Talbot Lab Single (non-fluorescent) *in situ* protocol

This protocol combines elements of the Amacher Lab protocols with Yi-Lin Yan's protocols, plus some additional modifications by Jared Talbot

Day 1:

1. Rehydrate the embryos through a methanol/PBST series (75%, 50%, 25% methanol mixed with PBST) 3-5 minutes per wash. (See note *, above)
2. Wash 5 x 5 mins in PBST (can be stored at 4˚C for an afternoon)
3. Treat the embryos with 1µg/ml proteinase K in PBST to increase the permeability of the membrane. Greater than 24 hours: stagger start times, so all fish end treatment concurrently. Time of ProK treatments are as follows:

Embryonic stage	**Length of ProK**	**Concentration**
24 hours	5 min	1 µg/ml
36 hours	30 min	1 µg/ml
48 hours	45 min	1 µg/ml
55 hpf	1 hour	1 µg/ml
72 hpf	1.5 hours	1 µg/ml

4. Fix the embryos in 4% PFA/1XPBS for 20 minutes to halt ProK activity.
5. Wash 5 X 5 mins in PBST
6. Split embryos into appropriate tubes. Tubes should contain <20 embryos for stages lacking tails, or <30 embryos for stages with tails.
7. Replace with Pre-warmed (to room temp) 0.5ml Pre-Hyb solution
8. Incubate at 65˚C for one to four hours.
9. Towards the end of step 7, prepare probes @1:400 in Pre-Hyb, preheat @ 65˚C
10. Add diluted preheated (450µl) probes to tubes.
11. Incubate overnight @ 65˚C

Probes: *Reuse old probes, because they have been pre-absorbed. Use 200 µl per tube, when possible. For new probe, we mix 2.5 µl probe with 1 ml pre-hyb.*

Store probes at -80˚C when undiluted.

Store probes at -20kC when diluted in Pre-Hyb

Day 2:

1. Preheat Post-hyb wash solutions, if you have not already done so
2. Remove probes, and **save them for reuse**
3. Wash 5 mins in "5X" (5X SSC, 50% formamide, 0.25% tween-20) @ 68-70˚C
4. Wash 5 mins in 3:1 5X:2X @ 68-70˚C
5. Wash 5 mins in 1:1 5X:2X @ 68-70˚C
6. Wash 5 mins in 1:3 5X:2X @ 68-70˚C
7. Wash 5 mins in "2X" (2x SSC, 0.25% tween-20) @ 68-70˚C
8. Wash 1 X 20 mins in 0.2X SSC, 0.25% tween-20 @ 68-70˚C
9. Wash 2 X 20 mins in 0.1X SSC, 0.25% tween-20 @ 68-70˚C
10. Wash 5 mins in 66% 0.1X SSC : 33% PBST @ room temp (RT)
11. Wash 5 mins in 33% 0.1X SSC : 33% PBST @ RT
12. Wash 5 mins in PBST at RT
13. Wash in blocking solution (5% BSA in PBST) for one hour @ RT
14. Replace blocking solution with blocking solution containing 1:5000 anti-digoxigenin-AP Fab fragments.
15. Rock 2 hours @ RT (or overnight @ 4˚C)
16. Wash 2X rapidly with PBST
17. Wash 5 X 15 mins with PBST
18. Store overnight at 4˚C

Day 3:

1. Wash 3 x 5 mins in coloration buffer (NTMT). Make NTMT solution fresh each time you do the in situ. Do not leave embryos in NTMT for extended periods of time.
2. Make fresh coloration solution
    a. *Use gloves when handling NBT/BCIP, and embryos treated with these chemicals- they are toxic!*
    b. Add 4.5µl NBT stock per ml of coloration buffer
    c. Mix by tilting
    d. Then, add 3.5µl BCIP stock per ml coloration buffer
3. Add 500µl coloration solution to each tube, and transfer embryos to white viewing dishes. Be sure to diagram the plate layout before transferring embryos! Also, mirror plate-layout with epi-layout in an epi rack.

    Hgfa takes 7-8 hours to develop. Cmet takes ~3 hours to develop.
4. During development, view embryos intermittently with epi-illumination. Take careful notes on probe development; 5 mins, 15 mins, 30 mins, 45 mins, 1 hour (then every 30 mins). Stop reactions when signal is climbing slower than noise.
5. Stop reactions by washing quickly 2 x with sterile water (*discard NBT/BCIP and follow up washes in the waste bottle in the fume hood!*)
6. Dehydrate in a MeOH/PBST series (25%, 50%, 75%, 100%, 100%)
7. Store at -20˚C.

Solution Recipes

PBST **(make new)**
___100 ml 10X PBS ___ 5 ml 20% Tween-20 ___ Sterile H2O to 1L
___ Mix ___ Store @ RT

Pre-Hyb: **(make new)**
___ 25 ml Formamide ___25 µl Heparin (100mg/ml) ___ 12.5 ml 20X SSC
___ 250 µl Yeast tRNA (100mg/ml) ___250µl 20% Tween ___ Sterile H2O to 50 ml
___Then, add 460µl 1M Citric Acid.
___ Mix ___ Test pH: (ideal: 6.3. Range: 6.0 to 6.5)
___Store @ -20˚C

"5X": 5X SSC / 0.1% Tween-20 / 50% formamide **(reuse, 12/12/16)**
___ 100 ml Formamide ___ 50 ml 20X SSC ___ 1ml 20% Tween
___49 ml sterile H2O ___ Mix ___Store @ 4˚C

"2X": 2X SSC/0.1% Tween-20 **(reuse, 2/7/17)**

"0.2X": 0.2X SSC/0.1% Tween-20 **(reuse, 8/2/16)**

"0.1X": 0.1X SSC/0.1% Tween-20 **(make new)**
___ 2 ml SSC (20X) ___ 2ml 20% Tween-20 ___ Sterile H2O to 400 ml ___ mix
___Store @ RT

Post-Hyb washes:
___add 20 ml 5X to a conical
___3:1 Add 12 ml 5X ___4 ml 2X to a conical
___1:1 Add 8 ml 5X ___8 ml 2X to a conical
___1:3 Add 4 ml 5X ___12 ml 2X to a conical
___Add 20 ml 2X to a conical
___Add 20 ml 0.2X to a conical
___Add 35 ml 0.1X to a conical
___Preheat the above conicals to 65˚C

33% 0.1X 66% PBST **(reuse, 10/24/14)**
66% 0.1X 33% PBST **(reuse, 10/24/14)**

Coloration Buffer (NTMT)
___Make this fresh, the day you do the coloration step!
___10 ml 1M Tris-HCL, pH 9.5
___ 5 ml 1M MgCl2
___ 2 ml 5M NaCl
___ 500 µl 20% Tween-20
___ Sterile H2O to 100 ml
___ Mix