IN SITU HYBRIDIZATION DATA PRODUCTION

Overview

The processes and protocols used by the Marmoset Gene Atlas (the Atlas) were designed based upon the methods developed by Dr. Cliff Ragsdale's laboratory at the University of Chicago (https://ragsdalelab.uchicago.edu/), (Grove EA. et al., 1998: Agarwala A. et al., 2001: Sanders TA. et al., 2002). The data production laboratory (the Shimogori Lab) is located at RIKEN Brain Science Institute in Wako, Saitama, Japan. ISH is performed by some modification to a previously reported method (Mashiko H. et al., 2012).

Methods

1. Sample collection

Early postnatal marmosets are anesthetized with a lethal dose of pentobarbitone (100 mg/kg), and after three failed attempts to elicit a foot withdrawal reflex the animals are transcardially perfused with 4% paraformaldehyde in PBS. Brains are removed and fixed in 30% sucrose/4% PFA in PBS for several days.

2. Sectioning

Brains are sectioned in the coronal plane on a freezing microtome at 28 µm. One hemisphere of the brain is marked with DiI solution (dissolved in 4M Sucrose/PBS) and each section is individually mounted on slides and processed for ISH.

3. Preparation of DNA template for cRNA synthesis

Primers for polymerase chain reaction (PCR) targeting common marmoset genes are designed with (primer3, ver. 0.4.0) software based on the common marmoset genome sequence. Total RNA is isolated from frozen embryonic and postnatal marmoset brains and collected in Trizol reagent (Invitrogen, Carlsbad, CA). cDNA is generated from total RNA using prime script 1st strand cDNA synthesis kit (Takara, Shiga, Japan) according to the manufacturer's recommended procedure.

4. Preparation of linearized templates:

Prepare plasmid DNA using Qiagen miniprep kit or equivalent. Following plasmid preparation, all steps should be performed using RNase-free solutions. Digest 5-10 µg of template DNA to completion using restriction enzyme of choice. Confirm completeness of digestion using gel electrophoresis. Following digestion, add 5 µL protease K (19 mg/mL stock) and 5 µL of 10% SDS and adjust total volume to 110 µL with ddH_2O and incubate at 37ºC for 15 minutes. Extract with 110 µL phenol/chloroform/isoamyl alcohol (25:24:1) once and 110

µL of chloroform/isoamyl alcohol (24:1) twice. Precipitate DNA by adding 200µL of 100% EtOH and 4µL of 5M NaCl. Store at -20ºC at least 30 minutes. Centrifuge for 15 minutes at 4ºC at maximum speed to collect pellet, then wash once with 200 µL 70% EtOH. Air-dry the pellet and resuspend to 0.5 ~ 1.0 µg/µL in TE.

5. Generation of cRNA probe:

- Following purification of template DNA, synthesize probe by mixing the following components in the order indicated. Use RNase-free aerosol tips and RNase-free solutions for all procedures: 2 µL 10x RNA polymerase buffer, 2 µL 10x DIG NTP mix, 11 µL ddH_2O, 1 µL 0.1M DTT, 1 µL RNase inhibitor, 1 µL RNA polymerase (T7, T3 or Sp6) to 18 µL final volume. Generate a master mix using these specifications when screening multiple probes. Finally, add 1 µg of template in 2 µL TE. This reaction can be performed in RNAse-free Eppendorf tubes or in 96 well RNAse-free PCR plates
- Incubate 3 hours at 37ºC.
- Precipitate by adding 180 µL ddH_2O, 400 µL 100% EtOH, and 8 µL 5M NaCl. Vortex at maximum speed for 5 seconds. If probe synthesis is performed in 96 well plate format, transfer the product to RNAse free Eppendorf tubes prior to precipitation.
- Store at -20ºC for at least 1 hour.
- Centrifuge for 15 minutes at 4ºC at maximum speed to collect pellet. Wash pellet once in 200 µL 70% EtOH. Air-dry the pellet for 5-10 minutes and dissolve in 100 µL TE. Run denaturing gel with RNA size marker to check probe yield and integrity.

6. Pre-hybridization treatment and hybridization

- Fix in fresh 4% paraformaldehyde in PBS (aka PFA) for 15 minutes at room temperature.
- Wash 3x 5 minutes in DEPC-PBS at room temperature. In this and all subsequent washes, place mailers on rotating platform to ensure good mixing.
- Incubate in Proteinase K solution (1µg/mL) at 37°C for 30 minutes. [Add 3 µl of 17 mg/mL Proteinase K stock per 50 ml 100 mM Tris-HCl pH 8, 50 mM EDTA pH 8.0.] Use a fresh aliquot of Proteinase K every 2 weeks.
- Fix in PFA for 15 min.
- Wash 3x 5 minutes in DEPC-PBS at room temperature.
- Hybridization: add 90~100 µL of DIG and/or FL-labeled probe to 14 mL hybridization solution per mailer. Mix well. Place slides into mailer and fill empty slots with RNase-free dummy slides. Incubate at 72°C overnight in dry oven or water bath.

