Appendix A7-1. Microinjection to medaka embryos

1. Overview
   Microinjection is a basic technique for various biological research, the establishment of transgenic lines, gene over-expression using mRNA, gene knockdown using morpholinos, and so on. These techniques utilize common equipment and protocols, except for preparation of each injectate.
   In this section, we will introduce the experimental flow of microinjection to medaka embryos using many photos and illustrations.
   A stereo microscope is used in this protocol. When you want to use a normal microscope (upright type), refer to Appendix 7-2 together with this protocol.
2. Flowchart of microinjection

- **Equipment and tools**
  - Make the following:
    - Injection needles (3-2)
    - Egg holder (3-1)

- **Fish and eggs**
  - To adjust spawning time, fix the fish conditions (Chapter 3.5 and Movie 7-1b).

- **Material for injection**
  - Prepare the materials.
  - Add phenol red and adjust concentration. Finally, centrifuge.

**The day before experiment**
- Collect and sort fertilized eggs (Chapter 3.8 and Movies 3-3a–d).

**The day of injection**
- Using a stereo microscope (4)
  - Check tools and reagents.
  - Align eggs on egg holder.
  - Fill the injectate into needle.
  - Start microinjection.

- Transfer the injected eggs to a culture dish filled with embryo water.
- Record the experimental data.

**The day after injection**
- Incubate embryos according to the standard protocol for embryo nursing (Chapter 3.9).
- Observation according to the purpose of each study
- mRNA injection, morpholino knockdown or other studies
- Transgenic screening by each strategy
- Establishment of transgenic line
3. Tools you must make yourself
3-1. How to make an egg holder (agar type)
For microinjection into many eggs, you can utilize an egg holder for serial injection. This egg holder is made of 2% agar gel, and about 100–200 eggs can be aligned into its channels. To make an egg holder, you have to prepare an egg holder mold (Figure A7-1) made of plastics (acrylic resin). Then, you can make an agar-type egg holder according to the procedure described in Figure A7-2.

Figure A7-1.
A diagram and a photo of an egg holder mold and preparation. (A) A draft of an egg holder mold. Numbers indicate lengths in millimeters. (B) Photo of an egg holder mold. The mold was made from an acrylic resin board with precision cutting.
Figure A7-2.
Egg holder preparation. (A) Float the egg holder mold on hot 2% agar solution, and cool the dish (this photo shows the hardened agar). (B) Slowly remove the mold with tweezers. (C) Separation of egg holder and mold. (D) Magnified image of the agar channels holding eggs.
3-2. How to make glass needles for microinjection

Glass needles are used for microinjection to medaka eggs. Normally, the injection is executed through the egg envelope. Therefore, the needles must be of the required strength and sharpness (Figure A7-3). We pull grass capillaries to make needles using a needle puller (Figure A7-4). You must determine the optimal pulling conditions of each puller individually. A photo of our needles is shown as a sample. If you have a needle grinder (Figure A7-5), sharpening the needle tip is one of the best ways to make a glass needle for microinjection to medaka eggs (M7-3).

Figure A7-4. A needle puller (M7-2).
Figure A7-5. A needle grinder (M7-3).

Figure A7-3. Glass needles for microinjection. (A) The ideal shape of the tip. (B) The needles in a storage box.
4. Instruments

Figure A7-6.
Typical instruments for microinjection. (A) A stereo microscope (Olympus SZX12). (B) An air pressure generator (Eppendorf FemtoJet transjector). (C) A micromanipulator (Narishige) with a needle holder.
5. Microinjection procedure
   Stage 1, Setup of equipment and material

Prepare the following:
1. Fertilized eggs (refer to “Egg collection”)
2. Egg holder (refer to “3-1. How to make an egg holder”)
3. Injection needles (refer to “3-2. How to make glass needles for microinjection”)
4. Injectate (DNA, RNA, or other materials. Refer to each term.)
5. Embryo water
6. Two pairs of tweezers
7. Dishes, microloader tips, and a pipetter

Setup of the injection needle into the needle holder
1. Measure 1–2 μL injectate using a microloading tip.
2. Insert the nose of the loading tip through the open end of the injection needle (Figure A7-7A).
3. Fill the tip of capillary slowly with the injectate.
4. If air bubbles are formed, remove the bubbles by tapping the open end of the needle.
5. Set the needle into the needle holder on a manipulator.

Setup of fertilized eggs into the egg holder
1. Pour in just enough embryo water to cover the agar pad.
2. Align eggs in the channels of the holder.

Figure A7-7.
Loading injectate into a needle. (A) Insert the nose of a loading tip into the open end of a glass needle. (B) A red injectate has been loaded in the needle tip.
Preparation of a broken-tip needle

1. Set an egg into the channel using tweezers.
2. Adjust the manipulator and set the needle tip to the center of the view.
3. Shift the needle tip into the water surface of the channel (Figure A7-9).
4. Press once and confirm the injectate outflow. (Normally, there is no outflow before breaking the tip.)
5. If there is no outflow (normal), break the tip by touching the chorion surface of the held egg. (Slightly insert the tip into the chorion and slowly vibrate the egg with your left hand.)
6. Press once and confirm the outflow again (Figure A7-10).
7. Repeat steps 5–6.
8. Proceed to “Injection” stage.

continue…

**Figure A7-8.**
Tensile property of the needle. The tip is bowing (arrowhead). These needles are unsuitable for microinjection to medaka eggs. You must remake the needle with other pulling conditions.

**Figure A7-9.**
The needle (tip is in the water) and the aligned eggs in channels.

**Figure A7-10.**
The outflow of the injectate (red spreading material). Adjust injection pressure by confirming the outflow repetitively.
5. Microinjection procedure

Stage 2, Injection

1. Find the cytoplasm (arrow in A)
2. Rotate egg with tweezers and turn up the cytoplasm to face the needle (circle in B).
3. Approach the needle to the egg surface.
4. Apply pressure. (push “inject” button if you use injector)
   *The pressure must be lasted until the sting out the needle.
5. Sting needle through chorion to cytoplasm.
   *Then the infusion starts spontaneously.
6. When the injection volume reaches desired volume, sting out the needle from the egg (B).
7. Stop pressure.
8. Move to the next egg and repeat 1–7 steps.
9. Once all eggs have been injected, retrieve the injected eggs from agar channels to new dish filled with embryo water.
10. Incubate at 27.5 °C.

Figure A7-11.
Procedure of microinjection into medaka eggs. A target of injection (A). The direction of the egg and needle approach to the target cell (B).
Figure A7-12.
Positional relationship of egg, needle, and egg holder.

Side view of the egg holder. The egg is held by agar channel and injection needle is approached in about 45° right upper.

To avoid dry and to keep the clear observation, water level must be keep above the bank.

Inject into the cytoplasm. (We can not discern the nucleus in the cell used the normally collected eggs. If you need injection into nucleus, refer “Column 7.1” mentioned in the book.)

The benchmark of microinjection volume is the apparent size of the oil droplets. Continue the injection until the size of the red ball is grown up to the same size of the oil droplet.
Figure A7-13.
One-cell stage eggs. An early 1-cell stage egg (A) and a late 1-cell stage egg (B). Green arrows indicate cytoplasm of each cell. These are the target of microinjection.