Protocol to detect RNAs from tissue sections in mice using Y-branched probe in situ hybridization

Here, we describe a fluorescent in situ hybridization protocol named Yn-situ, standing for Y-branched probe in situ hybridization, to detect RNAs from mice tissue sections. We provide steps for the synthesis and quantification of preamplifier probe using nickase. We also detail the preparation of tissue section, probe hybridization, signal development using hybridization chain reaction (HCR), and quantification of the signals. This approach avoids the use of proprietary in situ hybridization techniques, therefore reducing costs.

Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol
detect RNAs from tissue sections in mice
using Y-branched probe in situ
hybridization

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SUMMARY
Here, we describe a fluorescent in situ hybridization protocol named Yn-situ, standing for Y-branched probe in situ hybridization, to detect RNAs from mice tissue sections. We provide steps for the synthesis and quantification of preamplifier probe using nickase. We also detail the preparation of tissue section, probe hybridization, signal development using hybridization chain reaction (HCR), and quantification of the signals. This approach avoids the use of proprietary in situ hybridization techniques, therefore reducing costs. For complete details on the use and execution of this protocol, please refer to Wu et al. (2022).

BEFORE YOU BEGIN
The protocol below describes the specific steps for performing Yn-situ on fresh frozen developing mouse olfactory epithelium. Please see notes for using the protocol on other type of tissues. We describe the probe design steps using Benchling, plasmid purification steps using QIAGEN maxi-prep kit, and gel extraction steps using the Zymo Research gel purification kit. Other software and kits that produce good results can also be used. Please see notes for comparison of the alternative reagents.

Institutional permissions
Experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee at Stowers Institute and in compliance with the NIH Guide for Care and Use of Animals.

CRITICAL: Any experiments on live vertebrates or higher invertebrates must be performed in accordance with relevant institutional and national guidelines and regulations.

Designing probes

⭐ Timing: 30 min

This part provides a protocol to view target gene sequence, design specific targeting probes, and provides instructions for purchasing the probes. We routinely use Benchling to view and annotate DNA sequences (Figures 1A–1E). Other software such as SnapGene and Vector NTI can also be used. This protocol assumes the users are familiar with these DNA sequence editing software, Excel, and BLAST. For instructions on how to use these software, see: https://help.benchling.com/en/
Figure 1. Screenshots of the software used in the probe design
(A–E) Screenshots from Benchling. Numbers indicates the functions used in the corresponding steps in the protocol. Red square in D indicates the designed probes and their targeting region on the transcript (shown with the Omp gene).
(F) Screenshot of the probe design template file. Red square indicates where the sequence to be targeted should be pasted into.

Excel video training from Microsoft (https://support.microsoft.com/en-us/office/excel-video-training-9bc05390-e94c-46af-a5b3-d7c2216990bb); Comparative Genomics: Volumes 1 and 2. Chapter 9 BLAST QuickStart (Wheeler and Bhagwat, 2007). Experts of these software packages may follow the first level major steps. Beginners may follow the detailed sub-steps. The goal of this part is to design probes that are specific to the target gene by avoiding similar sequences in the same genome. For the probes to work under the current protocol, the GC content should be
between 30 and 90%. More than five repeats of the same nucleotide or di-nucleotides (such as “AAAAA” and “ATATATAT”) should be avoided. To detect all the transcripts from the same gene, we recommend designing probes against the shared exons and 3’ UTR region.

1. Import the gene sequence to Benchling for annotation.
   a. Login to Benchling at benchling.com.
   b. Create a folder for storing gene sequences.
   c. Enter the folder.
   d. Import the target gene sequence to Benchling by clicking the “plus” button, then “DNA sequences”, then “Import DNA sequences”.
   e. In the pop-up window, go to “import from databases” tab.
   f. Type the gene name.
   g. Select a mouse genome database. We use mm10.
   h. Select a transcript to be detected under “Transcript” drop down menu. For detecting all the transcripts from the same gene, we recommend choosing a transcript with the shared 3’ UTR.
   i. Rename the imported sequence under “Import As”.
   j. Complete the import by clicking “IMPORT” button.

2. BLAST the target gene to be detected to identify homologous regions to other genes.
   a. In Benchling, copy the region of the gene to design the probes from.
   b. Go to https://blast.ncbi.nlm.nih.gov/Blast.cgi.
   c. Select “nucleotide blast”.
   d. Paste the sequencing under “Enter Query Sequence”.
   e. Choose “Genomic + transcript databases” under “Choose Search Set” options.
   f. In the dropdown menu that appears, choose mouse genomic plus transcript.
   g. Click “BLAST” button to start the search.
   h. Based on the BLAST result, annotate any regions that are similar to other genes in Benchling.

3. Select 5–20 regions that contain 52 nucleotides (nt) sequences in the regions that do not have similarity to other genes. These are the sequences of the targeting probes.

4. BLAST the 52 nt sequences again to identify potential off-targeting transcripts that can be detected by the probes.

5. Copy the 52 nt to the column H of the Probe Design Template Table (Data S1; Figure 1F). The template will generate the sequences for the probes.

6. Order the probes that appear in column B. Each region in the target gene is targeted by a pair of probes, namely the odd and even probes. The odd probe targets the 5’ part of the transcript and using its 3’ end to hybridizes to the target whereas the even probe is the opposite. Once hybridized, the 5’ portion of the odd probe and the 3’ portion of the even probe provides a sequence complementary to the preamplifier for it to bind.

   Note: The probes do not need high purity purification such as HPLC purification. Standard de-salted oligos will suffice. We have obtained successful results using oligos from IDT (www.idtdna.com) and Synbio Tech (https://www.synbio-tech.com/).

Preparing targeting probes

© Timing: 2 h

This step dissolves targeting probes into IDTE buffer for long term storage. IDTE is Tris-EDTA buffer offered from IDT and may be replaced by custom-made buffers.

7. Upon arrival, dissolve individual oligos to 200 μM with IDTE buffer.
Note: A simple way to calculate the volume of IDTE buffer (in μL) to be added to the tube is to multiply the amount of oligos (in nmole) by 5. For example, for a tube containing 10 nmole oligos, dissolve it in 50 μL of IDTE buffer.

