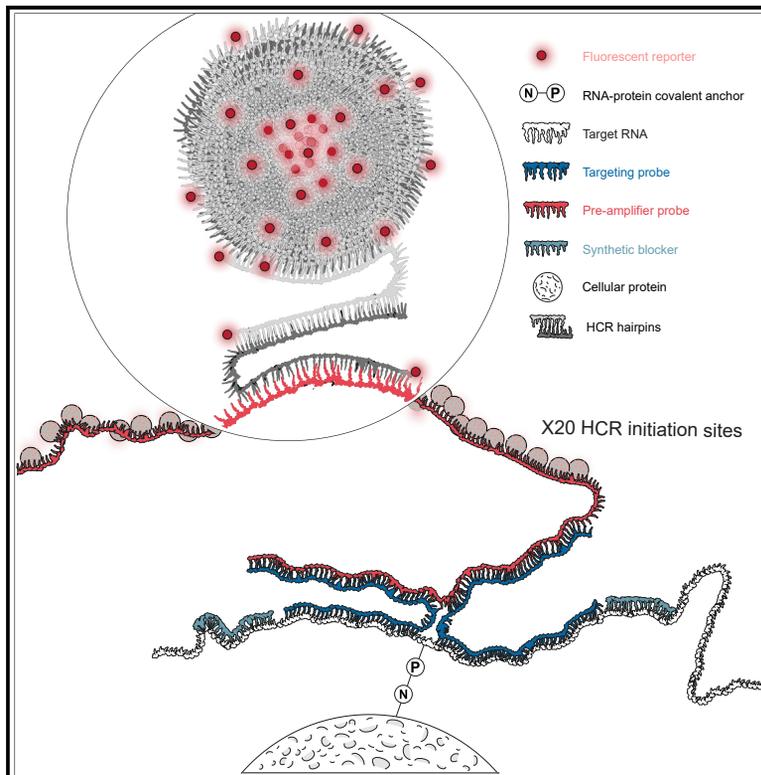


Robust and sensitive *in situ* RNA detection using Yn-situ

Graphical abstract



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In brief

Wu et al. develop Yn-situ, an *in situ* RNA detection method that is easy to use and cost effective. The authors introduce a preamplifier and a fixation step to improve the sensitivity and robustness of *in situ* hybridization methods.

Highlights

- Introduction of preamplifier improves detection of cellular RNA
- Preamplifier improves sensitivity and signal-to-noise ratio with reduced puncta size
- Yn-situ is robust, cost-effective, and easy to use
- Simultaneous detection of protein and RNA is achieved



Report

Robust and sensitive *in situ* RNA detection using Yn-situYunming Wu,^{1,3,*} Wenjing Xu,¹ Limei Ma,¹ Zulin Yu,¹ Yongfu Wang,¹ and C. Ron Yu^{1,2,4,*}¹Stowers Institute for Medical Research, Kansas City, MO 64110, USA²Department of Anatomy and Cell Biology, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160, USA³Present address: Department of Biology, Howard Hughes Medical Institute, Stanford University, Stanford, CA 94305, USA⁴Lead contact*Correspondence: yunmingw@stanford.edu (Y.W.), cry@stowers.org (C.R.Y.)<https://doi.org/10.1016/j.crmeth.2022.100201>

MOTIVATION *In situ* hybridization is widely used for detection of cellular RNA. Modern methods using short oligonucleotide probes have eliminated the use of long RNA probes, but the requirement of many short probes has limited the application to long transcripts and incur high cost when proprietary reagents are used. In the current study, we aimed to improve the robustness, sensitivity, and ease of use and to reduce the cost of current single-molecule fluorescent *in situ* hybridization methods.

SUMMARY

We describe a cost-effective, highly sensitive, and quantitative method for *in situ* detection of RNA molecules in tissue sections. This method, dubbed Yn-situ, standing for Y-branched probe *in situ* hybridization, uses a single-strand DNA preamplifier with multiple initiation sites that trigger a hybridization chain reaction (HCR) to detect polynucleotides. By characterizing the performance of this method, we show that the Yn-situ method, in conjunction with an improved fixation step, is sensitive enough to allow detection of RNA molecules using fewer probes targeting short nucleotide sequences than existing methods. A set of five probes can produce quantitative results with smaller puncta and higher signal-to-noise ratio than the 20-probe sets commonly required for HCR and RNAscope. We show that the high sensitivity and wide dynamic range allow quantification of genes expressed at different levels in the olfactory sensory neurons. We describe key steps of this method to enable broad utility by individual laboratories.

INTRODUCTION

Detecting nucleotide acid using *in situ* hybridization has been an important methodology in biological sciences since it was first invented in 1968, and it remains the gold standard of RNA detection in the cell (Gall and Pardue, 1969). Over the decades, various techniques have been developed to improve sensitivity, specificity, resolution, and quantification and to simultaneously detect multiple targets (Chen et al., 2015; Choi et al., 2010; Femino et al., 1998; Ishii et al., 2004; Shah et al., 2016; Wang et al., 2012, 2018). The primary challenge to *in situ* detection of polynucleotides is multi-fold. First, RNA is unstable in biological samples because of the ubiquitous presence of RNases. Degraded RNAs can lead to diffusive signals that increase background noise. Second, hybridization conditions may vary depending on the length and composition of the probes. The length of the target also limits its detectability. Small RNA and short open reading-frame transcripts have fewer specific targeting sequences. Third, there usually is a trade-off between sensitivity and specificity. For example, high-intensity signal from methods

based on catalyzed reporter deposition (CARD) is usually accompanied by high background noise (Ishii et al., 2004). Detection using directly labeled nucleotide acids has high specificity, but the signal is relatively weak (Trcek et al., 2012). To improve probe stability and specificity, short DNA oligos, especially split probes, have been adopted in both RNAscope and hybridization chain reaction (HCR) protocols (Choi et al., 2018; Wang et al., 2012). These new methods also employ multiple probes for the same target to improve sensitivity (Trcek et al., 2012), enhance signals through amplification (Larsson et al., 2010), or both (Choi et al., 2018; Wang et al., 2012). These significant improvements have allowed the quantification of single molecules using fluorescent signals. However, the high number of specific probes required by these methods incurs high costs and can be limiting, because only long polynucleotide molecules can provide sufficient target sites. Here, we present a new, cost-effective method of *in situ* hybridization that requires significantly fewer probes while achieving equal or superior sensitivity, specificity, spatial resolution, and dynamic range compared with other contemporary methods.



