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A simple and cost-effective technique to quench autofluorescence in formalin-fixed paraffin-embedded cnidarian tissues

Lorenzo GALLUS[1], Chiara MAZZARINO[1], Daniel CROCE[1], Chiara GAMBARDELLA[2], Carlo CERRANO[3] and Sara FERRANDO[1*]

[1]DiSTAV, University of Genoa, Italy
[2]ISMAR-CNR, Genoa, Italy
[3]DiSVA, Marche Polytechnic University, Italy

Corresponding author, e-mail:sara131274@gmail.com

Immunofluorescence is a first-choice technique for the study of neuroanatomy and, in general, of very fine structures. A stumbling block to the application of immunofluorescence is autofluorescence in tissues. This problem has different origins, as it can be related to the structures that are being examined, the fixation protocol, and the general condition of the organism which has been studied. Several techniques are available to help reduce autofluorescence in vertebrate tissues, particularly in mammalian tissues, and invertebrate tissues alike. The study of animals from different phyla generates additional problems regarding autofluorescence because of the presence of peculiar substances (e.g., chitin). In the present work, we present a simple technique to quench autofluorescence in sections of paraformaldehyde-fixed, paraffin-embedded cnidarians of three species: Paramuricea clavata, Eunicella cavolinii *and* Savalia savaglia. *The technique is based on the histological dye Sudan Black, which is already used in mammalian tissue samples; however, to our knowledge, this work represents the first use of this method in cnidarians. This method is perfectly compatible with immunofluorescence, as it does not appear to interfere with fluorochromes that are conjugated to secondary antibodies and appears to reduce background autofluorescence.*

Key words: histology, immunofluorescence, octocoral, hexacoral

INTRODUCTION

In optical microscopy, fluorescence techniques are very common; they are first-choice techniques to study fine structures.

Fluorescence microscopy is continuously evolving and therefore many innovations are being reported monthly in journals devoted to methods description. Nevertheless, the use of fluorescence in microscopy presents several problems, such as the bleaching of fluorochromes, phototoxicity (in cases of living specimens), and autofluorescence (AF) of biological tissue (which is sometimes increased after fixation). These difficulties have been partially resolved following the introduction of sophisticated systems, such as the recently introduced super-resolution microscopes. Nevertheless, the problem of AF, especially in select tissues and organisms, continues to pose a hurdle with respect to the epifluorescence microscope, which is still a very common and useful device in the

laboratory. Different animal phyla relay different degrees of AF because of differences in various substances that are accumulated in cells and tissues. Known fluorescent substances in biological tissues include flavins, reduced NADH and NADPH, lipofuscins, reticulin fibers, collagen, elastin, and chitin, among others (VIEGAS *et al.,* 2007). Fluorescent substances result in variably intense levels of background fluorescence in microscopy preparations, which is often very problematic for the observation of samples that are stained using fluorochrome-based techniques. Methods devoted to achieving dimming of AF have been developed and described in the literature for both vertebrates and invertebrates, especially for mammalian tissue: among other methods, the use of copper sulfate and of the histological stain Sudan Black B have been proposed (FRONTALI, 1968; SCHNELL *et al.,* 1999; HEANEY *et al.,* 2011; VIEGAS *et al*., 2007).

The bright colors of Anthozoans, a class of cnidarians, are due to pigments that can be intensely fluorescent under ultraviolet-A (UVA) and blue light, with emission at 420-620 nm (SALIH *et al*., 2000).

Here, we applied the Sudan Black method in an attempt to dim AF in formalin-fixed, paraffin-embedded samples taken from three Mediterranean cnidarian (Anthozoa) species: two octocorals, *Paramuricea clavata* (Risso, 1826) and *Eunicella cavolinii* (Koch, 1887), and one hexacoral, *Savalia savaglia* (Bertoloni, 1819). The alcyonacean *P. clavata* has a key structural and functional role in coralligenous biocenosis (LINARES *et al.,* 2007; PONTI *et al.,* 2014). The gorgonian *E. cavolinii* is one of the most frequent alcyonacea and is particularly significant in precoralligenous and semi-dark cave biocenoses (PONTI *et al.*, 2014). Both *P. clavata* and *E. cavolinii* have been subjected to massive mortality events related to climate change (VEZZULLI *et al.,* 2013), but they are resilient and have shown good recovery rates, at least in some populations (CUPIDO et al., 2012). In cases of partially damaged colonies with scattered necrotic tissue on their coenechyme, there is a loss of coordination among polyps (CERRANO & BAVESTRELLO, 2008). This aspect can strongly affect the effectiveness of the recovery of injured colonies. It is therefore necessary to acquire greater understanding of their biology and nervous system organization. Immunofluorescence has been performed on these species to investigate their neuroanatomies (GIROSI *et al.*, 2005; 2007).

