

Cell and Tissue Transplantation in Zebrafish Embryos

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1. Introduction

Zebrafish (*Danio rerio*) embryos have gained considerable popularity in recent years because they offer several advantages for developmental studies. The embryos are easy to manipulate, develop quite rapidly, and many genetic mutations are now becoming available. Classical cell and tissue transplantation techniques have been frequently applied to zebrafish embryos to analyze the state of cell commitment, inductive interaction between embryonic tissues and defective tissues in mutant embryos. This chapter introduces three kinds of transplantation techniques useful for the analysis of early inductive events in zebrafish embryos, such as mesoderm and neural induction.

In the first, the technique for yolk cell transplantation is described. In the teleost embryo, a large yolk cell is located vegetally, under the blastoderm which forms the embryo proper. It has been suggested that substances are passed from the yolk cell to the blastoderm to induce embryonic axes (1). To examine the inductive properties of the yolk cell, we have developed a transplantation method. By use of this technique, it has been demonstrated that, as in amphibian vegetal cells, the yolk cell of the teleost is responsible for induction and dorsoventral patterning of the mesoderm (2). Thus, normal activity of the yolk cell is essential for the early development of zebrafish. The technique will be useful in analyzing mutants showing defects in the embryonic axes, as the inductive activity of the yolk cell could be affected in some of those mutants.

The second technique has been developed in order to produce ventralized fish embryos. Ventralized embryos, in which maternal dorsal determinants have been inactivated or removed, have been an effective tool for analyzing

the mechanism underlying dorsoventral axis formation. In *Xenopus*, the embryos resulting from ultraviolet (UV) irradiation to the vegetal hemisphere of fertilized eggs show a ventralized phenotype, in which little or no axial structures are formed (3). By contrast, UV irradiation also causes incomplete epiboly in zebrafish embryos (4). Thus, until recently, no reliable method of producing ventralized embryos was available in zebrafish. We found, however, that ventralized fish embryos were reproducibly obtained by the removal of the vegetal yolk cell mass soon after fertilization. This method was developed based on the fact that teleost cytoplasmic determinants involved in induction of dorsal tissues are localized at the vegetal end of the yolk cell at the time of fertilization (5). They are then translocated from the vegetal end to the future dorsal side under the blastoderm during cleavage stages. This movement of the determinants is reminiscent of cortical rotation in amphibian embryos which occurs soon after fertilization and is blocked by UV irradiation (6). This technique assures a complete removal of dorsal determinants and can be used to analyze dorsoventral patterning in the fish embryo.

Finally, we describe a tissue transplantation technique similar to that described elsewhere (7). We, therefore, focus on the transplantation of organizer tissues which can be used for the analysis of neural induction in zebrafish. Furthermore, we found that, when transplanted into zebrafish embryos, mammalian cultured cells producing organizer factors mimicked the endogenous organizer. The transplantation of cultured cells is widely applicable. If a gene of interest encodes a secreted factor, its role *in vivo* can be easily assessed by transplanting cultured cells which have been transfected with the appropriately expressing cDNA into embryos.

2. Materials

1. Micropipet: The glass capillaries (blunt end tip, $\varnothing = 1$ mm (e.g., Narishige [Tokyo, Japan], G-1) are pulled to fine tips on a electrode puller (e.g., Narishige, PN-3). The tips are broken off at an angle using a hand-held razor blade. Capillary glass which contains an internal filament cannot be used because the filament may destroy cells during the transplantation procedure. The tips can be fire polished with a microforge (e.g., Narishige, MF-9), or the micropipet can be used without fire polishing the tip. The diameter of the tip for shield transplantation is 30–50 μm .
2. Phosphate-buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 2.9 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.2 g KH_2PO_4 in 1 L (pH 7.2).
3. 1X Ringer's solution: 116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl_2 , 5 mM HEPES (pH 7.2).
4. (1/3)X Ringer's solution: 39 mM NaCl, 0.97 mM KCl, 0.6 mM CaCl_2 , 1.67 mM HEPES (pH 7.2).
5. Calcium-free (1/3)X Ringer's solution: 39 mM NaCl, 0.97 mM KCl, 10 mM EDTA, 1.67 mM HEPES (pH 7.2).

