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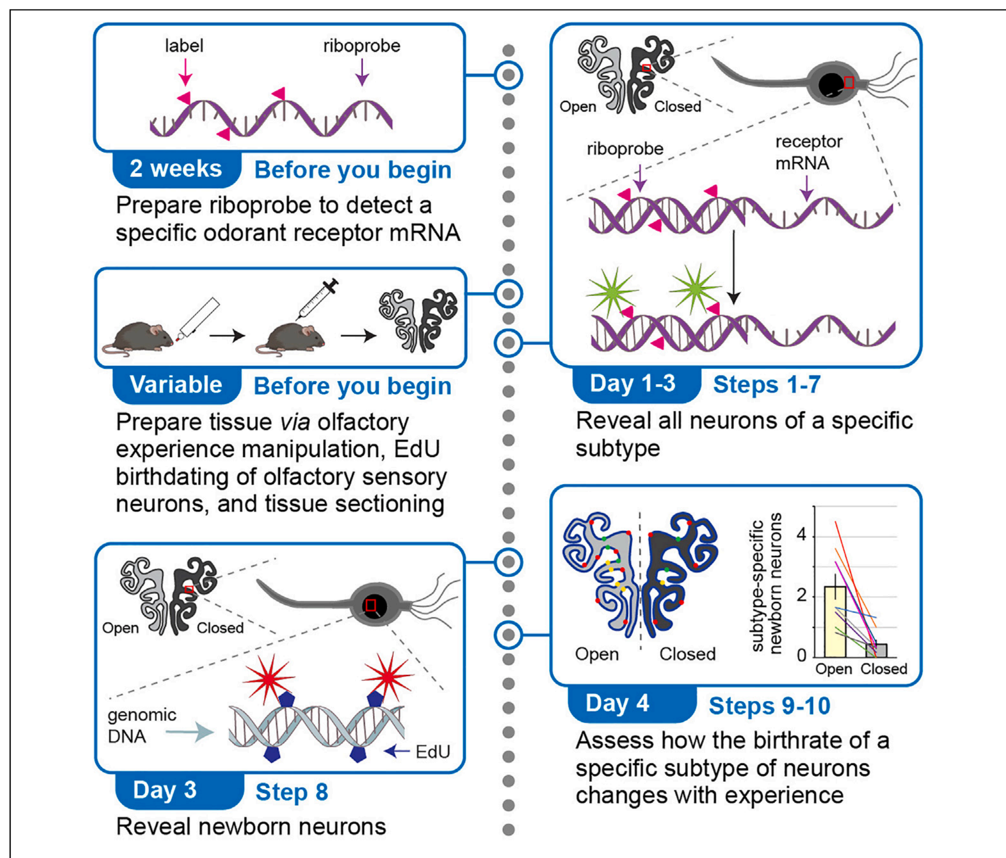
Protocol

A histological protocol for quantifying the birthrates of specific subtypes of olfactory sensory neurons in mice



Mammals typically have hundreds of distinct olfactory sensory neuron subtypes, each defined by expression of a specific odorant receptor gene, which undergo neurogenesis throughout life at rates that can depend on olfactory experience. Here, we present a protocol to quantify the birthrates of specific neuron subtypes *via* the simultaneous detection of corresponding receptor mRNAs and 5-ethynyl-2′-deoxyuridine. For preparation prior to beginning the protocol, we detail procedures for generating odorant receptor-specific riboprobes and experimental mouse olfactory epithelial tissue sections.

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

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Highlights

Riboprobes are designed to identify specific olfactory sensory neuron subtypes

New olfactory sensory neurons are birthdated using EdU

Subtype-specific birthrates are quantified via combined RNA-FISH and EdU staining

Experience-driven changes in the birthrates of specific neuron subtypes are assessed

Hossain et al., STAR Protocols 4, 102432
September 15, 2023 © 2023 The Author(s).
https://doi.org/10.1016/j.xpro.2023.102432

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A histological protocol for quantifying the birthrates of specific subtypes of olfactory sensory neurons in mice

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https://doi.org/10.1016/j.xpro.2023.102432

SUMMARY

Mammals typically have hundreds of distinct olfactory sensory neuron subtypes, each defined by expression of a specific odorant receptor gene, which undergo neurogenesis throughout life at rates that can depend on olfactory experience. Here, we present a protocol to quantify the birthrates of specific neuron subtypes *via* the simultaneous detection of corresponding receptor mRNAs and 5-ethynyl-2′-deoxyuridine. For preparation prior to beginning the protocol, we detail procedures for generating odorant receptor-specific riboprobes and experimental mouse olfactory epithelial tissue sections.
For complete details on the use and execution of this protocol, please refer to van der Linden et al. (2020).[1]

BEFORE YOU BEGIN

The following protocol describes a procedure for quantifying, within the olfactory epithelia (OE) of mice, the neurogenesis rates of specific olfactory sensory neuron (OSN) subtypes, each of which is defined by the expression of a single one of the > 1000 odorant receptor (OR) genes encoded in the mouse genome.[2–5] Here we outline how this protocol may be used to quantify changes in the birth-rates of specific OSN subtypes as a result of olfactory deprivation, exposure to discrete odorants, sex, or age, and how it may be used to quantify the subtype-specific survival of OSNs following neurogenesis.

Institutional permissions

All procedures involving mice were carried out in accordance with NIH standards and approved by the University of Colorado, Anschutz Medical Campus Institutional Animal Care and Use Committee (IACUC). Users of this protocol should note that approval for conducting experiments on mice must be acquired in advance from the relevant institutions.

Generation of riboprobes for specific odorant receptor mRNAs

Timing: 1–2 weeks

The following procedure can be used to design and generate antisense riboprobes for detecting mRNAs encoding specific ORs. The procedure involves the design of OR-gene specific PCR primers, extraction of RNA from the mouse OE, reverse transcription-PCR amplification of a fragment of cDNA corresponding to an OR gene of interest, molecular cloning of the PCR amplicon into a plasmid vector, sequencing and linearization of the plasmid to generate a template, and

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in vitro transcription of the template to generate a labeled antisense riboprobe. As an example, we outline how to design and generate riboprobes that detect the *Or9k7* (*Olfr827*) mRNA, but similar steps may be used to design riboprobes that detect transcripts corresponding to almost any OR or non-OR gene. Moreover, the protocol for quantifying the birthrates of specific OSN subtypes could potentially be adapted for use with alternative types of fluorescence *in situ* hybridization (FISH) probes that can be used with tyramide signal amplification (TSA) (e.g., RNAscope),[6] in which a fluorophore is covalently linked to biomolecules in the vicinity of mRNA targets. FISH detection methods that require the preservation of nucleic acids (e.g., hybridization chain reaction) may not be compatible with EdU labeling, which has been found to result in the degradation of nucleic acids.[7]

1. Design primer pairs to amplify riboprobe template sequences.
   a. Go to the Ensembl genome browser (https://ensembl.org/index.html) and select "Mouse" in the "All genomes" menu on the left side of the browser.
   b. Type "Or9k7" in the "Search Mouse" search bar and select "Go".
   c. On the next page, select "Or9k7 (Mouse Gene, Strain: reference (CL57bl6))" from the options list.

   Note: To find the *Or9k7*/*Olfr827* gene for mouse strains other than C57bl6, enter "Olfr827" in the "Search Mouse" bar and click "Go". You will be directed to a page where you can select the Olfr827 gene for the desired mouse strain from the options list.

   d. On the next page, select the "Show transcript table" option. In the "transcript ID" column, select the longest transcript that codes for a protein.
   e. On the following page, select "cDNA" in the "Transcript-based displays" column at the left side of the browser; select "Download sequence".
   f. In the new window under "Settings", deselect all "Included sequences" except "cDNA (transcripts)"; select "Download".

   Optional: We recommend importing the sequence into a DNA editing software application such as SnapGene Viewer (https://www.snapgene.com/snapgene-viewer) or Benchling (https://www.benchling.com/), free applications that enable the analysis and annotation of DNA sequences. Alternatively, the downloaded sequence can be opened using a standard text editing software application (e.g., Microsoft Word or Notepad).

   g. Analyze the target mRNA to identify regions that are not highly similar (<70% nucleotide identity) to transcripts corresponding to other OR genes using NCBI Nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov/).

   Note: Many mouse ORs show high sequence homology at the mRNA level, particularly within the CDS region. In such cases, targeting the 3′- or 5′-untranslated regions of mRNAs may result in more specific probes, as these regions tend to exhibit less sequence homology between ORs. Although it is possible to target intronic regions of a nascent OR mRNA, we have found that this approach typically yields small punctate signals within the nucleus that can be challenging to visualize. In our experience, targeting mRNA regions with <70% identity to off-target transcripts yields probes that are usually highly selective for the target. However, we advise testing probes prior to final use. The observation of homogeneous staining intensities across labeled OSNs indicates that a probe is selective for a single OR mRNA target, while extensive heterogeneity across OSNs may indicate inadequate probe selectivity. Examples of DNA oligonucleotide primers for generating riboprobes that we have found to be highly selective are listed in the KRT and in our previous publication.[1]

h. Using the Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), design primer pairs to generate amplicons of a desired length (typically 500–1000 bp, but see note below; defined using the "PCR product size" parameters) within desired regions of the transcript (defined using the "Range" parameters), with the organism set to "Mus musculus (taxid:10090)" and other parameters kept default.

Note: We have found that probes between 500 and 1000 bp in length yield robust signals. For targets that are highly similar to other mRNAs (>70% identity), we recommend designing probes between 500 and 600 bp in length in order to minimize the detection of off-target mRNAs.

i. Order primers from an oligonucleotide manufacturer such as Integrated DNA Technologies (https://www.idtdna.com/).

Note: We have found that the minimum synthesis scale of 25 nmole with standard desalting purification yields primers that work efficiently and are sufficient for hundreds of PCR reactions.

Optional: To increase the likelihood of generating a riboprobe that robustly and specifically detects a single OR mRNA, we recommend designing two or three riboprobes that target non-overlapping regions within the target.

2. Extract total RNA from a mouse OE (see "preparation of mouse OE tissue") using the Trizol Reagent according to the manufacturer's protocol. For troubleshooting, see problem 1.
3. Purify a portion of the extracted RNA using the RNeasy MinElute Cleanup Kit according to the manufacturer's protocol, taking care not to exceed the capacity of the column (45 μg), and store the purified RNA at −80°C.

Pause point: Purified RNA can be stored at −80°C for several years.

4. Using the purified RNA as template, generate cDNA using the QuantiTect Reverse Transcription Kit according to the manufacturer's protocol.

Pause point: cDNA samples can be stored at −20°C for several years.

5. Using the primer pairs designed in step 1 and cDNA (0.25 μL/ reaction) prepared in step 4, amplify the riboprobe template sequences *via* PCR using the Expand™ High Fidelity PCR System according to the manufacturer's protocol.

Note: If primers were designed to target intronic regions, mouse genomic DNA (e.g., from a tail snip) should be used as input. Genomic DNA may also be used as input to amplify sequences contained within single exons.

6. Analyze PCR amplicons (1–3 μL) alongside a DNA ladder *via* electrophoresis on a 1% agarose gel to verify that the sizes of the amplicons are correct.
7. Purify PCR amplicons using the MinElute Reaction Cleanup Kit according to the manufacturer's protocol.
8. Analyze the purified amplicons (0.5 μL) alongside a DNA ladder *via* electrophoresis on a 1% agarose gel to verify that the purification was successful.

Pause point: PCR amplicons can be stored at −20°C for several years.

