In Situ hybridization
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Preparation of ^{35}S-labeled riboprobes:

Materials and solutions:
1. RNA labeling kit (Amersham, RPN3100)
2. ProbeQuant^{TM}G-50 Micro Columns (Pharmacia, 27-5335-01)
3. ^{35}S-UTP (1000 Ci/mmol, 20 mCi/ml, Amersham, SJ603)
4. 5 M ammonium acetate (NH_4Ac):
5. STE: add 30 µl of NaCl to 970 µl TE, pH 8.0
6. 2.5 M DTT:
7. Formamide (Sigma)

Transcription of riboprobes:

1 Plasmids containing the subclone of interesting fragment are linearized by proper restriction enzyme as below:

Riboprobes		Restriction Enzyme	RNA polymerase
5-HT$_{1A}$ III intracellular loop (mouse)	Sense	Sac I	T7
695-1110 bp	Antisense	Kpn I	T3
5-HT$_{1A}$ 3' uncoding region (mouse)	Sense	Sac I	T7
1481-1860 bp	Antisense	Kpn I	T3
5-HT$_{2C}$ III intracellular loop (rat)	Sense	Kpn I	T7
1355-1630 bp	Antisense	Hinc II	T3
5-HT$_{2C}$ 3' uncoding region (mouse)	Sense	Bam H I	T3
2078-2512bp	Antisense	Hind III	T7
5-HT$_{2A}$ (rat)	Sense	Apa I	SP6
1281-1573 bp	Antisense	Pst I	T7
GPCR 21 (mouse)	Sense	Not I	T3
	Antisense	BamH I	T7

For example:

30 ml plasmid (~ 100 ng/µl) + 4 µl buffer (10x) + 6 µl restriction enzyme

5-HT$_{2C}$ III intracellular loop	NEB 1 + 0.4ml BSA (100x)	Kpn I	Sense
	React 4 (BRL)	Hinc II	Antisense
5-HT$_{2C}$ 3' uncoding region	Univ (Stratagene)	Bam H I	Sense
	Buffer 3 (Stratagene)	Hind III	Antisense
5-HT$_{2A}$ (rat)	React 4	Apa I	Sense
	React 2	Pst I	Antisense
5-HT$_{1A}$ III intracellular loop	Buffer 1(Stratag.)or A (BMG)	Sac I	Sense
	Buffer 1 (Stratagene)	Kpn I	Antisense
5-HT$_{1A}$ 3' uncoding region	Buffer 1(Stratag.)or A (BMG)	Sac I	Sense

	Buffer 1 (Stratagene)	Kpn I	Antisense
30 ml plasmid (~ 100 ng/µl) + 4 µl buffer (10x) + 6 µl restriction enzyme			
GPCR 21	React 3 (BRL)	Not I	Sense
	Univ (Stratagene)	Bam H I	Antisense

Incubate at 37°C for 2 hours or overnight.

2 Purify the linearized plasmid by separating with 1% agarose gel followed by gel extraction by QIAquick gel extraction kit (Qiagene) (Concert gel extraction kit from GIBCO RBL is better than Qiagene one).

3 Dry the extracted plasmid by using speed vacuum.

4 In vitro transcription: use RNA labeling kit (Amersham). Add following solutions in the tube that contains linearized plasmid (from step 3):

Transcription buffer	4 µl
0.2 M DTT	1 µl
HPRI	1 µl
GTP	0.5 µl
ATP	0.5 µl
CTP	0.5 µl
^{35}S-UTP	10 µl
RNA polymerase	2 µl
H_2O	0.5 µl
Total	20 µl

Mix and incubate at 37°C for at least 1 hour.

5 To remove template DNA, add 5 µl RNA-free DNase (10 Units). Mix and incubate at 37°C for 15 min.

6 Stop the reaction by add 25 µl STE and place the tube in ice.

7 Separate labeled riboprobe from free ^{35}S-UTP by using ProbQuant G-50 micro column.

a) Prepare column: Resuspend the resin in the column by vortexing. Loosen the cap one-fourth turn and snap off the bottom closure. Then, put the column in a 1.5 ml tube and spin for 1 min at 3000 rpm (735 x g).

b) Place the column in a new 1.5 tube and apply the 50 µl of the sample to the top-center of resin carefully. Then, spin the column at 3000 rpm for 2 min. The purified sample is collected in the support tube.

