Preface

In the last 20 years, research activity using the zebrafish *Danio rerio* has increased dramatically. Their contribution to modern genetic and molecular research originates with their use as a vehicle for testing ideas concerning the genetic basis of vertebrate brain formation and function at the University of Oregon's Institute for Neuroscience. Their research use has expanded into their becoming a leading model system for understanding the basic genetics, cell biology, and physiology of vertebrate development and human disease states in hundreds of labs around the world. It has been a heady time for the little fish! There are good reasons for this rapid rise of popularity, both practical and technical. Practically, zebrafish are easy and inexpensive to keep, breed, and raise, and—similar to yeast, mice, and fruit flies—zebrafish like being around humans. Technically, the genetic tractability, embryonic accessibility, and imaging potential of the zebrafish are, in our opinion, the features that have tempted so many people to push the boundaries of zebrafish research so far in such a short time. Although each model organism has its strengths and weaknesses, we now regard zebrafish as sitting alongside mouse, worm and fruit fly as key animal model systems in modern biology.

There are already a number of excellent books and papers dealing with zebrafish experimental techniques, which begs the question—why another one? In choosing the contributions to this book, we were guided by three principles as we sought to make sure that this volume made a useful contribution to the field. First, because of the rapid development of techniques and reagents, we looked for material that was not yet well known or widely distributed. Second, we sought experience from newer labs with approaches that had not received exposure. Third, we tried to avoid duplicating familiar, well tested, and trusted material. The material in this volume is organized loosely along three strengths of the zebrafish: genetic modification, accessibility for manipulation, and ease of in vivo live imaging.

With a nearly complete sequenced genome, with significant genetic homology to that of humans, and with ease of mutagenesis and housing of sufficient numbers to enable forward genetic screens, the zebrafish is a natural candidate for genetic analysis of biologic processes. Chapters 1 and 2 describe dense chemical and retroviral mutagenesis, Chapter 3 covers resource-efficient haploid screening, and Chapter 4 discusses effective cryopreservation of zebrafish sperm for the precious mutants harvested from these techniques.

External fertilization and the production of large numbers of embryos from each mother have made practical the microinjection of lineage dyes, mRNA for protein overexpression, and DNA for transgenesis, as well as the transplantation of cells for genetic cell-autonomy studies. It has also made possible large-scale screens for gene expression using *in situ* hybridization, and enhancer traps. Part II of this volume develops these themes, describing the use of transposons in Chapters 5 and 6, or homologous recombination in bacterial artificial chromosomes in Chapter 7 to modify zebrafish chromosomal DNA for transgenic analysis of gene expression, as well as efficient single-copy transgenesis in

Chapter 8. Having thus created reporter strains of zebrafish with fluorescently-labeled cells, a novel method of ablating these cells specifically with nitroreductase allows their role in the organism to be tested, and is discussed in Chapter 9. Such cellular-level precision is also found in Chapter 10, which focuses on the focal electroporation of dyes or DNA into cells deep within the fish. However, sometimes a slightly larger specific region of the embryo must be manipulated, and zebrafish surgical techniques along the lines of those utilized in chick experimental embryology are presented in Chapter 11. Having plentiful embryonic material also facilitates the use of microarrays to analyze mRNA expression. Chapters 12 and 13 describe their synthesis and use for the zebrafish. The recent emergence and importance of microRNA biology has been underscored by pioneering work in the zebrafish; Chapter 14 outlines methods for validating microRNA targets in vivo.

It is perhaps the optical transparency of the zebrafish embryo that has most tipped the balance in its favor. In this volume, we included chapters showcasing methods that most labs with access to the equipment of a modern biology department can use. Chapter 15 describes a protocol for following tissue-scale morphogenesis simultaneously in multiple embryos that allows for the estimation of precision and variability. The striking beauty and power of single-cell resolution in the living zebrafish is seen in Chapters 16 and 17, which focus on imaging the early immune system using laser confocal scanning microscopy and the deeper cells of the gastrula using two-photon. The significant technical challenges of imaging the late-developing gut are tackled in Chapter 18 with a range of methods that include principles with application to other larval organ systems. Finally, Chapter 19 presents methods for achieving the lofty goal of following every cell in an organ, or indeed an entire organism, during development.

We hope that these chapters not only meet experimental needs that already exist, but also that they might inspire approaches that were not previously considered, and finally that they might give close insight and perspective into the emerging literature. The editors would like to thank John Walker and the staff at Humana Press and Springer for their continuous assistance, and the authors for their hard work and flexibility.

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Chapter 2

Production of Pseudotyped Retrovirus and the Generation of Proviral Transgenic Zebrafish

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Summary

This chapter describes a method for generation of the high-titer pseudotyped Moloney murine leukemia virus (MLV) that efficiently infects zebrafish embryos (i.e., more than 25 retroviral copies per cell). Injection techniques are also described for production of the retrovirus-infected mosaic "founder" fish. We describe a quantitative PCR (qPCR)-based assay as a quick way to assess the infectivity after each round of viral production and injection. Most of the required equipment is commercially available and commonly present in most research laboratories.

Key words: Zebrafish, Retrovirus, Pseudotyped Moloney murine leukemia virus, Insertional mutagenesis, Injection.

