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In Situ Hybridization for Detection of AAV-Mediated Gene Expression

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Abstract

Techniques to localize vector transgenes in cells and tissues are essential in order to fully characterize gene therapy outcomes. In situ hybridization (ISH) uses synthesized complementary RNA or DNA nucleotide probes to localize and detect sequences of interest in fixed cells, tissue sections, or whole tissue mounts. Variations in techniques include adding labels to probes, such as fluorophores, which can allow for the simultaneous visualization of multiple targets. Here we provide the steps necessary to: (1) label probes for colorimetric visualization and (2) perform ISH on OCT cryo-preserved fixed frozen tissues.

Keywords

Riboprobes; In situ hybridization; ISH; Fluorescence in situ hybridization; FISH; Cell-specific labeling; Hybridization; DIG labeling

1 Introduction

In situ hybridization (ISH) of RNA-DNA hybrid probes in cytological specimens was first reported in the late 1960s [1]. A decade later, the use of fluorescent in situ hybridization (FISH) was published [2]. The introduction of various tags and non-isotopic visualization methods, such as fluorophores and colorimetric development, allowed for advances in resolution while enhancing speed and safety of the technique, and eventually gave rise to simultaneous visualization of multiple targets and live-cell imaging [3]. Today, ISH, and various alterations of the original technique, is among the most common protocols of any laboratory needing to localize DNA or messenger (m)RNA in cytological preparations of fixed cells, histologic tissue sections, or even whole mounts. This technique is invaluable for assessing the distribution of transduced cells after gene delivery, and can detect adeno-associated virus (AAV)-mediated gene expression with greater accuracy and sensitivity than standard immunolabeling of the expressed protein, which can vary depending on the

intracellular localization of the protein, the efficiency of antibody binding to the protein, the half-life of the protein, and other factors. ISH is particularly important when AAV is used to deliver a protein that is secreted or axonally transported, because in this case the presence of protein within a cell does not indicate that it was transduced by the AAV vector, and thus protein immunolabeling cannot be used to examine cellular transduction. The protocol outlined in this chapter has been used extensively in our laboratory and others for the past 20 years to assess various therapeutic methods, including viral vector-mediated gene therapy, whole cell delivery, and ex vivo gene therapy [4–23].

The design of RNA probes (riboprobes) for ISH has been commercialized, and a range of optimized riboprobes that efficiently bind to common target sequences can be purchased. The design of probe sequences will thus not be discussed in this chapter, although methods for generating and purifying digoxigenin (DIG)-UTP labeled riboprobes by in vitro transcription of template DNA are provided. The ISH protocol outlined here has three components: (1) antigen retrieval; (2) denaturation and hybridization of labeled RNA probes to the corresponding DNA followed by stringent washes; and (3) detection by antibody staining. This protocol focuses on ISH methods for cryo-preserved, paraformaldehyde (PFA)-fixed, OCT-embedded tissue preparations, but techniques using paraffin-embedded whole mount tissues and embryos have been established and can be found elsewhere [24].

Fluorescent ISH (FISH) can also be combined with immunofluorescent antibody (IFA) detection of markers for cell type or other functions. However, the reader is cautioned that the conditions of fixation and preparation of cells or tissues for IFA and ISH can work against each other, thus it is important to establish specific use of reagents for each combination of FISH and IFA reagents. Since FISH requires permeabilization for the probes to access mRNA, the sensitivity of detection of cell surface proteins may be reduced. In our experience, the detection of cytosolic proteins usually works well, such as combining FISH with IFA for tyrosine hydroxylase or neuron-specific enolase [9, 14].

2 Materials

Use sterile, ultrapure water for all solutions, unless otherwise noted.

