Immunofluorescence in *Pleurobrachia pileus* adult tissues

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– PROTOCOL –

Day 1 – Sample preparation

> *In each step, samples can be rocked on a nutator or on an orbital (horizontal) shaker.*

1. Dissect the desired adult tissue in 100% methanol.
2. Wash samples for 15 min each with a graded series of 500 µL (or 1 mL) of **methanol (60 and 30 %) in PBSTx** at RT.
3. Wash samples for 15 min with 500 µL (or 1 mL) of **PBSTx** at RT.
4. *(optional)* Rock samples for 2 min with 500 µL (or 1 mL) of **2.6 µg/mL proteinase K** at RT.
5. *(optional)* Wash samples for two times without incubation with 500 µL (or 1 mL) of **PBST** at RT.
6. *(optional)* Post-fix samples for 30 min with 500 µL (or 1 mL) of **3.7 % PFA** at RT.
7. *(optional)* Wash samples for 2 × 10 min with 500 µL (or 1 mL) of **PBSTx** at RT.
8. Incubate samples for 2 h with 500 µL (or 1 mL) of **PBSTx** at RT.
9. Incubate samples for >12 h (overnight) with 500 µL (or 1 mL) of **a blocking solution of 6 % goat serum in PBSTx** at RT.

Day 2 – Primary antibody reaction

1. Incubate samples for 24–48 h with 500 µL of **1:40 primary antibody in 6 % goat serum solution** at 4 °C.

Day 3 – Primary antibody reaction

1. *ongoing incubation with primary antibody solution*

Day 4 – Secondary antibody reaction

1. Wash samples for 6 × 30 min with 500 µL(or 1 mL) of **TBSTx** at RT.
2. *optional* Incubate samples for 1 h with 500 µL (or 1 mL) of **a blocking solution of 6 % goat serum in PBSTx** at RT.
3. Incubate samples for >12 h (overnight) with 500 µL of **1:20 secondary antibody in 6 % goat serum** at RT.

Day 5 – IF conclusion

1. Wash samples for 2 × 5 min, 5 × 15 min, and 1 × 5 min with 500 µL (or 1 mL) of **PBSTx** at RT.
2. Incubate samples for 30–60 min with 1 mL of **50 % glycerol in 1× PBS** at RT.
3. Incubate samples for 30–60 min with 1 mL of **75 % glycerol in 1× PBS** at RT.
4. Collect samples from the 75 % glycerol solution and place them on a cleaned (bridged, if necessary) slide.
5. Add ~15–20 µL of mounting medium (or VECTASHIELD® PLUS Antifade Mounting Medium with DAPI [H-2000]) directly on samples.

> *Adjust the amount of mounting medium according to the cover glass dimensions, the quantity of samples, and the thickness of the bridge.*

6. Seal the slide with nail polish and store in the dark at 4 °C.

– RECIPES –

1× PBS with 0.01 % Triton X-100 (PBSTx or PTx)

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 50 mL)
Triton X-100	5.0 µL	647.00	0.01 %
20× PBS	5.0 mL	–	1×
Ultrapure water	–	–	–

1. Add 5 mL of 20× PBS to a graduated cylinder.
2. Add 5 µL of Triton X-100.
3. Fill up to 50 mL with ultrapure water.

6 % goat serum in PBSTx

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 5 mL)
Normal goat serum	300.0 µL	–	6 %
Triton X-100	0.5 µL	647.00	0.01 %
20× PBS	250.0 µL	–	1×
Ultrapure water	–	–	–

1. Add 250 µL of 20× PBS to a beaker.
2. Add 300 µL of normal goat serum.
3. Add 0.5 µL of Triton X-100.
4. Fill up to 5 mL with ultrapure water.

Proteinase K working solution

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 10 mL)
20 mg/mL proteinase K stock solution	1.30 µL	–	2.6 µg/mL
PBST	–	–	–

1. Add 1.3 µL of 20 mg/mL proteinase K stock solution to 8 mL of PBST.
2. Fill up to 10 mL with PBST.

1:500 DAPI staining solution

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 500 µL)
DAPI	1.0 µL	–	1:500
1× PBS	499.0 µL	–	–

– RESOURCES –

1. **IHC in various ctenophore species.**

- Burkhardt, P., Colgren, J., Medhus, A., Digel, L., Naumann, B., Soto-Angel, J. J., ... & Kittelmann, M. (2023). Syncytial nerve net in a ctenophore adds insights on the evolution of nervous systems. *Science*, *380*(6642), 293-297. 10.1126/science.ade5645
- Jager, M., Chiori, R., Alié, A., Dayraud, C., Quéinnec, E., & Manuel, M. (2011). New insights on ctenophore neural anatomy: immunofluorescence study in *Pleurobrachia pileus* (Müller, 1776). *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *316*(3), 171-187. 10.1002/jez.b.21386
- Moroz, L. L., Kocot, K. M., Citarella, M. R., Dosung, S., Norekian, T. P., Povolotskaya, I. S., ...& Kohn, A. B. (2014). The ctenophore genome and the evolutionary origins of neural systems. *Nature*, *510*(7503), 109-114. 10.1038/nature13400
- Sachkova, M. Y., Nordmann, E. L., Soto-Àngel, J. J., Meeda, Y., Górski, B., Naumann, B., ... & Burkhardt, P. (2021). Neuropeptide repertoire and 3D anatomy of the ctenophore nervous system. *Current Biology*, *31*(23), 5274-5285. 10.1016/j.cub.2021.09.005