Whole Mount Antibody Staining I

(For ≤E14.5 and younger embryos, whole lungs, whole hearts)

Courtesy of Ross Metzger PhD.
Modified by W.P. Devine MD, PhD.

Dissection and Fixation

Embryos were dissected in cold (4°C) Ca^{2+} - and Mg^{2+} - free phosphate-buffered saline, pH 7.4 (PBS). For E14.5 and younger lungs, specimens were fixed in 4% paraformaldehyde/PBS (w/v) at 4°C, with gentle rocking for 1 hour. Paraformaldehyde (Sigma) was dissolved in PBS at 60°C, and aliquots were stored at -20°C and thawed immediately before use. Fixed lungs were washed in PBS twice for 10 minutes at room temperature, with gentle rocking. Lungs were then dehydrated by washing once as above in 25% methanol/PBS (v/v), once in 50% methanol/PBS, once in 75% methanol/PBS, and twice in 100% methanol. Dehydrated lungs were stored at -20°C in 100% methanol and used within a few months for best results.

Day 0: (can be done on Day 1, with 5 hour, rather than overnight, incubation or you can do this overnight at 4°C the day before starting the staining protocol):

1. Place dehydrated specimens in 100% methanol into 5 ml polystyrene round-bottom tubes (Falcon), according to genotype (and age).
2. Incubate tissue overnight in 5% H_20_2 / 95% methanol at 4°C, or for > 5 hours at room temperature with gentle rocking.

Day 1:

1. Wash tissue twice for 10 minutes in 100% methanol at room temperature, with gentle rocking.
2. Wash tissue at room temperature, with gentle rocking, for 10 minutes in 75% methanol/PBT (PBT: 0.1% Tween-20/PBS).
3. Wash tissue at room temperature, with gentle rocking, for 10 minutes in 50% methanol / PBT.
4. Wash tissue at room temperature, with gentle rocking, for 10 minutes in 25% methanol / PBT.
5. Wash tissue twice at room temperature, with gentle rocking, for 10 minutes in 100% PBT.
6. Optional: Permeabilize twice for 10 minutes in PBS / 1.0% Triton-X-100 at room temperature.
7. Block by incubating specimens twice, for 1 hour, in 2 mL 5% heat-inactivated rabbit serum/PBS/0.5% Triton-X-100 at room temperature, with gentle rocking*.
8. After blocking, for primary antibody staining incubate tissue overnight at 4°C with primary antibody diluted 1 mL of blocking solution (5% heat-inactivated rabbit serum/PBS/0.5% Triton-X-100), with gentle rocking.

Day 2:

1. Wash tissue four times, for 1 hour each wash, at 4°C, then once at room temperature for one hour, with 2 mL 5% rabbit serum/ PBS / 0.5% Triton-X-100, with gentle rocking.
2. For secondary antibody staining incubate tissue overnight at 4°C with biotin-conjugated rabbit anti-rat IgG (Vector) diluted 1:125* in 1 mL blocking solution (5% heat-inactivated rabbit serum/PBS/0.5% Triton-X-100).

Day 3:

1. Wash tissue five times as on Day 2.
2. Prepare ABC Elite reagent (Vector; VECTASTAIN Elite ABC Kit) by adding 2 drops (100 μL reagent A) to 5 mL blocking solution (5% heat-inactivated rabbit serum/PBS/0.5% Triton-X-100), mixing, then adding 2 drops (100 μL reagent B per 5 mL), mixing again, and letting the mixture stand for 30 minutes at room temperature. (You will need 1 mL per tube)
3. Incubate tissue with ABC elite reagent for 2 hours at room temperature, with gentle rocking.
4. Wash tissue two times for 30 minutes in PBT at room temperature, with gentle rocking. Following the second wash, replace PBT and leave specimens overnight at 4°C on shaker in cold room. Or wash three times for 30 minutes and continue with protocol.

Day 4:

1. Prepare Tyramide solution by first thawing Fluorescein Tyramide Reagent (stored at -20°C). Then dilute 30% H_2O_2 stock in amplification buffer to obtain a final concentration of 0.0015% H_2O_2.

