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Short Communication

Staining protocol for the histological study of sea anemones (Anthozoa: Actiniaria) with recommendations for anesthesia and fixation of specimens

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ABSTRACT. Many of the characteristics used in sea anemone taxonomy can only be examined through histological sections. Since there is no standardized procedure for this purpose, various anesthesia and fixation techniques applied to specimens of the intertidal species *Anthopleura hermaphroditica* and *Bunodactis hermafroditica* are discussed. Additionally, further modifications are proposed to the Masson's trichrome method according to the results obtained on these species. The combined effect of the short application of menthol crystals, together with small doses of $MgCl_2$ were the most satisfactory anesthetics for maintaining the specimens expanded. The best preparations were obtained from samples fixed for several months in 8% seawater formalin; however, in order to achieve a good differentiation of the tissue, mordanting the samples with Bouin's fixative was necessary. Besides being a fast method, the modified Masson's trichrome gives very good contrasts between the epithelia and the mesoglea, and allows controlling the timing of differentiation during staining. The present paper includes suggestions and precautions and thus offers practical help for the histological study of sea anemones.

Keywords: Actiniidae, *Anthopleura*, *Bunodactis*, Masson's trichrome, histology.

Protocolo de tinción para el estudio histológico de anémonas (Anthozoa: Actiniaria), con recomendaciones para la anestesia y fijación de especímenes

RESUMEN. Muchos de los caracteres empleados en la taxonomía de anémonas sólo pueden ser examinados en cortes histológicos. Dado que no existe un procedimiento estandarizado para este fin, se analizan distintas técnicas de anestesia y fijación aplicadas a ejemplares de las especies intermareales *Anthopleura hermaphroditica* y *Bunodactis hermafroditica*. Adicionalmente, se proponen nuevas modificaciones al método tricrómico de Masson acorde a los resultados obtenidos en estas especies. Los efectos combinados de los cristales de mentol en lapsos cortos con pequeñas dosis de $MgCl_2$, fueron los anestésicos más satisfactorios para conservar a los organismos en posición expandida. Las mejores preparaciones fueron obtenidas de muestras fijadas por varios meses en formalina al 8% en agua de mar, no obstante, para lograr una buena diferenciación de los tejidos fue necesario mordentar las muestras en fijador de Bouin. Además de ser un método rápido, el tricrómico de Masson modificado genera muy buenos contrastes entre los epitelios y la mesoglea, permitiendo controlar los tiempos de diferenciación a lo largo de la tinción. Haciendo tanto sugerencias como precauciones, este trabajo ofrece una ayuda práctica para el estudio histológico de anémonas.

Palabras clave: Actiniidae, *Anthopleura*, *Bunodactis*, tricrómico de Masson, histología.

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Permanent histological preparations play a fundamental role in taxonomic studies of sea anemones. Many of the characteristics used in the identification of species must go through a dying process to be analyzed. Basilar musculature, for instance, has been traditionally employed on taxonomic keys (Stephenson, 1935; Carlgren, 1949) to distinguish large groups within Actiniaria. Certain muscles such as the sphincter not only vary in shape and degree of development, but also in the tissue derivation (endodermal or mesogleal), which, until today, has been particularly useful to define families. Even at genus level, there are

specific biological characteristics (*e.g.*, type of reproduction) that can only be verified through histology. In all of these cases, descriptions depend on tested protocols in this group of organisms that can acquire a clear differentiation between the mesoglea and epithelial and muscle areas.

Since there is no standardized method for analyzing these structures, there are now as many histological techniques as sea anemone studies. Given the problems this creates to the taxonomic work, some authors have compared the different methods of anesthesia (see Moore, 1989), fixation (see Dunn, 1975) and staining (see Stotz, 1981), to establish which techniques are the most appropriate for these organisms. Parts of these procedures, however, depend largely on the specimens characteristics (*e.g.*, body size), information that usually is not considered in the methodologies. In fact, many of the protocols used are often unpublished modifications of the classical histological techniques. In Chile, only Stotz (1981) has compared different methods of anesthesia and staining, studying its effects on *Anthothoe chilensis* (Lesson, 1830), *Antholoba achates* (Drayton, 1846) and *Phymactis papillosa* (Lesson, 1830). This short communication introduces new observations for the narcotization and preservation of intertidal species *Anthopleura hermaphroditica* (Carlgren, 1899) and *Bunodactis hermafroditica* (Calgren, 1959), including some modifications to the Masson's trichrome method according to the results obtained by Spano *et al.* (*in press*). Therefore, this paper aims to promote the histological study of sea anemones, providing practical recommendations and a staining protocol suitable even in other marine invertebrates, such as flatworms, polychaetes and nematodes.

