# Chapter 9

# Gene Expression Patterns in the Ctenophore *Pleurobrachia bachei*: In Situ Hybridization

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## Abstract

In situ hybridization is a powerful and precise tool for revealing cell- and tissue-specific gene expression and a critical approach to validating single-cell RNA-seq (scRNA-seq). However, applying it to highly fragile animals such as ctenophores is challenging. Here, we present an in situ hybridization protocol for adult *Pleurobrachia bachei* (Cydippida)—a notable reference species representing the earliest-branching metazoan lineage, Ctenophora, sister to the rest of Metazoa. We provided expression patterns for several markers of cell phenotypes, as illustrated examples. The list includes predicted small secretory molecules/neuropeptides, WntX, genes encoding RNA-binding proteins (Musashi, Elav, Dicer, Argonaut), Neuroglobin, and selected transcription factors such as BarX. Both cell- and organ-specific expression of these genes further support the convergent evolution of many ctenophore innovations, which are remarkably distinct from tissue and organ specification in other basal metazoan lineages.

**Key words** In situ hybridization, Gene expression, Ctenophores, Genome, Wnt signaling, Dicer, Argonaut, Neuroglobin, Musashi, ELAV, Neuropeptides, *Pleurobrachia*, *Mnemiopsis*, Neurons, Evolution

## 1 Introduction

Despite the recent progress in comparative biology, comb jellies or ctenophores are still one of the most elusive clades among the basal Metazoa [1–3]. The phylum Ctenophora is critically important to understand the origins of animal innovations and signaling as well as to resolve the relationships among early-branching metazoans [1, 4–16].

Following the genome sequencing and assembly, we broadly used in situ hybridization (ISH) to validate genomic predictions and characterize tissue-specific expression profiling in the ctenophore *Pleurobrachia bachei* [17, 18]. However, *Pleurobrachia*, in particular, and ctenophores, in general, are incredibly challenging preparations to perform ISH and even to fix and process [19]. Their tissues are very fragile, and ISH in adults is virtually

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impossible for most species. Unsurprisingly, most efforts focused on larval stages using *Mnemiopsis leidyi* as a model [20].

ISH was first described in 1969 by Joseph G. Gall [21, 22]. ISH is based on a simple principle where a nucleic acid probe with a reporter localizes the complementary RNA or DNA sequence within a preserved biological tissue. The nucleic acid probe can be a double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), RNA (riboprobes), or synthetic oligonucleotides such as peptide nucleic acid (PNA), morpholino, and locked nucleic acid (LNA). The molecular reporter for the probes can be radioactive isotopes such as $^{32}P$, $^{35}S$, or, $^{3}H$ or nonradioactive labels including biotin, digoxigenin (DIG), and fluorescent dyes (for fluorescent in situ hybridization—FISH). Detection of the reporter attached to the in situ probes allows visualization of a specific DNA or RNA sequence in a cell, tissue section, or whole animal. As a result, cell/tissue gene expression can be estimated using highly specific riboprobes, and numerous approaches have been successfully used both in developmental biology and neuroscience.

We present an ISH protocol successfully tested using several fragile and gelatinous ctenophores like *Pleurobrachia bachei* as an illustrative example (*see* also [23, 24]). This protocol is derived from work on other invertebrates [25], updated and revised [26], and validated [17, 18]. Our ISH protocol can be easily adapted to other fragile animals as well.

## 2 Materials

### 2.1 Reagents

#### 2.1.1 Probe Generation Reagents

- Not I-HF with Buffer 4, BSA (Cat # R0189S, New England BioLabs).
- Pme1 with Buffer 4, BSA (Cat # R0560S, New England BioLabs).
- pCR®4-TOPO Vector (Cat # K4575-J10, Invitrogen/Life Technologies).
- DIG (digoxigenin) RNA Labeling Mix (Cat # 11277073910, Sigma Aldrich).
- Fluorescein RNA Labeling Mix (Cat # 11685619910, Sigma Aldrich).
- T3 RNA polymerase (Cat # 11031163001, Sigma Aldrich).
- T7 RNA polymerase (Cat # 10881767001, Sigma Aldrich).
- Turbo DNaseI (Cat # AM1907, ThermoFisher Scientific).
- 7.5 M Lithium Chloride (Cat # 9480, ThermoFisher Scientific).
- MiniElute PCR Purification Kit (Cat # 28004, Qiagen).
- Qubit® RNA Assay Kit (Cat # Q32852, ThermoFisher Scientific).

- Qubit® DNA Assay Kit (Cat # Q32850, ThermoFisher Scientific).
- RNAse Inhibitor (Roche # 03-335-399-001).

### 2.1.2 In Situ Hybridization Reagents

- 10× phosphate buffered saline (PBS) (Cat # BP399-1, ThermoFisher Scientific).
- Formaldehyde 37% (Cat # BP531-500, ThermoFisher Scientific).
- Methanol (MeOH) (Cat # BP1105-1, ThermoFisher Scientific).
- Ethanol, 100% (EtOH) (Cat # NC9789925, ThermoFisher Scientific).
- Triton X 100 (Cat # NC9903183, ThermoFisher Scientific).
- Tween-20 (Cat # BP337-100, ThermoFisher Scientific).
- 1 M $MgCl_2$, RNase-free (Cat # 9530G, ThermoFisher Scientific).
- 1 M Tris pH 8.0 (Cat # AM9856, ThermoFisher Scientific).
- 0.5 M EDTA pH 8.0 (Cat # 9260G, ThermoFisher Scientific).
- 5 M NaCl, RNase-free (Cat # 9759, ThermoFisher Scientific).
- Yeast tRNA (Cat # 15401-029, ThermoFisher Scientific).
- SSC 20× (saline-sodium-citrate buffer) (Cat # 15557-036, ThermoFisher Scientific).
- Denhardt Solution 50× (Cat # D2532, Sigma Aldrich).
- Goat Serum (Cat # G9023-10ML, Sigma Aldrich).
- Levamisole (Cat # 31742, Sigma Aldrich).
- Albumin from bovine serum, BSA (Cat # A9647-50G, Sigma Aldrich).
- Sodium dodecyl sulfate (SDS), 20% solution (Cat # 75832, Affymetrix).
- NBT (4-nitro-blue-tetrazolium-chloride)/BCIP (5-bromo-4-chloro-3-indolyl-phosphate) stock solution (Cat # 11681451001, Sigma Aldrich).
- Anti-digoxigenin-alkaline phosphatase (AP), Fab fragments (Cat # 11093274910, Sigma Aldrich).
- Anti-fluorescein-AP, Fab fragments (Cat # 11426338910, Sigma Aldrich).
- BM Purple AP substrate (Cat # 11 442 074 001, Sigma Aldrich).
- Fast Red TR/Naphthol AS-MX AP substrate tablet set (Cat # F-4523, Sigma Aldrich).
- Vector Red AP Substrate kit (Cat # SK-5100, Vector Laboratories).

- TSA Plus Fluorescein kit (Cat # NEL741E001KT, PerkinElmer).
- Methyl salicylate (Cat # M6752-250ML, Sigma Aldrich).
- Permount (Cat # SP15-100, ThermoFisher Scientific).
- VECTASHIELD Mounting Medium (Cat # H-1000, Vector Laboratories).
- RNase-free and DNAase-free water (Cat # 10977-015, ThermoFisher Scientific).

