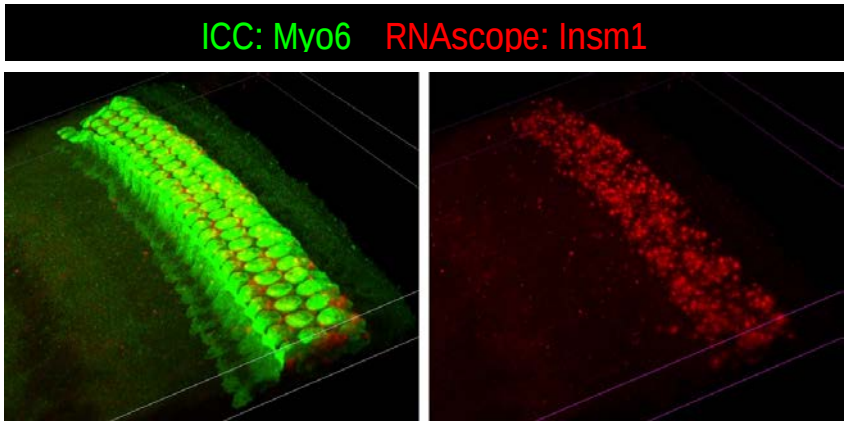
RNA Scope on neonatal mouse cochlear tissue



RNAscope Red manual kit for Insm1 on P0 mouse cochlea

NOTES:

1) The fresher the tissue, the better your results (even if you use RNAlater, we see decreased efficacy after ~2-3 weeks, definitely after 1 month).
2) Kits and probes tend to work better when they are new as well (though their shelf life is generally 6 months or more)
3) If using sections, thicker cut seems to work better (> 15 um).
4) For ALL steps, make sure the tissue is completely immersed in the solution. If the tissue is floating or stuck to a side-wall and not submerged during any step, it will not work.
5) We have protocols for embryonic tissue, for adult tissue, and for sections. If you run into any problems or want any assistance, feel free to contact me (bwalters2@umc.edu).
6) ACDBio (BioTechne) also have a great team who have generously supported this course with a number of reagents and technical assistance and they are a tremendous resource if you need help with your assays: www.ACDBio.com/RNAscope

Dissection and Whole Mount

Reagents Needed: Sterile DPBS,
Sterile 4% PFA
RNA-Later solution

1. For neonatal/postnatal mice (< P14), euthanize by rapid decapitation (use isoflurane first if > P7), immediately remove the temporal bones and immerse them in 4% PFA at room temperature on the paddle rotator for 3 hours. Replace the PFA with RNA-Later and store at 4º C until ready to proceed to fine dissection. **NOTE:** for highly expressed transcripts, round window perfusion of PFA is not necessary, however, for lower abundance transcripts, or, if quantifying differences in expression, perfusion may work better. Sometimes things also work a little better if you dissect the tissue in cold HBSS and HEPES before fixing with 4% PFA.

2. Fine dissection of inner ear tissues: In a petri dish filled with cold DPBS-RNA-later solution (20% RNA-Later in DPBS), dissect the cochlea (or utricle, etc.) out from the temporal bone and store each sample in a well of a round bottom 96 well plate. Fill the wells with several hundred microliters of RNA-later and store samples in 4º C until ready to perform RNAscope. **NOTE:** For this lab, you will want at least 3 samples (positive ctrl, negative ctrl, and a test sample). To accomplish this, you can cut one cochlea into 3 turns, or dissect more than one cochlea.

RNAscope Pre-treatment (Day 1)

Reagents needed: RNAscope H2O2 & Protease Plus Reagents (Cat# 322330)
Vector Labs Low pH Antigen Unmasking Solution (Cat# H3300)
RNAscope probe(s) of interest (see list of probes below)

1. Set a hybridization/baking oven (Fisher IsoTemp) to 65°C.

2. Remove any RNA-Later and then add **250 uL** of 50% EtOH to each well and incubate for 5 min at RT. Remove and follow with 70% EtOH, 95% EtOH, and then 100% EtOH for 5 min each (**NOTE**: sometimes an extra 100% EtOH step helps if you have low signal or high background).

3. Aspirate off as much EtOH as possible, BUT **DO NOT** DRY THE SAMPLE IN THE OVEN (and do not let it dry out completely by air drying, only 2-5 min air drying is usually required). Add 3 drops of Pre-treat 1 (peroxide) to each well of the dish and incubate for 30 min at RT (If not using peroxide from the kit, use 0.3% H2O2 solution, and add enough to completely immerse the tissue). Wash 2X with MQ H2O.

4. While the 30 min H2O2 step is proceeding: prepare low pH antigen unmasking solution (Vector Labs H-3300) in a 50ml Falcon tube by diluting 1:100 in MQ H2O. Aliquot several mLs into a 15mL conical tube and preheat in the oven at 65°C. **NOTES:** The antigen unmasking solution tends to work best if it is diluted same day... do not use diluted antigen unmasking solution that has been sitting around for more than a week. Also, save what you made up in the 50 mL tube as you will be using this as your wash buffer in subsequent steps.)

5. Remove the last H2O wash, then add **200-250 uL** of low pH antigen unmasking solution to each well and place the 96 well plate directly on the metal surface in the bottom of the oven. Incubate for 5 min. Wash immediately 2X with MQ H2O and **then repeat step 5. NOTE:** For this lab, it would be great if you could share plates so we are not trying to put 19 plates in the oven at the same time.

6. Remove the MQ H2O, then add 150 uL of pretreat III (protease plus) to each well (make sure the tissue is completely covered). Incubate for 25 min at RT. Wash 2X with MQ H2O.

7. At this point, the tissue can be stored in MQ H2O overnight at 4º C (sitting overnight in the residual protease helps).

