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Double Phosphorylated MAPK Staining Protocol

Rationale and Background:
To visualize the localization of MAPK activation in developing embryos of *Crepidula fornicata* and other calyptraeid snails.

Protocol:

1. Fix embryos in 3.7% formaldehyde for 30-35 minutes (diluted in FSW or 1X PBS) as above.
2. For longer term storage: Dehydrate in methanol and store at -20∘C. Embryos can be stored for short periods in PBS in the fridge.
3. Transfer specimens to spot-well dishes. For specimens that have been stored in methanol, rehydrate with PBTw (1X PBS with 0.1% Tween 20) at RT, 5-10 minutes.
4. Rinse twice more with PBTw 5-10 minutes at RT.
5. Pre-block embryos in PBTw with 4% BSA (or goat serum) for 2 hours at RT. It is very important to pre-block for the full amount of time.
6. Incubate in mouse anti-MAPK diluted 1:200 in PBTw with 4% BSA overnight at 4°C (or 2h at RT). Keep embryos in a moist chamber for overnight incubation.
7. Wash 6 times over 1-2 hours at RT with PBTw.
8. Incubate in secondary goat-anti-mouse AlexaFluor 546 (red) diluted 1:250 in PBTw + 4% BSA for 1-2 hours at RT. Protect from light from this point on.
   (Alternatively, use goat-anti-mouse HRP diluted 1:250 in PBTw + 4% BSA for 1-2 hours at RT. Develop DAB stain according to Pierce DAB-HRP staining instructions. Mount in 70% glycerin + PBS + Hoechst. Make sure to look at and photograph the HRP staining before exposing to UV for the Hoechst staining as the UV light will cause the embryos to become brown and discoloured.)
9. Wash 6 times over 1-2 hours at RT with PBTw.
10. Mount in 80% glycerin + PBS + Hoechst (1:10,000), view and image.

Solutions:

10X PBS (Mark Martindale recipe)
2.56g NaH2PO4-H2O (18.6mM NaH2PO4)
11.94g Na2HPO4-H2O (84.1mM Na2HPO4)
102.2g NaCl (1.75mM NaCl)
Mix phosphates with 800 mL dH2O.
Check that pH is within 0.4 of 7.4.
Add NaCl. Bring up to 1L.

PBTw
1X PBS + 0.1% Tween 20
Mix 5ml 10X PBS (pH 7.4) and 50 µL Tween-20. Bring up to 50 mL with dH_2O. Do NOT use

DEPC treated dH_2O for antibody staining.

PBTw + 4% BSA
Add 0.4g BSA (Bovine Serum Albumin: Sigma #A7906) to 10 mL PBTw. Mix well in a 15mL conical tube; BSA can be difficult to dissolve in PBTw.

80% glycerin + PBS + Hoechst
Add 2mL of 1X PBS to a 15mL tube. Add 1µL of Hoechst 33342 and mix well. Then add 8mL of glycerin and invert many times to mix. Store at 4°C and protect from light.