primary antibody only. Additional controls utilizing secondary antibodies only, were also performed. No immunohistochemical labeling was performed on *A. cervicornis*.

Image acquisition

Tissue sections were observed using appropriate filter sets for Alexa 546, phalloidin 488, and TO-PRO-3® (642/661nm) in a Nikon Eclipse 800 (E800) optical microscope equipped with the modular Nikon C1 Plus laser scanning confocal system that is fully controlled by Nikon Elements AR (NISO) software. The E800 is equipped with differential interference contrast (DIC) and 10X Plan Fluor (N.A.=0.30), 20X Plan Fluor (N.A.=0.45), 40X Plan Fluor (N.A.=0.65), 60X S Fluor with correction collar ring (N.A.=0.85), and 100X Plan Apo Oil immersed (N.A.=1.40) objectives. Images were acquired with a QImaging Retiga Exi camera (QImaging, Canada) with color filter. Image calibration and measurements were performed using Nikon NIS-Elements. Adobe Photoshop CS4 was used for figure plate preparation.

Results

The methods described in this report provided high quality longitudinal and cross tissue slides from corals (e.g., polyps). For instance, a longitudinal section of a sea fan polyp retracted into its calyx, with 4 extended tentacles surrounding the mouth is shown in **Figure 2a**. Although an artifact is observed in the separation of the basal tissue from the axial skeleton, this artifact is also frequently observed on histological preparations of octocoral using conventional techniques [**16**,**17**]. In fact, the microwave technique seems to minimize this effect. A cross sectional view of a polyp showing the mouth surrounded by 8 hollow radial chambers lined by gastrodermal tissue is also observed (**Figure 2b**). Many minute mesoglea openings surrounding the polyp are shown, some of which have spindle-like shapes [**18**]. These openings are lacunae remaining after sclerites were decalcified. In addition, another cross sectional view of a polyp shows a mouth surrounded by 8 tentacles filled with gastrodermal tissue (**Figure 2c**), each of which are separated by a septum also lined with gastrodermal tissue (**Figure 2d**).

Healthy coenenchyme (tissue between polyps) of sea fan and associated tissues and cells were also successfully

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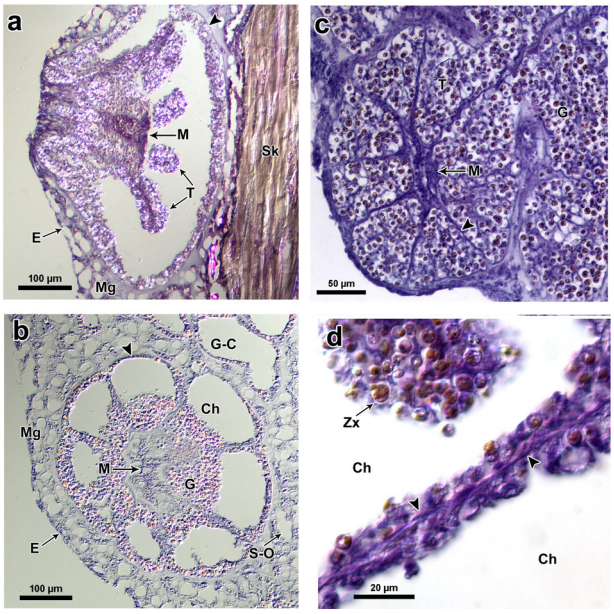

Figure 2. Bright-field and differential contrast (DIC) low and high magnification images of H&E stained *G. ventalina* polyps.
(**a**) Lateral view differential interference constrast (DIC) image of cross section of a polyp (10x). (**b**) DIC image of cross section of polyp (10x). Arrowhead shows membrane boundaries between gastrodermal tissue and mouth and polyp and mesoglea (10x). (**c**) Cross section of a polyp (40x); arrowhead shows membrane boundaries. (**d**) 60x magnification DIC image of membrane (arrowhead) separating chamber of a polyp. Abbreviations: Ch: Chamber; E: Epithelium; G-C: Gastrodermal channel; G: Gastrodermis; Membrane (arrowhead); Mg: Mesoglea ; M: Mouth; S-O: Sclerite opening; Sk: Skeleton; T: Tentacle; Zx: Zooxanthellae.

preserved and readily defined at low (**Figure 3a**) and high magnification (**Figures 3b** and **3c**). An epithelium ranging in thickness from 29 to 37µm is clearly observed separating the external environment from the gastrodermal tissue (**Figures 3a** and **3c**). Within the gastrodermal tissue, round zooxanthellae ranging from 5 to 7µm in diameter are surrounded by vacuoles within host cells (**Figure 3b**). In the mesoglea, sclerite openings are shown, surrounded by sclerocytes with round basophilic positive nuclei ranging in diameter from 3 to 6µm (**Figure 3c**).