2.1 DIG Labeling of Riboprobes

1. DIG RNA Labeling Kit (SP6/T7), containing:
    - **a.** 10× NTP labeling mixture
    - **b.** 10× Transcription buffer
    - **c.** 20 U/μL Protector RNase A Inhibitor
    - **d.** 20 U/μL SP6 RNA polymerase
    - **e.** 20 U/μL T7 RNA polymerase
    - **f.** 20 U/μL T3 RNA polymerase
    - **g.** 10 U/μL DNase I, RNase-free

2. Riboprobe template DNA.
3. 0.5 M EDTA, pH 8.0.
4. 20 mg/mL glycogen.
5. 4 M LiCl.
6. 100% Ethanol.
7. 5× MOPS gel running buffer: 0.1 M MOPS, 0.04 M sodium acetate, 0.005 M EDTA (pH 8). Dissolve MOPS and sodium acetate in DEPC-treated water. Adjust to pH 7 with NaOH. Add EDTA. Filter-sterilize through a 0.2 μm filter. Wrap the bottle in aluminum foil to protect it from light. Store at room temperature.
8. 10× RNA gel loading buffer: 50% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF, 0.01 M EDTA (pH 8). Store at 4 °C.
9. DEPC-treated water: fill large 2 L glass bottles with water (almost to top). In fume hood, add 2 mL of diethyl pyrocarbonate (DEPC) to each jug, seal tightly, and shake. USE A GLASS PIPETTE, as DEPC can melt plastic. Add nitrogen gas to the DEPC bottle, cap, and store at 4 °C. Do not store longer than 9 months. Loosen water caps and incubate at 37 °C for at least 30 min (overnight is usually used). Autoclave to degrade the remaining DEPC, leaving cap loosened. Tighten cap and store at 4 °C.
10. Agarose.
11. 12.3 M formaldehyde.
12. 100% formamide, deionized.
13. 0.4 mg/mL ethidium bromide.
14. Spectrophotometer.

2.2 In Situ Hybridization

1. RNase Zap (RNase decontamination solution).
2. 10 mg/mL Proteinase K enzyme: dissolve 100 mg of Proteinase K in 10 mL of nuclease-free water. Store at −20 °C in 200 μL aliquots.
3. 10× PBS: dissolve 80 g of NaCl, 2 g of KCl, 14.4 g of Na_2HPO_4, and 2.4 g of KH_2PO_4 in 800 mL of nuclease-free water. Adjust pH to 7.4 with HCl and bring volume to 1 L with nuclease-free water. To make 1× PBS, dilute 100 mL of 10× PBS with 900 mL of nuclease-free water.
4. 3% Hydrogen Peroxide: add 20 mL of 30% hydrogen peroxide and 20 mL of 10× PBS to 160 mL of nuclease-free water.
5. 1× Proteinase K buffer: 0.10 M Tris–HCl (pH 8), 0.05 M EDTA (pH 8). Combine 20 mL of 1.0 M Tris–HCl (pH 8) and 20 mL of 0.5 M EDTA (pH 8). Bring to 200 mL with nuclease-free water.

6. 0.1 M Triethanolamine (TEA) buffer, pH 8: weigh 7.43 g of TEA and add to 400 mL of DEPC-treated water. Add 11.5 mL of 2 N NaOH (*see* Note 1).

7. 0.1 M TEA/0.25% Acetic Anhydride wash: add 500 μL of Acetic Anhydride to 200 mL of 0.1 M TEA buffer. Recap acetic anhydride immediately after taking aliquot (*see* Note 2).

8. 2× SSC: 20× SSC diluted in nuclease-free water.

9. 1.0 M Tris–HCl, pH 7.5: dissolve 121.1 g of Trizma base in 800 mL of nuclease-free water. Adjust pH to 7.5 with ~68 mL of 12 N HCl. Bring volume to 1 L with nuclease-free water. Filter-sterilize.

10. Tris-EDTA-NaCl buffer: 4.200 M NaCl, 0.140 M Tris–HCl (pH 7.5), 0.014 M EDTA (pH 8). Dissolve 24.55 g NaCl in 70 mL of nuclease-free water. Add 14 mL of 1 M Tris–HCl (pH 7.5) and 2.8 mL of 0.5 M EDTA (pH 8). Bring volume to 100 mL with nuclease-free water. Filter-sterilize. Make 1 mL aliquots and store at −20 °C.

