

Whole Mount Immunohistochemistry With Tyramide Amplification

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- Protocol works well on embryos from E7.5 to E9.5.
- Avoid methanol fixation or tritonX100.

The following antibodies/reagents have been successfully tested for blood vessel staining with this protocol

- Rat anti-mouse PECAM1. Mec13.3 clone (PharMingen, 553370) – use @ 5 µg/mL
- Rat anti-mouse Endomucin. eBioV.7C7 clone (eBioscience, 14-5851-82) – use @ 5 µg/mL
- Goat anti-rat HRP (Biosource, cat#ARI3404 – now Invitrogen cat# A10549) or Donkey anti-Rat HRP (Jackson IR cat#712-035-153) – use @ ~1:2000 (must titrate for optimum results).
- Single colour staining: Cy3 TSA kit (PerkinElmer NEL744001)
- Two colour staining: Cy3/Cy5 TSA kit (PerkinElmer NEL752)

Method:

1. Dissect embryos in PBS (with Mg^{2+} and Ca^{2+}).
Open any membranes such as yolk sac and amnion, puncture hindbrain of embryos with closed neural tube to facilitate penetration otherwise uneven staining will occur.
2. Fix in 4% PFA @ 4°C for 4 hr with gentle agitation. Pre-coat tubes by rinsing quickly with 1% BSA solution in PBS to prevent embryos sticking.
4 hr fixation works well for anti-PECAM and anti-GFP antibodies. Other epitopes may tolerate longer fixation. Shorter fixation eg. 1 hr, leaves embryos quite fragile.
3. Wash 3 x 10 min PBT @ 4°C with gentle agitation.
4. Incubate 20 min with 50 mM sodium azide (NaN_3) in PBT, @ RT with gentle agitation.
Inactivates catalase
5. Incubate 20 min in 3% H_2O_2 + 50 mM NaN_3 in PBT @ RT with gentle agitation.
Inactivates endogenous peroxidases
6. Wash 3 x 10 min PBT with gentle agitation.
7. Incubate 2 hr @ RT in block solution with gentle agitation.
8. Incubate with primary antibody in block solution, 1 hr @ RT then O/N @ 4°C with gentle agitation (500 µl works well).

9. Wash embryos with PBT + 1% HI-FCS (without NaN₃ preservative) as follows with gentle agitation:
 - 1 x 1 mL 1 hr @ RT
 - 4 x 4 mL 1 hr @ RT
 - add an additional O/N 4 ml washes as needed or for embryos older than E9.0
10. Incubate with secondary antibody, O/N @ 4^oC in block solution (use HI-FCS without NaN₃ preservative).
11. Wash with PBT (without HI-FCS) as in step 11.
12. Wash 3 x 4 mL TNT buffer, 10 min ea @ RT with gentle agitation.
13. Transfer embryos to 1.5 mL tubes pre-coated with 1% BSA solution. Pre-equilibrate embryos in amplification diluent, 2 x 100 μL 10 min @ RT on rollers. Use larger volume if necessary, depending on number of embryos.
14. Incubate embryos with 200 μl working strength Tyramide-fluorophore solution (1:50 dilution) for 1 hr with agitation (eg. on rollers). Use larger volume if necessary.
 - Gentle agitation is absolutely essential to facilitate even staining. For larger embryos eg E9.5, longer incubation may be necessary to facilitate even penetration and staining.
15. Wash embryos 3 x 5 min 1 mL TNT buffer @ RT.
16. Incubate 30 min in 3% H₂O₂ in TNT @ RT with gentle agitation to stop reaction.
17. Wash 1 x 4 mL TNT O/N @ 4^oC. More washes can be done but normally are not necessary.
 - Unincorporated tyramide washes out very quickly, usually after the first few 5 min washes.
 - Specific pink signal should be visible in vessels such as yolk sac or dorsal aorta, even under bright field.
18. Store embryos in PBT at 4^oC. For storage lasting more than 1 week, add 10 mM NaN₃ as a preservative, then wash 3 x in PBT immediately before processing for OPT.
 - Embryos should be imaged as soon as possible after staining as they will slowly degrade.

For two colours, add both primary antibodies at step 8. Detect the strongest signal first by continuing through to step 16. To detect the second signal, repeat steps 10-17 for the second signal using the appropriate secondary reagent (must not recognize the primary antibody for the first signal) and tyramide conjugate.

Materials:

PFA:

Always handle PFA in a fume hood. Batch to batch variation in PFA can greatly affect staining results. For optimal consistency, use commercially available formaldehyde solution (eg: Polyscience cat#04018) and dilute to 4% final using dH₂O and 10% PBS stock solution (eg: Sigma cat#D1408).

From powder: dissolve 20 g of paraformaldehyde powder in 450mL PBS (without Mg²⁺ or Ca²⁺) on a heated stirrer at 60°C. Do not overheat, make sure powder is fully dissolved. Adjust pH to 7.4 with NaOH (approximately 100 µl 10 N NaOH per 500 mL PFA) and make to 500 mL with PBS (without Mg²⁺ or Ca²⁺). Aliquot into single use aliquots (4 mL) and store at -20°C. Avoid repeated freeze/thaws. Filtering PFA is recommended to remove particulates that can stick to embryos and cause background or damage.

PBT:

PBS (without Ca²⁺ or Mg²⁺) containing 0.1% tween20.

Block solution:

PBS containing 0.1% tween20, 1% HI-FCS, 1% normal serum from species matching 2^o reagent (eg. goat, donkey etc). Filtering block solution (eg. 0.45µm filter) is recommended to remove particulate matter that sticks to embryos and causes background

HI-FCS:

Heat inactivated FCS. Heat 50 mL FCS to 56°C for 30 min. Store sterile. Do not add sodium azide preservative to FCS as this may reduce HRP activity during staining and washing. Filter block and wash solutions containing FCS to remove particulate matter that can stick to embryos and cause background (eg. add 1ml FCS to 4 mL PBT, pass through 0.45µm filter and add to 95 mL PBT)

TNT buffer:

0.1 M Tris pH7.5, 0.15 M NaCl, 0.1% tween20 in dH₂O.

Tyramide-Fluorophore working solution:

Dilute tyramide fluorophore 1:50 in amplification diluent provided in the TSA kit. Lower dilutions (eg: 1:100) give weaker signal.