Chapter 5

Illustrated Neuroanatomy of Ctenophores: Immunohistochemistry

Tigran P. Norekian and Leonid L. Moroz

Abstract

Ctenophores or comb jellies are representatives of an enigmatic lineage of early branching metazoans with complex tissue and organ organization. Their biology and even microanatomy are not well known for most of these fragile pelagic and deep-water species. Here, we present immunohistochemical protocols successfully tested on more than a dozen ctenophores. This chapter also illustrates neural organization in several reference species of the phylum (*Pleurobrachia bachei*, *P. pileus*, *Mnemiopsis leidyi*, *Bolinopsis microptera*, *Beroe ovata*, and *B. abyssicola*) as well as numerous ciliated structures in different functional systems. The applications of these protocols illuminate a very complex diversification of cell types comparable to many bilaterian lineages.

Key words Ctenophora, Electrophysiology, Behavior, Neurotransmitters, Neuropeptides, Nitric oxide, *Pleurobrachia*, *Bolinopsis*, *Mnemiopsis*, *Beroe*, Neurons, Gap junctions

1 Introduction

For over a century, ctenophores were often considered the sister group of cnidarians and received less attention than other basal metazoans. The recent genomic revolution and sequencing of ctenophore genomes [1–4] and multiple transcriptomes [1, 5] started hot debates about their identity, relationships with other animals, and origins of ctenophore innovations. The consensus has not been reached [6–9]. However, the emerging conclusion is that ctenophores are the earliest surviving animal lineages, independently developing enormous complexity in their tissue and organ organization and behavior [10–12]. There is a reasonable case that ctenophores independently evolved neurons, synapses, muscles, and mesoderm [1, 12, 13].

Despite the recent rise in interest, we know very little about the microanatomical organization in ctenophores, with the latest

Authors Tigran P. Norekian and Leonid L. Moroz have equally contributed to this chapter.

Leonid L. Moroz (ed.), *Ctenophores: Methods and Protocols*, Methods in Molecular Biology, vol. 2757,
https://doi.org/10.1007/978-1-0716-3642-8_5,

systematic review of their ultrastructure published in 1991 [10]. Even basic microanatomy is unknown for most of these species. Plus, many of these pelagic organisms are highly fragile, which by itself presents a significant challenge for histochemical and molecular characterization in situ [14].

Immunohistochemistry is a relatively simple and powerful tool for any comparative biological study. This approach is still very needed to characterize ctenophore cells and tissues. Protocols and recommendations were reported for larval stages of *Mnemiopsis* [15, 16]. Here, we summarize practical immunohistochemistry protocols for ctenophores. We successfully tested this methodological approach on 14 adult ctenophore species and their larvae, focusing on their neuromuscular organization [17–21].

This chapter also provides illustrative examples of neural nets and receptor types in several reference species of the phylum (Figs. 2, 3, 4, 5, 6, 7 and 8). These are *Pleurobrachia bachei*, *P. pileus*, *Mnemiopsis leidyi*, *Bolinopsis microptera*, *Beroe ovata*, and *B. abyssicola* collected in the Northwestern Pacific and Atlantic (Fig. 1). In addition, we show ciliated structures in the aboral, swimming, digestive systems, and meridional canals of ctenophores (Figs. 3 and 9). The applications of these protocols illuminate a very complex diversification of cell types in ctenophores. The level of anatomical complexity within this phylum is comparable to many bilaterian lineages.

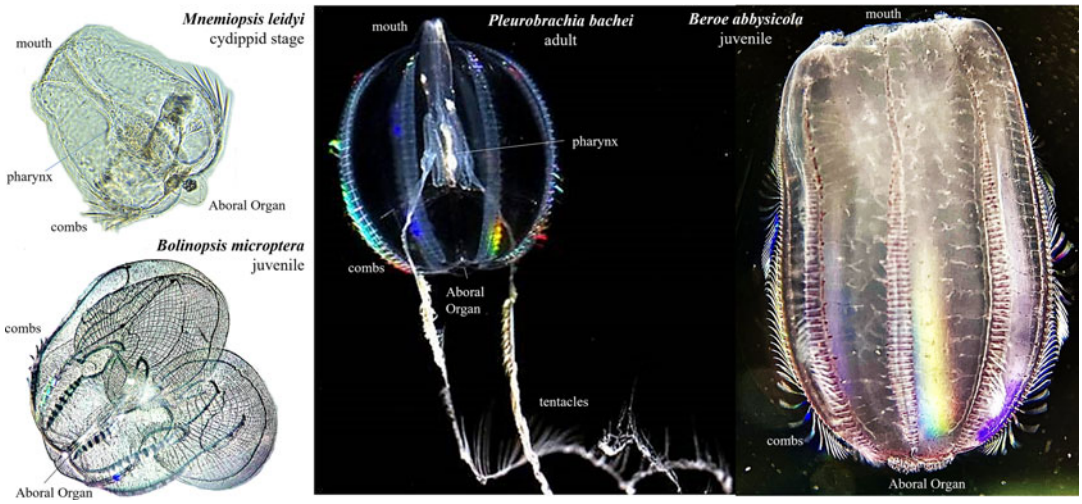

Fig. 1 *Mnemiopsis leydi* cydippid stage, juvenile and adult stages of *Bolinopsis microptera, Pleurobrachia bachei, and Beroe abbysicola* images with main organs

