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High-Throughput RNA In Situ Hybridization in Mouse Retina

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Abstract

The introduction of large-scale gene expression profiling studies has greatly increased the need to rapidly obtain high-quality cellular expression patterns of genes found to exhibit differential expression. The use of large-scale nonradioactive RNA in situ hybridization makes this possible, and greatly increases the general usefulness of this data. Here, we describe protocols for parallel analysis of up to 50 different gene-specific probes in mouse retinal sections.

Keywords

RNA; Gene expression; Cellular resolution; Hybridization; Digoxigenin; Riboprobe; Chromogenic; Retina; Photoreceptor; Development

1. Introduction

RNA in situ hybridization (1, 2) can be used to characterize the cellular expression pattern of any RNA species. By designing anti-sense probes that can undergo complementary base pairing with a target sequence of interest, one can readily design a probe that can strongly and selectively bind to virtually any target RNA, whether or not it codes for protein. The high stability of RNA–RNA hybrids means that hybridization conditions can be made especially stringent, thus resulting in both low background signal and high sensitivity. Complementary RNA (cRNA) probes are typically generated from linear DNA templates, using recombinant viral RNA polymerases to directly incorporate modified bases that contain a label of choice to allow detection of probe–target hybrids. Radioactive nucleotide triphosphates can be used to allow for direct detection of bound probe. Alternatively, chemically modified bases can be used, allowing indirect probe detection using immunodetection. Critical improvements came with the use of bases conjugated to the highly antigenic small molecule digoxigenin for probe labeling, which allowed the use of highly specific alkaline phosphatase-conjugated antibodies for immunohistochemistry (2, 3). These modifications improved the specificity and sensitivity of the protocol to the point where it could be used to analyze many different probes in parallel. One of the most spectacular uses of this approach has been the effort of the Allen Brain Atlas consortium to map the expression of all annotated mouse genes in the adult brain (4).

In the retina, large-scale in situ hybridization has been instrumental in analyzing the results of SAGE (5, 6) and microarray (7, 8) data obtained by profiling different developmental stages, or mutant animals exhibiting developmental defects or retinal degeneration. While global expression profiling of this sort generates vast amounts of data, it is very hard to interpret meaningfully unless one also knows the cellular expression pattern of any differentially expressed genes. Even in cases where isolated cell subtypes or even individual cells are profiled (9, 10), it is important to use a different experimental approach to confirm the validity of any results obtained. The regular structure of the retina enables the major cell

types that express a given gene to be identified on the basis of their laminar position, making this data particularly useful. Since up to 50 different cRNA probes can be run in parallel by a single investigator, with results obtained within 3–5 days, it is now feasible to rapidly sort through all of the most potentially interesting hits obtained in such experiments.

2. Materials

2.1. RNase-Free Solutions

1. DEPC-treated water: Add DEPC to 0.1% final concentration in MilliQ ddH₂O. Shake solution to mix, and leave overnight at room temperature. Inactivate DEPC by autoclaving (15–25 min at 15 psi) prior to use (see Note 1).
2. DEPC-treated PBS, SSC, EDTA, LiCl: Prepare these as described above for DEPC-treated water. Do not treat any buffer containing amines (e.g., triethanolamine, Tris, etc.) with DEPC (see Note 2).

2.2. Probe Preparation

1. 10× RNA polymerase buffer (Roche).
2. 10× DIG NTP mix (Roche).
3. RNase-free ddH₂O (not DEPC treated).
4. RNase inhibitor (Roche).
5. RNA polymerase (T7, T3, or Sp6).
6. RNase-free DNase to degrade probe.
7. RNase-free 1.5 ml Eppendorf tubes or RNase-free 96-well plates.
8. DEPC-treated 3 M NaOAc.
9. RNase-free 100% EtOH.
10. 70% EtOH prepared with DEPC ddH₂O.

