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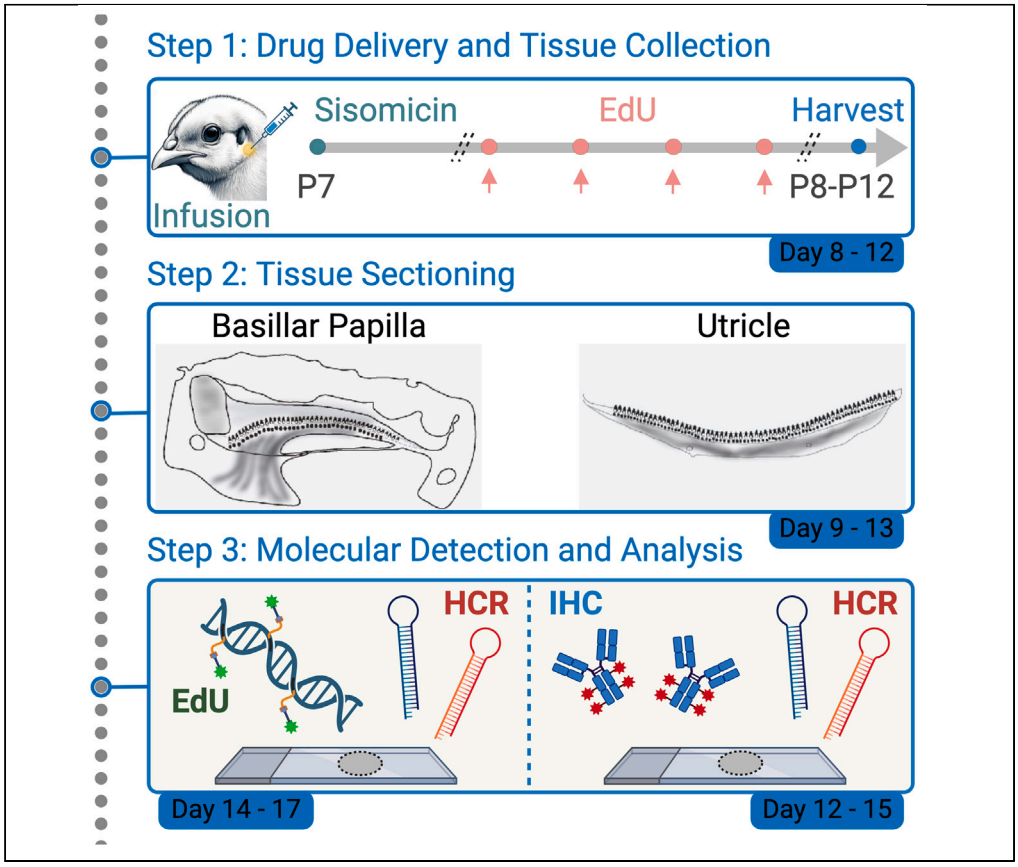

The avian inner ear can naturally regenerate sensory hair cells and is therefore an ideal candidate for investigating mechanisms leading to hair cell regeneration and functional recovery. Here, we present a surgical protocol for eliminating auditory hair cells via sisomicin injection into the lateral semicircular canal. We describe steps for multiplex mRNA detection in chicken basilar papilla and utricle sections. We then detail procedures for integrating immunohistochemistry for concurrent mRNA and protein visualization, complemented by S-phase labeling with EdU.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Infusion of ototoxin into the chicken inner ear

Vibratome sectioning of the chicken basilar papilla

Multiplexed mRNA detection in inner ear vibratome sections

Combination of mRNA and protein detection or S-phase tracing using a thymidine analog

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Protocol for *in vivo* elimination of avian auditory hair cells, multiplexed mRNA detection, immunohistochemistry, and S-phase labeling

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SUMMARY

The avian inner ear can naturally regenerate sensory hair cells and is therefore an ideal candidate for investigating mechanisms leading to hair cell regeneration and functional recovery. Here, we present a surgical protocol for eliminating auditory hair cells via sisomicin injection into the lateral semicircular canal. We describe steps for multiplex mRNA detection in chicken basilar papilla and utricle sections. We then detail procedures for integrating immunohistochemistry for concurrent mRNA and protein visualization, complemented by S-phase labeling with EdU.

For complete details on the use and execution of this protocol, please refer to Benkafadar et al., Benkafadar et al., Sato et al., Janesick et al., Scheibinger et al.[1–5]

BEFORE YOU BEGIN

Eliciting sensory hair cell loss *in vivo* in the avian inner ear is essential for investigating the regenerative mechanisms leading to hair cell regeneration and functional recovery.[3–6]

This paper presents a comprehensive protocol for inducing sensory hair cell loss and monitoring gene expression changes in the chicken inner ear using methods previously described in our published works.[1–3,5,7,8] Specifically, we provide a detailed description of local sisomicin infusion *via* the lateral semicircular canal in seven-day-old (P7) chickens,[1] which is, in birds, the most accessible canal from the head's surface. Additionally, we describe the preparation of transverse vibratome sections for the basilar papilla and utricle, enabling detailed histopathological analysis of undamaged or damaged and regenerating auditory and balance sensory organs. Detection of mRNA is performed using *in situ* hybridization chain reaction (HCR) technology, which optimizes signal amplification and background noise suppression.[9] HCR is used alongside immunohistochemistry to detect mRNA and proteins simultaneously. Combining HCR with the Click-iT EdU assay allows the identification of nuclei of cells that underwent the S-phase of the cell cycle.

Institutional permissions

All animal care and procedures complied with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The hatching, housing, and handling of chickens received approval from the Stanford University Institutional Animal Care and Use Committee (Protocol #28379).

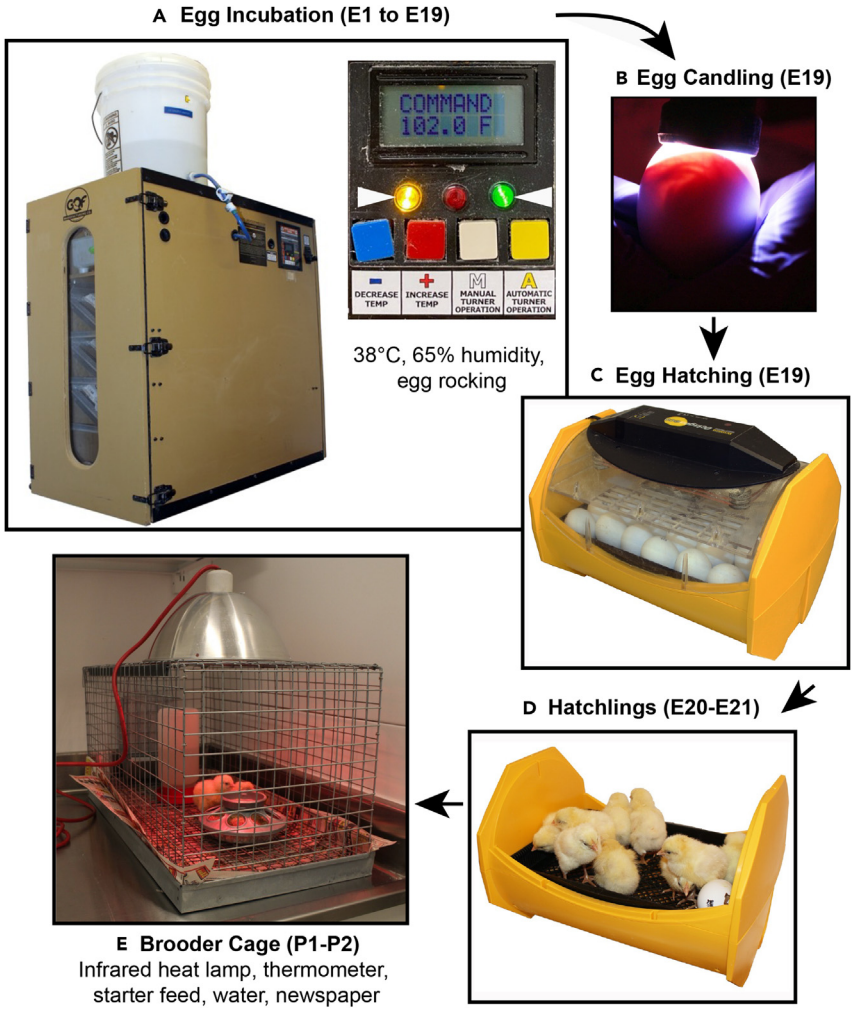

Figure 1. Step-by-step guide for incubating fertilized chicken eggs and hatching the chicks

We took all necessary steps to minimize animal suffering, including the use of anesthesia and analgesia during surgical procedures, and careful monitoring of animal health and welfare. Both male and female animals were included in all analyses. Tests were performed using the contralateral untreated ear as a control.

