Video Article

Localization of Odorant Receptor Genes in Locust Antennae by RNA *In Situ* Hybridization

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Abstract

Insects have evolved sophisticated olfactory reception systems to sense exogenous chemical signals. These chemical signals are transduced by Olfactory Receptor Neurons (ORNs) housed in hair-like structures, called chemosensilla, of the antennae. On the ORNs' membranes, Odorant Receptors (ORs) are believed to be involved in odor coding. Thus, being able to identify genes localized to the ORNs is necessary to recognize OR genes, and provides a fundamental basis for further functional *in situ* studies. The RNA expression levels of specific *OR*s in insect antennae are very low, and preserving insect tissue for histology is challenging. Thus, it is difficult to localize an OR to a specific type of sensilla using RNA *in situ* hybridization. In this paper, a detailed and highly effective RNA *in situ* hybridization protocol particularly for lowly expressed OR genes of insects, is introduced. In addition, a specific OR gene was identified by conducting double-color fluorescent *in situ* hybridization experiments using a co-expressing receptor gene, *Orco*, as a marker.

Video Link

The video component of this article can be found at https://www.jove.com/video/55924/

Introduction

Insect antennae, which are the most important chemosensory organs, are covered with many hair-like structures – called sensilla – that are innervated by Olfactory Receptor Neurons (ORNs). On the membrane of insect ORNs, Odorant Receptors (ORs), a type of protein containing seven transmembrane domains, are expressed with a coreceptor (ORco) to form a heteromer that functions as an odorant-gated ion channel[1,2,3]. Different ORs respond to different combinations of chemical compounds[4,5,6].

Locusts (*Locusta migratoria*) mainly rely on olfactory cues to trigger important behaviors[7]. Locust ORs are key factors for understanding molecular olfactory mechanisms. Localizing a specific locust OR gene to the neuron of a morphologically specific sensillum type by RNA *In Situ* Hybridization (RNA ISH) is the first step in exploring the ORs function.

RNA ISH uses a labeled complementary RNA probe to measure and localize a specific RNA sequence in section of tissue, cells or whole mounts *in situ*, providing insights into physiological processes and disease pathogenesis. Digoxigenin-labeled (DIG-labeled) and biotin-labeled RNA probes have been widely used in RNA hybridization. RNA labeling with digoxigenin-11-UTP or biotin-16-UTP can be prepared by *in vitro* transcription with SP6 and T7 RNA polymerases. DIG- and biotin-labeled RNA probes have the following advantages: non-radioactive; safe; stable; highly sensitive; highly specific; and easy to produce using PCR and *in vitro* transcription. DIG- and biotin-labeled RNA probes can be chromogenically and fluorescently detected. DIG-labeled RNA probes can be detected with anti-digoxigenin Alkaline Phosphatase (AP)-conjugated antibodies that can be visualized either with the highly sensitive chemiluminescent substrates nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate toluidine salt (NBT/BCIP) using an optical microscope or with 2-hydroxy-3-naphtoic acid-2'-phenylanilide phosphate (HNPP) coupled with 4-chloro-2-methylbenzenediazonium hemi-zinc chloride salt (Fast Red) using a confocal microscope. Biotin-labeled RNA probes can be detected with anti-biotin streptavidin Horse Radish Peroxidase (HRP)-conjugated antibodies that can be visualized with fluorescein-tyramides using a confocal microscope. Thus, double-color fluorescent *in situ* hybridization can be performed to detect two target genes in one slice using DIG- and biotin-labeled RNA probes.

RNA ISH with DIG- and/or biotin-labeled probes has been successfully used to localize olfactory-related genes, such as *OR*, ionotropic receptor, odorant-binding protein and sensory neuron membrane protein, in insect antennae of, but not limited to, *Drosophila melanogaster*, *Anopheles gambiae*, *L. migratoria* and the desert locust *Schistocera gregaria*[8,9,10,11,12,13,14,15,16]. However, there are two substantial challenges when performing RNA ISH for insect ORs: (1) OR genes (except *ORco*) are expressed at low levels and only in a few cells, making signal detection very difficult, and (2) preserving insect tissue for histology, such that the morphology is preserved and the background noise is low, can be challenging. In this paper a detailed and effective protocol describing RNA ISH for localizing OR genes in insect antennae is presented, including both chromogenic and Tyramide Signal Amplification (TSA) detection.

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Protocol

NOTE: To limit RNA degradation, prepare solutions using wet-autoclaved distilled water (at 121 °C for 60 min) and also wet-autoclave materials.