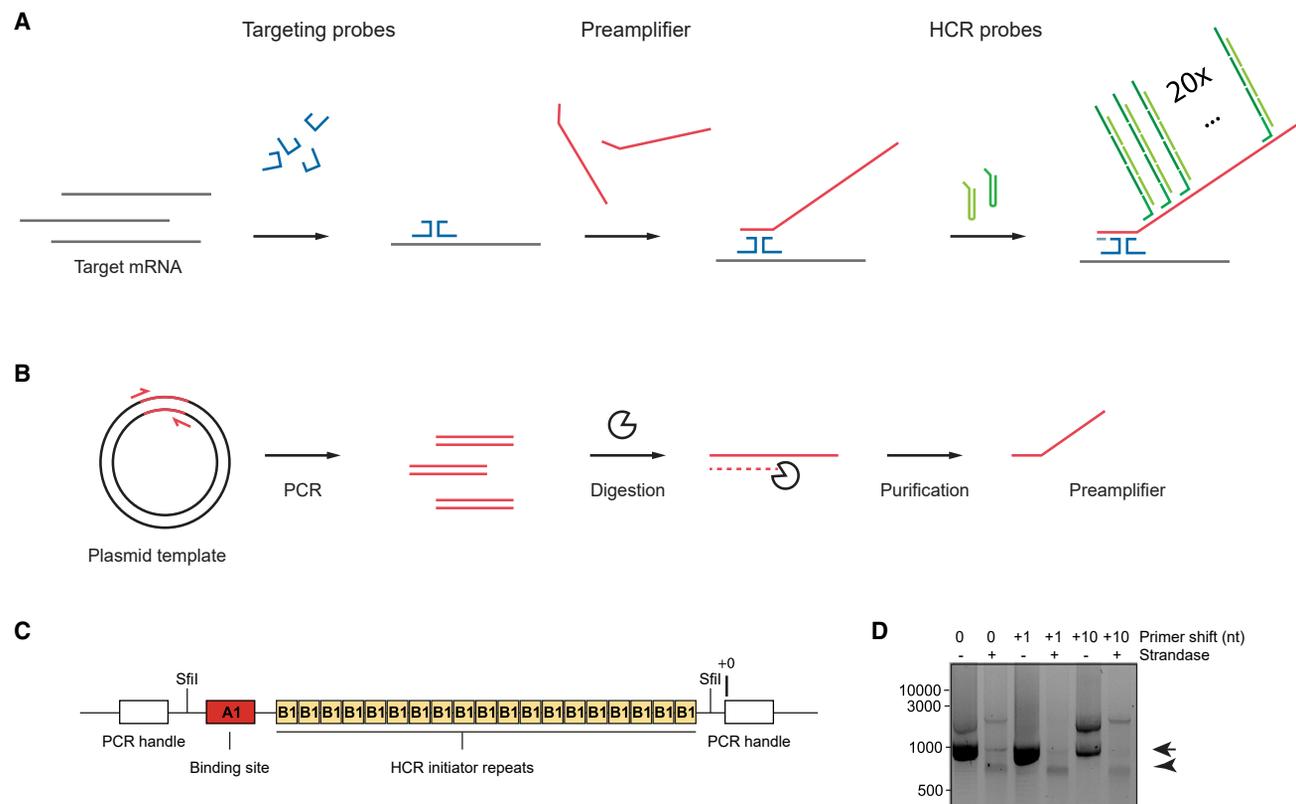


Figure 1. Schematic illustration of Yn-situ hybridization

(A) Steps involved in the hybridization processes. The target RNA is fixed to the cellular proteins by covalent bonds to prevent degradation. A pair of targeting probes (dark blue) recognizes a consecutive 52-nt sequence of the target. A preamplifier probe (red) recognizes the tail sequences only when the two targeting probes are aligned next to each other with head-to-head orientation. Each preamplifier probe carries 20 HCR initiation sites. Upon incubation with fluorescently labeled metastable HCR hairpins (green and dark green), the HCR initiation sites trigger enzyme-independent amplification through HCR, resulting in bright fluorescent signals.

(B) Process of synthesizing the preamplifier. PCR amplicons are digested with strandase to release single-stranded preamplifier probes.

(C) Schematic illustration of the preamplifier probe. The preamplifier probe contains a targeting probe-binding site (A1) and 20 HCR initiator sequences (B1). The sequence is flanked by two *SfiI* sites for verification purpose. The sequence including the *SfiI* sites is flanked by two primer-binding sites that allow for exponential amplification using PCR.

(D) Preamplifier probe synthesis with different PCR primers. The expected PCR product and ssDNA probe are indicated by arrow and arrowheads, respectively.

RESULTS

Design of Yn-situ

The design of Yn-situ and general procedures are illustrated in Figure 1. The method has improved upon previous approaches in three aspects. First, we have adopted a preamplifier design to allow a single probe to amplify signal multi-fold. In third-generation HCR, multiple pairs of target probes are used to increase sensitivity. Each pair of target probes hybridizes specifically to their binding sites on the target mRNA. The two probes are adjacent to each other such that the un-hybridized portion of the oligos forms an initiator to enable cooperative initiation of the HCR reaction. We have modified this design by redesigning the probe pairs such that the un-hybridized sequence is targeted by the preamplifier. The preamplifier probe, when hybridized to the target-specific probes, forms a Y-shaped structure, hence the name of this method (Figure 1A). Each preamplifier carries 20 initiator repeats, which can simultaneously trigger 20 HCR reactions (Figures 1A and 1C). This design maintains the use of

short oligo sequences (52 nt) and paired probes as in HCR to improve specificity. On the other hand, the use of a preamplifier significantly increases sensitivity while avoiding the requirement of many probe pairs to generate significant signals. Second, we have designed a strategy to generate preamplifier that can be readily made with basic molecular biology (Figures 1B–1D).

Design and synthesis of the preamplifier probe

The preamplifier contains a binding site to the paired probes and 20 repeats of HCR initiators (Figure 1C). Although the design is simple, it presents a challenge to generate the oligo. Because each preamplifier is approximately 1 kb long and contains repetitive sequences that serve as initiation sites, it is difficult to synthesize directly. We designed a plasmid that contains the double-stranded version of the preamplifier sequence (Figure 1B). The double-stranded preamplifier sequence on the plasmid is flanked by sequences recognized by the restriction enzyme *SfiI* and a pair of PCR handles that are used to amplify the fragment. *SfiI* digestion can be used to verify the total length

of the preamplifier. Double-stranded amplicons are generated using asymmetric PCR primers (one with 5'-phosphate, one without). The 5'-phosphate on the reverse primer allows the strandase to digest the antisense strand and produce the single-stranded preamplifier. An alternative approach using nickase is described in the [STAR Methods](#) section.

