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Whole mount *in situ* hybridization and immunohistochemistry for studying retinoic acid signaling in developing amphioxus

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Abstract

Retinoic acid (RA) is a vitamin A-derived signaling molecule acting during development and in the adult. This chapter provides protocols to characterize the role of RA signaling during development of the invertebrate chordate amphioxus. As sister group to all other chordates and characterized by the most vertebrate-like RA signaling system of all invertebrates, amphioxus is an important model for studying the evolution of RA signaling. Focusing on the development of GABAergic neurons in the amphioxus central nervous system, we provide detailed protocols for maintaining and breeding adult animals, for performing pharmacological treatments of embryos and for analyzing the effects of these treatments by whole mount *in situ* hybridization and immunohistochemistry coupled to confocal microscopy.

Keywords: amphioxus; cephalochordate; retinoic acid signaling; pharmacological treatments; immunohistochemistry; *in situ* hybridization; fluorescent and confocal imaging; neurobiology; GABA; GAD

1. Introduction

Retinoic acid (RA) is a signaling molecule that plays key roles in animal development and adult physiology (Laudet, Zieger, & Schubert, 2015). In the body, RA is produced from retinol (vitamin A) in a two-step oxidative process: (1) retinol is reversibly oxidized to retinaldehyde and (2) retinaldehyde is irreversibly oxidized to RA. This irreversible step is catalyzed by retinaldehyde dehydrogenase (RALDH) enzymes. Endogenous RA levels are further tightly regulated by tissue-specific degradation mediated by enzymes of the cytochrome p450 subfamily 26 (CYP26) (Ghyselinck & Duester, 2019). The cellular response to RA is generally controlled by heterodimers of two nuclear receptors, the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). RA binding to RAR induces conformational changes in the heterodimer and ultimately leads to the transcriptional regulation of target genes. Although different RA isomers have been identified *in vivo*, all-*trans* retinoic acid (ATRA) is considered the natural endogenous ligand of RAR (Mic, Molotkov, Benbrook, & Duester, 2003). RA signaling controlled by RAR evolved early in animal evolution, likely at the base of bilaterians, as a low-affinity sensor system. RARs with high affinity for RA, as seen in mammals, probably evolved only later and specifically in the chordate lineage (Fonseca et al., 2019; Handberg-Thorsager et al., 2018). This marks the origin of chordates as a milestone in the evolution of RA signaling.

Cephalochordates, commonly referred to as amphioxus or lancelets, are small, filter-feeding animals that live burrowed in the bottoms of temperate and tropical seas. Amphioxus are characterized by an elongated, fish-like body that, depending on the species, can reach a maximum length of between 2.5-8.5 cm (Poss & Boschung, 1996). According to their phylogenetic position as sister group to all other chordates (i.e. tunicates and vertebrates) (Putnam et al., 2008), amphioxus have a typical chordate body plan with a dorsal hollow nerve cord, a notochord that extends axially from rostrum to tail, metameric muscles and an anterior perforated pharynx (Bertrand & Escriva, 2011). Furthermore, in addition to a RAR with high RA affinity, amphioxus also has the most vertebrate-like RA signaling system of all invertebrates (Holland et al., 2008; Laudet et al., 2015). For example, in both amphioxus and vertebrates, RA signaling regulates anteroposterior patterning of the central nervous system by directly controlling colinear expression of *Hox* genes (Koop et al., 2010; Schubert, Holland, Laudet, & Holland, 2006). These features make amphioxus a key model for understanding the evolution of RA signaling during chordate diversification.

In this chapter, we describe how to maintain adult amphioxus in the laboratory, how to obtain gametes and how to manipulate RA signaling during embryonic development by pharmacological treatments. We also provide detailed protocols to assess the effects of these treatments on the developing neural architecture by whole mount *in situ* hybridization (ISH) and immunohistochemistry (IHC). These two approaches are complementary as the former detects mRNAs while the latter targets antigens. ISH tends to be more widely used in non-model organisms because species-specific probes can be synthesized rather easily (for details on probe synthesis see Yong, Kozmikova, & Yu, 2019). Compared to ISH, IHC is easier and faster to perform and yields higher anatomical resolution. For instance, IHC allows the detection of both cell bodies and projections of neurons, while ISH generally only stains cell bodies, a major drawback for anatomical analyses (Zieger, Candiani, Garbarino, Croce, & Schubert, 2018; Zieger, Garbarino, et al., 2018). A primary limitation of IHC is that specific antibodies are required, most of which have been developed for model organisms, such as mice and fruit flies. Although some of these antibodies have been shown to cross-react with conserved antigen epitopes in a large variety of species (Zieger et al., 2018), targeting non-conserved epitopes by IHC requires the production and subsequent validation of novel antibodies (Bozzo, Pergner, Kozmik, & Kozmikova, 2017). Thus, in addition to characterizing RA signaling functions, the protocols we provide will also be useful for the validation of novel antibodies in amphioxus, by allowing comparisons of IHC and ISH patterns.

2. Maintaining adult amphioxus in the laboratory

Sexually mature adults of the European amphioxus (*Branchiostoma lanceolatum*) are collected in Argelès-sur-Mer (France) (Fuentes et al., 2007) before the beginning of the spawning season and kept in a seawater facility in the laboratory (Carvalho, Lahaye, & Schubert, 2017; Theodosiou et al., 2011). 20-25 amphioxus adults are kept in 5 l tanks containing around 3 cm of autoclaved sand on a 14/10 h inverted light/dark cycle (light: 9 pm-11 am; dark: 11 am-9 pm). Each tank is continuously aerated. Open systems with seawater flowing continuously through the tanks are preferable, but closed systems with filtration devices can also be used (Carvalho, Lahaye, & Schubert, 2017).

Animals are fed with a mixture of live microalgae (such as *Tisochrysis lutea* and *Chaetoceros gracilis*). Algae are collected in 50 ml centrifuge tubes, pelleted by centrifugation at 4,000 x g for 10 min, washed twice with seawater and resuspended in fresh seawater. The resuspended algae are pooled to a final volume of 50 ml and half a teaspoon of powdered green algae (such as *Spirulina*) is added to the mixture. Each tank receives 1 ml of concentrated algae, corresponding to 15 ml of unspun algal culture, twice a day.

3. Spawning induction, gamete collection and *in vitro* fertilization

During the spawning season (May through July), amphioxus embryos can be obtained in large numbers by *in vitro* fertilization using gametes released in response to a thermal shock (Fuentes et al., 2007).

3.1 Equipment

- 15 ml centrifuge tubes
- 5 l tank
- 6 and 15 cm plastic Petri dishes. Eggs and embryos tend to stick to plasticware. To avoid this, all Petri dishes must be rubbed with wet tissue paper and rinsed three times with artificial seawater shortly before adding eggs or embryos.

- Plastic cups
- Red-light flashlight
- Room with controlled temperature and light/dark cycle
- Seawater refractometer, for instance, HANNA Instruments (HI96822)
- Stereomicroscope
- Water bath

3.2 Reagents

- Artificial seawater salt, Red Sea

3.3 Working solutions

- Artificial seawater: dissolve 400 g Red Sea seawater salt in 10 l of reverse osmosis or distilled water. Vigorously agitate using an aquarium pump for a couple of days to dissolve the salt. Measure salinity with a refractometer and adjust to 36-38 ppt by adding either seawater salt or water. Keep artificial seawater continuously agitated to prevent salt precipitation.

