# **Chapter 11**

# **Tissue Micromanipulation in Zebrafish Embryos**

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#### Summary

Although a common approach in large vertebrate embryos such as chick or frog, manipulation at the tissue level is only rarely applied to zebrafish embryos. Despite its relatively small size, the zebrafish embryo can be readily used for micromanipulations such as tissue and organ primordium transplantation, explantation, and microbead implantation, to study inductive tissue interactions and tissue autonomy of pleiotropic, mutant phenotypes or to isolate tissue for organotypic and primary cell culture or RNA isolation. Since this requires special handling techniques, tools, and tricks, which are rarely published and thus difficult to apply without hands-on demonstration, this article provides detailed instructions and protocols on tissue micromanipulation. The goal is to introduce a broader scientific audience to these surgical techniques, which can be applied to a wide range of questions and used as a starting point for many downstream applications in the genetically tractable zebrafish embryo.

Key words: Zebrafish, *Danio rerio*, Transplantation, Bead implantation, Presomitic mesoderm, Optic vesicle, Dissection, Micromanipulation.

#### 1. Introduction

The classical experimental approach to study inductive cellular interactions in the vertebrate embryo is to transplant tissue. If a tissue comprising cells with strong inductive potency is transplanted from a donor embryo to an ectopic site in a host embryo, creating a chimera composed of tissue from two individuals, it can, depending on the competence state of the host and developmental timing of the transplantation, change the fate of neighboring cells and tissue. The ectopic transplantation of dorsal blastopore lip tissue and secondary axis induction in the amphibian

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embryo by Hans Spemann and Hilde Mangold in the 1920s is the famous prototype of these experiments (1). Transplantations can also serve to test other fundamental properties of developing tissue, e.g., commitment and competence. Can transplanted tissue autonomously develop according to the position and fate it had in the donor, or is it recruited into the developing host and subsequently changes its fate in a host-dependent respecification process? A similar, but even further reaching question asks what degree of self-organization does morphogenesis and differentiation of a tissue or whole organ primordium show upon explantation from its normal developmental site. What are the extrinsic and intrinsic contributions to these processes and what is their timing? All these questions have classically been addressed by tissue micromanipulation in the embryo.

With the advances in biochemical, molecular biological, and genetic approaches to animal development, a vast canon of methods to study inductive tissue properties have emerged. Nonetheless, experimental manipulation of embryonic tissue is not an outdated approach but a powerful technical complement to genetic and molecular approaches: the protein microbead implantation procedure described in this article is a technique at the interface between biochemistry and tissue micromanipulation. On one hand, it requires purified proteins to be tested for their activity; on the other, it applies experimental techniques that were developed long before the discovery of inductive molecules. Bead implantations have proven to be a powerful tool to study the inductive nature of proteins, dosage effects, tissue responsiveness, and timing (2-6). The optic vesicle transplantation technique described in this article was originally developed to dissect the tissue autonomy of pleiotropic phenotypes of zebrafish mutants isolated in large-scale, forward genetic screens (3, 7-11).

Although performed routinely in chicken and frog embryos, tissue micromanipulation is rarely done in the zebrafish embryo, probably due to its relatively small size. Nevertheless, features such as the complete transparency and the easy access to large numbers of embryos and genetic mutants available make the zebrafish an ideal model for tissue manipulation. The focus of this article is *manual* micromanipulation techniques that can be applied to zebrafish embryos despite their small size: the surgical methods described here are all performed freehanded, under a conventional stereomicroscope with transillumination. Although they require very steady hands, they can be performed without sophisticated micromanipulators, which clearly enhances throughput. Protocols describing cell transplantation and injection with the aid of micromanipulators have been previously published (12-14). Importantly, the concrete experimental

procedures outlined here exemplify the principles and key techniques of tissue micromanipulation in zebrafish embryos in such a way that enable scientists to vary and adopt the protocols to their particular need and application.