### 2.2 Equipment

- Qubit® 2.0 Fluorometer (Cat # Q32866, Life Technologies).
- Belly Dancer orbital shaker (Cat # Z377554, Sigma Aldrich).
- Shaking incubator (Cat # H-7700-5, BioExpress).
- 24-well plate (Cat # 720084, ThermoFisher Scientific).
- Nalgene sterile disposable filter units 0.2 μM (Cat # 097403A, ThermoFisher Scientific).
- Nalgene syringe filter, 0.2 μM (Cat # 192-252-0, ThermoFisher Scientific).
- 60 mL syringes (Cat # 13-689-8, ThermoFisher Scientific).
- Corning 50 mL centrifuge tubes (Cat # 05-538-68, ThermoFisher Scientific).
- Dissecting tools.
- Microscope of choice.

### 2.3 Stock Solution Preparation

Since this protocol involves working with RNA, all solutions need to be RNase-free (*see* **Note 1** for tips on working with RNA). For dilutions, Milli-Q $H_2O$ can be used because it is RNase- and DNase-free. We prepare most working solutions in Corning 50 mL centrifuge tubes and store them at the appropriate temperature, 4 °C or −20 °C.

#### 2.3.1 Stock Solutions

- Prepare a liter of filter sea water (FSW) (*see* **Note 2** for preparation of FSW).
- Make three separate concentrations of methanol (MeOH). However, we prefer to use PTW, not water, to dilute the MeOH.
- 30%, 50%, 70% MeOH
- Add 10 mL, 20 mL, and 30 mL MeOH to different Corning 50 mL tubes.
- Fill each to 40 mL Milli-Q $H_20$.

- 1× PBS
- 5 mL 10× PBS
- Fill to 50 mL with Milli-Q $H_2O$.

- Four percent formaldehyde/paraformaldehyde in FSW or 1× PBS (*see* **Note 3** for a comparison of formaldehyde and paraformaldehyde)
- 5.4 mL of 37% formaldehyde/paraformaldehyde
- Fill to 50 mL with FSW or 1× PBS.

- PTW
- 50 μL Tween-20
- Fill to 50 mL with 1× PBS.
- Hybridization buffer
- 50% formamide: 25 mL formamide
- 5 mM EDTA: 0.5 mL 0.5 M EDTA
- 5× SSC: 12.5 mL 20× SSC
- 0.1% Tween-20: 50 μL Tween-20
- 0.5 mg/mL tRNA: 25 mg tRNA (*see* **Note 4** for preparation of tRNA)
- 1× Denhardt: 1 mL of 50× Denhardt (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA).
- Fill to 50 mL with Milli-Q $H_2O$.

- PBT (250 mL)
- 1× PBS: 25 mL (10× PBS)
- 0.1% Triton X 100: 0.25 mL
- BSA (albumin) 2 mg/mL: 500 mg
- Fill to 250 mL with Milli-Q $H_2O$.

- 10% goat serum (*see* **Note 5** for preparation and storage of goat serum)
- 0.4 mL of GS
- Fill to 4 mL with PBT.

- 1% goat serum
- 0.04 mL GS
- Fill to 4 mL with PBT.

- Detection Buffer (**Critical; Always Make Fresh**)
- 100 mM NaCl: 1 mL of 5 M NaCl.
- 0.1% Tween-20: 50 μL of Tween-20
- 50 mM $MgCl_2$: 2.5 mL of 1 M $MgCl_2$
- 100 mM Tris: 5 mL of 1 M Tris pH = 8

- 1 mM levamisole (*see* **Note 6** for preparation of levamisole)
- Fill to 50 mL with Milli-Q $H_2O$ and then filter.
- NBT (4-nitro-blue-tetrazolium-chloride)/BCIP (5-bromo-4-chloro-3-indolyl-phosphate) Stock solution (*see* **Note 7** for alternative preparations of NBT/BCIP)
- *Fluorescent Label*
- Developing solution
- 100 mM NaCl: 1 mL of 5 M NaCl
- 0.2% Tween-20: 100 μL of Tween-20
- Fill to 50 mL with Milli-Q $H_2O$.
- Adjust pH to 8.2–8.5 with 10 M NaOH; filter.
- Detection buffer—Vector Red Alkaline Phosphatase kit
- To 5 mL of developing solution, add the following:
- Two drops of reagent 1; mix.
- Two drops of reagent 2; mix.
- Two drops of reagent 3; mix.
- Inactivation Buffer
- 10 mM HCl: 100 μL 5 N HCl
- 0.2% Tween-20: 100 μL Tween-20
- Fill to 50 mL Milli-Q $H_2O$.
- Prepare hazardous waste bottles for all reagents according to regulations.

## 3 Methods

### 3.1 Riboprobe Generation for ISH

The generation of an ISH riboprobe depends on the vector used to clone the transcript and eventually make a probe. The vector determines the restriction sites used for linearizing the plasmid and the choice of polymerase to synthesize the RNA. One can use a PCR product to generate a probe too, but PCR amplification would be required every time to synthesize a probe. This is impractical if many repetitive experiments are designed at different intervals. We recommend making bacterial stocks of all clones, so bacterial culture can be grown and plasmid isolated to make the probes as needed. All riboprobes are constructed in the antisense direction.