3.4 Protocol for obtaining and rearing amphioxus embryos

1. Select an adequate number of males and females with full gonads shortly before the end of the light period. The number of animals to be selected depends: (1) on the total number of embryos required for performing the planned experiments and (2) on the spawning efficiency of amphioxus adults in the facility. For the experiments detailed in this chapter, four animals (two females and two males) should be sufficient. Ripe animals can be sexed using a stereomicroscope: eggs are clearly visible as small spheres inside the ovary, while testes are homogeneously filled with sperm.
2. Put the selected animals into a clean 5 l tank containing 2-3 cm of fresh seawater and no sand.
3. Place the tank into a water bath set at 23°C with the same light/dark regime used in the animal facility. Leave overnight without food and aeration.
4. The following day, shortly before the start of the dark period, transfer each animal into an individual plastic cup with 15 ml of artificial seawater and keep them at 19°C in the dark.
5. Check for spawning every 30 min using a red-light flashlight to avoid startling the animals. Adults typically spawn 1-4 h after the beginning of the dark period.
6. Collect eggs by gently pouring them into a 6 cm plastic Petri dish. Keep at 19°C until fertilization.
7. Collect sperm by pouring into a 15 ml centrifuge tube and promptly move to 4°C. Sperm remains active for about 12 h, with a marked decrease in fertilization efficiency after about 2 h.
8. Fertilize eggs by adding a few drops of sperm to the Petri dish. Gently swirl the dish to homogeneously distribute the sperm, hence ensuring synchronous fertilization.
9. Verify fertilization after 2-3 min: fertilized eggs are characterized by a clearly visible fertilization envelope.
10. Immediately transfer the fertilized eggs into a new 15 cm plastic Petri dish filled with artificial seawater to prevent embryos from sticking together.
11. Let develop until the desired stage. Note that cultures need to be kept at a constant temperature to ensure development according to predefined staging systems. The culture temperature most widely used for *B. lanceolatum* is 19°C, which was also used here.
12. After spawning, return the adults to the animal facility. Separate animals that did not spawn from those that did. While the former can be shocked again during the ongoing spawning season, the latter can be kept in the animal facility: around a third of the animals that spawned out will redevelop gonads ahead of the next spawning season (Carvalho et al., 2017).

4. Pharmacological manipulation of retinoic acid signaling in amphioxus embryos

To interfere with RA signaling, embryos were treated from the early gastrula (6 hpf, i.e. hours post-fertilization) until the late embryo stage (36 hpf) with the compounds listed in Table 1. The following protocol, presented in graphical

form in Fig. 1, can be applied to the pharmacological manipulation of any signaling pathway by changing compounds and adapting treatment windows.

4.1 Equipment

- 15 ml centrifuge tubes
- 25 ml serological pipettes: wet the inside of the pipette with artificial seawater before use on embryos to avoid sticking
- 6 cm plastic Petri dish rubbed with wet tissue paper and rinsed three times with artificial seawater, as described in section 3.1
- Filtered pipette tips
- Micropipettes
- Pipet aid
- Room with controlled temperature, set at 19°C
- Stereomicroscope

4.2 Reagents

- Dimethyl sulfoxide (DMSO), Sigma-Aldrich (D8418)
- all-*trans* retinoic acid (ATRA), Sigma-Aldrich (R2625)
- R115866/Talarozole, Sigma-Aldrich (SML2092)
- BMS493, Sigma-Aldrich (B6688)
- 4-Diethyl-aminobenzaldehyde (DEAB), Sigma-Aldrich (D86256)

4.3 Stock solutions

- Artificial seawater, as described in section 3.3
- 0.1 mM ATRA in DMSO. Can be stored in aliquots in the dark at -20°C for several months
- 0.5 mM R115866/Talarozole in DMSO. Can be stored in aliquots in the dark at -20°C for several months
- 1 mM BMS493 in DMSO. Can be stored in aliquots in the dark at -20°C for several months
- 10 mM DEAB in DMSO. Can be stored in aliquots in the dark at +4°C for several weeks

4.4 Protocol for the pharmacological treatment of amphioxus embryos

1. Following fertilization, check the quality of the embryos by monitoring the first cell divisions with a stereomicroscope. Reference images of amphioxus early development can be found elsewhere (for instance, Bertrand & Escriva, 2011).
2. Ahead of the first treatment, prepare and label the required number of dishes: number of compounds plus one control for each solvent multiplied by the number of treatment windows to be analyzed. In our case, four compounds and one solvent are used with a single treatment window (6 hpf to 36 hpf), so prepare and label five dishes.
3. Using a pipet aid and a 25 ml serological pipette, carefully transfer four times 2 ml of embryos from the culture plate into each dish, with each dish thus receiving a total of 8 ml. It is important to use a gentle pipetting stream to avoid damaging or removing the fertilization envelopes.
4. Verify the volume in each dish by pipetting (using the serological pipette) and, if needed, adjust to exactly 8 ml. Again, make sure to gently pipette the embryos.
5. At the desired stage (6 hpf for this experiment), add 10 µl of the appropriate 1,000x compound stock solution to 2 ml of artificial seawater in a 15 ml centrifuge tube. Mix well and add to the corresponding treatment dish. Swirl gently. Since the treatment volume is 10 ml, the resulting final treatment concentration is a 1,000-fold dilution of the compound stock solution. Always remember to include control dishes containing the corresponding concentration of the solvents used to prepare the compound stock solutions.
6. Incubate the embryos at 19°C in the dark until the moment of fixation, 36 hpf in this study.

[Insert Table 1 here]

[Insert Figure 1 here, full-page]

4.5 Expected phenotypes

If the phenotypes of the pharmacological treatments are known prior to the experiment, efficiency of the treatments can be assessed directly after fixation and prior to further analyses. For the treatments described here, it is known that upregulation of RA signaling activity in developing amphioxus shortens the pharynx, while its downregulation expands the pharynx (Escriva, Holland, Gronemeyer, Laudet, & Holland, 2002). The expected phenotypes are shown in Fig. 2.

[Insert Figure 2 here]

4.6 Troubleshooting & Optimization

Problem	Solution
Pharmacological compound does not dissolve in solvent.	Check solubility of the compound in different solvents. Note that solubility might vary greatly, and solvent must thus be chosen accordingly.
Pharmacological compound precipitates in artificial seawater.	Mix well after adding the compound to the artificial seawater. If necessary, vortex the stock solution tube before use. If problem persists, change stock solution aliquot. Eventually, prepare new stock solution using a different solvent.
Solvent control embryos do not develop normally.	Do not overcrowd treatment plates with embryos: a 6 cm Petri dish should hold about 150 embryos. Make sure to use the lowest possible solvent concentration. If abnormal development persists, verify embryo culture conditions.
Treated embryos stop developing.	Verify the concentration of the compound used. If you are testing a new molecule, consider lowering its concentration.
Embryos do not show the expected phenotype.	Verify the concentration of the compound used. If you are testing a new molecule, consider increasing its concentration. For retinoids, perform treatments in the dark and make sure that stock solutions were not exposed to light for a prolonged period of time. Try fresh aliquots of stock solutions. Eventually, prepare new stock solutions.

5. Fixation of amphioxus embryos

Embryos for ISH are fixed with paraformaldehyde (PFA) in MOPS-EGTA buffer and stored in RNase-free 70% ethanol. Embryos for GABA immunohistochemistry are fixed in PFA/glutaraldehyde in MOPS-EGTA buffer and stored in PBS/sodium azide.