To determine the optimal condition and kit for PCR amplification of the preamplifier, we first tested five commercial PCR polymerases: PrimeSTAR HS DNA polymerase, KAPA HiFi DNA polymerase, Q5 high-fidelity DNA polymerase, LongAmp Taq DNA polymerase, and GoTaq long PCR master mix. We found that KAPA HiFi and Q5 polymerase generated non-specific products. PrimeSTAR and GoTaq long PCR mix generated the desired band at a low yield. LongAmp polymerase generated the desired band with the highest yield ([Figure S1A](#)). We chose LongAmp for further optimization. We tested various PCR parameters, including annealing temperature, primer concentration, template concentration, and the choice between two-step or three-step PCR for LongAmp. We found that the desired product can be generated at almost any annealing temperature tested except 72°C ([Figures S1B and S1C](#)). The three-step PCR generated some smear bands below the target band ([Figure S1C](#)). This did not affect the experiment, since the band was further purified by gel extraction. The optimal primer concentration was 0.5 μM. The optimal template concentration was 0.05 ng/μL among the conditions tested ([Figure S1D](#)). Finally, we found that the strandase activity was influenced by the sequences at the priming site. This influence was not clearly understood. We therefore empirically tested a series of reverse primers to determine the optimal site for strandase digestion. We identified that priming at +1 and +40 nt away from the initial PCR handle site (+0) produced the most complete digestion ([Figure S1E](#)).

Note that the PCR amplification process produced preamplifiers containing the PCR handle sequences. This may increase background noise if these parts of the probe bind to complementary sequences in the cell. To reduce this background, short oligos corresponding to the PCR handle sequences were used at 10 times the preamplifier concentration as a blocking reagent in further experiments.

Determining the optimal condition for hybridization

For Yn-situ, we have adopted a chemical modification of cellular RNAs to reduce RNA degradation and effectively improve staining quality. Previous studies had identified carbodiimide fixatives that effectively crosslink the phosphate group of the cellular RNA with amine groups from the proteins ([Pena et al., 2009](#); [Sylwestrak et al., 2016](#)). We thus developed a protocol to irreversibly immobilize RNA molecules by crosslinking them to formaldehyde-fixed proteins using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). In 6-month-old tissue stored at -80°C, we observed a dramatic improvement in signals detected by our method ([Figure S2](#)).

We next performed a series of tests to determine the optimal experiment condition for Yn-situ using probes against olfactory marker protein (Omp), a marker gene expressed by the mature olfactory sensory neuron (mOSN; [Figure 2](#)). Since the probe design and hybridization condition were the same as HCR, we used the same concentration of target probes specified by the commercial

product protocol. We found that five pairs of target probes of Yn-situ produced highly specific and stronger signals than the 20-probe pairs for HCR ([Figures 2A and 2B](#)). Increasing the number of probe pairs further increased signal intensity ([Figures S3A and S3B](#)). However, we also observed signal saturation, which made it more difficult to resolve single fluorescent spots. The variation of intensities associated with individual spots also increased. Using the five probe pairs, we varied the concentration of the preamplifier. Signals can be detected with preamplifier concentration as low as 0.002 ng/μL, but lowering preamplifier concentrations significantly reduced the number of spots detected ([Figures 2C and 2D](#)). There was no signal produced when the antisense sequence of the preamplifier was used, indicating that the signals produced by Yn-situ were highly specific ([Figure 2E](#)).

We sought to determine the minimal pairs of targeting probes required for producing visible signals. Strong signals were detected using three pairs of probes ([Figure 2F](#)). Even one pair of targeting probes produced signals for Omp, but the signal was weak and not suitable for quantitative studies ([Figure 2G](#)). The best results were achieved at 0.2 ng/μL preamplifier at room temperature for HCR with 60 nM of each hairpin ([Figure 2B](#)). Hairpin concentration lower than 60 nM did not produce any visible signal (data not shown). Finally, we tested the impact of HCR reaction time on the signals ([Figures S3C and S3D](#)). Within 30 min, we detected signals in the tissue. Signal intensity increased with increasing incubation time but did not further increase after overnight.

Characteristics of the Yn-situ signals

We compared the signals generated by Yn-situ with those by conventional CARD reactions and the contemporary methods ([Figure 3](#)). As recommended by manufacturers, 20 probe pairs were used for RNAscope and third-generation HCR *in situ*. We conducted super-resolution microscopy using Leica Hyvolution, which is a deconvolution method based on the point-spread function (PSF) to allow high-speed multicolor imaging with a resolution down to 140 nm ([Borlinghaus and Kappel, 2016](#)). Unlike the diffuse signals developed using alkaline phosphatase (AP; [Figure 3A](#)) and horseradish peroxidase (HRP; [Figure 4A](#)), the fluorescent signals generated by Yn-situ were small puncta like those found with HCR and RNAscope ([Figure 3A](#)). Moreover, unlike non-specific signals from AP or HRP reactions, few signal puncta were observed for Yn-situ in the cells that did not express the target gene. We calculated the signal-to-noise ratio (SNR) of the four methods ([Figure 3B](#)). Because the experimental conditions are different for each method, for comparison, we normalized the signal intensity and used the variance of background signals to calculate SNR. We found that AP-generated signals had the highest relative background noise; RNAscope and Yn-situ had the narrowest distributions of background noise signal. They also had similar distributions of signals detected in the puncta, which were tighter than those generated by AP and HCR. Yn-situ had the highest SNR even with fewer pairs of targeting probes.

We also determined the size of the fluorescent foci for the three contemporary methods ([Figure 3C](#)). The puncta sizes for Yn-situ were significantly smaller than those for RNAscope. They were also smaller than the puncta from third-generation HCR, although the difference was not statistically significant. In previous