8. Mix equal volume of all odd probes into one tube and dilute the mixture using IDTE to make each probe 2 μM.

Note: Preparation will depend on the number of probes to be mixed. For a 10 nmole scale synthesis, if 5 pairs of primary probes are used, add 50 μL of each 200 μM odd probe into a 15 mL conical tube. Add 4.75 mL IDTE buffer to a final volume of 5 mL. A quick look up table is provided below step 9.

9. Mix equal volume of all even probes into one tube and dilute the mixture using IDTE to make each probe 2 μM.

| Reagent                          | 5 pairs | 10 pairs | 20 pairs |
|----------------------------------|---------|----------|----------|
| Each 200 μM probe (odd or even)  | 50 μL   | 50 μL    | 50 μL    |
| IDTE                             | 4.75 mL | 4.5 mL   | 4 mL     |
| Total                            | 5 mL    | 5 mL     | 5 mL     |

10. Aliquot the 2 μM stock solution into 10–20 tubes.
11. Store the aliquots in –20°C.

**Preamplifier synthesis**

⊙ Timing: 2 days

This process is for the synthesis of preamplifier probes used in Yn-situ. An illustration of the procedure is provided in Figure 2.

Note: This protocol uses QIAGEN Maxiprep kit for plasmid purification. The following reagents refer to the components in the QIAGEN Maxiprep kit.

12. Prepare preamplifier from plasmid A1-20XB1 (P004). The plasmid is available from Addgene (www.addgene.org, Stock number 184056).
   a. Upon arrival, streak a plate containing 50 μg/mL of kanamycin.

   ▲ CRITICAL: P004 confers kanamycin resistance. It is critical to use the correct antibiotics in the agar plate and LB media during the plasmid preparation.

   b. Select a few single colonies to perform miniprep and make glycerol stocks of the selected clones.
   c. Verify the number of B1 initiator by sequencing.

   ▲ CRITICAL: P004 contains 20 repeats of the B1 initiator sequence and can potentially result in recombination in the bacteria. It is critical to verify that the sequence is intact.

   d. Select a clone with the correct number of B1 initiators.
   e. Inoculate 20 μL of glycerol stock in 300 mL of L.B. medium containing 50 μg/mL of kanamycin overnight (12–18 h) at 37°C, 220 rpm.
Note: For convenience, NEB stable cells containing P004 can be grown at 37°C to shorten the growth time and increase maxiprep yield. Although it is recommended to grow NEB stable cells at 30°C, we do not find high frequency of recombination among the B1 initiator repeats when the cells are growth at 37°C.

f. Pellet the cells at 12,000 × g, 4°C, for 5 min.
g. Resuspend the pellet in 10 mL buffer P1.
h. Add 10 mL of buffer P2.
i. Gently mix the solution by slowly inverting the tube.

j. Lyse the cells on ice for 5 min.

**Note:** The preamplifier is eventually purified by gel extraction. The residual genomic DNA and RNA are removed during the gel excision step. However, significant amount of genomic DNA does affect the quantification of plasmid, which may affect estimation of plasmid quantity for the nicking step. Therefore, we recommend following the specified lysis timing.

k. Add 10 mL of buffer P3.

l. Gently mix the solution by slowly inverting the tube 6 times.

m. Centrifuge at 12,000 × g at 4°C, for 5 min.

n. Prepare a QIAGEN tip on a vacuum manifold.

o. Add 20 mL buffer QBT to the QIAGEN tip and pass through the column.

p. Pass the supernatant from step m to the column.

q. Wash with 20 mL buffer QC.

r. Repeat step q.

s. Place the QIAGEN tip onto a 50 mL conical tube using the tip adapter.

t. Elute the plasmid with 5 mL buffer QF.

u. Add 5 mL isopropanol to the flow-through.

v. Centrifuge at 12,000 × g at 4°C, for 5 min.

⚠️ **CRITICAL:** A glassy pellet should appear at the bottom of the tube.

w. Remove supernatant.

x. Add 2 mL of 80% ethanol.

y. Centrifuge at 12,000 × g at 4°C, for 5 min.

z. Remove supernatant.

aa. Air dry the pellet for 5 min.

bb. Resuspend the pellet in 500 μL of ddH2O.

c. Measure the concentration using Nanodrop. See troubleshooting: problems 1.

**Alternatives:** We have also purified plasmids suitable for preamplifier synthesis using ZymoPURE II Plasmid Maxiprep (Zymo Research D4202).

13. Nick the double stranded preamplifier template.

a. Prepare digestion with the following formula.

| Reagent                  | Final concentration | Amount (μL) |
|--------------------------|---------------------|-------------|
| P004 template            | 80 ng/μL            | variable    |
| Nt.BspQI enzyme          | 0.25 unit/μL        | 10          |
| 10× NEBuffer r3.1        | 1×                  | 50          |
| ddH2O                    | N/A                 | variable    |
| Total                    | N/A                 | 500         |

b. Digest plasmid for 4 h at 50°C, then deactivate the enzyme for 20 min at 80°C.

⚠️ **CRITICAL:** The digestion should be performed at 50°C, not 37°C. The reaction should not be performed overnight (12–18 h) to avoid star activity.

**Note:** This step can be performed in a thermocycler.

**Note:** The DNA is purified using Zymo DNA clean and concentrator kit. The following reagents refer to the components in the kit.
c. Pool the solution into a 1.7 mL tube.
d. Add 1 mL DNA binding buffer.
e. Vortex the solution briefly.
f. Install 2 Zymo DNA clean columns on a vacuum manifold.

△ CRITICAL: Each column should have a binding capacity larger than 20 μg.

g. Pass the solution through the 2 columns.
h. Wash the columns with 500 μL wash buffer.
i. Repeat step h.

△ CRITICAL: The remaining salt from buffer r3.1 inhibits the melting of double stranded DNA using DMSO. Salt needs to be removed completely before DMSO is added.

Note: We find the column purification is very effective in removing salt. If the user chooses to use ethanol precipitation to purify the digested DNA, one should not add sodium acetate to the 80% ethanol used for washing the pellet.

j. Place the columns on collection tubes.
k. Centrifuge at 12,000 × g for 2 min.
l. Place the dried columns into 1.7 mL tubes.
m. Add 75 μL ddH2O into each column.
n. Centrifuge at 12,000 × g for 2 min.
o. Pool the 150 μL eluate into a 1.7 mL tube.