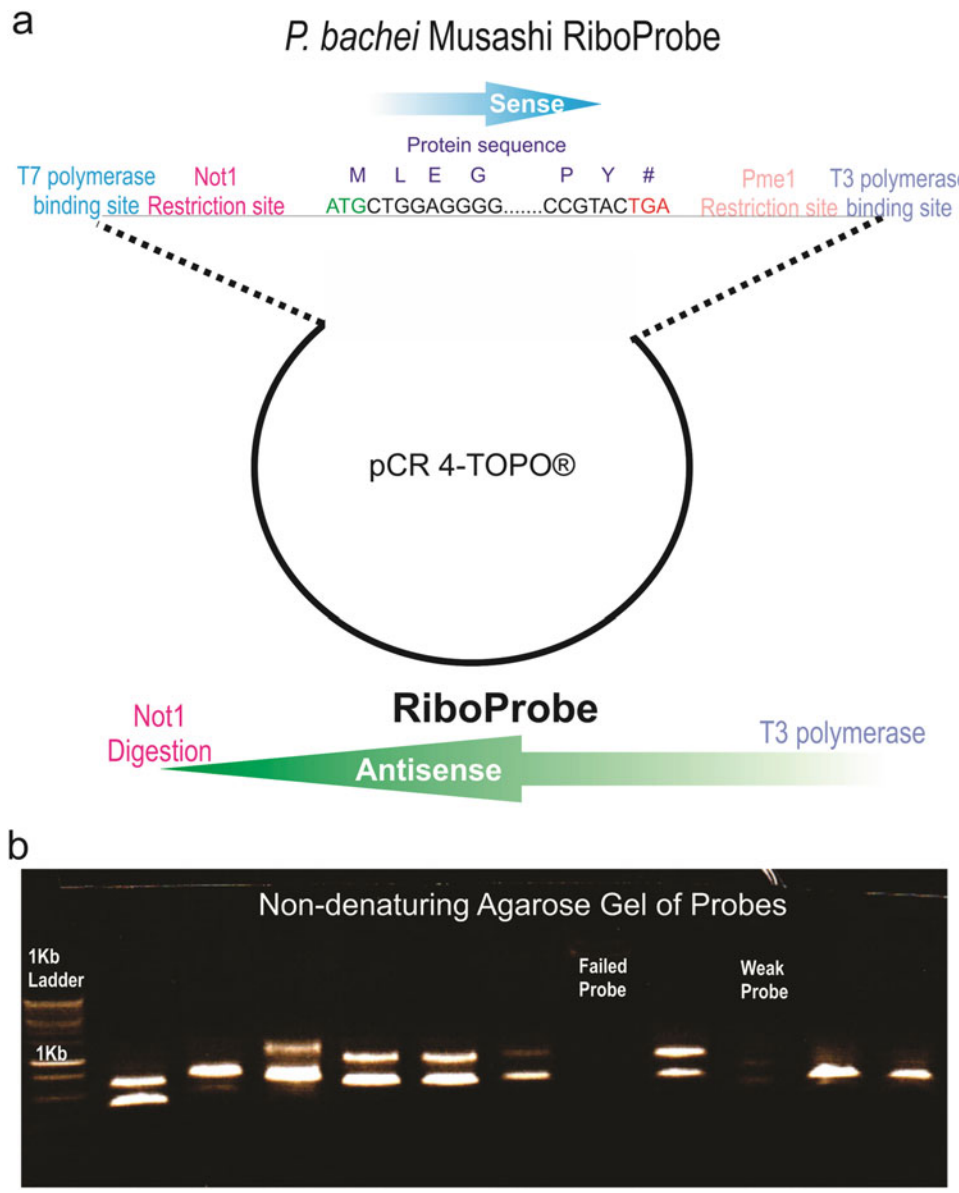


**Fig. 1** Strategy for construction of a probe and quality control. (**a**) Probe construction. There are three key steps to making a probe. (**1**) The selection of a target sequence and the vector for cloning: Here, we use pCR®4-TOPO. (**2**) Sequencing with either the T7 or T3 polymerase primer (these sites are specific to the vector). Then verify the direction of the insert. Is it in the sense or antisense direction relative to the primer used to sequence? To determine the sense or antisense direction, the predicted amino acid sequence is obtained from the cloned sequence. In this case, the sequence primer was T7, and the direction of the insert is in the sense direction relative to the predicted protein sequence. (**3**) Making the probe. An antisense riboprobe is generated with the opposite side polymerase, T3. The plasmid is first linearized with the restriction site at the 3′ end of the coding sequence. In this example, Not1 is used. Now, if the sequence would had been in the antisense direction relative to the sequencing primer, the riboprobe would had been made with the T7 polymerase and the plasmid and would had been linearized with Pme1. (**b**) Quality control of a probe. The integrity of the probe is evaluated on a non-denaturing agarose gel. Some probes will have two bands because

**Day 1**

1. Determine the strategy for making the probe as in Fig. 1a
   (a) First, determine the vector the clone is in. We use pCR®4-TOPO most frequently. Determine the direction of the insert relative to the vector. Antisense must be selected for riboprobe.
   (b) Determine the RNA polymerase: T3 polymerase or T7 polymerase for pCR®4-TOPO.
   (c) Determine restriction enzyme: Not1 for T3 polymerase and PmeI for T7 polymerase for pCR®4-TOPO.
   (d) Determine if restriction enzyme cuts insert.
2. Inoculate a 3 mL culture of Luria broth with appropriate antibiotic and clone of interest for the probe, then grow overnight at 37 °C with gentle shaking.

**Day 2**

3. Isolate the plasmid with miniprep from culture (use miniprep kit of choice, e.g., Qiagens, QIAprep) elute with 50 μL of Milli-Q $H_2O$ (30 μL if low concentration).
   (a) Run 1 μL on a 1% agarose gel to check for concentration, or check 2 μL on the Qubit® 2.0 fluorometer with DNA assay kit.
4. Linearized plasmid DNA with restriction enzymes using Not1or PmeI depending on direction.

   × μL for 1 μg plasmid DNA

   5.0 μL 10× buffer #4

   1.0 μL Not-HF (200 units) or PmeI (200 units) enzyme both using buffer 4

   0.5 μL of 100× BSA

   × μL Milli-Q $H_2O$ (add to a total of 50 μL)

   **50 μL total**
5. Incubate at 37 °C for 1 h.
   (a) Run a 2 μL on a 1% agarose gel to see if it is linearized; also run 2 μL of uncut plasmid to compare.
   (b) Purify the linearized plasmid to remove enzyme and salts using MiniElute PCR Purification Kit.

**Fig. 1** (continued) the RNA is not denatured, and the true size cannot be determined. One should look to see degradation and weak or the absence of the signal (if the probe failed). An alternative and convenient way is to use the Qubit® 2.0 fluorometer to determine the concentration and the Agilent TapeStation to determine the quality of the probe

(c) Add five volumes (250 μL) of PB; mix well and place in the column provided.

(d) Centrifuge for 1 min at 1000 g, and then turn to maximum speed for 1 min discarding flow-through (*see* **Note 8** pertaining to centrifuge timing).

(e) Place column back in the tube and add 750 μL of PE.

(f) Centrifuge at maximum speed for 1 min; discard flow-through.

(g) Then centrifuge at maximum speed for an additional 1 min to dry column.

(h) Place column in a new 1.5 mL tube and add 10–14 μL of Milli-Q $H_2O$, depending on concentration.

(i) Incubate for 1–3 min.

(j) Centrifuge for 1 min at 1000 g, and then turn to maximum speed for 1 min (*see* **Note 8** pertaining to centrifuge timing).

(k) The tube now contains linearized plasmid and template for transcription; store at −20 °C.

6. Run 1 μL on a 1% agarose gel to check for concentration or check 2 μL on Qubit® 2.0 fluorometer with DNA assay kit (can stop here).

**Day 3 (can also be done on Day 2 if time permitting)**

7. In vitro transcription reaction (for digoxigenin or fluorescein).

   × μL for 1 μg linearized plasmid DNA

   2.0 μL 10× transcription buffer (in polymerase kit)

   2.0 μL 10× DIG RNA labeling mix

   Or 2.0 μL 10× fluorescein RNA labeling mix

   2.0 μL RNA polymerase, 20 units/μL (T3 or T7)

   1.0 μL RNAse inhibitor

   × μL Milli-Q $H_2O$ (add to total of 20 μL)

   **20 μL total**

8. Incubate at 37 °C for 2 h.

   (a) Check 1 μL on a 1 % non-denaturing agarose gel for concentration.

9. Add 2 μL TURBO 10× buffer and 1 μL of TURBO DNase (two units/μL); incubate for 20–30 min at 37°C (*see* **Note 9** for reducing background).

10. Add 2.3 μL of resuspended DNase inactivation reagent, and incubate for 5 min at room temperature (RT). Centrifuge at 10,000 g for 1.5 min and transfer to clean 1.5 mL tube.