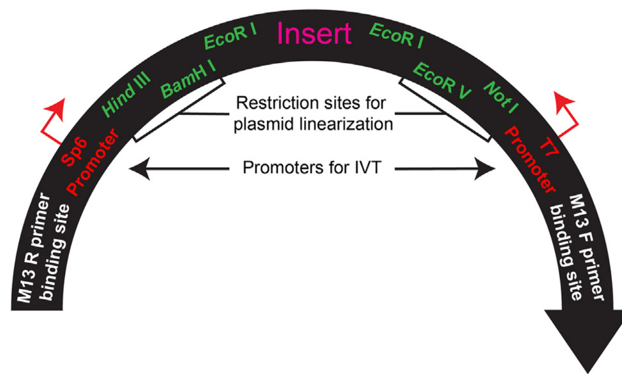

Figure 1. Schematic of the cloning region of the pCR™II-TOPO® vector
Promoters and restriction sites are shown in red and green, respectively. If the insert is in the forward orientation relative to the M13 Reverse (M13 R) primer binding site, HindIII or BamHI may be used to linearize the plasmid and the T7 promoter should be used for IVT. If the insert is in the reverse orientation, NotI or EcoRV may be used to linearize the plasmid and the Sp6 promoter should be used for IVT. The EcoRI sites may be used to excise the insert in order to confirm its presence and size. Additional details about the vector and cloning region may be found in the TOPO ® TA Cloning® Kit user guide.

9. Using the TOPO™ TA Cloning™ Kit, Dual Promoter, with One Shot™ TOP10 chemically competent *E. coli* cells, clone the purified PCR amplicons into the pCRII-TOPO vector and transform chemically competent *E. coli* cells according to the manufacturer's protocol. For troubleshooting, see problem 2.
10. Isolate plasmid DNA from at least three clones per target riboprobe using the QIAprep Spin Miniprep Kit according to the manufacturer's protocol, with an elution volume of 100 μL.
11. Quantify plasmid DNA concentrations using a spectrophotometer (e.g., NanoDrop).

Optional: We recommend confirming the presence and correct sizes of inserts *via* EcoRI restriction digestion of the plasmid DNA (500 ng) (Figure 1), followed by analysis of the digested products alongside a DNA ladder *via* electrophoresis on a 1% agarose gel.

12. Submit plasmids for Sanger sequencing using the M13R primer.

Note: We have found that sequencing from one end of the insert is usually sufficient to read the entire insert. For longer inserts, sequencing from the other end of the insert (using, e.g., the M13F primer) may be necessary to read the whole sequence. Alternatively, whole plasmid sequencing may be used.

13. Using the "Align two or more sequences" function within the NCBI Nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov/), with each clone sequence as the "Query Sequence" and the target mRNA (obtained in step 1f) as the "Subject Sequence", analyze each clone to confirm the presence of the desired insert and its orientation relative to the M13R primer binding site within the vector.

Note: If the insert sequence is in the same (sense) orientation as the target mRNA, it is in the forward orientation relative to the M13R primer binding site. If the sequence is in the orientation opposite (antisense) that of the target mRNA, it is in the reverse orientation relative to M13R.

⚠ CRITICAL: Identifying the orientation of the insert within the TOPO™ vector is critical for generating a riboprobe that is antisense to the target mRNA, as it will determine which restriction sites can be used to properly linearize the plasmid DNA (step 14) and which RNA polymerase should be used to generate the riboprobe (step 15) (Figure 1).

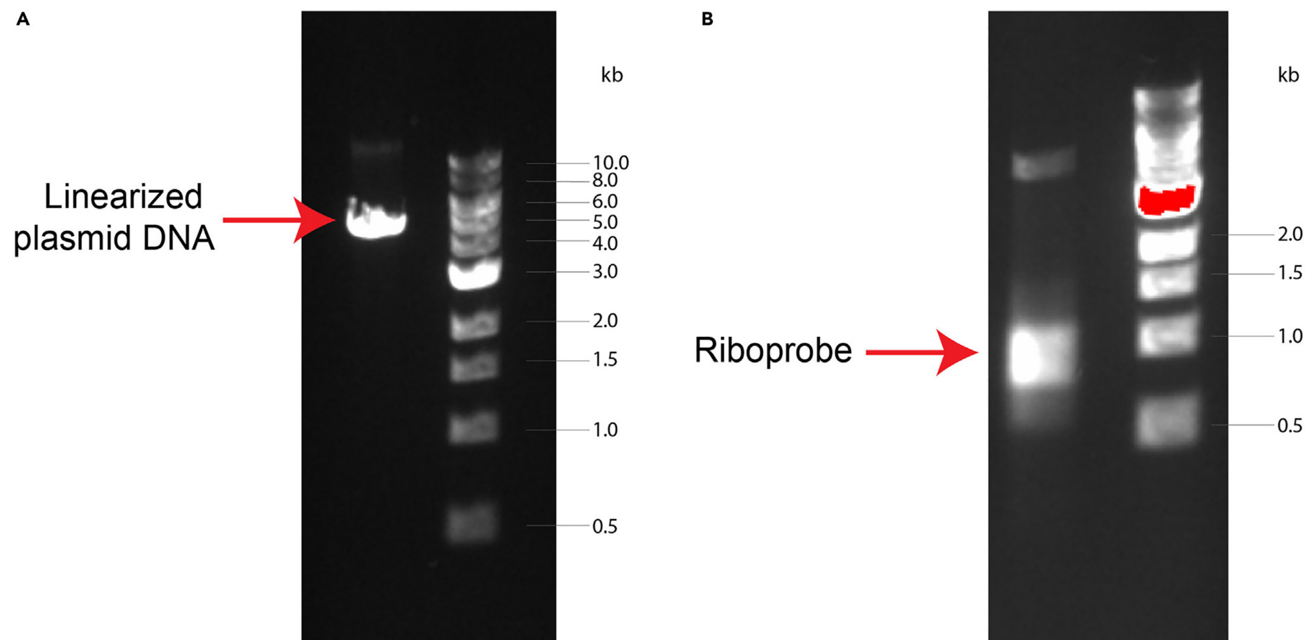

Figure 2. Agarose gel images of a linearized plasmid DNA and riboprobe
(A) The linearized plasmid DNA should appear as a narrow band at ~ 4.5 kb.
(B) The riboprobe should appear as a broad band at ~ 1 kb.

Pause point: Plasmid DNA can be stored at −20°C for several years.

14. Prepare DNA templates for generating riboprobes.
    a. For each target riboprobe, linearize 5 μg of plasmid DNA from a single clone using 100 units of an appropriate restriction enzyme (Figure 1).

Note: We recommend using BamHI or HindIII to linearize plasmids with inserts in the forward orientation and NotI to linearize those with inserts in the in the reverse orientation (Figure 1). For more details on restriction enzymes that can be used, refer to the TOPO ® TA Cloning® Kit user guide. To ensure complete linearization of plasmid DNA, reaction conditions are designed to over-digest the plasmid 20-fold. However, the presence of residual circular plasmid DNA is not expected to adversely affect downstream processes.

⚠ CRITICAL: Care should be taken to ensure that the selected restriction enzyme does not cut within the insert. This can be done by searching for potential restriction sites within the insert sequence using a DNA analysis application such as SnapGene Viewer or Benchling.

    b. Purify linearized plasmid DNA using the MinElute Reaction Cleanup Kit according to the manufacturer's protocol, with elution of purified DNA using 2 × 20-μL volumes of EB buffer.
    c. Analyze the purified DNA (0.5 μL) alongside a DNA ladder *via* electrophoresis on a 1% agarose gel to verify that linearization and purification were successful and to estimate the concentration of purified template by comparison to a 1 kb ladder, which has rungs of known concentrations (Figure 2A).

Note: In our experience, purification of linearized template DNA *via* the MinElute Reaction Cleanup Kit is highly efficient, resulting in a concentration that is typically 100–125 ng/μL.

Optional: The concentration of purified template may be measured using a spectrophotometer (e.g., NanoDrop).

⏸ **Pause point:** Linearized template DNA can be stored at −20°C for several years.

15. Generate DIG-labeled antisense riboprobes.
    a. For each riboprobe to be generated, combine the following reagents to make a 20-μL *in vitro* transcription (IVT) reaction mixture.

Reagent	Amount
Linearized DNA (0.9–1.1 μg)	10 μL
Transcription buffer (10×; included with RNA Polymerase)	2 μL
DTT (0.1 M)	2 μL
DIG RNA Labeling Mix (10×)	2 μL
RNA polymerase enzyme (see note)	2 μL
RNAase-free water	2 μL
Total	**20 μL**

Note: T7 RNA polymerase should be used for inserts in the forward orientation relative to M13R; Sp6 RNA polymerase should be used for inserts in the reverse orientation (Figure 1).

    b. Mix components thoroughly by pipetting.
    c. Incubate at 37°C for 2 h.
    d. Add 2 μL RQ1 RNase-Free DNase.
    e. Mix components thoroughly by pipetting.
    f. Incubate at 37°C for 20 min.
    g. Purify the DIG-labeled riboprobe *via* ethanol precipitation by combining the following in a 1.5-mL microcentrifuge tube.

Reagent	Amount
RNAase-free water	400 μL
Ammonium acetate solution (7.5 M)	44 μL
IVT reaction mixture	22 μL
Glycogen (5 mg/mL)	1 μL
Ethanol (100%)	1 mL
Total	**1.467 mL**

    h. Mix components thoroughly by vortexing.
    i. Incubate at –20°C for at least 1 h.

⏸ **Pause point:** Incubation at –20°C may be extended indefinitely.

    j. Spin microcentrifuge tubes containing the precipitated riboprobes at 4 °C at 21,000 × *g* for 20 min in a fixed-angle microcentrifuge, with the hinge of the lid oriented outward.
    k. Immediately following centrifugation, place tubes on ice and carefully decant supernatant by pipetting, taking care not to disrupt pellet, which should be located near the bottom of the tube on the side closest to the hinge but may not be visible.
    l. Carefully wash the pellets and sides of tubes with 100 μL of cold 75% ethanol.

Note: Removal of as much liquid as possible is recommended to avoid extended drying times.

    m. With tube lids open, allow pellets to air-dry at 22°C–26°C until no liquid is visible (typically 15 min).
    n. Dissolve pellets containing riboprobes in 30 μL/ tube of RNAase-free water.
    o. Incubate tubes for 15 min at 22°C–26°C.
    p. Vortex tubes for 30 s.

q. Measure riboprobe concentrations using a spectrophotometer (e.g., NanoDrop).

Note: Stock riboprobe concentrations obtained are typically 1–2 μg/μL. For most probes, hybridization at a final concentration of 1.3–2.6 ng/μL (750-fold dilution) yields robust FISH signals.

r. Analyze 0.5 μL of each riboprobe alongside a DNA ladder *via* electrophoresis on a 1% agarose gel (Figure 2B).

Note: Riboprobes should be visible as bright narrow smears with apparent lengths of 0.8–1.2 kb relative to a double-stranded DNA ladder (Figure 2B).

s. Store riboprobes at –80°C.

Pause point: Riboprobes can be stored at –80°C for several years.

Preparation of mouse OE tissue

Timing: Variable

The following steps outline procedures for experience manipulation (if necessary), EdU-birthdating of newborn OSNs, dissection and freezing of OEs, and preparation of slides containing OE cryosections. EdU is a thymidine analog used to label cells that are undergoing division at the time of EdU exposure.[8–10] When analyzed ~7 days-post injection, EdU can be used to identify newborn OSNs by click-chemical conjugation of a fluorophore.[1]

16. If necessary for the experimental question being addressed, perform experiments to manipulate mouse olfactory experience.

Note: Olfactory experience may be manipulated by reducing airflow on one side of the olfactory epithelium *via* unilateral naris occlusion (UNO)[1,11–13] or by exposing mice to specific odorants or olfactory environments,[14–18] typically for 2–3 weeks (Figures 7A and 10A). UNO can be performed on young mice (P0–P14) by permanently closing one naris *via* electrocauterization.[1] Alternatively, UNO can be performed reversibly on older mice using nose plugs.[19,20] Appropriate controls (e.g., non-occluded or unexposed mice) should be included in the experimental design.

