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Hybridization Histochemistry of Neural Transcripts

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Abstract

Expression of genes is manifested by the production of RNA transcripts within cells. Hybridization histochemistry (or *in situ* hybridization) permits localization of these transcripts with cellular resolution or better. Furthermore, the relative amounts of transcripts detected within different tissues or the same tissues in different states (e.g., physiological or developmental) may be quantified.

This unit describes hybridization histochemical techniques using either oligonucleotide probes (see Basic Protocols 1 and 2) or RNA probes (riboprobes; see Basic Protocols 3–5). These protocols include the more recent approaches using commercially available sets of oligonucleotide pairs for colorimetric and fluorescent detection (see Basic Protocol 2); the use of probes labeled with digoxigenin for colorimetric detection of RNA transcripts (see Basic Protocol 4); and an alternative method for detecting the digoxigenin-labeled probes via tyramide signal amplification (TSA) is included (see Alternate Protocol). Detection of the Y chromosome using either mouse or human riboprobes is then described (see Basic Protocol 5). Finally, a procedure is presented for the autoradiographic detection of radiolabeled probes (see Basic Protocol 6). Methods are provided for labeling oligodeoxynucleotide (see Support Protocol 1) and RNA probes (see Support Protocol 2) and performing northern analyses using these probes (see Support Protocol 3).

BASIC PROTOCOL 1

HYBRIDIZATION HISTOCHEMISTRY WITH OLIGODEOXYNUCLEOTIDE PROBES

This protocol is a good initial approach to hybridization histochemistry. It can be used when less sensitivity is needed than that provided by oligonucleotide probe pairs (Basic Protocol 2) or RNA probes (see Basic Protocol 3). Also, less molecular-biological expertise is necessary for this procedure.

Materials

50% (v/v) formamide in 4× SSPE (*APPENDIX 2A*)

Slides containing 12-μm tissue sections prepared by cryostat, mounted on Superfrost Plus slides (Fisher), and defatted (*UNIT 1.1*)

Hybridization solution (see recipe) containing $\sim 1 \times 10^6$ dpm/50 μ l ^{35}S -labeled oligodeoxynucleotide probe or 1 to 5 μ l/50 μ l digoxigenin-labeled oligonucleotide probe (see Support Protocol 1)

1 $\times$ SSPE/1 mM DTT, room temperature and 55°C

70% ethanol

Sterile Bio-Assay dishes (Nunc; 245 $\times$ 245 $\times$ 30 mm)

Whatman 3MM chromatography paper

Glass coverslips

Staining dishes and tubs

Slide rack

55°C water bath

NOTE: Use DEPC-treated water (*APPENDIX 2A*) for all reagents in pretreatment and hybridization steps.

- 1 Cover the inside of the Bio-Assay dish top (which has a larger surface area than the bottom) with a piece of Whatman 3MM chromatography paper. Wet paper with 50% formamide/4 $\times$ SSPE and lay the slides containing the tissue sections on the paper with the sections facing up.

Each dish top can hold 20 slides.

- 2 Place hybridization solution containing $\sim 1 \times 10^6$ dpm/50 μ l ^{35}S -labeled (or 1 to 5 μ l/50 μ l digoxigenin-labeled) oligodeoxynucleotide over the tissue section on each slide and cover with a coverslip.

To cover two adult rat coronal sections under an 18 $\times$ 30-mm coverslip, 45 μ l of hybridization solution is needed. The amount of hybridization solution used should be scaled up proportionately if a larger coverslip is used to cover a larger tissue section. The authors have found no need to pretreat the coverslips, but they should be dust-free. If background is a problem, use of silanized coverslips should be considered.

- 3 Cover the slides with the bottom of the Bio-Assay dish and incubate 20 to 24 hr at 37°C.
- 4 Remove slides from incubator and place with frosted ends up into a staining dish containing 1 $\times$ SSPE/1 mM DTT. Gently slide the coverslip until it partially overhangs the slide, then pry off the coverslip with a forceps.
- 5 Place the slide without a coverslip in a slide rack and immerse in a tub containing 1 $\times$ SSPE/1 mM DTT. Repeat step 4 with each slide until all the slides have had their coverslips removed and been placed in the same rack.

Do not allow the sections to dry until step 7.

- 6** Wash the slides by immersing successively for 15 min each time in four changes of 55°C 1× SSPE/1 mM DTT, then for 5 min each time in two changes of room temperature 1× SSPE/1 mM DTT.
- 7a** *For ³⁵S-labeled probes:* Rapidly dip the slides into water and then into 70% ethanol, then blow them dry while the slides are oriented with their frosted ends down. Proceed to detection (see Basic Protocol 5).
- 7b** *For digoxigenin-labeled probes:* Using slides directly from the SSPE/DTT wash in step 6, proceed to detection (see Basic Protocol 3 or Alternate Protocol).

BASIC PROTOCOL 2

HYBRIDIZATION HISTOCHEMISTRY WITH SETS OF OLIGONUCLEOTIDE PROBE PAIRS

This protocol relies on commercially available kits and probe sets that enable rapid, sensitive and low background detection of RNA transcripts. Generally, each probe set contains 15–20 pairs of oligonucleotides that must hybridize to adjacent stretches of the RNA in order to generate a signal through branched DNA amplification. The approaches taken by both Advanced Cell Diagnostics, Inc., (RNAscope®) and Affymetrix, Inc., (ViewRNA®) rely on the same underlying patents (e.g., US Patent No. 7,709,198). We present our protocol using the ViewRNA kit and probes. Very little molecular biological expertise is needed for this approach.

Materials

Slides containing 16-μm tissue sections prepared by cryostat and mounted on Superfrost Plus slides (Fisher) (*UNIT 1.1*). Stored at –80°C until needed.

37% formaldehyde

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

0.1 M Tris-HCl, pH 7.4

De-ionized water

70%, 80%, 100% ethanol

Tissue Tek staining dishes or tubs

Slide rack

Small 40°C humidified incubator

Oven at 60°C

Slide humidifying holder

Hydrophobic Barrier Pen

Bacterial Type XXIV Proteinase (Sigma-Aldrich P8038, 100MG)

DAPI (0.3μg/ml 4',6-diamidino-2'-phenylindole dihydrochloride)

Quantigene ViewRNA ISH Tissue 2-plex Assay kit (Affymetrix) that includes:

100× Pretreatment solution

Protease QF

Probe Set Diluent QT

Label Probe Diluent QF

PreAmplifier Mix QT

Amplifier Mix QT

Label Probe 1-AP

Label Probe 6-AP

Blue Buffer

Blue Reagent 1

Blue Reagent 2

Blue Reagent 3

AP Enhancer Solution

Fast Red tablets

Naphthol Buffer

AP Stop QT

Wash Buffer Component 1

Wash Buffer Component 2

Custom designed Probe Set(s) from Affymetrix

NOTE: Each probe set is made to appear either red (Fast Red, Type 1) or blue (Fast Blue, Type 2) under incandescent light upon amplification. They appear red or far red (not visible to unaided eye – camera-sensitive only), respectively, with fluorescent illumination.

Hybridization histochemistry for detection of a single transcript

- 1 Prepare 4% formaldehyde in a 200 ml capacity container (add 22 ml of 37% formaldehyde to 178 ml of PBS. Refrigerate at 4°C for a minimum of 1 hour in a slide holder such as a Coplin jar).
- 2 Put the slides in the cold formaldehyde for 16–18 hours at 4°C (overnight).
- 3 Prepare the following reagents: PBS, 50% ethanol, 70% ethanol, 100% ethanol, Wash Buffer (mix 27 ml Wash Component 1 and 7.5 ml Wash Component 2 in 2.5 L de-ionized H₂O).
- 4 Prewarm 40 ml of PBS and Probe Set Diluent QF to 40°C.
- 5 Thaw Probe Set(s) and place on ice until used.
- 6 Remove slides from 4% formaldehyde and wash twice in PBS for 1 min with constant agitation up and down, making sure tissues are completely submerged.

- 7 Transfer slides to 50% ethanol for 10 min at room temperature (RT).
- 8 Transfer slides to 70% ethanol for 10 min at RT.
- 9 Transfer slides to 100% ethanol for 10 min at RT.
- 10 Remove the slides and heat them at 60°C for 30 min (begin protease digestion within 1 hour of this step).
- 11 When cooled, using the hydrophobic pen, draw a thick rectangle surrounding the tissues. Let dry and repeat several times to ensure a solid boundary. Let the barrier dry at RT for a minimum of 20 min.
- 12 Prepare the working protease at a dilution of 1:100, with a recommended working volume of 400 µl, using the prewarmed PBS.

NOTE: The Quantigene ViewRNA kit does provide Protease QF; we recommend, instead, using Bacterial Type XXIV Proteinase (Sigma-Aldrich P8038, 100MG) that one has previously optimized and made into 1 ml aliquots at a concentration of 20 units/ml PBS. They are stored at –20°C between uses. A final, working concentration of 2 units/ml PBS should be a good starting point but you should try some different concentrations to get your best signal.

