Video Article

A High Throughput *in situ* Hybridization Method to Characterize mRNA Expression Patterns in the Fetal Mouse Lower Urogenital Tract

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Abstract

Development of the lower urogenital tract (LUT) is an intricate process. This complexity is evidenced during formation of the prostate from the fetal male urethra, which relies on androgenic signals and epithelial-mesenchymal interactions[1,2]. Understanding the molecular mechanisms responsible for prostate development may reveal growth mechanisms that are inappropriately reawakened later in life to give rise to prostate diseases such as benign prostatic hyperplasia and prostate cancer.

The developing LUT is anatomically complex. By the time prostatic budding begins on 16.5 days post conception (dpc), numerous cell types are present. Vasculature, nerves and smooth muscle reside within the mesenchymal stroma[3]. This stroma surrounds a multilayered epithelium and gives rise to the fetal prostate through androgen receptor-dependent paracrine signals[4]. The identity of the stromal androgen receptor-responsive genes required for prostate development and the mechanism by which prostate ductal epithelium forms in response to these genes is not fully understood. The ability to precisely identify cell types and localize expression of specific factors within them is imperative to further understand prostate development. *In situ* hybridization (ISH) allows for localization of mRNAs within a tissue. Thus, this method can be used to identify pattern and timing of expression of signaling molecules and their receptors, thereby elucidating potential prostate developmental regulators.

Here, we describe a high throughput ISH technique to identify mRNA expression patterns in the fetal mouse LUT using vibrating microtome-cut sections. This method offers several advantages over other ISH protocols. Performing ISH on thin sections adhered to a slide is technically difficult; cryosections frequently have poor structural quality while both cryosections and paraffin sections often result in weak signal resolution. Performing ISH on whole mount tissues can result in probe trapping. In contrast, our high throughput technique utilizes thick-cut sections that reveal detailed tissue architecture. Modified microfuge tubes allow easy handling of sections during the ISH procedure. A maximum of 4 mRNA transcripts can be screened from a single 17.5dpc LUT with up to 24 mRNA transcripts detected in a single run, thereby reducing cost and maximizing efficiency. This method allows multiple treatment groups to be processed identically and as a single unit, thereby removing any bias for interpreting data. Most pertinently for prostate researchers, this method provides a spatial and temporal location of low and high abundance mRNA transcripts in the fetal mouse urethra that gives rise to the prostate ductal network.

Video Link

The video component of this article can be found at http://www.jove.com/details.php?id=2912

Protocol

1. Synthesis of a Digoxigenin-11-UTP-Labeled Riboprobe from a PCR-Generated Template

1. To synthesize a gene-specific riboprobe, use Entrez Gene (*http://www.ncbi.nlm.nih.gov/sites/entrez*) to obtain the gene cDNA reference sequence (RefSeq). Use the Primer3 program (http://frodo.wi.mit.edu/primer3/)[5] to design gene-specific PCR primers against the 3'-region of the cDNA sequence. Recommended parameters for PCR primer selection are described elsewhere (http://www.gudmap.org/Research/Protocols/Vezina/Riboprobe_Syn.html).
2. Use the MegaBLAST Program (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=10090)[6] to assess specificity of the selected DNA sequence. The DNA sequence is considered specific when, using an EXPECT threshold of 0.01, it does not align with other sequences in the RefSeq database.
3. PCR amplify the riboprobe template. PCR reaction components and thermocycling conditions should be optimized for each primer set. A typical 50 µl reaction contains: 1X buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 1X Q solution, 1µg cDNA, 2.5U Taq DNA polymerase, 0.25µm primers, and nuclease-free H_2O. A typical thermocycling protocol includes an initial denaturation at 94°C for 2min followed by 40 cycles of 94°C for 30sec, 57°C for 30sec, 72°C for 1min and a final extension at 72°C for 10min. The cDNA used in the PCR reactions is synthesized from mouse urogenital mRNA.
4. Separate the PCR product by agarose gel electrophoresis, purify using the Gel Extraction Kit, and quantify the purified products by spectrophotometry. The expected yield is 1.2 - 3.6µg.
5. Transcribe the PCR product into a labeled riboprobe. The transcription reaction (40 µl) contains: 400ng purified PCR product, 1X nucleotide labeling mix containing digoxigenin 11-UTP, 1X transcription buffer, 5U RNase inhibitor, 80U T7 RNA polymerase, and nuclease-free H_2O. Incubate 3-4hr at 37°C and agitate samples every 30min.

