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Technical Note

Injection of DNA into the medaka oocyte nucleus

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I. Preparation of glass tools

1. *Microneedles for microinjection*

Apparatus Puller (NARISHIGE PN-3, supplied by Narishige Co., Tokyo).
Grinder (NARISHIGE EG-4).
Materials ϕ1 mm glass tube (NARISHIGE G-1).

1) Soak glass tubes in concentrated nitric acid overnight.
2) Rinse in distilled water.
3) Boil 3 times in distilled water for 20 min each time and dry.
4) Stretch glass tubes with the puller to make microneedles.
5) Hone microneedles on a whetstone (Fig. 1A) to sharpen them like a bamboo spear (Fig. 1B), which make it easier to puncture oocytes. Let drops of distilled water fall on the whetstone surface during sharpening. Blow out microneedles with air from a syringe to eliminate splinters of glass, dust, and water from microneedles (Fig. 2).
6) Check microneedles under microscope. Select microneedles with tips of 5 μm inner diameter and free from dust.
7) Sterilize microneedles for 40 min at 180°C.

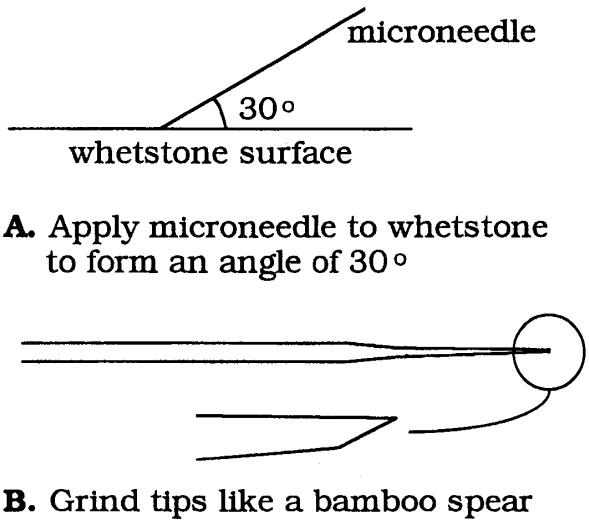

Fig. 1. Sharpening tips of microneedles.

2. *Capillary pipets for loading DNA*

Apparatus 2 mm diameter gas microburner.
Materials Glass microhematocrit tube (ϕ2 mm).

1) Treat glass tubes as described in 1–3 in I-1. *Microneedles.*
2) Holding both ends of the tube by hand, heat the centre of the tube while rotating it.
3) Pull the tube out to 20 cm long.
4) Cut at the centre.
5) The outer diameter should be 0.2–0.3 mm. Confirm whether the capillary will enter a microneedle from the thick end to the narrowest part.
6) Sterilize capillaries for 40 min at 180°C.

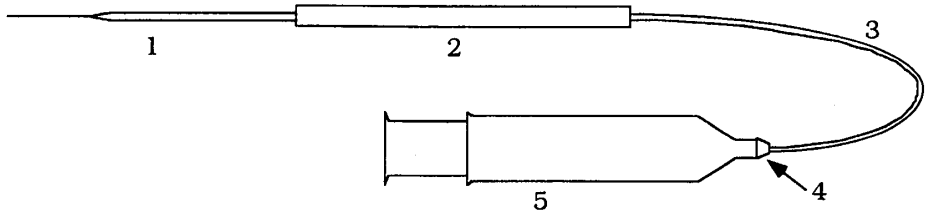

Fig. 2. Assembly of microneedles. 1. Microneedle; 2. Needle holder; 3. Polyethylene tube; 4. Joint; 5. Syringe (5 ml).

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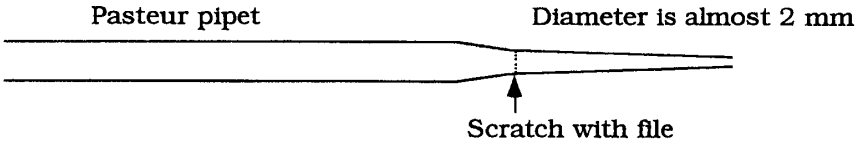

Fig. 3. Pipet for oocytes.

3. *Pipet for oocytes*

Apparatus Gas burner; File.
Materials Pasteur pipet.

