Published in final edited form as:

Curr Protoc Neurosci. ; 82: 1.3.1–1.3.27. doi:10.1002/cpns.39.

Hybridization Histochemistry of Neural Transcripts

W. Scott Young¹, June Song¹, and Éva Mezey²

¹Section on Neural Gene Expression, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland ²Adult Stem Cell Section, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland

Keywords

In situ hybridization; mRNA; Riboprobes; ViewRNA; Digoxigenin

INTRODUCTION

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 in different tissues or in the same tissues in different states (e.g., physiological or developmental) may be quantified. This unit describes hybridization histochemical techniques using either oligodeoxynucleotide probes (see Basic Protocols 1 and 2, Alternate Protocol 1) or RNA probes (riboprobes; see Basic Protocols 3 and 5). These methods include a more recent approach using commercially available sets of oligodeoxynucleotide pairs for colorimetric and fluorescent detection (see Basic Protocol 2), as well as a method for detection of the Y chromosome using either mouse or human riboprobes (see Basic Protocol 5). Additional methods include colorimetric detection (see Basic Protocol 4) and tyramide signal amplification (TSA) of digoxigenin-labeled probes (see Alternate Protocol 2), and autoradiographic detection of radiolabeled probes (see Basic Protocol 6). Finally, methods are provided for labeling oligodeoxynucleotide (see Support Protocol 1) and RNA (see Support Protocol 2) probes, and verifying the probes by northern analysis (see Support Protocol 3). © 2016 by John Wiley & Sons, Inc.

HYBRIDIZATION HISTOCHEMISTRY WITH OLIGODEOXYNUCLEOTIDE

PROBES

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

Materials

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

12- μ m cryostat tissue sections mounted on Superfrost Plus slides (Fisher) and defatted (*UNIT 1.1*; Gerfen, 2003)

Hybridization solution (see recipe)

^{35}S - or digoxigenin-labeled oligodeoxynucleotide probe (see Support Protocol 1)

1 $\times$ SSPE/1 mM dithiothreitol (DTT)

70% (v/v) ethanol

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

Whatman 3MM chromatography paper

Glass coverslips

Staining dishes with slide racks

55°C water bath

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

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

The top of the dish has a larger surface area than the bottom. Each top can hold 20 slides.

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

For example, to cover two adult rat coronal sections under an 18 $\times$ 30-mm coverslip, use 45 μ l hybridization solution. The amount of hybridization solution used should be scaled up proportionately if a larger coverslip is used to cover a larger tissue section.

There is generally no need to pretreat the coverslips, but they should be dust-free. If background is a problem, the use of silanized coverslips should be considered.

3. Cover 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.

5. Gently slide the coverslip from the first slide until it partially overhangs the slide, then pry it off with forceps. Place the slide in a slide rack and immerse in a staining dish containing 1 $\times$ SSPE/1 mM DTT.

6. Repeat until all the slides are in the same rack.

Do not allow the sections to dry prior to step 8.

7. Wash slides by immersing successively as follows:

four times for 15 min each in 55°C 1× SSPE/1 mM DTT

two times for 5 min each in room temperature 1× SSPE/1 mM DTT.

8a. *For ³⁵S-labeled probes*: Rapidly dip the slides in water and then 70% ethanol. Hold slides with their frosted ends oriented down and blow them dry. Proceed to detection (see Basic Protocol 6).

8b. *For digoxigenin-labeled probes*: Use slides directly from the SSPE/DTT wash for detection (see Basic Protocol 4 or Alternate Protocol 2).

HYBRIDIZATION HISTOCHEMISTRY FOR DETECTION OF A SINGLE TRANSCRIPT USING SETS OF OLIGODEOXYNUCLEOTIDE 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 to 20 pairs of oligodeoxynucleotides that must hybridize to adjacent stretches of the RNA in order to generate a signal through branched DNA amplification. This protocol uses the ViewRNA kit and probes (Affymetrix), but RNAscope (Advanced Cell Diagnostics) relies on the same underlying patents (e.g., U.S. Patent No. 7,709,198). Very little molecular biological expertise is needed for this approach.

Probe sets are custom designed by Affymetrix. Each probe set is made to appear either red (Fast Red, Type 1) or blue (Fast Blue, Type 2) under incandescent light upon amplification. With fluorescent illumination, they will appear red or far red (not visible to the naked eye, camera-sensitive only), respectively. For a modified method for simultaneous detection of two transcripts using both labels, see Alternate Protocol 1.

Materials

37% (w/v) formaldehyde

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

16-μm cryostat tissue sections (*UNIT 1.1*; Gerfen, 2003) mounted on Superfrost Plus slides (Fisher)

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

Wash Buffer Components 1 and 2

Probe Set Diluent QT

Protease QF

PreAmplifier Mix QT

Amplifier Mix QT

Label Probe Diluent QF

AP Enhancer Solution

Fast Red tablets

Naphthol Buffer

Blue Buffer

Blue Reagents 1–3

Label Probe 1-AP

Label Probe 6-AP

50%, 70%, and 100% (v/v) ethanol

Custom-designed probe set(s) (Affymetrix)

Bacterial Type XXIV Proteinase (Sigma-Aldrich, cat. no. P8038)

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

M Tris-Cl, pH 7.4 (*APPENDIX 2A*)

Coplin jars

Staining dishes with slide racks

Oven at 60°C

Hydrophobic barrier pen

HyBEZ Oven (Advanced Cell Diagnostics) with slide holder and covered tray

Whatman 3MM chromatography paper

Coverslips

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

Fix tissue sections

1. Prepare 4% formaldehyde by adding 22 ml of 37% formaldehyde to 178 ml PBS. Fill the required number of Coplin jars and refrigerate at least 1 hr at 4°C.
2. Fix slides with tissue sections in cold formaldehyde overnight (16 to 18 hr) at 4°C.

The protocol is described for standard-size microscope slides with two rat or four mouse 16-µm-thick coronal brain sections placed closely together. Slides can be stored indefinitely at –80°C before use.

Prepare reagents and washes

3. Prepare wash buffer by mixing 27 ml Wash Buffer Component 1 and 7.5 ml Wash Buffer Component 2 in 2.5 liters deionized H₂O.
4. Prepare a set of Coplin jars containing the following reagents:
PBS (two jars)

50%, 70%, and 100% ethanol

Wash buffer.

5. Prewarm 40 ml PBS and Probe Set Diluent QT to 40°C.

6. Thaw custom-designed probe set(s) and place on ice.

Dehydrate and denature sections

7. Remove slides from fixative and wash twice for 1 min each in PBS with constant agitation up and down, making sure tissues are completely submerged.

8. Transfer slides successively to 50%, 70%, and 100% ethanol for 10 min each.

9. Remove slides and heat in a 60°C oven for 30 min.

IMPORTANT NOTE: *Begin protease digestion within 1 hr of this step.*

10. Allow slides to cool, then use a hydrophobic pen to draw a thick rectangle surrounding the tissues. Let dry and repeat several times to ensure a solid boundary. Let final barrier dry at least 20 min at room temperature.

Digest proteins and fix tissues

11. Dilute stock protease solution in PBS to give 400 µl of 1× working solution per slide.

For ViewRNA Protease QF, dilute 1:100

For optimized Bacterial Type XXIV Proteinase, dilute 1:16

Bacterial Type XXIV Proteinase (Sigma-Aldrich) is recommended by Affymetrix.

