CHAPTER 8

Hemichordate Embryos: Procurement, Culture, and Basic Methods

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I. Introduction

The phylum Hemichordata is composed of two extant classes: the solitary burrowing marine worms, or enteropneusts, and the pterobranchs, which are colonial sessile organisms. Bateson originally placed this group within the chordates, due to many of the adults morphological affinities between the two groups (Bateson, 1885) and it was not until much later that they were reclassified into their own phylum (Hyman, 1959). Current molecular phylogenies place them as the sister group of the echinoderms (Adoutte et al., 2000; Cameron et al., 2000; Turbeville et al., 1994; Wada and Satoh, 1994). Despite the close relationship of these two groups, their respective adult body plans are highly divergent, and even gross axial comparisons between the groups are problematic. The bilateral symmetry, gill slits, stomochord, post-anal tail, and hollow dorsal nerve cord of hemichordates provide strong morphological affinities with the chordates, making them a particularly interesting group for testing hypotheses of chordate origins (Lowe et al., 2003; Nubler-Jung and Arendt, 1999; Tagawa et al., 2001). For any consideration of deuterostome evolution, hemichordates are a critical component: their key phylogenetic placement and proposed morphological affinities with the chordates make them an intriguing but poorly described group. Developmental studies on this phylum are beginning to generate a substantial amount of gene expression data for conserved developmental regulatory genes (Harada et al., 2000, 2001, 2002; Lowe et al., 2003; Ogasawara et al., 1999; Okai et al., 2000; Peterson et al., 1999; Tagawa et al., 1998a,b, 2000, 2001; Taguchi et al., 2000, 2002; Takacs et al., 2002) allowing a more comprehensive phylogenetic sampling of developmental data within the deuterostome lineage. These results are beginning to provide critical insights into early deuterostome evolution. Only by a comprehensive understanding of the evolution of all three phyla can we hope to reconstruct the early evolutionary history of the deuterostomes and understand the transitions that led to the unique and unusual body plans of this group.

Within the enteropneusts, there are two contrasting developmental strategies, one indirect and the other direct. Indirect-developing species, the Ptychoderids and Spengellidae, produce a larva that swims and feeds in the plankton for months before transforming into the adult worm and settling. Direct-developing species (the Harramanids) bypass the larval stage and develop to the adult worm from the egg. Comparative studies of these contrasting developmental strategies is particularly interesting as current diverse hypotheses of chordate origins alternatively propose emergence of the dorsal nervous system of the chordate body plan either from progenitor larval structures (Garstang, 1928; Nielsen, 1999; Tagawa
et al., 2000) or from the basic invertebrate adult nervous system (Arendt and Nubler-Jung, 1996; DeRobertis and Sasai, 1996; Lowe et al., 2003). Since extant chordates lack larval stages, investigation of both life history modes will probably be necessary to comprehensively analyze the implications that various molecular observations have for these hypotheses.

This chapter focuses on practical considerations for collection of hemichordate adults and procurement of gametes for carrying out developmental studies. We describe methods for collecting, spawning, and rearing embryos and larvae from two species of hemichordate—Saccoglossus kowalevskii and Ptychodera flava, the latter an indirect developer producing a true larval form and the former a direct-developing species. In addition, we present two protocols for whole mount in situ hybridization.

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II. Procurement, Spawning, and Culture of S. kowalevskii

A. Background

Despite the general recognition of hemichordates as a phylogenetically key group, very few studies have focused on their development. In large part, this is due to some of the technical challenges associated with collecting adults and reliably obtaining viable gametes in large enough quantities for molecular studies. Artificial triggers for completion of meiosis and germinal vesicle breakdown have not been determined for hemichordates thus far, and experimental manipulation of gravid animals to stimulate natural spawning is required to induce oocyte maturation (Colwin and Colwin, 1962).

S. kowalevskii is found along the eastern seaboard of the United States from South Carolina to New Hampshire. It is an intertidal species found mainly in sheltered bays and forms its burrows in coarse organic sand, often in clam beds. The worms are approximately 6 to 9 inches long and form U-shaped burrows. Figure 1A shows a diagram of this species with major body regions labeled. Figure 1B,C shows pictures of a gravid adult male and female.

B. Method

1. Reproductive Season

Our experience is limited to animals collected on Cape Cod close to the Marine Biological Laboratory at Woods Hole, MA. The animal collection staff of the Marine Resources Center at the Marine Biological Laboratory has extensive experience in both collecting and shipping these animals (www.mbl.edu). From late August to the end of September, ripe animals of both sexes are abundant in the area. The peak of spawning tends to be toward the middle of September, with few ripe animals being found by the beginning of October. In addition to the main spawning season in the fall, mature individuals of both sexes have been reliably collected in May, though we have not thoroughly investigated this period of reproductive activity.
Fig. 1  Adult enteropneust hemichordates, *Saccoglossus kowalevskii* and *Ptychodera flava*. (A) Model of an acorn worm adult based on *S. kowalevskii* outlining some of the characteristic features of the adult morphology. (B) Adult *S. kowalevskii* female. White arrowheads indicate the position of the green ovary. Black scale bar = 3 cm. (C) Adult *S. kowalevskii* male. White arrowheads indicate the position of the orange/white testes. (D) High magnification of branchial region of metasome on *S. kowalevskii*. Lateral view. White arrowheads indicate the position of two of the multiple pairs of gill slits on the dorsal side of the worm. Scale bar = 0.5 cm. (E) Spawning female of *S. kowalevskii*. Oocytes are green/blue-colored and are spawned along the length of the gonad. (F) Adult *P. flava*. Black arrowhead indicates the position of the proboscis. White arrowheads indicate the position of the genital wings. Scale bar = 1 cm. (See Color Insert.)

