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TITLE:
Heterologous Expression and Functional Analysis of *Aedes aegypti* Odorant Receptors to human odors in *Xenopus* Oocytes

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SHORT ABSTRACT:
A protocol is presented that functionally characterizes mosquito ORs in response to human odors using a *Xenopus* oocyte expression system coupled with a two-electrode voltage clamp, providing a powerful new technique for exploring the responses of mosquitoes ORs to exposure to human odors.

ABSTRACT
The mosquito *Aedes aegypti* (Linnaeus), a vector of many important human diseases including yellow fever, dengue fever and Zika fever, shows a strong preference for human hosts over other warm-blooded animals for blood meals. Olfactory cues play a critical role for mosquitoes as they explore their environment and seek a human host to obtain blood meals, thus transmitting human diseases. Odorant receptors (ORs) expressed in the olfactory sensory neurons are known to be responsible for the interaction of mosquito vectors with human odors. To gain deeper insights into *Ae. aegypti*'s olfactory physiology and investigate their interactions with humans at the molecular level, we used an optimized protocol of *Xenopus* Oocytes heterologous expression to functionally

analyze *Aedes aegypti* odorant receptors in response to human odors. Three example experiments are presented: 1) Cloning and synthesizing cRNAs of ORs and odorant co-receptors (Orc) from four-to-six day-old *Ae. aegypti* antennae; 2) Microinjection and expression of ORs and Orc in *Xenopus* oocytes; and 3) Whole-cell current recording from *Xenopus* oocytes expressing mosquito ORs/Orco with a two-electrode voltage-clamp. These optimized procedures provide a new way for researchers to investigate human odor reception in *Aedes* mosquitoes and reveal the underlying mechanisms governing their host-seeking activity at a molecular level.

INTRODUCTION

The yellow fever mosquito *Ae. aegypti* can transmit many deadly diseases including yellow fever, dengue fever and Zika fever, causing tremendous distress and loss of life. Mosquitoes make use of multiple cues such as CO_2, skin odor, and body heat to locate their hosts[1]. Given that both humans and other warm-blooded animals produce CO_2 and have similar body temperatures, it seems likely that female *Ae. aegypti* rely primarily on skin odor for host discrimination[2]. This creates a complex picture, however, with one early study isolating more than 300 compounds from human skin emanations[3]. Further behavioral assays have indicated that a number of these compounds evoke behavioral responses in *Ae. Aegypti*[4-7], but precisely how these compounds are detected by the mosquito remains largely unknown. Recent research by our group has identified several human odorants that may be involved in *Ae. aegypti* host-seeking activity, though their roles have yet to be confirmed by further behavioral assays[8]. How these essential human odorants are decoded in the peripheral sensory system of *Ae. aegypti* has yet to be established.

Insects detect odorants through the chemosensory sensilla on their olfactory appendages. Inside each of the sensilla, different olfactory receptors, including odorant receptors (ORs), ionotropic receptors and gustatory receptors, are expressed on the membrane of olfactory sensory neurons[9]. These ORs are responsible for sensing most of the odorants encountered by insects, especially the odors associated with food, hosts and mating partners[10-13]. A previous study focusing on deorphanizing the function of ORs in *Anopheles gambia* using the *Xenopus* expression system coupled with a two-electrode voltage clamp has found that *Anopheles* ORs are specifically tuned to the aromatics that are the major components in human emanations[14]. A recent genome study identified up to 117 *OR* genes in *Ae. aegypti*[15], a large portion of which were found to be *Aedes* specific. Consequently, a way to systematically address the functions of these *Aedes* ORs in response to biologically or ecologically important odorants such as human odors or oviposition stimuli would provide useful information for those seeking to further understand the chemical ecology or neuroethology of *Ae. aegypti*.