5.1 Equipment

- 4-well cell culture plates
- Baskets with a 35 μ m mesh bottom, prepared by cutting the sieve out of the cap of a Corning flow cytometry tube (352235), as illustrated in Fig. 1C
- Filtered pipette tips, RNase-free
- Glass bottle, RNase-free
- Gloves
- Magnetic stirrer with hot plate and stir bar
- Micropipettes
- pH meter
- Screw-cap, low-binding microcentrifuge tubes, RNase-free
- Tweezers

- Vacuum suction system and filters

5.2 Reagents

- 3-(N-morpholino)propanesulfonic acid (MOPS), Sigma-Aldrich (69947)
- Diethyl pyrocarbonate (DEPC), Sigma-Aldrich (40718)
- Ethanol, absolute, molecular biology grade, Millipore (100983)
- Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), Sigma-Aldrich (E3889)
- Glutaraldehyde solution, 50%, Sigma-Aldrich (G7651)
- Hydrochloric acid (HCl), 37%, Sigma-Aldrich (30721)
- Magnesium sulfate (MgSO_4), Sigma-Aldrich (M2643)
- Paraformaldehyde (PFA), Sigma-Aldrich (P6148)
- RNaseZAP, Sigma-Aldrich (R2020)
- Sodium azide, Sigma-Aldrich (71289)
- Sodium chloride (NaCl), Sigma-Aldrich (S9888)
- Sodium hydroxide solution (NaOH), 1 N, Sigma-Aldrich (79724)
- Sodium phosphate dibasic, Sigma-Aldrich (S9638)
- Sodium phosphate monobasic, Sigma-Aldrich (S3264)

5.3 Working solutions

- 4% PFA/MOPS-EGTA: weigh 4 g of PFA in an RNase-free glass bottle. Add 10 ml of 1 N NaOH and dissolve on a magnetic stirrer with hot plate by heating to 60°C. Add 100 ml of MOPS-EGTA and leave stirring at 60°C overnight. Bring to pH 7.4 with 37% HCl and filter. Perform the whole process under a fume hood. Prepare fresh at the beginning of the spawning season. Can be stored at 4°C for several weeks
- 70% ethanol: dilute molecular biology grade absolute ethanol to 70% (v/v) with DEPC-treated water
- Artificial seawater, as described in section 3.3
- DEPC-treated water: add DEPC to distilled water to a final concentration of 0.1% (v/v), shake vigorously. Incubate overnight under a fume hood and autoclave. Can be stored at room temperature for several months
- MOPS-EGTA buffer: 0.1 M MOPS, 1 mM EGTA, 2 mM MgSO_4 , 0.5 M NaCl, pH 7.5, in DEPC-treated water. Filter. Prepare fresh at the beginning of the spawning season. Can be stored at +4°C for several weeks
- PBS/sodium azide: 2.6 mM sodium phosphate monobasic, 17.4 mM sodium phosphate dibasic, 0.9% (wt/v) NaCl, 0.1% (wt/v) sodium azide, pH 7.4. DEPC-treat and autoclave. Can be stored at room temperature for several months
- PFA/glutaraldehyde: add glutaraldehyde to 0.3% (v/v) to 4% PFA/MOPS-EGTA

5.4 Protocol for fixing amphioxus embryos

1. Perform the whole process under a fume hood.
2. Fill a 1.5 ml screw-cap microcentrifuge tube with 1 ml of ice-cold fixative, label it appropriately and place it on ice.
3. Take a dish containing treated embryos, open it, turn the lid upside down and place a basket inside.
4. To concentrate the embryos in a small volume, transfer them from the dish to the basket using a micropipette with a 1,000 μl tip. Make sure that the basket does not overflow. Repeat until all the embryos have been moved from the dish into the basket. If needed, pipette the embryos up and down inside the basket to remove the fertilization envelope.
5. Using tweezers, move the basket into a well of a 4-well plate containing 600 μl of artificial seawater.
6. Using a micropipette with a 200 μl tip, transfer the embryos from the basket into the tube placed on ice and containing the fixative. Carefully add the embryos drop by drop to the tube. Do not transfer more than 600 μl to one tube.
7. Invert the tube several times and place on ice until the embryos have settled at the bottom of the tube.
8. Remove the solution and replace with fresh ice-cold fixative. Invert the tube several times. Fix at +4°C overnight in an upright position to let the embryos settle.
9. Remove the fixative and replace with ice-cold storage solution. Invert the tube several times.

10. Place the tube on ice and wait until the embryos have settled. Remove the solution and replace with fresh ice-cold storage solution. Invert the tube several times. Leave at +4°C overnight.
11. Remove the solution and replace with fresh ice-cold storage solution. Keep embryos at +4°C, if stored in PBS/sodium azide, or at -20°C, if stored in 70% ethanol. Samples stored in PBS/sodium azide should be used within weeks, while samples stored in 70% ethanol can be conserved for months.

5.5 Troubleshooting & Optimization

Problem	Solution
Embryos are damaged.	Minimize volume transferred from basket to fixation tube. Remove as much solution as possible when replacing the fixative.
Embryos are damaged, especially mid-gastrula and early neurula stages.	From mid-gastrula to early neurula stages, embryos produce hatching enzymes. For these stages, fertilization envelopes must thus be removed before fixation. Make sure to remove as much liquid as possible when changing the fixative, eventually performing additional washes.
Cleavage stages are dissociated.	Blastomeres do not adhere very strongly before the blastula stage and might dissociate after removal of the fertilization envelope. Fix these stages with intact fertilization envelopes and remove them only before staining either by pipetting up and down in the well or by using insect pins.

6. Whole mount *in situ* hybridization

Whole mount ISH allows the detection of mRNAs in entire embryos with cellular resolution. This technique is widely used to determine the spatiotemporal expression patterns of developmental genes. Standard protocols are based on the hybridization of the target sequence with a complementary riboprobe labeled either with digoxigenin or fluorescein. These antigens are subsequently recognized by specific antibodies that are conjugated with a reporter activity. For amphioxus embryos, protocols for both chromogenic (Yu & Holland, 2009) and fluorescent (Yong et al., 2019) ISH have already been described. In addition, double ISH approaches are available, allowing the analysis of two different mRNAs within a single embryo (Candiani, Garbarino, & Pestarino, 2015). While the protocol below uses two chromogenic substrates, NBT/BCIP and Vector® Blue, the distribution of the riboprobes within the embryo will be assessed using a confocal microscope. Compared to exclusively fluorescent ISH methods, this approach has the advantage that the progress of the staining reaction can be monitored under a stereomicroscope and stopped when the signal is fully developed. We apply this protocol to the detection of mRNA transcripts of the *glutamate decarboxylase (GAD)* gene that encodes an enzyme involved in the biosynthesis of the neurotransmitter GABA.

6.1 Equipment

- 200 µl and 1,000 µl filtered micropipette tips, RNase-free
- 24-well cell culture plates, RNase-free
- 4-well cell culture plates, RNase-free
- Baskets, prepared as described in section 5.1
- Gloves
- Horizontal orbital shaker
- Hybridization oven
- Micropipettes
- Paper tape
- pH meter
- Stereomicroscope
- Syringes with 0.45 µm filters
- Tweezers
- Vortex