Hybridization (Day 2)

1. Preheat oven to 40°C. Place a humidifying chamber in the oven to warm to 40°C. We use a black freezer box (Fisher cat # 14-100-F) as our humidifying chamber, just fill the bottom with some ddH2O or MQH2O. For this and all subsequent steps, place the humidifying chamber on the metal rack in the oven, **DO NOT** place the chamber on the metal floor of the oven.

2. Add probes, 150 uL to each well, enough to cover the tissue. Place the lid on the plate then put the plate in the humidifying chamber and hybridize probes for 2h at 40°C. **NOTE**: it is

generally a good idea to fill some of the wells around your sample wells with MQ H2O so that the 96 well plate acts as its own humidifying chamber

Amplification and detection (Day 2 cont'd)

Reagents Needed: RNAscope 2.5 HD Detection Reagents – RED (Cat# 322360)
Vector Labs Low pH antigen unmasking solution (H-3300)

NOTE: again it is critical that the tissue be completely covered by all of the solutions for all of the steps.
NOTE: we have shortened the washes for the protocol here at BIE, but in general if you increase the time and number of washes, you can get better signal to noise

AMP1: Wash 2 x 2 min in low pH antigen unmasking solution (Vector labs H-3300, diluted 1:100 in ultrapure H2O, 200 – 250 uL per well). While washing, warm AMP1-6 reagents to room temperature. Then add 150 uL of AMP1 to each well and place in the humidifying chamber in the oven at 40°C for 30 min.

AMP2: Wash 2 x 2 min in low pH antigen unmasking solution. Then add 150 uL of AMP2 to each well and place in the humidifying chamber in the oven at 40°C for 15 min.

AMP3: Wash 2 x 2 min in low pH antigen unmasking solution. Then add 150 uL of AMP3 to each well and place in the humidifying chamber in the oven at 40°C for 30 min.

AMP4: Wash 2 x 2 min in low pH antigen unmasking solution. Then add 150 uL of AMP4 to each well and place in the humidifying chamber in the oven at 40°C for 15 min.

AMP5: Remove the humidifying chamber from the oven and leave to cool on the lab bench. Wash the tissue 2 x 2 min in low pH antigen unmasking solution. Then add 150 uL of AMP5 to each well and place in the humidifying chamber for 25 - 30 min **at Room Temp**.

AMP6: Wash 2 x 2 min in low pH antigen unmasking solution. Then add 150 uL of AMP6 to each well and place in the humidifying chamber for 10 - 15 min **at Room Temp**. Then wash 2 x 2 min in low pH antigen unmasking solution.

Briefly spin down the contents of the Fast RED-B tube to be sure contents are at the bottom of the tube before opening the cap. Prepare sufficient RED working solution per section by using a 1:75 ratio of Fast RED-B to Fast RED-A. For example, add 2 µL of Red B to 150 µL of Red A into a tube. Mix well.
IMPORTANT! Use the Fast RED solution within 5 MIN of mixing A and B. Do not expose to direct sunlight or UV light.

Pipette ~150 µL RED solution into each well. Ensure tissue is covered, and incubate for 4 - 10 MIN at RT (best to watch the color reaction under the stereomicroscope to see how long).

Remove the RED working solution from the slides, and wash 3 x 5 min in MQ water.

Samples can be stored in MQ Water or other aqueous buffer at 4ºC if further processing is required (e.g. immunostaining). If no other processing is required, mount the tissue to slide and

coverslip. **DO NOT** expose the tissue to EtOH or any other organic solvents, or mounting media containing ethanol or other organics as this will dissolve the Fast RED. **NOTE:** If signal is faint, you may be able to increase it by lengthening the AMP5 step. AMP6 may also be lengthened to 15 min.

Immunofluorescence

NOTE: If performing immunofluorescence subsequent to Fast RED reaction, it is important to note that the Fast Red has an emission spectra that extends into the far-red channel and so any antigens you wish to detect with Alexa-647 antibodies (or similar) will have to have a good signal to noise ratio as the Fast Red can cause bleedthough in this channel. If you are attempting to show co-localization, it would be best for the protein of interest to be labeled in the blue or green channels.

Wash tissue 2X in PBS.

Apply your primary antibodies in 0.05% TritonX in PBS and incubate for 15hrs (i.e. overnight) at 4°C. Wash 3X in PBS. **NOTE:** Lina Jensen in Alan Cheng's lab recommends not using blocking buffer as it seems to worsen rather than improve immunostaining after RNAscope. We have found that immunostaining with all of our antibodies so far works very well without performing a blocking step.

Apply secondary antibodies in 0.1% TritonX in PBS and incubate at RT in the dark for 2h.

Wash 3X in PBS.

Optional: Incubate in Hoechst at 1:1500 for 25 min. Then wash 3 x 5 in in PBS

Mount coverslips on slides using 50% glycerol in PBS (or other aqueous mounting media) and seal the edges with clear nail polish. NOTE: If you used the FastRED, do not use mounting media that contains EtOH or other organic solvents as it will dissolve the FastRED.

NOTES:

GFP/EGFP/tdTomato are mostly quenched by the in situ procedure so endogenous fluorophores can only be simultaneously detected via immunostaining. Abcam's anti-GFP (rabbit, IP grade, or the chicken anti-GFP) and rabbit anti-mCherry are excellent antibodies for this.

It is important to use the low pH antigen unmasking solution instead of the kit wash buffer as the kit wash buffer contains LDS detergent and may disintegrate the tissue or we often find cause it to stop sticking to the slide if you are doing sections. If you are not doing mouse cochlea, but have a thicker tissue with more ECM, you may want to use the kit wash buffer instead.

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