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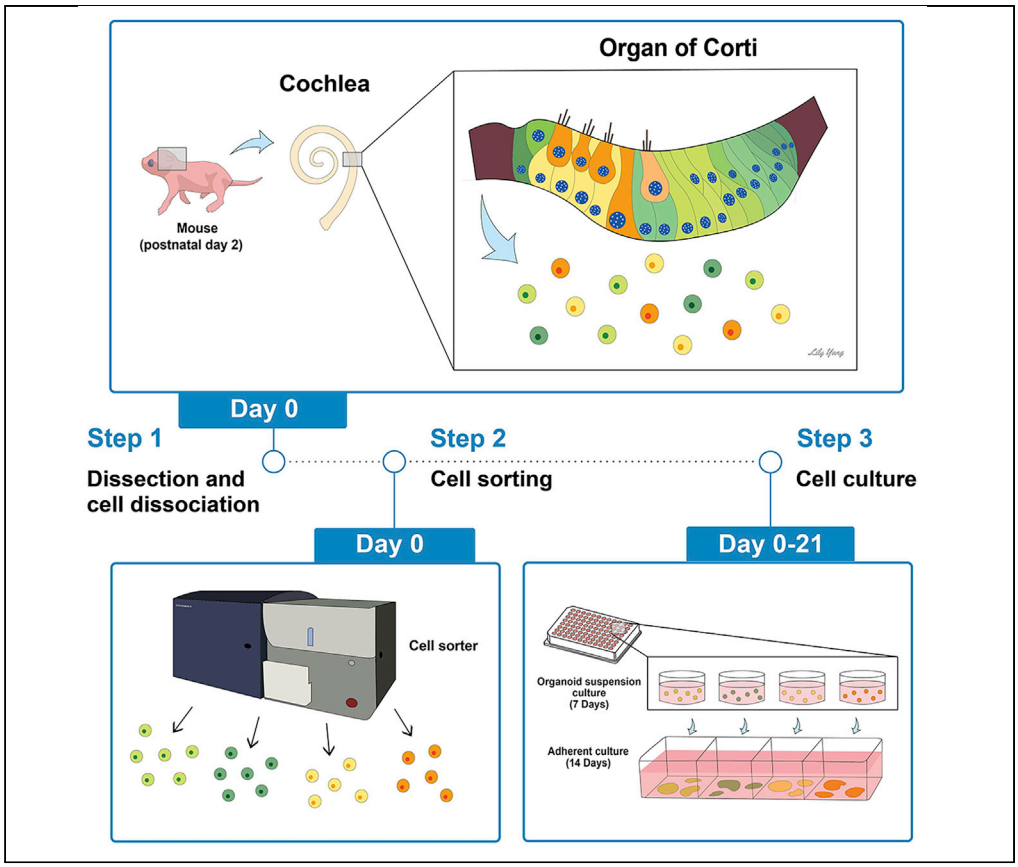
Murine cochlear cell sorting and cell-type-specific organoid culture



Neonatal mouse cochlear duct cells can proliferate and grow *in vitro* into inner ear organoids. Distinctive cochlear duct cell types have different organoid formation capacities. Here, we provide a flow cytometric cell-sorting method that allows the subsequent culture of individual cochlear cell populations. For the efficient culture of the sorted cells, we provide protocols for growing free-floating inner ear organoids, the adherence of organoids to a substrate, and the expansion of organoid-derived inner ear colonies.

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Highlights

Flow cytometric sorting of mouse cochlear cells

Culture of sorted cochlear cell populations and growth of inner ear organoids

Adherent growth of inner ear organoid-derived colonies

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Murine cochlear cell sorting and cell-type-specific organoid culture

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SUMMARY

Neonatal mouse cochlear duct cells can proliferate and grow *in vitro* into inner ear organoids. Distinctive cochlear duct cell types have different organoid formation capacities. Here, we provide a flow cytometric cell-sorting method that allows the subsequent culture of individual cochlear cell populations. For the efficient culture of the sorted cells, we provide protocols for growing free-floating inner ear organoids, the adherence of organoids to a substrate, and the expansion of organoid-derived inner ear colonies.

For complete details on the use and execution of this protocol, please refer to Kubota et al. (2021).

BEFORE YOU BEGIN

Preparation of mice

Timing: 5 min for step 2

Timing: 10 min for step 3

1. Reporter mice are used that express fluorescent proteins for distinction of cochlear cell subtypes. The example presented in this protocol utilizes transgenic mice that carry homozygous Fgfr3-CreERT2 and Ai14-tdTomato alleles (Fgfr3-tdTomato). Fgfr3-tdTomato mice are mated with heterozygous Sox2-GFP transgenic mice to create double-fluorescent reporter mice.

   Note: Homozygous Sox2-GFP mice are not viable. Mating heterozygous Sox2-GFP mice with homozygous Fgfr3-tdTomato mouse results in half of the litter carrying Sox2-GFP, Fgfr3-CreERT2, and Ai14-tdTomato alleles (Fgfr3-tdTomato/Sox2-GFP). It is critical to obtain at least three Fgfr3-tdTomato/Sox2-GFP pups in a litter for successful cell sorting.

