Development of Marine Invertebrates

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Abstract

Marine invertebrates are used in developmental, cell, and evolutionary biology, and some critical biological phenomena have been found using these organisms. For example, one of the most important cell-cycle regulator proteins, cyclin, was found in a sea urchin (Evans et al., Cell 33:389–396, 1983); the cell-fate determinant, macho-1, was first identified in ascidians (Nishida and Sawada, Nature 409:724–729, 2001); and it was clearly demonstrated, using spiralians, that the acquisition of a novel gene set could produce new developmental processes (Morino et al., Nature Ecology & Evolution 1:1942–1949, 2017). Due to the easy accessibility of their habitats and easily obtained gametes, marine invertebrates have been used for science and education in marine biological stations worldwide. In this chapter, we summarize methods for the use of marine invertebrates to study developmental biology with a focus on sea urchins, ascidians, and gastropods.

10.1 Sea Urchins

Two species are widely used for developmental biology courses in marine stations in Japan. *Hemicentrotus pulcherrimus* (Fig. 10.1, left) breeds between December and April, and *Heliocidaris crassispina* (Fig. 10.1, right) breeds between June and September. Other species, such as *Temnopleurus hardwickii*, *Temnopleurus*

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Fig. 10.1 Commonly used sea urchins in developmental biology courses in Japanese marine stations. Left, *Hemicentrotus pulcherrimus*; right, *Heliocidaris crassispina*. Bar = 1.0 cm

reevesii, *Scaphechinus mirabilis,* and *Clypeaster japonicus,* are also used, each with different breeding seasons.

10.1.1 How to Collect Sea Urchins

Hemicentrotus pulcherrimus and *Heliocidaris crassispina* live in the intertidal and shallow subtidal zone, so students are able to collect the adult sea urchins by hand at low-tide time under supervision by marine station staff. If a particular number of sea urchins are required for the course, it is better to ask the staff to collect prior. Because some sea urchin species, including *Hemicentrotus pulcherrimus* and *Heliocidaris crassispina*, are protected under fishing rights, permission from the Japan Fisheries Cooperative is required.

10.1.2 How to Collect and Fertilize Gametes and Grow Embryos

The eggs and sperm of sea urchins are easily obtained in marine laboratory courses. In addition, the fertilization and development of embryos/larvae are easily observed under a microscope. The following protocol explains how to grow sea urchin embryos/larvae from eggs and sperm.

Methods

1. Remove Aristotle's lantern (mouth/teeth) and discard the body cavity fluid.
2. Set the adult upside down in a beaker filled with filtered sea water (FSW).
3. Pour 0.5 M KCl into the body cavity and wait for a few minutes.
   (a) If yellowish eggs are spawned, leave the adult in the beaker until the egg stream stops. Wash the eggs discarding the supernatant seawater and pouring clean seawater into the beaker, repeating three times. Store the eggs at 4 °C

for *Hemicentrotus pulcherrimus* and about 20–25 °C for *Heliocidaris crassispina* until use.

(b) If white sperm appear, transfer the adult to a 6–10 cm plastic or glass dish and wait until the dry sperm accumulates on the dish. Transfer the dry sperm to a 1.5 ml microtube using a pipette and store at 4 °C until use.

5. To fertilize the eggs, suspend the stored eggs in a beaker filled with FSW at a final concentration of less than 10% volume/volume. Add 1 ml of FSW-diluted sperm (0.2–0.5 µl dry sperm) to 100 ml of egg suspension, and wait for 10 min.
6. Discard the supernatant and add new FSW to wash out the extra sperm, repeating several times. Store the fertilized eggs at the appropriate temperature (15–18 °C for *Hemicentrotus pulcherrimus*, and 20–25 °C for *Heliocidaris crassispina*). Because too many eggs interfere with normal development, eggs should be settled as a single layer at the bottom of a glass beaker with plenty of FSW.
7. Observe and sketch the development of sea urchins under a microscope (ref. Fig. 10.2 for *Hemicentrotus pulcherrimus* development). The following are examples of observation and sketching points:
   - Compare the unfertilized and fertilized eggs.
   - When and how are the fertilized eggs divided?
   - Characterize the blastomeres.
   - When and how do the embryos start to move?
   - When and how do they hatch?
   - Characterize the mesenchyme cells.
   - Characterize the gut structure.
   - Characterize the spicules.