S. savaglia is a zoanthid species and, as other zoanthids, its anatomy and histology have not been well described in the literature. This species has a wide distribution in the Mediterranean Sea (GIUSTI *et al.,* 2014) and is characterized by very high longevity and by its important ecological role in the mesophotic zone (CERRANO *et al.,* 2010). As with *P. clavata* and *E. cavolinii*, it is extremely important to obtain information about the biology of *S. savalia* (SINNIGER *et al.*, 2013; ALTUNA *et al.*, 2010).

MATERIAL AND METHODS

Sampling and histological techniques

Specimens of *P. clavata*, *E. cavolinii* and *S. savaglia* were collected from the Ligurian Sea. They were anesthetized with 0.01% Tricainemethanesulfonate (Sigma-Aldrich, USA; diluted 1:1000 in sea water). Small fragments of the colonies were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4, 8% NaCl) for 6-12 h, rinsed in PBS and decalcified in Osteodec (Bio-Optica, Italy) for 24 h. After careful rinsing in PBS, the specimens were dehydrated, Paraplast (Bio-Optica, Italy) embedded and sliced into 6-µm-thick sections.

Slides for the evaluation of autofluorescence

The sections were deparaffinized at room temperature by two consecutive immersions in xylene for 15 min each. The deparaffinized sections were rehydrated with a series of ethanol alcohol: two consecutive immersions in 100% ethanol for one min each, followed by four passages of one min each in 95%, 90%, 80% and 70% ethanol. Finally, the sections were washed with bi-distilled water for one min and placed into a fresh PBS bath. Deparaffinized and rehy-

drated control sections were directly mounted with 50:50 glycerol:PBS. After brief observation and micrograph acquisition using an epifluorescence microscope, slides were opened again using a fresh PBS bath and then treated with Sudan Black (see the following protocol) before being mounted again to acquire photographs of the same sections.

Alternatively, subsequent slides were used as control and Sudan Black-treated samples to avoid the "re-opening and re-mounting" procedure.

Immunofluorescence

Deparaffinized and rehydrated sections were used for immunohistochemistry. A monoclonal mouse anti-acetylated tubulin antibody (Sigma, USA, Cat. No. T7451) was used as a primary antibody. A secondary goat anti-mouse antibody conjugated with Alexa Fluor 488 was supplied by Molecular Probes, Invitrogen (Carlsbad, CA). For immunostaining, deparaffinized sections were rinsed in PBS and incubated overnight with the primary antibody (1:200 in PBS) in a moist chamber at room temperature. After rinsing in PBS, the sections were incubated with the secondary antibody (1:400 in PBS) for 2 hours in a moist chamber at room temperature. After rinsing in PBS, the slides were mounted with 50:50 glycerol:PBS to be briefly observed and photographed using an epifluorescence microscope. The slides were again opened using a fresh PBS bath, after which they were treated with Sudan Black (see the following protocol) before being re-mounted to acquire photographs of the same sections. Alternatively, subsequent slides were used to visualize control and Sudan Black-treated immunofluorescence. Standard negative controls were performed by omitting the primary antibody.

Sudan Black protocol

Deparaffinized and rehydrated histological sections were treated for 20 minutes in a moist chamber at room temperature with 0.1% Sudan Black in 70% ethanol. Following this, the slides were washed three times for 5 minutes each with 0.02% Tween 20 in PBS and then washed a final time for 1 minute in PBS before being mounted with 50:50 glycerol:PBS. The Sudan Black solution is stable at room temperature.

This identical procedure was also applied to re-opened slides after brief observation and to immunofluorescently stained slides.

Microscopy

Slides were observed through a Leica DMRB light microscope that was equipped for epifluorescence with a lamp (Mercury Short Arc Photo Optic Lamp HBO 100 W. Osram, Germany) and proper filter cubes, including A (blue fluorescence), I3 (green fluorescence) and N2.1 (red fluorescence). Micrographs were acquired with a Leica CCD camera DFC420C (Leica, Germany) and Leica Application Suite (LAS) software.

All of the software settings (e.g., gain, saturation and gamma) were kept constant throughout the work. Exposure times differed according to specimen, as there were substantial variations in fluorescence brightness between them. Exposure times were recorded and used to normalize the fluorescence measurements for the next step of image analysis.

All of the tissue sections were evaluated with each of the three filter sets.

Fluorescence measurements

Both AF from cnidarian tissues and fluorescence due to the presence of the Alexa 488 fluorochrome were quantified using the open source software ImageJ (RASBAND, 1997-2014). A simple method for determining the level of fluorescence in a given region is the evaluation of corrected total area fluorescence (CTAF).

Either the exact same areas or very similar areas (see above) were photographed before and after Sudan Black treatment according to the method described. Ten different areas were randomly chosen within the cnidarian tissues in each micrograph. The same areas were chosen to compare the control and Sudan Black treated tissues of the same species.