2. At postnatal day 0 (P0), perform phenotyping for the presence of the Sox2-GFP allele. Positive pups display GFP expression inside the head that is observable through the skin using fluorescent light illumination and a stereomicroscope equipped with appropriate light filters (Figure 1).
3. At P0, inject tamoxifen (0.2 mg/g, 15 µL/animal) subcutaneously into Fgfr3-tdTomato/Sox2-GFP pups with a 1 mL syringe and 30 $^{1}/_{2}$ G needle.

   Note: Keep the Sox2-GFP-negative and non-recombinant Fgfr3-tdTomato (not tamoxifen injected) pups; the cochlear duct cells from these pups are used as a negative gating control for fluorescence-activated cell sorting (FACS).

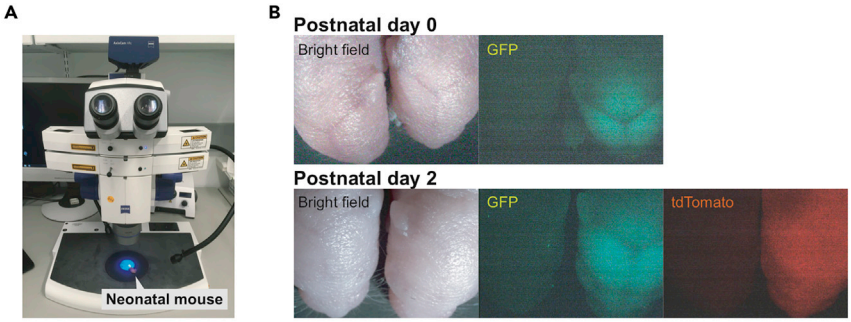

Figure 1. Phenotyping of mouse pups

(A) Fluorescence dissection microscope used for phenotyping.

(B) Bright field and fluorescence illumination of P0 transgenic mice with and without Sox2-GFP allele as well as P2 Fgfr3-tdTomato / Sox2-GFP transgenic pups after cre-recombination. Fluorescence of brain tissue is visible through the skull.

Preparation of a 96-well suspension culture plate for cell sorting

Timing: 15 min

4. Prepare DMEM/F-12 supplemented with 1× N-2 Supplement, 1× B-27 Supplement, and 100 μg/mL ampicillin (DMEM/F-12/N-2/B-27/Amp).
5. Prepare the maintenance medium; add growth factors, small molecules, and recombinant proteins to DMEM/F-12/N-2/B-27/Amp.

Note: Reagents should be added at the time of use. An example of reagent combinations known for promoting the organoid formation of cochlear cells is epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), insulin growth factor 1 (IGF1), CHIR99021, valproic acid, 2-phospho-L-ascorbic acid, and 2-(3-(6-methylpyridin- 2-yl)-1H-pyrazol-4-yl)-1,5-naphthyridine) (McLean et al., 2017). Details are provided below under materials and equipment.

6. Add 200 μL maintenance medium per well into a 96-well flat-bottom suspension culture plate. The collection plate is kept at 4°C until sorting.

Preparation of cochlear duct cells for sorting

Timing: 2 h

7. At postnatal day 2 (P2), dissect the cochlear ducts (Oshima et al., 2009) from the recombinant Fgfr3-tdTomato/Sox2-GFP mice and the non-recombinant Fgfr3-tdTomato (negative control) mice. Male and female mice are used.

⚠ CRITICAL: Six to ten cochlear ducts from Fgfr3-tdTomato/Sox2-GFP pups (three to five animals) need to be dissected for successful sorting of GER cells and pillar/Deiters' cells for subsequent organoid cultures. At least four cochlear ducts from Fgfr3-tdTomato Sox2-GFP-negative pups (two animals) are required for the negative control.

⚠ CRITICAL: Sterilize the microdissection forceps and scissors with 70% ethanol before starting the dissection to avoid contamination. Also, decapitated mouse heads should be submerged for a few seconds in 70% ethanol, and then transferred into a dish filled with sterile Hanks' Balanced Salt Solutions (HBSS) for rapid washing. Dissection is then

performed in a dish filled with phosphate-buffered saline (PBS) in a laminar flow hood at 18°C–24°C.

Note: Cre recombination results in tdTomato expression in the brain, which can be verified with fluorescent light illumination and a stereomicroscope equipped with appropriate light filters.

8. Dissociation of cochlear duct cells (Oshima et al., 2009).
   a. Place a 50 μL drop of PBS without calcium and magnesium (PBS (-)) into a well of a 6-well suspension culture dish. Rinse the cochlear ducts by soaking them in the drop.
   b. Place another 50 μL drop of PBS (-) into a different well. Carefully transfer the cochlear ducts into the drop using forceps.
   c. Add 50 μL of 0.25% trypsin/EDTA solution for a combined volume of 100 μL, and incubate at 37°C for 10 min.
   d. Add 50 μL Dulbecco's Modified Eagle Medium; Nutrient Mixture F-12, without phenol red (DMEM/F-12/phenol (-)) to the drop.
   e. Add 50 μL of trypsin inhibitor (20 mg/mL)/ DNAseI (2 mg/mL) solution.
   f. Mechanically dissociate the cochlear ducts by pipetting up to 80 times.

