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Technical Note

Production of chimeric medaka (*Oryzias latipes*)

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I. Preparation

1. *Fish*

Two medaka (*Oryzias latipes*) strains different in the body color are used; a wild-type (donor) and an albino (recipient) strains.

2. *Reagents and Equipments*

90% calcium- and magnesium-free phosphate-buffered saline (90% CMF-PBS) (Table 1); Balanced salt solution for medaka (BSS) (Iwamatsu, 1983) (Table 2); BSS supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (BSS + PS); BSS supplemented with 2 ppm methylene blue (BSS + MB). Wide-mouthed pipet (Fig. 1); Stereoscopic microscope (magnification of 60–70); Plastic suspension culture dish, diameter 60 mm (SUMILON, Sumitomo Bakelite Co., Tokyo); Fine forceps (INOX No. 5, A. Dumont & Fils, Switzerland); Micromanipulator (NARISHIGE MN-2, Narishige Co., Tokyo); Microneedle (Fig. 2); Loading capillary (Fig. 3); Degassed water; Liquid paraffin; Orientation needle (glass tube with plastic thread at tip) (Fig. 4).

Table 1. Composition of 90% CMF-PBS. To adjust to osmotic pressure of fish, Ca^{2+}- and Mg^{2+}-free PBS is diluted to 90% with distilled water.

PBS [Dulbecco's formula without magnesium and calcium (Takara, Kyoto)]	1 pellet
Distilled water	111 ml

Table 2. Composition of BSS. Dilute 25 ml of solution A with 475 ml of distilled water and autoclave. Add 1 ml of solution B to this solution.

Solution A:	NaCl	65 g
	KCl	4 g
	$MgSO_4 \cdot 7H_2O$	2 g
	$CaCl_2 \cdot 2H_2O$	2 g
	Phenol red	5 mg
	Add distilled water to make 500 ml.	
Solution B:	5% $NaHCO_3$ in distilled water. Sterilize by filtration.	

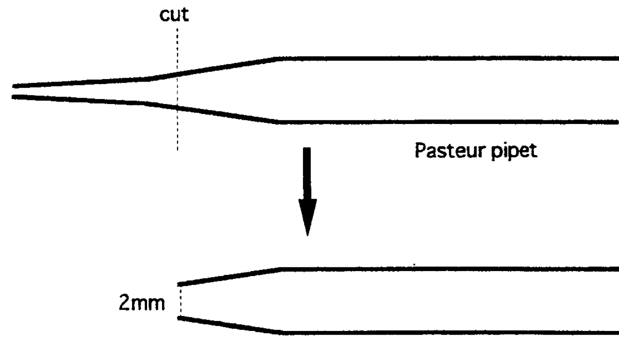

Fig. 1. Wide-mouthed pipet. Wide-mouthed pipets are made by cutting Pasteur pipets where the inner diameter is 2 mm and fire-polishing the cut end.

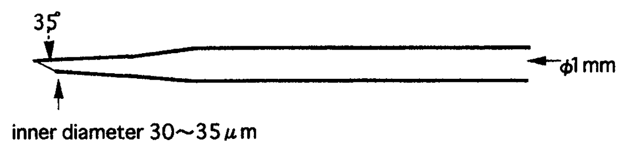

Fig. 2. Microneedle. Microneedles are prepared by pulling 1 mm (outer diameter) glass capillary tubing with a puller (NARISHIGE PN-3, supplied by Narishige Co., Tokyo). The tip of microneedle is sharpened with a needle sharpener (NARISHIGE EG-4). The inner diameter at the opening is 30–35 μm and the angle is 35°.

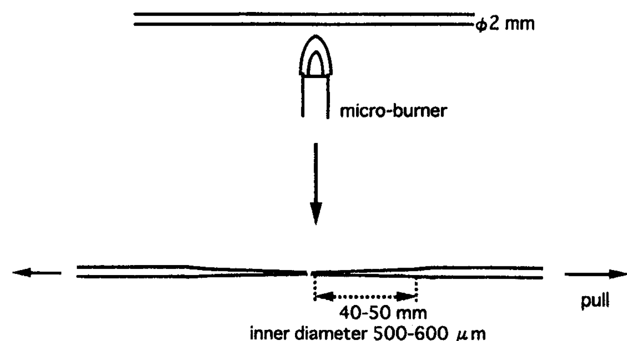

Fig. 3. Loading capillary. Loading capillaries are made by heating 2 mm (outer diameter) glass capillary tubing by hand to a length of 40–50 mm. The inner diameter at the opening is 500–600 μm, which is slightly smaller than the inner diameter of capillaries for microneedles.