- 13 Apply the protease solution within the hydrophobic pen barrier and incubate the slides at 40°C in the humidified incubator for 10 min. Do not use a coverslip for this or subsequent steps.
- 14 Wash slides in PBS twice for 1 min each with a few gentle up and down motions during that time.
- 15 Place slides in 4% formaldehyde for 5 min at RT.
- 16 Wash slides in PBS twice for 1 min each with constant agitation (approximately an up and down motion every second). Slides may sit in this buffer until next step.
- 17 Prepare the working Probe Set solution by diluting Probe Set(s) at a dilution of 1:40 in prewarmed Probe Set Diluent QT and then vortexing. Each slide (containing about 2 rat or 4 mouse closely placed coronal brain sections) is incubated with 10 µl of desired Probe in 390 µl of Probe Set Diluent QT.
- 18 Remove the slides, gently flick to remove the PBS (make sure tissues do NOT dry), and add 400 µl of probe solution to each one.

NOTE: Always flick the slides to remove excess liquid.

- 19 Transfer slides to the 40°C humidified incubator and allow hybridization to occur for 2 hours.
- 20 Prewarm kit reagents during the 2 hour hybridization period: Preamplifier Mix QT, Amplifier Mix QT, and label probe Diluent QF buffers to 40°C. Label Probe 1-AP, Label Probe 6-AP, and Blue Reagents 1, 2, and 3 are left at 4°C until used.

The Fast Red tablets, Naphthol Buffer, AP Enhancer Solution and Blue Buffer are brought to RT.

- 21** Wash slides with Wash Buffer three times for 2 min each with constant agitation at RT.
- 22** Remove slides and flick to remove the Wash Buffer and then quickly proceed.
- 23** Add 400 μ l of the PreAmplifier Mix QT to each slide. Incubate the slides for 25 min at 40°C.
- 24** Wash in Wash Buffer three times for two min each with constant agitation.
- 25** Remove the slides, and add 400 μ l of Amplifier Mix QT. Incubate the slide for 15 min at 40°C.
- 26** Wash in Wash Buffer three times for two min each with constant agitation.
- 27** Prepare either working Label Probe 6-AP solution OR working Label Probe 1-AP solution at a dilution of 1:1000. (for a 400 μ l working volume per slide, add 0.4 μ l of Label Probe 6-AP OR Label Probe 1-AP in 399.6 μ l of Label Probe Diluent QF and vortex.) Apply 400 μ l of the solution to each slide and incubate at 40°C for 15 min.
- 28** Wash in Wash Buffer three times for three min each with constant agitation.

If using a Type 6 Probe Set:

- 29a** Prepare Fast Blue Substrate: Add 105 μ l of Blue Reagent 1, 105 μ l of Blue Reagent 2, and 105 μ l of Blue Reagent 3 to 5 ml of Blue Buffer; and vortex. Make sure the solution is protected from light. Add 400 μ l of fast blue substrate to each slide and incubate for 30 min at RT.
- 30a** Wash slides in PBS for 1 min.
- 31a** Counterstain with DAPI for 1 min and briefly rinse with de-ionized H₂O.
- 32a** Place a temporary coverslip over the tissues with 0.1 M Tris-HCl, pH 7.4, and view under a microscope. After analysis, remove the coverslips, briefly dip in de-ionized H₂O, and allow slides to remain dry in a slide box.

If using a Type 1 Probe Set:

- 29b** Prepare Working Label Probe 1-AP Solution in a dilution of 1:1000 with prewarmed label Probe Diluent QF. (for a 400 μ l working volume per slide, add 0.4 μ l of label probe 1-AP to 399.6 μ l of Label Probe Diluent QF and vortex). Apply 400 μ l of the solution to each slide and incubate at 40°C for 15 min.
- 30b** Wash in Wash Buffer three times for three min each with constant agitation.
- 31b** Remove each slide and add 400 μ l of AP Enhancer Solution. Incubate at RT for 5 min. During this time the Fast Red substrate is made. Dissolve 1 Fast Red tablet in 5 ml of Naphthol Buffer and vortex. Make sure solution is protected from light.

- 32b** Add 400 μ l of Fast Red substrate to each slide, and incubate for 30 min at 40°C.
- 33b** Wash the slides in PBS for 1 min.
- 34** Counterstain with DAPI for 1 min and then briefly wash with de-ionized H₂O.
- 35** Place a temporary coverslip with 10mM Tris-HCl, pH 7.4, and view under a microscope. After analysis, remove the coverslips, briefly rinse in de-ionized H₂O, and allow slides to remain dry in a slide box.

Hybridization histochemistry for simultaneous detection of two transcripts

1. Prepare 4% formaldehyde in a 200 ml capacity container (add 22 ml of 37% formaldehyde to 178 ml of PBS. Refrigerate at 4°C for a minimum of 1 hour in a slide holder such as a Coplin jar).
2. Put the slides in the cold formaldehyde for 16–18 hours at 4°C (overnight).
3. Prepare the following reagents: PBS, 50% ethanol, 70% ethanol, 100% ethanol, Wash Buffer (mix 27 ml Wash Component 1 and 7.5 ml Wash Component 2 in 2.5 L de-ionized H₂O).
4. Prewarm 40 ml of PBS and Probe Set Diluent QF to 40°C.
5. Thaw Probe Set(s) and place on ice until used.
6. Remove slides from 4% formaldehyde and wash twice in PBS for 1 min with constant agitation up and down, making sure tissues are completely submerged.
7. Transfer slides to 50% ethanol for 10 min at room temperature (RT).
8. Transfer slides to 70% ethanol for 10 min at RT.
9. Transfer slides to 100% ethanol for 10 min at RT.
10. Remove the slides and heat them at 60°C for 30 min (begin protease digestion within 1 hour of this step).
11. When cooled, using the hydrophobic pen, draw a thick rectangle surrounding the tissues. Let dry and repeat several times to ensure a solid boundary. Let the barrier dry at RT for a minimum of 20 min.
12. Prepare the working protease at a dilution of 1:100, with a recommended working volume of 400 μ l, using the prewarmed PBS.

NOTE: The Quantigene ViewRNA kit does provide Protease QF; we recommend, instead, using Bacterial Type XXIV Proteinase (Sigma-Aldrich P8038, 100MG) that one has previously optimized and made into 1 ml aliquots at a concentration of 20 units/ml PBS. They are stored at –20°C between uses. A final, working concentration of 2 units/ml PBS should be a good starting point but you should try some different concentrations to get your best signal.

13. Apply the protease solution within the hydrophobic pen barrier and incubate the slides in 40°C in the humidified incubator for 10 min. Do not use a coverslip for this or subsequent steps.

14. Wash slides in PBS twice for 1 min each with a few gentle up and down motions during that time.
15. Place slides in 4% formaldehyde for 5 min at RT.
16. Wash slides in PBS twice for 1 min each with constant agitation (approximately an up and down motion every second). Slides may sit in this buffer until next step.
17. Prepare the working Probe Set solution by diluting Probe Set(s) at a dilution of 1:40 in prewarmed Probe Set Diluent QT and then vortexing. Each slide (containing about 2 rat or 4 mouse closely placed coronal brain sections) is incubated with 10 μ l of desired Probe in 390 μ l of Probe Set Diluent QT.
18. Remove the slides, gently flick to remove the PBS (make sure tissues do NOT dry), and add 400 μ l of probe solution to each one.

NOTE: Always flick to remove excess liquid.

19. Transfer slides to the 40°C humidified incubator and allow hybridization to occur for 2 hours.
20. Wash slides in Wash Buffer three times for 2 min each with constant agitation.
21. Store slides in Storage Buffer (60 ml of Wash Comp 2 and 140 ml of de-ionized H₂O) for up to 24 hours at RT.
22. Before starting, Prewarm kit reagents: Preamplifier Mix QT, Amplifier Mix QT, and Label Probe Diluent QF buffers to 40°C. Label Probe 1-AP, Label Probe 6-AP, and Blue Reagents 1, 2, and 3 are left in 4°C until used. The remaining reagents of Fast Red tablets, Naphthol Buffer, AP Enhancer Solution and Blue Buffer are brought to RT.
23. Take the slides out of the Storage Buffer and wash in Wash Buffer twice for 2 min each with constant agitation.
24. Flick the slides to remove the Wash Buffer and then quickly add 400 μ l of the PreAmplifier Mix QT. Incubate the slides for 25 min at 40°C.
25. Wash in Wash Buffer three times for two min each with constant agitation.
26. Remove slides and add 400 μ l of Amplifier Mix QT. Incubate the slides for 15 min at 40°C.
27. Wash in Wash Buffer three times for two min each with constant agitation.
28. Prepare working Label Probe 6-AP Solution in a dilution of 1:1000 with prewarmed Label Probe Diluent QF. (for a 400 μ l working volume per slide, add 0.4 μ l of Label Probe 6-AP and 399.6 μ l of Label Probe Diluent QF and vortex) Apply 400 μ l of the solution to each tissue and incubate at 40°C for 15min.
29. While the sections are incubating in the Label Probe 6-AP Solution, prepare Fast Blue Substrate: add 105 μ l of Blue Reagent 1, 105 μ l of Blue Reagent 2, and 105 μ l of Blue Reagent 3 to 5 ml of Blue Buffer; and vortex. Make sure the solution is protected from light.