Note: A conventional cell culture incubator is sufficient for the incubation at 37°C.

Note: Pipetting should be done carefully to avoid the introduction of air bubbles and foaming. A simple strategy for preventing air bubbles is to set the pipette to ≈20% less than the total sample volume of 200 μL (e.g., 160 μL) and to keep the tip submerged at all times. The use of low retention pipet tips with a barrier filter minimizes cell loss.

9. Transfer the dissociated cells into 5 mL round bottom polystyrene tubes.
10. Wash the cells with 1 mL DMEM/F-12/phenol (-).
    a. Centrifuge the samples at 300 × *g* for 3 min in a swing-out rotor at 18°C–24°C.
    b. Locate the cell pellet and carefully remove the supernatant.
    c. Add 1 mL DMEM/F-12/phenol (-) per sample and resuspend carefully.
    d. Repeat step a-c twice for a total of three washes.
    e. Resuspend the cells in DMEM/F-12/phenol (-) in a total volume of a multiple of 80 μL per each cochlear duct.

⚠ CRITICAL: Use phenol red-free media. Phenol red interferes with the fluorescence-based detection in flow cytometry.

⚠ CRITICAL: Bovine serum albumin (BSA) is often used in cell suspensions for flow cytometry. We noticed that BSA agglutinates cochlear duct cells, and consequently, BSA should not be used.

⚠ CRITICAL: Cochlear dissection, tissue dissociation, and cell washing should be done in less than 90 min. Immediately proceed with flow cytometry and cell sorting.

11. Filter each sample through the 35 μm mesh of a cell strainer snap cap of a Falcon 5 mL Round Bottom Polystyrene Test Tube.
12. Add dead cell stain (SYTOX Red) to the sample tubes.
    a. Dilute SYTOX Red stock solution 10-fold with DMSO.
    b. Add 1/100 volume of the diluted SYTOX Red to each sample.
    c. Gently mix the sample.

Note: We typically perform steps 9–12 at 18°C–24°C, and immediately proceed to flow cytometry.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti Myosin 7a (1:1000)	Proteus	Cat#25–6790; RRID:AB_10015251
Chemicals, peptides, and recombinant proteins		
1× DPBS without Ca/Mg	Corning	Cat# 21-031-CV
1× DPBS with Ca/Mg	Corning	Cat# 21-030-CV
HBSS	Corning	Cat# 21-021-CV
Tamoxifen	Sigma-Aldrich	Cat# T5648
Trypsin-EDTA(0.25%), phenol red	Gibco	Cat# 25200056
Soybean Trypsin Inhibitor	Gibco	Cat#17075029
DNAseI	STEMCELL Technologies	Cat# 07469
DMEM/F12, HEPES	Gibco	Cat# 11330032
DMEM/F12, HEPES, no phenol red	Gibco	Cat# 11039021
Bovine Serum Albumin	Fisher Scientific	Cat# BP1600-00
SYTOX Red Dead Cell Stain	Invitrogen	Cat# S34859
DMSO	Sigma-Aldrich	Cat# D2650
N-2 Supplement (100×)	Gibco	Cat# 17502048
B-27 Supplement (50×)	Gibco	Cat# 17504044
Ampicillin	Sigma-Aldrich	Cat# A9518
Recombinant Mouse EGF	R&D Systems	Cat# 2028-EG
Recombinant Mouse FGF basic (FGF2)	R&D Systems	Cat# 3139-FB
Recombinant Mouse IGF-1	R&D Systems	Cat# 791-MG
CHIR99021	LC Laboratories	Car# C-6556
Valproic acid sodium salt	Sigma-Aldrich	Cat# P4543
2-Phospho-L-ascorbic acid trisodium salt	Sigma-Aldrich	Cat# 49752
2-(3-(6-Methylpyridin-2-yl)-1H-pyrazol-4-yl)-1, 5-naphthyridine, ALK 5 inhibitor II	Millipore	Cat# 616452
Matrigel Growth Factor Reduced Basement Membrane Matrix	Corning	Cat# 356230
16% Paraformaldehyde Aqueous Solution	Electron Microscopy Sciences	Cat# 15710
Experimental models: organisms/strains		
Sox2-GFP mice	The Jackson Laboratory	Stock # 017592; RRID: IMSR_JAX:017592
Fgfr3-CreERT2 mice	The Jackson Laboratory	Stock # 025809; RRID: IMSR_JAX:025809
Ai14-tdTomato mice	The Jackson Laboratory	Stock # 007908; RRID: IMSR_JAX:007908
Software and algorithms		
FlowJo	BD	https://www.flowjo.com
Other		
Clean bench	NA	NA
Cell culture incubator	NA	NA
Falcon 5 mL Round Bottom Polystyrene Test Tube	Corning	Cat# 352058
Falcon 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap	Corning	Cat# 352235
CELLSTAR Clear F-Bottom 96-well suspension culture plates	Greiner Bio-One	Cat# 655185
BD FACSAria II/ FACSDiva, v8.0.1	BD Biosciences	
BD FACS Clean Solution	BD Biosciences	Cat# 340345
BD FACS Rinse Solution	BD Biosciences	Cat# 340346
Lab-Tek Chamber Slide (8 wells)	Thermo Fisher Scientific	Cat# 62407-315

MATERIALS AND EQUIPMENT

Ampicillin		
Reagent	Final concentration	Amount
Ampicillin (powder)	100 mg/mL	1 g
MilliQ water	n/a	10 mL
Total	**100 mg/mL**	**10 mL**

The stock solution of 100 mg/mL ampicillin should be filtered to sterilize. The stock solution can be stored in convenient aliquots for at least two years at −20°C.