   Example: add 1 µL of 30% H_2O_2 to 200 µL of amplification buffer, then add 1 µL of this intermediate dilution (0.15% H_2O_2) to a further 100 µL of amplification buffer. We recommend preparing 100 µL of this working solution per sample.

   Prepare a tyramide working solution by diluting the thawed tyramide stock solution 1:100 in amplification buffer/0.0015% H_2O_2 (prepared above) just prior to labeling. Prepare 250-350 µL of working solution per specimen. The diluted reagent should be used immediately.

2. Incubate tissue with the diluted Tyramide reagent for 30 minutes at room temperature, with vigorous rocking to equally cover embryos (stand tubes upright, covered from light).
3. Wash tissue with PBSTw at least 3-5 times for 15-30 minutes at room temperature with gentle rocking.
4. Check stain under scope. Stained tissue were mounted and stored in Vectashield (Vector) at 4°C or -20°C.
5. Inactivate primary peroxidase (and tyramide amplification reaction) by incubating in 5.0% H_2O_2 / PBS (could try PBSTween, but avoid triton(?)) overnight at 4°C**.
6. Wash embryos in PBSTw three times, 10 minutes each wash, at room temperature.
7. Wash embryos in blocking solution for 1 hour, or more, at room temperature.
8. Incubate with second, secondary HRP-conjugated antibody (diluted at 1:125) in blocking solution, overnight at 4°C.

Day 5:

1. Wash tissue five times as on Day 2.
2. Prepare ABC Elite reagent (Vector; VECTASTAIN Elite ABC Kit, catalog number PK-6100) by adding 2 drops (100 μL reagent A) to 5 mL blocking solution (5% heat-inactivated rabbit serum/PBS/0.5% Triton-X-100), mixing, then adding 2 drops (100 μL reagent B per 5 mL), mixing again, and letting the mixture stand for 30 minutes at room temperature. (You will need 1 mL per tube)
3. Incubate tissue with ABC elite reagent for 2 hours at room temperature, with gentle rocking.
4. Wash tissue two times for 30 minutes in PBT at room temperature, with gentle rocking. Following the second wash, replace PBT and leave specimens overnight at 4°C on shaker in cold room. Or wash three times for 30 minutes and continue with protocol.

Day 6:

1. Prepare Tyramide solution by first thawing Fluorescein Tyramide Reagent (stored at -20°C). Then dilute 30% H_2O_2 stock in amplification buffer to obtain a final concentration of 0.0015% H_2O_2.

   Example: add 1 µL of 30% H_2O_2 to 200 µL of amplification buffer, then add 1 µL of this intermediate dilution (0.15% H_2O_2) to a further 100 µL of amplification buffer. We recommend preparing 100 µL of this working solution per sample.

   Prepare a tyramide working solution by diluting the thawed tyramide stock solution 1:100 in amplification buffer/0.0015% H_2O_2 (prepared above) just prior to labeling. Prepare 250-350 µL of working solution per specimen. The diluted reagent should be used immediately.

2. Incubate tissue with the diluted Tyramide reagent for 30 minutes at room temperature, with vigorous rocking. (Stand tubes upright, covered)
3. Wash tissue with PBT at least 3-5 times for 15-30 minutes at room temperature with gentle rocking.
4. Check stain under scope.
5. Inactivate primary peroxidase (and tyramide amplification reaction) by incubating in 1.0% H_2O_2 / PBS (could try PBSTween, but avoid triton(?)) for no less than 20 minutes at room temperature or overnight at 4°C.
6. Wash embryos in PBT two times, 10 minutes each wash, at room temperature.
7. Wash embryos in blocking solution for at least 1 hour, or more, at room temperature (up to overnight at 4°C)
8. Once background staining is gone from washing, post-fix tissue in 4% PFA/PBS for at least one hour at 4°C. Wash tissue extensively in PBTw.
9. Stained tissue were mounted and stored in Vectashield (Vector) at 4°C or processed for vibratome sectioning.