14. Denature and purify single strand preamplifier.
   a. Add 350 μL DMSO to the sample.
b. Vortex mix for 10 s.
c. Incubate at 95°C for 2 min.
d. Prepare a 0.5% agarose gel using sodium borate buffer with 25 wells of more than 25 μL loading volume each.
e. Load 25 μL sample in each well.

△ CRITICAL: Do not add loading dye while loading the sample to the gel. DMSO serves as a loading buffer.

f. Run the gel at 5 V/cm (100 V for a standard 20 cm long gel tank) for 1 h. See troubleshooting: problems 2, 3, and 4.

Note: it is not necessary to run a denaturing gel. We find the ssDNA separates as long as they are denatured before loading into the gel.

Note: The DNA is purified using Zymo gel extraction kit. The following reagents refer to the components in the kit.

g. Extract the bands containing preamplifiers. Collect every 3 bands into one 2 mL tube.
h. Add 1 mL agarose dissolving buffer (ADB) to each tube.
i. Melt the gel at 55°C. Vigorously invert the tubes intermittently to facilitate the melting.
j. Install 2 new DNA binding columns onto a vacuum manifold.

△ CRITICAL: The column should have a binding capacity larger than 5 μg.

k. Pass the melt gel solution through the column.
l. Warm 100 μL IDTE buffer to 95°C.
m. Wash the column with 400 μL wash buffer.

n. Transfer the columns to collection tubes.

o. Centrifuge at 12,000 × g for 2 min.

p. Transfer the columns to 1.7 mL tubes.

q. Elute in 50 μL 95°C IDTE buffer.

15. Measuring the concentration of purified preamplifier.

a. Make a 1:10 dilution of the 1 kb plus ladder by adding 1 μL of ladder to 9 μL of ddH2O.

b. Make a 1:100 dilution of the ladder by adding 1 μL of the 1:10 dilution ladder into 9 μL of ddH2O.

c. Make a 1:10 dilution of preamplifier by adding 1 μL of purified preamplifier to 9 μL of H2O.

d. In an 8-tube PCR strip, mix according to the table below by adding the specified amount of sample and DMSO to the tubes.

Note: The concentration of the 850 bp band is 30 ng/μL in undiluted molecular marker mix. Following this protocol, 0.3 ng, 0.6 ng, 1.5 ng, 3 ng, 6 ng, and 15 ng of the 850 bp band will be present in the gel. To measure the concentration of preamplifier accurately, it is optimal that 1–10 ng of preamplifier is loaded to the gel. Without considering loss, the theoretical yield of preamplifier from 40 μg plasmids is around 80 ng/μL.

| Tube | Sample content (DNA amount referring to the 850p band) | Sample to be added to the mix | Amount (μL) | DMSO (μL) |
|------|--------------------------------------------------------|------------------------------|-------------|-----------|
| 1    | 0.3 ng ladder                                          | 1:100 diluted ladder         | 1           | 3         |
| 2    | 0.6 ng ladder                                          | 1:100 diluted ladder         | 2           | 5         |
| 3    | 1.5 ng ladder                                          | 1:100 diluted ladder         | 5           | 12        |
| 4    | 3 ng ladder                                            | 1:10 diluted ladder          | 1           | 3         |
| 5    | 6 ng ladder                                            | 1:10 diluted ladder          | 2           | 5         |
| 6    | 15 ng ladder                                           | 1:10 diluted ladder          | 5           | 12        |
| 7    | 1:10 diluted preamp                                    | 1:10 diluted preamplifier    | 1           | 3         |
| 8    | Undiluted preamplifier                                 | Undiluted preamplifier       | 1           | 3         |

e. Heat to 95°C for 2 min.

f. Run all the samples in a 1% GelRed agarose gel at 100 V for 60 min.

g. Image the gel. See troubleshooting: problems 5.

h. Open the gel image in FIJI.

Note: Steps i to r measure the preamplifier band intensity using FIJI’s gel quantification function. The same function can be accessed through Analyze/Gels/Select First Lane,… For more instructions for using FIJI, see https://imagej.net/learn/.

i. Use the “Rectangle” tool to select the first lane of ladder.

j. Press “control” + “1”.

k. Select the second lane of ladder.

l. Press “control” + “2”.

m. Repeat k to l for all the remaining lanes except the last lane.

n. Select the last lane of sample.

o. Press “control” + “3”.

p. In the resulting traces, use the “Make Straight Line” tool to draw a line at the bottom of all the peaks.

q. Use “Wand” tool to select the peaks that is sealed by the line.

r. A chart will show the area of the peaks.

s. Copy the areas of the peaks from both ssDNA ladder and samples to the Concentration Calculation Table (Data S2).
Note: Supplementary file Data S2 performs linear regression in excel based on the 850 bp bands from the ladder lanes and automatically calculate the concentration of the preamplifier. It is designed for non-expert to use. Alternative methods can also be used.

t. The concentration of the preamplifier band will appear.
u. Dilute preamplifier to 20 ng/μL according to the measured concentration of the stock solution.

△ CRITICAL: The concentration of the preamplifier is critical to Yn-situ and must be measured accurately. We recommend performing gel quantification as it detects ssDNA with high sensitivity. Alternatively, a Qubit fluorometer with a ssDNA quantification kit can be used. However, a typical yield of preamplifier under the current protocol is between 2-10 ng/μL which is at the lower end of the Qubit ssDNA detection range. Quantification by Nanodrop spectrometer is frequently not accurate because of the residual agarose melting buffer.

