



Development of Marine Fish: Several Procedures for the Observation of Embryonic Development

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Abstract

The technologies of developmental biology have been improving over the past 30 years. Full genome sequencing enables between species comparisons, and genomics have revealed continuous genetic relationships between species. Genetic manipulation, such as modification of specific genes and genetic improvements, has been getting easier by gene editing technology, if fresh fertilized fish eggs are prepared and cultured. On the other hand, micro-manipulation, such as micro-operation of embryos, also opens a new possibility of surrogate production. Gametes have been induced through germ-line chimera induced artificially by germ-cell transplantation between different species. In this context, the intercellular interaction between species with different genomes is an important subject to be analyzed. In this section, general methods are described for artificial fertilization and cultivation in teleosts, and a few methods useful for experimental embryology are introduced. Then, observation procedures and development are described in several species. Finally, some experiments are introduced to study embryonic mechanisms in freshwater species. These experiments may be possible in other species if chorion can be removed at the early embryonic stage.

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11.1 Introduction

The technologies of developmental biology have been improving over the past 30 years. Particularly, full genome sequencing enables between species comparisons, and genomics have revealed continuous genetic relationships between species. In teleosts, full genome sequences have been performed in several species, such as pufferfish (*Fugu rubripes*), rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Danio rerio*), and medaka (or Japanese rice fish, *Oryzias latipes*). Genetic information from these organisms is useful for analyzing the genetic relationships with other species.

In teleosts, germ-line chimerism has been induced by germ-cell transplantation between different species. By using this technique, it is possible to develop breeding methods, such as genetic improvement and cellular selection techniques. In surrogate propagation, transplanted donor germ cells are required to differentiate the gametes in the host gonadal environments. Therefore, the intercellular interaction between germ cells and somatic cells is an important subject to be analyzed between species with different genomes. It is important to elucidate the mechanism(s) of embryonic induction during morphogenesis.

Fertilized eggs have holoblastic cleavage in some ancient teleost species, such as *Polypterus* spp. and sturgeon, while almost all other teleosts have discoidal cleavage during the early stages of development. The embryonic development of holoblastic species is very similar to that of amphibians. The embryonic development of discoidal species is different to that of reptilian and avian species. In addition, discoidal eggs show two types of yolk distribution: a single yolk mass and yolk granules in a single egg. The yolk in the former type is enclosed with a limited membrane and separated from the egg cytoplasm. In the latter type, yolk granules, which are also enclosed with a limiting membrane, are scattered in the egg cytoplasm. This difference in yolk distribution affects the cleavage pattern of development, especially the formation of the yolk syncytial layer (YSL), described later in this chapter. After fertilization, the egg cytoplasm moves to the animal pole side, while the yolk granules move to the vegetal pole, resulting in the blastodisc with egg cytoplasm being formed around the animal pole.

The embryonic development of discoidal eggs is subdivided into the following periods in zebrafish: cleavage, blastula, gastrula, segmentation, pharyngula, and hatching. During these periods, epiboly, the movement and spreading out of blastoderm cells over the yolk, occurs. As egg sizes vary between species, the differentiation of organs depends on the period, not on the percentage of epiboly. The order of normal organogenesis is similar in the normal development of teleosts, but the speed is variable because of the egg size and environmental conditions, such as temperature and oxygen concentration. Therefore, observing the detailed staging of embryonic development with student experiments at marine stations is difficult. The comparative analysis of different stage embryos is effective to understand the differentiation of tissues and/or organs.

Synchronous cleavages occur throughout the blastoderm, then change to asynchronous cleavages at the mid-blastula transition. Three cell lineages, namely the enveloping layer (EVL), deep cell layer (DCL), and yolk syncytial layer (YSL), are

established around this stage. The EVL is an extra-embryonic epithelial monolayer covering the blastoderm. The DCL is the origin of the embryonic body itself. The YSL is thought to be the organizing center, inducing the mesoderm and endoderm cells in the teleost blastoderm. The YSL and underlying yolk are collectively called the yolk cell. Without the yolk cell, the blastoderm could not differentiate the normal embryonic body with the central nervous system. The establishment of the YSL during the early cleavage stage is different between eggs with one single yolk and those with many yolk granules. Observation is monotonous during the cleavage stage, but it is important in the differentiation of the three germ layers to partition the maternal cytoplasm with its determinants.

The blastoderm, with the EVL, DCL, and YSL, begins to spread out of yolk, and forms the germ ring and embryonic shield, called the gastrula. DCL cells around the marginal part internalize under the peripheral part of the blastoderm and form a thickened germ ring. The mesoderm and endoderm cells are internalized, and the remaining upper cells are ectoderm cells. One part of the internalized cells moves toward the animal pole, forming the embryonic shield. The embryonic shield marks the dorsal side of the embryo. The side of the animal pole is anterior and the marginal part of the embryonic shield is posterior. The dorsal/ventral, anterior/posterior, and right/left axes are clearly distinguishable after the appearance of the embryonic shield. Thereafter, the shield cells move toward the animal pole, and the upper ectoderm cells and internalized mesoderm and endoderm cells move dorsally and form the embryonic body. The dorsal part of the embryonic body, the ectoderm, is called the epiblast, and the ventral part, the mesoderm and endoderm, is called the hypoblast. The central part of the hypoblast, the axial mesoderm, differentiates the notochord along the anteroposterior axis.

Somites differentiate from the paraxial part of the hypoblast and increase in number from the anterior to posterior. This process is called somitogenesis. The number of somites is an indicator of the developmental staging during somitogenesis. The skeletal muscle, dermis, and spine are the main derivatives of somite. The differentiation of muscle is observable, but that of the latter two is difficult to distinguish from the outside. The differentiation of muscle is distinguishable by comparison between the anterior and posterior somites during somitogenesis, because of the striped pattern of the muscle fiber. The embryo begins to become motile after muscle differentiation.

Simultaneously, in the epiblast, the brain and sensory organs begin to differentiate during the somitogenesis stage. The brain first divides into three parts: the forebrain, midbrain, and hindbrain, and following this, the spinal cord is differentiated. Thereafter, the telencephalon and diencephalon, mesencephalon, and rhombencephalon and myelencephalon differentiate from the forebrain, midbrain, and hindbrain, respectively. Morphological observation of these brain derivatives is easy at the early stage but becomes difficult with the expansion of the brain ventricle. Morphogenesis of the eye and ear, the sensory organs, is fascinating. The eyes are originated from a horizontal extrusion of the forebrain, called the optic vesicle. The optic vesicle consists of two cell walls, and the cavity between the walls links the brain ventricle. The outer wall of the optic vesicle becomes thickened and invaginated, converting into an

optic cup. The lens originates from the thickening of the epithelium over the optic vesicle and falls into the cup. The outer and inner walls of the optic cup differentiate the sensory and pigmented retinas, respectively. The inner ear originates from the thickening of the sensory layer of the ectoderm, known as the otic placode. The placode cells invaginate and then form a vesicle. Thereafter, the ventral part of the vesicle thickens and forms a sensory layer, and an otolith located on it. Three semi-circular canals differentiate around the dorsal part of the vesicle. These processes can be observed from outside using a binocular microscope.

The establishment of the cardiovascular system is a very impressive period of development, but the initiation of the heartbeat and blood flow around the pharynx is difficult to detect from outside using a binocular microscope. The heartbeat starts just ventrally to the pharynx. Blood cells begin to differentiate around several parts of body, namely the central mesoderm and on the yolk sac.