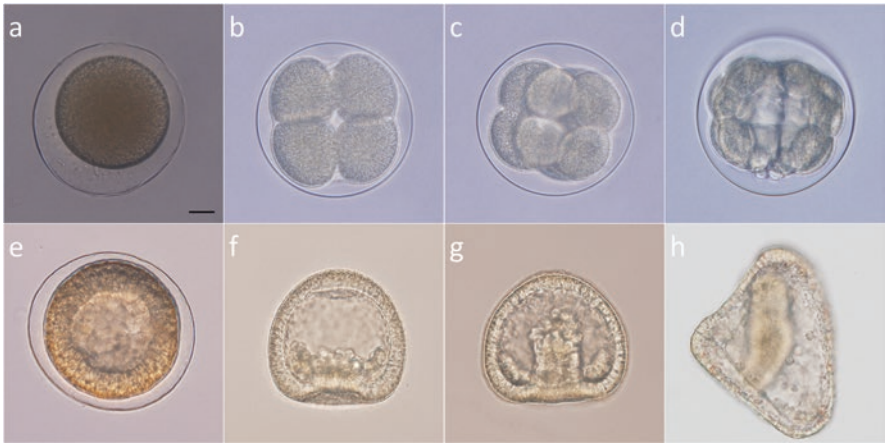

Fig. 10.2 Early development of *Hemicentrotus pulcherrimus*. (**a**) Fertilized egg, (**b**) 4-cell stage, (**c**) 8-cell stage, (**d**) 32-cell stage, (**e**) unhatched blastula, (**f**) early gastrula, and (**g**) prism larva. Bar = 20 µm

10.1.3 Experiment 1

Observation of the Jelly Coat and Jelly Canal

Unfertilized eggs of sea urchins are covered with a jelly coat, the main components of which are a fucose sulfate polysaccharide and a sialoprotein (SeGall and Lennarz 1979). The jelly coat is invisible under normal conditions because of its transparency. In addition, it is reported that there is a jelly canal at the animal pole of the eggs (Schroeder 1980). The following protocol is to visualize the jelly coat and jelly canal (modified from Maruyama et al. 1985).

Methods (Fig. 10.3)

1. Obtain eggs directly (dry eggs) from the gonopores of a 0.5 M KCl-treated adult female with a glass pipette, and transfer them onto a small glass/plastic dish.
2. Apply one drop of black ink (Bokuju in Japanese) around the eggs.
3. Gently pour FSW into the dish.
4. Observe the eggs under a microscope.

The following are examples of observation points:

- How thick is the jelly coat?
- How can you decide the location of the jelly canal on the egg?
- Without ink, you might realize the presence of jelly coat. Explain this.
- What happens to the jelly coat at fertilization?

10.1.4 Experiment 2

Fertilization Between Different Species

Unfertilized sea urchin eggs are activated by multiple stimuli. For example, the fertilization envelope is elevated with the intracellular introduction of FSW by

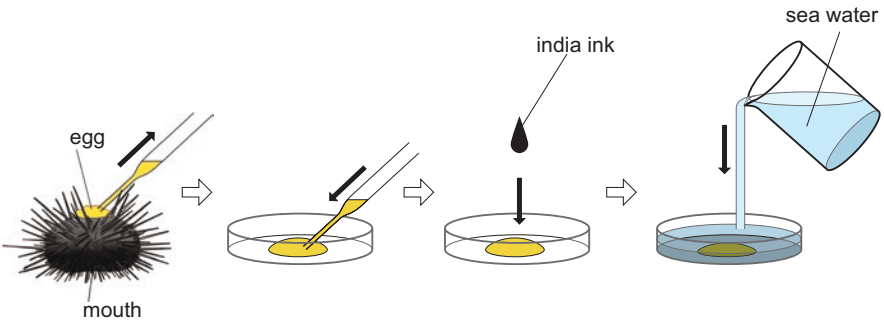

Fig. 10.3 How to visualize the jelly coat and canal. (**a**) Collect dry eggs with a pipette. (**b**) Transfer the eggs onto a glass/plastic dish. (**c**) Add a drop of black ink (Bokuju in Japanese). (**d**) Gently pour FSW into the dish, and observe the eggs under the microscope

scratching the cell membrane. An attack by sperm of different species could be an alternative. In addition, when several unknown conditions are in place, cleavage and development occur normally, resulting in swimming embryos being obtained. In fact, hybrid species are sometimes reported in both natural and experimental conditions (MacBride 1912; Brandhorst and Davenport 2001). The following protocol can be used to make a hybrid between different species.

Methods

1. Prepare dry sperm and eggs of species A (e.g., *Heliocidaris crassispina*) as described in Sect. 10.1.2.
2. Prepare dry sperm and eggs of species B (e.g., *Clypeaster japonicus*).
3. Fertilize egg A with sperm A (control) or B.
4. Fertilize egg B with sperm B (control) or A.
   Examples of experimental conditions:
   - Change the concentration of sperm or eggs.
   - Change the temperature of the FSW.
   - Change the pH of FSW.
   - Change the concentration of ions (e.g., Na^+, Ca^{2+}, or Mg^{2+}) in the FSW.
5. If a fertilization envelope is observed, pick up and transfer the fertilized eggs to a new dish or glass beaker filled with FSW.
6. Observe the development of the hybrids, compared to that of the controls.
   Examples of experimental conditions and observation points:
   - Change the temperature for culturing hybrids.
   - How does the cleavage occur?
   - Are there any differences in the morphology, gastrulation, mesenchyme cells, and spicule patterns between the hybrids and controls?