1. Introduction

The use of zebrafish as a vertebrate model organism in research has increased substantially in the past two decades, following the demonstration that zebrafish are amenable to large-scale forward mutagenesis screens *(1, 2)*. Typically, forward genetic screens were generally limited to studies using invertebrates such as flies, worms, and yeast. Because of its small size, fecundity, and fast embryonic development *ex utero*, zebrafish made it possible to apply large-scale genetic screens to the study of vertebrate-specific processes that affect development and disease. Two large-scale forward genetic screens used the chemical ethylnitrosourea (ENU) as a mutagen and generated more than 6,600 observable embryonic mutations. However, because ENU mainly produces point

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mutations, laborious positional cloning becomes necessary for identifying most of the mutated genes. To date, only about 160 of the genes responsible for the corresponding phenotypes identified in these two large-scale ENU-based screens have been cloned.

Insertional mutagenesis is a method complimentary to chemical mutagenesis, where foreign DNA is inserted into the genome, disrupting gene expression. The major advantage of insertional mutagenesis is the ease of identifying the mutated genes. The inserted foreign DNA (e.g., transposons or retroviruses) can act as a molecular landmark that allows for rapid cloning of the adjacent flanking genomic sequences. Moloney murine leukemia virus (MLV) pseudotyped with the envelope glycoprotein from vesicular stomatitis virus (VSV-G) has been developed as an insertional agent in zebrafish *(3)*. Pseudotyping renders MLV the ability to infect a broader range of hosts, including zebrafish cells. It also increases the stability of viral particles, allowing for the increase of the viral titers 1000-fold through ultracentrifugation *(4, 5)*. A large-scale forward insertional mutagenesis screen based on this pseudotyped MLV system was performed successfully, identifying approximately 500 observable embryonic recessive mutations. These mutants represent about 385 different genes; 335 of which have been identified *(6–10)*.

The pseudotyped MLV system can also be used as a transgenesis tool for the purpose of gene delivery. For example, a large-scale enhancer detection screen has used this MLV system to deliver an “enhancer-trap” vector into zebrafish and generated more than 1,000 transgenic lines expressing the reporter yellow fluorescent protein (YFP) in various tissues and cells *(11)*.

The pseudotyped MLV system provides the advantage that it is the most efficient insertional agent in vertebrates to date. Using a high-quality preparation of virus, almost all injected founders will transmit integrations through the germline with an average of ten copies per cell in the F1 progeny *(12, 13)*. In this chapter, we describe protocols to generate highly infective pseudotyped MLV particles and techniques to inject zebrafish embryos with the pseudotyped MLV. A quick quantitative polymerase chain reaction (PCR) assay is also described for the early assessment of infectivity after injection.

2. Materials

2.1. Cell Culture

1. 600-mL cell culture flasks (Nalge Nunc International, Rochester, NY).
2. Poly-L-lysine, 0.01% *(w/v)* (Sigma, St. Louis, MO).

3. 1X phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA, Carlsbad, CA).
4. 0.25% Trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Invitrogen).
5. DMEM growth medium: A 500-mL bottle of Dulbecco's modified Eagle' medium (DMEM, Invitrogen) is supplemented with fetal bovine serum (FBS; Hyclone, Logan, UT) to 10% *(v/v)* and 1X penicillin-streptomycin (Invitrogen).
6. Lipofectamine™ transfection reagent (Invitrogen).
7. pHCMV-G plasmid *(5)*.
8. Opti-MEM I medium (Invitrogen).
9. Bottle top filters, 0.2 μm pore size (Nalge Nunc International).
10. HEK293-based viral packaging cell line, for best results with stable MLV gag-pol expression and stable proviral mRNA expression.

2.2. Virus Concentration

1. Ultracentrifuge (XL-90, Beckman Coulter, Fullerton, CA).
2. SW28 (or equivalent ultracentrifuge rotor) (Beckman Coulter).
3. Ultracentrifuge tubes, 40 mL (Beckman Coulter).

2.3. Embryo Preparation

1. One 2-L Erlenmeyer flask (for preparing 1X Holtfreiter's).
2. One 1-L Erlenmeyer flask (for preparing 1X Holtfreiter's solution containing polybrene).
3. Three to four 100-mL beakers (for collecting embryos).
4. 25-mL pipets (for washing embryos).
5. 1 M HEPES solution (Sigma), stored at 4°C.
6. 80 mg/mL polybrene (10,000X stock): This stock solution is made by adding appropriate amount of water to the lyophilized powder of Sequa-brene (Sigma). Store stock solution at −20°C.
7. 10X modified Holtfreiter's solution: For 2 L of 10X stock solution, mix 70 g NaCl, 2.6 g $CaCl_2 \cdot (2H_2O)$, and 1 g KCl, filter-sterilized or autoclaved. This 10X stock is stable for months at room temperature. For preparing 1X Holtfreiter's working solution, dilute the 10X stock into 1X and buffer it with HEPES to a final concentration of 5 mM at pH 7.0. This 1X Holtfreiter's solution should be made freshly each time as HEPES is not stable at room temperature. Two to three liters of 1X Holtfreiter's solution is usually required for preparing 3–4 clutches of embryos. Set aside 0.5–1 L of 1X Holtfreiter's and add polybrene to a final concentration of 8 μg/mL.

8. 10 mg/mL Pronase: This stock is prepared by adding an appropriate amount of water to the pronase powder (Roche Applied Science) and incubating the solution at 37°C for 1 h (self-digestion step). The solution is then aliquoted (e.g. 500 μL per tube) and stored at –20°C.

