In Situ Hybridization of Labeled RNA Probes to Fixed *Parhyale hawaiensis* Embryos

E. Jay Rehm, Roberta L. Hannibal, R. Crystal Chaw, Mario A. Vargas-Vila and Nipam H. Patel

Cold Spring Harb Protoc; doi: 10.1101/pdb.prot5130

Protocol

In Situ Hybridization of Labeled RNA Probes to Fixed *Parhyale hawaiensis* Embryos

E. Jay Rehm, Roberta L. Hannibal, R. Crystal Chaw, Mario A. Vargas-Vila, and Nipam H. Patel[1]

Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720-3140, USA
Department of Integrative Biology, University of California, Berkeley, CA 94720-3140, USA
Howard Hughes Medical Institute, University of California, Berkeley, CA 94720-3140, USA

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 in situ hybridization of fluorescein- or digoxigenin (DIG)-labeled RNA probes to fixed *P. hawaiensis* embryos. Standard techniques of molecular biology should be used to produce an appropriate template for generation of antisense RNA probes. RNA-labeling mixes designed to produce fluorescein- or DIG-labeled RNA probes using T3, T7, or SP6 RNA polymerases are commercially available. Probes should be purified using QIAGEN RNeasy columns or similar means. Considerations for double-labeling experiments using both fluorescein- and DIG-labeled RNA probes are included.

RELATED INFORMATION

The fixed embryos used in this protocol were prepared as described in ***Fixation and Dissection of* Parhyale hawaiensis *Embryos*** (Rehm et al. 2009a). These embryos can also be stained with antibodies to investigate protein localization, as in ***Antibody Staining of* Parhyale hawaiensis *Embryos*** (Rehm et al. 2009b). Cell-lineage analysis can be performed following injection of *P. hawaiensis* embryos with fluorescent tracers, which is detailed in **Injection of *Parhyale hawaiensis* Blastomeres with Fluorescently Labeled Tracers** (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).

MATERIALS

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

[1]Corresponding author (nipam@uclink.berkeley.edu)
This article is also available in *Emerging Model Organisms: A Laboratory Manual*, Vol. 1. CSHL Press, Cold Spring Harbor, NY, USA, 2009.
Cite as: Cold Spring Harb. Protoc.; 2009; doi:10.1101/pdb.prot5130

Reagents

Antibodies (anti-DIG and/or anti-fluorescein)
<R>AP reaction buffer for *Parhyale*

Prepare immediately before use.

<R>BCIP/NBT solution

<!>*Fast Red reaction solution can be used as an alternative for the color reaction. Prepare 1 mL Fast Red TR/Naphthol AS-MX (Sigma-Aldrich F4648) according to the manufacturer's instructions. Filter through a 0.22-µm syringe filter.*

<R>DAPI solution (for DAPI staining only; see Step 33)
<!>Formaldehyde (37%)
<R>Glycerol solutions (50% and 70%)
<R>Glycine buffer
<!>Methanol (30%, 50%, and 70%; all dilutions in PT)
P. hawaiensis embryos

This protocol assumes that P. hawaiensis *embryos have been fixed for 15 min or longer and dehydrated for storage in 100% methanol as described in* ***Fixation and Dissection of* Parhyale hawaiensis *Embryos*** *(Rehm et al. 2009a).* P. hawaiensis *embryos should always be dehydrated before using them for in situ hybridizations, even if they have been freshly dissected. Dehydration and rehydration mitigate against their tendency to float in solution, greatly speeding up the protocol. Fixed* Drosophila melanogaster *embryos can be added to tubes of* P. hawaiensis *embryos to reduce general background and help prevent floating. For further details, see* ***Antibody Staining of* Parhyale hawaiensis *Embryos*** *(Rehm et al. 2009b).*

<R>PT
<R>PT + BSA
RNA probe(s)
<R>SDS hybridization (hyb) buffer
<R>SSC (20X, diluted to 2X before use)

Equipment

Centrifuge tubes (50 mL; BD Biosciences)
Dissection microscope
Light source (see Step 1)
Microcentrifuge tubes
Pipettes (glass)
Test tube rack for microcentrifuge tubes
Water bath pre-set to 65°C (and 85°C, optional; see Step 9)

METHOD

Perform all incubations and washes in microcentrifuge tubes at room temperature using ~1 mL of solution, unless otherwise indicated. Add wash volumes gently down the sides of the tubes, rather than directly onto the embryos. No agitation is necessary during washes or incubations.

