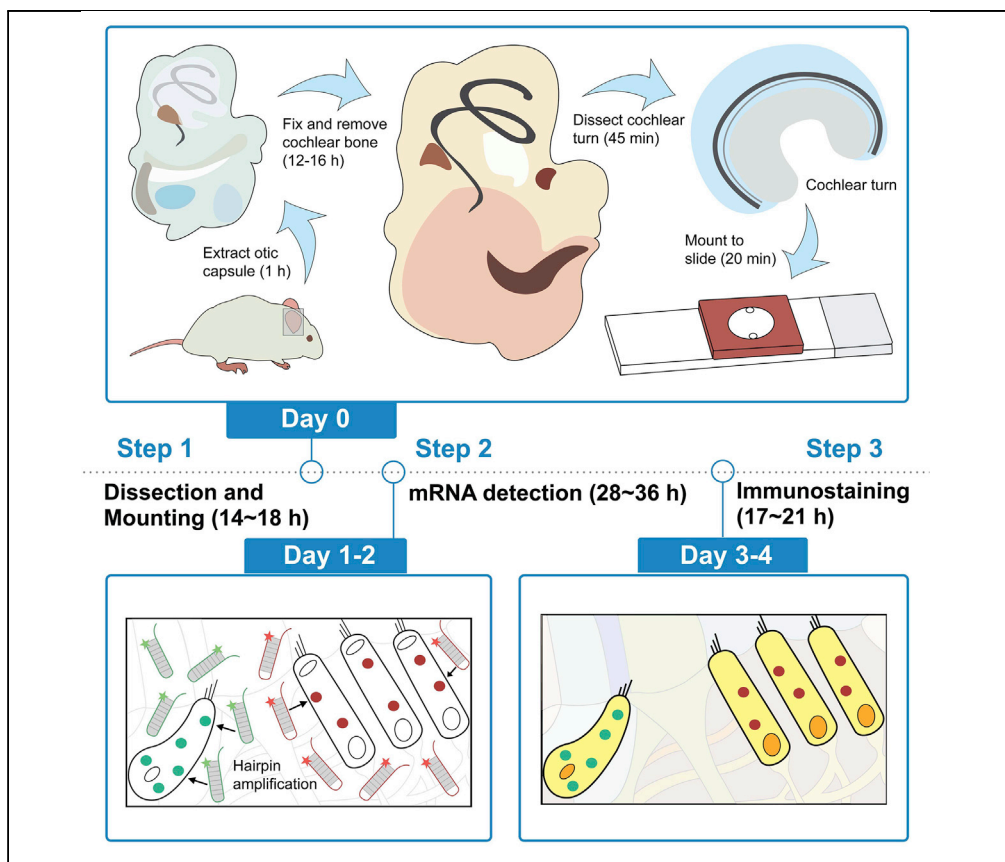


Protocol

Fluorescent *in situ* mRNA detection in the adult mouse cochlea



Ossification and the delicateness of the cochlear duct make histologic assessments of the mature cochlea a challenging endeavor. Treatments to soften the bone facilitate sectioning and dissection of the cochlear duct but limit *in situ* mRNA detection in such specimens. Here, we provide a protocol for *in situ* mRNA detection using hybridization chain reaction in whole-mount preparations of the adult mouse cochlea. We show examples for multi-probe detection of different mRNAs and describe combination of this method with conventional immunohistochemistry.

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Highlights

Instructions for preparing the adult mouse cochlea for whole-mount histology applications

Modifications for hybridization chain reaction protocol

Adult cochlea mRNA *in situ* detection paired with immunostaining and imaging

Protocol

Fluorescent *in situ* mRNA detection in the adult mouse cochleaGiovanni H. Diaz^{1,2,3,4,*} and Stefan Heller^{1,2,3,5,*}¹Department of Otolaryngology – Head & Neck Surgery, Stanford University School of Medicine, Stanford, CA 94305, USA²Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA 94305, USA³Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305⁴Technical contact⁵Lead contact*Correspondence: ghdiaz@stanford.edu (G.H.D.), hellers@stanford.edu (S.H.)<https://doi.org/10.1016/j.xpro.2021.100711>

SUMMARY

Ossification and the delicateness of the cochlear duct make histologic assessments of the mature cochlea a challenging endeavor. Treatments to soften the bone facilitate sectioning and dissection of the cochlear duct but limit *in situ* mRNA detection in such specimens. Here, we provide a protocol for *in situ* mRNA detection using hybridization chain reaction in whole-mount preparations of the adult mouse cochlea. We show examples for multi-probe detection of different mRNAs and describe combination of this method with conventional immunohistochemistry.

BEFORE YOU BEGIN

Preparation of mice

⌚ Timing: [15 min]

1. Reporter mice that express fluorescent tdTomato protein in cochlear hair cells are used in this protocol for visualization of the organ of Corti during cochlear dissection. Using fluorescent reporter proteins is helpful but not absolutely necessary. The example presented in this protocol utilizes transgenic mice that carry heterozygous Myo15Cre and Ai14tdTomato alleles.
2. Mice were euthanatized in accordance with an IACUC-approved animal study protocol, which included CO₂ inhalation for 8 min, followed by cervical dislocation.

Note: Homozygous Myo15Cre mice are deaf (Pepermans et al., 2014). Mating homozygous Myo15Cre mice with homozygous Ai14tdTomato mice results in mice with normal hearing thresholds.