2.3. Tissue Pretreatment, Probe Hybridization, and Washes

1. 4% Paraformaldehyde (PFA): 45 ml ddH₂O, 4 g PFA.
To prepare, heat to 60–70°C, add 1 drop 10 N NaOH, stir to dissolve. Once PFA has fully dissolved, add 5 ml 10× PBS, pH 7.5. Sterile filter and store on ice. Use PFA solution on the day of preparation. Alternatively, stocks of 20% PFA in water (dissolve as above) can be prepared ahead of time. These can be thawed, and brought to 4% in 1× PBS prior to use.
2. Hybridization buffer (50 ml volume): 25 ml 100% ultrapure formamide, 12.5 ml 20× SSC, pH 6.0, 5 ml 50× Denhardt's solution, 250 µg/ml final yeast tRNA in DEPC water (store resuspended aliquots at –80°C), 500 µg/ml final salmon sperm DNA, DEPC ddH₂O (to 50 ml).
3. 20× SSC, pH 6.0 (1 l): Add 175.9 g NaCl and 88.2 g Na₃(C₃H₅O(COO)·2H₂O) to 800 ml of ddH₂O. Adjust pH to 6.0 with concentrated HCl. Adjust volume to 1,000 ml final.

¹DEPC is highly toxic. Use caution when preparing solutions, and do not breathe vapor. Autoclaving will fully inactivate 0.1% DEPC, and the faint smell detectable after autoclaving reflects residual ethanol contamination.

²DEPC reacts with amine, hydroxyl, and thiol groups of proteins, thus inactivating RNase, as well as any other protein with which it comes into contact. As a result, it is highly toxic and should be handled with great care.

4. 50× Denhardt's solution (1 l): 900 ml ddH₂O, 5 g Ficoll 400, 5 g polyvinylpyrrolidone, 5 g BSA (Fraction V), adjust volume to 1,000 ml with ddH₂O. Filter through a 0.2 µm filter. Store in aliquots at −20°C.
5. Protease K solution: Dissolve at 0.5 mg/ml in DEPC-treated water. Freeze in aliquots and store at −20°C. Do not reuse aliquots.
6. RNase buffer: 0.5 M NaCl, 10 mM Tris pH 7.5, 5 mM EDTA.
7. B1 buffer: 0.1 M Tris pH 7.5, 0.15 M NaCl.
8. B2 buffer: B1 + 5% heat-inactivated normal sheep serum (HISS).
Place serum in a water bath at 56°C, 30 min to heat inactivate. Store HISS at −20°C in aliquots.
9. B3 buffer: 0.1 M Tris–Cl pH 9.5, 0.1 M NaCl, 50 mM MgCl₂.
Filter through a 0.45 µm filter (see Note 3).
10. B4 buffer: 3.375 µl/ml NBT (100 mg/ml in 70% dimethyl-formamide), 3.5 µl/ml BCIP (50 mg/ml in ddH₂O), 0.24 mg/ml levamisole in B3 buffer.
11. Gelvatol mounting media: 21 g PVA, 42 ml, 52 ml, 0.2 M Tris, pH 8.5, 3–5 crystals of NaN₃, ddH₂O.
Preparation of Gelvatol: Add PVA to glycerol followed by ddH₂O. Add 3–5 crystals of NaN₃. Stir with low heat for a few hours or until reagents dissolved. Clarify the mixture by centrifugation at 5,000 × g for 15 min. Aliquot and store at 4°C. See Note 4 for details on Gelvatol preparation.
12. 3 M NaOH.
13. 1% SDS in ddH₂O.
14. Tissue-Tek plastic slide boxes and slide holders (Fisher).
15. Siliconized 24 × 60 mm coverslips.
To prepare, load coverslips onto 24-slot Tissue-Tek slide holder. In a fume hood, dip twice into 3% Sigmacote (Sigma) in chloroform (dip 2×), then dip twice into 100% EtOH. Air-dry coverslips in hood. Prepare several hours in advance to allow sufficient time for drying.

3. Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1. Probe Preparation for Linearized Templates (See Note 5)

1. Prepare plasmid DNA using Qiagen miniprep kit or equivalent. Digest 5–10 µg of template DNA to completion using restriction enzyme of choice. Confirm completeness of digestion using gel electrophoresis.

³The filter unit used to prepare B3 can be wrapped with parafilm and reused.