The two-electrode voltage clamp (TEVC) technique was originally developed to examine the function of membrane ion channels in the mid-1990s[16,17]. Since then, TEVC has been used to investigate ORs from a number of different insect species[14,18-34]. This functional examination of ORs has substantially contributed to answering important ecological questions in insects, including: 1) How do insects locate food sources? 2) How are they attracted by the volatile sex pheromones released by their mating partners? 3) How do they find a perfect oviposition site for their offspring? and 4) Are there any compounds, plant-derived or synthetic, that can efficiently protect humans from biting bugs? Answers to these questions are crucial for controlling important disease vectors such as mosquitoes.

A number of other approaches, including those based on the human embryonic kidney cell line 293 (HEK293), the *Drosophila* empty neuron system, zinc-finger nuclease, transcription activator-like effector nuclease, and the CRISPR/Cas9 gene editing system, have also been used in OR functional studies[12,20,35-37]. However, these techniques all require the skills of an experienced molecular biologist and involve multiple potentially confounding factors. TEVC/oocyte expression is capable of directly measuring odor-evoked receptor currents and ion conductance and has the added advantage of the speedy quick setup time required to express receptors from cRNA. In this study, we therefore used TEVC to examine the responses of one *Ae. aegypti* OR55 (AaOR55) against several odorants with potential biological relevance, revealing that oocytes expressed with AaOR55•AaOrco showed a dose-dependent response to the human odorant benzaldehyde.

PROTOCOL

The protocol for this procedure, the Care and Use of Laboratory Animals, is approved and monitored (Auburn University's Institutional Animal Care and Use Committee: approved protocol # 2016-2987.

NOTE: Custom gene synthesis is a viable alternative to cloning for mosquito antennae.

1. Mosquito and Olfactory Appendages (Antennae) Collection

1.1 Maintain *Ae. aegypti* mosquitoes (obtained from Dr. James Becnel, USDA, ARS, Mosquito and Fly Research Unit) at 25 ± 2°C and a photoperiod of 12: 12 (L:D) h (lights on 8 am).

1.2 Anesthetize ~800 4-6-day old female mosquitoes without blood feeding using CO_2. Cut the mosquito antennae (olfactory tissues) under a microscope (40x) using a pair of scissors and collect in a 1.5 mL centrifuge tube kept on dry ice.

NOTE: The collected antennae can be stored in a freezer at -80 °C or be used immediately.

2. *OR* Cloning from Antennae of *Ae. aegypti*

2.1 Extract total RNA from the antennae using a commercially available total RNA kit following the manufacturer's protocol.

2.2 Digest the total RNA using the commercial DNA-free kit in order to remove DNase and other ions following the manufacturer's protocol.

2.3 Synthesize cDNA from 1.5 µg of DNA-free RNA using the Oligo d(T)20-primed SuperScript IV First-Strand Synthesis System[13,30].

2.4 Design PCR primers for the *OR* genes according to the sequences available at VectorBase (https://www.vectorbase.org/) and the special requirements of the enzyme cutting site. Add a Kozak sequence (GCCACC), which initiates translation in most eukaryotic organisms, between the cutting site and the start codon in each forward primer[30].

2.5 Clone the coding sequences of the *OR* genes by PCR using gene-specific primers containing restriction endonuclease sites and the Kozak sequence following the manufacturer's protocol.

2.6 Detect PCR products through a 1% agarose gel and purify using a commercial gel extraction kit following the manufacturer's protocol.

2.7 Perform recombinant plasmid construction through cloning of PCR products into a pT7Ts vector after digestion by restriction enzymes following the manufacturer's protocol.

2.8 Verify the recombinant plasmids by Sanger sequencing and confirm using the VectorBase database.

3. cRNA Synthesis

3.1 Linearize the constructed plasmids with specific restriction enzyme(s).

NOTE: Plasmid DNA must be linearized with a restriction enzyme downstream of the gene to be transcribed.

3.2 Use the linearized plasmids to synthesize cRNAs with a commercial T7 kit following a previous description[13,30].

3.3 Premix cRNA of OR and ORCO (1:1) in a centrifuge tube and aliquot the mixed cRNA into a 2 µL volume (500 ng/µL) for each PCR tube. Store the aliquoted cRNA at -80 °C before use.