2.4. Pre-injection Preparation

1. Needle puller (Sutter Model P-2000, Sutter Instrument, Novato, CA).
2. Quartz or glass capillaries (Sutter Instrument).
3. 10-cm Petri dishes.
4. Microscope slides (75 X 25 mm, 1 mm thick).
5. 2% *(w/v)* agarose made in 1X Holtfreiter's solution (for making injection ramps).
6. Six-well tissue culture dishes (Corning, Lowell, MA).

2.5. Virus Injection

1. Injection apparatus (*see* **Subheading 3**).
2. Scalpel blade (for cutting off the tip of the injection needle).
3. Injection hood with dissecting microscope.
4. 160 μg/mL polybrene (20X stock).
5. 1% *(w/v)* phenol red in 1X PBS.
6. 5.75-in. Wide bore pasteur pipet (cat. no. 13–678–30, Fisher Scientific, Pittsburgh, PA).
7. 0.22-μm-filtered system water.
8. 10-cm Petri dishes (for raising the injected embryos during the first 5 d).

2.6. Virus Evaluation

1. Proteinase K lysis buffer: 10 mM Tris–HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 0.4% sodium dodecyl sulfate [SDS], and 100 μg/mL proteinase K (freshly added from a 200X stock, Invitrogen). This solution can be prepared beforehand with all the components added except proteinase K. Lysis buffer without proteinase K can be stored at room temperature for months.
2. Isopropanol.
3. 70% ethanol.
4. Heating blocks set at 50°C and 37°C.
5. Viral (SFG) probe: 5′-FAM-CTGCTGACCACCCCCAC-CGC-TAMRA-3′, stored at –20°C and protected from light.
6. RAG1probe:5′-HEX-GCGCAACGGCGGCGCTC-TAMRA-3′, stored at –20°C and protected from light.
7. SFG primers:
   5′-CGCTGGAAAGGACCTTACACA-3′
   5′-TGCGATGCCGTCTACTTTGA-3′.

8. RAG1 primers:
   5′-ATTGGAGAAGTCTACCAGAAGCCTAA-3′
   5′-CTTAGTTGCTTGTCCAGGGTTGA-3′.
9. Platinum® *Taq* DNA polymerase and buffers (Invitrogen) or equivalent PCR reagents: 10X PCR buffer, 50 mM $MgCl_2$, and Platinum® *Taq* polymerase (5 U/μL).
10. 10 mM dNTP Mix (Invitrogen).
11. iCycler (Bio-Rad, Hercules, CA) or equivalent real-time PCR machine
12. 96-well PCR plates for iCycler (Bio-Rad).
13. Reference fish DNA samples with known numbers of proviral copies (e.g., reference DNA with one and four proviral copies per cell).

3. Methods

3.1. Cell Culture

Pseudotyped retroviruses are typically generated in a packaging cell line while the cells are transiently transfected with vectors expressing the pseudotyped envelope proteins. We used a cell line, termed GT186 *(12)*, for packaging the pseudotyped MLV. The GT186 cell line was derived from 293 human embryonic kidney cells that stably express the *gag* and *pol* genes of MLV under the control of the human cytomegalovirus (CMV) promoter *(14)*. There is also a stable chromosomal integration of the proviral genome whose RNA transcript is packaged into the MLV active viral core particles. The proviral genome contains full MLV long terminal repeats (LTRs), the packaging signal (ψ), and a reporter, *lac*Z gene, used for titering purposes. Plasmid pHCMV-G (the VSV-G protein driven by the CMV promoter) is transiently transfected into GT186 cells. The expressed VSV-G proteins encapsulate the viral core particles at the plasma membrane and the active pseudotyped retroviruses are released into the tissue culture medium and collected. It is critical to express the VSV-G proteins only transiently because they are cytotoxic when expressed at the levels necessary to generate high-titer pseudotyped viruses. A detailed protocol for generating a large batch of virus sufficient for a 1-wk schedule of injections follows.

3.1.1. Day 1: Grow the Cells on Poly-Lysine-Coated Tissue Culture Flasks

1. Coat the 600-mL flasks with poly-L-lysine. Draw 25 mL of 0.01% *(w/v)* poly-L-lysine solution to cover the surface of each flask for few seconds and then transfer the solution to the next flask. Repeat the step until all flasks are “rinsed.” Leave the “rinsed” flasks horizontally in the hood for 5 min and then

stand all flasks up to let the excess solution flow down to the bottom of the flasks (*see* **Note 1**).

2. Use a sterile aspirating pipet connected to a vacuum source to dry the surface of flasks until no liquid can be seen.
3. To have the appropriate density of cells at the time of transfection, typically we grow GT186 cells in five 600 mL flasks (surface area 185 cm^2, without the poly-L-lysine coating) until about 80% confluence and then split the cells into 10 poly-L-lysine-coated 600-mL flasks the day before transfection (*see* **Note 2**).
4. To trypsinize the cells, the cells in each flask are washed with 20 mL of 1X PBS once. 3 mL of trypsin–EDTA solution is then added slowly to cover the entire cell monolayer. Sit the flasks under the hood for about 3–5 min to ensure that all cells detach from the surface and from each other (HEK293 cells actually adhere to each other better than to the flask).
5. While waiting for trypsinization to complete, aliquot 20 mL of DMEM growth medium to the poly-L-lysine-coated flasks. Stop trypsinization by adding 7 mL of DMEM growth medium into each flask. Resuspend thoroughly, and pool all cells into one flask (the total volume should be around 50 mL). Mix thoroughly and aliquot 5 mL of the cell suspension into each poly-L-lysine-coated flask containing 20 mL of DMEM growth medium (*see* **Note 3**).