Methods of anesthesia

Before preservation, specimens of many taxa (especially invertebrates) must be relaxed to prevent contraction of the body and mucus secretion (Häussermann & Försterra, 2009). Menthol crystals, magnesium salts, chloral hydrate, tricaine methane-sulfonate (MS-222), alcohol or formalin (drip) and even relaxation by freezing are documented narcotization methods for sea anemones. Of these, few have yet proven to be suitable for histological studies (Moore, 1989). Today, the most accepted anesthetics for anemones are menthol crystals and magnesium salts ($MgCl_2$ and $MgSO_4$ at 3.5-7.5%); nevertheless, they can equally macerate the tissue depending on the time of exposure (Häussermann, 2004). In fact, although menthol crystals are especially effective in small organisms (column diameter 0.5-2 cm) (Stotz, 1981), very large amounts or very long exposure times always had deleterious effects on the internal anatomy of *Anthopleura hermaphroditica* and *Bunodactis hermafroditica*, species with a pedal disc near to a centimeter of diameter. Particularly in *B. hermafroditica*, 23 of the 25 specimens used for this study (>90% of the sample) wholly or partially contracted when anesthetized for over an hour. Because the amount of anesthetic depends both on the specimens as well as the volume of water used, the crystal size and the exposure time (Stephenson, 1928), a standardized protocol cannot be formulated since parameters are always species-specific. For *A. hermaphroditica*, for example, it was found that exposing the specimens to more than 10 g of menthol crystals in 50 mL of water for more than three hours, irreversibly damaged smaller individuals (<2 mm diameter pedal disc) and caused most of the large specimens to evert the actinopharynx. On the other hand, even when the animals do not respond to tactile stimuli, very short relaxation times can cause a delayed contraction of the organisms when immersed in the fixative. For *A. hermaphroditica*, the best results in terms of expansion were obtained by anesthetizing the specimens in less than 100 mL of water with 50 g of menthol crystals for no more than one hour. In both species, very good results were observed by additionally administering small doses (1 to 2 mL) of 6% $MgCl_2$ directly into the gastrovascular cavity of the animal, right before being immerse in the fixative. If they are kept in optimal conditions (*e.g.*, low light, water temperature below zero degrees), a 50 mL jar can satisfactorily relax up to 10 small specimens (<6 mm pedal disc diameter). While simple measures can be taken to control some of these variables (Stotz, 1981), in order to effectively elaborate a narcotization method, the anesthesia parameters must first be related to the ontogenetic characteristics of each species to be studied.

Methods of fixation

The selection of the fixative is particularly important for samples that are intended for histological processing. Although the most frequently used fixatives for sea anemones are seawater formalin and picric acid solutions such as aqueous Bouin, good results have also been achieved with heavy-metal based fixing fluids such as Heidenhain (Susa), Zenker and Helly. Each one influences the quality of the preparations differently according to the tissue characteristics of the species and the dyes used. The trichrome techniques, for example, it is recommended for organisms that were fixed in formalin to mordant for at least two days in picric acid before staining. For species with a thin mesoglea layer (<100 µ column),

Table 1. Detail of Masson's trichrome method (1928) with its respective modifications. *Check results under microscope.

Masson Trichrome Stain (1928)	Humason's modification (1962)	Luna's modification (1968)	Spano & Flores's modification (this study)
Deparaffinize and hydrate to water.	Deparaffinize and hydrate to water.	Deparaffinize and hydrate to distilled water.	Deparaffinize and hydrate to distilled water.
Mordant with iron alum 5%: 5 min (45°-50°C).	Mordant with iron alum 4%: ½ h.	Stain in Weigert's iron hematoxylin: 10 min.	Stain in Harri's hematoxylin: 3-5 min.
Wash in water	Wash in running water: 5 min.	Wash in tap water: 10 min.	Rinse (several times) in tap water.
Stain in Regaud's hematoxylin: 5 min (45°-50°C).	Stain in Delafield's hematoxylin: ½ h.	Wash in distilled water.	Differentiate in hydrochloric alcohol 1%: 30 seconds*.
Rinse in 95% alcohol.	Wash in running water: 5 minutes.	Stain in solution Biebrich Scarlett 1% - acid fuchsin 1%: 2 min	Rinse (several times) in tap water.
Differentiate in picric alcohol.	Differentiate in saturated aqueous picric acid.	Wash in distilled water.	Wash in distilled water: 5 min.
Wash in running water.	Wash in running water: ≥10 min.	Differentiate with solution of phosphomolybdic acid 2.5% - phosphotungstic acid 2.5%: 10-15 min.	Stain in solution acid fuchsin 0.1% - Ponceau de xylidine 0.2%: 6-10 min.
Stain in solution acid fuchsin 0.3% - Ponceau de xylidine 0.3%: 5 min.	Stain in acid fuchsin 1%: 5 min.	Rinse in distilled water (quick step).	Rinse in distilled water (two times).
Rinse in distilled water.	Rinse in distilled water until excess stain is removed.*	Stain in acetic aniline blue 2%: 5 min.	Differentiate with phosphotungstic acid 5%: 4-8 min*.
Differentiate in phosphomolybdic acid 1%: 5 min.	Stain in Ponceau de xylidine 1%: 1-5 min.	Wash in distilled water.	Rinse in distilled water (two times).
Stain in acetic aniline blue 2%: 5 min.	Rinse in tap water.*	Differentiate in acetic acid 1% rinse: 3-4 min.	Stain in acetic aniline blue 2%: 5-10 min.
Rinse in distilled water.	Differentiate in phosphomolybdic acid 1%: 5 min.	Dehydrate, clear and mount.	Differentiate in acetic acid 1% rinse until excess stain is removed.*
Back to phosphomolybdic acid 1%: 5 min.	Stain in acetic fast green 2%: 2 min.		Dehydrate (quickly), clear and mount.
Differentiate in acetic acid 1%: 5 min.	Differentiate in acetic acid 1% rinse: 1-2 min.		
Dehydrate, clear and mount.	Dehydrate, clear and mount.		

