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Yasunori Sasakura *Editor*

Transgenic Ascidians

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Foreword

The ascidian *Ciona* provides one of the best experimental systems in developmental biology, developmental genomics, and evolutionary developmental biology. We ascidian developmental biologists are justifiably proud of the fact that we established the *Ciona* system.

In traditional embryology, ascidians were recognized as organisms exhibiting a "mosaic-mode of embryogenesis", in contrast to the regulatory mode of embryogenesis seen, for example, in sea urchins. A detailed description of embryonic cell lineage by Edwin G. Conklin revealed that the mosaic-mode of embryogenesis is determined by determinate cleavage of the embryo and a robust pattern of differentiation and morphogenesis. Maternal factors and cellular communications that play essential roles in gastrulation, neurulation, tailbud-embryo formation, and tadpole-type larval development work together under the harmonious, but rigid control of a gene regulatory network. In addition, newly hatched larvae comprise only about 2600 cells, including 40 notochord cells and 38 muscle cells. Thus, ascidians are considered embryonically simple compared to vertebrates and other deuterostomes, such as sea urchins. In other words, ascidians represent a system to challenge the most basic question of developmental biology: How does a complex, multicellular, metazoan body arise from a single cell, the fertilized egg?

Decoding of the draft genome of *Ciona intestinalis* in 2002 identified almost all of the developmentally relevant genes in its genome, which represent the basic set of gene components in chordates prior to the two rounds of genome duplication that occurred in the vertebrate lineage. The absence of redundancy in ascidian regulatory gene functions makes it easier to ascertain developmental roles of individual genes. In association with the genome sequencing project, a cDNA sequencing project was also carried out, providing a great quantity of information on expression profiles of regulatory genes. Many whole-mount in situ hybridization studies reveal distinct gene expression profiles in embryos at the single-cell level. Furthermore, results obtained from embryological research provide straightforward insights into chordate evolution as well as the origin of vertebrates, because ascidians are the closest relatives to vertebrates.

In this research environment, it is desirable to introduce new, state-of-the-art techniques into the *Ciona* system, with the most valuable at this time being "transgenic" techniques. These include microinjection and/or electroporation of exogenous DNA to reveal mechanisms involved in gene regulatory networks, germ-line transgenesis to create various marker lines for

studies of gene regulation, and TALEN-based and CRISPR/Cas9-based knockout lines to facilitate studies of gene function.

This book, edited by Prof. Yasunori Sasakura at the University of Tsukuba, constitutes a very timely discussion of recent advances in this field. In addition, most authors of this book are new or mid-career researchers. Indeed, we owe them further development of the ascidian systems. Lastly, I wish to express once again my conviction that *Ciona* is the most promising system to explore molecular and genetic mechanisms involved in animal embryogenesis at the single-cell level, the tissue and organ level, and the individual level.

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Noriyuki Satoh

Preface

Undoubtedly, transgenic technologies are inevitable for studying molecular functions during development. In several model ascidian species, creation of transgenic animals has been routinely performed, and the methods are easily retrieved from many articles and from some book chapters. However, the details of the methods have not always been provided in the literature. On the one hand, microinjection is basically a simple method for introducing exogenous DNAs without the requirement of an expensive machine. At the same time, because microinjection can be a relatively difficult process, many laboratories have their own practical tips that are suitable for their study of species. These tips are usually not seen in original articles whose main purpose is describing development mechanisms. To solve the issue, this book has been purposed to gather and describe the devices that have arisen from our own great interest and enthusiasm, and that are not usually seen or known as major methods for performing transgenesis in ascidians.

I hope this book will be useful for all researchers, including beginners in tunicate research who wish to introduce a tunicate in their laboratories and also specialists of tunicates who wish to find a clue to improve their methods.

Shimoda, Japan — Yasunori Sasakura

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Introduction

1

Yasunori Sasakura

Abstract

The chordate ascidians, the major group of tunicates, is the best animal group for studying molecular and cellular processes underlying formation of a chordate body plan. For these studies, transgenic technologies are powerful. Transgenesis of ascidians has a long history of more than 20 years, and many practical tips have been accumulated. This book is aimed at summarizing the accumulated techniques in ascidians in addition to concrete research in which transgenic techniques have played pivotal roles. This book is useful for fast assimilation of the techniques and for learning the unique devices developed by the enthusiasm of ascidian researchers.