Note: Heterozygous Myo15Cre/Ai14tdTomato mice express tdTomato at equivalent levels between cochlear inner and outer hair cells in neonates. At four weeks of age, the red fluorescence in outer hair cells is much lower when compared with inner hair cells. Additionally, a subset of neurons in the spiral ganglia expresses tdTomato.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-Myosin7a (1:1000)	Proteus	Cat# 256790; RRID: AB_10015251
Mouse anti-Ctbp2 (1:200)	Becton Dickinson	Cat# 612044; RRID: AB_399431

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Alexa Fluor 647 donkey anti-rabbit IgG(H+L) secondary (1:200)	Thermo Fisher Scientific	Cat# A31573; RRID: AB_2536183
Alexa Fluor 647 donkey anti-mouse IgG(H+L) secondary (1:200)	Thermo Fisher Scientific	Cat# A31571; RRID: AB_162542
DAPI (1:1000)	Thermo Fisher Scientific	Cat# D1306, RRID: AB_2629482
Oligonucleotides		
Ocm HCR DNA probe	NM_033039.3	Lot# PRC932
Otof HCR DNA probe	NM_001100395.1	Lot# PRC934
Efh2 HCR DNA probe	NM_025994.3	Lot# PRC935
Chemicals, peptides, and recombinant proteins		
1× DPBS without Ca ²⁺ /Mg ²⁺	Corning	Cat# 21-031-CV
10× DPBS without Ca ²⁺ /Mg ²⁺	Corning	Cat# 46-013-CM
Nuclease-Free Water	Thermo Fisher Scientific	Cat# AM9932
RNAlater™ Solution	Thermo Fisher Scientific	Cat#AM7021
Tween-20	Sigma-Aldrich	Cat# P9416
20× Sodium chloride citrate (SSC)	Thermo Fisher Scientific	Cat# AM9770
Proteinase K, Molecular Biology Grade (800 units/mL)	New England Biolabs	Cat# P8107S
Bovine Serum Albumin (BSA)	Millipore Sigma	Cat# 9048-46-8
Triton X-100	Sigma-Aldrich	Cat# X100
4,6-Diamidino-2-phenylindole (DAPI, 1 µg/mL PBS)	Thermo Fisher Scientific	Cat# D1306
FluorSave™ Reagent	Calbiochem	Cat# 345789-20 mL
16% Paraformaldehyde Aqueous Solution	Electron Microscopy Sciences	Cat# 15710
HCR probe hybridization buffer	Molecular Instruments	N/A
HCR probe wash buffer	Molecular Instruments	N/A
HCR amplification buffer	Molecular Instruments	N/A
Experimental models: organisms/strains		
Myo15-Cre mice	Dr. Christine Petit, Pasteur Institute	N/A
Ai14-tdTomato mice	The Jackson Laboratory	Stock# 007908; MGI:3817869; RRID: IMSR_JAX:007908
Software and algorithms		
Fiji/ImageJ	Fiji	RRID:SCR_002285 http://fiji.sc
Black Zen	Zeiss	RRID:SCR_018163 http://www.zeiss.com/microscopy/en_us/products/microscope-software/zen.html#introduction
Blue Zen	Zeiss	RRID:SCR_013672 http://www.zeiss.com/microscopy/en_us/products/microscope-software/zen.html#introduction
Other		
Heratherm OMS60 Lab Oven	Thermo Fisher Scientific	Cat# 51028121
Secure-Seal™ hybridization sealing system, (9 mm diameter, Electron Microscopy Sciences)	Electron Microscopy Sciences	Cat# 70333-40
Adhesive Port Seal Tabs	Electron Microscopy Sciences	Cat# 70328-00
Eppendorf tube revolver rotator (Hematology/Chemistry Mixer 346)	Thermo Fisher Scientific	N/A
Falcon 50 mL conical centrifuge tube	Corning	Cat# 352070
Microcentrifuge tube storage box for Eppendorf tubes (Axygen brand)	VWR	Cat# TR8300-BLK
Micro Dissecting Curette/Spoon	Biomedical Research Instruments	Cat# BRI 15-1020
SYLGARD® 184 silicone	World Precision Instruments	Cat# SYLG184

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Vannas Micro Dissecting Spring Scissors; Straight; 2 mm Cutting Edge; 0.05 mm Tip Width; 3.5" Overall Length	Roboz	Cat# RS-5640
Scalpel Blades - Breakable	Fine Science Tools	Cat# 10050-00
Blade Holder & Breaker - Flat Jaws	Fine Science Tools	Cat# 10052-11
Superfrost Plus microscope slides	Thermo Fisher Scientific	Cat# 12-550-15
18G 1 Sterile Precision Glide Needles	Becton Dickinson	Cat# 305195
30G 1/2 Hypodermic needle	Becton Dickinson	Cat# 305106
1 mL Syringe Luer Lok Tip	Becton Dickinson	Cat# 309628
35x10 mm Dish	Corning	Cat# 353001

MATERIALS AND EQUIPMENT

Preparation of 4% paraformaldehyde (PFA) solution

⌚ Timing: [5 min]

Reagent	Final concentration	Amount
10x PBS (without Ca ²⁺ /Mg ²⁺)	1x	4 mL
RNase-free water	N/A	26 mL
16% PFA	4% PFA	10 mL
Total		40 mL

Note: 4% PFA solution is made fresh before each cochlear dissection.

Note: Using PBS with Ca²⁺ and Mg²⁺ will result in increased autofluorescence.

Preparation of 5x saline sodium citrate buffer with Tween-20 (SSCT)

⌚ Timing: [5 min]

Reagent	Final concentration	Amount
20x SSC	5x	10 mL
10% Tween-20	0.1%	400 μL
DEPC-treated water	N/A	30 mL
Total		40 mL

Note: Prepare a stock solution of 10% Tween-20 in RNase-free water. This solution can be stored at 4°C for several months.

Preparation of PBS with Tween-20 (PBST)

⌚ Timing: [5 min]

Reagent	Final concentration	Amount
PBS without Ca ²⁺ /Mg ²⁺	N/A	50 mL
10% Tween-20	0.1%	500 μL
Total		50 mL

Note: This solution can be stored at 4°C for several months.