10.1.5 Experiment 3

Isolation of Blastomeres and Culturing the Embryoids

Although sea urchin embryos undergo regulative development, the cell fate of each blastomere is gradually restricted during development. To analyze the developmental potency of each blastomere, a number of researchers have performed blastomere-isolating experiments, in which they use finely pulled glass needle to detach each blastomere under a microscope. This technique is powerful but also requires plenty of experience. Therefore, not all people could succeed in this type of experiment. On the other hand, the following protocol, modified from the method described in Wikramanayake et al. (1995), is relatively easy for students on marine biology courses.

Methods (Fig. 10.4)

1. Fertilize the eggs with sperm under the conditions of 1 mM 3-amino-1,2,4 triazole (ATA) FSW to keep the fertilization envelope soft.
2. Collect 8-cell stage embryos in a 15 ml tube.

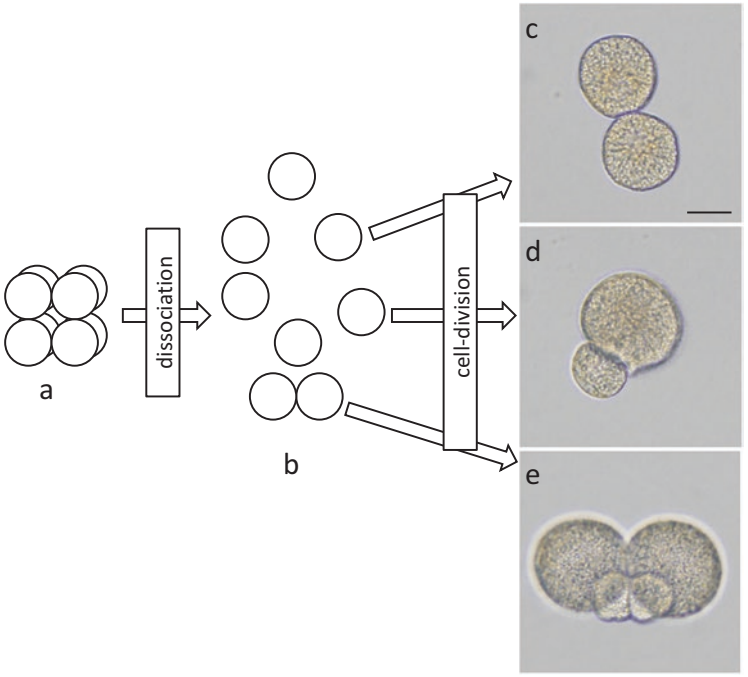

Fig. 10.4 Blastomere isolation. (**a**) Schematic image of the 8-cell stage. (**b**) Schematic image for the dissociated 8-cell-stage embryo into each blastomere(s). After one more cell division, the blastomere will be cleaved into a (**c**) mesomere pair and a (**d**) macromere and micromere pair. Embryoids derived from two or more blastomere sets are sometimes obtained. Bar = 20 μm

3. Remove the FSW, and wash eggs with $Ca^{2+}Mg^{2+}$-free SW twice.
4. Wait for about 5 min (depending on the batch).
5. Remove the $Ca^{2+}Mg^{2+}$-free SW and add 5 ml of Ca^{2+}-free SW and close the lid.
6. Shake the tube a few times by hand.
7. Check a part of the dissociated blastomeres under the microscope. If the dissociation is not enough, incubate the sample for another 5 min in Ca^{2+}-free SW and repeat shaking. If the cell membrane is broken, shorten the $Ca^{2+}Mg^{2+}$-free SW incubation time.
8. Transfer the dissociated blastomeres with Ca^{2+}-free SW to a 10 cm or 15 cm plastic dish filled with FSW. This dish should be coated with a thin layer of serum (e.g., lamb, bovine, goat, and horse) and dried before use.
9. Pick up the blastomeres with a micropipette and transfer to a FSW-filled 96-well plate coated with 0.5% agarose gel. It is recommended that the FSW contains 50 μg/ml kanamycin.
10. Wait until the occurrence of the next cleavage, judge the original tier of the blastomere(s), and label it on the well.
11. Let the embryoids grow for as long as possible and observe them under the microscope.

12. Discuss the cell-fate and developmental potency of each blastomere at the 8-cell stage.

10.2 Observation of Ascidian Embryogenesis

Tunicates are marine invertebrate chordates that are the closest living relatives of vertebrates (Dehal et al. 2002; Delsuc et al. 2006). As the phylogenetic position suggests, tunicates have the characteristics specific to chordates, such as a dorsally located central nervous system and tubular notochord (Fig. 10.5). Because all tunicate species found so far live in the ocean, the observation of tunicates provides students with the precious opportunity to understand the chordate body plan, its developmental mechanisms, and evolution.

Ascidians form the largest group in the tunicates, and collecting reproductively mature adults, isolating gametes, and performing in vitro fertilization are easy for many species. Thus, ascidians are frequently used as materials for developmental biology. Here, we introduce a basic program using ascidians in our marine course.