17. Birthdate newborn OSNs using EdU.
   a. Remove a mouse from its home cage and weigh it.
   b. Clean the mouse's abdominal fur and skin with 70% ethanol.
   c. Inject 50 mg/kg EdU intraperitoneally using a 31-gauge Insulin syringe. A 10-gram mouse, for example, would require 50 μL of 10 mg/mL EdU solution.
   d. Replace the mouse in its home cage.
   e. 3–4 h after the first EdU injection, repeat steps a–d to administer a second dose of EdU.
   f. For adult mice (> 8 weeks), repeat steps a–e for two additional days.

Note: Although toxicity has not been observed using the doses of EdU described in this protocol,[21–23] mice should be carefully monitored both during and after EdU administration for adverse effects, including hunched posture and weight loss greater than 15%. Animals exhibiting adverse effects should be euthanized in accordance with animal care and use standards. The timing and number of EdU injections depend on an animal's age, which is inversely correlated with OSN birthrate. Thus, while we administer two doses of EdU to juveniles (e.g.,

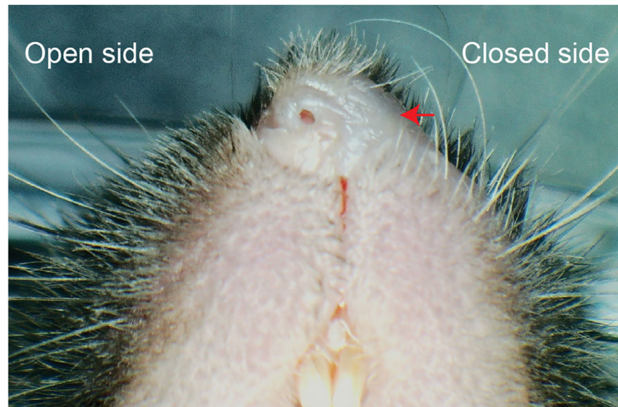

Figure 3. Image of the nose of a 5-week-old mouse that was UNO-treated *via* electrocautery at 2 weeks of age
Complete closure of the electrocauterized nostril (arrow) indicates that UNO treatment was successful.

P28; Figures 7A and 8A), we typically administer six doses to adults (e.g., P56; Figure 8B) to compensate for their lower OSN birthrate.

18. Prepare cryomolds, one for each OE to be dissected, by marking with sample information, including the dorso-ventral orientation in which the tissue will be placed within the cryomold.
19. Prepare a freezing apparatus.
    a. Add 0.8–1.2 L of liquid nitrogen to a 1900-mL glass Dewar flask.
    b. Fill a 500-mL glass beaker with 100 mL of isopentane.
    c. Place the beaker into the Dewar flask to submerge the bottom portion in liquid nitrogen.

    Note: The precise sizes of the flask and beaker are not critical, but the beaker should be able to fit inside the flask and accommodate the cryomold. The volume of isopentane needed depends on the size of the beaker and should be sufficient to submerge the cryomold to a depth of 1 cm.

    d. Fill an ice bucket half-way with dry ice pellets or powder and cover.
20. Euthanize experimental mice *via* isoflurane inhalation, followed by cervical dislocation or decapitation.

    Note: To quantify subtype-specific OSN birthrates, we typically euthanize mice 7 days post-EdU. However, the time point may be varied depending on the experimental question being addressed.

21. If applicable, assess and document the completeness of unilateral naris closure using a dissecting microscope.

    Note: If UNO was successful, the electrocauterized nostril should appear completely closed (Figure 3).

22. Dissect the olfactory epithelium (Figure 4).

    Note: Successful dissection of a mouse OE requires a basic level of dissection experience. We recommend practicing on non-precious samples prior to dissecting experimental ones. Equipment required for this step include a dissecting microscope, small surgical scissors, and fine-tipped forceps.

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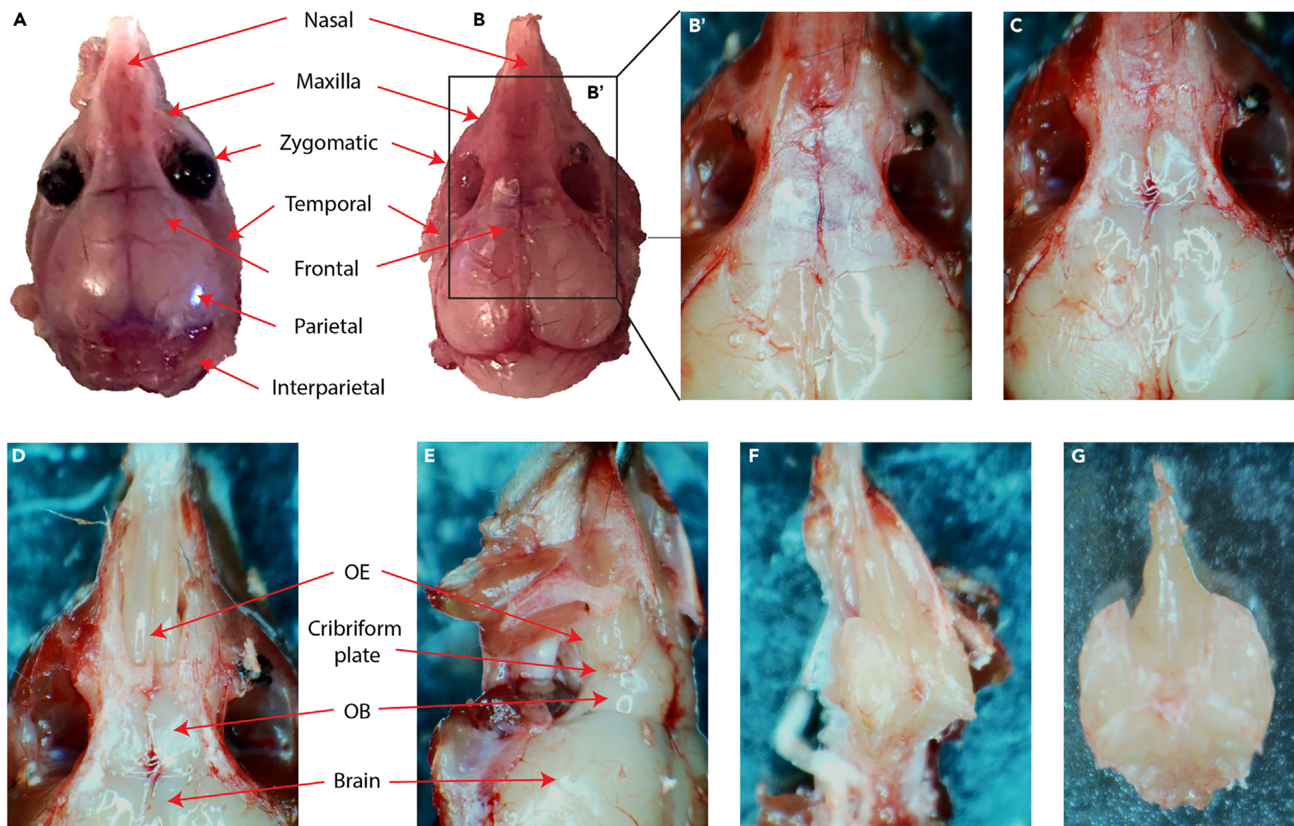

Figure 4. Dissection of an OE from a 5-week-old mouse

(A–F) Representative images of the head following removal of the skin and incisors (A), interparietal and parietal bones (B), frontal bone (C), nasal bone (D), temporal bone (E), and left zygomatic bone (F).

(G) Representative image of a dissected OE.

a. Using scissors, detach the head from the body by cutting between the maxilla (upper jaw) and mandible (lower jaw) and through the neck.
b. Remove the skin from the head and cut the incisors (front teeth) flush with the jawbone (Figure 4A).
c. Place the head, dorsal side up, in a new polystyrene petri dish under a dissecting microscope.
d. Remove eyes and associated connective tissue with scissors and forceps, respectively.
e. Using fine scissors, carefully cut the skull along the midline starting from the occipital bone at the base of the skull to the middle of the frontal bone.
f. Remove the interparietal and parietal bones from the two sides of the midline using forceps (Figures 4B and 4B′).
g. Gently remove the rest of the frontal bone, which covers the olfactory bulb on both sides of the midline by lifting with closed forceps (Figure 4C).
h. Remove the nasal bone that resides on the top of OE with forceps (Figure 4D).
i. Remove the temporal bone and gently lift the posterior portion of the brain from of the base of the skull and turn the head 90° along the anterior-posterior axis to allow the posterior brain to detach and place tension on the nerve fibers connecting the OE to the OB (Figure 4E).
j. Carefully detach the fibers between the OE and olfactory bulb (OB) at the cribriform plate with closed forceps. Remove the OB and brain.
k. Loosen the connections between the OE and zygomatic bones and pull out only the left zygomatic bone (Figure 4F).

l. Detach the maxilla bone from the anterior side of the OE, hold the right zygomatic bone with one pair forceps, and remove the OE with another pair of forceps by pushing from the left.
m. Remove any connective tissue adhered to the OE using forceps (Figure 4G).

Note: Special care should be taken when dissecting OEs from younger (< 2 weeks of age) or older mice (> 6 weeks of age), as the hardness of the bones inside and surrounding the OE varies with age.

23. Freeze the dissected OE.
    a. Fill the labeled cryomold (from step 18) with optimal cutting temperature (OCT) compound to a depth of 1 cm.
    b. Holding the OE by the anterior end with forceps, submerge it, posterior end-down, into the OCT with a dorso-ventral orientation corresponding to labels on the cryomold.
    c. Adjust the OE position with forceps so that its posterior end is flush with the bottom of the cryomold and the anterior end points up.

Note: The presence of air within the OE can cause it to float out of position within the liquid OCT. This can be avoided by freezing the block quickly after the OE has been properly positioned.

    d. When the isopentane within the freezing apparatus (prepared in steps 19b, c) has begun to freeze, remove the beaker from the liquid nitrogen and place it on dry ice.
    e. Partially submerge the cryomold into the cold isopentane and allow it to completely freeze (5–7 min).

⚠ CRITICAL: Avoid allowing isopentane to flow into the cryomold and mix with OCT, as this can result in cracking of the tissue block and accumulation of isopentane at the bottom of the cryomold. Rapid and complete freezing of the tissue block is critical for obtaining tissue sections with good morphology. Partial or prolonged freezing may result in the formation of ice crystals in the tissue, which can cause tearing and adversely affect the morphology of the resulting cryosections.

    f. Place the frozen tissue block into a zip-locked plastic bag.
    g. Store frozen tissue blocks at –80°C until ready for cryosectioning.

Pause point: Tissue blocks can be stored in tightly-sealed plastic bags at –80°C for several years.

24. Cryosection the frozen OE.
    a. For each OE to be sectioned, use a mechanical pencil to label 20–25 slides with sample information.
    b. Check that the chamber temperature of the cryostat is set to –20°C and the section thickness is set to 12 μm.
    c. Place an anti-roll plate into and a new sectioning blade in their respective holders at least 15 min prior to sectioning.
    d. Place a new razor blade and a specimen chuck within the cryostat chamber at least 15 min prior to sectioning.

Note: An OE section thickness of 12 μm has been found to work well in this protocol, irrespective of animal age at the time of OE dissection.