2. Adult Collection

a. Their populations are patchy and can be found most commonly in sheltered bays. Populations seem to be most common in brackish water and are present in the mid to low intertidal zone.

b. The burrows of the adults are most easily identified by the presence of fragile, spiral fecal castings, 3 to 4 cm in diameter, which are very obvious in calm water, but are easily disturbed by water flow.
c. Adults are collected by digging vertically with a shovel close to the burrows, which are often in groups, at low tide, carefully transferring the sand into a large sieve (mesh diameter of approximately 0.5 cm). Both digging and sieving are best done in shallow water, which aids in locating the burrows and also allows sand to easily pass through the sieve, causing less damage to the fragile adults. The sieve should remain in the water to avoid entangling the animals in the sieve and damaging the epidermis. The worms are transferred into widemouth, screw-top plastic bottles by placing the mouth of the bottle next to worm on the mesh and submerging the sieve, washing the worm into the vessel. Avoid washing in additional debris and ensure there is no sharp shell debris or algae in the vessel with the worm.

3. Transportation

Adult animals are extremely fragile and are easily damaged in transport. Fine, clean sand should be added to the vial, just enough to cover the animal. Once collected, the animals should be kept cool and not left in the sun to heat up above 25 °C. Animals can be shipped by FeDEX®, after filling the vials with fresh seawater aerated with oxygen for a few seconds before sealing the vials. Add 20 µg/ml gentamycin if warm. Twenty or so vials of animals can be shipped in one box by double-packing the vials with large plastic bags, with the inner one filled with seawater. Add two cool packs to the box before shipping. It is imperative that the sand be free of sharp shells or filamentous algae to avoid damaging the animals during shipment.

4. Adult Maintenance in the Lab

Ideally, the adults should be kept in running seawater aquaria at a marine station, but a recirculating aquarium would also be adequate. Animals remain healthier when entirely covered by sand and allowed to establish burrows. However, this makes egg collection impractical, so maintaining the adults in a small amount of clean sand that is changed daily is the best compromise to ensure animals can be effectively monitored for spawning. If mucus accumulates around the collar of the animals, it should be removed with forceps as such “nooses” can slice through the epithelium of the collar within a day or two, leading to a rapid decline in health. We have not attempted to maintain a long-term population of worms in the lab. For short-term storage of several days to two weeks, animals may be kept in their shipping vessels after replacing the seawater with filtered seawater and gentamycin (20 µg/ml) and kept at 4 °C.

The next section describes the treatment of animals following either collection from the field or receipt of a shipment.

a. Animals can be tipped with their sand into a much larger container filled with fresh seawater to expose the worm, and transferred into a small glass dish using a turkey baster or spoon.
Fig. 2  Embryological, larval, and juvenile stages of *S. kowalevskii* and *P. flava*. A through E reflect stages of *S. kowalevskii* development. F through I reflect stages of *P. flava* development. (A) Early cleavage stage within the vitelline envelope. Animal blastomeres are slightly smaller than vegetal ones. a = animal pole, v = vegetal pole. Scale bar = 100 μm. (B) Late blastula. (C) Post-gastrula with prominent telotroch at the posterior end of the embryo swimming within the vitelline envelope. (D) SEM micrograph of midstage embryonic development (day 4), lateral view. The tuft of cilia at the far left of the panel is the apical tuft. The ectoderm is evenly ciliated except for the dense telotroch at the posterior of the embryo. Scale bar = 75 μm. (E) Late juvenile 14 days old, lateral view. All three body regions clearly visible. White arrowhead indicates the position of the anus and, posterior to
b. Examine the animal under a dissecting scope to check for damage. The long gut should be amputated with fine scissors just anterior to any region where there is damage, and we routinely remove large stretches of post-branchial gut, as the animals tend to tie themselves in tight knots without large amounts of sand to burrow in. If the proboscis is damaged at the tip, then a clean cut should be made with a scalpel posterior to the damage. Much of the proboscis can be removed without compromising the health of the animal significantly in the short term or inhibiting gamete production. If the entire proboscis is removed, females are unable to spawn or regenerate new tissue. Ovary that is damaged during shipping or storage rapidly turns orange and should be amputated immediately anterior to the damage to avoid degeneration in animal health. Removal of this tissue does not compromise the spawning ability of the animal from the remaining ovary. Gently rinse each animal free of residual sand from the collection site as sperm are sometimes contained in it.

c. Transfer animals into small, white plastic containers: we have found disposable plastic weigh boats of approximately 10 cm diameter to be useful for this purpose. The white background helps to identify spawned oocytes that are hard to see among sand grains. The worm should only just be covered with clean sand, free of any sharp shell debris or algae, and placed into a running seawater aquarium. Adult males and females can be distinguished only when reproductive by the color of their gonads (Fig. 1B, C). Males have a milky white gonad visible through the ectoderm, whereas females have a green/blue color to their gonad. Oocytes can clearly be seen through the ectoderm during their reproductive season (Fig. 1B, D, E). Males and females should be kept in separate tanks. Animals should be checked every day for general health and body knots, and mucus strands removed from their collars. Sand should be changed every other day to avoid buildup of mucus secreted during their feeding and burrowing, which can attract ciliates and bacteria.

5. Spawning

All further steps of the protocol should be carried out in dedicated embryological glassware that should be washed in water only with no detergent. Any glassware that has been exposed to fixative, detergent, or heavy metals should not be used to culture embryos.
a. Active sperm can be obtained by rupturing the testes of a ripe male with fine forceps. Concentrated sperm will ooze out of the wound and can be collected with a Pasteur pipette.

b. Oocytes are arrested in meiosis in the female gonad and, in order to achieve successful fertilization, the females must be induced to spawn. This can be achieved by a heat treatment in seawater to 29 °C for 8 h, followed by reimmersion in the seawater at ambient temperature (22 °C) (Colwin and Colwin, 1962). Do not extend the heat treatment for any longer than 8 h. Often, females will start spawning 6 to 8 h after heating and continue for 3 to 4 h, so we recommend heating overnight to avoid animals spawning late at night. At peak spawning season, females may spawn unpredictably in the seawater tables several hours after collection, purely from the stress of transport, without any heat stimulus. Regular animal inspections are advised during these peak times, both in the holding aquaria and during the heat treatment, to ensure spawning females are identified quickly. Egg collection is most effectively carried out using a Pasteur pipet to separate eggs from sand. Oocytes are released along the length of the ovary and often line the burrow in the sand. If the sand is kept to a minimum in the containers, then these burrows are easily cracked open to reveal the oocytes. It is not advised to heat worms without any sand as they will become entangled in mucus threads, leading to rapid decline in health.