Keywords

Chordate · Ascidian · Vertebrate · *Ciona* · Genome · Transgenesis

1.1 The Aim of This Book

Marine invertebrate chordates, tunicates, is an important animal group for understanding the mechanisms of chordate evolution (Satoh 2003; Lemaire 2011). This is because tunicates are regarded as the sister group of vertebrates (Delsuc et al. 2006; see Chaps. 2, 7 and 8 for details). Tunicates have characteristics that define chordates, such as a notochord, a dorsal neural tube, a gill in the pharynx, and an endostyle/thyroid gland. Moreover, it has been recently suggested that tunicates have some features that were previously thought to be specific to vertebrates, such as neural crest cells and neurogenic placodes (Jeffery et al. 2004; Abitua et al. 2015). Therefore, tunicates are the key organisms for understanding how the chordate/vertebrate-specific features evolved. Among the tunicates, which include ascidians, larvaceans, and thaliaceans, ascidians have been centered on molecular development and genetic studies in this animal group. In addition to their importance from the point of view of phylogenetic position, ascidians possess advantageous features as experimental systems. The advantage of ascidians can be summarized in their simplicity. In the case of *Ciona intestinalis*, a representative ascidian species, the simplicity can be seen in three aspects:

- Fast development, which starts with gastrulation at 5 hours postfertilization (hpf), neurulation at 7 hpf. The larval stage starts within 1 day of fertilization, and metamorphosis is completed within a few days.
- Small cell numbers constituting embryonic and larval bodies. Early gastrula embryos

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consist of only 110 cells. Ascidian larvae possess about 3000 cells, and cell lineages of most cells have been described.

- Compact genome. The genome size of *Ciona intestinalis* is ~160 mega base pairs with ~16,000 genes (Dehal et al. 2002). The scores indicate that a gene is present in every 10 kilobase pairs of the *Ciona* genome. *Cis* elements controlling transcription of genes are usually located near the transcription start sites. The gene sets in the ascidian genomes are basically nonredundant (Satou et al. 2003; Sasakura et al. 2003), because ascidian genomes are thought not to have experienced whole genome duplication, which has occurred twice during vertebrate evolution.

Owing to these characteristics, ascidians are regarded as the best animal for studying molecular and particularly cellular processes underlying formation of the chordate body plan. For these studies, transgenic technologies are powerful and nowadays almost inevitable. In some model ascidians, the techniques have been reported more than 20 years ago, and thus have a long history (Hikosaka et al. 1992). Since then, the methods have been used in many applications, some of which are unique in ascidians (Iitsuka et al. 2014). These technological innovations are exceptional, particularly in marine invertebrates. This book is aimed at summarizing the accumulated transgenesis technologies in ascidians, in addition to concrete examples of research in which transgenic techniques have played pivotal roles. The information provided will be useful for ascidian researchers for fast assimilation of the techniques, and for biologists working with other organisms to learn the unique techniques and ingenious attempts specific to ascidians, in addition to knowledge from recent studies.

I would like to state here a potential issue that may confuse readers of this book. Recently, a few manuscripts have been published that propose new classification of *Ciona intestinalis*. In some locations such as Europe, so-called *Ciona intestinalis* populations have been suggested to be divided into type A and B (Caputi et al. 2007). The detailed comparison of morphology and some developmental features of these types further suggested that they should be divided into two species (Pennati et al. 2015). The authors claimed that *Ciona intestinalis* type A, which is the common type of *Ciona intestinalis*, should be renamed "*Ciona robusta*", and that *Ciona intestinalis* type B, which lives in more restricted areas, is *Ciona intestinalis*. Because the renaming proposal is under consideration for *Ciona* (and the ascidian) community, I decided not to rigidly determine which name, *Ciona intestinalis* or *Ciona robusta*, should be used in this book. Therefore, although some chapters simply use *Ciona intestinalis*, others mention the rename issue in the first section of the chapters and then use *Ciona robusta*. Because all authors took so much care with regard to dealing with the name, I believe that there is no confusion when reading this book; however, please keep this potential issue in your mind.

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2 Microinjection of Exogenous Nucleic Acids into Eggs: *Ciona* Species

Kenji Kobayashi and Yutaka Satou

Abstract

Microinjection is a common technique used to deliver nucleic acids into eggs and embryos in *Ciona* species. There are three *Ciona* species that are commonly used for research—*Ciona intestinalis* type A (*C. robusta*), *C. intestinalis* type B (*C. intestinalis*), and *C. savignyi*. Here, we present the microinjection methods using eggs and embryos of *C. intestinalis* type A and *C. savignyi*; however, our methods would also be applicable to eggs and embryos of *C. intestinalis* type B. Microinjection is a classical and widely used delivery method, which involves the use of a glass micropipette, a hollow glass needle with a microscopic tip, to inject nucleic acids into eggs and embryos under a stereo microscope. The required amount of nucleic acids is much smaller for microinjection than for electroporation, another delivery method. Proteins, and other chemicals, such as fluorescent dye, can be introduced with nucleic acids using a microinjection.