6. Agar (e.g., DIFCO [Franklin Lakes, NJ] BACTOAGAR): dissolved in distilled water or the desired Ringer's solution.
7. Antibiotics: penicillin and streptomycin solution (10000 U/mL penicillin and 10,000 µg/mL streptomycin, Gibco BRL [Rockville, MD] 15140-122) are added to all media used for operations at a final concentration of 1% to 2%.
8. Methyl cellulose (e.g., 3500–5600 cps, Sigma [St. Louis, MO] M-0387).
9. Lipofectamine™ (Gibco BRL 18324-012).
10. Rhodamin-dextran (10,000 MW, e.g., Molecular Probes, [Eugene, OR] D-1816).
11. Biotin-dextran (10,000 MW, lysine fixable; e.g., Molecular Probes, D-1956).
12. Albumen, prepared from egg white: Addition of egg albumen to Ringer's solution sometimes increases the survival rate of embryos which have been manipulated, especially when the embryos have sustained some damage to the yolk membrane by the removal of the yolk or fusion of two embryos (8). In addition to nutritive components, the albumen contains *lysozyme*, a bacteriostatic agent. For this reason, egg albumen is often used in embryo cultures to prevent the growth of microorganisms as well as for nutrition.
13. Embryo transfer pipet: Pasteur pipets and rubber teats.
14. 35-mm, 60-mm, and 100-mm plastic culture dishes with lids.
15. Agar-coated dishes for dechorinated embryos: Pour an appropriate amount of hot 1% agar in the desired Ringer's solution into culture dishes and wait until it is completely solidified. Fill the dishes about three-quarters full with the desired Ringer's solution. Agar-coated dishes help to prevent the embryos from sticking to the dish.
16. Micromanipulator: A simple manual micromanipulator works well for cell transplantation (e.g., Narishige, MM-3).
17. Watchmaker's forceps.
18. Sharpened glass needle: The end of a Pasteur pipet is pulled to a fine tip on a small gas burner or spirit lamp.
19. Blunt glass needle: Burn the tip of sharp glass needle for a while.
20. Tungsten needle: sharpened from a fine tungsten filament (0.2 mm in diameter, e.g., Nilaco Corp., Tokyo). To sharpen, mount into a Pasteur pipet or needle holder, then insert repeatedly in the side of a very hot flame; further sharpen by repeatedly soaking the tip of the filament in melted sodium nitrite. For melting, heat the crystal in a quartz melting pot with a gas burner. Do not use ceramic pots, which cannot withstand the heat of melting sodium nitrite. This process is very dangerous and great care should be taken.
21. Mold for making holes in agar-coated dishes (**Fig. 1A**): agar-coated dishes containing multiple holes are required for holding embryo/yolk cell combinations to ensure complete adhesion between the donor and host tissues. The holes in the agar should just fit the recombinants. The best diameter for the hole is approximately 1.2 mm. To make these dishes, we use a silicone rubber mold. The silicone mold is made by pouring liquid silicone mixed with a hardener onto a stainless plate containing holes ($\varnothing = 1.2$ mm), in which one end of the hole has been sealed with tape (**Fig. 1B**).

