Protocol

Embryonic In Situ Hybridization for the Tardigrade *Hypsibius exemplaris*

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In situ hybridization is a method for visualizing embryonic gene expression that is amenable to non-model systems. Here, an in situ hybridization protocol is presented for the tardigrade *Hypsibius exemplaris*. This method allows gene expression to be visualized directly and with fluorescence microscopy.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Acetic anhydride

Anti-DIG antibody

AP developing solution <R>

Blocking buffer for *Hypsibius* in situ hybridization <R>

BM Purple AP substrate solution (Roche 11442074001)

Chymotrypsin/chitinase solution <R>

DIG-labeled riboprobe

Fixative solution for *Hypsibius* in situ hybridization <R>

Hybridization buffers

Hypsibius in situ hybridization buffer (hyb buffer) <R>

Plain *Hypsibius* in situ hybridization buffer (plain hyb buffer) <R>

Hyb buffer is used during prehybridization and hybridization (Steps 22–25); plain hyb buffer is used during posthybridization (Step 29).

Maleic acid buffer (MAB) <R>

Methanol (25%, 50%, 70%, and 90% [v/v] in 0.5× PBTw; 100%)

Mounting medium (e.g., Fluoromount-G [Invitrogen 00495802])

PBTween (PBTw; 0.5×) <R>

Salmon sperm DNA solution (Invitrogen 15632011), diluted in H₂O to a concentration of 100 µg/mL

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From the Emerging Model Organisms collection.

Supplemental material is available for this article at cshprotocols.cshlp.org.

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Spring water

Use commercial bottled spring water found in grocery stores. Do not use tap water; the chlorine and/or chloramine common in tap water is harmful to many microscopic animals.

SSC plus 0.1% CHAPS/Tween <R>

Tardigrade (*H. exemplaris*) maternal exuviae

For general advice on maintaining tardigrades in the laboratory, see Protocol: **Laboratory Culture of *Hypsibius exemplaris*** (McNuff 2018).

Triethanolamine (1% in 0.5× PBTw)

Equipment

Boeckel Rocker II

Collection tubes (2 mL)

Compound microscope using DIC optics or laser scanning confocal microscope

Depression slides

Dissecting microscope

Heat block at 100°C

Hybridization oven at 60°C

Microcentrifuge

Microcentrifuge tubes (1.5 mL, low-retention)

Mobicols (Boca Scientific)

Mobicol mini-columns with Luer-lock cap, closing cap, and plug (M1002)

Large filters for Mobicols, 10-μm pore size (M2210)

Bottom filters for Mobicols, 10-μm pore size (M2110)

Needles (25 gauge)

Pasteur pipettes (9 inch, glass)

Petri dishes (35 mm × 10 mm)

Syringes (1 mL)

U-bottom culture plate (96 well)

Vortexer (VWR Genie 2 G560)

Water bath at 60°C

METHOD

This protocol is based on *in situ* protocols for insects (Tomoyasu et al. 2009), the nematode *Caenorhabditis elegans* (Seydoux and Fire 1995), and small embryos (Irvine 2007).

Throughout the protocol, make sure that the embryos remain submerged in liquid. Perform all steps at room temperature unless otherwise indicated.

The protocol is long, but most wash steps can be extended overnight if needed. To extend a room-temperature wash overnight, wash at 4°C.

Embryo Collection

1. In a 35 mm × 10 mm Petri dish filled with spring water, slice through the middle of maternal exuviae filled with embryos with a 25-gauge needle attached to a sterile 1-mL syringe (see Supplemental Movie S1).
2. Transfer embryos to a 1.5-mL tube filled with 0.5× PBTw.

We routinely follow this protocol with 20–50 embryos per 1.5-mL tube.

Primary Permeabilization

3. Centrifuge the 1.5-mL tube containing embryos at 18,500g for 3 min. Remove most of the liquid, leaving embryos suspended in $\sim 20 \mu\text{L}$ of $0.5\times$ PBTw.
4. Add $20 \mu\text{L}$ of chymotrypsin/chitinase solution, and let stand for 1 h. Centrifuge at 3000g for 3 min, and remove most of the liquid.
5. Wash three times as follows: Add $500 \mu\text{L}$ of $0.5\times$ PBTw, let stand for 5 min, centrifuge at 3000g for 3 min, and remove most of the liquid.

