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Whole Mount In Situ Hybridization in Zebrafish

D R Hammond-Weinberger[1]

[1]Murray State University

D R Hammond-Weinberger: Protocol based on Thisse et al., 1993

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Weinberger Lab

D Hammond-Weinberger
Murray State University

ABSTRACT

Whole mount in situ hybridization protocol optimized for single gene detection using chromogenic substrates NBT/BCIP in zebrafish (Danio rerio). Options are included for bleaching and permeabilization. This protocol beings with tissue preparation and ends with glycerol mounting for imaging. Probe synthesis is not included. This protocol has been used for embryos/larvae from 24 - 72 hpf.

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Tissue Prep

1 Dechorionate embryos, if needed.

2 Fix embryos in **500 µL** 4% paraformaldehyde for **02:00:00** at **Room temperature** 2h or overnight at **4 °C** .

2.1 Wash in **1 mL** 100% MeOH at **Room temperature** for **00:10:00** . 10m (1/3)

2.2 Wash in **1 mL** 100% MeOH at **Room temperature** for **00:10:00** . 10m (2/3)

2.3 Wash in **1 mL** 100% MeOH at **Room temperature** for **00:10:00** . 10m (3/3)

2.4 Store at **-20 °C** long-term (can be months or longer)

Day 1 6h

3 Wear gloves and treat surfaces for RNAses.

All reagents should be nuclease-free. Use barrier pipet tips.

4 Rehydrate the embryos

4.1  5m

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Wash embryos in **0.5 mL** 75% Methanol/25% PBTween, rocking, for **00:05:00** at **Room temperature** in 1.5 mL centrifuge tubes.

PBTween is 1x PBS + 0.1% Tween20

Methanol is hazardous waste. All liquids and contaminated materials must be collected and disposed of properly.

4.2 Wash embryos in **0.5 mL** 50% MeOH / 50% PBTween, rocking, for **00:05:00** at **Room temperature** 5m

4.3 Wash embryos in **0.5 mL** 25% MeOH / 75% PBTween, rocking, for **00:05:00** at **Room temperature** 5m

4.4 Wash embryos in **0.5 mL** PBTween, rocking, for **00:05:00** at **Room temperature** (1/3) 5m

4.5 Wash embryos in **0.5 mL** PBTween, rocking, for **00:05:00** at **Room temperature** (2/3) 5m

4.6 5m

Wash embryos in **0.5 mL** PBTween, rocking, for **00:05:00** at **Room temperature** (3/3)

5 5m

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Optional: Bleach embryos in **0.5 mL** freshly-made 3% H_2O_2 + **1.79 millimolar (mM)** KOH for up to **00:05:00** . Leave the tube caps open and monitor bleaching.

5.1 Rinse in **0.5 mL** PBTween (1/2)

5.2 Rinse in **0.5 mL** PBTween (2/2)

6

Permeabilize tissue. Option 1: proteinase K - proceed to step 6.1. Option 2: acetone - proceed directly to step 6.3.

Timing of this step is critical.

6.1 Option 1: Digest with **1 mL** **10 µg /mL** Proteinase K in PBTween at **Room temperature** for **00:05:00 (24 hpf)** or **00:20:00 (48 hpf)** or **00:30:00 (72 hpf)** . Proceed to step 6.2 55m

Time is variable by a few minutes depending on proteinase K stock

6.2 20m

Refix tissue in **0.5 mL** 4% PFA, rocking, at **Room temperature** for **00:20:00** . Skip to step 6.4.

Paraformaldehyde (PFA) is hazardous. All liquids and contaminated materials must be collected and disposed of properly.

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6.3 20m

Option 2: Incubate in **1 mL** 80% acetone/ 20% diH_2O at **Room temperature** for **00:20:00** .

6.4 5m

Wash in **0.5 mL** PBTween, rocking, at **Room temperature** for **00:05:00** (1/3)

6.5 5m

Wash in **0.5 mL** PBTween, rocking, at **Room temperature** for **00:05:00** (2/3)

6.6 5m

Wash in **0.5 mL** PBTween, rocking, at **Room temperature** for **00:05:00** (3/3)

6.7 Separate embryos into designated tubes (example: sense vs. anti-sense tubes) if not done previously.

7 4h

Incubate in **250 µL** prehybe in hybe oven set to **65 °C** , rocking, for at least **04:00:00**

Formamide is hazardous. Liquids and contaminated materials must be collected and disposed of properly.

8

Incubate with (0.1-1µg/mL) probe diluted in **250 µL** warmed prehybe **Overnight** , **65 °C** , rocking.

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Prehybe Recipe (**10 mL**):

Mix together: **5 mL** formamide, **1.5 mL** 20x SSC, **50 µL** 20% Tween20, **185 µL** **0.5 Molarity (M)** Citric acid, **10 µL** heparin, **500 µL** **10 mg / mL** tRNA, and **2.75 mL** nuclease-free water

OPTIONAL: mix in **0.5 g** dextran sulfate

Day 2 1h 40m

9 Remove probes. Probes can be stored at **-20 °C** and reused up to 3 times.

10 Post-hybridization washes

10.1  10m

Wash in **0.5 mL** 100 % (50% 5x SSC / 50% formamide) for **00:10:00** at **75 °C** rocking