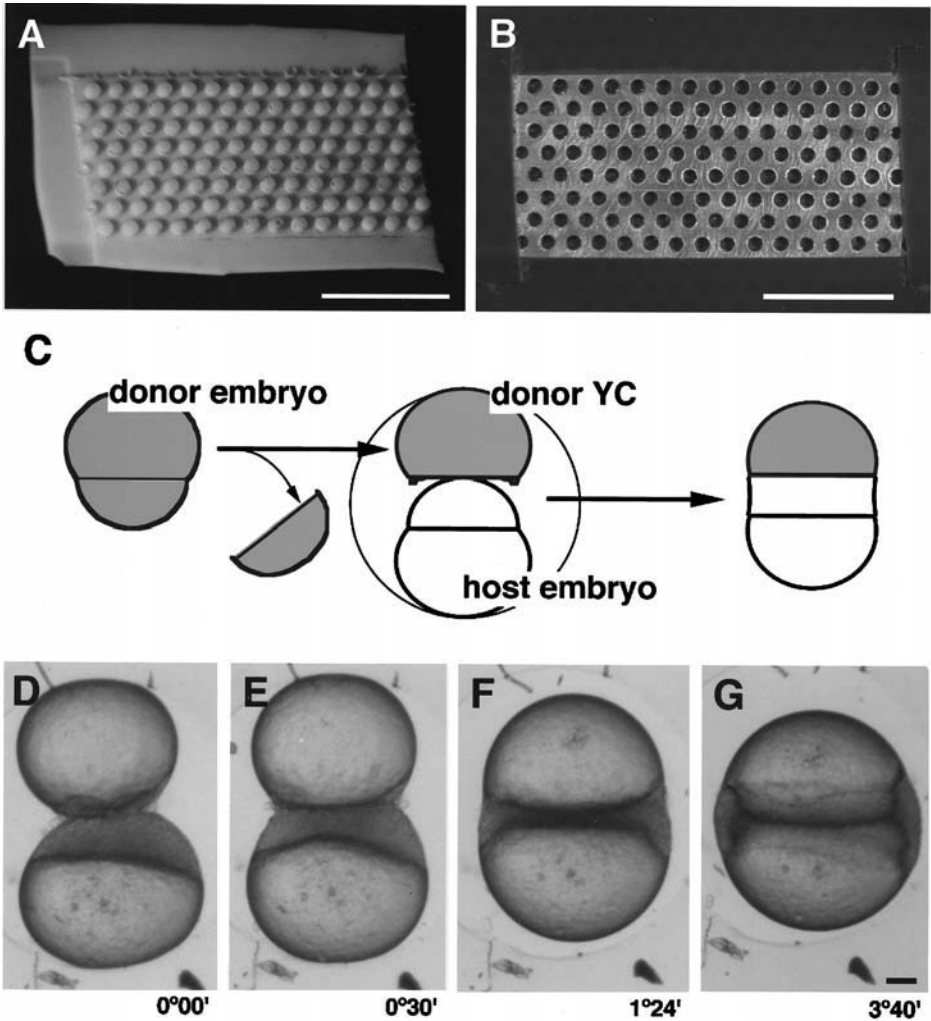


Fig. 1. Transplantation of the yolk cell. (A) A silicone rubber mold for agar holes. Scale bar = 10 mm. (B) A stainless steel plate used for production of the silicone mold shown in A. The diameter of the hole is 1.2 mm. (C) Schematic representation of the experiment. (D–G) The process of adhesion between the donor yolk cell (upper) and the host embryo (lower) which are kept in an agar hole. Scale bar = 100 μ m.

22. Hooked glass needle (**Fig. 2A**) used for removal of the yolk mass: Glass capillaries are pulled to fine tips on an electrode puller. The tips are then fire polished with a microforge. To make a hooked shape, heat the center of the pulled capillary with a microforge.

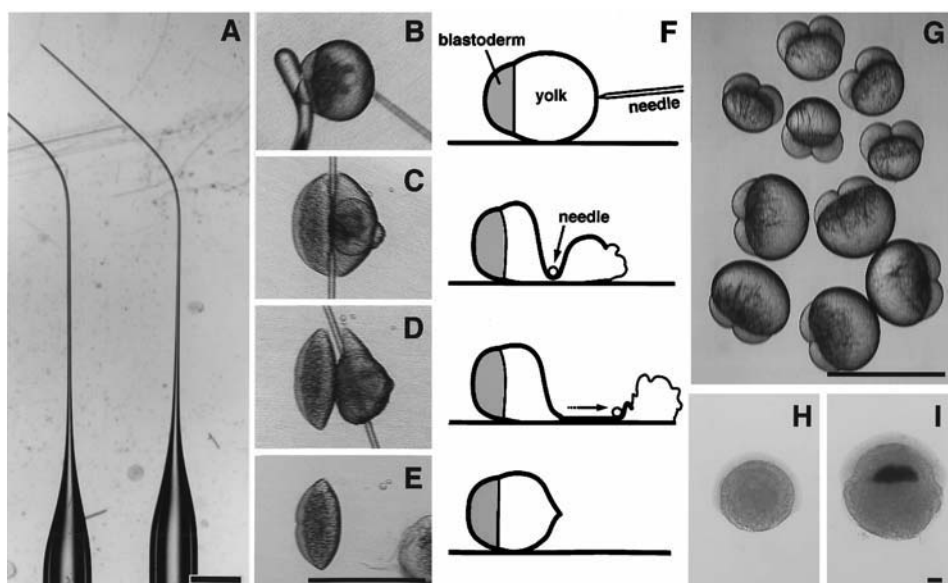


Fig. 2. Removal of the vegetal yolk hemisphere. (A) Hooked glass needles used in the operation. (B–E) The process of the operation. The vegetal yolk mass is squeezed out through a small hole made in the vegetal yolk membrane. The operation should be finished in a few seconds. (F) Schematic representations of the operation shown in B–E. (G) Two-cell stage embryos. As compared with normal embryos (lower five), the experimentally manipulated embryos (upper five) are smaller in size but undergo a normal cleavage. (H,I) *In situ* hybridization with *goosecoid* probe at the 50% epiboly stage. The manipulated embryo does not express *goosecoid* (H) whereas the control embryo (I) shows a positive signal in the future dorsal region. Scale bar = 1 mm (A–G), 100 μm (H,I).