1. Preparation of RNA ISH Antisense and Sense Probes

1. **Target gene amplification and purification**
    1. First, produce a 387 bp double-stranded fragment of *L. migratoria OR1* (*LmigOR1*, GenBank: JQ766965) from the plasmid containing the full-length cDNA of *LmigOR1* with a *Taq* DNA Polymerase that adds adenines to both ends of the fragments.
        1. Use a 100 µL final reaction volume, containing 50 µL of 2x reaction mix, 4 µL each of sense and antisense primers (**Table 1**), 1 µL of plasmid template, and 41 µL of RNase-free H_2O. Use the following PCR protocol: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, followed by 72 °C for 10 min.
        2. Repeat these steps to produce the 1,303 bp double-stranded fragment of *LmigOR2* (GenBank: JQ766966) and 1,251 bp double-stranded fragment of *LmigORco* (GenBank: JN989549). The primers used in these experiments are presented in **Table 1**.
        3. Run PCR products on 1.2% agarose gels in 1x Tris-acetate-EDTA buffer and visualize using ethidium bromide staining.
    2. Extract these PCR products using a gel extraction kit.
        1. Following electrophoresis, excise DNA bands from the gel and place the gel slices in a 1.5 mL tube. Then, add 100 µL of binding buffer (Buffer PN) per 0.1 g of gel slice. Vortex and incubate at 50 °C until the gel slice is completely dissolved.
        2. Insert a CA2 adsorption column into the collection tube and add 500 µL of balance buffer (Buffer BL). This improves the absorption capability and stability of the silica membrane. Centrifuge at 12,400 x g for 1 min.
        3. Transfer the dissolved gel mixture to the adsorption column assembly and incubate at room temperature for 2 min. Then, centrifuge tubes at 12,400 x g for 1 min. Discard the flow-through and reinsert the adsorption column into collection tube.
        4. Add 600 µL of wash solution (ethanol added, Buffer PW) twice. Centrifuge at 12,400 x g for 1 min. Discard flow-through and reinsert the adsorption column into collection tube.
        5. Empty the collection tube and recentrifuge the column assembly at 12,400 x g for 2 min to allow evaporation of any residual ethanol.
        6. Carefully transfer adsorption column to a clean 1.5 mL centrifuge tube. Air-dry the pellet for 5-10 min and redissolve the DNA in a suitable volume (*e.g.,* 30 µL) of nuclease-free water.
        7. Incubate at RT for 2 min. Centrifuge at 12,400 x g for 2 min. Discard adsorption column and store the DNA at 4 °C or -20 °C.

2. **Construct the recombinant plasmids**
    1. Individually ligate the 387 bp fragment of *LmigOR1*, 1,303 bp fragment of *LmigOR2* and 1,251 bp fragment of *LmigORco* into a T vector that contains promoters for T7 (upstream) and SP6 (downstream) RNA polymerases adjacent to the inserted DNA using T4 DNA ligase. Prepare the following 10 µL reaction: 5 µL of 2x ligation buffer, 1 µL of T vector, 3 µL (#100 ng) of the inserted gene's DNA and 1 µL of T4 DNA ligase, and incubate O/N at 4 °C.
    2. Add 5 µL of each recombinant plasmid containing the 387 bp fragment of *LmigOR1*, 1,303 bp fragment of *LmigOR2* and 1,251 bp fragment of *LmigORco* separately into 50 µL of competent *Escherichia coli* DH5α cells in sterile 1.5 mL tubes.
        1. Mix gently and put the tubes into ice for 30 min and then incubate them for 90 s at 42 °C. Transfer them back into ice for 3 min.
        2. Add 450 µL of Luria-Bertani (LB) liquid medium without ampicillin to every tube and incubate them in a shaker at 150 rpm for 1 h at 37 °C to restore the *E. coli* DH5α.
        3. Take a 100 µL aliquot of each transformant and use them to inoculate LB solid substrate plates containing 50 µg/mL ampicillin, 24 µg/mL isopropyl β-D-1-thiogalactopyranoside (IPTG), and 40 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).
        4. Incubate these plates in a constant incubator at 37 °C for 18 h. Screen the target recombinant plasmids using the blue-white screening method. Sequence the target recombinant plasmids using Sanger sequencing and identify the three OR genes' insert directions.

3. **Select restriction endonucleases to linearize recombinant plasmids**
    1. Use restriction endonucleases that not only digest in the multiple cloning site of the vector but also do not cut the insert DNA. In this experiment, all 3 genes are inserted into the T vector in the direction corresponding to that of the vector.
    2. Use *Nco* I restriction endonuclease to linearize the recombinant plasmids containing the 387 bp fragment of *LmigOR1*, 1,303 bp fragment of *LmigOR2* and 1,251 bp fragment of *LmigORco* to produce the antisense probes. Use *Spe* I restriction endonuclease to produce the sense probes.
    NOTE: If the insert direction corresponds to that of the vector, then a unique restriction site from the end of T7 is selected to linearize the recombinant plasmid and SP6 RNA polymerase is used to prepare the antisense probe. A unique restriction site from the end of SP6 is selected to linearize the recombinant plasmid, and the T7 RNA polymerase is used to prepare the sense probe. Otherwise, if the insert direction does not correspond to the vector's direction, then a unique restriction site from the end of SP6 is selected to linearize the recombinant plasmid and T7 RNA polymerase is used to prepare the antisense probe. The unique restriction site from the end of T7 is selected to linearize the recombinant plasmid, and the SP6 RNA polymerase is used to prepare sense probe.