- Water bath
- Zipper bag

6.2 Reagents

- 4-Nitro blue tetrazolium chloride (NBT), Roche (11383213001)
- 5-Bromo-4-chloro-3-indolyl phosphate 4-toluidine salt (BCIP), Roche (11383221001)
- Acetic anhydride, Sigma-Aldrich (320102)
- Anti-DIG AP-conjugated Fab fragments, Roche (11093274910)
- Bovine serum albumin (BSA), Sigma-Aldrich (A9647)
- Denhardt's solution, 50x, Sigma-Aldrich (D9905)
- Diethyl pyrocarbonate (DEPC), Sigma-Aldrich (D5758)
- Ethylenediaminetetraacetic acid (EDTA), Sigma-Aldrich (E6758)
- Formamide, Fisher Scientific (BP228-100)
- Glycerol, Sigma-Aldrich (G7893)
- Glycine, Sigma-Aldrich (50046)
- Heparin, Sigma-Aldrich (H4784)
- Hoechst 33258, Sigma-Aldrich (14530)
- Hydrochloric acid (HCl), 1 N, Fisher Scientific (11993123)
- Levamisole, Sigma-Aldrich (BP212)
- Magnesium chloride (MgCl_2), Sigma-Aldrich (M9272)
- Proteinase K, Sigma-Aldrich (P2308)
- RNase A, Sigma-Aldrich (R6513)
- RNase T1, Sigma-Aldrich (R1003)
- RNaseZAP, Sigma-Aldrich (R2020)
- Sheep serum, Sigma-Aldrich (S3772)
- Sodium acetate, 3 M, pH 5.5, Invitrogen (AM9740)
- Sodium azide, Sigma-Aldrich (71289)
- Sodium chloride (NaCl), Sigma-Aldrich (S9888)
- Sodium citrate, Sigma-Aldrich (71497)
- Sodium dodecyl sulfate (SDS), Sigma-Aldrich (L3771)
- Sodium phosphate dibasic, Sigma-Aldrich (S9638)
- Sodium phosphate monobasic, Sigma-Aldrich (S3264)
- Torula yeast RNA, Sigma-Aldrich (R3629)
- Triethanolamine, Sigma-Aldrich (90279)
- Tris-EDTA (TE) buffer, pH 7.4, Sigma-Aldrich (93302)
- Triton X-100, Sigma-Aldrich (T8787)
- TRIZMA base, Sigma-Aldrich (93362)
- Tween-20, Sigma-Aldrich (P9416)
- Vector® Blue alkaline phosphatase substrate kit, Vector Laboratories (SK-5300)

6.3 Stock solutions

- 0.1 M triethanolamine (TEA): dilute TEA to 0.1 M in DEPC-treated water. Adjust to pH 8 with 1 N HCl. Do not treat TEA with DEPC and do not autoclave. Can be stored at +4°C for several months
- 1 M Tris-HCl, pH 8.2 or pH 9.6: dissolve RNase-free TRIZMA base in DEPC-treated water and adjust to either pH 8.2 or pH 9.6 with 1 N HCl. Do not treat with DEPC. Filter. Can be stored at 4°C for several months
- 10% BSA: dissolve to 10% (wt/v) in PBS. Can be stored in aliquots at -20°C for several months
- 10% glycine: dissolve RNase-free glycine to 10% (wt/v) in DEPC-treated water. Can be stored in aliquots at -20°C for several months
- 10% Tween-20: dilute to 10% (v/v) with distilled water. DEPC-treat and autoclave. Can be stored at room temperature for several months

- 10x PBS (phosphate buffer saline): 26 mM sodium phosphate monobasic, 174 mM sodium phosphate dibasic, 9% (wt/v) NaCl, pH 7.4. DEPC-treat and autoclave. Can be stored at room temperature for several months
- 20x saline sodium citrate (SSC): dissolve NaCl to 3M and sodium citrate to 300 mM. Adjust to pH 7 with 1 N HCl, DEPC-treat and autoclave. Can be stored at room temperature for several months
- DEPC-treated water: prepare as described in section 5.3
- Hoechst stock: dissolve to 10 mg/ml in distilled water. Can be stored in the dark at +4°C for several months
- Proteinase K stock: dissolve to 10 mg/ml in DEPC-treated water. Can be stored in aliquots at -20°C for several months
- RNase A stock: dissolve to 10 mg/ml in TE buffer. Boil for 10 min then quickly cool on ice. Can be stored in aliquots at -20°C for several months
- RNase T1 stock: dilute RNase T1 to 10,000 U/ml in 0.1 M sodium acetate, pH 5.5. Boil for 10 min then quickly cool on ice. Can be stored in aliquots at -20°C for several months
- Sheep serum: inactivate by heating to 55°C for 30 min. Cool on ice and prepare 1 ml aliquots that can be stored at -20°C for several months

6.4 Working solutions

- 0.25% or 0.5% acetic anhydride in TEA. Prepare fresh. Do not store
- 1x PBS: Prepare diluting 10x PBS stock with DEPC-treated water. Can be stored at room temperature for several months
- 2 mg/ml glycine: dilute 10% glycine 1:50 with PBSTw
- 80% glycerol: dilute glycerol with PBS/sodium azide. Can be stored at room temperature for several months
- Alkaline phosphatase (AP) buffer with MgCl_2 : 100 mM NaCl, 50 mM MgCl_2 , 100 mM Tris-HCl pH 9.6, 0.1% Tween-20, 1 mM levamisole in DEPC-treated water. Prepare fresh and filter
- Antibody solution: anti-DIG AP-conjugated antibody 1:3,000, 2 mg/ml BSA, 6.5% heat-inactivated sheep serum, 1x PBS, 0.1% TritonX-100. Can be stored in 1 ml aliquots at -20°C for several months
- AP buffer without MgCl_2 : 100 mM NaCl, 100 mM Tris-HCl pH 9.6, 0.1% Tween-20, 1 mM levamisole in DEPC-treated water. Prepare fresh and filter
- Blocking solution: 2 mg/ml BSA, 10% heat-inactivated sheep serum in PBSTw. Prepare fresh
- Hybridization buffer (HB): 50 % (v/v) formamide, 100 $\mu\text{g}/\text{ml}$ heparin, 5x SSC, 0.1 % (v/v) Tween-20, 5 mM EDTA, 1 mg/ml torula yeast RNA, 1x Denhardt's solution in DEPC-treated water. Warm solution to 60°C before adding torula yeast RNA. Can be stored in aliquots at -20°C for several months
- PBS/sodium azide: 20 mM sodium phosphate buffer, pH 7.4, 0.9 % (wt/v) NaCl, 0.1% (wt/v) sodium azide. Can be stored at room temperature for several months
- PBSTw: 20 mM sodium phosphate buffer, pH 7.4, 0.9 % (wt/v) NaCl, 0.1 % (v/v) Tween-20 in DEPC-treated water. Prepare fresh from 10x PBS and 10% Tween-20 stocks and filter
- Proteinase K working solution: 7.5 $\mu\text{g}/\text{ml}$ in PBSTw prepared fresh from the stock. We recommend a two-step dilution as follows. First, dilute 1 μl proteinase K stock in 99 μl of PBSTw and mix vigorously. Second, add 75 μl of this solution to 925 μl of PBSTw and mix vigorously
- RNase mix: 20 $\mu\text{g}/\text{ml}$ preboiled RNase A, 10 U/ml preboiled RNase T1 in WS3
- Staining solution: 2.5 $\mu\text{l}/\text{ml}$ NBT, 3.5 $\mu\text{l}/\text{ml}$ BCIP in AP buffer with MgCl_2 . Mix vigorously and keep in the dark.
- Vector® Blue staining solution: add 80 μl of Reagent 1, 80 μl of Reagent 2 and 40 μl of Reagent 3 to 5 ml of 100 mM Tris-HCl pH 8.2 + 0.2% Tween-20. Mix vigorously and keep in the dark
- Wash solution 1 (WS1): 50 % (v/v) formamide, 5x SSC, 1% SDS in DEPC-treated water. Mix formamide, SSC and water and heat to 60°C before adding the SDS. Can be stored in aliquots at -20°C for several months
- Wash solution 2 (WS2): 50% formamide, 2x SSC, 1% SDS in DEPC-treated water. Mix formamide, SSC and water and heat to 60°C before adding the SDS. Can be stored in aliquots at -20°C for several months
- Wash solution 3 (WS3): 2x SSC, 0.1% Tween-20 in DEPC-treated water. Filter. Can be stored in aliquots at -20°C for several months
- Wash solution 4 (WS4): 0.2x SSC, 0.1% Tween-20 in DEPC-treated water. Filter. Can be stored in aliquots at -20°C for several months
- Wash solution 5 (WS5): 2 mg/ml BSA in PBSTw. Prepare fresh

6.5 Protocol for whole mount *in situ* hybridization

Before starting, clean bench, pipettes and tweezers with RNaseZAP. Always use gloves to manipulate solutions, reagents and equipment. Use only RNase-free filtered tips. If not otherwise stated, all steps of the protocol are performed in 500 µl of solution, at room temperature and rotating the embryos horizontally at 35-40 rpm. For changing solutions, use tweezers to move baskets between two wells.

