Journal of Biological Methods | **2021** | **Vol**. 8(3) | e153
DOI: 10.14440/jbm.2021.354

PROTOCOL

Getting two birds with one stone: Combining immunohistochemistry and Azan staining in animal morphology

Patrick Beckers*, Claudia Müller, Christiane Wallnisch, Thomas Bartolomaeus

Institute of Evolutionary Biology, University of Bonn, An der Immenburg 1 53121 Bonn, Germany

*Corresponding author: Patrick Beckers, Email: pbeckers@evolution.uni-bonn.de

Competing interests: The authors have declared that no competing interests exist.

Received December 7, 2020; Revision received April 7, 2021; Accepted April 7, 2021; Published September 3, 2021

ABSTRACT

Classical histological stained sections have the disadvantage that fine structures, like individual neurites, or specific macromolecules, like neurotransmitters cannot be visualized. Due to its highly specific staining of only one target molecule within the cell, the visualization of delicate structures, which would be superimposed by other tissue layers in classical Azan staining, is possible with immunohistochemistry. However, using immunohistological methods not all tissues of a specimen can be visualized at once. In contrast, density specific stains like Azan allow for a whole staining of the tissues. We provide a step by step protocol of how to combine immunohistochemistry and Azan staining in the same serial paraffin sections. The combination of both methods allows for a highly detailed investigation of structures of interest. The spatial detection of the previous, to Azan staining, gained antibody-labeled signal allows for a much better understanding of animal organ systems. By using serial sections, it is possible to create an aligned image stack that is both Azan stained and also antibody-labeled. Thus enabling a correlative approach that bridges traditional histology with immunohistochemistry in animal morphology.

Keywords: animal morphology, Azan staining, correlative approach, immunohistochemistry, serial sections

BACKGROUND

The great advantage of immunohistochemistry is the visualization of specific molecules, like neurotransmitters, in animal cells using antibodies directed against the molecule of interest. Due to the highly specific "staining" of only one target molecule per antibody the resulting picture gained in confocal scanning laser microscopy (CLSM) only shows the structure of interest and all other tissue is ideally not visible. This is, however, also a disadvantage of immunohistochemistry. The whole tissue cannot be labeled at once and thus information in unstained tissue is lost. Determining the spatial orientation of detected signals within the animal is often difficult. Cross-reaction or fail-detection of signal might take place and lead to misinterpretations [1].

This is never the case in density-dependent tissue-visualization staining like Azan trichrome staining. We use a modified Azan-staining (Azocarmin G and Aniline blue-Orange G) to visualize specific tissues. In our staining, Azocarmin is replaced by Carmalaun. Here, the color pigments of the different staining solutions are of different sizes and thus adhere to different tissues specifically. This allows immediate tissue identification across all phyla, from sponges to humans.

In this modified Azan staining, the neurites of the nervous system for example, are always stained in grey. Since this staining is density specific, the musculature may stain in orange or blue depending on the contraction of muscles. The extracellular matrix and collagen stain in dark blue. Nuclei are stained in shades of red.

Density specific staining, however, has the disadvantage that fine, delicate structures may be superimposed upon other structures. For instance, fine neurites of the nervous system are masked by prominent muscle fibers. Additionally, single components of tissues cannot be highlighted. Therefore, we tested several possibilities to combine Immunohistochemistry with Azan staining.

In polychaete morphology, histological sections were classically used to investigate, for example, the nervous system *e.g.*, [2-7]. Since the late 1990's, the nervous system of polychaetes was, for the most part, investigated solely by antibody-labeling [8-11]. If both methods were used to better understand the composition of the elements of the nervous system, these were applied to different specimen [12]. However, by using different specimens the co-localization of the immunohistological result is very difficult and sometimes impossible. The solution is to use the same serial sections for both methods. Here we present a

How to cite this article: Beckers P, Müller C, Wallnisch C, Bartolomaeus T. Getting two birds with one stone: Combining immunohistochemistry and Azan staining in animal morphology. *J Biol Methods* 2021;8(3):e153. DOI: 10.14440/jbm.2021.362

protocol combining antibody-labelling with Azan staining to get most information out of both techniques.

We exemplified our approach by using antibody labelling against commonly used neurotransmitters and Tubulin followed by Azan staining in the model organism *Platynereis dumerilii*.