30. Wash slides in Wash buffer twice for 2 min with constant agitation.
31. Add 400 μ l of Fast Blue Substrate to each slide and incubate for 30 min at RT.
32. Remove each slide and add 400 μ l of AP Stop QT and incubate in the dark for 30 min at RT.
33. Wash each slide twice for 1 min in PBS with constant agitation, and then wash slides in Wash Buffer for 1 min with gentle agitation.
34. Prepare working Label Probe 1-AP Solution in a dilution of 1:1000 with prewarmed Label Probe Diluent QF (for a 400 μ l working volume per slide, add 0.4 μ l of Label Probe 6-AP to 399.6 μ l of Label Probe Diluent QF) Apply 400 μ l of the solution to each slide and incubate at 40°C for 15min.
35. Wash in Wash Buffer three times for three min each with constant agitation.
36. Remove each slide and add 400 μ l of AP Enhancer Solution and incubate at RT for 5 min.
37. During this time the Fast Red substrate is made. Dissolve 1 Fast Red tablet in 5 ml of Naphthol Buffer and vortex. Make sure the solution is protected from light.
38. Add 400 μ l of Fast Red substrate to each slide and incubate for 30 min at 40°C.
39. Wash the slides in PBS for 1 min.
40. Counterstain with DAPI for 1 min.
41. Brief rinse with de-ionized H₂O.
42. Place a temporary coverslip with 0.1 M Tris-HCl, pH 7.4, and view under the microscope. After analysis, remove coverslips, briefly dip in de-ionized H₂O, and allow slides to remain dry in a slide box.

BASIC PROTOCOL 3

HYBRIDIZATION HISTOCHEMISTRY WITH RNA PROBES

RNA probes offer great sensitivity for hybridization histochemistry. This can be important in initial mapping surveys so that the results are as inclusive as possible. Although this approach may not be as rapid or as sensitive as the one provided in Basic Protocol 2, it is a cheaper alternative allowing many more sections to be analyzed for the same cost.

Materials

50% (v/v) formamide in 4 $\times$ SSPE (*APPENDIX 2A*)

Slides containing 12- μ m tissue sections prepared by cryostat, mounted on Superfrost Plus slides (Fisher), and defatted (*UNIT 1.1*)

Hybridization solution (see recipe) containing $\sim 1 \times 10^6$ dpm/50 μ l ³⁵S-labeled riboprobe or 3 to 10 μ l/100 μ l digoxigenin-labeled riboprobe (see Support Protocol 2)

4 $\times$ SSPE/1 mM DTT

RNase A solution (see recipe), 37°C

0.1× SSPE/1 mM DTT, room temperature and 65°C

1× SSPE

70% ethanol

Sterile Bio-Assay dishes (Nunc; 245 × 245 × 30 mm)

Whatman 3MM chromatography paper

Glass coverslips

Staining dishes and tubs

Slide rack

55°C incubator

65°C water bath

NOTE: Use DEPC-treated water (*APPENDIX 2A*) for all reagents in pretreatment and hybridization steps.

- 1** Cover the inside of the Bio-Assay dish top (which has a higher surface area than the bottom) with a piece of Whatman 3MM chromatography paper. Wet paper with 50% formamide/4× SSPE and lay the slides containing the sections on the paper.
- 2** Place hybridization solution containing $\sim 1 \times 10^6$ dpm/50 μ l 35 S-labeled (or 1 to 5 μ l/50 μ l digoxigenin-labeled) riboprobe onto the tissue section on each slide and cover with a coverslip.

To cover two adult rat coronal sections under a 18 × 30-mm coverslip, 45 μ l of hybridization solution is needed. The amount of hybridization solution used should be scaled up proportionately if a larger coverslip is used to cover a larger tissue section. The authors have found no need to pretreat the coverslips, but they should be dust-free. If background is a problem, use of silanized coverslips should be considered.
- 3** Cover the slides with the bottom of the Bio-Assay dish and incubate 20 to 24 hr at 55°C.
- 4** Remove slides from incubator and place with frosted ends up into a staining dish containing 1× SSPE/1 mM DTT. Gently slide the coverslip until it partially overhangs the slide, then pry off the coverslip with a forceps.
- 5** Place the slide without a coverslip in a slide rack and immerse in a tub containing 4× SSPE/1 mM DTT. Repeat step 4 with each slide until all the slides have had their coverslips removed and been placed in the same rack.

Do not allow the sections to dry until step 9.
- 6** Wash the slides by immersing successively for 5 min each time in four changes of room temperature 4× SSPE/1 mM DTT.

- 7 Incubate slides in RNase A solution 30 min at 37°C. Rinse twice, each time by immersing 5 min in 0.1× SSPE/1 mM DTT at room temperature.
- 8 Wash the slides by immersing successively for 30 min each time in two changes of 65°C 0.1× SSPE/1 mM DTT (kept at 65°C in a water bath), then for 5 min each time in two changes of room temperature 1× SSPE.
- 9a *For ³⁵S-labeled probes:* Rapidly dip the slides into water and then into 70% ethanol, then blow them dry while the slides are oriented with their frosted ends down. Proceed to detection (see Basic Protocol 6).
- 9b *For digoxigenin-labeled probes:* Using slides directly from the 1× SSPE wash in step 8, proceed to detection (see Basic Protocol 4 or Alternate Protocol).

BASIC PROTOCOL 4

DETECTION OF DIGOXIGENIN-LABELED PROBES USING AP-CONJUGATED ANTI-DIGOXIGENIN ANTIBODIES

Digoxigenin-labeled probes are detected by using antibodies directed toward the digoxigenin moiety. These primary antibodies are usually conjugated to alkaline phosphatase (AP) or horseradish peroxidase (HRPO), either of which will deposit a colored reaction product in the presence of the appropriate substrate at the site of hybridized probe. The steps here are for AP-conjugated antibodies; see Alternate Protocol for discussion of the use of the HRPO-conjugated antibody, along with tyramide signal amplification (TSA).

Materials

Digoxigenin-labeled sections on slides (see Basic Protocol 1, step 6, or Basic Protocol 3, step 8)

TBS, pH 7.5 (*APPENDIX 2A*; room temperature)

Detection buffer (see recipe)

Alkaline phosphatase-conjugated sheep polyclonal anti-digoxigenin antibody (Sigma-Aldrich)

Development buffer (see recipe)

NBT/BCIP substrate working solution (see recipe)

1× SSPE (*APPENDIX 2A*)

Cytoseal 60 (Stephens Scientific) or similar organic-basic mounting medium

Staining dishes or tubs

Slide warmer

1. Transfer slides from the final SSPE wash after digoxigenin labeling (see Basic Protocol 1, step 6, or Basic Protocol 3, step 8) to a staining dish or tub containing TBS, pH 7.5. Incubate 5 min at room temperature, then replace the solution with fresh TBS, pH 7.5, and incubate an additional 5 min.

2. Transfer slides to a staining dish or tub containing detection buffer and incubate 30 min.
3. Prepare a 1:2000 dilution of alkaline phosphatase–conjugated anti-digoxigenin antibody in detection buffer. Incubate slides with this solution 5 to 16 hr at room temperature with gentle rocking.

The slides may be incubated in slide mailers at this step and step 6 to conserve reagents.

4. Transfer slides to a staining dish or tub containing TBS, pH 7.5, and incubate 10 min, then replace the solution with fresh TBS, pH 7.5, and incubate an additional 10 min.
5. Transfer slides to a staining dish or tub containing substrate buffer and incubate 5 min.
6. Incubate slides at room temperature in the dark with NBT/BCIP substrate working solution.

For most probes, 1 to 2 hr of development is sufficient, and the background is reduced compared with overnight incubation.

7. Wash slides four times, each time by immersing 30 min in fresh 1× SSPE.

These long washes eliminate residual NBT (and BCIP), which interacts nonspecifically with nuclear emulsion.

8. Dip slides briefly in water and blow dry. Thoroughly dry slides on a slide warmer.

Slides should be thoroughly dried on a slide warmer before mounting of coverslips; otherwise the signal may be lost.

9. Apply coverslips to slides using Cytoseal 60 or similar organic-based mounting medium, or proceed to autoradiographic detection (see Basic Protocol 6).