6. Fertilization

One drop of concentrated sperm solution should be diluted in 100 ml of seawater before fertilization of oocytes. If oocytes are not immediately fertilized, then they can be stored for up to 12 h at 10 °C (and possibly longer) before fertilization. The oocytes should be washed with several rinses of filtered seawater to remove the mucus and debris carried over during collection. Approximately 3 ml of sperm suspension prepared as has been described should be added to washed oocytes and stirred in 100 ml of filtered seawater. Fertilization success can be monitored by observation of the zygotes under a dissecting scope. A thick fertilization envelope should begin to rise 1 to 2 minutes following the addition of sperm if successful fertilization occurs.

7. Culture of Embryos

Figure 2A–E shows a range of developmental stages of *S. kowalevskii* from cleavage (Fig. 2A) through to juvenile (Fig. 2E). At 22 °C, first cleavage begins at approximately 120 min, and then every 30 min subsequently (Bateson, 1884; Colwin and Colwin, 1953). Gastrulation begins at approximately 18 h and is completed by 36 h. At this stage, the telotroch (ciliated band) forms and the embryo begins to swim within the vitelline (Fig. 2C). One ectodermal, circumferential groove forms at the boundary of the prospective proboscis and collar, and is closely followed by a second groove marking the boundary between the collar and metasome. At this stage, approximately 3 to 4 days postfertilization (Fig. 2D), the telotroch is very prominent and all three body regions are clearly visible. Embryos
hatch at 7 days and swim for up to 24 h before searching on the bottom of the glass dish for a settlement site. At this stage, the juveniles begin to develop their postanal ventral tail (Fig. 2E) and actively burrow in fine sand. The following section describes the basic protocol required for culturing embryos and juveniles.

a. Embryos are cultured in small glass dishes at room temperature (22°C). Do not crowd the dishes with too many embryos, as this will result in slower development and asynchrony of the culture. There should be no more than a loose monolayer of embryos on the bottom of the dish. Examine cultures at advanced cleavage stages: any damaged or irregular embryos and unfertilized eggs should be discarded from the culture. Some embryos will have small yolk particles in the perivitelline space which have no effect on the viability of the embryos. Post-gastrula embryos can be kept at 4°C for several days to slow down development without adverse effects; however, earlier embryonic stages tolerate temperatures lower than 16°C poorly. Transfer the embryos once they begin to hatch into a larger glass dish with a thin layer of fine reptile sand coating the base of the dish. The sand is very important as the worms begin to secrete mucus at this stage and will become entangled in mucus threads if cultured without a substrate to burrow in.

b. Two days following hatching, juveniles should be fed by adding a suspension of phytoplankton (see Chapter 4 by Greg Wray or Lowe and Wray, 2000). We have used Dunaliella tertiolecta, Rhodomonas lens, and Isochrysis galbana. These can be prepared ahead of time, spun down to a concentrated pellet, aliquoted, and frozen at −20°C, or added live as a suspension. Filtered seawater should be replaced every day, and the cultures should be very gently aerated at this stage with an air pump to keep water circulating in the culture dish. Juveniles have been successfully cultured for at least 20 days after hatching. Harvesting the juveniles out of the sand before fixation can be problematic, but if MgSW is added to anaesthetize the juveniles and the sand disturbed by squirting forcefully with a turkey baster, then the juveniles become dislodged. When the culture is swirled inside the dish, juveniles will move into the center on top of the sand. This procedure should be repeated several times to recover the majority of animals.

III. Procurement, Spawning, and Culture of Ptychodera flava

A. Background

Ptychodera flava was the first hemichordate described (Eschscholtz, 1825), although the original classification, based only on adult structure, placed it within the holothuroids. The species has an extensive range throughout the IndoPacific. The worms are approximately 10 to 25 cm long and 1 to 2 cm in diameter. Fig. 1F is a photograph of a gravid adult female. Like all the ptychoderid species, Ptychodera flava, is an indirect developer. Females spawn large numbers of small oocytes that develop into tornaria larvae. The larvae remain in the water column for several months, feeding and growing until they become competent to transform into the juvenile
worm. Unlike echinoderm metamorphosis, the larval body plan of the tornaria is
directly inherited by the adult without the loss of larval tissue or cells (Agassiz,
1873). This suggests the possibility of comparing meaningfully gene expression
patterns among embryonic, larval, young juvenile, and adult, stages that all
 preserve the bilateral body plan laid down during gastrulation. This body plan
is also directly comparable with the vertebrate body plan.

B. Method

1. Adult Collection

_Ptychodera flava_ adults are abundant in sand flats and are easily collected by
snorkeling in shallow water around the islands of Hawaii. We have collected
primarily on the island of Oahu at either the large sandbar across the entrance
to Kanohe Bay on the windward (east) coast or at the shallow reef that extends
off Paiko on the leeward (south) coast. These habitats are strikingly different, the
former constituted by deep, coarse, clean sand while at Piko, the animals are
found in shallow, small collections of muddier, fine sand in pockets in the reef. At
Piko, when the sand layer is very thin, the animals may even be found exposed.
The animals do not seem to maintain fixed burrows but to move continuously in
the top few inches of the sand.

In both locations, animals are found by using a vigorous wave of the hand in the
water to stir up the top one or two inches of sand. The exposed animals are caught
in the hand and placed into Ziplock bags of seawater. During collection, we often
place 10 to 20 animals in a sandwich bag full of seawater and transport them in
this manner for the 2 or 3 h required to reach the lab.