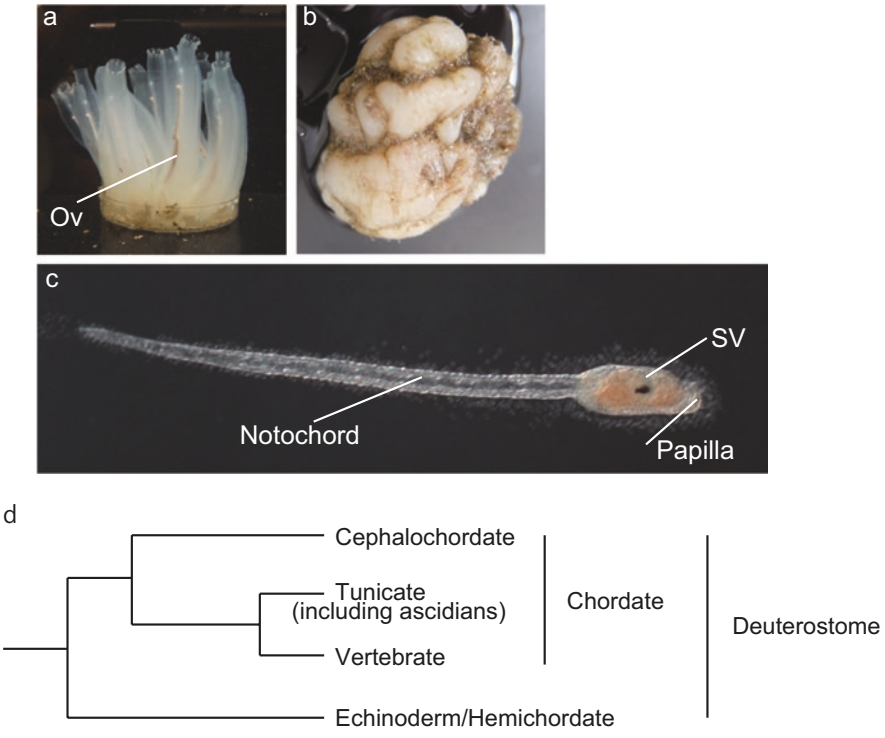

Fig. 10.5 Ascidians. (**a**) Adults of *Ciona intestinalis*. *Ov* oviduct. (**b**) An adult of *Styela plicata*. (**c**) A larva of *Ciona intestinalis*. *SV* sensory vesicle with otolith and ocellus. (**d**) The phylogenetic relationship of deuterostomes

The purpose of this program is for students to learn the body plan of chordates, mosaic model of cell-fate decision, morphogenetic movement, and metamorphosis; all of which are representative topics in developmental biology.

10.2.1 Observation of Myoplasm

In the mosaic model of ascidian embryogenesis, the cell fates of some tissues are determined by substances found in specific regions of eggs and early embryos (reviewed in Nishida 2005). The larval muscle is the representative tissue whose fate is determined by such a maternal factor. Fertilized ascidian eggs and cleaving embryos have a specific cytoplasm named myoplasm. Myoplasm is segregated into the blastomeres that are destined to be larval muscle (Conklin 1905).

The color of myoplasm is different among ascidian species. The myoplasm of *Styela plicata* exhibits a vivid yellow color and is easy to observe in live embryos (Fig. 10.6a). In Japan, *Styela plicata* is a common ascidian and its collection is easy in the summer. Therefore, we recommend using this ascidian for myoplasm observation. If it is difficult to collect this species, you can use alternative species, such