Day 1

Rehydration of Embryos

1. Rehydrate *P. hawaiensis* embryos stored in 100% methanol by incubating them for 5 min each in 70%, 50%, and 30% methanol. During rehydration and all subsequent wash steps, gently mix the embryos by spinning the microcentrifuge tubes as they rest in a tube rack. When changing solutions, pipette away as much as possible without allowing the embryos to dry. Avoid shaking or flicking the tubes because the embryos will splash up the tube sides, dry out, and not stain properly.

   P. hawaiensis *embryos often sink slowly (or not at all) to the bottom of the tube. Be careful to avoid pipetting away these embryos when changing solutions. It is often necessary to illuminate the tube with a strong localized light source (such as that provided by a typical fiber optic lamp designed for a dissection microscope) to ensure that the translucent embryos are not inadvertently withdrawn.*

Glass pipettes can be used to change solutions, but embryos will stick to glass.
See Troubleshooting.

Post-fixation

2. Wash the *P. hawaiensis* embryos with PT three times for 5 min.
3. Prepare a solution of 9 parts PT to 1 part 37% formaldehyde. Fix the embryos in this solution for 30 min.
4. Wash the embryos with PT twice for 5 min.
5. (Optional) Add ~10 µL of rehydrated fly embryos to each tube of *P. hawaiensis* embryos.
   The fly embryos help to reduce the background by increasing the tissue mass in the tube.
6. Wash the embryos with 500 µL of hyb buffer once for 5 min.
   Amphipods tend to float in hyb buffer. Be particularly careful when placing a pipette into a tube with Parhyale *embryos in hyb buffer.*

Hybridization

7. Add 500 µL of fresh hyb buffer, and pre-hybridize the embryos by incubating for 10-60 min (or longer if preferred) at 65°C.
8. Dilute the RNA probe to a final concentration of 1 ng/µL in hyb buffer.
   If using two differently labeled probes, mix them together at this point.
9. (Optional) Heat the hyb buffer containing the probe for 5 min at 85°C. Cool to 65°C.
   This will relax RNA probes that contain high degrees of secondary structure, allowing them to hybridize more effectively to targets in situ. The presence of 50% formamide in hyb buffer typically makes this step unnecessary.
10. Add 300-500 µL of hyb buffer containing probe to each tube of embryos.
11. Hybridize without shaking in a water bath for 20-24 h at 65°C.

Day 2

Washes

Perform initial washes at 65°C, with subsequent washes at room temperature. Hyb buffer and 2X SSC can be stored in 50-mL screw-cap tubes in a water bath set at 65°C. All wash volumes are ~1 mL, unless otherwise indicated.

12. Recover the probe and store at -20°C.
    Probes can be reused many times.
13. Wash the samples with hyb buffer once for 30 min at 65°C.
14. Wash the samples with 2X SSC four times for 30 min at 65°C. The 2X SSC used at this step, and in Step 15 below, should be adjusted to a pH of between 5 and 6 to keep the embryos from disintegrating.
15. Wash the samples with 2X SSC twice for 10 min at room temperature. Remove only ~500 µL of the 2X SSC from the final wash.
16. Wash the samples once for 20 min by adding 500 µL of PT at room temperature to tubes containing 500 µL of 2X SSC.
17. Wash the samples with PT three times for 20 min at room temperature.
18. Wash the samples with PT + BSA once for 30 min at room temperature.

Probe Visualization I

Antibody reactions are performed to detect localized RNA probes. React the stronger probe first when performing double-labeling reactions. Antibodies should only be added for one probe at a time. The anti-fluorescein antibody typically results in weaker staining than the anti-DIG antibody for a given probe. BCIP/NBT reactions produce stronger staining than Fast Red.