4. **Purifying the linearized recombinant plasmids**
    1. Digest 20 µg each of the three recombinant plasmids containing the 387 bp fragment of *LmigOR1*, 1,303 bp fragment of *LmigOR2* and 1,251 bp fragment of *LmigORco* using the *Nco* I restriction endonuclease in a 100 µL reaction that contains 10 µL of 10x buffer, 40 µL of 0.5 µg/µL plasmid, 3 µL *of Nco* I and 47 µL of RNase-free H_2O, followed by a 3 h incubation at 37 °C.
    2. Similarly, digest 20 µg of each of these three recombinant plasmids using the *Spe* I restriction endonuclease.

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3. Purify these digested plasmids using a gel extraction kit as described in 1.1.2. Determine the concentrations of these extracted linearized recombinant plasmids by spectrophotometry and adjust to 0.5 μg/μL.

5. **Prepare antisense and sense probes**
   1. Use the T7/SP6 RNA transcription system to generate antisense and sense probes. SP6 RNA polymerase is used to transcribe double-stranded RNA for DIG- and biotin-labeled antisense probes for these three OR genes using linearized recombinant plasmids as templates *in vitro*. The T7 RNA polymerase is used to produce sense probes. Perform the reaction in a 20 μL final volume, containing 2 μL of 10x NTP mix, 2 μL of 10X transcription buffer, 1 μL of RNase inhibitor, 2 μL of T7 or SP6 RNA polymerase, 4 μL of 0.5 μg/μL linearized plasmid and 9 μL RNase-free H_2O, followed by a 3 h incubation at 37 °C.
   2. Incubate the DIG- and biotin-labeled antisense and sense probes for 30 min with 1 μL DNase at 37 °C. Add 2.5 μL of 5 M LiCl and 75 μL of absolute ethanol, then incubate the reactions for 30 min at -70 °C.
   3. Centrifuge at 15,000 x g for 30 min at 4 °C. Carefully decant the supernatant. Add 150 μL of 70% ethanol to wash the precipitant. Prepare the 70% ethanol (1 L) by adding 95% ethanol (736.8 mL) to sterile distilled water (263.2 mL).
   4. Centrifuge at 15,000 x g for 30 min at 4 °C again and air-dry the pellet for 5-10 min at RT. Add 25 μL of RNase-free H_2O to dissolve the RNA antisense and sense probes.
   5. If the length of the inserted gene is longer than 1 kb, subsequently fragment RNA antisense and sense probes to an average length of #300 bp by incubation in a bicarbonate-carbonate buffer solutions. Perform the reaction in a 50 μL final volume, containing 25 μL of the RNA probe and 25 μL of the bicarbonate-carbonate buffer solution (80 mM $NaHCO_3$, 120 mM Na_2CO_3, pH 10.2) at 60 °C. The hydrolysis time required is given by a previously published formula[17].
   6. Add 5 μL of 10% acetic acid to stop the reaction. In this experiment, fragment the *LmigOR2* and *LmigORco* antisense and sense probes for 24 and 23 min, respectively.
      $t = (L_o-L_f)/kXL_oXL_f$
      L_o= the initial fragment lengths in kb
      L_f=the final fragment lengths in kb
      k=the rate constant for hydrolysis, approximately 0.11 $kb^{-1}min^{-1}$
      t=the hydrolysis time in min
   7. Finally, add 250 μL of hybridization buffer and store at -80 °C.

2. Preparation of Cryostat Sections

1. Precool the freezing microtome to -24 °C.
2. Select new molting adult locusts that are active and have intact antennae. Cut the antennae into 2-3 mm pieces using sterile razors.
3. Put O.C.T. compound on a freezing microtome holder and put one or two samples on the compound horizontally. Then, transfer the holder into the freezing microtome at -24 °C to equilibrate until the compound freezes. Take out the holder and cover the samples with a little compound. Transfer the holder into the freezing microtome at -24 °C again for at least 10 min (**Figure 1**).
   NOTE: Avoid getting bubbles in the compound.
4. Fix the holder with the samples into the freezing microtome, then section the frozen samples into 12 μm-thick slices at -24 °C.
5. Thaw mount the slices one by one on slides (25 x 75 mm; nuclease-free) and air dry for 10 min.

3. Fixing Sections

1. After preparing cryostat sections, put the slides in a plastic container (#100 x 40 x 80 mm), and fix the tissues by incubating the slides in 4% paraformaldehyde solution (PFA) for 30 min at 4 °C.
2. Wash the slides in 1X Phosphate-Buffered Saline (PBS) for 1 min.
3. To eliminate the alkaline proteins, transfer the slides to 0.2 M HCl for 10 min.
4. To eliminate the surface protein of nucleic acid, transfer the slides to 1XPBS with 1% Triton X-100 for 2 min.
5. Wash the slides twice for 30 s in 1x PBS.
6. Finally, rinse the slides in formamide solution for 10 min at 4 °C.

