Protocol

Injection of *Parhyale hawaiensis* Blastomeres with Fluorescently Labeled Tracers

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INTRODUCTION

The great diversity of arthropod body plans, together with our detailed understanding of fruit fly development, makes arthropods a premier taxon for examining the evolutionary diversification of developmental patterns and hence the diversity of extant life. Crustaceans, in particular, show a remarkable range of morphologies and provide a useful outgroup to the insects. The amphipod crustacean *Parhyale hawaiensis* is becoming established as a model organism for developmental studies within the arthropods. This protocol describes the injection of *P. hawaiensis* blastomeres with fluorescently labeled tracers for the purpose of cell-lineage analysis. The total (holoblastic) cleavages that characterize early embryogenesis in *P. hawaiensis* generate an eight-cell embryo with a stereotypical arrangement of blastomeres, each of which already possesses an invariant cell fate. Fluorochrome-conjugated dextran solutions, mRNAs encoding fluorescent proteins, and biotin-dextran have all proven to be useful lineage markers. The relative merits of various tracers are considered.

RELATED INFORMATION

Microinjection has also been used to generate genetically transformed lines of *P. hawaiensis* by coinjecting one-cell embryos with a Minos transposable element together with mRNA encoding the Minos transposase (see Pavlopoulos and Averof 2005). *P. hawaiensis* embryos can be fixed as described in **Fixation and Dissection of *Parhyale hawaiensis* Embryos** (Rehm et al. 2009a). The fixed embryo tissue can be used to study protein localization as described in **Antibody Staining of *Parhyale hawaiensis* Embryos** (Rehm et al. 2009b) or for in situ hybridization to probe mRNA expression, as in **In Situ Hybridization of Labeled RNA Probes to Fixed *Parhyale hawaiensis* Embryos** (Rehm et al. 2009c). An introduction to *P. hawaiensis* that discusses husbandry, the developmental stages of this crustacean, and its utility as a model organism for studies of arthropod development and evolution, is presented in **The Crustacean *Parhyale hawaiensis*: A New Model for Arthropod Development** (Rehm et al. 2009d).

The Sutter "Pipette Cookbook" (http://sutter.com/contact/faqs/pipette_cookbook.pdf) provides information on the preparation and use of micropipettes. Program parameters for pulling needles suitable for injecting *P. hawaiensis* blastomeres can be designed using this cookbook.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

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Reagents

Labeled dextrans (50 mg/mL stocks; Sigma Aldrich):
<!>FITC (Fluorescein isothiocyanate)-dextran (MW = 250,000)
<!>TRITC (Tetramethylrhodamine isothiocyanate)-dextran (MW = 155,000)
Biotin-dextran (MW = 70,000)

See "Tracer Choice" in the Method section.

Halocarbon oil 700
mMessage mMachine SP6 Kit (Ambion)
mRNA encoding fluorescent proteins (see Step 2)
<!>Nitrogen gas
P. hawaiensis pairs

*Materials for collecting embryos from gravid females are also required (see **Fixation and Dissection of Parhyale hawaiensis Embryos** [Rehm et al. 2009a]).*

<!>Phenol red solution (0.5%; Sigma-Aldrich P0290)
<R>Seawater (filter-sterilized)
Sylgard 184 Silicone Elastomer Kit (World Precision Instruments)

Equipment

Capillary glass tubing with filament (1-mm OD; World Precision Instruments TW100F4)
Dissection microscope with light source (20X-50X total magnification)

An inverted compound microscope with a stage-mounted micromanipulator may also be necessary (see "Injection Rig Set-Up" in the Method section).

Forceps (blunt)
Glass microscope slide
Incubator pre-set to appropriate temperature (see Step 7)
Injection holder (for 1-mm OD tube; Narishige HI-7)
Iron plate (Narishige IP)
Joystick micromanipulator (Narishige MN-151)
Magnetic base (Narishige GJ-1)
Microinjector (Narishige IM300)
Micropipette puller (Sutter P-97)
Modeling clay
Pasteur pipettes (5.75 in.; VWR)
Petri dish (large, plastic, 150 × 15 mm)
Petri dish (medium, plastic, 60 × 15 mm)
Petri dishes (small, plastic, 35 × 10 mm)
Pipette bulb
Plastic containers with lids (e.g., 14- × 10- × 10-cm plastic food storage containers)
Razor blade
Silicone tubing (1/32-in. ID; 3/32-in. OD; Cole-Parmer 06411-60)