Ripe animals may be recognized by fuller, more distended genital wings which
are a bit brighter yellow in color. The genital wings are a yellowish tan, reflecting
the color of the underlying gonad, when compared with the tan color of the rest of
the animal. Generally, there is not a great distinction between the females and
males, but the males at Piko exhibit a darker, greenish gray color in their genital
wings and usually can be distinguished from females.

Gravid animals can be collected from late October through January in Hawaii.
The adults can be maintained in a healthy state for several months in running
seawater tanks with 3 to 4 cm sand from their native habitat. We often keep the
animals in small groups placed in 10 × 15 cm or 20 × 20 cm glass baking dishes with a
few cm of sand stacked in the running seawater aquaria. In the Kewalo Marine Lab
seawater system, artificial sources of food are not required if the running seawater is
not filtered. Tanks should be kept clean and algal growth kept to a minimum.

2. Spawning

Oocytes are stored in the ovary arrested in meiosis with prominent germinal
vesicles. Attempts to artificially induce _P. flava_ oocyte maturation after dissec-
tion from the ovary have been unsuccessful (Tagawa et al., 1998a). Unlike
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*S. kowalevskii*, active sperm cannot be dissected from the ripe testes, and natural spawning must be induced in both males and females to achieve fertilization (Tagawa et al., 1998a). Spontaneous spawning of individuals generally occurs in the evening after sundown, suggesting that spawning in *P. flava* may be stimulated by onset of darkness (Tagawa et al., 1998a). Ripe individuals can be kept in holding tanks for at least 2 weeks without loss of fecundity if they do not spawn spontaneously, which is most frequent a day or two following collection.

Spawning is reliably induced at about sundown by placing individual worms into glass or plastic containers with sand covering the bottom, raising the seawater temperature from ambient temperature of 22 to 26 °C and maintaining the containers in a dark room. The room must be kept dark until the animals spawn. Spawning usually occurs within 2 h of the temperature increase. Unlike *S. kowalevskii*, spawning occurs during, rather than following, the heat treatment. Gametes are shed through the epidermis along the length of the genital wings. Females usually produce 0.05 to 0.2 ml of eggs, but some females shed up to 1 ml of eggs.

3. Fertilization

a. The oocytes of *P. flava* are translucent yellow, 120 µm in diameter, and surrounded by a thick, sticky jelly coat that makes them difficult to collect with a simple Pasteur pipette. We find a 5 or 10 ml Komagome Pipette (Iwaki Glass Co. Ltd, Tokyo) works best because you can suck up the eggs with a constant flowing stream of water. Transfer the oocytes to a 15 ml centrifuge tube and pellet the eggs using a hand centrifuge. Wash the eggs several times with filtered seawater. Concentrated sperm can be collected by Pasteur pipette from the genital wing area of spawning males or from the bottom of the vessel where the males have spawned.

b. Transfer the eggs into a petri dish. A few drops of semen should be diluted in approximately 100 ml of filtered seawater and 2 to 3 ml of this suspension should be swirled with the washed eggs. Fertilization success can be assayed by the appearance of the first polar body between 30 and 90 min later; first cleavage begins approximately 3 h post-fertilization.

4. Culture of Embryos and Larvae

a. Fertilized eggs are transferred into glass beakers up to a density that creates a monolayer on the bottom of the beaker. Wash the zygotes with filtered seawater several times and allow them to develop at room temperature (22 to 24 °C). Cultures should be washed daily with several rinses of fresh, filtered seawater containing streptomycin (50 mg/l). Before hatching, the embryos will settle to the bottom of the container and the water can be decanted easily.
b. Gastrulation begins at 18 h post-fertilization and is completed before hatching occurs at 44 h post-fertilization. After hatching, we culture the larvae at a density of about 1 per ml of seawater. Following hatching, the ciliated embryos and larvae disperse throughout the seawater and further seawater changes must be carried out by an alternative procedure using 100 μm Nitex® mesh. Do not collect the larvae on the mesh. We remove the bottom of a plastic beaker and replace it with the Nitex® mesh. (A hot glue gun works well to stick the Nitex® to the base; solvent-based adhesives are not recommended.) We immerse this modified beaker in a slightly larger beaker into which the culture has been transferred and aspirate as much old medium as possible. Add fresh filtered seawater, repeat once or twice, and transfer the culture to a clean culture container.

c. We have not successfully fed *P. flava* larvae. We have attempted to feed tornaria with cultured algal strains of *Tetraselmis*, *Nanochloropsis*, *Nanochloris*, *Isochrysis*, and *Rhodomonas*. Young tornaria after 72 h ingest and swallow all of them; the algal cells can be observed to enter the stomach. They also seem to be able to digest all of them except *Tetraselmis*, which remains intact and even appears viable in the stomach. However, the tornaria do not seem to thrive when fed with any one or a mixture of these strains.

d. We have been successful culturing larvae for 2 months, from early tornaria through the Metschikoff stage tornaria (Fig. 2F) (Stiansny-Wijnhoff and Stiasny, 1926; 1927) by standing cultures in a sunlit window. However, attempts to culture the tornaria through to late larvae that will transform to young juvenile worms have so far proven unsuccessful.

Larvae competent to transform to juvenile worms are easily collected in plankton tows during the months of April to July. Figure 2G shows a Krohn stage tornaria, the stage after the Metschikoff stage, collected in a plankton tow (Stiansny-Wijnhoff and Stiasny, 1926, 1927). Collected animals soon begin to transform as shown in Fig. 2H. When they are placed in a petri dish with a single layer of sand to provide an attractive substrate, the transforming larvae shed the cilia of their telotroch and burrow in the sand as young juvenile worms, as shown in Fig. 2I. At this point, the two pigmented eye spots of the larva are still evident at the tip of the proboscis and one or two pairs of gill slits are present in the pharynx of the young worm. By 2 weeks, the trunk region has extended considerably, more than doubling the length of the animal, and four or five gill slits are evident.