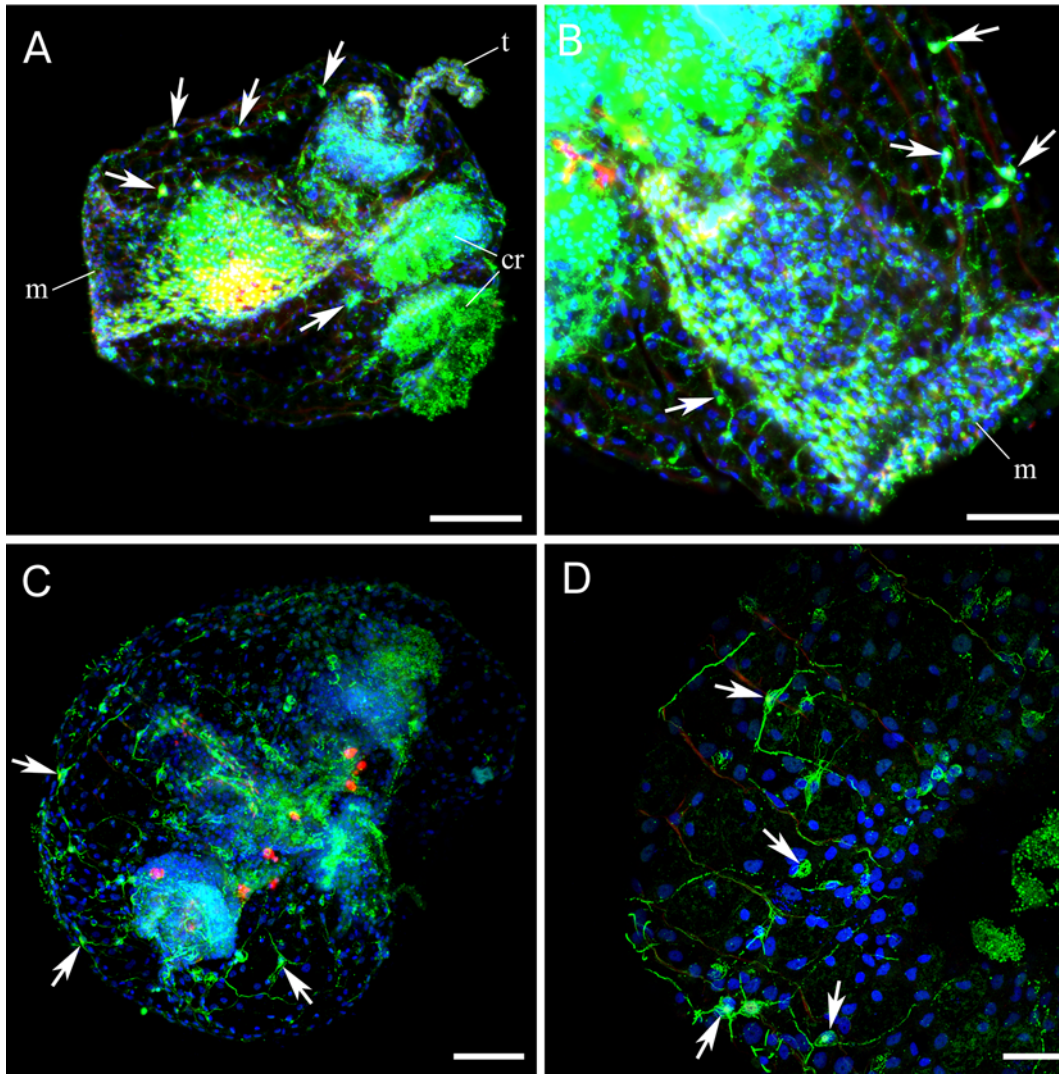

Fig. 2 The nervous system in early ctenophore development (revealed by anti-tubulin immunoreactivity in green; see text and [17, 21]). (**a**, **b**) Hatched 4-day-old *Mnemiopsis* cydippid stage. Note individual neurons (arrows) that started forming the subepithelial neural network. (**c**, **d**) *Bolinopsis* 3-day cydippid after hatching. Arrows point to the subepithelial neurons that begin to form the neural network. Abbreviations: *cr* comb row, *m* mouth, *t* tentacle. Scale bars: (**a**) 50 μm; (**b**) 30 μm; (**c**) 50 μm; (**d**) 20 μm

2 Materials

1. Phosphate-buffered saline (PBS)—0.2 M stock solution, pH = 7.6.
2. Stock 16% paraformaldehyde aqueous solution in 10 mL ampules (Ted Pella, Catalog # 18505, EM grade). Working concentration—4% paraformaldehyde in 0.1 M PBS.

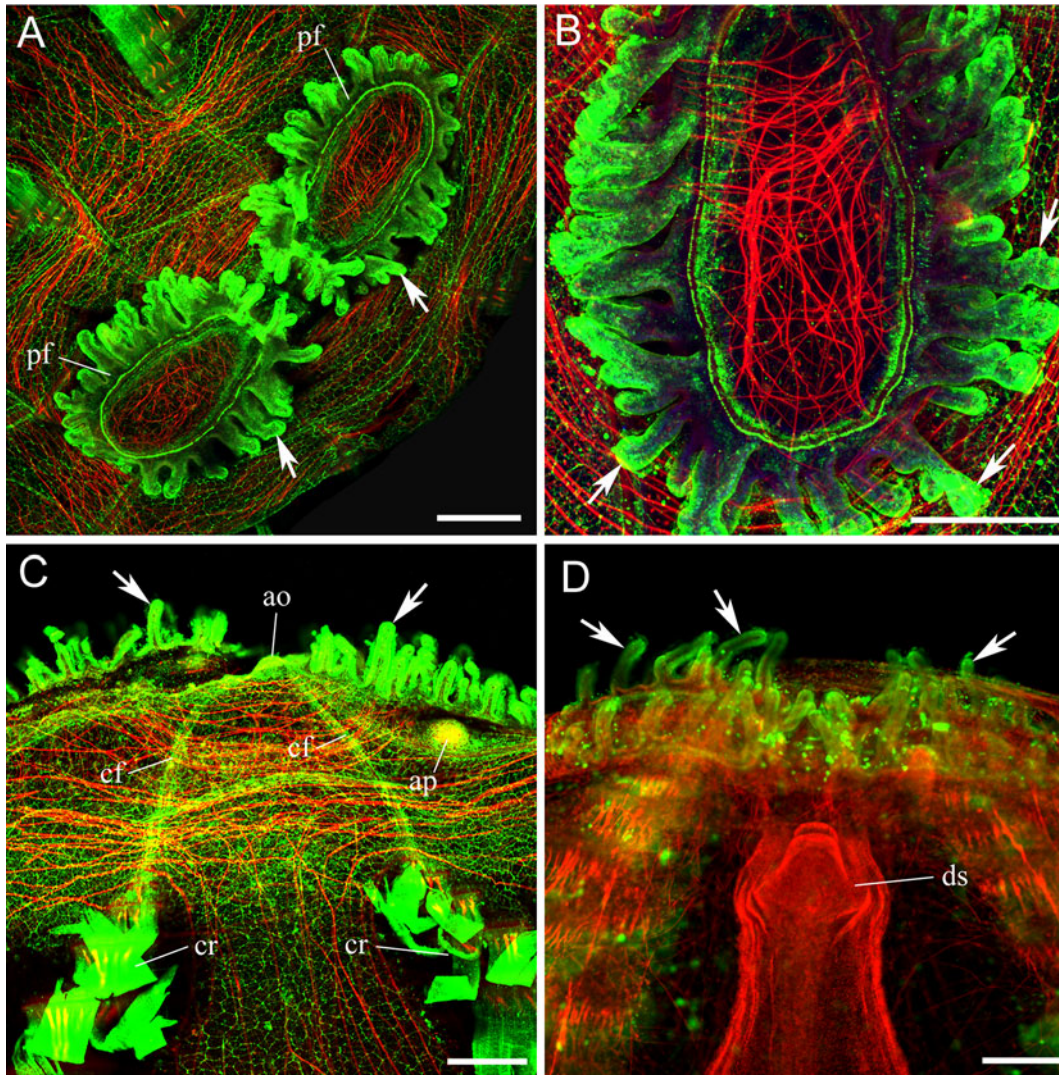

Fig. 3 Aboral organ and polar fields in *Beroe ovata*. (**a**, **b**) Polar fields with tall lobes (arrows) forming a crown are brightly labeled by tubulin AB (green), horizontal view. Note a subepithelial neural network covering the entire surface area, which is also stained by AB in green. Phalloidin-labeled muscle fibers are red. (**c**, **d**) Optical cross-section of the aboral area. Arrows point to the polar field lobes. See more details in [18, 20]. Abbreviations: *ao* aboral organ, *ap* anal pore, *cf* ciliated furrow, *cr* comb row, *ds* digestive system, *pf* polar field. Scale bars: 200 μm