8 Precipitate the ^{35}S-labeled riboprobe:

a) Add 5 µl of 5 M (NH_4)Ac (1/10 vol of the sample) and 150 µl of 100 % ethanol (3 vol. of the sample) to the purified sample.

b) Place the tubes in –20°C for at least 2 hr or overnight.
c) Centrifuge the tube at 14,000 rpm, 4°C for 30 min. and decant the supernatant by pipette. Leave the tube up side down until all the solution is dried.

9 Add 50 µl DEPC-treated H_2O to the tube and dissolve the riboprobe. Or, 23 µl H_2O + 2□µl 2.5M DTT + 25 µl formamide.

10 Take 1 µl of the riboprobe to a scintillation vial, add 5 ml scintillation fluid and count at ^{14}C channel in scintillation counter. The counts should be 2,000,000-6,000,000 cpm. The riboprobe can store in –80°C for weeks.

Hybridization

Materials and solutions:

1 4% paraformaldehyde: add 24 g paraformaldehyde in 600 ml 1X PBS, heat to 60°C and add a pellet of NaOH until the solution become clear. Filter the solution and cool to room temperature. The solution has to make freshly.

2 0.25% acetic anhydride in 1 M triethanolamine (TEA): Add 9 ml TEA and 2.52 ml conc. HCl to 600 ml DEPC-treated H_2O. Immediately before use add 1.5ml acetic anhydride. (You can add acetic anhydride after the slides are placed in the 1 M TEA).

3 70% ethanol: mix 420 ml 100% Ethanol and 180 ml H_2O.

4 80% ethanol: mix 480 ml 100% ethanol and 120 ml H_2O.

5 Hybridization solution (150 µl/slide):

a) Hybridization buffer: To make 8.8 ml buffer, mix:

Stock solution	Vol	Final conc.
1M Tris pH 7.4	200 µl	20 mM
formamide	5 ml	50%
5 M NaCl	600 µl	0.3 M
0.5 M EDTA	20 µl	1 mM
50 X Denhard's sol	200 µl	1X
50% dextrane sulfate	2 ml	10%
DEPC-treated H_2O	780 µl	

b) Ribomix: Mix:

Salmon Sperm (10 mg/ml)	250 µl
tRNA (50 mg/ml)	250 µl
DEPC-treated H_2O	500 µl

c) Denatured riboprobe solution: To make 1ml hybridization solution, 40 µl ribomix are added proper vol of riboprobe to make 20,000 - 40,000 cpm/□µl of hybridization solution. (For example, if the riboprobe is 4,000,000 cpm/□µl, add 10 µl of the riboprobe into 40 µl ribomix). Heat the mixture at 65°C for 10 min and cool in ice for at least 2 min.

d) Hybridization solution: To make 1 ml solution, add

		Final conc.
Hybridization buffer	880 µl	
2.5 M DTT	60 µl	150 mM
10% SDS	20 µl	0.2%
Denatured riboprobe sol.	40 µl	

6 4X SSC: add 200 ml 20X SSC into 1000 ml H_2O.

7 1X SSC: 30 ml 20X SSC to 600ml

8 0.5 X SSC: 15 ml 20X SSC to 600 ml.

9 0.1X SSC: 5 ml 20X SSC to 1000ml.

10 2 mM DTT in 0.1X SSC: 250 µl 5M DTT into 600 ml 0.1X SSC (65°C) immediately before use.