The chosen areas were selected using the

rectangle selection tool. From the Analyze>Set measurements menu, the Area, Integrated density and Mean gray value options were selected. The ten areas within the cnidarian tissues were measured (Analyze>Measure), in addition to an area within an empty zone of the photograph to set the background value.

The measurements were saved in Microsoft Excel format and then the CTAF for each area was calculated using the following formula: Integrated Density - (Area*Background Fluorescence).

The mean and standard deviation for each species and each filter set, and for Alexa 488 through the I3 filter set, were calculated and displayed as histograms.

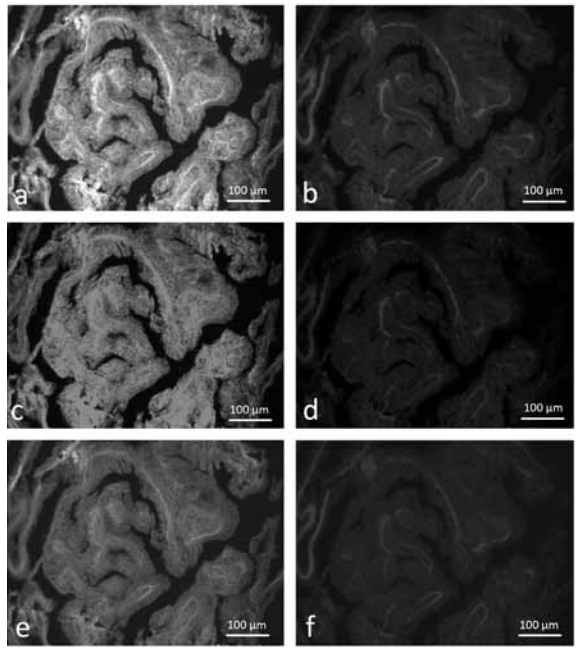

Fig. 1. Histological sections of a P. clavata *polyp. a, c, e) Control section. b,d,f) The same section after Sudan Black treatment. a,b) I3 filter set. c,d) N2.1 filter set. e,f) A filter set. Scale bars: 100 µm*

RESULTS AND DISCUSSION

All the specimens showed a strong to weak AF with each of the three filter sets. In almost each case, the Sudan Black treatment dimmed the AF, with different levels of effectiveness. The AF in *P. clavata* tissues was bright through

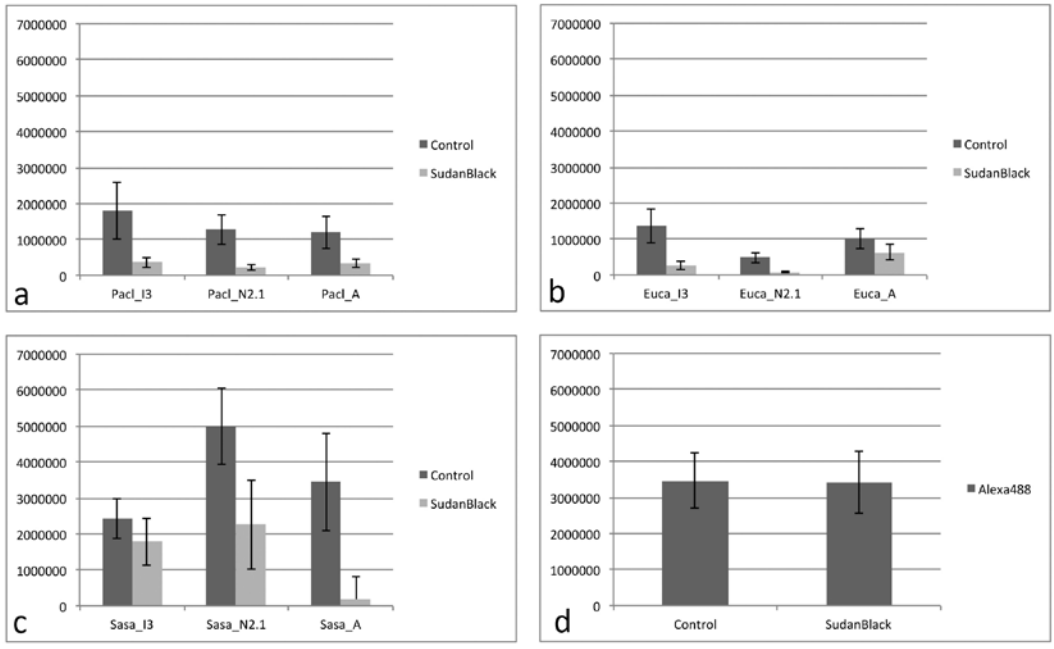

Fig. 2. Histograms of average values (± standard deviations) of the corrected total area fluorescence (CTAF) for each species through each filter set and for Alexa 488 through the I3 filter set. Pacl = Paramuricea clavata*; Euca* = Eunicella cavolinii*; Sasa* = Savalia savaglia*. Unit in the ordinate: CTCF = integrated density − (selected area × background fluorescence)*

all the filter sets and was strongly dimmed by Sudan Black treatment (Figs. 1 & 2a).