Paraformaldehyde Fixation

6. Wash with 1 mL of fixative solution while shaking vigorously on a VWR Genie 2 G560 Vortexer set to shake speed 3 for 30 min. Centrifuge at 3000g for 3 min, and remove most of the liquid.
7. Wash five times as follows: Add $500 \mu\text{L}$ of $0.5\times$ PBTw, let stand for 5 min, centrifuge at 3000g for 3 min, and remove most of the liquid.

Cleaning Mobicol Mini-Columns

8. Set up mini-columns as in Figure 1A and B, and then wash them four times as follows:
 - i. Unscrew the Luer-lock cap, add $600 \mu\text{L}$ of $0.5\times$ PBTw to the top of the mini-column, and then screw the Luer-lock cap and syringe back on.
 - ii. Push the syringe plunger all the way down, and discard the liquid that collects in the tube.

Methanol Dehydration

For all mini-column washes below, unscrew the Luer-lock cap, add the liquid to be used for washing the embryos, and then screw the Luer-lock cap and syringe back on. After letting the embryos stand in the liquid for the specified time,

FIGURE 1. (A) The Mobicol mini-column configuration used in this protocol. The black dashed line denotes the approximate level to which we reduce the embryo suspension during wash steps. (B) The mini-columns are kept in a tube rack for this protocol. (C) The mini-column configuration while the embryos are stored in methanol at -20°C .

push the liquid through the mini-column with a 1-mL syringe (see the black dashed line in Fig. 1A). The embryos must remain submerged in liquid at all times, so during these washes, pull back on the syringe plunger to form a vacuum in the mini-column to ensure that not all of the liquid is lost.

9. Transfer the embryos to a clean mini-column using a 9 inch glass Pasteur pipette.
10. Wash embryos for 5 min in 500 μ L of 25% methanol in 0.5 $\times$ PBTw.
11. Repeat Step 10 with 50%, 70%, and 90% methanol in 0.5 $\times$ PBTw.
12. Quickly wash the embryos three times in 500 μ L of 100% methanol. After adding the final wash of 100% methanol, insert a bottom plug into the column and replace the Luer-lock cap with a closing cap (Fig. 1C). Leave the tube at -20°C for at least 20 min.

Methanol Rehydration

13. Remove the bottom plug, and replace the closing cap with the Luer-lock cap and syringe. Wash the embryos for 5 min each in 500 μ L of 90%, 70%, 50%, and 25% methanol in 0.5 $\times$ PBTw, followed by three quick washes with 500 μ L 0.5 $\times$ PBTw.

Secondary Permeabilization

14. Use a 9 inch glass Pasteur pipette to transfer the embryos from the mini-column into a 35-mm dish filled halfway with 0.5 $\times$ PBTw.
15. Cut the embryos out of the eggshells with a 25-gauge needle attached to a 1-mL syringe (see Supplemental Movie S2).
16. Re-collect the embryos in a clean mini-column in 500 μ L of 0.5 $\times$ PBTw, and then push the excess liquid through the mini-column with a 1-mL syringe.

Acetylation

17. Quickly wash the embryos with 500 μ L of 0.5 $\times$ PBTw.
18. Quickly wash the embryos twice with 500 μ L of 1% triethanolamine in 0.5 $\times$ PBTw.
19. In a 1.5-mL tube, combine 1.3 μ L of acetic anhydride and 500 μ L of 1% triethanolamine in 0.5 $\times$ PBTw, and vortex briefly. Wash the embryos for 5 min in this solution.
20. Repeat Step 19.
21. Quickly wash the embryos twice with 500 μ L of 0.5 $\times$ PBTw.

Prehybridization

22. Repeat Step 21 one more time, but at the end of the wash, pass half of the 0.5 $\times$ PBTw through the mini-column and replace it with room-temperature hyb buffer. Rock gently on a Boekel Rocker II for 20 min at room temperature.

The syringe remains attached during the washes in Steps 22–24. Ensure that the embryos remain submerged in liquid at all times by pulling back on the syringe plunger to form a vacuum in the mini-column.