== IV. Removal of Vitelline Envelope in *S. kowalevskii*

A. Background

Following fertilization, a thick vitelline envelope is raised around the embryo. This envelope can be removed manually with fine forceps post-gastrulation, but is very difficult to remove manually without damaging the embryo before the onset
of gastrulation. We have developed a protocol to remove the vitelline chemically in *S. kowalevskii*. Removal of the membrane has been crucial for carrying out embryological manipulations and for microinjection. Denuded oocytes and embryos should be handled with extreme care following this procedure, as they become extremely fragile and sticky. Embryos are cultured in small petri dishes coated with agar, and seawater should be filtered and supplemented with antibiotic and calcium. The blastomeres tend to disaggregate without the vitelline in place.

**B. Method**

1. Obtain unfertilized eggs, as described in Section II. The egg’s color is dark grey/green to cream colored, depending on the female. The unfertilized egg’s diameter is 0.35 to 0.40 mm. Fertilize eggs as described in Section II. Add filtered SW at 22 °C. After fertilization, the fertilization envelope lifts from the surface, at least doubling the diameter within 30 min. The transparent fluid space between the egg surface and envelope is readily apparent.

2. Dejelly eggs at 60 min post-fertilization (second polar body forms by 50 min; first cleavage at about 120 min), as follows:

   3. Prepare a small dish of 3.5 ml DTT-NaOH-FSW as described in Section VII.8. The pH will be approximately 8.0. Add eggs (up to 100) with minimal seawater carryover, pipet up and down gently with a Pasteur pipet at 15-s intervals to get uniform exposure and reduce sticking of the eggs to the glass surface. Within 3 min, the fertilization envelope dissolves. Materials on the egg surface (hyalin-like?) are also removed. Take care not to break the bare eggs by passing them through the meniscus.

3. Transfer bare eggs to 10 ml FSW (fresh seawater) in a dish and rinse by slow swirling. Do a second FSW rinse if there are many eggs (>30). Transfer eggs to a holding dish with thin agar coating on the surface (that is, rinse the dish surface with 1% agarose and decant the excess) in 10 ml FSW to which 10 mM CaCl₂ has been added.

**V. Whole-Mount In Situ Hybridization**

**A. *S. kowalevskii***

*In situ* hybridization is a critical technique for determining the expression of developmental genes. The deployment of conserved developmental genes during the development of the hemichordate body plan can potentially give key insights into axial similarities and differences between the deuterostome taxa. The protocol for *S. kowalevskii* is based largely on the *Xenopus* protocol developed initially by Harland (1991) and modified by Salic *et al.* (1997) and Lowe *et al.* (2003). Some of the steps have been changed due to the smaller size of
the embryos. The signal-to-noise ratio varies between different genes but, in general, is excellent. Probe quality is particularly important and probe should be free of all unincorporated nucleotides. We do not remove the vitelline for this protocol and have not noticed an attenuation of signal or increase in background as a result of its presence.

1. Fixation
   a. Harvest embryos or juveniles and rinse in filtered seawater, then incubate for 10 min in MgSW to anaesthetize in a 50 ml falcon tube (only embryos after day 2 of development).
   b. Draw off most of the seawater and pipet the anaesthetized specimens into a 50 ml falcon tube. Add 20 ml of fixative A and incubate on a shaking stage at low speed for 1 h at room temperature. The vitellines will stick to the wall of the tube if the embryos are allowed to settle.
   c. Drain the fixative and wash embryos 3× for 10 min in buffer of fixative A (without fixative). Do not use PBST to wash the embryos as embryos become rapidly deformed.
   d. Dehydrate the fixed embryos by adding 100% EtOH directly without stepping through an ethanol series. The tube should be continuously agitated during the first few minutes following addition of EtOH to avoid the vitellines sticking to the side of the tube. Incubate for 10 min and repeat 2× before storing the embryos in 100% EtOH –20°C.

2. Rehydration
   Transfer embryos into 1 gill screw to glass vials and rehydrate through an ethanol series into PBST. Five minutes for each wash
   - 75% EtOH/25% H2O
   - 50% EtOH/50% H2O
   - 25% EtOH/75% H2O
   - 3X 100% PBST

3. Proteinase K Treatment
   a. Incubate samples for 5 min in a 1 µg/ml solution of Proteinase K in PBST at 37°C.
   b. Rinse 2×, 5 min each in 0.1 M Triethanolamine.

4. Acetic anhydride treatment
   a. Incubate for 10 min in 0.1 M Triethanolamine and acetic anhydride (250 µl in 100 ml of 0.1 M Triethanolamine).
   b. Rinse 2×, 5 min each in PBST.

5. Refix in formaldehyde
   a. 10 min in 4% formaldehyde in PBST.
   b. Rinse 3× for 5 minutes each in PBST.
Fig. 3 Examples of patterns of expression in hemichordate embryos of genes implicated in specifying vertebrate structures. Whole mount in situ hybridization in *S. kowalevskii* and *P. flava*. A and B show *S. kowalevskii*, C and D show *P. flava*. (A) Retinal homeobox expression in the developing proboscis ectoderm of a 2-day-old early embryo. Optical frontal section of a cleared specimen. Scale bar = 150 μm. (B) Expression of BarH, a neural determining gene, in the mesosome ectoderm of a 3-day-old early embryo. Frontal optical section of a cleared embryo. (C) Expression of the forebrain gene, T-brain, in a 3-day-old tornaria larva at the apical organ, as marked by a white arrowhead. Scale bar = 50 μm. (D) Expression of Brachyury, a gene important in notochord development, in a 2-day-old tornaria larva. White arrowhead indicates the position of the mouth and black, the position of the anus. (See Color Insert.)
6. Prehybridization.
   a. Wash embryos with small amount of hybridization A solution for 10 min.
   b. Replace solution in each vial with approximately 1 ml of fresh hybridization buffer A.
   c. Incubate for 1 to 3 h at 60 °C in a shaking water bath.

7. Hybridization
   a. Replace solution in vials with 1 ml of hybridization A solution with probe diluted to approximately 1 μg/ml. Riboprobes should be prepared using standard protocols and purified using G50 columns (Ambion).
   b. Hybridize overnight at 60 °C in shaking water bath.