Before use, it should be optimized in trial hybridizations to determine the best

signal. A final working concentration of 2 U/ml should be a good starting point.

Once optimized, the proteinase should be stored in 1-ml aliquots at a 10× stock concentration in PBS. Aliquots can be stored indefinitely at –20°C between uses.

Aliquots may be thawed and frozen at least three times. It is recommended to make many aliquots of the optimized protease to avoid potentially inconsistent batches in each kit and because this protocol uses more than the kit recommends.

12. Apply 400 µl protease solution within the hydrophobic barrier on each slide. Place the slides on the HybEZ Oven slide holder and place that on wet Whatman 3MM chromatography paper in the HybEZ tray with a lid. Place the tray in the oven prewarmed to 40°C for 10 min.

13. Wash slides twice in PBS for 1 min each, agitating with a few gentle up and down motions.

14. Place slides in 4% formaldehyde for 5 min at room temperature.

15. Wash slides twice in PBS twice for 1 min each with constant agitation (approximately one up and down motion every second). Allow slides to sit in second wash until next step.

Perform hybridization and labeling

16. Prepare 400 μ l working probe solution for each slide by diluting probe set(s) 1:40 in prewarmed Probe Set Diluent QT and vortexing.

17. Remove one slide at a time from PBS, gently flick to remove PBS, and apply 400 μ l probe solution.

Always flick slides after washing to remove excess liquid, but make sure tissues do NOT dry.

18. Return slides to the humidified slide holder and place in the hybridization oven for 2 hr.

19. During hybridization, prewarm the following kit reagents:

To 40°C:

Preamplifier Mix QT

Amplifier Mix QT

Label Probe Diluent QF

To room temperature:

AP Enhancer Solution, Fast Red tablets, and Naphthol Buffer (for Type 1 probe set)

or

Blue Buffer (for Type 6 probe set).

Label Probe 1-AP, Label Probe 6-AP, and Blue Reagents 1–3 are left at 4°C until use.

20. Wash slides with wash buffer three times for 2 min each at room temperature with constant agitation.

If desired, slides can be stored at this point for up to 24 hr at room temperature in storage buffer consisting of 60 ml Wash Buffer Component 2 and 140 ml deionized H₂O. This is generally a convenient place to pause and continue the next day if detecting two transcripts. Before use, the slides should be washed twice in wash buffer for 2 min each with constant agitation. All reagents should be prewarmed (step 19) just prior to use.

21. Remove slides, flick to remove buffer, and immediately apply 400 μ l PreAmplifier Mix QT. Incubate 25 min at 40°C.

22. Wash slides with wash buffer three times for 2 min each at room temperature with constant agitation.

23. Remove slides, flick, and apply 400 μ l Amplifier Mix QT. Incubate 15 min at 40°C.

24. Wash slides with wash buffer three times for 2 min each at room temperature with constant agitation.

For Type 6 probe set

25a. For each slide, prepare 400 μ l working solution of Label Probe 6-AP by diluting 1:1000 in prewarmed Label Probe Diluent QF. Vortex solution.

26a. Remove slides, flick, and apply 400 μ l Label Probe to each slide. Incubate 15 min at 40°C.

27a. Wash slides with wash buffer three times for 3 min each at room temperature with constant agitation.

28a. While slides are in the wash, prepare Fast Blue substrate by combining the following:

105 μ l Blue Reagent 1

105 μ l Blue Reagent 2

105 μ l Blue Reagent 3

5 ml Blue Buffer.

Vortex and protect solution from light.

29a. Wash slides with wash buffer three times for 3 min each at room temperature with constant agitation.

30a. Remove slides, flick, and apply 400 μ l Fast Blue substrate. Incubate 30 min at room temperature, protected from light.

31a. Wash slides in PBS for 1 min before counterstaining.

For Type 1 probe set

25b. For each slide, prepare 400 μ l working solution of Label Probe 1-AP by diluting 1:1000 in prewarmed Label Probe Diluent QF. Vortex solution.

26b. Remove slides, flick, and apply 400 μ l Label Probe to each slide. Incubate 15 min at 40°C.

27b. Wash slides with wash buffer three times for 3 min each at room temperature with constant agitation.

28b. Remove slides, flick, and apply 400 μ l AP Enhancer Solution. Incubate 5 min at room temperature.

29b. During incubation, dissolve one Fast Red tablet in 5 ml Naphthol Buffer. Vortex and protect solution from light.

30b. Flick off the AP Enhancer Solution. Apply 400 μ l Fast Red substrate to each slide. Incubate 30 min at 40°C.

31b. Wash slides in PBS for 1 min before counterstaining.

Counterstain and view slides

32. Counterstain with 400 μ l DAPI for 1 min.
33. Briefly rinse with deionized H₂O.
34. Place a temporary coverslip over the tissues with 0.1 M Tris-Cl, pH 7.4, and view under a microscope.
35. Remove coverslip, briefly dip slide in deionized H₂O, and allow to dry. Store in a slide box indefinitely at room temperature.

Uncoverslipped slides may be stored in a box for many months and perhaps indefinitely.

HYBRIDIZATION HISTOCHEMISTRY FOR SIMULTANEOUS DETECTION OF TWO TRANSCRIPTS USING SETS OF OLIGODEOXYNUCLEOTIDE PROBE PAIRS

The protocol for detection of two transcripts is essentially the same as for a single transcript, except that labeling is carried out sequentially. Below, the first transcript is labeled and visualized using Label Probe 1-AP and Fast Red substrate, the AP reaction is stopped using AP Stop QT solution (provided in the ViewRNA kit), and then the second transcript is labeled and visualized using Label Probe 6-AP and Fast Blue substrate.

For materials, see Basic Protocol 2.

Perform hybridization

1. Perform all steps through hybridization and signal amplification as described for a single transcript (see Basic Protocol 2, steps 1 to 24).

Given the time involved with two transcripts, it is convenient to pause overnight after step 20.

Label first transcript

2. Prepare a working solution of Label Probe 1-AP (400 μ l/slide) by diluting 1:1000 in prewarmed Label Probe Diluent QF. Vortex solution.
3. Remove slides from wash buffer, flick, and apply 400 μ l Label Probe 1-AP to each slide. Incubate 15 min at 40°C.
4. Wash slides in wash buffer three times for 3 min each with constant agitation.
5. Remove slides, flick, and apply 400 μ l AP Enhancer Solution. Incubate 5 min at room temperature.
6. During incubation, dissolve one Fast Red tablet in 5 ml Naphthol Buffer. Vortex and protect solution from light.

7. Flick off the AP Enhancer Solution. Apply 400 μ l Fast Red substrate to each slide. Incubate 30 min at 40°C.
8. Wash slides with wash buffer three times for 2 min each at room temperature with constant agitation.
9. Remove from wash, flick, and apply 400 μ l AP Stop QT. Incubate 45 min at room temperature, protected from light.
10. Wash slides three time for 1 min each in PBS with constant gentle agitation, and then once in wash buffer for 1 min with constant gentle agitation.