6. Use the Qiagen RNEASY Mini kit to purify riboprobes based on instructions for RNA cleanup with on-column DNase digestion. Quantitate riboprobes by spectrophotometry. The expected yield is 4-20 µg. Assess probe quality by separating an aliquot by electrophoresis on a 1.5% non-denaturing agarose gel. High quality probes migrate as distinct bands with minimal smearing.
7. To ensure the riboprobe specifically recognizes its target, include a positive control tissue for the first ISH experiment. The riboprobe's target mRNA pattern should be known in this positive control tissue.

2. Preparation of Vibrating Microtome Blade (Based on Previously Described Protocol)[7]

1. Prepare a 4% low-melt agarose solution in phosphate buffered saline (PBS). Microwave solution to dissolve agarose and maintain solution at 62°C.
2. Prepare a polystyrene ring mold by removing the membrane from a 12mm diameter Millicell culture plate well insert and retain polystyrene ring to use as an agarose mold. Soak the rings overnight in RNase inhibitor solution prior to each use.
3. To ensure a smooth cutting surface, remove rust inhibitor and other additives from the surface of the Wilkinson blade by rinsing with the following solvents, at 100% concentration: petroleum ether, xylene, chloroform, methanol, and MilliQ water. Separate the double blade lengthwise into two single blades.
4. Adhere the Wilkinson blades to a microtome blade with Loctite adhesive. The microtome blade is not used for cutting; it adds rigidity to the Wilkinson blade. The microtome blade should be cut to the length of the Wilkinson blade using metal shears and, once adhered, should be offset by 3 – 4mm from the cutting edge of the Wilkinson blade.

3. Dissection, Storage, and Preparation of Urogenital Tissues for Sectioning

1. Prepare PBSTw solution (PBS containing 0.1% Tween™ 20 and 0.2mM sodium azide, filtered through the 0.22µm Stericup® filter unit). Solution can be prepared in advance and stored at 25° C.
2. Incubate a freshly dissected mouse LUT (bladder, pelvic urethra, and associated Wolffian and Müllerian duct-derived structures) overnight at 4°C in PBS containing 4% paraformaldehyde fixative.
3. Dehydrate tissues by washing for 10min at 25°C in a series of graded methanol/PBSTw (1:3, 1:1, 3:1 v/v) solutions. Store samples at –20°C in 100% methanol at least overnight. Archived tissues may be kept at least 2yr.
4. Prepare tissues for sectioning by rehydrating archived tissues. Wash for 10min at 25°C in a series of graded methanol/PBSTw (3:1, 1:1, 1:3 v/v) solutions.
5. Dissect and discard approximately two-thirds of the bladder, leaving most of the trigone region attached to the urethra.

4. Embedding Urogenital Tissue in Agarose

1. Place the polystyrene ring mold, flat surface down, on a 25°C plain glass microscope slide.
2. Fill the ring mold with 62°C agarose solution and cool for about 2min.
3. Remove LUT tissue from PBSTw and blot dry on an absorbent wipe.
4. Transfer the tissue into agarose solution.
5. Use forceps to orient the LUT tissue in agarose so that it is suspended halfway between the top and bottom of the ring mold and incubate the tissue at 4°C until the agarose has solidified.
6. If the tissue sinks completely during the process of agarose solidification, it can be excised from the agarose and re-embedded. Adjust the cooling time of the agarose as needed during the re-embedding process.

5. Sectioning Urogenital Tissue With a Vibrating Microtome (Based on Previously Described Protocol)[7]

1. Mount the reinforced Wilkinson blade in the vibrating microtome and set the blade angle to 35°. Fill deluxe specimen bath with PBS and pack wet ice around the specimen bath.
2. Remove the solidified agarose plug from the ring mold and blot the bottom surface with an absorbent wipe. Verify that the tissue is oriented correctly. Tissues orientation can be adjusted by using a razor blade to bevel the flat edge of the agarose plug.
3. Adhere the agarose plug onto a vibrating microtome specimen mounting disk with Loctite adhesive as shown in **Fig. 1A**.
4. Insert the specimen mounting disk into the vibratome.
5. Adjust the microtome section thickness to 50µm, the speed to 2, and the blade amplitude to 4 and begin cutting tissue sections.
6. Use blunt forceps to transfer each tissue section (**Fig. 1B**) to a 24-well culture plate well that contains ice-cold 0.5mL PBSTw.
7. To prepare samples for *in situ* hybridization, excise most of the agarose around each tissue section (the remaining agarose will melt during the ISH procedure) and remove all associated debris. Store sections up to 48hr at 4°C in PBSTw.