4. Hybridization

1. **Prepare the antisense and sense probes**
   1. Use hybridization buffer to dilute RNA antisense or sense probes in the 1.5 mL nuclease-free tubes. In this experiment, for all of the antisense and sense probes (*LmigOR1, LmigOR2,* and *LmigOrco*), add 1 μL of probe to 99 μL hybridization buffer per slide. Generally, the use of 1:100 dilutions of antisense probes produces significant positive signals and low backgrounds using this protocol.
   2. For chromogenic detection, dilute DIG-labeled probes individually.
   3. For TSA detection, dilute DIG-labeled *LmigOR1* with biotin-labeled *LmigOrco* probes or DIG-labeled *LmigOR2* with biotin-labeled *LmigOR1* probes together in the hybridization buffer.
   4. Heat the dilutions for 10 min at 65 °C and put them on ice for at least 5 min.

2. **Hybridization**
   1. Drain the slides and add 100 μL of diluted antisense and sense probes (Step 4.1) to the tissue sections. Then, place coverslips (24 mm x 50 mm; nuclease-free) on the tissue sections.
   2. Place the covered slides horizontally into a humid box (#300 x 180 x 50 mm^3; **Figure 2**) and incubate at 55 °C for 22 h. Add formamide solution or 1X PBS to the bottom of the box to keep the environment moist, but do not submerge the slides in the liquid.

3. **Washing and blocking.**

1. After hybridization, remove the coverslips carefully. Wash the slides twice for 30 min in 0.1x Saline Sodium Citrate (SSC) at 60 °C.
   NOTE: Washing means putting the slides in a slide holder that is then placed into a plastic container and gently agitated on a rocker (# 50 rpm; **Figure 2**).
2. Rinse the slides in 1x Tris-Buffered Saline (TBS) for 30 s.
3. Add 1 mL of 1% blocking reagent in TBS supplemented with 0.03% Triton X-100 on each slide, and incubate for 30 min. Then, discard the blocking solution.

4. **Immunohistochemistry.**
   1. For chromogenic detection, use blocking reagent in TBS to dilute 750 units (U)/mL anti-digoxigenin AP-conjugated antibody to 1.5 U/mL AP solution. Add 100 µL of AP solution per covered (24 mm x 50 mm) slide.
   2. For TSA detection, use blocking reagent in TBS to dilute 750 U/mL of anti-digoxigenin AP- conjugated antibody and anti-biotin streptavidin HRP- conjugated antibody to the AP/HRP solution. Add 100 µL of AP/HRP solution per covered (24 mm x 50 mm) slide.
   3. Incubate the slides in a humid box (**Figure 2**) for 60 min at 37 °C. Use the formamide solution or 1X PBS to keep box moist but not soggy.

5. Staining

1. Remove the coverslips carefully. Wash the slides three times for 5 min in 1x TBS supplemented with 0.05% Tween-20. Rinse the slides in DAP-buffer (chromogenic detection: pH 9.5; TSA detection: pH 8.0) for 5 min.
2. **Staining (chromogenic detection)**
   1. Add 100 µL of NBT (375 µg/mL)/BCIP (188 µg/mL) substrate solution (diluted in DAP; pH 9.5) to every slide. Carefully place coverslips (24 x 50 mm) onto the slides.
   2. Incubate the slides in a humid box with substrate solution for 10 min - O/N at 37 °C.
      NOTE: Check the development by looking at the slides from time to time under the microscope.
   3. When the development is ready, stop the reaction by transferring the slides into water.

3. **Staining (TSA detection)**
   1. Use a syringe to move the HNPP (100 µg/mL)/Fast Red (250 µg/mL) substrate from the syringe filter (0.22 µm; **Figure 2**).
   2. Add 100 µL of HNPP/Fast Red substrate per slide covered with a coverslip (24 x 50 mm). Incubate the slides for 30 min with HNPP/Fast Red substrate at RT.
   3. Remove the coverslips carefully, and wash the slides three times for 5 min in 1X TBS supplemented with 0.05% Tween-20.
   4. Use 100 µL of TSA substrate/covered (24 x 50 mm^2) slide. Incubate the slides with TSA substrate for 10 min at RT.
   5. Remove the coverslips carefully, and wash the slides three times for 5 min in 1x TBS supplemented with 0.05% Tween-20.

4. Embed the slides in PBS/glycerol (1:3).
   NOTE: After finishing these procedures, the slides should be observed as soon as possible because the fluorescent signal will quench quickly.

6. Observation

1. **Chromogenic detection**
   1. Observe tissue sections using an optical microscope. Choose the 10X, 20X, and 40X objective lenses to observe the results of chromogenic detection.
   2. Use DP-BSW software to analyze the results and capture the images.