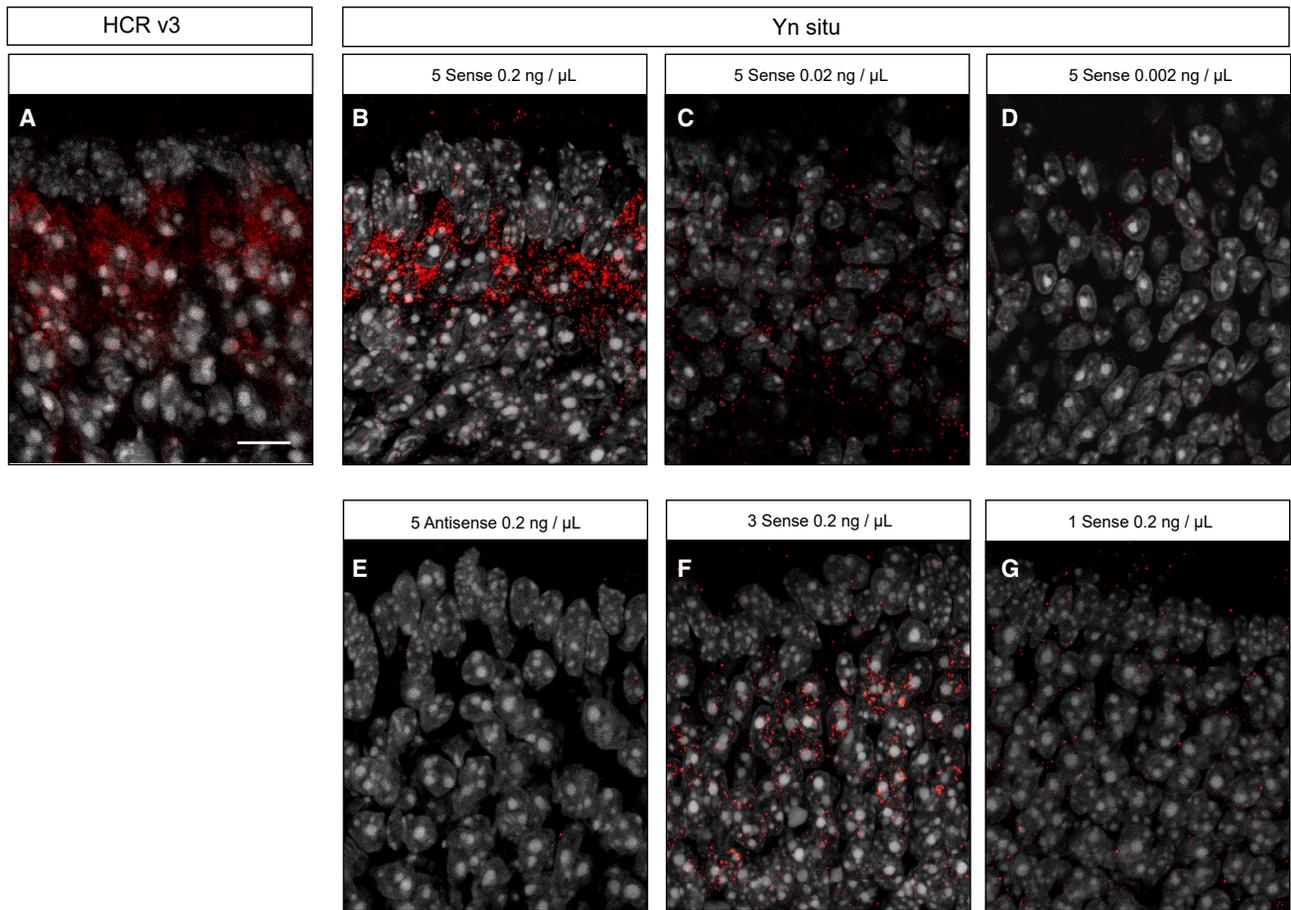


Figure 2. Optimal condition for Yn-situ hybridization

(A) Representative image showing the spatial localization of Omp mRNA detected in the olfactory epithelium (postnatal days 0–3) by third-generation HCR *in situ* hybridization.

(B–D) Representative images showing the spatial localization of Omp mRNA detected in the olfactory epithelium by Yn-situ hybridization using five probe pairs with different preamplifier concentrations.

(E) Representative image showing the Yn-situ hybridization signals using an antisense preamplifier probe as a negative control.

(F and G) Detection of Omp signal using three pairs (F) and a single pair (G) of probes. Scale bar, 10 μm .

publications, single puncta have been inferred as from single RNA molecules (Choi et al., 2018; Femino et al., 1998; Wang et al., 2012). We further analyzed the distribution of the brightness of the puncta (Figures S3B and S3D). The intensities fell into normal distributions (Shapiro-Wilk normality test, $p = 0.18$; Figure S3D), as expected from stochastic interactions between the probes and the targets. The brightest signals were well below 2-fold of the mean. If some puncta were from more than one molecule, we would expect many puncta with two or more folds of the intensities of the average puncta, and the signals would follow a multimodal distribution. This analysis indicated that the single puncta detected with Yn-situ were likely to be from single mRNA molecules, which was in line with the consensus of the field.

We performed Yn-situ hybridization experiments against several genes that had variable expression levels to test the dynamic range (the amount of RNA puncta that can be detected from a cell). In the olfactory epithelium, all mature OSNs expressed Omp at the intermediate level. Individual olfactory

receptor (OR) genes were expressed at high levels by a very few neurons. A small population of cells expressed the gene Cochlin at a moderate level. In CARD experiments, signals amplified from enzymatic reactions often obscured the quantity of RNA in these cells. The signal in neurons expressing an OR appeared similar to those expressing Cochlin (Figure 4A). Yn-situ, on the other hand, allowed a wide range of expression levels of RNA transcription to be quantified (Figures 4B–4D). Cochlin signals were comparatively lower than that of Omp. For Olfr855, we detected nearly 400 puncta in a single cell without the signals being overcrowded. This result demonstrated a high dynamic range of detection by Yn-situ.

DISCUSSION

In this study, we conducted a proof-of-principle study of the Yn-situ method using perinatal olfactory epithelium sections. Although it has not been tested against other tissues or at

