3 Practical Guide for Ascidian Microinjection: *Phallusia mammillata*

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Abstract

Phallusia mammillata has recently emerged as a new ascidian model. Its unique characteristics, including the optical transparency of eggs and embryos and efficient translation of exogenously introduced mRNA in eggs, make the *Phallusia* system suitable for fluorescent protein (FP)-based imaging approaches. In addition, genomic and transcriptomic resources are readily available for this ascidian species, facilitating functional gene studies. Microinjection is probably the most versatile technique for introducing exogenous molecules such as plasmids, mRNAs, and proteins into ascidian eggs/embryos. However, it is not practiced widely within the community; presumably, because the system is rather laborious to set up and it requires practice. Here, we describe in as much detail as possible two microinjection methods that we use daily in the laboratory: one based on an inverted microscope and the other on a stereomicroscope. Along the stepwise description of system setup and injection procedure, we provide practical tips in the hope that this chapter might be a useful guide for introducing or improving a microinjection setup.

Keywords

Microinjection · Ascidian · *Phallusia mammillata* · Live imaging · Fluorescent protein

3.1 Introduction

3.1.1 *Phallusia mammillata* as a Model for Imaging-Based Studies from Egg to Tadpole

To take advantage of fluorescent microscopy techniques, it is standard practice to express fluorescent protein (FP)-fused constructs in living cells. In the case of ascidian models, electroporation or microinjection techniques are used to introduce either plasmid or mRNA constructs encoding FP-fusion proteins. Electroporation is not possible when the egg is covered by extracellular layers of cells, vitelline coats, egg shells, etc., and the removal of these vestments prevents correct embryonic development in many invertebrates. Remarkably, however, removing the extracellular chorion of ascidian eggs to expose

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the naked plasma membrane does not prevent their development. This has made it possible to simultaneously electroporate plasmids into hundreds of fertilized ascidian eggs, allowing the transient expression of FPs in a lineage-specific manner in the embryos of *Ciona intestinalis* (Corbo et al. 1997). Transient transgenesis is a powerful technique that has now been exploited in *Ciona* by many laboratories in the community (Christiaen et al. 2009; Matsuoka et al. 2005; Zeller et al. 2006). However, FP fluorescence, even when the FP is expressed under the control of an early acting promoter such as the *Fog* promoter, is not measurable until around the time of gastrulation (Roure et al. 2007). The technique is also dependent on the availability of promoters suitable for expressing FPs in the time and space of interest. Therefore, although it has revolutionized ascidian experimental studies, electroporation-based transient transgenesis is not useful for live-imaging events taking place from fertilization up to gastrulation.

To monitor FP fluorescence in unfertilized eggs and early embryos, mRNA or protein microinjection is necessary. As protein production is time-consuming and more challenging than making mRNA, we usually microinject mRNA. Unfortunately, microinjected mRNAs are not translated efficiently in unfertilized eggs or early embryos of some species of ascidian. Fortunately though, species such as *Phallusia mammillata* and *Ascidiella aspersa* do translate mRNAs efficiently in the unfertilized egg and during cleavage divisions (*Ascidiella*: Levasseur and McDougall 2000; *Phallusia*: Negishi and Yasuo 2015; Prodon et al. 2010). Interestingly, both these species have exceptionally transparent eggs and embryos, making them additionally useful for fluorescence microscopy (Fig. 3.1). We have been developing fluorescent GFP-based markers for use in *Phallusia* eggs/embryos for cell biology studies for many years, e.g., markers for the cytoskeleton (actin/microtubules), chromosomes/kinetochores, nucleus, centrosomes, and plasma membrane (Fig. 3.1) (McDougall et al. 2015). Here, we describe in detail two microinjection techniques we use to introduce exogenous molecules into *Phallusia mammillata* eggs: an inverted microscope setup and a stereomicroscope setup.