Alternatives: We have compared several dyes including SYBR safe, Midori green, ethidium bromide, and GelRed. We find the staining sensitivity of SYBR safe and Midori green is significantly lower than GelRed. We also find ethidium bromide and Midori green migrate toward cathode, causing the low molecular weight bands to be lower in brightness. We do not recommend using them as alternatives.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Bacterial and virus strains | | |
| One Shot TOP10 Electrocomp E. coli | Thermo Fisher Scientific | CAT: C404052 |
| NEB Stable Competent E. coli (High Efficiency) | New England Biolabs | CAT: C3040H |
| Chemicals, peptides, and recombinant proteins | | |
| 1-methylimidazole | MilliporeSigma | CAT: M50834, CAS: 616-47-7 |
| 5-(Ethylthio)-1H-tetrazole | MilliporeSigma | CAT: 493805, CAS: 89797-68-2 |
| 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide | MilliporeSigma | CAT: 39391, CAS: 1892-57-5 |
| Hydrochloric acid | MilliporeSigma | CAT: 320331, CAS: 7647-01-0 |
| Sodium hydroxide | MilliporeSigma | CAT: 1310-73-2 |
| Paraformaldehyde | MilliporeSigma | CAT: 441244, CAS: 30525-89-4 |
| Sodium chloride | MilliporeSigma | CAT: 59888, CAS: 7647-14-5 |
| Citric acid | MilliporeSigma | CAT: C0759, CAS: 77-92-9 |
| TWEEN 20 | MilliporeSigma | CAT: P1379, CAS: 9005-64-5 |
| Dextran sulfate | MilliporeSigma | CAT: D6001, CAS: 9005-64-5 |
| Heparin sodium salt | MilliporeSigma | CAT: H3393 |
| 50× Denhardt’s solution | Thermo Fisher Scientific | CAT: 750018 |
| Protease K | Thermo Fisher Scientific | CAT: AM2546 |
| Deionized formamide | VWR | CAT: 97062-008, CAS: 75-12-7 |
| Tissue-Tek O.C.T. Compound | VWR | CAT: 25608-930 |
| rt.BspQI | NEB | CAT: R06445 |
| DMSO | MilliporeSigma | CAT: 276855, CAS: 67-68-5 |
| GelRed Nucleic Acid Gel Stain | Biotum | CAT: 41003 |
| PBS (10x), pH 7.4 | Thermo Fisher Scientific | CAT: 70011044 |
| UltraPure 20× SSC Buffer | Thermo Fisher Scientific | CAT: 1557-044 |
| IDTE | IDT | CAT: 11-05-01-05 |
| Kanamycin sulfate | MilliporeSigma | CAT: K1377, CAS: 25389-94-0 |
| LB Agar, powder (Lennox L agar) | Thermo Fisher Scientific | CAT: 22700041 |

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Continued

**Critical commercial assays**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| QIAGEN Plasmid Maxi Kit | QIAGEN | CAT: 12162 |
| Zymppy Plasmid Miniprep Kit | Zymo Research | CAT: D4019 |
| DNA Clean & Concentrator-25 (Capped) | Zymo Research | CAT: D4033 |
| Zymoclean Gel DNA Recovery Kit | Zymo Research | CAT: D4007 |
| ImmEdge Hydrophobic Barrier PAP Pen | Vector Laboratories | CAT: H-4000 |
| 1 Kb Plus DNA Ladder | Thermo Fisher Scientific | CAT: 10787018 |
| ProLong Gold Antifade Mountant | Thermo Fisher Scientific | CAT: P36934 |

**Deposited data**

| Raw data | Stowers Original Data Repository | http://www.stowers.org/research/publications/libpb-1743 |

**Experimental models: Organisms/strains**

| Mouse | CD1-Elite (SOPF) Mouse, male and female, P0 to 6 weeks old. | Charles River | Crl:CD1 (ICR) |
|-------|------------------------------------------------------------|--------------|--------------|
| Mouse | Fos^tm2.1CreERT2/LacZ (Fos2A-CreER,TRAP2), male and female, P0 to 6 weeks old. | The Jackson Laboratory | JAX: 030323 |
| Mouse | B6.Cg-Gt(Rosa)26Sor^tm14(CAG-tdTomato)Hay/J, male and female, P0 to 6 weeks old. | The Jackson Laboratory | JAX: 007914 |

**Oligonucleotides**

| HCR hairpin B1H1-594: CgTAAAggAAgACTCTTTCCTgTccAggAgggCAgCAAACgggAAgAg /C9-Alexa Fluor 594-3/ | Molecular Instruments | N/A |
| HCR hairpin B1H2-594: /5 TCTTCCTTACgCTCTTCCCgTTTgCTgCCCTCCTCCTACgAAAgAATgC | Molecular Instruments | N/A |
| OMP-A1P1E, CACCGGCCACT | Wu et al. (2022) | N/A |
| OMP-A1P2E, GAGCTGAAACTTCTTGCCTTCGCGCATTaaCGTCAACGACAAGC, Targeting probe for Omp | Wu et al. (2022) | N/A |
| OMP-A1P3E, GACCCCTCTAACTCTGCTACACCATTaaCGTCAACGACAAGC, Targeting probe for Omp | Wu et al. (2022) | N/A |
| OMP-A1P4E, ATGAGCGTGGGTGGCTAGAGTTGTTTaaCGTCAACGACAAGC, Targeting probe for Omp | Wu et al. (2022) | N/A |
| OMP-A1P5E, ATCTCTCAGTCCCGCTCTTCACCACTaaCGTCAACGACAAGC, Targeting probe for Omp | Wu et al. (2022) | N/A |
| OMP-A1P1O, AGAGAATCATACGTtaTGAGACAGAGGCACCGGCTCAAG, Targeting probe for Omp | Wu et al. (2022) | N/A |
| OMP-A1P2O, AGAGAATCATACGTtaTTTCCTGTCAGTTAGGCATCACA, Targeting probe for Omp | Wu et al. (2022) | N/A |
| OMP-A1P3O, AGAGAATCATACGTtaAGTCTCAGTCTCCCAGTTCAAAAA, Targeting probe for Omp | Wu et al. (2022) | N/A |
| OMP-A1P4O, AGAGAATCATACGTtaGGGGCCCATCCATCTTCCCACGTGG, Targeting probe for Omp | Wu et al. (2022) | N/A |

**Recombinant DNA**

| Plasmid: A1-2081 (P004) | Wu et al. (2022) | Addgene #184056 |

**Software and algorithms**

| FIJI | Schneider et al. (2012) | https://imagej.nih.gov/ij/ |
| Illustrator | Adobe | www.adobe.com |

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MATERIALS AND EQUIPMENT

**Alternatives:** This protocol uses Boekel Shaking Hybridizing Incubator for hybridization steps. We have also acquired good results using Stratagene PersonalHyb incubator and ACD HybEZ Hybridization System.

| MATERIALS AND EQUIPMENT | DESCRIPTION |
|-------------------------|-------------|
| **Methylimidazole buffer** | Final concentration Amount |
| 1-methlyimidazole | 1% (v/v) 1 mL |
| 3 M NaCl | 300 mM 10 mL |
| 12 M HCl | N/A to pH 8 |
| ddH₂O | variable |
| Total | N/A 100 mL |

△ CRITICAL: This buffer needs to be prepared fresh for each experiment.