ALTERNATE PROTOCOL

TYRAMIDE SIGNAL AMPLIFICATION (TSA) DETECTION OF DIGOXIGENIN-LABELED PROBES

This alternative procedure for digoxigenin detection uses peroxidase-conjugated antibody in an amplification scheme involving biotinyl tyramide or fluorochrome-conjugated tyramide and subsequent detection with streptavidin-conjugated fluorochromes, horseradish peroxidase (HRPO), or alkaline phosphatase (AP). This technique offers greater sensitivity than the one in Basic Protocol 4, approaching that of radiolabeled probes. It also enables the researcher to develop the signal as a fluorescent product compatible with a second nonradioactive hybridization histochemical or immunohistochemical labeling.

Additional Materials (also see Basic Protocol 4)

Peroxidase-conjugated sheep polyclonal anti-digoxigenin antibody (Sigma-Aldrich)

TSA Plus Biotin kit (Perkin-Elmer) containing:

Plus Biotin TSA reagent

1xPlus Amplification Diluent

Streptavidin– Fluorochrome (Perkin-Elmer)

Streptavidin-HRPO conjugate (Perkin-Elmer)

TBS, pH 8.0 (*APPENDIX 2A*; room temperature)

Diaminobenzidine (DAB) tablets (Sigma)

Urea/hydrogen peroxide tablets (Sigma)

Cyto seal 60 (Stevens Scientific) or similar organic-basic mounting medium

1. Wash slides with TBS, pH 7.5, then with detection buffer, as for AP detection (see Basic Protocol 4, steps 1 and 2).
2. Prepare a 1:300 to 1:600 dilution of peroxidase-conjugated anti-digoxigenin antibody in detection buffer. Incubate slides with this solution 2 hr at room temperature with gentle rocking, and then overnight at 4°C.
3. Wash slides three times, each time by immersing for 5 min in fresh TBS, pH 7.5.
4. Follow the instructions of the kit
5. At the end you will have attached an abundance of biotin molecules to your bound probe

Detection of the biotin labeled probe may now proceed in several ways—through the use of streptavidin–Texas red or streptavidin-fluorescein conjugate (steps 8a to 10a), streptavidin-HRPO conjugate (steps 8b to 10b), or streptavidin–alkaline phosphatase conjugate (steps 8c to 10c)—in increasing order of sensitivity.

To label with Texas red or fluorescein

- 8a Prepare a 1:2000 dilution of streptavidin–Texas red or streptavidin-fluorescein in TBS, pH 7.5.
- 9a Transfer slides to this solution and incubate 60 min at room temperature.
- 10a Wash slides four times, each time by immersing 5 min in fresh TBS, pH 7.5.

For detection with HRPO and substrate

- 8b Prepare a 1:3000 dilution of streptavidin-HRPO conjugate in TBS, pH 7.5 containing 0.5% blocking reagent. Transfer slides to this solution, incubate 60 min at room temperature, then wash slides four times, each time by immersing 5 min in fresh TBS, pH 8.0.
- 9b Dissolve one DAB tablet and one urea/hydrogen peroxide tablet in 15 ml of TBS, pH 8.0. Transfer slides to this solution and incubate until a signal develops (~5 to 10 min).

Sometimes the signal becomes apparent while looking directly at the tissue section. With some other mRNAs, however, the sections need to be examined microscopically.

- 10b** Wash slides twice, each time by immersing 5 min in fresh TBS, pH 8.0.

For detection with alkaline phosphatase and substrate

- 8c** Prepare a 1:25,000 dilution of streptavidin-alkaline phosphatase conjugate in detection buffer.
- 9c** Transfer slides to this solution and incubate 60 min at room temperature.
- 10c** Develop with NBT/BCIP substrate and wash with SSPE (see Basic Protocol 4, steps 4 to 7).
- 11** Dip slides briefly in water and blow dry. Thoroughly dry slides on a slide warmer.

Slides should be thoroughly dried on a slide warmer before application of coverslips; otherwise the signal may be lost.

- 12** Apply coverslips to slides using Cytoseal 60 or similar organic-based mounting medium, or proceed to autoradiographic detection (see Basic Protocol 6).

One can also use fluorescein-12-UTP (Sigma-Aldrich) instead of digoxigenin-labeled UTP at the same concentrations to label RNA for nonradioactive hybridization histochemistry (see Support Protocol 2 and Basic Protocol 3). The tyramide signal amplification method described here is then used with a HRPO-conjugated sheep polyclonal anti-fluorescein antibody (Sigma-Aldrich), followed by biotinylated tyramide and streptavidin-fluorochrome or streptavidin-enzyme. The biotinylated tyramide and streptavidin-alkaline phosphatase detection is very sensitive, close to that of radiolabeled probes (the new TSA Plus system from Perkin-Elmer is about ten times more sensitive, but increases background). Furthermore, this permits the simultaneous detection of two different transcripts by nonradioactive means. After detection of the digoxigenin-labeled probe (see Basic Protocol 4), proceed without drying to tyramide signal amplification and use the HRPO-conjugated sheep polyclonal anti-fluorescein antibody in step 2 of this protocol (in place of the anti-digoxigenin antibody used here); follow this with the biotinyl tyramide and streptavidin-fluorochrome or streptavidin-enzyme detection steps.

BASIC PROTOCOL 5

DETECTION OF CHROMOSOMAL DNA (Y CHROMOSOME) USING RIBOPROBES

Gender-mismatched bone marrow and tissue transplants have been widely used to follow cell fate. This approach requires the identification of the typically male donor tissue in the presence of the typically female host tissue. Thus, localization of the Y chromosome permits

this identification. This protocol provides a reliable means of detecting the Y chromosome, in combination with antigen detection if desired.

Due to the high temperature needed to “melt” DNA there is a chance that sections might detach from the slides. The authors have found that the use of Superfrost (positively charged) slides will generally avoid this problem.

Materials

Slides containing defatted 12- μ m fresh-frozen or paraffin-embedded sections prepared by cryostat (*UNIT 1.1*) or whole fixed cells

Paraformaldehyde–picric acid solution (see recipe)

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

0.1 $\times$, 0.5 $\times$, 1 $\times$, and 2 $\times$ SSC (*APPENDIX 2A*)

Citrisolv (Fisher) or xylene

70%, 80%, 95%, and 100% ethanol

Citra Plus, pH 6 (BioGenex)

Optional: Reagents for combined immunocytochemistry/immunohistochemistry including:

Blocking solution: PBS containing 1% (w/v) BSA and 0.6% (v/v) Triton X-100

Biotinylated secondary antibody (from Vectastain ABC kit, Vector Laboratories, or Jackson Immunoresearch)

0.1 M Tris-Cl, pH 8 (*APPENDIX 2A*)

Streptavidin-HRPO conjugate (Perkin-Elmer)

Biotinylated tyramide (Perkin-Elmer)

Fluorochrome-labeled streptavidin of different color from that used to develop the hybridization histochemical signal

Hybridization solution (see recipe) containing 3 to 10 μ l/100 μ l digoxigenin-labeled riboprobe (Support Protocol 2) to a mouse repeat sequence (Bishop and Hatat, 1987; Mezey et al., 2000) or human Y-chromosomal marker DYZ1 (Mezey et al., 2003a)

70% formamide/2 $\times$ SSC

Plastic Coplin jar

Staining dishes and tubs

Slide rack

Sterile Bio-Assay dishes (Nunc, 245 $\times$ 245 $\times$ 30 mm)

55°, 65°, and 80°C incubators

65° and 80°C water bath

Prepare specimens

For fresh-frozen sections and whole fixed cells

- 1a** Immerse slides for 5 min in 1% paraformaldehyde–picric acid solution.
- 2a** Wash slides three times, each time for 5 min, in 1× PBS, then once for 5 min in 1× SSC.

For fresh-frozen tissues, go to step 4 for combined immunocytochemistry/immunohistochemistry or to step 5 for hybridization histochemistry alone. For whole fixed cells, go to step 3.

For paraffin-embedded sections

- 1b** Using a slide rack, immerse slides containing tissue sections in staining dishes containing Citrisolv (or xylene) three times, each time for 7 min, to deparaffinize.
- 2b** Immerse slides in 100% ethanol three times, each time for 3 min. Rehydrate by immersing slides successively, each time for 5 min, in 95%, 80%, and 70% ethanol, then rinsing briefly in water. Go to step 3.

Extract with Citra Plus

- 3** Place slides containing deparaffinized sections or whole fixed cells in a plastic Coplin jar filled with Citra Plus and transfer to a microwave oven. Bring to a boil at 600 W and then adjust power to 450 W for 15 min.

Paraffin-embedded sections as well as whole fixed cells need to be microwave-pretreated to allow access to nuclear DNA. Cooking times must be determined individually for each paraffin block, due to differing fixation conditions (initially perform a time course starting with 1 min to determine correct conditions for the particular tissue). Citra Plus is the optimal buffer for antigen unmasking.