6. Sample Basket Preparation For *In Situ* Hybridization

1. Cut the bottom of a microcentrifuge tube at the 100µL mark.
2. Heat the cut edge of the tube in a flame until the plastic is softened, then press the microcentrifuge tube firmly onto the center of a 0.5in polyester mesh square.
3. Trim excess mesh and use a heated 18 gauge needle to pierce two holes into each tube lid to complete basket preparation (**Fig. 1C**).
4. Remove the lid of a 24 well plate and drill a 12mm hole centered over each well. Use the lid to transfer sample baskets between washes of the ISH protocol (**Fig. 1D**).

7. Embryo Powder Preparation For *In Situ* Hybridization (Based on Previously Described Protocol)[8]

1. Collect mouse embryo tissue from mice that are the same stage as the tissue sections that are being assessed and store at –80°C. Place frozen tissue into a ceramic mortar, submerge tissue in liquid nitrogen, and use a pestle to grind tissue into a fine powder.
2. Combine embryo powder with 4 volumes of acetone and homogenize with several strokes of a dounce homogenizer.

3. Transfer homogenate to a 15mL glass screw-top vial and extract overnight at 4°C.
4. Pellet embryo powder by centrifugation at 5000rpm for 10min at 4°C. Remove and discard the lipid-containing supernatant. Resuspend the tissue pellet in 4vol of fresh acetone and extract for 2hr at 4°C.
5. Pellet the embryo powder by centrifugation at 5000rpm for 10min at 4°C. Remove and discard the supernatant.
6. Air dry the pellet on a #2 Whatman filter paper. Crush pellet to yield a fine powder and store in a tightly sealed glass vial at 4°C. The approximate yield is 50 mg powder per 1 g embryo wet weight.

8. *In Situ* Hybridization Day 1

1. Preheat prehybridization solution (50% formamide, 5x SSC, 1% Blocking reagent, 10µg/mL yeast tRNA, 10µg/mL heparin store at -20°C) to 60.5°C. This solution can be prepared in advance and stored at -20°C.
2. Prepare a humidified hybridization chamber by filling a small plastic storage container with about 0.5 in of tap water. Cover container and preheat to 60.5°C.
3. Add 2mL PBSTw to the wells of a 24-well culture plate. Place sample baskets in the holes of the 24-well plate lid and transfer tissue sections into the baskets (up to 10 sections per basket have been used).
4. Incubate tissue sections for 30min at 25°C in 6% H_2O_2. This and all subsequent incubations should be carried out with gentle agitation on an orbital shaker, unless otherwise indicated. All incubations and washes are conducted in 24-well plates and use a total solution volume of 2mL/well.
5. Wash tissue sections 4 x 5min at 25°C in PBSTw.
6. Incubate tissue sections for 12min at 25°C in PBSTw containing 5µg/mL proteinase K.
7. Wash tissue sections 1 x 5min at 25°C in PBSTw.
8. Post-fix tissue sections for 20min at 25°C in PBS containing 4% paraformaldehyde and 0.2% glutaraldehyde.
9. Wash tissue sections 2 x 5min at 25°C in PBSTw.
10. Add 2mL/well of the prewarmed prehybridization buffer and incubate tissue sections inside humidified hybridization chamber for at least 1hr at 60.5°C.
11. Add 0.65µg labeled riboprobe to the prehybridization buffer in each well and incubate tissue sections overnight in the humidified hybridization chamber at 60.5 °C.