3.1.2. Day 2: Transfection Using Lipofectamine™ (LF)

1. We found that the optimal cell density just prior to transfection is 70–80% confluence. The optimal LF:DNA ratio is 15:1 (μL:μg). For each 600 mL flask, 8 μg of pHCMV-G plasmid is used (and thus 120 μL of LF is needed). In total 80 μg of pHCMV-G plasmid and 1200 μL of LF are needed for ten flasks.
2. To prepare the DNA/LF complex, in one 15-mL conical tube, mix 80 μg of pHCMV-G plasmid with 5 mL of Opti-MEM I medium without serum and antibiotics. In another 15-mL conical tube, mix 1,200 μL of LF with 4 mL of Opti-MEM I medium without serum and antibiotics.
3. Combine the solutions from these two 15-mL conical tubes and mix by gently pipeting up and down three times.
4. Incubate the DNA/LF solution for 15–20 min at room temperature.
5. During this incubation, rinse the cells with 20 mL of Opti-MEM I medium without serum or antibiotics and replenish the cells with 19 mL of Opti-MEM I medium without serum or antibiotics in each flask (*see* **Note 4**).
6. Aliquot 1 mL of DNA/LF mix to each flask (for a final total volume of 20 mL). Mix gently and return the cells to the 37°C incubator.

7. Incubate at 37°C for 8–12 h.
8. Discard the DNA/LF mix and replenish the cells with 20 mL of DMEM growth medium (containing 10% FBS and antibiotics). Return to the 37°C incubator.

3.1.3. Day 3: First Collection and Change Medium

1. Between 21 and 24 h post-transfection, collect the medium and filter through a 0.2-μm filter, and replenish the cells with fresh 20 mL of DMEM growth medium. Return to the 37°C incubator. The filtered, collected medium is stored at 4°C (first collection).

3.1.4. Day 4: Second Collection

1. Between 44 and 48 h post-transfection, collect the medium again through a 0.2-μm filter. Combine the second collection with the first. The medium is then subjected to ultracentrifugation (*see* **Note 5**).

3.2. Virus Concentration

The volume of collected medium (~400 mL) requires two rounds of centrifugation in an SW28 rotor. To reduce the sample loss, we use the same centrifuge tubes (five tubes) for both rounds of centrifugation.

1. 38 mL of collected medium is added into each SW28 ultracentrifuge tube. The tubes need to be balanced to within 0.1 g of each other. The medium is centrifuged in a SW28 rotor at 27,000 rpm for 1.5 h at 4°C (131,453 × *g*).
2. The supernatant is gently poured into a beaker containing bleach and the tube is inverted briefly on a piece of paper towel to allow liquid to collect near the top rim of the tube.
3. Aspirate the rim of the tubes briefly and return the tubes to the tube adapters.
4. Load the second half of the medium to the same five centrifuge tubes and repeat the centrifugation step.
5. After the second centrifugation, a Pasteur pipet is used to aspirate away the excess liquid. It is important to ensure that all the excess liquid is removed from the side of the tube; small amounts of medium running back into the pellet can significantly dilute the virus, but also be careful not to over-dry the sample (*see* **Note 6**).
6. For each tube, gently resuspend the virus pellet in 30 μL of 1X PBS by pipeting up and down several times gently; avoid introducing bubbles (*see* **Note 7**).
7. Place a small piece of Parafilm over the tube and leave the sample at 4°C for 4 h to overnight.
8. Pool all the samples into a 1.5-mL microtube. Typically about 100 μL of virus suspension can be collected from five centrifuge tubes.

3.3. Embryo Preparation

The embryos are injected at the blastula stage, approximately 3 h after fertilization. Because of this small window of time for injection and the many embryos to be injected, it is essential to generate clutches of embryos in "waves" by setting up crosses at different times throughout the morning. We typically set up three to four tanks of adult fish for crosses the evening before injection. The next morning female and male fish are crossed to generate embryos at 45- to 60-min intervals. Before injection, the chorions of embryos need to be removed. The detailed protocol for embryo preparation follows.

1. The evening before injection, 6–8 females and 3–4 males are kept separate in a large breeding box. The most common large breeding box is made by cutting out the bottom of a "double-width" mouse cage and gluing a piece of wire screen over the hole. The cut-out mouse cage is stacked into another mouse cage, which is filled with system water. Place the males in the lower section by putting them in before putting in the insert. Place the females in the "upper" chamber. The belief is that keeping the fish together, but unable to breed, will maintain the "interest" to breed longer than if they are kept completely separate. It is unclear whether this is actually true. Typically three to four breeding boxes are set up for a day's injections.
2. At "dawn" when the lights come on in the fish facility, mix the females and males from the first breeding box in the top portion of the breeding box. The fish are allowed to mate for no more than 15 min after the first embryos are released.
3. After the fish are removed, the embryos are collected by pouring the water containing the embryos through a small tea strainer. The embryos are rinsed with sterile methylene-blue-containing system water briefly, and rinsed into a 100-mL beaker.
4. Replace the methylene-blue-containing system water with 20 mL of freshly made 1X Holtfreiter's solution.
5. Add 150 μL of 10 mg/mL pronase solution to the embryos and swirl gently. The pronase will begin digesting away the chorions of the embryos. Periodically swirl the beaker gently to help separate the chorions from the embryos.
6. It is critical to stop the pronase digestion promptly so that the embryos do not suffer unwanted damage. Usually we stop the pronase treatment when about 15–20 embryos appear to be dechorionated. To estimate the number of dechorionated embryos, gently swirl the beaker and the embryos that have lost their chorions will collect in the middle of the beaker. Over-digestion will result in fragile embryos and poor survival.
7. To stop the pronase digestion, slowly add 60 mL of 1X Holtfreiter's solution to the beaker and gently pour off. Do not pour off all the buffer as surface tension will damage the