⁴The viscosity of the Gelvatol requires optimization for each batch prepared. When making the solution, add PVA in step 4 until the solution is clear and is slightly less viscous than molasses. Then refrigerate the beaker of Gelvatol overnight at 4°C (after step 5 and before step 6), and check it the next morning to be sure that the viscosity is that of molasses. If it is, continue on to step 6. If it is too viscous, add a little more glycerol to lower the viscosity and then go on to step 6. If it is not viscous enough, add more PVA with heat and refrigerate for a few more hours, again checking the viscosity before going on to step 6. Continue until viscosity is optimal. Gelvatol can be stored at 4°C indefinitely, or at room temperature for 1 month.

2. Following digestion, add 0.5 µg protease K to the enzyme digestion buffer and incubate at 37°C for 15 min.
3. Increase volume to 200 µl with DEPC-treated TE.
4. Extract once with 200 µl TE-buffered phenol, and then once with 200 µl chloroform.
5. Precipitate with 600 µl volumes of EtOH and 20 µl DEPC-treated 3 M NaOAc.
6. Spin for 15 min at maximum speed to collect pellet, and then wash twice in 200 µl 70% EtOH prepared with DEPC-treated water.
7. Air-dry the pellet and resuspend at 1 µg/µl in TE.

3.2. Alternative Protocol: Probe Preparation for PCR-Generated Templates

1. Amplify the probe template using primers that include the promoter sequence for the RNA polymerase used for probe generation. Primers commonly used for this include M13 forward and reverse, and primers targeting the T7, T3, and Sp6 RNA polymerase promoter sequences (see Note 6). Use approximately 0.1 ng of plasmid template, running 25–30 cycles of amplification.
2. Confirm that a correctly sized band is amplified using agarose gel electrophoresis.
3. Purify amplified DNA using a Qiagen spin column, eluting in TE.
4. Following purification of template DNA, synthesize probe by mixing the following components in the order indicated. Use RNase-free aerosol tips for all procedures: 2 µl 10× RNA polymerase buffer, 2 µl 10× DIG NTP mix, RNase-free ddH₂O (to 17 µl final), 1 µl RNase inhibitor, and 1 µl RNA polymerase (T7, T3, or Sp6) to 19 µl final volume. Generate a master mix using these specifications when screening multiple probes. Finally, add 0.5–1 µg of template in 1 µl TE. This reaction can be performed in RNase-free Eppendorf tubes or in 96-well RNase-free PCR plates.
5. Incubate for 60 min at 37°C.
6. Add 2 µl RNase-free DNase. Incubate for 15 min at 37°C to degrade probe template.
7. Run denaturing gel with RNA size marker to check probe yield and integrity.
8. If probe yield and integrity are satisfactory, add 2.5 µl 4 M DEPC-treated LiCl and 75 µl 100% EtOH to precipitate. Vortex at maximum speed for 5 s. If probe

⁵DNA templates used for cRNA probe synthesis should be between 300 and 2,000 bp in length, with 700–1,000 bp being optimal. Probe sequences should lack any repeat sequences longer than 40 bp, and should not show more than 90% identity over any continuous stretch of 150 bp or more, or cross-reactivity will result. Probe templates must be cloned into vectors (such as pBluescript) in which the insert is flanked by T7, T3, or Sp6 RNA polymerase promoters, so as to allow generation of labeled cRNAs. Many 3' directed ESTs, such as those from the BMAP project or other large-scale cDNA sequencing efforts, work very well as ISH probes, and can be easily ordered from companies such as Thermo-Fisher. Alternatively, PCR amplification followed by cloning into an appropriate vector (such as the TOPO-TA vector) can be used if repeat-free ESTs are not already available as probe templates. Only cloned sequences should be used as templates for probe generation. While PCR can be used to simultaneously amplify template sequences from complex target preparations (e.g., reverse-transcribed cDNA), this introduces a strong possibility of contamination. Once a clone containing the probe sequence has been obtained, a linearized template must be generated in order to conduct run-off transcription for cRNA synthesis. All experiments should include both a positive and a negative control sample. Positive control probes should robustly recognize target mRNAs in retina, but not be so abundant that contamination of other slides is a real risk. Good negative control probes should target transcripts that are not detectably expressed in retina (such as albumin, cardiac albumin, GFP, etc.). Use of sense control probes is not usually recommended, as a sizeable minority of mammalian genes have associated antisense transcripts (11).