Chicken husbandry

Timing: 1 month

This section outlines proper chicken husbandry techniques, starting with egg incubation and leading up to chick hatching. It includes steps for maintaining egg viability, embryo development checks, hatching preparations, and care for the newly hatched chicks.

1. Place fertilized chicken eggs (*Gallus gallus domesticus*) into a humidified Sportsmen 1502 egg incubator set to 38°C, with automatic rocking switched on (Figure 1A).

   Note: One of the most critical issues for hatching is to avoid contamination with bacteria. Always store fertilized eggs in clean containers and keep the incubator clean.

Note: Store the eggs and incubate them with the more pointed end facing downwards. This ensures the air-filled compartment inside the egg will not become dislodged, which affects viability.

Note: If you need to keep the fertilized eggs for a few days before incubating, store them at a 25°–30° angle in a cool and dry place (like a wine cooler, set to 14°C–15°C) for up to one week. Every 8–12 h, turn the rack so that the storage angle "rocks" from 25°–30° to –(25°–30°), which preserves viability.

2. On day 19 of incubation, identify developed embryos by candling (Figure 1B).

Note: You will need to turn the room light off; consider having a flashlight available if the switch is any distance from the candling table.

Note: Always candle with the pointed end facing downwards. Avoid jolting the egg and keep the eggs outside the incubator only for as long as it takes to candle them.

3. Move the eggs into a Brinsea Octagon incubator for hatching (Figure 1C).

Note: Turn on the egg incubator at least 1 h before you put the eggs in. This allows the air inside the incubator to reach the correct temperature and humidity before the eggs are placed inside.

Note: The correct temperature in a forced air incubator is 37.5°C. The correct humidity level in preparation for hatching should be 65%.

4. When the hatchlings' bodies are dry, transfer them to a brooder cage equipped with an infrared heat lamp, digital thermometer, chick starter feed, water, and newspaper (Figures 1D and 1E).

Note: The chickens are raised in a calm environment, with an artificial 12-h day-night cycle and low ambient sound levels.

Note: We record and document daily the temperature, weight, food and water supply, and animal number. The newspaper is changed every other day.

Practice localization of, and infusion into, the lateral semicircular canal

Timing: 1–2 days

This section covers the technique for accurately locating and infusing substances into the lateral semicircular canal. It includes steps for preparation, infusion practice, and verification of successful inner ear delivery.

5. Euthanize P7 chicken and proceed with head fixation.
   a. Decapitate the chicken.
   b. Place the head in a 50 mL Falcon tube containing 15 mL of 4% paraformaldehyde (PFA) solution (w/v).
   c. Store at 4°C for 12–16 h.

Note: It is recommended to practice on multiple specimens. Here, we assume that 6 animals are sufficient for gaining principal expertise.

Note: Chicken husbandry is explained below in the step-by-step method details.

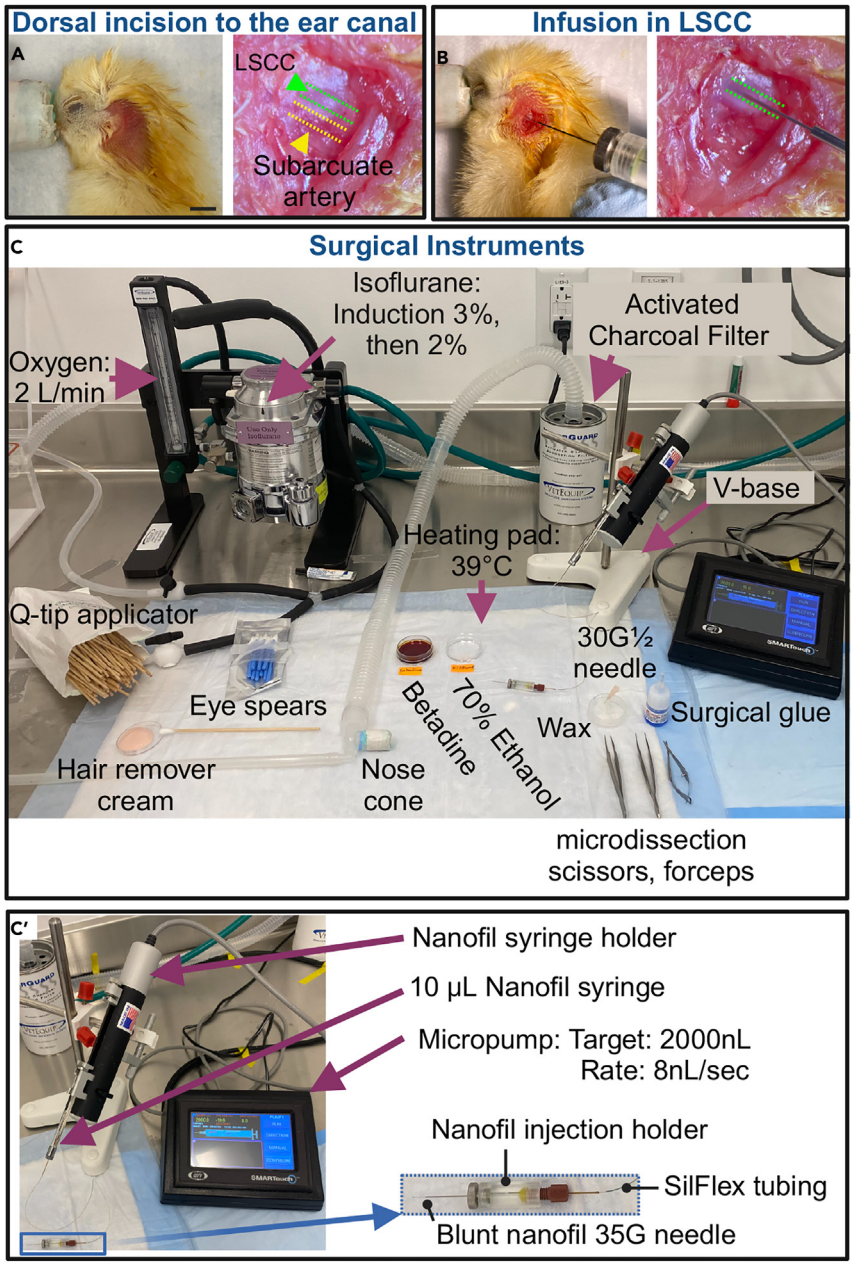

Figure 2. Surgical instruments for infusion into the lateral semicircular canal in post-hatch chickens
Scale bar = 1 cm. LSCC: lateral semicircular canal.

6. Rinse the fixed head in a 50 mL Falcon tube using 25 mL of 1X PBS for 2 h at 20°C–22°C, with gentle agitation.
7. Locate the lateral semicircular canal on the left side of the head (Figure 2A).

Note: The surgical landmark is the junction of the V-shaped subarcuate artery with the mastoid air cells. Caudal to the subarcuate artery, and below the mastoid cells is the lateral canal near the surface. The lateral semicircular canal in a P7 chicken is about 200 µm in diameter (Figures 3A and 3B).