1) Scratch to cut a Pasteur pipet with a file at the fine point (Fig. 3). The inner diameter of the end should be about 2 mm.
2) Heat the cut end to polish.
3) Confirm whether it can draw up oocytes in practice.

4. *Pipet for fertilized eggs*

Makes as described in **3.** *Pipet for oocytes.* However, the diameter must be a little larger to accommodate the size of fertilized eggs.

5. *Glass holder for fixing oocytes*

Apparatus Glass cutter; Minitor (micro-grinder).
Materials Micro-slideglass, 76 × 26 mm; Glass bar, 5 × 2 mm; Glass bar, 5 × 3 mm; Hemacytometer cover-glass; Micro-standard coverglass; Lead weight; Glass glue.

1) Make a glass holder as illustrated in Fig. 4.
2) Notches are made with a micro-grinder. Be careful not to make notches too big. The size of a slightly caved-in oocyte is suitable.
3) The holder is 0.6 mm thick.
4) Glue materials with glass glue.

6. *Glass bar for orientation of oocytes*

Apparatus Gas burner.
Materials Glass tube, ϕ5 mm; Fishing line, size 0.3.

1) Heat glass tubes in gas flame and pull.
2) Divide them into two at the finest part.
3) Pass fishing line into glass tubes from the thick end. Knot end of fishing line so it won't go all the way through (Fig. 5).
4) Glue to the tip of glass tubes with quick-dry glue. The fishing line is 15 mm long at the tip.

II. Setting up of manipulator and injector system

1) Construct a manipulator system by combining a light-weight three-dimensional manipulator (NARISHIGE M-4) with a joy stick hydraulic micro-manipulator (NARISHIGE MO-102) (Fig. 6).
2) Construct an injector system by connecting each part with polyethylene tube as shown in Fig. 7.
3) Fill tubes with distilled water, taking care not to let air bubbles enter tube. Air bubbles disturb transmission of pressure.

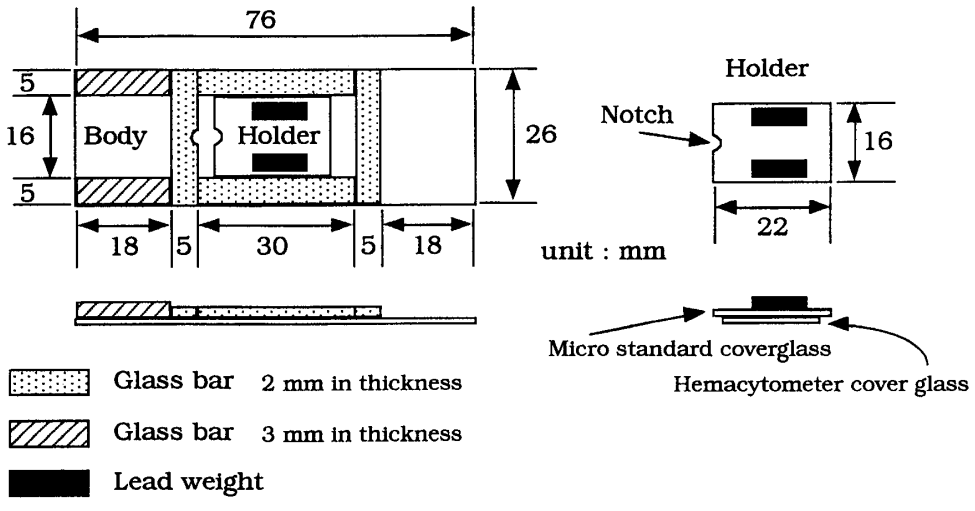

Fig. 4. Preparation of glass holder.

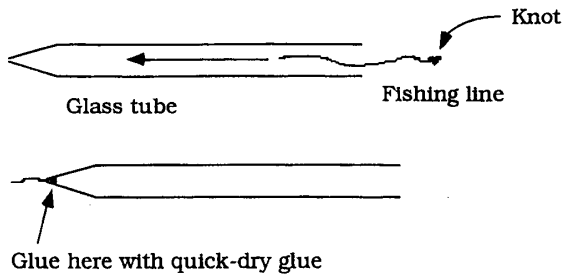

Fig. 5. Glass bar for orientation of oocytes.