- 4** Immerse slides in 70% ethanol at 4°C for 5 min, then rinse quickly in water.

If combined immunocytochemistry/immunohistochemistry is to be performed:

- a.** Incubate slides in blocking solution for 10 min at room temperature.
- b.** Incubate slides in appropriately diluted primary antibody 1 hr at 37°C or overnight at 4°C.
- c.** Wash three times in PBS, each time for 5 min at room temperature.
- d.** Dilute biotinylated secondary antibody 1:1000 in blocking solution. Incubate slides in diluted secondary antibody 1 hr at room temperature.
- e.** Wash three times in PBS, each time for 5 min at room temperature.
- f.** Dilute streptavidin-HRP conjugate 1:250 in blocking solution. Incubate slides in diluted streptavidin-HRP conjugate 30 min at room temperature.

- g. Wash three times in 0.1 M Tris·Cl, pH 8, each time for 5 min at room temperature.
- h. Prepare a 1:50 to 1:100 dilution of biotinylated tyramide in 1× diluent (provided in Renaissance TSA-Indirect kit). Incubate slides in the biotinylated tyramide solution 5 min at room temperature.
- i. Wash three times in 0.1 M Tris·Cl, pH 8, each time for 5 min at room temperature.

Continue with step 5. After step 11, add fluorochrome-labeled streptavidin (of a different color from that used to develop the hybridization histochemical signal) at a dilution of 1:500 to 1:2500 (see Alternate Protocol, step 8a) to visualize the antigen of interest (Tran et al., 2000).

- 5 Transfer to 0.2 N HCl at room temperature for 20 min, then rinse quickly in water.
- 6 Place slides in 2× SSC at 80°C (in a water bath) for 25 min and then transfer them to 2× SSC at room temperature to cool.

Perform hybridization histochemistry

- 7 Heat the hybridization buffer containing the probe at 80°C for 10 min. Simultaneously, place the slides in 70% formamide/2× SSC at 80°C for 10 min.
- 8 Incubate the sections in 50 to 80 µl of hybridization buffer containing the probe at 80°C for 12 min, then transfer the slides to a Bio-Assay dish at 55°C for 30 min.
- 9 Remove the coverslips in 2× SSC. Wash slides by immersing successively in 1×, 0.5×, and 0.1× SSC at room temperature, each time for 5 min.
- 10 Transfer the slides to 0.1× SSC at 65°C (in a water bath) for 30 min.

To block endogenous peroxidase prior to tyramide staining, the authors use DAKO Peroxidase Blocking Reagent (usually 3% hydrogen peroxide will also suffice) for 5 min at room temperature.
- 11 Perform tyramide signal amplification (TSA) detection of digoxigenin-labeled probes (see Alternate Protocol).

With human brain tissue, the autofluorescence of lipofuscin granules is a serious problem when using fluorescence hybridization histochemistry. After testing a variety of techniques, the authors have found that a quick staining with Sudan Black will completely eliminate autofluorescence without significantly affecting specific fluorescence. The technique is described in Romijn et al. (1999); in essence, it is a 2-min staining in 70% ethanol/Sudan Black at the end of the hybridization procedure.

BASIC PROTOCOL 6

DETECTION OF RADIOLABELED PROBES

Detection of radiolabeled probes entails apposition of the samples to X-ray film or phosphorimaging plates and subsequent “development.” Higher cellular resolution entails coating the sample with a nuclear emulsion as described here. These steps are performed *after* detection of the digoxigenin-labeled probes if the two types of probes are used simultaneously.

Materials

Ilford K5.D or Kodak NTB-3 nuclear emulsion

7.5 M ammonium acetate

³⁵S-labeled samples, slide-mounted (see Basic Protocols 1 and 3)

Kodak D-19 photographic developer, 17°C

Kodak Rapid Fix (without hardener), 17°C

Counterstain (optional): 0.4% toluidine blue, 2 µg/ml ethidium bromide, hematoxylin/eosin, or other stain of choice

Cytoseal 60 (Stephens Scientific) or similar organic-based mounting medium

Darkroom with safelight

Coplin jars

Spatula

40°C water bath

Black slide boxes

Desiccant capsules (e.g., Humi-caps from United Desiccants–Gates)

Black photography tape

Slide racks

Slide warmer

Prepare the nuclear emulsion for coating the sections

- 1 In a darkroom under safelight conditions, scoop out 40 ml of emulsion with a spatula and transfer into a Coplin jar containing 1.6 ml of 7.5 M ammonium acetate (for final ammonium acetate concentration of 300 mM).
- 2 Place the Coplin jar in a 40°C water bath for 20 to 30 min to allow air bubbles to rise. Mix gently and test for the complete elimination of bubbles by examining a clean slide after dipping it into the emulsion.
- 3 Dip ³⁵S-labeled, slide-mounted sections into the emulsion and allow to dry for several hours standing up.

Typically, place five slides in red plastic slide grips and dip them five at a time into the emulsion, then hang them from a custom-made Plexiglas holder.

- 4 Place the emulsion-coated slides in black slide boxes with desiccant capsules. Tape the edges of the box with black photography tape and store the boxes at 4°C in the dark.

Develop the emulsions and stain the tissue sections

- 5 Put the emulsion-coated slides in slide racks and develop as follows.
 - a. Immerse 2 min in 17°C Kodak D-19 developer with agitation every 30 sec.
 - b. Immerse 15 sec in running tap water with slight agitation.
 - c. Immerse 2 min in Kodak Rapid Fix (without hardener) with agitation every 30 sec.

The room lights may be turned on after all slides are fixed.

- 6 Rinse slides 8 min in running tap water. Counterstain, if desired, for 30 sec in 0.4% toluidine blue, 2 µg/ml ethidium bromide, hematoxylin/eosin, or other stain of choice, then rinse again briefly to remove excess stain.

Some stains may obscure colorimetric detection of the digoxigenin probe or destroy silver grains (e.g., periodic acid/Schiff reagent).

- 7 Dip slides very briefly into deionized water, then in 70% ethanol, and place on slide warmer to thoroughly dry.

Slides should be thoroughly dried on a slide warmer before application of coverslips; otherwise the signal may be lost.

- 8 Apply coverslips to slides using Cytoseal 60 or similar organic-based mounting medium.

SUPPORT PROTOCOL 1

PREPARATION OF OLIGONUCLEOTIDE PROBES FOR HYBRIDIZATION HISTOCHEMISTRY

Oligonucleotides, generally ~48 bases long, are labeled using terminal deoxynucleotidyl transferase (TdT) and either [α -³⁵S]dATP or digoxigenin-dUTP. When the radioactive label is used, the resultant probes have specific activities (>10,000 Ci/mmol) of approximately ten times the specific activity of the [α -³⁵S]dATP. The radioactive and nonradioactive probes produced by this protocol are then used in Basic Protocol 1.

Materials

5× tailing buffer (see recipe)

Oligonucleotide to be used as probe

[α -³⁵S]dATP (>1000 Ci/mmol; Perkin-Elmer)

Terminal deoxynucleotidyl transferase (TdT; Roche or Invitrogen)

250 μ M digoxigenin-dUTP/1 mM dNTP (or dATP) mix (ingredients available from Roche; store mix indefinitely at -20°C)

TE buffer, pH 7.6 (*APPENDIX 2A*)

4 M NaCl

25 $\mu\text{g}/\mu\text{l}$ yeast tRNA

70% and 100% ethanol

5 M dithiothreitol (DTT)

For radiolabeled probes

1a Prepare the following reaction mix on ice:

10 μl 5 $\times$ tailing buffer

Oligonucleotide to 0.1 μM

[^{35}S]dATP to 1 μM

70 to 100 U TdT

H₂O to 50 μl .

The ^{35}S -dATP should not be added in the form of a solution containing EDTA or similar chelator that will interfere with the tailing buffer (a color change indicates that this has happened). ^{32}P -dATP is easily substituted for ^{35}S -dATP at the same concentration in this reaction to make probes for northern analysis (see Support Protocol 3). For a 48-base oligonucleotide, 0.1 μM is ~ 15.6 ng/ μl (780 ng for a 50- μl reaction volume).

2a Incubate reaction ~ 2 to 5 min at 37°C to add 10 to 15 bases.

The enzyme lot may be examined to assess activity and number of nucleotides added per unit time. Recent experience with recombinant TdT indicates that reaction times need to be increased to upwards of 15 min.

For digoxigenin-labeled probes

1b Prepare the following reaction mix on ice:

10 μl 5 $\times$ tailing buffer

Oligonucleotide to 0.1 μM

1 μl 250 μM digoxigenin-dUTP/1 mM dNTP (or dATP) mix

70 to 100 U TdT

H₂O to 50 μl

2b Incubate 2 hr at 37°C .