3. Goat serum as a blocking solution (Sigma, Catalog#: G9023). Working concentration—6% goat serum in 0.1 M PBS.
4. Primary antibodies. Rat monoclonal anti-tubulin antibody (AbD Serotec, Cat# MCA77G).
5. Secondary antibodies. Goat anti-rat IgG antibodies: Alexa Fluor 488 conjugated (Molecular Probes, Invitrogen, Cat#

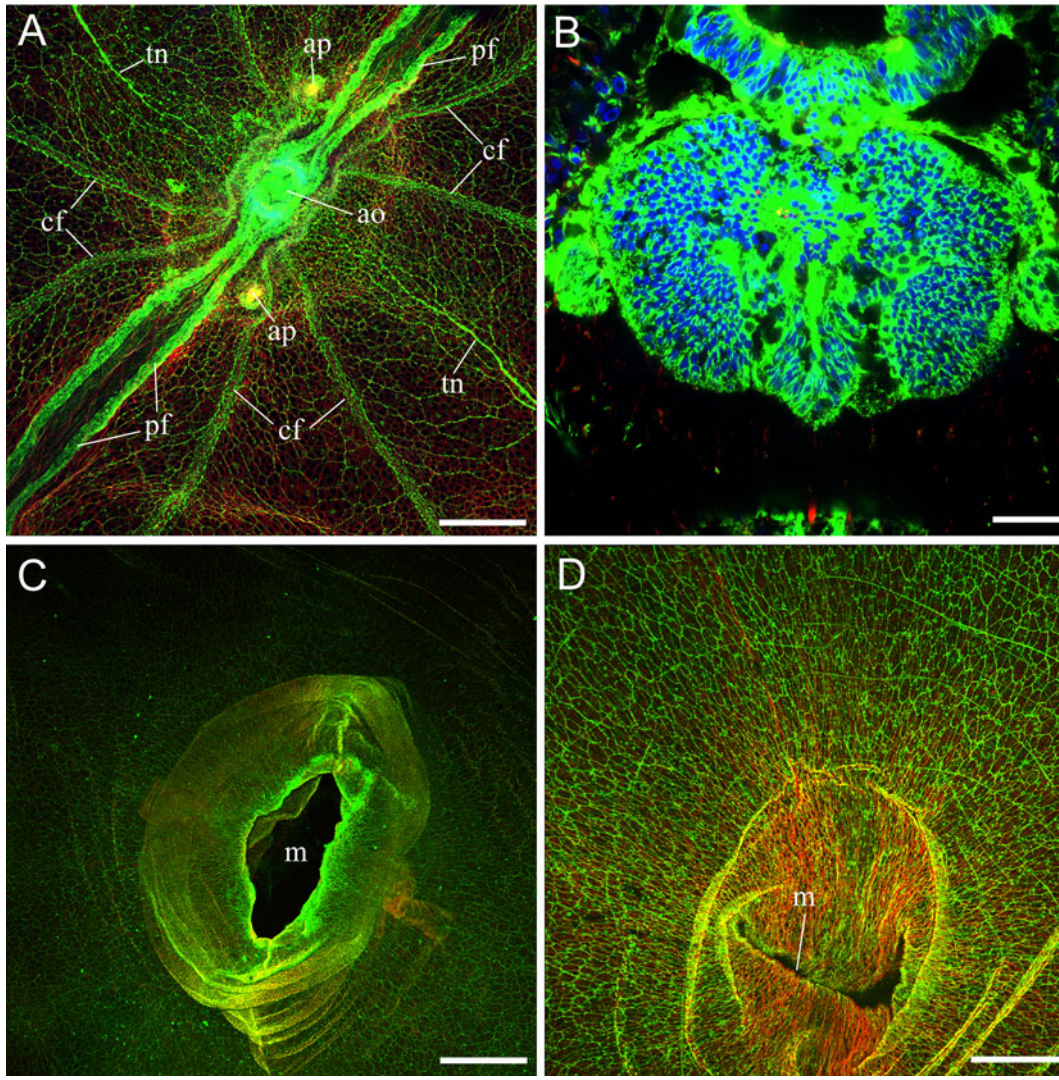

Fig. 4 The aboral organ and mouth in *Pleurobrachia*. (**a**) Aboral organ (*ao*) and polar fields (*pf*) in *Pleurobrachia pileus* (horizontal view). (**b**) Side view of the aboral organ in *Pleurobrachia pileus*. The aboral organ consists of many tightly packed and immunoreactive to tubulin AB cells, whose nuclei are stained blue by DAPI. (**c**, **d**) Mouth (*m*) area of the *Pleurobrachia bachei*. Note a subepithelial neural network covering the entire surface area, which is labeled by tubulin AB in green; phalloidin—red. See additional details in [19, 20]. Abbreviations: *ao* aboral organ, *ap* anal pore, *cf* ciliated furrow, *m* mouth, *pf* polar field, *tn* tentacular nerve. Scale bars: (**a**) 200 μm; (**b**) 20 μm; (**c**) 500 μm; (**d**) 300 μm

A11006) and Alexa Fluor 568 conjugated (Molecular Probes, Invitrogen, Cat# A11077).

6. Phalloidin (Alexa Fluor 488 and Alexa Fluor 568 phalloidins from Molecular Probes, Catalog#: A-12379 and A-12380) for labeling muscle fibers.