III. General procedure of experiments

Experiments are carried out as shown diagrammatically in Fig. 8. *Preparation of oocytes* includes dissection of females, selection of oocytes, placing in glass holder, and orientation of the direction of oocyte nuclei, each of which is explained in detail in section IV. *Microinjection* includes loading DNA solution into microneedles, adjusting micromanipulator, and microinjection. *Culture of oocytes* includes removal of follicles. *Artificial insemination* includes dissection of males and preparation of sperm suspension.

IV. Preparation of oocytes

1. *Modification of spawning time*

Medaka lay eggs at dawn in nature. For experiments, medaka are maintained under lighting conditions of 14-h light and 10-h darkness. Under these conditions, medaka spawn at the time lights go on. In the case shown in Fig. 8, the light turns on at 18:00 and off at 8:00. Spawning time can be adjusted to the experimental plan by shifting the lighting time. About 2 weeks after the shift, medaka will lay eggs adaptively to the new light condition.

2. *Dissection of female fish*

1) Isolate female fish which are laying eggs and put them in separate tanks at 18:00 (light on) the day before experiments. These fish are expected to spawn on the following day too.
2) Sacrifice female fish by cutting the back part of the head with scissors at 8:00 (light off). Dissect females (Fig. 9) and remove ovaries.
3) Place ovaries in dish filled with culture medium (below).

Oocyte culture medium: Earle's 199 medium = 2% bovine serum albumin / 2 mM $NaHCO_3$ (pH 8.0).

3. *Preparation of oocytes*

1) Isolate oocytes which are due to be laid that day under a dissecting microscope. Largest and most transparent are suitable for microinjection.
2) Remove small immature oocytes and tissues surrounding the oocytes.
3) Transfer oocytes to the glass holder with pipet for oocytes.
4) Adjust the direction of an oocyte nuclei to the front of the microneedle with orientation glass bar under a dissecting microscope (Fig. 10A).

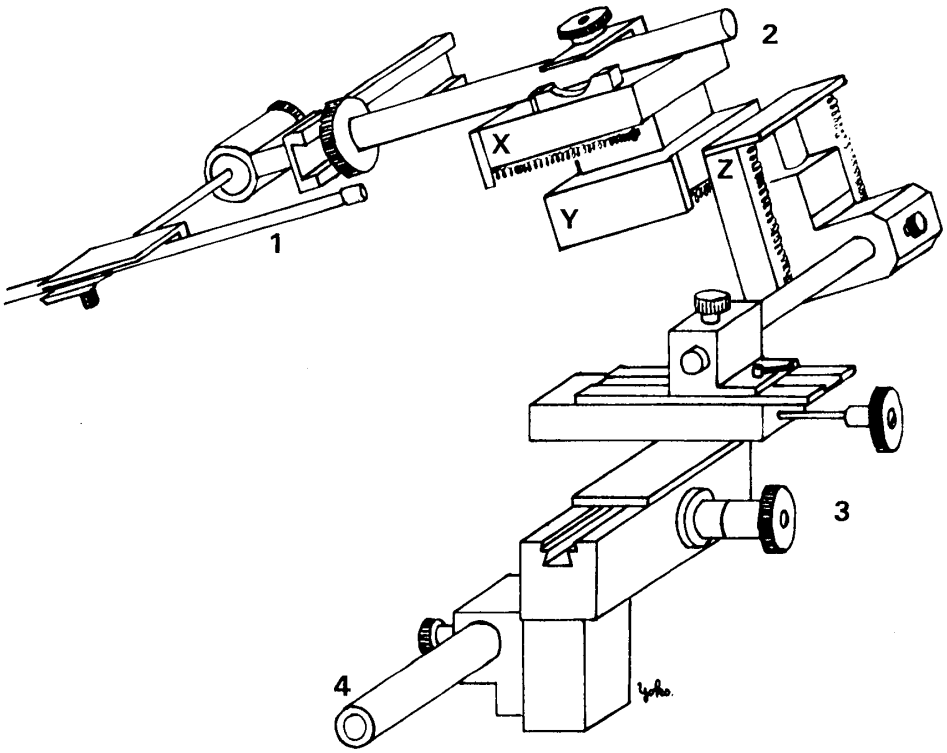

Fig. 6. Construction of a manipulator system. 1. Needle holder; 2. Jot stick hydraulic micro-manipulator (NARISHIGE MO-102); 3. Light-weight three-dimensional manipulator (NARISHIGE M-4); 4. Stainless pipe. Adjust 1 to be parallel with X-axis of 2. Fix 4 to the stage of the microscope using joints.