## 2. Materials

2.1. Media and Reagents

1	Ringer medium	116 mM NaCl, 3 mM KCl, 4 mM CaCl <sub>2</sub> anhydrous, 1 mM MgCl <sub>2</sub> , 5 mM HEPES.
2	E3 medium	10 L of 60 × E3 (dilute in deionized water): 175-g NaCl (5 mM), 7.6-g KCl (0.17 mM), 29-g CaCl <sub>2</sub> ·6H <sub>2</sub> O (0.33 mM), 49-g MgSO <sub>4</sub> ·7H <sub>2</sub> O (0.33 mM).
;	L15 medium	Leibovitz's L-15 medium (1×), liquid – with L-glutamine, without phenol red (Invitrogen Cat. No. 21083-027).
4	Trizol	TRIZOL® reagent, 100 mL (Invitrogen).
5	AMP-PNP, 40 mM in water	<ul> <li>AMP-PNP adenosine 5'-(β,γ-imido)</li> <li>triphosphate tetralithium salt hydrate,</li> <li>~95% (HPLC), powder (Sigma-Aldrich Cat. No. A2647).</li> </ul>
6	4% Paraformaldehyde	Paraformaldehyde, reagent grade, crystal- line (Sigma-Aldrich Cat. No. P6148); dilute to a working concentration of 4% in PBST.
7	Microbeads	Polybead Polystyrene 45.0-μm micro- spheres, 2.51% solids-latex (Polysciences, Inc. Cat. No. 07314) ( <i>see</i> <b>Note 1</b> ).
8	Fgf8 protein (see Note 2)	Recombinant Mouse FGF-8b, CF (R&D Systems Cat. No. 423-F8-025/CF).
9	Low melting point (LMP) agarose	LMP-agarose (Invitrogen).
10	Mineral oil	Mineral oil, mouse embryo tested, light oil (neat) (Sigma Cat. No. M8410) ( <i>see</i> <b>Note 3</b> ).
11	0.5 M NaCl in water.	
12	PBS.	
13	Ethanol.	
14	Electrophoresis-grade ag	garose.

#### 2.2. Instrumentation

1	Micropipette puller e.g., Flar puller, ments	ning/Brown micropipette Model P-97 (Sutter Instru- Co.)
2	Water bath, 42°C	
3	Standard laboratory power supply w or a 9-V block battery	ith cables and alligator clip
4	Benchtop centrifuge	
5	Heating block, 50°C	
6	Bunsen burner	

#### 2.3. Other Materials

1	Borosilicate glass tubing	World Precision Instruments Cat. No. TW100F-3
2	Microelectrode holder with a male Luer port	World Precision Instruments Cat. No. MPH3
3	Handle for microelectrode holder	World Precision Instruments Cat. No. 2505
4	Pasteur pipettes, glass	
5	Plastic pipettes	
6	Micropipette set	
7	0.5-mL reaction tubes, siliconiz	ed
8	1.5-mL reaction tubes	
9	15-mL Falcon tubes	
10	Diamond glass knife	
11	Superglue	
12	Dumont forceps	
13	Tungsten wire	e.g., a set of 5, 20, 100, and 200-µm diameter wires ( <i>see</i> <b>Note 4</b> )
14	Blunt needle on holder	e.g., a sewing needle
15	27G needles	
16	Petri dishes, 35 mm	
17	Petri dishes, 35 mm, coated wit	h silicone elastomer ( <i>see</i> <b>Note 5</b> )
18	Scalpel, pointed blade	(World Precision Instruments)
19	Plastic tubing (see Note 6)	(World Precision Instruments)

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## 3. Methods

3.1. Immobilizing	For microbead implantation and tissue manipulation at late stages
Embryos in Agarose	( <i>see</i> Note 7). A protocol was developed to mount embryos at somitogenesis stages for bead implantation and manipulation of the optic vericle
	<ol> <li>Melt 1.2% Ringer–LMP agarose in a 15-mL Falcon tube using a microwave oven (the tube has to be almost full). Place the tube into a water bath at 42°C in reach of your stereomicroscope and let it "cool" there for at least 30 min.</li> </ol>
	<ol> <li>Dechorionate embryos using Dumont forceps in a Petri dish (coated with 1–2% E3 agarose) containing E3 medium.</li> </ol>
	3. Place the dish with the dechorionated embryos next to your stereomicroscope.
	4. Open the Falcon with LMP-agarose and place it back into the water bath.
	5. Draw an embryo into a fire-polished Pasteur pipette with as little medium as possible ( <i>see</i> <b>Notes 8</b> and <b>9</b> ).
	6. Pick up the LMP-agarose tube from the water bath; carefully pipette the embryo into it and quickly draw the embryo with agarose back into it ( <b>Fig. 1a</b> ).
	<ol> <li>Pipette the embryo in an agarose drop to the center of a 35-mm Petri dish cover (<i>see</i> Note 10, Fig. 1b).</li> </ol>
	8. Rapidly orientate the embryo with a blunt needle and hold the embryo until the agarose solidifies.
	9. Let the agarose solidify for another 2–5 min at room temperature ( <i>see</i> Note 11).
	10. Use a pointed scalpel to cut a wedge pointing toward the embryo into the agarose ( <i>see</i> Note 12, Fig. 1b).
	<ol> <li>Follow the contours of the wedge with a blunt tungsten needle up to the embryo surface to loosen it (<i>see</i> Note 13, Fig. 1c, top).</li> </ol>
	12. Fill the dish with Ringer medium until the agarose block is fully covered.
	13. Use the blunt tungsten needle to remove the agarose wedge: insert the needle under the agarose wedge and flap it upward as one piece ( <b>Fig. 1c</b> , bottom).
	14. Check if the embryo is fully exposed in the wedge – if not, use a blunt tungsten needle to remove any remaining agarose pieces. Now the embryo is ready for further manipulation ( <i>see</i> <b>Note 14</b> ).