Label second transcript

11. Prepare a working solution of Label Probe 6-AP (400 μ l/slide) by diluting 1:1000 in prewarmed Label Probe Diluent QF. Vortex solution.
12. Remove slides from wash buffer, flick, and apply 400 μ l Label Probe 6-AP to each slide. Incubate 15 min at 40°C.
13. Wash slides with wash buffer three times for 3 min each at room temperature with constant agitation.
14. While slides are in the wash, prepare Fast Blue substrate by combining the following:
 - 105 μ l Blue Reagent 1
 - 105 μ l Blue Reagent 2
 - 105 μ l Blue Reagent 3
 - 5 ml Blue Buffer.Vortex and protect solution from light.
15. Remove slides, flick, and apply 400 μ l Fast Blue substrate. Incubate 30 min at room temperature, protected from light.
16. Wash slides in PBS for 1 min.
17. Proceed with counterstaining and visualization (see Basic Protocol 2, steps 32 to 35).

IMPORTANT NOTE: The order of labeling (red Type 1 Probe plus Label Probe 1-AP before blue Type 6 probe plus Label Probe 6-AP) is the opposite of what the manufacturer recommends. However, we observed that the manufacturer's order can lead to cross-contamination labeling of the red Type 1 probe with blue substrate in addition to the expected red substrate.

It is also possible to use either color substrate after the alkaline phosphatase is attached to the probe.

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/4× SSPE (*APPENDIX 2A*)

12-μm cryostat tissue sections mounted on Superfrost Plus slides (Fisher) and defatted (*UNIT 1.1*; Gerfen, 2003)

Hybridization solution (see recipe)

³⁵S- or digoxigenin-labeled riboprobe (see Support Protocol 2)

1× SSPE/1 mM dithiothreitol (DTT)

4× SSPE/1 mM DTT

RNase A solution (see recipe)

0.1× SSPE/1 mM DTT

1× SSPE

70% (v/v) ethanol

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

Whatman 3MM chromatography paper

Glass coverslips

55° and 37°C incubators

Staining dishes with slide racks

65°C water bath

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

1. Cover the inside of a Bio-Assay dish top with a piece of Whatman 3MM chromatography paper. Wet the paper with 50% formamide/4× SSPE and lay the slides containing tissue sections on the paper with the sections facing up.

The top of the dish has a larger surface area than the bottom. Each top can hold 20 slides.

2. Apply hybridization solution containing $\sim 1 \times 10^6$ dpm/50 μl ³⁵S-labeled or 1 to 5 μl/50 μl digoxigenin-labeled riboprobe onto the tissue section on each slide and cover with a coverslip.

For example, to cover two adult rat coronal sections under an 18×30 -mm coverslip, use 45 μ l hybridization solution. The amount of hybridization solution used should be scaled up proportionately if a larger coverslip is used to cover a larger tissue section.

There is generally no need to pretreat the coverslips, but they should be dust-free. If background is a problem, the 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 $1 \times$ SSPE/1 mM DTT.
5. Gently slide the coverslip from the first slide until it partially overhangs the slide, then pry it off with forceps. Place the slide in a slide rack and immerse in a staining dish containing $4 \times$ SSPE/1 mM DTT.
6. Repeat until all the slides are in the same rack.

Do not allow the sections to dry prior to step 9.
7. Wash slides by immersing four times for 5 min each in room temperature $4 \times$ SSPE/1 mM DTT.
8. Incubate slides in RNase A solution for 30 min at 37°C.
9. Wash slides by immersing successively as follows:
 - two times for 5 min each in $0.1 \times$ SSPE/1 mM DTT at room temperature
 - two times for 30 min each in $0.1 \times$ SSPE/1 mM DTT in a 65°C water bath
 - two times for 5 min each time in $1 \times$ SSPE at room temperature.
- 10a. *For ^{35}S -labeled probes:* Rapidly dip the slides in water and then 70% ethanol. Hold slides with their frosted ends oriented down and blow them dry. Proceed to detection (see Basic Protocol 6).
- 10b. *For digoxigenin-labeled probes:* Use slides directly from the SSPE wash for detection (see Basic Protocol 4 or Alternate Protocol 2).

DETECTION OF DIGOXIGENIN-LABELED PROBES USING AP-CONJUGATED 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 (HRP), 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 employ AP-conjugated antibodies.

Materials

Digoxigenin-labeled sections on slides (see Basic Protocol 1 or 3)

TBS, pH 7.5 (*APPENDIX 2A*)

Detection buffer (see recipe)

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

Development buffer (see recipe)

NBT/BCIP working solution (see recipe)

1× SSPE (*APPENDIX 2A*)

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

Staining dishes with slide racks

Slide warmer

NOTE: Carry out all steps at room temperature.

1. Transfer slides directly from the final wash after digoxigenin labeling to a staining dish containing fresh TBS, pH 7.5. Wash 5 min, then repeat with a second wash in fresh TBS, pH 7.5.

2. Transfer slides to a staining dish containing detection buffer and incubate 30 min.

3. Transfer slides to a staining dish containing a fresh 1:2000 dilution of AP-conjugated anti-digoxigenin antibody in detection buffer. Incubate 5 to 16 hr with gentle rocking.

To conserve reagents, the slides may be incubated in slide mailers here and in step 6.

4. Wash twice for 10 min each in fresh TBS, pH 7.5.

5. Transfer slides to a staining dish containing development buffer and incubate 5 min.

6. Transfer slides to a staining dish containing NBT/BCIP working solution and incubate 1 to 2 hr in the dark.

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 for 30 min each 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, then dry thoroughly on a slide warmer.

Signal may be lost if slides are not thoroughly dried on a slide warmer before coverslips are applied.

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

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

This alternate procedure for digoxigenin detection uses peroxidase-conjugated antibody in an amplification scheme involving biotinylated or fluorochrome-conjugated tyramide and subsequent detection with streptavidin-conjugated fluorochromes, horseradish per-oxidase (HRP), 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. A schematic of the various detection options is shown in Figure 1.3.1.

Additional Materials (also see Basic Protocol 4)

Horseradish peroxidase (HRP)–conjugated sheep polyclonal anti-digoxigenin antibody (Sigma-Aldrich)

TSA Plus Biotin kit (Perkin-Elmer) containing:

Plus Biotin TSA reagent

1×Plus Amplification Diluent

Streptavidin-fluorochrome (Perkin-Elmer)

0.5% bovine serum albumin (BSA)

Streptavidin-HRP conjugate (Perkin-Elmer)

Diaminobenzidine (DAB) tablets (Sigma)

Urea/hydrogen peroxide tablets (Sigma)

TBS, pH 8.0 (*APPENDIX 2A*)

Streptavidin–alkaline phosphatase (AP) conjugate (Perkin-Elmer)

Perform amplification and detection

1. Wash slides with TBS, pH 7.5, and then detection buffer as described for AP detection (see Basic Protocol 4, steps 1 and 2).
2. Prepare a 1:300 to 1:600 dilution of HRP-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. Carry out amplification following the instructions of the TSA kit.

At the end, there will be an abundance of biotin molecules attached to the 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 5a to 7a), streptavidin–HRP (steps 5b to 7b), or streptavidin–AP (steps 5c to 7c)—in increasing order of sensitivity.

For detection with Texas Red or fluorescein

5a. Prepare a 1:2000 dilution of streptavidin–Texas Red or streptavidin–fluorescein in TBS, pH 7.5.

6a. Transfer slides to this solution and incubate 60 min at room temperature.