embryos. Repeat the wash steps 6–8 times until most chorions are washed away. However, it is normal to see a small portion of embryos with chorions still attached (in fact, when all embryos have lost their chorions, it usually indicates that the embryos have suffered over-digestion by pronase). For the final wash, use 1X Holtfreiter's solution containing 8 μg/mL of polybrene (*see* **Note 8**).

8. Incubate the embryos in 1X Holtfreiter's solution containing 8 μg/mL of polybrene in a 28°C incubator for approximately 2.5–3 h.
9. To inject as many embryos as possible on a single day, space crosses 45 min to 1 h apart for the remaining breeding boxes. If all three or four rounds of crosses give significant numbers of embryos (>300 embryos per cross), this will allow the injector 3–4 h of injection time in the afternoon with appropriately timed embryos available throughout the period of injection; 1000–2000 embryos can be injected per person in an afternoon.

3.4. Pre-injection Preparation

3.4.1. Needle Preparation

Microinjection needles may be pulled in any commercial needle puller. We use the Sutter Model P-2000 (Sutter Instrument). The needles we use are pulled from quartz capillaries with 1.0 mm outer and 0.7 mm inner diameter. The pulled needle should be long and thin as the injections are made into the intercellular space instead of into the cells directly. Thus, a long, thin needle can slip in between cells with a minimal amount of damage to the integrity of the cell cap.

3.4.2. Injection Ramps

Agarose ramps are used to secure and position embryos during injection. To make an injection ramp, place a regular microscope slide in the bottom of a 10-cm Petri dish and then pour about 15 mL of 2% *(w/v)* agarose (made with 1X Holtfreiter's solution) to just cover the slide. A second slide is then placed on the top of the first slide and rested on the rim of the Petri dish at an angle (**Fig. 1**). After the agarose hardens, carefully remove the second slide to create a ramp and a groove in the bottom of the dish. Fill the dish with 1X Holtfreiter's solution containing 8 μg/mL of polybrene and incubate at 32°C for at least 1 h before injection.

3.4.3. Recovery Dishes

After injection, embryos will be transferred from the ramp into the recovery dishes, which are regular six-well tissue culture dishes filled with 1X Holtfreiter's solution containing 8 μg/mL of polybrene. Prepare several of those buffer-filled dishes and place them in a 37°C incubator at least 1 h before the injections begin.

3.5. Virus Injection

3.5.1. Injection Apparatus

The injection apparatus we use is a simple device. It consists of a 20-mL syringe with a 20-gauge syringe needle, connected to a needle holder by polypropylene tubing (**Fig. 2**). The front of

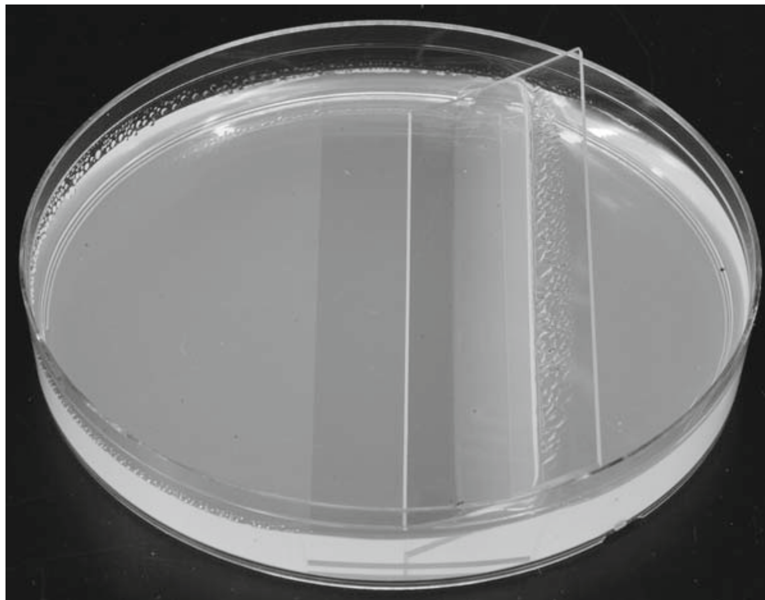

Fig. 1. Injection ramp.

the needle holder has a small silicon gasket, which is used as an airtight seal when the needle is inserted. The end of the pulled needle is sealed and needs to be removed with a scalpel blade. This cut is made under a dissecting microscope by lowering the blade straight down onto the needle. The location of the cut should be close to the point as we want the needle hole to be very small. However, because the viral preparations are slightly particulate, the hole needs to be large enough so that it does not get clogged with debris. The needle is then put into the silicon gasket and held in place by a screwed cap that fits over the end of the needle holder.