The hatching stage is different among species. Embryos of many pelagic species, such as flatfish, hatch before the opening of the mouth and anus, while those of some demersal species, such as salmonids, hatch after the establishment of some juvenile characters. The timing of hatching is affected by environmental conditions, such as temperature and high density of eggs, even in the same batch of the same species.

During embryogenesis, germ-line cells differentiate from the blastomeres with maternal germplasm in teleosts. The germplasm is distributed throughout the zygote cytoplasm, colonized on the cleavage furrows at the early cleavage stages. Founders of germ cells, primordial germ cells (PGCs), are destined from the blastomeres that inherited germplasm and are established around the blastula stage. PGCs migrate from their original appearance position to the final gonadal region. It is impossible to observe the germplasm and PGCs during embryogenesis using a normal or binocular microscope. Recently, however, it has been possible to visualize these with GFP fluorescence by injecting artificial mRNA into the cytoplasm at the early cleavage stages. Visualized PGCs show fascinating movement, extrusion of filopodia and lamellipodia, under a fluorescent microscope during migration.

In this section, we describe the general method for artificial fertilization and cultivation, at first, and introduce a few methods useful for experimental embryology. Then, we describe the observation procedures and development of anchovy, herring, smelt fish, and ice goby, as some examples. Finally, we show some experiments to study embryonic mechanisms in freshwater species, such as zebrafish and goldfish. These experiments may be carried out on other species with demersal eggs whose chorions are removable at the early embryonic stage.

11.2 General Method

11.2.1 Parent Fish and Gametes

In the spawning season, gametes are obtained from mature fish captured at spawning grounds or cultured at fishery experimental stations. As egg properties, such as transparency, adhesion, ocean zone (pelagic/demersal), isolation/aggregation,

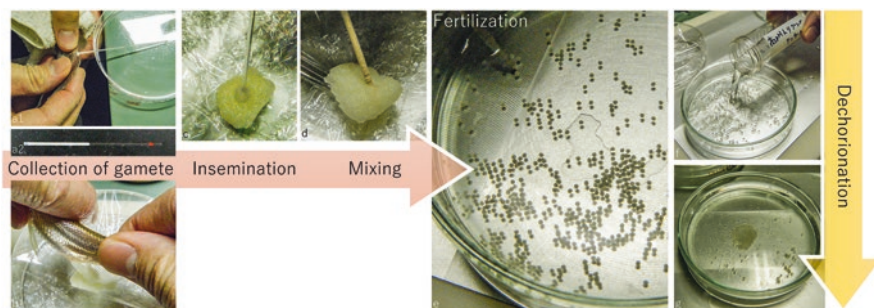


Fig. 11.1 Flowchart of the fertilization (a–e) and dechoriation (f, g) of smelt fish. (a1) Collection of sperm from mature male into a microcapillary tube. (a2) Collected sperm. (b) Squeezed eggs on plastic wrap. (c) Insemination of sperm into the eggs. (d) Gentle mixing with a tooth pick. (e) Fertilization of eggs in the environmental water. (f) Treatment with enzyme solution. (g) Denuded eggs without chorions

and so on, are highly diverse, the choice of species is also important to observe morphogenesis.

Eggs are squeezed from mature females onto a sheet or into a vessel without contamination with environmental water, which activates the development (Fig. 11.1). Sperm are collected into a capillary tube or plastic cup, also without contamination. Generally, artificial fertilization is performed as follows: eggs inseminated with sperm are scattered into environmental water (seawater or freshwater, depending on the spawning habitat). After fertilization, additional washing with environmental water is recommended in order to remove excess sperm.

During cultivation, the daily removal of dead embryos and changing of water should be performed in order to prevent the deterioration of water quality. In the case of pelagic eggs, adding a small amount of salt to the culture medium makes the medium exchange easier, because of the floatation of surviving embryos. The more seawater allowed for cultivation, the higher the embryo survival rate. In the case of adhesive eggs, as the removal of dead eggs is laborious, a low-density cultivation is recommended. In both cases, as the hatching stage is subject to mass mortality, embryos should be cultivated in a large vessel. When observing the floating eggs with a microscope, it is difficult to observe from the side because of the oil droplet in the yolk. Better results may be obtained by incubating the embryos in Ringer's solution (see Goto et al. 2019).

11.2.2 Softening and Dechoriation

The chorions of several species are removable by hand and/or enzymatic procedures. In eggs without chorions, detailed observation of morphogenesis and organogenesis is easy. Embryonic manipulation, such as blastoderm and blastomere transplantation, is also available in denuded embryos. In addition to this, you can try to inject some artificial material, such as a fluorescent reagent,

artificial mRNA, or gRNA for genome editing, into the egg cytoplasm, if chorion softening and/or dechoriation are performed. Some methods for chorion softening and dechoriation are described for example species in following subsections. In closely related species, with the exception of those with floating eggs such as anchovy, the chorion may be removable using a similar treatment with slight modifications. We also summarize modifications to the dechoriation and/or chorion softening methods (Tables 11.1) and the developmental schedule (Table 11.2) for several fish species.

11.2.3 Microinjection

We have already described the procedures for microinjection for a few types of eggs, including the preparation of the injection needle, operation of manipulator, and treatment of fertilized eggs (Goto et al. 2019). Please refer to the relevant description for microinjection. Microinjection enables us to label some blastomeres or specific cells, such as PGCs. For tracing cell lineage, FITC-dextran is available, according to Kimmel and Low (1985). Five percent of FITC-dextran solution in 0.2 M KCl should be injected into one of the upper tiers of the blastomeres that are isolated. For histological tracing, a mixed solution of FITC-dextran and biotin-dextran-lysine is useful. Labeled cells are stained on

Table 11.1 Handling procedures of chorion for micro-injection and micro-surgery

		References
1. Injection after removal		
Gobius fish, medaka	Manual removal	Saito et al. (2006), Iwamatsu (1988)
Zebrafish, loach, dace	Trypsin pH 7	Saito et al. (2006, 2010)
Goldfish, common carp	0.1% trypsin and 0.4% urea pH 7	Yamaha et al. (1986), Ito et al. (1999)
Medaka	Hatching enzyme	Sakai (1961)
Smelt fish	0.1% trypsin after high pH treatment	Takahashi et al. (2016)
2. Injection after softening or after inhibition of hardening		
Herring	Softening by 0.1% trypsin and 1% urea pH 7	Saito et al. (2006)
Salmonid species	Inhibition of hardening by glutathion reduced	Yoshizaki et al. (1989)
3. Direct injection without treatment just after fertilization		
Eel, barfin flounder, anchovy	Direct injection through chorion	Saito et al. (2011), Goto et al. (2015)
Salmonids species	Direct injection through micropyle	Guyomard et al. (1989)
4. Difficult		
Brown sole	Too small	
Greenling	Tough adhesive materials	

Table 11.2 Rough developmental schedule of each fish species described in this chapter