11. 100% Deionized Formamide: store 5 mL aliquots at −20 °C.

12. 40% Dextran Sulfate: heat 10 mL of Nuclease-Free water to 65 °C in a water bath for 15–20 min. Dissolve 5 g of dextran sulfate while stirring under low heat. Final volume will be 12 mL. Store in 3–4 mL aliquots at 4 °C (*see* Note 3).

13. 5× Maleic Acid buffer, pH 7.5: 0.50 M Maleic acid, 0.75 M NaCl. Dissolve 58.05 g of Maleic acid, 43.83 g of NaCl, and 40 g of NaOH pellets in 800 mL of nuclease-free water. Adjust pH to 7.5 with ~16 mL of 2 N NaOH. Bring volume to 1 L with nuclease-free water. Filter-sterilize.

14. 10% Blocking solution: dissolve 50 g of Blocking Reagent (Roche) in 500 mL of 1× maleic acid buffer by microwaving for 5 min. Check every 30 s after the first 3 min to prevent boil-over. Allow to cool on benchtop. Add 500 μL of DEPC and stir at room temperature for 10 min, then incubate in a 37 °C oven overnight. On the next day, autoclave for 45 min. Make 100 aliquots of 500 μL and store at −20 °C. These 500 μL aliquots are to be used in the hybridization solution. Aliquot the remaining solution into 40 mL aliquots and store at −20 °C. These 40 mL aliquots are used to make the colorimetric blocking buffer.

15. 5× RNase buffer: 2.50 M NaCl, 0.05 M Tris (pH 7.5), 5 mM EDTA (pH 8). Dissolve 146.1 g of NaCl in 800 mL of water (see Note 4). Add 50 mL of 1 M Tris (pH 7.5) and 5 mL of0.5 M EDTA. Bring volume to 1 L with water.

16. Formamide Wash buffer: 50% deionized formamide, 2× SSC.

17. TSA Cyanine 3 System (Perkin Elmer). Contains the blocking powder, the streptavidin-HRP conjugate, the Cy3 substrate, and the Cy3 dilution buffer.

18. Cy3 Substrate: The Cy3 substrate comes lyophilized. Reconstitute the pellet by adding 300 μL of water. Store the substrate at 4 °C in the dark (wrap the tube in aluminum foil for protection).

19. 1× TNT Wash Buffer: 0.100 M Tris–HCl (pH 7.5), 0.150 M NaCl, 0.05% Tween 20. Dissolve 8.7 g of NaCl in 850 mL of water, then add 100 mL of 1 M Tris–HCl (pH 7.5) and 5 mL of 10% Tween 20. Bring final volume to 1 L with water.

20. 1× TNB Block (for immunofluorescent ISH): 0.10 M Tris–HCl (pH 7.5), 0.15 M NaCl, 0.5% blocking solution. Dissolve 5.26 g of NaCl in 450 mL of water and add 60 mL of 1 M Tris–HCl (pH 7.5). Heat solution to 60 °C in a water bath for approximately 30 min. Slowly add 3 g of blocking powder (*see* **item 17**) to the warm solution while stirring. Bring final volume to 600 mL with water. Prepare 10–15 mL aliquots and store at −20 °C.

21. Blocking buffer (for colorimetric ISH): 2× SSC, 0.05% Triton X-100, 2% blocking solution (*see* **item 14**).

22. Anti-Digoxigenin alkaline phosphatase primary antibody (Roche #1093274). Use at a 1:2500 dilution for colorimetric ISH, or 1:150 for immunofluorescent ISH.

23. Biotinylated anti-sheep IgG secondary antibody.

24. Antibody Dilution buffer: 1× Maleic Acid buffer, 1% blocking solution, 0.3% Triton X-100.

25. 10× Tris/NaCl: 1 M Tris–HCl (pH 9.5), 1 M NaCl. Dissolve 121.1 g of Trizma Base and 58.44 g of NaCl in 800 mL of water, then adjust pH to 9.5 with HCl. Bring volume to 1 L with water. Filter-sterilize.