The average AF in *E. cavolinii* tissues was brightest with the A and I3 filter sets. The Sudan Black treatment efficiently quenched green and red AF, whereas blue AF (A filter sets) was not efficiently dimmed (Fig. 2b).

The average AF in *S. savaglia* tissues was brightest with the N2.1 and A filter sets, and in both the cases the Sudan Black treatment was very effective, especially for blue AF. Nevertheless, the green AF (I3 filter set) was not efficiently dimmed (Fig. 2c). The Alexa 488 fluorochrome was not affected (Fig. 2d).

Comparing the AF from all the species and with all three filter sets showed that *S. savaglia* was the most autofluorescent species, whereas the two octocoral species had lower AF; the intensity of AF in *E. cavolinii* and *P. clavata* was roughly the same, particularly for the I3 and A filter sets (Fig. 2a, b and c). The Sudan Black treatment was generally most effective on *P. clavata* tissues, whereas it was generally less effective on *S. savaglia*. It was possible to distinguish a similar pattern of AF quenching in the octocoral species, in which the AF that was visible with the I3 and N2.1 filter sets was reduced in Sudan Black treated sections to approximately 16-20% of the AF in the untreated sections of the same species. Blue AF was quenched with less effectiveness in *E. cavolinii* than in *P. clavata*. In the hexacoral species *S. savaglia*, the Sudan Black treatment was very effective only for the A filter set, whereas the green background remained bothersome after the Sudan Black treatment.

CONCLUSIONS

The Sudan Black treatment described herein is likely to be useful in many cnidarian species, although with a great variability in effectiveness from one species to another and through different filter sets. The effectiveness of the method is generally lower than what has been observed in mammalian tissues (e.g., OLIVEIRA *et al.,* 2010), wherein autofluorescence was completely abolished; nevertheless, due to the specifically intense autofluorescence of cnidarian tissue, the use of Sudan Black treatment can be recommended.

Of the species investigated here, we can conclude that *S. savaglia* has a substantial quantity of autofluorescent substances in its tissues and that red fluorochromes in particular should not be used with standard epifluorescence microscopy in this context. The very different responses of green and red AF in samples from octocoral and hexacoral species to Sudan Black treatment could indicate that different types of AF are caused by different types of substances.

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Jednostavna i isplativa metoda potiskivanja autofluorescencije u histološkim rezovima paraformaldehid fiksiranih i parafin uklopljenih tkiva žarnjaka (Cnidaria)

Lorenzo GALLUS[1], Chiara MAZZARINO[1], Daniel CROCE[1], Chiara GAMBARDELLA[2], Carlo CERRANO[3] i Sara FERRANDO[1*]

[1]*DiSTAV, Sveučilište u Genovi, Italija*

[2]*ISMAR-CNR, Genova, Italija*

[3]*DiSVA, Sveučilište Marche, Politehinčki Fakultet, Ancona, Italija*

Kontakt adresa, e-mail:sara131274@gmail.com

SAŽETAK

Imunofluorescencija je optimalna metoda za istraživanje neuroanatomije i, općenito, vrlo finih struktura. Kamen spoticanja u primjeni imunofluorescencije je autofluorescencija u tkivima. Ovaj problem ima različito podrijetlo, povezanost sa strukturama koje se ispituju, protokol fiksiranja, te opće stanje organizma koji se istražuje.

Nekoliko metoda se koristi za smanjenje autofluorescencije u tkivima kralježnjaka, te osobito u tkivima sisavaca i tkivima koja su slična tkivima beskralježnjaka. Istraživanje životinja iz različitih skupina stvara dodatne probleme u vezi autofluorescencije zbog prisutnosti specifičnih tvari (npr. hitina).

U ovom radu je predstavljena jednostavna tehnika potiskivanja autofluorescencije u histološkim rezovima paraformaldehid-fiksiranim i parafin uklopljenim tkivom kod tri vrste žarnjaka: *Paramuricea clavata*, *Eunicella cavolinii* i *Savalia savaglia.*

Metoda se temelji na histološkim bojilima Sudan Black, koji se već koristi u uzorcima tkiva sisavaca. Međutim, prema saznanjima, ovaj rad predstavlja prvu primjenu ove metode kod žarnjaka. Ova metoda je savršeno kompatibilna s imunofluorescencijom, jer ne ometa fluorokrome koji se vežu na sekundarna protutijela, a čini se da smanjuje pozadinsku autofluorescenciju.

Ključne riječi: histologija, imunofluorescencija, oktokoralije, heksakoralije