MATERIALS AND METHODS

Reagents

Azan

- ✓ Formol (Roth, Cat. # 7398)
- ✓ Acetic acid (Roth, Cat. #6755)
- ✓ Ethanol (Roth,Cat. # T171.1)
- ✓ Methylbenzoate (Roth, Cat. # 6944.1)
- ✓ n-Butanol (Roth, Cat. # 7724)
- ✓ Histoplast PE (impregnate) (Thermo-Scientific, Cat. # 8330)
- ✓ Histoplast IM (embedding) (Thermo-Scientific, Cat. # 8331)
- ✓ Xylene (Roth, Cat. # 9713)
- ✓ Carminic acid (Waldeck, Cat. # 5A-380)
- ✓ Phosphotungstic acid hydrate (Sigma-Aldrich, Cat. # 79690)
- ✓ Orange G (Waldeck, Cat. # 1B-221)
- ✓ Aniline-blue (Fluka,Cat. # 1B-501)
- ✓ Potassium alum (Roth, Cat. # CN781)
- ✓ Malinol (Waldeck, Cat. # 3C-242)

Immunohistology

- ✓ Poly-L-Lysin (Sigma, Cat. # P8920)
- ✓ Liquid barrier marker (Roth, Cat. # AN91.1)
- ✓ Disodium phosphate (Roth, Cat. # P030.1)
- ✓ Monosodium phosphate (Roth, Cat. # T878.1)
- ✓ Sodium chloride (Chem-solutions, Cat. # 721816)
- ✓ Triton (Serva, Cat. # 39795.01)
- ✓ Normal goat serum (Sigma, Cat. # G9023)
- ✓ FMRF (rabbit) (Immunostar, Cat. # 20091)
- ✓ Anti-α-Tubulin (mouse) (Sigma, Cat. # T5168)
- ✓ Cy2 (goat anti-rabbit) (Immunostar, Cat. # 111-225-144)
- ✓ Cy5 (goat anti-mouse) (Immunostar, Cat. # 115-175-146)
- ✓ Glycerine (Merck, Cat. # 1370281000)

Recipes

- ✓ Phosphate-buffer 0.2 M, stock solution (1000 ml): Disodium phosphate 20, 6 g, Monosodium phosphate 6, 52 g, dissolve in 900ml H_2O Milli Q, fill up the solution to 1000 ml with H_2O Milli Q
- ✓ SoCl 1.2 M, stock solution (1000 ml): 70,13 g SoCl dissolve in 900 ml H_2O Milli Q, fill up the solution to 1000 ml with H_2O Milli Q
- ✓ Phosphate-buffered saline 0.2 M (500 ml): *e.g.* 500 ml 0.2 M PB/ 0.3 M SoCl; pH 7.2
- ✓ 125 ml 0.2 M PB, 125 ml 1.2 M SoCl, fill up solution to 490 ml with H_2O Milli Q, alter the pH-value to 7.2, fill up solution to 500 ml with H_2O Milli Q
- ✓ PBTR (0.1%) (v/v): 100 ml 0.2 M PBS dilute 0.1 ml of Triton while stirring
- ✓ Antibody mastermix: 400 µl PBTR (0.2%) (v/v), Tubulin-acetylated 1:400, FMRF 1:2000
- ✓ Secondary antibody mastermix: 400 µl PBTR (0.1%) (v/v), Cy2: 1:200; Cy5: 1:200
- ✓ 5% (v/v) Phosphotungstenacid (100 ml): 5 g of phosphotungstenacid in 100 ml Aqua dest.
- ✓ Carmalaun (1000 ml): 50 g potassium alum, 5 g carminic acid, 1000 ml Aqua dest, dissolve color pigments while warming, filtrate after cooling
- ✓ Aniline-blue Orange G 1:1 (1000 ml): 15 g Aniline blue, 15 g Orange G, 80 ml Acetic acid, 1000 ml Aqua dest. boil until color pigments dissolve, filtrate after cooling, for staining dilute 1:1 with Aqua dest.

Equipment

- ✓ Standard laboratory equipment, cuvettes, wet chamber, glass pipettes, microtome, light microscope, CLSM

Animals

- ✓ *Platynereis dumerilii* (Audouin & Milne Edwards, 1833) (**Fig. 1A**) was collected from the culture of the Institute of evolutionary biology and ecology/University of Bonn.