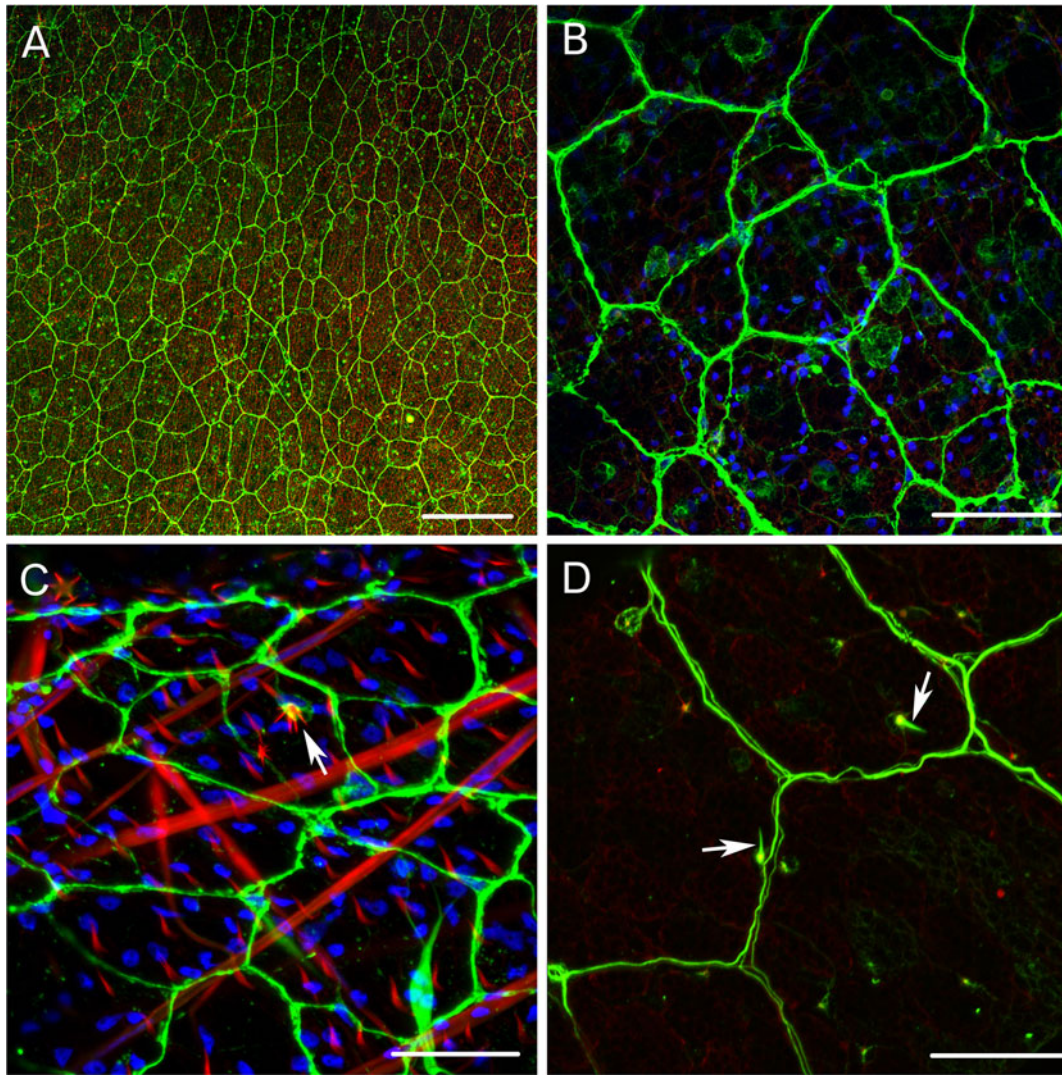

Fig. 5 Subepithelial neural network in different ctenophore species labeled by tubulin AB (green). The phalloidin labeling is red, while DAPI is blue. (**a**, **b**) Neural network in the body wall of adult *Pleurobrachia bachei*. (**c**) Subepithelial neural network in *Beroe abyssicola* pharynx wall (green) with parietal muscle fibers shown in red. The arrow points to the receptor with multiple stereocilia. (**d**) Subepithelial neural network in *Euplokamis* with surface receptors identified by arrows. Scale bars: (**a**) 200 μm; (**b**) 35 μm; (**c**) 50 μm; (**d**) 20 μm

7. Mounting medium VECTASHIELD Hard-Set Mounting Medium with DAPI (Vector Labs, Cat# H-1500).
8. Glass microscope slides and coverslips.

2.1 Equipment

1. Nikon Research Microscope Eclipse E800 with Epi-fluorescence using standard TRITC and FITC filters.
2. Nikon C2 laser scanning confocal microscope.

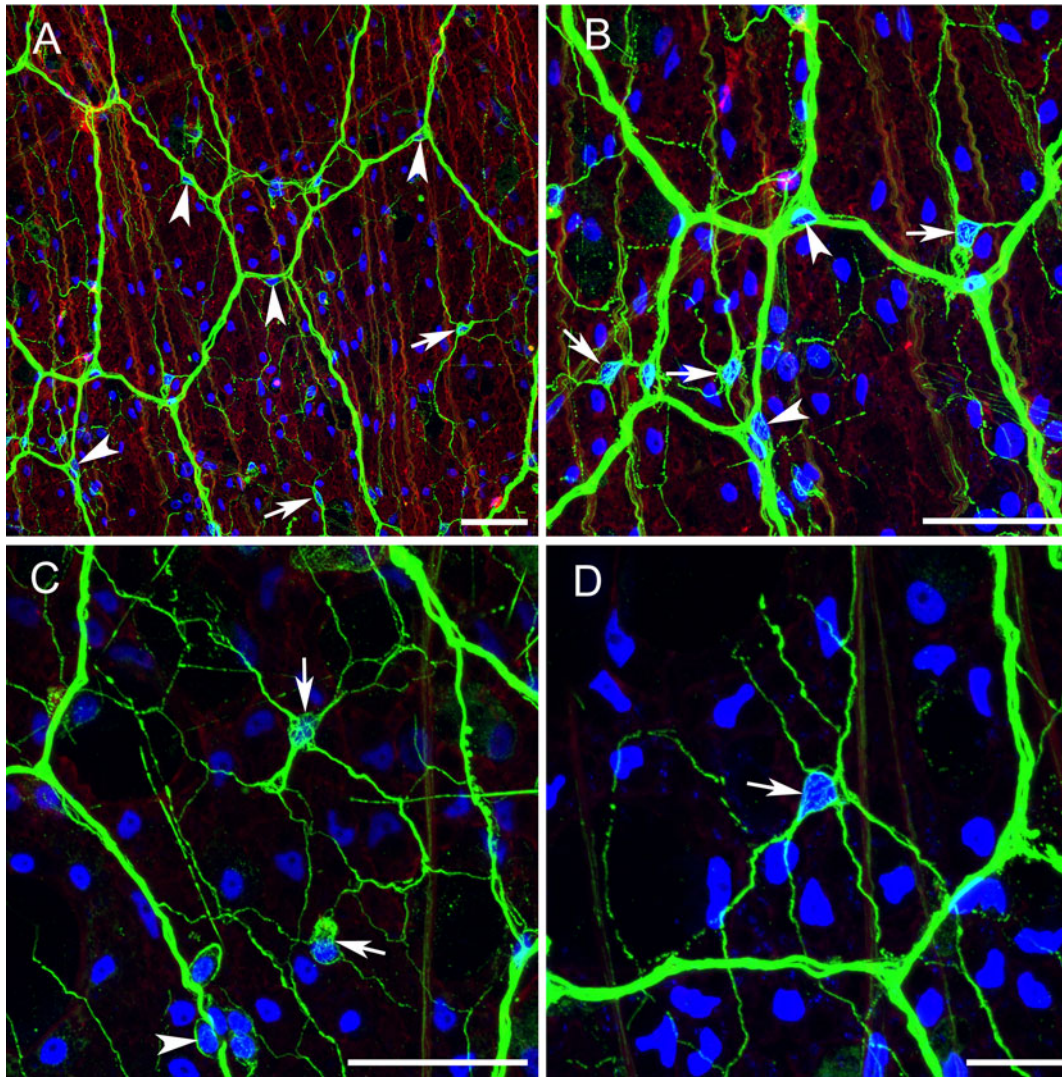

Fig. 6 Subepithelial neural network in *Pleurobrachia pileus* (tubulin AB, green; phalloidin labeling is red, while DAPI is blue). Note the individual neurons with long processes between major strands of the network (arrows). Arrowheads point to the nuclei inside the strands of the network. Scale bars: (**a**, **b**, and **c**) 25 μm; (**d**) 10 μm