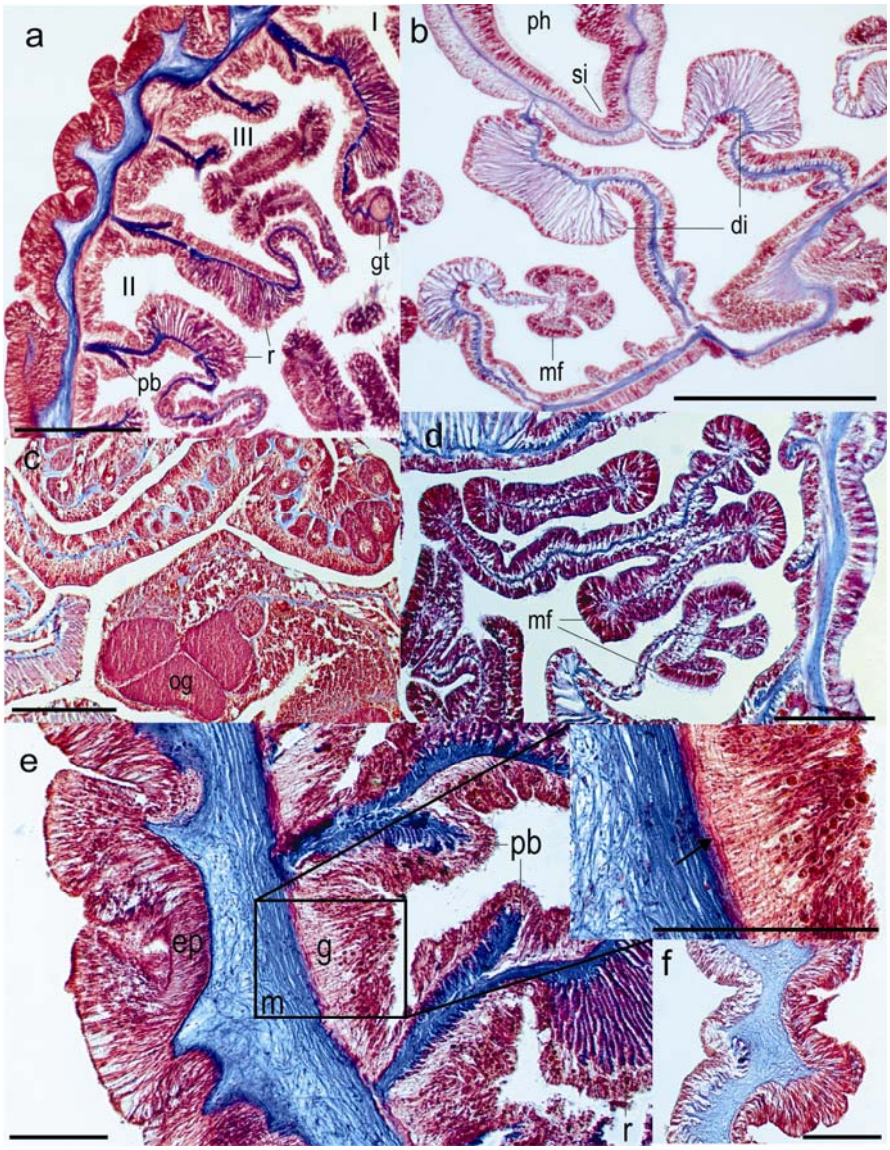

Figure 1. Histological slides of *Anthopleura hermaphroditica* (a, c, e) and *Bunodactis hermafroditica* (b, d, f) stained through Masson's trichrome method (modified). a, b) Cross sections of upper part of column, c) cross section of a fertile mesentery, d) cross section of mid-column exposing uni and trilobulated mesenterial filaments, e) cross section of the column with a close up of the connective portion between the mesogleal fibres and the endodermal epithelia-muscular cells (arrow), f) longitudinal section through a verrucae. I, II and III: 1st to 3rd cycle of mesenteries, di: directives, ep: epidermis, g: gastrodermis, gt: gametogenic tissue, mf: mesenterial filaments, m: mesoglea, og: oogonia, pb: parietobasilar muscle, ph: actinopharynx, r: retractor muscle, si: siphonoglyph. Scale: a, b = 500 µm; c, d, e, f = 100 µm.