PROCEDURE

Immunohistochemistry

1. Specimens are anaesthetized for 1 h using a 7% (m/v) $MgCl_2$—solution mixed in seawater (1:1) in the fridge.

   NOTE: Anaesthetization should take place in the fridge to prevent muscle contraction during fixation. Animals are orientated ideally in a straight way to perform sagittal, cross or horizontal sections.

2. Animals are fixed in a 7% (v/v) Formalin-solution in seawater (ultrafiltrated) in a petridish with a little amount of the prior used relaxing solution at room temperature. Fixation time is 12 h.

   NOTE: Alter fixation time to 12–24 h depending on the size of the specimen. The use of 4% (v/v) paraformaldehyde is also possible. However, for subsequent Azan staining, 7% (v/v) Formalin has the better tissue conservation properties. Bouin's fluid should not be used, due to the strong cross linkage of proteins and lipids, which may inhibit the penetration of the tissue with antibodies.

3. Fixative must be carefully removed using PBS with several medium changes on a shaker. Washing time is at least 24 h.

   NOTE: Time depends on the size of the specimen.

4. Transfer the specimen to 40% (v/v) Ethanol for 1 h and change medium 3 times.
5. Transfer to 70% Ethanol (v/v) (short time (1 d) storage possible). Animals are prepared for standard paraffin embedding.
6. 80% (v/v) Ethanol for 1 h.
7. 90% (v/v) Ethanol for 1 h.

   NOTE: optional: 90% (v/v) Ethanol with 0.5% (m/v) eosin 10–30 min (stains tissue red, as needed for very small samples to be found in the paraffin block).

8. 95% (v/v) Ethanol for 1 h.
9. 100% (v/v) Ethanol 3 times for 1 h.
10. Methyl benzoate 24 h (change several times).

    CAUTION: Samples may float in the beginning.

    NOTE: You may alter the time up to 72 h. However, prolonged time would lead to a complete hardening of the tissue and section artefacts will occur.

11. Butanol 3 times for 1 h.
12. Butanol heat up in oven to 60°C, then replace with
13. ⅓ Histoplast PE 8 h, then replace with
14. ⅓ Histoplast PE 8 h, then replace with
15. ⅔ Histoplast PE 8 h.
16. Histoplast IM 3 times for 24 h.

    NOTE: Times frames are given for the size of the specimen investigated. If unsure use prolonged times. If there is still a smell of butanol recognizable, the paraffin must be replaced several times again.

17. Embedding in casting molds in the orientation of the preferred section angle (cross-, sagittal-, or horizontal sections).
18. Serial sectioning, 10 µm (**Fig. 1B**).

    NOTE: Sections down to 4µm are possible. Thicker sections are also possible but turned out to be less easy to handle and coiling of sections may occur. Additionally for subsequent Azan staining and investigations with the light microscope sections of 4–10 µm are the size of choice.

19. Slides are coated with 0.06% (w/v) Poly-L-Lysin and dried.

    NOTE: The coating sticks the sections to the slides and has itself no autofluorescence.

20. Sections are placed on the slide (**Fig. 1C**).

    NOTE: Take care that there is enough space around the sections on the slide to prepare the tray for applying the antibody-solution (**Fig. 1D**). Sections are deparaffinized.

21. Xylene 3 times (100%) (v/v), 3 min.
22. Mixture of Xylene/Ethanol (100%) (v/v) (1:1), 3 min.
23. Ethanol 100, 95, 90, 80, 70, 40% (v/v), all for 3 min.
24. Aqua dest. 2 times, 3 min.

25. Carefully remove the water from the slides with a lens cleaning tissue without touching the sections
26. Use the liquid barrier marker to apply a border around the sections which prevents the antibody solution to run of the slide (**Fig. 1D**)
27. PBS (0.2 M) wash 2 times with and 1 time with PBS and Triton (0.1%) (v/v).
28. Apply the blocking solution and incubate for 2 h at room temperature in a wet chamber (**Fig. 1E**).
29. Apply the antibody master mix to the sections, incubate overnight at 4°C in a wet chamber.
30. PBS and Triton (0.1%) (v/v) wash for one hour with 4 times medium change.
31. Apply the secondary antibody master mix and incubate for 2 h at room temperature in a wet chamber.
32. PBS and Triton (0.1%) (v/v) wash 2 times for 15 min.
33. PBS wash 2 times for 15 min.
34. Remove barrier marker by peeling off.
35. Cover with Glycerine and store in the fridge in darkness.