7a. Wash slides four times, each time by immersing 5 min in fresh TBS, pH 7.5.

For detection with HRP and substrate

5b. Prepare a 1:3000 dilution of streptavidin–HRP conjugate in TBS, pH 7.5, containing 0.5% BSA. 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.

6b. 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 mRNAs, however, the sections need to be examined microscopically.

7b. Wash slides twice, each time by immersing 5 min in fresh TBS, pH 8.0.

For detection with AP and substrate

5c. Prepare a 1:25,000 dilution of streptavidin–AP conjugate in detection buffer.

6c. Transfer slides to this solution and incubate 60 min at room temperature.

7c. Develop with NBT/BCIP substrate and wash with SSPE (see Basic Protocol 4, steps 4 to 7).

Dry and mount slides

8. Dip slides briefly in water and blow dry, then dry thoroughly on a slide warmer.

Slides should be dried thoroughly on a slide warmer before application 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).

Slides may also be stored dry without mounting. For subsequent viewing, they may be coverslipped with 0.1 M Tris–Cl, pH 8, and then dried again for storage. This may lead to longer preservation of the labeling.

One can also use fluorescein-12-UTP (Sigma-Aldrich) instead of digoxigenin-labeled UTP at the same concentrations to label RNA for nonradioactive hybridization histo-chemistry (see Support Protocol 2 and Basic Protocol 3). The TSA method described here is then used with HRP-conjugated sheep polyclonal anti-fluorescein antibody (Sigma-Aldrich), followed by biotinylated tyramide and streptavidin-fluorochrome or streptavidin-enzyme. The biotinylated tyramide and streptavidin-AP 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 simultaneous detection of two different transcripts by nonradioactive means. After detection of the digoxigenin-labeled probe (see Basic Protocol 4), proceed without drying to TSA and use the HRP-conjugated sheep polyclonal anti-fluorescein antibody in place of the anti-digoxigenin antibody in step 2 above; follow this with the biotinylated tyramide and streptavidin-fluorochrome or streptavidin-enzyme detection steps.

DETECTION OF Y CHROMOSOME DNA 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. The following protocol provides a reliable means of detecting the Y chromosome, and can be used in combination with antigen detection, if desired.

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

Materials

Defatted 12- μ m fresh-frozen or paraffin-embedded cryostat sections (*UNIT 1.1*; Gerfen, 2003) or whole fixed cells, mounted on Superfrost Plus slides (Fisher)

1% (w/v) 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% (v/v) ethanol

Citra Plus, pH 6 (BioGenex)

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

Primary antibody of interest

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

Streptavidin-HRP (Perkin-Elmer)

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

Biotinylated tyramide (Renaissance TSA Indirect kit; Perkin-Elmer)

0.2 N HCl

70% (v/v) formamide/2× SSC

Hybridization solution (see recipe)

Digoxigenin-labeled riboprobe (see Support Protocol 2) to a mouse repeat sequence (Bishop & Hatat, 1987; Mezey, Chandross, Harta, Maki, & McKercher, 2000) or human Y-chromosomal marker DYZ1 (Mezey et al., 2003a)

Fluorochrome-labeled streptavidin

Staining dishes with slide racks

Plastic Coplin jars

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

55°, 65°, and 80°C incubators

65° and 80°C water bath

80°C hot plate

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 for 5 min each in 1× PBS, then once for 5 min in 1× SSC.

For whole fixed cells, proceed to step 3. For fresh-frozen tissue, proceed to step 4 for immunocytochemistry/immunohistochemistry, or to step 14 for hybridization histochemistry alone.

For paraffin-embedded sections

1b. Immerse slides three times for 7 min each in Citrisolv (or xylene) to deparaffinize the tissue.

2b. Wash and rehydrate tissue by immersing slides successively in the following:

three times for 3 min each in 100% ethanol

once for 5 min each in 95%, 80%, and 70% ethanol

brief rinse in water.

Proceed 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, then adjust power to 450 W and continue heating for 15 min.

Citra Plus is the optimal buffer for antigen unmasking. Paraffin-embedded sections and whole fixed cells need to be microwave-pretreated to allow access to nuclear DNA. The optimal time must be determined individually for each paraffin block, due to differing fixation conditions. An initial time course starting with 1 min can be used to determine *correct conditions for a particular tissue*.

Perform immunocytochemistry/immunohistochemistry (optional)

4. Incubate slides in blocking solution for 10 min at room temperature.
5. Incubate slides in appropriately diluted primary antibody 1 hr at 37°C or overnight at 4°C.
6. Wash three times for 5 min each in PBS, room temperature.
7. Dilute biotinylated secondary antibody 1:1000 in blocking solution. Incubate slides in diluted secondary antibody 1 hr at room temperature.
8. Wash three times for 5 min each in PBS, room temperature.
9. Dilute streptavidin-HRP 1:250 in blocking solution. Incubate slides in diluted streptavidin-HRP 30 min at room temperature.
10. Wash three times for 5 min each in 0.1 M Tris·Cl, pH 8, at room temperature.
11. 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.
12. Wash three times for 5 min each in 0.1 M Tris·Cl, pH 8, room temperature.

The final step in immunodetection is performed after hybridization histochemistry.

Perform hybridization histochemistry

13. Transfer slides to 0.2 N HCl for 20 min at room temperature, then rinse quickly in water.
14. Transfer slides to 2× SSC in an 80°C water bath for 25 min.
15. Transfer slides to 2× SSC at room temperature and allow to cool.
16. Place slides in 70% formamide/2× SSC for 10 min at 80°C. At the same time, heat hybridization solution containing probe for 10 min at 80°C.
17. Incubate sections in 50 to 80 µl hybridization solution containing probe (under a coverslip) for 12 min at 80°C on a hot plate, then transfer the slides to a Bio-Assay dish and incubate 30 min at 55°C.

18. Remove the coverslips in $2\times$ SSC, then wash slides by immersing successively, for 5 min each, in $1\times$, $0.5\times$, and $0.1\times$ SSC at room temperature.

19. Transfer slides to $0.1\times$ SSC in a 65°C water bath for 30 min.

20. Perform detection of digoxigenin-labeled probes using the TSA method (see Alternate Protocol 2).

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

21. To block endogenous peroxidase prior to tyramide staining, incubate slides in DAKO Peroxidase Blocking Reagent for 5 min at room temperature.

A 3% hydrogen peroxide solution will also usually suffice.

Perform final immunodetection step

22. Add fluorochrome-labeled streptavidin at a dilution of 1:500 to 1:2500 to visualize the antigen of interest (see Alternate Protocol 2, steps 6a to 7a; Tran et al., 2003). Use a different color from that used to develop the hybridization signal.

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 can be obtained by coating the sample with a nuclear emulsion as described here. These steps are performed *after* detection of digoxigenin-labeled probes if both radiolabeled and digoxigenin-labeled probes are used simultaneously.