DMEM/F-12/N-2/B-27/Amp		
Reagent	Final concentration	Amount
N-2 Supplement (100×)	1×	400 µL
B-27 Supplement (50×)	1×	800 µL
Ampicillin (100 mg/mL)	100 µg/mL	40 µL
DMEM/F12, HEPES	n/a	38.8 mL
Total	**n/a**	**40 mL**

100× N-2 and 50× B-27 supplements are commercially available. DMEM/F-12/N-2/B-27/Amp can be stored at least for 2 weeks at 4°C.

Reagents		
Reagent	Stock concentration	Final concentration
EGF	40 µg/mL, in 0.1% BSA/PBS(-)	20 ng/mL (1:2000)
FGF2	20 µg/mL, in 0.1% BSA/PBS(-)	10 ng/mL (1:2000)
IGF1	100 µg/mL, in 0.1% BSA/PBS(-)	50 ng/mL (1:2000)
CHIR99021	3 mM, in DMSO	3 µM (1:1000)
Valproic acid	1 M, in PBS	500 µM (1:2000)
2-phospho-L-ascorbic acid	100 mg/mL, in PBS	100 µg/mL (1:1000)
2-(3-(6-methylpyridin- 2-yl)-1H-pyrazol-4-yl)-1,5-naphthyridine	2 mM, in DMSO	2 µM (1:1000)

Optimized based on (McLean et al., 2017).
*BSA: Bovine serum albumin.
*PBS(-): phosphate-buffered saline without calcium and magnesium.

Trypsin inhibitor/DNaseI			
Reagent	Stock concentration	Final concentration	Amount
Soybean trypsin inhibitor	40 mg/mL, in DMEM/F12, HEPES	20 mg/mL	25 µL
DNaseI	4 mg/mL, in DMEM/F12, HEPES	2 mg/mL	25 µL
Total	n/a	n/a	50 µL

Stock solutions of soybean trypsin inhibitor and DNaseI are mixed 1:1, filtered to sterilize and stored in 50µL aliquots at -80°C.

STEP-BY-STEP METHOD DETAILS

Timing: 2 h

Cell sorting into 96-well plates

Cochlear duct cells from Fgfr3-tdTomato/Sox2-GFP mice are sorted into cell subgroups based on the fluorescence intensities of GFP and tdTomato (Waldhaus et al., 2015, Kubota et al., 2021). This cell sorting protocol is optimized for subsequent cell-type-specific organoid culture, and the gating steps are outlined. A 100 µm nozzle is used with a BD FACSAria II flow cytometer. The