9. *In Situ* Hybridization Day 2

1. Prepare the following solutions for post-hybridization washing steps: Solution 1 (50% formamide, 5x SSC, 1% SDS), Solution 2 (10mM Tris-HCL pH 7.5, 0.5M NaCl, 0.1% Tween™ 20, 0.2mM sodium azide, 0.22µm filtered), and Solution 3 (2x SSC, 50% formamide). These solutions can be prepared in advance. Solutions 1 and 3 are stored at -20°C and Solution 2 is stored at 25°C. The storage life of the solutions is at least 3 months.
2. Wash tissue sections 3 X 30min at 60.5°C with pre-warmed Solution 1. Use the humidified chamber during washes.
3. Wash tissue sections 1 X 10min at 60.5°C with pre-warmed Solution 1/Solution 2 (1:1 v/v) solution. Use the humidified chamber during the wash.
4. Wash tissue sections 4 X 10min at 25°C with Solution 2.
5. Incubate tissues sections for 15min at 37°C in Solution 2 containing 0.25µg/mL RNase.
6. Wash tissue sections 1 X 10min at 25°C with Solution 2 (without RNase).
7. Wash tissue sections 1 X 10min at 25°C with Solution 3, followed by 2 X1hr washes at 60.5°C with Solution 3. Use the humidified chamber during the 60.5°C washes.
8. Prepare the following solutions for immunohistochemical detection of the DIG-labeled riboprobes: Tissue Blocking Buffer (TB, 1X TBS, 10% sheep serum, 1% blocking reagent, 1% BSA, 0.1% Tween™ 20, 0.22µm filtered), Antibody Dilution Buffer (AD, 1xTBS, 5% sheep serum, 1% blocking reagent, 1% BSA, 0.1% Tween™ 20, 0.2mM sodium azide, 0.22™m filtered) Antibody Absorption Buffer (AA, 1xTBS, 5% sheep serum, 1% blocking reagent, 1% BSA, 6mg/mL embryo powder), and TBSTw (1xTBS, 0.1% Tween™ 20, 0.2mM sodium azide, 0.22µm filtered). These solutions can be prepared in advance. TBSTw is stored at 25°C, all other solutions are stored at -20°C.
9. Wash 3 X 10 min at 25°C with TBSTw.
10. Incubate the tissue sections at least 2hr at 25°C in TB buffer.
11. While tissues are incubating in TB buffer, add 1.1µL anti-DIG antibody per 200µL AA buffer for each well. Incubate AA buffer + antibody at least 2hr at 4°C, then centrifuge at 10,000rpm for 1min. Remove the AA buffer supernatant and add it to 2mL of AD buffer.
12. Remove tissue sections from TB buffer and incubate them overnight in a humidified chamber at 4°C in AD buffer containing antibody.

10. *In Situ* Hybridization Day 3

1. Prepare color development solution NTMT (100mM Tris-HCL pH 9.5, 100mM NaCl, 50mM $MgCl_2$, 0.2mM sodium azide, 0.22µm filtered). This solution can be prepared in advance and stored at 25°C. Immediately prior to use, add 2mM levamisole and 0.1% Tween™ 20.
2. Remove the antibody solution (AD buffer + antibody) from the wells and store solution at 4°C. It can be reused up to two additional times.
3. Wash the tissues 8 X 10 min at 25°C with TBSTw containing 2mM levamisole.
4. Carefully transfer tissues from the baskets to a Petri dish containing TBSTw. Use forceps to remove visible debris. Transfer the tissues into clean microcentrifuge tubes.
5. Wash tissues 1 X 10 min at 25°C with 1mL NTMT.
6. Remove the NTMT and add 1mL/tube of a mixture containing 50% NTMT (containing 2mM levamisole) and 50% BM Purple, place tubes in a light protected box and incubate at 25°C. Color development time ranges from several hours to several days. If color is slow to develop, 100% BM Purple can be used.
7. Monitor color development and change NTMT/BM Purple solution if it accumulates precipitated crystals or if it undergoes color change from yellow to purple. After full color development (4-250 hours), wash tissues 2 X 5min at 25°C with 1mL/tube of NTMT containing 2mM levamisole.
8. Incubate tissues overnight at 4°C in 1mL/tube of PBS containing 4% paraformaldehyde post-fixative.
9. To bleach the tissues, incubate tissues for 30min at 25°C in 1mL/tube of PBSTw containing 3% H_2O_2. Then wash the tissues 1 X 10min at 25°C in 1mL/tube of PBSTw and store at 4°C in 1mL/tube of PBS containing 4% paraformaldehyde post-fixative.
10. Tissue sections are mounted on glass slides, coverslipped, and imaged with a compound microscope.

