Sep 06, 2022

Double Whole Mount In Situ Hybridization in Zebrafish

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dx.doi.org/10.17504/protocols.io.4r3l2onnxv1y/v1

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ABSTRACT

This protocol has been optimized for serial detection of two chromogenic substrates in embryonic zebrafish (Danio rerio). Several stain pairings are included as options. Protocol begins with tissue preparation and ends with a glycerol series in preparation for imaging. This protocol has been successfully used on 24 hpf zebrafish embryos.

DOI

dx.doi.org/10.17504/protocols.io.4r3l2onnxv1y/v1

PROTOCOL CITATION

D R. Hammond-Weinberger 2022. Double Whole Mount In Situ Hybridization in Zebrafish. **protocols.io** https://dx.doi.org/10.17504/protocols.io.4r3l2onnxv1y/v1

FUNDERS ACKNOWLEDGEMENT

NIGMS
Grant ID: 8P20GM103436

McNair Grant
Grant ID: #P217A090330

CREATED

Apr 24, 2022

LAST MODIFIED

Sep 06, 2022

PROTOCOL INTEGER ID

61319

Tissue Prep 3h

1 Dechorionate embryos, if needed.

2  2h

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

2.1  10m

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

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

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

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

Day 1 5h

3 Wear gloves and treat surfaces for RNAses.

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

4 Rehydrate the embryos

4.1 

5m

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

5m

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

4.3

5m

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

4.4

5m

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

4.5

5m

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

4.6

5m

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

5 5m

Optional: Bleach embryos in **0.5 mL** freshly-made 3% H2O2 + **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 permeabilization is critical.

6.1 55m

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)**

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**

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

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)

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.

Prehybe Recipe (**10 mL**):

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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 5h

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

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

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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

Can sit overnight in this step

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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Staining with DAB requires the use of a peroxidase-conjugated enzyme, such as 1:200 POD-FLU.

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

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rocking (7/10)

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

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)

Can sit overnight in this step

If staining with DAB, skip directly to step 13

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

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18 Prepare fresh stain solution. Choose **one** of the following:

18.1 

NBT/BCIP - Indigo stain: Add **4.5 µL /mL** NBT and **3.5 µL / mL** BCIP to NTMT buffer. Protect from light. Jump to step 16.

18.2 FastRed - Red stain - Dissolve buffer tablet(s) in **1 mL /tablet** /tablet dH_2O 10m and sonicate **00:05:00** . Dissolve FastRed tablet(s) in buffer and sonicate for **00:05:00** . Jump to step 16.

18.3 

DAB - brown stain - Diaminebenzidine requires a peroxidase-conjugated antibody. Prepare 1x Pierce™ DAB Substrate Kit **Thermo Fisher Catalog #34002** in DAB buffer.

18.4  15m

FR/BCIP - cyan stain - Dissolve buffer tablet(s) in **1 mL** /tablet dH_2O and sonicate **00:05:00** . Dissolve FastRed tablet(s) in buffer and sonicate for **00:05:00** . Add **3.5 µL /mL** BCIP and **5.6 µL /mL** FastRed to fresh NTMT. Vortex and let sit upright for **00:05:00**

19 Replace NTMT in culture plates with **1.5 mL** of the freshly prepared stain solution.

19.1 Cover with foil

19.2 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.

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Day 4 5h

20 Transfer embryos back to tubes. Protect from light in this and all subsequent steps.

21 Incubate 00:30:00 , rocking, in [M]0.1 Molarity (M) glycine HCl pH2.2 plus 0.1% Tween at Room temperature to remove first antibody. 30m

22 Wash away glycine

22.1 Wash in 0.5 mL PBTween for 00:05:00 at Room temperature , rocking (1/4) 5m

22.2 Wash in 0.5 mL PBTween for 00:05:00 at Room temperature , rocking (2/4) 5m

22.3 Wash in 0.5 mL PBTween for 00:05:00 at Room temperature , rocking (3/4) 5m

22.4 ⏸ 5m

Wash in 0.5 mL PBTween for 00:05:00 at Room temperature , rocking 4/4)

Can sit overnight in this step

23 Incubate embryos in 100 µL preabsorbed sheep AP-conjugated anti-DIG Fab fragments at a 1:2000 dilution in block. You can reuse the antibody 3x. Rock for 02:00:00 Room temperature or Overnight 4 °C . 4h

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Staining with DAB requires the use of a peroxidase-conjugated enzyme, such as 1:200 POD-FLU.

Day 5 2h 30m

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

25 Post-antibody washes

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

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

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

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

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

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

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

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

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

25.10 ⏸

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

> Can sit overnight in this step
>
> If staining with DAB, skip directly to step 24

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

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

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

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

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28 Wash in **1 mL** NTMT buffer for **00:05:00** at **Room temperature**

29 Prepare fresh stain solution. Choose **one** of the following:

Stain colors for first and second sequence must be compatible.

29.1 

NBT/BCIP - Indigo stain: Add **4.5 µL /mL** NBT and **3.5 µL / mL** BCIP to NTMT buffer. Protect from light. Jump to step 27.

29.2 FastRed - Red stain - Dissolve buffer tablet(s) in **1 mL /tablet** /tablet dH_2O 10m and sonicate **00:05:00** . Dissolve FastRed tablet(s) in buffer and sonicate for **00:05:00** . Jump to step 27.

29.3 VectorRed - Red/yellow stain - To **5 mL** of **0.1 Molarity (M)** Tris-HCl **8.2** + 0.1% Tween, add 2 drops each of reagents 1, 2, and 3 of Vector Red Substrate kit. Mix well. Jump to step 27.

VectorRed cannot be used as the first stain, per vendor instructions.

29.4 

DAB - brown stain - Diaminebenzidine requires a peroxidase-conjugated antibody. Prepare 1x Pierce™ DAB Substrate Kit **Thermo Fisher Catalog #34002** in DAB buffer. Jump to step 27.

15m

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29.5

FR/BCIP - cyan stain - Dissolve buffer tablet(s) in **1 mL** /tablet dH_2O and sonicate **00:05:00** . Dissolve FastRed tablet(s) in buffer and sonicate for **00:05:00** . Add **3.5 µL /mL** BCIP and **5.6 µL /mL** FastRed to fresh NTMT. Vortex and let sit upright for **00:05:00** . Jump to step 27.

Hurtado R, Mikawa T (2006). Enhanced sensitivity and stability in two-color in situ hybridization by means of a novel chromagenic substrate combination.. Developmental dynamics : an official publication of the American Association of Anatomists.

30 Replace NTMT in culture plates with **1.5 mL** of the freshly prepared stain solution.

30.1 Cover with foil

30.2 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.

31 Fix tissue after staining

31.1 Transfer embryos back to their tubes

20m

31.2

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

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

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

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

32

Prepare embryos for glycerol imaging

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

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

32.3 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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