6.5.1 Day 1

1. For each experimental condition, select 10-15 embryos and transfer them to a basket placed inside a well of a 24-well plate.
2. Move the basket into a new well containing PBSTw and rotate for 5 min to rehydrate the embryos. Repeat twice with fresh PBSTw.
3. Digest with freshly diluted proteinase K. Additional details for this step are provided in the “Troubleshooting & Optimization” section.
4. Add 10 µl of 10% glycine to each basket to stop the digestion and incubate for 1 min. Quickly transfer into 2 mg/ml glycine and incubate for 5 min.
5. Wash with 0.1 M TEA for 1 min, followed by a second wash of 5 min.
6. Transfer into freshly prepared 0.25% acetic anhydride. Incubate for 5 min. Do not rotate.
7. Transfer into freshly prepared 0.5% acetic anhydride. Incubate for 5 min. Do not rotate.
8. Wash with PBSTw for 1 min, followed by a second wash of 5 min.
9. Transfer to HB, pre-warmed to 60°C. Incubate for 1 min.
10. Transfer into fresh pre-warmed HB. Seal the lid of the plate with paper tape then place the plate into a disposable zipper bag. Seal the bag and rock in a hybridization oven at 60°C for at least 2 h.
11. Dilute the probe to 1 ng/µl in pre-warmed HB. Denature at 70°C for 5 min.
12. Remove the plate from the hybridization oven, add the probe solution to a new well and transfer basket into probe solution. If more than one basket is used, repeat the procedure, cleaning the tweezers with RNaseZAP between each basket move to prevent the transfer of probe and/or embryos between wells.
13. Re-seal the plate with paper tape, place the plate into a zipper bag and rock in the hybridization oven at 60°C overnight.

6.5.2 Day 2

14. Pre-warm WS1 and WS2 to 60°C. Thaw WS3 and WS4. Work quickly in steps 15-18 to keep embryos at hybridization temperature.
15. Unseal the plate and remove the probe solution, which can be stored at -20°C for reuse.
16. Wash twice with pre-warmed WS1 at 60°C for 5 min.
17. Wash twice with pre-warmed WS1 at 60°C for 15 min.
18. Wash with pre-warmed WS2 at 60°C for 5 min, then move the plate to room temperature and rotate for 10 min.
19. Wash with WS2 for 15 min.
20. Wash with WS3 for 1 min, followed by a second wash of 5 min.
21. Incubate with RNase mix at 37°C for 20 min.
22. Wash twice with WS3 for 20 min.
23. Wash with WS4 for 20 min.
24. Wash with freshly prepared WS5 for 5 min.
25. Transfer into freshly prepared blocking solution and incubate for at least 2 h.
26. Transfer into freshly thawed antibody solution and incubate at 4°C overnight.

6.5.3 Day 3 (for NBT/BCIP staining)

27. Remove antibody solution and store at -20°C. Can be re-used once, if supplemented with fresh antibody solution.
28. Wash four times with fresh PBSTw for 20 min.
29. Transfer into freshly prepared AP buffer without MgCl₂ for a quick wash, then transfer into fresh solution and rotate for 10 min.
30. Transfer into freshly prepared AP buffer with MgCl₂ and rotate for 10 min. Repeat twice with fresh buffer.

31. Take embryos out of the basket and transfer them into a 4-well plate. Pipet embryos up and down in a 200 μ l tip to prevent them from sticking to the bottom of the well. Remove fibers and debris that might have accumulated in the well.
32. Change for 300 μ l of staining solution. Incubate at room temperature in the dark or at 37°C with gentle rotation.
33. Monitor the progress of the staining reaction by observing the embryos with a stereomicroscope on a white background every 15-30 min. Staining time can vary significantly between probes, from 15 min to several days.
34. Every 3 h, if the staining reaction requires more time, wash briefly with AP buffer with $MgCl_2$ and change for freshly prepared staining solution. The staining reaction needs to be stopped before the appearance of conspicuous background staining. A correctly stained embryo is thus transparent or slightly pinkish, and stained cells are dark blue or purple. To optimize the signal-to-noise ratio, do not incubate in staining solution overnight. Instead, wash briefly with AP buffer with $MgCl_2$ and leave at +4°C in fresh AP buffer with $MgCl_2$. The following day pick up the protocol at step 32.
35. When the signal is fully developed, stop the staining reaction by washing briefly with AP buffer without $MgCl_2$.
36. Change for fresh AP buffer without $MgCl_2$ and wash for 10 min.
37. Wash with PBSTw for 10 min.
38. To counterstain nuclei, incubate with freshly diluted 1:5,000 Hoechst stock in PBSTw in the dark for 15 min.
39. Wash twice with PBSTw for 10 min.
40. Wash with PBS/sodium azide for 10 min.
41. Change solution for 80% glycerol. Stained embryos can be stored at +4°C for several months.

6.5.4 Alternative day 3 (for Vector® Blue staining)

27. Remove antibody solution and store at -20°C. Can be re-used once, if supplemented with fresh antibody solution.
28. Wash four times with fresh PBSTw for 20 min.
29. Transfer to 100 mM Tris-HCl pH 8.2 + 0.2% Tween-20 and incubate for 10 min. Repeat twice with fresh buffer.
30. Take embryos out of the basket and transfer them into a 4-well plate. Pipet embryos up and down in a 200 μ l tip to prevent them from sticking to the bottom of the well. Remove fibers and debris that might have accumulated in the well.
31. Change for 300 μ l of Vector® Blue staining solution. Incubate at room temperature in the dark or at 37°C with gentle rotation.
32. Monitor the progress of the staining reaction by observing the embryos with a stereomicroscope on a white background every 15-30 min. Staining time can vary significantly between probes, from 15 min to several days.
33. Every 3 h, if the staining reaction requires more time, wash briefly with 100 mM Tris-HCl pH 8.2 + 0.2% Tween-20 and change for freshly prepared Vector® Blue staining solution. The staining reaction needs to be stopped before the appearance of conspicuous background staining. A correctly stained embryo is yellowish, and stained cells are blue. To optimize the signal-to-noise ratio, do not incubate in staining solution overnight. Instead, wash briefly with 100 mM Tris-HCl pH 8.2 + 0.2% Tween-20 and leave at +4°C in fresh buffer. The following day pick up the protocol at step 31.
34. When the signal is fully developed, stop the staining reaction by washing briefly with 100 mM Tris-HCl pH 8.2 + 0.2% Tween-20.
35. Wash with PBSTw for 10 min.
36. To counterstain nuclei, incubate with freshly diluted 1:5,000 Hoechst stock in PBSTw in the dark for 15 min.
37. Wash twice with PBSTw for 10 min.
38. Wash with PBS/sodium azide for 10 min.
39. Change solution to 80% glycerol. Stained embryos can be stored at +4°C for several months.

6.6 Troubleshooting & Optimization

Proteinase K treatment is a critical step of the ISH protocol and crucial for optimizing the signal-to-noise ratio.

Digestion times depend on developmental stage, fixation quality and proteinase K batch and thus need to be adapted

accordingly. The following digestion times are meant to serve as an orientation: 0-15 hpf – 5 min; 15-36 hpf – 10 min; over 36 hpf – 15 min. The digestion should not damage the embryos, with tail and snout regions being particularly sensitive to overdigestion.