⚠ CRITICAL: The cryosectioning temperature is critical for obtaining sections with good morphology. The optimal cutting temperature can vary among cryostats. We recommend

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testing the temperature using a non-precious tissue sample. For more information, see troubleshooting, problem 3, 4, and 5.

e. Remove the tissue block from the cryomold.
f. Dispense a thin layer of OCT onto the specimen chuck and then immediately place the tissue block on the liquid OCT layer, with the anterior end of the OE down.
g. If necessary, dispense additional OCT to secure the block to the specimen chuck.
h. Place the tissue-mounted specimen chuck on the freezing shelf for at least 30 min to allow its temperature to equilibrate with that of the cryostat chamber.
i. Place the tissue-mounted specimen chuck into the chuck holder.
j. Adjust the orientation of the tissue block so that the dorsal side of the OE is facing up and the posterior end is perpendicular to the blade.
k. If the posterior OE is not visible, slowly cut and discard sections until it can be seen.
l. If necessary, adjust the angle of the specimen chuck so that the posterior end of the OE is level and not tilted to the right, left, dorsal, or ventral sides relative to the microtome blade.
m. Using a cold razor blade, carefully trim excess OCT from the sides of the block to a 1-cm square encompassing the OE.
n. Sequentially collect OE sections on slides (prepared in step 24a) such that each slide contains a series of 8–10 sections spanning the posterior to anterior ends of the OE.

Note: Slides should be kept at 22°C–26°C while acquiring sections.

o. Immediately after cryosectioning, place the tissue-mounted slides in a secure slide box and store at –80°C until use.

Pause point: Tissue-mounted slides can be stored at –80°C for several years.

Preparation of equipment for combined RNA-FISH and EdU staining

Timing: 40–50 min

25. Pre-heat a hybridization oven and a heat block to 65°C and 95°C, respectively.
26. Pre-cool a microcentrifuge to 4°C.
27. Clean a slide submersion chamber with ultrapure (e.g., Milli-Q-purified) water before use and allow to air dry.
28. Prepare a humidified horizontal slide chamber (Figure 5).

Note: The horizontal slide chamber can be prepared by cutting two pairs of 2-mL serological pipettes to a length of 20 cm each (from the top) and gluing them using a cyanoacrylate adhesive (e.g., ''Super Glue'') to the bottom of a 24.5 cm × 24.5 cm × 2.5 cm bioassay dish such that each pair is spaced 3 cm apart and 3 cm from the chamber edge (Figure 5). Optionally, the lid can be wrapped with aluminum foil to protect the chamber from light. Commercial horizontal slide chambers can also be purchased (please see key resources table).

a. Clean the horizontal slide chamber with ultrapure water.
b. Cut absorbent paper (e.g., filter paper or paper towel) into strips (20 cm × 2 cm) and place them between the glued cut pipettes (Figure 5).
c. Add 20–30 mL of an appropriate buffer, as detailed in the protocol, to the absorbent paper.
d. Close the chamber until ready to load experimental slides.

29. Prepare a humidified hybridization chamber (Figure 6).

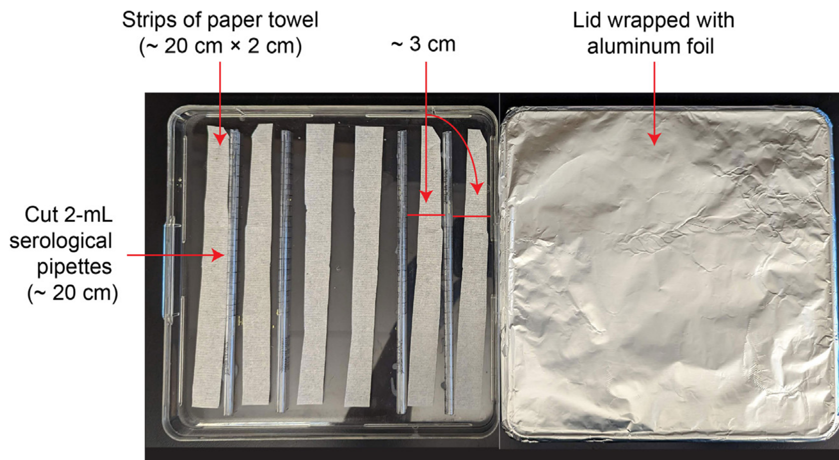

Figure 5. Horizontal slide chamber

To prepare the chamber, two pairs of 2-mL serological pipettes are cut to a length of 20 cm each from the top and are attached to the bottom of a 24.5 cm × 24.5 cm × 2.5 cm bioassay dish using a cyanoacrylate adhesive. The chamber's lid is covered with aluminum foil to shield it from light. Before loading slides, the chamber is humidified by placing strips of absorbent paper that are wetted with 20–30 mL of buffer. Processing one-at-a-time, slides are transferred from the slide submersion chamber to the humidified horizontal slide chamber and immediately covered with the appropriate solution. After all slides are processed, the chamber is closed.

a. Clean a hybridization chamber with ultrapure water and allow it to air-dry completely.
b. Place a clean, blank slide in position 1 and slip two tightly folded Kim Wipes between the slide and the bottom of the box (Figure 6).
c. Add 4 mL of 50% formamide in 5× SSC to the folded Kim Wipes.

Optional: If using multiple riboprobes in the same experiment, we recommend separating slides receiving different riboprobes by creating partitions within the chamber consisting of two adjacent blank slides sandwiching a folded Kim Wipe (Figure 6) or preparing multiple chambers.

d. Close the chamber until ready to load experimental slides.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-digoxigenin-POD, Fab fragments (1:1000)	Roche	Cat# 11207733910; RRID: AB_514500
Chemicals, peptides, and recombinant proteins		
Trizol	Invitrogen	REF# 15596026
1,4-Dithio-DL-threitol, 98% (DTT)	Thermo Scientific	Cat# A15797-03
DIG RNA Labeling Mix	Roche	Cat# 11277073910
Ammonium acetate solution	MilliporeSigma	Product# A2706
Glycogen	Invitrogen	Cat# AM9510
5-ethynyl-2′-deoxyuridine (EdU)	Carbosynth	Cat# NE08701
Isoflurane	Piramal Pharma	NDC# 66749-017-25
Isopentane	Honeywell	Cat# M32631-500ML
Tissue-Tek® O.C.T. Compound (OCT)	Sakura	Product code# 4583
32% Paraformaldehyde aqueous solution	Electron Microscopy Sciences	Cat# 15714S
Triton X-100	Acros Organics	Cat# 21568-2500

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sodium lauryl sulfate (sodium dodecyl sulfate)	Ward's Science	Cat# 470302-616
Triethanolamine hydrochloride	Chem-Impex International, Inc.	Cat# 00423250G
SSC, 20× Buffer	Quality Biological	Cat# 351-003-131
Formamide, 99%	Thermo Scientific Chemicals	Cat# A1107636
Denhardt's solution	Thermo Scientific	Cat# AAJ63135AE
Ribonucleic acid, yeast (Yeast tRNA)	MilliporeSigma	SKU 55714-1GM
Herring sperm DNA	Promega	Cat# D1811
Heparin sodium salt (heparin)	BeanTown Chemical	Cat# 139975-1G
EDTA 0.5 M (pH 8.0), Molecular Biology Grade	Promega	REF# V4231
TWEEN 20 (Tween-20)	MilliporeSigma	Product# P1379.
CHAPS detergent (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) (CHAPS)	Thermo Scientific	Cat# 28300
Tris hydrochloride (1 M), pH 7.5	Quality Biological	Cat# 351-006-131
Hydrogen peroxide (30% in water)	Fisher BioReagents	Cat# BP2633500
Blocking reagent	Akoya Biosciences	SKU FP1012
Bovine serum albumin	Fisher BioReagents	Cat# BP9706100
Copper(II) sulfate, anhydrous	BeanTown Chemical	Cat#121535-100G
Sulfo-Cyanine3 azide	Lumiprobe	Cat# A1330
L-Ascorbic acid sodium salt, 99% (Sodium ascorbate)	BeanTown Chemical	Cat# 219180-100G
DAPI	Biotium	Cat# 40043
Vectashield	Vector Laboratories	Cat# H1000
Dimethyl sulfoxide	Alfa Aesar	Cat# J66650
EcoRI	NEB	Cat# R0101S
HindIII	NEB	Cat# R0104S
BamHI	NEB	Cat# R0136S
NotI	NEB	Cat# R0189S
T7 RNA polymerase	NEB	Cat# M0251L
Sp6 RNA polymerase	NEB	Cat# M0207S
RQ1 RNase-free DNase	Promega	Cat# M6101
Critical commercial assays		
RNeasy MinElute Cleanup Kit	Qiagen	Cat# 74204
QuantiTect Reverse Transcription Kit	Qiagen	Cat# 205311
Expand™ High Fidelity PCR System	Roche	Cat# 11732650001
MinElute Reaction Cleanup Kit	Qiagen	Cat# 28206
TOPO™ TA Cloning™ Kit, Dual Promoter, with One Shot™ TOP10 chemically competent *E. coli* cells	Thermo Fisher	Cat# K460001
QIAprep Spin Miniprep Kit	Qiagen	Cat# 27106
Tyramide Signal Amplification Plus Fluorescein Kit	Akoya Biosciences	SKU NEL741001KT
Deposited data		
Table S1	Present study	Mendeley Data: https://doi.org/10.17632/jpj9gpxzr4.2
Experimental models: Organisms/strains		
Mouse: C57bl/6J (females and males, age 5–9 weeks)	The Jackson Laboratory	Strain #:000664
Oligonucleotides		
Olfr827 forward primer: TCTTGTGCAAGCAAAAGGTT Olfr827 reverse primer: TGAACTCAAAGCCTGCCTCT	van der Linden et al., 2020	https://www.sciencedirect.com/science/article/pii/S2211124720311992
Other DNA oligonucleotide primers for amplifying sequences to generate antisense fluorescent *in situ* hybridization (FISH) probes	van der Linden et al., 2020	https://www.sciencedirect.com/science/article/pii/S2211124720311992
Software and algorithms		
SnapGene Viewer	Dotmatics	https://www.snapgene.com/snapgene-viewer
NCBI BLAST tool	NIH	https://blast.ncbi.nlm.nih.gov/Blast.cgi
Primer-BLAST	NIH	https://www.ncbi.nlm.nih.gov/tools/primer-blast/
ZEN microscopy software (Blue edition)	Zeiss	https://www.zeiss.com

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Fisherbrand™ Biology Dissecting Kit-Vinyl Case	Fisher Scientific	Cat# 13-820-095
SPECIMEN BLOCK CRYOMOLD	Andwin Scientific	Product# 25608-916
16-Place Microscope Slide Staining Jar/Staining Dish (slide submersion chamber)	Kartell	Part# 235505
Slide Moisture Chamber (commercial horizontal slide chamber)	Newcomer Supply	Part# 68432A
Fisherbrand™ Microscope Slide Box (hybridization chamber)	Fisher Scientific	Cat# 22363400
Fisherbrand™ Lab Grade Cylindrical Dewar Flask with Aluminum Housing and Handle (glass Dewar flask)	Fisher Scientific	Cat# FB101969
VWR® Micro Cover Glasses, Rectangular, 60 × 24 mm, #1 (#1 coverslip)	VWR	Cat# 48393-106
VWR® Micro Cover Glasses, Rectangular, 60 × 22 mm, #1.5 (#1.5 coverslip)	VWR	Cat# 16004-360
Polyester Film Electrical Tape 56 (tape with a thermosetting rubber adhesive)	3M	Cat# 7000133148
Nail polish; Clear	Electron Microscopy Sciences	Cat# 72180

MATERIALS AND EQUIPMENT

EdU solution		
Reagent	Final concentration	Amount
5-ethynyl-2′-deoxyuridine (EdU)	10 mg/mL	200 mg
1× PBS	1×	20 mL
Total	**N/A**	**20 mL**

Note: Dissolve EdU by incubating at 37°C with agitation. Divide the solution into 1 mL aliquots and store at –20°C for up to 6 months. Before use, thaw one aliquot and redissolve by heating at 37°C with agitation.