Materials

Ilford K5.D or Kodak NTB-3 nuclear emulsion

7.5 M ammonium acetate

^{35}S -labeled sections on slides (see Basic Protocol 1 or 3)

Kodak D-19 photographic developer, 17°C

Kodak Rapid Fix (without hardener), 17°C

Counterstain (*optional*): e.g., 0.4% toluidine blue, 2 $\mu\text{g}/\text{ml}$ ethidium bromide, hematoxylin/eosin

70% (v/v) ethanol

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

Darkroom with safelight

Coplin jars
40°C water bath
Clean slide
Black slide boxes
Desiccant capsules (e.g., Humi-caps from United Desiccants–Gates)
Black photography tape
Slide racks
Slide warmer

Coat sections with nuclear emulsion

1. In a darkroom under safelight conditions, scoop out 40 ml emulsion with a spatula and transfer to a Coplin jar containing 1.6 ml of 7.5 M ammonium acetate (to give a final 300 mM ammonium acetate).
2. Place 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 dipped in the emulsion.
3. Dip ^{35}S -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 emulsion-coated slides in black slide boxes with desiccant capsules. Tape the edges of the box with black photography tape and store until there is a good signal-to-noise ratio at 4°C in the dark.

This duration will depend on the specific activity of the probe and abundance of the mRNA, but 4 months is the practical limit.

Develop emulsion and stain tissue

5. Place emulsion-coated slides in slide racks and immerse for 2 min in 17°C Kodak D-19 developer, agitating every 30 sec.
6. Immerse 15 sec in running tap water with slight agitation.
7. Immerse 2 min in Kodak Rapid Fix (without hardener), agitating every 30 sec.

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

8. Rinse slides 8 min in running tap water.
9. If desired, counterstain 30 sec in 0.4% toluidine blue, 2 µg/ml ethidium bromide, hematoxylin/eosin, or other stain of choice. 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).

10. Dip slides very briefly in deionized water and then 70% ethanol.

11. Place on a slide warmer to dry thoroughly.

Signal may be lost if slides are not thoroughly dried on a slide warmer before coverslips are applied.

12. Apply coverslips using Cytoseal 60 or similar organic-based mounting medium.

PREPARATION OF OLIGODEOXYNUCLEOTIDE PROBES FOR HYBRIDIZATION HISTOCHEMISTRY

Oligodeoxynucleotides (generally ~48 bases long) are labeled using terminal deoxynucleotidyl transferase (TdT) and either [α - ^{35}S]dATP or digoxigenin-dUTP. The radioactive probes have specific activities >10,000 Ci/mmol (approximately ten times that of [α - ^{35}S]dATP).

Materials

5× tailing buffer (see recipe)

Oligodeoxynucleotide to be used as probe

Labeled nucleotide (select one):

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

mix of 250 μM digoxigenin-11-UTP/1 mM dNTP (or dATP; components from Roche; store mix indefinitely at -20°C)

Terminal deoxynucleotidyl transferase (TdT; Roche or Invitrogen)

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

4 M NaCl

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

70% and 100% (v/v) ethanol

5 M dithiothreitol (DTT)

1a. *For radiolabeled probes.* Prepare the following reaction mix on ice:

10 μl 5× tailing buffer

5 pmol oligodeoxynucleotide (final 0.1 μM)

50 pmol [^{35}S]dATP (final 1 μM)

70–100 U TdT

H₂O to 50 μl .

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

For a 48-base oligodeoxynucleotide, 0.1 μ M is ~15.6 ng/ μ l (780 ng for a 50- μ l reaction).

[³⁵S]dATP should not be added in a solution containing EDTA or similar chelator that will interfere with the tailing buffer. A color change will indicate that this has happened.

When making probes for northern analysis, [³²P]dATP is easily substituted for [³⁵S]dATP at the same concentration.

The enzyme lot may be examined to assess activity and number of nucleotides added per unit time.

1b. *For digoxigenin-labeled probes:* Prepare the following reaction mix on ice:

10 μ l 5 $\times$ tailing buffer

5 pmol oligodeoxynucleotide (final 0.1 μ M)

1 μ l 250 μ M digoxigenin-11-UTP/1 mM dNTP (or dATP) mix

70–100 U TdT

H₂O to 50 μ l.

Incubate 2 hr at 37°C.

2. Add the following to the labeling reaction:

375 μ l TE buffer, pH 7.6

25 μ l 4 M NaCl

50 μ g 25 μ g/ μ l yeast tRNA

1 ml 100% ethanol.

3. Mix thoroughly and incubate 10 min on wet ice.

4. Microcentrifuge 10 min at maximum speed and remove supernatant.

5. Rinse pellet with 1 ml of 70% ethanol, microcentrifuge briefly at maximum speed, and remove ethanol.

6a. *For radiolabeled probes:* Add 50 μ l TE buffer, pH 7.6, and 1 μ l of 5 M DTT, and dissolve pellet with gentle vortexing. Count 1 μ l in a scintillation counter and record the number of dpm.

Radiolabeled probes may be stored indefinitely at –80°C, but the sensitivity of the probe decreases as the radiolabel decays.

Approximately 500,000 dpm/ μ l can be expected. From this value, the specific activity can be estimated based on the total amounts of [³⁵S]dATP and oligodeoxynucleotide added and the amount of radioactivity recovered, conservatively assuming 50% recovery.

6b. *For digoxigenin-labeled probes:* Add 50 μ l TE buffer, pH 7.6, and dissolve pellet with gentle vortexing.

Digoxigenin-labeled probes can be stored indefinitely at 4°C.

PREPARATION OF RNA PROBES FOR HYBRIDIZATION HISTOCHEMISTRY

Riboprobes are generated by transcription in the presence of [α -³⁵S]UTP or digoxigenin-11-UTP. Typically, the plasmid containing the cDNA to be transcribed is linearized with a restriction enzyme. In this case, it may be useful to digest a large amount of plasmid (~10 μ g) if a particular probe will 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 instead of linearized plasmid.

Materials

Linearized plasmid or PCR product containing cDNA to be transcribed

Labeled nucleotide (select one):

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

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

5 $\times$ transcription buffer (see recipe)

100 mM and 5 M dithiothreitol (DTT)

10 mM individual solutions of ATP, CTP, and GTP RNasin (Promega)

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

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

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

4 M NaCl

25 μ g/ μ l yeast tRNA

70% and 100% (v/v) ethanol

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

TE buffer/5% (w/v) SDS

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

1a. *For radiolabeled probes:* Combine ~250 ng linearized plasmid (or 10 to 100 ng PCR product) with [³⁵S]UTP at a final concentration of 20 to 30 μ M in a microcentrifuge tube. Dry in a Speedvac evaporator at room temperature.

When making probes for northern analysis, [³²P]dATP can be substituted for [³⁵S]dATP. Use 5 μ l [³²P]dATP (>3000 Ci/mmol; 10 mCi/ml), and add 0.5 μ l of

150 μM UTP to the components in step 2. This will give final concentrations of $\sim 3 \mu\text{M}$ [^{32}P]UTP (500 Ci/mmol) and 15 μM cold UTP.

1b. *For digoxigenin-labeled probes:* Combine ~ 250 ng linearized plasmid (or 10 to 100 ng PCR product) with 0.5 μl of 1 mM UTP/4 mM digoxigenin-11-UTP mix in a microcentrifuge tube.

2. Add the following and mix thoroughly (especially where [^{35}S]UTP and DNA have been pelleted):

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 10 to 20 U/ μl SP6, T3, or T7 RNA polymerase H₂O to 5 μl .

The choice of RNA polymerase depends on the RNA polymerase recognition sequence present in the DNA template.