⁶When using PCR primers that target an RNA polymerase promoter sequence (i.e., T3, T7, or Sp6), it is important to include 2–3 bases of 5' overhang outside the primer sequence. This substantially improves the efficiency of cRNA synthesis.

synthesis is performed in 96-well plate format, transfer the product to RNase-free Eppendorf tubes prior to precipitation.

9. Store at -80°C for at least 2 h. Precipitate by centrifuging at maximum speed.
10. Wash twice in 200 μl 70% EtOH prepared with DEPC-treated water. Air-dry the pellet and resuspend at 1 $\mu\text{g}/\mu\text{l}$ in TE. The probe can be stored in EtOH indefinitely, or in TE for at least 2 years at -80°C .

3.3. Preparation of Fresh-Frozen Tissue Sections (See Note 7)

1. Remove eyes and embed directly in O.C.T. compound (VWR) in Peel-A-Way disposable plastic mold (Polysciences) and snap freeze on dry ice. Store block at -80°C prior to sectioning.
2. Allow block to warm to cutting temperature for a minimum of 20 min.
3. Cut 15–20 μm sections using a cryostat or freezing microtome onto Superfrost Plus slides (VWR, 48311-703) (see Note 8).
4. Air-dry sections for at least 20 min. Slides can be dried for several hours if necessary. Do not allow to dry overnight, however. Use dried slides immediately for in situ hybridization analysis or store at -70°C in sealed slide box (can store for 1–2 years without appreciable loss of signal). If using stored sections for analysis, allow them to equilibrate to room temperature in a closed slide box.

3.4. Alternative Protocol: Preparation of Immersion Fixed Sections

1. Remove cornea, lens, and sclera from dissected eyes to create eyecup preparation. Fix tissue by overnight immersion at 4°C in 4% PFA in $1\times$ PBS.
2. Transfer to 30% sucrose in $1\times$ PBS for 24 h at 4°C .
3. Mount in O.C.T. compound and section as described for Method 1.
4. Air-dry for at least 20 min. Slides can be dried overnight if needed. Use immediately or freeze at -20°C as described above.

3.5. Pretreatment of Sections

1. Before beginning: Remove all RNase contamination from slide racks and chambers by rinsing with 0.3 M NaOH, rinsing with MilliQ water, treating with RNaseZap or 1% SDS in MilliQ water, and then rinsing once again with MilliQ water. Wipe clean with Kimwipes (see Note 9). Use DEPC-treated solutions for all treatments prior to hybridization (see Note 10).
2. Fix in fresh 4% PFA in $1\times$ PBS for 10 min.
3. Wash $3\times$ with PBS, 5 min each (see Note 11).

⁷Fixed tissue generally produces weaker signal intensity than fresh-frozen, though use produces superior morphology and better overall signal–noise ratio. Fresh-frozen tissue is often easier to section consistently than fixed tissue, and is particularly useful for analyzing embryonic samples.

⁸It is advantageous to fill as much as the slide as possible with sectioned samples (although make sure that O.C.T. from other sections does not overlap your tissue). The more sections present on a slide, the greater the likelihood of obtaining high-quality data in a given experiment. It is also the best way to directly compare results from different samples, since placing them on the same slide effectively eliminates slide-to-slide variation in signal intensity and probe spreading.

⁹RNases are very resistant to inactivation, so pretreatment is designed to simply denature and remove any proteins that might come into contact with the slides. In this step, this is accomplished by treatment with detergent and a strong base.

4. If tissue was fixed in 4% PFA before embedding, treat with 2 μ g Protease K in PBS for 10 min, followed by 2 $\times$ 5-min washes in PBS, a 5-min refix in 4% PFA/PBS (see Note 12), and 2 $\times$ 5-min PBS washes.
5. Incubate for 10 min in a mixture of 270 ml DEPC-treated water/30 ml 1 M triethanolamine, pH 8.0/0.75 ml acetic anhydride. Mix solution in an RNase-free glass bottle and mix well by shaking after addition of acetic anhydride (see Note 13).
6. Wash 3 $\times$ 5 min with PBS.