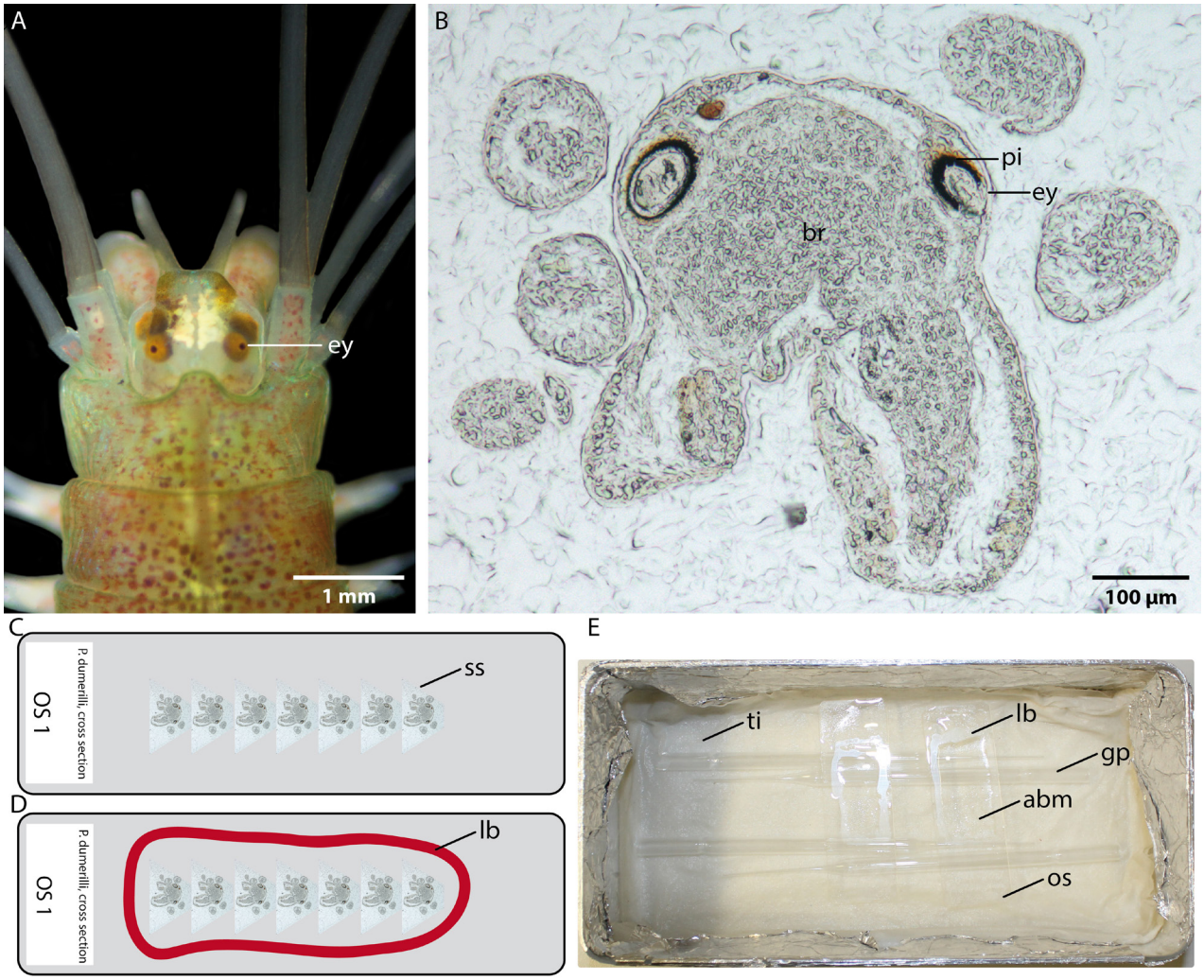

Figure 1. Specimen and Methods. A. Living specimen of *Platynereis dumerilii*. **B.** Section 10 µm. In the unstained section only the pigments surrounding the eyes are visible. **C.** Slide showing the ideal position of the serial sections. **D.** Slide showing the liquid barrier produced by the liquid barrier marker. **E.** Wet chamber. The slides are placed upon glass pipettes into the wet chamber. A wet tissue provides high humidity in the chamber. The antibody master mix is directly applied inside the liquid barrier to the sections. abm, antibody master-mix; br, brain; ey, eye; gp, glass pipettes; lb, liquid barrier; os, object slides; pi, pigments; ss: serial sections; ti: tissue.