3.5.2. Loading Virus into the Needle

Just before injection, add polybrene to a final concentration of 8 μg/mL from a 20X polybrene stock and a trace amount of 1% phenol red (2 μL every 100 μL of concentrated virus) to the virus suspension prepared in **step 8 of Subheading 3.2** to help visualize the virus during injection. The virus stock now is ready for use in injections (*see* **Note 9**). To load the virus into the needle, on a clean microscope slide, add a drop (~15 μL) of the virus stock under the dissecting microscope, which is mounted in an injection hood (*see* **Note 10**). The virus is then drawn up into the needle by applying a vacuum with the 20-mL syringe. The concentrated virus often contains a significant amount of small debris, which inevitably will clog the needle. When the needle is clogged, apply the pressure on syringe while lifting the needle out of the surface near the edge of the virus drop. This helps

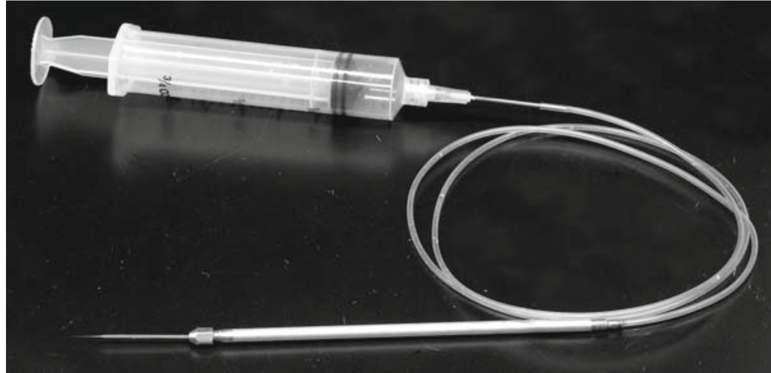

Fig. 2. Injection apparatus.

clear up the clog and also leaves the debris on the edge of the drop so that the same debris will not be sucked into the needle again. By repeating the "drawing and pushing" steps, the needle should be filled up within 5–10 min. Be careful not to introduce air bubbles into the needle by always keeping the needle under the liquid surface while applying a vacuum. We find it easier to use a hand-held needle holder rather than a micromanipulator for both aspiration of the virus as well as for injection (*see* **Note 11**). The main reason the virus is aspirated into the needle instead of being loaded with a "gel-loading" pipet tip from the back end, is to prevent clogging of the needle during injections.

3.5.3. Injection Procedure

Once the needle is filled with virus, take out the agarose ramp from the 32°C incubator and place the embryos in a single row at the bottom edge of the ramp using a 5.75-in. wide-bore Pasteur pipet. Caution should be taken while transferring the embryos because they are very fragile without the protection of the chorions (*see* **Note 12**). The embryos placed on the ramp should be between the 512- and 2000-cell stages. This is the optimal time frame for infecting the germ cells. At this stage there are four primordial germ cells, which will divide into 20–30 cells in the next few hours *(15)*, and the cell cap provides a "space" where the virus can be injected and retained long enough for infection. The injection ramp is then placed on a dissecting microscope in an injection hood. To start the injection, set the plunger of the 20-mL syringe half way along the syringe barrel and gently push the syringe to initiate the virus slowly flowing out of the needle point. The needle point is then inserted into the cell mass (**Fig. 3**). Only a very gentle "touch" is needed to get the point into the cell mass. Avoid penetrating the yolk with the point, for this is frequently fatal to the embryo. Each embryo is injected 5–6 times at different locations around the cell cap. The injector should

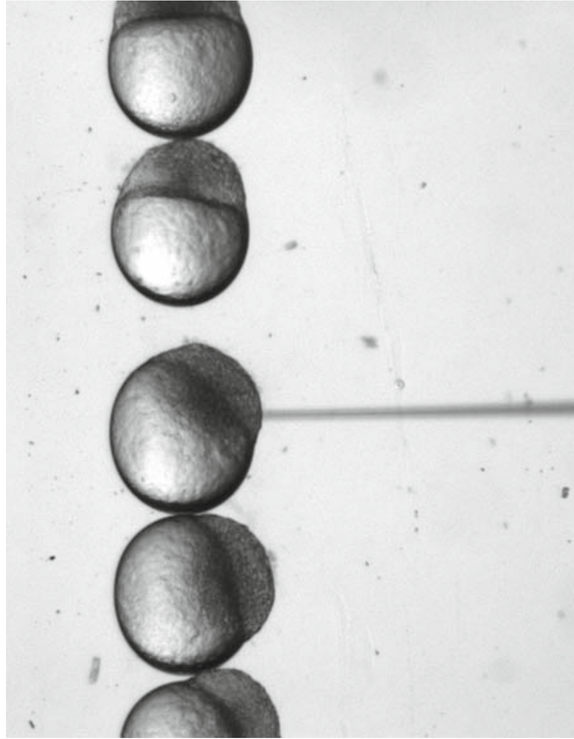

Fig. 3. Injection procedure. Dechorionated embryos (512- to 2,000-cell stages) are placed at the bottom edge of the agarose ramp. Viruses are injected into each embryo at different locations around the cell cap.

try to distribute the phenol red color evenly over the entire cell mass. The flow rate of the virus out of the needle tip should be kept as constant as possible to ensure that every embryo receives a similar amount of virus. It usually takes 15–20 min to inject one row of embryos (~100 embryos in an agarose ramp). After every embryo on the ramp has been injected, the injector returns to the beginning of the ramp and repeats the injection process. But this time only 2–3 "pokes" are given to each embryo. Tests in our laboratory have shown that there is generally an increase in overall virus infection when the injections are done twice to each embryo (*see* **Note 13**).