3 Add 375 μ l TE buffer, pH 7.6, 25 μ l of 4 M NaCl, 50 μ g yeast tRNA, and 1 ml ethanol to the reaction mix from step 2a or 2b. Mix thoroughly, incubate 10 min on wet ice, then microcentrifuge 10 min at maximum speed.

4a *For radiolabeled probes:* Remove the supernatant and rinse the pellet with 1 ml of 70% ethanol. Microcentrifuge briefly at maximum speed, remove the ethanol, and add 50 μ l TE buffer, pH 7.6, and 1 μ l of 5 M DTT. Dissolve pellet with gentle vortexing and count 1 μ l of the radiolabeled probe in a scintillation counter and record the number of dpm. Store probe up to 3 months at 4°C or at -80°C for longer periods.

Expect ~500,000 dpm/ μ l. At this point, one can estimate the specific activity based on the total amounts of [³⁵S]dATP and oligonucleotide added and the amount of radioactivity recovered, conservatively assuming 50% recovery.

4b *For digoxigenin-labeled probes:* Remove the supernatant and rinse the pellet with 1 ml of 70% ethanol. Microcentrifuge briefly at maximum speed, remove the ethanol, and add 50 μ l TE buffer, pH 7.6. Dissolve pellet with gentle vortexing. Store indefinitely at 4°C.

Use 3 to 10 μ l of digoxigenin-labeled oligodeoxynucleotide probe to 100 μ l hybridization solution (see Basic Protocol 1). The exact amount must be determined empirically.

SUPPORT PROTOCOL 2

PREPARATION OF RNA PROBES (RIBOPROBES) FOR HYBRIDIZATION HISTOCHEMISTRY

Typically, the plasmid containing the cDNA to be transcribed is linearized with a restriction enzyme. It may be useful to subject a large amount (~10 μ g) of plasmid to restriction digestion if that particular probe is to be made repeatedly. Another approach is to generate PCR fragments of the desired cDNA using primers that contain different RNA polymerase transcription initiation sequences. The PCR fragments are gel-purified and used below instead of the linearized plasmid. The resulting radioactive or digoxigenin-labeled riboprobes are then used in Basic Protocol 3.

Materials

Linearized plasmid containing cDNA to be transcribed

[α -³⁵S]UTP (>1000 Ci/mmol; Perkin-Elmer)

5 $\times$ transcription buffer (see recipe)

100 mM dithiothreitol (DTT)

10 mM ATP, CTP, and GTP

RNasin (Promega)

10 to 20 U/ μ l RNA polymerase (SP6, T3, or T7)

1 mM UTP/4 mM digoxigenin-11-UTP mix (ingredients available from Roche; store mix indefinitely at -20°C)

1 U/ μl RNase-free DNase I (*APPENDIX 2A*)

TE buffer (*APPENDIX 2A*)

4 M NaCl

25 $\mu\text{g}/\mu\text{l}$ yeast tRNA

70% and 100% ethanol

5 M DTT

10% (w/v) sodium dodecyl sulfate (SDS)

TE buffer/5% (w/v) SDS

NOTE: Use DEPC-treated water (*APPENDIX 2A*) for all reagents.

For radiolabeled probes

- 1a** Combine ~250 ng of linearized plasmid (or 10 to 100 ng of PCR product) with [^{35}S]UTP at a final concentration of 20 to 30 μM in a microcentrifuge tube. Dry in a Speedvac evaporator at room temperature.

If [^{32}P]UTP (>3000 Ci/mmol; 10 mCi/ml) is used to make probes for northern analysis (see Support Protocol 3), use 5 μl of the 10 mCi/ml solution of the radiochemical and add 0.5 μl of 150 μM UTP to the components in step 2a below (15 μM cold UTP and ~3 μM [^{32}P]UTP, or 500 Ci/mmol, final concentrations).

- 2a** Add, at room temperature, to the tube with the dried [^{35}S]UTP and DNA:

1 μl 5 $\times$ transcription buffer

0.5 μl 100 mM DTT

0.5 μl 10 mM ATP

0.5 μl 10 mM CTP

0.5 μl 10 mM GTP

0.5 μl RNasin

0.5 μl of 10 to 20 U/ μl SP6, T3, or T7 RNA polymerase

H₂O to 5 μl .

Mix thoroughly, especially where the [^{35}S]UTP and DNA has pelleted.

The version of RNA polymerase to use depends on which RNA polymerase recognition sequence is present in the DNA.

For digoxigenin-labeled probes

- 1b** Combine at room temperature in a microcentrifuge tube:

~250 ng linearized plasmid
 0.5 μ l 1 mM UTP/4 mM digoxigenin-11-UTP mix
 1 μ l 5 $\times$ transcription buffer
 0.5 μ l 100 mM DTT
 0.5 μ l 10 mM ATP
 0.5 μ l 10 mM CTP
 0.5 μ l 10 mM GTP
 0.5 μ l RNasin
 0.5 μ l SP6, T3, or T7 RNA polymerase
 H₂O to 5 μ l.

- 2b** Mix thoroughly and proceed to step 3.
- 3** Incubate reaction mixture 30 min at 37°C and add another 0.5 μ l of the appropriate (SP6, T3, or T7) RNA polymerase.
- 4** Incubate 30 min at 37°C, then add 0.5 μ l RNasin and 0.5 μ l of 1 U/ μ l RNase-free DNase and continue incubating 10 min at 37°C.
- 5** Add 420 μ l TE buffer, 25 μ l of 4 M NaCl, 2 μ l of 25 μ g/ μ l tRNA, and 1 ml of 100% ethanol. Mix thoroughly, place on wet ice for 10 min, then microcentrifuge 10 min at maximum speed.
- 6** Remove the supernatant and rinse the pellet with 1 ml of 70% ethanol. Microcentrifuge briefly at maximum speed.
- 7a** *For radiolabeled probes:* Remove the ethanol and add 485 μ l TE buffer, 10 μ l of 10% SDS, and 5 μ l of 5 M DTT to dissolve the pellet. Count 1 μ l of radiolabeled probe in a scintillation counter and record the number of dpm. Freeze at -80°C until used.

Expect $\sim 1 \times 10^6$ dpm per μ l. One can estimate the specific activity based on the percentage of U in the transcript.

The reaction can be further miniaturized to a 1- μ l volume if several probes are labeled at once with the same enzyme. Dry 2 to 3 μ l of ³⁵S-UTP per reaction, then make up a cocktail with transcription buffer, ATP, CTP, GTP, RNasin, RNA polymerase, DTT, and water to 0.75 μ l per reaction (using the same relative amounts of the different reagents as in step 2a). Use this cocktail to redissolve the ³⁵S-UTP, then put 0.75- μ l aliquots in 0.5-ml screw-top microcentrifuge tubes. Add 50 ng linearized DNA template and a drop of mineral oil. Microcentrifuge briefly, then incubate 60 min at 37°C, then add 0.25 μ l each of DNase and RNasin, spin briefly, and incubate another 10 min. Add 400 μ l TE buffer, 25 μ l 4 M NaCl, and 50 μ g of tRNA to the tube. Extract once with chloroform to remove oil, and precipitate supernatant with 1 ml

ethanol on wet ice. Dissolve the pellet in 94 μ l TE buffer, 5 μ l 10% SDS, and 1 μ l 5 M DTT.

- 7b** *For digoxigenin-labeled probes:* Remove the ethanol and add 50 μ l TE buffer/0.5% SDS to dissolve the pellet. Store in aliquots indefinitely at -80°C .

Use 3 to 10 μ l of digoxigenin-labeled riboprobe to 100 μ l of hybridization solution (see Basic Protocol 3). The exact amount must be determined empirically.

SUPPORT PROTOCOL 3

NORTHERN ANALYSIS USING OLIGODEOXYNUCLEOTIDE PROBES AND RIBOPROBES

Northern analysis of RNA samples using the same probe sequences is a useful control to determine if the probe is detecting previously characterized transcripts. Although DNA/RNA or RNA/RNA hybridizations on synthetic membranes are unlikely to be kinetically identical to those performed on tissue sections, they are likely to be similar under the same temperature, salt, and formamide conditions. Furthermore, if bands are observed representing mRNA sizes different from what have been reported previously (probably using double-stranded cDNA probes), then one must consider the likelihood that the hybridization histochemistry is also detecting these other transcripts which may or may not be transcribed from the same gene.

Materials

RNA sample

GeneScreen membrane (Perkin-Elmer)

Northern hybridization solution (see recipe)

Northern hybridization solution containing $\sim 2 \times 10^6$ dpm/ml ^{35}S -labeled oligodeoxynucleotide probe (see Support Protocol 1) or riboprobe (see Support Protocol 2) $1\times$ SSPE (*APPENDIX 2A*)/0.2% SDS, room temperature and 55°C (for oligodeoxynucleotide probes)

$0.1\times$ SSPE/0.2% SDS, 65°C (for riboprobes)

UV cross-linker (e.g., Stratalinker from Stratagene)

70°C vacuum oven

Plastic wrap

Autoradiography cassettes

Intensifying screens

Autoradiography film (e.g., Kodak Biomax MR)

NOTE: Use DEPC-treated water (*APPENDIX 2A*) for all reagents.

