In situ hybridization on vibratome sections using DIG-labeled RNA probes, anti-DIG-AP and NBT/BCIP

by Tatsuya Okafuji, Kevin Mitchell Lab, Smurfit Institute of Genetics, Trinity College Dublin

Updated on 20/10/2009

Preliminary Notes for *in situ* hybridization

- Handle sections in RNase-free conditions until hybridization is completed.
  - Wear gloves during operation. Change them when they become dirty.
  - Use DEPC-treated solution or reagents dissolved in DEPC-treated or nuclease-free water.
  - Use new clean plasticware (pipettes, etc.). Keep them clean during operation; put them back in the case after operation, for example.
  - Clean forceps, razor blades, brushes, etc. using RNase AWAY or equivalents to make and keep them RNase-free.

Day 0. Preparation of vibratome sections

1. Dissect out brains in DEPC-PBS and fix in 4% paraformaldehyde (PFA)/DEPC-PBS at 4°C overnight. For brains at postnatal stages, fix by perfusion before dissection.
2. Wash briefly with PBS, trim (if necessary) and embed in 2.5% Select Agar (Sigma) or 4% low melting-point agarose/PBS in a 6- or 12-well plate.
3. Cut sections on a vibratome. Collect them in 6-well plates filled with DEPC-treated PBS until all sections are prepared.
   - Thickness of the sections should be changed according to ages of samples.
   - Up to 6 sections/well would be appropriate.

Proceed to the next steps. For long-term storage, replace PBS with cryoprotection solution and store at -20°C until use.

Day 1.1 Pre-hybridization treatments

- **Use 3 ml solution per well for each treatment.**
- **For efficient solution exchange, it is recommended that you transfer the section into another well filled with new solution, rather than changing the solution in the same well.**
- **Use paintbrushes cleaned with RNase AWAY to transfer the sections into another well.**

1. Dehydrate in a 50% (in PBT)-100% methanol series. Allow sections to rock gently at room temperature for 5 min in each solution. The sections can be stored at this point at –20°C.

- Rock the plates on an orbital shaker, not on a Nutator shaker during pre-hybridization treatments. Sections may stick to the wall of the wells if they are rocked on a Nutator shaker.

2. Rehydrate in a 75%-50%-25% methanol/PBT series for 5 min each. Make sure that the sections fit in the solution, especially in 75% and 50% methanol/PBT.

4. Wash twice with PBT for 5 min each.

5. Permeabilize in RIPA buffer three times for 30 min each.

- During the first RIPA buffer treatment, start warming equilibration buffer and prehybridization solution at 65°C.
- Prepare postfix solution (see below) during the third RIPA buffer treatment.
- Prepare a tight-sealed box humidified with 50% formamide in water and incubate at 65°C during these treatments.

6. Postfix with 4% PFA-0.2% glutaraldehyde/PBT for 20 min.

7. Wash with PBT two times for 5 min each.

8. Wash with PBT/equilibration buffer (1:1) for 10 min at room temperature (RT).

9. Wash with equilibration buffer for 10 min at RT.

Day 1.2 Prehybridization and Hybridization

1. Incubate the sections in prehybridization solution (2 ml/well) for ≥1 h at 65°C in a tight-sealed box humidified with 50% formamide in water (see above). Add 1.8 ml of prehybridization in another wells for probe solution.

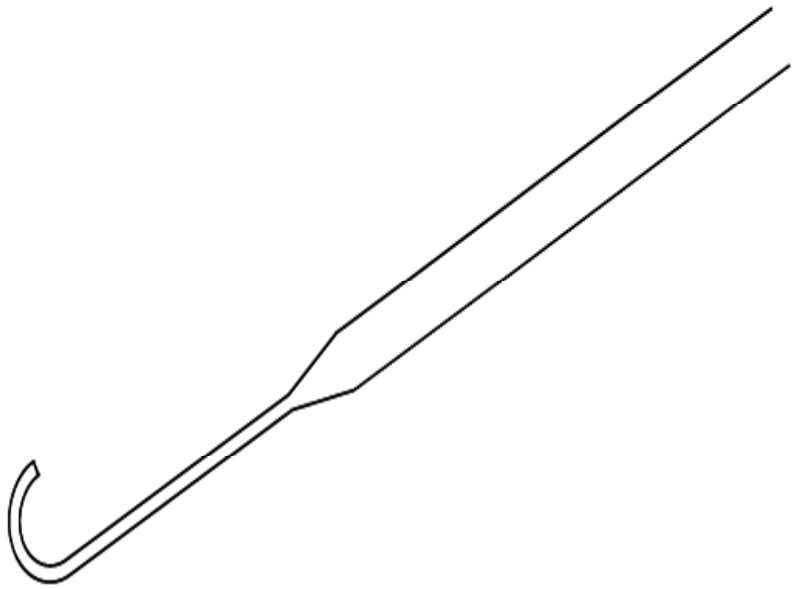
2. Prepare probe solution.