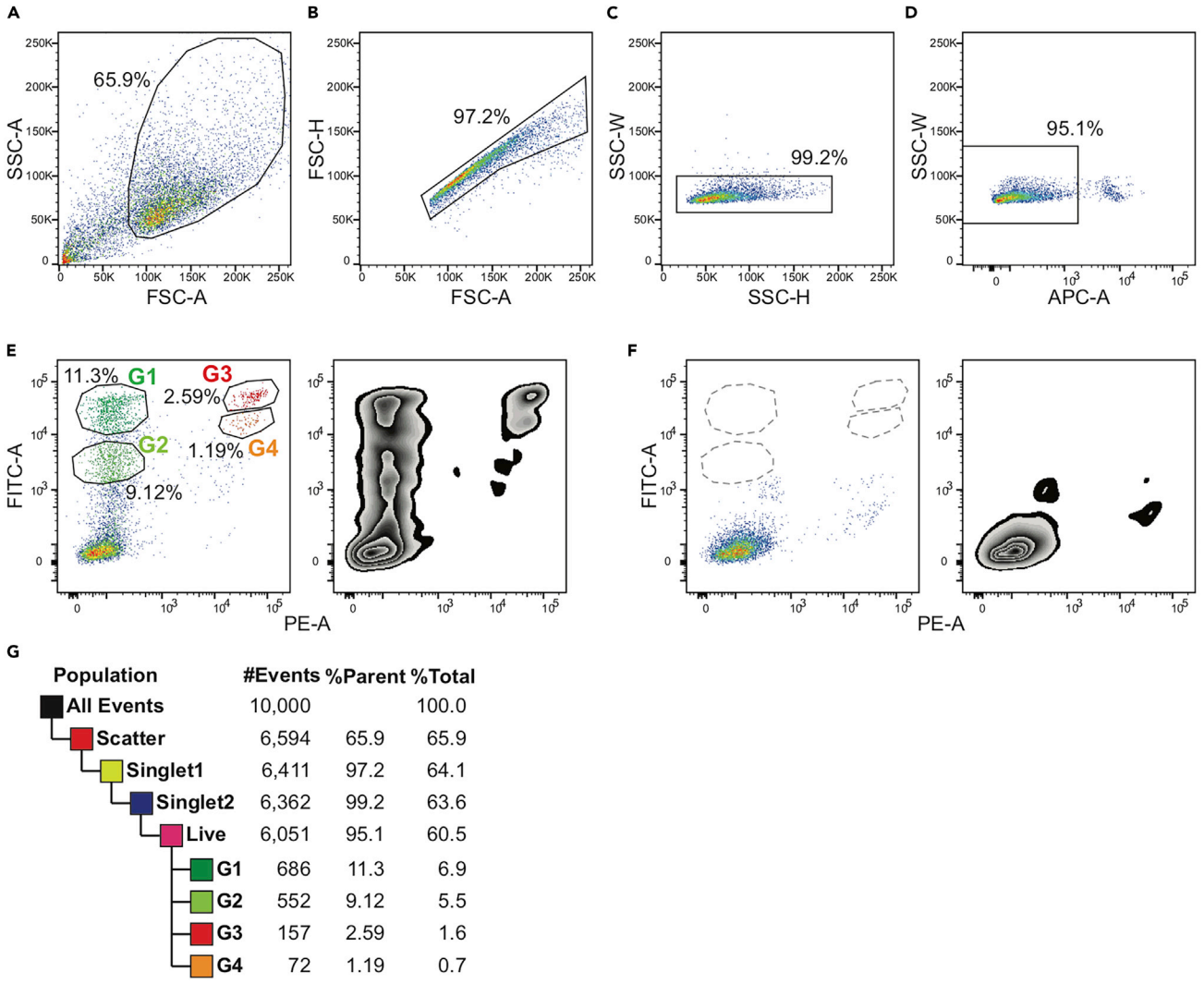

Figure 2. Flow cytometry analysis and gating

(A) Scatter gate for debris exclusion.

(B) Singlet gate 1.

(C) Singlet gate 2.

(D) Dead cell exclusion based on SYTOX Red fluorescence intensity.

(E) Fgfr3-tdTomato/Sox2-GFP sample data. Gates are drawn for sorting pillar/Deiters' cells (G3 and G4) and GER cells (G1 and G2). Dot plot (left) and zebra plot (right) are shown.

(F) Negative control data recorded on the same setting as in (E). Dot plot (left) and Zebra plot (right) are shown. G1–G4 are projected on the dot plot (dotted lines).

(G) Population hierarchy.

cochlear duct cells from Sox2-GFP-negative and non-recombinant Fgfr3-tdTomato (not tamoxifen injected) mice are used as a negative control.

⚠ CRITICAL: To ensure sterile sorting, the nozzle needs to be rinsed with 70% ethanol. Also, clean the sample path by running FACSClean (10% bleach), FACSRinse, and dH_2O prior to loading the samples.

1. Exclude debris by setting cell size (forward scatter) and granularity (side scatter) parameters so that small and low granular particles are excluded (Figure 2A).

2. Exclude multiplets.
   a. Forward-scatter area [FSC-A] *versus* forward-scatter height [FSC-H] (Figure 2B).
   b. Side-scatter height [SSC-H] *versus* side-scatter width [SSC-W] (Figure 2C).
3. Exclude dead cells. Cells labeled with SYTOX Red are detected with 640 nm red laser excitation and using the allophycocyanin (APC) filter (Figure 2D).

   Note: Start with the negative control tube in steps 1–3 to adjust parameter voltages and gates.

4. Use phycoerythrin (PE) and fluorescein isothiocyanate (FITC) emission filters for detecting tdTomato and GFP excited with the 561 nm yellow-green laser line and the 488 nm blue laser line, respectively (Figure 2E).
   a. Load negative sample control tube and acquire data until a few thousand events are represented on the FACS plot (Figure 2F).
   b. Load Fgfr3-tdTomato/Sox2-GFP sample tube and acquire the data. Optimize PE and FITC voltages for proper event separation.
   c. Load negative control tube and record the data to document specificity (Figure 2F).
   d. Load Fgfr3-tdTomato/Sox2-GFP sample tube and record the data (Figure 2E).
   e. Define sorting gates (Figure 2E).
      i Gate G1 isolates greater epithelial ridge (GER) cells (mainly lateral- intermediate GER cells).
      ii Gate G2 isolates medial GER cells.
      ii Gate G3 contains pillar cells and Deiters' cells.
      iv Gate G4 contains pillar cells and hair cells.

   Note: Gates G1–G4 need to be placed into areas where no cells are detected in the negative control dot plot (Figure 2F).

   Note: The specific cell groups obtained in G1–G4 have been validated (Kubota et al., 2021), but composition of the groups ultimately depends on the gating of each specific sort.

   Note: Depending on the flow cytometer's configuration and capabilities, you might encounter that excitation of tdTomato and GFP fluorescence can only be achieved with the 488 nm blue laser. In this case, color compensation must be performed. We use compensation beads because it is challenging to prepare single-colored cell-based compensation controls. Compensation by hand might lead to better results over the built-in compensation algorithm, which tends to overcompensate. Consultation with facility staff is recommended. A simple solution is to use an instrument that has specific laser lines for tdTomato and GFP.