7. Post-hybridization and color development

- Transfer slides from hybridization mailer to mailers containing Solution X.
- Wash 3x 45 minutes in Solution X at 72°C. Use of rotating platform is unnecessary for this step.
- Wash for 3x 15 min in TBST at room temperature.
- Block for 1 hour in 10% lamb serum in TBST at room temperature (or overnight at 4°C).
- Incubate in pre-absorbed alkaline phosphatase-conjugated anti-digoxygenin antibody [1:5000 in 1% lamb serum in TBST] for 2 hours at room temperature or overnight at 4°C.
- Wash 3x 15 min in TBST at room temperature to remove excess antibody.
- Wash for 15 min in freshly prepared NTMT at room temperature.
- Begin color reaction by adding 35µL NBT (100mg/mL in 100% DMF) and 35µL BCIP (50mg/mL in 100% DMF) per 10 mL NTMT. Incubate at room temperature in the dark. Continue color reaction overnight at room temperature or 4°C as appropriate.
- When color has developed, rinse quickly in NTMT buffer, then wash 3x 15 min in TBST at room temperature to remove background. Stop reaction by adding TE Stop Buffer.

8. Cover slip and image acquisition

After color reaction is completely stopped in TE Stop Buffer, slides are dehydrated with ethanol, tissue clearing is performed using Histo-Clear (National Diagnostic. GA) and cover slips are placed using Eukitt (Electron Microscopy Science. PA). Images of whole slides are collected by scanning with the Leica SCN400 Slide Scanner.

9. Solutions

- 4% paraformaldehyde (PFA): 45 mL ddH_2O, 4g paraformaldehyde
  To prepare, heat to 60-70ºC, add 1 drop 10N NaOH, stir to dissolve. Once PFA has fully dissolved, add 5mL DEPC-treated 10xPBS, pH 7.5. Sterile filter and store on ice.
- 20x SSC, pH 4.5 (1L): Add 175.9g NaCl and 88.2g $Na_3(C_3H_5O(COO)).2H_20$) to 800 mL of ddH_2O. Adjust pH to 4.5 with concentrated HCl. Adjust volume to 1000 mL final.
- 0.2M Phosphate buffer, pH 7.5: Dissolve 3.84g of KH_2PO_4 and 45.1 g Na_2HPO_4 into 800 mL ddH_2O. Adjust pH to 7.5 with HCl. Adjust volume to 1000 mL final.
- Hybridization Solution: 50% formamide, 5 x SSC (pH 4.5), 1% SDS, 500 µg/mL tRNA, 200 µg/mL acetylated BSA, 50 µg/mL heparin. For 500mL of hybridization solution: 250 mL ultrapure formamide, 125 mL 20xSSC (pH

4.5), 50 mL 10% SDS, 2.0 mL tRNA (125 mg/mL in DEPC ddH_2O), 4.0 mL acetylated BSA (25 mg/mL in DEPC ddH_2O), 2.5 mL heparin (10 mg/mL in DEPC ddH_2O), and 68.5 mL DEPC ddH_2O.

- Solution X: 2 x SSC (pH 4.5), 50% formamide, 1% SDS For 500 mL of Solution X: 50 mL 20xSSC (pH 4.5), 250 mL ultrapure formamide, and 50 mL 10% SDS, and 150 mL DEPC ddH_2O.
- NTMT: 100 mM NaCl, 100 mM Tris-HCl (pH 9.5), 50 mM $MgCl_2$, 1% Tween 20. For 250 mL of NTMT: Combine 12.5 mL 2M NaCl, 25 mL 1M Tris-HCl (pH9.5),12.5 mL 1M $MgCl_2$, 2.5 mL Tween-20, 197.5 mL ddH_2O.
- TE Stop Buffer. 10 mM Tris-HCl (pH 7.5). 10 mM EDTA (pH 8.0)

10. References

Grove EA, Tole S, Limon J, Yip L, Ragsdale CW. (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 125:2315-25.

Agarwala S, Sanders TA, Ragsdale CW. (2001) Sonic hedgehog control of size and shape in midbrain pattern formation. Science. 291:2147-50.

Sanders TA, Lumsden A, Ragsdale CW. (2002) Arcuate plan of chick midbrain development. J Neurosci. 22:10742-50.

Mashiko H, Yoshida AC, Kikuchi SS, Niimi K, Takahashi E, Aruga J, Okano H, Shimogori T. (2012) Comparative anatomy of marmoset and mouse cortex from genomic expression. *J Neurosci.* 32:5039-53.

Shimogori T and Blackshaw S. In Situ Hybridization Methods. PartIII, 10: 207-220. Springer Protocols. Human Press.