- Add probe stock solution to 0.2 ml of prehybridization solution in 2-ml centrifuge tubes.
- Heat the tubes for 5 min at 80°C.
- Transfer the probe solution into pre-warmed prehybridization solution (see above). Mix the solution by gentle agitation.

3. Transfer the sections into the probe solution. Use a sterilized glass hook, which is made by sealing and bending the tip of a Pasteur pipette (see the figure below).

4. Incubate overnight at 65°C in a tight-sealed container humidified with 50% formamide in water.

- You can start warming post-hybridization wash solution after this operation.



Day 2. Post-hybridization washes, blocking and antibody incubation

1. Prewarm wash solution I and solution III at 65°C.
2. Transfer the sections in prewarmed solution I (2 ml/well) with a sterilized glass hook (see above). Sterilize the hook when transferring the sections hybridized with different probes to avoid cross-contamination of probes. Wash the sections with solution I three times for 30 min each at 65°C.
   - Collect probe solution into microcentrifuge tubes. Probe solution can be reused several times.
   - The following procedures do not have to be done in RNase-free conditions.
   - To wash the sections efficiently and to avoid loss of the sections, it is preferred that you transfer the sections to a well filled with new wash solution, instead of changing the solution in the same well.
3. Wash with solution III (2 ml/well) three times for 30 min each at 65°C.
4. Cool down the sections to RT on an orbital shaker. Add an equal volume of TBS containing 1% Tween 20 (TBST).
   - You can use a Nutator shaker from this wash.
5. Wash with TBST three times for 5 min each at RT.
6. Incubate in blocking solution (10% heat-inactivated sheep serum in TBST; 2 ml/well) ≥1 h at RT.
7. Incubate in antibody solution [1: 2000 diluted anti-DIG AP Fab fragment (Roche) in blocking solution; 2 ml/well]. Incubate overnight at 4°C with gently rocking on an orbital shaker in the cold room.

Day 3. Post antibody treatment washes and Color development

1. Wash with TBST (3 ml/well) three times for 5 min each time at RT.
2. Wash with TBST (3 ml/well) three times for 2 h each.
3. (Optional) Wash with TBST (3 ml/well) overnight at 4°C with gently rocking.
4. Wash with NTMT (3 ml/well) three times for 10min each time.
5. Prepare NBT/BCIP solution during the final NTMT. Filter the solution to remove small particles, which may cause spot-like background on the sections. Add the filtered NBT/BCIP solution into the clean wells of a 6-well plate (2 ml/well).
6. Transfer the sections into NBT/BCIP solution and incubate the plate in a humidified box in the dark. If you want to obtain the results quickly (normally within 2h), add 20 μl of NBT/BCIP stock solution (Roche)/ml NTMT. If you want to develop the color slowly (recommended; overnight-4 days), add 2-5 μl of the stock solution/ml NTMT.
7. Throughout the development reaction, check the sections periodically to monitor the progress of color development. You may want to change NBT/BCIP solution if the color of the solution is turned into violet/purple before staining is fully developed.
8. Wash with PBS to stop reaction. Postfix with 4% PFA/PBS or buffered 3.7% formaldehyde/PBS (pH 7.4).
9. Store at 4°C in a humidified box until the sections are mounted on glass slides.

Day 4. Mounting sections

1. Wash sections in PBS, rinse briefly in deionized water to remove the salt on the sections and mount on SuperFrost glass slides. Completely dry the sections (at least several hours; overnight or longer is fine).
2. Wash the glass slides briefly with PBS. Rinse briefly in deionized water to remove the salt and coverslip with aqueous mount media such as Aqua/Poly Mount.

Reagents

4% paraformaldehyde/PBS (200 ml)

Add 8 g of paraformaldehyde into ~150 ml of DEPC-H_2O.

Heat to 65°C.

Add 50-100 μl of 5M NaOH.

When the solution becomes clear, add 20 ml of 10x PBS.

Add DEPC-H_2O to 200 ml.

