Two-color FISH Protocol

Steps 1 - 13 are based on Andy McMahon's “Double Fluorescent Section in situ hybridization”. Steps 14 - 38 are based on the Gaido lab's bench “TSA Fluorescent Section in situ hybridization” method.

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Probes were labeled based on the protocol found on the GUDMAP website (GAIDO lab). Tissue fixation method based on the McMahon lab protocol located on the GUDMAP website. (PFA to sucrose, frozen sections cut then air dried). 10µm slides air dried for 30 minutes before freezing at -80C.

Hybridization - DAY 1

Note: Throughout the procedure, try to remove as much previous solution as possible before going into a new solution; however, DO NOT let the tissue completely dry out.

1. Air dry previously frozen slides under an air-flow hood for 15 minutes.

(Step 1 has been optimized by the Gaido lab.)

2. Fix sections in 4% PFA/PBS for 10 min. Do not discard, save for step 6.
3. Rinse in DEPC PBS, 3 x 5 min each.
4. Treat with 10µg/ml of Proteinase K in PBS for 10 min.

Be careful as to the strength of the PK as it varies from lot to lot – this timing/concentration should be adjusted every time a new stock is made.

5. Rinse quickly in DEPC PBS.
6. Fix in 4% PFA/PBS for 5 min.
7. Rinse in DEPC PBS, 3x 5 in each.
8. **De-acetylation**:

In a glass histology jar on a stirrer combine:

DEPC H2O	200ml
Triethanolamine	2.66ml

37% HCl 0.35ml

Begin stirring and add:

*Acetic anhydride 0.75ml

Place rack of slides in jar while stirring, stir for 10 min.

*Add 375ul of acetic anhydride, wait 5 min, then add the second 375ul.

Acetic anhydride is only good for 5 minutes, adding another aliquot is necessary.

9. Rinse in DEPC PBS, 2x 3 min each.
10. Rinse with 0.85% NaCl for 3 min.
11. Wash with 70%EtOH/0.85%NaCl for 5 min.
12. Wash with 95% EtOH for 5 min.
13. Air dry for 10 min.
14. **Hybridization**:

Dilute probes with hyb buffer such that the probe concentration is between 300ng/ml to 500ng/ml.

Heat the diluted probe to 80ºC for 30 seconds, then heat to 65ºC for 10 min, cool to room temp.

Arrange slides horizontally in a humidified chamber (PBS), remove excess solution, apply ~150µl of hybridization buffer with probe to each slide, and apply parafilm.

Incubate at 55º - 58º C O/N.

Note: typically use 300ng/ml for strong probes, 400ng/ml for medium probes and 500ng/ml for weak probes.

15. Hyb - added 150µl of probe to each slide, cover with parafilm, incubated O/N at 55ºC. (Make humidified chamber for o/n incubation with PBS.)

DAY 2

NOTE: keep reagents at 55 - 58ºC during incubation.

16. 5x SSC - 55ºC - 25 min
17. Formamide I - 55ºC - 50 min
18. Formamide II - 55ºC - 1 hour
19. 0.1x SSC - 55ºC - 25 min
20. TN wash 3x 5 min each, RT with agitation
21. 1% H2O2 in TN - 20 min (no Tween)
22. TN wash 3x 5 min each, RT with agitation
23. Block (PE) in TNB - RT - 30 min
24. ** αDig POD or anti-DNP HRP (1:500) - RT - 1 hour
25. TNT wash - 3x - 5 min each - RT with agitation
26. ** Apply ~100µl TSA Cy3 or TSA fluorescein (1:50) - 15 min incubation (use humidified chamber (PBS))
27. TNT wash 3x 5 min each - RT with agitation
28. TN wash 3x 5 min each, RT with agitation (gets rid of Tween)
29. 1% H2O2 in TN - 30 min (no Tween) - optimized by the Gaido lab
30. TN wash 3x 5 min each, RT with agitation
31. Wash with TNT, 1x 1 min (just enough to add back some Tween)
32. ** αDig POD or anti-DNP HRP (1:500) - RT - 1 hour (use humidified chamber (PBS))
33. TNT wash 3x 5 min each - RT with agitation
34. ** TSA-Cy3 (1:50) or TSA-Fluorescein (1:50) - 15 min incubation (use humidified chamber (PBS))
35. TNT wash 3x 5 min each - RT with agitation
36. H2O rinse
37. Coverslip once dry & use VectaShield Mounting media for flourescence with Dapi (Vector Laboratories, Cat# H-1200, 10ml)

Reagents

Volumes can be adjusted for the type of container used.

4% PFA/PBS

Proteinase K (10ug/ml) in PBS

De-Acetylation:

DEPC H2O	200ml
Triethanolamine	2.66ml
37% HCl	0.35ml
on a stirrer add:	
*Acetic anhydride	0.75ml

After 5 min add another acetic anhy.

