Talbot Lab Triple Fluorescent in situ Protocol

This protocol was published 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.

Troubleshooting:

The largest factor that dictates whether the in situ will work well is the quality of probes used. When I've had problems with this protocol, I've generally been able to solve them with new, cleaner, probes. In my hands, DNP works as well, if not better, than DIG for probe labeling. Fluorescein labeled probes have the reputation for giving background, so I put my nicest probe in fluorescein, and develop this first so it has more time to H2O2 bleach. The first step of this protocol generally produces better label than the probe developed second. I have found that Tyr-Cy3 works considerably better than using fast red protocols. Both Tyr-Fluor and Tyr-Cy3 produce comparable signal to noise ratios and good labeling under similar conditions. I have yet to closely troubleshoot Tyr-Cy5, but it seems to work quite nicely in the trials I've done so far. Cy5 (far red) wont show up under many fluorescent dissecting microscopes, so put a very consistent probe in this color. The Welton et al protocol recommends doing hybridization at 55˚C, which I have not yet tried. As per the norm with in situ, it is critical that the pH of pre-hyb is around 6-6.5.

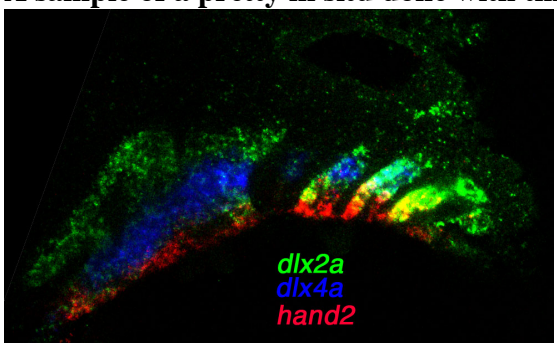
Double In situ:

To do a double in situ, instead of a triple in situ, simply add anti-DIG on day 2, and skip day 3. Develop DIG with tyr-Fluor, and develop DNP with tyr-Cy3. This is to avoid using fluorescein probe, which some people don't like.

Conceptualized protocol:

Permeabilize embryos → hybridize probes →Destroy native peroxidase activity → bind anti-Fluor-peroxidase →develop Tyr-Fluorescein → destroy peroxidase activity → bind anti-DIG-peroxidase → develop Tyr-Cy3 → destroy peroxidase activity → bind anti-DNP-peroxidase → develop Tyr-Cy5 → H2O2 treat to reduce background.

A sample of a pretty in situ done with this method:



Probe synthesis:

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

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
The DIG probe sysnthesis 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.
Fluorescein Labeling Mix: Roche cat# 11685619910
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
TSA Cyanine 5 system: Perkin Elmer Cat# NEL745001KT

Embryo preparation:

1. Fix embryos overnight at 4˚c in 4% PFA/1X PBS
2. Wash twice in PBST (1X PBS, 0.25% tween-20)
3. Dechorionate using a pair of watchmakers forceps
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

Recipes:

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.

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

"5X": (5X SSC, 50% formamide, 0.25% tween-20)
100ml formamide 50ml 20X SSC 2.5ml 20%tween 47ml sterile H2O
store at 4˚C

"2X": (2x SSC, 0.25% tween-20)
40ml 20X SSC 5ml 20% tween-20 Sterile H2O to 400ml
store at room temp

"0.2X": (0.2X SSC, 0.25% tween-20)
4ml 20X SSC 5ml 20% tween-20 Sterile H20 to 400ml
store at room temp

In situ hybridization:

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. Stagger start times, so all fish end treatment concurrently.

Time of ProK treatments are as follows:

Embryonic stage	**Length of ProK**
24 hours	10 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 (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])
7. Incubate at 68-70˚C for at least 2 hours. I've always done this at least four hours.
8. Replace prehyb with 200µl fresh Pre-Hyb.
9. Add no more than 200ng of each probe to this hyb solution.
10. Incubate overnight @ 68-70˚C

Day 2:

11. Remove probes, and save them for reuse
12. Wash 5 mins in "5X" (5X SSC, 50% formamide, 0.25% tween-20) @ 68-70˚C
13. Wash 5 mins in 3:1 5X:2X @ 68-70˚C
14. Wash 5 mins in 1:1 5X:2X @ 68-70˚C
15. Wash 5 mins in 1:3 5X:2X @ 68-70˚C
16. Wash 5 mins in "2X" (2x SSC, 0.25% tween-20) @ 68-70˚C
17. Wash 3 X 20 mins in 0.2X SSC, 0.25% tween-20 @ 68-70˚C
18. Wash 2 X10 mins in PBST @ room temp
19. Replace with 2% H202 in PBST
20. Shake 60 mins @ room temp
21. Wash 4 X 5 mins in TNT (0.1 M Tris-Hcl pH 7.5; 0.15 M NaCl; 0.5% Tween20)
22. Block at least two hours (I've typically done four hours) in 400µl TBSTB
23. Replace with 400µl 1:5000 anti-Fluorescein-POD in TBSTB.
24. Rock overnight @ 4˚C

Day 3:
25. Wash 8 times in TNT at room temp over the course of 1-2 hours
26. Wash five minutes in 50µl Perkin Elmer Amplification Diluent
27. Prepare 1:50 Tyr-Fluorescein in Amplification Diluent for 50µl*(n+1) samples.
28. Replace amplification diluent with 50µl 1:50 Tyr-Fluorescein
29. Shake one hour in the dark, tubes upright. Don't exceed one hour. All steps from here on out are done in the dark.
30. Wash 2 X 5 mins in TNT
31. Wash one hour in 2%H2O2/TNT
32. Wash 4 X 5 mins in TNT
33. Block with 400µl TBSTB 1-4 hours
34. Replace with 1:1000 anti-DIG-peroxidase in TBSTB.
35. Rock overnight at 4˚C.

Day 4:
36. Wash 8 times in TNT at room temp over the course of 1-2 hours
37. Wash five minutes in 50µl Amplification Diluent
38. Prepare 1:50 Tyr-Cy3 in Amplification Diluent for 50µl*(n+1) samples.
39. Replace amplification diluent with 50µl 1:50 Tyr-Cy3
40. Shake one hour, tubes upright.
41. Wash 2 X 5 mins in TNT
42. Wash one hour in 2%H2O2/TNT
43. Wash 4 times in TNT
44. Block with 400µl TBSTB 1-4 hours
45. Replace with 1:500 anti-DNP-peroxidase in TBSTB.
46. Rock overnight at 4˚C.

Day 5:
47. Wash 8 times in TNT at room temp over the course of 1-2 hours
48. Wash five minutes in 50µl Amplification Diluent
49. Prepare 1:50 Tyr-Cy5 in Amplification Diluent for 50µl*(n+1) samples.
50. Replace amplification diluent with 50µl 1:50 Tyr-Cy5
51. Shake one hour, tubes upright.
52. Wash 2 X 5 mins in TNT
53. Wash one hour in 2%H2O2/TNT (optional. I find this improves the second stain's signal:noise somewhat)
54. Wash 4 times in TNT
55. Store fish in TNT @4˚C. Staining lasts several months in these conditions.