Fig. 1. Immobilizing embryos in agarose. (a) Transfer dechorionated donor (*red*) and host (*white*) embryos with a fire-polished Pasteur pipette into 42°C LMP agarose. (b) Place and orient embryos side-by-side in a Petri dish cover until agarose solidifies and cut a wedge shape into the agarose at the side of the embryos with a scalpel (*top view*). (c) Follow the wedge contours with a blunt tungsten needle to loosen the agarose block; cover the preparation with medium and lift off the agarose (*side view*).

This protocol can also be used for embedding embryos for in vivo imaging purposes: mount embryos (depending on developmental stage) in agarose between 0.5 and 1.5% in a dish, fill the dish with a medium, and image with a dipping lens. Try to spread the agarose in a way that only a thin film covers the embryo.

**3.2. Opening the Embryonic Epidermis** To access deep manipulation sites at late stages of development, it is necessary to locally remove the primitive embryonic epidermis. The method of choice to achieve this, without destroying the embryo as a whole, is to locally destroy and remove the epidermis with mineral oil.

- 1. Embed the embryo in agarose (see Subheading 3.1).
- 2. Use the handheld micropipette (*see* Subheading 3.6.2 and Fig. 5) to apply a droplet of mineral oil onto the epidermis at the site that you want to open (*see* Notes 15 and 16 for oil application prior to optic vesicle transplantation).
- Incubate the embryos for 3–5 min at room temperature during that time the oil will destroy the epidermis underneath the drop (*see* Note 17).
- 4. Using a fine tungsten needle, carefully lift off the destroyed patch of epidermis (*see* **Note 18**). Now the embryo is ready for further manipulation (*see* **Note 19**).

**3.3. Microbead** This section covers a method of microbead implantation into zebrafish embryos. It has proven to be a very useful technique, e.g., to deliver inductive proteins, with high spatial and temporal resolution and control over concentration. The procedure differs for embryonic stages before and after gastrulation – therefore, two different protocols are outlined. The method is exemplified by the implantation of Fgf8-protein beads (a) at the animal pole of embryos at the 50% epiboly stage (b) and into the eye primor-dium at the 15-somite stage.

3.3.1. Implantation into Embryos at Blastula Stages

- 1. Dechorionate embryos using Dumont forceps in a Petri dish (coated with 2% E3 agarose) containing E3 medium.
- 2. Transfer embryos into a premade mold of 2% E3 agarose (Fig. 2a, *see* Note 20) using a fire-polished Pasteur pipette (*see* Note 21).
- 3. Once in the mold, orient the embryos with the desired implantation site facing upward, using a blunt needle.



Fig. 2. Microbead implantation into blastula-stage embryos. (a) Plastic form for casting agarose molds [see (11) for details]. (b) Opening of the embryos with a blunt tungsten needle and bead implantation. (c) Live embryos, 1 h after bead implantation at the 50% epiboly stage.

	<ol> <li>Resuspend beads in the tube by carefully pipetting up and down. Transfer 0.5 μL of bead suspension (<i>see</i> Subheading 3.3.3) slowly into the dish and let the beads sink onto the agarose bed before you continue (<i>see</i> Note 22).</li> </ol>
	5. Open the first embryo by gently poking it with a blunt tung- sten needle. The hole has to be at least the size of the bead. If too small, try to make it bigger by gentle sideward movements with the needle inserted in the embryo ( <i>see</i> <b>Note 23</b> ).
	6. Approach the beads lying on the agarose bed with the needle and move one bead onto the opened implantation site ( <i>see</i> <b>Note 24</b> ).
	7. Once the bead lies on top of the opening in the embryo, gently push it in with the needle ( <i>see</i> <b>Note 25</b> and <b>Fig. 2b, c</b> ).
	8. After implantation, transfer the embryos with a fire-polished Pasteur pipette into a fresh, agarose-coated Petri dish with E3 medium; place it in a 28.5°C incubator and let the embryos heal for 30–60 min.
	9. Now, the embryos can be handled like normal, dechorionated embryos.
3.3.2. Implantation into Embryos at Somitogenesis Stages	Bead implantation into somitogenesis stage embryos requires prior agarose mounting and opening of the epidermis. The protocol was developed for implantation of Fgf8-protein-loaded microbeads into the optic vesicle between the 5- and 15-somite stages. 1. Embed embryos in agarose ( <i>see</i> <b>Subheading 3.1</b> ).
	2. Open the embryos (see Subheading 3.2 and Note 26).
	3. Continue with step 4 of Subheading 3.3.1.
	4. Insert a fine tungsten needle through the hole in the epider- mis and gently push the cells at the implantation site apart. This loosens the tissue connectivity and allows implanting the bead deep into the tissue.
	5. Continue with steps 6 and 7 of Subheading 3.3.1.
	6. Continue with steps 10 and 11 of Subheading 3.4.1.
3.3.3. Loading Microbeads with Protein	Microbeads can serve as carriers for various substances. Different bead diameters and materials exist. The protocol describes the principles of this method for 45-µm polystyrene microbeads ( <i>see</i> <b>Note 27</b> ) loaded with recombinant mouse Fgf8 protein:

- 1. Resuspend beads in the container that they are delivered in and transfer one drop (*see* Note 28) of bead suspension to 1-mL tube.
- 2. Add 1 mL of 100% ethanol and mix by inverting the tube a few times.
- 3. Split into four ~250 μL samples by transferring into new, siliconcoated (*see* **Note 29**) 500-μL tubes. Carefully resuspend the

beads before splitting to achieve an equal distribution over the four samples.

- 4. Pellet the beads by 5-min centrifugation in a standard benchtop centrifuge at 13,000 rpm and remove supernatant.
- 5. Dry the bead pellets in opened tubes for ~20 min on a 50°C heating block. All ethanol needs to evaporate before the next step. Test if the beads are dry: they should fall as white powder to the bottom of the tube, when you gently flick it.
- 6. Add protein solution at the desired concentration (e.g., 250  $\mu$ g/mL Fgf8 in PBS, prepared according to manufacturer's guide) to the beads (*see* **Note 30**).
- 7. Suspend the bead pellet by pipetting up and down (see Note 31).
- 8. Incubate at  $4^{\circ}$ C for 24 h to let the protein solution completely soak the beads.
- 9. Control beads are loaded accordingly with, e.g., BSA or PBS.

**3.4. Tissue Transplantation** This protocol can also be used as a starting point for explant culture or isolation of embryonic cells or as a source for tissue-specific RNA preparations (*see* **Subheading 3.5**).

*3.4.1. Optic Vesicle* The following protocol was developed for explantation/trans-*Transplantation* plantation of the optic vesicle between the 5- and 20-somite stages but can be easily adapted to other developmental tissues and stages.

- 1. Mount two embryos (one donor and one host for the transplantation) at the 5–10-somite stages in 1.2% LMP-agarose in Ringer medium (*see* Subheading 3.1) (Fig. 1). Mount the embryos, lying on their side (right body half facing up), close to each other, with their heads down.
- 2. Locally remove the agarose to expose the optic vesicle to be transplanted (*see* Subheading 3.1), and then open the host embryo with mineral oil (*see* Subheading 3.2): apply only a small oil drop to the epidermis posterior to the optic vesicle.
- 3. Lift off the destroyed patch of epidermis with a fine tungsten needle.
- 4. Using a tungsten hook (*see* **Subheading 3.6.1** and **Fig. 4c**), increase the size of the opening in the epidermis if necessary: the opening needs to be a little bigger than the grafted tissue block.
- 5. Use the tungsten hook to cut the optic vesicle loose off the forebrain primordium (*see* **Note 32**).
- 6. Once the optic vesicle tissue lies loose in the embryo use the tungsten loop (*see* Subheading 3.6.1 and Fig. 4b) like a spoon to take it out. Remnant host tissue can be removed by scraping the created cavity with the loop.