Preparation of immunostaining solutions

⌚ Timing: [120 min]

Antibody wash buffer		
Reagent	Final concentration	Amount
PBS (without Ca ²⁺ /Mg ²⁺)	N/A	20 mL
10% Triton X-100	0.2%	400 μL
Total		20 mL

Note: Prepare a stock solution of 10% Triton X-100 in PBS without Ca²⁺/Mg²⁺. This solution can be stored at 4°C for several months.

Antibody blocking buffer		
Reagent	Final concentration	Amount
PBS with 0.2% Triton X-100	N/A	20 mL
BSA	1%	0.2 g
Total		20 mL

Antibody incubation buffer		
Reagent	Final concentration	Amount
PBS (without Ca ²⁺ /Mg ²⁺)	N/A	10 mL
PBS with 0.2% Triton X-100	N/A	10 mL
BSA	0.5%	0.1 g
Total		20 mL

Note: PBS with 0.2% Triton X-100 can be stored at 4°C for several months.

Note: Prepare blocking and incubation buffers fresh prior to each immunostaining for consistent results. Store solutions at 4°C between primary and secondary antibody application.

Note: BSA dissolves slowly. Preparing the solution and then placing the tube on a shaker set to gentle rocking for 90–120 min is recommended.

STEP-BY-STEP METHOD DETAILS

Extraction of the otic capsule

⌚ Timing: [60 min, followed by incubation for 12–16 h]

1. Euthanize the four-week-old mice (postnatal day (P) 28). Male and female mice are used.

Note: The workflow and timing information assumes that three mice (6 cochleae) are used.

2. Decapitate the mouse and bisect the skull with two scissor cuts, the first from the base of the brainstem above the skull to the nose and the second from the base of the brainstem to the lower jaw.
3. Remove the brain tissue to expose the otic capsules (Figure 1A).

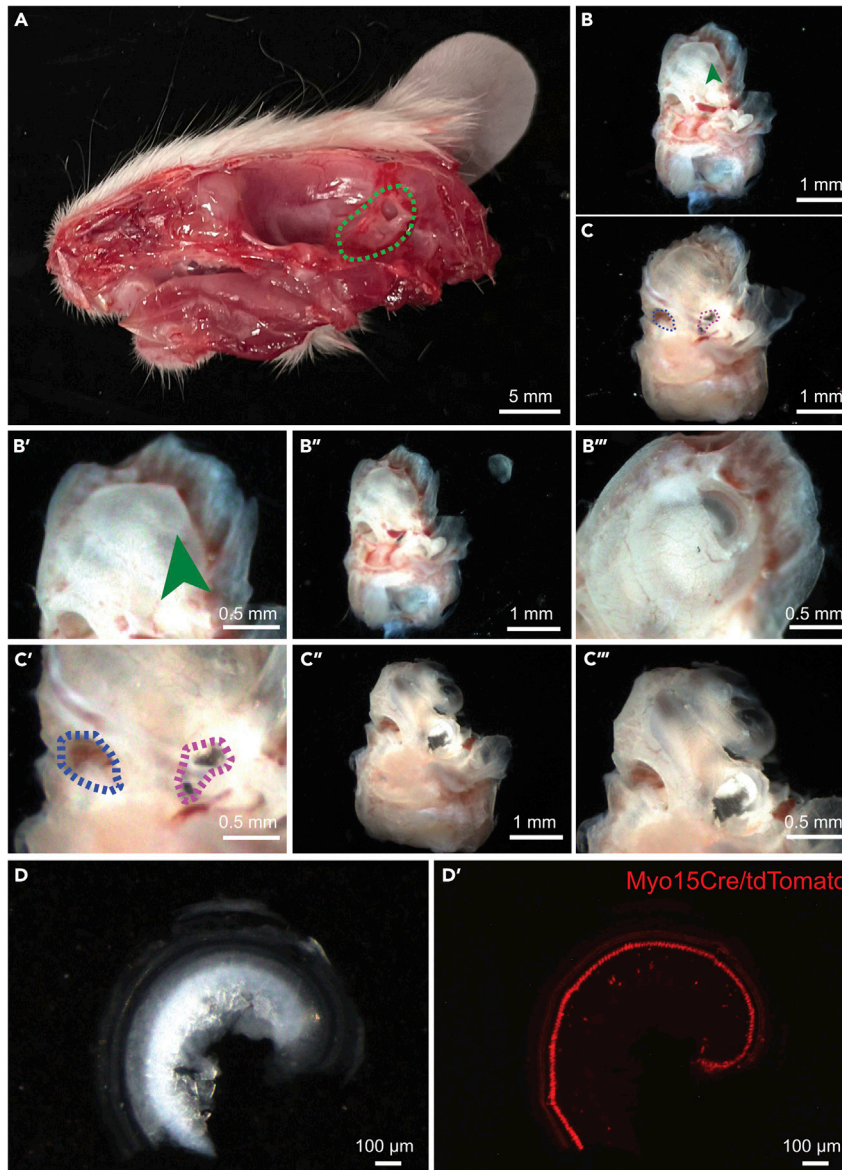


Figure 1. Dissection of the cochlear apical turn

- (A) Bisected P28 mouse head. The otic capsule containing the cochlea and the vestibular system is outlined with a green dashed line.
- (B) Dissected mouse otic capsule. Green arrowhead denotes the apical turn crease that is targeted to create a window in the apex.
- (B') Magnified image of the cochlea's apex. The arrow points to the crease of the apical turn that is used as a toehold for creating a window in the apex.
- (B'') Otic capsule with the apical bone removed. The pried-off bone piece is seen to the right of the otic capsule.
- (B''') A higher magnified view of the window made at the apex of the otic capsule.
- (C) Otic capsule after removal of bone to create window in the apex. The location of the round window is indicated by a blue dashed line and the oval window is outlined with a purple dashed line.
- (C') Magnified image of the round window (blue dashed line) and oval window (purple dashed line).
- (C'') Otic capsule after the removal of the bone covering the sensory epithelium.
- (C''') Magnified image of the exposed cochlear turns after bone removal.
- (D) Dissected apical turn after excess stria vascularis and spiral ganglion tissue was removed.
- (D') Strong Myo15Cre-tdTomato expression is associated with P28 inner hair cells and lower levels are observed in outer hair cells in the shown apical turn. Some spiral ganglion cells also express tdTomato.