METHOD

Tracer Choice

Both fluorochrome-conjugated dextran solutions and mRNAs encoding fluorescent proteins have proven to be useful for continuous in vivo observation of clones following microinjection of early blastomeres. Biotin-dextran injections require subsequent fixation and enzymatic development and thus provide data for only a single time point. However, biotin-dextran tracers result in a superior signal-to-noise ratio, are permanent, and allow for simultaneous DAPI staining. For details on the fixation, dissection, and histochemical reaction of embryos injected with biotin-dextran, see Gerberding et al. (2002). Fluorescent tracers provide excellent spatial resolution through gastrulation, and diminishing

resolution thereafter. FITC-dextran injections have higher background because of embryonic tissue autofluorescence. Injection of high concentrations of FITC-dextran (25-50 mg/mL) results in photoablation when the tissue is subsequently irradiated with blue light. TRITC-dextran suffers from fewer autofluorescence issues and does not cause cell death. Injection of mRNA encoding DsRed.T1 results in detectable fluorescence after 10 h. DsRed.T1 mRNA injections result in very strong fluorescence and can also be detected with anti-DsRed antibodies for permanent preparations. Enhanced green fluorescent protein (EGFP) mRNA injections typically result in weaker fluorescence.

Preparing Reagents for Injection

Fluorochrome-coupled dextrans must persist stably as high-molecular-weight molecules in a cellular environment. Some dextrans break down rather quickly following injection and subsequently pass readily across cell membranes. The dextrans described here have all proved to be effective tracers.

1. Prepare the dextrans at the following working concentrations:

FITC-dextran	2.0 mg/mL
TRITC-dextran	2.0 mg/mL
Biotin-dextran	1.0 mg/mL

2. Prepare mRNAs for the fluorescent proteins EGFP-1 (Clontech) and DsRed.T1 (Bevis and Glick 2001) in the pSP64T expression vector, which contains 5′- and 3′-flanking sequences from a *Xenopus laevis* β-globin gene and includes a poly(dA) region (Krieg and Melton 1984; Gerberding et al. 2002). Generate capped transcripts by run-off in vitro transcription using the mMessage mMachine SP6 Kit. Inject them at a concentration of 1.0 mg/mL.

Injection Rig Setup

Injections of P. hawaiensis *embryos from the one- to eight-cell stages can be accomplished using dissection microscopes with sufficient magnification (20X-50X). When attempting to inject micromeres at the eight-cell stage, some researchers prefer to use an inverted compound microscope with a stage-mounted micromanipulator. This section describes a tabletop micromanipulator used in conjunction with a dissection microscope. Fitting the dissection microscope with an ocular reticle that has been scaled with a stage micrometer allows the calculation of injection volumes and enables the microinjector to be adjusted such that various needles deliver consistent injection volumes. The microinjector must be capable of precise and reproducible delivery of very small volumes (~50 pL).*

3. Position the iron plate adjacent to a dissection microscope, and secure the magnetic stand to the plate by activating the magnet. Connect the microinjector to a source of nitrogen gas. Mount the joystick micromanipulator onto the secured magnetic stand. Use silicone tubing to connect the injection holder to the output of the microinjector, ensuring that the tubing is free of kinks.

4. Prepare a Sylgard-coated injection dish by coating a 60-mm plastic Petri dish with 2-3 mm of Sylgard 184 following instructions for the Silicone Elastomer Kit. After the Sylgard hardens, use a razor blade to make a shallow V-shaped trough in the Sylgard that is approximately the width of a *P. hawaiensis* embryo (0.25-0.75 mm). Rinse the dish twice in H_2O to prevent the embryos from sticking.

5. Pull the needles by placing capillary glass tubing in the micropipette puller and selecting an appropriate program.

A good needle has a tip sturdy enough to penetrate the eggshell and maintains a large enough bore to prevent frequent clogging, but is small enough in diameter to avoid excessive damage to the embryo. Needles can be stored by pressing them into a strip of clay in a large Petri dish.

Typical program parameters for pulling needles suitable for injecting P. hawaiensis *blastomeres using a Sutter P-97 micropipette puller are heat 615, pull 100, velocity 20, time 250. Trial and error will be required to optimize these settings. The Sutter "Pipette Cookbook" is a good source for program design help.*

Collecting *P. hawaiensis* Pairs

6. Collect *P. hawaiensis* pairs in amplexus the night before injecting.

   Pairs can be placed in lidded containers (such as 14- × 10- × 10-cm plastic food storage containers) and left overnight at room temperature without aeration.