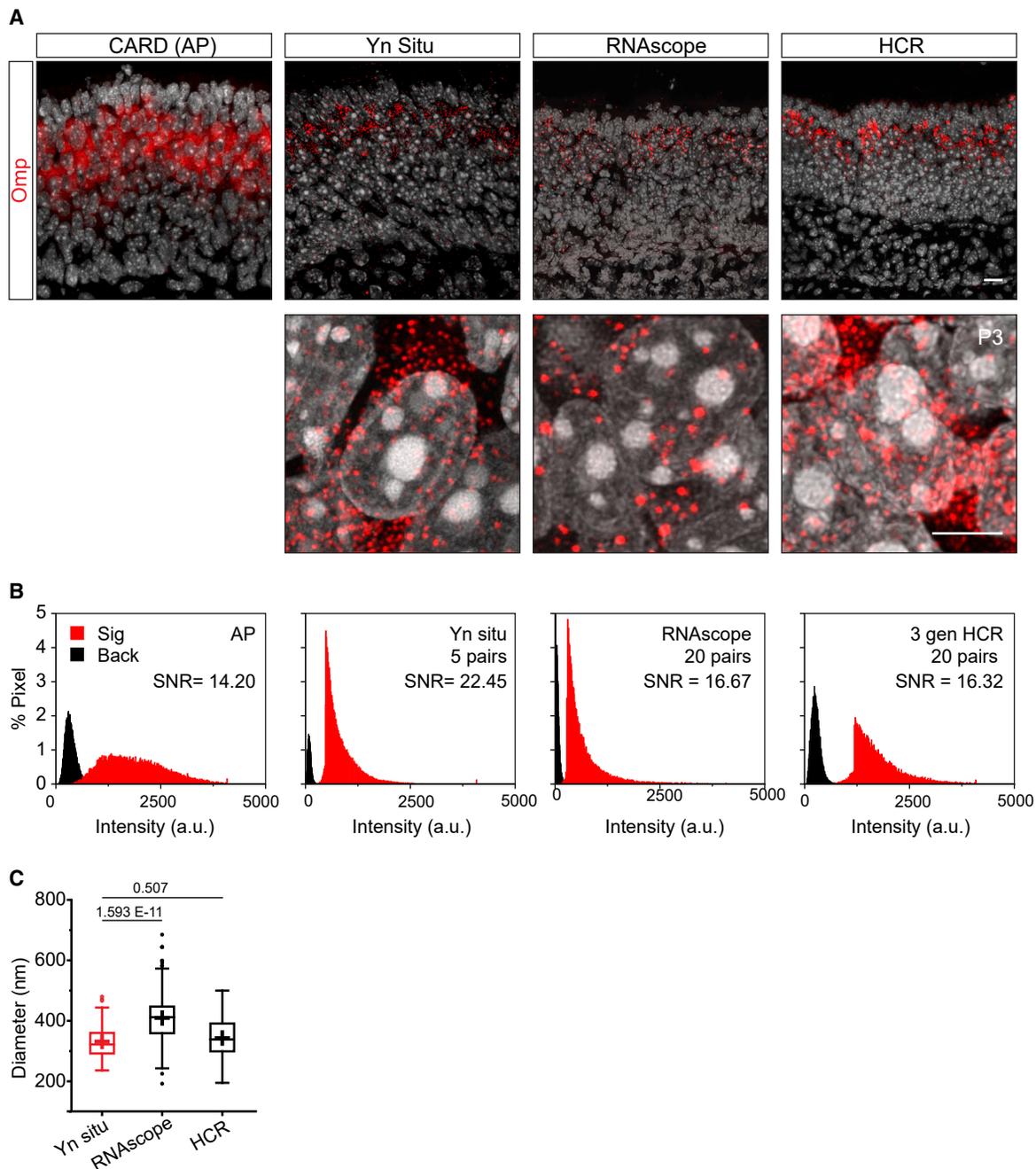


Figure 3. Characterization of the Yn-situ signals

(A) Representative images showing the spatial localization of Omp mRNA detected in the olfactory epithelium by conventional FISH using AP (left), Yn-situ, RNAscope, and HCR. High-magnification pictures are shown at the bottom. Scale bar, 10 μ m.

(B) Histograms of signal strength from pixels in the puncta (red) and background (black). Signal-to-noise ratio (SNR) of the methods were calculated accordingly. The pixel intensity was normalized between 0 and 4,095 for comparison between different experiments.

(C) Boxplot showing the puncta size measurements for the three methods examined. Statistical analysis was performed using one-way ANOVA. p values are shown above the boxes. N = 106, 107, and 105 for HCR, RNAscope, and Yn situ, respectively.

different developmental time points, the data we have collected demonstrate that the method can produce high-quality and quantitative detection of RNA species. The size and discrete distribution of single-signal puncta are similar to what is found in

HCR and RNAscope, suggesting that they are likely from single RNA molecules.

Yn-situ offers five advantages over current approaches. First, the Yn-situ signal is highly specific. Few puncta were observed in

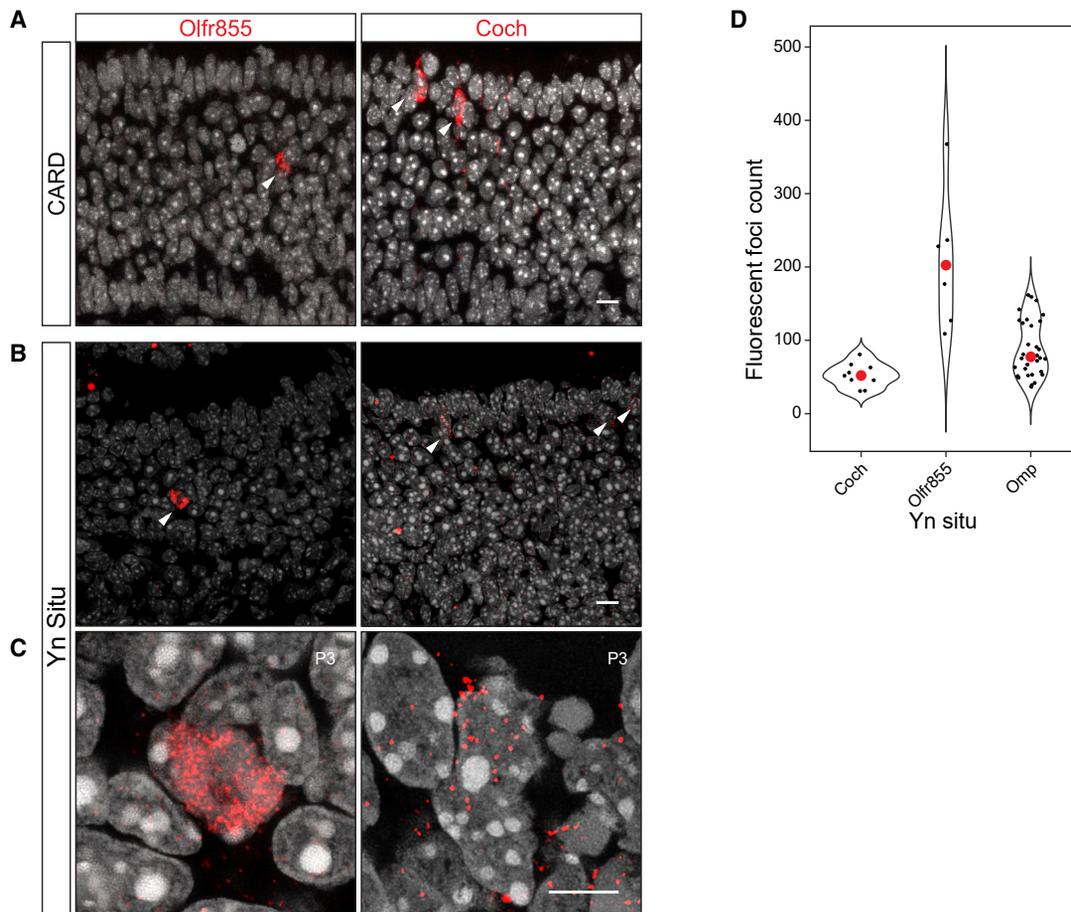


Figure 4. Dynamic range of the Yn-situ signals

(A) Representative images showing signals from traditional RNA *in situ* hybridization in detecting Omp and Cochlin (Coch), respectively, in the olfactory epithelium using HRP. The signal strengths in individual cells appear similar for the two genes.

(B and C) Representative images showing the Yn-situ signal for the same genes (B). Individual signal puncta are clearly visible in the high-resolution images (C). Scale bar, 10 μ m.