4. Extract each otic capsule by applying pressure with a gloved thumbnail above the apex of the cochlea. Hold a bisected skull in the left hand and apply pressure to the tissue behind the otic capsule so that the capsule is pushed upwards, while simultaneously pushing down on the semi-circular canal with the other thumb. This will lift the otic capsule so that it can be removed from the surrounding tissues using RNase-free forceps and placed directly into 4% PFA solution.

△ **CRITICAL:** Transfer the extracted otic capsules directly into 4% PFA solution at room temperature (18°C–24°C).

Caution: PFA is a hazardous material, and the following steps need to be conducted using a dissection microscope inside a chemical hood.

5. Perfuse 4% PFA solution into the round and oval windows of the isolated otic capsule.
 - a. Transfer the isolated otic capsules into 4% PFA solution in a 35 × 10 mm dish, coated with black Sylgard for dissections (Montgomery and Cox, 2016).
 - b. Using a curved pair of sharp forceps or an 18-gauge needle that has been curved on a sharpening/grinding block, place the curved sharp tip along the crease of the apical cochlear turn and apply gentle pressure to remove a fragment of the apical bone (Figures 1B and 1B'). This movement generates a small window at the apex (Figures 1B' and 1B'').
 - c. Using a 30-gauge needle, slowly perfuse about 100 μL of 4% PFA solution into the round and oval windows (blue and purple dotted circles in Figure 1C, respectively). Slowly push the solution through the cochlea using gentle pressure, at a flow of ≈ 50 μL per minute. The solution will flow out of the window made in the apex.

Note: Remove the stapes with forceps to allow for easier perfusion.

△ **CRITICAL:** Thorough perfusion with 4% PFA solution is essential for completely fixing the tissue. Additionally, this process makes the sensory epithelium more rigid, mediating easier removal from the bone.

- d. Place the perfused cochleae into a 2 mL Eppendorf tube containing 1.8 mL of 4% PFA solution.
- e. Incubate 12–16 h on a shaker set to gentle rocking at 4°C.

Note: Separate the left and right cochleae into different Eppendorf tubes to help with subsequent orientation of the tissue.

△ **CRITICAL:** Limit the number of otic capsules that are placed in the 1.8 mL of fixative to six to ensure thorough fixation.

Dissection of all three cochlear turns from decalcified otic capsules

⌚ Timing: [4 h 30 min]

6. After overnight fixation, transfer the cochleae into RNase-free 0.5 M EDTA solution pH 8.0.
7. Incubate for 3 h with slow agitation with an Eppendorf tube revolver rotator (Fisher Scientific Hematology/Chemistry Mixer 346) at room temperature.
8. Rinse the cochleae once in RNase-free PBS and then transfer the cochleae into RNase-free PBS at room temperature for dissection.

△ **CRITICAL:** The cochlear bone will not be fully decalcified after 3 h, however it will be softened enough to remove the cochlear turns. Additional decalcification will result in easier dissection, but prolonged EDTA treatment can reduce downstream mRNA detection efficacy (Belluoccio et al., 2013, Shao et al., 2006). For immunostaining, we have not noticed adverse effects of extended decalcification times (for up-to 6 days).

Note: Even with a short 3 h EDTA decalcification time, we have noted that *Otof* mRNA detection levels were affected when directly compared to non-decalcified tissue.

Note: Protocols have suggested that co-incubation of EDTA with RNAlater solution (Invitrogen) improves RNA preservation and detection (Belluoccio et al., 2013). We have not systematically investigated the effects of RNAlater on wholemount samples, but have noted no clear improvement of mRNA detection in experiments on cryosectioned cochlear tissue. Future optimization is warranted to see if this protective step can further improve mRNA detection in wholemount cochleae.

Note: Decalcified otic capsule dissection was done according to (Montgomery and Cox, 2016).

9. After decalcification, insert one blade of Vannas Spring Scissors into the oval window (purple dotted line in Figures 1C and 1C') and cut along the turn towards the apex.
10. To separate the basal turn from the middle and apical turn, place one scissor blade into the region cut in step 9 with the other scissor blade on the back of the cochlea and cut.
11. Hold the apical/middle turn so that the apical end points down. Cut along the scala media.
12. To separate the middle and apical turns, place one scissor blade into the region that was cut in step 11 and place the other blade on the outside of the bony labyrinth and cut.
13. To remove the basal turn, cut the spiral ganglion fibers that connect to the basal turn and cut along the lateral wall.
14. Use the scissors to remove excess bone and spiral ganglion tissue.
15. To help with removal of surrounding tissue, as it is sometimes challenging to get the correct angle with the scissors, transfer turns to a 35×10 mm plastic Petri dish filled with RNase-free PBS and use a 0.1 mm thick scalpel blade mounted to a blade holder to remove excess tissue.

Dissection of the apical turn, without decalcification

⌚ Timing: [45 min]

We describe in the following an alternative dissection that focuses on the apical turn. The benefit of this dissection is that it can be done without decalcification, which increases the sensitivity of subsequent mRNA detection. The following steps should be conducted after step 5 and instead of steps 6–15.

16. Following the overnight fixation, transfer the cochleae into RNase-free PBS without Ca^{2+} / Mg^{2+} for dissection.

Note: The samples should be processed immediately for best signals and not stored at 4°C.

17. Use the sharp tip of a curved 18-gauge needle to chip away the bone from the cochlear apical turn (Figures 1B and 1B').
 - a. Use the curved tip to gently hook the bone and to chip it away from the apical window opening that was previously made (Figures 1B'' and 1B''') until all the bone surrounding the sensory epithelium is removed. An example of a cochlea after the bone surrounding the sensory epithelium is removed is shown in (Figures 1C'' and 1C''').