3. Incubate 30 min at 37°C.

4. Add another 0.5 μl polymerase and incubate 30 min at 37°C.

5. Add 0.5 μl RNasin and 0.5 μl of 1 U/ μl RNase-free DNase I and incubate 10 min at 37°C.

6. Add the following to the labeling reaction:

420 μl TE buffer, pH 7.6

25 μl 4 M NaCl

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

1 ml 100% ethanol.

7. Mix thoroughly and incubate 10 min on wet ice.

8. Microcentrifuge 10 min at maximum speed and remove supernatant.

9. Rinse pellet with 1 ml of 70% ethanol, microcentrifuge briefly at maximum speed, and remove ethanol.

10a. *For radiolabeled probes:* Add the following and dissolve the pellet:

485 μl TE buffer, pH 7.6

10 μl 10% SDS

5 μl 5 M DTT.

Count 1 μ l in a scintillation counter and record the number of dpm.

Radiolabeled riboprobes may be stored indefinitely at -80°C , but the sensitivity decreases as the radiolabel decays.

Approximately 1×10^6 dpm/ μ l can be expected. The specific activity can be estimated based on the percentage of U in the transcript.

The reaction can be scaled down to 1 μ l if several probes are labeled at once with the same enzyme. Dry 2 to 3 μ l [^{35}S]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/reaction. Use this cocktail to redissolve the [^{35}S]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 and 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 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.

10b. *For digoxigenin-labeled probes:* Add 50 μ l TE buffer/0.5% SDS to dissolve the pellet.

Digoxigenin-labeled riboprobes can be stored indefinitely in aliquots at 4°C .

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 and 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 different mRNA sizes from those 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)

^{35}S -labeled oligodeoxynucleotide probe (see Support Protocol 1) or riboprobe (see Support Protocol 2)

$1\times$ SSPE (*APPENDIX 2A*)/0.2% SDS (for oligodeoxynucleotide probes)

$0.1\times$ SSPE/0.2% SDS (for riboprobes)

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

70°C vacuum oven

Hybridization bags or roller bottles

Water bath(s) or hybridization oven(s) at 37° and 55°C (for oligodeoxynucleotide probes) or 65°C (for riboprobes)

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.

For detailed methods, see Brown, Mackey, and Du (2004).

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

For a discussion on optimizing UV cross-linking, see Perry-O'Keefe and Kissinger (1994).

3. Add ~0.5 to 1 ml northern hybridization solution per 10 cm² of membrane and incubate:

3 hr at 37°C for oligodeoxynucleotide probes or

18 hr at 65°C for riboprobes.

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.

4. Remove northern hybridization solution and replace with an equal volume of northern hybridization solution containing $\sim 2 \times 10^6$ dpm/ml ³⁵S-labeled probe. Incubate:

18 hr at 37°C for oligodeoxynucleotide probes or

24 hr at 65°C for riboprobes.

5. Wash membrane with the appropriate stringency:

For oligodeoxynucleotide probes:

twice briefly with 1× SSPE/0.2% SDS at 55°C

five times for 15 min each in fresh 1× SSPE/0.2% SDS at 55°C.

For riboprobes:

twice briefly with 0.1× SSPE/0.2% SDS at 65°C

four times for 30 min each in fresh 0.1× SSPE/0.2% SDS at 65°C.

When using riboprobes, membranes must not be allowed to cool below 65°C. To keep them from cooling, place plastic hybridization bags in 65°C water in a large beaker, cut open the ends of the bags above the water's surface, then pull the membranes out and immediately place them in 65°C wash buffer. These washes are more easily performed with roller bottles.

6. Place 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 to make solutions for pretreatment and hybridization. Deionized water may be used for subsequent wash and digoxigenin development steps. For DEPC treatment and for common stock solutions, see APPENDIX 2A.

Detection buffer

100 mM Tris·Cl, pH 7.5 (APPENDIX 2A)
150 mM NaCl
5% (v/v) normal goat serum (Vector Laboratories)
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 (APPENDIX 2A)

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

Working solution:

Appropriate quantity of labeled probe (see Basic Protocol 1 or 3)

4 µl solution A

Water to 12 µl

Mix well, then heat 5 min at 65°C

Cool rapidly on ice to room temperature

Add the following:

84 µl solution B

2 µl 5 M DTT

1 µl 10% (w/v) sodium thiosulfate

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

Mix well and use immediately

Prepare using DEPC-treated water for all solutions.

NBT/BCIP working solution

Development buffer (see recipe) containing:

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

Stock solutions of nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) are prepared in dimethylformamide (Invitrogen) and can be stored for at least a year at 4°C.

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

Northern hybridization solution

20 ml formamide

2 ml 20× SSPE (APPENDIX 2A)

8 ml 50% (w/v) dextran sulfate (Sigma)
4 ml 50× Denhardt's solution (*APPENDIX 2A*)
400 µl 25 mg/ml yeast tRNA (Sigma)
500 µl 20 mg/ml yeast total RNA (Sigma)
400 µl 10 mg/ml single-stranded DNA (Sigma)
400 µl 10% (w/v) sodium dodecyl sulfate (SDS)
H₂O to 40 ml
Store indefinitely at −20°C
Prepare using DEPC-treated water for all solutions.

Paraformaldehyde/picric acid solution, 1% (w/v)

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

RNase A 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
Just before use, warm 500 ml buffer to 37°C, then add 1 ml of 10 mg/ml RNase A
Use immediately
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
mM dithiothreitol (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 describes protocols for examining the expression of genes within tissue sections at a light-microscopic resolution (Bradley, Towle, & Young, 1992; Pagani et al., 2015; Young, 1992; Young, Mezey, & Siegel, 1986, 1990). A number of excellent resources are available for readers interested in localizing transcripts in whole-mount tissues, to chromosomes, or at the electron microscopic level (e.g., Albertson, Fishpool, & Birchall, 1995; Morey, 1995; Rosen & Bedington, 1993; Swiger & Tucker, 1996; Wilkinson, 1992).

Hybridization histochemistry is generally amenable to combination with other techniques such as immunohistochemistry (*UNIT 1.2*; Volpicelli-Daley & Levey, 2004), tract-tracing (Burgunder & Young, 1988), and in vitro receptor autoradiography (Westlake, Howlett, Bonner, Matsuda, & Herkenham, 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 hybridization histochemistry. The immunohistochemical steps are usually performed after the hybridization histochemical steps 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 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, Krause, & Sharkis, 2003).

Critical Parameters and Troubleshooting

Tissue treatment—Successful hybridization histochemistry seeks a balance between preserving tissue morphology and permeabilizing the tissue to allow access of the probe to the transcripts. A number of protocols utilize HCl and/or proteases to permeabilize the tissue sections. The approach described here avoids these harsh treatments through the use

of chloroform to remove fat from sections. However, paraffin-embedded tissue sections require the use of protease.

Choice of probe—Longer riboprobes offer greater sensitivity because they target longer stretches of transcripts than single oligodeoxynucleotide probes. Theoretically and in practice, ribo-probes are more sensitive than labeled, double-stranded cDNA probes covering the equivalent stretch of bases. Some researchers employ alkaline hydrolysis of riboprobes to increase the ease of tissue penetration. This treatment has not been found to help with the protocols in this unit, and it 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. The use of multiple oligodeoxynucleotide pairs that must hybridize next to each other for amplification to occur (e.g., ViewRNA, RNAscope) is extremely rapid and sensitive, and offers high resolution.