△ CRITICAL: 1-methlyimidazole is a toxic chemical and should be handled and disposed of safely.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| ilastik | Berg et al. (2019) | https://www.ilastik.org/ |
| R | The R Foundation | https://www.r-project.org/ |
| Rstudio | RStudio, PBC | https://www.rstudio.com/ |
| Benchling | Benchling | https://benchling.com/ |
| BLAST | Altschul et al. (1990) | https://blast.ncbi.nlm.nih.gov/Blast.cgi |
| Other | Leica | CM3050 S |
| Humid chamber for slides | Caplug Evergreen | 240-9020-Z10 |

**100 x ETT**

| REAGENT | FINAL CONCENTRATION | AMOUNT |
|---------|---------------------|--------|
| 5-(Ethylthio)-1H-tetrazole (ETT) | 100 x | 2 g |
| ddH₂O | N/A | 1.5 mL |
| Total | N/A | 1.5 mL |

Note: The solution can be stored at −20°C for 1 year. It may be warmed up to 37°C to dissolve ETT.

△ CRITICAL: ETT is a toxic and self-reactive chemical and should be handled and disposed of safely.

| REAGENT | FINAL CONCENTRATION | AMOUNT |
|---------|---------------------|--------|
| 100 x ETT | 1 x | 100 μL |
| 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) | 300 mM | 176 μL |
| 12 M HCl | N/A | to pH 8 |
| Methylimidazole buffer | N/A | to 10 mL |
| Total | N/A | 10 mL |
\[\text{CRITICAL: This buffer needs to be prepared fresh for each experiment.}\]

\[\text{CRITICAL: EDC is a toxic chemical and should be handled and disposed of safely.}\]

### 1 M citric acid

| Reagent         | Final concentration | Amount |
|-----------------|---------------------|--------|
| citric acid     | 1 M                 | 19.2 g |
| ddH₂O           | N/A                 | to 100 mL |
| **Total**       | N/A                 | 100 mL |

*Note:* This solution can be stored at room temperature (18°C–25°C) for 1 year.

### Probe hybridization buffer

| Reagent                        | Final concentration | Amount     |
|--------------------------------|---------------------|------------|
| Deionized formamide            | 30%                 | 12 mL      |
| 20x SSC                        | 5x                  | 10 mL      |
| 1 M citric acid                | 9 mM                | 360 µL     |
| 10% Tween 20                   | 0.1%                | 400 µL     |
| 10 mg/mL Heparin               | 50 µg/mL            | 200 µL     |
| 50x Denhardt’s solution        | 1x                  | 800 µL     |
| 50% dextran sulfate            | 10%                 | 8 mL       |
| ddH₂O                          | N/A                 | variable   |
| **Total**                      | N/A                 | 40 mL      |

*Note:* This solution can be stored at –20°C for 1 year.

### Probe wash buffer

| Reagent                        | Final concentration | Amount     |
|--------------------------------|---------------------|------------|
| Deionized formamide            | 30%                 | 300 mL     |
| 20x SSC                        | 5x                  | 250 mL     |
| 1 M citric acid                | 9 mM                | 9 mL       |
| 10% Tween 20                   | 0.1%                | 10 mL      |
| 10 mg/mL Heparin               | 50 µg/mL            | 5 mL       |
| ddH₂O                          | N/A                 | variable   |
| **Total**                      | N/A                 | 1 L        |

*Note:* This solution can be stored at –20°C for 1 year.

\[\text{CRITICAL: Formamide and the buffer containing formamide are toxic and should be handled and disposed of safely.}\]

### Amplification buffer

| Reagent                        | Final concentration | Amount   |
|--------------------------------|---------------------|----------|
| 20x SSC                        | 5x                  | 10 mL    |
| 10% Tween 20                   | 0.1%                | 400 µL   |
| 50% dextran sulfate            | 10%                 | 8 mL     |
| ddH₂O                          | N/A                 | variable |
| **Total**                      | N/A                 | 1 L      |
Note: This solution can be stored at 4°C for 1 year.

STEP-BY-STEP METHOD DETAILS

Tissue preparation

© Timing: 1 h

Tissue is collected from the animal, snap frozen in O.C.T. using liquid nitrogen vapor, and stored at −80°C.

1. Sacrifice the animal by cervical dislocation.
2. Dissect and embed the tissue in O.C.T.

△ CRITICAL: Process the tissue quickly to avoid RNA degradation by the endogenous RNase.

3. Float a PCR tube rack on the surface of liquid nitrogen.
4. Place the O.C.T. embedded tissue block on the tube rack.
5. Wait until O.C.T. solidifies. The O.C.T. appears white after it solidifies.
6. Drop the O.C.T. block into liquid nitrogen until there are no more bubbles.

△ CRITICAL: Do not directly drop the tissue into liquid nitrogen when O.C.T. is still in liquid form.

7. Store the block at −80°C until use. The frozen sample can be stored up to a year.

Note: This step is optional. The tissue block can be processed directly to the next step if the user chooses not to store the sample.

Tissue sectioning and fixation

© Timing: 4 h

Frozen tissue is sectioned into 10 μm sections and mounted on slides. The tissue sections are fixed for long term storage.

8. Section the tissue at 10 μm using a cryostat at −25°C.

Note: The number of sections that can fit onto a single slide depends on the size of the tissue and the skill of the experimenter. For example, for sections 0.5 cm in length and width, 4–8 sections may be processed on one slide.

9. Dry slides at 100°C for 2 min and proceed directly to fixation.

Note: To avoid drying of the solution in the following steps, the slides are processed in a humidifying chamber, a small plastic box with added water or wet Kimwipe at the bottom, as indicated in the Figure 3.

10. Add water into the humidifying chamber.
11. Place the slides into the humid chamber. The slides should be elevated above the water at the bottom and leveled.
12. Fix slides in 4% PFA for 1 h at room temperature (18°C–25°C).
13. Wash slides 3× with PBS, 5 min each.
Figure 3. A schematic illustration of the step-by-step method details
14. Wash slides with freshly prepared 1-methylimidazole buffer for 5 min.
15. Fix slides in EDC fixative for 1 h.
16. Wash slides with PBS for 5 min.

△ CRITICAL: PFA and EDC are toxic and should be handled and disposed of safely.

Optional: Permeabilize slides in 100% methanol at room temperature (18°C–25°C) for 1 h. For eye tissue, this step improves probe penetration.