3. Methods

3.1. Transplantation of Yolk Cell: Analysis of Mesoderm Induction

A schematic representation of the experiment described below is shown in Fig. 1C.

1. Label donor embryos at the 1–8 cell stages: inject a rhodamine–biotin mixture (1.65% rhodamine–dextran and 1% biotin–dextran in 0.2 M KCl) into the yolk (microinjection into zebrafish embryos, see Chapter 11). The injected dye spreads through intercellular cytoplasmic connections to all cells of the blastoderm. This ensures that the cells used for transplantation are labeled, and hence recognizable from those of the host embryos.

2. Preparation of agar holes: pour the appropriate amount of hot 1.5% agar in 1X Ringer's solution into culture dishes and immediately place the silicon mold (*see item 21* under **Subheading 2.**) onto the hot agar. When the agar is completely solidified, carefully remove the mold and fill the agar-holed dish with 1X Ringer's solution (referred to as an "agar-hole dish").
3. Dechorionate labeled donor and host embryos (removing embryos from their chorions, *see Chapter 11*). Wash them three times with fresh (1/3)X Ringer's solution, transfer dechorionated donor or host embryos with a Pasteur pipet into agar-coated dishes containing (1/3)X Ringer and agar-hole dishes containing 1X Ringer, respectively.
4. Preparation of donor yolk cells: Donor yolk cells are usually prepared from midblastula embryos (1000 cell stage to sphere stage). Place labeled donor embryos in an agar-coated dish containing calcium-free (1/3)X Ringer's solution. Remove the blastoderm cells from the yolk cell mechanically with a sharpened glass needle. Gently pipet isolated yolk cells up and down in order to remove marginal cells that are tightly attached to the yolk cell. Make sure that most of the blastoderm cells have been removed (*see Note 1*). Carefully transfer isolated yolk cells to the agar-hole dish containing host embryos in 1X Ringer.
5. Before transplanting the yolk cell, make a small incision in the enveloping layer of the animal-pole region of the host embryo with a sharpened glass needle. This helps rapid adhesion between the donor and host tissues. Transplantation should then be carried out immediately. By use of a blunt glass needle, push both donor the yolk cell and the host embryo into a hole made in the agar, with the donor's yolk syncytial layer facing the host animal pole. Let the recombined embryos sit for about 30 min in 1X Ringer's solution, during which time the host blastoderm cells start to cover the donor yolk cell (**Fig. 1D–G**). The higher salt concentration in an agar-hole dish helps the manipulated embryos to heal, but it needs to be exchanged to a lower-salt-concentration (1/3)X Ringer's solution before the onset of epiboly.
6. Thirty minutes after the operation, replace 1X Ringer's solution with (1/3)X Ringer's solution by washing three times with (1/3)X Ringer's, taking care that the recombinants do not come out of their holes. Incubate them until they reach the appropriate developmental stage.
7. The recombinants may then be then fixed and examined for gene expression. For example, ectopic expression of mesodermal genes such as *no tail* and *gooseoid* is induced in the host cells around the grafted yolk cell (2). It is difficult to culture these recombined embryos beyond the bud stage, probably due to a shortage of the cell number required for formation of two body axes (*see Notes 2–5*).

3.2. Removal of the Vegetal Yolk Mass: Production of Ventralized Embryos

A schematic representation of the method described next is shown in **Fig. 2B–F**.

1. Preparation of egg albumen: stir egg albumen with an eggbeater to make it dissolved easily. Leave it overnight at 4°C and use this liquefied egg albumen as a 100% concentration.
2. Prepare embryos by in vitro fertilization as described in (7).
3. Transfer the fertilized embryos to an agar-coated dish containing 1X Ringer (without albumen). To produce ventralized embryos at a high frequency, the operation should be carried out within 30 min. of fertilization (*see Note 6*).
4. Soon after fertilization (5–10 min), yolk-free cytoplasm begins to segregate to the animal pole. Locate the vegetal end of the embryos. Stick the tip of a hooked glass needle into the vegetal yolk membrane (**Fig. 2B**).
5. Place the hooked glass needle in the equatorial region of the yolk mass. Gently push the needle, trying to squeeze the vegetal yolk mass out of the embryo (**Fig. 2C**). For complete removal, move the needle slowly toward the vegetal end while applying continuous pressure against the agar bed (**Fig. 2D**).
6. Let the operated embryo sit for a few minutes. The operated embryos resume a round shape and start to recover from the damage to the yolk membrane. (**Fig. 2E,F**).
7. Transfer these manipulated embryos to an agar-coated dish containing 1X Ringer's supplemented with 1.6% egg albumen.
8. If culture of the embryos for an extended period is required replace the 1X Ringer's with (1/3)X Ringer's without albumen at 50% epiboly.
9. Fix the embryos at the appropriate developmental stage and examine gene expression. For example, these manipulated embryos show no *goosecoid* mRNA expression at the onset of gastrulation (**Fig. 2H,I**) whereas *no tail* is normally expressed in the germ ring (*see Note 7*).