Keywords

Developmental biology · Microinjection · Ascidian · *Ciona* · Nucleic acid · DNA · RNA · Morpholino oligonucleotide · Egg · Embryo

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2.1 Introduction

Microinjection is a common technique used to deliver nucleic acids into eggs and embryos of *Ciona* species. The diameter of eggs of *Ciona* species typically ranges from 140 to 150 μm (Fig. 2.1); therefore, microinjection can be performed under a stereo microscope. The technique is not difficult to learn; in our experience, most persons are able to perform it after several days of training. Skilled persons can inject nucleic acids into over 100 eggs within 1 h.

Among the species belonging to the genus *Ciona*, *Ciona intestinalis* and *Ciona savignyi* have been widely used for manipulation. It has been considered that *C. intestinalis* is grouped into two subspecies—type A and type B (Suzuki et al. 2005; Nydam and Harrison 2007; Caputi et al. 2007). However, recently, a report proposed that these are indeed different species and not subspecies (Brunetti et al. 2015). According to that report, *C. intestinalis* type A is *C. robusta*, and *C. intestinalis* type B is *bona fide C. intestinalis*. However, most of the previous studies on *Ciona* species did not clearly discriminate between these two species. In addition, the reproductive systems of these two species are undoubtedly isolating, but still not completely isolated (Suzuki et al. 2005; Caputi et al. 2007; Nydam and Harrison 2011; Sato et al. 2014). Therefore, we would not discriminate between these two species, and simply describe them as *C. intesti-*

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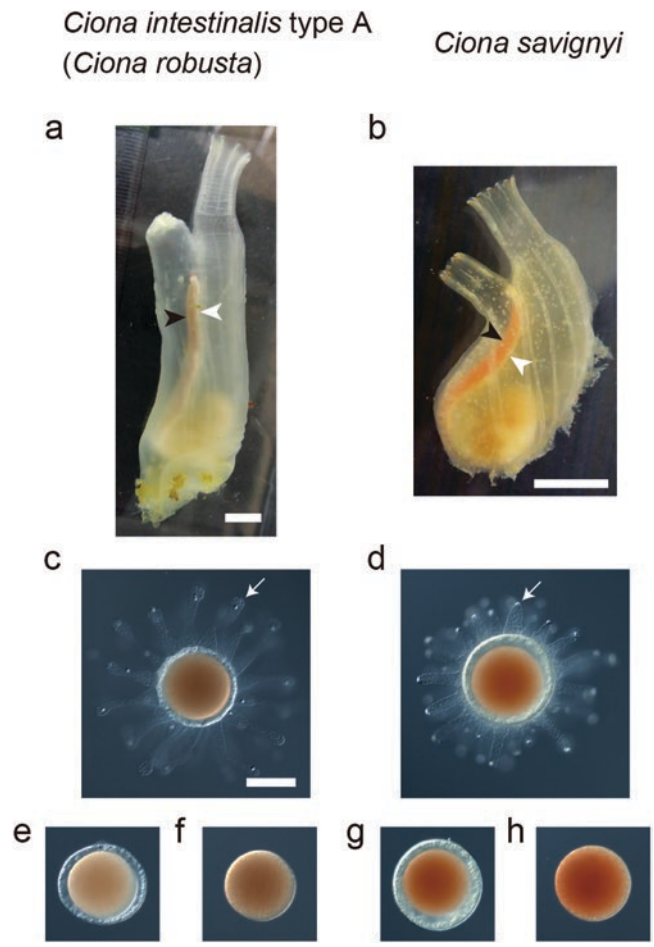

Fig. 2.1 Two *Ciona* species commonly used for experiments. Adult animals of (**a**) *Ciona intestinalis* type A (*Ciona robusta*) and (**b**) *Ciona savignyi*. *Black arrowheads* indicate oviducts, and *white arrowheads* indicate sperm ducts. Unfertilized eggs of (**c**, **e**, **f**) *C. intestinalis* type A, and (**d**, **g**, **h**) *C. savignyi*. *White arrows* indicate follicle cells. Follicle cells can easily be removed by pipetting, and such eggs with chorion (**e**, **g**) are sometimes used for microinjection. However, in most cases, the chorion is removed and used for microinjection (**f**, **h**). Scale bars, 10 mm (**a**, **b**); 100 μm (**c**)

nalis in this chapter. Animals available in our laboratory are *C. intestinalis* type A and *C. savignyi* (Fig. 2.1); therefore, the methods described here are based on these two species. However, our methods would also be applicable to eggs and embryos of *C. intestinalis* type B.