Problem	Solution
No specific signal.	Make sure you work in an RNase-free environment. Check riboprobe integrity on denaturing gel. Verify that the riboprobe was correctly synthesized.
Embryos look damaged.	Reduce incubation time in proteinase K. If the problem persists, consider post-fixing with 4% PFA/PBS for 1 h at room temperature immediately after the second glycine treatment.
High background, especially in the tail region.	Reduce incubation time in proteinase K and/or reduce probe concentration.
Unspecific staining in the gut with NBT/BCIP.	Make sure to include levamisole in AP buffer and staining solution.
Very weak Vector® Blue signal under a stereomicroscope.	Image the embryos with a confocal microscope. If the problem persists using fluorescence, try repeating color reaction with NBT/BCIP. If the expression of a gene is being analyzed for the first time, we suggest using NBT/BCIP staining, as it is more sensitive than Vector® Blue staining.

7. Whole mount immunohistochemistry

Whole mount IHC allows the detection of target antigens in entire embryos with subcellular resolution using antibodies that are either labeled (direct method) or subsequently recognized by labeled secondary antibodies (indirect method). The protocol presented below is optimized for the detection of the neurotransmitter GABA and uses a fluorochrome-conjugated secondary antibody to detect the primary anti-GABA antibody.

7.1 Equipment

- Filtered micropipette tips
- 4-well cell culture plates
- Gloves
- Horizontal orbital shaker
- Micropipettes
- pH meter
- Stereomicroscope
- Syringes with 0.45 µm filters

7.2 Reagents

- Bovine serum albumin (BSA), Sigma-Aldrich (A9647)
- Glycerol, Sigma-Aldrich (G7893)
- Goat anti-rabbit IgG Alexa Fluor 488, Invitrogen (A-11008)
- Goat serum, Sigma-Aldrich (G6767). Inactivate by heating to 55°C for 30 min. Cool on ice and prepare 1 ml aliquots, that can be stored at -20°C for several months
- Hoechst 33258, Sigma-Aldrich (14530)
- Rabbit anti-GABA antibody, Abcam (ab8891)
- Sodium azide, Sigma-Aldrich (71289)
- Sodium chloride (NaCl), Sigma-Aldrich (S9888)
- Sodium phosphate dibasic (NaH₂PO₄), Sigma-Aldrich (S9638)
- Sodium phosphate monobasic (Na₂HPO₄), Sigma-Aldrich (S3264)
- Triton X-100, Sigma-Aldrich (T8787)

7.3 Stock solutions

- 10x PBS (phosphate buffer saline): 26 mM sodium phosphate monobasic, 174 mM sodium phosphate dibasic, 9% (wt/v) NaCl, pH 7.4. DEPC-treat and autoclave. Can be stored at room temperature for several months
- Hoechst stock: dissolve to 10 mg/ml in distilled water. Can be stored in the dark at +4°C for several months

7.4 Working solutions

- 1x PBS: Prepare from 10x PBS stock and filter
- 80% glycerol: dilute glycerol with PBS/sodium azide. Can be stored at room temperature for several months
- Blocking solution: 10% heat-inactivated goat serum, 1.5% BSA in PBSTx. Prepare fresh. Can be stored at +4°C for a few days
- PBS/sodium azide: 1x PBS, 0.1% (wt/v) sodium azide. Can be stored at room temperature for several months
- PBSTx: 1x PBS, 0.3% (v/v) Triton X-100
- Secondary antibody solution: dilute Alexa-conjugated secondary antibody 1:800 and Hoechst stock 1:5,000 in blocking solution. Prepare fresh. Do not store

7.5 Protocol for whole mount immunohistochemistry

If not otherwise stated, all steps of the protocol are performed in 350 µl of solution, at room temperature and rotating the embryos horizontally at 35-40 rpm. Each time you change a solution, remove fibers and debris that might have accumulated in the wells.

1. Place embryos into a 4-well plate and pipet them up and down in a 200 µl tip to prevent them from sticking to the bottom of the well.
2. Wash five times with PBSTx for 5 min.
3. Change to freshly prepared blocking solution and incubate for at least 1 h.
4. Change to blocking solution containing anti-GABA antibody diluted 1:300 and incubate at room temperature overnight.
5. Wash five times with PBSTx for 20 min.
6. Change to freshly prepared secondary antibody solution and incubate at 4°C overnight with rotation. From this step onward, do not expose the plate to direct light, unless absolutely necessary. Perform washes in the dark, for instance by putting the plate inside a small cardboard box on the shaker.
7. Wash five times with PBSTx for 20 min.
8. Wash with PBS/sodium azide for 5 minutes.
9. Change for 80% glycerol.
10. Mount embryos on slides and image them as soon as possible. Slides and plates can be kept in the dark at +4°C for a few weeks.

7.6 Troubleshooting & Optimization

Problem	Solution
No signal is detected.	If using a new primary antibody, optimize the protocol by varying antibody concentrations and incubation conditions. Verify whether particular fixatives and/or fixation protocols are required.
Only superficial cells are stained.	Antibody did not penetrate properly: incubate primary antibody at +4°C for at least 72 h.

8. Mounting embryos for microscopy

To avoid damaging the embryos, it is important to use spacers between slide and coverslip when mounting the amphioxus material for microscopy. We routinely use three layers of Scotch tape.

8.1 Equipment

- 200 µl micropipette tips
- Coverslips 24 x 32 mm

- Insect pins size 00
- Magic tape (3M Scotch)
- Micropipette
- Microscope slides 26 x 76 mm
- Transparent nail polish

8.2 Protocol for mounting amphioxus embryos for microscopy

1. On a clean microscope slide, create two spacers delimitating a central space of approximately 2 cm by placing three pieces of Scotch tape on top of each other (see Fig. 3).
2. Using a micropipette with a 200 μ l tip, transfer 40-50 μ l of 80% glycerol containing 5-10 embryos to the center of the slide, between the two spacers.
3. Using the tip of an insect pin, orient the embryos as desired by gently moving the glycerol around them.
4. To minimize air bubble formation, place one short edge of the coverslip on one of the spacers and carefully lower the coverslip toward the other spacer.
5. For confocal microscopy, glue the coverslip to the spacers with transparent nail polish to minimize movement of the samples during stack acquisition.

[Insert Figure 3 here]

8.3 Troubleshooting & Optimization

Problem	Solution
Embryos are too close to the spacers.	Use less mounting medium. While orienting the embryos, gently push them towards the slide. Floating embryos will inevitably be moved towards the spacers.
Coverslip detaches easily when using an inverted microscope.	Use more mounting medium and verify its concentration. Improve fixation of the coverslip using nail polish.
Embryos are deformed/damaged.	Verify distance between the two spacers on the slide, they might be too far apart. Tape thickness might vary between brands. If you are using a tape other than 3M Scotch, a different number of tape layers might be required to appropriately mount the embryos.

9. Confocal microscopy for chromogenic *in situ* hybridization and fluorescent immunohistochemistry

Chromogenic ISH and fluorescent IHC can be visualized, respectively, with a brightfield and an epifluorescence microscope. In addition, to increase the cellular resolution of the staining, the outcome of both techniques can be analyzed with a confocal microscope. While Vector® Blue, Alexa dyes and Hoechst are intended for use in fluorescence microscopy and are thus bright fluorochromes, the acquisition of NBT/BCIP fluorescence is more challenging. Two approaches are possible. Since NBT/BCIP forms crystals that reflect light, it can be visualized by setting a narrow acquisition window that includes the excitation wavelength (Jékely & Arendt, 2007). Although the signal-to-noise ratio is generally good, this method should be used with caution, as some anatomical structures (such as the amphioxus pigment spots) are also reflective and thus indistinguishable from the NBT/BCIP-derived signal. Moreover, some fluorochromes can also be reflective, limiting the use of this approach when analyzing different stainings in a single embryo. For these reasons, we suggest acquiring NBT/BCIP fluorescence in the far-red spectrum (Trinh et al., 2007). However, since the emission spectrum of NBT/BCIP peaks at 850 nm, which is beyond the maximum wavelength measurable by most commercial confocal microscopes, the signal tends to be weak and needs to be acquired several times, averaging the obtained values. It is thus very important to optimize the signal-to-noise ratio of the NBT/BCIP ISH procedure. Should this not be possible, NBT/BCIP can be replaced by Vector® Blue, which emits mostly in the visible spectrum. The parameters used to acquire the images presented in Figs. 4 and 5 are listed in Table 2. To avoid bleed-through artifacts, sequential scans have been performed.