11. Representative Results:

The spatial orientation of LUT tissue in agarose determines the plane of tissue sections. For sagittal sections, at least two-thirds of the bladder is excised and the remaining LUT tissue is embedded in agar such that the urethral midline is parallel to the flat surface of the agarose plug (**Figure 1A**). Minor adjustments in the tissue plane can be made by beveling the flat edge of the agarose plug. A representative sagittal section from a 17.5dpc male LUT tissue that is oriented in this plane is shown in **Figure 1B**.

Sample baskets protect the delicate tissue sections from loss and from accumulating dust and particulate matter during the multi-day ISH procedure. Sample baskets are prepared by melting polyester mesh to the cut end of a 1.5mL microfuge tube (**Figure 1C**). A small hole is pierced into the lid of each sample basket to facilitate solution flow into and out of the baskets. Sample baskets are suspended in ISH solutions by placing them in 12mm holes drilled into 24-well plate lids (**Figure 1D**). The modified plate lids support baskets when they are transferred between 24-well plates during solution changes.

It is challenging to limit non-specific background staining during the long incubation periods required for detection of low abundance mRNAs. The addition of 0.2mM sodium azide to sample buffers and their subsequent filtration through 0.22µm filters appeared to limit background staining **(Figure 2)**. Using the method described here, there does not appear to be visible differences in background staining when samples are incubated in color development solution for a prolonged time periods (**Figure 3**).