11. Run 1 μL on a 1% non-denaturing agarose gel for concentration (*see* Fig. 1b for examples of good and bad probes).
12. Check 1 μL on Qubit® 2.0 fluorometer with RNA assay kit; use 0.1–1.0 μg/mL in next step.
13. The timing of the probe construction can be performed concurrently with the first day of the ISH protocol. However, caution needs to be taken that a high-quality probe is prepared and ready when the prehybridization is started because the protocol cannot be delayed after this point.

### 3.2 Whole-Mount ISH of the Ctenophore, Pleurobrachia bachei

We used adult *P. bachei* animals, which can be small and sturdy to withstand fixation. These animals have very little connective tissue; therefore, challenges with permeability issues are minimal. Our protocol is broken up into 5 days which can be shortened if the time dedicated to the protocol is flexible. We include colorimetric and fluorescent development. This protocol was validated using more than 200 in situ hybridizations.

Make all stock solution (Subheading 2.3) in advance of starting this protocol except where noted. All washes are at room temperature (RT) unless otherwise noted. A flow diagram of the whole protocol with a timeline is in Fig. 2.

**Day 1 (fixation of the specimen)**

1. Fix the whole specimen in 4% formaldehyde in filtered sea water (FSW) overnight at 4 °C (*see* **Note 3**). The FSW should be, if possible, the same water animals are found in the wild.
2. Place no more than ten animals in a 50 mL conical tube. Critical is all the animals are covered with liquid. To mix, hold on the side and rotate gently (*see* **Note 10** for mixing strategy).

**Day 2 (dehydration of specimen)**

3. Rinse 3× for 10 min in PTW (PBST) at RT.
4. To mix, hold on side and rotate gently. Dispose of all solutions in hazardous waste properly.
5. Wash in 1:1 methanol (MeOH)/PTW (to equilibrate to MeOH) 10 min at RT.
4. Place specimen tube on its side in 100% MeOH and store at −20 °C for at least 2 h, but no more than a week (*see* **Note 11** for reducing background)

**Day 3 (rehydration of specimen and hybridization)**

6. Rehydrate specimens for 10 min each in MeOH/PTW 3:1, 1:1, 1:3, and 0:1 at RT.

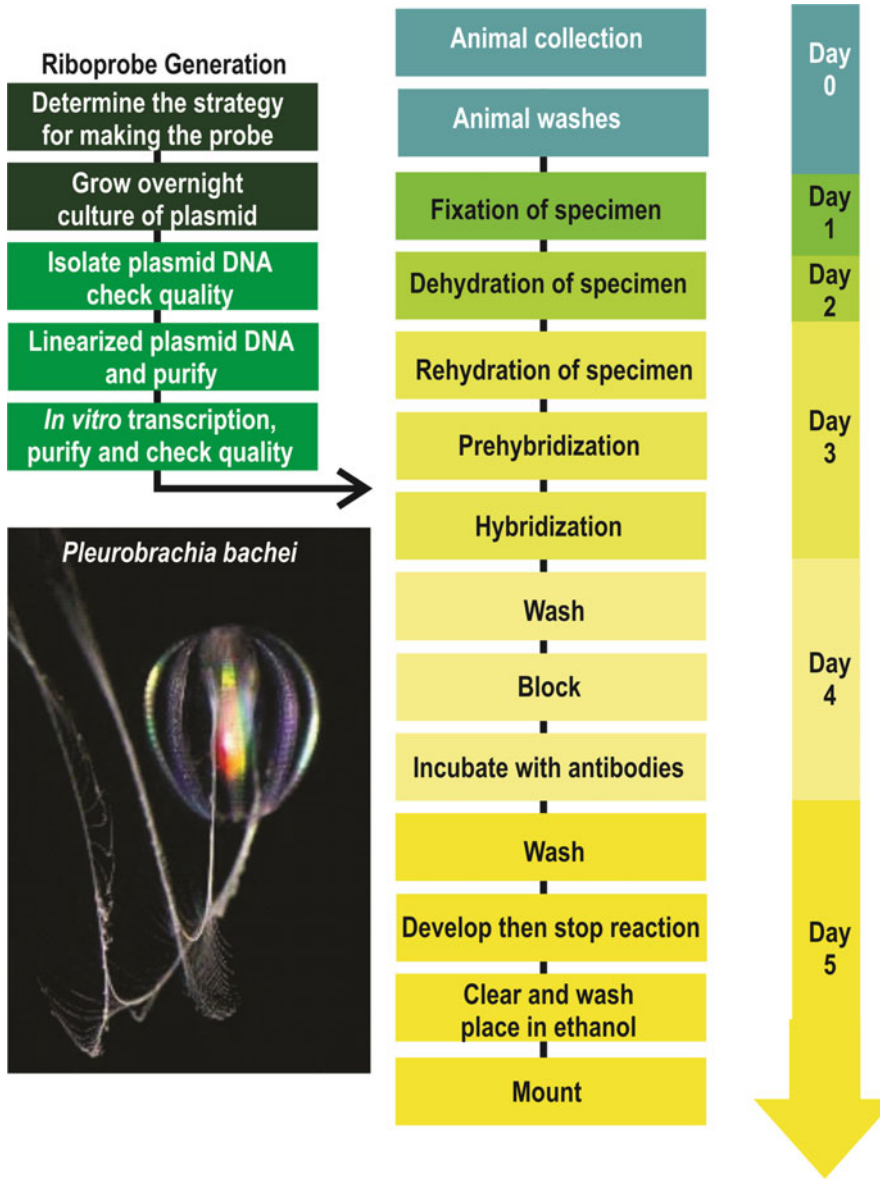


**Fig. 2** Flow diagram for in situ hybridization with a timeline. Different steps to the ISH are indicated in the boxes. Colors correlate to timing and the appropriate days. The timing of this protocol can be variable. For first-time users, it is best if the probe is made and ready to be used before the in situ hybridization protocol starts because if your probe is not of acceptable quality, nothing can proceed. Once one is experienced, probe generation can be done in parallel with the Day 1 of the in situ hybridization protocol. Day 1 can be a combination of collecting and washing animals followed by fixation of the specimens overnight. The next day can be a combination of Day 2 and 3, so the dehydration, hydration, and hybridization can be performed on the same day as long as the appropriate times are followed as suggested in the protocol. The next day is a short day with washing and incubation of antibodies. We do not suggest trying to shorten this step. The development is going to be the most variable for timing. Development can be very rapid for something like tubulin or a neuropeptide or very slow for a receptor or channel and depends on the expression level of your transcript of interest. Be prepared to have a long day of development for a successful result

7. Wash in 1:1 solution of hybridization buffer (HB) and PTW for 15 min at RT.
8. Prehybridization, incubate in HB (no probe) buffer for 1 h at 60 °C (*see* **Note 12**).
9. Hybridization, incubate in HB with DIG-RNA probe O/N at 60 °C and rock gently if possible.
   (a) Mix 1 mL HB with 2–10 μL of probe (add 0.1–1.0 μg/mL) (*see* **Note 13** on probe concentration).
   (b) Remove prehybridization buffer from tube with animals, and add freshly prepared HB to cover specimen.