Confocal microscopy presents several advantages. In addition to increasing the cellular resolution, confocal microscopes acquire stacks of focal planes (layers) that span the whole embryo. These stacks can be used to compile 3D reconstructions of whole embryos or to project sets of a limited number of focal planes, using open-source software packages like Fiji (Schindelin et al., 2012). Whenever possible, it is advisable to acquire stacks of the entire sample during confocal imaging. Projecting the information of several stacks into a single layer during post-production generally yields the best results. An example of the usefulness of this stack selection is given in section 11.

[Insert Table 2 here]

[Insert Figure 4 here]

9.6 Troubleshooting & Optimization

Problem	Solution
Fluorescent signal fades quickly.	Make sure that the glycerol is buffered, as some fluorochromes are pH-sensitive. Consider using a mounting medium containing an anti-fading agent instead of glycerol.

10. Safety considerations

When handling chemicals, validated health and safety regulations need to be respected at all times. Although this protocol generally requires the manipulation of only small volumes, some substances are particularly hazardous and need to be handled with extra care. These include solutions containing fixatives, formamide and sodium azide as well as concentrated acids and bases. Also, the compounds used to interfere with RA signaling are bioactive. Confocal microscopes are equipped with powerful lasers that represent a potential danger to the human eye. Common laser safety protocols must thus always be respected.

11. Retinoic acid signaling and GABAergic neurons in amphioxus

Pharmacological treatments represent a straightforward approach for manipulating the activity of molecular signaling pathways. The ease of these treatments is particularly appealing for work on emerging animal models, for which genetic approaches remain technically challenging. Amphioxus falls into this category of emerging animal models, with several signaling pathways having already been studied using pharmacological approaches (Bertrand, Le Petillon, Somorjai, & Escriva, 2017). For RA signaling, a number of different compounds have been shown to upregulate or downregulate its activity during amphioxus development. These compounds either target the RAR/RXR heterodimer or components of RA metabolism, such as RALDH and CYP26 (see Table 1). Here, we made use of some of these compounds to compile protocols for the characterization of phenotypes obtained by the pharmacological modulation of RA signaling activity in amphioxus.

Previous work has demonstrated that RA signaling controls the regionalization of the amphioxus embryo along its anteroposterior axis (Escriva et al., 2002), with RA signaling being active in different developmental tissues, such as the pharyngeal endoderm, the general ectoderm and the central nervous system (Koop et al., 2010). In the central nervous system, for example, RA signaling mediates the patterning of GABAergic neurons (Zieger et al., 2018). During normal development, three clusters of GABAergic neurons are formed in the anterior central nervous system of late embryos and early larvae (Candiani, Moronti, Ramoino, Schubert, & Pestarino, 2012). Upregulation of RA signaling activity, by either ATRA or R115866, leads to the complete loss of the posterior clusters and a shortening of the remaining neurites (Fig. 4). Conversely, downregulation of RA signaling activity, by either BMS493 or DEAB, results in the formation of supernumerary clusters at more posterior levels and a concomitant posterior extension of neurites (Fig. 4). In addition to perturbing the patterning of GABAergic neurons, the treatments also severely disrupt muscular swimming, affecting both the intensity of muscular contractions and the coordination of left-right body flexing (Zieger et al., 2018). The GABAergic system in the anterior amphioxus central nervous system might thus be required for the coordination of sinusoidal swimming by inhibiting contralateral muscle contractions (Lacalli & Candiani, 2017).

In this chapter, we assayed the number of GABAergic neurons in the amphioxus central nervous system by ISH (using a *GAD*-specific riboprobe) and IHC (using a GABA-specific antibody). Although, for any given treatment, both approaches yielded similar results, there were marked differences in the cellular resolution. For example, *GAD* ISH only stained cell

bodies, while GABA IHC marked cell bodies and neurites (Fig. 4). Thus, only the IHC results allowed us to conclude that the pharmacological treatments affected not only the number of GABAergic neurons, but also the length of their projections. Furthermore, only IHC revealed the presence of GABAergic neurons in the amphioxus ectoderm (Fig. 5). Given that this ectodermal IHC signal is very conspicuous, careful stack selection during image data analysis is required for optimizing the presentation of the obtained results.

[Insert Figure 5 here]

12. Summary

RA signaling can be easily manipulated in amphioxus embryos using the protocols presented in this chapter. Prior to more refined analyses, treatment success can be verified visually by comparing pharynx lengths between control and experimental conditions. Although ISH and IHC are used routinely in a wide variety of models, the execution of both techniques presents specific challenges that were addressed in this chapter. Our results show that both ISH and IHC are powerful techniques to study the roles of RA signaling in developing amphioxus. The choice between the two depends on several factors, including the required cellular resolution and the available imaging equipment. In addition, the IHC technique is strictly dependent on the existence of specific antibodies and efforts have recently been undertaken to develop novel antibodies for IHC experiments in amphioxus (Bozzo et al., 2017). Our protocols, allowing detailed correlations of IHC and ISH patterns, will be useful for the validation of these novel antibodies.

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References

- Bertrand, S., & Escriva, H. (2011). Evolutionary crossroads in developmental biology: amphioxus. *Development*, 138, 4819–4830.
- Bertrand, S., Le Petillon, Y., Somorjai, I. M. L., & Escriva, H. (2017). Developmental cell-cell communication pathways in the cephalochordate amphioxus: actors and functions. *The International Journal of Developmental Biology*, 61, 697–722.
- Bozzo, M., Pergner, J., Kozmik, Z., & Kozmikova, I. (2017). Novel polyclonal antibodies as a useful tool for expression studies in amphioxus embryos. *The International Journal of Developmental Biology*, 61, 793–800.
- Candiani, S., Garbarino, G., & Pestarino, M. (2015). Detection of mRNA and microRNA expression in basal chordates, amphioxus and ascidians. *Neuromethods*, 99, 279–292.
- Candiani, S., Moronti, L., Ramoino, P., Schubert, M., & Pestarino, M. (2012). A neurochemical map of the developing amphioxus nervous system. *BMC Neuroscience*, 13, 59.
- Carvalho, J. E., Lahaye, F., & Schubert, M. (2017). Keeping amphioxus in the laboratory: an update on available husbandry methods. *The International Journal of Developmental Biology*, 61, 773–783.
- Escriva, H., Holland, N. D., Gronemeyer, H., Laudet, V., & Holland, L. Z. (2002). The retinoic acid signaling pathway regulates anterior/posterior patterning in the nerve cord and pharynx of amphioxus, a chordate lacking neural crest. *Development*, 129, 2905–2916.
- Fonseca, E. S. S., Hiromori, Y., Kaite, Y., Ruivo, R., Franco, J. N., Nakanishi, T., ... Castro, L. F. C. (2019). An orthologue of the retinoic acid receptor (RAR) is present in the Ecdysozoa phylum Priapulida. *Genes*, 10, 985.
- Fuentes, M., Benito, E., Bertrand, S., Paris, M., Mignardot, A., Godoy, L., ... Escriva, H. (2007). Insights into spawning behavior and development of the European amphioxus (*Branchiostoma lanceolatum*). *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 308, 484–493.
- Ghyselinck, N. B., & Duester, G. (2019). Retinoic acid signaling pathways. *Development*, 146: dev167502