The new protocol is also useful for describing the cytology of diseased tissues. For instance, the characteristic features of diseased sea fan tissue: a complete absence of epithelium, broken mesoglea, and gastrodermal channels with their zooxanthellae outside their host gastrodermal cells are readily observed (**Figure 4a**). Hyphae–like structures surrounded by skeletal material as well as capsules, spores, and filamentous-like structures are also observed within the diseased tissue (**Figures 4b** and **4d**).

Additionally, the use of the accelerated microwave procedure assures the antigenicity of proteins, enabling us to perform fluorescence immunohistochemistry. For instance, **Figure 5**

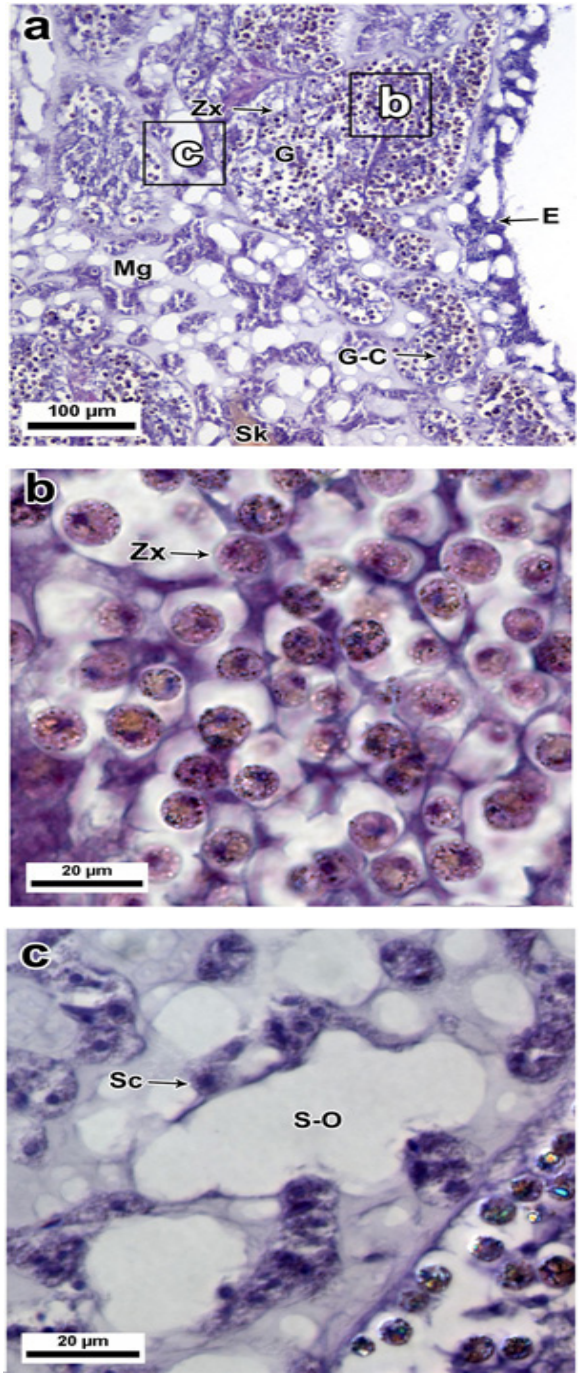

Figure 3. Bright-field low and high magnification images of healthy H&E stained coenenchyme tissue from *G. ventalina*.
(**a**) Healthy coenenchyme (10x). (**b**) Magnified view of **Figure 4a** showing rounded zooxanthellae encaged in vacuoles within the gastrodermis (100x oil immersion). (**c**) Magnified view of **Figure 4a** showing sclerite scarring and amoebocyte-like cells within the mesogleal tissue (60x oil immersion). Abbreviations: Sc: Sclerocytes-like cells; E: Epithelium; G-C: Gastrodermal channel; G: Gastrodermis; Mg: Mesoglea; S: Sclerite opening; Sk: Skeleton; Zx: Zooxanthellae.