17. Remove PBS.

Pause point: The slides can be store at –80°C after removing PBS. If methanol permeabilization is used, the tissue sections can be stored directly in methanol.

**Yn-situ hybridization**

© Timing: 1–2 days

This step is the core procedure for detection of RNA using Yn-situ. The target RNA first hybridizes with targeting probes. The preamplifier then hybridizes with targeting probe pairs that bind close to each other. Fluorescent signals are developed by HCR reaction *in situ*.

18. Air dry the slides and draw a well around the tissue with ImmEdge hydrophobic barrier PAP pen.

**Note:** The hydrophobic barrier should be complete dry before the next step to ensure its proper function (Figure 4).

19. Digest the tissue with 10 μg/mL proteinase K in IDTE buffer for 10 min at 40°C.

△ CRITICAL: The optimal proteinase K concentration and digestion time need to be determined for different tissue type and age. High concentration of proteinase K results in the loss of tissue. Low concentration of proteinase K results in weak signals.

20. Wash slides with PBS for 5 min.
21. Wash slides with hybridization solution for 5 min.
22. Prepare probe mixture (4 pmol per probe) by adding 2 μL of each of the odd and even probes (2 μM stock solution) into 1 mL hybridization buffer.
23. Hybridize sections with probe mixture at 40°C, overnight (12–18 h). See troubleshooting: problems 6 and 7.
24. Remove probe mixture by flicking the slide to shake the droplet off.
25. Wash slides in probe wash buffer, 4 × for 30 min each, at 40°C.
26. Wash slides in 5× SSCT for 5 min, 3 times at room temperature (18°C–25°C).
27. Wash slides in hybridization buffer for 5 min at room temperature (18°C–25°C).
28. Add 5 μL preamplifier (20 ng/μL) into 500 μL probe hybridization buffer (final concentration 0.2 ng/μL).
29. Incubate sections with preamplifier for 5 h, 40°C.

**Note:** This step can be shortened if the user wants to check whether a new probe can produce signals. A minimum of 2 h is required to produce visible signals.

30. Wash slides in probe wash buffer for 30 min at 40°C.
31. Equilibrate sections in amplification buffer for 5 min at room temperature (18°C–25°C).
32. Anneal 2 μL of each HCR Hairpins (3 μM) in separate tubes in a thermocycler, start at 95°C for 90 s, decrease temperature 2°C/min for 35 min until the temperature reaches 25°C.  

△ CRITICAL: The two HCR hairpins need to be annealed separately.  

33. Add both annealed hairpins into 100 μL amplification buffer (1:50 dilution).  

Note: A minimum of 30 μL of diluted hairpin solution is required for one slide. For hairpin annealing, we do not recommend volume below 2 μL to avoid problem caused by evaporation in the thermocycler. The 100 μL diluted working solution prepared here from the 2 μL of stock solution is sufficient for 3 slides. If the user has more than 3 slides, the amount used need to be scaled up accordingly.  

34. Incubate sections in hairpin solutions overnight (12–18 h), in the dark, at room temperature (18°C–25°C).  

△ CRITICAL: The incubation should be performed in dark.  

35. Wash slides in 5× SSCT for 5 min, 3 times.  
36. Mount the coverslip with ProLong gold antifade mounting media.  

¶¶ Pause point: the slides can be stored at 4°C for a month before imaging.  

Imaging  

☉ Timing: 2 h  

This step is for imaging the samples prepared in the above steps. We will provide general guidance for this process, not specific parameters because the users likely have different microscopes and the specific optics, lasers, age of the microscopes will influence how the parameters are determined.  

37. View the signals under an epifluorescence microscope with a low magnification lens (any 10× or 20× lens) and locate the cells of interest to image. See troubleshooting: problems 8–11.  
38. Image the slides with a confocal microscope using a high magnification lens (Plan-Apochromat 40×/1.4 Oil DIC M27 or Plan-Apochromat 63×/1.4 Oil DIC M27 for Zeiss microscopes, and HC PL APO 100×/1,40 OIL STED WHITE for Leica microscopes).  

Note: Empirically determine the laser power to avoid photobleaching. Take Z-stack images that cover the entire cell of interest. Use a Z step distance that is half of the z resolution. Save both individual z images and the maximal intensity projection image. When multiple areas need to be imaged, use a tile scan to generate a larger image.  

Note: We have used a Leica SP8 confocal microscope with HyVolution (Borlinghaus and Kappe, 2016) and a Zeiss 780 confocal microscope when super resolution imaging is not
performed. We encourage the users to use super resolution imaging when the density of the puncta within the cell of interest is too high to be resolved by regular confocal microscope.

⚠ CRITICAL: To facilitate quantification of foci, the user should acquire the image with sufficient resolution. This can be achieved by zooming into individual punctum and counting the number of pixels contained by the punctum. Users should avoid using the resolution below 5 pixels/μm as it is hard to distinguish individual puncta under this resolution (Figure 5).

Note: The intensities of the brightest and weakest puncta should be within the detection range of the sensor. The users should avoid overexposing and underexposing the signals regardless of the microscope being used. In most modern fluorescent microscopes, histograms of the pixel intensities are plotted. One can adjust system gain, background threshold, and laser power to set a dynamic range that has the pixel intensity to be well distributed with few pixels beyond this range. Some software, such as Zeiss Zen, has a “range indicator” to show overexposed and underexposed pixels.

39. Export the images as tiff files for analysis.

EXPECTED OUTCOMES

Figure 2 panels B and C show expected gel digestion patterns. Examples of Yn situ results can be found in the original publication (Wu et al., 2022).

QUANTIFICATION AND STATISTICAL ANALYSIS

Manual spot counting in FIJI

We describe the process of counting fluorescent foci in FIJI to quantify the expression level of targeted RNA. For more information on using FIJI, see https://imagej.net/learn/. For users who do not have access to high resolution confocal microscope or super resolution microscope, one can image the experiment and control samples with an epifluorescence microscope with the same settings including excitation intensity, camera exposure time, detector gain, and filters, then quantify the average fluorescence intensity within the cells like in the traditional in situ hybridization methods whose signals are not dot-like.

1. Import the images to FIJI by using function “File/Open...” (Ctrl + O).
Alternatives: The images can be opened by dragging into FIJI directly.