11 300 mM NH_4Ac, ethanol solutions:

Ethanol Conc	100% Ethanol	6 M NH_4Ac	H_2O
50%	300 ml	30 ml	270 ml
70%	420 ml	30 ml	150 ml
90%	540 ml	30 ml	30 ml
95%	570 ml	30 ml	

12 40 µg/ml RNase A solution:
   a) NTE solution: add 50 ml 5M NaCl, 5 ml 1M Tris, pH 8.0 and 1 ml 0.5 M EDTA, pH 8.0 onto 500ml H_2O.
   b) RNase A solution: add 1ml 20mg/ml RNase A into 500 ml NTE.

13 5M DTT:
   a) 0.01 M NaAc solution: dissolve 68 mg NaAc (M.W 136.08) into 50 ml H_2O.
   b) Dissolve 0.77g DTT into 1 ml 0.01M NaAc.

14 6 M NH_4acetate: dissolve 231g ammonium acetate onto 500 ml H_2O.

15 50% formamide in 2X SSC solution:
   500 ml formamide + 100 ml 20X SSC + 400 ml H_2O

16 Chloroform

Procedure:

On the day of the assay, the slides contained brain sections are taken out of –80ºC freezer and take out plastic wrap. The slide boxes then are placed in a dessicator until they are reached to room temperature. Arrange the slides in metal slide racks (50 slides or 25 slides) and treat them as below:

1 Fix the sections in 4% paraformaldehyde for 10 min. (the time is important).
2 In 1 X PBS for 1 min.
3 In 1 X PBS for 1 min.
4 0.25% acetic anhydrite in 0.1M TEA solution for 10 min with stirrer. (put the slide rack in 0.1M TEA first, then add acetic anhydrite and mix well).
5 70% Ethanol for 1 min.
6 80% ethanol for 1 min.
7 95% ethanol for 2 min.
8 100% ethanol for 1 min.
9 Chloroform for 5 min.
10 100% ethanol for 1 min.
11 95% ethanol for 1 min.
Air-dry the section for 30-60 min before hybridization.

Hybridization:

1 Pipette 150 µl of hybridization solution on each slide and then carefully place a coverslip on the brain sections. Make sure there is no air-bubble and the hybridization solution evenly spread on all the brain sections.

2 Prepare humidified-chambers: In a plastic box, put two layers of filter paper and wet them with 50% formamide in 2X SSC. Place several pipettes on the filter paper to support slides.

3 Place slides in the humidified-chambers with coverslip facing up. Cover the chamber and incubate the slides in 54ºC for 16-20 hr. To keep humidity, put boxes with 50% formamide in 2X SSC in the top and bottom of the incubator.

Wash:
1. Wash off coverslips by holding slide vertically and up and down several times in 4X SSC solution. Then, place the slides back to metal racks.
2. Wash the slides in 4X SSC at RT for 5 min with shaking.
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4. Wash the slides in 4X SSC at RT for 5 min with shaking.

5. Incubate the slides in RNase A solution for 30 min at RT.

6. Wash slides in 1X SSC at RT for 5 min. with shaking.
7. Wash slides in 0.5X SSC at RT for 5 min. with shaking.
8. Wash slides in 0.1X SSC at RT for 5 min. with shaking.

9. Incubate slides in 2 mM DTT in 0.1X SSC at 65ºC for 15 min. Add DTT right before the incubation and same for the step 10-12.
10. Incubate slides in 2 mM DTT in 0.1X SSC at 65ºC for 15 min.
11. Incubate slides in 2 mM DTT in 0.1X SSC at 65ºC for 15 min.
12. Incubate slides in 2 mM DTT in 0.1X SSC at 65ºC for 15 min.

13. Wash slides in 2 mM DTT in 0.1X SSC at RT for 1 min.

14. 300 mM NH_4Ac, 50% ethanol solutions 1 min.
15. 300 mM NH_4Ac, 70% ethanol solutions for 1 min.
16. 300 mM NH_4Ac, 90% ethanol solutions for 1 min.
17. 300 mM NH_4Ac, 95% ethanol solutions for 1 min.
18. Rinse with 100% ethanol.

Air-dry the slides and expose to Kodak BIOMAX MR film (Amersham) with a ^{14}C-standard strip for 7-14 days. Develop the film manually. Analysis the image by using NIH Image program.