Microinjection is a classical delivery method. On the other hand, electroporation, as explained in Chap. 5, is easier to learn, and nucleic acids can be introduced into a greater number of eggs in one experiment. However, microinjection is still widely used for several reasons—the primary reason is probably that the required amount of nucleic acids is much smaller for microinjection than for electroporation. Preparation of RNAs and synthetic oligonucleotides at amounts sufficient for electroporation is often costly; additionally, simultaneous introduction of DNA, RNA, protein, and other chemicals, such as a fluorescent dye, is possible with a microinjection.

2.2 Preparation of the Microinjection

Room temperature should be adjusted to around 18 °C. Seawater and agar-coated dishes should be equilibrated to the room temperature before use.

2.2.1 Preparation of Nucleic Acids

Deoxyribonucleic acid (DNA) is typically microinjected at final concentrations of 1–20 μg/mL. While preparing the injection solution,

nucleic acids are commonly mixed with a dye; therefore, the DNA stock solutions have to be prepared at a higher concentration. Injection of higher concentrations of DNA may nonspecifically inhibit development (Hikosaka et al. 1992). Plasmid DNA can be prepared from *Escherichia coli* using a conventional alkaline-lysis method. Most of the commercially available kits could be used for this purpose. Circular plasmid DNAs are sometimes linearized with a restriction enzyme, which may improve their transcriptional efficiency. It is also possible to introduce PCR products after purification. Columns with a silica membrane are convenient for this purpose. DNA is typically eluted or dissolved using 1 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA (Hikosaka et al. 1994; Satou and Satoh 1996).

Ribonucleic acid (RNA) is typically microinjected at final concentrations of 0.05–2 mg/mL, which is much higher than the concentration of DNA, but that has no effect on embryogenesis. While preparing the injection solution, nucleic acids are commonly mixed with a dye; therefore, RNA stock solutions have to be prepared at a higher concentration. RNA is prepared using in vitro transcription from DNA templates using T3, T7, or SP6 promoter. For efficient translation, a cap structure analog 7mGpppG is usually incorporated as the 5'terminal or the first G of the transcript during in vitro transcription. It is convenient to use a commercially available kit, such as mMessage mMachine Transcription Kits (Thermo Fisher Scientific, Waltham, MA, USA), for this synthesis. Template DNAs need to be removed before injection following the manufacturer's protocol. RNAs are typically eluted or dissolved in nuclease-free water.

Morpholino oligonucleotides (MOs; Gene Tools, Philomath, OR, USA) are used for knocking down gene function (Satou et al. 2001), and can be injected in the same way as DNA or RNA at final concentrations of 0.25–1.5 mM; the concentration is usually determined empirically.

2.2.2 Preparation of Manipulators and a Stereo Microscope

Any stereo microscopes can be used for microinjection. Systems with zoom magnification up to ×100 are convenient. The working distance is important for setting up manipulators under the objective lens.

In our laboratory, manipulators produced by Narishige Corporation (Setagaya-ku, Tokyo, Japan) are used and shown here as a typical set (Fig. 2.2). However, the following example also works as a reference for setting up manipulators that are available from several other companies.

2.2.3 Preparation of Injection Needles

We usually prepare needles from glass capillaries with an external diameter of 1.0 mm (e.g., GD-1 or GDC-1; Narishige). Capillaries with a filament are convenient, because an aliquot (~1 μL) of nucleic acid solution is loaded at the back end of a needle with a micropipette, and then the filament leads the nucleic acid solution to the tip of the needles. For preparation of the needles, a machine called a puller is required (we use Model P-97, from Sutter Instrument, Novato, CA, USA). The puller heats a glass capillary and then pulls it to make two needles. The strength of heating and pulling power are adjustable, and greatly influence the shape of the needles. The shape is important for successful injection, but this is a matter of preference. Start with the shape of a needle that we use (Fig. 2.3a, b), and change it, if you want, by adjusting the settings of your puller (our typical setting for the puller is—heat, 550 (filament lamp test, 565); pull, 20; velocity, 90; time, 255; we adjust these values every time before use). Keep needles on a needle stand, which can be easily hand-made (Fig. 2.3c).