8. Post-hybridization washing
   a. Remove hybridization solution with probe and discard. Replace with hybridization buffer A and fill vial with 2× SSC.
   b. Invert each vial into one Netwell sample vial in a 12-well tissue culture plate. Fill the reservoirs with 2× SSC and rinse 3×, 20 min each at 60 °C.
   c. Rinse 2× in 0.2× SSC, 30 min each at 60 °C.

9. Blocking
    Transfer the Netwell vials into a fresh 12-well culture plate. Add 1× MAB with 2% Roche blocking agent for 1 h at room temperature.

10. Antibody incubation
    a. Replace blocking solution with the same solution containing a 1:1000 dilution of anti-digoxigenin, alkaline phosphatase-conjugated (Roche), or 1:2000 dilution of an antifluorescein, alkaline phosphatase-conjugated antibody (Roche).
    b. Incubate for 4 h at room temperature.

11. Washes
    Wash 4×, 30 min each in 1× MAB at room temperature, and an additional 60 min at room temperature or O/N at 4 °C.

12. Chromogenic reaction
    a. Rinse 2×, 5 min each in alkaline phosphatase buffer.
    b. Remove embryos from the Netwells and transfer into the reservoirs of the 12-well plate directly. Remove as much of the buffer as possible and add 1 ml of BM purple.
    c. Allow to develop in the dark and monitor every 30 min for staining intensity.

13. Post fixation
    a. Once embryos have reached the desired level of staining, they should be post-fixed overnight in Bouins fixative.
b. Following fixation, embryos should be repeatedly rinsed in 80% EtOH and 0.1M Tris pH 8.0 until all yellow stain from the embryos has disappeared.

14. Clearing

a. Further dehydrate samples in sequential rinses into 100% EtOH and 3×5 min in 100% MeOH.

b. Transfer embryos to a glass depression slide and remove all but a trace amount of MeOH.

c. Immediately add several drops of a 1:2 mix of benzyl alcohol and benzyl benzoate. Allow embryos to become transparent.

d. Make permanent mounts of embryos by replacing clearing agent with Permount and covering specimens with a cover slip with thin clay feet on each corner.

B. General Considerations

The critical parameters in the protocol are: (1) Proteinase K digestion should be very light as the embryos will not tolerate long incubations or high concentrations. Adequate results can be achieved by eliminating the step altogether albeit with a slightly higher background staining. The collar of late juveniles (2–3 gill slits) is particularly sensitive to this treatment. (2) Probe quality: we use full-length probes and generally have not found it necessary to hydrolyze. We also routinely use fluorescein-labeled probe rather than digoxigenin, as background is generally slightly lower with fluorescein. We use G-50 columns from Ambion® to remove unincorporated nucleotides and check probe quality on an agarose gel. The fluorescein-labeled probes glow green/blue under UV illumination and give a rough indication of fluorescein incorporation in the probe.

We use Netwell vials®, which are small plastic vials with a Nytex mesh that fit into the wells of a 12-well tissue culture dish. These vials ensure minimal fluid carryover between each wash step, and minimize the loss of embryos through accidental uptake during aspiration of the post-hybridization washes. They are not critical to the procedure and washes may be carried out in the hybridization vials. The fixation in Bouins is only necessary if the embryos are to be cleared in benzyl benzoate/benzyl alcohol. The blue precipitate from the AP reaction is unstable in this clearing agent and will rapidly disperse without the post-fixation in Bouins.

C. P. flava

The in situ hybridization protocol for P. flava was developed largely from the protocol established for ascidian embryos and larvae (Tagawa et al., 1998a; Yasuo and Satoh, 1994). The level of background varies with different genes but, in general, is quite low. We do not remove the vitelline membrane from prehatching stages because we have not noticed an attenuation of signal nor an increase in
background as a result of its presence. After hatching, the handling of tornaria larva requires more care because the larvae settle slowly in the reaction tube and it is easy to lose the sample during changes of solutions.

To reduce background, we use a preparation of powered adult hemichordate tissue prepared and used much as the one developed for Amphioxus (Holland et al., 1996). The hybridization solution becomes viscous during hybridization because of the mucus from young juveniles. To reduce background, it should be removed when you start washing the hybridized preparations.

1. Fixation
   a. Embryos, larvae, and juveniles are fixed in freshly made 4% formaldehyde in Fixation buffer B at 4 °C overnight or for 1 h at room temperature in a 1.5 ml Eppendorf tube. (The formaldehyde fixative is made from 4% paraformaldehyde powder that is dissolved in the fixation buffer by heating.)
   b. Fixative is removed and embryos washed 3× for 10 min each in buffer B.
   c. Samples are dehydrated in an ethanol series up to 80%, 30 min each step.
      50% EtOH/50% H₂O
      70% EtOH/30% H₂O
      80% EtOH/20% H₂O
   d. Embryos in 80% EtOH can be stored for over a year at −20 °C.

2. Rehydration
   Transfer embryos into transparent 1.5 ml tube (Fukae Kasei Co., Ltd) and rehydrate through EtOH into PBST, 5 min for each wash.
   75% EtOH/25% H₂O
   50% EtOH/50% H₂O
   25% EtOH/75% H₂O
   3X 100% PBST

3. Proteinase K Treatment
   a. Incubate in a solution of Proteinase K in PBST at 37 °C.
      Embryos: 2 µg/ml for 30 min
      Tornaria larva: 2 µg/ml for 10 min
      Transforming larva: 5 µg/ml for 30 min

4. Refix in paraformaldehyde
   a. 1 h in 4% paraformaldehyde in PBST
   b. Rinse 3× for 5 min each in PBST.

5. Acetic anhydride treatment
   a. Incubate for 5 min in 0.1 M Triethanolamine and acetic anhydride (250 µl in 100 ml of 0.1 M Triethanolamine).
   b. Rinse 4× for 10 min each in PBST.
6. Prehybridization
   a. Wash embryos with equal parts of hybridization B solution and PBST for 10 min.
   b. Replace solution in each vial with approximately 1 ml of fresh hybridization buffer B and incubate at room temperature for 10 min.
   c. Replace with fresh hybridization buffer and incubate for 1 h at 42 °C for embryos and tornaria larvae and at 60 °C for metamorphosing larva and young juveniles.