26. 10× $MgCl_2$: 0.5 M $MgCl_2$. Dissolve 101.7 g of $MgCl_2$ in 800 mL of water. Bring volume to 1 L and filter-sterilize.

27. BCIP/NBT reaction Buffer: 100 mM Tris (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$. Add 20 mL of 10× Tris/NaCl and 20 mL of 10× $MgCl_2$ to a graduated cylinder, then bring to 100 mL with water.

28. BCIP/NBT tablets.

29. 10× Stop Buffer: 0.10 M Tris–HCl (pH 7.5), 0.01 M EDTA (pH 8), 1.50 M NaCl. Dissolve 88 g of NaCl in 800 mL of water. Add 10 mL of 1 M Tris (pH 7.5) and 2 mL of 0.5 M EDTA. Bring volume to 1 L with water and filter-sterilize.

30. 4% Paraformaldehyde.

31. HybriSlip Hybridization Covers (Grace Bio-Labs).

32. CoverWell Incubation Chambers (Grace Bio-Labs).

33. Aqua Poly/Mount mounting medium (Polysciences, Inc.).

34. Vectashield Antifade Mounting Medium with DAPI (Vector Labs).

35. 24 × 60 mm, no. 1.5 glass coverslips.

36. Humid chamber (*see* Note 5).

37. Hybridization oven.
38. Slide holders and solution containers.
39. 100% Ethanol.
40. DEPC-treated water (*see* **item 9** in Subheading 2.1).

3 Methods

3.1 DIG Labeling of Riboprobes

3.1.1 RNA Labeling Reaction

1. Add 1 μg of riboprobe template DNA or 4 μL of control DNA to a sterile, RNase-free reaction vial. Bring the total sample volume to 13 μL with water (sterile, RNase-free, DEPC-treated).
2. Place the reaction vial on ice, then add each reagent as shown in Table 1.
3. Mix and centrifuge briefly.
4. Incubate for 2 h at 37 °C.
5. Pulse spin, then add 2 μL of DNase I, RNase-free (10 U/μL) to remove template DNA.
6. Incubate for 15 min at 37 °C.
7. Stop the reaction by adding 2 μL of 0.5 M EDTA (pH 8.0).
8. Ethanol precipitate probes overnight at −80 °C as shown in Table 2.
9. Centrifuge the RNA precipitate at 4 °C for 20 min in a microfuge at full speed.
10. Wash the RNA pellet once with 1 mL of 70% ethanol, centrifuge for 15 min, then air-dry.
11. Resuspend the RNA pellet in 75 μL of DEPC-treated water.
12. Visualize the labeled RNA by running 5 μL on a 1% agarose/formaldehyde gel at 80 V (see Subheading 3.1.2).
13. Quantify RNA using a spectrophotometer.
14. Aliquot riboprobe at 4 μg/tube and store at −80 °C.

3.1.2 Visualization of Probes Usinga Formaldehyde Gel

1. Prepare a 1% agarose gel by microwaving 1 g of agarose in 62 mL of DEPC-treated water, then cooling to 60 °C. Add 20 mL of 5× MOPS gel running buffer and 18 mL of 12.3 M formaldehyde (2.2 M final concentration) to the dissolved agarose. Pour the mixture into a gel caster.
2. Prepare the sample for denaturation of RNA as shown in Table 3.

3. Vortex and spin samples, then incubate at 65 °C for 15 min. Remove samples from water bath and place on ice. Spin samples briefly on a microfuge and return to ice.
4. Add 2.4 μL of 10× RNA gel loading buffer, then vortex and spin. Place samples on ice.
5. While samples are incubating, pre-run the gel at 80 V (5 V/cm gel) for 30 min.
6. Load samples onto gel along with control RNA and RNA marker on separate lanes.
7. Run until dye is halfway through the gel.
8. View under UV light.