1. Size-fractionate RNA and transfer to a GeneScreen membrane.

CPMB UNIT 4.9 provides detailed instructions on how to perform this transfer.

2. Cross-link RNA to the membrane using a UV cross-linker. Bake filter 2 hr at 70°C in a vacuum oven.

CPMB UNIT 3.19 describes procedures for optimizing UV cross-linking.

For northern analysis using oligodeoxynucleotide probes

- 3a** Add ~0.5 to 1 ml northern hybridization solution per 10 cm² of membrane and incubate 3 hr at 37°C.

The prehybridizations, hybridizations, and washes may be performed conveniently in small roller bottles in an oven. Prewarmed solutions may simply be decanted and added to the bottles containing the membranes.

- 4a** Remove northern hybridization solution and replace with the same amount of northern hybridization solution containing $\sim 2 \times 10^6$ dpm/ml ³⁵S-labeled oligodeoxynucleotide probe. Incubate 18 hr at 37°C.
- 5a** Rinse membrane twice briefly with 1× SSPE/0.2% SDS at 55°C, then wash five times, each time by immersing the membrane 15 min in fresh 1× SSPE/0.2% SDS at 55°C.

For northern analysis using riboprobes

- 3b** Add ~0.5 to 1 ml northern hybridization buffer per 10 cm² of membrane and incubate 18 hr at 65°C.

The prehybridizations, hybridizations, and washes may be performed conveniently in small roller bottles in an oven. Prewarmed solutions may simply be decanted and added to the bottles containing the membranes. If hybridizations are performed in plastic bags, the membranes should be double-bagged.

- 4b** Remove northern hybridization solution and replace with the same amount of northern hybridization solution containing $\sim 2 \times 10^6$ dpm/ml ³⁵S-labeled riboprobe. Incubate 24 hr at 65°C.
- 5b** Rinse twice briefly with 0.1× SSPE/0.2% SDS at 65°C and then wash four times, each time by immersing the membrane 30 min in fresh 0.1× SSPE/0.2% SDS at 65°C.

The membranes must not be allowed to cool below 65°C. The washes are therefore done by placing the plastic bags in 65°C water in a large beaker, cutting open the ends of the bags above the water's surface, then pulling the membranes out and immediately placing them in the 65°C wash buffer. These washes are more easily performed with roller bottles.

- 6** Place the membrane between sheets of plastic wrap in an autoradiography cassette with intensifying screens and appose to film at –80°C.

REAGENTS AND SOLUTIONS

Use DEPC-treated water (**APPENDIX 2A**) to make solutions for pretreatment and hybridization. Deionized water may be used for the subsequent wash and digoxigenin development steps; for suppliers, see **SUPPLIERS APPENDIX**.

Detection buffer

100 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)

150 mM NaCl

5% (v/v) normal goat serum (Vector Labs)

0.6% (v/v) Triton X-100

Store indefinitely at −20°C

Development buffer

100 mM Tris·Cl, pH 9.5 (*APPENDIX 2A*)

100 mM NaCl

50 mM MgCl₂

Store up to 1 year at room temperature

Hybridization solution

Solution A:

100 µg/ml salmon sperm DNA (Sigma)

250 µg/ml yeast total RNA (Sigma)

250 µg/ml yeast tRNA (Sigma)

Store indefinitely at −20°C

Solution B:

23.8 ml formamide (Ultrapure; Invitrogen)

0.95 ml 1 M Tris·Cl, pH 7.4 (*APPENDIX 2A*)

0.19 ml 250 mM EDTA, pH 8.0

3.75 ml 4 M NaCl

9.52 ml 50% (w/v) dextran sulfate (Sigma)

0.95 ml 50× Denhardt's solution (*APPENDIX 2A*)

H₂O to 40 ml

Store indefinitely at −20°C

Solution C: Combine appropriate quantity of labeled probe (see Basic Protocol 1 or 3) with 4 μ l solution A and water to 12 μ l. Mix, then heat 5 min at 65°C and cool rapidly on ice to room temperature.

Working solution:

Add to solution C as prepared above:

84 μ l solution B

2 μ l 5 M DTT

1 μ l 10% (w/v) sodium thiosulfate

1 μ l 10% (w/v) sodium dodecyl sulfate (SDS)

Mix well and use immediately

Prepare all solutions with DEPC-treated water. The recipe may be scaled up appropriately.

NBT/BCIP substrate solution

Stock solutions:

75 mg/ml nitroblue tetrazolium chloride (NBT) in dimethylformamide (Invitrogen)

50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in dimethylformamide (Invitrogen)

Working solution:

Prepare in development buffer (see recipe):

0.34 mg/ml NBT (from 75 mg/ml stock)

0.18 mg/ml BCIP (from 50 mg/ml stock)

Prepare fresh just prior to use

Levamisole (Vector Labs) may be added to a concentration of 1 mM to block peripheral-type endogenous alkaline phosphatase (see Critical Parameters).

Northern hybridization solution

20 ml formamide

2 ml 20 $\times$ SSPE (*APPENDIX 2A*)

8 ml 50% (w/v) dextran sulfate (Sigma)

4 ml 50 $\times$ Denhardt's solution (*APPENDIX 2A*)

400 μ l 25 mg/ml yeast tRNA (Sigma)

500 μ l 20 mg/ml yeast total RNA (Sigma)

400 μ l 10 mg/ml single-stranded DNA (Sigma)

400 μ l 10% (w/v) sodium dodecyl sulfate (SDS)

H₂O to 40 ml

Store indefinitely at –20°C

Use DEPC-treated water for all solutions.

Paraformaldehyde–picric acid solution—Add 40 g paraformaldehyde to 500 ml water and heat to 60°C. Add 10 N NaOH until paraformaldehyde dissolves (e.g., until the mixture clears). Cool to below 30°C and add 100 ml of 10× PBS (*APPENDIX 2A*), 150 ml saturated picric acid, and water to 1 liter. Dilute 1 part of this solution with 3 parts PBS, to obtain 1% paraformaldehyde working solution. Prepare fresh or freeze at –20°C until use.

RNase A solution

Stock solution:

62.5 ml 4 M NaCl

2.5 ml 2 M Tris·Cl, pH 8.0 (*APPENDIX 2A*)

0.5 ml 0.25 M EDTA, pH 8.0 (*APPENDIX 2A*)

Store up to 1 year at room temperature

Working solution: Just prior to use, prewarm 500 ml of RNase buffer (see above) to 37°C and add 1 ml of 10 mg/ml RNase A. Use immediately.

In the authors' experience, some lots of RNase A are more potent than others. When a new lot of RNase A is obtained, a range of concentrations should be tested to determine the best signal-to-noise ratio.

Tailing buffer, 5×

500 mM potassium cacodylate, pH 7.2

10 mM CoCl₂

1.0 mM DTT

Store indefinitely at –20°C

Transcription buffer, 5×

200 mM Tris·Cl, pH 7.9 (*APPENDIX 2A*)

30 mM MgCl₂

50 mM NaCl

10 mM spermidine

Store indefinitely at –20°C

Prepare using DEPC-treated water.

COMMENTARY

Background Information—Hybridization histochemistry provides a method for detecting specific mRNAs in tissue sections. Furthermore, as mRNA levels may change from one state to another (e.g., during development or after physiological manipulations), hybridization histochemistry can provide “snapshots” through the course of a dynamic situation. This unit reprises the authors’ protocols for examining the expression of genes within tissue sections at a light-microscopic resolution (Young et al., 1986, 1990; Bradley et al., 1992; Young, 1992; Pagani et al., 2015). There are a number of excellent sources of information for the reader interested in localizing transcripts in whole-mount tissues, to chromosomes, or at the electron microscopic level (Wilkinson, 1992; Rosen and Beddington, 1993; Albertson et al., 1995; Morey, 1995; Swiger and Tucker, 1996).

Hybridization histochemistry is generally amenable to combination with other techniques such as immunohistochemistry (*UNIT 1.2*), tract-tracing (Burgunder and Young, 1988), and in vitro receptor autoradiography (Westlake et al., 1994). The combination of hybridization histochemistry with immunohistochemistry and/or tract-tracing may necessitate perfusion fixation of the animal (in order to preserve immunoreactivity and/or tracer deposition) prior to freezing the specimen and sectioning it. These sections, however, generally have a reduced “signal-to-noise” ratio for the hybridization histochemistry. The immunohistochemical steps are usually performed after the hybridization-histochemical ones to avoid loss of mRNA from exposure to RNases present in the antibody and development solutions. To use the same tissue for in vitro receptor autoradiography and hybridization histochemistry, alternate fresh-frozen sections are used.