Fix solution		
Reagent	Final concentration	Amount
32% paraformaldehyde (PFA)	4% (w/v)	5 mL
1× PBS	1×	35 mL
Total	**N/A**	**40 mL**

Figure 6. Hybridization chamber
To prepare the chamber for hybridization, it is humidified by adding 4 mL of 50% formamide in 5× SSC to two tightly folded Kim Wipes held in place by a clean slide positioned at the bottom of the box. If slides with different probes are to be incubated simultaneously, partitions can be created by sandwiching two adjacent blank slides around a folded Kim Wipe. The chamber should be positioned vertically, and the slides should be placed into it like shelves in a bookcase. After coverslipped slides containing probe solution are loaded, the chamber is sealed with thermosetting rubber adhesive tape and placed vertically inside a preheated hybridization oven for 12–16-h incubation.

Note: Prepare immediately before use.

⚠ CRITICAL: To avoid exposure to formaldehyde gas and liquid, fix solution should be prepared in a fume hood with use of appropriate personal protective equipment.

0.5% Triton X-100 in 1× PBS		
Reagent	Final concentration	Amount
Triton X-100	0.5% (v/v)	2.5 mL
1× PBS	1×	497.5 mL
Total	**N/A**	**500 mL**

Note: Store at 22°C–26°C for up to 6 months.

20% SDS		
Reagent	Final concentration	Amount
Sodium dodecyl sulfate (SDS)	20% (w/v)	20 g
Ultrapure water	N/A	Up to 100 mL
Total	**N/A**	**100 mL**

Note: Ultrapure water is purified using a water purification system such as Milli-Q®. Store 20% SDS at 22°C–26°C for up to 6 months.

1% SDS in 1× PBS		
Reagent	Final concentration	Amount
20% SDS	1% (w/v)	2.5 mL
1× PBS	1×	47.5 mL
Total	**N/A**	**50 mL**

Note: Store at 22°C–26°C for up to 6 months.

1 M TEA, pH 7.5		
Reagent	Final concentration	Amount
Triethanolamine hydrochloride (TEA)	1 M	92.85 g
10 N sodium hydroxide (NaOH)	0.23 N	11.7 mL
Ultrapure water	N/A	Up to 500 mL
Total	**N/A**	**500 mL**

Note: Store at 22°C–26°C for up to 6 months.

0.1 M TEA, pH 7.5		
Reagent	Final concentration	Amount
1 M TEA, pH 7.5	0.1 M	50 mL
Ultrapure water	N/A	450 mL
Total	**N/A**	**500 mL**

Note: Store at 22°C–26°C for up to 6 months.

Acetylation solution		
Reagent	Final concentration	Amount
0.1 M TEA, pH 7.5	0.1 M	30 mL
Acetic anhydride	0.25% (v/v)	75 μL
Total	N/A	30 mL

⚠ CRITICAL: Components should be combined and thoroughly mixed immediately before use. To prevent exposure to acetic anhydride vapors, acetylation solution should be prepared in a fume hood using appropriate personal protective equipment.

5× SSC		
Reagent	Final concentration	Amount
20× SSC	5×	125 mL
Ultrapure water	N/A	375 mL
Total	N/A	500 mL

Note: Store at 22°C–26°C for up to 6 months.

Hybridization solution		
Reagent	Final concentration	Amount
Formamide	50% (v/v)	25 mL
20× SSC	5×	12.5 mL
50× Denhardt's solution	5×	5 mL
Yeast tRNA (100 mg/mL)	250 μg/mL	125 μL
Herring sperm DNA (10 mg/mL)	500 μg/mL	2.5 mL
Heparin (50 mg/mL)	50 μg/mL	50 μL
500 mM EDTA	2.5 mM	250 μL
Tween-20	0.1% (v/v)	50 μL
10% CHAPS (w/v)	0.25% (w/v)	1.25 mL
Molecular biology grade water	N/A	3.275 mL
Total	N/A	50 mL

Note: Molecular biology grade water, which is available commercially, is certified to be highly pure, sterile, and nuclease-free. Hybridization solution should be stored at −20°C for up to one year.

50% formamide in 5× SSC		
Reagent	Final concentration	Amount
Formamide	50% (v/v)	20 mL
20× SSC	5×	10 mL
Ultrapure water	N/A	10 mL
Total	N/A	40 mL

Note: Store at 22°C–26°C for up to 6 months.

0.20× SSC		
Reagent	Final concentration	Amount
20× SSC	0.20×	1 mL
Ultrapure water	N/A	99 mL
Total	N/A	100 mL

Note: Prepare immediately before use.

TN, pH 7.5		
Reagent	Final concentration	Amount
1 M Tris HCl, pH 7.5	100 mM	100 mL
5 M NaCl	150 mM	30 mL
Ultrapure water	N/A	870 mL
Total	**N/A**	**1000 mL**

Note: Store at 22°C–26°C for up to 6 months.

3% hydrogen peroxide		
Reagent	Final concentration	Amount
30% hydrogen peroxide (w/v)	3% (w/v)	5 mL
TN, pH 7.5	N/A	45 mL
Total	**N/A**	**50 mL**

Note: Prepare immediately before use using 30% hydrogen peroxide that is no older than 6 months from initial opening.

TNT		
Reagent	Final concentration	Amount
1 M Tris HCl, pH 7.5	100 mM	100 mL
5 M NaCl	150 mM	30 mL
Ultrapure water	N/A	869.5 mL
Tween-20	0.05% (v/v)	500 μL
Total	**N/A**	**1000 mL**

Note: Store at 22°C–26°C for up to 6 months.

TNB		
Reagent	Final concentration	Amount
Blocking reagent	0.5% (w/v)	250 mg
TN, pH 7.5	N/A	50 mL
Total	**N/A**	**50 mL**

Note: Add blocking reagent slowly to TN with agitation, incubate at 55°C with continuous agitation (30–60 min), and store at –20°C for up to 1 year. Before use, thaw an aliquot and centrifuge the required volume at 4°C for 1 min at 21,000 × *g* to remove insoluble precipitates.

Anti-DIG-POD antibody in TNB		
Reagent	Final concentration	Amount
Anti-DIG-POD antibody (150 U/mL)	0.15 U/mL	1 μL
TNB	N/A	999 μL
Total	**N/A**	**1000 μL**

Note: Immediately before use, centrifuge at 4°C for 1 min at 21,000 × *g* to remove insoluble precipitates.

TSA Plus Fluorescein stock solution

Dissolve TSA Plus Fluorescein in 150 μL dimethyl sulfoxide (DMSO) per tube.

TSA Plus Fluorescein working solution		
Reagent	Final concentration	Amount
TSA Plus Fluorescein stock solution	1×	20 μL
1× amplification reagent	1×	980 μL
Total	N/A	1000 μL

Note: Prepare immediately before use using 1× amplification reagent at a temperature of 22°C–26°C.

BSA wash buffer		
Reagent	Final concentration	Amount
Bovine serum albumin (BSA)	3% (w/v)	3 g
1× PBS	1×	Up to 100 mL
Total	N/A	100 mL

Note: Store at –20°C for up to one year.

200 mM copper sulfate		
Reagent	Final concentration	Amount
Copper sulfate ($CuSO_4$)	200 mM	160 mg
Ultrapure water	N/A	5 mL
Total	N/A	5 mL

Note: Store at 4°C indefinitely.

4 mM sulfo-Cyanine3 azide		
Reagent	Final concentration	Amount
Sulfo-Cyanine3 azide	4 mM	1 mg
Ultrapure water	N/A	330 μL
Total	N/A	330 μL

Note: Store at –20°C indefinitely.

1 M sodium ascorbate		
Reagent	Final concentration	Amount
Sodium ascorbate	1 M	198 mg
Ultrapure water	N/A	1 mL
Total	N/A	1 mL

Note: Prepare immediately before use.

Click chemistry reaction cocktail		
Reagent	Final concentration	Amount
1× PBS	1×	879 μL
200 mM copper sulfate	4 mM	20 μL
4 mM sulfo-Cyanine3 azide	4 μM	1 μL
1 M sodium ascorbate	100 mM	100 μL
Total	N/A	1000 μL

Note: Add components in the order listed and immediately before use.

DAPI solution		
Reagent	Final concentration	Amount
DAPI (10 mg/mL)	1 μg/mL	5 μL
TN	N/A	50 mL
Total	N/A	50 mL

Note: Prepare immediately before use.

STEP-BY-STEP METHOD DETAILS

Combined RNA-FISH and EdU staining

Timing: 3–5 days

The following is a detailed protocol for the simultaneous detection of subtype-specific OR mRNA transcripts, *via* FISH, and EdU incorporated into the genomic DNA of birthdated cells *via* click-chemical conjugation of a fluorophore. This protocol enables the identification of OSNs of specific subtypes born at defined times. Using appropriate tissues and riboprobes, it can be applied to the study of the birthrates and/or survival rates of OSNs of essentially any subtype and how they change as a function of experience.

Note: Steps of this protocol involving the transfer of slides from one solution to another can be performed either by decanting the solution from the slide submersion chamber and adding new solution, or by moving the slides to a different submersion chamber containing new solution. Steps involving the transfer of slides from the submersion chamber to the humidified horizontal slide chamber should be done with care to avoid drying of tissue sections. For these steps, we recommend removing one slide at a time, gently tapping an edge of the slide on a paper towel to remove excess wash solution, wiping the bottom and side edges of the slide on a dry paper towel, placing the slide into the horizontal chamber, carefully adding the required solution dropwise to cover all sections on the slide as evenly as possible, and, If applicable, placing a #1 coverslip on the slide by gently letting it fall onto the solution starting from the bottom edge. After all slides have been processed, the chamber should be closed. The steps involved in detection of FISH riboprobes were adapted and from the TSA Plus Fluorescence Kits manual.

1. Re-hydrate OE tissue sections.
   a. Transfer slides from the –80°C freezer to an open horizontal slide chamber.
   b. Incubate slides: 5 min, 37°C.
   c. Equilibrate slides in 1× PBS (pH 7.2) in a slide submersion chamber: 3 min, 22°C–26°C.

   CRITICAL: For this step, do not allow slides to exceed 3 min in 1× PBS, as this may result in the diffusion of mRNA transcripts out of the unfixed sections and a reduced FISH signal intensity.

2. Fix and permeabilize tissue sections.
   a. Immerse slides in fix solution: 10 min, 22°C–26°C.

   CRITICAL: All steps involving fix solution should be performed in a fume hood with appropriate personal protective equipment.

   b. Wash slides in 1× PBS: 3 min, 22°C–26°C.

c. Immerse slides in 0.5% Triton X-100 in 1× PBS: 10 min, 22°C–26°C.
d. Wash slides in 1× PBS: 3 min, 22°C–26°C.
e. Immerse slides in 1% SDS in 1× PBS: 3 min, 22°C–26°C.
f. Wash slides in 1× PBS: 3 × 3 min, 22°C–26°C.
g. Immerse slides in acetylation solution: 10 min, 22°C–26°C.

⚠ CRITICAL: Steps involving acetylation solution should be performed in a fume hood with appropriate personal protective equipment.

h. Wash slides in 1× PBS: 5 × 3 min, 22°C–26°C.

3. Prepare tissues for FISH riboprobe hybridization.
   a. Transfer slides to a horizontal slide chamber humidified with 5× SSC (see step 28 in the "before you begin" section).
   b. Pre-hybridize tissues by applying 500 μL of hybridization solution to each slide.
   c. Cover horizontal chamber.
   d. Incubate slides: 30 min, 22°C–26°C.
4. Prepare FISH riboprobe for hybridization.
   a. Dilute the stock FISH riboprobe to a concentration of 1.3–2.6 ng/μL (typically, 1:750) in hybridization solution and mix thoroughly by vortexing.
   b. Denature the diluted riboprobe: 2.5 min, 95°C, then place immediately on ice.
5. Hybridize FISH riboprobe to OR-specific mRNAs in OE tissue sections.
   a. Decant hybridization solution from slides.
   b. Apply 115 μL of the diluted riboprobe to each slide and cover with a #1 coverslip.