3.2 In Situ Hybridization

3.2.1 Hybridization

1. Before beginning, clean solution containers with RNase Zap and rinse with DEPC-treated water to eliminate all RNase. Be careful not to introduce RNase throughout Day 1. Clean gloves with RNase Zap periodically and conduct all procedures with RNase-free reagents on a dedicated and clean RNase-free bench.
2. Warm 200 mL of Proteinase K buffer (without Proteinase K enzyme) to 37 °C in a water bath (see Note 6). Be sure to use autoclaved, RNase-free glassware.
3. Remove slides from −80 °C and allow to dry on lab bench for *at least* 1 h (*see* Note 7). Load slides in a slide holder after they are dry. Take out two slides that are known to be positive for your target sequence and label as controls: one positive control (label “Antisense” and treat the same as all other slides) and one negative control (label “Sense” and use a sense riboprobe in **steps 13** and **14**).
4. Dip the slides for 1 min into 200 mL of each of the following solutions in the given order to rehydrate the tissue sections (*see* Note 8):
    a. 100% Ethanol
    b. 100% Ethanol
    c. 95% Ethanol
    d. 70% Ethanol
    e. 50% Ethanol
    f. 2× SSC
5. For colorimetric ISH, skip to **step 7**. For fluorescent ISH, place slides in 200 mL of 3% hydrogen peroxide in 1× PBS for 25 min at room temperature.
6. Wash slides twice in 1× PBS for 5 min each.
7. Add 200 μL of 10 mg/mL Proteinase K enzyme to the pre-warmed 200 mL Proteinase K buffer and mix. Transfer slides immediately after adding Proteinase

K. Dip the slide holder 2 quick times and then incubate at 37 °C for 3 min for tissue sections less than 5 μm thick, 4 min for 6–10 μm, or 5 min for 11–20 μm.

8. Dip slides in 200 mL of RNase-free water twice for 10 s each time.

9. Incubate the slides in 200 mL of freshly made 0.1 M TEA buffer (pH 8) for 3 min.

10. Incubate the slides in 0.1 M TEA/Acetic Anhydride solution for 10 min. DO NOT add acetic anhydride until immediately before use. Dip slide holders up and down to mix.

11. Dip the slides for 1 min into 200 mL of each of the following solutions in the given order to dehydrate the tissue sections (use the same solutions used for rehydration in **step 4**):

    a. 2× SSC

    b. 50% Ethanol

    c. 70% Ethanol

    d. 95% Ethanol

    e. 100% Ethanol

    f. 100% Ethanol

12. Air-dry the slides for at least 1 h at room temperature.

13. Prepare the hybridization solution as shown in Table 4. Remember to prepare a second hybridization solution containing a sense riboprobe for negative control. While the slides are air-drying, combine the riboprobe and nuclease-free water in a1.7 mL microcentrifuge tube, then place the tube in boiling water for 10 min. Place the tube directly onto ice after boiling. While on ice, combine with the remaining reagents as shown in Table 4 in a 15 mL tube (*see* Note 9). Probe aliquots are 4 μg, so the final concentration will be 0.8 μg/mL. After combining all reagents, the mixture can be vortexed and returned to boiling water for 2–5 min to remove bubbles (this is not necessary if no bubbles are present) (*see* Note 10).

14. Prepare HybriSlip hybridization coverslips (coverslips with peel-off backing). Pipette 90 μL of hybridization solution onto each coverslip.

15. Carefully roll the slide over the coverslip so that all sections are in contact with the hybridization solution. This works best if a long line of solution is laid across the coverslip, then rolled onto the slide gently without pushing. The slide can then be picked up and the coverslip adjusted so that all sections are covered. Apply gentle pressure to the coverslip to remove any remaining bubbles and affix it to the slide.

16. Place the slides in a humid chamber along with 1 wet paper towel and 50 mL of dH_2O. Do not allow slides to touch the paper towel. Incubate in a hybridization

oven at 63 °C overnight (at least 8 h). Place a beaker filled with water in the oven (*see* Note 11).

3.2.2 Post-hybridization—An RNase-free environment is no longer required from this stage onward. Do not use the dedicated RNase-free bench for any of the below steps.

