Whole mount double RNA in situ hybridisation protocol

Niko Prpic, Gabi Büttner, Martin Klingler 11/01

(based on double-flourescent in situ protocoll by Christian Wolff (Wolff et al. 1998); please cite: Prpic et al. 2001, Dev. Genes Evol. 211, 467, and Hauptmann 1999, Dev Genes Evol 209:317–321

Principle: Two antisense RNA probes are used, probe#1 labelled with digoxigenin (DIG), probe#2 with fluorescein (FL).
After simultaneous hybridisation, the DIG-probe is detected by a peroxidase-coupled anti-DIG antibody (anti-DIG-POD) and the signal is amplified by local biotin-residue deposition, which then is detected by streptavidin-beta-Gal. This amplification is necessary to compensate for the low processivity of beta-Gal).
The FL-probe is detected directly by an alkaline phosphatase-coupled anti-fluorescein antibody (anti-FL-AP).

in situ hybridisation and signal detection

- transfer fixed embryos to an Eppendorf tube and proceed the following steps in 1ml volume unless stated otherwise;
  "**rinse (r)**" means: remove supernatant, add fresh buffer such that embryos are swirled up, let embryos settle down again.
  "**wash (w)**" means: after addition of fresh buffer, close lid of Eppendorf vial and mix by rolling on rotating weel. w15 means: roll for 15 minutes.
- r r with methanol
- rinse with methanol/PBT 1:1
- **post-fix** with 140 µl formalin (37%, Sigma) in 1ml PBT for 15min
- r r w w with PBT
- perform **proteinase K digest** (Boehringer, about 15mg/ml); make 1:10 dilution of PK and use 5 ul for 1ml PBT (=1:2000 dilution) and incubate for 4min on wheel, let embryos settle down for another minute
- rr with PBT
- post-fix again as above
- rrwr
- remove supernatant, add 500 µl PBT/hybe-B (1:1)
- remove supernatant, add 250 µl hybe-B
- replace with 250 µl hybe-A
- **prehybridize** at 65°C for 60-120min (waterbath)
- remove as much of Hybe-A supernatant as possible
- add both probes (typically 0.5 to 1µl of probe are sufficient, but you have to test the optimal amount for each probe generated),
  mix embryos and probes by moving yellow tip in circles (don't pipett up, down)
- **hybridize** at 65°C over night (12-16h)
- (optional: start preabsorption of anti-DIG-POD, anti-FL-AP, streptavidin-CY2 (1hour is sufficient)
- prewarm hybe-B and add 500µl to embryos, incubate 20min at 65°C
- r with 500mikrol. hybe-B and incubate again for 20min at 65°C

- rinse with 1:1 hybe-B:PBT at room temperature (RT)
- rw w15 with PBT
- **blocking**: to 800 ul PBT add 200 ul of a 2,5% stock of Boehringer blocking reagent (-> 0,5%), incubate 20 min on wheel
- incubate with **anti-DIG-POD** 1:2000 in PBT at RT for 1hour
- rrw w20 w30 w30 (PBT)
- w5 with **Blast amplification** buffer (AB, TSA Biotin system)
- incubate 6 min at RT in 435 µl Blast amplification buffer plus 65 µls of biotinyl-tyramide (the optimal amount of biotinyl-tyramide is critical, if necessary, test different concentrations)
- rw w15 w15 with PBT/20% DMSO
- rr (PBT)
- incubate with **anti-FL-AP** and **streptavidin-betaGal** (1:2000 each, simultanously, for 1hour at room temperature, or at 4°C over night)
- rrw w20 w30 w30 (PBT)
- rw5 with AP-staining buffer
- **develop AP-signal**: use 1ml of AP-staining buffer with 4,5 µl NBT and 3,5 µl B-Cip (=X-Phosphate)
- stop reaction with PBT
- rw w15 w15
- rw with beta-Gal staining buffer
- **develop AP-signal**: to 1 ml beta-Gal staining buffer add 30 ul X-Gal (0,5 µg/µl)
- stop reaction with PBT
- w w15 w30
- dissect embryos in glycerol, embedd for microscopy in 50% glycerol

Materials

- Boehringer DIG-/FL-RNA labeling kits (#1175025/#1685619)
- NEN/Perkin-Elmer TSA Biotin System (#NEP-700A, containing 2xAB buffer to be diluted 1:1 with water, and biotinyl-tyramide solution)
- anti-DIG-POD (Boehringer #1207733)
- anti-FL-AP (Boehringer #1426338)
- anti-Streptavidin-beta-Gal (Dianova)

Solutions

- PBT: PBS plus 0.02% Tween 20; stirred with 0.5ml DEPC/l for 30min and autoclaved (10x PBS: 320g NaCl + 8g KCl + 8g KH_2PO_4 + 46g Na_2HPO_4, add dH_2O to 4l, pH to 7.4)
- hybe-B: 50% formamide, 5xSSC pH 5.5
- hybe-A (20 ml): to 10 ml deionized formamide add 5 ml of 20x SSC, make to pH 5.5 (yes!) with HCl, fill to 20 ml with dest. water. add:
  0.4 ml of 10 mg/ml of boiled/chilled sonicated salmon testis DNA

100 ul of 20 mg/ml tRNA
20 ul of 50 mg/ml heparin

-beta-Gal staining buffer:
3mM K3 Fe(CN)6 (1,5 ml of 0,1M stock)
3mM K4 Fe(CN)6 (1,5 ml of 0,1M stock)
0.9 mM MgCl2 (45 ul of 1M stock)
in PBT (add to 50 ml)
keep at RT

AP Staining buffer:
20ml of 0.1M Tris pH 9.5
add 1ml of 1M $MgCl_2$, mix
add 400ul of 5M NaCl, mix
add 100ul of 20% Tween20, mix

NBT solution: 4-nitro blue tetrazolium chloride, 75mg/ml in 70% dimethylformamide (and water); store at -20^{o}C

X-phosphate solution: 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt, 50mg/ml in 100% dimethylformamide (DMF); store at -20^{o}C