Embryo antibody staining protocol

1. Fix embryos according to standard methods (i.e., dechorionating in 50% bleach, fixing in PFA-heptane, MeOH popping, store at -20 degrees)
2. When ready to perform staining, remove embryos from the freezer and transfer to an eppendorf tube (I use the 0.6 mL tubes for staining- it helps to cut down on the amount of antibody used).
3. Rinse embryos at least 3x in PBT-BSA, allowing them to settle each time. This step removes excess Methanol, which can destroy the primary antibody.
4. Rock embryos 30' (or longer) in PBT-BSA at room temperature (RT).
5. Add primary antibody, diluted in PBT-BSA. Each primary antibody has a different dilution necessary for it to use. Please ask for each new primary antibody that you might use.
6. Rock embryos overnight in primary antibody dilution at 4 degrees. (*stopping point*)
7. The next day, remove and save the primary antibody for later use. Store used antibodies at 4°C. Be sure to label tubes appropriately. Transfer the embryos to a 1.5mL eppendorf tube (this will aid washing steps below).
8. Rinse embryos 3x in PBT-BSA
9. Wash embryos 3x for 10' in PBT-BSA at RT.
10. Add secondary antibody, diluted in PBT-BSA. For DAB staining, use biotinylated secondary antibodies at 1:200 dilution.
11. Rock embryos at RT for at least one hour. (*stopping point- if you're going to stop here, incubate at 4°C*)
12. Rinse embryos 3x in PBT.
13. Following this step, mix A+B reagent:
    1. Dilute A 1:100 and B 1:100 together in a tube using PBT. Plan on a final volume of around 400µL for each staining.
    2. Allow this tube to rock for at least 30'
14. Wash embryos 3x for 10' in PBT
15. Add mixed A+B reagent to embryos. Rock embryos at RT for <u>exactly</u> 30 minutes.
16. Remove A+B reagent and discard. Rinse embryos 3x in PBT.
17. Wash embryos 3x for 10' in PBT at RT.
18. Following the last wash, remove as much PBT as possible. (*stopping point- if you stop here, add fresh PBT and store at 4°C*).
19. Add 600µL PBT, 20µL Nickel Chloride, and 30µL DAB to each tube. **DAB is a potent carcinogen!!! <u>Use gloves whenever handling DAB! Always discard any solutions and labware that has come in contact with DAB in the appropriate container!</u>**
20. Invert each tube 1-2 times to mix.

21. Remove the embryos and place them into a watch glass. Also be sure to get as much of the DAB solution as possible and put it with the embryos in the watch glass.
22. Dilute hydrogen peroxide 1:500 in PBT. Add 2µL of diluted hydrogen peroxide solution to embryos in the watch glass. Gently "stir" the embryos in the well with the pipet tip by gently pipetting the staining solution once or twice.
23. Monitor DAB color development under the microscope. Stop the reaction just before you think it's done.
24. Stop DAB development by washing 3-5 times in PBT. Remove the embryos to a 1.5mL Eppendorf tube and wash using the full volume of the tube. Discard all DAB washes in DAB waste. Rinse watch glass with PBT to clean it. Save all cleaning washes and discard in DAB waste.
25. NOTE: At this point, you have a choice to make:
    (A) If performing a secondary stain (i.e. for another primary antibody), then block for 30' in PBT-BSA and return to step 10.
        I. When you reach step 19 for the second time, omit the Nickel Chloride- the DAB development will give a brown, not black precipitate.
    (B) If done with antibody staining, proceed to step #26
26. Wash embryos in 70% Ethanol for 10 minutes at RT. Repeat this step.
27. Was embryos in 90% Ethanol for 10 minutes at RT.
28. Wash embryos in 100% Ethanol for 10 minutes at RT.
29. Wash embryos in 100% dry Ethanol for 10 minutes at RT. Repeat this step.
30. Wash embryos in Acetone for 5 minutes at RT. Repeat this step.
31. Remove as much acetone as you can. Add 300µL of 1:1 Araldite:Acetone. Avoid getting the araldite on your skin. Place embryos at 4°C overnight to allow embryos to sink in the acetone:araldite.
32. Mount embryos in araldite.