Make aliquots and store at -20°C

10x PBS (1000 ml)

NaCl	80 g
KCl	2 g
Na_2PO_4	14.4 g
KH_2PO_4	2.4 g
H_2O	to 1000 ml

Cryoprotection Solution (200 ml)

0.1M Phosphate buffered saline, pH 7.2		100 ml	(50%)
For 500 ml of 0.1 M phosphate buffer;			
$NaH_2PO_4 \cdot H_2O$	1.59 g		
Na_2HPO_4	5.47 g		
NaCl	9.0 g		
DEPC-treated H_2O	to 500 ml		
Sucrose		60 g	(30% w/v)
Polyvinylpyrrolidone (PVP-40)		2 g	(1% w/v)
Ethylene glycol		60 ml	(30% v/v)
DEPC-treated H_2O		to 200 ml	

RIPA buffer (200 ml)

5 M NaCl	6 ml	(150 mM)
NP40	2 ml	(1%)
Sodium deoxycholate	1 g	(0.5%)
10% SDS	2 ml	(0.1%)
0.2 M EDTA, pH 8.0	1 ml	(1 mM)
1M Tris-HCl, pH 8.0	10 ml	(50 mM)
DEPC-H_2O	to 200 ml	

Re-fixation solution (10 ml)

4% paraformaldehyde/PBS	10 ml	(4%)
10% Tween 20	0.1 ml	(0.1%)
25% glutaraldehyde	80 µl	(0.2%)

Equilibration solution (50 ml)

Formamide	25 ml	(50%)
20x SSC, pH 4.5	12.5 ml	(5x)
10% Tween 20	0.5 ml	(0.1%)
DEPC-H_2O	12 ml	

Prehybridization solution (50 ml)

Formamide	25 ml	(50%)
20x SSC, pH 4.5	12.5 ml	(5x)
10% SDS	5 ml	(1%)
10% CHAPS	0.5 ml	(0.1%)
50 mg/ml yeast RNA (Roche)	0.25 ml	(250 µg/ml)
50 mg/ml heparin	50 µl	(50 µg/ml)
DEPC-treated H_2O	6.7 ml	

Solution I for post-hybridization wash (50 ml)

Formamide	25 ml	(50%)
20x SSC, pH4.5	12.5 ml	(5x)
10% SDS	5 ml	(1%)
H_2O	7.5 ml	

Solution III for post-hybridization wash (50 ml)

Formamide	25 ml	(50%)
20x SSC, pH4.5	5 ml	(2x)
10% Tween 20	0.5 ml	(0.1%)
H_2O	19.5 ml	

10x TBS (1000 ml)

NaCl	80 g	(8%)
KCl	2 g	(0.2%)
1 M Tris-HCl, pH 7.5	250 ml	(250 mM)
H_2O	to 1000 ml	

NTMT (50 ml)

1 M Tris-HCl, pH9.5	5 ml	(0.1 M)
5 M NaCl	1 ml	(0.1 M)
1 M $MgCl_2$	2.5 ml	(50 mM)
10% Tween-20	5 ml	(1%)
H_2O	36.5 ml	

References

Core protocol for *in situ* hybridization

1. RNA whole-mount *in situ* hybridization: Core protocol for both mouse and chick embryos Cepko/Tabin lab http://genetics.med.harvard.edu/~cepko/protocol/ctlab/ish.ct.htm

RIPA buffer for permeabilization

2. **Rosen, B. and Beddington, R. S. P.** (1993), Whole-mount *in situ* hybridization in the mouse embryo: gene expression in three dimensions. *Trends Genet*. **9**, 162-167.

3. **Grove, E. A., Tole, S., Limon, J., Yip, L-w., and Ragsdale, C. W.** (1998), The hem of the embryonic cerebral cortex is defined by the expression of multiple *Wnt* genes and is compromised in *Gli3*-deficient mice. *Development* **125**, 2315-2325.

4. **Tole, S., Christian, C., and Grove, E. A.** (1997), Early specification and autonomous development of cortical fields in the mouse hippocampus. *Development* **124**, 4959-4970.

Cryoprotection solution

5. **Hoffman, G. E., and Le, W. W.** (2004), Just cool it! Cryoprotectant anti-freeze in immunocytochemistry and in situ hybridization. *Peptides* **25**, 425-431.

6. **Lu, W., and Haber, S. N.** (1992), In situ hybridization histochemistry: a new method for processing material stored for several years. *Brain Res*. **578**, 155-160.

7. **Watson Jr., R. E., Wiegand, S. J., Clough R. W., and Hoffman, G. E.** (1986), Use of Cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. *Peptides* **7**, 155-159.