Stage name	Time	Pond smelt	Ice goby (19°C)	Goldfish	J. anchovy ^a	Zebrafish	Characteristics (mainly referred from pond smelt)
Species	Herring						
Temperature	8–10 °C	10 °C	19 °C	20 °C	24 °C	28.5 °C	
<i>Cleavage period</i>							
Fertilization							Attach to matrix by outer layered adhesive membrane, forming perivitelline space
1-cell	2 h	2 h 30 min		40 min	0 min		Formation of the blastodisc on the animal pole
2-cells	3 h 40 min	5 h	45 min	1 h	24 min	45 min	Formation of the first cleavage furrow
4-cells		6 h 30 min	1 h 35 min	1 h 30 min	51 min	1 h	Blastodisc formed by 2 × 2 array blastomeres
8-cells		8 h	2 h 30 min	2 h 10 min	1 h 18 min	1 h 15 min	Blastodisc formed by 2 × 4 array blastomeres
16-cells		9 h 30 min	3 h 30 min	2 h 40 min	1 h 45 min	1 h 30 min	The fourth cleavage plane divided the monolayered blastomeres horizontally
32-cells		11 h	4 h 40 min	3 h 10 min	2 h 12 min	1 h 45 min	
64-cells		12 h 30 min	5 h 55 min	3 h 50 min	2 h 39 min	2 h	The number of blastomeres became uncountable by external appearance
Early blastula period							
128-cells		14 h		4 h 20 min	3 h 6 min	1 h 45 min	
256-cells		15 h 30 min		4 h 50 min	3 h 33 min	2 h 30 min	

(continued)

Embryonic body	3 days	1 day 3 h	20 h	12 h	10 h	Completion of the blastoderm covering on the yolk cell, embryonic body was formed
Bud	3 days 12 h	1 day 6 h				Embryonic body expand rapidly by drastic conversion and extension movement
<i>Segmentation period</i>						
5-somite	2 days 12 h	4 days	1 days 2 h	14 h	11 h 40 min	Beginning of somitogenesis, optic vesicle formation
9-somite	2 days 17 h	4 days 12 h	2 days 4 h	16 h	16 h	Kupffer's vesicle formation, beginning of optic cup formation
15-somite	3 days	5 days	2 days 11 h	20 h	14 h	Optic cup and lens placode formation, otic placode formation started
22-somite		5 days 12 h	3 days 8 h			
28-somite	3 days 13 h	6 days	4 days 12 h		17 h 30 min	Lens formation
34-somite		6 days 12 h				Beginning of eye pigmentation, extinction of Kupffer's vesicle
39-somite		7 days	5 days	2 days		Otoliths and nasal placode formation, tail part began to bend to right side of the body, mobility
44-somite		7 days 12 h				Actively moved tail part
50-somite	5 days 13 h	8 days	6 days	3 days	21 h 30 min	Pectoral fins formation, beginning of heart beating

(continued)

Table 11.2 (continued)

Stage name	Time	Pond smelt	Ice goby (19°C)	Goldfish			Characteristics (mainly referred from pond smelt)
Species	Herring					J. anchovy ^a	Zebrafish
Temperature	8–10 °C	10 °C	19 °C	20 °C	24 °C	24 °C	28.5 °C
54-somite		8 days 12 h					Beginning of caudal fin fold and intestinal tract formation
58-somite		9 days					Beginning of median fin fold formation
62-somite		9 days 12 h					Completion of somitogenesis, lumen of intestinal tract became observable from outside
<i>Pharyngula period</i>							
		10 days					Beginning of hatching glands formation at the middle region of dorsal body trunk and pigmentation at lower part of intestinal tract
		11 days					A lot of hatching glands appeared at dorsal body trunk
		12 days					Semicircular canals formation, anterior part of gut's lumen became large
		13 days					Many pigments were formed at the lower part of gut tract, liver could be observed from outside, formation of lateral line, tail part became straight shape
	11 days	15 days	8 days				Mouth opening and gill arch formation
		16 days					Weak peristaltic motion was observed in part of intestinal tract, hatching glands with reflective granules
		18 days					Open anus, beginning of hatching and swimming
<i>Hatching period</i>							
	11 days 6 h	20 days 12 h	13 days	5 days	27 h 40 min	2 days	More than half of embryos cultivated with chorion hatched

^aFertilized eggs of Japanese anchovy were obtained in the tank by natural spawning at around 35 min before embryo reached one cell stage

the histological sections by histochemical procedures, using an avidin-peroxidase complex. To visualize the germplasm and PGCs, artificially synthesized mRNA (*bucky ball*-GFP and *GFP-nos3* 3'UTR, respectively) should be injected, according to Saito et al. (2006, 2014).

The actual process of the injection is as follows. First, place the dish with eggs onto the stage of a stereomicroscope and position the tip of the injection needle. The needle tip should be leveled to the top end of the eggs. Then, apply small pressure with the microinjector to let the needle leak a small amount of the solution into the seawater. The operations of microinjection are very simple. Just move the egg to the center of the microscopic view and insert the needle into it. Move the tip of the needle back and forth using a micromanipulator. Repeat these steps until you obtain enough injected embryos. To identify the injected embryos, you may want to add tracer dye, such as FITC-dextran or rhodamine-dextran, into the solution. If the injector's pressure is properly applied to the needle, the leaking solution can be injected into the egg. Remove the needle from the egg when the diameter of the injected solution reaches 1/10–1/5 that of the egg. When the internal pressure of the egg is too high to inject the solution, we recommend using an injection needle with 'break', as described by Goto et al. (2019). You can see the process in barfin flounder (Fig. 11.2) and anchovy (Fig. 11.3a). There is also a movie showing the actual process of microinjection in goldfish (Movie 11.1).

11.3 Experimental Procedures of Eggs and Embryos in Several Species

11.3.1 Japanese Anchovy (Floating Pelagic Eggs)

Japanese anchovy, *Engraulis japonicus*, is a marine pelagic fish that inhabits the north western and central Pacific, including the coastal areas of Japan. The reproductive characteristics of the Japanese anchovy differ, depending on the distribution area and are correlated with temperature and condition factor (Funamoto et al. 2004; Takasuka et al. 2005). The size at first maturity is estimated to be around 7 cm, and most fish mature at around 9 cm in body length (Funamoto and Wada 2004). Under captive conditions, sexual maturation occurs within 3–4 months at 22–28 °C (Yoneda et al. 2015). Although the reproductive characteristics of Japanese anchovy vary among stocks or rearing conditions, fertilized eggs can be reliably obtained at certain times, usually a few hours after dark, when fish are reared at a temperature of 20–26 °C (Yoneda et al. 2013; Pandey et al. 2017a, b). According to Yoneda et al. (2013), the range of ovulation cycle in each female varies from two to more days, depending on sea water temperature and nutritional condition. In our observations, during active spawning season, the cycle becomes shorter; the females likely spawn 3 or 4 days. The control of the full life-cycle of the Japanese anchovy is relatively easy compare to that of other marine species; therefore, this species is a suitable marine fish for developmental studies.