**Day 4 (incubation with antibodies)**

10. Transfer specimens to 24-well plate and label wells or if specimens are too many in number or too large, continue in the larger conical capped tubes (*see* **Note 14** on 24-well plates).
11. Wash in HB for 30 min at 60 °C.
    (a) Remove old HB buffer and replace with 1 mL fresh HB in the same well.
12. Wash in 1:1 HB/PTW for 30 min at 60 °C.
    (a) Remove old HB buffer and replace with 1 mL 1:1 HB/PTW in the same well.
13. Wash in PTW for 30 min at RT.
    (a) Remove old 1:1 HB/PTW and replace it with 1 mL PTW in the same well.
14. Block in 10% goat serum (GS) for 60 min at RT.
    (a) Remove old PTW and replace it with 1 mL of 10% goat serum in the same well.
15. Incubate in anti-DIG 1/2000 at 4 °C O/N (*see* **Note 15** on the concentration of antibody).
    (a) Remove 10% goat serum, and replace with 1 mL 1% GS and 1:2000 of alkaline phosphatase-conjugated DIG antibodies in the same well.

**Day 5 (development)**

16. Wash 4× 30 min in PBS at RT.
    (a) Make detection buffer and aliquot 1 mL into a clean well for each sample. When ready to develop, add 20 μL of NBT/BICP mix until dissolved. It should be yellow in color! Be sure the NBT/BCIP is fully dissolved before adding samples. *Now* add samples. **Put on ice and cover with tin foil** (*see* **Note 16** about detection buffer).

17. Watch for appropriate color development (*see* **Note 17** for light considerations).
18. Prepare wells with PBS transfer animals to fresh wells with PBS to stop (*see* **Note 18** about crystal formation).
19. Wash in 4% formaldehyde in MeOH 30 min at RT (*see* **Note 19** for clearing times).
    (a) The timing of this step depends on the strength of the probe signal and the background. This time is for a high probe signal with low background.
20. Wash 3× 10 min in 100% ethanol (EtOH) at RT.
21. Store in 100% EtOH at 4 °C.
22. Mount.
    (a) Add animals to methyl salicylate until they sink in a vial (*see* **Note 20** for working with methyl salicylate).
    (b) Put animal onto microscope glass, clean, absorb methyl salicylate leftovers, add a drop of Permount, and put on the coverslip.

Figures 3, 4, 5, and 6 illustrate in situ hybridization experiments with *permanent* markers for selected genes.

#### 3.2.1 Fluorescent Detection

An alternative to the colorimetric single labeled DIG system is fluorescent detection using Vector Red Alkaline Phosphatase kit. Fluorescent detection can be with direct or indirect detection. This protocol is quick and gives excellent results. The TSA Plus Fluorescein kit also gave excellent results.

**Day 4 (a direct method of detection)**
Follow the above protocol for Day 4 up to **step 14** and proceed with the following:

1. Wash 5× 15 min with PBT at RT.
2. Make detection buffer and aliquot 1 mL into a clean well for each sample at RT.
3. Watch for appropriate color development. The vector red product is visible by the eye (it can be used for nonfluorescent ISH) and in the rhodamine, Cy3 channel (*see* **Note 21**).
4. Stop the reaction by placing PBT.
5. Wash 6× 20 min in PBT (~2 h of washes).
6. Mount in VECTASHIELD mounting medium.
7. For viewing counterstains with DAPI to visualize nuclei.

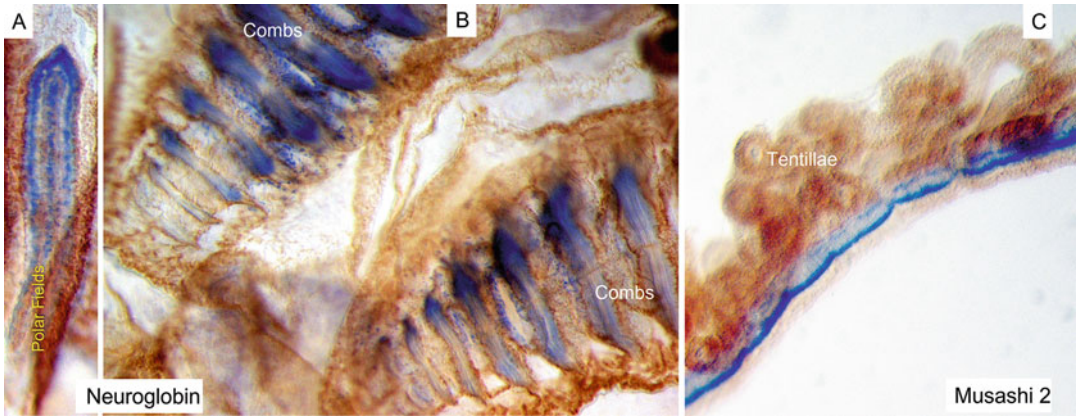


**Fig. 3** *Pleurobrachia* Neuroglobin (**a**, **b**) and Musashi-2 (**c**) gene expression patterns. In situ hybridization was performed on whole-mount preparations using DIG-labeled probes (blue staining) for two specific genes (*see* details in [18]). High expression levels are observed in nonneuronal cells within comb plates and tentacles. Neuroglobin is also expressed in the cells of the polar fields, known as putative chemosensory structures in ctenophores

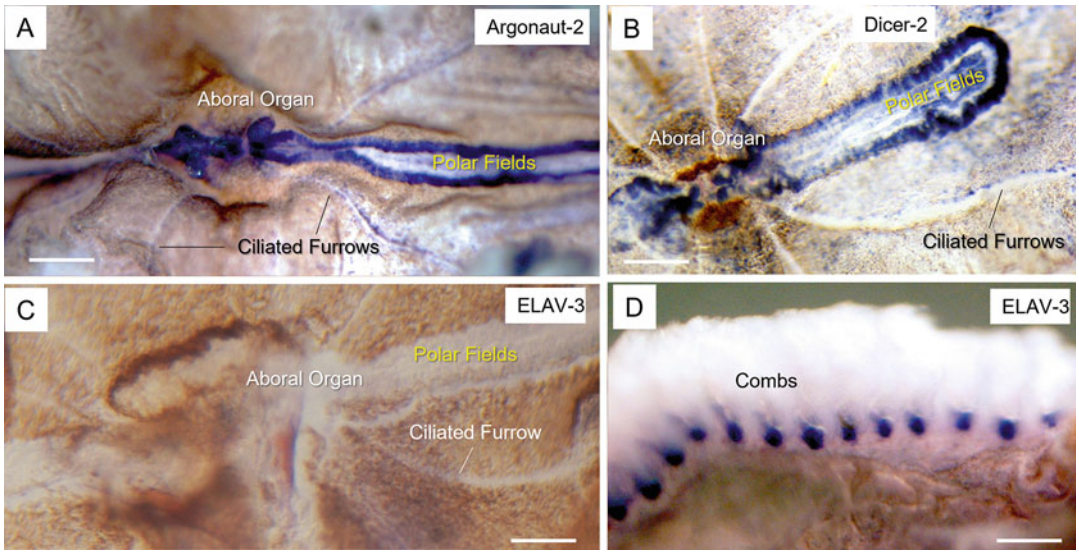


**Fig. 4** Argonaute (**a**, JN202326) and Dicer-2 (**b**, JN202325) genes are selectively expressed in the aboral organ, polar fields, and cells close to the ciliated furrows and skin (weak expression) of *Pleurobrachia bachei*. All these structures are associated with sensory and integrative functions. *Pleurobrachia* ELAV-3 (JN202319, RNA-binding protein recognized as pan-neuronal markers in many bilaterians) is not expressed in neurons or organs enriched with neurons such as the aboral organ and polar fields or cells with a neuronal-like appearance (**c**). However, the highest levels of ELAV-3 expression were detected in the adult comb plates (**d**); modified from [17]. In situ hybridization was performed on whole-mount preparations using DIG-labeled probes (blue staining). Scale bars: 500 μM