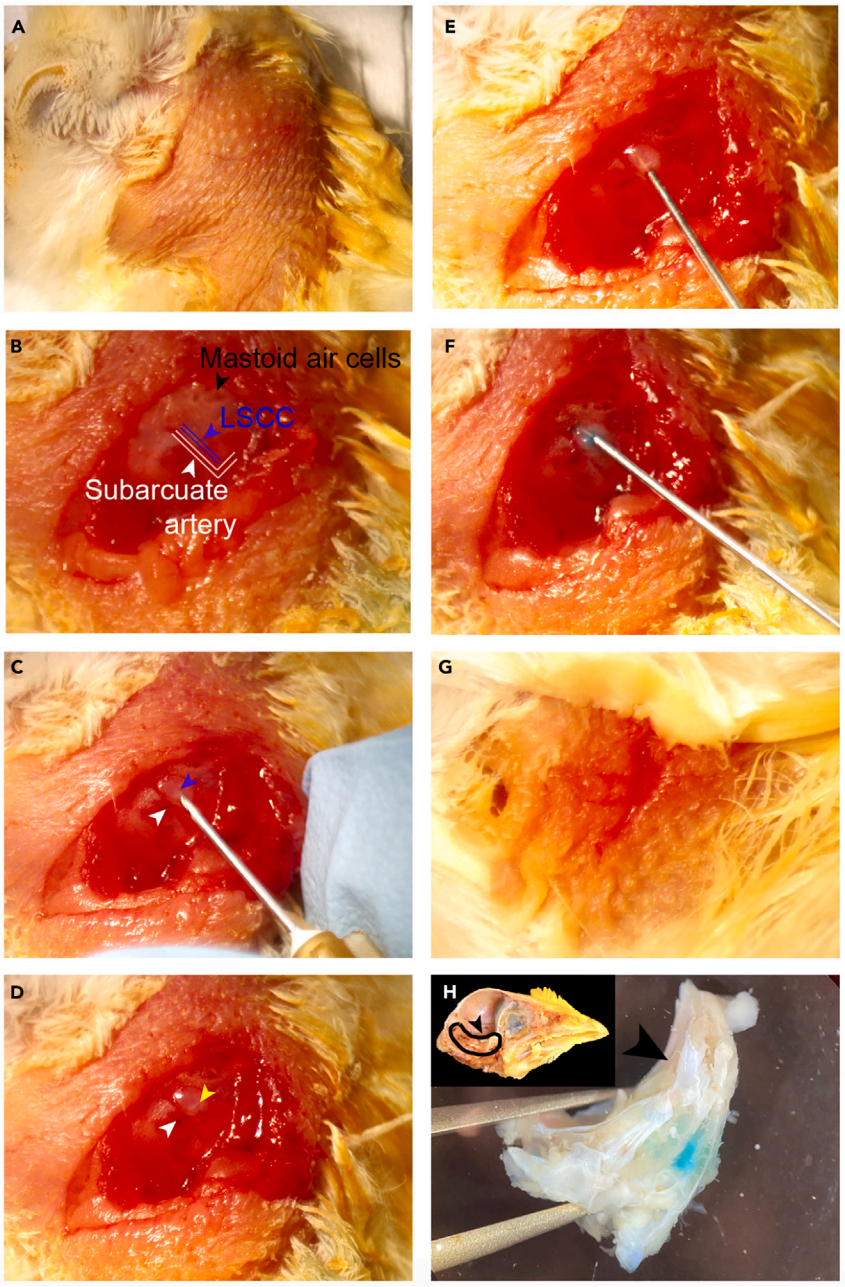

Figure 3. Surgical exposure and identification of key anatomical features

(A) Initial presentation of the surgical site after feather removal and before incision.

(B) Post-incision exposure of the subarcuate artery (white arrowhead and dashed white line) and mastoid air cells (black arrowhead). The lateral semicircular canal (LSCC) is highlighted in blue.

(C) Localization of the semicircular canal designated for puncture with a $30^{1}/_{2}$-gauge needle (blue arrowhead) adjacent to the subarcuate artery (white arrowhead; the blood-filled artery is distinctively visible).

(D) Visualization of the surgical puncture on the semicircular canal (yellow arrowhead) with the subarcuate artery indicated by a white arrowhead.

(E) Mid-procedure image depicting a blunt Nanofil needle inserted into the semicircular canal.

(F) Appearance of a blue stain indicating the infusion of a solution containing methylene blue and minimal leakage at the insertion site.

(G) Post-procedure image showing the cleared surgical site after skin closure/wound sealing with surgical superglue.

Figure 3. *Continued*
(H) Trimmed temporal bones containing the basilar papilla and vestibular apparatus extracted from half of the head. The black arrowheads point to the same anatomical region near the top of the basilar papilla (adjacent to the lagena). Note that the trimmed temporal bone is viewed from the lateral perspective of the region outlined in black.

Note: In our initial publications, there was a misidentification of the lateral semicircular canal as the posterior semicircular canal. Despite the process and landmarks remaining consistent, this has resulted in a mismatch in our descriptions. It's important to recognize this discrepancy to accurately understand and replicate the procedure.

8. Puncture the surface of the semicircular canal using a $30^{1}/_{2}$-gauge needle (Figures 3C and 3D).
   a. Insert a 35-gauge blunt Nanofil needle into the puncture at an angle of 20 degrees, gently pushing it approximately 2–3 mm deep into the canal.

   Note: If resistance is encountered, retract the needle by 1 mm.

   b. Proceed to infuse 2 μL of artificial perilymph mixed with 100 mM methylene blue at a ratio of 1/10 into the semicircular canal, administering the solution at a controlled rate of 8 nL/s (Figures 2B, 2C, 3E, and 3F).
9. Trim the heads to obtain the temporal bones containing the basilar papilla and vestibular apparatus (Figure 3H).

   Note: Because the goal of this injection is to determine its efficacy, we will immediately dissect the vestibular apparatus and the basilar papilla to determine whether the blue buffer solution has reached the inner ear.

10. Confirm visually that the labyrinth is filled with blue perilymph (Figure 3H).

    Note: The injection requires a surgical microscope and suitable illumination. For conducting injections on live chickens, please see Figure 2, which outlines additional essential equipment needed to ensure precision and accuracy.

    Note: A step-by-step dissection guide is provided in the method details below.

Order target mRNA probes for the hybridization chain reaction

Timing: 1 h

This section provides guidance on ordering specific mRNA probes for hybridization chain reaction (HCR) analysis. It involves selecting genes for study, procuring HCR probes, and choosing the correct fluorophores for multiple probe detection.

11. Identify each gene to be detected on NCBI, obtain the corresponding accession number, and order the HCR probes from Molecular Instruments (www.molecularinstruments.com).

    Note: Probes for mRNA detection are made-to-order and usually ship within 2–3 weeks.

    Note: The use and implementation of HCR are described in detail by.[9]

12. Select the correct combination of HCR reagents for the detection of multiple probes in parallel. One fluorophore must be assigned to each probe channel (B1, B2, or B3). In this protocol, we use B1 488 (green), B2 546 (red), and B3 647 (far red) reagents.

Note: Ensure your microscope has appropriate filters to visualize the selected fluorophores.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-Myosin7a (1:500)	Proteus	Cat# 256790; RRID: AB_10015251
Mouse anti-SOX2 (1:200)	Becton Dickinson	Cat# 612044; RRID: AB_399431
Anti-PV3	Self-prepared. The antigen consists of the entire sequence of bullfrog PV3. https://link.springer.com/content/pdf/10.1007/s10162-002-2050-x.pdf	https://www.ncbi.nlm.nih.gov/nuccore/AY049967
Donkey anti-rabbit IgG, Alexa Fluor 546 (1:250)	Thermo Fisher Scientific	Cat# A10040, RRID: AB_2534016
Donkey anti-mouse IgG, Alexa Fluor 488 (1:250)	Thermo Fisher Scientific	Cat# A-21206, RRID: AB_ 141607
DAPI (1:1,000)	Thermo Fisher Scientific	Cat# D1306, RRID: AB_2629482
Oligonucleotides		
CALB2 HCR DNA probe	NM_205316.2	Lot# PRJ052
RAB3IP HCR DNA probe	XM_046909870.1	Lot# RTB846
CDH1 HCR DNA probe	NM_001039258.2	Lot# PRD698
TMEM255B HCR DNA probe	XM_040657646.2	Lot# RTB850
CABP1 HCR DNA probe	XM_004934386	Lot# PRD383
B3GNTL HCR DNA probe	NM_001277651.1	Lot # RTA482
ATOH1 HCR DNA probe	XM_004941130.5	Lot# PRJ062
SLC1A3 HCR DNA probe	XM_046935476.1	Lot# PRJ066
Chemicals, peptides, and recombinant proteins		
1X DPBS without Ca^{2+}/Mg^{2+}	Corning	Cat# 21-031-CV
10X DPBS without Ca^{2+}/Mg^{2+}	Corning	Cat# 46-013-CM
Nuclease-free water	Thermo Fisher Scientific	Cat# AM9932
Tween 20	Sigma-Aldrich	Cat# P9416
20X 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)	MilliporeSigma	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
Click-iT EdU Kit 647	Thermo Fisher Scientific	Cat# C10340
Experimental models: Organisms/strains		
Fertilized eggs	Charles River	Cat# 10100326
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