4. *Xenopus* Oocyte Collection

NOTE: The procedure is following the instruction of Schneider et al.[38].

4.1 Anesthetize *Xenopus laevis* for 20-30 min in 1 L of ultra-purified water with 1 g of ethyl 3 amino-benzoate methanesulfonate salt (**Figure 1A**). Pinch one of the frog's toes using fingers; if there is no response, the frog is sufficiently anesthetized and ready for surgery. Transfer the anesthetized frog on the ice bed.

4.2 The surgery

4.2.1 Rub the frog ventral area with 10% povidone-iodine solution and wipe the solution with clean cotton balls. Repeat this step 2 times.

4.2.2 Make a small abdominal incision (10-15 mm, **Figure 1B**) with a disposable scalpel. Penetrate skin, fascia-muscle layer separately. The incision is in the lower part of the abdomen, lateral to the midline.

NOTE: Do not injury internal organs.

4.2.3 Grasp parts of ovarian lobes with forceps and pull to the exterior gently. Lift the ovary and cut it off at the body wall level with scissors. Repeat several times until get enough ovarian materials (~200 eggs, **Figure 1C**). Transfer the ovarian materials into washing buffer (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$•$6H_2O$, 5 mM HEPES sodium salt, 10 µg/mL gentamycin, pH 7.6).

4.2.4 Close the surgery wounds (both muscle and skin) with absorbable sutures that typically degrade and fall out naturally in 10-14 days.

4.2.5 After each surgery, place the frog in 1 L of recovery solution (containing 3.4 g of canning & pickling salt and 2 g of instant ocean sea salt) until it wakes up and then return it back to the frog facility.

NOTE: Up to 4 surgeries can be performed on each frog. The operation interval of each frog should be more than 2 months.

4.3 Enzymatic defolliculated digestion and oocytes culturing

4.3.1 Separate the oocytes (~200 eggs, **Figure 1C**,) into small pieces.

4.3.2 Transfer all oocytes into a digestion solution, including 10 mL of washing buffer (Section 4.2.3) and 2 mg/mL collagenase B for 40-60 min at ambient temperature in order to remove follicular cell layers. Examine the samples under a microscope to determine whether 80% of the oocytes have been fully defolliculated; if not, check every 10 min till most of the oocytes are ready.

4.3.3 Rinse the oocytes with 1x Ringer's solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2 \cdot 6H_2O$, 5 mM HEPES sodium salt, pH 7.6), washing buffer (Section 4.2.3), modified Barth's saline (78 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.41 mM $CaCl_2 \cdot 2H_2O$, 0.82 mM $MgSO_4 \cdot 7H_2O$, 2.4 mM $NaHCO_3$, 10 mM HEPES sodium salt, 1.8 mM sodium pyruvate, 10 µg/mL gentamycin, 10 µg/mL streptomycin, pH 7.6) in sequence (5 times for each buffer, using 10 mL each time). Rinsing the oocytes extensively with buffers removes immature and poor-quality oocytes.

4.3.4 Harvest oocytes at stage V-VI according to Dumont's classification. Transfer health/good quality oocytes at stage V-VI with a clear white/dark division and no visually obvious damage (**Figure 1D**) into a 90 mm Petri dish containing modified Barth's saline (Section 4.3.3). Discard the bad quality oocytes (**Figure 1E**). Maintain digested oocytes in insect growth chambers at 18 °C for about 24 hours before microinjection.

5. cRNA Microinjection and Expression of Odorant Receptors (ORs) and OR Co-receptors (Orco) in *Xenopus* Oocytes

5.1 Put a metal matrix into the 100 mm x 15 mm Petri dish, adding modified Barth's saline (Section 4.3.3) until the matrix is submerged. Transfer oocytes on the matrix, and discard the oocytes that do not meet the size. Arrange the oocytes with the vegetal poles on the right side (**Figure 1F**).

