Immunocytochemistry Followed by FISH (Version 1)

Section of Cancer Genomics, Genetics Branch, NCI
National Institutes of Health

*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-1 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 (MgCl₂) 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 Technologies, Cat. 10010-023

Potassium chloride (KCl)
Macron Chemicals, Cat. 1-28050

Potassium phosphate, monobasic (KH$_2$PO$_4$)
Sigma-Aldrich, Cat. P5379

Rabbit polyclonal antibodies (ICC 1\° Ab)
Specific for desired protein

RNase A
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**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>54 mg</td>
<td>f.c. [20mM]</td>
</tr>
<tr>
<td>NaCl</td>
<td>152 mg</td>
<td>f.c. [130mM]</td>
</tr>
<tr>
<td>KCl</td>
<td>30 mg</td>
<td>f.c. [20mM]</td>
</tr>
<tr>
<td>0.5M EGTA</td>
<td>400 µl</td>
<td>f.c. [10mM]</td>
</tr>
<tr>
<td>2M MgCl$_2$</td>
<td>100 µl</td>
<td>f.c. [10mM]</td>
</tr>
<tr>
<td>10% Triton X-100</td>
<td>200 µl</td>
<td>f.c. [0.1%]</td>
</tr>
<tr>
<td>25% EM glutaraldehyde</td>
<td>120 µl</td>
<td>f.c. [0.15%]</td>
</tr>
</tbody>
</table>

*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 dH₂O  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
dH₂O  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
dH₂O 498 ml
*Pre-warm to 60˚C

4X SSC/Tween 20
20X SSC  200 ml
dH₂O 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]
dH₂O 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.