	7. Repeat steps 2–6 for the donor embryo (see Note 33).
	8. Transfer the donor optic vesicle and place it onto the opening in the host embryo using the tungsten loop ( <i>see</i> <b>Note 34</b> ).
	9. Gently push the graft into the cavity in the host embryo using a blunt tungsten needle.
	10. Incubate the embedded embryos in Ringer medium at 28.5°C until the hole in the epidermis has completely healed.
	11. Carefully remove donor and host embryos from the agarose (by cutting the embedding blocks apart with forceps) and transfer them to a new agarose-coated dish with E3 medium.
	The success of tissue transplantation can be followed using fluorescently labeled embryos as donors. This allows monitoring the amount, extent, and integrity of the graft at different time points after transplantation $(7)$ . An alternative protocol for optic vesicle transplantation has been published before $(11)$ .
3.5. Flat-Mount Tissue Dissection	Removing an optic vesicle, as described earlier (Subheading 3.4), takes advantage of the natural tissue boundaries in the embryo. However, if a subsection of a tissue without naturally occurring internal boundaries or structures is required, a more aggressive technique must be used to obtain it. As an example in this section we describe the dissection of one side of the posterior part of the embryonic presomitic mesoderm (PSM). This micromanipulation was not practical using the whole embryo mounting technique described earlier, but rather uses a live flat-mounting technique after yolk cell removal (15). The protocol combines the advantages of high precision in dissecting the same part of the PSM of similarly staged embryos, a significant reduction of contamination by other tissues, e.g., lateral plate mesoderm (LPM), and the ability to exert increased force upon the immobilized embryonic tissue. It can be simply adopted to dissect other parts of the embryonic body, especially mesenchymal tissue. The dissected tissue can be used for subsequent RNA isolation to study stage- and tissue-specific gene expression (16).
3.5.1. "Deyolking" and Flat Mounting of Live Embryos for Dissection	To be able to flatten the embryo on a stable silicone surface the yolk cell needs to be removed. When cutting with a fine tungsten tool, this should yield just enough to stabilize the embryo and to not break the tools.
	<ol> <li>Transfer three to four 12–13-somite stage embryos (<i>see</i> Note 35) to L15 medium-covered silicone plates and dechorion-ate using Dumont forceps.</li> </ol>
	2. Position one embryo in a small dell in the silicone surface to stabilize it ( <i>see</i> <b>Note 36</b> ).
	<ol> <li>Inject an 8-nL bolus of AMP-PNP solution (<i>see</i> Note 37) into the yolk cell with a micropipette attached to a plastic tube for mouth aspirating, or the handheld micropipette (without oil)</li> </ol>

(*see* **Subheading 3.6.2** and **Note 38**). Yolk will start to float out of the yolk cell.

- 4. After waiting 30–60 s for the yolk cell cortex to paralyze, extrude most of the yolk by gently streaking along the ventral side with a blunt tungsten needle (*see* **Subheading 3.6.1**).
- 5. Cut open the ventral yolk cell epidermis (between head and tail) by holding the embryo with a thicker tungsten needle and cutting with a fine, pointed scalpel.
- 6. By holding the embryo still via the head, pull off the yolk cell enveloping layer and epidermis with a slightly hooked tungsten needle (*see* **Note 39**).
- 7. Cut off the head of the embryo with a fine, pointed scalpel and remove the periderm from the caudal part of the embryo (*see* **Note 40**).
- Remove the LPM using a sharp tungsten needle (Fig. 3a; see Note 41).



Fig. 3. Flat-mount presomitic mesoderm (PSM) dissection. (a) Embryo is shown in a dorsal and a cross-sectional view. In a L15 medium-containing, silicone-coated Petri dish, the deyolked and decapitated embryo is held ventral side down, and the lateral plate mesoderm is removed locally by cutting along its border with the PSM. (b) The embryo is transferred to a new silicone-coated Petri dish in a drop of L15 medium. While holding the embryo ventral side down with a blunt tungsten needle, the medium is aspirated until the embryo is trapped between the silicone surface and the liquid layer, as seen in cross-sectional view (*bottom*). Dissection can now be completed along the *red dotted* lines.

3.5.2. Dissection and Transfer of PSM and Embryo

- 9. Using a fire-polished glass pipette, transfer the embryo to a fresh silicone plate in a drop of L15 medium and position the ventral side down by holding it with a tungsten needle in the anterior trunk area.
- 10. Aspirate the L15 medium with the handheld micropipette (without oil) (see Subheading 3.6.2) or mouth aspirate using a micropipette attached to a plastic tube until the embryo is trapped between the silicone surface and the water surface that immobilizes it (Alexander Aulehla, personal communication; Fig. 3b; see Note 42).
- 1. With a very fine sharp Tungsten needle, make an incision in the PSM perpendicular to the end of the notochord (Fig. 3b; see Note 43).
- 2. Make a second cut at a distance two-thirds from the anterior end of the PSM (see Note 44).
- 3. Complete the first cut perpendicular to the notochord end.
- 4. Make a third cut along the PSM-neural tube boundary (see Note 45).
- 5. Draw the dissected PSM piece into a fresh micropipette with the handheld micropipette with as little medium as possible and expel it into a prepared 1.5-mL reaction tube containing ice-cold 500-µL Trizol.
- 6. Immediately transfer the tube onto dry ice or immerse in liquid nitrogen.
- 7. To replenish the dissection area, carefully apply L15 medium next to the embryo using the handheld micropipette or by mouth pipetting to lift it from the silicon surface (see Note 46).
- 8. Transfer the embryo to a prepared 0.5-mL reaction tube containing ice-cold 4% paraformaldehyde for fixation.

The embryo is then used for standard whole-mount in situ hybridization (14). The PSM piece can now be used for RNA isolation and subsequent DNA microarray analysis.