3.3. Transplantation of Organizer Tissues: Analysis of Neural Induction

3.3.1. Transplantation of the Embryonic Shield

A schematic representation of the experiment described below is shown in **Fig. 3**.

1. Label donor embryos at the 1–8 cell stages by injecting a rhodamine–biotin mixture (1.65% rhodamine–dextran and 1% biotin–dextran in 0.2 M KCl) into the yolk.
2. Dechorionate the labeled donor and host embryos. After washing three times with fresh (1/3)X Ringer's, transfer dechorionated embryos with a Pasteur pipet into agar-coated cultured dishes containing (1/3)X Ringer's. Incubate them (at 28.5°C) until use.
3. Place a shield-stage donor embryo into the well of a depression slide containing PBS. Then, 2% methyl cellulose in (1/3)X Ringer's is spread on the surface of the well to hold the embryo, which is then overlaid with a drop of PBS (**Fig. 3A**).

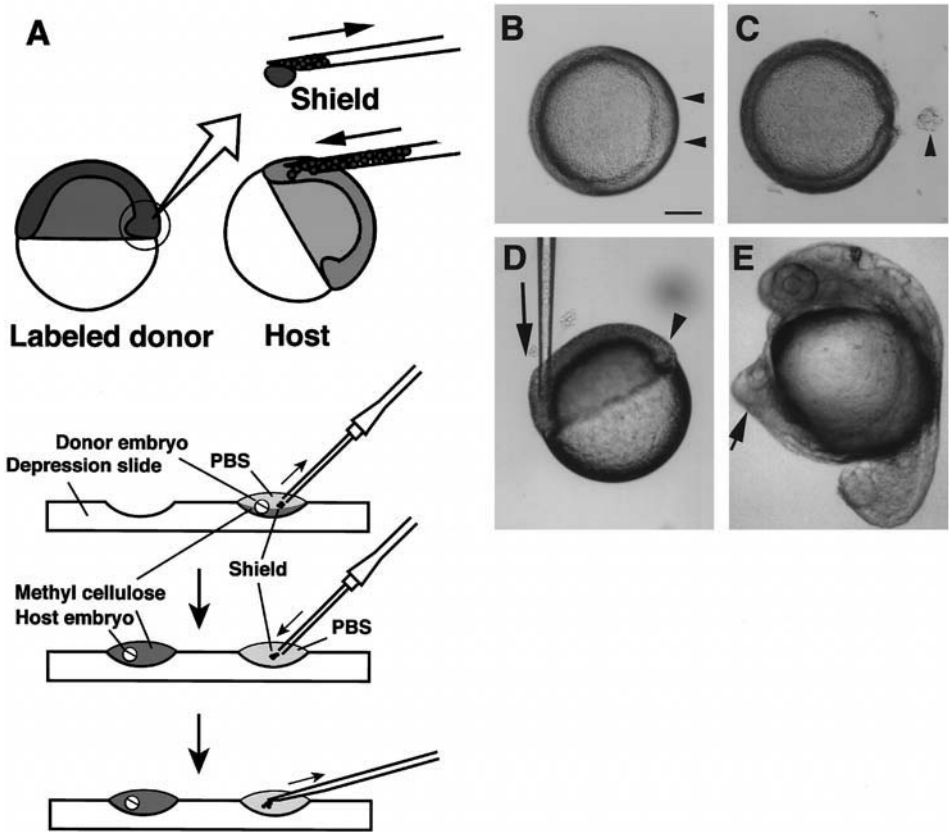


Fig. 3. Transplantation of the embryonic shield. (A) Schematic representation of the experiment. (B) Animal-pole view of a shield-stage embryo (6 h). The shield region (thickened germ ring) is indicated by a pair of arrowheads. (C) Animal-pole view of the shield-stage embryo in which the embryonic shield has been removed, the arrowhead indicates the isolated shield tissue. (D) The host embryo (shield-stage) into which is inserted on the ventral side the micropipet containing donor tissue. The arrowhead indicates the host shield region. (E) The secondary axis with anterior head structures (arrow) induced by the transplanted shield in a 20-h host embryo. Scale bar = 100 μm .