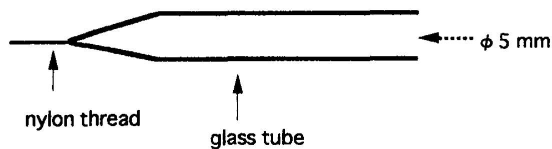

Fig. 4. Orientation needle. Orientation needles are made by fixing the plastic thread to the tip of 5 mm (outer diameter) glass tubing.

3. *Preparation of hatching enzyme solution*

1. Culture embryos in 0.5 ppm methylene blue solution at 26°C and harvest them just before hatching (8–9 days after fertilization). To remove methylene blue, transfer them to distilled water one day before collection.
2. Put harvested embryos in a homogenizer and add equivalent volume of 90% CMF-PBS. Homogenize embryos on ice and store at 4°C overnight.
3. Centrifuge the homogenate at 15,000 rpm for 10 min at 4°C.
4. Dispense the supernatant by 100 μl aliquots into 1.5 ml centrifuge tubes and store at –80°C.

4. *Preparation of agar plates*

Embryonic cell transplantation is carried out on an asceptically prepared agar plate.

1. Pour sterilized BSS containing 2% agar into a 6 cm plastic dish to a depth of 3 mm.
2. After the agar hardens, make two V-shaped grooves 1 mm wide and 1 mm deep for orientation of embryos with a sterile razor blade (Fig. 5). It is easy to make a groove using a razor blade held by a forceps.

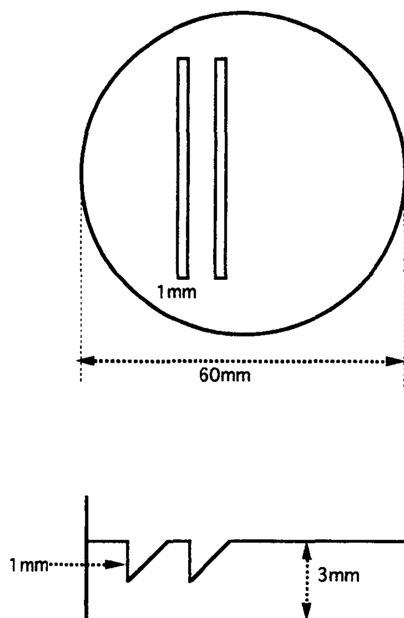

Fig. 5. Agar plate. Two V-shaped grooves are made at one side of the agar plate.

II. Collection of embryos

Embryos at midblastula-stage (Iwamatsu, 1994) are used for transplantation. Collect embryos before morula stage because embryos continue to develop during the hatching enzyme treatment.

1. Scoop females up with a net and collect clusters of eggs for the abdomen.
2. Place egg clusters in the center of a paper towel and roll them, pressing gently with another paper towel. Do not press the clusters firmly, as embryos at the early stage are easily injured. The attaching filaments are broken and removed by this treatment, and egg clusters separate into single eggs.
3. Transfer single eggs to dishes to distilled water. Remove unfertilized (lacking fertilization membrane) and dead eggs. Eggs whose chorion has been dented in the process of removal of attaching filaments return to normal after a short time, but eggs whose blastoderm has become blackish soon die.

III. Removal of chorion

Medaka embryos are covered with a 2-layer chorion that has a hard inner layer and a soft outer. Glass needles penetrate the hard chorion with difficulty so that operations such as aspiration and injection of blastodermal cells cannot be performed smoothly. Therefore, embryos dechorionated with medaka hatching enzyme are used for transplantation. The hatching enzyme is protease, which is secreted from the hatching enzyme gland and dissolves the inner layer of the chorion (Yasumasu, *et al.*, 1994). Dechorionated embryos develop normally and at the same rate as embryos with a chorion.

1. Arrange eggs in a monolayer in a well of a 24- or 96-well plastic plate. If eggs are piled on top of each other, eggs at the bottom are crushed when the chorion dissolves. Immerse eggs in the hatching enzyme solution and keep at 26°C. Check periodically under a stereoscopic microscope for dissolution of chorion. When the enzyme begins to work, a number of lunar crater-like holes open in the inner layer, and the inner layer soon disappears, generally within 30–60 min. After this treatment, use sterile tools and reagents.
2. Add BSS + PS gently after the inner layer dissolves.