3.6. Custom-Made The earlier protocols require a few custom-made dissection and manipulation tools. Using standard workshop and laboratory Tools for materials they can be easily built. Micromanipulation

Tools made of tungsten wire can be sharpened to very fine-pointed 3.6.1. Tungsten Tools for needles and bent into different shapes ideal for microdissection (see Note 47). These protocols describe how to make them.

**Electrolytic Sharpening:** 

Microdissection

- 1. Cut a 2-cm piece off the tungsten wire.
- 2. Attach two cables to a standard laboratory power supply (see Note 48) that you place next to a stereoscope. Clamp the piece of tungsten wire into an alligator clip attached to the anode (+) of the power supply.

	3. Fill a small glass beaker with 0.5 M NaOH (see Note 49).
	4. Turn the power supply to $5-10$ V.
	5. Insert the cathode (-) into the NaOH solution.
	<ol> <li>Repeatedly dip the tungsten wire in and out of the solution by hand. The solution will bubble with ongoing electrolysis. Check the progress of sharpening under the stereomicroscope. You can determine the degree of sharpening and shape of the needle tip by adjusting the voltage and dipping rate.</li> </ol>
Fire-Polishing	A less sophisticated way to sharpen tungsten tools is to fire-polish them. For thinner tungsten wires, that works well. Since it is a slow process, it leaves time to adjust the thickness and sharpness precisely.
	1. Attach a 2-cm piece of tungsten wire to a needle and handle ( <i>see</i> <b>Subheading "Attaching to Holders and Shaping</b> ") and hold it into a Bunsen burner flame until it glows orange for 30–60 s.
	2. Check the progress of sharpening under a stereomicroscope.
Attaching to Holders and Shaping	For holding the tungsten needles a simple and cheap design can be used ( <b>Fig. 4a</b> ):
	1. Cut the cannula of a 27G needle with pliers to a length of ~0.5 cm.
	2. Use flat nose pliers to carefully reopen the now flat cannula tip by squeezing on the short edges.
	3. Insert the piece of tungsten wire a few millimeters into the cut cannula.
	4. Fix the tungsten wire in the cannula by strongly clamping the tip with pliers.
	a
	tungsten wire 2/G heedle syringe
	b c 100μm
	Ø20μm ₩100μm Ø5μm wire Øire

Fig. 4. Tungsten tools for microdissection and transplantation. (a) Holder for tungsten tools, consisting of a 1-mL syringe and a cut 27G needle. (b) Tungsten loop for optic vesicle transplantation. (c) Tungsten "hook" for microdissection.

	5. If the tungsten is not completely fixed (this will be the case with small-diameter wire), apply a drop of superglue.
	6. The tungsten needle can now be attached via its female Luer lock port to many holders (e.g., use a 1-mL syringe as a very cheap but good handle).
	7. To bend the tip of the needle to different tool shapes (e.g., a sharp hook or a loop) attach the holder to a micromanipulator and place the needle tip into the field of view. Use a pair of Dumont forceps to bend the needle tip to the desired shape ( <i>see</i> <b>Note 50</b> ).
	8. To electrolytically sharpen a needle after use, take it off the handle and hold the cannula with the alligator clip ( <i>see</i> Subheading "Electrolytic Sharpening").
	9. Store needles in the cannula on a piece of modeling clay in a Petri dish.
	10. Used tungsten needles can be conveniently cleaned and ster- ilized by flaming with a lighter.
3.6.2. Handheld Micropipette	To apply mineral oil as small droplets onto the surface of the embryo a custom-made, handheld micropipette, which is simple to put together, is ideal. It can also be used to transfer small pieces of explanted tissue in a small liquid volume ( <i>see</i> <b>Subheading 3.5.2</b> ).
Parts	The device consists of three parts (Fig. 5a):
	• Glass needle, pulled from borosilicate glass tubing with internal filament, pulled to the shape of a standard injection micropipette on a micropipette puller and broken to a controlled tip diameter.
	• Microelectrode holder with a male Luer port.
	• Handle (World Precision Instruments, 2505).
Assembly and Operation	1. Attach the microelectrode holder to the handle.
	2. Cut a 15-cm piece of flexible tubing that fits with its inner diameter to the male Luer port on the microelectrode holder ( <i>see</i> <b>Note 51</b> ).
	3. Attach one end of the tubing to the male Luer port on the microelectrode holder ( <i>see</i> Note 52).
	4. Bend the other end of the tubing back toward the end of the handle.
	5. Loop a piece of thick wire around the tubing and handle and twist-lock it in place using pliers. This air-seals the tubing.
	6. Use flexible tape to loosely glue the tubing at a mid level to the handle.
	7. Fill a micropipette by attaching it (e.g., using a piece of mod- eling clay as holder) to an oil-filled reaction tube with the



Fig. 5. Handheld micropipette. (a) A commercial micropipette holder is attached to a handle and an airtight piece of plastic tubing serves to expel and aspirate liquids through a pulled, glass micropipette. (b) The device is held like a pen and operated by compressing the plastic tubing. (c) For oil application, glass micropipettes (with internal filament) are filled by capillary force, by inserting into an oil-filled reaction tube, attached via a piece of modeling clay.