19. Dilute the anti-DIG (1:3000) or anti-fluorescein (1:4000) antibodies in PT + BSA.

20. Add 300-500 µL of diluted antibody per tube and incubate overnight at 4°C.

Day 3

Probe Visualization II

Room temperature washes are followed by visualization reactions.

21. Remove the antibody, and wash the samples with PT four times for 30 min at room temperature.

22. Wash the samples with AP reaction buffer for *Parhyale* three times for 5 min at room temperature.

23. React by adding 500 µL of the appropriate reaction solution (BCIP/NBT or Fast Red) to each tube. Store the tubes in the dark at room temperature.

    Monitor reactions by briefly examining the embryos under a dissection microscope. Color frequently develops within 1 h, although the reactions may need to be left overnight. Reaction solutions can be replaced after 3-4 h, if necessary.

24. Stop the reactions by washing with PT three times for 5 min at room temperature.

25. For single-labeling experiments, wash the embryos in PT overnight at room temperature and continue with Steps 32-34.

Day 4

Double-Labeling Experiment: Second Probe Visualization

To continue with a double-labeling experiment, follow the washes by the addition of an antibody specific to the second probe.

26. Wash the embryos with PT three times for 5 min at room temperature.

27. Rinse the embryos briefly with glycine buffer at room temperature.

28. Wash the embryos with glycine buffer once for 10 min at room temperature.

29. Wash the embryos with PT three times for 5 min at room temperature.

30. Wash the embryos with PT + BSA once for 1 h at room temperature.

31. React by adding 500 µL of the appropriate reaction solution (BCIP/NBT or Fast Red) to each tube. Store the tubes in the dark at room temperature.

    Monitor reactions by examining the embryos under a dissection microscope. Reactions may need to be left overnight. Reaction solutions can be replaced after 3-4 h, if necessary.

32. Stop the reactions by washing with PT three times for 5 min at room temperature.

33. If DAPI staining is desired, place the reacted embryos in 200 µL of DAPI solution for 30 min or longer. If no DAPI staining is required, simply place the embryos in 50% glycerol for 30 min.

34. Transfer the embryos to 70% glycerol.

    Embryos can be stored in 70% glycerol for several weeks at room temperature, several years at 4°C, and several decades at -20°C.

TROUBLESHOOTING

Problem: Embryos stick readily and permanently to the inner surfaces of glass pipettes.
[Step 1]
Solution: Consider the following:

1. Pass yolk from dissected embryos into pipettes that will be used in proximity to the embryos, thus coating the pipettes with a nonstick layer of yolk proteins.

2. Alternatively, rinse pipettes with PT before using them near embryos.

ACKNOWLEDGMENTS

We thank P. Liu, R. Parchem, and M. Protas for helpful comments and C. Reiss for incisive edits.

REFERENCES

Rehm, E.J., Hannibal, R.L., Chaw, R.C., Vargas-Vila, M.A., and Patel, N.H. 2009a. Fixation and dissection of *Parhyale hawaiensis* embryos. *Cold Spring Harb. Protoc.* (this issue). doi: 10.1101/pdb.prot5127.

Rehm, E.J., Hannibal, R.L., Chaw, R.C., Vargas-Vila, M.A., and Patel, N.H. 2009b. Antibody staining of *Parhyale hawaiensis* embryos. *Cold Spring Harb. Protoc.* (this issue). doi: 10.1101/pdb.prot5129.

Rehm, E.J., Hannibal, R.L., Chaw, R.C., Vargas-Vila, M.A., and Patel, N.H. 2009c. Injection of *Parhyale hawaiensis* blastomeres with fluorescently labeled tracers. *Cold Spring Harb. Protoc.* (this issue). doi: 10.1101/pdb.prot5128.

Rehm, E.J., Hannibal, R.L., Chaw, R.C., Vargas-Vila, M.A., and Patel, N.H. 2009d. The crustacean *Parhyale hawaiensis*: A new model for arthropod development. *Cold Spring Harb. Protoc.* (this issue). doi: 10.1101/pdb.emo114.