⚠ CRITICAL: Scrape the region between the stria vascularis and the bone with the sharp needle to gently separate the two compartments. This step decreases the chance of removing the sensory epithelium along with the chipped bone.

Note: Carefully bend the tip of the 18-gauge needle to generate a sharp curved "hook".

- b. Use Vannas Spring Scissors to dissect the apical turn.

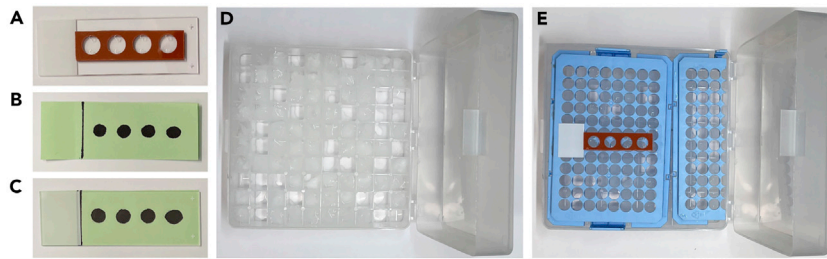


Figure 2. Mounting Hybridization chambers and humidifying chamber

- (A) One stripe of four hybridization chambers placed onto a microscope slide.
 (B) A piece of Post-it paper is cut to the dimensions of a microscope slide and four ≈ 9 mm circles or dots are made that align with the circular hybridization chambers to create a visual guide. The sticky section of the Post-it aligns with the frosted end of the slide.
 (C) A Superfrost Plus microscope slide is placed on top of the visual guide and the sticky part of the Post-it paper is used to hold the visual guide in place.
 (D) Microcentrifuge storage box used as humidifying chamber. The slots are filled with pieces of paper towel and water.
 (E) A plastic inset of a pipette tip box is used as a slide holder.

- c. Remove excess stria vascularis tissue with the scissors.
- d. To aid with removal of surrounding tissue, as it is sometimes challenging to get the correct angle with the micro scissors, transfer apical turns to a 35×10 mm plastic Petri dish filled with RNase-Free PBS, and use a 0.1 mm thick scalpel blade mounted to blade holder to remove excess tissue. After excess tissue is removed you will be left with an apical turn with hair cells visualization as presented in (Figures 1D and 1D').

Note: A disposable scalpel may not be thin enough to aid in this dissection step. The thinner 0.1 mm breakable FST blades (Cat# 10050-00) are recommended.

Note: It is possible to remove parts of the middle and basal turns from non-decalcified cochleae. However, these turns are difficult to reliably extract because the tissue tends to tear between the outer and inner hair cells resulting in removal of outer hair cells. For analyses of inner hair cells only and potentially also spiral ganglion cells, one can use such tissue for downstream RNA detection.

△ CRITICAL: Use a micro spoon to transfer fully immersed tissue.

Mounting of cochlear turns to slide

⌚ Timing: [20 min]

18. Cut in half a sheet of Secure-Seal™ hybridization chamber system (hybridization chamber) for two stripes of four chambers each (Figure 2A).
19. Use a marker to outline four 9 mm diameter circles on a piece of paper that align with the four hybridization chambers. (Figure 2B). A Post-It note is useful for this because it can be temporarily attached to the back of a microscopy slide, preventing the visual guide from moving (Figure 2C).
20. Place 10 μ L of RNase-free PBS without Ca^{2+} / Mg^{2+} onto the front of the Superfrost Plus microscope slide corresponding to each marked region (Figure 2C).
21. Transfer the sensory epithelium turns into the 10 μ L PBS drop using a micro spoon and orient the turns using a pair of forceps using a dissection microscope.

Note: It is possible to place and orient up to five cochlear turns into each 10 μ L drop as long as they do not overlap when mounted.

△ **CRITICAL:** Orient the tissue so that the hair cells are facing upwards with the bundles pointing toward the surface.

22. After the tissue specimens are correctly oriented, carefully remove excess PBS with a pipette, and allow to air dry for about 5 min.

△ **CRITICAL:** Carefully monitor sample drying, allowing just enough time for the solution to evaporate and avoiding over-drying of the samples.

23. Attach the hybridization chambers over the designated regions and fill the wells carefully with 50 μ L RNase-free PBS.

Hybridization chain reaction (HCR) protocol modifications

The HCR v3.0 Protocol (Choi et al., 2018) was used for mRNA detection in the presented examples. HCR has been commercialized and additional information is available at www.molecularinstruments.com.

For mRNA detection in cochlear whole mount tissues, we use a modified HCR protocol. The original protocol can be found in the supplemental section of the HCR publication (Choi et al., 2018) and an updated version of the protocol can be found at <https://files.molecularinstruments.com/MI-Protocol-HCRv3-frozentissue-Rev1.pdf>.

Reagents	Composition
HCR probe hybridization buffer	30% formamide 5 \times sodium chloride citrate (SSC) 9 mM citric acid (pH 6.0) 0.1% Tween 20 50 μ g/mL heparin 1 \times Denhardt's solution 10% dextran sulfate
HCR probe wash buffer	30% formamide 5 \times sodium chloride citrate (SSC) 9 mM citric acid (pH 6.0) 0.1% Tween 20 50 μ g/mL heparin
HCR amplification buffer	5 \times sodium chloride citrate (SSC) 0.1% Tween 20
PBST	1 \times PBS without Ca ²⁺ /Mg ²⁺ 0.1% Tween 20
Proteinase K solution	Proteinase K (800 units/mL) 1:400 in PBST

Details of HCR probes/amplifiers and other reagents are provided in the key resource table.