4. Prepare another depression slide for transplantation (transplantation slide). It is better to use a depression slide containing two wells (**Fig. 3A**). Fill one of the wells with 2% methyl cellulose in $(1/3)\text{X}$ Ringer's for the host embryo and the other with PBS for the donor tissues. Place a host embryo (dome to shield stage) into the well containing 2% methyl cellulose in $(1/3)\text{X}$ Ringer's.
5. Under a dissecting microscope, isolate the embryonic shield by cutting the embryo with a sharpened tungsten needle while the embryo is being held by a

watchmaker's forceps (**Fig. 3B,C**). Make sure that isolated tissues are free of yolk if the yolk membrane is damaged.

6. Transfer the shield tissue to the well of the transplantation slide containing the host embryo with a capillary glass (Narishige, G-1) equipped with a rubber aspirator tube to the mouth.
7. Place the transplantation slide on the stage of a microscope equipped with a micromanipulator. It is best if the microscope has a fixed stage; otherwise, the micromanipulator will need to be mounted on the stage. The operation can be carried out under a dissecting microscope if high magnification (X40–X60) is available.
8. Position a glass micropipet with a broken tip near the dissected shield under the objective and pipet up a little of the PBS solution. Try to keep zero pressure at the tip of the micropipet.
9. Suck the cells gently from the shield tissue into the micropipet.
10. Withdraw the micropipet and move the slide or stage so that the micropipet is now located next to the host embryo, while watching under the objective.
11. Insert the micropipet into the appropriate position of the host embryo, on the ventral side if the shield is visible (**Fig. 3A,D**). Do not damage the yolk cell membrane (*see* **Notes 8–12**).
12. Expel the cells with gentle pressure.
13. Withdraw the micropipet from the host embryo.
14. Add gently a small aliquot of (1/3)X Ringer's to the well containing the host embryo.
15. Place the slide containing the hosts in a plastic culture dish ($\varnothing = 10$ cm) and incubate it. You may pour 10 to 20 mL of (1/3)X Ringer's gently into the dish so as to completely cover the slide.
16. After a few hours' incubation, the methyl cellulose solution becomes less viscous and the host embryos become detached from the bottom of the depression slides. Transfer them carefully with a Pasteur pipet to a culture dish containing fresh (1/3)X Ringer's and incubate them for an appropriate period. The secondary axis becomes visible during the late gastrula to 24-h stages (**Fig. 3E**).

3.3.2. Transplantation of COS7 Cells Secreting Organizer Factors

A schematic representation for the experiment described below is shown in **Fig. 4**. For making cell aggregates of COS7 cells, we essentially follow the protocol described elsewhere (**9**).

1. Three days before the transplant will take place, plate COS7 cells (approximately 5×10^5) on a small culture dish ($\varnothing = 35$ mm) so that they will be 70–80% confluent on the next day. The culture medium used is Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS).
2. Two days before the operation. Transfect the cells with plasmid DNAs with LipofectamineTM following the manufacturer's protocol. Briefly, 12 μ L of LipofectamineTM and 2 μ g of plasmid DNA (purified by cesium chloride banding) are diluted separately into 100 μ L of aliquots of serum-free DMEM (with-