Adult fish can be maintained in a circular tank, with a recommendation of 100 fish/1000 L. A tank larger than 1000 L is preferable since fish can damage themselves by swimming into the walls of the tank. To maintain spawning adults, fish

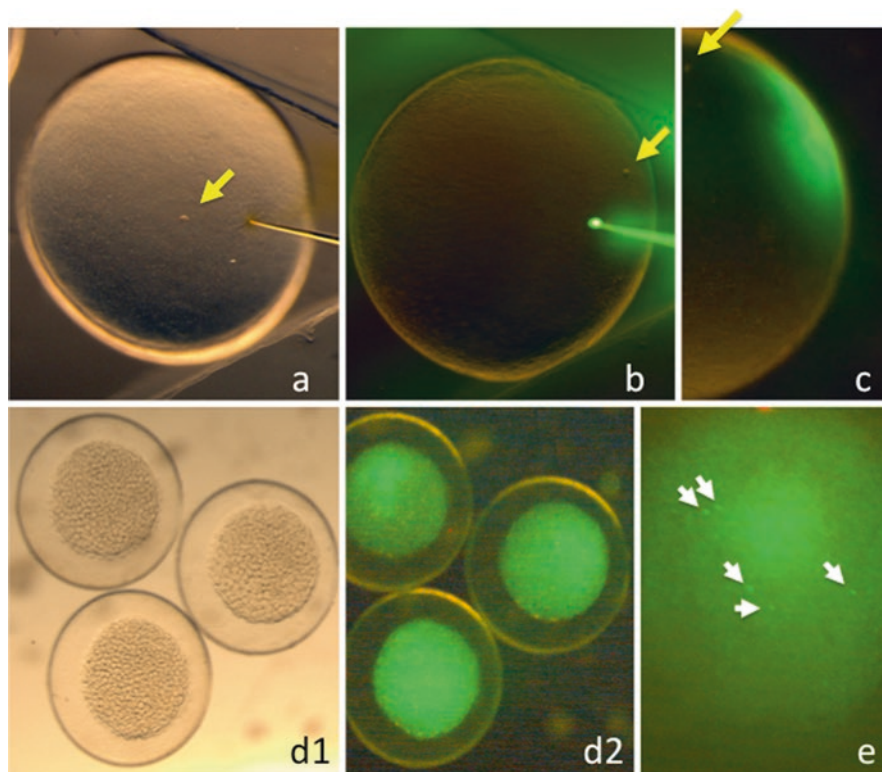
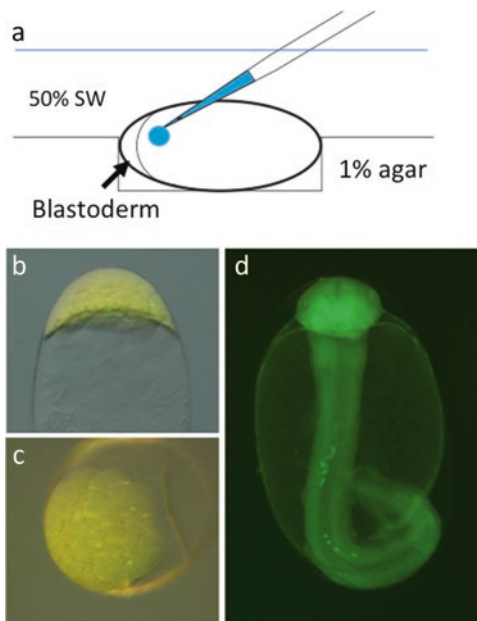


Fig. 11.2 Injection with fluorescent reagent into a fertilized egg of barfin flounder. **(a)** Backlight view, showing the injection point of the fertilized egg. **(b)** Fluorescent view showing an injection with FITC-dextran. **(c)** Lateral fluorescent view showing the distribution of FITC-dextran in a fertilized egg. FITC solution is only distributed in the blastodisc. Yellow arrows indicate micro-pyles. **(d)** Blastula stage developed from injected eggs with GFP-*nos3* 3'UTR mRNA. **(d1)** Backlight view. **(d2)** Fluorescent view. Blastomeres with visualized germplasm are shown in the eggs when the chorions are removed. **(e)** Visualized primordial germ cells (PGCs) (white arrows) in dissociated blastomeres

should be reared in a flow-through seawater system with 300–500% exchange in a day, enough aeration with/without oxygen generator to keep dissolved oxygen between 5 and 7 mg/L and photoperiod of 14:10 L:D (Pandey et al. 2017a, b). Pellets were fed at about 3–5% of body weight per day over 12 h with an automatic feeder. The main spawning time was between 21:00 and 22:00 h when fish reared temperature at 19–21 °C and daylight from 6:00 to 20:00 h (Pandey et al. 2017a). Therefore, checking the spawning time by scooping surface seawater from rearing tank the day prior to the experiment is recommended. It is not suggested that a fish net is used to collect the eggs since the fertilized eggs of the Japanese anchovy are quite fragile and are easily damaged once exposed to air.

Once the spawning time is determined, observe the fish carefully and transfer a mature male and female (with swollen belly, a sign of ovulation) to the 1000 L tank

Fig. 11.3 (a) Schematic illustration of microinjection in an anchovy egg, and (b and c) visualization of primordial germ cells (PGCs) by injecting bucky ball-GFP RNA or (d) GFP-*nos3* RNA. (b) A side view and (c) an animal polar view of an embryo at the 128-cell stage. (d) A ventral view of an embryo just before hatching. Green dots in the images are labeled as PGCs



15–30 min before the spawning time to prevent natural spawning in the original tank. To collect the sperm and ovulated eggs for artificial fertilization, anesthetize the fish with MS-222, and then place on a plastic sheet. To obtain the sperm, gently squeeze the abdomen of the male by hand, then collect the sperm with a pipette and dilute by 100 times with Hank's solution at 4 °C (Pandey et al. 2017b). In most cases, fish excrement appears before the sperm; wipe the excrement away before collecting the sperm. Sperm can be collected from the mature male at any time of day. However, Japanese anchovy sperm have a time-specific activation (circadian rhythm). Therefore, the best time to collect sperm with the highest mobility and movement velocity for fertilization is at spawning time. To collect the ovulated eggs, push the abdomen of the female and squeeze gently. Collected eggs should be kept in a 90 mm dish with a lid to prevent them drying out before fertilization. Mix the ovulated eggs with the diluted sperm in a container, then add seawater to activate the sperm and wait for 5 min. Remove the seawater from the container, add FSW supplemented with 0.01% streptomycin and penicillin, and incubate. Keep a maximum of a few hundred eggs in a 1 L beaker to avoid embryo deformity and delaying embryonic development.

The external appearance of each embryonic development stage is shown in Fig. 11.4. The developmental time described here for each stage is determined from the 1-cell stage. After fertilization, cytoplasmic movement begins and the formation of the blastoderm takes about 45 min at 16 °C, 40 min at 22 °C, and 33 min at 26 °C. The highest blastoderm with a flattened bottom is defined as the 1-cell stage. Cleavages repeat about every 15 min at 24 °C until the early blastula stage. It is noteworthy that the bottom of the blastoderm is slightly slanted in the embryo at the