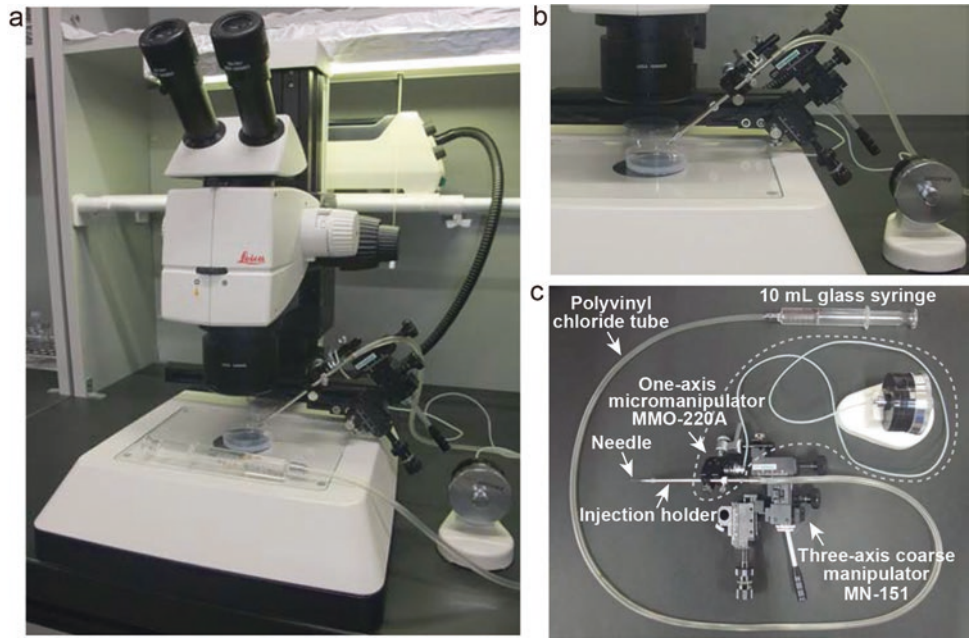

Fig. 2.2 A stereo microscope and a set of manipulators for microinjection. (**a**) A photograph of the whole microinjection system. (**b**) A high magnification view of the manipulators. (**c**) The whole set of manipulators

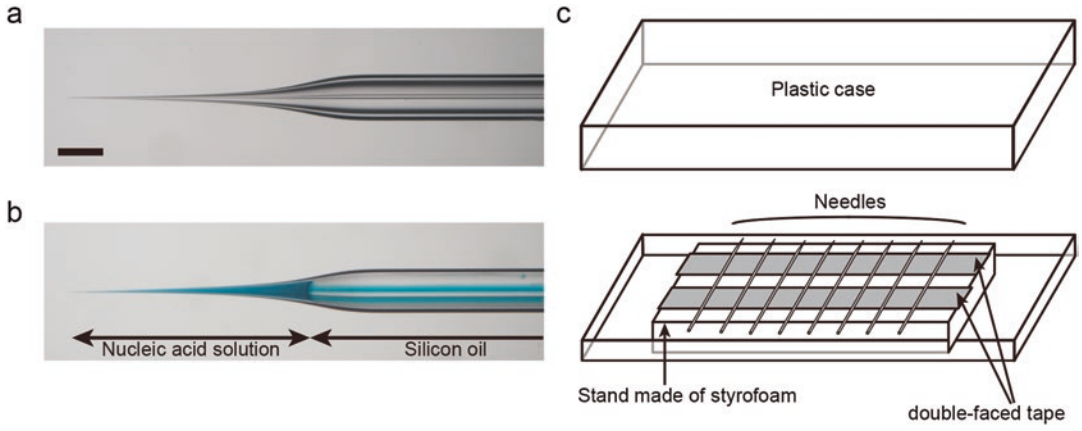

Fig. 2.3 Injection needles. (**a**) An empty glass needle with a filament. (**b**) Nucleic acid solution with green dye was loaded at the tip of the needle, and silicone oil filled the remaining part. (**c**) A needle stand used for keeping needles and filling nucleic acid solutions into needles. Scale bar, 1 mm (**a**)

Glass capillaries without a filament are also used widely. In this case, an additional type of needle is required for filling the nucleic acid solution into the tip of injection needles from the back end, which is not described here.

2.2.4 Preparation of Agar-Coated Petri Dishes

2.2.4.1 Agar-Coated Petri Dishes for Incubation of Embryos

For the incubation of dechorionated embryos, dishes need to be coated with agar, because dechorionated embryos are easily broken by sticking to the naked surface of dishes. Agar is commonly dissolved in seawater at a concentration of 1% (w/v) by heating in a microwave. To dissolve agar completely, agitate the mixture occasionally during heating. Then pour an adequate amount of 1% agar into the dishes. Keep the agar layer thin so that agar-coated dishes can hold a sufficient amount of seawater. After agar is cooled, pour seawater onto the agar gel to prevent the agar gel from drying out. Agar-coated dishes may be stored in a refrigerator. Dishes with a diameter of 6- or 9-cm would be easy to use.