(D) Quantification of the number of signal puncta in each cell for three genes.

the cells not expressing Omp and Olfr855. The SNR is highest among all of the methods tested, even though it uses the fewest pairs of probes. The signal puncta are spherical. With a diameter of \sim 300 nm (five pairs of primary probes), and they are also smaller than in other methods. The signal is bright and that makes it visible directly under the microscope.

Second, the small puncta size enables digital quantification of the RNA transcripts even for highly expressed genes. Yn-situ does not produce large, aggregated signals even after overnight reaction. The size of the signal is determined by the structure of the HCR amplification complex formed *in situ*, not by the sequence or the length of the target RNA. Because the HCR reaction is saturated overnight (Figure S3), the size of the Yn-situ signal is constant across different targets, allowing more quantitative measurement at the single-puncta level at a larger dynamic range. The expression of the odorant receptor gene is expected at \sim 1%–2% of the total mRNA produced. At this level, we can still resolve individual puncta for quantification purposes. For third-generation HCR to resolve a single molecule, 20 or

more probe pairs and precise timing of the reaction are required. In comparison, Yn-situ can resolve single puncta with as few as three pairs of targeting probes. This significantly decreases the cost and investigation time. We have not fully optimized the probe design; it is possible that a single probe pair may produce enough signal for quantification purposes.

Third, the method is simple to perform. Conventional fluorescent *in situ* hybridization using long RNA probes requires molecular cloning and the synthesis of long RNA probes. The single-stranded RNA probes used for *in situ* hybridizations are prone to degradation by both endogenous RNase from the tissue itself and exogenous RNase from contamination of common reagents used in the laboratory. By taking advantage of the use of synthetic short DNA oligos as with other modern approaches, Yn-situ not only overcomes the problems by removing the requirement of an RNase-free environment but also makes the timing requirement more relaxed. A timed HCR step is not required to resolve single puncta; a short incubation of 30 min is sufficient to produce visible signals. Long incubation

improves the signal but does not cause overcrowding of signals. At the longer timescale, Yn-situ takes a similar amount of time as third-generation HCR (3 days). At the shorter timescale, Yn-situ is comparable to RNAscope (overnight).

Fourth, Yn-situ needs a length of only 52 nt to detect the target. This length is smaller than most coding RNAs and primary microRNAs (miRNAs). This creates the possibility to detect small RNA species, such as miRNAs and RNAs that are only targetable by short sequences, such as circular RNA (circRNA). Indeed, the Yn-situ method was successfully used to detect small nuclear RNA U1 and U2 in cultured cells (Chen et al., 2021).

Finally, Yn-situ offers a significant reduction in cost and time. Only standard desalted oligos are required as primary probes. The cost is less than one cent for the primary probes per assay. This method does not require any additional equipment other than the existing molecular cloning and histology devices. A small-scale synthesis of the preamplifier is sufficient for hundreds of tests. These factors make the method cost efficient, significantly lower than any commercially available single molecule *in situ* hybridization method. One consideration is the fluorescent metastable hairpin for HCR, which can run up the cost if purchased from the commercial sources. On the hand, the hairpins can be made through well-established amine-NHS ester reactions (Choi et al., 2010). Thus, the cost of this method can be further reduced.

Although Yn-situ is simple and cheap, it is not merely a poor man's *in situ* hybridization method. Because of the low cost, robustness, and binary nature of the signals, Yn-situ has the potential for further advanced applications such as high-throughput automation and multiplexing. The multiplexing includes the simultaneous detection of multiple RNA species and the simultaneous detection of different molecular classes. Simultaneous detection of multiple RNAs can be implemented by the synthesis of additional preamplifiers that do not bind to the same sequence and initiate different pairs of HCR hairpins. Five orthogonal HCR hairpins have been demonstrated (Choi et al., 2010). In theory, it is possible to design and synthesize at least four more orthogonal preamplifiers. To detect proteins simultaneously with RNAs, Yn-situ needs to be performed prior to immunohistochemistry. This is because the formamide in the probe hybridization buffer is a denaturant that affects antibody-protein binding. The detection of protein after *in situ* hybridization has been demonstrated previously (Meyer et al., 2017). The Yn-situ protocol does not involve the high annealing temperature of RNA probes at 65°C. Low temperature is more feasible for detection of proteins afterward. Alternatively, immunostaining of proteins can be performed first with an added step of PFA fixation to crosslink antibodies to the target. Using the later approach, we were able to simultaneously detect protein and RNA transcript in the same tissue sample (Figures S3E–S3G). It is possible to perform sequential Yn-situ to increase the detection scale.

Limitations of the study

The current study is a proof-of-principle experiment. There are several limitations. The experiment in this study was performed primarily on postnatal olfactory epithelia. Additional optimization may be needed to achieve good results from other types of tis-

sue, tissues with different fixation methods, and tissues of other ages. A few key steps, including proteinase treatment, permeabilization conditions, and preamplifier washing conditions are primary targets for optimization. Currently, we have not tested the lower bound in detecting RNA transcripts. Although we infer from the puncta sizes that Yn situ detects single mRNA molecules, a direct proof is missing. Finally, the method is currently limited to detecting only one gene at a time. The realization and scalability of multiplex detection warrant further investigation.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.crmeth.2022.100201>.

ACKNOWLEDGMENTS

We would like to thank David Cheng-Hao Wang for sharing the TRAP2;Ai14 sections. We also thank Dr. Lu Chen, Liang Li, Dr. Andrew Shuster, and URee Chon for testing the method and providing input. This work was supported by funding from the National Institutes of Health (R01DC008003, R01DC014701, R01DC016696) and funding from Stowers Institute for Medical Research to C.R.Y.

AUTHOR CONTRIBUTIONS

Conceptualization, Y. Wu.; methodology, Y. Wu.; investigation, Y. Wu., W.X., L.M., Z.Y., and Y. Wang; writing – original draft, Y. Wu; writing – review & editing, Y. Wu, W.X., and C.R.Y.; funding acquisition, C.R.Y.; supervision, C.R.Y.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: November 22, 2021