2. **TSA detection**
   1. Observe tissue sections using a confocal microscope. DIG-labeled genes should be observed under 543 nm light, presenting a red color, and biotin-labeled genes should be observed under 488 nm light, presenting a green color. When they emerge, they present yellow color.
   2. Choose the 20X and 40X objective lenses to observe the results of TSA detection, respectively.
   3. Use FV1000 software to analyze the results and capture the images.

Representative Results

With chromogenic detection, a small subset of the antennal cells in every adult antennal section was denoted by the DIG-labeled *LmigOR1* and *LmigOR2* antisense probes (**Figure 3**). RNA ISH on consecutive sections to localize *LmigOR1* and *LmigOR2* showed that antennal cells expressing the two genes were located in ORN clusters expressing *LmigORco*, indicating that the putative *LmigOR1* and *LmigOR2* were actually expressed in ORNs (**Figure 4a-4d**). Occasionally, labeled dendritic-like structures were visualized (**Figure 4e-4f**). *LmigOR1* and *LmigOR2* were both localized to neurons in the basiconic sensilla (**Figure 5a-5b**), but they were not co-expressed in individual sensilla, indicating that they were present in different basiconic sensillium subtypes (**Figure 5c**-**5d**).

In TSA detection, double-color fluorescent *in situ* hybridization showed that *LmigOR1*-expressing cells (red color) were located to ORN clusters expressing *LmigORco* (green color), indicating that the putative *LmigOR1* was expressed in ORNs (**Figure 6a**). A close view of the boxed areas in **Figure 6a** are shown in **Figure 6b**. *LmigOR1*- expressing neurons (green color, **Figure 7a**) and *LmigOR2*-expressing neuron (red color, **Figure 7b**) were located to different basiconic sensillium subtypes (**Figure 7c**).

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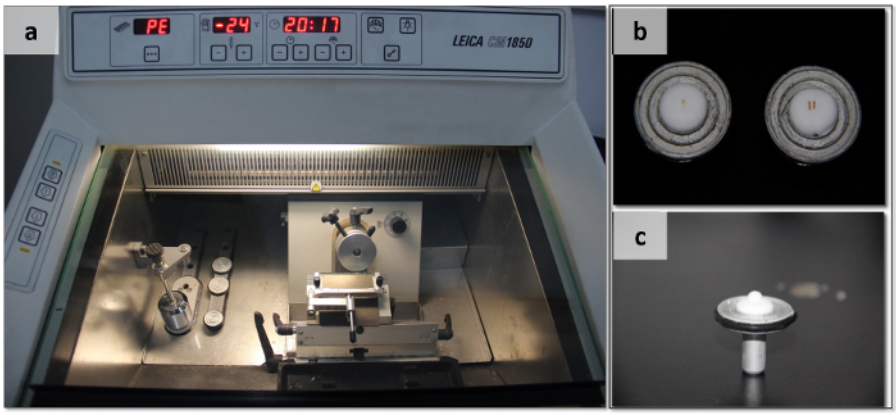

Figure 1: Preparation of Insect Antennal Sections for RNA *In Situ* Hybridization. (**a**) The freezing microtome; (**b-c**) The samples are embedded in freezing O.C.T. Compound. Please click here to view a larger version of this figure.

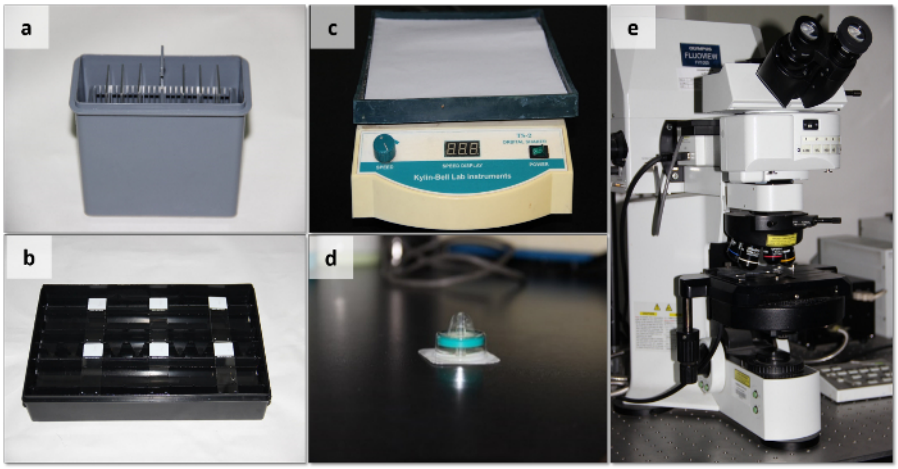

Figure 2: Experimental Items for RNA *In Situ* Hybridization. (**a**) The slide holder and the plastic container. (**b**) The humid box. (**c**) The rocker. (**d**) The membrane filter. (**e**) Confocal microscope. Please click here to view a larger version of this figure.