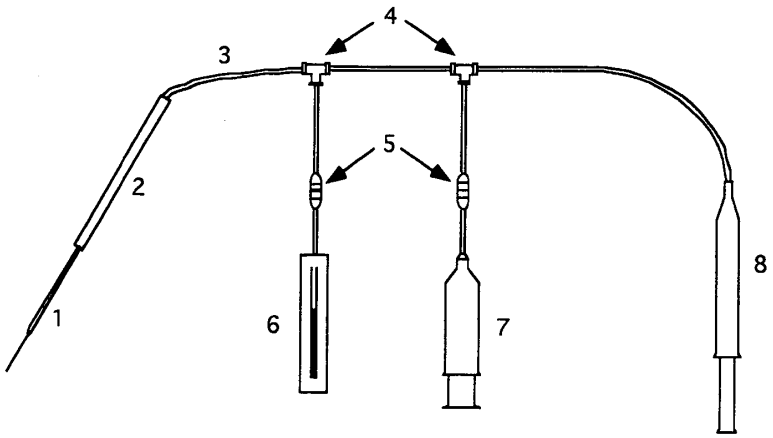

Fig. 7. Connection of injector. 1. Microneedle; 2. Needle holder; 3. Polyethylene tube; 4. Three-way joint; 5. Open and shut valves; 6. Water-pressure meter; 7. Syringe to supply water and adjust water pressure to atmospheric pressure; 8. Injector. When connecting parts, be careful not to let air bubbles enter.

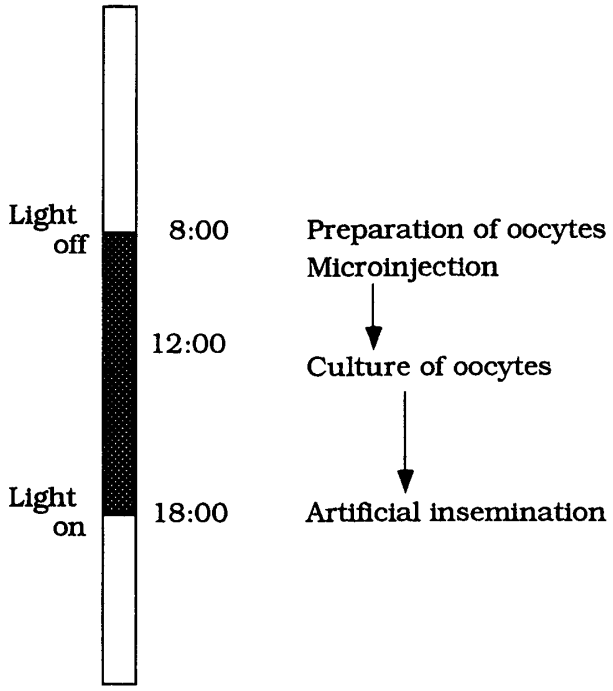

Fig. 8. A schema for experimental procedures to show the time schedule of oocyte isolation, DNA injection, and maintenance and activation of injected oocytes.

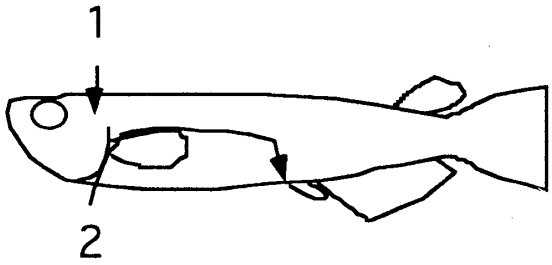

Fig. 9. Dissection of fish. Sacrifice the fish by cutting at 1 with scissors. Cut open along with 2 and remove an ovary or a testis.