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
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
Moria Spoon-11 cm	Fine Science Tools	Cat# 10321-11
1.5 mm Acu-Punch	Acuderm	Cat# P1550
Sylgard 184 silicone	World Precision Instruments	Cat# SYLG184
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
PAP Pen	Sigma-Aldrich	Cat# Z377821-1EA
35 × 10 mm dish	Corning	Cat# 353001
Other: Chicken husbandry		
Fertilized Rhode Island Red eggs	Charles River	Cat# 10100326
Egg incubator	GQF Manufacturing Company	Cat# 1502
Brinsea Octagon 20 Advance	Amazon	Cat# B00DPMZ37K
Chick starter feed	Farmers Warehouse Company	NA
Galvanized chick feeder	My Pet Chicken	NA
Bantam waterer	My Pet Chicken	NA
Infrared heat lamp 75 Watts	Amazon	Cat# B01BIBMY9E
Small animal cage	Circle K Industries	NA
Digital Hygro-thermometer	Amazon	Cat# B000NI4AQY
Egg candler	Amazon	Cat# B07FQNX9WF
Newspaper	NA	NA
Other: Surgical instruments		
Oxygen regulator (CGA870)	Cramer Decker Medical, Inc.	Cat# AREG8725-B2D
Oxygen	NA	NA
Isoflurane w/ Anti-spill adapter	Stanford VSC Pharmacy	NA
V-1 Tabletop anesthesia system	VetEquip, Inc.	Cat# 901806
Activated charcoal filter VaporGuard	VWR	Cat# 89012-608
Heated small animal pad	K&H Pet Products	NA
Carprofen	Stanford VSC Pharmacy	NA
Scissors	Roboz	Cat# RS-5610
Surgical microscope	Leica	Cat# M320
Eye Spears (Beaver-Visitec)	Fisher Scientific	Cat# NC0972725
UMP3 Microinjection syringe pump	World Precision Instruments	Cat# UMP3T-1
Nanofil needle	World Precision Instruments	Cat# NF35BL-2
SilFlex tubing	World Precision Instruments	Cat# SILFLEX-2
Nanofil injection holder	World Precision Instruments	Cat# NFINHLD
Nanofil syringe (10 µL)	World Precision Instruments	Cat# NANOFIL
FrameWorks V-Base Kit	World Precision Instruments	Cat# 503207
Sisomicin	MedChemExpress	Cat# HY-B1222
Methylene blue	Sigma-Aldrich	Cat# M9140
Bone wax (Ethicon)	Stanford VSC Pharmacy	NA
Surgical superglue (VetClose)	Stanford VSC Pharmacy	NA
Leg Bands (1/4 inch)	Amazon (Chicken Hill)	Cat# B078BHHLSH
Digital scale (Ozeri)	Amazon	Cat# B004164SRA

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Kaytee Exact formula	Amazon	Cat# B0002DGJH8
DMSO	Sigma-Aldrich	Cat# D8418
Nose Cone	Stanford Veterinary Service Center	N/A
Nair	Amazon	Cat# B000TJT65M
Surgical scissors	Fine Science Tools	Cat# 14002-13
Disposable scalpel (size 11)	VWR	Cat# 89176-380
5-ethynyl-2′-deoxyuridine (EdU)	Thermo Fisher Scientific	Cat# A10044

MATERIALS AND EQUIPMENT

Employing RNase-free techniques and solutions ensures the preservation of all options for detecting mRNA in future applications.

Sisomicin solution at 75 μg/μL		
Reagent	Final concentration	Amount
Sisomicin	75 μg/μL	40 mg
1X PBS (without Ca^{2+}/Mg^{2+})	N/A	500 μL
Total		**500 μL**
Make fresh before each series of injections. Filter sterilize. Store at 20°C–22°C.		

Note: For simplicity, to achieve the desired concentration of 75 μg/μL, calculate the volume of 1X PBS needed by multiplying the sisomicin mass "m" in milligrams by 12.5.

Sisomicin solution at 50 μg/μL		
Reagent	Final concentration	Amount
Sisomicin	50 μg/μL	60 mg
1X PBS (without Ca^{2+}/Mg^{2+})	N/A	500 μL
Total		**500 μL**
Make fresh before each series of injections. Filter sterilize. Store at 20°C–22°C.		

Note: For simplicity, to achieve the desired concentration of 50 μg/μL, calculate the volume of 1X PBS needed by multiplying the sisomicin mass "m" in milligrams by 8.33.

5-ethynyl-2′-deoxyuridine (EdU) solution		
Reagent	Final concentration	Amount
5-ethynyl-2′-deoxyuridine	16.7 mg/mL	50 mg
DMSO	N/A	1.5 mL
1X PBS (without Ca^{2+}/Mg^{2+})	N/A	1.5 mL
Total		**3 mL**
Filter sterilize. Make 500 μL aliquots. Store at −20°C for up to six months.		

4% paraformaldehyde (PFA) solution		
Reagent	Final concentration	Amount
10X PBS (without Ca^{2+}/Mg^{2+})	1X	4 mL
RNase-free water	N/A	26 mL
16% PFA (w/v)	4% PFA (w/v)	10 mL
Total		**40 mL**
Make fresh each time.		

4% agarose solution		
Reagent	Final concentration	Amount
Low-gelling temperature agarose	4% (w/v)	2 *g*
1X PBS (without Ca^{2+}/Mg^{2+})	N/A	50 mL
Total		**52 *g***

Microwave the mixture until the agarose is completely dissolved, resulting in a clear solution.
The solution is stable at 20°C–22°C and can be reused. For the next utilization, remicrowave the solution.

Click-iT EdU reaction cocktail

Note: This kit is commercially available, and all required solutions are provided.

Reagent	Final concentration	Amount	Notes to achieve final concentration
10X Buffer	1X Buffer	430 μL	Dilute 10X Buffer to 1X by mixing 43 μL 10X Buffer with 407 μL MilliQ water to prepare 450 μL
$CuSO_4$	N/A	20 μL	N/A - used as is
Azide-647	N/A	1.2 μL	N/A - used as is
10X Buffer Additive	1X Buffer Additive	50 μL	Dilute 10X Buffer to 1X by mixing 5 μL 10X Buffer Additive with 50 μL MilliQ water to prepare 55 μL
Total		**501.2 μL**	—

Add reagents in this order to avoid aggregate formation.
The reaction cocktail should be used within 15 min of making it.
Make fresh each time.

5X saline sodium citrate buffer with Tween-20 (SSCT)		
Reagent	Final concentration	Amount
20X SSC	5X	12.5 mL
Tween-20	0.1%	50 μL
Diethyl pyrocarbonate (DEPC)-treated water	N/A	37.45 mL
Total		**50 mL**

This solution can be stored at 4°C and remains stable for up to six months.

PBS with Tween-20 (PBST)		
Reagent	Final concentration	Amount
1X PBS (without Ca^{2+}/Mg^{2+})	N/A	49.9 mL
Tween-20	0.2%	100 μL
Total		**50 mL**

This solution can be stored at 4°C and remains stable for up to six months.

Note: The HCR v3.0 Protocol[9] was used for mRNA detection in the presented examples. 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.[9] For convenience and reproducibility, we used the commercially available premade buffers from Molecular Instruments. Additional information is available at www.molecularinstruments.com.

HCR probe hybridization buffer		
Reagent	Final concentration	Amount
Formamide	30%	3 mL
5X Sodium chloride citrate (SSC)	1X	2 mL
Citric acid (pH 6.0)	9 mM	0.27 mL

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Continued		
Reagent	Final concentration	Amount
Tween-20	0.1%	0.01 mL
Heparin	50 μg/mL	0.05 mL
Denhardt's solution	1X	1 mL
Dextran sulfate	10%	1 mL
Total		**10 mL**
Store at −20°C for up to six months.		

HCR probe wash buffer		
Reagent	Final concentration	Amount
Formamide	30%	3 mL
5X Sodium chloride citrate (SSC)	1X	2 mL
Citric acid (pH 6.0)	9 mM	0.27 mL
Tween-20	0.1%	0.01 mL
Heparin	50 μg/mL	0.05 mL
Water	N/A	4.67 mL
Total		**10 mL**
Store at −20°C for up to six months.		

HCR amplification buffer		
Reagent	Final concentration	Amount
5X Sodium chloride citrate (SSC)	5X	2 mL
Tween-20	0.1%	0.01 mL
Dextran sulfate	10%	1 mL
Water	N/A	7.99 mL
Total		**10 mL**
This solution can be stored at 4°C and remains stable for up to six months.		

Proteinase K solution	
Reagents	Composition
Proteinase K (800 units/mL)	1:2000 in PBST
Make fresh each time.	

Immunostaining solutions

Antibody permeabilization buffer		
Reagent	Final concentration	Amount
1X PBS (without Ca^{2+}/Mg^{2+})	N/A	49.75 mL
Tween-20	0.5%	250 μL
Total		**50 mL**
This solution can be stored at 4°C and remains stable for up to six months.		

Antibody blocking buffer		
Reagent	Final concentration	Amount
1X PBS (without Ca^{2+}/Mg^{2+})	N/A	49.25 mL
Tween-20	0.5%	250 μL
BSA	1% (w/v)	500 mg
Total		**50 mL**
This solution can be stored at 4°C and remains stable for up to six months.		

Antibody wash buffer (PBST)		
Reagent	Final concentration	Amount
1X PBS (without Ca^{2+}/Mg^{2+})	N/A	49.9 mL
Tween-20	0.2%	100 μL
Total		50 mL

This solution can be stored at 4°C and remains stable for up to six months.

STEP-BY-STEP METHOD DETAILS

Surgical method: Pre-operation

Timing: 10 min

This section details the pre-operation steps for a surgical method involving sisomicin, outlining solution preparation and equipment setup. It emphasizes the importance of concentration accuracy and injection technique for successful hair cell depletion.