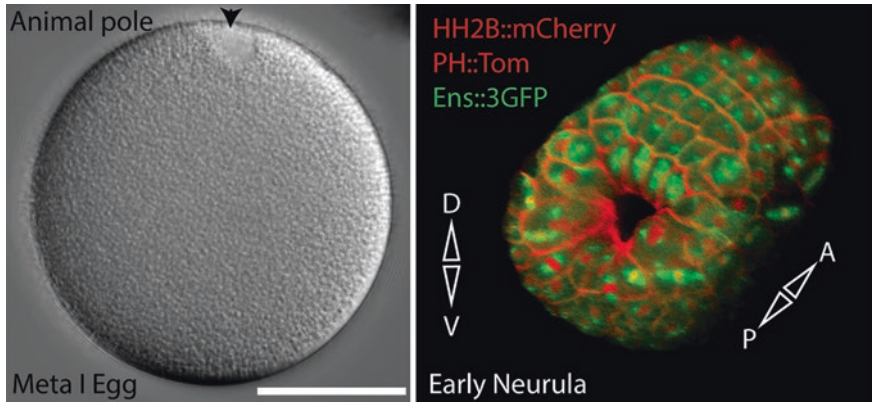

Fig. 3.1 Unfertilized *Phallusia* egg and early neurula. A differential interference contrast image of an unfertilized *Phallusia* egg at metaphase I. The animal pole containing the meiotic spindle appears as a clear zone (*arrowhead*). Early neurula embryo showing fluorescence from three mRNAs that were co-injected into the egg. PH::Tom (Tom = tdTomato) labels the plasma membrane (*red*), histone H2B::mCherry labels the chromatin (*red*) and Ens::3GFP labels the microtubules (*green*). The image is a projected z-stack from a confocal time-lapse that has been rendered in 3D using the VTK module in ICY software. *D* dorsal, *V* ventral, *A* anterior, *P* posterior. Scale bar = 50 μm

3.1.2 General Information About *Phallusia* and Transparent Ascidians

For those interested in *Phallusia* as an experimental model, there are now resources available including the genome sequence (Aniseed), several RNAseq transcriptomic datasets (Aniseed) and an arrayed cDNA library (searchable in Aniseed) (Brozovic et al. 2016). *Phallusia* is found exclusively in Europe. It is possible to obtain eggs and embryos from *Phallusia* from February through October and, if kept in the laboratory and fed, they will produce eggs during the winter, making it possible to work with them all year round (although springtime and fall is best). We have grown *Phallusia* at Villefranche-sur-Mer and the life cycle is about 6 months from the fertilized egg to the production of eggs capable of being fertilized. For those researchers wishing to use a transparent ascidian such as *Phallusia* outside of Europe, one can find *Ascidiella* on the east coast of the USA (e.g., Woods Hole), and in northern Japan (e.g., Asamushi Marine Station). Finally, note that other species of *Phallusia* such as *P. nigra* also produce transparent eggs.

3.2 Part 1: Inverted-Microscope Microinjection Setup (the McDougall Lab Setup)

3.2.1 Materials

3.2.1.1 Gelatin/Formaldehyde Coating

Gelatin crosslinked with formaldehyde produces a thin transparent film of gelatin on the glass or plastic surface when dry. Treatment of glassware and plastic dishes with 1% gelatin/formaldehyde (GF) solution (in water) makes them nonsticky for *Phallusia* eggs/embryos. To coat glass or plastic surfaces, apply a thin layer of GF solution, dry and rinse in water (Sardet et al. 2011).

3.2.1.2 Dechorionated *Phallusia* Eggs

Add 1 ml of 10× trypsin solution to 9 ml of seawater/10 mM TAPS (pH 8.2) containing chorionated *Phallusia* eggs in a GF-coated plastic petri dish (5 cm in diameter). Agitate (rotation, seesaw, etc.) gently for about 1½ to 2 h at 18–22 °C. When most eggs are "dechorionated" and have settled at the bottom of the dish, wash them several times with seawater. Transfer dechorionated eggs to a GF-coated dish.

- TAPS buffer stock solution: 500 mM TAPS, pH 8.2. Store at room temperature.
- 10× trypsin solution: 1% trypsin (Sigma, T-9201) in seawater/10 mM TAPS.

3.2.1.3 Injection Mix

We routinely microinject a number of reagents including proteins, synthetic mRNAs, plasmids, morpholino oligonucleotides, and fluorescent dyes into unfertilized eggs, fertilized eggs, and blastomeres. Generally, these reagents are diluted in water or injection buffer (180 mM KCl, 10 mM PIPES, 100 μM EGTA, pH 7.1). We dilute all mRNAs for microinjection in Molecular Biology Grade Water (5 Prime, Hamburg, Germany). We dilute dyes such as Fura-2 in injection buffer. To estimate injection volume, we measure the displacement of the cytoplasm upon microinjection (typically one tenth to one fifth of the diameter of an egg, which is roughly equivalent to 0.1% to 1% of the egg volume).