Note: The riboprobe concentration may require adjustment to optimize the signal-to-noise ratio. If FISH signals are reproducibly weak and background is low, raising the probe concentration by 1.5–2-fold is recommended. In cases in which background is high, reducing probe concentration by 1.5–2-fold is recommended. For more information, see troubleshooting, problem 6.

⚠ CRITICAL: We recommend that gloves be changed when switching between different riboprobes to avoid cross-contamination.

c. Carefully transfer coverslipped slides to a hybridization chamber humidified with 50% formamide in 5× SSC (see step 29 in the "before you begin" section).

Note: With the chamber standing on-end vertically, slides should be placed into the chamber like shelves in a bookcase.

d. Carefully seal the chamber using tape with a thermosetting rubber adhesive.
e. Carefully place the sealed hybridization chamber vertically inside the pre-heated hybridization oven.
f. Incubate slides: 12–16 h, 65°C.
g. Allow coverslips to detach from slides by immersing coverslipped slides in prewarmed (65°C) 5× SSC in a slide submersion chamber.
h. Rinse slides in 0.2× SSC: 15 s, 22°C–26°C.

Note: 0.2× SSC can be kept at 65°C between washes by storing in the 65°C hybridization oven.

i. Wash slides in 0.2× SSC: 4 × 20 min, 65°C.
j. Wash slides in 0.2× SSC: 5 min, 22°C–26°C.
k. Wash slides in TN: 5 min, 22°C–26°C.

6. Prepare tissues for detection of FISH riboprobes.
   a. Quench endogenous peroxidases by incubating slides in freshly prepared 3% hydrogen peroxide in TN: 10 min, 22°C–26°C.
   b. Rinse slides in TNT: 15 s, 22°C–26°C.
   c. Wash slides in TNT: 5 × 3 min, 22°C–26°C with gentle rocking (70 RPM).
   d. Processing slides one-at-a-time, transfer each to a horizontal chamber humidified with TN and apply 500 μL TNB.
   e. Cover horizontal chamber and incubate slides: 30 min, 22°C–26°C.
   f. Processing slides one-at-a-time, remove each slide from the horizontal chamber, drain TNB, place slide back into the horizontal chamber, and apply 500 μL of anti-DIG-POD antibody in TNB.
   g. Cover horizontal chamber and place on a level surface: 12–16 h, 4°C.
   h. Decant antibody solution and rinse slides in TNT: 15 s, 22°C–26°C.
   i. Wash slides in TNT: 4 × 15 min, 22°C–26°C, with gentle shaking (70 RPM).
7. Generate fluorescent signals corresponding to OR mRNA.
   a. Processing slides one-at-a-time, transfer each to a horizontal chamber humidified with TN.
   b. Apply 200 μL of TSA Plus Fluorescein working solution.
   c. Cover horizontal chamber and incubate slides: 12 min, 22°C–26°C.

   Note: If processing multiple slides, stagger reaction start and end times by 30–40 s so that all slides have the same reaction time.

   d. Wash slides in TNT: 2 × 5 min, 22°C–26°C with gentle shaking (70 RPM).
8. Generate fluorescent signals corresponding to EdU.
   a. Wash slides in BSA wash buffer: 2 × 5 min, 22°C–26°C, with gentle rocking (70 RPM).
   b. Processing slides one-at-a-time, transfer each to a horizontal chamber humidified with 1× PBS.
   c. Apply 500 μL of click chemistry reaction cocktail.
   d. Cover horizontal chamber and incubate slides: 30 min, 22°C–26°C.
   e. Rinse slides in BSA wash buffer: 2 × 15 s, 22°C–26°C.
   f. Wash slides in TNT: 2 × 5 min, 22°C–26°C, with gentle shaking (70 RPM).
   g. Counterstain nuclei by immersing in DAPI solution: 5 min, 22°C–26°C.
   h. Wash slides in TNT: 2 × 3 min, 22°C–26°C, with gentle shaking (70 RPM).
   i. Processing slides one-at-a-time, transfer each to a horizontal chamber.
   j. Apply 50 μL of Vectashield dropwise around tissue sections.
   k. Cover with a #1.5 coverslip.
   l. Seal all edges with clear nail polish.
   m. Allow to dry for at least 20 min before imaging.

   Alternatives: For detecting the signals of OR-specific mRNAs and EdU, we typically use FITC and Cy3 fluorophores, respectively. Alternative reagents with compatible excitation/emission spectra may be used.

   Pause point: Sealed slides can be stored at 4°C for up to 1 month before imaging.

9. Acquire images.

   Note: For imaging we use the Zeiss LSM 900 Airyscan confocal microscope with a 10× objective and the ZEN software application.

   a. Open the "ZEN" software application, select the "Locate" tab and choose "Eyepiece" to enable observation of the slide through the microscope oculars.
   b. In the "Microscope" module on the right, select the 10× objective.

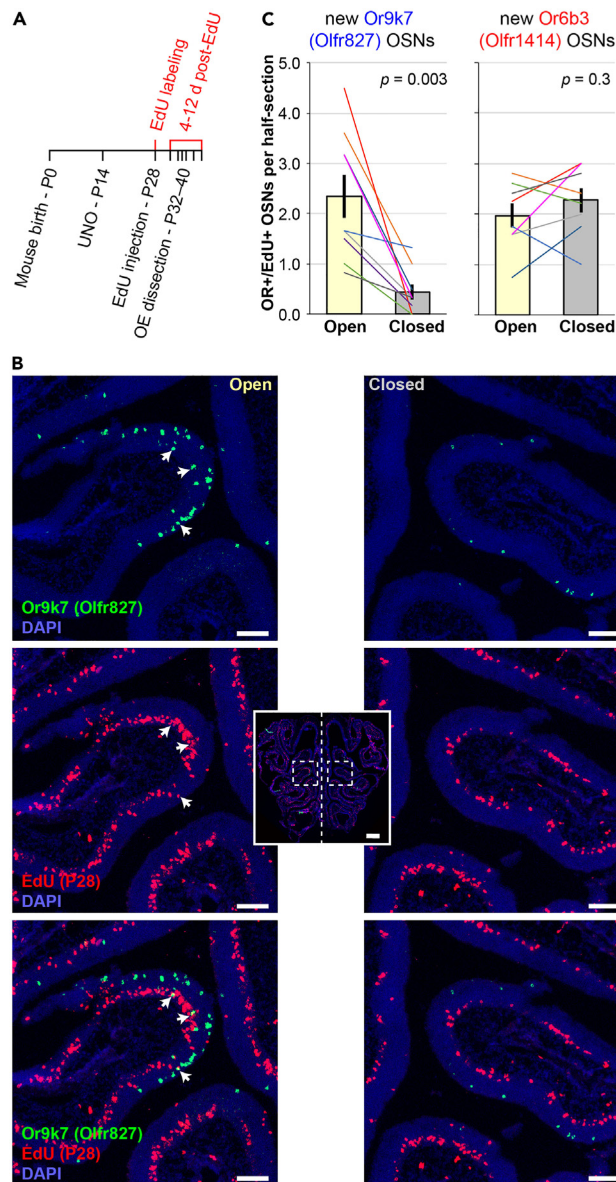

Figure 7. Quantification of the birthrates of OSN subtypes *Or9k7* (*Olfr827*) and *Or6b3* (*Olfr1414*) as a function of olfactory stimulation

(A) Experimental timeline. C57bl6 mice were UNO-treated at P14, EdU-injected at P28, and dissected at P32–40.

(B) Representative OE section from a UNO-treated and EdU-injected mouse stained for *Or9k7* mRNA and EdU. *Scale bars, low magnification panel*: 500 μm; *high magnification panels*: 100 μm.

(C) Quantification of the frequencies of newborn *Or9k7* and *Or6b3* OSNs on the open and closed side of the OE. Each line represents a different mouse; *error bars*: SEM. P values were determined using a paired t test.

c. Clean the slide with ultrapure water and place it on the stage.
d. On the left panel, select "DAPI" and focus on a tissue section by viewing through the oculars.
e. Assess the quality of OR mRNA and EdU signals by successively observing tissue sections using the green and red channels, respectively.

Note: High-quality staining is indicated by the distinct and bright labeling of a subset of cells located within the neuroepithelium (see Figure 7).

f. To capture images, select the "Acquisition" tab and use the "Smart Setup" option on the left side panel to configure the channels corresponding to OR mRNA, EdU, and nuclear DNA (typically FITC, Cy3, and DAPI, respectively).
g. Check the signal intensity of each channel by selecting "Live" in the "Acquisition" tab and adjust the laser power as needed.

Note: We typically use the following initial laser settings so that most of the dynamic range of the detector is used and most pixels are maintained on-scale. FITC (400 nm laser): 1%–3% power, master gain: 700 V; Cy3 (560 nm laser): 1%–2% power, master gain: 650 V; DAPI (405 nm laser): 0.5%–1% power, master gain: 650 V. For all channels, the pinhole is typically set to 460 μm/21.63 AU but may be reduced to enhance optical resolution.

h. Acquire mosaic images of stained tissue sections using tile scanning (9 × 9) and stitch the mosaic images.

Note: Ensure that the focus is adjusted separately for each section of the slide based on OR staining (FITC). As tile scans are used for imaging, a good strategy is to scroll across each section to ensure that all areas are in focus.

Alternatives: Other confocal or epifluorescence microscopes that are equipped with an automated stage (to enable the acquisition of mosaic images) and the appropriate filters (to enable the detection of the appropriate fluorophores) may be used with this protocol.

10. Count EdU-labeled OSNs of a specific subtype.
a. Open a CZI image file using the ZEN (lite or full) software application.

Note: ZEN lite, which can be freely downloaded and installed on Windows computers, can be used to visualize and annotate CZI files.

b. From each slide, select for quantification all OE sections that have highly intact and symmetrical morphologies.

Note: For analysis of mice that have been manipulated *via* UNO, both halves of the OE sections should be symmetrical and intact.

Alternatives: Other imaging software applications that allow adjusting white/black levels may be used for image visualization and quantification. We typically use green, red, and blue pseudo-colors to visualize OR mRNA, EdU, and DAPI staining, respectively (Figure 7B), but alternative color combinations may also be used.

c. To enhance signal visualization, adjust the gamma levels for FITC (OR mRNA) and DAPI (DNA) channels to 1.5 and 0.45, respectively.
d. Adjust the black levels to 2%–5% of the dynamic range for all channels.
e. Adjust the white levels to 12%–15% of the dynamic range for FITC (OR mRNA), 14%–16% for Cy3 (EdU), and 35%–40% for DAPI (DNA).