Note: a detailed composition of the reagents used including probe hybridization buffer, probe wash buffer, and amplification buffer are provided by Molecular Instruments and can be found in their protocol (Choi et al., 2018). For convenience and reproducibility, we used the commercially available buffers from Molecular Instruments.

Note: Detection step modifications:

We used 2 \times the probe concentration that is recommended in the original protocol.

Note: Amplification step modifications:

Snap-cool amplifier hair pins on ice for 5 min, instead of the suggested 30 min at room temperature.

Sample preparation

⌚ Timing: [20 min]

24. Gently remove the PBS from each well in the hybridization chamber and add 50 μ L of diluted Proteinase K solution to each chamber and incubate for 2.5 min at room temperature.

Note: We dilute Proteinase K (800 units/mL) 1:400 in PBST and prepare the solution fresh for each use.

⚠ CRITICAL: Proteinase K concentration and duration may need to be optimized as each batch is slightly different. A final concentration of 2 units/mL is a good starting point.

25. Gently remove the Proteinase K solution using a pipette and perform three 30 s washes with 50 μ L PBST followed by 50 μ L PBST for 5 min.

⚠ CRITICAL: Manually aspirate the solution with a P200 pipette and be careful not to dislodge the mounted tissue.

Detection

⌚ Timing: [30 min, followed by incubation for 12–16 h]

⌚ Timing: [90 min] for step 34

26. Pre-warm a humidified plastic chamber to 37°C.

Note: A plastic microcentrifuge tube storage box makes a good humidifying chamber when the individual compartments are filled with a piece of paper towel and water is added ([Figure 2D](#)). A plastic inset from a P1000 pipette box is used as a slider holder and avoids excess condensation on the slide ([Figure 2E](#)).

27. Replace the PBS in each hybridization chamber with 50 μ L of HCR probe hybridization buffer for pre-hybridization.

Note: Pre-heat probe hybridization buffer to 37°C.

Caution: Probe hybridization buffer contains formamide, a hazardous material.

28. Pre-hybridize for 10 min inside the humidified chamber at 37°C.
29. During pre-hybridization, prepare probe hybridization solution by adding 2 μ L of 1 μ M probe stock to 250 μ L of hybridization buffer.

Note: if multiple probes are used, combine them all into the 250 μ L hybridization buffer.

30. Remove the pre-hybridization solution with a P200 pipette.
31. Immediately add 50 μ L of the probe hybridization solution into each of the hybridization chambers.
32. Place sealing tabs over the chamber port holes, press them tightly onto the holes, and incubate overnight (12–16 h) at 37°C in the humidified chamber.
33. The next day, remove the probe hybridization solution and replace with HCR probe wash buffer that has been pre-heated to 37°C.

△ **CRITICAL:** Be sure to use different pipette tips to avoid cross contamination of probe combinations if performing multiple HCR probe combinations.

△ **CRITICAL:** It is important to perform buffer exchange steps carefully using a pipette. Vacuum aspiration increases the likelihood of dislodging and aspirating the samples.

Caution: Probe wash buffer contains formamide, a hazardous material.

34. Remove excess probes by incubating sample hybridization chambers at 37°C in 50 µL of:
 - a. 75% of probe wash buffer / 25% 5× SSCT for 15 min
 - b. 50% of probe wash buffer / 50% 5× SSCT for 15 min
 - c. 25% of probe wash buffer / 75% 5× SSCT for 15 min
 - d. 100% 5× SSCT for 15 min

Note: Pre-heat probe wash buffer and 5× SSCT solutions to 37°C.

35. Incubate samples in 5× SSCT for 5 min at room temperature.

Amplification

⌚ **Timing:** [60 min, followed by incubation for 12–16 h]

⌚ **Timing:** [75 min] for step 44

36. Add 50 µL of amplification buffer into each hybridization chamber and incubate in a humidified chamber (Figures 2D and 2E) for 30 min at room temperature.

Note: Equilibrate an aliquot of HCR amplification buffer to room temperature before use.

Note: The final amount of hairpin solution needed depends on the number of experiments/hybridization chambers used; each hybridization chamber requires 50 µL to fill.

37. For each hybridization chamber, aliquot 1 µL of hairpin H1 (3 µM stock) of desired fluorophore into a fresh tube.
38. For each hybridization chamber, aliquot 1 µL of hairpin H2 (3 µM stock) of desired fluorophore into a fresh tube.

Note: Use at least 4 µL of solution for each tube for heat shock treatment.

Note: We have used up to 3 different hairpins-fluorophore combinations simultaneously.

39. Heat the tubes containing hairpins at 95°C for 90 s and then snap-cool on ice in the dark for 5 min.
40. Prepare hairpin solution by adding snap-cooled H1 hairpins and snap-cooled H2 hairpins to amplification buffer at room temperature. Add 1 µL of each hairpin per 50 µL.
41. Remove the amplification buffer from the hybridization chambers.
42. Add 50 µL of the hairpin solution in amplification buffer into each of the hybridization chambers.
43. Seal the hybridization chambers with adhesive seal tabs that come with Secure-Seal™ hybridization chamber system and incubate overnight (12–16 h) in a dark humidified chamber at room temperature.
44. Remove excess hairpins by incubating slide hybridization chamber in 5× SSCT at room temperature twice for 30 min, followed by a single wash for 15 min.