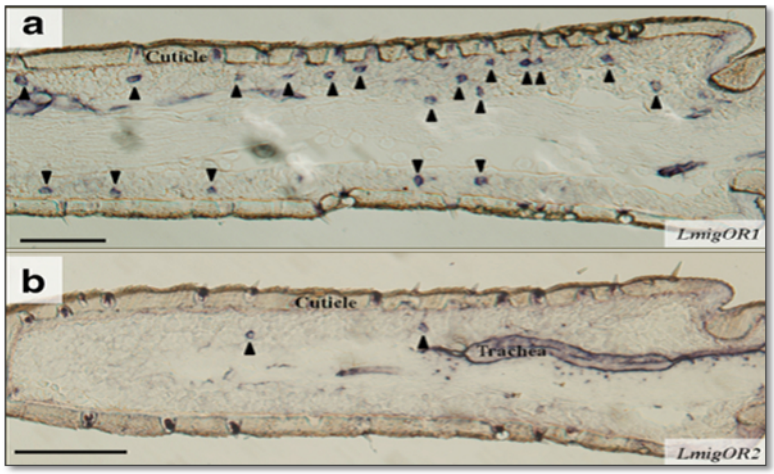

Figure 3: Cellular Localization of *LmigOR1* & *LmigOR2* in Olfactory Organs. (**a**) Overview of *LmigOR1*-expressing cells in a locust antennal segment. (**b**) Overview of *LmigOR2*-expressing cells in a locust antennal segment. Arrowheads indicate cells expressing *LmigOR1* (**a**) and *LmigOR2* (**b**). Scale bars = 100 μm (a and b). Figures were adapted from Xu *et al.*[12] Please click here to view a larger version of this figure.

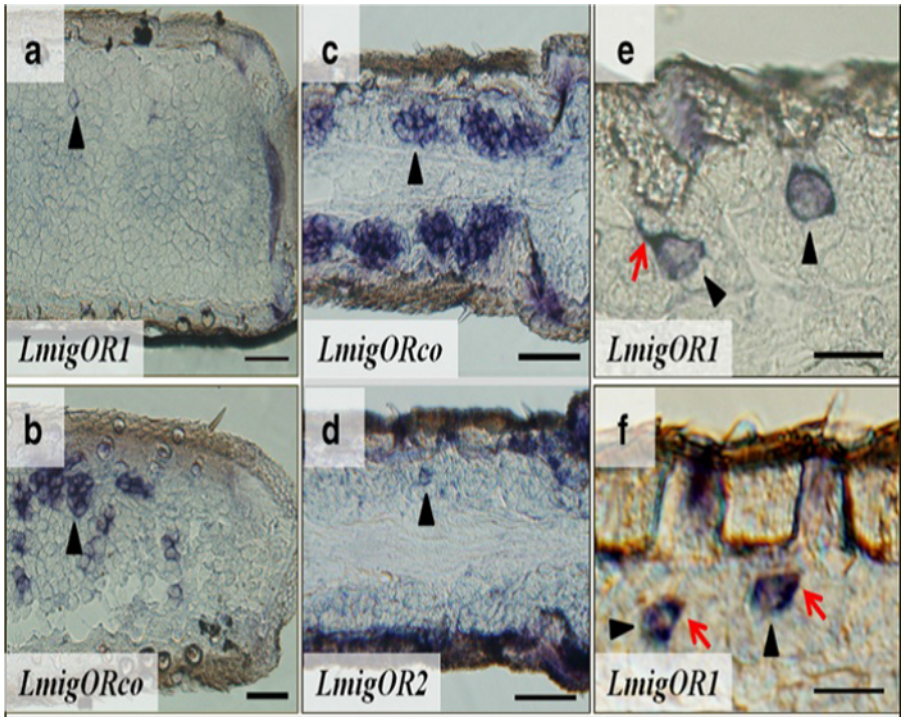

Figure 4: Neuronal Identity of Antennal Cells Expressing *LmigORs* by Chromogenic Detections. (**a-b**) The labeling pattern of *LmigOR1* (**a**) and *LmigORco* (**b**) antisense probe on consecutive sections of locust antenna. (**c-d**) The labeling pattern of *LmigORco* (**c**) and *LmigOR2* (**d**) antisense probe on consecutive sections of locust antenna. (**e-f**) Illustration of occasionally labeled dendritic-like structures (indicated by red arrows). Scale bars = 50 µm (a-d); 20 µm (e and f). Figures were adapted from Xu *et al.*[12] Please click here to view a larger version of this figure.