10.2 Wash in **0.5 mL** 75% (50% 5x SSC / 50% formamide) / 25% 2x SSC for **00:10:00** at **75 °C** rocking 10m

10.3 Wash in **0.5 mL** 50% (50% 5x SSC / 50% formamide) / 50% 2x SSC for **00:10:00** at **75 °C** rocking 10m

10.4 Wash in **0.5 mL** 25% (50% 5x SSC / 50% formamide) / 75% 2x SSC for **00:10:00** at **75 °C** rocking 10m

10.5 Wash in **0.5 mL** 2x SSC for **00:10:00** at **75 °C** rocking 10m

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10.6 Wash in **0.5 mL** 0.2x SSC for **00:15:00** at **75 °C** rocking 15m

10.7 Wash in **0.5 mL** 0.2x SSC for **00:15:00** at **75 °C** rocking 15m

10.8 Wash in **0.5 mL** 75% 0.2x SSC / 25% PBTween for **00:10:00** at **Room temperature** rocking 10m

10.9 Wash in **0.5 mL** 50% 0.2x SSC / 50% PBTween for **00:10:00** at **Room temperature** rocking 10m

10.10 Wash in **0.5 mL** 25% 0.2x SSC / 75% PBTween for **00:10:00** at **Room temperature** rocking 10m

10.11 10m

Wash in **0.5 mL** 100% PBTween for **00:10:00** at **Room temperature** rocking

11 Incubate in **0.5 mL** block for at least **02:00:00** **Room temperature** , rocking 2h

Block solution: is 5% sheep serum, 2mg/mL BSA, and 1% DMSO in PBTween
For **10 mL** : Mix **500 µL** normal sheep serum, **0.2 g** BSA, **100 µL** DMSO, and **9.4 mL** PBTween

12 4h

Incubate **Overnight** **4 °C** with **0.5 mL** 1:5000 sheep AP-conjugated anti-DIG Fab fragments (or 1:2000 sheep AP-conjugated anti-FLU Fab fragments)

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Day 3 2h 30m

13 Remove antibody. Antibody can be stored at **4 °C** and reused up to 3 times.

14 Post-antibody washes

14.1 Wash in **0.5 mL** PBTween for **00:10:00** at **Room temperature**, 10m
rocking (1/10)

14.2 Wash in **0.5 mL** PBTween for **00:10:00** at **Room temperature**, 10m
rocking (2/10)

14.3 Wash in **0.5 mL** PBTween for **00:10:00** at **Room temperature**, 10m
rocking (3/10)

14.4 Wash in **0.5 mL** PBTween for **00:10:00** at **Room temperature**, 10m
rocking (4/10)

14.5 Wash in **0.5 mL** PBTween for **00:10:00** at **Room temperature**, 10m
rocking (5/10)

14.6 Wash in **0.5 mL** PBTween for **00:10:00** at **Room temperature**, 10m
rocking (6/10)

14.7 Wash in **0.5 mL** PBTween for **00:10:00** at **Room temperature**, 10m
rocking (7/10)

14.8 Wash in **0.5 mL** PBTween for **00:10:00** at **Room temperature**, 10m
rocking (8/10)

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14.9 Wash in **0.5 mL** PBTween for **00:10:00** at **Room temperature**, rocking (9/10) 10m

14.10 ⏸ 10m

Wash in **0.5 mL** PBTween for **00:10:00** at **Room temperature**, rocking (10/10)

15 Make fresh NTMT buffer. Mix **1 mL** **1 Molarity (M)** Tris **9.5**, **200 µL** **5 Molarity (M)** NaCl, **500 µL** **1 Molarity (M)** $MgCl_2$, **50 µL** Tween20 and **8.25 mL** water

15.1 Equilibrate embryos in **0.5 mL** NTMT buffer for **00:05:00** at **Room temperature** (1/2) 5m

15.2 Equilibrate embryos in **0.5 mL** NTMT buffer for **00:05:00** at **Room temperature** (2/2) 5m

16 Transfer embryos to multiwell culture plate (keep the tubes)

17 Wash in **1 mL** NTMT buffer for **00:05:00** at **Room temperature** 5m

18 Prepare fresh stain solution.

18.1 ☠

Add **4.5 µL /mL** NBT and **3.5 µL / mL** BCIP to NTMT buffer. Protect from light.

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NBT and BCIP are hazardous. Liquids and contaminated materials must be collected and disposed of properly.

18.2 Replace NTMT in culture plates with **1 mL** of the freshly prepared NBT/BCIP stain solution.

18.3 Cover with foil

18.4 Stain in the dark until staining reaches desired intensity, typically when color begins to appear in the sense controls. This step can last hours to days.

18.5 Stop the reaction by rinsing in **2 mL** PBTween

19 Fix tissue after staining

19.1 Transfer embryos back to their tubes

19.2  20m

Fix embryos in **0.5 mL** 4% PFA, rocking, at **Room temperature** for **00:20:00**

19.3 Wash in **0.5 mL** PBTween, rocking, at **Room temperature** for **00:05:00** (1/3) 5m

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19.4 Wash in **0.5 mL** PBTween, rocking, at **Room temperature** for **00:05:00** (2/3) 5m

19.5 Wash in **0.5 mL** PBTween, rocking, at **Room temperature** for **00:05:00** (3/3) 5m

20

Prepare embryos for glycerol imaging

20.1 Wash embryos in **1 mL** 30% glycerol / 70% PBTween at **Room temperature** for **00:10:00** while rocking and covered in foil. 10m

20.2 Wash embryos in **1 mL** 50% glycerol / 50% PBTween at **Room temperature** for **00:10:00** while rocking and covered in foil. 10m

20.3 Wash embryos in **1 mL** 80% glycerol / 20% PBTween at **Room temperature** for **00:10:00** while rocking and covered in foil. 10m

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