Once the injections are complete, the embryos are carefully transferred from the injection ramp to the pre-warmed six-well dishes. Each well of the six-well dish is loaded with a similar number of embryos. A single six-well dish usually holds embryos from one or two ramps of injected embryos (i.e., ~100–200 embryos). The six-well dish is then placed in a 37°C incubator for a 90-min heat shock period. After heat shock, the dish is transferred to a 32°C incubator and left for about 20 min. The dish is then taken out of the incubator again; the broken, unfertilized

embryos, and their debris are removed from each well under the dissecting microscope. This cleanup process is critical to ensure the proper development of the remaining healthy-looking embryos. After the cleanup, the dish is returned to a 32°C incubator and left overnight. The next morning embryos that appear to have developed normally are transferred into filtered system water in a 10-cm Petri dish and raised in the normal fashion at 28°C.

3.6. Virus Evaluation

Because the production of pseudotyped virus depends on transient transfection of the packaging cells with the plasmid encoding VSV-G, the titer of the virus can vary significantly between different viral preparations. The most direct assessment of the efficacy of the virus from different viral preparations is to determine the level of infectivity (i.e., average proviral copies per cell) in the injected founders. Although this value does not indicate the actual number of proviral copies that will be transmitted to the next generation, it is a fairly good predicator of overall transgenicity, which correlates with germline transmission. To have a quick assessment on the efficacy of the virus, we randomly select two to three embryos from each batch of injected embryos, isolate their genomic DNA, and determine the average proviral copies in the isolated DNA by using a multiplex qPCR-based assay (designated as Embryo Assay) *(7)*. The number of proviral insertions per cell is computed by measuring the amplification rate of the *SFG* locus, which is specific to proviral DNA, and comparing the ratios of threshold values between founder embryo DNA and DNA with known copy numbers of proviral insertions; the results are normalized to the control *RAG1* locus, which is simultaneously measured. A detailed procedure of a typical Embryo Assay is as follows.

1. Select 2–3 healthy-looking 2- to 3-d-old injected embryos and put them into a 1.5-mL microtube (*see* **Note 14**).
2. Remove the residual water and add 100 μL of lysis buffer (with freshly added 100 μg/mL proteinase K) to the embryos.
3. Incubate the microtube in a heat block at 50°C. Resuspend the solution every 10 min to help the lysis process until the solution becomes homogenous without any visible tissue. This step usually takes 30–40 min.
4. Add 100 μL of isopropanol to the solution, vortex, and centrifuge in a benchtop centrifuge at the maximum speed for 5 min at room temperature to precipitate the genomic DNA. A black pellet should be seen in the bottom of the tube after centrifugation.
5. Discard the supernatant and wash the pellet with 200–400 μL of 70% ethanol. Repeat the centrifugation step.
6. Discard the supernatant and dry the pellet by applying a gentle suction over the pellet using a vacuum attached to a Pasteur pipet with the tip covered by a 200-μL pipet tip.

7. A 50 μL *Bam*HI restriction enzyme digestion reaction is setup to digest the genomic DNA in the pellet by adding the reaction mix directly into the tube with the dried pellet.
8. Incubate at 37°C for 30 min with occasional resuspension by pipeting up and down to break up the pellet.
9. During the 30 min restriction enzyme digestion, setup the qPCR "master mix" without the DNA in a volume that after the addition of 3 μL of the *Bam*HI-digested genomic DNA into the master mix, the final concentrations of the reaction components will be as follows and the total reaction volume per well will be 25 μL: 1X PCR buffer containing 5 mM $MgCl_2$, 0.4 mM dNTP, 0.2 μM of each *SFG* and *RAG1* primer, 0.5 μM *RAG1* probe, 0.25 μM *SFG* probe, 2.5 U of Platinum® *Taq* polymerase. We usually setup the reaction in duplicates for each DNA sample tested in a 96-well PCR plate. Each run also contains wells of a reference control from a fish with known copy number of inserts.
10. The PCR is carried out with an iCycler (Bio-Rad) using HEX-530 and FAM-490 channels for *RAG1* and *SFG* probes, respectively. The cycle profile is 2 min at 95°C, followed by 40 cycles of (15 s at 95°C, 30 s at 60°C).
11. At the end of the run, the *RAG1* and *SFG* threshold cycles (Cts; the cycle at which the amount of product passed a certain threshold in the linear amplification range) are calculated for each sample. A ΔCt value is defined by subtracting the *SFG* Ct from the *RAG1* Ct. The larger the ΔCt value, the greater the number of proviral copies for any given sample is. By subtracting the reference's ΔCt from each sample's ΔCt (the reference contains known *N* proviral copies per cell), we calculate the ΔΔCt, which can then be used in the following formula to estimate the number of average proviral copies per cell in any given fish: the average proviral copies per cell in a given fish = $N \times 1.9^{\Delta\Delta Ct}$.

4. Notes

1. Coating the cell-growing surface with the positively charged poly-L-lysine molecules helps the GT186 cells remain firmly attached to the surface, making it ready for the subsequent transfection procedure. 600-mL flasks can be replaced with the 15-cm diameter cell culture dishes for cell culture. Using flasks makes the handling easier during the collection of medium, which contains the viral particles. However, flasks are significantly more expensive than dishes.