1. Pre-warm 200 mL of the following solutions at the temperatures indicated below:

Formamide wash solution	65 °C
RNase buffer #1	37 °C
RNase buffer #2	65 °C
2× SSC #1	RT
2× SSC #2	37 °C
2× SSC #3	37 °C

2. Remove the slides from the humid chamber and carefully remove the hybridization coverslip. Place the slides onto a slide holder that is immersed in SSC (to prevent drying while loading slides). Wash in the following order:
   a. 2× SSC for 30 min with low-speed shaking at room temperature.
   b. Formamide wash solution for 30 min at 65 °C. Gently agitate the slides (move up and down in container) periodically during the first 5 min of incubation.
   c. 2× SSC #2 for 10 min at 37 °C.
   d. 2× SSC #3 for 10 min at 37 °C.
3. Incubate the slides in RNase buffer #1 containing 400 μL (10 mg/mL) of RNase A for 30 min at 37 °C. Add slides immediately after adding RNase to buffer.
4. Wash slides in RNase buffer #2 (no RNase A added) for 30 min at 65 °C. For colorimetric immunohistochemical detection, continue to Subheading 3.2.3. For immunofluorescent detection, skip to Subheading 3.2.4.

3.2.3 Colorimetric Immunohistochemical Detection

1. Place slides in 200 mL of blocking buffer while shaking at low speed for 2 h at room temperature. If desired, slides can remain in blocking buffer at 4 °C overnight (no shaking) (*see* Note 12).
2. Prepare antibody dilution buffer as described in Table 5 (*see* Note 13). Add anti-digoxigenin AP antibody last, then mix gently.
3. Using CoverWell coverslips (rubber side up), pipette 220 μL of antibody solution in a line from the left to the right edges of the coverslip. Remove any bubbles with a micropipette.
4. Press the bottom edge of the slide to the middle of the bottom rubber edge of the CoverWell, and gently roll the CoverWell onto the slide, allowing solution to fill

the entire CoverWell from the bottom. Keep the slide and CoverWell in contact with the bench to prevent the coverslip from sliding off. The coverslip can then be moved gently to an optimal position (*see* Note 14). If bubbles form, they can be moved to a corner of the coverslip by tilting the slide, or can be pressed out by gently easing the corner of the CoverWell that contains bubbles over the edge of the slide, releasing the bubbles (be careful not to lose solution).

5. Place the slides in a humid chamber along with 1 wet paper towel and 50 mL of dH_2O. Do not allow slides to touch the paper towel. Close humid chamber and incubate for 3 h at RT or overnight at 4 °C. Quickly check all slides for infiltration of bubbles before incubating, as bubbles can stick to the edges initially but move to the middle once slides are laid flat in the chambers.

6. Carefully remove the CoverWell coverslips and load slides onto a slide holder immersed in 1° Maleate buffer (to prevent drying while slides are loaded). After removing, place CoverWell coverslips in deionized water with soap (*see* Note 15). While the slides are washed in **steps 7** and **8**, clean the coverslips by rinsing off soap thoroughly, first with warm tap water, then with deionized water. Gently rub the plastic under water to remove all residual antibody, which can cause background. Dry CoverWell coverslips with paper towels and lay out in a dark room for **step 10**.

7. Wash slides twice in 200 mL of 1× Maleate buffer at room temperature for 10 min each, with low-speed shaking.

8. Wash in BCIP/NBT reaction buffer at room temperature for 10 min with low-speed shaking.

9. Dissolve 1 BCIP/NBT tablet in 10 mL of sterile distilled water.

10. Place 220 μL of dissolved BCIP/NBT onto a CoverWell coverslip, then roll slide onto coverslip as described in step 4. Follow any notes on the slide labels to ensure that the same sections are coverslipped (*see* Note 14).

11. As described in **step 5**, place slides in humid chamber and check all slides for infiltration of bubbles. Incubate for 12–14 h in the dark at room temperature (*see* Note 16).

12. If 4% paraformaldehyde has already been made and is stored at 4 °C, pour into solution container(s) before beginning so that it can warm to room temperature during **steps 13** and **14**.