- tip inserted the internal filament will draw the oil into the needle. This can take a few hours (**Fig. 5c**).
- 8. Fit the oil-filled micropipette into the microelectrode holder.
- 9. Hold the device like a pen using the index finger to compress the tubing on the handle to expel small oil droplets (*see* Note 53 and Fig. 5b). Test the oil gun by expelling small droplets into a medium-filled Petri dish under a stereomicroscope. The size of the droplet can be regulated by breaking the tip to different diameters.

#### 4. Notes

- 1. Polysciences, Inc. offers a broad range selection of microbeads. Polystyrene beads with a diameter down to  $25 \mu m$  have been successfully used for manual implantation.
- 2. Or any other protein/substance that you want to test by local, time-controlled application through microbeads.
- 3. Important: use exactly this oil not every mineral oil from you lab stock will work.

- 4. Tungsten wire of different lengths and thicknesses can be bought from various suppliers, e.g., World Precision Instruments (WPI), Clark Electromedical Instruments/Harvard Apparatus, or California Fine Wire.
- 5. The Petri dish should be coated up to 1.5 mm under the rim, making it easier to reach the embryo with dissection tools.
- 6. For example, Tygon tubing, ID 1/32, OD 3/32, 50' is distributed by VWR.
- 7. Only by so doing can you exert force against the embryo, which is necessary if you want to explant or implant.
- 8. As soon as you manage to rapidly orientate embryos in agarose you can do parallel embeddings with two or more embryos in one drop this enhances throughput. Regarding fire-polishing of Pasteur pipettes, *see* Note 21.
- 9. It is important to avoid transfer of excess medium into the agarose to prevent gradual dilution. Excess medium at ambient temperature also tends to clog the melted agarose in the Falcon tube. Hold the tube against the light to find embryos.
- 10. Use the cover, not the dish's bottom, because the high rims of the bottom hinder manual access with the dissection tools during manipulation.
- 11. Embedded embryos can be stored in a "moist chamber" (e.g., a parafilm-sealed Petri dish with water-soaked pieces of tissue).
- 12. The embryo will be exposed in this wedge. The size and width of the wedge depend on the subsequent surgery. For eye manipulation, cut a wedge that forms a 45–60 angle this leaves the embryo embedded in enough agarose to not become loose during surgery.
- 13. This needs to be done before adding medium otherwise, the contours cut into the agarose will not be visible and cannot be followed with the needle.
- 14. Embryos will grow into the wedge during further incubation and thus should be manipulated rapidly after completed mounting. If you want to store mounted embryos, do so before cutting the wedges.
- 15. Gently press the tip of the oil-filled micropipette to the embryo and then press the handheld micropipette until a droplet sticks to the epidermis. Alternatively, first nick the epidermis with a sharp, fine-pointed tungsten needle and then place the droplet.
- 16. For all manipulation steps that require very precise manual operation, let the working hand rest on the stereomicroscope stage. Support the working hand using the other hand.
- 17. After this step the epidermis detaches from the embryo as a necrotic "blister."

- 18. If it does not detach easily, incubate for longer after application of the oil.
- 19. Healing happens rapidly thus, directly proceed with manipulation after this step, before the wound closes.
- 20. It is best if the mold size is a little smaller than the diameter of the embryos. Thus "squeezed-in," embryos do not roll during implantation.
- 21. For fire-polishing, nick the pipette with a diamond knife at the shank position that corresponds to the desired tip diameter. Then, gently break off the tip. Pull the pipette a few times through the flame of a Bunsen burner to polish it unpolished pipettes can damage dechorionated embryos easily.
- 22. After drawing beads into the pipette tip, clean outside of the tip by dipping it once into a Petri dish containing embryo medium this removes beads from the outside of the tip, which otherwise would float on the surface of the medium in your manipulation dish.
- 23. Use a fairly blunt needle this ensures that the cells are pushed apart rather than destroying them.
- 24. For this, use a kind of "underwater hockey" technique, generating swirls that move the bead ahead.
- 25. It is sometimes favorable to push the bead a little sideward after inserting it, which assures that during healing it does not slip out. This might require making the initial opening with a little spatial offset to the desired, final implantation site.
- 26. For late bead implantations, create only a small hole by applying a smaller oil droplet. Beads tend to slip out of the tissue if the hole is too big.
- 27. Often, heparin-coated beads (e.g., heparin-acrylic beads, Cat. No. H5263, Sigma) are used. But these beads are variable in size and shape and therefore get loaded with unpredictable amounts of protein. Comparison of implantation results of two beads from the same preparation is not possible. Sizecontrolled polystyrene beads have very reproducible effects.
- 28. The beads are delivered in drop dispensers (one drop corresponds to approximately  $35 \ \mu$ L).
- 29. Protein and protein-loaded beads can stick to the wall of uncoated plastic ware, decreasing effective protein concentration during loading and poor retrieval of beads.
- 30. Depending on how much protein solution is added, the amount of beads/volume is controlled. Following the instructions of this protocol, adding volumes as small as 10  $\mu$ L/tube is fine.
- 31. Do not introduce bubbles otherwise, beads will stick to the wall of the reaction tube.