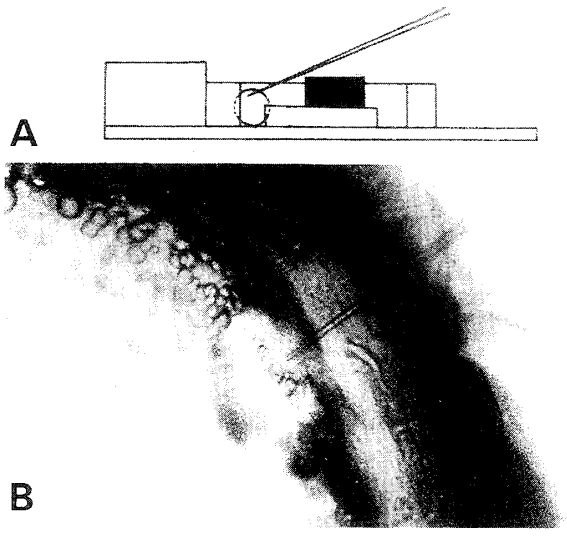

Fig. 10. **A.** Adjust the direction of an oocyte nuclei to the front of the microneedle. **B.** Injection of DNA solution into an oocyte nuclei.

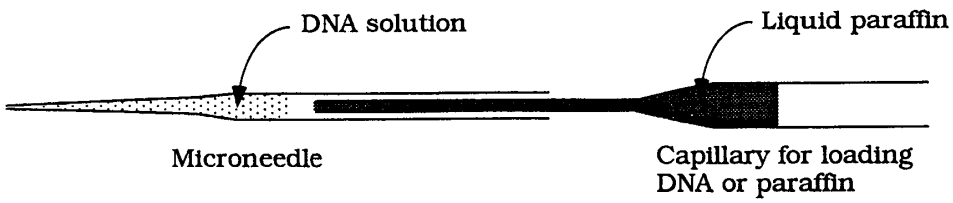

Fig. 11. Loading DNA solution and fluid paraffin into a microneedle.

V. Microinjection

1. *Loading DNA solution into a microneedle*

1) Draw up DNA solution with a loading capillary pipet and inject it into the microneedle from the thick end (Fig. 11).
2) Inject liquid paraffin next to DNA solution in the same way.
3) Connect the microneedle to the needle holder. Be careful not to let air bubbles enter.

2. *Microinjection*

1) Place the glass holder on the microscope.
2) Adjust the location of the microneedle using the micro-manipulator.
3) Inject DNA solution into the oocyte nucleus using the injector (Fig. 10B). Before experiments using DNA solution, practice injection using colored solution such as phenol red.
4) Remove injected oocytes to dish filled with medium. Oocytes at room temperature are suitable for injection until almost 12:00 noon, when progressive flattening of nuclei makes injection difficult. About 100 oocytes can be injected in this period. The period for microinjection can be prolonged by storing them at 4°C to retard development.

3. *Culture of oocytes*

1) Incubate injected oocytes in medium at 26°C. Around the time of lighting (18:00), ovulation begins and follicles peel off.
2) Peel off remaining follicles with tweezers.

4. *Artificial insemination*

1) Dissect male fish (Fig. 9) prior to the time of artificial insemination at 18:00.
2) Take the testis out and suspend sperm in medium on a hole-slideglass.
3) Remove oocytes to another hole-slideglass and add sperm suspension.
4) Wash fertilized eggs with distilled water when chorion hardens, about 1 hr after fertilization.
5) Incubate in 0.5 ppm methylene blue solution at 26°C.

VI. Staining for detecting the β-galactocidase gene expression

When the β-galactocidase gene is injected, the transgene expression is detected by staining with X-gal.

1) Fix eggs in 3% glutaraldehyde/PBS for 15 hr at 15°C.
2) Wash 3 times with PBS.
3) Place in X-gal solution overnight at 37°C to develop color.
4) Wash 3 times with 1 mM EDTA/PBS.

X-gal solution: 1 mM $MgCl_2$/PBS, 740 μL; 50 mM $K_3[Fe(CN)_6]$, 120 μL; 50 mM $K_4[Fe(CN)_6]$, 120 μL; 10% Triton X-100, 10 μL; 20 mg/mL X-gal, 25 μL.

Reference

Ozato, K., H. Kondoh, H. Inohara, T. Iwamatsu, Y. Wakamatsu and T.S. Okada (1986) *Cell Differ.*, **19**: 237–244.