3 Methods

Fixation—12 h

1. Before fixation and dissection, animals were incubated in high Mg^{2+} seawater (0.3 M $MgCl_2$ in filtered seawater in 1:1 ratio) for 30–40 min. It was essential for such muscular animals like *Beroe* with strong withdrawal reactions (*see* **Note 1**).

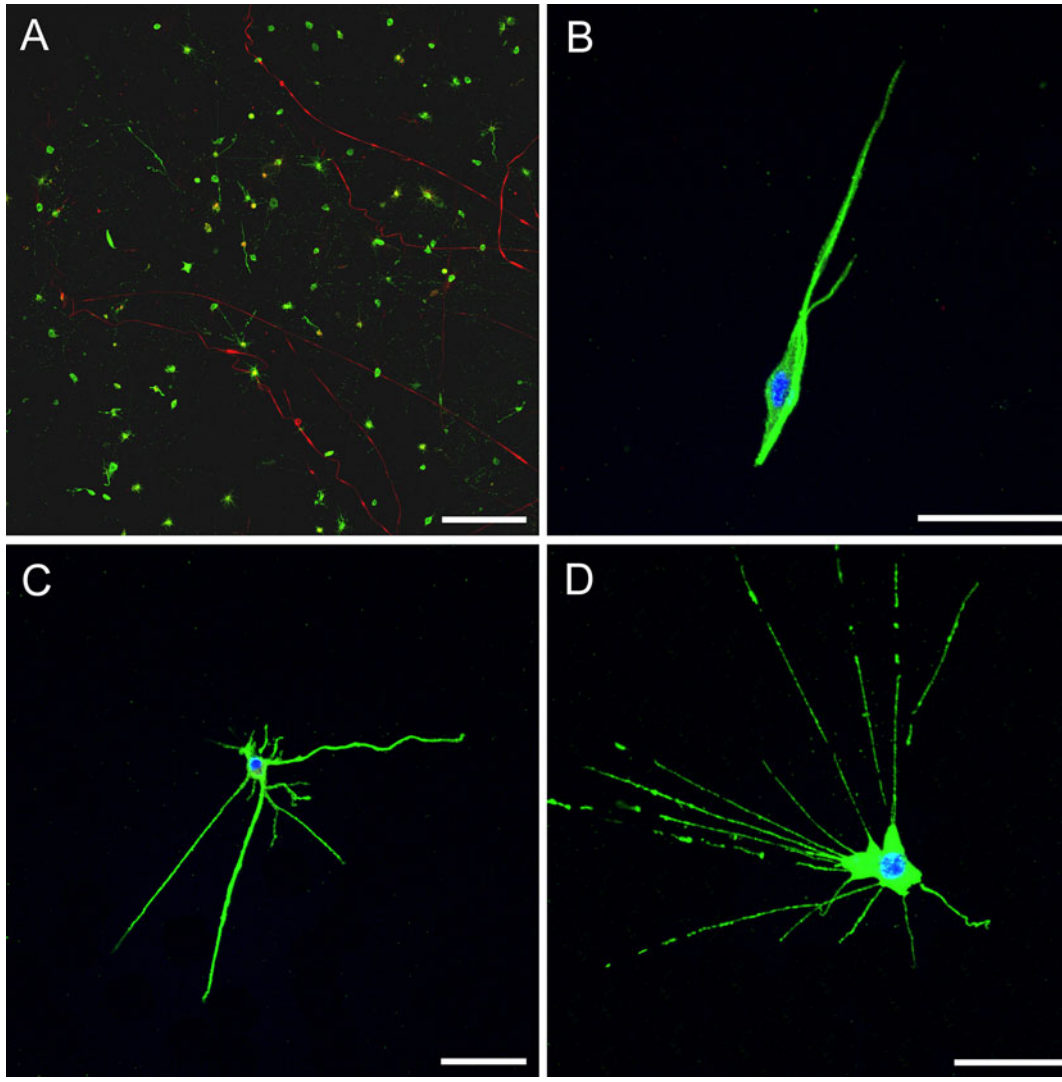

Fig. 7 Mesogleal neurons in *Pleurobrachia pileus* (tubulin AB, green; phalloidin labeling is red, while DAPI is blue). (**a**) Numerous tubulin-immunoreactive neural type cells in the mesogleal region. (**b**) Bipolar mesogleal neuron-like cell. (**c**, **d**) Multipolar mesogleal neural type cells. Scale bars: (**a**) 100 μm; (**b**, **c**, and **d**) 20 μm

2. Large ctenophores were dissected in smaller pieces, while small animals were fixed whole (all *Pleurobrachia* and smaller *Beroe*). The focus during dissecting was on specific organs and areas—like the aboral organ with polar fields, comb rows, mouth, etc.
3. The tissue was placed in 20–30 mL vials with closed lids.
4. Seawater or high Mg^{2+} seawater was removed from the vials with a suction pipette, and 4% paraformaldehyde in PBS was immediately added into vials (*see* **Note 1**).