2.2.4.2 Agar-Coated Petri Dishes for Microinjection

As for the above-mentioned procedure, prepare 1% agar solution using seawater. Pour the agar solution into a dish, and then float a cover glass on the agar solution in the dish (Fig. 2.4). Use tweezers to move the cover glass to an appropriate position. After the agar is cooled, remove the cover glass using tweezers. A small step would be formed on the surface of the agar in the dish, which would be useful during injection. Pour seawater onto the agar gel to prevent the agar gel from drying out.

2.2.5 Preparation of a Micro-Glass Pipette

Although it is not mandatorily required, a micro-glass pipette (Fig. 2.5) is extremely useful for transferring eggs one by one. Cut a glass tube with an external diameter of 4.0 mm and a length of 100–200 mm. Using a gas burner, heat the center of the glass tube until it is softened. Pull the glass with both hands, which will form two pipettes. Cut the tips of these pipettes with a file; the ideal pore size will be around 200 μm in diameter. Then, smooth the tip using a gas burner. A silicone tube, one end of which is plugged or tied, can be used as a bulb.

2.2.6 Preparation of Solutions

2.2.6.1 Seawater

Natural seawater should be purified using a 0.2-μm pore size filter before use. Commercially available artificial seawater may be used. We usu-

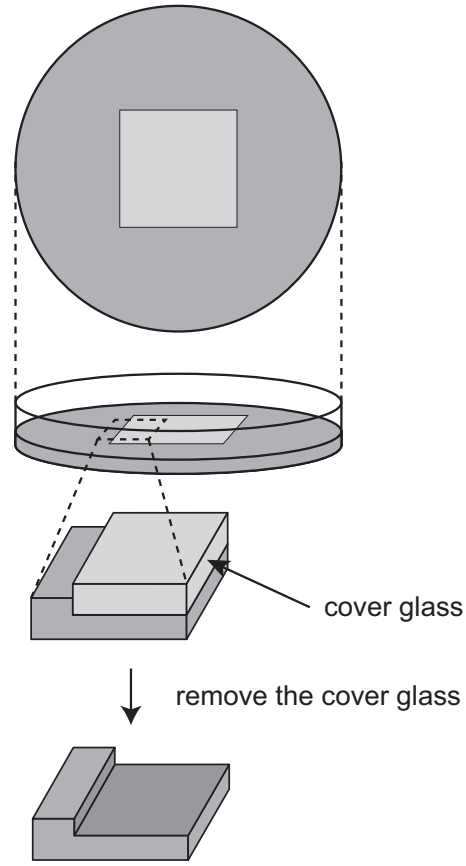

Fig. 2.4 Preparation of an agar-coated Petri dish for microinjection. By floating a cover glass before the agar is cooled, a small step can be made on the agar surface

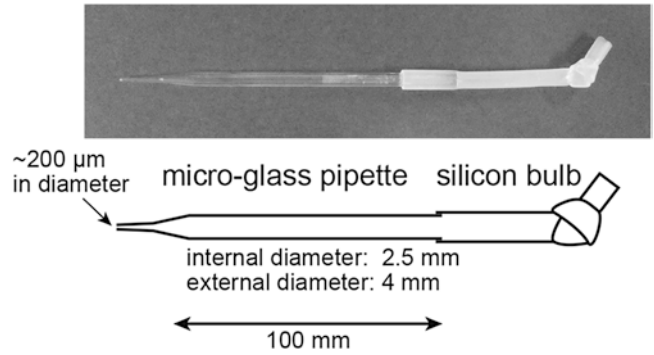

Fig. 2.5 A photograph and illustration of a micropipette, which is useful for manipulation of embryos

ally use Marine Art BR (Tomita Pharmaceutical, Naruto, Tokushima, Japan). Streptomycin can be added at the concentration of 50 µg/mL.

2.2.6.2 Dechorionation Solution

The dechorionation solution is prepared at ×2 concentration just before use.

- ×2 dechorionation solution
- 2% sodium thioglycolate
- 0.1% actinase (pronase) E
- 1 M NaOH

2.2.6.3 A Dye Indicator for Microinjection

- ×10 Fast Green FCF solution

Dissolve Fast Green FCF at a concentration of 10 mg/mL in nuclease-free water. This solution can be stored at room temperature.