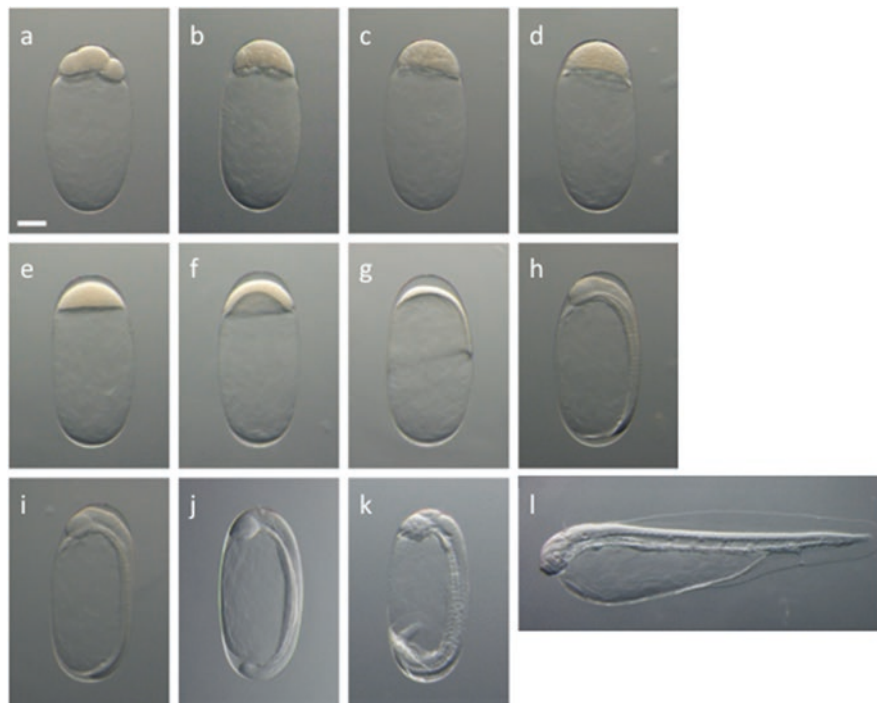


Fig. 11.4 External appearance of each embryonic development stage in Japanese anchovy, *Engraulis japonicus*. The (a) 4-cell, (b) 16-cell, (c) 128-cell, (d) 512-cell, (e) sphere, (f) germ ring, (g) 50% epiboly, (h) 100% epiboly, (i) 17–18-somite, (j) 32-somite, (k) pre-hatching, and (l) hatching stages

64-cell stage. The reason for this nonuniform shape and its developmental importance are unknown. The YSL formation is a good indicator for the embryonic staging of the blastula period in Japanese anchovy, as reported in other species. Because of the transparent body, the somites are difficult to determine during epiboly. The earliest somite observed here was in the 9-somite stage at 90% epiboly. Some of the embryos were hatched at 23 h 45 min at 24 °C and all embryos were hatched at 26 h.

11.3.2 Pacific Herring (Adhesive, Demersal Eggs)

Mature Pacific herring (*Clupea pallasii*) are caught around the northern coasts of the Pacific Ocean from winter to early spring. In nature, they scatter eggs and sperm in the seaweed beds of shallow seas, near to the coast. We obtain fish from the National Center of Stock Enhancement, Akkeshi, Hokkaido, Japan. It will likely be possible to obtain mature fish from a local fish market or fishermen. In our experience, it is possible to obtain eggs and sperm that are capable of fertilization from the fish in a fish market if they are caught on the same day as the experiment and kept on ice.

Fish must be kept on ice until use. You can easily distinguish between female and male by pushing their belly if they are fully matured: females and males will ooze yellowish eggs and white milt from their genital pores, respectively. To obtain eggs, squeeze a female abdomen while positioning its genital pore over a polypropylene sheet-lined plastic dish. Generally, you can obtain a lot of eggs from a single female, but in most cases 1 g of eggs is more than enough for experiments in the laboratory. The eggs need to be covered with the polypropylene sheet by folding it in order to avoid them drying out. Sperm are also obtained in a similar fashion, by squeezing the abdomen of the male. We used a 15 or 50 ml centrifuge tube for storage. The eggs and sperm must be kept on ice until use. In our experience, they keep their fertilization capability for several hours in this condition, but we did not test the limits of this. The dry method can be used to perform artificial fertilization. Transfer a small amount of eggs (100–200 eggs) to a new polypropylene sheet with a plastic tea spoon, add a small amount of sperm (1–5 μ l), and mix gently with the spoon. After that, place the mixture in FSW in a plastic dish (90 mm) and mix instantly so that they spread throughout the dish. The egg chorions of herring are very sticky and strongly adhere to the wall and bottom of the plastic dish just after transfer. Incubate at 4 °C in an incubator for 10 min and replace the fertilization solution twice with pre-chilled seawater to remove the excess sperm.

The stickiness of the eggs can be removed by incubating them with 1% urea and 0.1% trypsin in FSW for a few hours at 4 °C (Saito et al. 2006). With this treatment, the egg chorions become thin and the stickiness is removed, although the eggs remain attached to the bottom of the dish. After the treatment, replace the urea/trypsin solution with FSW three times. The microinjection of a solution, such as GFP-*nos3* 3'UTR mRNA, to visualize primordial germ cells (as described by Saito et al. 2006), will be possible after the de-stickiness treatment. For the microinjection, eggs should be attached to the central part of the plastic dish, preventing the vertical wall of the dish disturbing the approach of the needle to the eggs. Herring eggs contain 'yolk granules' rather than a single yolk, and the cytoplasm moves toward the animal pole as the embryo develops, so the solution can be injected into any part of the yolk at the 1- to 2-cell stage (Goto et al. 2019).

The embryonic development of the herring is very slow compared to that of model fish species, such as zebrafish and medaka. The embryo can be kept at a range of 3.5–15 °C (Kawakami et al. 2011), while replacing the FSW daily. Increasing the temperature accelerates the speed of embryonic development. Thus, it is relatively easy to adjust the developmental stage to your experiment/lecture plan, although there is no information about the speed of embryonic development for specific temperatures. Although it is slightly difficult, embryos can be dechorionated using a pair of fine forceps after the early somitogenesis period. Dechorionated embryos should be placed on an agar-coated (1% in seawater) dish filled with FSW supplemented with antibiotics (0.01% penicillin and 0.01% streptomycin) to prevent being damaged by the hard bottom of the dish. After the mid-somitogenesis period, the embryos will start to move. For photography and observation, you can add tricaine solution to the FSW at the same dose as the experiment for zebrafish (400 mg/100 ml).

11.3.3 Smelts (Adhesive Membrane, Demersal Eggs)

During the spawning season (spring), mature pond smelts can be obtained from river mouths at night. In Japan, eggs from the following four related species belonging to Osmeriformes are available for artificial fertilization: ayu (*Plecoglossus altivelis*), wakasagi (*Hypomesus nipponensis*), kyuriuo (*Osmerus mordax dentex*), and capelin (shishamo) (*Spirinchus lanceolatus*). Mature fish of kyuriuo can be also collected during the spawning season in Hokkaido, while ayu and shishamo cannot, because they are commercially important species. As several prefectural fisheries experimental stations propagate ayu seedlings, refer to your local stations.

The collection of gametes and their fertilization are carried out by the dry method, the same as for Pacific herring. Inseminated eggs are scattered in a Petri dish filled with tap water. In this condition, fertilized eggs can be cultured under wide temperature conditions around 0–18 °C. Therefore, the rate of embryogenesis can be controlled by the culture temperature. Fertilized eggs can be dechorionated by treatment with 15 ml of smelt's Ringer's solution (SRS) containing 0.1% trypsin and 100 µl of 1 N NaOH for 1 min, followed by 30 ml of SRS containing 0.1% trypsin without NaOH. Three hours after treatment, the chorions of almost all of the eggs were removed. Therefore, the injection of artificial materials and operation of the embryo are possible for these denuded eggs. Please refer to Takahashi et al. (2016, 2017) for details of the experimental methods.

The embryonic development of pond smelt is shown in Fig. 11.5. During embryogenesis, the endodermal organs, e.g., the liver, pancreas, and digestive tract, can be easily observed because of the transparent body and late pigmentation. During the gonad formation, primordial germ cells first accumulate in the center of the body and then distribute asymmetrically as they interact with the endodermal organs. This characteristic makes pond smelt useful experimental animals because the interaction between the gonad and endodermal organs has not been studied in zebrafish due to their lack of stomach.