5. Select appropriate configurations in Sort Layout; After running the test sort and adjusting the stream for the wells' centers, sorting into 96-well plates is selected in Menu item "Device". "Purity" is selected in Menu item "Precision".
6. Load the sample tube and sort cells.

   Note: At least 1,000 cells per well need to be targeted for efficient growth of the organoids from the cells in G1 and G2 (see problem 1). The cells in G3 and G4 are viable at a lower concentration of 500 cells per well and grow into small organoids consisting of a few dozen of the cells.

7. Cells can be validated with an inverted fluorescent microscope for expression of GFP- and/or tdTomato to ensure that they were accurately sorted.

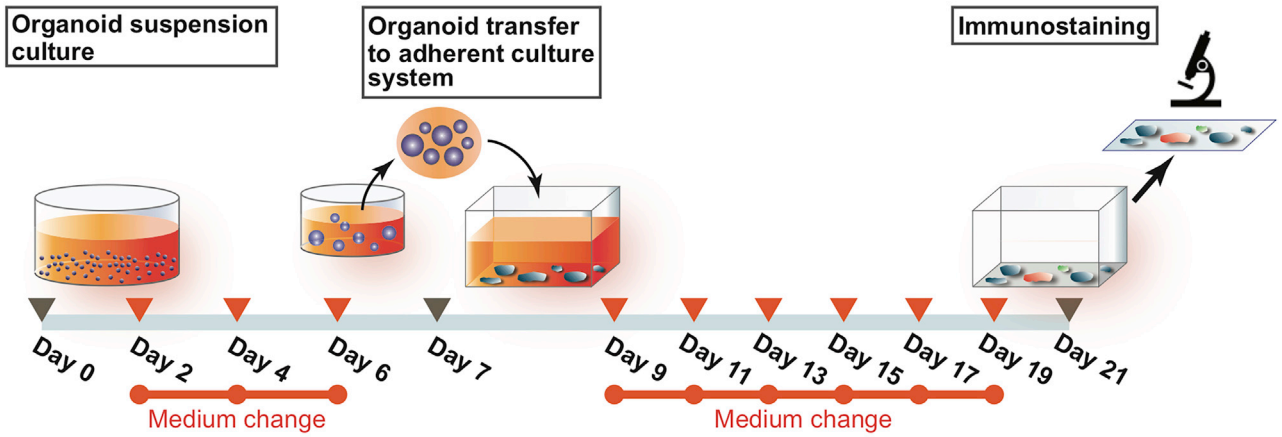

Figure 3. Timeline of cochlear duct cell sorting and culture
21 days of organoid and colony growth illustrated along the timeline.

Organoid suspension culture

Timing: 30 min

The sorted cells in a 96-well plate are incubated for 7 days at 37°C in a humidified atmosphere with 5% CO_2 to grow organoids. The medium is changed every other day as described in steps 8–11 (Figure 3).

8. Prepare maintenance medium with freshly added growth factors, small molecules, and recombinant proteins to DMEM/F-12/N-2/B-27/Amp medium. Prewarm the medium to 37°C in the cell culture incubator for 20 min.
9. Remove 80 μL of the old medium per well.

Note: The organoids tend to loosely attach to the dish's bottom. Carefully aspirate the media from the surface.

10. Add 100 μL of the fresh medium per well.
11. Place the dish back into the incubator.

Organoid transfer to adherent culture system

Timing: 2 h

At culture day 7, the organoids are transferred to an 8-well Lab-Tek glass chamber slide pre-coated with Matrigel (Figure 3). The organoids will attach and spread out on the chamber bottom during the following 14 days and grow into substantially sized colonies.

12. Prepare maintenance medium with freshly added growth factors, small molecules, and recombinant proteins to DMEM/F-12/N-2/B-27/Amp medium. Prewarm the medium to 37°C in the cell culture incubator for 20 min.
13. Coat the chamber bottom with Matrigel.
    a. Thaw the Matrigel on ice.
    b. Dilute Matrigel ten times with ice-cold DMEM on ice using pre-cooled pipette tips.
    c. Fill the chamber wells with the diluted and cold Matrigel until it covers the chamber bottom.
    d. Incubate the chamber at 18°C–24°C for 1 h.