3.2.1.4 Injection Chamber, Glass Pieces, and Associated Reagents

Injection chambers are custom-made of Perspex (Fig. 3.2). VALAB (Vaseline/Lanolin/Beeswax 1:1:1) is used to attach small pieces of glass to coverslips when creating a microinjection wedge. Dow Corning Vacuum grease is used to fix coverslips to a Perspex mounting chamber.

3.2.1.5 Injection Needles, Puller, and High-Pressure Injection Box

We use glass capillaries without a filament (GC-100 T10, Harvard Apparatus) and pull needles with a Narishige horizontal puller (PN-30). Both needles are used following a single two-stage pull. Perhaps the most important factor in microinjection is that the needles are appropriate. For

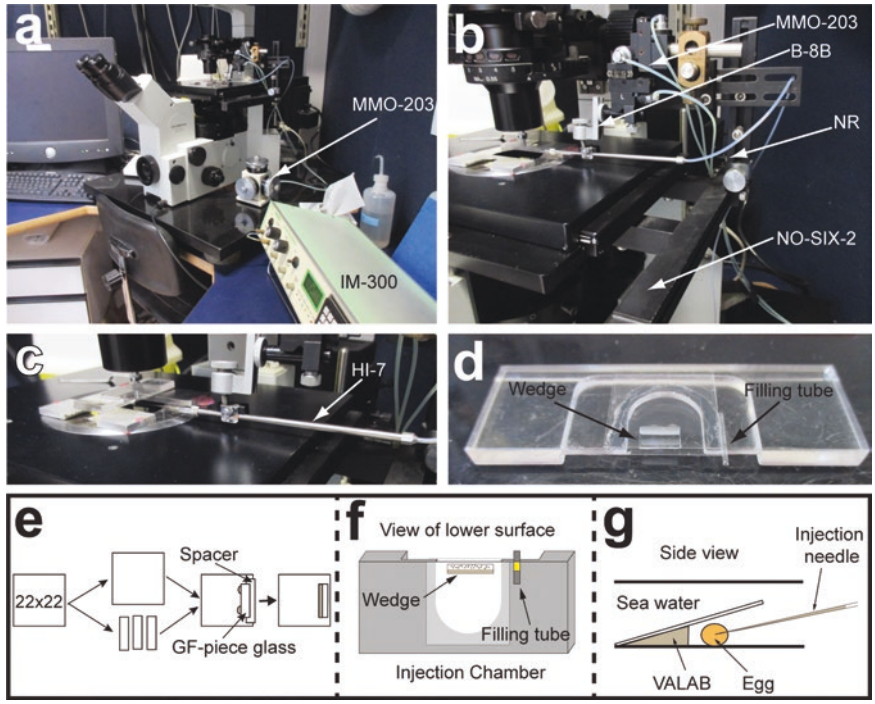

Fig. 3.2 Inverted microscope setup. (**a**) Olympus inverted microscope (IX70), with the three-way hydraulic micromanipulator highlighted (MMO-203). The high-pressure injection box is also visible (IM-300). The stage control is left mounted, allowing the use of the right hand for the micromanipulator and the left for the stage. (**b**) Close-up of the injection setup, showing the stage side mounting (NO-SIX-2), the mounting adaptor with rotation mechanism (NR), the ball joint for holding the injection needle, and the hydraulic three-way micromanipulator (MMO-203). (**c**) Close-up of the stage, injection needle holder (HI-7), and the injection chamber. (**d**) View of the lower surface of an injection chamber with a wedge and a filling tube attached. Note: as the microscope is inverted, both the wedge and the filling tube are on the side that face the objective lens (the lower side). (**e**) Schematic showing the preparation of a wedge. Briefly, a 22 × 22 no. 1.5 coverslip is coated with gelatin and cut into small pieces. A spacer is laid upon a GF coverslip. Then some VALAB is scraped onto a GF piece of glass, which is attached parallel to the spacer. Next, the coverslip is moved to a heating block to melt the VALAB, and the Spacer removed once the melted VALAB has solidified. (**f**) Schematic of the injection chamber with wedge and filling tube viewed from the lower surface. (**g**) Side view of an egg in a wedge being injected. The VALAB holds the GF piece of glass of the coverslip, but note that the angle of the wedge is sufficiently acute so that the egg does not touch the VALAB. If the eggs touch the VALAB they may die, so avoid this. If it proves difficult to get the perfect wedge at first, you could try using no. 1 or no. 0 coverslips to make the spacer form a more acute angle. Note: the position of the injection needle close to the center of the egg