2. Open ROI manager by using function “Analyze/Tools/ROI manager…”.
3. Select the “Multi-point tool” by right clicking the “Point tool” icon on the tool panel.
4. Click on all puncta within a cell.

Note: Cell boundaries are difficult to define based on fluorescent puncta and nuclear staining alone. To determine the boundaries more accurately, we recommend labelling the cells with other tools. For example, the cells can be sparsely labeled with genetic tools. In the corresponding paper, we used TRAP2;Ai14 mice to label the individual neurons by taking advantage of the sparse expression pattern of c-Fos during development. Similar methods such as adenovirus and AAV can also be used.

5. Press “T” on the keyboard. This step adds the punctum as a ROI in the ROI manager.
6. Repeat steps 4 and 5 until all cells of interest are analyzed.
7. In the ROI manager, click “More>>/List”.
8. In the resulting window, save the list into a “.csv” file by using “File/Save as” (Ctrl + S).
9. Save the ROI selection by clicking “More>>/Save” in the ROI manager.

Note: The resulting count table can be further analyzed using statistical software such as R and Origin. “Name” column represents the names of the cells. “Points” column represents the number of puncta in the given cell. Additional information includes the position of the cell (“X” and “Y” columns) and the area of the cell (“Width” and “Height”). While the original image is open, clicking the cells in the ROI manager will re-select all the puncta selected for that cell. This quantification is usually performed on Z-projected 2-D images. High density puncta may complicate quantification. In this case, one can analyze individual Z-stack images. One should avoid overcounting by ensuring that puncta with the same X-Y coordinates are not counted more than once.

Semi-automated spot counting using Ilastik and FIJI
This step uses Ilastik, an interactive machine learning based image analysis software, to identify the fluorescent foci in the image, and quantify them with higher throughput (Figure 6). For more instructions on using Ilastik, see https://www.ilastik.org/documentation/index.html.

10. Open Ilastik.
11. Select “Pixel Classification” to start a new project.
12. Name the project and save the project in a folder.
13. In the Input Data tab, click “Add New…” button.
14. Select 1–2 images for training.
15. In the “Feature Selection” tab, click “Select Features…” button.
16. Select all the features.
17. In the “Training” tab, click “Label1”.
18. Draw 5–10 dots over the fluorescent puncta.

Note: It is beneficial to select the puncta with different intensities and densities.

19. Click “Label2”.
20. Draw 2–5 lines over background.

Note: It is beneficial to draw over the area closely surrounding the puncta.

21. Click “Live Update”.
22. Examine if the two colors are masking the puncta and background correctly. If not repeat 9–12 until the masking is accurate.

**Note:** The user should pay attention to whether the boundaries of the puncta are split. Non-split puncta will be counted as one in the later steps in FIJI. The trained model can be reused.

23. In the “Prediction Export” tab, select “Simple segmentation” option from the source drop-down.
24. Click “Choose Export Images Settings...”.
25. Choose “tiff” format from the “format” drop-down.
26. Click “ok”.
27. In the “Batch processing” tab, click “Select Raw Data Files...”.
28. Select all the images to be processed.
29. Click “Process all files” button.
30. Import the exported images containing the segmented puncta to FIJI.
31. Open ROI manager by using function “Analyze/Tools/ROI manager...”.
32. Threshold the images into binary form using function “Image/Adjust/Threshold...”.
33. Choose a method from the drop-down menu that accurately selects the puncta.
34. Click “Apply”.
35. Separate connected puncta using function “Process/Binary/Watershed”.
36. Use “Freehand” tool to select a cell of interest.
37. Select the puncta within the cell by using function “Process/Find Maxima...”.
38. In the popup window, choose “Point selection” as output type.
39. Select “Preview point selection”.
40. Change “Prominence” value until all the puncta are selected.
41. Click “OK”.

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**Figure 6. Example screenshots of the puncta quantification process**

(A) The image used to perform the quantification.
(B) The same image after manual counting with multi-point tool at step 4.
(C) Screenshot from Ilastik showing the training step 12. Blue lines are drawn over the background. Yellow lines are drawn over the puncta.
(D) The appearance of the image after step 25.
(E) The appearance of the image after step 32. Scale bar, 5 μm.
42. Press “T” on the keyboard.
43. Keep doing steps 35–41 until all cells of interest are analyzed.
44. Follow steps 16–18 in the manual counting method to acquire the count table.

**LIMITATIONS**

The probe design method in this protocol is time consuming and there is no guarantee that the probes will always generate specific and robust signals. The probes need to be tested in experiment to know their performance. This protocol is optimized for neonatal olfactory epithelium, eye, and brain tissue. We have not tested the protocol using other tissue types and at other developmental stages. The users may consider optimizing the length of fixation, the strength of proteinase treatment, and adding the methanol treatment. This protocol is developed using thin cryostat sections. Additional optimization is needed for thick sections or paraffin embedded tissue sections.

**TROUBLESHOOTING**

**Problem 1**
When measuring the DNA concentration of the maxiprep, no or little DNA is detected by the nanodrop (before you begin, step 12, cc).

**Potential solution**
This may be caused by three reasons. First, wrong antibiotic is used during the inoculation. Use kanamycin rather than ampicillin. The culture will not look cloudy after overnight inoculation if a wrong antibiotic is used. Second, the precipitated plasmid pellet is lost during the removal of supernatant. There should be an opaque pellet after step u. When the DNA purity is high, the pellet looks translucent. The users should be careful when removing the supernatant and keep the pellet. Third, a large volume of culture growing directly from glycerol stock sometimes does not work well. Adding intermediate steps, such as streaking out colonies or growing with a smaller starter volume might also help.

**Problem 2**
After running the gel using the digestion product, no or little DNA is observed (before you begin, step 14, f).

**Potential solution**
To identify the problem, we provide several diagnosis methods and solutions to various causes of the issue.

DNA concentration is not accurately determined after the maxiprep. If there is significant among of RNA left in the prep, it can cause miscalculation of the DNA concentration and result in insufficient amount of DNA used in the digestion. The users may first check the expiration date of the P1 buffer, and whether RNase is added to it. Insufficient RNA removal can happen when the RNase in the P1 buffer has expired or not present. If this is the case, the problem can be solved by using P1 buffer containing fresh RNase and storing P1 with RNase at 4°C. The users can then measure the DNA absorption spectrum by Nanodrop. A 1.8 ratio between the absorption at 260 nm and 280 nm generally indicate good quality DNA. The users can then run the maxiprep product on a gel to examine whether contaminants exist. A strong signal around 100–200 bp area would indicate large amount of residual RNA. A strong signal around the loading vial indicates genomic DNA contamination. Second, too much DNA is added to the column. If the QIAGEN tip is overloaded, the yield can be low. To avoid adding too much DNA, less culture should be used. Third, ethanol is not added to the Zymo wash buffer. This will result in DNA being washed away from the column.