2.3 Microinjection

2.3.1 Filling Needles with Nucleic Acid

Prepare an injection solution containing your nucleic acid and ×1 Fast Green FCF. You can mix multiple nucleic acids, if required. Using a micropipette, dispense up to 1 µL of the injection solution to the back end of a needle, which is placed on a needle stand. The filament within the needle guides the solution to the tip of the needle. Next, you need to fill up the rest of the needle with silicone oil using a 1- or 5-mL glass syringe with a fine metal needle (30-gauge, 2″ backfilling needle; Drummond Scientific, Broomall, PA, USA) behind the injection solution. Do not introduce air bubbles into the needle. We usually prepare five or more needles for one experiment. Keep the filled needles in a refrigerator or at room temperature.

2.3.2 Fertilization and Dechorionation

2.3.2.1 Eggs and Sperm

Pick up two healthy adult animals with a sufficient amount of eggs and sperm (ascidians are hermaphrodites), because self-fertilization rarely occurs. Because their body is transparent, you can easily find eggs (brown in *C. intestinalis* type A and orange in *C. savignyi*) and sperm along the intestine (see Fig. 2.1).

To obtain eggs and sperm, remove the tunic that covers the whole body manually using scissors. Then, carefully dissect the body to expose the oviduct and sperm duct. First, cut the oviduct and softly push out the eggs into a Petri dish filled with seawater. Second, cut the sperm duct and softly push out sperm into a different empty dish that is not filled with seawater (alternatively, you can use a Pasteur pipette to collect sperm). Repeat the same process for another ascidian. Now you will have two dishes with eggs and two dishes with sperm. Sperm can be mixed in a tube and kept in a refrigerator for several hours.

Eggs are viscous. Using a Pasteur pipette, continuously withdraw and dispense eggs into

seawater to minimize their viscosity (step 1 in Fig. 2.6). Swirl the dish to collect eggs around the center. Transfer eggs with a small amount of seawater into a new dish with fresh seawater.

2.3.2.2 Fertilization Before Injection

If you inject nucleic acid solutions into unfertilized eggs, skip this step. Add an aliquot of sperm to the dish in which eggs are kept (step 2 in Fig. 2.6). Sperm from a different individual (or a mixture of sperm from multiple individuals) should be used, because *Ciona* eggs are hardly ever self-fertilized. Mix the contents with a Pasteur pipette. Incubate a mixture of eggs and sperm for 5–10 min.

If you need to remove the chorion (vitelline membrane) of the eggs before injection, proceed to the next step. If you need to inject nucleic acid solutions into the eggs with the chorion, eggs should be washed with fresh seawater at least twice, the dish should be swirled to gather the eggs around the center, and transfer them with a minimum amount of seawater to a new dish.

Activation of sperm may be helpful for synchronized fertilization. Add 25 μL 1 M Tris-HCl (pH 9.5) to 500 μL seawater, and mix well. Then, add 10 μL sperm to the mixture, and mix well again. You will see actively moving spermatozoa under the stereo microscope. To fertilize the eggs, add 50 μL of the solution containing activated spermatozoa to a 6-cm Petri dish containing eggs and seawater.

2.3.2.3 Dechorionation

If you need to inject your nucleic acid solutions into eggs with the chorion, skip this step. In most experiments, dechorionated eggs are used for injection, because they are more amenable for injection than eggs with the chorion. Injection to *C. savignyi* eggs with the chorion is much easier than that to *C. intestinalis* type A eggs with the chorion (Hikosaka et al. 1992). However, it is known that ascidian embryos lose the left–right axis without the chorion (Yoshida and Saiga 2011), and larvae developed from eggs with the chorion may metamorphose at a higher rate.