Phallusia microinjection, success is improved with long thin needles (the shanks should be as narrow as possible). We pull using the following settings: second pull set to 10, heater 65.1, submagnet 13.8, main magnet 78.3 (however, heater and magnet settings alter as the platinum loop becomes older; thus, the shape of the needles should be inspected regularly). We use an IM-300 Narishige high-pressure microinjection box connected to a compressor providing around 100 psi air pressure to the injection box. Injection needles are tip-filled.

3.2.1.6 Injection Setup

Our injection setup is shown in Fig. 3.2 and comprises the following equipment.

- Inverted microscope (Olympus IX70) using a ×10 objective and transmitted light.
- Three-axis hydraulic micromanipulator (Narishige MMO-203)
- Stage-mounting equipment (Narishige NO-SIX-2)
- Needle holder (Narishige HI-7)

3.2.2 Method

3.2.2.1 Prepare Glass Wedge Setup for Microinjection

1. Glass coverslips (22 × 22, no. 1.5) are dipped in GF solution. Coat and air dry about 20 coverslips. Once dry, wash with tap water and air dry. Once dried, store for later use.
2. Using one of these gelatin coverslips, cut small pieces (about 10 mm long by 4 mm wide) with a diamond knife. Store for later use. For convenience, we call these GF pieces.
3. Prepare similar sized glass pieces (15 mm long, 4 mm wide) from virgin coverslips and store (we call these spacers).
4. To prepare the wedge, place a gelatin coverslip on a clean surface (a black plastic mat is ideal).
5. Using forceps, add a spacer to one side of the coverslip so that it lies parallel to one edge, slightly overhanging.
6. Using forceps, scrape some VALAB onto one GF piece of coverslip to create two small feet on one of the long edges (Fig. 3.2).
7. Place the GF piece parallel to the spacer so that the VALAB feet of the GF piece of coverslip attach to the coverslip next to the spacer (but avoid touching the spacer with the VALAB; Fig. 3.2).
8. Tap the GF piece of glass with the forceps so that it lies on the spacer at an angle.
9. Place immediately on a heated block to melt the VALAB, which flows along the edge of the GF piece of glass. Remove from the heated block and the VALAB solidifies, becoming opaque (this takes about 30 s).
10. Now, carefully remove the spacer leaving the small GF piece of coverslip glass attached to the coverslip. This creates a wedge-shaped chamber coated in gelatin to which the eggs can be added (See Fig. 3.2).

3.2.2.2 Fill the Filling Tube with Injection Material

1. Place the filling tube on the edge of a 5-ml petri dish and add 1 µl mineral oil.
2. Add 1 µl mRNA (or other injection material).
3. Add 1 µl mineral oil, forming an oil sandwich with mRNA in the middle.

Note: the filling tube containing mRNA can be kept for several weeks at 4 °C and reused.

3.2.2.3 Attach Glass Wedge to the Perspex Injection Chamber, Fill with Eggs, and Attach Filling Tube

1. Apply Dow Corning High Vacuum Grease to the upper and lower sides of the injection chamber where the coverslips will be attached.
2. Attach the wedge to the lower surface of the injection chamber.
3. Using a binocular microscope and a mouth pipette, add eggs to the wedge. With practice, up to about 100 eggs can be loaded into a wedge.
4. Once the eggs have been added to the wedge, a second coverslip is attached to the upper surface of the injection chamber, with the grease forming a sandwich.
5. Fill the resultant space between the wedge and coverslip with seawater (approximately 400 µl).
6. Attach the filling tube to the lower surface of the injection chamber parallel to the coverslip with the attached wedge so that the filling tube protrudes a little in front of the seawater opening (Fig. 3.2).
7. Move the injection chamber with the eggs to the inverted microscope for injection.

