Talbot lab two color fluorescent in situ

This protocol has been adapted with minor modifications from the protocol described in Talbot et al., 2010: *hand2 and Dlx genes specify dorsal, intermediate and ventral domains within zebrafish pharyngeal arches.*

The Talbot et al 2010 protocol was itself derived from MCM Welten et al. 2006. ZebraFISH: Fluorescent in situ hybridization protocol and three dimensional imaging of gene expression patterns. Zebrafish v3 (4) 465-476, and modifiers taken from Julia Dalcq, and Yi-Lin Yan's protocols, and then modified slightly over subsequent years.

Conceptualized protocol:

Permeabilize embryos → hybridize probes →Destroy native peroxidase activity → bind anti-DNP-peroxidase →develop Tyr-Fluorescein → destroy peroxidase activity → bind anti-DIG-peroxidase → develop Tyr-Cy3 → H2O2 treat to reduce background.

Pertinent Materials:

Anti-DNP-POD: Perkin-Elmer Cat#NEL747A001KT. Comes with blocking powder.
Anti-DIG-POD: Roche cat# 1207733
Anti Fluor-POD: Invitrogen cat# A-21253
DIG probe synthesis materials can be obtained from Roche in a kit (Cat# 11175025910), or individually. With the exception of the DIG mix, this kit can also be used for Fluor probe synthesis, or DNP probe synthesis.
20XNTP mix: 100mM NTP's obtained from Amersham Bioscience (cat# 27202501).
Mix: 10µl each of ATP, GTP, CTP with 6.5µl UTP in 13.5µl nuclease free H2O
Resulting in: 20mM each ATP, GTP, CTP, 13mM UTP stock.
20X DNP-11-UTP stock: Obtain a 250nmol/25µl stock of DNP-11-UTP from Perkin-Elmer (cat# NEL555001EA). Add 10.7µl nuclease free H2O for a 7mM stock
TSA Cyanine 3 and Fluorescein system: Perkin Elmer Cat# NEL753001KT

Ty-Fluorescein and Tyr-Cy3 arrive as a powder from Perkin elmer; for most kits, re-suspend these pellets in 150 µl DMSO, and then dilute as indicated.

Embryo preparation:

1. Dechorionate using a pair of watchmakers forceps, or using Pronase.
   for embryos 20 hpf and younger, dechorionation can be done after fixation and PBST washes
2. Fix embryos overnight at 4˚c in 4% PFA/1X PBS, or 2-4 hours at room temperature.
3. Wash twice in PBST (1X PBS, 0.25% tween-20)
4. Dehydrate with a series of methanol/PBST solutions (25%, 50%, 75% methanol mixed with PBST), then twice with 100% methanol. Shake 3-5 minutes in each solution.
5. Store the embryos in 100% methanol at –20˚C up to several months

Probe synthesis:

DNP Probes:	DIG probes:
1-2µg linear plasmid	1-2µg linear plasmid
1µl 20X NTP	2µl DIG NTP mix
1µl 20X DNP-11-UTP	
2µl 10X TXN buffer	2µl 10X TXN buffer
1µl RNAse inhibitor	1µl RNAse inhibitor
2µl T7/SP6/T3	2µl T7/SP6/T3
Nuclease free H2O to 20µl	Nuclease free H2O to 20µl

1. Prepare the above mixtures
2.Incubate 2 hours @ 37˚C
3. Mix in 2µl DNAse I
4. Incubate 15 minutes at 37˚C
5. Add 0.8µl 0.5M EDTA pH 8.0
6. Add 2µl 5M LiCl
7. Add 75µl Prechilled 100% Etoh
8. Place @ -80˚C for 45 mins to overnight
9. Centrifuge 10 mins @ 4˚C
10. Remove liquid
11. Rinse with 200µl Prechilled 80% Etoh
12. Centrifuge 5 mins @ 4˚C
13. Remove as much liquid as possible
14. Air dry 5-10 mins at room temp
15. Resuspend in 60µl nuclease free H2O
16. Mix in 1µl RNAse inhibitor
16. Store at –20˚C ASAP.

Diagnostic gel:
1. Mix 2µl probe with 5µl formamide, 3µl nuclease free H2O.
2. Heat this aliquot 3 mins @ 68-70˚C
3. Run products 20 mins on a 1.5% gel @ 130V

Day 1:

1. Rehydrate the embryos through a methanol/PBST series (___75%, ___50%, ___25% methanol mixed with PBST) 3-5 minutes per wash.

___ ____ ___ ___2. Wash five minutes in PBST, four times.

3. Treat the embryos with 1µg/ml proteinase K in PBST to increase the permeability of the membrane.

Time of ProK treatments are as follows:

Embryonic stage	**Length of ProK**
16 som	5 min
20 som	5 min
24 hours	7 min
30 hours	20 min
36 hours	30 min
48 hours	45 min
55 hours	1 hour

4. Fix the embryos in 4% PFA/1XPBS for 20 minutes to ensure the ProK has stopped.

___ ____ ___ ___ ___5. Wash 5 X 5 mins in PBST

___6. Replace with 1ml Pre-Hyb solution
___7. Incubate at 68-70˚C for at least 2 hours. I've always done this at least four hours.
___8. Prepare probe mixtures in fresh pre-hyb. Let warm up at least 15 minutes.

Add at least 200 µl solution to each tube.
Add 2µl each probe per 200 µl pre-hyb.
Allow probes to heat up before adding to each tube.

