Immunocytochemistry Followed by FISH (Version 1)

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***We present multiple versions of the Immunocytochemistry / FISH protocols because the conditions under which each antibody and DNA probe work can be different and must be determined empirically.**

Reagents

Antifade (1,4-phenylene-diamine)
Sigma-Aldrich, Cat. 78460
Bovine Serum Albumin (BSA)
Roche Diagnostics, Cat. 100350
Cot-1 DNA (Human)
Life Technologies, Cat. 15279-011
Cot-I DNA (Mouse)
Life Technologies, Cat. 18440-016
DAPI
Sigma-Aldrich, Cat. 18860
Dextran sulfate (50%)
Millipore Cat. S 4031
Dimethyl sulfoxide (DMSO)
Sigma-Aldrich, Cat. D2650
EGTA
Sigma-Aldrich, Cat. E3889
Ethylene glycol bis(succinimidyl succinate)
Sigma-Aldrich, Cat. E3257
EM glutaraldehyde, 25% EM grade
Polysciences, Inc., Cat. 01909
Formamide
Sigma-Aldrich, Cat. 47670
Formamide, deionized
Ambion, Cat. 9342
Goat anti-mouse-FITC (FISH 2˚ Ab)
Sigma-Aldrich, Cat. F0257
Goat anti-rabbit-TRITC (ICC 2˚ Ab)
Sigma-Aldrich, Cat. T-5268
Normal Goat Serum
Sigma-Aldrich, Cat. G6767
HCl, 1M
Magnesium chloride ($MgCl_2$) 2M
Quality Biological, Inc., Cat. 340-034-721EA

Mouse anti-biotin-FITC (FISH 1˚ Ab)
Sigma-Aldrich, Cat. F4024
1X Phosphate Buffered Saline, pH 7.4
Life Technolgies, Cat. 10010-023
Potassium chloride (KCl)
Macron Chemicals, Cat. 1-28050
Potassium phosphate, monobasic (KH_2PO_4)
Sigma-Aldrich, Cat. P5379
Rabbit polyclonal antibodies (ICC 1˚ Ab)
Specific for desired protein
RNaseA
Roche Diagnostics, Cat. 10109169001
20X SSC
Salmon testes DNA
Sigma-Aldrich, Cat. D-7656
Sodium borohydride ($NaBH_4$)
Sigma-Aldrich, Cat. 452882
Sodium chloride (NaCl)
Fischer Scientific, Cat. S271-500
Sodium hydroxide (NaOH)
Triton X-100
Calbiochem, Cat. 648462
Tween 20
Sigma-Aldrich, Cat. P1379

Preparation

Fixation Permeabilization Buffer

KH_2PO_4	54 mg	f.c. [20mM]
NaCl	152 mg	f.c. [130mM]
KCl	30 mg	f.c. [20mM]
0.5M EGTA	400 µl	f.c. [10mM]
2M $MgCl_2$	100 µl	f.c. [10mM]
10% Triton X-100	200 µl	f.c. [0.1%]
25% EM glutaraldehyde	120 µl	f.c. [0.15%]

*Bring to 20 ml with sterile distilled water

0.1% Sodium Borohydride solution
Prepared fresh 1mg/ml in 1X PBS

Blocking Solution I (5% NGS/ 5% BSA/1X PBS)

NGS	500 µl
BSA	0.5 g
1X PBS	10 ml

*Store at 4˚C

Antibody Solution I (1% NGS/1% BSA/1X PBS)

Blocking Solution I	200 µl
1X PBS	800 µl

Ethylene glycol bis(succinimidyl succinate) (EGS) Solution

Weigh volume of EGS powder [i.e., 100 µl powder] in eppendorf tube
Add equal volume of DMSO [i.e., 100 µl DMSO]
Incubate at 37°C until dissolved and re-determine volume
Calculate concentration based on weight of EGS used, molecular weight of EGS, and final volume of solution (should be ~ 500-650 mM)
Store at RT <1 month
Dilute stock into 1X PBS immediately prior to use for final conc. 50 mM, discard unused portion

RNase A (DNase-free)

20mg/ml in sterile water
Boil 15', cool to RT, aliquot and store at -20˚C

Master Mix

Dextran sulfate, 50%	40 ml	f.c. 20%
20X SSC, pH 7.0	20 ml	f.c. 4X SSC
Sterile dH_2O	40 ml	
Total	100 ml	

Vortex solution and place tube on a shaking platform overnight to insure proper mixing.
*Aliquot, and store at -20˚C.