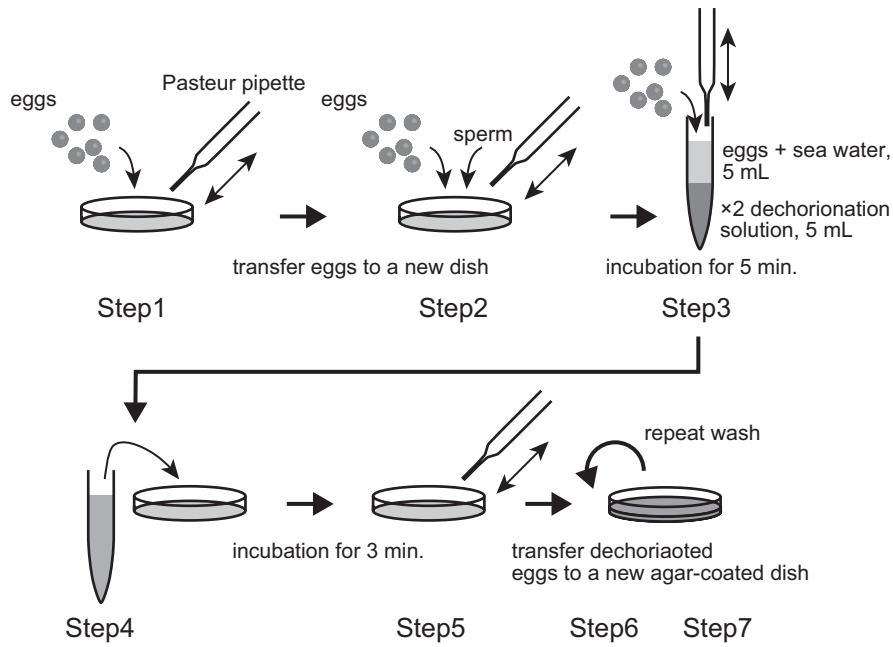

Fig. 2.6 Dechorionation procedure

Dispense 5 mL 2× dechorionation solution and 375 μL 1 M NaOH into a plastic 15-mL tube. Swirl the dish to collect eggs around the center, and transfer 5 mL seawater containing as many eggs into the plastic tube (now it becomes ×1 solution; step 3 in Fig. 2.6). Mix the content gently, and then transfer the entire content to a new 6-cm Petri dish. Incubate the reaction for at least 3 min (step 4 in Fig. 2.6). Observe the eggs under the stereo microscope to confirm that the vitelline membrane is beginning to break down. After that, mix the contents softly using a Pasteur pipette, and observe eggs under the stereo microscope (step 5 in Fig. 2.6). Immediately after chorions of almost all (~80%) the eggs are removed, swirl the dish and transfer the dechorionated eggs to an agar-coated dish filled with fresh seawater (step 6 in Fig. 2.6). After gentle mixing, swirl the content, and transfer the eggs into another agar-coated dish filled with fresh seawater (step 7 in Fig. 2.6). Repeat this wash one more time. It is important to reduce the time of the dechorionation step. Longer incubation in the dechorionation solution is harmful for normal development.

2.3.3 Microinjection Technique

2.3.3.1 Aligning Eggs

Align up to about 130 eggs along the step that was formed by a cover glass on the agar of an injection dish (step 1 in Fig. 2.7). A micro-glass needle is useful for this procedure.

2.3.3.2 Set Up the Instrument

Set a needle filled with the nucleic acid and silicone oil into an injection holder, the back end of which is connected to a polyvinyl chloride tube and a 10-mL glass syringe filled with water (see Fig. 2.2c). Take care not to introduce air bubbles into both a polyvinyl chloride tube and a 10-mL glass syringe. Then, fix the holder to the one-axis fine manipulator (micromanipulator), and fix it to the three-axis coarse manipulator.

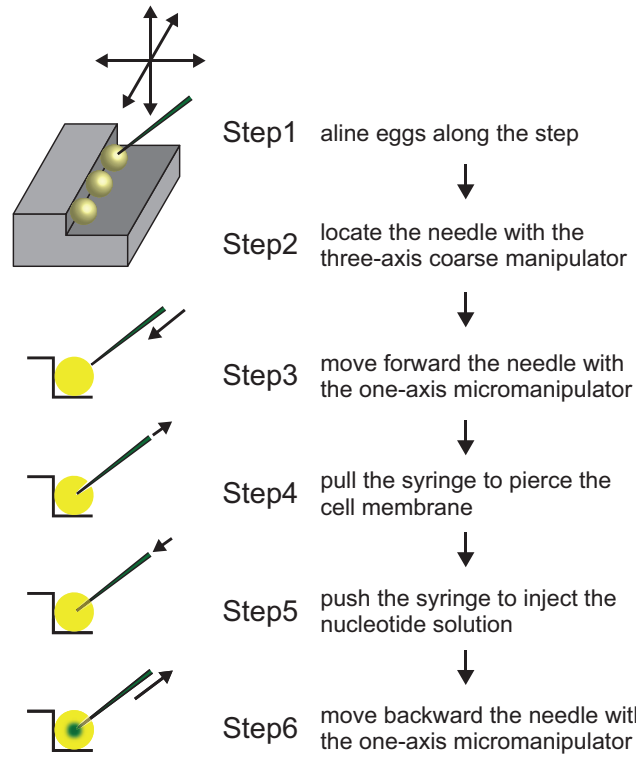

Fig. 2.7 Microinjection procedure. The step in this figure is the one created on the surface of agar, as explained in Fig. 2.4