11.3.4 Ice Goby (Adhesive Threads)

The ice goby, *Leucopsarion petersii*, is a goby species that inhabits the inshore areas of Japan and Korea. They undertake anadromous migration to spawning in rivers from January to June. They are thought to be neotenic; they retain their larval form (transparent body, swim bladder, no scales, and a small pelvic fin) even when they reach the sexually mature stage. They spawn eggs on the top of small rocks in rivers. In Japan, you can purchase the fish from local fish dealers or directly from fishermen. Adult ice gobies are captured by a four-armed scoop net during their migration upstream in the spawning season. If you purchase them from a remote location, you can ask the dealer to put them in a plastic bag with oxygen and send them by refrigerated courier service (4 °C).

It is possible to induce the final maturation artificially in the laboratory. First, after receiving the fish, separate the males and females into separate tanks according

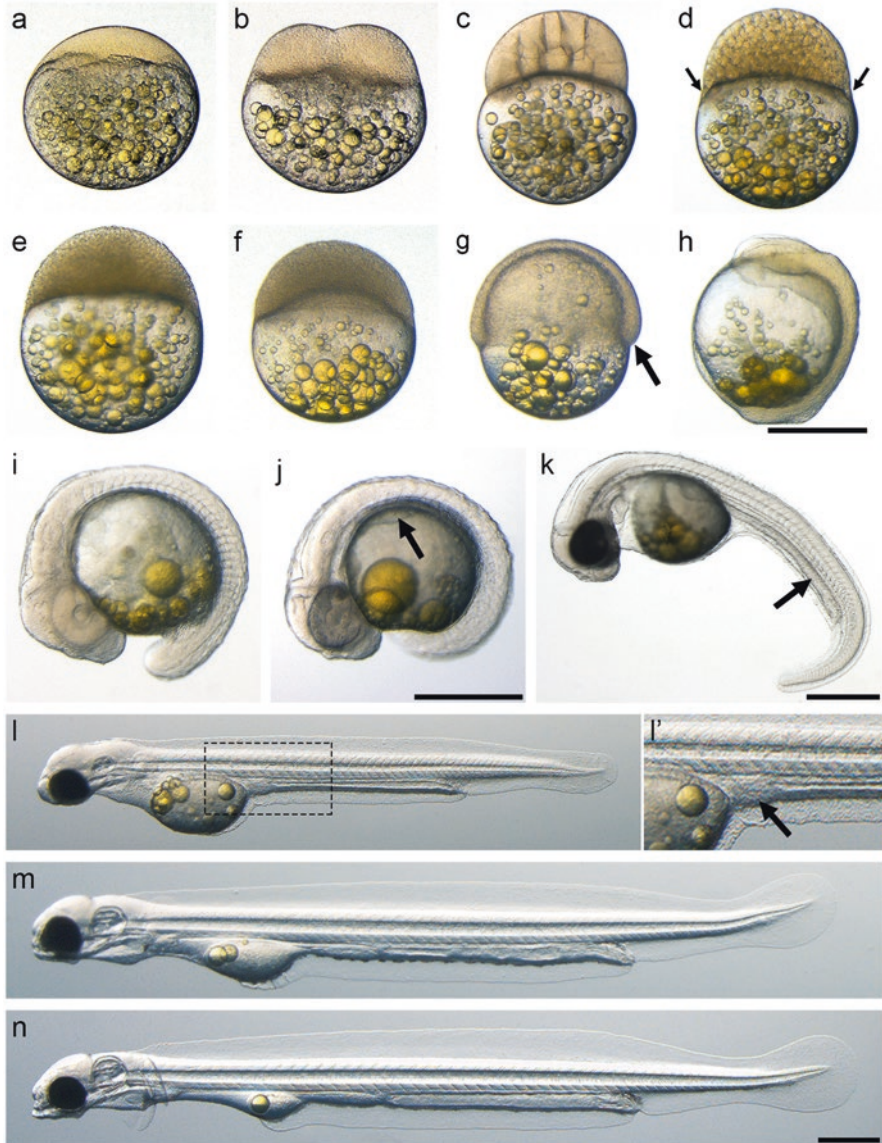


Fig. 11.5 External appearance of the embryonic development stages of pond smelt, *Hypomesus olidus*. (a) 1-cell, (b) 2-cell, (c) 16-cell, (d) mid-blastula, (e) sphere, (f) 30% epiboly, and (g) embryonic shield (arrow) stages. (h) Appearance of the embryonic body, (i) optic cup, and (j) pigmentation of eye. (k) Hatching, (l) 14 days post-fertilization (dpf), lateral line primordium observed. (l') Higher magnification of (l). (m) Liver formation 18 dpf. (n) Opening of anus

to their pigmentation patterns: females have a line of pigment on both sides of their belly (see Fig. 11.1 in Arakawa et al. 1999). Then, the males must be kept in darkness in freshwater for 2 weeks at 19 °C in order to induce gonadal maturation.

Regarding the females, inject 25 units of human chorionic gonadotropin (hCG) into the body cavity to induce ovulation and culture in freshwater at 19 °C. Within 10 days, the eggs in the ovarian sac became round and swollen; this is a sign of ovulation. Anesthetize some females in tricaine solution in freshwater, and gently squeeze the belly to obtain unfertilized eggs, collecting them on a polypropylene sheet. Then, cover the eggs with the polypropylene sheet by folding it to avoid them drying out until use. Because it is quite difficult to squeeze the milt from mature males due to their small testes size, they should be sacrificed for insemination using an overdose of anesthesia, such as tricaine or 2-phenoxyethanol. Remove the testes and mince them into small pieces in a small amount of Hank's solution (about 50 µl). Then drop the sperm solution onto the unfertilized eggs and activate the sperm by adding small amount of freshwater (100–200 µl).

After fertilization, the chorion absorbs water and elongates to form an eggplant shape. The chorion can be removed easily with a pair of fine forceps in Ringer's solution. The embryos are very fragile compared to those of cyprinid species, such as goldfish and zebrafish; thus, microinjection should be performed in Ringer's solution supplemented with 1.6% albumen (chicken egg white solution). This albumen–Ringer's solution mixture is expected to work as an antibiotic (because of the lysozyme in the albumen) and aid the recovery of the embryos from injection damage. The ice goby eggs contain “yolk granules” rather than a single yolk. Usually, the embryo develops normally without problem with a volume of injectant 1/10 of the diameter of the egg (Saito et al. 2006). The dechorionated embryos need to be cultured in the same solution until the completion of epiboly. Without the albumen solution, the dechorionated embryos will sometimes adhere to the bottom of plastic dishes for an unknown reason, which causes the rupture of the yolk ball. Most ice goby embryos show latitudinal cleavage at the third cleavage to form eight cells, as seen in frogs and sea urchin embryos. Thus, it is possible to divide the tiers of blastomeres by using a fine glass-fiber needle and culturing them to study the function of the embryonic patterning determinants (Saito et al. 2002). For culturing embryos, temperature of 15–22 °C seems sufficient, although there is no literature confirming this. The normal developmental stages of ice goby are described in Arakawa et al. (1999).