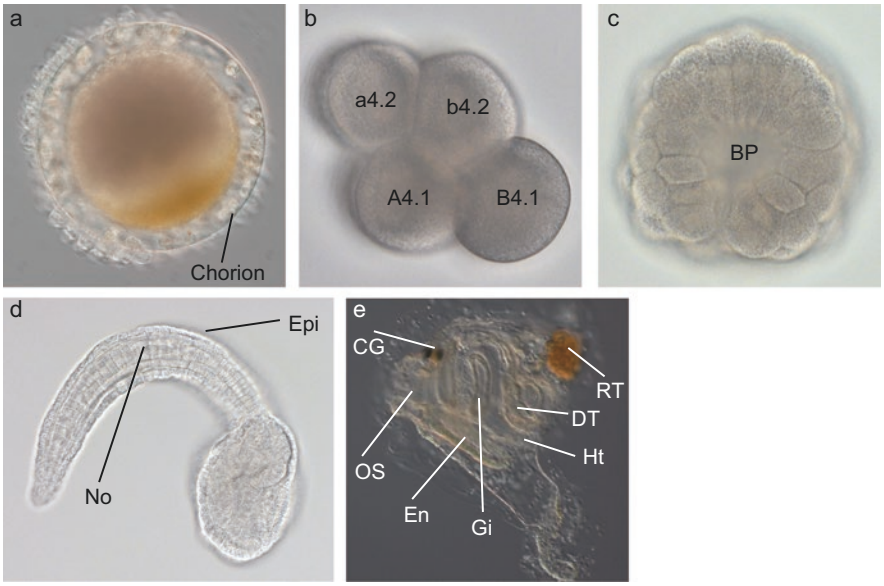

Fig. 10.6 Embryogenesis of ascidians. (**a**) A fertilized egg of *Styela plicata* with the chorion. (**b**) An 8-cell-stage embryo of *Ciona intestinalis*, lateral view. The names of the blastomeres are shown. B4.1 is a posteriorly located blastomere, some of whose daughter cells are differentiated into muscle. Note that the chorion was removed prior to photographing in (**b**–**d**). (**c**) A gastrula embryo. *BP* blastopore. (**d**) A tail bud embryo. *No* notochord, *Epi* epidermis. (**e**) A juvenile. *CG* cerebral ganglion, *DT* digestive tube, *En* endostyle, *Gi* gill, *Ht* heart, *OS* oral siphon, *RT* regressed tail

as *Styela clava* or *Boltenia villosa*. Here, we describe a method for the collection of gametes from *Styela plicata* adults.

Methods

1. Cut an adult along its midline.
2. Isolate the gonad and place it in a 9-cm plate filled with seawater. The gonad of *Styela plicata* can be recognized by the white color of the testis. The ovary is the brownish zones between the testes.
3. Leave for several tens of minutes. During the incubation, mature sperm will exude around the gonad. Collect dense sperm with a pipette and store on ice.
4. Both mature and immature eggs will be ejected around the gonad. Transfer the mature eggs onto another plate with seawater. Immature eggs have a large germinal vesicle at the center. Mature eggs are somewhat bigger than immature eggs and the germinal vesicle is invisible.
5. Mix the eggs and sperm to start fertilization. Ascidians are hermaphroditic and have self-sterility. It is thus usually better to mix sperm and eggs isolated from different individuals. However, surgically isolated *Styela plicata* eggs are frequently fertilized with self-sperm during isolation.
6. Observe myoplasm and its segregation pattern to specific blastomeres that are fated to differentiate into muscle.

10.2.2 Observation of Embryogenesis of *Ciona intestinalis*

The model ascidian species, *Ciona intestinalis*, is used worldwide as experimental material for molecular biology, and the delivery system of its wild-type animals has been established in some nations, including Japan (Sasakura et al. 2009). Because the system guarantees us to be able to obtain reproductively mature *Ciona* adults in a reproducible manner, we recommend using *Ciona* to observe ascidian embryogenesis.

Methods

1. Prepare two reproductively mature *Ciona* adults. The white and brownish lines are the spermiduct and oviduct, respectively (Fig. 10.5a). Cut the body along the oviduct with fine scissors while taking care not to injure the oviduct or spermiduct. Pierce the oviduct with the tip of some scissors and collect the eggs on a plate filled with seawater.
2. Repeat the procedure to collect the eggs from two animals. Before dealing with the other animals, make sure to wash your hands and instruments with tap water to inactivate any contaminated sperm. This is necessary to prevent untimely fertilization.
3. Collect sperm from the spermiduct with a pipette and preserve it on ice until use.
4. Divide eggs between a few 9-cm plates. Usually, one *Ciona* adult has too many eggs to culture on one plate. Reducing the number of eggs per plate will prevent embryogenesis from going wrong due to a high density of embryos.

5. Fertilize the eggs to start embryogenesis. Dilute the sperm with seawater to be somewhat cloudy. Add a few drops of diluted sperm to the plate with the unfertilized eggs. Immediately pipette the eggs to facilitate simultaneous fertilization. Record the time of insemination.
6. Check for the occurrence of fertilization. Unfertilized eggs are a perfect sphere. After fertilization, eggs change their shape due to the ooplasmic segregation (Sardet et al. 1989). After this event, fertilized eggs become oval and the shape change is a signature of fertilization. If the morphological change does not occur, repeat insemination with sperm from a different individual.
7. Observe under a microscope (Fig. 10.6b–d).
   Examples of observation points are as follows:
   - Shape of unfertilized eggs with chorion, follicle cells, and test cells;
   - Measure time of cleavages after fertilization to make a developmental table;
   - Shape of embryos at the cleaving stages;
   - Gastrulation;
   - Neurulation;
   - Tail bud formation.
8. Because *Ciona* larvae have representative characteristics specific to chordates (Fig. 10.5c), it is important to sketch the larva to understand the chordate body plan.
   The points of the sketch will be as follows:
   - The number of adhesive papillae.
   - Shape of otolith and ocellus.
   - Sensory vesicle.
   - Endoderm.
   - Muscle.
   - Primordia of the oral and atrial siphons.
9. Observation of juvenile (Fig. 10.6e). The transparency of *Ciona* juveniles, with functional organs, such as the endostyle, gill, heart, stomach, and digestive tube, provides students with an excellent chance to deepen their understanding of tissue/organ composition in adult ascidians. Ascidian hearts do not possess the cardiac valve and the direction of blood circulation changes periodically; this can also be observed. When using *Ciona* juveniles in a marine course, you may need to prepare them prior to the course; it takes at least 1 week for *Ciona* to reach the stage appropriate for observation.