23. Wash with 500 μ L of room-temperature hyb buffer, rocking gently for 20 min at room temperature.
24. Wash with 500 μ L of hyb buffer heated to 60°C in a water bath. Let the embryos stand in a hybridization oven for 2 h at 60°C .

Hybridization

25. Add DIG-labeled riboprobe to a final concentration of ~ 0.5 $\mu\text{g/mL}$ in 500 μ L of hyb buffer. Incubate for 5 min at 100°C on a heat block.

26. Boil salmon sperm DNA, and shear by pulling in and out of a 25-gauge needle attached to a 1-mL syringe. Add 1.0 μ L of 100 μ g/mL boiled and sheared salmon sperm DNA to the DIG-labeled riboprobe solution.
27. Pass most of the buffer that the embryos are suspended in through the mini-column, and add the riboprobe solution.
28. Hybridize overnight at 60°C in a hybridization oven.

The syringe continues to remain attached during Steps 28–32. Ensure that the embryos remain submerged in liquid at all times by pulling back on the syringe plunger to form a vacuum in the mini-column.

Posthybridization Washes

For posthybridization washes, all buffers should be preheated to 60°C and the embryos should be incubated in a 60°C hybridization oven.

29. Wash the embryos five times quickly and five times for 20 min each with 500 μ L of plain hyb buffer.
30. Wash the embryos five times quickly and once for 30 min with 500 μ L of 2 $\times$ SSC plus 0.1% CHAPS/Tween.
31. Wash the embryos twice for 30 min each with 500 μ L of 1 $\times$ SSC plus 0.1% CHAPS/Tween.
32. Wash the embryos twice for 30 min each with 500 μ L of 0.2 $\times$ SSC plus 0.1% CHAPS/Tween.

Immunohistochemistry

33. Remove the embryos from the hybridization oven. Quickly wash the embryos twice with 500 μ L of room-temperature 0.5 $\times$ PBTw.
34. Incubate the embryos for 2 h at room temperature in 500 μ L of blocking buffer.
35. Incubate the embryos overnight at 4°C in 500 μ L of 1:1500 anti-DIG antibody:blocking buffer.

Post-Antibody Washes

36. Wash the embryos five times quickly and five times for 10 min each with 500 μ L of MAB.

Detection

Perform the transfers in Steps 39, 40, and 42 under a dissecting microscope.

37. Wash the embryos three times quickly and once for 10 min with 500 μ L of AP developing solution.
38. Transfer embryos to a clean depression slide using a 9 inch glass Pasteur pipette.
39. Transfer all of the embryos to one well of a 96-well plate filled with 200 μ L AP developing solution.

Transfer the embryos carefully; this may take some time. The embryos can remain in the well for up to 1 h.

40. Transfer the embryos to a well with 200 μ L of Roche BM Purple AP substrate solution.
41. Develop in the dark at room temperature.

Development times vary; 4–16 h is typical for my probes.

42. To stop the reaction, transfer the embryos to a well filled with 0.5 $\times$ PBTw.

The embryos can stay in 0.5 $\times$ PBTw until they are mounted.

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Imaging

43. Mount embryos on microscope slides in Fluoromount-G or other appropriate mounting medium.
44. Image on a compound microscope using DIC optics or on a laser scanning confocal microscope using specifications from Smith et al. (2016).

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 44): The in situ signal is low.

Solution: Increase probe concentration (Step 25) and/or decrease temperature of hybridization and posthybridization washes (Steps 28–32).

Problem (Step 44): Background staining is high.

Solution: Decrease probe concentration (Step 25) and/or increase temperature of hybridization and posthybridization washes (Steps 28–32).

RECIPES

AP Developing Solution

1 M Tris-HCl (pH 9.5)	5 mL
1 M NaCl	5 mL
1 M MgCl ₂	2.5 mL
10% (v/v) Tween 20 in DEPC-treated H ₂ O	500 µL
ddH ₂ O	37 mL
Final volume	50 mL

Prepare on the day of use.

Blocking Reagent Solution (BRS; 10%)

Maleic acid buffer (MAB) <R>	50 mL
Blocking Reagent (Roche 11096176001)	5 g

Boil 50 mL of MAB in a sterile 250-mL glass bottle. Slowly add 5 g of Blocking Reagent to the MAB, while intermittently swirling the solution. Autoclave. Store for up to 1 mo at 4°C.