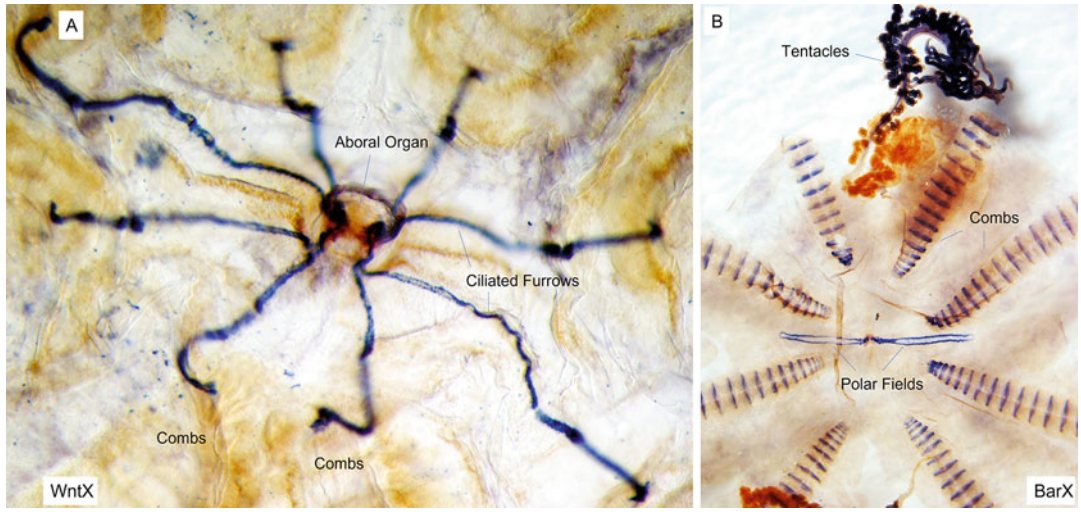


**Fig. 5** WntX is selectively expressed in the aboral organ (AO) and major conductive pathways of *Pleurobrachia* (**a**), indicating its involvement in integrative and neural-like functions (in situ hybridization on a whole-mount preparation using DIG-labeled probes (blue staining)). One of the highest WntX expressions is found in AO and ciliated furrows, whereas the polar fields showed a moderate expression level associated with their central regions. (**b**) The homeobox transcription factor BarX is differentially expressed in tentacles, comb plates, and polar fields. (Modified from [17])

*3.2.2 Double/Fluorescent Label*

Multiple simultaneous hybridizations can be performed with this same protocol by using combinations of digoxigenin-, biotin-, and fluorochrome-labeled (fluorescein) probes to different transcripts of interest. Such multiprobe experiments are possible because of the different fluorescent dye coupled antibodies, including fluorescein or FITC (fluorescein isothiocyanate; yellow), rhodamine or TRITC (tetramethylrhodamine isothiocyanate; red), and AMCA (amino-methylcoumarin acetic acid; blue).

Figures 7 and 8 illustrate in situ hybridization experiments with fluorescent markers for selected genes.

## 4 Notes

1. Obtaining high-quality, intact RNA is the first and often the most critical step in performing successful ISH experiments. Labeled RNA is produced during the in vitro transcription reaction. It is crucial to be aware of the risk of RNase contamination. RNases are found in practically every cell type and can be tough enzymes to inactivate. The primary sources of RNases within most laboratory environments are microorganisms such as bacteria, fungi, their spores, and human contamination. Some basic precautions include wearing gloves throughout the experiment and changing them if they have come in contact with skin. Designate an RNase-free area of work to wash the

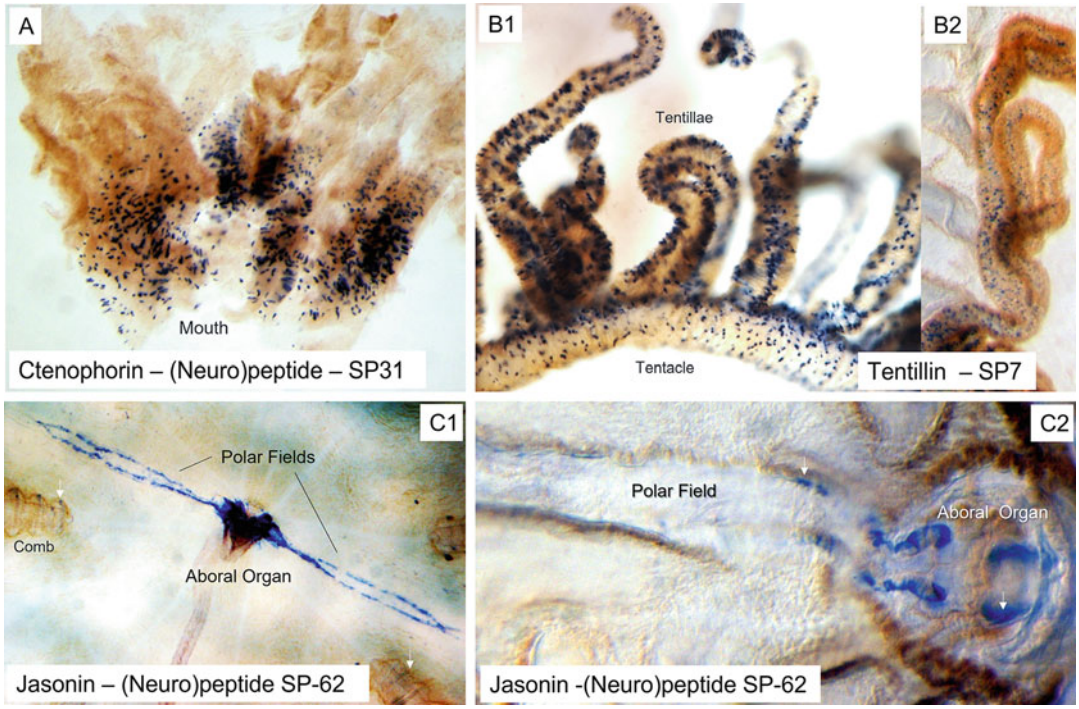


**Fig. 6** Expression of genes encoding putative secretory molecules in *Pleurobrachia bachei* (DIG-labeled in situ hybridization (ISH)). We selected these transcripts using a computational pipeline and RNA-seq profiling from different adult tissues. (Modified from [17]). Ctenophorin (**a**), or secretory (neuro)peptide 31 (SP-31, JQ700341), is uniquely expressed in polarized cells around the mouth of *Pleurobrachia*, and we found its homologs in all ctenophore species we sequenced [17]. Tentillin, (**b1**, **b2**) (SP-7, JQ700317) is a *Pleurobrachia*-specific gene, uniquely expressed in polarized secretory-like cells in tentillae and tentacles. Jansonin (**c1**, **c2**) (SP-62, JQ700372) is also a species-specific gene, primarily expressed in the aboral organ and polar fields

benchtop after each experiment with RNaseZap or similar products. It is good to have a dedicated set of pipettors and equipment used solely for RNA work. Use fresh packaged filtered pipette tips and tubes guaranteed to be RNase-free. It is also possible to order and use RNase-free chemicals and reagents. Liquid solutions can be treated with diethylpyrocarbonate (DEPC); however, DEPC degradation products can inhibit in vitro transcription; therefore, obtaining RNase-free (non-DEPC treated) reagents is best. By definition, Milli-Q water is RNase-free if the equipment is maintained correctly.