10.2.3 Electroporation

Introduction of exogenous DNA into a living organism is used to examine gene functions, for labeling cells, and indicating tissue development and function. Generally, transgenic techniques require a long time and advanced skills, such as microinjection; these aspects make it difficult to carry out the experiments during a marine course. An important advantage of using *Ciona* is the ease of introducing exogenous DNA by electroporation (Corbo et al. 1997). Thus, *Ciona* is an ideal

animal to learn the methodology of transgenic techniques. Various methods of transgenic techniques in ascidians are available in Sasakura (2018).

Methods

1. Collect the unfertilized eggs and sperm as described above.
2. Pour 10 ml of dechorionation solution (1% sodium thioglycolate and 0.05% actinase E in seawater) into a 6-cm plate coated with gelatin. Then, add 300 μl of 2 M NaOH. Homogenize the liquid by pipetting.
3. Transfer unfertilized eggs onto the plate with the activated dechorionation solution. Gently mix the eggs while avoiding a bubbly appearance. Continue pipetting periodically.
4. Observe the eggs with a stereoscopic microscope. The chorion becomes yellowish in the solution. Dechorionated eggs are brownish smooth balls.
5. When most eggs are dechorionated, gather the dechorionated eggs by slowly rotating the plate. Then, quickly transfer the eggs into another gelatin-coated plate filled with seawater. Repeat this step once more.
6. Dissolve a few microliters of dry sperm into seawater to activate the sperm. Add a few milliliters of diluted sperm into the plate containing the dechorionated eggs.
7. Observe fertilization with a stereoscopic microscope. The eggs will rotate due to the collision with the sperm. If no action is observed add diluted sperm from another animal. The occurrence of fertilization can be recognized by the morphological change in the eggs. Leave for 15 min after insemination if fertilization successfully occurs.
8. Collect the fertilized eggs and wash them with 0.693 mM D-mannitol in seawater (Mannitol-SW) once. Use a 10 ml glass centrifuge tube and hand rotator for this purpose. After the eggs sink by gentle rotation, discard the Mannitol-SW and add an appropriate volume of fresh Mannitol-SW by calculating as follows: one electroporation requires eggs in 300 μl of Mannitol-SW.
9. Mix 30–60 μg of DNA (in 80 μl of 1× Tris-EDTA [TE] buffer) with 420 μl of Mannitol-SW. Add the DNA solution to a 4 mm cuvette. Then, add 300 μl of Mannitol-SW with eggs into the cuvette. Mix gently by pipetting several times and insert the cuvette into the shock pod. Push the start button to start pulsing. When using the Gene Pulser II (Bio-Rad, CA, USA), the recommended parameters of the pulse are 50 V and 20 ms.
10. Recover the eggs into a gelatin-coated plate filled with seawater. Exchange the seawater once to reduce the remaining DNA. Culture overnight at 18 °C.

When a fluorescent protein is used as the reporter gene in the electroporated plasmid, students can observe its expression in living embryos using a fluorescent microscope. When *lacZ* is used as the reporter gene, staining is necessary prior to observation (Fig. 10.7; Hikosaka et al. 1992).

Methods

1. Collect embryos in a 1.5 ml test tube. Add a 1/10 volume of formalin. After gentle mixing, incubate for 15 min.

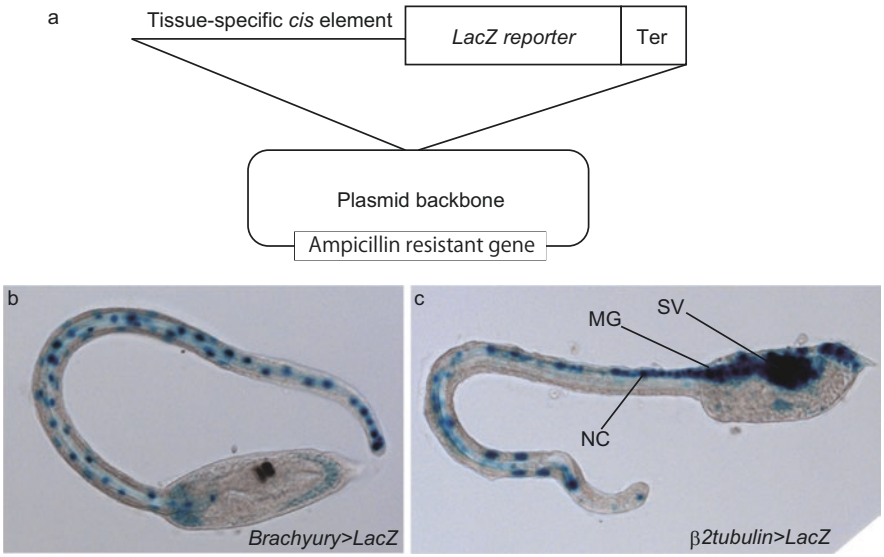

Fig. 10.7 Electroporation. (**a**) The schematic illustration of the reporter construct. Ter, transcription termination signal sequence. (**b**–**c**) Expression of *ß*-galactosidase in larvae into which a lacZ expression construct was electroporated at the 1-cell stage. The name of the construct is shown at the bottom of the panels. (**b**) *ß*-galactosidase is expressed in the notochord. (**c**) *ß*-galactosidase is expressed in the nervous system. *MG* motor ganglion, *NC* nerve cord, *SV* sensory vesicle