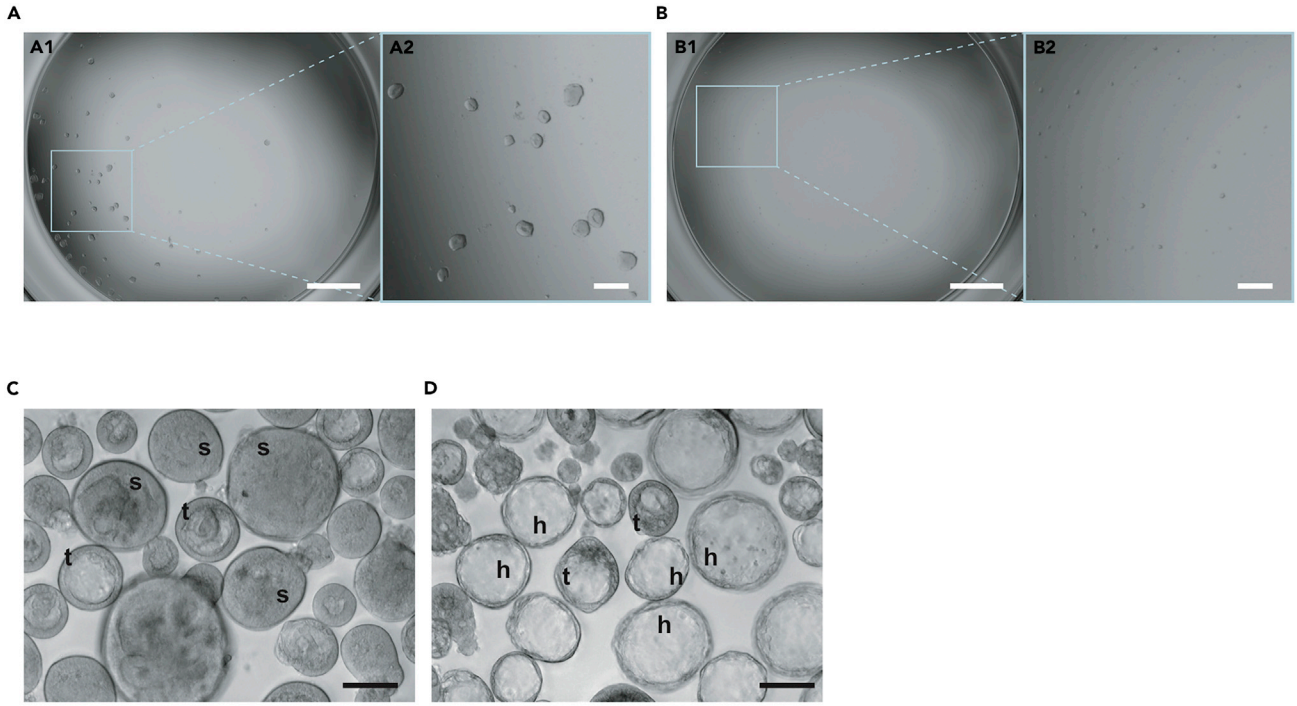

Figure 4. Morphology of organoids after 7 days suspension culture

(A) Whole well image of organoids grown from GER cells in a 96-well dish. The organoids are generally large and of solid morphology. Scale bars: 1 mm (a1) and 200 μm (a2).

(B) Whole well image of organoids grown from pillar cells and Deiters' cells. The organoids are generally smaller than GER-derived organoids. Scale bars: 1 mm (b1) and 200 μm (b2).

(C) Example of an organoid culture with many solid type organoids. s = solid, t = transitional. Scale bar: 100 μm.

(D) Example of an organoid culture with many hollow type organoids. h = hollow, t = transitional. Scale bar: 100 μm.

e. Aspirate Matrigel and rinse the chamber with 400 μL DMEM/F12.

f. Add 400 μL fresh maintenance medium per well.

⚠ CRITICAL: Steps e and f need to be performed with care so that the pipette tips do not scratch the chamber bottom.

14. Remove 100 μL medium from a single well of the 96-well plate with the 7-days-old organoids (step 11). Transfer the remaining 100 μL of medium with all organoids from a single well into a single chamber well (step 13f). A regular 200 μL pipette tip works well for this transfer procedure. The final volume in the chamber well will be 500 μL.

Note: Solid-type organoids, densely packed with cells inside, have features of otic progenitors, whereas hollow-type organoids do not (Kubota et al., 2021, Diensthuber et al., 2009). Most organoids that originate from the FACS purification described in this protocol are of the solid type at culture day seven (Figure 4). However, when the culture is initiated with a less defined population of cochlear cells (e.g., organoids grown directly from the dissociated cochlear duct cells without sorting), the grown organoids will have different morphologies (Figures 4C and 4D). In such cases, solid-type organoids can be picked with a pipette using an inverted microscope and selectively transferred to the chamber wells in order to grow colonies that efficiently give rise to otic epithelial cells. In case that the organoids are grown from undefined cochlear duct cells seeded at 0.5×10^5 cells in 1 mL medium per well of a 12-well suspension culture dish, transfer ten solid-typed organoids into a chamber well. With this