13. Carefully remove the CoverWell coverslips and load slides onto a slide holder immersed in stop buffer to prevent drying.

14. Wash slides in Stop Buffer for at least 30 min at room temperature with low-speed shaking.

15. Fix in 200 mL of 4% paraformaldehyde for 20 min at room temperature with low-speed shaking.

16. Wash 3× with 200 mL of Stop Buffer for 1 min each to remove paraformaldehyde.
17. Place in fresh Stop Buffer for up to 24 h until ready to coverslip.
18. Coverslip in Aqua Polymount. Take a rectangular glass coverslip (24 × 60 mm, no. 1.5) and place an even line of Polymount along the entire bottom edge of the coverslip, leaving no space between the Polymount and the edge (space can cause bubbles to form). Remove any bubbles with a micropipette. Take the slide out of stop buffer, dry the sides and back, and place the bottom edge against the bottom edge of the coverslip with Polymount. Allow the Polymount to slowly and evenly move up the slide so that no bubbles form. Wipe off any excess, press out any bubbles, and air-dry. Once dried, excess Polymount can be cleaned off with 95% ethanol.

3.2.4 Immunofluorescent Detection

1. Using CoverWell coverslips, incubate in 1× TNB for 1 h at room temperature with shaking at low speed. Use 200 µL per slide.
2. Dilute the anti-Digoxigenin primary antibody 1:150 in 1× TNB, cover each slide with 200 µL (use CoverWell coverslips), and incubate for 30 min at room temperature.
3. Wash 3× with TNT for 5 min each.
4. Dilute the biotinylated anti-sheep IgG secondary antibody 1:100 in 1× TNB. Cover each slide with 200 µL (use CoverWell coverslips) and incubate for 30 min at room temperature.
5. Wash 3× with TNT for 5 min each.
6. Dilute the streptavidin-HRP conjugate 1:100 in 1× TNB. Cover each slide with 200 µL (use CoverWell coverslips) and incubate for 30 min at room temperature.
7. Wash 3× with TNT for 5 min each.
8. Dilute the Cy3 substrate 1:50 in Cy3 dilution buffer. Cover each slide with 200 µL (use CoverWell coverslips) and incubate for 5 min in the dark at room temperature. Do not allow the reaction to proceed for more than 5 min.
9. Wash 3× with TNT for 5 min each.
10. Place slides in 1× PBS and store in the dark at 4 °C until ready to coverslip. Alternately, cell type-specific immunofluorescent staining may be performed at this point.
11. Coverslip using Vectashield mounting medium with DAPI.

4 Notes

1. Always prepare TEA fresh.

2. Acetic anhydride is rapidly hydrolyzed to acetic acid upon exposure to air. Keep closed tightly when not in use.

3. Dextran sulfate aliquots are good for up to 1 year. This solution can also be prepared by vortexing dextran sulfate with heated water in a 50 mL tube.

4. Do not use DEPC-treated water.

5. Use a commercially available humid chamber. Alternatively, a humid chamber can be constructed by gluing cut plastic pipettes to the base of a plastic chamber (such as the Corning 245 mm Square BioAssay Dish). Glue pipettes in pairs spaced 1 inch apart. Before adding slides, add a wet paper towel and 50 mL of dH_2O to the chamber. Place slides on the pipettes, ensuring that slides do not touch paper towels.

6. If using a full slide holder (48 slides), 160 mL of solution can be used instead of 200 mL for all steps. 180 mL can be used for a half-full slide holder.

7. Tissue sections should be mounted on RNase-free adhesion microscope slides. To avoid introduction of RNase during adhesive coating, we recommend purchasing precoated adhesion slides that are certified RNase-free.

8. For **steps 4–10** (Subheading 3.2.1), if using multiple slide holders, run both slide holders simultaneously: proceed through each wash with the second slide holder following one container behind the first. The same ethanol and SSC solutions (**step 4**) and RNase-free water washes (**step 8**) can be used for both slide holders. However, a fresh 3% hydrogen peroxide solution (step 5, if needed), Proteinase K solution (step 7), TEA buffer (**step 9**), and TEA/acetic anhydride wash (step 10) should be used for each slide holder.