3.2.2.4 Microinjection

1. Lay the injection chamber with the wedge containing the eggs on the stage of an inverted microscope for injection. Focus on the filling tube using a ×10 objective lens. Note: the injection chamber needs to be raised about 1 cm above the surface of the stage, as the injection is horizontal.
2. Place a pre-pulled needle into the injection holder, clip into place, and advance the needle toward the filling tube by eye. As the viewer first focuses on the filling tube, both needle and tube are visible in the field of view using a ×10 objective.

3. View the needle and filling tube using oculars and with the three-way hydraulic micromanipulator, gently break the tip of the needle against the side of the filling tube, and move the needle into the filling tube to fill with mRNA, etc.
4. Go to the fill program on the IM-300 injection box and fill the needle. We use two programs: a fill program and an injection program. Both should be programmed using the instruction manual before starting. Fill the needle so that the tip of the needle and the meniscus both remain visible in the field of view with a ×10 objective.
5. Stabilize the movement of the meniscus with the balance pressure.
6. Once the meniscus is stable, activate the inject program, remove the needle from the filling tube, and advance it toward the eggs in the adjacent wedge.
7. Insert the needle into an egg such that the tip of the needle is about half way into the egg (Fig. 3.2).
8. Apply a short pulse of suction pressure using the fill button (100-ms pulse) to break the plasma membrane.
9. Inject by tapping the foot pedal, activating the injection program: our injection program consists of one 100-ms pulse each time the foot pedal is tapped. Adjust the injection pressure knob to select the appropriate pressure (typically this is between 10–20 psi [pounds per square-inch]). Note: the cytoplasm is displaced by the high-pressure rapid injection, allowing estimation of the injection size.
10. Remove the needle from the egg. Caution: the most delicate part of injecting *Phallusia* eggs is removing the needle. There is no single method for this, as it depends on the batch of eggs, some being more fragile than others. However, a good rule of thumb is to remove the needle slowly until the egg starts to move together with the needle, using the three-way hydraulic micromanipulator. At this point, the needle should be removed very rapidly using the stage control.
11. If the injected eggs are to be recovered and moved to a petri dish they can be pushed out of the wedge so that they lie at the edge of the wedge and can easily be recovered later using a mouth pipette or similar device on a binocular microscope.

With practice, about one egg per minute can be injected using this technique (or fewer eggs if the eggs are particularly fragile). If injecting unfertilized eggs proves difficult at first, fertilized eggs at the pronucleus stage are a little easier to inject, but there is a time constraint, because once they enter mitosis, it is not advisable to inject them, as this often perturbs cell division.

3.3 Part 2: Stereomicroscope Microinjection Setup (the Yasuo Lab's Setup)

3.3.1 Materials

3.3.1.1 Injection Mix

We routinely inject morpholino oligonucleotides, plasmids, in vitro synthesized mRNAs, and proteins into ascidian eggs/blastomeres. Solutions containing these molecules are mixed with Fast Green (see below), which allows the injected solution to be visualized in eggs/blastomeres and its volume to be estimated. When it is important to trace descendants of injected blastomeres, the injection mix is supplemented with fluorescent dextrans (see below; e.g., Haupaix et al. 2013).

- Fast Green FCF (Sigma–Aldrich): 1 mg/ml in distilled water (×2 to ×4 stock). Keep the working aliquot at room temperature, storing the remaining aliquots at −20 °C.
- Fluorescent dextrans, 10-kDa molecular weight (Texas-Red coupled, fluorescein-coupled, rhodamine-coupled, etc.; Molecular Probes): 2 mM in distilled water (×2 stock). Keep aliquots at −20 °C.
- Mineral oil (Sigma–Aldrich)
- Artificial seawater (ASW): 420 mM NaCl, 9 mM KCl, 10 mM $CaCl_2$, 24.5 mM $MgCl_2$,

25.5 mM $MgSO_4$, 2.15 mM $NaHCO_3$, and 10 mM HEPES buffer, pH 8.0. Sterilize with a 0.2-μm filter and add 0.05 g/l of kanamycin sulfate.