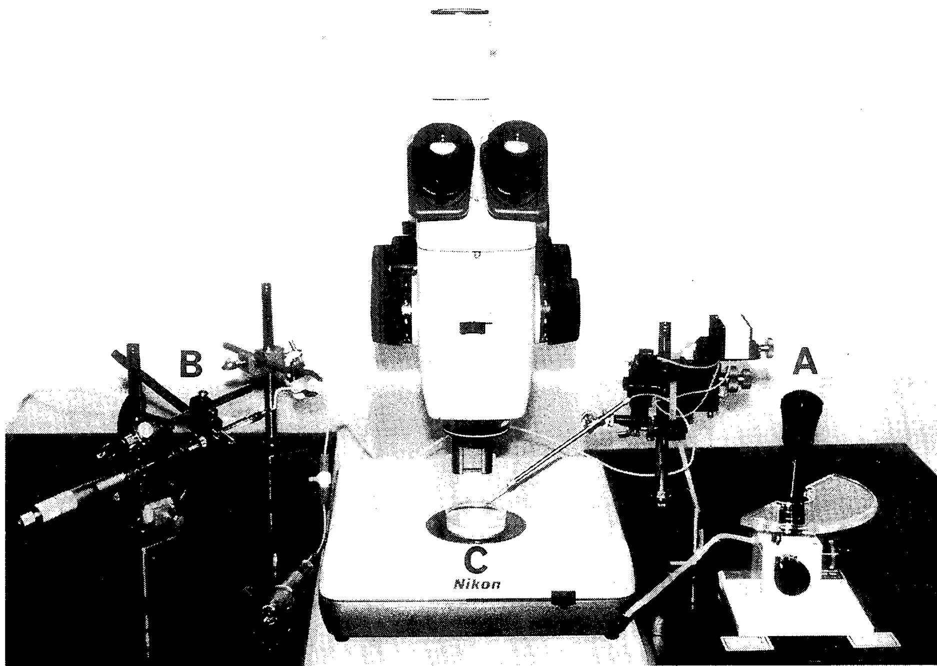

Fig. 6. Injection apparatus. A, Micromanipulator. B, Injection system. C, Agar plate.

3. Pipet gently with a wide-mouthed pipet to float embryos. Draw up slowly floating embryos and transfer gently to BSS + PS in a plastic suspension culture dish. Use this type of dish after this step to prevent dechorionated eggs from adhering to dishes.
4. Transfer embryos to fresh BSS + PS to remove hatching enzyme.
5. Pinch the outer layer with a fine forceps sterilized in 70% alcohol and agitate gently it in BSS + PS. The outer layer of the chorion can be easily removed.
6. Transfer dechorionated eggs only to fresh BSS + PS with a wide-mouthed pipet. Do not make dechorionated eggs come into contact with air. When dechorionated eggs are drawn up with air bubbles, they collapse instantly.
7. Incubate embryos at 26°C until they develop to midblastula-stage, and store them at 4°C to stop development until use.

IV. Transplantation

Transplantation is carried out under a stereoscopic microscope with a micromanipulator (Fig. 6).

1. Draw degassed water up in a loading capillary. Insert the loading capillary into a microneedle from the back and fill the microneedle with degassed water. Don't allow air bubbles to enter the microneedle.
2. Place the microneedle filled with degassed water in the needle holder of the micromanipulator. Draw liquid paraffin up to 10–15 mm from the top of the microneedle. Liquid paraffin prevents intense pressure changes in the needle.
3. Pour BSS + PS onto agar plate. Place midblastula-stage donor embryos in one groove and same-stage recipient embryos in the other. With an orientation needle, arrange embryos with blastoderms facing the microneedle (Figs. 7 and 8).