Note: Signals corresponding to OR mRNA (FITC) and EdU (Cy3) should appear as distinct, bright, round cell-sized structures surrounding a DAPI-labeled nucleus within the epithelial region of the OE. FITC-positive cells represent OSNs of a specific subtype (green puncta in Figure 7B, top panel) and Cy3-positive cells represent newly generated cells (red puncta in Figure 7B, mid panel). Cells positive for both FITC and Cy3 represent newly generated OSNs of a specific subtype (yellow puncta marked by white arrows in Figure 7B, bottom-left

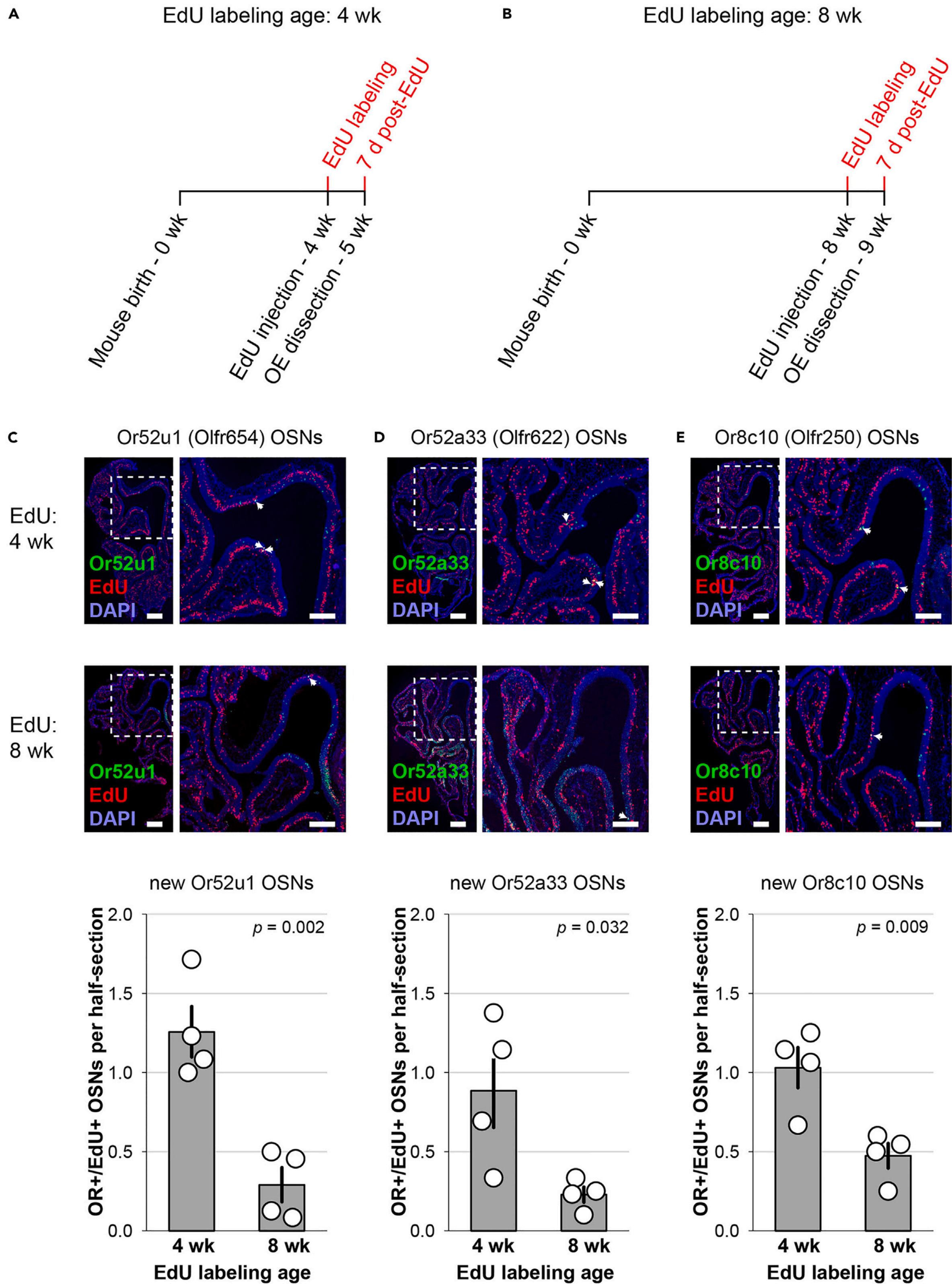
A
EdU labeling age: 4 wk
EdU labeling
7 d post-EdU
Mouse birth - 0 wk
EdU injection - 4 wk
OE dissection - 5 wk
B
EdU labeling age: 8 wk
EdU labeling
7 d post-EdU
Mouse birth - 0 wk
EdU injection - 8 wk
OE dissection - 9 wk
C
Or52u1 (Olfr654) OSNs
D
Or52a33 (Olfr622) OSNs
E
Or8c10 (Olfr250) OSNs
EdU:
4 wk
EdU:
8 wk
Or52u1
EdU
DAPI
Or52a33
EdU
DAPI
Or8c10
EdU
DAPI
new Or52u1 OSNs
new Or52a33 OSNs
new Or8c10 OSNs
OR+/EdU+ OSNs per half-section
p = 0.002
p = 0.032
p = 0.009
4 wk
8 wk
EdU labeling age

Figure 8. Quantification of the birthrates of OSN subtypes as a function of age

(A and B) Experimental timelines. Mice were EdU-injected at either at 4 (A) or 8 weeks (B).

(C–E) Representative images of (*top*) and quantification of newborn OSNs within (*bottom*) OE sections from mice that were EdU-injected at 4 or 8 weeks and stained for *Or52u1* (*Olfr654*) (C), *Or52a33* (*Olfr622*) (D), and *Or8c10* (*Olfr250*) (E) mRNA and EdU. *White arrows*: newly generated OSNs of the corresponding subtypes. *Scale bars, low magnification panels*: 500 μm; *high magnification panels*: 250 μm. Each circle represents a different mouse; *error bars*: SEM. P values were determined using an unpaired t test.

panel). For common problems and solutions related to OR mRNA and EdU staining, refer to troubleshooting problem 6, 7, and 8.

f. Count and record the number of FITC-positive cells in each half-OE section to determine the abundance of all OSNs of the subtype of interest.
g. Count and record the number of cells positive for both FITC and Cy3 in the epithelial region of each half-OE section to determine the abundance of newly generated OSNs of the subtype of interest.
h. Repeat steps f and g for each selected section.

Note: To count a cell as a newly generated OSN of a specific subtype, we require that the OR mRNA staining encompass at least 50% of the pixels corresponding to an EdU-labeled nucleus. This threshold is based on the expected incomplete colocalization of OR mRNA, which is concentrated in the cytoplasm, and EdU, which is confined to the nucleus. To minimize potential bias, we recommend that quantification be performed with the investigator blinded to the identities of experimental groups.

EXPECTED OUTCOMES

The protocol outlined above enables quantification of the neurogenesis rates of specific OSN subtypes and the measurement of potential changes in these rates resulting from alterations in olfactory experience. For example, we have used this technique to investigate the effects of olfactory deprivation on the birthrates of specific OSN subtypes (Figure 7). Olfactory deprivation *via* UNO was found to reduce the birthrate of *Or9k7* (*Olfr827*) OSNs by ~ 5-fold (Figure 7C, *left*), but had no effect on the birthrate of *Or6b3* (*Olfr1414*) OSNs (Figure 7C, *right*).[1]

The protocol may also be used to evaluate how the birthrates of distinct OSN subtypes change as a function of developmental or life stage (e.g., infancy, adolescence, adulthood, etc.). For instance, quantification of the birthrates of three OSN subtypes [*Or52u1* (*Olfr654*), *Or52a33* (*Olfr622*), and *Or8c10* (*Olfr250*)] in mice that were EdU-injected at 4 or 8 weeks of age (Figures 8A and 8B) revealed varying degrees of birthrate reduction with age (Figures 8C–8E). Specifically, while *Or52u1* and *Or52a33* OSNs exhibited a ~ 4-fold reduction at 8 weeks compared to 4 weeks (Figures 8C and 6D), *Or8c10* OSNs exhibited only a ~ 2-fold reduction (Figure 8E). These findings suggest that although OSN neurogenesis rates generally decrease with age, as has been reported previously,[24–26] this decrease may not be not uniform across all OSN subtypes.

The protocol presented here may also be used to analyze the relative neurogenesis rates of different OSN subtypes. For example, quantification of the birthrates of 10 subtypes revealed differences spanning ~ 7-fold, from the subtype with the lowest birthrate, *Or4f60*, to the one with highest, *Or6z3* (Figure 9). These results are consistent with previous studies reporting substantial variability among OR expression levels and subtype-specific OSN abundances within the mouse OE.[16,27,28]

The protocol outlined above may also be adapted to investigate the survival rates of newborn OSNs of specific subtypes and how they are affected by experience. For example, we have varied the time between EdU injection and OE dissection to assess the effects of olfactory deprivation on the survival rates of newborn OSNs of five subtypes: three previously found to undergo stimulation-dependent

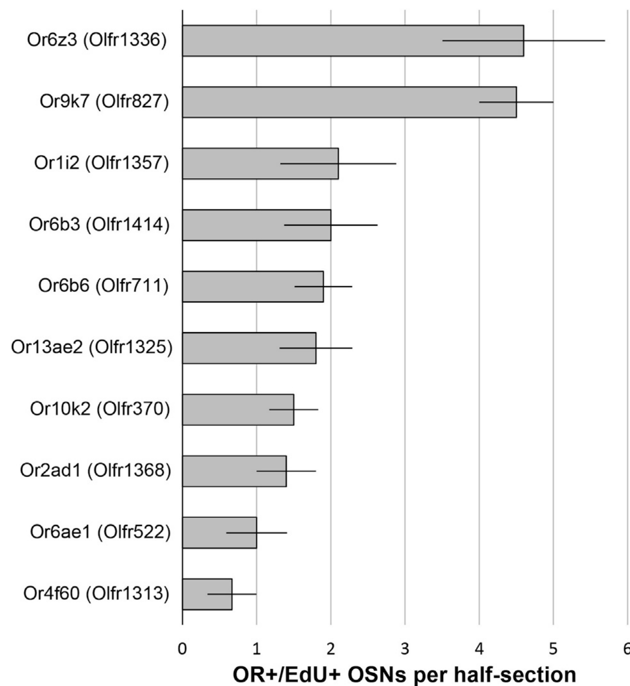

Figure 9. Quantification of the birthrates of different OSN subtypes
Mice were EdU-injected at P28 and dissected at P35. *Error bars*: SEM; n = 4 mice.

neurogenesis [*Or7g35* (*Olfr855*), *Or9k7 (Olfr827), and Or13ae2* (*Olfr1325*)], and two previously found to undergo stimulation-independent neurogenesis [*Or6z3* (*Olfr1336*) and *Or6b3* (*Olfr1414*)] (Figure 10).[1] Subtypes that undergo stimulation-dependent neurogenesis tended to show lower frequencies of newborn OSNs on the closed side of the OE compared to the open beginning from the earliest time point at which robust OR expression is observable (4 days post-EdU labeling) (Figures 10B–10D), while stimulation-independent subtypes showed no such differences throughout the time course (Figures 10E and 10F). On both sides of the OE, frequencies of newborn OSNs of all subtypes peaked 7–12 days post-EdU and declined sharply thereafter (Figure 10). Interestingly, open-side survival rates appear to differ among subtypes, with *Or7g35* and *Or6b3* OSNs showing a greater surviving fraction compared to the other three subtypes at 21–28 days post-EdU. While these results agree with previous findings that a majority of adult-born OSNs do not survive past 4 weeks of age,[25] they indicate that survival rates may differ among subtypes.

QUANTIFICATION AND STATISTICAL ANALYSIS

The following are step-by-step details for the statistical analysis of subtype-specific neurogenesis rates on the open and closed sides of the OE of a UNO-treated mouse (Table 1; Table S1).

1. Enter the number of cells that are double-positive for EdU and a specific OR mRNA on the open and closed sides of selected OE sections from experimental mice (Table 1, columns 3 and 4).

   Note: To ensure adequate statistical power, at least 4 representative OE sections from each of at least 4 mice are required for comparisons within animals (e.g., open versus closed sides of the OE) for an OR expression frequency of at least 0.1% and an effect size of at least 2-fold. For comparisons between animals, 8 representative half-sections from each of at least 5 mice are required for the same OR expression frequency and effect size. These sample size estimations are based on our previous studies.[1,17] If the OR expression frequency and/or effect size are lower, additional samples may be required.