*Acetic anhydride is only good for 5 minutes, add another 0.375ml after 5 minutes.

0.85% NaCl (in DEPC H2O)

70% EtOH/0.85% NaCl (in DEPC H2O)

95% EtOH (in DEPC H2O)

Hybridization buffer
Ambion hybridization buffer - use 1ml per probe
heat to 65ºC for 10 minutes, cool to RT
15µl 100mg/ml DTT
15µl 10mg/ml tRNA

5X SSC	preheat to 65ºC for 10 minutes before use.
37.25ml	DEPC H2O
12.5ml	20x SSC
250µl	10% Tween 20
50ml	Total volume

0.1x SSC preheat to 65ºC for 10 minutes before use.

49.5ml DEPC H2O
250µl 20x SSC
250µl 10% Tween 20
50ml Total volume

Formamide I preheat to 65ºC for 10 minutes before use.
14ml DEPC H2O
3.5ml 20X SSC
175µl 10% Tween 20
17.5ml formamide
35ml Total volume

Formamide II preheat to 65ºC for 10 minutes before use.
15.75ml DEPC H2O
1.75ml 20X SSC
175µl 10% Tween 20
17.5ml formamide
35ml Total volume

TNB preheat to 65ºC for 10 minutes before use.
5ml 10X TN
250µl 10% Tween 20
45ml DEPC H2O
0.25g blocking reagent*
50ml Total volume
*Add blocking reagent to solution then heat so that it goes into solution.

1x TN
250ml 10x TN
2250ml DEPC H2O
2500ml Total volume

1% H2O2 (x2)
Make 2 different solutions to accommodate the 2 steps.
500µl H2O2 Do not reuse the same 1% H2O2 for the second step.
50ml 1x TN
50.5ml Total volume

Anti-digoxigenin-POD 1:500 in TNB

Anti-DNP HRP 1:500 in TNB

1X TNT

125ml	10x TN
6.25ml	10% Tween 20
1118.75ml	DEPC H2O
1250ml	Total volume

Tyramide-Cy3 (1:50)
- followed instructions in kit for diluting, incubated for 15 minutes.

Tyramide-Fluorescein (1:50) followed instructions in kit for diluting, incubated for 15 minutes.

10x TN
For 1L add the following:
Tris 121.5g
NaCl 87.95g
Add ultra pure water up to a volume of 800ml and stir until solid components dissolve. Adjust pH to 7.5 and add more ultra pure water up to a final volume up to 1L.
Store at RT.

10% Tween 20 - dilute Tween 20 to 10% with DEPC H2O

** The label of the strongest probe should be added first. Example: the strongest probe is labeled with DIG, the Anti-Dig-POD (step 24) should be added first then TSA-Cy3 (step 26). The medium or weak probe labeled with DNP, the anti-DNP-HRP should be added second (step 32) followed by TSA-fluorescein (step 34). If the strongest probe was labeled with DNP then add the anti-DNP first followed by TSA-Fluorescein then add the anti-dig for step 32 and TSA-Cy3 for step 34. Optimized by the Gaido lab.

Materials list

Chemical	Company	Catalog #
DEPC	Sigma	D5758
Triethanolamine	Sigma	90279
37% HCl	Sigma	258148
Acetic Anhydride	Sigma	A6404
PF	EMS (Electron Microscopy Sciences)	19208
PK	Roche	3115836001
5M NaCl	Ambion (ABI)	AM9759
PBS	Sigma	P3813-10Pak
Ethanol	Sigma	E7023
Hybridization buffer	Ambion (ABI)	B8807G
DTT	Sigma	43815
yeast tRNA	Ambion (ABI)	AM7119
20x SSC	Ambion (ABI)	AM9763
Tween 20	Sigma	P1379
Formamide	ISC Bioexpress	606
Blocking reagent	Perkin Elmer	FB1012
anti-DIG POD	Roche	11207733910
anti-DNP HRP*	Perkin Elmer	NEL747A001KT
TSA-Cy3	Perkin Elmer	NEL744B001KT
TSA-fluorescein	Perkin Elmer	NEL741001KT

Tris	Sigma	T1503
NaCl	Sigma	S3014
Superfrost Plus Slides	Fisher	12-550-15
H2O2	Sigma	216763
DMSO	Sigma	D8418
Filter Paper (Whatman)	Sigma	Z240109
Parafilm	Sigma	P7543
Coverslips	Corning	12531C (2935-224)
Dapi mounting media	Vector Labs, Inc	H-1200

*TSA plus DNP (HRP) System kit (only need the anti-DNP HRP conjugate from kit but have to buy whole kit.)