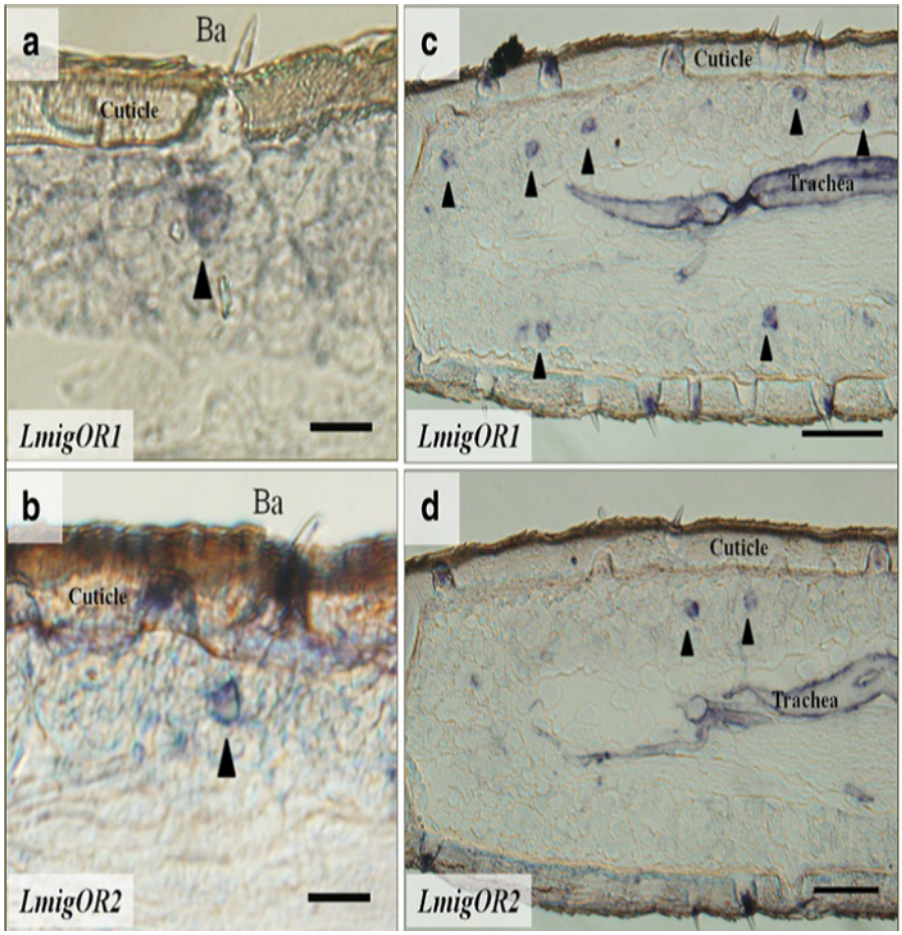

Figure 5: *LmigOR1* & *LmigOR2* are Expressed in ORNs Housed in Basiconic Sensilla. (**a-b**) Basiconic sensillum housed ORNs expressing *LmigOR1* (**a**) and *LmigOR2* (**b**). (**c-d**) The expression of *LmigOR1* and *LmigOR2* in distinct subset of antennal ORNs was verified on consecutive sections (c-d). Arrowheads denote antennal cells expressing *LmigOR1* (a, c) and *LmigOR2* (b, d). Ba: basiconic sensillum. Scale bars = 20 µm (a and b); 50 µm (c and d). Figures were adapted from Xu *et al.*[12] Please click here to view a larger version of this figure.

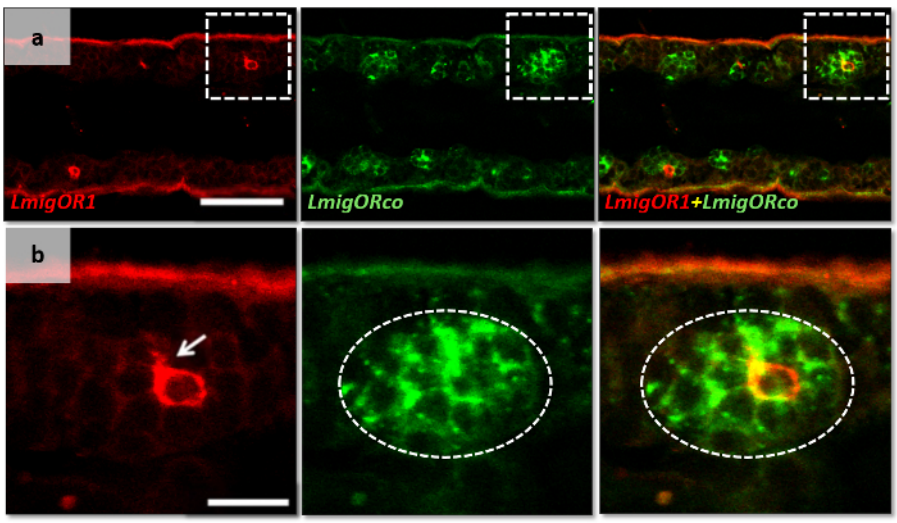

Figure 6: Neuronal Identity of Antennal Cell Expressing *LmigOR1* by TSA Detections. (**a**) Two-color *in situ* hybridization was performed on longitudinal antennal section to illustrate the expression of *LmigOR1* (Red) and *LmigORco* (Green). Localization of *LmigOR1*-expressing cells in cell clusters expressing *LmigORco* confirmed its neural identity. (**b**) Close view of boxed areas in a. Occasionally labeled dendritic like structures were indicated by arrow. Circled areas indicate ORNs cluster expressing *LmigORco* and sharing the same sensillum. Scale bars = 50 µm (a); 20 µm (b). Figures were adapted from Xu *et al.*[12] Please click here to view a larger version of this figure.