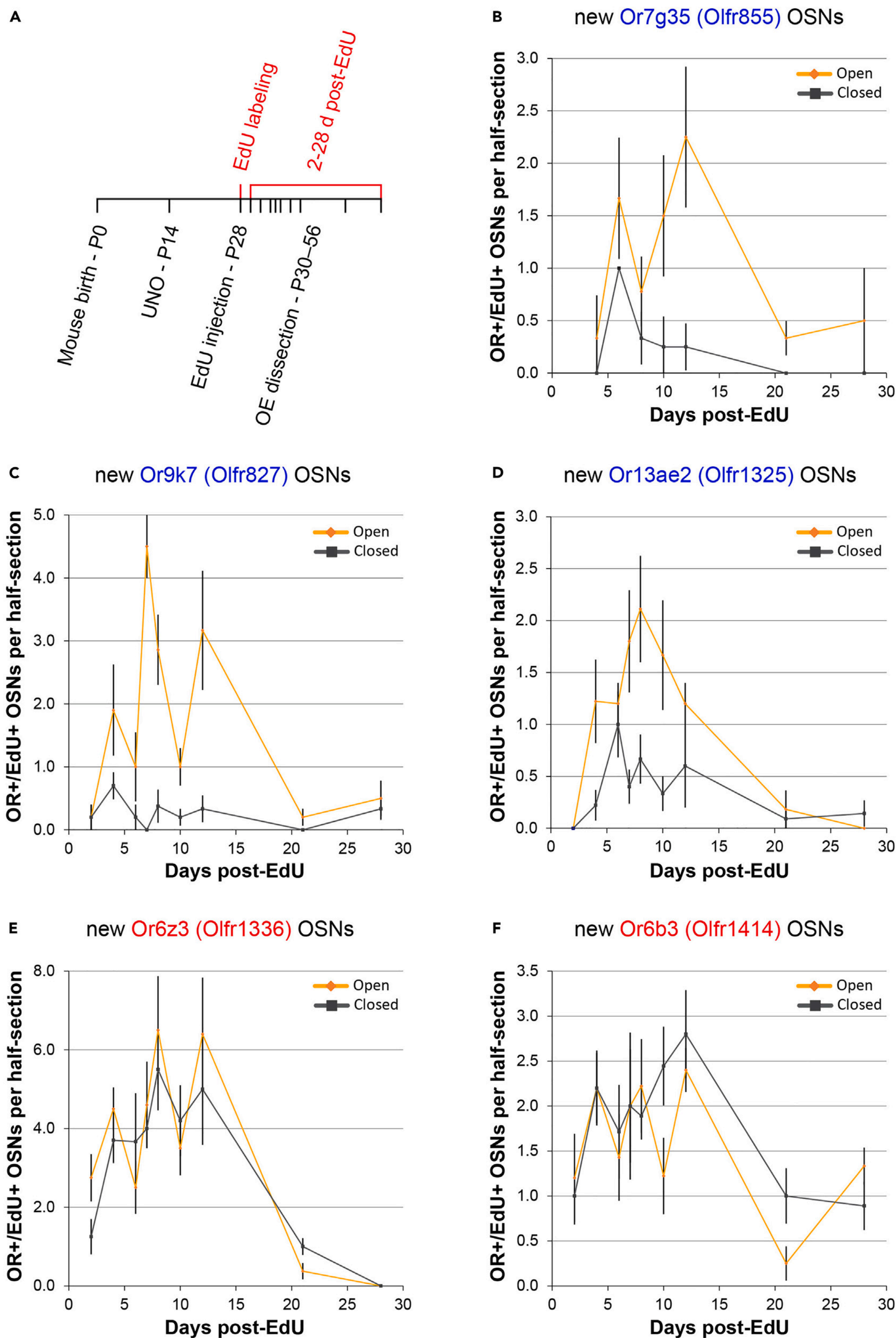
A
EdU labeling
2-28 d post-EdU
Mouse birth - P0
UNO - P14
EdU injection - P28
OE dissection - P30–56
B
new Or7g35 (Olfr855) OSNs
C
new Or9k7 (Olfr827) OSNs
D
new Or13ae2 (Olfr1325) OSNs
E
new Or6z3 (Olfr1336) OSNs
F
new Or6b3 (Olfr1414) OSNs
Open
Closed
OR+/EdU+ OSNs per half-section
Days post-EdU

Figure 10. Quantification of the subtype-specific survival of newborn OSNs

(A) Experimental timeline. Mice were UNO-treated at P14, EdU-treated at P28, and sacrificed between P30–P56.

(B–F) Quantification of the frequencies of EdU-labeled OSNs of subtypes previously found to either undergo (B–D) or not undergo (E, F) stimulation-dependent neurogenesis. *Error bars*: SEM; n = 3 mice.

2. Calculate the mean number of double-positive cells/ half OE section for each side and mouse (Table 1, column 5 and 6) and use these data for following analyses:
   a. Calculate the mean number of double-labeled cells/ half OE section/ mouse for each side of the OE (Table 1, columns 7 and 8).
   b. Calculate the standard error of the mean (SEM) for the number of double-labeled cells on each side of the OE (Table 1, columns 9 and 10).
   c. Using a two-tailed, paired t-test (pairing the open and closed sides of the OE of each mouse), test for a subtype-specific difference in OSN birthrate on the two sides (Table 1, column 11).
   d. Based on a p-value threshold of 0.05, determine whether there is a statistically significant subtype-specific difference in OSN birthrate. With p = 0.001, the data in Table 1 support the conclusion that the birthrate of *Or9k7* OSNs is lower on the closed side of the OE compared to the open.

Note: For experimental questions requiring comparisons of OEs from mice in different treatment or age groups, statistical significance can be tested using a two-tailed, unpaired t-test. Table 1 is available as Table S1, which may be used as a template for data analysis.

LIMITATIONS

Limitations of this protocol include the following: 1) The targeting of OR mRNAs with high (>70%) nucleotide identity throughout the transcript may result in the staining of off-target OSN subtypes. 2) FISH detection methods that require the preservation of nucleic acids (e.g., hybridization chain reaction) may not be compatible with EdU detection, which has been found to result in the degradation of nucleic acids.[7] 3) As described, this protocol can be used to assess the birthrates of only

Table 1. Quantification of EdU-labeled *Or9k7* (*Olfr827*) OSNs on open and closed sides of the OEs of UNO-treated mice

		Newborn *Or9k7* OSNs/ half OE section		Mean newborn *Or9k7* OSNs/ half OE section		Mean newborn *Or9k7* OSNs/ half OE section/ mouse		Standard error of mean (SEM)		
Mouse ID	Section number	Open	Closed	Open	Closed	Open	Closed	Open	Closed	P-value
100b-F1	1	4	2	3.60	0.40	2.92	0.37	0.40	0.05	0.001
	2	2	0							
	3	1	1							
	4	6	1							
	5	5	1							
100b-F5	1	3	2	3.17	0.50					
	2	2	0							
	3	1	0							
	4	4	0							
	5	5	0							
	6	4	1							
100d-M6	1	1	0	1.75	0.25					
	2	2	0							
	3	2	0							
	4	2	1							
100d-M7	1	6	1	3.17	0.33					
	2	2	0							
	3	6	0							
	4	3	0							
	5	1	1							
	6	1	0							

a single OSN subtype in each experiment. However, it is possible that the protocol may be adapted for use with multiplexable FISH probes to enable simultaneous quantification of the birthrates of multiple subtypes. 4) This protocol enables subtype-specific assessment of OSN birthrates no earlier than 2–4 days post-EdU, the time frame during which the expression of a single dominant OR begins to be robustly detectable.[1,29–31] Prior to this time frame, OSN precursors have been found *via* scRNA-seq to express low levels of multiple OR transcripts,[32–35] which we have found to be undetectable using the methods outlined in this protocol.

TROUBLESHOOTING

Problem 1

Poor RNA quality (step 2 in "before you begin").

Potential solution

Poor RNA quality may result from the use of an excessive amount of tissue, RNase contamination, prolonged isolation time, or extended exposure of purified RNA to elevated temperatures. This problem may be resolved by.

- Using no more than the recommended amount of tissue in the RNA isolation protocol.
- Using RNase-free reagents and maintaining a clean workspace.
- Minimizing RNA isolation time.
- Avoiding prolonged exposure of purified RNA to elevated temperatures.

Problem 2

Insert cloning yields few or no colonies (step 9 in "before you begin").

Potential solution

Insufficient insert concentration, suboptimal ligation conditions, and poor-quality competent cells can cause this issue. This problem may be resolved by.

- Increasing the amount of purified PCR product in the ligation reaction.
- Always using purified PCR products for cloning.
- Strictly following the ligation conditions outlined in the kit manual.
- Always storing competent cells in –80°C and avoiding freeze-thaw cycles.

Problem 3

Tissue cryosections are folded or curled (step 24 in "before you begin").

Potential solution

This problem may be caused by a below-optimal cryostat temperature set point or failure to use an anti-roll plate. This problem may be resolved by.

- Raising the set point by 1 or 2°C and then allowing the chamber and tissue block to equilibrate for 10–15 min before resuming cryosectioning.
- Ensuring the use of an anti-roll plate.

Problem 4

Tissue cryosections are crumpled (step 24 in "before you begin").

Potential solution

This problem may be caused by an above-optimal cryostat temperature. It may be resolved by lowering the set point by 1 or 2°C and then allowing the chamber and tissue block to equilibrate for 10–15 min before resuming cryosectioning.

Problem 5

Tissue cryosections are torn or shredded (step 24 in "before you begin").

Potential solution

This problem may be caused by the formation of ice crystals during tissue freezing, especially if the freezing process is prolonged. It may be alleviated by ensuring that the isopentane within the freezing apparatus is sufficiently cold and that tissue blocks are allowed to remain submerged within the isopentane until completely frozen.

Problem 6

Poor FISH signal-to-noise ratio (steps 5 and 10).

Potential solution

This problem may be caused by a variety of factors, including non-specific binding of the riboprobe to tissue sections, insufficient riboprobe concentration, poor tissue quality, excessive drying of tissue sections between experimental steps, spilling or wicking of riboprobe or antibody solutions from slides, and a non-optimal TSA reaction temperature. This problem may be resolved by.

- Optimizing the riboprobe concentration and/or design. For example, if the background signal is high, try reducing the probe concentration 2-fold. If the FISH signals are weak with minimal background, try increasing the probe concentration 2-fold.
- Redesigning riboprobes to target different regions of the OR mRNA.
- Ensuring that hybridization and horizontal chambers are properly humidified during incubation steps.
- Properly sealing hybridization chambers prior to 12–16-h incubation.
- Using care when placing coverslips on slides prior to hybridization to avoid loss of hybridization solution.
- Transferring hybridization chambers to the hybridization oven with care to prevent the loss of hybridization solution or the displacement of coverslips from slides.
- Ensuring that chambers are on a level surface during incubation to prevent solution drainage.
- Removing excess liquid from the bottom and sides of the slides before applying riboprobe and antibody solutions to prevent wicking.
- Preparing TSA plus working solution using 1× amplification reagent that has been allowed to come to 22°C–26°C.

Problem 7

Poor EdU signal-to-noise ratio (step 10).

Potential solution

The issue may be due to the use of sodium ascorbate that was not prepared immediately before addition to the reaction cocktail, or from combination of reaction components out of the recommended order, which could cause inactivation of the reaction cocktail. This problem may be resolved by.

- Preparing 1 M sodium ascorbate immediately before use.
- Preparing the click chemistry reaction cocktail by adding components in the order listed and immediately before use.

Problem 8

Cellular OR mRNA staining intensity and/or area vary excessively within a section (step 10).

Potential solution

This issue may result from riboprobe cross-reactivity with other highly similar OR transcripts (> 70% nucleotide identity). This problem may be resolved by.

- Designing riboprobes from regions of the target mRNA that have <70% nucleotide identity with other transcripts.
- Excluding OR-positive cells for which the intensity or area of OR mRNA signals are <50% of maximal.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Stephen Santoro (stephen.santoro@cuanschutz.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze new datasets/code. Table S1 has been deposited in Mendeley Data as Supplementary File 1 (https://doi.org/10.17632/jpj9gpxzr4.2).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at Mendeley Data: https://doi.org/10.1016/j.xpro.2023.102432.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation under project IOS-1943528.

AUTHOR CONTRIBUTIONS

Conceptualization, K.H., S.W.S.; investigation, K.H., M.S.; writing, K.H., M.S., S.W.S.; supervision, funding acquisition, S.W.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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