2. Prepare at least a liter of filter sea water (FSW) with a Nalgene sterile disposable filter unit and 0.2 μM filter. If possible, use the same water the animals were obtained or use the water the animals were collected and shipped. Store capped containers at 4 °C for a week, and check each time for any bacterial growth.

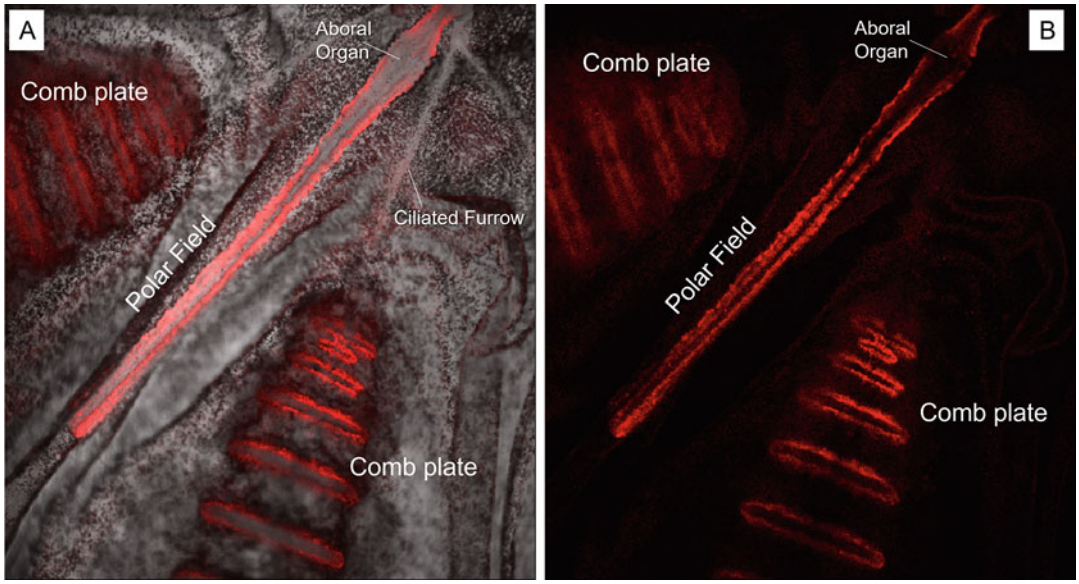


**Fig. 7** β-Tubulin mRNA is differentially expressed in the polar fields (PFs) and comb rows of *Pleurobrachia bachei*. We recommend that the fluorescent in situ hybridization (ISH) (FISH) protocol should be first tested with highly abundant genes (e.g., β-tubulin) as a positive control of the procedure. The bright staining of the same structures can be observed even with background illumination using a convenient transmission light source (**a**) to visualize all structures in this whole-mount preparation from the aboral side. The protocol was performed with a probe labeled with Dig. However, the Vector Red Alkaline Phosphatase kit was used for development and then visualized on the confocal microscope (**b**)

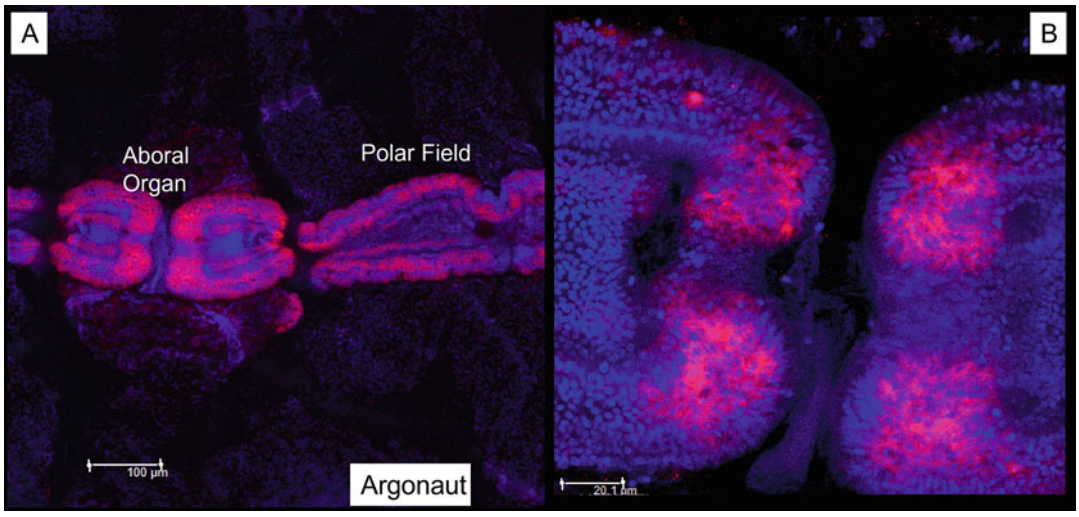


**Fig. 8** Differentially expressed Argonaute in *Pleurobrachia bachei* (red labeling with fluorescent in situ hybridization (FISH) protocol). Dark blue fluorescence is DAPI nuclear staining. Low magnifcation of the aboral organ and polar field (**a**) and high magnification of the aboral organ (**b**)

3. The distinction between paraformaldehyde (PFA) and formaldehyde is that PFA is the polymerization product of formaldehyde. PFA comes in small glass ampoules and is more expensive than formaldehyde, but both have similar results for ISH. Working with PFA or formaldehyde requires a well-ventilated area or hood.
4. ThermoFisher Scientific tRNA is convenient because 25 mg of tRNA comes in individual vials. We add 1 mL of RNase-free $H_2O$ and dissolve it according to manufacture recommendations.
5. Goat serum arrives frozen in large quantities. Make 1.0 mL aliquots in 1.5 mL centrifuge tubes, and keep frozen at −20 °C until ready to use. This eliminates the time of freeze-thaw and possible contamination. We do the same thing for 50× Denhardt.
6. Prepare 200 mM levamisole in the receiving bottle, and then aliquot 0.25 mL in 1.5 mL centrifuge tubes; keep frozen at −20 °C until ready to use.
7. We prefer the NBT (4-nitro-blue-tetrazolium-chloride)/BCIP (5-bromo-4-chloro-3-indolyl-phosphate) stock solution because everything is premixed to the appropriate concentration, and it is in ready to use liquid form. Keep reagent in the dark.
8. We centrifuge first at a slower rate to allow the liquid to absorb into the filter. Then we increase the speed to elude the product.
9. DNAase treatment of the probe helps remove background in ISH.
10. Gently rotating the animals in the Corning 50 mL tubes will provide better results.
11. The longer the animals are stored (usually after 2 weeks) in 100% MeOH at −20 °C, the greater chance of background developing.
12. We store about ten animals (depending on size) per Corning 50 mL tube in 100% MeOH at −20 °C until prehybridization. We do not use more than three animals per probe. We place one to three animals in autoclaved test tubes 16 × 125 mM or new plastic tubes similar in size. After the prehybridization, the animals/tissue samples might shrink.
13. The amount of probe added to the detection buffer is variable. It will depend on the quality, G-C content, length, and concentration of the probe. It will also depend on the abundance of the transcript of interest. Sigma Aldrich recommends between 0.1 and 1.0 μg/mL. We usually start with 0.2–0.4 μg/mL.