3.3.1.2 Injection Setup

The stereomicroscope injection setup is as shown in Fig. 3.3a, b using the following materials:

- Stereomicroscope (Leica S8APO with Leica TL BFDF, a brightfield–darkfield transmitted light base)
- Three-axis hydraulic micromanipulator (Narishige MMO-203)
- Three-axis coarse manipulator (Narishige M-152 or Harvard apparatus MM-33)
- Magnetic stand (Narishige GJ-1)
- Needle holder (Narishige HI-7)
- Iron plate

3.3.1.3 Tubing

We use the following materials to connect a glass syringe to the Narishige HI-7 needle holder via a Teflon tube (Fig. 3.3c). The Bio-Rad Tefzel tube that we use has an inner diameter (ID) of 0.5 mm, whereas the HI-7 needle holder is compatible with a tube of 1 mm ID. It is thus necessary to enlarge one end of the tube using the tip of a fine forceps. The other end of the tube is fitted with a set of Delrin nut/ferrule/lock ring (Bio-Rad 1.6 mm OD Post-Pump Fittings 750-0554) and can be connected to the male luer of glass syringe via a 1/4–28 female to male luer (Bio-Rad Luer to BioLogic System Fittings Kit, 732-0113; Fig. 3.3d). The entire system is filled with mineral oil.

- Teflon tube (Bio-Rad 1.6 mm OD Tefzel Tubing, 750-0602).
- Tube fittings (Bio-Rad Luer to BioLogic System Fittings Kit, 732-0113; Bio-Rad 1.6 mm OD Post-Pump Fittings, 750-0554).
- 2-ml glass syringe with a male Luer–Lock connection fitting.

3.3.1.4 Injection Needles

We use glass capillaries with filament and pull needles with a Narishige needle puller (PN-31). We use both needles resulting from a single pull. Needle pulling is one of the critical steps for a successful injection: the efficiency of the injection can change dramatically depending on the shape of the needles. The current setting of our PN-31 is: heater 84.4, sub-magnet 32.7, and main magnet 103.3. However, the optimal setting should be obtained for each machine and regularly revised. Needles are cut so that the straight capillary part (=pre-tapering part) is about 3 cm long. The blunt end should be rounded using the flame of a lighter so that the inner silicone gasket of the needle holder is not damaged.

- Glass capillaries (Harvard Apparatus Borosilicate Thin Wall with Filament, 1.0 mm OD, 0.78 mm ID, 100 mm L, 30-0038).
- Needle puller (Narishige PN-31)
- Glass cutter
- 5-ml glass syringe fitted with a metal 25-gauge hub needle. This syringe is used to fill injection needles with mineral oil after they are loaded with the injection mix.
- Needle carrier: we make this carrier using a plastic petri dish measuring 140 × 20 mm. Stick a polystyrene block of 15 mm × 15 mm × 150 mm on the petri dish lid with double-sided tape. Stick a strip of double-sided tape on the surface of the polystyrene block. Needles are stuck to the tape while they are loaded with injection mix and mineral oil and are held on the block until used for injection.

3.3.2 Injection Chamber

We make the injection chamber with 1.5% agarose in ASW using a mold (see Fig. 3.3e for how to make the mold and injection chamber. Note: refer also to Gregory and Veeman (2013) for a 3D printing-based method of making a mold.

- Coverslip (#1).
- Plastic block (about 15 mm × 15 mm × 5 mm).
- Double face tape.
- Plastic petri dishes (5 cm diameter).
- 1.5% agarose in ASW. Note: do not over-boil the agarose/ASW in a microwave so as not to change the salt concentration of ASW.