- 32. Do so by following the contours of the optic vesicle in a stepwise fashion, first superficially and then increasingly deeper in the tissue. This step needs to be done with extreme care to avoid disruption of the optic vesicle. Do not disrupt the epidermis above the optic vesicle: once the host vesicle is removed it will serve as a "pocket" for the donor vesicle. Without this epidermal pocket, the graft will not heal in. This also applies to other tissue grafts – always place the tissue under an intact piece of epidermis; otherwise, it will fall off and disintegrate.
- 33. For the donor embryo, less care to preserve the epidermis above the optic vesicle is necessary. Rather, the tissue integrity of the optic vesicle to be grafted is more important, which is sometimes better achieved if larger parts of the head epidermis are removed by applying several small oil droplets.
- 34. **Steps 8** and **9** have to be done rapidly to avoid the disintegration of the graft.
- 35. This stage was picked because the PSM is compact enough to dissect it as a whole piece and contamination by endodermal cells is almost negligible since they occur as a scattered, single-cell layer ventral to the PSM.
- 36. The dell does not need to be deep, the embryo should fit in half way – it is only needed to facilitate injection. Make the dell with a nonpulled micropipette.
- 37. AMP-PNP is needed to inhibit contractions of the yolk cell by paralyzing the actomyosin skeleton. This reduces curling of the embryo and facilitates surgery. AMP-PNP is membrane impermeable and will not diffuse into the embryonic cells.
- 38. Mouth pipetting was always used since it works more accurately than the handheld micropipette. Mouth pipetting is not allowed in every country, so consult your local regulations.
- 39. It can often be taken off conveniently as one piece.
- 40. The periderm is very flexible and difficult to cut with a tungsten wire and this makes it difficult to dissect the PSM properly. That is already apparent when cutting off the head. The advantage is that it can be pulled off in one piece and it will already detach from the embryo trunk when cutting off the head. When trying to culture the embryo, the periderm needs to remain in place, otherwise the embryo will lose its normal morphology.
- 41. The border between PSM and LPM is clearly visible at this stage from dorsal or ventral. The embryo was positioned with the ventral side down and was stabilized with the other hand.
- 42. Positioning the embryo always in this way facilitates dissection and increases precision. Also, do not aspirate too much

fluid, otherwise the preparation will dry out by the heat produced by the microscope lamp.

- 43. Do not cut all the way through the tissue because it will rapidly split open causing a morphological change of the tissue, which makes it difficult to make a precise second cut.
- 44. The position for the second cut was defined using an eyepiece reticule. Position the embryo with its midline parallel to the reticule and the perpendicular notochord incision line at the null position of the reticule. Based on test dissections and subsequent ISH stainings the mark on the reticule had been defined that would demarcate the two-third PSM piece for that orientation at the given magnification.
- 45. Try to follow that line all the way through to the ventral side, ending next to the notochord, since in the posterior end of the PSM the neural tube covers the PSM on the dorsal site.
- 46. This is a critical step because if working too slowly the surface will dry off, which will damage the tissue. Also, when adding L15 to the embryo, it can stick to the water/air interface, which destroys the embryo.
- 47. Fine tungsten needles can also be used for dissection and thick sectioning of fixed embryos, e.g., for imaging of whole-mount stainings. A good set of tungsten needles is very precious and can be used for many years.
- 48. Alternatively use a 9-V block battery.
- 49. The NaCl solution can be recycled many times.
- 50. The loop for optic vesicle transplantation is made of  $5-\mu m$  diameter tungsten wire and bent to a diameter slightly bigger than the tissue block to be transplanted (90  $\mu m$ ). The sharp hook for tissue loosening and dissection is made of sharpened 20- $\mu m$  diameter tungsten wire.
- 51. If the tubing is too soft it will fold and close. To prevent this, you can insert a piece of more rigid, small-diameter tubing into the soft tubing to support it.
- 52. Silicon grease helps to make the connection airtight.
- 53. If the oil does not expel, the system is either not airtight or the tip of the glass needle is too small.

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