2. GT186 cells will gradually lose the efficacy of packaging viral particles, possibly because of the instability of the integrated viral genome and/or *gag-pol* genes after prolonged propagation. It is thus critical to use GT186 cells with a limited number of passages. After a batch of cells has been propagated for a while (~3–4 months) and the infectivity starts to drop, we thaw a new vial of early-passage cells from the frozen stock.
3. 293 cells attach to each other more strongly than they do to the bottom of the flask. To ensure even plating of the cells, you must actively dissociate the cells from each other. To get the cells into a single-cell suspension, we rest the pipet tip against the bottom of the flask while pipeting up and down the cell suspension. In this way the solution is passing through a narrow space, resulting in a greater shearing force that can separate the cell aggregates more efficiently.
4. Opti-MEM I medium can be replaced with serum-free DMEM medium from **steps 2** to **5 of Subheading 3.1.2**. The washing step (**Subheading 3.1.2**, **step 5**) may also be carried out using 20 mL of 1X PBS.
5. At 48-h post-transfection, you should see many cell-to-cell fusions in the VSV-G-transfected GT186 cells. This usually indicates a good production of infectious MLV particles as the expressed VSV-G proteins tend to induce cell fusion.
6. It is helpful to prevent the liquid running down the sides of the centrifuge tube, by scratching the inner side of the tube with the Pasteur pipet to make a spiral path while aspirating the excess liquid. Any residual liquid will thus be trapped by the scratched grooves instead of running back to the virus pellet when the tube is upright and the pellet is suspended in PBS.
7. Usually the whiter the suspension at this point, the higher the viral titer will be.
8. Promptly stopping the pronase digestion appears to be one of the most critical steps to have good quality embryos for injection. Most chorions will come off of the embryos during the subsequent washing steps, not during the digestion period. Thus, do not hesitate to stop digestion even when only few embryos have lost their chorions. It takes some practice to get the timing right, but generally when about 20 embryos have lost their chorions, the washing step should be begun. During the washing step, it is convenient to use a 25-mL pipet. After drawing the solution into the pipet, usually we detach the pipet from the pipettor and use the thumb to hold and release the solution slowly into the beaker in an effort trying not to overly disturb the embryos, minimizing the potential damage to the delicate embryos.

9. The virus stock can be stored at 4°C for up to 5 d without significantly losing titer. We do not recommend freezing down the virus since the viral titer will decrease by approximately 50% after freezing.
10. VSV-G pseudotyped MLV can also infect humans. Proper precautions should be used in handling this type of virus.
11. Loading the needle with virus can be one of the most difficult steps in the whole procedure because the needle tip will inevitably be clogged by the small debris in the viral preparation. We have found the best way to load the needle is to move the syringe plunger almost, but not all the way down to the bottom of the syringe. This allows a strong vacuum to be applied to the needle, but leaves a small space to apply pressure in the other direction if the needle becomes clogged. We also found that it is helpful not to start applying the pressure to clear the clog until the needle is completely clogged or the virus just drawn into the needle will almost be completely pushed out during the clearing process, resulting in almost no gain in the loading process. It is also important not to draw in any air bubbles because the small bubbles also clog the needle tip. Once the needle is full, it is better to disconnect the hose again (it is also a good idea to learn how to do the unplugging just using one hand) and move the plunger to about half way up the syringe. This gives more control over applying pressure during injection.
12. It is important to use the Pasteur pipet with an opening wide enough (e.g. at least 2 mm in diameter) to transfer embryos to prevent them from crushing each other as they pass through the opening. To place the embryos on the ramp as a neat line, slowly draw the embryos into the pipet from the beaker (it is important to fully release the bulb before retrieving the pipet tip out of the surface so that the pipet is completely filled with the solution without trapping any air in the very front of the pipet tip). Immerse the pipet tip just under the surface of the solution in the ramp. Let gravity draw the embryos down and out of the pipet without squeezing the bulb. While the embryos are dropping out of the pipet, move the pipet along the ramp to have the embryos drop as a single line.
13. The injection of virus into embryos is a learned skill. It takes time for an injector to get acquainted with the technique. It is a delicate balance between giving the embryo enough virus to achieve high infectivity, and fatally damaging the embryo with excessive pokes and virus. Several pointers below should help a novice shorten the learning process: (1) Because the virus is injected between cells, the needle needs to be very thin to reduce the chance of poking into the cells directly.

However, using a fine needle also means an increased difficulty of loading the virus. The injector should resist the urge to cut a larger needle hole, which will not only easily disrupt the embryo but also cause needless waste of virus. (2) Try to do several small injections, evenly throughout the entire cell mass. The tip of the needle can be used to orient the embryos. A very gentle "touch" should be enough to penetrate the needle into the cell mass. Always avoid damaging the yolk. The hand holding the injection apparatus should rest firmly on the stage of the microscope to increase stability. While doing the second round of injections, pay extra attention to those embryos with lighter dye tracer. One or two extra injections can be given to those lighter-colored embryos. (3) Pay attention to the virus flow out of the needle tip. Use the hand on the syringe plunger to control the flow rate by gently pushing or pulling the plunger when needed during injection. If the needle hole is fine enough, the virus flow should stay constant for quite a while after a gentle push. (4) Micromanipulators are not recommended. It is difficult at first to inject "freehand," but eventually it is much faster than the micromanipulator, and high injection numbers are desirable to offset reduced survival.

14. You should wait until the embryos are at least 2 d-old before performing the embryo infection assay to prevent measuring the "unintegrated" viral DNA, which is still present in the early embryos. This will result in artificially high estimates of infection. It is also important to avoid selecting the unhealthy looking embryos to assay because the measured values from those fish do not represent the infectivity of the healthy population (again, they tend to be inflated values).

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