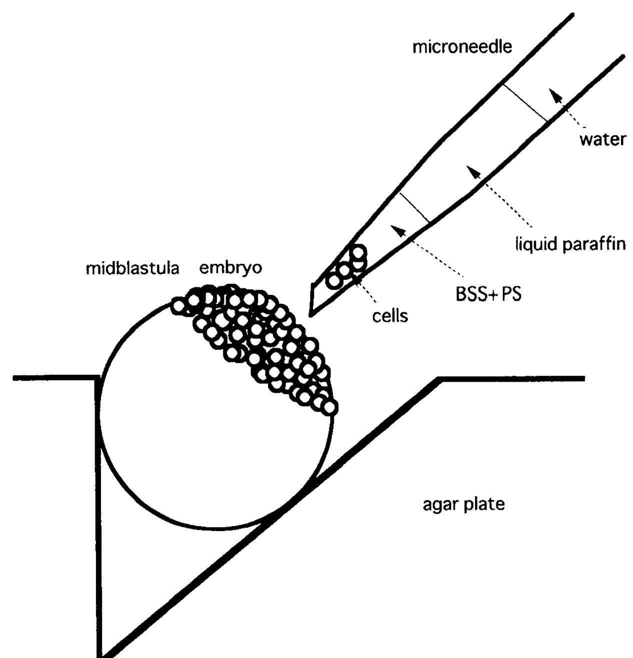

Fig. 7. Schema of transplantation. Approach of the microneedle to an embryo from the side without grooves.

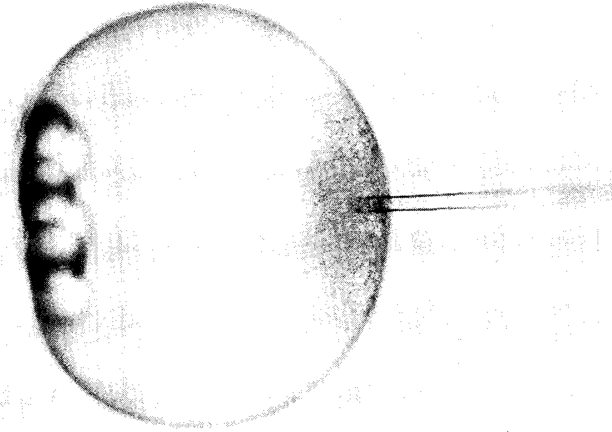

Fig. 8. Photograph of transplantation.

4. Puncture the donor blastoderm with microneedle and slowly draw up donor cells from deep in blastoderm core.
5. Pull microneedle out of donor blastoderm and wait a few minutes while aspirated cells fall down and gather at the tip of the microneedle.
6. Insert microneedle into the recipient blastoderm and inject harvested cells slowly.

V. Culture and observation of transplanted embryos

1. Incubate transplanted embryos in BSS + MB at 26°C.
2. Change BSS + MB every 3 days.
3. Remove dead embryos. Dead embryos stain blue with methylene blue. Handle the dish gently because dechorionated embryos are sensitive to mechanical shock. Transplanted embryos develop at nearly the same speed as control embryos.
4. After 2–3 days, melanophores appear on the yolk sac, head, eyes, and trunk of transplanted embryos (Fig. 9). Appearance of melanophores is a sign of successful formation of chimeras.
5. After 7–10 days, embryos begin swimming, which shows that embryos have hatched.

See Wakamatsu *et al.* (1993) for description of general methods.

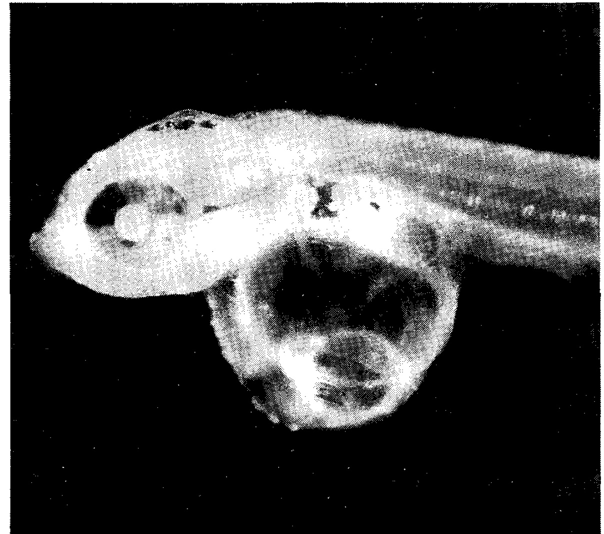

Fig. 9. A 6-day-old chimeric embryo. Melanized cells are found on the head, eyes, and trunk.

References

Iwamatsu, T. (1983) *J. Exp. Zool.*, **228**: 83–89.
Iwamatsu, T. (1994) *Zool. Sci.*, **11**: 825–839.
Wakamatsu, Y., K. Ozato, H. Hashimoto, M. Kinoshita, M. Sakaguchi, T. Iwamatsu, Y. Hyodo-Taguchi and H. Tomita (1993) *Mol. Marine Biol. Biotech.*, **2**: 325–332.
Yasumasu, S., I. Iuchi and K. Yamagami (1994) *Dev. Growth Differ.*, **36**: 241–250.