2. Wash embryos with phosphate buffered saline with 0.1% Tween 20 (PBST) twice. Usually, 200 µl is sufficient to wash the embryos. Spin down the embryos gently before removing the liquid. Carrying out the washing step under a stereoscopic microscope will prevent the loss of embryos.
3. Wash once with the staining solution (1 mM $MgCl_2$, 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide in PBST. Store at 4 °C before use).
4. Add 200 µl of staining solution, and then add 2 µl of X-gal (10 mg/ml).
5. Incubate for 30–60 min at 37 °C. If the signal is weak, extending the incubation time will yield a better result without increasing the background signal.

In our marine course, we use the reporter constructs expressing *lacZ* in the notochord, neural tissue, and muscle (Fig. 10.7). The constructs are available from the National BioResource Project in Japan (http://marinebio.nbrp.jp/ciona/).

10.3 Spiralian Development

The Spiralia are a superphylum of protostomes, characterized by stereotypical early development, such as spiral cleavage and conserved fate of blastomeres (Lambert 2010; Laumer et al. 2015). Many molluscan species have orthodox spiralian development. Here, we introduce a method of artificial fertilization and early

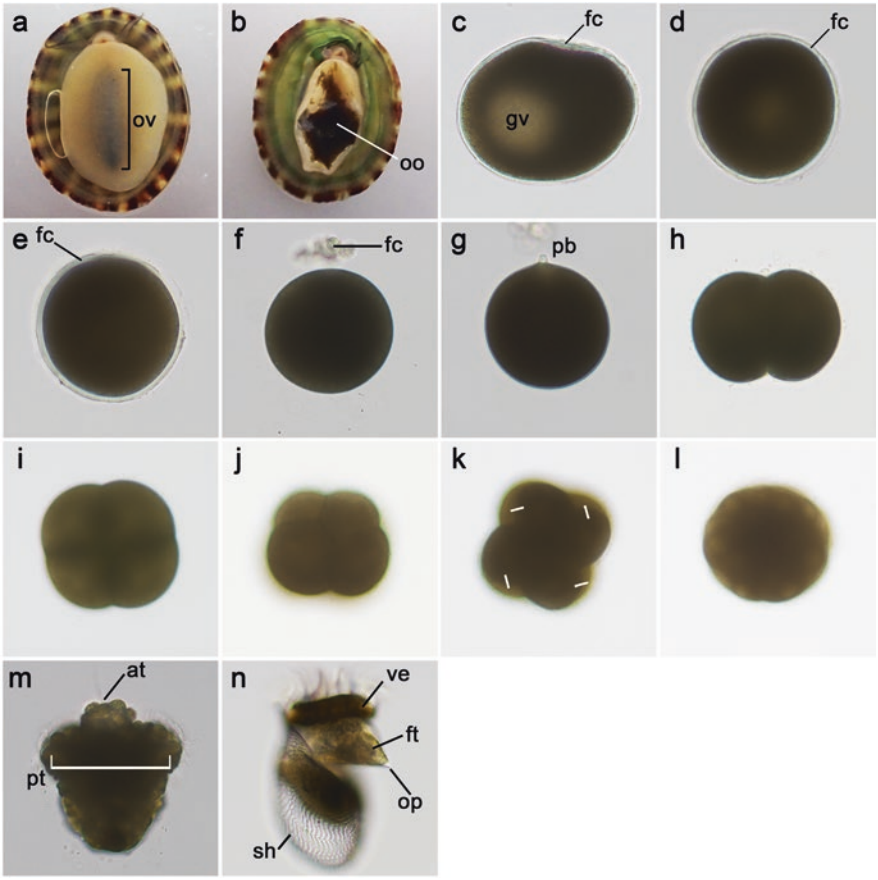

Fig. 10.8 Early development of *Nipponacmea fuscoviridis* (**a**, **b**) Adult female specimen of *Nipponacmea fuscoviridis* with mature ovary. *ov* ovary, *oo* oocytes. (**c**) Untreated, immature egg. *fl* follicle cells, *gv* germinal vesicle. (**d**) Egg 10 min after ammonia treatment. The eggs become spherical and the germinal vesicle fades. (**e**) Egg 30 min after treatment. The germinal vesicle has broken down and is invisible. (**f**) Egg 50 min after treatment. The follicular cells have disappeared. (**g**) Egg 30 min after fertilization. The first polar body is released. pb, polar body. (**h**) The 2-cell stage, 1 h post-fertilization (hpf). (**i**) The 4-cell stage, 1 h and 20 min post-fertilization (mpf). (**j**) The 8-cell stage, 1 h and 45 mpf, lateral views with the animal pole uppermost. The animal blastomeres are smaller than the vegetal blastomeres. (**k**) The 8-cell stage; view from the animal pole. The shifted arrangement of the animal and vegetal blastomeres is caused by the oblique direction of cleavage. (**l**) The morula stage, 3 hpf, lateral view. (**m**) Early trochophore larva, 5.5 hpf. *at* apical tuft, *pt* prototroch. (**n**) Veliger larva, 24 hpf. *ve* velum, *ft* foot, *op* operculum, *sh* larval shell

development for the limpet *Nipponacmea fuscoviridis* (phylum Mollusca, class Gastropoda), which inhabit the intertidal zone of rocky shores in Japan (Fig. 10.8a). *Nipponacmea fuscoviridis* is useful for observing the early development of spiralians as they are common on Japanese coasts and it is easy to culture adult specimens in laboratory aquaria (Deguchi 2007). Unlike many spiralian species, which

have a limited reproductive season, *Nipponacmea fuscoviridis* can be fertilized artificially for more than half of the year. The artificial fertilization procedure for this species is established and simple (Deguchi 2007) and the speed of development is rapid; therefore, it is possible to observe development within a short period of time.

10.3.1 Obtaining Gametes