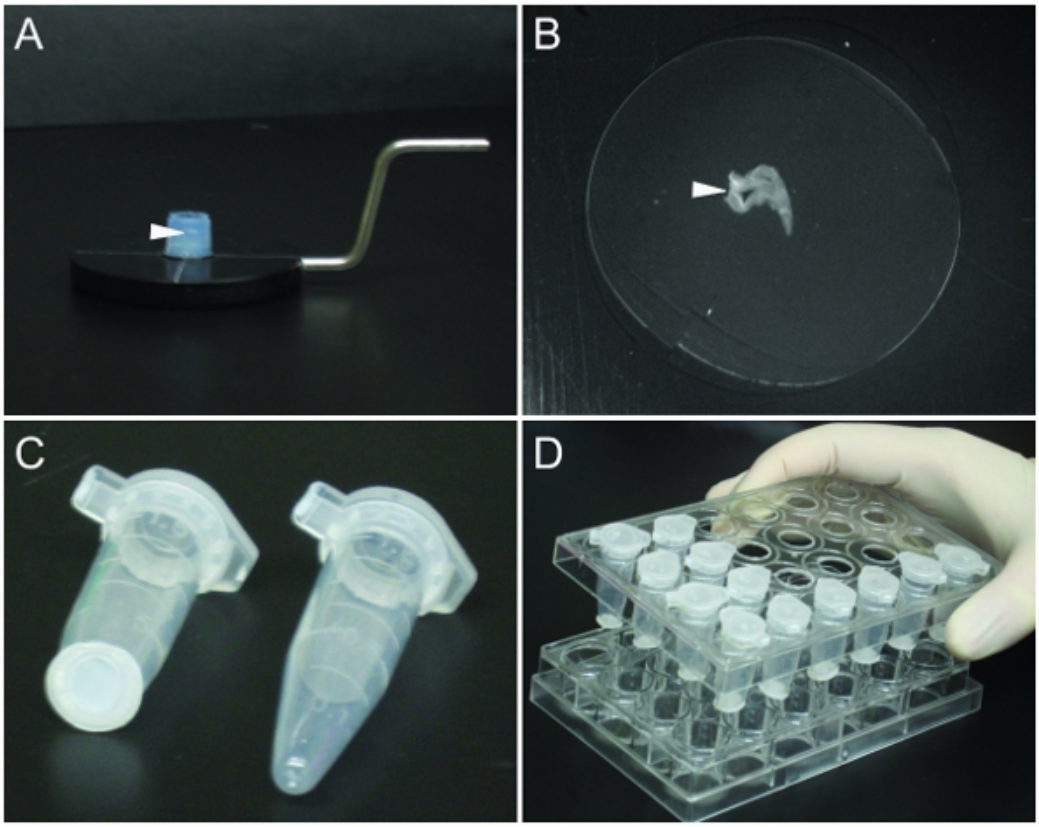

Figure 1. Preparation of a mouse lower urogenital (LUT) tract tissue section and microcentrifuge tube a basket for ISH. A LUT containing part of the bladder, pelvic urethra and associated Wolffian and Müllerian duct-derived structure) is embedded in a cylindrical plug of 4% low-melt agarose. (A) The plug is glued to a specimen mounting disk and (B) cut into 50µm sections with a vibrating microtome. (C) An LUT section is transferred into a microcentrifuge tube basket that is prepared by piercing a hole into the tube lid and fusing polyester mesh to the cut bottom end of the tube. (D) The microcentrifuge tube is inserted into 12mm holes drilled into a 24-well plate lid so that tissue sections are suspended in buffer solution during the ISH protocol. Arrowheads indicate the LUT tissue in the agarose plug.

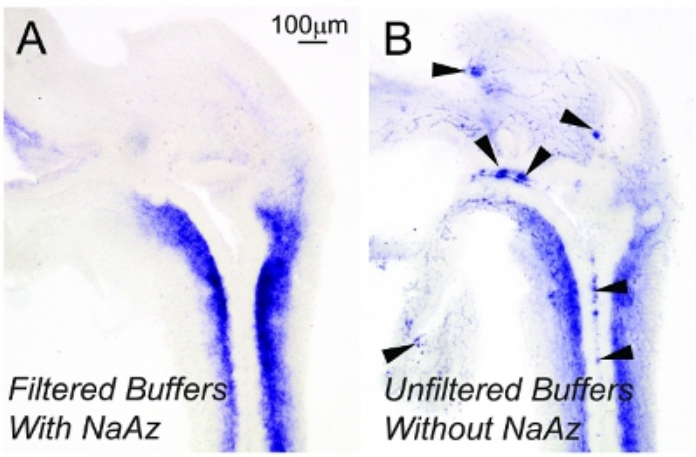

Figure 2. Incorporation of 0.2mM sodium azide into 0.22µm filtered solutions improves tissue quality and reduces background staining. 17.5dpc male mouse lower urogenital tracts (LUTs) were sectioned in a sagittal plane to a thickness of 50µm. Tissue sections were stained by ISH using a probe directed against twist homolog 1. Buffers used for ISH were either (A) 0.22µm filtered and supplemented with 0.2mM sodium azide (NaAz) or (B) unfiltered and not supplemented with NaAz. Arrowheads indicate background staining. Images were captured at the same magnification. Results are representative staining patterns for *n* = 3 litter-independent mice.