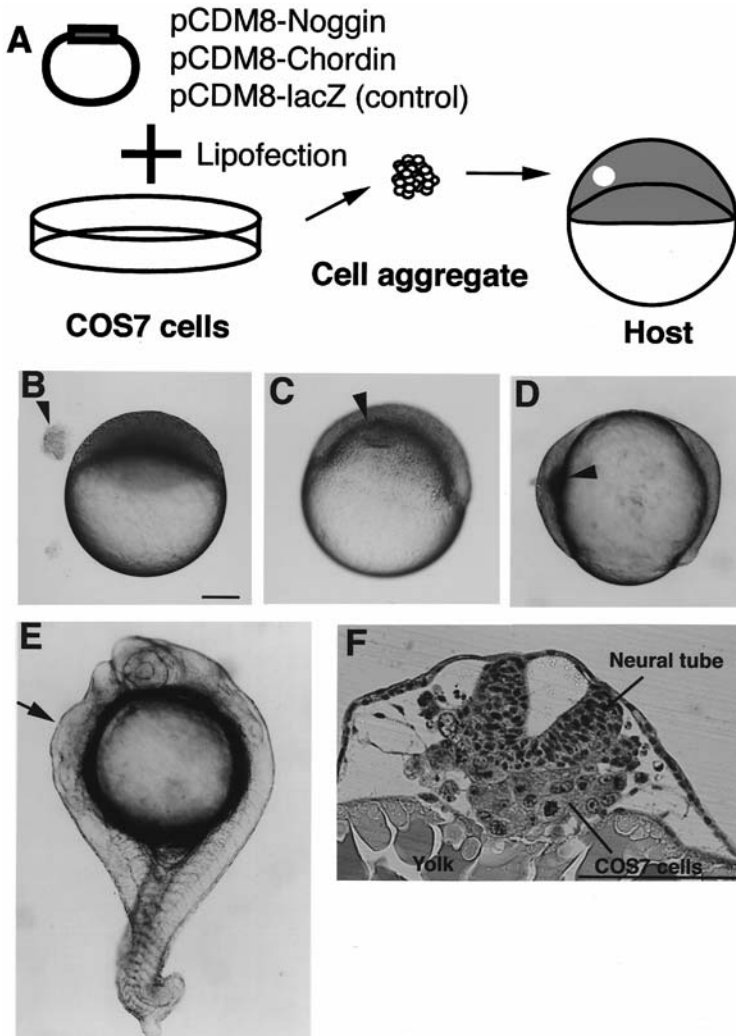


Fig. 4. Transplantation of COS7 cells secreting organizer factors. **(A)** Schematic representation of the experiment. **(B)** The cell aggregate (arrowhead) placed near the host embryos (dome stage, $4\frac{1}{3}$ h). **(C)** The host embryo (50%-epiboly, $5\frac{1}{4}$ h) grafted with the cell aggregate (arrowhead, about 1 h after transplantation). **(D)** The host embryo (80% epiboly, 8 h) grafted with the cell aggregate (arrowhead, about 8 h after transplantation). The ventral epiblast around the graft becomes thick, indicating neural plate formation on the ventral side. **(E)** Secondary axis (arrowhead) induced by Noggin/Chordin COS7 at 24 h. The secondary axes induced by COS7 tend to show a cyclopic phenotype (one-eyed head), probably because of a lack of axial mesoderm. **(F)** Cross sections of the secondary axis at the level of the hindbrain. The COS7 cell mass is located under the induced neural tube. Scale bars = 100 μ m.

out antibiotics). These aliquots are gently mixed and incubated at room temperature for 15 min to form complexes. The complexes are diluted with 0.8 mL of serum-free DMEM (without antibiotics) and the mixture (transfection medium) is added to subconfluent cells in a small culture dish ($\varnothing = 35$ mm). The cells are rinsed twice with serum-free DMEM (without antibiotics) prior to the addition of the diluted complexes. We use 1 μ g each of pCDM8 containing *Xenopus* noggin and chordin cDNAs or 1–2 μ g of pCDM8 containing *lacZ* cDNA as a control.

3. After a 6-h incubation in 1 mL of transfection medium, add 0.8 mL of DMEM and 0.2 mL of FCS to the dish and incubate overnight.
4. On the morning of the day before the transplant, change the medium to fresh DMEM/10% FCS. In the evening, harvest the cells and replat them on a culture dish coated with 1% agar. Incubate them overnight in DMEM/10% FCS. To make the agar dish, pour 0.5–0.6 mL of hot 1% agar in distilled water or PBS into a small culture dish ($\varnothing = 35$ mm) and wait until completely solidified.
5. On the morning of the day of the transplant, make sure that cell aggregates are formed. The size of the cell aggregates depends on the density of the cells plated and/or to what degree they are dissociated at the stage of plating.
6. Dechorionate host embryos (sphere to shield stage) and place them in the well of a depression slide containing 2% methyl cellulose in (1/3) X Ringer's.
7. Transfer a small group of cell aggregates from the culture dish into the well containing the host embryos, using a glass capillary equipped with an aspirator tube or a Pasteur pipet.
8. Under a dissecting microscope, pick up a cell aggregate of the appropriate size or cut out a small piece from a bigger aggregate with a sharpened tungsten needle (approximately 50 μ m in diameter is preferable). Move the aggregate near the host using the needle (**Fig. 4B**).
9. Make a small incision (*see Note 11*) in the enveloping layer of the host with a sharpened needle. Insert the cell aggregate into the deep cell layer using the needle, taking care not to damage the yolk membrane (**Fig. 4C**). It is essential to make the incision as small as possible, otherwise the cell aggregate will be pushed out during epiboly.
10. Add gently a small aliquot of (1/3)X Ringer's to the well containing the host embryos.
11. Place the slide in a plastic culture dish ($\varnothing = 100$ mm) and incubate it. You may pour 10–20 mL of (1/3)X Ringer's gently into the dish so as to completely cover the slide.
12. After a few hours' incubation, the methyl cellulose solution becomes less viscous and the host embryos detach from the bottom of the depression slides. Transfer them carefully with a Pasteur pipet into fresh (1/3)X Ringer's and incubate them for an appropriate period. The secondary axis becomes visible during late gastrula to 24-h stages (**Fig. 4D–F**) (*see Note 12*).