Blocking Buffer for *Hypsibius* In Situ Hybridization

Bovine serum albumin	1 g
Blocking reagent solution (BRS; 10%) <R>	25 mL
PBTween (PBTw; 0.5×) <R>	25 mL
Final volume	~50 mL

Store for up to 1 mo at 4°C.

Chymotrypsin/Chitinase Solution

Chitinase (Sigma-Aldrich C6137)	5 units
Chymotrypsin (Sigma-Aldrich C4129)	10 mg
0.5× PBS (diluted from 10× PBS [pH 7.4] <R>)	1 mL
Final volume	~1 mL
Store for up to 1 mo at 4°C in the dark.	

Denhardt's Solution (50×)

Ficoll	5 g
Polyvinylpyrrolidone	5 g
Bovine serum albumin	5 g
ddH ₂ O	to 500 mL
Final volume	500 mL
Store for up to 5 yr at −20°C.	

Fixative Solution for Hypsibius In Situ Hybridization

Heptane	333.33 µL
16% formaldehyde (Electron Microscopy Sciences 15700)	250 µL
10% (v/v) Tween 20 in DEPC-treated H ₂ O	10 µL
PBTween (PBTw; 0.5×) <R>	406.67 µL
Final volume	1 mL
Prepare immediately before use.	

Hypsibius In Situ Hybridization Buffer (Hyb Buffer)

100% formamide	25 mL
20× SSC <R>	12.5 mL
100 mg/mL heparin	50 µL
10% (v/v) Tween 20 in DEPC-treated H ₂ O	500 µL
10 mg/mL yeast RNA	500 µL
10% (w/v) CHAPS in DEPC-treated H ₂ O	500 µL
Denhardt's solution (50×) <R>	1 mL
DEPC-treated H ₂ O	9.95 mL
Final volume	50 mL
Store for up to 5 yr at −20°C.	

Maleic Acid Buffer (MAB)

Maleic acid	11.61 g
NaCl	8.77 g
NaOH pellets	7.2 g
Add the above ingredients to 500 mL of sterile H ₂ O, and adjust the pH to 7.5. Fill to 1 L with H ₂ O. Store for up to 1 yr at room temperature.	

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Phosphate-Buffered Saline (PBS; 10×, pH 7.4)

Na ₂ HPO ₄ ·7H ₂ O	25.6 g
NaCl	80 g
KCl	2 g
KH ₂ PO ₄	2 g
H ₂ O	1 L
Final volume	~1 L

Mix the ingredients and adjust the pH to 7.4. Sterilize by autoclaving. Store for up to 1 yr at room temperature.

PBTween (PBTw; 0.5×)

Phosphate-buffered saline (PBS; 10×, pH 7.4) <R>	2.5 mL
Tween 20 (10% [v/v] in DEPC-treated H ₂ O)	500 μL
DEPC-treated H ₂ O	47 mL
Final volume	50 mL

Store for up to 1 mo at room temperature.

Plain Hypsibius In Situ Hybridization Buffer (Plain Hyb Buffer)

100% formamide	25 mL
20× SSC <R>	12.5 mL
10% (v/v) Tween 20 in DEPC-treated H ₂ O	500 μL
DEPC-treated H ₂ O	12 mL
Final volume	50 mL

Store for up to 5 yr at −20°C.

SSC

For a 20× solution: Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL of H₂O. Adjust the pH to 7.0 with a few drops of a 14 N solution of HCl. Adjust the volume to 1 L with H₂O. Dispense into aliquots. Sterilize by autoclaving. The final concentrations of the ingredients are 3.0 M NaCl and 0.3 M sodium citrate.

SSC Plus 0.1% CHAPS/Tween

SSC <R>	0.2×, 1×, or 2×
CHAPS	0.1% (w/v)
Tween 20	0.1% (v/v)

Use ddH₂O to dilute 20× SSC to the desired working concentration (0.2×, 1×, or 2×), and add CHAPS to 0.1% (w/v) and Tween 20 to 0.1% (v/v). Store for up to 1 mo at room temperature.

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