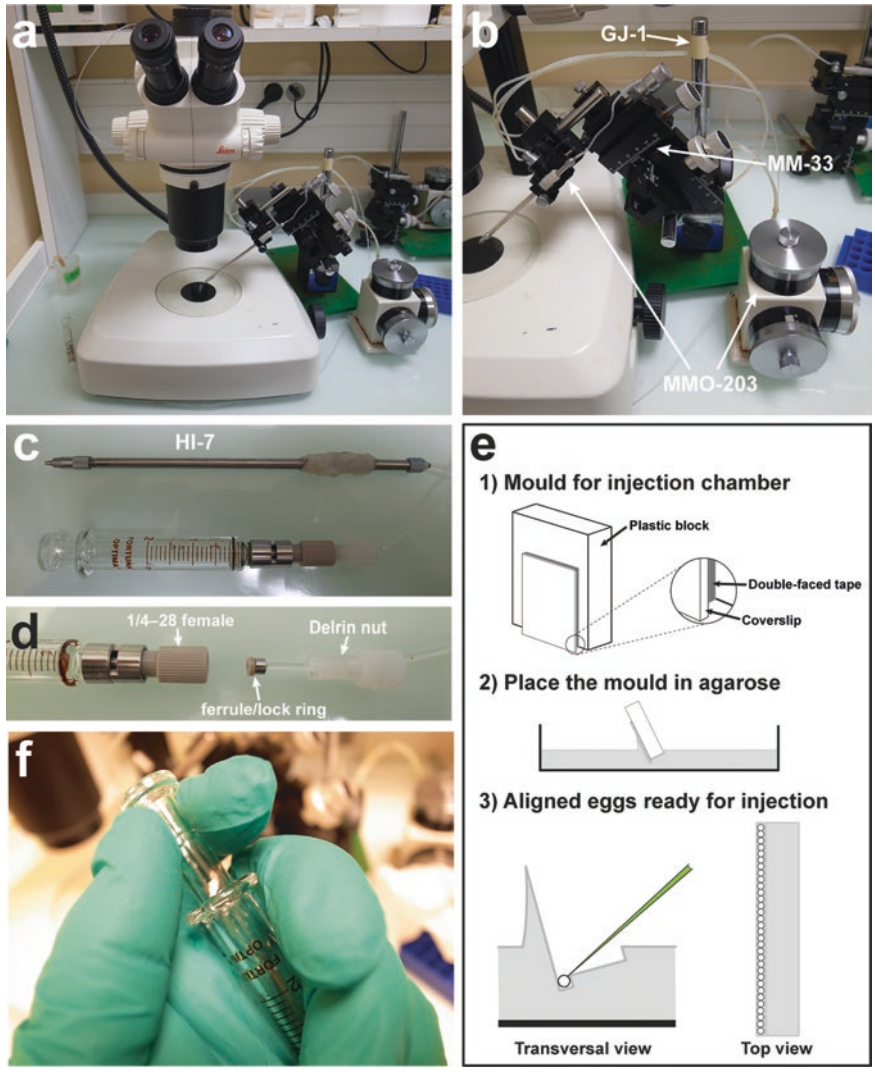

Fig. 3.3 Stereomicroscope microinjection setup. (**a**) Overview of our stereomicroscope microinjection setup. (**b**) Micromanipulator setup. (**c**) The assembly of the needle holder, Teflon tube and glass syringe. (**d**) Close-up of the connection of a Teflon tube to a glass syringe with male luer lock using Bio-Rad fittings kit components. (**e**) Schematics showing (*1*) how to make a mold for the injection chamber, (*2*) how to place the mound in melted 1.5% agarose/ASW, and (*3*) aligned eggs in the injection chamber. To obtain a perfect mold, the coverslip has to stick out from the edge of the plastic block, ideally by about 120 μm. This is not an easy task and requires much trial and error. Gregory and Veeman (2013) recently reported their successful application of a 3D–printing technology to make a microwell mold suitable for ascidian microinjection (Gregory and Veeman 2013). Their method represents a sophisticated alternative to mold making. To make the injection chamber using our mold, pour melted 1.5% agarose/ASW using a plastic pipette into a petri dish 5 cm in diameter and float the petri dish on ice-cold water. Before the agarose hardens, place the mold slightly tilted, as shown in the drawing, using forceps: make sure that the edge of the coverslip is not in direct contact with the bottom of the petri dish. Keep the mold in place until the agarose hardens and then let the petri dish float on ice-cold water for a few minutes. Take the mold gently out of the agarose. Cover the agarose with ASW and keep injection chambers at 4 °C. We use an injection chamber for several times over 1 week before disposing it. To align eggs into the groove of the injection chamber, first place dechorionated unfertilized eggs on the platform next to the groove and, using a gentle water flow from the micropipette, displace the eggs toward the groove and let them fall into it. (**f**) Image showing how to hold the injection syringe. This holding position allows both slight pulling and pushing of the plunger

- Micropipette: we use a hand-made micropipette to handle eggs/embryos. A glass tube (OD 5 mm, ID 3 mm) is pulled under the flame, so that one end becomes tapered to around 1 mm in diameter. To the large end, attach a rubber tube with the other end stapled (Sardet et al. 2011). Soak newly made micropipettes in water overnight; this treatment renders glass pipettes less sticky.