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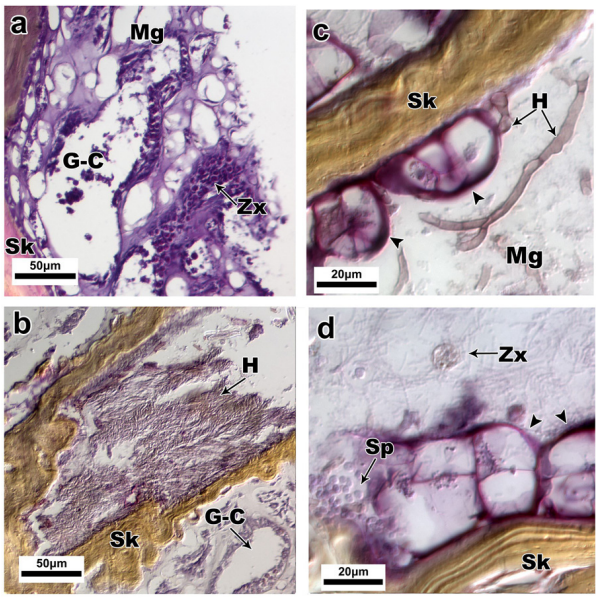

Figure 4. Bright-field images of H&E stained diseased tissue from *G. ventalina*.
(**a**) Disrupted gastrodermal and mesogleal tissue (40x). (**b**) Hyphae-like structures within the axis (40x). (**c**) Capsule-like structures (arrowheads) and fungal hyphae within the mesogleal tissue (60x oil immersed). (**d**) Capsule-like structures (arrowheads), spore-like cells, and zooxanthellae within mesoglea (60x oil immersed). Abbreviations: G-C: Gastrodermal chamber; H: Hyphae-like structures; Mg: Mesoglea; Sk: Skeleton; Sp: Spore-like structures; Zx: Zooxanthellae.

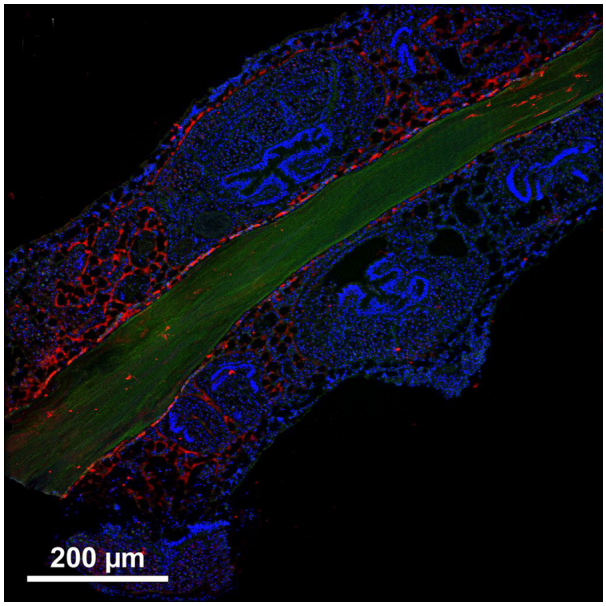

Figure 5. Confocal images of *Aspergillus* spp. identified by using fluorescence immunohistochemistry in healthy *G. ventalina* tissue.
Actin (green; phalloidin); *Aspergillus* (red; Alexa Fluor 546); Nuclei (blue; To-Pro-3®). Scale bar=200µm.

shows immunohistochemistry staining of a healthy sea fan tissue. Filaments of actin are primarily detected on the axial skeleton, whereas *Aspergillus* spp. is detected across the coenenchyme, bordering the polyps, and the axial skeleton. Nuclei are detected throughout the cells found across coenenchyme and polyps. Moreover, this result adds further evidence supporting the argument that fungi and in particular *Aspergillus* fungi are common to healthy sea fans [19-22].