such as *A. hermaphroditica* and *B. hermafroditica*, Bouin's fixative penetrated tissue in less than 48 h, but, due to the high concentration of formalin (Bouin weak: 15% and strong: >20%), the specimens hardened and deteriorated at cellular level. A maceration of muscle fibers caused by the section on poorly preserved and, presumably, badly anesthetized specimens were observed several times in preparations. To favor a good fixation of the internal structures, especially in large organisms, it is sometimes desirable to inject small doses of the fixative solution through the mouth (Stephenson, 1928). Furthermore, specimens can dilute a lot of the preservative medium due to the water retained inside the tissues. In this regard, it is recommended to cover 20 times the volume of the body in the fixative (Lopez *et al*., 1982)

or at least replace it after a couple of days of use (Häussermann & Försterra, 2009). At tissue level, the best results were observed in preparations fixed for long periods in 8% seawater formalin. When requiring trichrome techniques, it is suggested to fix the specimens first in seawater formalin for a period exceeding two months and then mordant them in Bouin's solution (very weak, ideally 8%) for one or two weeks. Formalin concentration (5 to 15%) as well as the fixation time (two to six months) will depend primarily on the size of the specimens to be preserved (Häussermann, 2004).

Methods of staining

Several staining methods have been applied in sea anemones for taxonomic work. Among the protocols reported in the literature for this group, highlights the trichromics of Mallory (Pantin, 1948), Azocarmine (Humason, 1967), Heidenhain Azan (Presnell & Schreibman, 1997) and the Nuclear Red Picroindigocarmine (Romeis, 1948); the latter being recommended by Stotz (1981). For general purposes, bicomponent staining of eosin with Weigert's iron, Harris's or Ehrlich's hematoxylin have also reported good results in anemones. Even *in toto* staining (prior sectioning), such as borax carmine (Carter, 1965), has proven to be a useful way to gain primary insights into these animals' anatomy. Masson's trichrome (Sumner & Sumner, 1969) was suggested by Manuel (1981) as a general stain for Anthozoa, allowing for a good contrast between the mesoglea and the cellular regions. The original method of Masson (1928) was initially thought for carcinoids analysis, integrating four dyes (Regaud's hematoxylin, acid fuchsin with Ponceau de xylidine and aniline blue) which have been repeatedly combined on later modifications. Among the many changes made to this method, it is worth mentioning the modifications yielded by Humason (1962) and Luna (1968) (*fide* Lopez *et al*., 1982) by facilitating the control of the acid dyes. From the last protocol, some modifications were made according to the results obtained from histological preparations of *Anthopleura hermaphroditica* and *Bunodactis hermafroditica* (Table 1).

The entire procedure (from deparaffinize to mount) takes no more than two hours, utilizes relatively few reagents and offers the possibility of controlling the times during each stage of differentiation. Following this protocol, the mesoglea turns to a deep blue color while the cytoplasmic elements and epithelia differentiate to pink-reddish tones, clearly highlighting loaded nematocysts structures (*e.g.*, mesenterial filaments, acontia, acrorhagi). To prevent discoloration of the tissue, dehydration prior to mounting must be direct (to 100% ethanol) and preferably fast (at two steps of 5 min each). Likewise, it is very important to renew the dyes after several months of use, as this also influences the quality of the preparations. Although good results were obtained with *A. hermaphroditica* and *B. hermafroditica* (Fig. 1), it is always advisable to proceed initially with a full protocol test to verify the results on each step of the method according to the species of study and the laboratory equipment. If the sections are stretched on a hotplate, for example, it is very important to control the times of adhesion because fine tissues, such as mesenteries, are very sensitive to temperature. Also note that for samples fixed in aqueous Bouin, the wash must be in 70% ethanol. If working with a strong Bouin solution, it is also desirable to keep the specimens in the final ethanol wash for a longer period of time (overnight) and then clear the samples with butanol according to Gabe (1968). Given the small size of these species, many of these details finally determined the specimen's resistance to the histological process. Taking into account these recommendations, this staining method has demonstrated very good results for the histological study of sea anemones.

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