Choice of label—There are a number of reasons to label hybridization histochemistry probes with ^{35}S . It provides greater resolution and higher efficiency of grain production compared to either ^{32}P or ^{33}P . It also 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. Digoxigenin-labeled probes are useful for simultaneous detection of two different transcripts within the same tissue sections (and within the same cells). For a single transcript, however, radiolabeled probes are more sensitive and permit more accurate quantitation of transcript levels.

Signal specificity—Controls for specificity are the essence of any experiment. Unfortunately, there is no single, absolute control in hybridization histochemistry, so the researcher must rely on as many different checks as possible. Recommended controls include (in roughly descending order of usefulness):

1. Observing the same signal distribution using probes against different portions of the same transcript.
2. Blocking the signal by prior hybridization with unlabeled probe.
3. Correlation of signal with immunocyto-chemical results.
4. Observing different signal distribution using probes against unrelated transcripts, including sense probes. It should be noted, however, that sense probes occasionally detect mRNA transcribed from the opposite DNA strand, and a signal with the sense probe does not necessarily invalidate the findings obtained with the antisense probe.
5. Observing band(s) of the expected size in northern analysis using the probe under the same degrees of stringency.

An especially good control, although 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 also be aware of artifacts that may arise from autoradiography and/or colorimetric 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 were hybridized with a sense probe. Grains are especially susceptible to loss during staining or after coverslip-ping 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 can be blocked with levamisole. Intestinal alkaline phosphatase is more refractory, and requires a brief treatment (10 min) with 0.1 M HCl at room temperature (Kiyama & 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.

The use of multiple oligodeoxynucleotide probe pairs (ViewRNA, RNAscope) provides higher sensitivity because of the need for probe pairs to hybridize in close proximity. This approach may also be less prone to some of the above concerns, because designers of the proprietary probes are able to make use of extensive genome and transcript databases to avoid any cross-hybridization.

Anticipated Results

Hybridization histochemistry should enable the researcher to determine whether a given gene is expressed in particular cells. Figure 1.3.2 shows the simultaneous detection of two transcripts using radiolabeled and digoxigenin-labeled probes. Riboprobes are more sensitive than single oligodeoxynucleotide probes, enabling detection of five or fewer transcripts per cell. Traditionally, ³⁵S-labeled probes are more sensitive than colorimetrically detected probes. TSA (also known as catalyzed reporter deposition or CARD; Brown, Harris, Shaughnessy, & Litt, 1989) offers a number of branch points for varying degrees of amplification and different reaction products (Hunyady, Krempels, Harta, & Mezey, 1996; Kerstens, Poddighe, & Hanselaar, 1995; Toth & Mezey, 2007). However, the use of multiple oligodeoxynucleotide pairs whose members need to hybridize immediately adjacent to each other to generate signal may supplant the use of the other techniques in time. Their principle drawback is the relatively higher cost per slide (i.e., design and purchase of probe sets as well as kit reagents).

For quantitative analysis of autoradiograms, X-ray or ^3H -sensitive films may provide the easiest means of quantitation if the signal is sufficient and the cells are closely grouped. By simultaneously exposing brain-paste standards that incorporate known amounts of radioisotope, the film optical densities can be used to calculate the number of copies of hybridized probe. Phosphor imaging devices (e.g., from Fuji Medical Systems or Molecular Dynamics) offer two advantages over film: up to 40-fold-greater sensitivity and signals that are directly proportional to the amount of hybridized radiolabeled probe. A good general practice is to examine sections with a phosphor imaging system prior to dip-ping them in nuclear emulsion. Detailed protocols for quantitative analysis of autoradiograms are available (Gerfen, 1989; Young, 1992). The use of multiple oligodeoxynucleotide pairs results in colored dots that may be readily counted (Fig. 1.3.3). These signals are often detectable under bright field (or even dark field) illumination, but fluorescent illumination will be best if the level of transcripts is low.

Time Considerations

Hybridization histochemistry is composed of three main steps: preparation of tissue sections, hybridization and washing of the sections, and detection of the hybridization signal. After collection of the tissue specimens, preparation of sections depends, of course, on the number of sections needed and the size of the region(s) studied. This may take hours to weeks. Hybridization and washing steps take 2 consecutive days for radiolabeled probes or 3 consecutive days for digoxigenin-labeled probes. Detection of digoxigenin-labeled probes is completed by the end of day 3. 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, or may take months after coating with nuclear emulsion. Results using multiple oligodeoxynucleotide pairs can be obtained in 2 or 3 days for single or duplex analysis, respectively. Using this approach, it may be necessary to extend the times of the hybridization or amplification development steps to see if the signal improves.

Slides hybridized with radiolabeled probes can be kept for many years (even decades) with little change. It is important that they be thoroughly dried before coverslipping. The authors have not had success with permanent mounting media for sections labeled with non-radioactive oligonucleotide pairs, and thus recommend only temporarily coverslipping them while examining and photographing the sections. Uncoverslipped signals should last for many months, if not indefinitely, when stored in slide boxes.

Acknowledgments

This research was supported by the intramural research program of the NIMH (ZIA-MH-002498–24) and the NIDCR (ZIA DE000714 and ZIA DE000676).

Literature Cited

- Albertson DG, Fishpool RM, & Birchall PS (1995). Fluorescence in situ hybridization for the detection of DNA and RNA. *Methods in Cell Biology*, 48, 339–364. doi: 10.1016/S0091-679X(08)61395-3. [PubMed: 8531734]
- Bishop CE, & Hatat D (1987). Molecular cloning and sequence analysis of a mouse Y chromosome RNA transcript expressed in the testis. *Nucleic Acids Research*, 15, 2959–2969. doi: 10.1093/nar/15.7.2959. [PubMed: 3562242]