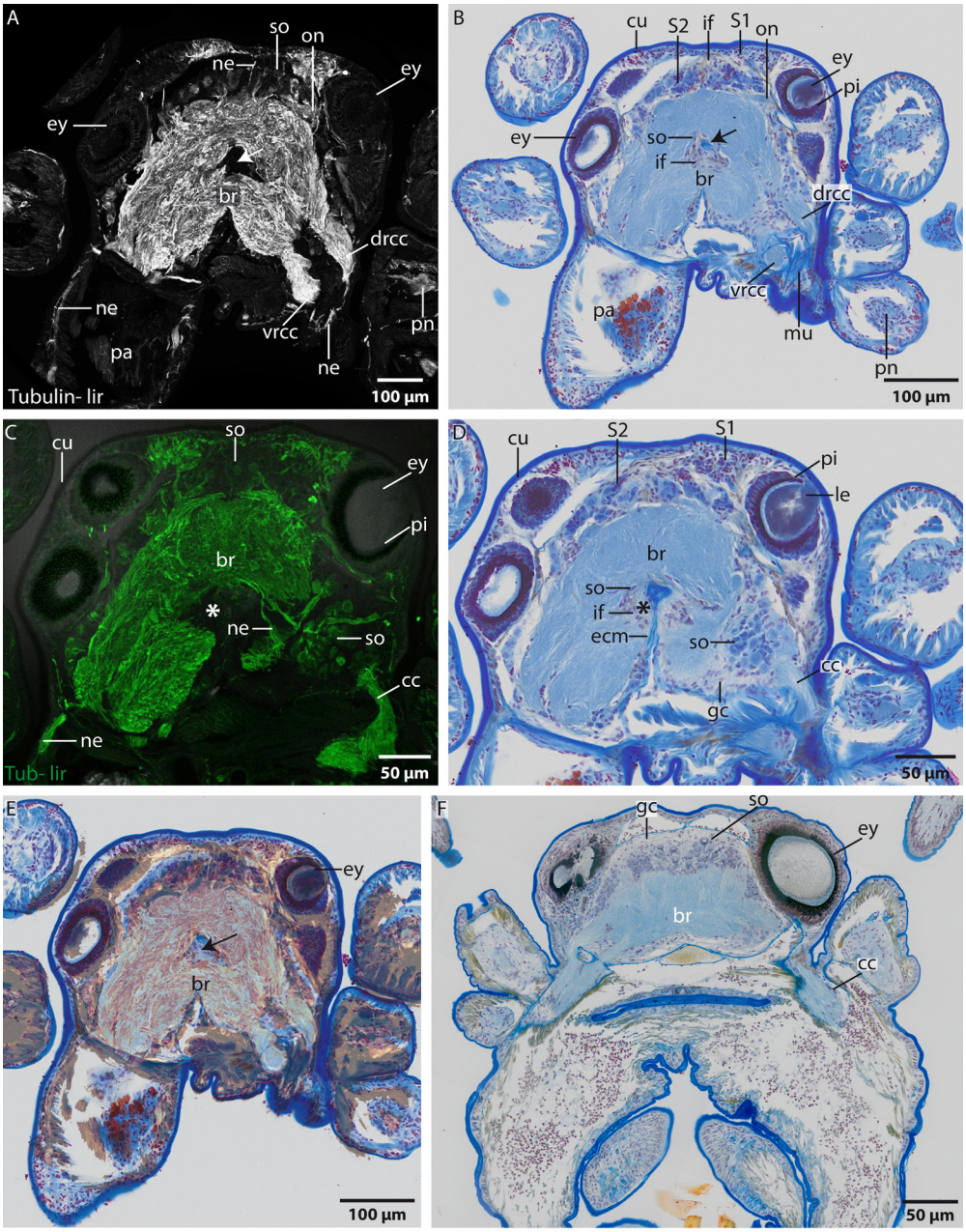

Figure 2. Immunohistochemistry (max. intensity) and histological sections (Azan) (section thickness: A-E: 10 µm; F: 5 µm). A. Tubulin-lir: The neuropil of the brain is visible. The neurites of the ventral root of the circumesophageal are continuously connected to the brain in staining against Tubulin compared to (B). Fine neurite bundles are visible. An optical nerve connects the sensory cells of the eye to the brain. Neuronal somata are partly visible. Pigment cell of the eye are not visible. A Parapodial nerve is present. Note the unstained structure (white arrow) in the median part of the brain. **B.** Same section as in (A): The optical nerve and the pigments cells of the eye are visible. The ventral root of the circumesophageal connective seems not to be connected to the brain. In contrast to (A), the cuticle, the musculature and intermediate filaments are visible. The black arrow points to the same region as in (A) (white arrow). This region is composed of radial glial cells containing intermediate filaments and somata of glial cells or neurons. S1-S2 (neuronal somata type one and two) **C.** Tubulin-lir/green and transmission channel image. The neuropil of the brain and some neuronal somata are labeled. Pigments of the eye and the cuticle are only visible in the transmission channel image. Small neurite bundles are visible, which are to fine to be differentiated in the Azan stained section. Note the unstained region in the brain neuropil (asterisk). **D.** Same section as in (C). Different types of neuronal somata (S1, S2) can be differentiated. The eye is composed of pigment cells and a lens. The region in (C) (asterisk) is composed of somata, intermediate filaments and extracellular matrix. **E.** Composite image of Tubulin-lir and Azan staining. The combination of both pictures shows that all neurites of the brain are Tubulin-lir positive. **F.