9. Cut off the end of the pipette used to transfer dextran sulfate, as it is typically too thick to transfer with a standard pipette tip.

10. Adjust the total volume according to the number of slides needed (90 μL per slide). Extra can be stored at −20 °C. When using next, vortex and boil for 10 min, then put on ice and allow to cool.

11. The incubation temperature may need to be optimized for the specific riboprobe that is used.

12. If you have more than 48 slides, it is *strongly recommended* that you leave them at 4 °C overnight and continue the next day, as coverslipping can be very time consuming.

13. Use 220 μL per slide. CoverWells can be difficult to use, so prepare extra volume. 15 mL is recommended for one full slide holder (48 slides), 25 mL for two full slide holders (96 slides).

14. CoverWell coverslips may not cover all tissue sections on the slide. To ensure that the same tissue sections are coverslipped in each step, we recommend always coverslipping the sections that are closest to the frosted slide label. If these sections are damaged or folded, coverslip the sections furthest from the

label instead, and clearly label the slide to ensure that these same sections are coverslipped in all subsequent steps.

15. Do not use soap that contains lotion, such as hand soap.

16. Try not to exceed 16 h, as areas with strong signal can be overexposed.

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Table 1

Preparation of riboprobe DIG labeling reaction

Reagent	Volume	Control #1	Control #2	Template (1×) Sense	Template (1×) Antisense	Template (2×) Antisense	Template (5×) Antisense
10× NTP mixture	2 μL	2 μL	2 μL	2 μL	2 μL	4 μL	10 μL
10× Transcription buffer	2 μL	2 μL	2 μL	2 μL	2 μL	4 μL	10 μL
Protector RNase A Inhibitor (20 units/μL)	1 μL	1 μL	1 μL	1 μL	1 μL	2 μL	5 μL
SP6 RNA polymerase (20 units/μL), OR	2 μL	-	2 μL	-	-	-	-
T7RNA polymerase (20 units/μL), OR	2 μL	2 μL	-	2 μL	-	-	-
T3 RNA polymerase (20 units/μL)	2 μL	-	-	-	2 μL	4 μL	10 μL
Total reaction volume	20 μL	20 μL	20 μL	20 μL	20 μL	40 μL	100 μL

Table 2

Preparation of ethanol precipitation

Riboprobe labeling reaction mixture	24 μL	44 μL	144 μL
Glycogen (20 mg/mL) (optional)	1 μL	1 μL	1 μL
4 M LiCl	2.5 μL	5 μL	12.5 μL
100% EtOH (2.5× volume to be precipitated)	75 μL	125 μL	400 μL

Table 3

Preparation of RNA denaturation reaction

RNA	5 μL
5× MOPS Gel running buffer	2 μL
12.3 M Formaldehyde	3.5 μL
100% Formamide, deionized	10 μL
0.4 mg/mL ethidium bromide	1 μL
Total reaction volume	21.5 μL

Table 4

Preparation of hybridization solution

Reagent	Amount (μL)
digoxigenin (DIG)-labeled riboprobe, 4 μg	X
Nuclease-free water	440-X
Tris-EDTA-NaCl buffer	360
100% deionized Formamide	2500
40% Dextran sulfate	1200
10% Blocking solution	500
Total	5000

Table 5

Preparation of antibody dilution buffer

Stock solutions	Vol. stock solution	Final concentration (antibody solution)
dd water	6.7/10.05/13.4/16.75 mL	
5× Maleic acid buffer	2/3/4/5 mL	1× Maleate
10% Blocking solution	1.0/1.5/2.0/2.5 mL	1% Blocking solution
10% Triton X-100	0.3/0.45/0.6/0.75 mL	0.3% Triton X-100
Anti-Digoxigenin AP (ADD LAST)	4/6/8/10 μL	1:2500 dilution
Total volume	10/15/20/25 mL	