3.3.3 Method

3.3.3.1 Preparation of Injection Needles

1. Place the needle carrier with the needles vertical on the bench with the cut end of needles pointing upward. We normally prepare two needles per one injection chamber.
2. With a Gilson P2 pipette (or equivalent), place a tiny drop of injection mix (0.25 to 0.5 µl) onto the cut end of the needle. The injection mix should quickly go down along the inner filament toward the needle tip.
3. Tap the needle gently with a pencil to remove most of the air bubbles present in the tapered part of the needle.
4. Using the 5-ml glass syringe filled with mineral oil, fill up the needles with mineral oil. It is acceptable to leave small air bubbles at the interface between the injection mix and mineral oil, but make sure that there are no air bubbles in the straight capillary part of the needle up to the cut end.
5. Needles are now ready to be fitted into the needle holder.

3.3.3.2 Microinjection

1. Make sure that there are no air bubbles in the mineral oil in the assembly of the needle holder/tubing/syringe. Note: a small bubble in the syringe can be tolerated.
2. Insert a needle into the needle holder, making sure to avoid air bubbles. Note: push a little oil out of the needle holder using the syringe while pushing the needle into the holder; it gets a little messy with mineral oil spilling onto fingers.
3. Align dechorionated-unfertilized eggs into the groove of the injection chamber using a micropipette (Fig. 3.3e).
4. Cut a coverslip into a strip about 5 mm wide. Mark a spot (or line) on one side of the strip so that the mark is under the seawater. Stick the strip into the agarose of the injection chamber so that it leans on the wall of the petri dish with the marked surface facing toward you.
5. Set the injection chamber under the stereomicroscope and position and adjust the manipulator setup so that the needle tip is in the seawater around the centre of the injection chamber. Displace the injection chamber so that the needle tip comes very close to the marked surface of the coverslip strip. Under the stereomicroscope, focus on the needle tip and bring the needle tip "very slowly" toward the marked surface by controlling the oil hydraulic micromanipulator. During this process, maintain a gentle pressure on the glass syringe. When the needle tip makes contact with the coverslip, it breaks and the green injection solution oozes out of the tip. It is important to keep the opening of the needle tip as small as possible: the opening should not be seen. It is now ready to inject. Note: wear a powder-free nitrile glove on the hand handling the glass syringe.
6. Position the needle tip against the middle of the first egg.
7. Push the needle into the egg with the oil hydraulic micromanipulator so that its tip is placed around the center of the egg.
8. Pull back the syringe gently to break the egg membrane (nothing can be seen happening; this step takes trial and error). See Fig. 3.3f for how to hold the syringe.
9. Push the syringe gently to dispense the injection mix into the egg. A colored sphere of injected solution forms around the needle tip, but it should quickly disperse. Injection volume can be controlled based on the diameter of the sphere before it disperses: we aim to control the diameter of the sphere to about one quarter of the egg's diameter. Note: if the

injected solution remains as a distinct sphere, the egg membrane was not broken at step 8. If this is the case, repeat the step.

10. Pull the needle gently out from the egg. Position the needle at the second egg and repeat steps 7 to 10. Note: during injection, keep the eggs in focus.

3.3.3.3 Troubleshooting

1. In our experience, using the stereomicroscope injection setup, *Phallusia* eggs are more fragile than *Ciona* eggs, making it more challenging to inject them (e.g., around 20 injected eggs can be obtained from one injection chamber of lined-up *Phallusia* eggs compared with about 50 with *Ciona* eggs). It is clear when *Phallusia* eggs are killed by injection as they become opaque (Movie 3.1).
2. The plunger of the injection syringe has to move "very smoothly." Tiny dusts between the plunger and barrel could prevent its smooth movement, which significantly hinders successful injection. Make sure that the plunger and inner wall of the barrel are clean and move smoothly (always use powder-free gloves to handle the syringe).
3. Make sure that there are no air bubbles in the mineral oil in the assembly of the needle/needle holder/Teflon tubing.
4. When the needle tip starts to become clogged, there are three options: (a) stab the needle tip into the agarose of the injection chamber to remove cellular debris from the needle tip, (b) repeat step 5 to reopen the needle tip or (c) change to a new needle.

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