** Pure Azan staining without previous antibody-labeling. The whole tissue is perfectly contrasted. br, brain; cc, circumesophageal connective; cu, cuticle; drcc, dorsal root of circumesophageal connective; ecm, extracellular matrix; ey, eye; gc, glial cells; if, intermediate filaments; le, lens; mu, musculature; ne, neurites; on, optical nerve; pa, palps; pi, pigments; pn, parapodial nerve; so: somata; S1, neuronal somata type 1; S2, neuronal somata type 2; vrcc: ventral root of circumesophageal connective.

Start your investigation with the CLSM on the sections latest the next day and try to be fast. The coverslip must be removed prior to subsequent Azan staining. The glycerine may harden and the coverslip removal might prove difficult. After scanning directly place the slides into a cuvette filled with distilled water to ease the removing of the cover slip. Ideally, it is removed by gravity only. Avoid mechanically removing the coverslip, since sections may be scratched of the slide.

Azan staining

All solutions are filled into cuvettes and are placed under a fume.

36. Wash slides with distilled water two times for 3 min
37. Carmalaun stain for 24 min
38. Aqua dest. dip in one time
39. 5% (m/v) Phosphotungstic acid for 3 min
40. Aqua dest. wash one time
41. Aniline blue-Orange G 1:1 stain 14 min
42. drain on paper towel
43. Aqua dest. dip in two times
44. 80% (v/v) Ethanol dip in 3 times
45. 90% (v/v) Ethanol dip in 4 times
46. 95% (v/v) Ethanol dip in 5 times
47. 100% (v/v) Ethanol 3 times for 2.5 min
48. Xylene/ Ethanol (1:1) for 2.5 min
49. Xylene 3 times for 2.5 min
50. Covering with Malinol for long time storage

The slides can be covered with Malinol and a cover slide, as soon as they reach the last Xylene cuvette. When stored for longer periods the level of Xylene in the cuvette must be checked at least once a day. Drying out of the Xylene bath will damage the sections.

ANTICIPATED RESULTS

The staining of antibody-labeled paraffin sections with Azan provides insights into both localized detection of macromolecules and the composition of the remaining tissue (**Fig. 2A-2E**).