1. Prepare the sisomicin solution.

Note: Varying concentrations of sisomicin are necessary to achieve total hair cell depletion in the basilar papilla compared to the utricle. Our standard practice involves administering 75 μg/μL of sisomicin in a total volume of 2 μL for the basilar papilla, and 50 μg/μL in a volume of 0.8 μL for the utricle.

⚠ CRITICAL: Outcomes can be affected by factors such as the seal and placement of the needle, as well as the techniques employed by different surgeons. Please consult problem 1 in the troubleshooting section.

⚠ CRITICAL: Occasionally, sisomicin infusion can result in a flattened epithelium, characterized by the lack of SOX2-positive supporting cells and a thin cellular layer over the basement membrane. At such highly destructive doses, homogene cells may be absent, and the tegmentum vasculosum may appear abnormal. For these instances, refer to problem 2 in the troubleshooting section.

Note: Adding 100 mM methylene blue allows for visual feedback during the injection. We did not notice dye-related adverse effects; however, needle clogging is more likely. When the surgery becomes routine, conducting the injection without dye is advisable.

Note: Make fresh sisomicin solution before each series of injections.

2. Front-load the 35-gauge Nanofil needle, SilFlex tubing, and syringe with sisomicin solution (Figure 2C).
3. Attach the pump to a V-base (Figure 2C).

Note: For optimum control, keep the Nanofil injection holder ready.

Surgical method: Infusion and post-operation

Timing: 15–30 min

This section outlines the steps for the surgical infusion process and subsequent post-operative care. The procedure includes setting up the heating pad, administering anesthesia, preparing the surgical

site, and carefully delivering the sisomicin solution into the cochlea. Post-operative instructions involve monitoring and ensuring the well-being of the chickens.

4. Place the chicken on a thermostatically controlled heating pad set to the chicken's natural body temperature of 39°C (Figure 2A).
5. Anesthetize P7 chickens.
   a. Induce lateral recumbency with 2 L/min oxygen and 3% isoflurane with a nose cone.
   b. Maintain with 2 L/min oxygen and 2% isoflurane (Figure 2A).

   Note: It is important to ensure that the anesthesia machine is properly set up and calibrated before initiating anesthesia. This procedure should be carried out under the guidance and supervision of a veterinarian due to the anesthesia involved with live animals. Our institution relies on the services of the veterinary service center, which offers support and expert guidance for such procedures, in accordance with institutional permissions and protocols.

6. Remove feathers on the surgical site using a hair remover cream (Nair), followed by blotting the exposed skin with three alternating rotations of betadine and 70% isopropanol (Figures 2A and 2C).
7. Make a 6 mm dorsal incision to the ear canal with microdissection scissors, carefully avoiding the superficial blood vessel.
   a. Locate the bump near this vessel to use as a landmark.
   b. Retract the muscle to reveal the temporal bone (Figure 2B).
8. Use eye spears to soak up any blood and visualize the subarcuate artery closely beneath the temporal bone with a surgical microscope (Figures 2A, 2C, and 3B).

   Note: Bleeding should be minimal.

9. Locate the lateral semicircular canal located above the subarcuate artery (Figure 3B).
10. Carefully puncture into the canal to penetrate the bony labyrinth adjacent to the mastoid air cell junction using a $30^{1}/_{2}$ gauge needle (Figures 2A, 2B, 3C, and 3D).
11. Using the UMP3 Micropump and the blunt Nanofil needle, infuse 2 μL of the 75 μg/μL sisomicin solution or sterile control solution (vehicle) at 8 nL/s into the perilymphatic space between the membranous lateral semicircular canal and the bone (Figures 2B,C′; Figures 3E and 3F).
12. Seal the hole with bone wax and close the skin/muscle wound with surgical superglue (Figures 2C and 3G).
13. Inject 100 μL carprofen (1 mg/kg) subcutaneously under the wing near the breast tissue.

    Note: Monitor the respiratory rate and document it in the surgical record.

14. Switch to pure oxygen flowing at 4 L/min (Figure 2C).
15. After recovering from lateral recumbency, place the chicken in a small plastic container and closely monitor its vitals before returning it to the brooder cage.
16. House chickens after surgery in cages separate from chickens that did not receive surgery.

    Note: To distinguish chickens from each other, use colored $^{1}/_{4}$ inch leg bands.

    Note: Weigh the chickens daily and compare them to their natural growth curve. Ensure they eat and drink enough.

    Note: An indication of successful hair cell ablation is the onset of head-tilting towards the operated side, which can begin as soon as 12 h after the surgery. While the head tilt typically lessens over time, there are instances where the vestibular deficits may progressively

deteriorate, leading to an inability of the animal to feed itself. Should this occur, please consult problem 3 in the troubleshooting section.

EdU bioavailability and proliferative window

Timing: 5 min

This section describes how to make the thymidine analog EdU available to cells for marking DNA synthesis during the S-phase. It involves a simple procedure of sterilization and subcutaneous injection near the breast muscle.

EdU incorporation during DNA replication labels cells that pass-through S-phase.

17. Sterilize the underside of the wing in an area near the breast muscle with 70% isopropanol.
18. Inject 50 mg/kg EdU in 50:50 1X PBS:DMSO in a volume of 200 μL subcutaneously under the wing near the breast tissue with a $26^{1/2}$-gauge needle.

Note: Systemically injected EdU passes the blood-inner ear barrier. Its pharmacokinetic availability to inner ear cells is transient as it is ultimately absorbed and secreted. Therefore, unlike *in vitro* culture experiments, where EdU is available for days, *in vivo* experiments require that EdU is re-administered before its bioavailability diminishes. We determined EdU's bioavailability in P7-P9 chickens to be between 4–6 h. S-phase entry of supporting cells peaks at 48-h post-sisomicin, primarily in the basilar papilla's neural region.[4]

Note: Make 500 μL aliquots of EdU solution. Store them at −20°C for up to six months.

Preparation of chickens

Timing: 12–16 h

This section describes the euthanasia and preparation of chickens for inner ear dissection.

19. Euthanize the chickens with CO_2 inhalation for 5 min followed by decapitation, which is in accordance with our IACUC-approved animal study protocol (# 28379).

Note: Our method of euthanizing animals is consistent with the recommendations of the panel of Euthanasia of the American Veterinary Medical Association involving chamber CO_2 asphyxiation, which is a rapid depressant and leads to unconsciousness. Specific requirements and permissions for animal procedures need to be obtained through your institution.

20. Clean the box used for euthanasia thoroughly after each use to remove odors that might lead to stress in animals that will be euthanized in the future.
21. Dissect out the inner ear (Figure 4A).
    a. Place it in 4% PFA for fixation.

Note: Make a fresh 4% PFA solution each time.

    b. Ensure the specimen is shaken gently at 4°C for 12–16 h.