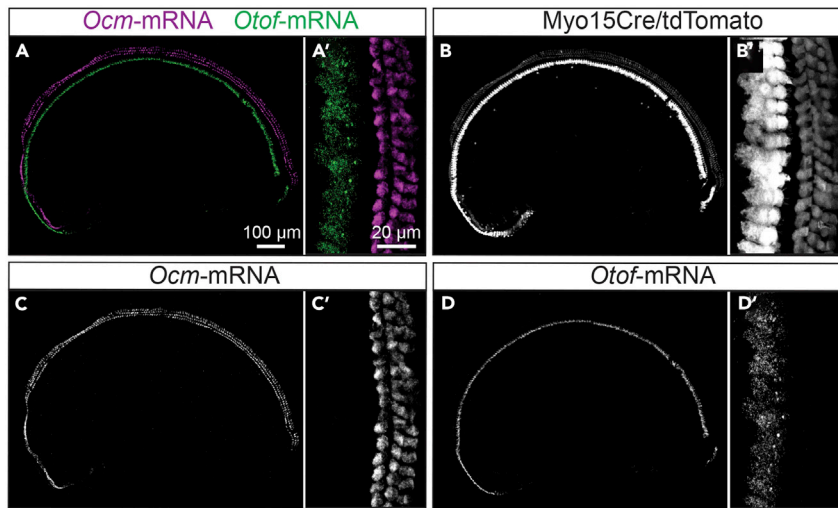


Figure 3. Wholemount hybridization chain reaction *in situ* mRNA detection in cochlear hair cells

(A) P28 mouse cochlear apical turn results after hybridization chain reaction protocol. *Ocm*-mRNA is shown in green and *Otof*-mRNA is shown in magenta.

The magnified panel (A') shows expression of *Ocm*-mRNA in outer hair cells and *Otof*-mRNA in inner hair cells.

(B) tdTomato fluorescence labels inner hair cells and outer hair cells.

(B') shows a magnified region.

(C) *Ocm*-mRNA expression.

(C') shows a magnified region.

(D) *Otof*-mRNA expression.

(D') shows a magnified region.

Note: At this stage, if the goal is to perform immunostaining along with the HCR protocol, continue to the **Immunostaining following HCR mRNA detection** section (below). Otherwise, complete the HCR only protocol with mounting the samples for microscopy (Figure 3).

Sample mounting for microscopy

⌚ Timing: [2 h]

45. Incubate with DAPI at 1:1000 in PBST for 15 min at room temperature.

46. Wash with PBST for 15 min at room temperature.

47. Replace PBST with 50 μ L of 50% glycerol for each hybridization chamber.

⚠ **CRITICAL:** Do not allow the samples to dry after removal of the hybridization chambers. Brief equilibration in 50% glycerol lowers the chances of drying out the sample.

48. Remove glycerol solution and then remove the adhesive Secure-Seal™ hybridization system.

49. Add a drop of FluorSave™ Reagent onto each sample.

50. Place cover slip and allow the mounting solution to dry in the dark at room temperature for 1 h.

⚠ **CRITICAL:** Gently lower coverslip to avoid bubble formation.

⚠ **CRITICAL:** Avoid excessive coverslip adjustments, as this will dislodge and possibly damage the sensory epithelium.

51. Add a small amount of clear nail polish along edge of the coverslip to permanently secure the coverslip.

52. Proceed with confocal microscopy image acquisition and analysis.

Note: Completion of the HCR only protocol results in detection of mRNA expression within the wholemount cochlea apical turn (Figure 3).

Immunostaining following HCR mRNA detection

Directly after the removal of excess hairpins with 5× SSCT in step 44 of the amplification stage HCR protocol, continue with the following steps.

Primary antibody incubation

⌚ Timing: [2 h, followed by incubation for 12–16 h]

53. Equilibrate the sample for 5 min in antibody wash buffer.
54. Replace the antibody wash buffer with blocking buffer and incubate at room temperature for 2 h.
55. Prepare diluted primary antibody solution by diluting the primary antibody in antibody incubation buffer.

Note: Briefly centrifuge the tube of primary antibody prior to taking aliquot to collect all solution at the bottom of the tube for easier pipetting.

Note: Dilute primary antibody as needed. We used mouse anti-Ctbp2 (1:200) and rabbit anti-Myo7a (1:1000) for the presented examples. Amount of primary antibody used is dependent on the number of hybridization chambers used; each hybridization chamber requires 50 μ L to fill.

56. Add 50 μ L of diluted primary antibody incubation solution to each hybridization chamber and incubate in a humidified box overnight (12–16 h) at 4°C.

Secondary antibody incubation

⌚ Timing: [2 h 30 min]

57. Remove excess primary antibodies by washing three times with 50 μ L of antibody wash buffer, each time for 15 min at room temperature.
58. Prepare a diluted secondary antibody solution by diluting the secondary antibody in antibody incubation buffer.

Note: Briefly centrifuge the tube of secondary antibody prior to taking aliquot to collect all solution at the bottom of the tube for easier pipetting.

Note: Dilute secondary antibody as needed. We used Donkey anti-mouse Alexa-fluor 647 (1:200) or donkey anti-rabbit Alexa-fluor 647 (1:200) to correspond with species of the primary antibody used for the presented examples.

59. Add 50 μ L of secondary antibody solution to each hybridization chamber and incubate at room temperature for 60 min.
60. Remove excess secondary antibodies by washing the samples three times with 50 μ L of antibody wash buffer, each time for 15 min at room temperature.
61. Add 50 μ L of DAPI solution (1:1000 DAPI dilution in PBST) and incubate for 15 min at room temperature.
62. Replace DAPI solution with 50 μ L of PBST and incubate for 15 min.