As with gorgonians, this technique can also be used, with excellent results, on scleractians coral. For instance, **Figure 6** shows tentacle epithelium of a healthy *A. cervicornis*. Numerous spirocyst across the epithelia are observed, while junction of mesoglea and myoepithelial cells are seemingly right underneath the epithelium.

Figure 6. Bright field images of healthy H&E stained *A. cervicornis* tissue.
Epithelium and gastrodermal tissue (20x). Complex interface of epithelial cells and mesoglea (arrow heads). Insert: Magnification of epithelium and gastrodermal tissue (40X). Abbreviations: E: Epithelium; G: Gastrodermis; Sp: Spyrocst-like cells.

Discussion

Fourteen *G. ventalina* tissue samples (11 healthy and 3 diseased) and one healthy *A. cervicornis* were collected, fixed, decalcified, dehydrated, embedded with paraffin, sliced, stained and prepared for light microscopy examination in approximately 5 hours (**Figure 1** and **Table 1**). This constitutes the shortest time ever reported for a cnidarians histopathology study. In contrast, common procedures of coral histopathology take several days to complete tissue slices for light microscopic examination. For example, the average time of tissue fixation in gorgonians is 28 hours, with times ranging from 12-48 hours [23-28]. We reduced the fixation time to 1 hour. Similarly, decalcification time in previous studies ranged between 12 to 94 hours with EDTA [24,26,29] or 12 hours in ascorbic acid [28] at room temperature. In this study, tissue decalcification with

the microwave oven took between 20 to 40 minutes (**Table 1**). Fragments between 10 to 20mm^2 were decalcified in 20 min, while larger fragments (e.g., <20mm^2) were decalcified in 40 min. Standard procedures of tissue processing generally take 9 hours, using 7 washes of alcohols and several washes of clearing solutions, i.e., xylene [**23**]. We reduced tissue processing to 1.47 hours**,** while at the same time reducing the treatments of alcohol and their volumes for tissue dehydration to 2, and substituting 2-propanol for xylene in the step of tissue clearing (**Table 1**). Unlike xylene, 2-propanol does not cause tissue samples to shrink or harden after clearing, and 2-propanol is less toxic to humans than xylene [**1**,**30**,**31**]. Furthermore, by substituting 2-propanol for xylene we improved the immunohistochemical staining of cells (**Figure 6**).

Conclusion

This technique not only accelerated the chemical processing of tissue, but also provided sections of high histological quality and resolution and, thereby, the capacity to distinguish between tissues and structures that are in close proximity. The microwave-accelerated tissue processing procedure also has several advantages over the conventional methods. First, it is versatile, as bright-field microscopy, immunohistochemistry and electron-microcopy (data not shown) can be conducted with the same equipment and tissue preparations. In addition, with minor modification, it can be used for other corals or invertebrates. Second, it is safer than the conventional method, because the reagents used with it are less toxic than those generally used [**1**,**30**,**32**]. Third, it is more cost efficient than the conventional method because it significantly reduces the number of steps and volumes of reagents used. Lastly, in contrast to the conventional method, microwave-accelerated processing is less time consuming: it takes approximately 5 hours to complete, as opposed to the 72 to 96 hours of the conventional method.

Additionally, in light of the current decline of corals due in part to diseases [**33**], this methodology may improve our understanding about their etiologies. Most coral diseases, including aspergillosis of gorgonian and white band syndromes (WBS) in acroporid corals, are diagnosed almost exclusively based on the macroscopic manifestation of the disease. However, macroscopic diagnoses of coral diseases may cause confusion and misinterpretation when different pathogenic agents induce similar macroscopic responses to the coral host. Yet, this technique should improve our capacity to diagnose these diseases accurately and rapidly, further improving our understanding of coral diseases, and thus our ability to effectively respond to them.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	CTH	IIRV	JLSV
Research concept and design	✓	✓	--
Collection and/or assembly of data	✓	--	✓
Data analysis and interpretation	✓	✓	✓
Writing the article	✓	✓	✓
Critical revision of the article	✓	✓	✓
Final approval of article	✓	✓	✓
Statistical analysis	✓	✓	✓

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