Double Fluorescent in situ hybridization (dfISH) Protocol for Tribolium embryos

If you use this protocol please cite: Buchta *et al*. "Patterning the dorsal-ventral axis of the wasp Nasonia vitripennis" (Dev Biol. 2013 Jun 2. doi:pii: S0012-1606(13)00298-4; PMID:23735637)

This protocol is a modified version from the one published in Lynch et al. 2010. The size and the large amount of yolk of Tribolium eggs (compared to Drosophila and Nasonia eggs) constitute a considerable signal/noise ratio problem for fluorescent in situ hybridizations in Tribolium embryos. We address this issue with efficient blocking, strong signal amplification and confocal like imaging (Apoptome) of the stained embryos.

Start with fixed eggs, stored in MeOH. DIG and DNP labeled probes have to be prepared before.

All steps should be done with 1ml volume, on a rotating wheel or softly shaking, if not indicated otherwise.

1. Wash in PBT 3 times 5min
2. Postfix in 5% Formaldehyde/PBT for 25min
3. Wash in PBT 4 x 5min
4. Wash in 1:1 PBT/Hyb for 5min
5. Incubate for one or more houre at 65°C in Hyb (blocking)
6. Remove Hyb, add 100-150µl prediluted DNP-lablled and DIG-labelled probes (1µl probe in 50µl Hyb), incubate over night at 60°C

7. Wash in Hybwash solution 3 time 25min, each at 60°C (preheat Hybwash solution)
8. Wash in 1:1 MABT/Hybwash for 5min at room temperature
9. Wash 4 times 10min in MABT
10. Block in 2%BBR MABT+10%NGS for one or more hours, 500µl are sufficient (blocking)
11. Prepare 1:2500 dilution of anti-DIG-AP antibody and 1:4000 dilution of anti-DNP-Rabbit antibody in, 2%BBR MABT+10%NGS
12. Remove antibody solution and add 200-400µl prediluted antibody. Incubate over night at 4°C, rotating

13. Wash 4 times 10min with MABT
14. Wash 3 times 5min with AP Buffer
15. Dissolve one fast red tablet in 2ml of AP buffer
16. Filter solution by using a syringe and filter (0,2µm)
17. Add 10µl HNPP solution per ml of filtered fast red solution
18. Add 300-400µl of this solution to each sample, after taking of the last AP buffer wash
19. Observe the development of the staining using a stereomicroscope. The red staining will be stronger when exposed to UV light. Be careful not to over stain.
20. Stop reaction by washing several times with MABT
21. Block in 2%BBR MABT+10%NGS for one or more hours, 500µl are sufficient (blocking)

22. Add anti-Rabbit-HRP antibody 1:100 prediluted in 2%BBR MABT+10%NGS (200-400µl) and incubate overnight at 4°C

23. Wash 4 X 10min with PBT
24. Wash 3 X 5min with PBS
25. Dilute 30% H_2O_2 1:100 in amplification buffer, add 2µl of this dilution to each 100µl amplification buffer for a final dilution of 0,0015%
26. Add 1µl tyramide 488 stock to above solution and immediately add to embryos (200-250µl). Stain in dark for 2h at room temperature. Protect from light
27. Wash 3 times 5min with PBT
28. Counter stain with DAPI (e.g. by mounting in Vectashield containing DAPI (Vector Laboratories)). Mount with two spacers and use a confocal microscope or Apoptom to take a Z-stack of the stained embryo. Combine the Z-stack to a projection.
29. Done ☺

PBT:
PBS with 0,1%Tween

MABT:
MAB with 0,1% Tween

Hyb:
50% formamide
5X SSC
2% SDS
2% BBR
250µg/ml tRNA
50µg/ml heparin
H2O

Hybwash:
50% formamide
2X SSC
1% SDS
0,1% Tween
H2O

2%BBR MABT+10%NGS:
1XMAB
0,1% Tween
10% NGS

2% BBR (solves best when rocked at 60°C)
H2O

5XMAB:
58g Maleic Acid
43,8g NaCl
36g NaOH pellets
Adjust pH to 7.5 using NaOH
Fill to with H_2O to 1L

AP Buffer:
100mM NaCl
100mM Tris pH9,5
50mM MgCl
0,1%Tween or no Tween

Probes:
The Probes were prepared by using the T7 Maxiscript kit (Ambion) in combination with DIG-labelling mix (Roche) or DNP-labelling mix (see recipe below). After precipitation the probe was dissolved in 80 – 100µl probe suspension solution (50% Formamid, 2X SSC in H_2O)

DNP Labeling mix:
20XNTP mix: Mix: 10µL each of ATP, GTP, CTP with 6.5µL UTP (all at 100mM) in 13.5µL nuclease free water.
20x DNP-11-UTP mix: Dilute DNP-11-UTP stock (Perkin-Elmer: NEL555001EA) with 10.7 µL nuclease free water.
Add 1µL each to 20 µL

Antibodies:
anti-digoxigen-AP antibody (Roche)
anti-DNP-rabbit antibody (Molecular Probes)
anti-rabbit-HRP antibody (included in TSA kit)

Staining reagents:
Fast-red tablets (Roche)
HNPP Fluorescent detection set (Roche)
AlexaFluor 488 TSA kit (Invitrogen)

Blocking reagents:
BBR: Blocking reagent for nucleic acid hybridization and detection (Roche)
NGS: Normal goat serum (Jackson ImmunoResearch)