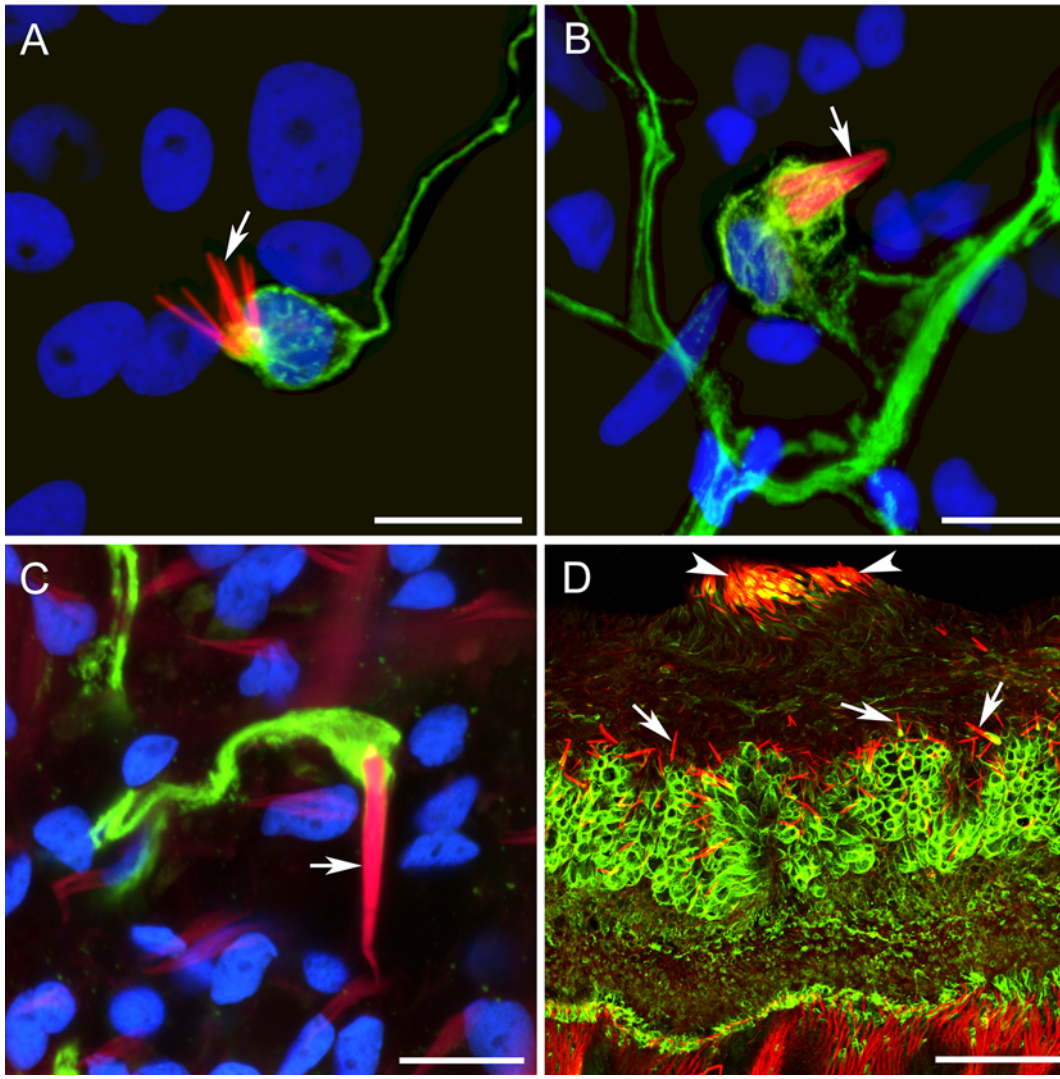

Fig. 8 Surface receptors in *Beroe abyssicola*. (**a**, **b**) Receptors with multiple stereocilia labeled by phalloidin in red (arrows), DAPI is blue. The cell body of the receptor is labeled by tubulin AB (green). (**c**) Receptor with a single large stereocilium labeled by phalloidin (arrow) on the top of tubulin-ir cell. (**d**) Numerous receptors with a single large and labeled by phalloidin cilium (arrows) are found on the lips and in large congregates (arrowheads) on the outside body surface. Scale bars: (**a**, **b**, and **c**) 10 μm; (**d**) 100 μm

5. The vials with tissue in fixative solution were placed in the refrigerator and left overnight or for 12 h at 5 °C. The tissue can stay in a fixative for a day. However, long fixations for 2 days or more are not advisable.
6. After fixation, the tissue was rinsed in 0.1 M PBS. The fixative was removed from vials with suction pipettes and properly disposed of—under the hood and using gloves. Then clean PBS was added for 40 min.