Transverse vibratome sectioning

Timing: 60 min

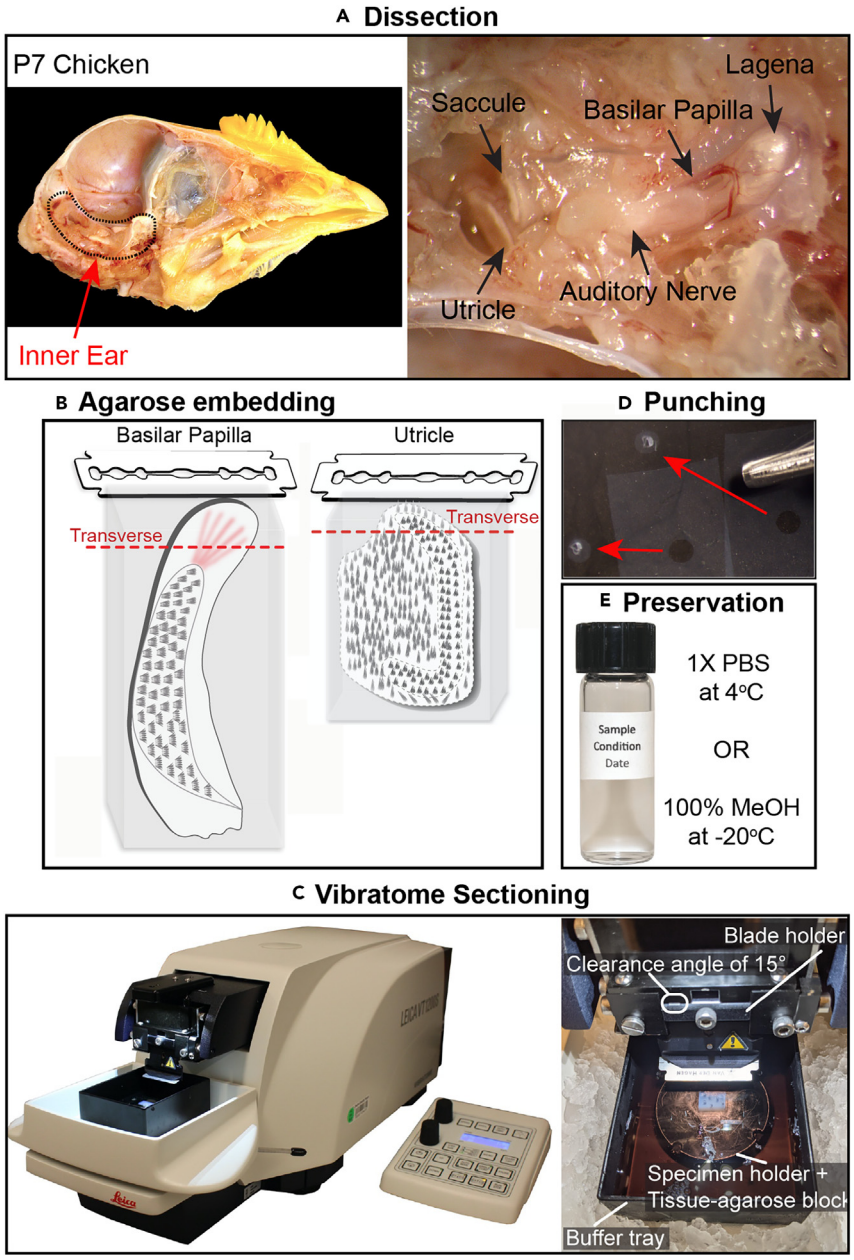

Figure 4. Step-by-step guide for obtaining vibratome sections from the chicken's basilar papilla
This figure is a partial reprint from Scheibinger et al., Neuromethods 2022, used with permission.

This section outlines the process of transverse vibratome sectioning, which includes preparing the agarose solution, positioning the sample for sectioning, and completing the sectioning process. Essential tips such as sterilization of molds, careful sample placement, and ensuring RNase-free conditions are highlighted to optimize the procedure for histological or imaging analysis.

22. Prepare a 4% agarose solution.
23. Bring the agarose solution to a water bath set to 55°C.

Note: The solution is stable at 20°C–22°C and can be reused. For the next utilization, remicrowave the solution and adjust it to 55°C.

24. Dissect the basilar papilla and utricle.
25. Sterilize the disposable molds by autoclaving or soaking them in 70% ethanol.

Note: To create RNase-free molds, soak them for 10 min in RNaseZap.

26. Add agarose to a small petri dish and transfer the basilar papilla or utricle sample using a pipette with the tip cut off. Minimize 1X PBS transfer and gently mix to integrate any 1X PBS with the agarose.
27. Carefully place the sample in a mold pre-filled with a sufficient layer of agarose at the bottom, using forceps. Ensure the sample is fully submerged and positioned for optimal sectioning (Figure 4B).[5] Mark the sample's position and orientation on the mold's exterior.

Note: The agarose should be poured slowly to avoid creating any air bubbles.

28. Place the mold in a refrigerator or on a cooling block to allow the agarose to solidify. This takes 30 min. Check to make sure that the sample has not moved during this time.

Note: If the samples are stored in a refrigerator for more than 6 h, ensure they are placed in a small bag to prevent the agarose from shrinking.

29. Once the agarose has solidified, remove the mold from the refrigerator or cooling block. Carefully remove the solidified agarose block from the mold by cutting open the edges of the mold. The agarose block can now be further processed for histological or imaging studies.
30. Wipe the surface of the blade holder, cutting head, buffer tray, and specimen plate with a Kimwipes brand wiper soaked in RNaseZap.
31. Precool the buffer tray and the specimen plate at 4°C for 15 min.
32. Mount the tissue-agarose block onto the center of the metal specimen holder plate using a drop of cyanoacrylate adhesive, the surface closer to the sample(s) facing up. Gently orient the block[5] and let the adhesive set for 5 min at 20°C–22°C.
33. Attach the specimen holder plate with the glued tissue-agarose block into the buffer tray (Figure 4C).
34. Fill the buffer tray with pre-ice-cooled RNase-free 1X PBS to submerge the agarose block and the blade (Figure 4C).

Note: Always keep track of the proper orientation of the specimen and correct the orientation if needed.

35. Place the pre-cooled buffer tray into the dovetail holder on the vibratome stage and push the buffer tray with the glued embedded specimen in as far as it will go (Figure 4C).
36. Insert a new single-edge razor blade (Schick Injector Blades 7) with the sharp side facing up in the blade holder (Figure 4C).
37. Return the blade holder to the cutting position by rotating the blade holder to a clearance angle of 15° (Figure 4C).
38. Use the following vibratome settings: Amplitude: 0.8 mm, Speed: 0.5–0.6 mm/s, Thickness: 50–60 μm.
39. Start sectioning at the desired thickness once the blade has cut into the sample.

Note: If the specimens' serial anatomical locations need to be documented, transfer each section into a single well of a multi-well plate filled with cold RNase-free 1X PBS using a micro spoon. Otherwise, sections are decanted into a Sylgard coated dissection dish.

40. Remove excess agarose with 1–5 mm diameter biopsy punches to obtain round agarose slices with the specimen slices inside (red arrows in Figure 4D).

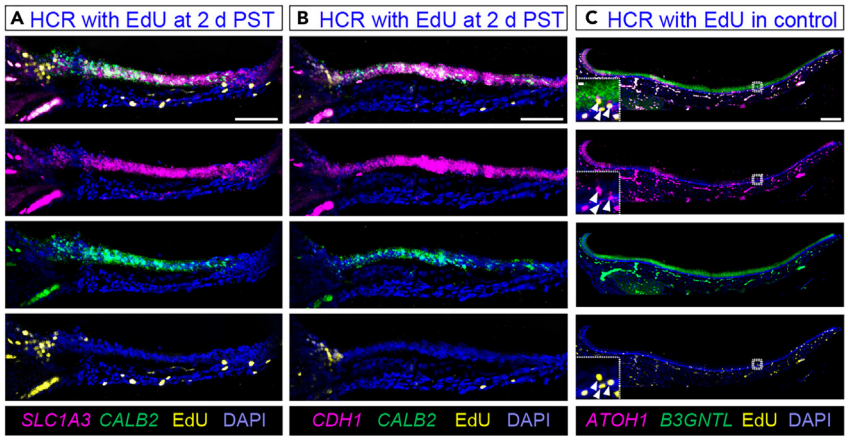

Figure 5. Examples of hybridization chain reaction *in situ* mRNA detection coupled with Click-It EdU reaction

(A and B) No hair cells were detected in a transverse vibratome section of the chicken basilar papilla at 2 days post-sisomicin treatment. Responding supporting cells are visualized with *SLC1A3*, *CALB2*, and *CDH1* mRNA, while some display EdU incorporation.

(C) All utricle hair cells are visualized with *B3GNTL*, whereas immature utricle hair cells are detected with *ATOH1*. EdU incorporation was found in the nuclei of immature hair cells and supporting cells during mitosis, indicated by arrowheads in the magnified image. Scale bar = 50 μm, 100 μm, and 5 μm in the basilar papilla, utricle, and magnified image of the utricle, respectively.

Note: For immunohistochemistry, transfer sections into cold RNase-free 1X PBS using a transfer pipette with the tip cut off. Before this step, ensure the pipette is immersed in the washing buffer. For *in situ* mRNA detection, sections should first be dehydrated by transferring them into 50% methanol in RNase-Free 1X PBS for 5 min on ice using a transfer pipette with the tip cut off, then stored in 100% methanol at −20°C in scintillation vials (Figure 4E).

Note: Sections in 100% methanol can be stored at −20°C for up to six months.

Click-It EdU reaction

Timing: 90 min (Figure 5)

This section describes the Click-It EdU reaction, a method for labeling cells in the S-phase of DNA replication. The process involves preparing tissue sections on microscope slides, creating a hydrophobic barrier to contain reagents, and applying the EdU reaction solution for visualization of cell proliferation.

41. Drop sections with methanol onto a Superfrost Plus microscope slide.
42. Arrange the sections using clean forceps into an 8 mm diameter circular space.
43. Allow sections to fully dry for 5 min, which aids in sticking.
44. Using a PAP pen, a tool that creates a hydrophobic barrier, outline the sections to confine staining reagents to the specified area.
45. Wash twice with 50% MeOH, 50% RNase-Free 1X PBS on ice, each time for 5 min.
46. Replace liquid by washing twice with 100% RNase-Free 1X PBS on ice, each time for 5 min.
47. Permeabilize sections for 30 min at 20–22°C in 3% Triton X-100, 1X PBS.
48. Replace the solution with 100 μL EdU reaction solution.
49. Incubate for 30 min at 20–22°C.

