

## Tardigrade Embryo Immunostaining Protocol

By Willow Gabriel, , for *Hypsibius dujardini* embryos

From Gabriel, W.N. and B. Goldstein. (2007) Segmental Expression of Pax3/7 and Engrailed Homologs in Tardigrade Development. Development Genes and Evolution 217: 421-433.

- use siliconized eppendorf tubes for all steps
  - wash by centrifugation at 4500 rpm for 3 min, followed by aspiration with a Pasteur pipet
  - carry out all steps with agitation
  - day 1 = 3.5-4 hours, after embryo collection; day 2 = 4.5-5 hours
1. Treat with chitinase (5 units/ml)/chymotrypsin (10-20 mg/ml) in 0.5x PBS for 1 hour.
  2. Wash 3X - 5 min in bottled spring water (we have used Deer Park and Crystal Geysers).
  3. Fix in ice-cold absolute methanol for 20 min at 4°C.
  4. 90% MeOH for 5 min at RT.
  5. 70% MeOH for 5 min at RT.
  6. 50% MeOH for 5 min at RT.
  7. Fix for 10 min in 4% PFA in 0.5X PBT (w/ 0.05% Triton, not Tween) = 250µl 16% EM grade PFA + 750µl 0.5X PBT (Cat. # 15710, EM Sciences, [www.emsdiasum.com](http://www.emsdiasum.com))
  8. Sonicate on a Branson 250 sonifier for 20 sec (5 sec pulses, 15 sec on ice between pulses) at an amplitude of ~2.2 with a constant duty cycle.
  9. Allow to recover on ice for 15 min.
  10. Wash 5X - 5 min in 0.5X PBT (w/ 0.05% Triton, not Tween).
  11. Block 15 min in 5% DMSO, 1% BSA in 0.5X PBT.
  12. Add 1° antibody to tube (1:200 for tubulin, e.g.), and agitate in cold room O/N or at RT for 2 hours (depends on antibody).
  13. Wash 3X - 5 min, then 2X - 20 min with 0.5X PBT.
    - All following steps should be carried out in dark.
  14. Add 2° antibody (1:200 for Alexa 488 G-M) in 0.5X PBT +5% DMSO → 45 min at RT.
  15. Wash 2X - 20 min, 3X - 5 min in 0.5X PBT;
  16. tyramide signal amplification (Molecular Probes) - 20 min (+ 2 µl of tyramide/100µl of 1:2000 dilution of H<sub>2</sub>O<sub>2</sub>)
  17. wash 3X - 5 min
  18. DAPI (1µg/ml solution diluted 1:200, final concentration) + topro-3 (2µl per 1 ml 0.5X PBT) for 20 min
  19. wash 3X - 5 min
  20. Mount on subbed slides with mounting media. Seal with nail polish.