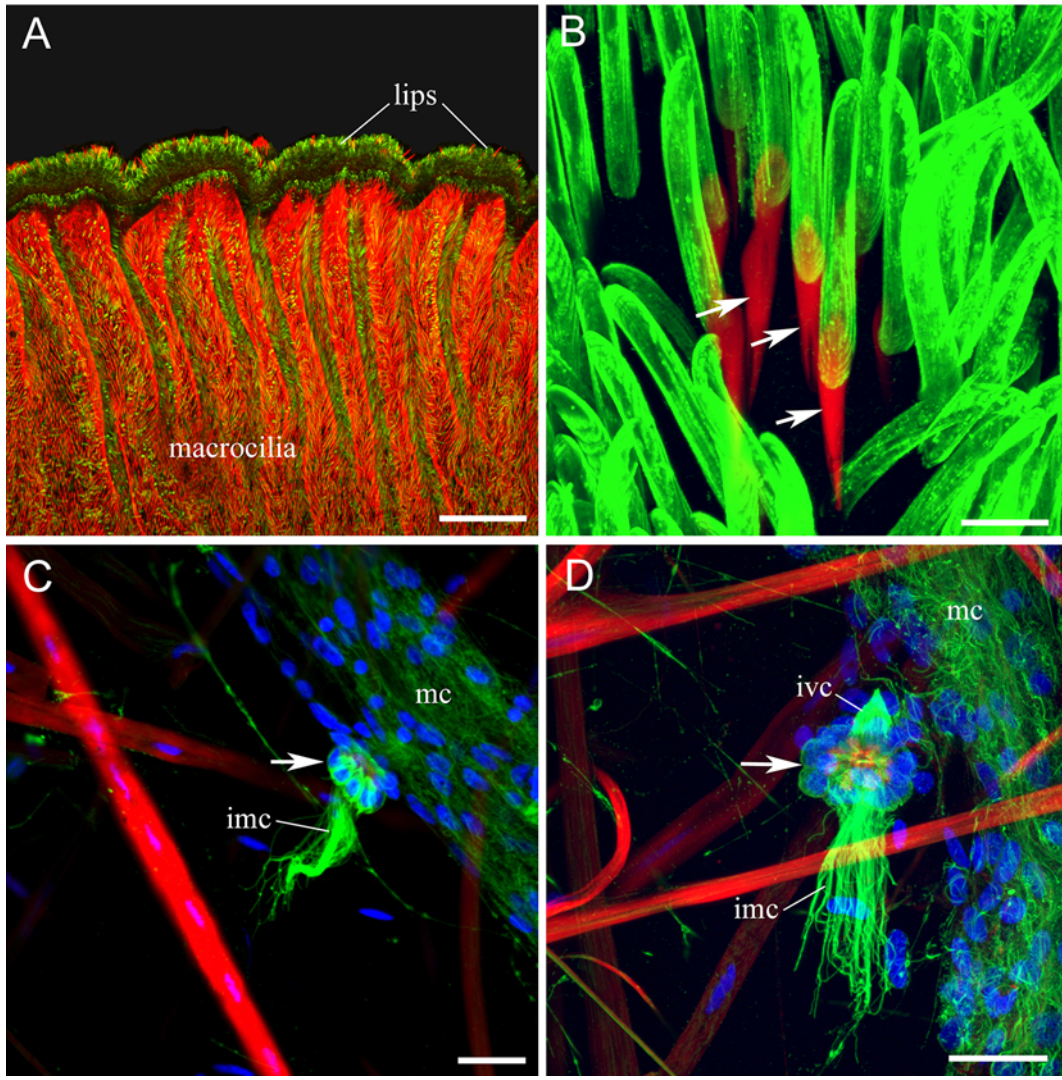

Fig. 9 Non-neuronal structures in ctenophores: Macrocilia and ciliated pores in meridional canals. Tubulin AB is green, phalloidin is red, while DAPI is blue. (**a**, **b**) Microcilia inside the mouth of *Beroe abyssicola*. The long cylindrical body of macrocilia is stained by tubulin AB and attached to the phalloidin-labeled bundles of actin (arrows) inside the macrociliary cells. (**c**, **d**) Meridional canal pores (ciliated rosettes) in *Beroe abyssicola.* Each pore consists of two superimposed rings (arrows) with eight ciliated cells. Two groups of cilia are labeled by tubulin antibody: shorter intravascular cilia, which project into the meridional canal, and much longer intramesogleal cilia that protrude into the mesoglea. Muscle fibers are stained in red. Abbreviations: *mc* meridional canal, *imc* intramesogleal cilia, *ivc* intravascular cilia. Scale bars: (**a**) 200 μm; (**b**) 10 μm; (**c**, **d**) 20 μm

7. There should be at least three rinses with a total minimum time of 2 h. At this stage, the tissue can be stored in a refrigerator for a few days.

Blocking solution—12 h to 1 day

8. After fixation and rinses, the large pieces of tissue and the whole fixed small animals were dissected and trimmed as necessary for the final viewing. The tissue was removed from the vials, placed in the small Petri dishes, and looked at under a dissecting microscope. The final size of tissues should be relatively flat and small to be viewed effectively under the confocal microscope later. At the same time, one should keep in mind the necessity to preserve the functional continuity of the data (e.g., not to cut polar fields in half).
9. The cleaned and trimmed pieces of tissue were then placed again in the 20–30 mL vials with the clean 0.1 M PBS containing 0.2% Triton-X 100. Incubation of the tissue in Triton-X 100 solution for 6–12 h was necessary for improving the subsequent antibody penetration (*see* **Note 2**).
10. PBS in vials was then replaced with 6% goat serum in PBS as a blocking solution. The specimens were kept in a refrigerator at 5 °C for 12 h (up to 1 day).

    Primary antibodies—2 days

11. The old blocking solution was removed from the vials.
12. The primary antibodies were then diluted in 6% goat serum in PBS (blocking solution) and added into vials with specimens. The final dilution depended on the type of primary antibodies. For example, we used the final dilution of 1:40 for the rat monoclonal anti-tubulin antibody. The tissue should stay in primary antibodies for about 2 days in a refrigerator at 5 °C. Depending on the tissue samples' size, the primary body incubation could be reduced to 1 day or increased to 3 days. From time to time, some shaking of the vials is advisable during incubation.
13. At the end of incubation, the primary antibody solution was removed from the vials and replaced with fresh 0.1 M PBS. The rinsing of specimens should last minimum 12 h, better 1 day, and include a minimum 3–4 rinses. All long-term steps are carried in a refrigerator.

    Secondary antibodies—1 day

14. Following the multiple rinses in PBS after primary antibody incubation, the specimens were placed in the secondary antibodies—one of two types of goat anti-rat IgG antibodies: Alexa Fluor 488 conjugated or Alexa Fluor 568 conjugated, at a final dilution 1:20 in 0.1 M PBS (*see* **Note 3**). Incubation in secondary antibody was carried in the refrigerator for minimum 12 h, up to 1 day.
15. The secondary antibodies were then removed from vials and replaced with 0.1 M PBS. There should be at least three rinses in PBS for 12 h.

    Phalloidin labeling—8 h to a day

16. In order to obtain additional information and visualize muscle fibers in ctenophores along with immunolabeling, we used the well-known marker phalloidin, which binds to F-actin. Depending on the type of secondary antibodies, we used Alexa Fluor 488 or Alexa Fluor 568 conjugated phalloidins. The tissue was incubated in phalloidin solution in PBS for 8 h at a final dilution 1:80 in a refrigerator at 5 °C.
17. The specimens were then washed in 0.1 M PBS several times for 6 h.

Mounting and Viewing

18. Following the PBS rinses, the pieces of tissue were transferred onto the microscope glass slides. Each piece of tissue was oriented for the best viewing position. The PBS was then carefully removed and a mounting medium was added to the slides. We preferred to use a hard-set mounting medium with DAPI to visualize the cell nuclei. The tissue was then covered with a cover glass.
19. For the mounting medium to harden sufficiently, it was necessary to wait for about 2 h. However, it was better to wait at least 6 h before viewing for DAPI staining to penetrate the entire thickness of the tissue.
20. The slides with mounted tissue were viewed first at the microscopes with epi-fluorescence to identify and select the best specimens and areas inside each specimen for the following detailed scanning on the confocal microscope.
21. The slides could be stored in closed booklets in a refrigerator for several weeks for later viewing. However, a signal loss would inevitably occur after prolonged exposure to the laser—especially fast the loss of signal developed for the phalloidin labeling.

The illustrated examples of immunolabeling in different ctenophore species are summarized in Figs. 2, 3, 4, 5, 6, 7, 8 and 9. For more detailed descriptions of microanatomy, we recommend several earlier and recent publications [10, 11, 17–36].

4 Notes

1. Paraformaldehyde fixation worked very well for many ctenophore groups such as *Pleurobrachia*, *Beroe*, *Hormifora*, and *Euplokamis*. However, ctenophores from two genera, *Mnemiopsis* and *Bolinopsis*, which are the most fragile, full of water, and least dense, were very difficult to fix. Their tissue was disintegrating during fixation in paraformaldehyde. Nevertheless, the larva stages of these species were much denser and tougher and could be easily fixed and processed for

immunolabeling (Figs. 1a and 2—*Mnemiopsis* and *Bolinopsis* larvae). For adult *Mnemiopsis* and *Bolinopsis*, we have developed a different fixation protocol, which showed promising results. The fixative solution contained 1 part of 6% paraformaldehyde in filtered seawater and 1 part of 100% ethanol with added 0.5% acetic acid. The prepared solution was then placed in the freezer to reach −20 °C. The specimens were cooled in a refrigerator to 5 °C; then the seawater was removed with a suction pipette, and a fixative solution at −20 °C was added into the vials with ctenophore tissue. The vials were kept at −20 °C in the freezer for 1 h and then transferred into the refrigerator at 5 °C for overnight (12 h) fixation. The following steps were usual, including rinses and incubations in primary and secondary antibodies.