Note: Ensure samples are protected from light at this point.

50. Continue with antibody staining (steps 51–52) or HCR *in situ* hybridization (steps 53–55).

Note: If sections were stored in 100% methanol, rehydration steps (45 and 46) should be followed. When sections are fresh and in 1X PBS, skip these steps.

Note: Use freshly made 1X buffer, 1X buffer additive, and EdU reaction solution.

Note: At this stage, if the goal is to perform *in situ* hybridization along with the EdU incorporation protocol, proceed to the hybridization chain reaction mRNA detection section (steps 53–55). Otherwise, move to the immunostaining (steps 51–52) and sample mounting for microscopy protocols.

Immunostaining

Timing: 18–24 h

This section outlines the process for immunostaining, starting with the primary antibody incubation, including washing, permeabilizing, and blocking steps, followed by the secondary antibody application. It emphasizes proper handling of antibodies, incubation times and conditions, and specific washing procedures to ensure accurate staining results.

51. Primary antibody incubation.
    a. Wash the sections twice with 1X PBS containing 0.1% Tween 20 (PBST), for 5 min each time, ensuring the solution is applied directly to the sections within the boundaries marked by the PAP pen in each hybridization chamber.

Note: 1X PBS solution can be stored at 4°C and remains stable for up to six months.

Note: If sections were stored in 100% methanol, rehydration steps (45 and 46) should be followed. When sections are fresh and in1X PBS, skip these steps.

    b. Permeabilize samples using PBST for 30 min with gentle agitation.

Note: Permeabilization buffer can be stored at 4°C and remains stable for up to six months.

Note: If the immunostaining follows the Click-It EdU reaction, a second permeabilization is not necessary.

    c. Block samples with antibody blocking buffer for 45 min at 4°C.

Note: Blocking buffer can be stored at 4°C and remains stable for up to six months.

    d. Prepare a working concentration of primary antibodies in the antibody blocking buffer.

Note: Centrifuge the tube of the primary antibody at 15,000 *g* for 30 s to collect all the solution at the bottom of the tube for easier pipetting.

Note: Dilute the primary antibody as needed in the blocking buffer. For the presented examples (Figure 6), we used Rabbit anti-MYO7A (1:100), Rabbit anti-PV3[10] (1:2000), and mouse anti-SOX2 (1:50). Make fresh each time.

    e. Remove the antibody buffer and add the primary antibody solution.

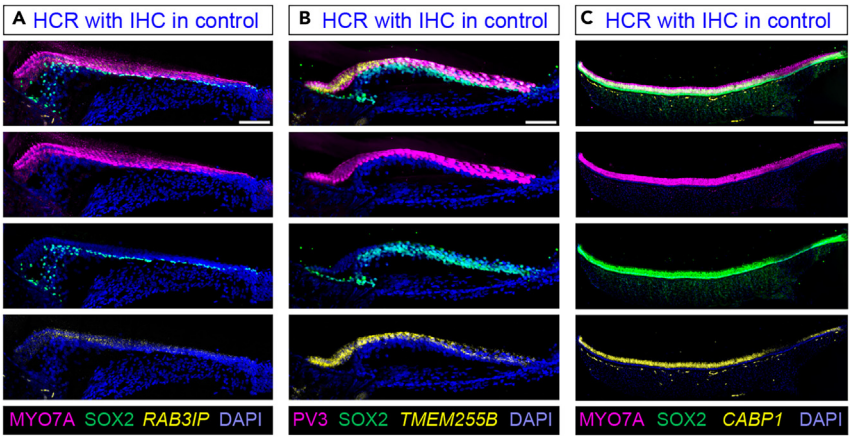

Figure 6. Examples of hybridization chain reaction *in situ* mRNA detection coupled with immunohistochemistry

(A) HCR *in situ* detection of mRNA for *RAB3IP*, a hair cell marker, and immunohistochemistry (IHC) for MYO7A and SOX2 in a representative cross-section of the basilar papilla in control. MYO7A immunostaining merges with *RAB3IP* signals, and SOX2 immunoreactivity is detectable in the supporting cells.

(B) HCR for *TMEM255B*, a hair cell marker, and IHC for PV3 and SOX2.

(C) HCR for *CABP1*, an extrastriolar type II hair cell marker, and IHC for MYO7A and SOX2 in the utricle. MYO7A-positive hair cells in striola are negative for *CABP1*. Scale bar = 50 μm and 100 μm in the basilar papilla and utricle, respectively.

i. Add 200 μL of primary antibody solution to each hybridization chamber directly on top of the sections, in the area defined by the PAP pen.
ii. Incubate in a humidified box for 12–16 h at 4°C with gentle rotation or 2.5 h at 20°C–22°C.

Note: Incubation may be optimized depending on sample type, antibody labeling properties, and thickness.

52. Secondary antibody incubation.
    a. Remove unbound primary antibodies by performing four washes with washing buffer (PBST) for 15 min each, at 20°C–22°C and with agitation, ensuring that the washing solution is directed onto the sections within the areas delineated by the PAP pen in each hybridization chamber.

Note: Washing buffer can be stored at 4°C and remains stable for up to six months.

    b. Prepare secondary antibodies in antibody blocking buffer (1:250).

Note: Centrifuge the tube of secondary antibodies at 15,000 *g* for 30 s to collect all solutions at the bottom of the tube for easier pipetting.

Note: Concentrations may be optimized depending on the primary antibodies' properties. Make fresh each time.

    c. Remove PBST and add 200 μL of secondary antibody solution directly onto the tissue sections within the hybridization chambers, ensuring coverage within the area marked by the PAP pen.
    d. Incubate at 20°C–22°C for 2 h with gentle agitation.

Note: Ensure samples are protected from light.

e. Remove excess antibodies by washing the samples four times with 200 μL of PBST, each time for 5 min at 20°C–22°C.

Note: At this stage, if the goal is to perform immunostaining along with *in situ* hybridization chain reaction mRNA detection, proceed to the section below. Otherwise, move to the sample mounting for microscopy step.

⚠ CRITICAL: If immunostaining results in inadequate staining intensity, consult problems 4 and 5 in the troubleshooting section for guidance on addressing this issue.

Hybridization chain reaction (HCR)

Timing: 68–72 h

This section is dedicated to detecting and amplifying specific mRNA sequences in tissue samples. It encompasses sample preparation, probe hybridization to the targeted mRNAs, and an amplification step that heightens signal clarity, enabling precise localization and quantification of gene expression.

53. Sample preparation.
    a. Gently replace the solution with 200 μL of diluted Proteinase K solution and incubate for 10 min at 20°C–22°C.

Note: We dilute Proteinase K (800 units/mL) 1:2000 in PBST and prepare the solution fresh for each use.

⚠ CRITICAL: Proteinase K concentration and duration may need to be optimized as each batch differs slightly. A final concentration of 0.4 units/mL is a good starting point.

    b. Gently remove the Proteinase K solution using a pipette and perform a 5-min fixation with 4% PFA at 20°C–22°C.
    c. Wash twice for 5 min with 200 μL PBST on ice.
    d. Wash with 50% PBST / 50% 5X SSCT for 5 min on ice.
    e. Wash with 5X SSCT for 5 min on ice.

Note: The 5X SSCT solution can be stored at 4°C and remains stable for up to six months.

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

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

Note: A rectangular food storage container filled with a piece of wet paper towel makes a good humidifying chamber. An inverted rack for Eppendorf type 80 × 1.5 mL tubes is used as a slide holder.

    b. Incubate with 200 μL of HCR probe hybridization buffer (pre-heated to 37°C) for pre-hybridization for 5 min on ice.

⚠ CRITICAL: Probe hybridization buffer contains formamide, a hazardous material.

Note: The probe hybridization buffer should be stored at −20°C for up to six months.

c. Replace the HCR probe hybridization buffer with HCR probe hybridization buffer that was pre-heated at 37°C and incubate for 30 min inside the humidified chamber at 37°C.
d. During pre-hybridization, prepare probe hybridization solution by adding 1 μL of 1 μM probe stock to 200 μL of hybridization buffer.

Note: If different probes are being multiplexed, combine them all into the 200 μL hybridization buffer.

Note: We use 1/200 probe concentration which is double the probe concentration that is recommended in the original protocol.

e. Remove the pre-hybridization solution with a P200 pipette and replace it immediately with 200 μL of the probe hybridization solution.

Note: Make sure the samples are surrounded by a PAP pen to prevent any leakage causing dry out.

f. Incubate for 12–16 h at 37°C in the humidified chamber.