14. 24-well plates are very convenient for ISH experiments. Typically, we use a row or column per sample and then transfer the sample in its dedicated row or column. Transfer the sample to a clean well when possible. Fragile specimens may be damaged with many transfers.
15. The amount of antibody added to the incubation can be quite variable and depends on many factors. With a probe concentration of 0.2–0.4 μg/mL, a 1 to 2000 dilution of alkaline phosphatase-conjugated DIG antibodies works well for us.
16. Detection buffer is one of the most important buffers in the ISH protocol. Not only is it essential to make at the correct concentrations, but pH is also critical. The pH should be as close to 9.5 ± 0.1 pH units. Plus, this solution needs to be filtered. We use a 60 mL syringe with a Nalgene syringe filter, 0.2 μM. Detection buffer can be prepared fresh on the day of detection.

    Do not keep detection buffer longer than 2 weeks as it will become cloudy, but we prefer freshly prepared buffer.
17. This is a light-sensitive reaction so try and keep in the dark until you need to monitor the development. We often wait for at least 30 min before visually examining the development. In general, we briefly examine the preparations under a stereo microscope and cover them for additional development either at +4 °C or at room temperature—the selection of developing conditions depends upon the abundance of a target transcript and the intensity of a signal.
18. In some ctenophores, we had seen crystal formation at times when animals were kept for any length of time in PBS at 4 °C. When this has been an issue, we have stopped the development reaction in 4% formaldehyde in MeOH. This is not the most efficient way to stop the reaction because this solution does not wash the samples and PBS, but it helps reduce the crystal formation.
19. Development times are highly variable. The 4% formaldehyde in MeOH and 100% EtOH are both clearing steps. A considerable amount of background/tissue coloration might be lost. For the first time using this protocol, try several development times to evaluate what works best for your transcript of interest and specimen.
20. Methyl salicylate is an organic ester found in natural products. However, caution should be applied when handling this chemical, and the use of a well-ventilated room and a hood is required.
21. We were successful with the direct method of detection and Vector Red AP Substrate kit. This provides both white light and fluorescence options to visualize expression.

The timing of this protocol can be variable. For first-time users, it is best if the probe is made and ready to be used before the start of the in situ hybridization protocol because if your probe is not of acceptable quality, nothing can proceed. Once one is experienced, probe generation can be done in parallel with the Day 1 of the in situ hybridization protocol. Day 1 can be a combination of collecting and washing animals followed by fixation of the specimens overnight. The next day can be a combination of Day 2 and 3, so the dehydration, hydration, and hybridization can be performed on the same day as long as the appropriate times are followed as suggested in the protocol. The next day is a short day with washing and incubation of antibodies. We do not suggest trying to shorten this step. The development is going to be the most variable for timing. Development can be very rapid for something like tubulin or a neuropeptide or very slow for a receptor or channel and depends on the level of expression of your transcript of interest. Be prepared to have a long day of development for a successful result.

## 5 Anticipated Results

In situ hybridization techniques can be long and arduous experiments, especially when optimizing conditions for perfect results. However, the information they provide is invaluable with cell-specific spatial resolution. We present the ISH protocol for fragile small gelatinous animals such as ctenophores. We defined six basic components to successful ISH experiments: the experimental design, fixation of cell/tissue/animal, pretreatment and permeabilization of cells, binding of specific RNAs to a labeled riboprobe, amplification of the reporter through immunological detection (for indirect detection only), and visualization. Although we successfully tested these protocols for dozens of different preparations, if poor initial results for new species are obtained, it is relatively straightforward to return to the six key stages of ISH to systematically evaluate and adjust required parameters starting with the detection of relatively abundant transcripts such as cytoskeleton proteins or secretory molecules (Figs. 6 and 7).

## 6 Illustrated Examples: Quest for Neuronal Genes in Ctenophores

As illustrated examples, we selected several genes as markers of distinct cellular populations focusing on the still ongoing quest to identify and characterize unique neural populations in ctenophores. The systematic genomic survey in *Pleurobrachia* and related

ctenophore species strongly suggested independent origins of neural systems in this lineage [7, 10, 17, 27, 28] and an apparent lack of pan-neuronal genes across Metazoa [9, 18]. Indeed, such ctenophore gene homologs as Neuroglobin, ELAV, and Musashi, known to be expressed in bilaterian nervous tissue, do not specifically label neurons in *Pleurobrachia* but many other cell types in combs and tentacles (Figs. 3b, c and 4c, d). Although Neuroglobin marked small cells in the polar field (putative chemosensory organ with neurons) and within the comp plates (Fig. 3a, b), their identity remains to be determined. The overall labeling is quite different from mapping neural cells in *Pleurobrachia* and other studied ctenophores, with most neurons localized in the skin and mesoglea [29–32]. Surprisingly, genes encoding different aspects of RNA processing (including small RNAs) such as Argonaute and Dicer (Figs. 4a, b and 8) are associated with the polar fields and the aboral organ (contains statocyst and might be an analog of the elementary brain)—structures with dense neuronal populations.

WntX is expressed specifically in ciliated furrows (Fig. 5a), which are nonneuronal elements (*see* also [23] for a closely related species—*P. pileus*). The homeobox transcriptional factor BarX known to be involved in vertebrate mesenchymal transitions (e.g., [33]) is differentially expressed in combs, polar fields, and tentacles (Fig. 5b).

It was indicated that ctenophore neural systems might be peptidergic in their nature with a diversity of neuron-specific small secretory peptides—neuropeptides [10, 17, 34–36]. Indeed, some predicted *Pleurobrachia* neuropeptides expressed in neural-type structures (Fig. 6), as reported earlier [17], are similar to recent data on *Mnemiopsis* [35]. Nevertheless, two predicted neuropeptides are *Pleurobrachia* innovations (SP-7, SP-62), and their homologs have not been identified in the *Mnemiopsis*. In contrast, SP-31 has a homolog in *Mnemiopsis* with similar patterns of expression in the mouth-gut areas in both species—this was the reason for its original name as Ctenophorin [17].

In conclusion, analysis of cell-specific gene expression patterns, especially in adult ctenophores, is critical in deciphering neuronal organizations in basal metazoan lineages and animal innovations in general. In situ hybridization was and will remain a helpful tool in this endeavor.

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