With the increasing interest in the fate of cell lineages in stem cell research, the technique of chromosomal hybridization to detect the Y chromosome in gender-mismatched transplants has become popular. This technique avoids many problems associated with gene silencing when using promoter-specific expression of fluorescent proteins or β -galactosidase (Mezey et al., 2003b; Theise et al., 2003).

Critical Parameters and Troubleshooting—Successful hybridization histochemistry seeks a balance between preserving tissue morphology and permeabilizing the tissue to allow access of the probe to the transcripts. Whereas a number of protocols utilize HCl and/or proteases to permeabilize the tissue sections, the approach described in this unit avoids these harsh treatments through the use of chloroform to remove fat from the sections. However, paraffin-embedded tissue sections require the use of protease.

The authors’ experience has shown that longer riboprobes offer greater sensitivity because they target longer stretches of the transcripts than single oligodeoxynucleotide probes. Theoretically, and in practice, riboprobes are more sensitive than the equivalent stretch of bases presented by labeled, double-stranded cDNA probes. Some researchers also employ alkaline hydrolysis of their riboprobes to increase the ease of tissue penetration. The authors have not found this treatment to help with the protocols in this unit, and such a step may result in inconsistency in the probe sizes produced. Other approaches and further discussion are presented in Valentino et al. (1987) and Wilkinson (1992). The use of multiple oligodeoxynucleotide probes targeted against the same transcript can significantly improve

sensitivity, compared to a single oligodeoxynucleotide probe. In fact, the more recently developed use of multiple oligonucleotide pairs (ViewRNA®, RNAscope®), that must hybridize next to each other for the amplification to occur, are extremely rapid and sensitive while offering high resolution.

The authors label probes for hybridization histochemistry with ^{35}S for a number of reasons. It provides greater resolution and higher efficiency of grain production than either ^{32}P or ^{33}P . Also, it has a half-life of 87 days, compared with 14 and 25 days for ^{32}P and ^{33}P , respectively. These considerations more than compensate for the lower specific activity of ^{35}S . Although ^3H provides greater resolution and has a much longer half-life, its specific activity is so low that it is not practical for labeling probes targeted against transcripts of relatively low abundance. The authors generally use digoxigenin-labeled probes to enable simultaneous detection of two different transcripts within the same tissue sections (and within the same cells). The radiolabeled probes permit more accurate quantitation of transcript levels and are still more sensitive.

Controls for specificity are, of course, the essence of any experiment. Unfortunately, there is no single, absolute control for hybridization histochemistry. Instead, the researcher relies on as many different checks as possible. The ones preferred by the authors of this unit are as follows (in a roughly descending order of usefulness).

1. Observing the same distribution of signal with probes directed against different portions of the same transcript.
2. Observing blockage of signal by prior hybridization with unlabeled probe.
3. Correlation of signal with immunocytochemical results.
4. Observing different distribution of signal with probes against unrelated transcripts, including sense probes. The investigator should be aware, however, that occasionally the “sense” probe detects mRNA transcribed from the opposite DNA strand. However, a signal with the sense probe does not necessarily invalidate the findings obtained with the antisense probe.
5. Observing that northern analysis using the probe under the same degrees of stringency shows band(s) of expected size(s).

An especially good control but of limited availability, is the use of knockout animals or tissues from humans with mutations that eliminate expression of the gene of interest. In these cases, one should not observe a transcript if the probes are specific (or there is not a duplication allowing expression).

Two commonly used “controls” are not recommended. The use of RNase prior to hybridization is analogous to using a protease prior to immunohistochemistry and the dilution of labeled probe with unlabeled probe only serves to reduce the specific activity of the probe. Both of these procedures are essentially of no use.

One should be aware of the potential artifacts that arise from autoradiography and/or color techniques. Positive and negative chemography—i.e., the spurious creation and destruction of grains, respectively—are constant concerns with autoradiography. Positive chemography

is probably more common, and is best assessed using sections that were not hybridized or that were hybridized with a sense probe. Grains are especially susceptible to loss during staining or after coverslipping if moisture still remains in the tissue sections. These and other aspects of autoradiography are expertly discussed by Rogers (1979). Color-development artifacts with alkaline phosphatase may be due to endogenous peripheral-type enzyme, which may be blocked with levamisole. Intestinal alkaline phosphatase is more refractory, and requires a brief (10-min) treatment with 0.1 M HCl at room temperature (Kiyama and Emson, 1991). Also, DTT that is present during the enzymatic development can impart a strong purplish color. The use of nonhybridized sections should reveal whether adventitious color formation is occurring. Loss of alkaline phosphatase staining occurs with exposure to ethanol.

As noted above, the more recently developed use of multiple oligonucleotide pairs (ViewRNA®, RNAscope®) must hybridize next to each other for the amplification to occur and provide high sensitivity. Furthermore, they may be less prone to some of the above concerns. This is because the proprietary probe designers make use of extensive genomic and transcript databases to avoid any cross-hybridizations.

Anticipated Results—Hybridization histochemistry should enable the researcher to determine whether a given gene is expressed in particular cells. Figure 1.3.1 shows the simultaneous detection of two different transcripts using radiolabeled and digoxigenin-labeled probes. Riboprobes are more sensitive than single oligodeoxynucleotide probes, enabling one to see five or fewer transcripts per cell, and traditionally, ³⁵S-labeled probes are more sensitive than colorimetrically detected ones. Furthermore, the recent introduction of the tyramide amplification system (TSA, also known as catalyzed reporter deposition or CARD; Bobrow et al., 1989) offers a number of branch points for varying degrees of amplification and different reaction products (Kerstens et al., 1995; Hunyady et al., 1996, Toth and Mezey, 2007). However, we believe that the use of multiple oligonucleotide pairs and the insertion of a non-mammalian sequence in the reaction for higher specificity (ViewRNA®, RNAscope®) may supplant the use of the other techniques in time. Their principle drawback is the relatively higher costs per slide (i.e., design and purchase of probe sets as well as kit reagents).

For quantitative analysis of autoradiograms, X-ray or tritium-sensitive films may provide the easiest means of quantitation if the signal is sufficient and the cells are closely grouped. In these cases, the film optical densities can be used to calculate the number of copies of probe hybridized through the use of simultaneously exposed brain-paste standards that incorporate known amounts of the radioisotope. Phosphorimaging devices (e.g., those sold by Fuji Medical Systems or Molecular Dynamics; see *SUPPLIERS APPENDIX*) offer two advantages over films: their sensitivity is up to 40-fold greater and the signals are directly proportional to the amount of hybridized radiolabeled probe. The authors generally examine sections with the phosphorimaging system prior to dipping them into nuclear emulsion. Detailed protocols for quantitative analysis of autoradiograms are available (Gerfen, 1989; Young, 1992). Multiple oligonucleotide pairs (ViewRNA®, RNAscope®) results in colored dots in the tissues that may be readily counted (Figure. 1.3.2). These signals are often

detected under brightfield (or even darkfield) illumination, but if the level of transcripts are low, fluorescent illumination will be best.

Time Considerations—Hybridization histochemistry may be viewed as being composed of three steps: preparation of the tissue sections, hybridization and washing of the sections, and detection of the hybridization signal. Preparation of the tissue sections, after collection of the tissue specimens, essentially consists of cutting the sections and, of course, depends upon the number of sections needed and the size of the region(s) studied. This may take hours to weeks.

The hybridization and washing steps take 2 consecutive days for radiolabeled probes or 3 consecutive days if digoxigenin-labeled probes are used. Detection of the digoxigenin-labeled probes is then complete at the end of the third day. Depending on the signal strength and degree of resolution needed, radiolabeled-probe deposition can be determined over the course of minutes using film or phosphorimaging plates, but may take months after coating with nuclear emulsion. Multiple oligonucleotide pairs (ViewRNA®, RNAscope®) results are obtained in 2 or 3 days for single or duplex results, respectively. Within this latter approach, the researcher may wish to extend the time of hybridization (Basic Protocol 2, step 18) or amplification development (Basic Protocol 2, step 27) to see if the signal improves.

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Figure 1.3.1.

Photomicrograph shows vasopressin and oxytocin neurons within the rat paraventricular nucleus of the hypothalamus labeled respectively, with ^{35}S -labeled and digoxigenin-labeled probes. The arrows indicate neurons containing both transcripts. Neurons containing the digoxigenin-labeled probe show a dark stain from development of the alkaline phosphatase on the anti-digoxigenin antibodies. The deposition of the radiolabeled probe is indicated by the gray-colored silver grains. Note that a nucleus (n) is clearly delineated by the alkaline phosphatase staining. (Basic Protocols 1, 4, and 6)

Figure 1.3.2.

Photomicrograph shows oxytocin (blue) and vasopressin 1b (red) receptors simultaneously in pyramidal neurons (DAPI, pseudocolored green) within the mouse CA2 region of the hippocampus. The transcripts were detected using the ViewRNA kit (Basic Protocol 2).