___9. Add Prehyb/probe to embryos
___10. Incubate overnight @ 68-70˚C
___11. Prepare solutions for Day 2 washes, and leave O/N at 68˚C:

Day 2:

___ Ensure that 5x/2x solutions are @ 68˚C. Thaw out TBSTB.

___ 12. Remove probes, **and save them for reuse**

___ 13. Wash 5 mins in “5X” (5X SSC, 50% formamide, 0.25% tween-20) @ 68-70˚C

___ 14. Wash 5 mins in 3:1 5X:2X @ 68-70˚C

___ 15. Wash 5 mins in 1:1 5X:2X @ 68-70˚C

___ 16. Wash 5 mins in 1:3 5X:2X @ 68-70˚C

___ 17. Wash 5 mins in “2X” (2x SSC, 0.25% tween-20) @ 68-70˚C

___ ___ ___ 18. Wash 3 X 20 mins in 0.2X SSC, 0.25% tween-20 @ 68-70˚C

___ ___ 19. Wash 2 X10 mins in PBST @ room temp

___ 20. Replace with 2% H202 in PBST

___ 21. Shake 60 mins @ room temp

___ ___ ___ ___22. Wash 4 X 5 mins in TNT (0.1 M Tris-Hcl pH 7.5; 0.15 M NaCl; 0.5% Tween20)

___ 23. Block at least two hours (I’ve typically done four hours) in 400µl TBSTB

___ 24. Replace with 400µl 1:500 anti-DNP-POD in TBSTB.

___ 25. Rock overnight @ 4˚C

Day 3:

___ ___ ___ ___ ___ ___ ___ ___26. Wash 8 times in TNT at room temp over the course of 1-2 hours
___ 27. Wash five minutes in 50µl Perkin Elmer Amplification Diluent
___ 28. Prepare 1:50 Tyr-Fluorescein in Amplification Diluent for 50µl*(n+1) samples
        ___X µl Diluent per sample   ___1/50X µl Tyr-Fluorescein per sample
___ 29. Replace amplification diluent with 50µl 1:50 Tyr-Fluorescein
___ 30. Shake one hour in the dark, tubes upright. Don't exceed one hour. All steps from here on out are done in the dark.
___ ___ 31. Wash 2 X 5 mins in TNT
___ 32. Wash one hour in 2%H2O2/TNT (___1/10X ml 30% H2O2   ___9/10X ml TNT)
___ ___ ___ ___ 33. Wash 4 X 5 mins in TNT
___ 34. Block with 400µl TBSTB 1-4 hours
___ 35. Replace with 1:1000 anti-DIG-peroxidase in TBSTB
___ 36. Rock overnight at 4˚C.

Day 4:

___ ____ ___ ___ ___ ___ ___ ___ 37. Wash 8 times in TNT at room temp over the course of 1-2 hours
___ 38. Wash five minutes in 50µl Amplification Diluent
___ 39. Prepare 1:50 Tyr-Cy5 in Amplification Diluent for 50µl*(n+1) samples.
    ___X µl Diluent   ___1/50 X µl Tyr-Fluorescein
___ 40. Replace amplification diluent with 50µl 1:50 Tyr-Cy5
___ 41. Shake one hour, tubes upright.
___ ___ 42. Wash 2 X 5 mins in TNT
___ 43. Wash one hour in 2%H2O2/TNT (optional. I find this improves the second stain's signal:noise somewhat)
___ ___ ___ ___ 44. Wash 4 times in TNT
___ 45. Store fish in TNT @4˚C. Staining lasts several months in these conditions.
___ 46. Add 1% PFA/TNT to the fish, and store.

DO NOT store in PBST!!!

Working solutions

Pre-Hyb: (50% formamide, 5X SSC, 100µg/ml yeast RNA, 50µg/ml Heparin, 0.25% tween-20, Citric acid to pH 6.0 [appx 0.02M citric acid final])
100ml formamide 50ml 20X SSC 2.5ml 20% tween-20 water to 200ml
200µl 50mg/ml Heparin 400µl 250mg/ml Yeast tRNA 1.9ml 1M citric acid
Test pH → 6.0
store at 4˚C after use

Probe mixturess: __200 µl Pre-Hyb __2 µl probe1-DNP __2 µl Probe2-DIG
Multiply by number of samples when making a master mix for multiple tubes

PBST: (1X PBS, 0.25% tween-20)
100ml 10X PBS 12.5ml 20% tween-20 Water to 1L
store at room temp

TNT: (0.1 M Tris-Hcl pH 7.5; 0.15 M NaCl; 0.5% Tween20)
100ml Tris pH 7.5 30ml 5M NaCl 25ml 20% tween-20 Water to 1L
store at room temp

TBSTB: (TNT with 0.5% Perkin-Elmer blocking powder)
50ml TNT 0.25g Perkin Elmer Blocking Powder
Mix well, and heat @ 68˚ for one hour to get powder into solution.
Store at -20˚C, and once thawed, never refreeze.

Wash solutions: *Place at 68˚C overnight on day 1, for quick work in the morning of day 2.*
"5X": ___ 20 ml "5X" (5x SSC 50% formamide 0.25% Tween-20)
"3:1": ___15 ml 5X ___5 ml 2X
"1:1": ___10 ml 5X ___10 ml 2X
"1:3": ___5 ml 5X ___15 ml 2X
"2X": ___ 20 ml "2X" (2X SSC 0.25% Tween-20)
"0.2X": ___50 ml 0.2X SSC 0.25% Tween-20