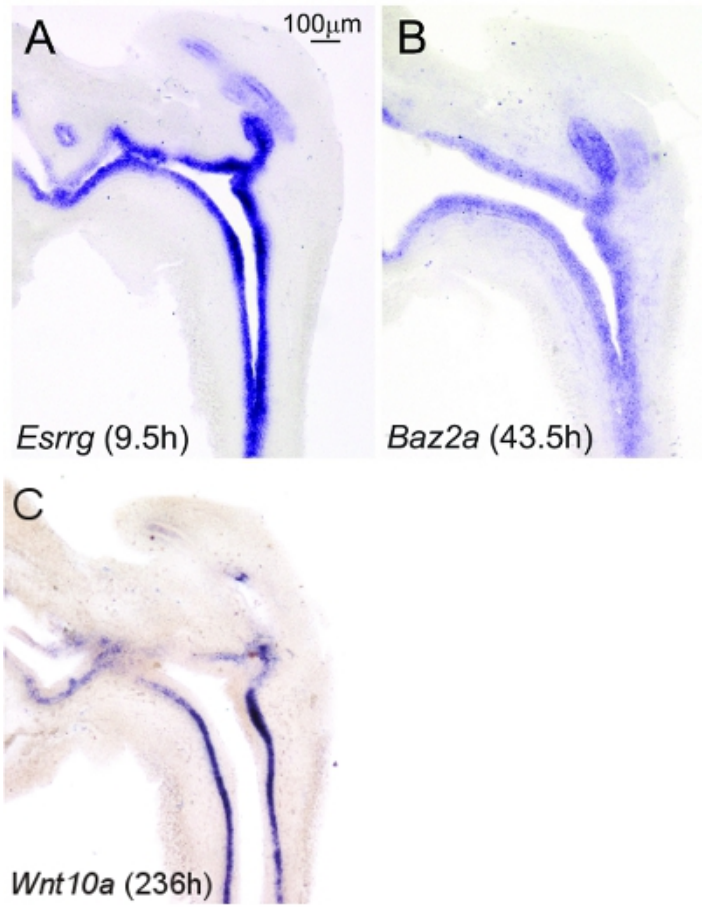

Figure 3. Background staining intensity does not appear to increase with prolonged color development. 17.5dpc male mouse lower urogenital tracts (LUTs) were sectioned in a sagittal plane to a thickness of 50µm. Sections were stained by ISH by incubating them in chromagen staining solution for (A) 9.5h using a probe that recognizes the high abundance transcript estrogen-related receptor gamma (*Esrrg*), (B) for 43.5h using a probe that recognizes the medium abundance transcript bromodomain adjacent to zinc finger domain, 2A (*Baz2a*), or (C) for 236h using a probe that recognizes the low abundance transcript wingless-type MMTV integration site family, member 10a (*Wnt10a*). Images were captured at the same magnification. Results are representative staining patterns for *n* = 3 litter-independent mice.

Discussion

Using the method described here, it is possible to detect mRNAs in all of the major cell types and tissue compartments of the fetal male and female mouse LUTs including the mesenchymal pads, urothelium, smooth muscle, prostatic buds, ejaculatory duct, and vagina. The 50µm sections used in this protocol have the advantage of being thick enough to resolve tissue architecture (such as blood vessels) but are thin enough to avoid probe trapping, which is a methodological problem commonly encountered during whole-mount ISH. Every new riboprobe is assessed on positive control tissue, where staining has been assessed in a previous published study. We ensure that staining patterns are specific in these tissues. Another advantage of our method is that patterns of multiple mRNAs can be assessed in adjacent tissue sections from the same LUT tissue. Furthermore, this method can be coupled with immunohistochemical techniques to visualize cell specific protein markers and identify cell types stained by the ISH protocol. This method is incompatible with traditional counterstaining methods, such as nuclear counterstaining with Fast Red or hematoxylin. However, we have successfully counterstained samples with fluorescent nuclear stains, including 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide.

The method described here includes several improvements over one that was previously described[7]. Almost all of the buffers and reagent stock solutions in the current manuscript are prepared in advance and can be stored for long periods of time, which increases efficiency. The addition of 0.2mM sodium azide to most reagents and their filtration through 0.22µm membranes greatly diminishes non-specific background staining, increases reagent shelf-life, and minimizes accumulation of particulate matter on the tissue sections during the ISH procedure. This manuscript also describes the manufacture of permeable microcentrifuge tube baskets that contain tissue sections during ISH and a modified multi-well culture plate lid that holds the baskets during buffer changes. We found that these devices, which are easily manufactured in the lab, reduce sample loss, minimize tissue section damage during processing, and improve efficiency. Furthermore, the use of a sealable plastic container as a humidity chamber helps to protect against evaporation. This is especially important for tissue sections in peripheral sample wells, where the so-called ‘edge effect’ can introduce an experimental variable. While using humidity chambers, we have not observed appreciable staining quality differences in peripheral versus inner sample wells.

While this protocol is an efficient mechanism to study mRNA expression patterns in mouse LUT tissues, there are some limitations with this method. Efficiency of mRNA detection varies among riboprobes and absolute abundance of mRNAs cannot be determined. Another limitation is that staining patterns can vary depending on the section plane. To minimize these limitations, we assess each mRNA pattern in multiple sections from multiple litter-independent fetuses.

This method can be optimized for visualizing mRNA expression patterns in other mouse tissue types or other species. Such a modification requires that the microtome blade amplitude and speed be optimized during tissue sectioning and that proteinase K concentration be optimized during the ISH procedure. Furthermore, it is necessary to pre-absorb the anti-digoxigenin antibody with embryo powder from the same species of tissue that is being assessed by ISH. Although this method is not useful for tissue sections thinner than 40 microns, because the sections disintegrate during ISH staining, it can be adapted for use in high-throughput whole-mount ISH staining. We have used this method successfully to conduct whole-mount ISH on fetal and neonatal mouse prostate as well as fetal gonad and kidney. For whole-mount ISH staining, it is necessary to optimize proteinase K tissue digestion as well as the quantity and duration of washes following overnight antibody incubation to reduce background staining caused by probe trapping.

Disclosures

No conflicts of interest declared.

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