11.3.5 Goldfish and Zebrafish (Adhesive, Demersal Egg)

Sperm are collected using an automatic micropipette with a crystal tip or glass capillary from anesthetized mature males of zebrafish or goldfish, respectively, and diluted with artificial seminal plasma at appropriate concentrations. Mature eggs are striped onto vinylidene chloride film and inseminated with diluted sperm. Inseminated eggs are scattered into the fertilization solution, tap water for zebrafish or urea water for goldfish, for fertilization. Fertilized eggs are easily dechorionated by using freshwater Ringer's solution containing 0.1% trypsin and 0.2% urea at about pH 7.0, adjusted using NaOH solution. Urea is not always required, but pH is an important factor for dechoriation in zebrafish. Goldfish chorions cannot be

removed without urea either in fertilization or dechoriation solution. Dechorionated eggs are incubated in 1% agar-coated Petri dishes filled with fresh-water Ringer's solution containing 1.6% albumin from chicken egg whites until the end of epiboly. Then, embryos can be cultivated in 1% agar-coated Petri dishes filled with 1.8 mM CaCl_2 and 1.8 mM MgCl_2 solution.

The embryonic stages of zebrafish and goldfish are precisely described in Kimmel et al. (1995) and Tsai et al. (2013), respectively. The simple developmental appearance of each stage for goldfish is shown in Fig. 11.6. As zebrafish embryos are highly transparent, the morphological changes are clearly observable during development. However, the optimal temperature for development is relatively limited; therefore, it is difficult to show the rich variety of developmental stages in student experiments over a limited time. In contrast, goldfish embryos can develop under a wide range of temperature conditions, from 10 to 30 °C, and thus it is possible to show these developmental stages to students. We cultured fertilized goldfish eggs at 10–24 °C condition and prepared the various developmental stages.

11.4 Observation and Experimental Analysis for Developmental Mechanisms

11.4.1 Cell Lineage

Cell labeling is useful for lineage tracing during development. For labeling, prepare an injection needle, from which a small amount of FITC solution is streaming out constantly, and settle its tip just at the surface of a single blastomere after the 64-cell stage in zebrafish and goldfish, and after the 8-cell stage in ice goby. After setting, tap the back of the needle holder gently, resulting that small amounts of fluorescent reagent is injected into the cell to label it (Fig. 11.7a). Descendants from a single-labeled cell can be traced thereafter under a fluorescence microscope. In the case of zebrafish, they begin to scatter after the beginning of epiboly, while in goldfish, they do so after the late-blastula stage (Fig. 11.7b). In goldfish, the labeled blastomeres begin to move just after the mid-blastula stage.

11.4.2 Showing YSL Function by Surgical Operation

Experimental embryology, such as experimental extirpation, has revealed the mechanisms controlling individual development by means of experiments on living organisms. It uses methods such as the marking, removal, transplantation, and isolation of body parts and organs. In some cases, these methods easily induce drastic morphological changes in development, disclosing the fundamental mechanisms of development. This is useful to raise the students' interest for development. In this sub-section, we describe an experiment to visualize YSL function during early development as an example of experimental embryology.

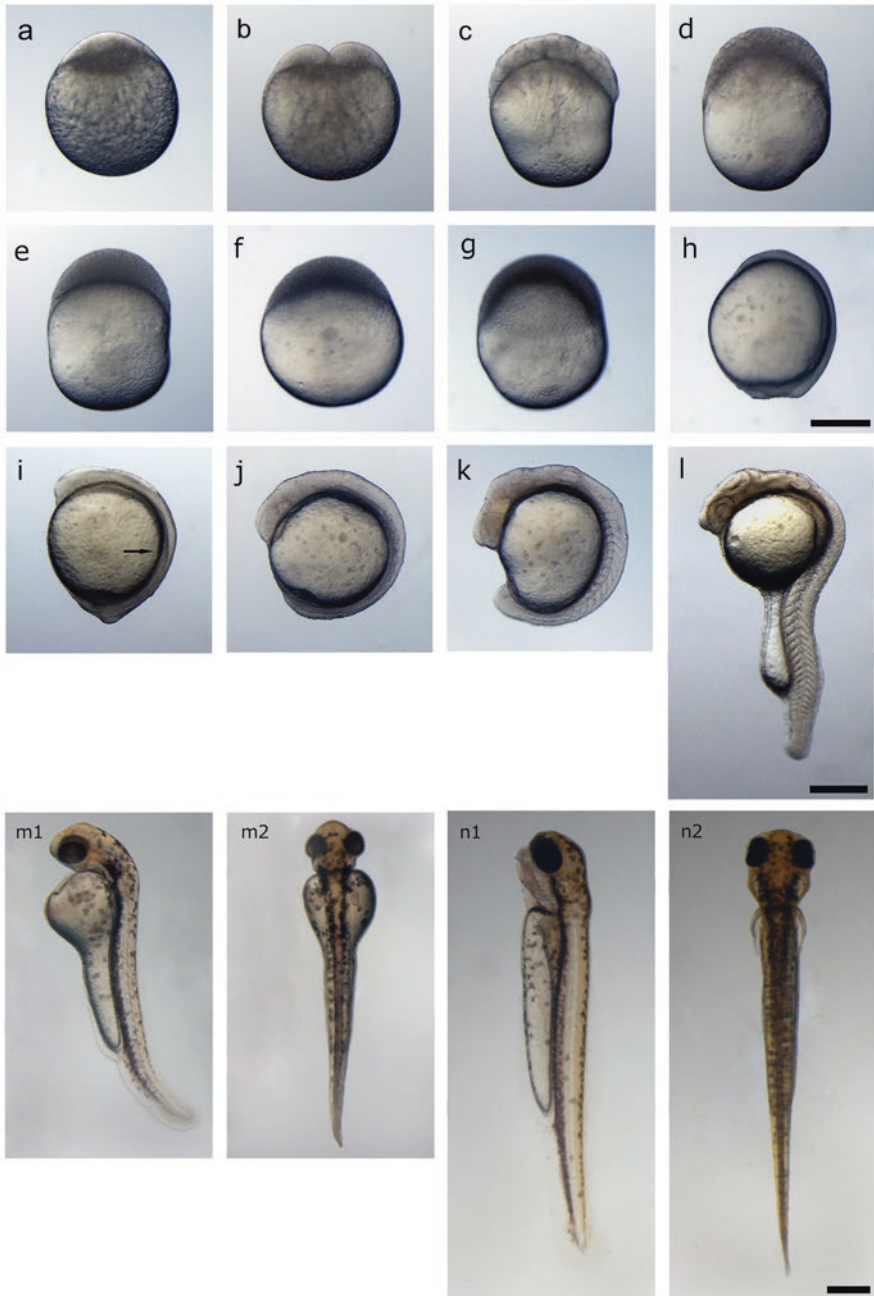


Fig. 11.6 External appearance of the embryonic development stages of goldfish, *Carassius auratus*. (a) 1-cell, (b) 2-cell, (c) 64-cell, (d) mid-blastula, (e) oblong, (f) sphere, (g) 50% epiboly, (h) 100% epiboly, (i) 2-somite, (j) 8-somite, (k) 17-somite, and (l) 30-somite stages. (m1, m2). Lateral and dorsal view of heartbeat, and (n1, n2) lateral and dorsal view of hatching (by courtesy of Mr. Hirotarō Urushibata)

