Chondrichthyan whole mount mRNA *in situ* hybridization protocol

Gillis Lab

Pretreatment and hybridization of embryos:

1. After dehydrating in 100% methanol at –20C…

[At this point, make up 50mL of PBT by combining 50mL of DEPC PBS with 100uL of 50% tween in a clean 50mL conical tube. Shake vigorously to mix before using].

2. Rehydrate embryo at room temp (5min each at 75%, 50%, 25% methanol in PBT, and 3 x 5min in PBT). [Make sure washes fill the vial].

3. Permeabilise with prot-K. Incubate in a 1:2000 dilution of 10mg/mL proteinase K in 15min at room temp.

4. Refix for 20min at RT with 4% PFA. Wash 3 x 5min in PBT at room temp.

5. Add prehyb [full vial] and wait until the embryo is on the bottom of the vial. [Do not do this step on the shaker – rather, gently invert the tube occasionally, until you can no longer see wavy lines of PBS coming form the tissue]. Remove and add fresh prehyb [full vial]. Incubate at least 60min at 70C.

    Prehyb:
    i. 5ml formamide
    ii. 2.5ml 20X SSC Ph 4.5
    iii. 1ml 10% SDS
    iv. 250ul of tRNA (2mg/ml stock) or 50ul of tRNA (if 10mg/ml stock)
    v. 5ul Heparin (50mg/ml stock)
    vi. 1.5ml water

6. Remove prehyb, and add 1-2ml of hybridization mixture + riboprobe. Probe concentration must be determined empirically, but 1ng/uL typically works well. Hybridize for 24-72 hours at 70C.

Posthybridization wash and antibody detection:

1. Prepare and prewarm these solutions before starting washes [do all these washes very gently, as post-hyb embryos are extremely delicate]:

    a. Sol'n 1:
        i. 20ml formamide
        ii. 8ml 20X SSC Ph 4.5
        iii. 4ml 10% SDS
        iv. 8ml water

b. Sol'n 3:
    i. 20ml formamide
    ii. 4ml 20X SSC Ph 4.5
    iii. 16ml water

c. 20% sheep + 1% roche blocking agent in MABT [for 10ml, combine 2ml of sheep serum, 1ml of 10X blocking reagent, and 7ml of MABT]

2. Wash 2 x 60min with prewarmed solution 1 at 70C.

3. Wash 2 x 30min in solution 3 at 65C.

4. Quickly rinse embryos with MABT, then wash 3 x 10min in MABT.

5. Block with 20% heat inactivated sheep serum + 1% Roche blocking agent for at least 2.5hr at RT on a horizontal shaker. [full vial]

6. Remove block and add antibody diluted in block (1:2000 Roche anti-Dig or anti-fluorescein AP fab fragments). [full vial] Incubate overnight (i.e. at least 18 hours) at 4C with gentle shaking.

Post antibody washed and detection:

1. Wash 3 x 5min in MABT at room temp.

2. Wash 5 x 60min in MABT at room temp. Embryos may be left in MABT in the fridge for several days, with regular MABT changes, before developing.

3. Wash 3 x 10min in NTMT at room temp:

    a. NTMT
        i. 2ml 5M NaCl
        ii. 5ml Tris/HCl 2M Ph 9.5
        iii. 5ml 1M MgCl2
        iv. 0.2ml 50% Tween20
        v. Water to 100ml

4. Incubate in BM Purple (monitor every hour for signal).

5. When reaction is complete:
    a. 2 x 30min PBT and then into 4% PFA for 1hr at room temp.
    b. Rinse 3 x 5min in PBS, and clear in glycerol for imaging (25%, 50% and 75% glycerol in PBS).