3.6. Riboprobe Hybridization

MilliQ water is adequate for this and all subsequent steps.

1. Place slides in a chamber constructed from a 245 $\times$ 245 mm BD Falcon* Square BioDish XL (Fisher, 02-667-21) square Petri dish on a raised platform constructed from two 2 ml polystyrene pipettes taped to the surface of the chamber with waterproof tape. Alternatively, these can be bonded directly to the surface of the dish using chloroform (see Note 14).
2. Place 500–1,000 μ l of hybridization buffer on slide. Cover sections completely (see Note 15).
3. Leave in humidified chamber (keep moist with strips of gel blot paper soaked in 5 $\times$ SSC) for at least 90 min.
4. Prepare siliconized 24 $\times$ 60 mm coverslips several hours in advance to allow sufficient time for drying.
5. Pour off prehybridization solution and blot off edges by direct touching to bench paper. To reduce costs, prehybridization solution can be saved, stored at -20°C , and reused 3–4 additional times. Add 75–100 μ l hybridization solution containing 200–300 ng/ml DIG RNA which has been heated at 80°C for 5 min, vortexed for 5 s, and then snap-chilled on ice. Add probe along bottom, long edge of the slide.
6. Clean coverslips using blown compressed air or manual tapping if visible dust is present.
7. Coverslip slides by slowly lowering down a siliconized coverslip. Place long edge, in contact with probe, down first and lower the rest slowly using fingers or a bent needle. Go slowly, to avoid trapping of air bubbles. Once on the slide, raise and

¹⁰Avoiding RNase contamination during the first day of the procedure is critical. RNases can come from human and animal skin and hair, or very often as contamination from other experimental procedures, particularly plasmid DNA preparation. The danger is greater to the probe than to the sample, as target RNA sequences in fixed tissue are much less accessible to RNases than are cRNAs in solution. Do everything possible to avoid these sources of contamination. Wear a clean lab coat to cover arms, tie back long hair, and avoid working in lab areas where animals are sacrificed. Use aerosol tips for all solutions that require pipetting. Put down fresh bench paper before beginning the procedure, and change gloves whenever the possibility of contamination arises. We have often used a fume hood to conduct all the steps up until the prehybridization step, which both protects your sample from airborne debris and removes any PFA vapors from the work area.

¹¹Dunk sections several times during each wash to ensure good mixing.

¹²This refixation is important to maintain tissue integrity following the protease K treatment.

¹³Acetic anhydride modifies positively charged amine groups in proteins and lipids, greatly reducing nonspecific binding by the negatively charged cRNA probe. However, acetic anhydride hydrolyzes rapidly in aqueous solution, and it is extremely important that the solution be applied immediately to the slides once mixed.

¹⁴It is important that slides rest above the surface of the box so as to avoid capillary transfer of solution away from the sections.

¹⁵Do not allow the tissue to dry out at this or any other successive stage. This will result in a loss of signal in the affected area. Chill prehybridization solution on ice prior to applying to reduce surface tension and ensure an even spread. Solution used for prehybridization can be reused 3–4 times. Pour into 50 ml conical tubes prior to adding probe and store at -80°C (reused solution should not be mixed with probe and used for hybridization).

lower the coverslip a couple of times to mix the probe with the prehybridization solution that remains on the slide (see Note 16).

8. Place slides horizontally in a humidified chamber (use 20 slide capacity microslide boxes) (VWR). If possible, place slides with different probes in separate boxes. However, if running many probes in one experiment, place abundant probes at the bottom. Make sure that long edge of slides is not in contact with the back of the box, as this can promote capillary transfer of the hybridization buffer away from the slide. Insert at least four blank slides (pushed all the way to the back of the box) to avoid this problem (see Note 17). Place a couple of Kimwipes (VWR) soaked in 5–10 ml 50% formamide/5× SSC in the bottom of the box to ensure that slides do not dry out.
9. Seal with waterproof tape (incubate at 65–72°C overnight) (see Note 18).