7. Hybridization
   a. Replace hybridization solution with diluted probe between 0.1 μg/ml – 1 μg/ml in hybridization buffer.
   b. Hybridize overnight (16 h) at 42 °C for embryos and tornaria larvae and at 60 °C for metamorphosing larva and young juveniles in a shaking water bath.

8. Post-hybridization washing
   Post-hybridization washes at 42 °C for embryos and larvae, and 65 °C for metamorphosis larva
   a. 4× SSC, 50% FA, 0.1% Tween 20 for 20 min, 2×
   b. 2× SSC, 50% FA, 0.1% Tween 20 for 20 min, 2×
   c. Solution A at 37 °C for 10 min, 3×
   d. 20 μg/ml Rnase A in Solution A at 37 °C for 30 min.
   e. Solution A at 37 °C for 15 min.
   f. 1× SSC, 50% FA, 0.1% Tween 20 at 42 or 65 °C for 20 min, 4×
   g. 1× SSC, 50% FA, 0.1% Tween 20: PBST @ 3:1 at RT for 10 min
   h. 1× SSC, 50% FA, 0.1% Tween 20: PBST @ 1:1 at RT for 10 min
   i. 1× SSC, 50% FA, 0.1% Tween 20: PBST @ 1:3 at RT for 10 min
   j. 4× PBST at RT for 20 min.

9. Blocking
   a. 0.5% Roche blocking agent in PBT at 4 °C for 20 min.
   b. 0.5% Roche blocking agent in PBT at 4 °C overnight.

10. Antibody incubation
    a. For embryos and early larvae, place in 1/2000 anti-digoxigenin-alkaline phosphatase in 0.5% blocking reagent in PBST and incubate at RT for 1 to 1.5 h.
    b. For transforming larvae juvenile worms, dilute the preparation of alkaline phosphatase-coupled anti-digoxigenin antibody pre-absorbed with adult powder (see Section VII) 1/3000 with 0.5% blocking reagent in PBST. Incubate at 4 °C overnight.
11. Washes
   a. Wash in PBST for 15 to 20 min 6× at RT. (During the first washes for juveniles when adult powder has been used to block the antisera, be sure to adequately swirl the larvae to disperse and subsequently remove the mucus that forms around them.)
   b. 2× in AP buffer (pH 8.0).
   c. 2× in AP buffer (pH 9.5).

12. Chromogenic reaction
   a. Develop color reaction in NBT 0.9 μl/ml/x-phos 0.7 μl in 200 μl AP buffer (pH 9.5) for 15 min to several hours until the desired level of color is achieved.
   b. Stop reaction by washing in PBST several times for 5 min each. Postfix with 4% paraformaldehyde in PBT for 1 h at RT. Then wash 2 to 3 times with PBST for 5 to 10 min.

VI. Preparation of Blocking Reagent for P. flava

A. Background

Anti-digoxigenin antisera used to immunolocalize the hybrids tends to react nonspecifically to the older P. flava developmental stages. To reduce background in the reactions, we have resorted to the tactic of blocking such nonspecific reactions with extracts of P. flava tissue. Our approach follows closely the procedure developed for Amphioxus (Holland et al., 1996) of mixing an aqueous preparation from an acetone powder of animal tissue with the antisera before the incubation with the experimental preparations.

B. Protocol to Prepare Pre-Blocked Antisera

1. Preparation of acetone extracted tissue powder.
   a. Grind frozen adult tissue in a cold glass pestle with the smallest possible volume of PBS.
   b. Add 4 volumes ice-cold acetone.
   c. Incubate on ice for 30 min.
   d. Transfer to a centrifuge tube and centrifuge at 10,000g.
   e. Collect pellet and wash twice in acetone.
   f. Air dry in a mortar and grind further with the pestle.
   g. Store dry at minus 20 °C.

2. Preparation of pre-blocked antiserum
   a. Weigh out 1.5 mg hemichordate powder.
   b. Add 400 μl PBS/0.1% Triton x-100.
c. Heat at 70 °C for 30 min.
d. Add 50 μl of 20 mg/ml bovine serum albumen.
e. Add 50 μl pretreated sheep serum.
f. Add 0.5 μl anti-digoxigenein antibody.
g. Mix and incubate on shaker for 1 h or more at room temperature or overnight at 4°C.
h. Add 1 ml PBS containing 0.1% Triton x-100.
i. Add 2 mg/ml bovine serum albumen powder.
j. Add 50 μl sheep serum.
k. Store frozen at −20 °C in 200 μl aliquots.
l. This solution can safely be reused in a second reaction.

VII. Materials and Reagents

A. Solutions for Fixation and in Situ Hybridization

1. Magnesium seawater (MgSW): 3.5% MgCl₂ in filtered seawater
2. Fixation Buffer A
   - 0.1 M MOPS pH 7.5
   - 0.5 N NaCl
   - 2 mM EGTA pH 8.0
   - 1 mM MgCl₂
   - 1X PBS
3. PBST: 1XPBS, 0.1% Tween 20
4. Hybridization buffer A
   - Heparin 100 μg/ml
   - Denhardt’s solution 1X
   - CHAPS 0.1%
   - Tween 20 0.1%
   - 20X SSC 5X
   - EDTA 5 mM
   - Formamide 50%
   - yeast tRNA 1 mg/ml
   - Water to volume
   - Store at −20 °C
5. 100X Denhardts
   - 2% nuclease-free BSA
   - 2% Ficoll 400
   - 2% PVP-40
   - Supplier: Calbiochem

6. Hybridization buffer B
   - Denhardt’s solution 5X
   - SSC 6X
   - Tween 20 0.1%
   - yeast tRNA 100 μg/ml
   - formamide 50%
   - Water to volume
   - Store at −20 °C
Make up in DEPC-treated water and store in aliquots at −20 °C.