50% FA/SSC

20X SSC	20 ml
dH_2O	80 ml
Formamide	100 ml
Total	200 ml

Adjust pH to 7.25 with 1M HCl
*Pre-warm to 45˚C

0.1X SSC

20X SSC	2.5 ml
dH_2O	498 ml

*Pre-warm to 60˚C

4X SSC/Tween 20

20X SSC	200 ml
dH_2O	799 ml
Tween 20	1 ml
Total	1000 ml

*Pre-warm to 45˚C

Blocking Solution II (3% BSA/4X SSC/Tween 20)

BSA	0.3 g
4X SSC/Tween 20	10 ml

*Store at 4˚C

Antibody Solution II (1% BSA/4X SSC/Tween 20)

Blocking Solution II	333 µl
4X SSC/Tween 20	666 µl
Total	1000 µl

DAPI (stock solution)

DAPI	2 mg	f.c. [0.2 mg/ml]
dH_2O	10 ml	

*Aliquot and store at -80˚C

DAPI (staining solution)

DAPI stock solution	40 µl	f.c. [80 mg/ml]
2X SSC	100 ml	

Antifade (1,4-phenylene-diamine)

See Antifade preparation procedure in CGH Protocols

Procedure

1. Grow adherent cells on coverslips or cytospin suspension cells onto poly-L-lysine coated coverslips.

2. Fix cells in Fixation Permeabilization Buffer for 30 min at RT.

3. Wash 3 x 5 min 1X PBS at RT.
4. Wash 2 x 15 min fresh 0.1% sodium borohydride solution.

5. Block coverslips with 25µl blocking solution I in hybridization chamber 30

min at 37˚C.

6. Incubate with rabbit polyclonal (ICC 1˚ Ab) in 25 µl antibody solution I in hybridization chamber at 37˚C for 60 min.

7. Wash 3 x 5 min with 1X PBS at RT.

8. Incubate with ICC 2˚ Ab [goat anti-rabbit-TRITC; 1:200 in 25 µl antibody solution I] in hybridization chamber at 37˚C for 60 min.

9. Wash 3 x 5 min with 1X PBS at RT.

10. Incubate with 25 µl EGS solution [dilute stock to 50 mM in 1X PBS prior to use and mix well (will be turbid)] in hybridization chamber at 37°C for 30 min to allow postfixation cross-linking of the Ab to the target protein.

11. Wash 3 x 5 min with 1X PBS at RT.

12. Incubate with RNaseA (1:200 in 1X PBS) in hybridization chamber 60 min at 37˚C.

13. Wash 3 x 5 min 1X PBS.

14. Denature chromosomal DNA by inverting coverslips (18 mm x 18 mm) onto 25 µl drop of NaOH (pH 13.0 - ~0.1M) for exactly 2 min.

15. Rinse immediately in cold 1X PBS

16. Hybridize denatured/pre-annealed biotin-labeled probe to coverslip (as per standard FISH Protocol, probe is denatured at 80°C, 5 min, in 50% deionized Formamide/50% Master Mix and pre-annealed if necessary at 37˚C in the presence of Cot-I DNA for 60-90 min).

17. Seal with rubber cement and incubate in hybridization chamber at 37˚C overnight.

18. Remove rubber cement.

19. Wash coverslips 3 x 5 min in FA/SSC (pre-warmed to 45˚C), shaking.

20. Wash coverslips 3 x 5 min in 0.1X SSC (pre-warmed to 60˚C), shaking.

21. Dip slides in 4X SSC/Tween 20 (pre-warmed to 45˚C); do not let dry.

22. Block with 25 µl blocking solution II in hybridization chamber 30 min at 37˚C.

23. Dip slides in 4X SSC/Tween20; do not let dry.

Note: Centrifuge all fluorescent-conjugated Ab for 3 min at 14,000 rpm.

24. Incubate with FISH 1˚ Ab [mouse anti-biotin-FITC, 1:200 in 25 µl antibody solution II] in hybridization chamber 45 min at 37˚C.

25. Wash coverslips 3 x 5 min in 4X SSC/Tween20 (pre-warmed to 45˚C), shaking.

26. Incubate with FISH 2˚ Ab [goat anti-mouse-FITC, 1:200 in 25 µl antibody solution II] in hybridization chamber 45 min at 37˚C.

27. Wash coverslips 3 x 5 min in 4X SSC/Tween20 (pre-warmed to 45˚C), shaking.

28. Stain for 2 min with DAPI.

29. Wash in 1X PBS for 10 min, shaking.

30. Mount coverslip with antifade on microscope slide.