4. Notes

1. During all procedures of operation, make sure that dechorionated embryos and isolated tissues do not touch the surface of any solution or they will burst because of the surface tension of the liquid.

2. If the donor tissues are labeled with biotin–dextran, they are visualized in the host by biotin–peroxidase staining as described elsewhere (7,10).
3. Yolk cells prepared from 512-cell to sphere stage embryos show the same inducing activity.
4. Under our experimental conditions, we cannot remove the marginal cells completely with the yolk cell intact, probably due to the tight adhesion of the marginal cells with the yolk syncytial layer. Thus, the yolk cells to be transplanted contain a few marginal cells. It is confirmed that a few marginal cells attached to donor yolk cells do not affect gene expression in the host cells (2,14).
5. Although the higher salt condition (1X Ringer's) is required for wound healing, replacement of 1X Ringer's with low-salt (1/3)X Ringer's is essential for successful transplantation. However, the timing of replacement differs for each experiment and even on batches of eggs. It is best to carry out the replacement either as soon as firm adhesion between the donor and host tissues is established, or when the manipulated embryos recover from their damage. It is known that the higher salt condition perturbs the gastrulation process in dechorionated zebrafish embryos.
6. Originally, this technique was developed for goldfish embryos (5,11). In these experiments, the embryos were bisected from the vegetal yolk hemisphere using nylon fibres crossing the equator. This method is only applicable for zebrafish embryos until approximately 15 min postfertilization, because the yolk membrane loses its softness after this stage. However, the modified method described here can be applied to zebrafish embryos at any developmental stage.
7. The embryos from which the vegetal yolk mass has been removed make no dorsal structures, such as notochord, somites, and neural tube. The frequency of abnormality decreases as the age at which the vegetal yolk hemisphere is removed increases (5). For zebrafish embryos, the frequency of a ventralized phenotype is highest when yolk mass removal is carried out 20 min after fertilization and no ventralized embryos are obtained by this manipulation after the 8-cell stage (14).
8. To avoid damage to the yolk membrane during shield transplantation, it is better to perform the injection by moving the stage. Once the position of the micropipet is fixed under the objective, we never touch the micromanipulator during transplantation.
9. Methyl cellulose, when contaminating the deep cell layer, inhibits normal development of the embryo, especially the process of epiboly. Thus, it is essential not to take up the methyl cellulose solution into the micropipet before insertion into the embryo. Similarly, too much PBS injected into the embryo leads to abnormal development. Try to transplant tissues or cells with as little medium as possible.
10. During the process of transplantation, the embryonic shield tends to disintegrate into small fragments or even single cells, because of weak cell adhesion at this stage. Because the inducing ability of the shield is displayed to the full when transplanted as a tissue mass, it is important to handle the shield tissue gently, taking care to avoid dissociation.

11. To obtain a secondary axis with anterior head structures, it is essential to graft organizer tissues halfway between the blastoderm margin and the animal pole. When the inducing tissues are grafted near the blastoderm margin, secondary axes are frequently induced, but those axes lack anterior head structures (**12,13**).
12. The fish organizer (embryonic shield) and mammalian COS7 cells transfected with *Xenopus* noggin and chordin cDNAs (Noggin/Chordin COS7) induce secondary axes equally when transplanted at mid-blastula to early gastrula stage on the ventral side of the fish embryo (**Fig. 3E** and **Fig. 4E**). However, these inducing tissues behave differently in terms of their contribution to the secondary axes produced. Grafted embryonic shield contributes to the axial mesoderm and the ventral part of the neural tube (**13**), whereas the Noggin/Chordin COS7 shows no sign of self-differentiation but is present in a cell mass under the neural tube (**Fig. 4F; 15**). No axial mesoderm is detectable in the secondary axis induced by the Noggin/Chordin COS7.

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