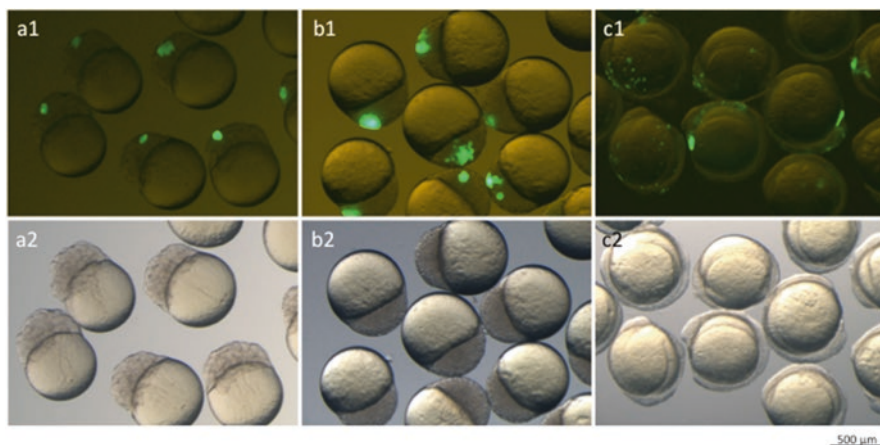


Fig. 11.7 Cell labeling and tracing in zebrafish. (a) Immediately after injection with FITC into the upper tier of blastomeres. (b) Results at the mid-blastula stage. Descendants of labeled cells begin to scatter in some blastulae. (c) Results at 100% epiboly. Descendants of labeled cells scatter in the embryos. (a1–c1) Fluorescent view. (a2–c2) Backlight view

For the surgical operation, prepare a dissecting needle with thin glass-wool attached to the top (SD2) and an agar plate, such as a 90 mm glass dish filled with a 1 mm layer of 1% agar in Ringer's solution. Transport the blastulae without chorions to the agar plate filled with an appropriate amount of Ringer's solution. Two types of operation are performed in the blastulae using a dissecting needle. First is the isolation of the blastoderm from the yolk cell. Cut off the blastoderm from just above the yolk cell. The resultant blastoderm form a spherical cell-mass covered with an enveloping layer (Supplemental Movie 11.2). Second is the removal of yolk by injuring the yolk membrane or cutting off the vegetal yolk hemisphere. After healing the injury, the resultant embryos are small yolk cells covered with large blastoderms (Supplemental Movie 11.3). These two types of operated embryos and a control (without operation) are cultured independently in separated wells of a culture plate filled with the appropriate culture medium. In the case of zebrafish, freshwater Ringer's solution containing 1.6% albumin from egg white is used for the operation and as a cultivation medium for 1 day after the operation. After the completion of epiboly, the denuded eggs are cultured in a simpler culture medium; for zebrafish and goldfish, freshwater containing 1.8 mM CaCl_2 and 1.8 mM MgCl_2 is sufficient for cultivation. When the surgical operation is carried out at around the blastula stage, the functions of YSL are visualized in the morphology of the resultant embryos (Fig. 11.8). The separated blastoderm, from which the yolk cell is removed just after the mid-blastula transition, develops a rotationally symmetrical embryo. At the late-blastula stage, it develops abnormal embryos with segmented tissues and without the anterior differentiation of the brain. Separated embryos with a partial YSL and a small amount of yolk, which have recovered from the injury to the yolk cell, develop as almost normal embryos with optic vesicles. These results

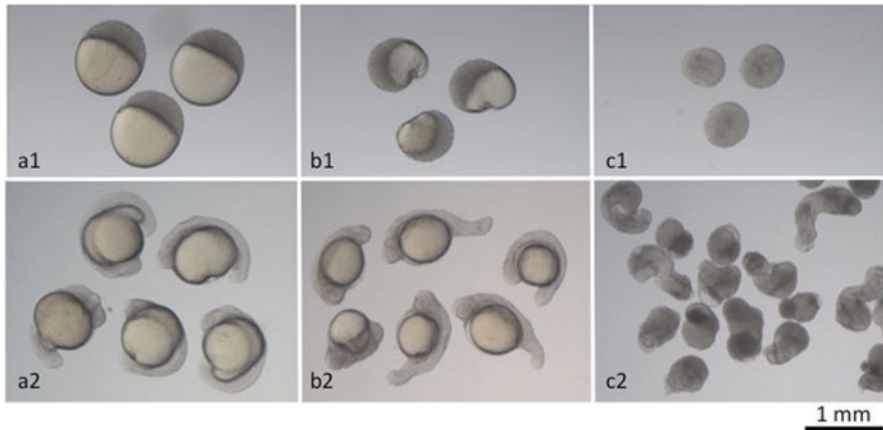


Fig. 11.8 Surgical operation at the blastula stage in zebrafish (**a1**, **b1**, **c1**) and the resultant embryos at the tail bud stage (**a2**, **b2**, **c2**). (**a1**, **a2**) Control embryos without extirpation of the yolk. (**b1**, **b2**) Operated embryos with extirpated vegetal half of yolk cell. (**c1**, **c2**) Operated embryos without yolk cell. This operation indicates the function of yolk cells with a YSL

suggest that yolk cells with a YSL induce the differentiation of the blastoderms gradually after the mid-blastula stage, and are required for normal development. This type of experiment is applicable to other species if the chorions are removable from the embryos.

11.4.3 PGC Visualization and Migration

The teleost germplasm and PGCs are visualized by injecting artificial mRNA, such as *bucky ball*-GFP mRNA and GFP-*nos3* 3'UTR mRNA, respectively, into the egg cytoplasm just after fertilization (Saito et al. 2008). Germplasm begin to visualize during the early cleavage stage along the ends of the cleavage furrows and the connected side between the upper and lower blastomeres (Fig. 11.3b, c). These signals disappear after the blastula stage. PGCs begin to visualize after the gastrula stage by GFP-*nos3* 3'UTR mRNA (Fig. 11.3d). Not all PGCs are always visualized by artificial mRNA; it is dependent on the distribution of the solution. The fluorescence strength of each PGC is different even in a single embryo, while auto-fluorescence is observed in somatic cells around the PGCs.

11.5 Conclusion

Recently, gene editing has attracted public attention, because of the easy modification of specific genes and rapid improvements to genetic breeding. Many marine teleosts, which are recognized as important species from a commercial viewpoint, have been gene-edited in order to introduce extraneous characters that are expected

to be useful in various applications for aquaculture. On the other hand, early-stage mortality is very high in marine teleosts. A limited number of modified embryos should be handled with care and grown to maturity. Therefore, handling techniques and observation abilities are required for researchers in this field. Marine stations are essential places for training to improve these techniques and abilities.

Solutions

Artificial seminal plasma (zebrafish, goldfish, common carp, and other cyprinid fish): NaCl 5.61 g, KCl 5.23 g, CaCl₂·2H₂O 0.33 g, MgCl₂·6H₂O 0.22 g, and NaHCO₃ 0.2 g/L DW.

Urea water (fertilization solution for goldfish): 0.2% urea and 0.24% NaCl in tap water.

Dechoriation solution (goldfish and zebrafish): 0.1% trypsin and 0.2% urea in freshwater Ringer's solution maintained at about pH 7.0 (adjusted by NaOH solution).

Culture solution for dechorionated eggs: Ringer's solution containing 1.6% albumin from egg-white precipitate.

Freshwater Ringer's solution: 128 mM NaCl, 2.8 mM KCl, and 1.8 mM CaCl₂.

SRS: 128 mM NaCl, 2.8 mM KCl, and 2.7 mM CaCl₂.

Dechoriation solution for smelts: SRS containing 0.1% trypsin and 100 µl of 1 N NaOH.

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