Revised: February 25, 2022

Accepted: March 22, 2022

Published: April 18, 2022

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal RFP antibody	Rockland	CAT: 600-401-379; RRID: AB_2209751
Donkey anti Rabbit Cy3	Jackson ImmunoResearch	CAT: 711-165-152; RRID: AB_2307443
Bacterial and virus strains		
One Shot TOP10 Electrocomp <i>E. coli</i>	Thermo Fisher Scientific	CAT: C404052
NEB Stable Competent <i>E. coli</i> (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: 493,805; 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: S5881; CAS: 1310-73-2
Paraformaldehyde	MilliporeSigma	CAT:441244; CAS: 30525-89-4
Sodium chloride	MilliporeSigma	CAT: S9888; 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
50x Denhardt's solution	Thermo Fisher Scientific	CAT: 750018
Proteinase K	New England Biolabs	CAT: P8107S
Deionized formamide	VWR	CAT: 97062-008; CAS: 75-12-7
Tissue-Tek O.C.T. Compound	VWR	CAT: 25608-930
Sfil	New England Biolabs	CAT: R0123S
nt.BspQi	New England Biolabs	CAT: R0644S
DMSO	MilliporeSigma	CAT: 276855; CAS: 67-68-5
Midori Green Direct	Bulldog Bio	CAT: MG06
GelRed Nucleic Acid Gel Stain	Biotium	CAT: 41003
PBS (10X), pH 7.4	Thermo Fisher Scientific	CAT: 70011044
UltraPure 20X SSC Buffer	Thermo Fisher Scientific	CAT: 1557-044
IDTE	IDT	CAT: 11-05-01-05
Critical commercial assays		
PrimeSTAR HS DNA Polymerase	Takara Bio	CAT: R010B
KAPA HiFi HotStart ReadyMix	Roche	CAT: 7958927001
Q5 High-Fidelity 2X Master Mix	New England Biolabs	CAT: M0492S
LongAmp Taq DNA Polymerase	New England Biolabs	CAT: M0323S
GoTaq Long PCR Master Mix	Promega	CAT: M4021
Guide-it Long ssDNA Production System v1	Takara bio	N/A
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
Deposited data		
Raw data	Stowers original data repository	Stowers ODR: http://www.stowers.org/research/publications/libpb-1691

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Mouse: CD1-Elite (SOPF) Mouse	Charles river	JAX: 006494
Mouse: TRAP2	The Jackson Laboratory	JAX: 030323
Mouse: Ai14	The Jackson Laboratory	JAX: 007908
Oligonucleotides		
HCR hairpin B1H1-594: CgTAAAggAAgAC TCTTCCCgTTTgCTgCCCTCCTCgCATTCT TTTCTTgAggAgggCAgCAAACgggAAgAg /C9-Alexa Fluor 594-3'/	Molecular Instrument	N/A
HCR hairpin B1H2-594:/5'- Alexa Fluor 594-C12/gAggAgggCAgCAAACgggAA gAgTCTTCCCTTTACgCTCTTCCCGTTTg CTgCCCTCCTCAAgAAAATgC	Molecular Instrument	N/A
HCR hairpin B1H1-647: CgTAAAggAAgAC TCTTCCCgTTTgCTgCCCTCCTCgCATTCT TTCTTgAggAgggCAgCAAACgggAAgAg/ C9-Alexa Fluor 647-3'/	Molecular Instrument	N/A
HCR hairpin B1H2-647:/5'- Alexa Fluor 647-C12/gAggAgggCAgCAAACgggA AgAgTCTTCCCTTTACgCTCTTCCCGTTT gCTgCCCTCCTCAAgAAAATgC	Molecular Instrument	N/A
Additional oligos, see Table S1	N/A	N/A
Recombinant DNA		
Plasmid: A1-20B1 (P001)	This paper	Addgene #161820
Plasmid: A1-20B1 (P004)	This paper	Addgene #184056
Software and algorithms		
ImageJ	Schneider et al., 2012	https://imagej.nih.gov/ij/
Illustrator	Adobe	www.adobe.com
Origin Pro	OriginLab	www.originlab.com
R	The R Foundation	https://www.r-project.org/
Rstudio	RStudio, PBC	https://www.rstudio.com/

RESOURCE AVAILABILITY

Lead contact

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

Materials availability

Plasmids generated in this study will be available from Addgene for distribution upon publication 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/libpb-1691>).
- No computer code was used for analysis.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Wildtype CD1 between postnatal day 0 (P0) and P21 pups are used for experiment. Both sexes are randomly assigned to the experiment. All animals were maintained in Stowers LASF with a 14:10 light cycle and provided with food and water *ad libitum*.

Experimental protocols 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.

METHOD DETAILS

Oligonucleotides

Oligonucleotides used in this study were listed in the [Table S1](#). Oligonucleotides used as PCR primers, HCR probes, Yn-situ blockers, and Yn-situ probes were purchased from IDT with standard desalting. Fluorescently labeled HCR hairpins were purchased from Molecular Technologies.

Synthesis of preamplifier using strandase

Plasmid A1-20B1 was synthesized by Thermo Fisher Scientific GeneArt gene synthesis. The plasmid carried kanamycin resistance. The synthesized plasmid was transformed into One Shot TOP10 Electrocomp *E. coli* (Thermo Fisher Scientific) for further use. Plasmid DNA was purified by miniprep (Zymo Research). Double stranded preamplifier synthesis template was synthesized by PCR using LongAmp PCR polymerase, non-phosphorylated forward primer, phosphorylated reverse primer. PCR reaction contained 0.05 ng/ μ L plasmid, 0.5 μ M each primer. The PCR program was 95°C for 2 minutes, 37 cycles of 94°C for 30 seconds and 65°C for 1 minute, a final extension at 72°C for 10 minutes. PCR product was purified using DNA spin column (Zymo Research). Single strand preamplifier was synthesized using Guide-it Long ssDNA Production System (Takara Bio) under manufacture's instruction. Briefly, 5 μ g purified PCR products, 5 μ L Strandase A Buffer (10X), and 5 μ L Strandase Mix A in 50 μ L volume was incubated at 37°C for 5 minutes, then 80°C for 5 minutes. The reaction mix was added with 50 μ L Strandase B Buffer (10X) and 1 μ L Strandase Mix B, incubated at 37°C for 5 minutes, 80°C for 5 minutes. The synthesized preamplifier was purified by DNA spin column (Zymo Research).

Synthesis of preamplifier using nickase

We have developed an alternative method to synthesize preamplifier. The method is similar to that used by [Yoshimi et al. \(2016\)](#). The preamplifier synthesized by this method was used in [Figures S2](#) and [S3](#). We find this method easier to use compared to the strandase method and recommend this as the primary method to synthesize preamplifier. We first synthesized a new plasmid A1-20B1 (P004). The preamplifier sequence was flanked by two BspQI sites. The plasmid was nicked using nt.BspQI and denatured using DMSO. The single strand preamplifier was separated from the remaining parts of the plasmid by electrophoresis and purified by gel extraction. A step-by-step protocol can be found in the supplementary material [Method S1](#).