3.7. Washes, Antibody Binding, and Signal Detection

1. Place slides in rack, submerged in 5× SSC to remove coverslips. If coverslips are slow in falling off, the solution temperature can be increased to 65–70°C. Carefully remove slides from solution with forceps, grasping the frosted end. Coverslips should fall off into the solution when slides are lifted up (see Note 19).
2. Transfer slides with forceps into metal racks.
3. Incubate in 0.2× SSC at 65°C for 1 h in a water bath. After 30 min, jostle the slides a bit to remove bubbles that may have accumulated on the slides. Slides can be washed longer if needed, but not longer than 3 h total (sections will often fall off the slide if heated longer) (see Note 20).
4. Wash with RNase buffer for 5 min at 37°C.
5. Wash in RNase buffer containing 10 µg/ml RNase A for 30 min at 37°C (see Note 21).
6. Wash in RNase buffer for 5 min at room temperature.
7. Wash 2× for 30 min in 0.2× SSC, 65°C.
8. Wash in 0.2× SSC for 5 min.
9. Wash in B1 for 5 min.
10. Place 1 ml buffer B2 on horizontal slides for 1 h (see Note 22).
11. Place 0.5 ml anti-DIG Ab (1:5,000 in buffer B2) on each slide. Incubate in humidified chamber at 4°C overnight (see Note 23).

¹⁶Probe must be well mixed to ensure even signal intensity across the slide.

¹⁷It is critically important to change gloves between probes. Failure to do so will result in cross-contamination.

¹⁸70–72°C is an optimal hybridization temperature for perfect match probes that are longer than 300 bases. 65°C is better for shorter probes or an imperfect match (i.e., rat probe used on mouse tissue).

¹⁹Do not use any force or pressure to remove the coverslip. Doing so will result in damage to the tissue.

²⁰In this and every subsequent step, preheat solutions in a water bath prior to washing.

²¹The RNase treatment degrades the single-stranded cRNA probe while leaving double-stranded RNA–RNA hybrids intact. This results in a considerable reduction in background signal. However, it requires great caution, as the RNase used in this step can contaminate materials used prior to the hybridization step. Every effort should be made to prevent this. Make sure that slide boxes and racks used for RNase treatment are treated with 0.3 M NaOH and RNaseZap after use. Change gloves after handling slides during this step.

²²To ensure even spread of solution across the slide and to reduce the amount of serum used, chill B2 solution on ice prior to application.

²³For abundant RNA a 1-h incubation is sufficient, but overnight incubation greatly enhances the signal and reduces color reaction time.

12. Wash with buffer B1 3× for 5 min.
13. Wash with buffer B3 for 5 min, keeping slides horizontal in humidified chamber (use the same prehyb chamber), puddle on buffer B4. Keep in the dark (reaction is photosensitive) at RT (cover chamber with foil). Check color after 15 min, 1 h, and then again after 3 h and 6 h using a low-power microscope (see Note 24). Can leave reaction for up to 3 days at either room temperature or 4°C, and for even longer if background is low.

3.8. Coverslipping and Mounting

1. Rinse slides in TE.
2. Rinse slides in ddH₂O.
3. Mount in 4 drops Gelvatol per slide, using 24 × 60 mm coverslips. Leave overnight before examining. Once dry, wash away excess Gelvatol with tap water, and then air-dry (see Note 25).

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²⁴Optimizing the ratio of target to background signal is crucial for obtaining high-quality data using this procedure. To this end, it is essential to vigilantly track the progress of the color reaction so as to assess when it should be terminated. The most efficient way to do this is to directly view the slides in their chamber against a white background using a dissecting microscope such as a Leica Stemi 2000-C or an Olympus BH-2 at roughly 10× magnification. Stop the reaction before any obvious background is visible, but slightly past the point at which the color exposure appears optimal to the eye, as the cellular signal intensity will look weaker at the 200× magnification usually used for photographing retinal sections.

²⁵Gelvatol automatically seals the slide once it dries, which reduces labor substantially when running large number of slides. One can also use Aquamount (Fisher, 14-390-5) or an equivalent water-based solution to mount the slides, but they must then be sealed with clear nail polish to prevent drying during long-term storage.

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