**Problem 3**
The digestion product forms continuous smear rather than discrete bands in the gel (before you begin, step 14, f).
Potential solution
This may be caused by loading too much DNA. The buffering capacity of sodium borate buffer is limited. When too much DNA is loaded and run in this buffer, the bands tend to elongate and become smears. This can be solved by loading the digestion product into multiple wells to reduce the DNA loaded into individual well.

Problem 4
When examine the ssDNA on the agarose gel, the bands look very bright and correspond to the sizes of possible dsDNA products resulting from partial melting. No or weak band corresponding to the size of preamplifier is observed. The band have a similar size of the undigested plasmid (before you begin, step 14, f).

Potential solution
This may be caused by two reasons. First, the sample was not fully desalted. Trace amounts of salt from NEBuffer r3.1 can inhibit the DMSO mediated melting. This can be solved by using the column based desalting method and two rounds of washing using wash buffer. Second, DMSO concentration is too low. The DMSO:sample ratio should be above 3:2.

Problem 5
ssDNA bands are observed when running the digestion product, but no ssDNA is observed in the quantification gel (before you begin, step 15, g).

Potential solution
This is normally caused by agarose being not fully dissolved. DNA remains in the undissolved agarose gel and is not eluted. This can be solved by fully melting the agarose gel using more ADB buffer. Using more ADB buffer and vigorous shaking can speed up the melting.

Problem 6
Tissue disappears from the slide after overnight hybridization (step-by-step method details, step 23).

Potential solution
The proteinase K treatment may be too harsh. When this happens, one can lower the concentration of proteinase K, use a shorter incubation time, or do both. If the user wants to optimize the proteinase digestion, we recommend the user test a series of different proteinase concentration and incubation time. The user may start with 100 μg/mL, 10 μg/mL, 1 μg/mL, 0.1 μg/mL, 30 min, 10 min, and 5 min, then proceed with the conditions where the tissue does not show obvious damage, compare the end results to determine the optimal condition.

Problem 7
The hybridization solution covering the tissue is dried after overnight hybridization (step-by-step method details, step 23).

Potential solution
Enough water should be added to the bottom of the humid chamber. The users should also make sure the hydrophobic boundary is working properly. The hybridization solution should not spill outside of the boundary after being added. Other common problems are the use of expired ImmEdge pen and not waiting until the boundary is dried before adding solutions.

Problem 8
No signal is observed after the procedure (step-by-step method details, step 37).
Potential solution
There are many reasons that can result in failed experiment. We list a few common problems we have experienced.

- Preamplifier is not successfully synthesized. The user may run the preamplifier used in the experiment on a gel similar to the one shown in Figure 2C. It is also important to examine the preamplifier in a gel at the synthesis step rather than simply measure the concentration using a Nanodrop. A high reading in the Nanodrop does not equal to high quality ssDNA. The preamplifier should be single stranded, contain 20 repeats of the HCR initiator, and of high concentration and purity. When too little or double stranded preamplifier is added to the Yn situ, no signal is developed.

- Another common problem is RNA degradation. It is hard to identify if this is the issue or which step this issue appears without going through complicated RNA extraction and microchip analysis of the RNA content from the tissue. In Yn situ, the RNase mediated degradation is likely coming from before step 7. The user should proceed directly to the following steps once the tissue is sectioned, mounted onto the slide and placed at room temperature.

- HCR hairpins are annealed in the same tube. When the HCR hairpins are heated in the same tube, they will bind to each other directly. No HCR reaction will happen on the tissue.

- Probe does not work. This can be identified by several diagnosis step. First, the user may check the expression pattern of the gene from literature and RNA-Seq data if it exists to make sure the gene is expressed by the tissue or cell type. Second, the user may try to perform Yn-situ using the same tissue but with a control probe. The control probe is a probe that works before, a probe designed against a highly abundant gene, or a probe designed with the same sequence used by another in situ method. It is possible for the designed probes to not bind to the target. The users can design different probes or increase the number of probe pairs to include more probes.

Problem 9
For genes with known expression pattern, signals can be seen in the regions where the gene is not expected to be expressed. Puncta are observed across the tissue with no specific patterns but are clearly distinguished from the background (step-by-step method details, step 37).

Potential solution
This may be caused by two reasons. First, too much primary probe was used. The correct concentration of primary probe in the hybridization solution is 4 nM. This concentration is for individual oligos, not for the oligo mixture, which is 40 nM when 5 pairs of probes are used. Second, the designed probe is not specific. Different probes should be designed when this is the case.

Problem 10
Specific signals are observed only in the cells or regions where the target gene is known to express. The signals are above the background but the signal to background ratio is not as good as described in the original paper. The signals are too weak to image (step-by-step method details, step 37).

Potential solution
Weak signals may result from insufficient hybridization. This can be improved by increasing the proteinase K treatment strength and adding the methanol permeabilization step. Another possibility is that the target probes do not hybridize efficiently. One can add more probes targeting to different regions of the same transcript.

Problem 11
Bright fluorescence comes from all areas in the tissue. These background signals do not appear to restrict in specific cells nor regions of the tissue. The fluorescence is not puncta-like but homogenous (step-by-step method details, step 37).
Potential solution
The hybridization solution may have dried during hybridization. We find the probes cannot be washed away efficiently when the solution is dried. It can also be cause by unspecific probes that bind to many species.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact C. Ron Yu (cry@stowers.org).

Materials availability
Plasmids used in this study are available from Addgene with the plasmid numbers listed in the key resources table.

Data and code availability
All original data are available through Stowers Institute for Medical Research Original Data Repository (http://www.stowers.org/research/publications/libp-1743). No computer code was used for analysis.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101686.

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AUTHOR CONTRIBUTIONS
Conceptualization, Y.W.; investigation, Y.W.; writing – original draft, Y.W.; writing – review & editing, Y.W. and C.R.Y.; funding acquisition, C.R.Y.; supervision, C.R.Y.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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