Collecting and Staging Embryos

For details concerning embryo collection, see ***Fixation and Dissection of*** **Parhyale hawaiensis** ***Embryos*** *(Rehm et al. 2009a).*

7. The following morning, pairs will have separated, and gravid females can be removed for embryo collection. Collect and stage embryos, and plan injection schedule accordingly.

   Note that freshly shed eggs lack a hard outer chorion and are extremely fragile. Do not remove the eggs from the brood pouch until they have hardened for at least 1 h. The following are elapsed times of early embryonic stages at 26°C:

Stage 1	*One-cell*	*0-4 h*
Stage 2	*Two-cell*	*4-6 h*
Stage 3	*Four-cell*	*6-7.5 h*
Stage 4	*Eight-cell*	*7.5-9 h*

   The rate of embryogenesis can be modulated by adjusting the incubation temperature between 18°C and 32°C. If one-cell embryos are required, for instance, they can be kept at 18°C until injections begin. If eight-cell embryos are needed, incubating at higher temperatures will speed development.

Injecting Embryos

Tracers are diluted and phenol red is added to assist needle filling. Embryos are injected with 30-70 pL of tracer solution.

8. Prepare a small (~5 µL) volume of injection solution using the desired tracer. Use 4.5 µL of tracer at the appropriate working concentration (see Step 1) and 0.5 µL of 0.5% phenol red solution.

9. Back-fill an injection needle. Pipette 0.5 µL of injection solution into the nonpulled end of a needle.

   Capillary action will draw the solution into the needle.

10. Insert the back-filled needle into the injection holder, and lower it onto a glass microscope slide with a drop of halocarbon oil on it. Adjust the micromanipulator and stand so that the needle enters the oil at an angle of ~45° and the needle tip is centered under the dissection microscope. Do not overtighten the collar on the injection holder; this will constrict the enclosed silicon gasket and prevent injection.

    The back end of the needle sits in the gasket, requiring little pressure to make a tight seal. This gasket should be inspected and replaced regularly.

11. Turn on the microinjector, and open the nitrogen gas supply valve. Use a regulator to adjust the input line pressure to 80-90 psi. Balance the needle and try to inject using an injection pressure of 25-40 psi with a duration of 20-50 msec. If no solution comes out of the needle, use a razor blade to carefully remove the tip of the needle while it remains in the injection holder.

12. Repeat Step 11 until the injections produce a consistent drop size of 3-5 µm (~33 pL) in halocarbon oil. Leave the needle tip in oil while assembling the embryos and between rounds of injections.

    Microinjector settings for injection pressure and duration can be adjusted to fine-tune the drop size. Injection durations of <20 msec are not reliably reproduced by the Narishige IM300. Injection pressures >40 psi can cause damage to the embryo and thus are also not recommended.

13. Place embryos into the trough of a Sylgard-coated injection dish filled with sterile seawater. Position the injection dish under the dissection microscope.

14. Use blunt forceps to maneuver an embryo into position so that the needle is aimed at the desired cell.

15. Gently push the needle into the embryo, using forceps if necessary to prevent the embryo from rolling.

    See Troubleshooting.

16. Inject.

    In general, one-cell embryos are easier to inject, so practice with them first. One-cell embryos can handle larger injection volumes, which are best achieved by multiply injecting a small volume. When injecting micromeres, try to inject just under the cell membrane to avoid penetrating the macromere underneath.

    See Troubleshooting.

17. Following injections, transfer the embryos to a 35- × 10-mm Petri dish filled with sterile seawater and store at the appropriate temperature.

TROUBLESHOOTING

Problem: The needle does not penetrate the eggshell.
[Step 15]
Solution: The needle is too thin. To make it thicker, cut it with a razor blade. If the problem persists, adjust the needle puller program to make stouter needles.

Problem: The needle clogs.
[Step 16]
Solution: Consider the following:

1. Use the Clear function on the microinjector to drive the debris from the needle. Gently flicking the needle tip with forceps while clearing may help. These steps can be done in seawater next to the embryos.
2. If the needle remains clogged, position the needle tip in a drop of halocarbon oil on a glass slide, and cut the end with a razor blade.

Problem: The needle leaves a large hole in the embryo that leaks yolk when the needle is withdrawn.
[Step 16]
Solution: Try another needle with a smaller-diameter tip. Leaking embryos, especially at the one- and two-cell stages, will often live, so keep damaged embryos as well.

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