- Handberg-Thorsager, M., Gutierrez-Mazariegos, J., Arold, S. T., Nadendla, E. K., Bertucci, P. Y., Germain, P., ... Schubert, M. (2018). The ancestral retinoic acid receptor was a low-affinity sensor triggering neuronal differentiation. *Science Advances*, 4, eaao1261.
- Holland, L. Z., Albalat, R., Azumi, K., Benito-Gutierrez, E., Blow, M. J., Bronner-Fraser, M., ... Holland, P. W. H. (2008). The amphioxus genome illuminates vertebrate origins and cephalochordate biology. *Genome Research*, 18, 1100–1111.
- Jékely, G., & Arendt, D. (2007). Cellular resolution expression profiling using confocal detection of NBT/BCIP precipitate by reflection microscopy. *BioTechniques*, 42, 751–755.
- Koop, D., Holland, N. D., Sémon, M., Alvarez, S., de Lera, A. R., Laudet, V., ... Schubert, M. (2010). Retinoic acid signaling targets *Hox* genes during the amphioxus gastrula stage: insights into early anterior-posterior patterning of the chordate body plan. *Developmental Biology*, 338, 98–106.
- Lacalli, T., & Candiani, S. (2017). Locomotory control in amphioxus larvae: new insights from neurotransmitter data. *EvoDevo*, 8, 4.
- Laudet, V., Zieger, E., & Schubert, M. (2015). Evolution of the retinoic acid signaling pathway. In *The Retinoids: Biology, Biochemistry, and Disease* (pp. 75–90). Hoboken, NJ: John Wiley & Sons, Inc.
- Mic, F. A., Molotkov, A., Benbrook, D. M., & Duester, G. (2003). Retinoid activation of retinoic acid receptor but not retinoid X receptor is sufficient to rescue lethal defect in retinoic acid synthesis. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 7135–7140.
- Poss, S. G., & Boschung, H. T. (1996). Lancelets (Cephalochordata: Branchiostomatidae): how many species are valid? *Israel Journal of Zoology*, 42, S13–S66.
- Putnam, N. H., Butts, T., Ferrier, D. E. K., Furlong, R. F., Hellsten, U., Kawashima, T., ... Rokhsar, D. S. (2008). The amphioxus genome and the evolution of the chordate karyotype. *Nature*, 453, 1064–1071.
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., ... Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods*, 9, 676–682.
- Schubert, M., Holland, N. D., Laudet, V., & Holland, L. Z. (2006). A retinoic acid-*Hox* hierarchy controls both anterior/posterior patterning and neuronal specification in the developing central nervous system of the cephalochordate amphioxus. *Developmental Biology*, 296, 190–202.
- Theodosiou, M., Colin, A., Schulz, J., Laudet, V., Peyrieras, N., Nicolas, J.-F., ... Hirsinger, E. (2011). Amphioxus spawning behavior in an artificial seawater facility. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 316, 263–275.
- Trinh, L. A., McCutchen, M. D., Bonner-Fraser, M., Fraser, S. E., Bumm, L. A., & McCauley, D. W. (2007). Fluorescent in situ hybridization employing the conventional NBT/BCIP chromogenic stain. *BioTechniques*, 42, 756–759.
- Yong, L. W., Kozmikova, I., & Yu, J. K. (2019). Using amphioxus as a basal chordate model to study BMP signaling pathway. *Methods in Molecular Biology*, 1891, 91–114.
- Yu, J. K., & Holland, L. Z. (2009). Amphioxus whole-mount in situ hybridization. *Cold Spring Harbor Protocols*, 4, pdb.prot5286.
- Zieger, E., Candiani, S., Garbarino, G., Croce, J. C., & Schubert, M. (2018). Roles of retinoic acid signaling in shaping the neuronal architecture of the developing amphioxus nervous system. *Molecular Neurobiology*, 55, 5210–5229.
- Zieger, E., Garbarino, G., Robert, N. S. M., Yu, J. K., Croce, J. C., Candiani, S., & Schubert, M. (2018). Retinoic acid signaling and neurogenic niche regulation in the developing peripheral nervous system of the cephalochordate amphioxus. *Cellular and Molecular Life Sciences*, 75, 2407–2429.

Tables

Table 1 List of chemicals used to interfere with retinoic acid signaling.

Chemical	Mechanism of action	Stock concentration	Final concentration
all- <i>trans</i> -retinoic acid (ATRA)	Endogenous RAR ligand	0.1 mM	0.1 μ M
R115866 (Talarozole)	CYP26 inhibitor	0.5 mM	0.5 μ M
BMS493	RAR inverse agonist	1 mM	1 μ M
4-Diethyl-aminobenzaldehyde (DEAB)	RALDH inhibitor	10 mM	10 μ M

Table 2 Parameters for fluorochrome acquisition using a confocal microscope.

Fluorochrome	Excitation	Emission	Beamsplitter
Hoechst	405 nm	420-480 nm	DD488/552
Alexa Fluor 488	488 nm	500-550 nm	TD488/553/638
NBT/BCIP	638 nm	700-800 nm	TD488/553/638
Vector® Blue	638 nm	700-800 nm	TD488/553/638

Figure Legends

Fig. 1 Schematic protocol for the pharmacological manipulation of retinoic acid signaling in amphioxus embryos. (A) Preparation of embryo plates for treatments. (B) Treatment and fixation. (C) Preparation of embryo baskets from the caps of flow cytometry tubes.

Fig. 2 Phenotype evaluation following pharmacological manipulation of retinoic acid signaling. Arrows indicate the anterior and posterior limits of the pharynx. Compound list is provided in Table 1. DMSO is solvent control. Brightfield images of embryos in lateral view with anterior to the left.

Fig. 3 Mounting whole mount amphioxus embryos for microscopy. Arrows indicate the zone of nail polish application, which corresponds to the shorter edges of the coverslip.

Fig. 4 Effects of pharmacological manipulation of retinoic acid signaling on the development of GABAergic neurons in the amphioxus central nervous system. Numbers indicate clusters of GABAergic neurons. Compound list is provided in Table 1. DMSO is solvent control. (A-E) Whole mount *in situ* hybridization targeting *GAD* mRNA. Confocal images of embryos in lateral views with anterior to the left. (A'-E') Whole mount immunohistochemistry revealing GABA immunoreactivity (GABA-ir). Embryos in lateral (top) and dorsal (bottom) views with anterior to the left.

Fig. 5 Layer selection for z-projections of GABA immunoreactivity (GABA-ir). Confocal images of DMSO control embryos in lateral views with anterior to the left. (A) Projection of all focal planes. (B) Projection of layers corresponding to the position of the ectoderm on one side of the embryo. (C) Projection of layers corresponding to the position of the central nervous system.

At 5 hpf, split embryo culture into separate dishes adding a total volume of 8 ml to each dish

A

At treatment time (6 hpf), prepare compound working solutions and add it to the dish containing the embryos

B

Baskets can be prepared by cutting the caps of flow cytometry tubes

C

At fixation time (36 hpf), concentrate embryos using a basket and fix them on ice

RA signaling upregulation

DMSO

ATRA

R115866

RA signaling downregulation

DMSO

BMS493

DEAB

	<i>GAD</i> mRNA	GABA-ir
DMSO	<p>1 2 3</p> <p>A</p> <p>50 μm</p>	<p>1 2 3</p> <p>A'</p>
ATRA	<p>1</p> <p>B</p>	<p>1</p> <p>B'</p>
R115866	<p>1</p> <p>C</p>	<p>1</p> <p>C'</p>
BMS493	<p>1 2 3 4 5</p> <p>D</p>	<p>1 2 3 4 5 6</p> <p>D'</p>
DEAB	<p>1 2 3 4</p> <p>E</p>	<p>1 2 3 4</p> <p>E'</p>

GABA-ir

50 μ m