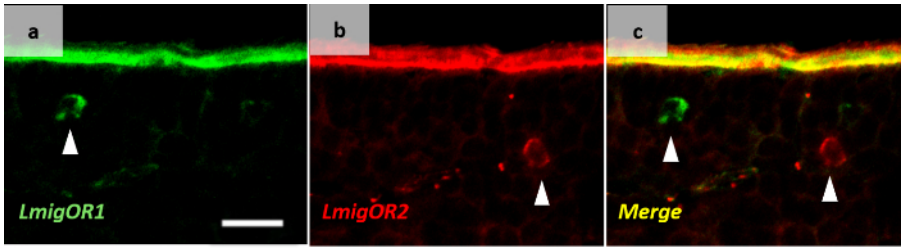

Figure 7: *LmigOR1* & *LmigOR2* were Located to Different Basiconic Sensilla ORNs by TSA Detection. Fluorescent signals were visualized using detection systems, indicating *LmigOR1*-labeled neurons by green fluorescence (**a**) and *LmigOR2* positive cells by red fluorescence (**b**) were expressed in different basiconic sensilla subtypes (**c**). Arrowheads denote antennal cells expressing *LmigOR1* and *LmigOR2*. Scale bars = 20 μm. Figures were adapted from Xu *et al.*[12] Please click here to view a larger version of this figure.

Name	Sequences
Lmig OR1-probe-s	5'-AAGGGGTGGGAGACGGCCTG-3'
Lmig OR1-probe-as	5'-CAGCTCCTCCCCAACGACAGC-3'
Lmig OR2-probe-s	5'-ATGGGTGAGCGTGGAGAGGC-3'
Lmig OR2-probe-as	5'-GGTCATCGCTGTGGACGTGG-3'
Lmig Orco-probe-s	5'-CTCGTCTGACAGCGTAACTCAC-3'
Lmig Orco-probe-as	5'-AAGACGCAGAAGAGGAAGACCT-3'

Table 1: The Sequences of the PCR Primers.

Discussion

It is hard to perform RNA ISH to localize OR genes in insect antennae because the expression levels of OR genes, except *ORco*, are very low and preserving histological slices of insect antennae is very difficult. In addition, TSA detection is also very tricky. To address these problems, the following measures should be taken. The antennae are selected from newly molting adult locusts that have thin and soft antennal cuticles, which maintain their morphology on the slide. The frozen samples are sectioned into 12 μm-thick slices. Highly sensitive and specific biotin- and DIG-labeled probes are used. Detergents, such as Tween-20 and Triton X-100, are used to decrease the background in many steps. In TSA detection, a highly expressed gene, relative to another lowly expressed gene, should be labeled with biotin-16-UTP. The slides should be observed as soon as possible because the fluorescent signals will quench quickly.

DIG- and biotin-labeled probes have the advantages of longer shelf lives, higher signal to noise ratios and better cellular resolutions than radioactive probes[18,19]. The protocol presented in this paper has some advantages over whole mount *in situ* hybridization. This protocol easily identifies the localizations of genes at the cellular level but cannot be easily used to investigate gene distribution patterns, which is more readily performed with whole mount *in situ* hybridization.

To identify an OR gene, two approaches were taken. One was the chromogenic detection in consecutive sections, and the other was fluorescent detection in one section. *ORco* is co-expressed with a specific *OR* as an ORN marker[10,20,21]. Cells expressing *LmigOR1* or *LmigOR2* were both located to clusters of *LmigORco*-expressing cells that unambiguously verified them as ORs (**Figure 4** and **Figure 6**). Using the same approaches, we found that *LmigOR1* and *LmigOR2* are not co-expressed in one sensillum. The results of these two approaches are corroborative.

This protocol was also successfully used to localize *LmigORco, SgreORco, SgreIR8a* and *SgreIR25a* in locust antennae[10,14]. Recently *LmigOR3* was localized to trichoid sensilla neurons in *L. migratoria* using the same protocol[15]. This protocol was used to localize two sensory neural membrane proteins *SgreSNMP1* and *SgreSNMP2* in the antennae of *S. gregaria*[16]. Thus, this protocol reliably localized chemosensory-related genes in locust antennae, which not only verified these candidate genes, but also localized these genes to specific cells housed in different types of sensilla.

In conclusion, this highly effective protocol of RNA ISH is specifically described to localize OR genes, as well as other genes expressed at low levels, in insect antennae.

Disclosures

The authors have nothing to disclose or any other conflicts of interest.

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