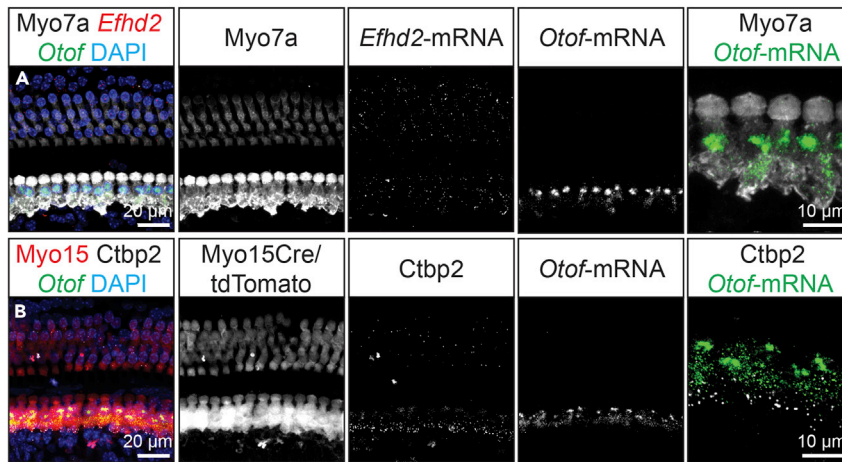


Figure 4. Examples for hybridization chain reaction *in situ* mRNA detection coupled with immunohistochemistry
(A) Immunolabeling and HCR *in situ* mRNA detection on whole mount P28 mouse cochlear apical turns without decalcification. Hair cells are labeled with antibodies against Myo7a (white). HCR detection of *Efh2*-mRNA (red) and *Otof*-mRNA (green). A magnified image of the inner hair cells that demonstrate detection of *Otof* mRNA (green) and immunolabelling for antibodies against Myo7a (white).
(B) Myo15Cre-tdTomato(red) transgenic mouse was used to label hair cells. Presynaptic ribbons in the inner and outer hair cells were stained with antibodies against Ctbp2 (white). Hybridization chain reaction provides individual cell mRNA resolution of the hair cell marker *Otof*-mRNA (green). A magnified image of the inner hair cells demonstrating mRNA expression of *Otof* mRNA (green) and immunolabelling for antibodies against Ctbp2 (white). Results are from an apical turn that was not decalcified.

Sample mounting for microscopy

⌚ Timing: [90 min]

63. Remove PBST and add 50 μ L of 50% glycerol to each hybridization chamber.
64. Remove glycerol solution and adhesive Secure-Seal™ hybridization system.
65. Add a drop of FluorSave™ Reagent onto each sample.

⚠ **CRITICAL:** Do not allow the samples to dry after removal of the hybridization chambers. Brief equilibration in 50% glycerol lowers the chances of drying out the sample.

66. Place cover slip and allow to dry in the dark for 1 h at room temperature.

⚠ **CRITICAL:** Avoid excessive coverslip adjustments, as this will dislodge and possibly damage the sensory epithelium.

67. Add a small amount of clear nail polish along edge of the coverslip to permanently secure the coverslip.
68. Proceed with confocal microscopy image acquisition and analysis (Figure 4).

EXPECTED OUTCOMES

The provided protocol will allow for the simultaneous detection of multiple mRNA transcripts and proteins of interest. Because HCR is a quantitative *in situ* mRNA detection method, it can be used to quantify mRNA expression levels using confocal microscopy.

LIMITATIONS

With this protocol, we can reproducibly dissect the apical turn of the P28 mouse cochlea for mRNA and protein detection without decalcification of the tissue. Although we can obtain pieces of the mid

and basal turns, we are not able to reliably obtain whole middle and basal turns without decalcifying. Decalcifying the adult cochlea enables dissection of apical, middle, and basal turns. It is possible to detect mRNA using HCR on decalcified cochlear tissue. However, by comparing decalcified with non-decalcified apical turns, we noted a decrease in the amount of mRNA HCR signal in the decalcified apical hair cells. Additional experiments are warranted to see if the addition of RNAlater (Beluoccio et al., 2013) will improve RNA preservation during decalcification. Mounting of cochlear tissue to a microscopy slide can be challenging as not all turns are oriented flat on the slide. However, when the imaged cochleae are visualized with confocal microscopy and the resulting volumes are analyzed with software capable of 3-dimensional manipulations, such as Imaris, it is possible to quantify mRNA expression in the organ of Corti.

TROUBLESHOOTING

Problem 1

Cochlear turns detach from the hybridization chamber bottom during medium change (step 30).

Potential solution

Avoid a vacuum aspiration system. Pipetting should be conducted manually using a 200 μ L pipette. The sample needs to dry sufficiently during the attachment step (step 22). We have also noted that prolonged Proteinase K treatment or too high a concentration of the enzyme results in poor attachment.

Problem 2

No HCR signal from any of the hairpin amplifiers (step 52).

Potential solution

Be sure to use a matching fluorescent H1 and H2 hairpin combination. Do not mix two different fluorescent H1 or H2 hairpins.

Problem 3

No HCR signal (step 52).

Potential solution

Samples may not have been fixed long enough. We have noted that fixation for a minimum of three hours is needed for HCR to work properly.

Problem 4

Low tdTomato fluorescence (step 52).

Potential solution

Samples may not have been fixed long enough. We have noted that fixation for less than one hour results in a low fluorescent protein signal.

Problem 5

Adhered cochlear turn becomes dislodged during solution changes (step 34).

Potential solution

The detached sample can be salvaged and moved through the HCR/immunostaining protocols. Careful pipetting and visualization with a dissection microscope can help with salvaging the dislodged tissue.

Problem 6

No HCR signal, even with highly expressed genes (step 52).

Potential solution

Use a different amplifier. For example, we have noticed performance issues with one batch of 647 nm hairpin amplifiers, as the mRNA signal was detectable with 488 nm and 546 nm amplifiers. In these experiments, we instead utilized the 647 nm channel for immunohistochemical detection of a hair cell marker protein.

Problem 7

No antibody signal (step 68).

Potential solution

The examples shown in [Figures 3](#) and [4](#) used antibodies that work well in a variety of applications and conditions. Each new antibody should be thoroughly validated before using it for a combined HCR/immunostaining procedure.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Stefan Heller (hellers@stanford.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new datasets/code.

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AUTHOR CONTRIBUTIONS

Conceptualization, G.D. and S.H.; methodology and analysis, G.D.; writing – original draft, G.D.; writing – review & editing, G.D. and S.H.; funding acquisition, S.H.

DECLARATION OF INTERESTS

S.H. is a paid consultant of Pipeline Therapeutics.

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