Polymerase chain reaction (PCR)

PCR performed in [Figure 1](#) was conducted under manufacture's instruction with changes detailed below. For PrimeSTAR, 10 μ L PrimeSTAR Max Premix (2X), 0.2 μ M of each primer, 1 ng template were used in a 20 μ L system. PCR cycles were 35 cycles of 98°C for 10 seconds, 55°C for 15 seconds, and 72°C for 1 minute. For KAPA HiFi, 10 μ L of 2X ReadyMix, 0.2 μ M of each primer, 1 ng template were used in a 20 μ L system. PCR cycles were 1 cycle of 95°C for 3 minutes, 35 cycles of 98°C for 20 seconds, 60°C for 15 seconds, 72°C for 1 minute, 1 cycle of 72°C for 10 minutes. For Q5, 10 μ L of 2X master mix, 0.5 μ M of each primer, 1 ng template were used in a 20 μ L system. PCR cycles were 1 cycle of 98°C for 30 seconds, 35 cycles of 98°C for 10 seconds, 60°C for 30 seconds, 72°C for 1 minute, 1 cycle of 72°C for 10 minutes. For GoTaq, 10 μ L of 2X master mix, 0.5 μ M of each primer, 1 ng template were used in a 20 μ L system. PCR cycles were 1 cycle of 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 65°C for 1 minutes, 1 cycle of 72°C for 10 minutes. For LongAmp, different conditions were used. For the experiment in [Figure S1A](#) 10 μ L of master mix, 0.5 μ M of each primer, 1 ng template were used in a 20 μ L system. PCR cycles were 1 cycle of 94°C for 30 seconds, 30 cycles of 94°C for 30 seconds, 65°C for 1 minute, 1 cycle of 65°C for 10 minutes. For the experiment in [Figure S1B](#), a gradient from 52°C to 65°C was used for annealing and extension. For the experiment in [Figure S1C](#), two different gradients were used as annealing temperature. One was 52°C to 65°C. The other was 65°C to 72°C. 65°C was used as extension temperature. For the experiment in [Figure S1D](#), 65°C was used for both annealing and extension. Different concentration of primers and templates were used as indicated in the figure. All PCRs were performed in a thermocycler (Biorad).

Gel electrophoresis

1% TopVision Agarose gel (Thermo Fisher Scientific) was used for gel electrophoresis analysis. dsDNA molecules were stained with Midori Green Direct DNA staining dye (Bulldog Bio) for gel loading. Electrophoresis was run at 130 V for 30 minutes. ssDNA molecules were stained with Gelred (Biotium). Electrophoresis was run at 100 V for 1 hour. Gels were imaged with Gel Logic 100 system (Carestream Health), ChemiDoc (Biorad) or SmartDoc gel imaging hood (Stellar Scientific) equipped with an iPhone X (Apple). Images were cropped and contrast enhanced in Fiji.

Yn-situ hybridization

For reproducibility purpose, we attached a step-by-step protocol for performing the procedures in the [supplementary material](#). Tissue sections were performed as previously described ([Wu et al., 2018](#)).

For simultaneous detection of proteins and RNAs, the animal was perfused with 10 mL PBS, followed with 10 mL 4% PFA in PBS. The brain was dissected and post-fixed in 4% PFA in PBS overnight at 4 °C. The sample was then embedded in 4% low melting point agarose and sectioned into 50 μm with a vibratome (Leica). The sections were mounted onto charged slides, air dried under room temperature, washed with methylimidazole buffer briefly, fixed with EDC fixative for 1 hour at room temperature, and permeabilized in methanol for 1 hour at room temperature, rehydrated by washing in PBST briefly. The sections were then stained with rabbit-anti-RFP antibody (Rockland) in PBST with 1:1000 dilution at room temperature overnight, then washed with PBST for 5 minutes three times, stained with donkey anti-rabbit-cy3 (Thermo Fisher Scientific) in PBST at 1:1000 dilution for 3 hours. After the staining, Yn-situ detection of Slc17a7 was performed in the same way as described in the supplementary protocol starting from the proteinase K treatment step.

Conventional fluorescent *in situ* hybridization (FISH)

Conventional fluorescent *in situ* hybridization was conducted following previously described method (Ishii et al., 2004). Briefly, the olfactory epithelia were dissected and embedded in O.C.T. (Sakura Finetek). The embedded samples were snap-frozen in liquid nitrogen. The samples were stored under –70°C until sectioning. The tissue blocks were cut into 10 μm sections using a cryostat (CryoStar NX70) and mounted on charged slides (Thermo Fisher Scientific). The sections were dried on a slide warmer at 100 °C for 2 minutes, fixed with 4% PFA in PBS for 1 hour, fixed with EDC fixative (Pena et al., 2009) for 1 hour before hybridization. Digoxigenin and fluorescein labeled ribonucleotide probes targeting 3' UTR regions were used. The hybridization was conducted at 65°C overnight. After washing with SSC, the probes were detected with anti-digoxigenin and anti-fluorescein antibodies conjugated with alkaline phosphatase (AP) and horseradish peroxidase (HRP) using AP detection kit (Roche) and tyramide signal amplification (TSA, Thermo Fisher Scientific) kits. Slides were mounted with No. 1.5 coverslip using Y-mount.

RNAscope

RNAscope was performed according to manufacturer's instruction using probes designed by the company.

3rd generation HCR *in situ* hybridization

3rd generation HCR was performed according to manufacturer's instruction using probes designed by the company.

Microscopy

Conventional FISH images were taken using Zeiss LSM700 confocal microscope using Plan-Apochromat 20X/0.8 M27 lens. HCR, RNAscope, and Yn-situ images were taken using Leica SP8 confocal microscope equipped with HyD hybrid detector using HC PL APO 100X/1.40 Oil lens. Hyvolution images were taken using Leica SP8 confocal microscope under the Hyvolution mode with 0.6 AU pinhole and deconvolved using prolong gold as mounting media in LAS X (Leica). Images were exported as tiff format and analyzed in Fiji. Pixel intensities were measured as procedure generated units from the microscope.

QUANTIFICATION AND STATISTICAL ANALYSIS

For Figure 3, SNR calculation, an area in the background was selected to extract pixel intensity values. For signals in the puncta generated by Yn-situ, RNAscope, and HCR, a threshold function in Fiji was used to create masks for the puncta, where the signal intensities for every pixel was extracted. For AP *in situ*, the signals were diffuse. A high signal intensity area was selected without thresholding to extract pixel intensities. The histograms for background and signals were plotted after the signals were normalized to 4096 grayscales. SNR was calculated using the mean values of the signal divided by the variance of the background signal. This calculation avoided the use of background signal intensity because imaging threshold may artificially change the values. The puncta sizes were measured using Hyvolution images. Statistical test was conducted using one-way ANOVA in R. Multiple pairwise-comparisons between the means of groups were performed using function TukeyHSD(). N represented fluorescent puncta. p-values were adjusted for the multiple comparisons. The statistical details can be found in the figure legend.

For Figure 4, quantification of signals within a cell, each signal punctum was treated as a single molecule and the number of spots detected in a cell was used to measure the number of RNA molecules in that cell.

For Figure S3, The brightness of puncta were measure in Fiji. Each data point represented one punctum.