- Bobrow MN, Harris TD, Shaughnessy KJ, & Litt GJ (1989). Catalyzed reporter deposition: A novel method of signal amplification. *Journal of Immunological Methods*, 125, 279–285. doi: 10.1016/0022-1759(89)90104-X. [PubMed: 2558138]
- Bradley DJ, Towle HC, & Young WS, III. (1992). Spatial and temporal expression of α and thyroid hormone receptor mRNAs, including the β 2 subtype, in the developing mammalian nervous system. *Journal of Neuroscience*, 12, 2288–2302. [PubMed: 1607941]
- Brown T, Mackey K, & Du T (2004). Analy-sis of RNA by northern and slot blot hybridization. *Current Protocols in Molecular Biology*, 67, 4.9.1–4.9.19.
- Burgunder J-M, & Young WS, III (1988). The distribution of thalamic projection neurons containing cholecystokinin messenger RNA, using in situ hybridization histochemistry and retro-grade labeling. *Molecular Brain Research*, 4, 179–189. doi: 10.1016/0169-328X(88)90024-1.
- Gerfen CR (1989). Quantification of in situ hybridization histochemistry for analysis of brain function. In Conn PM (Ed.), *Methods in Neuroscience* (pp. 79–97). New York: Academic Press.
- Gerfen CR (2003). Basic neuroanatomical methods. *Current Protocols in Neuroscience*, 00, 1.1:1.1.1–1.1.11. doi: 10.1002/0471142301.ns0101s23.
- Hunyady B, Krempels K, Harta G, & Mezey E (1996). Immunohistochemical signal amplification by catalyzed reporter deposition and its application in double immunostainings. *Journal of Histochemistry and Cytochemistry*, 12, 1353–1362. doi: 10.1177/44.12.8985127.
- Kerstens HMJ, Poddie PJ, & Hanselaar AGJM (1995). A novel in situ hybridization signal amplification method based on the deposition of biotinylated tyramine. *Journal of Histochemistry and Cytochemistry*, 43, 347–352. doi: 10.1177/43.4.7897179.
- Kiyama H, & Emson PC (1991). An in situ hybridization histochemistry method for the use of alkaline phosphatase-labeled oligonucleotide probes in small intestine. *Journal of Histochemistry and Cytochemistry*, 39, 1377–1384. doi: 10.1177/39.10.1682362.
- Mezey É, Chandross KJ, Harta G, Maki RA, McKercher SR (2000). Turning blood into brain: Cells bearing neuronal antigens generated in vivo from bone marrow. *Science*, 290, 1775–1779. doi: 10.1126/science.290.5497.1779. [PubMed: 11099418]
- Mezey É, Key S, Vogelsang G, Szalayova I, Lange GD, & Crain B (2003a). Transplanted bone marrow generates new neurons in human brains. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 1364–1369. doi: 10.1073/pnas.0336479100. [PubMed: 12538864]
- Mezey É, Nagy A, Szalayova I, Key S, Bratincsak A, Baffi J, & Shahrar T (2003b). Comment on “Failure of bone marrow cells to transdifferentiate into neural cells in vivo”. *Science*, 299, 1184. doi: 10.1126/science.1079318.
- Morey AL (1995). Non-isotopic in situ hybridization at the ultrastructural level. *Journal of Pathology*, 176, 113–121. doi: 10.1002/path.1711760204.
- Pagani JH, Williams Avram SK, Cui Z, Song J, Mezey E, Senerth JM, . . . Young WS (2015). Raphe serotonin neuron-specific oxytocin receptor knockout reduces aggression without affecting anxiety-like behavior in male mice only. *Genes, Brain, and Behavior*, 14, 167–176. doi: 10.1111/gbb.12202.
- Perry-O’Keefe H, & Kissinger C (1994). Chemi-luminescent detection of nonisotopic probes. *Current Protocols in Molecular Biology*, 26, 3.19.1–3.19.8.
- Rogers AW (1979). *Techniques of Autoradiography* New York: Elsevier Science Publishing.
- Romijn HJ, van Uum JFM, Breddijk I, Emmering J, Radu I, & Pool CW (1999). Double immunolabeling of neuropeptides in the human hypothalamus as analyzed by confocal laser scanning fluorescence microscopy. *Journal of Histochemistry and Cytochemistry*, 47, 229–235. doi: 10.1177/002215549904700211. [PubMed: 9889258]
- Rosen B, & Beddington RS (1993). Whole-mount in situ hybridization in the mouse embryo: Gene expression in three dimensions. *Trends in Genetics*, 9, 162–167. doi: 10.1016/0168-9525(93)90162-B. [PubMed: 8337752]
- Swiger RR, & Tucker JD (1996). Fluorescence in situ hybridization: A brief review. *Environmental and Molecular Mutagenesis*, 27, 245–254. doi: 10.1002/(SICI)1098-2280(1996)27:4%3c245::AID-EM1%3e3.0.CO;2-C.

- Theise ND, Krause DS, & Sharkis S (2003). Comment on "Little evidence for developmental plasticity of adult hematopoietic stem cells." *Science*, 299, 1317. doi: 10.1126/science.1078412. [PubMed: 12610282]
- Tóth ZE, & Mezey É (2007). Simultaneous visualization of multiple antigens with tyramide signal amplification using antibodies from the same species. *Journal of Histochemistry and Cytochemistry*, 55, 545–554. doi: 10.1369/jhc.6A7134.2007.
- Tran SD, Pillemer SR, Dutra A, Barrett AJ, Brownstein MJ, Key S, . . . Mezey É (2003). Differentiation of human bone marrow-derived cells into buccal epithelial cells in vivo: A molecular analytical study. *Lancet*, 361, 1084–1088. doi: 10.1016/S0140-6736(03)12894-2. [PubMed: 12672312]
- Valentino KL, Eberwine JH, & Barchas JD (1987). (Eds.), *In Situ Hybridization: Applications to Neurobiology* New York: Oxford University Press.
- Volpicelli-Daley LA, & Levey A (2004). Immunohistochemical localization of proteins in the nervous system. *Current Protocols in Neuroscience*, 25, 1.2.1–1.2.17.
- Westlake TM, Howlett AC, Bonner TI, Matsuda LA, & Herkenham M (1994). Cannabinoid receptor binding and messenger RNA expression in human brain: An in vitro receptor autoradiography and in situ hybridization histochemistry study of normal aged and Alzheimer's brains. *Neuroscience*, 63, 637–652. doi: 10.1016/0306-4522(94)90511-8. [PubMed: 7898667]
- Wilkinson DG (1992). (Ed.), *In Situ Hybridization: A Practical Approach* New York: Oxford University Press.
- Young WS, III (1992). In situ hybridization with oligodeoxyribonucleotide probes. In Wilkinson DG (Ed.), *In Situ Hybridization: A Practical Approach* (pp. 33–44). New York: Oxford University Press.
- Young WS, III, Mezey É, & Siegel RE (1986). Vasopressin and oxytocin mRNAs in adrenalectomized and Brattleboro rats: Analysis by quantitative in situ hybridization histochemistry. *Molecular Brain Research*, 1, 231–241. doi: 10.1016/0169-328X(86)90029-X.
- Young WS, III, Reynolds K, Shepard EA, Gainer H, & Castel M (1990). Cell-specific expression of the rat oxytocin gene in transgenic mice. *Journal of Neuroendocrinology*, 2, 917–925. doi: 10.1111/j.1365-2826.1990.tb00660.x. [PubMed: 19215438]

Figure 1.3.1.

There are many ways to use the TSA system for in situ hybridization histochemistry.

Tyramide can be added to any step that introduces HRP. Several routes are presented here, but the user must determine the optimal method for each specific tissue/probe combination. More amplification can be added, if necessary, by adding an anti-FITC-HRP or biotinylated tyramide to the streptavidin-HRP at the last step. However, the more rounds of amplification that are used, the more nonspecific background may appear. A control (using either a sense probe or a random probe) should always be run to make sure that the signal is specific. SA, streptavidin; TYR, tyramide.

Figure 1.3.2.

Vasopressin and oxytocin transcripts in neurons of the paraventricular nucleus of the rat hypothalamus hybridized with ^{35}S - and digoxigenin-labeled probes, respectively. Deposition of radiolabeled probe is indicated by gray-colored silver grains. Digoxigenin-labeled probe is indicated by a dark stain following development of alkaline phosphatase on anti-digoxigenin antibodies. Arrows indicate neurons containing both transcripts. Cell nuclei are generally clearly delineated by surrounding cytoplasmic alkaline phosphatase staining.

Figure 1.3.3.

Simultaneous labeling of oxytocin (blue) and vasopressin 1b (red) receptors in pyramidal neurons within the CA2 region of the mouse hippocampus. Transcripts were detected using multiple oligodeoxynucleotide pairs with the ViewRNA kit. DAPI counterstaining is shown in pseudocolor green.