Note: Ensure samples are protected from light.

g. Replace the probe hybridization solution with HCR probe wash buffer that has been pre-heated to 37°C.

⚠ CRITICAL: Use different pipette tips to avoid cross contamination 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.

⚠ CRITICAL: Probe wash buffer contains formamide, a hazardous material.

Note: The probe wash buffer should be stored at −20°C for up to six months.

h. Wash four times for 15 min with 200 μL of probe wash buffer at 37°C.

Note: During the fourth wash, begin with step 55c.

i. Wash samples twice for 5 min with 5X SSCT at 20°C–22°C.

55. Amplification.
    a. Replace 5X SSCT buffer with 200 μL amplification buffer.

Note: The amplification buffer should be stored at +4°C for up to six months.

Note: Equilibrate an aliquot of HCR amplification buffer to 20°C–22°C before use.

b. Replace the amplification buffer with 200 μL of amplification buffer and incubate in a humidified chamber for 30 min at 20°C–22°C.
c. Separately prepare hairpin H1 and hairpin H2 by snap cooling 10 μL of hairpin 3 μM stock in hairpin storage buffer.

Note: The final volume of hairpin solution needed depends on the number of experiments/hybridization chambers used.

Note: The snap cooling method serves to denature hairpin structures. To illustrate this technique, one could aliquot 1 μL from a 3 μM stock solution of hairpin H1 associated with the first fluorophore into an RNase-free tube. Similarly, for hairpin H2 tied to the same fluorophore, 1 μL from its 3 μM stock solution would be allocated to another RNase-free tube. Each tube is then heated to 95°C for 90 s. After heating, promptly cool the tubes to 20°C–22°C for 30 min. To preserve the integrity of the fluorophores, store the tubes in a dark place, such as a light-protected drawer. This example provides a reference point; however, actual volumes should be adjusted according to the specific requirements of each experiment.

Note: Use at least 5 μL of solution in each tube for heat shock treatment.

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

d. Prepare hairpin solution by adding snap-cooled H1 hairpins and snap-cooled H2 hairpins to the amplification buffer at 20°C–22°C. Add 1 μL of each hairpin per 200 μL.
e. Remove the amplification buffer from the hybridization chambers.
f. Add 200 μL of the hairpin solution.
g. Incubate for 12–16 h protected from light in a humidified chamber at 20°C–22°C.
h. Remove excess hairpins by incubating the slide hybridization chamber in 5X SSCT at 20°C–22°C twice for 30 min, followed by a single wash for 15 min.

⚠ CRITICAL: If HCR results in inadequate staining intensity, consult problems 4 and 5 in the troubleshooting section for guidance on addressing this issue.

Sample mounting for microscopy

Timing: 2 h

The purpose of this section is to prepare tissue samples for microscopic examination. It involves a series of steps that include the staining of cell nuclei with DAPI, thorough washing, and careful mounting on slides with a clear medium to preserve fluorescence, culminating with securing the samples for high-resolution imaging. This method ensures the samples are well-preserved and ready for detailed visual analysis under a microscope.

56. Incubate with DAPI at 1:1000 in PBST for 30 min at 20°C–22°C.
57. Wash three times with 1X PBS for 15 min at 20°C–22°C.

⚠ CRITICAL: Do not allow samples to dry out.

58. Gently remove the PAP pen marks with a wet paper towel and firmly attach a secure-seal spacer.
59. Remove extra 1X PBS carefully with a pipette and Kimwipes. Add a drop of FluorSave Reagent onto each sample.
60. Place a cover slip and allow the mounting solution to dry in the dark at 20°C–22°C for 1 h.

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

⚠ CRITICAL: Avoid excessive coverslip adjustment, which will dislodge and possibly damage the sample.

61. Add a small amount of clear nail polish along the edge of the coverslip to permanently secure the coverslip.
62. Proceed with confocal microscopy image acquisition and analysis.

⚠ CRITICAL: Sample degradation can occur during prolonged microscopic examination. For guidance on addressing this issue, please see problem 4 in the troubleshooting section.

EXPECTED OUTCOMES

The provided protocol enables the detection of proliferative cells and simultaneous mRNA expression (Figure 5), as well as the simultaneous detection of multiple mRNA transcripts and proteins of interest in controls and post-drug infusion (Figure 6). Since HCR is a quantitative *in situ* mRNA detection method, it can be used to quantify mRNA expression levels using confocal microscopy.

LIMITATIONS

Technical limitations in mRNA detection and immunohistochemistry techniques can compromise the accuracy and reliability of research findings. Common limitations include non-specific binding of probes or cross-reactivity between probes in mRNA detection, and non-specific binding of antibodies in immunohistochemistry, leading to false-positive results or background staining. However, these limitations can be minimized by optimizing experimental conditions, using appropriate controls, and validating results using complementary methods. Negative and positive controls can be used to verify the specificity and accuracy of staining.

TROUBLESHOOTING

Problem 1

Partial damage to the basilar papilla may occur after sisomicin infusion, leading to a gradient of damage. Here, complete damage is observed in proximal sections, with only abneural and far neural sides affected in middle sections, and distal regions remaining intact.

Potential solution

This happens when not enough sisomicin is infused. To achieve uniform hair cell loss while preserving supporting cells, fine-tune the sisomicin concentration within 50–100 μg/μL. Ensure needle seal integrity and positioning are consistent across procedures. Inspect surgical tools prior to each operation to prevent clogging issues and prioritize using new Nanofil needles to reduce variability.

Problem 2

Sisomicin infusion occasionally induces a flat epithelium identified by the absence of SOX2-positive supporting cells and the presence of a thin layer of cells covering the basement membrane. Homogene cells might disappear, and the tegmentum vasculosum shrinks at these highly destructive doses.

Potential solution

This happens when a too high concentration of sisomicin is infused. We encourage the experimenter to find the right concentration (50–100 μg/μL) that gives a complete loss of hair cells and preservation of supporting cells.

Variability in the needle seal and position, as well as differences in surgeons' approaches, influences the results. At the start of each surgery, the syringe, needle, and tubing must be carefully inspected for potential clogging. Furthermore, although Nanofil needles can be cleaned, they function best when new. We have noticed increasing variability with reused needles, presumably due to clogging.

Problem 3

After a sisomicin infusion, a chicken presents vestibular defects that progressively worsen until the animal cannot feed itself.

Potential solution

The animals need to be closely monitored for vestibular defects. If the vestibular deficiencies worsen, leading to body weight loss, the chicken can be fed 3x per day by oral gavage using Kaytee formula and injected subcutaneously with 5 mL lactated Ringer's solution for proper hydration. The chicken will eventually recover from vestibular defects around 2.5 weeks post-sisomicin treatment, and oral gavage can be discontinued. Based on our institution's veterinary staff's recommendations, chickens exhibiting weight loss greater than 25% should be euthanized.

Problem 4

Despite following the protocol, you may encounter inadequate staining intensity, which can hinder the observation and analysis of mRNA and protein expressions in tissue samples.

Potential solution

Ensure that the concentration and incubation time of HCR probes and primary antibodies are optimal. Increasing the concentration of probes or antibodies and extending incubation times can improve staining intensity. Additionally, verify that all reagents are fresh and that the samples are not washed excessively after application of the HCR probes or antibodies, which can diminish staining intensity. Always include positive controls to set a benchmark for expected staining results.

Problem 5

Over time, photobleaching and sample degradation may occur, especially during prolonged periods of microscopic examination, which can lead to loss of signal and decreased data quality.

Potential solution

Minimize exposure to intense light and use anti-fade mounting media to reduce photobleaching. Additionally, keep the examination sessions brief and capture images quickly after staining. When not observing the samples, store them in the dark at appropriate temperatures to prevent degradation. Consider using fluorophores with higher photostability or enhance your imaging system with photostable LED lights if frequent observation or re-imaging is required.

RESOURCE AVAILABILITY

Lead contact

For further information and requests for reagents, please direct your inquiries to the lead contact, Nesrine Benkafadar (kafadar@stanford.edu).

Technical contact

For technical questions or support regarding the protocol and experimental procedures, please contact Nesrine Benkafadar (kafadar@stanford.edu) or Mitsuo P Sato (qqd755pd@spice.ocn.ne.jp).

Materials availability

The study did not generate new unique reagents.

Data and code availability

- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.P.S. and N.B.; methodology, M.P.S. and N.B.; formal analysis, M.P.S. and N.B.; investigation, M.P.S. and N.B.; visualization, M.P.S., A.P.H., and N.B.; funding acquisition, M.P.S., N.B., and S.H.; project administration, S.H. and N.B.; supervision, N.B.; writing – original draft, M.P.S. and N.B.; writing – review and editing, M.P.S., A.P.H., S.H., and N.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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