2. Ctenophore tissue is not very dense and easily penetrable for primary and secondary antibodies. Therefore, we used Triton-X 100, widely accepted in immunochemistry protocols for improved antibody penetration, for only 6–12 h during incubation in the blocking solution. The only exception was the early larva stages, which had much tougher tissue. We used 0.1 M PBS with 0.2% Triton-X 100 during blocking solution and primary antibody incubations. We also used Triton-X 100 in our novel fixation protocol, which was used for adult *Mnemiopsis* and *Bolinopsis* and contained 1:1 paraformaldehyde and ethanol with 1% acetic acid at −20 °C.
3. Some obvious reminders. A blocking solution should be made from the serum of the animal, which was used to produce secondary antibodies. Secondary antibodies are made against the animal in which the primary antibody was produced. If the secondary antibody were conjugated with Alexa Fluor 488, then phalloidin should be labeled with a fluorescent label with a different wavelength—Alexa Fluor 568 (and vice versa).

Acknowledgments

This work was supported in part by the Human Frontiers Science Program (RGP0060/2017) and the National Science Foundation (IOS-1557923) grants to L.L.M. Research reported in this publication was also supported in part by the National Institute of Neurological Disorders and Stroke of the National Institutes of Health under award number R01NS114491 (to LLM). The content is solely the authors' responsibility and does not necessarily represent the official views of the National Institutes of Health.

References

1. Moroz LL et al (2014) The ctenophore genome and the evolutionary origins of neural systems. Nature 510(7503):109–114
2. Ryan JF et al (2013) The genome of the ctenophore *Mnemiopsis leidyi* and its implications for cell type evolution. Science 342(6164): 1242592
3. Hoencamp C et al (2021) 3D genomics across the tree of life reveals condensin II as a determinant of architecture type. Science 372(6545):984–989
4. Schultz DT et al (2021) A chromosome-scale genome assembly and karyotype of the ctenophore *Hormiphora californensis*. G3 (Bethesda) 11(11):jkab302
5. Whelan NV et al (2017) Ctenophore relationships and their placement as the sister group to all other animals. Nat Ecol Evol 1(11): 1737–1746
6. Telford MJ, Moroz LL, Halanych KM (2016) Evolution: a sisterly dispute. Nature 529(7586):286–287
7. Halanych KM et al (2016) Miscues misplace sponges. Proc Natl Acad Sci U S A 113(8): E946–E947
8. Redmond AK, McLysaght A (2021) Evidence for sponges as sister to all other animals from partitioned phylogenomics with mixture models and recoding. Nat Commun 12(1):1783
9. Li Y et al (2021) Rooting the animal tree of life. Mol Biol Evol 38(10):4322–4333
10. Hernandez-Nicaise M-L (1991) Ctenophora. In: Harrison FWFW, Westfall JA (eds) Microscopic anatomy of invertebrates: Placozoa, Porifera, Cnidaria, and Ctenophora. Wiley, New York, pp 359–418
11. Tamm SL (1982) Ctenophora. In: Electrical conduction and behavior in "simple" invertebrates. Clarendon Press, Oxford, pp 266–358
12. Nielsen C (2019) Early animal evolution: a morphologist's view. R Soc Open Sci 6(7): 190638
13. Moroz LL, Kohn AB (2016) Independent origins of neurons and synapses: insights from ctenophores. Philos Trans R Soc Lond Ser B Biol Sci 371(1685):20150041
14. Mitchell DG, Edgar A, Martindale MQ (2021) Improved histological fixation of gelatinous marine invertebrates. Front Zool 18(1):29
15. Pang K, Martindale MQ (2008) Ctenophore whole-mount antibody staining. CSH Protoc 2008:pdbprot5086
16. Sachkova MY et al (2021) Neuropeptide repertoire and 3D anatomy of the ctenophore nervous system. Curr Biol 31(23):5274–5285 e6
17. Norekian TP, Moroz LL (2016) Development of neuromuscular organization in the ctenophore *Pleurobrachia bachei*. J Comp Neurol 524(1):136–151
18. Norekian TP, Moroz LL (2019) Neural system and receptor diversity in the ctenophore *Beroe abyssicola*. J Comp Neurol 527(12): 1986–2008
19. Norekian TP, Moroz LL (2019) Neuromuscular organization of the Ctenophore *Pleurobrachia bachei*. J Comp Neurol 527(2):406–436
20. Norekian TP, Moroz LL (2020) Comparative neuroanatomy of ctenophores: neural and muscular systems in *Euplokamis dunlapae* and related species. J Comp Neurol 528(3): 481–501
21. Norekian TP, Moroz LL (2021) Development of the nervous system in the early hatching larvae of the ctenophore *Mnemiopsis leidyi*. J Morphol 282(10):1466–1477
22. Tamm SL (2014) Cilia and the life of ctenophores. Invertebr Biol 133(1):1–46
23. Jager M et al (2011) New insights on ctenophore neural anatomy: immunofluorescence study in *Pleurobrachia pileus* (Muller, 1776). J Exp Zool B Mol Dev Evol 316B(3):171–187
24. Jager M et al (2013) Evidence for involvement of Wnt signalling in body polarities, cell proliferation, and the neuro-sensory system in an adult ctenophore. PLoS One 8(12):e84363
25. Bullock TH, Horridge GA (1965) Structure and function in the nervous systems of invertebrates, vol 1719. Freeman, San Francisco
26. Chun C (1880) Die Ctenophoren des Golfes von Neapel. Fauna Flora Neapel, Monogr, pp 1–313
27. Hernandez-Nicaise ML (1973) The nervous system of ctenophores. II. The nervous elements of the mesoglea of beroids and cydippids (author's transl). Z Zellforsch Mikrosk Anat 143(1):117–133
28. Hernandez-Nicaise ML (1973) The nervous system of ctenophores. I. Structure and ultrastructure of the epithelial nerve-nets. Z Zellforsch Mikrosk Anat 137(2):223–250
29. Hernandez-Nicaise ML (1973) The nervous system of ctenophores. III. Ultrastructure of synapses. J Neurocytol 2(3):249–263
30. Hernandez-Nicaise ML, Amsellem J (1980) Ultrastructure of the giant smooth muscle

fiber of the ctenophore *Beroe ovata*. J Ultrastruct Res 72(2):151–168

31. Horridge GA (1964) The giant mitochondria of ctenophore comb plates. Q J Microsc Sci 105:301–310
32. Horridge GA (1964) Presumed photoreceptive cilia in a ctenophore. Q J Microsc Sci 105:311–317
33. Horridge GA (1965) Non-motile sensory cilia and neuromuscular junctions in a ctenophore independent effector organ. Proc R Soc Lond Biol 162:333–350
34. Horridge GA (1974) Recent studies on the Ctenophora. In: Muscatine L, Lenhoff HM (eds) Coelenterate biology. Academic, New York, pp 439–468
35. Horridge GA, Mackay B (1964) Neurociliary synapses in *Pleurobrachia* (Ctenophora). Q J Microsc Sci 105:163–174
36. Hyman LH (1940) Invertebrates: protozoa through Ctenophora, vol 1. McGraw-Hill, New York/London, p 726