Maleic Acid Buffer (MAB) 5X Stock: 500 mM maleic acid; 750 mM NaCl; pH with solid NaOH to 7.5.

Alkaline phosphate buffer (AP buffer): 0.1 M Tris pH 9.5 or 8.0; 50 mM MgCl2; 0.1 M NaCl; 0.1% Tween 20.

Solution A: 0.5 M NaCl, 10 mM Tris (pH8.0), 5 mM EDTA, 0.1% Tween 20.

Bouins fixative: For 100 ml; 75 ml of saturated picric acid, 25 ml of 40% aqueous formaldehyde, 5 ml of glacial acetic acid. **Attention: Powdered picric acid is highly explosive.**

DTT, NaOH-FSW; Prepare 3.5 ml DTT-NaOH-FSW by adding to FSW a volume of 30 µl 1.0 M DTT (made fresh), swirled to mix, and then adding and immediately mixing 20 µl 1N NaOH to partially ionize the DTT and raise the pH to 8.

**B. Materials**

1. Corning Netwells® plates 74 µm mesh size; Fisher Scientific cat #07-200-211
2. BM purple AP Substrate®; Roche CAT #1442074
3. Anti-Digoxigenin FAB fragments; Roche #1 093 274
4. Anti-Fluorescein FAB fragments; Roche #1426338
5. Algal F/2 Algae Food (Fritz Industries, Dallas, TX) or Alga grow® (Carolina Biologicals)
6. BCIP; Roche #1 383 221
7. NBT; Roche #1 383 313
8. 1.5 ml tube; Fukae Kasei Co., Ltd., #131-415C
9. 10 ml Nissui Spitz tube; P. Nissui Pharmaceutical Co., Ltd., #302064700
10. 5 ml Komagome pipette; Iwaki Glass Co., Ltd., Tokyo, http://www.tech-jam.com/items/2051-1a.phtml
11. Acrodisc®, 0.2 µm; Gelman Sciences, Ann Arbor, MI
12. Triethanolamine; Sigma T-1377
13. Roche blocking reagent; Roche #10057177

**References**


Chapter 7, Fig. 2  (A) Adult Cliona intestinalis cultured in a 10-cm petri dish. (B) Typical "cage" used for holding C. intestinalis. (C) Cages used for holding Cliona savignyi.
Chapter 8, Fig. 1  Adult enteropneust hemichordates, *Saccoglossus kowalevskii* and *Ptychodera flava*. (A) Model of an acorn worm adult based on *S. kowalevskii* outlining some of the characteristic features of the adult morphology. (B) Adult *S. kowalevskii* female. White arrowheads indicate the position of the green ovary. Black scale bar = 3 cm. (C) Adult *S. kowalevskii* male. White arrowheads indicate the position of the orange/white testes. (D) High magnification of branchial region of metasome on *S. kowalevskii*. Lateral view. White arrowheads indicate the position of two of the multiple pairs of gill slits on the dorsal side of the worm. Scale bar = 0.5 cm. (E) Spawning female of *S. kowalevskii*. Oocytes are green/blue-colored and are spawned along the length of the gonad. (F) Adult *P. flava*. Black arrowhead indicates the position of the proboscis. White arrowheads indicate the position of the genital wings. Scale bar = 1 cm.
Chapter 8, Fig. 2 Embryological, larval, and juvenile stages of S. kowalevskii and P. flava. A through E reflect stages of S. kowalevskii development. F through I reflect stages of P. flava development. (A) Early cleavage stage within the vitelline envelope. Animal blastomeres are slightly smaller than vegetal ones. a = animal pole, v = vegetal pole. Scale bar = 100 \( \mu \)m. (B) Late blastula. (C) Post-gastrula with prominent telotroch at the posterior end of the embryo swimming within the vitelline envelope. (D) SEM micrograph of midstage embryonic development (day 4), lateral view. The tuft of cilia at the far left of the panel is the apical tuft. The ectoderm is evenly ciliated except for the dense telotroch at the posterior of the embryo. Scale bar = 75 \( \mu \)m. (E) Late juvenile 14 days old, lateral view. All three body regions clearly visible. White arrowhead indicates the position of the anus and, posterior to that, the ventral post-anal tail. White arrows indicate the position of the first two gill slits. Scale bar = 150 \( \mu \)m. (F) Lateral view, DIC micrograph of 3-day-old tornaria larva. White arrowhead indicates the larval mouth and black arrowhead, the anus. Scale bar = 50 \( \mu \)m. (G) Krohn stage larva. Black arrowheads indicate the folds, or epaulets, in the dorsal ciliated bands, and white arrowhead marks the telotroch. Scale bar = 300 \( \mu \)m. (H) Larva during transformation with clearly formed proboscis. The black arrowhead marks the position of the eyespots, and white arrowhead, the telotroch. Scale bar = 300 \( \mu \)m. (I) Juvenile worn one week after the initiation of transformation. White arrowhead indicates the band that persists after the cilia of the telotroch are shed upon settlement. Scale bar = 500 \( \mu \)m. P = prosome, M = mesosome, and Me = metasome.
Chapter 8, Fig. 3  Examples of patterns of expression in hemichordate embryos of genes implicated in specifying vertebrate structures. Whole mount in situ hybridization in *S. kowalevskii* and *P. flava*. A and B show *S. kowalevskii*, C and D show *P. flava*. (A) Retinal homeobox expression in the developing proboscis ectoderm of a 2-day-old early embryo. Optical frontal section of a cleared specimen. Scale bar = 150 μm. (B) Expression of BarH, a neural determining gene, in the mesosome ectoderm of a 3-day-old early embryo. Frontal optical section of a cleared embryo. (C) Expression of the forebrain gene, T-brain in a 3-day-old tornaria larva at the apical organ, as marked by a white arrowhead. Scale bar = 50 μm. (D) Expression of Brachury, a gene important in notochord development, in a 2-day-old tornaria larva. White arrowhead indicates the position of the mouth and black, the position of the anus.