Antibodies against α-Tubulin visualize only the neurites and partly the somata of the nervous system (**Fig. 2A** and **2C**). Musculature, pigment cells, the cuticle, and intermediate filaments are not labeled and thus not visible. Fine neurites are detectable (**Fig. 2A** and **2C**). In the corresponding Azan stained sections, different types of neuronal somata can be differentiated (**Fig. 2B** and **2D**). In the median part of the brain an unstained region is visible in labeling against α-Tubulin and might be interpreted as a hole in the brain neuropil (**Fig. 2A**). However, Azan staining reveals the presence of somata, intermediate filaments, and extracellular matrix in that region (**Fig. 2B**). In **Figure 2B**, the dorsal and ventral roots seem to be not connected to the brain, since glial cells, somata, and musculature are superimposed on the neurites. In the same region in **Figure 2A**, the neurites of the roots are continuous. The same applies for **Figure 2C** and 2D. If, in addition to the antibody-labeling, the transmission channel picture is recorded, then pigment cells of the eyes are visible (**Fig. 2C**). However, the lens is only visible in Azan staining (**Fig. 2D**).

By using the same sections for both stainings, it is possible to merge information from both methods (**Fig. 2E**). Immunostaining has no influence on the quality of the subsequent Azan stained sections (**Fig. 2F**).

TROUBLESHOOTING

For step immunostaining, due to the much better function of Antibody-labeling, the use of "fresh" material is recommended.

For step Azan staining, be cautious that sections are not removed from the slide. Poly-L-Lysine is not the ideal media to attach paraffin sections to the slides.

Author's contribution

PB conceived the study, analyzed the data. PB and TB wrote the manuscript. CW and CM performed the laboratory work. All authors approved the manuscript.

Data availability

All data used in this research is included into the manuscript.

Acknowledgments

We are very grateful to Dr. Michael Andrew Jansen for language editing.

References

1. Mulisch M. Welsch U. Romeis-Mikroskopische Technik. 18th ed. Springer Verlag. Berlin. 2015. 551 p.
2. McIntosh WC. A monograph of the British annelids. London, The Ray society. 1910
3. Hanström B. Das zentrale und periphere Nervensystem des Kopflappens einiger Polychäten. Zeitschrift für Morphologie und Ökologie der Tiere. 1927;7:543–96. https://doi.org/10.1007/BF00540532.
4. Gustafson G. Anatomische Studien über die Polychäten-Familien Amphinomidae und Euphrosynidae. Zool Bidr fran Uppsala 1930;12:305–471.
5. Orrhage L. Über die Anatomie des zentralen Nervensystemes der sedentären Polychaeten. Ark Zool. 1966;19:99–133.
6. Orrhage L. Ueber die anatomie, histologie und verwandtschaft der Apistobranchidae (Polychaeta Sedentaria) nebst Bemerkungen ueber die systematische Stellung der Archianneliden. Zoomorphology. 1974;79(1):1–45. https://doi.org/10.1007/BF00298840.
7. Orrhage L. On the Innervation and Homologues of the Cephalic Appendages of the Aphroditacea (Polychaeta). Acta Zool. 1991;72(4):233–46. https://doi.org/10.1111/j.1463-6395.1991.tb01201.x.
8. Hessling R, Müller M, Westheide W. CLSM analysis of serotonin-immunoreactive neurons in the central nervous system of Nais variabilis, Slavina appendiculata and Stylaria lacustris (Oligochaeta: naididae). Hydrobiologia. 1999;406:223–33. https://doi.org/10.1023/A:1003704705181.
9. Müller MC, Westheide W. Structure of the nervous system of Myzostoma cirriferum (Annelida) as revealed by immunohistochemistry and cLSM analyses. J Morphol. 2000 Aug;245(2):87–98. https://doi.org/10.1002/1097-4687(200008)245:2<87::AID-JMOR1>3.0.CO;2-W PMID:10906744
10. Müller MC. Polychaete nervous systems: ground pattern and variations—cLS microscopy and the importance of novel characteristics in phylogenetic analysis. Integr Comp Biol. 2006 Apr;46(2):125–33. https://doi.org/10.1093/icb/icj017 PMID:21672729
11. Heuer CM, Loesel R. Immunofluorescence analysis of the internal brain anatomy of Nereis diversicolor (Polychaeta, Annelida). Cell Tissue Res. 2008 Mar;331(3):713–24. https://doi.org/10.1007/s00441-007-0535-y PMID:18071754
12. Beckers P, Helm C, Purschke G, Worsaae K, Hutchings P, Bartolomaeus T. The central nervous system of Oweniidae (Annelida) and its implications for the structure of the ancestral annelid brain. Front Zool. 2019 Mar;16(1):6. https://doi.org/10.1186/s12983-019-0305-1 PMID:30911320