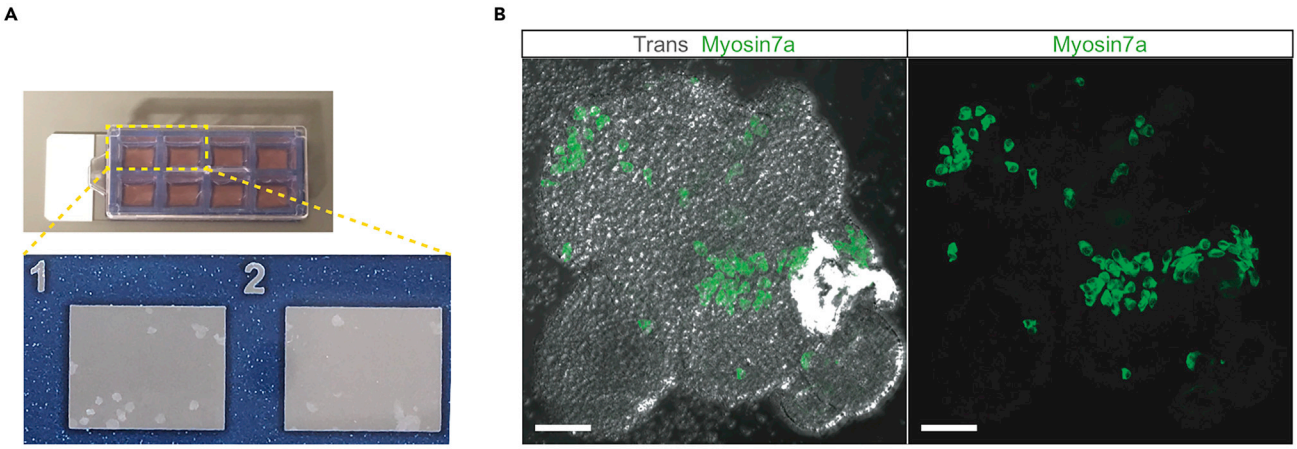

Figure 5. GER-cell derived colonies

(A) Colonies grown after 14 days in a chamber slide (upper). G1- and G2- derived colonies are detectable with the naked eye (Lower; 1: G1-derived colonies, 2: G2-derived colonies).

(B) An example of confocal microscopic images obtained from a G1-derived colony. Many cells can be immunolabeled with antibodies to the sensory hair cell marker Myosin7a. G2-derived colonies do not differ in their potential to generate hair cell marker-positive cells. Scale bars: 50 μm.

strategy, it is possible to successfully grow large colonies with hair cell-like cells (Kubota et al., 2021).

15. Incubate the chamber slides at 37°C in a humidified atmosphere with 5% CO_2.

Colony adherent culture

Timing: 30 min

The chamber slide is incubated for 14 days at 37°C in a humidified atmosphere with 5% CO_2. The medium is changed every other day as described in steps 16–19 (Figure 3). Colonies will grow during this time period.

16. Prepare new maintenance medium with freshly added growth factors, small molecules, and recombinant proteins to DMEM/F-12/N-2/B-27/Amp medium. Prewarm the medium to 37°C in the cell culture incubator for 20 min.
17. Aspirate ≈90% of the old medium from the wells.
18. Add 500 μL of fresh medium to each well.
19. Place the chamber slide back into the incubator.

EXPECTED OUTCOMES

By culture day 21, colonies will have grown on the chamber bottoms. G1- and G2-derived colonies are large and detectable with the naked eye (Figure 5A). G3-and G4-derived colonies are small, consisting of a few to several dozen cells. Additional details about organoid shapes and features of colonies grown from G1–G4 are described in Kubota et al. (2021). After washing with PBS and fixing with 4% paraformaldehyde (in PBS), the colonies are ready for immunostaining directly inside the chamber slides for imaging (Figure 5B). It is important to handle buffer exchange steps carefully because the fixed colonies do easily detach during the immunostaining procedures. We noticed that a blocking buffer containing 1% BSA improves the colonies' attachment. A benefit of growing organoids after day 7 as flattened colonies is the ease of imaging. Moreover, the continued growth of the colonies results in a relatively large population of cells with inner ear phenotypes for different kinds of downstream analyses.

It is possible to omit cell sorting and to grow organoids directly from dissociated cochlear duct cells. This will result in a mixed population of organoids because they are derived from a mixed population of source cells. We found that FACS sorting of GER cells generates a consistent organoid population that is likely to respond more homogeneously to subsequent experimental manipulations when compared to organoids generated from whole cochlear ducts.

LIMITATIONS

With this protocol, the sorted cells from each gate reproducibly generate organoids. However, it is difficult to generate more than 10–15 individual wells from one mouse litter due to the paucity of the cochlear duct cells. Upscaling the culture volumes from FACS-sorted cell-derived organoids therefore remains a challenge.

TROUBLESHOOTING

Problem 1

Cells in G1 and G2 tend to die when cultured at low concentration (< 500 cells/ well).

Potential solution

It is recommended to aim for 1,500–2,000 cells per well in step 6, so that the cells grow efficiently into organoids.

Problem 2

In step 15, organoids do not attach, and colonies fail to form.

Potential solution

Check the expiration date of the Matrigel stock. This can happen when old/expired Matrigel is used.

Problem 3

In step 18, colonies detach from the chamber bottom during medium change.

Potential solution

Detachment can be reduced by not completely replacing all of the old medium. The use of a vacuum aspiration system should be avoided, and pipetting should be conducted manually using a 1000 μL pipette.

Problem 4

After the final step, colonies fail to differentiate into mature inner ear cell types.

Potential solution

Make sure that FACS purified GER cells and organoids with solid morphology are used.

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

Cell compositions in FACS gates G1–G4 (step 4e) have been validated with single-cell RNA-seq; data are available at Gene Expression Omnibus (GEO: GSE162308) and the gene Expression Analysis Resource (gEAR) data depository (https://umgear.org/p?l=afd2eb77).

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AUTHOR CONTRIBUTIONS

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

DECLARATION OF INTERESTS

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

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