The gonads ripen several times per year. For example, specimens collected from Hiraiso shore (Ibaraki, Japan) in January–February, June–July, and October–November, 2018 had mature gonads. From the ventral side, the mature ovary appears black or dark green (Fig. 10.8a), and the testes look yellowish white.

Methods

1. Cut the midline of the body of a female specimen to obtain oocytes (Fig. 10.8b) and place them in 10 ml of ASW. These immature oocytes remain in the first meiotic metaphase. Immature eggs are not spherical, surrounded by follicular cells, and the germinal vesicle can be seen (Fig. 10.8c).
2. To obtain mature eggs, add 200 µl of 250 mM NH_4Cl and 500 mM Tris (pH 9.0) to ASW containing the oocytes (final concentration: 5 mM NH_4Cl and 10 mM Tris) and incubate for 10 min (Deguchi 2007).
3. Wash the oocytes three times in ASW.

The ammonia treatment induces egg maturation; the eggs become round, the germinal vesicle breaks down, and the follicular cells detach (Fig. 10.8d–f). The maturation process occurs rapidly, so the eggs should be observed immediately after starting the ammonia treatment. Most eggs become suitable for fertilization after 40–60 min of ammonia treatment at room temperature (20–25 °C).

Examples of observation points are as follows:

- Transition of egg shape;
- Follicular cells;
- Germinal vesicle breakdown (GVBD).

10.3.2 Fertilization

Methods

1. Cut the midline of the male body and dilute the sperm in ASW. Untreated sperm do not move actively.
2. To activate the sperm, add a few drops of 250 mM NH_4Cl and 500 mM Tris (pH 9.0) to ASW containing the sperm. The sperm will start swimming rapidly after this treatment.
3. Mix the activated sperm and eggs to fertilize the eggs. Incubate for 10 min.
4. Wash the fertilized eggs three times in ASW to remove the remaining sperm.

Development proceeds normally about 20–25 °C. We usually culture the embryos in ASW at 22 °C.

An example of an observation points is as follows:

- Comparison of sperm movement before and after activation by ammonia.

10.3.3 Early Development

The first polar body is released about 30 min post-fertilization (mpf) (Fig. 10.8g). The first cleavage occurs at about 1 h post-fertilization (hpf) (Fig. 10.8h). After the first cleavage, a new cleavage occurs every 20–25 min (Fig. 10.8h–k). In the 8-cell stage, two general features of spiralian development can be observed: (1) the animal daughter blastomeres are smaller than the vegetal daughter blastomeres (Fig. 10.8j) and (2) cleavage proceeds at an oblique angle relative to the animal–vegetal axis (Fig. 10.8j, k). The embryos reach the morula stage at about 3 hpf (Fig. 10.8l). At 5–6 hpf, the embryos start swimming with cilia cells on the prototroch (Fig. 10.8m). The larval shell forms on the dorsal side at 12–14 hpf. Maintaining a low density (<10 specimens/ml) decreases the failure rate of normal shell formation. The shells of veliger larvae cover their posterior bodies (Fig. 10.8n). Although adult limpets lack an operculum, the veliger larvae have an operculum on the foot (Fig. 10.8n).

Examples of observation points are as follows:

- Polar bodies;
- First and second cleavage;
- Spiral and unequal cleavage at the 8-cell stage;
- Start of swimming;
- The larval shell and velum at the veliger larval stage;
- Presence of the operculum.

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