5.2 Pull a fresh glass capillary (1.0 mm OD x 0.5 mm ID, length = 100 mm) for each sample using a micropipe puller (Heat = 525, Pull = 50, Velocity = 50, Time = 250) and sharpen with a micropipette beveler at an angle of 25°. Mount the capillary tube on the nanoliter injector.

5.3 Before injection, remove one tube of premixed cRNAs of OR and Orco (Section 3.3) from the freezer and place it on ice until completely dissolved. Fill the capillary tube with the OR/Orco mixture by pressing the **Fill** button of nanoliter injector.

5.4 Inject 10 ng (~23 nL) of the premixed cRNA for each oocyte with a nanoliter injector at a RNAse-free station. (**Figure 1F**).

NOTE: Before injection, we press the 'EMPTY' button to discharge little mixture to ensure the capillary tube is not blocked. Waiting for about 5 seconds to let the cRNAs enter the oocyte entirely.

5.5 After injection, store oocytes on a sterile 24-well cell culture plate with modified Barth's saline at 18°C for 3-7 days before whole-cell recording.

6. Whole-cell Current Recording suing a Two-electrode Voltage-clamp system (Figure 2)

6.1 Before the two-electrode Voltage-clamp recording, dissolve the individual odorant compounds in dimethyl sulfoxide (DMSO) to obtain a series of stock solutions (100 μg/μL). Dilute these stock solutions with 1x ND96 buffer (91 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2 \cdot 2H_2O$, 2 mM $MgCl_2 \cdot 6H_2O$, 5 mM HEPES sodium salt, pH 7.6) 1000-fold to obtain a set of working solutions (0.1 μg/μL).

6.2 Ensure that everything on the TMC vibration isolation table, including the metal shield, microscope, light source, and waste pump, is carefully grounded to reduce the effect of external electrical noise on the whole-cell signal recording.

6.3 Prepare microelectrodes by pulling a glass capillary (1.20 mm OD x 0.94 mm ID, length = 750 mm) using a micropipe puller (Heat = 525, Pull = 50, Velocity = 50, Time = 250). Fill the capillaries with 3 M KCl. Chloridize the Ag/AgCl wires for 2-5 min in 10% chlorine bleach until they turn a dark grey color (pale grey Ag/AgCl wires need to be chloridized).

6.4 Turn on the machine by switching on the Oocyte Clamp and Digidata Digitizer (**Figure 3A**). Open the software (e.g., Clampex 10.3) and click the **Record** button. Turn on the switch for the 1x ND96 buffer (Section 6.1) and the pump for the waste buffer.

6.5 Conduct a pre-test by inserting microelectrodes into the flowing buffer and then pressing the **V_m Electrode Test** button and the **V_e Electrode Test** button to check the resistance of the microelectrodes. V_m should be < 40 mV and V_e should be < 40 μA. Switch off the 1x ND96 buffer (Section 6.1) flow and the waste pump after the pretest.

6.6 Place each oocyte in the perfusion chamber (**Figure 2**) filled with 1x ND96 buffer (Section 6.1) and gently position the oocytes with the vegetal poles facing up by the glass caterpillar. Insert

the electrodes slowly into the oocyte (**Figure 3B**). Adjust the V_m parameter to 0 and then the V_e parameter to 0. Change the Clamp button from 'OFF' to 'FAST'. Adjust the Gain button to ~ -80 mV.

6.7 Check the value of V_e, which should be constant. If not, there is a leak in at least one of the two microelectrodes. If this happens, replace any faulty components with new capillaries.

6.8 Record whole-cell currents from the injected oocytes with an oocyte clamp at a holding potential of ~−80 mV (**Figure 2B**).

6.9 Perform data acquisition and analysis.

6.9.1 Resume the flow of the 1x ND96 buffer (Section 6.1) and turn on the waste pump.

6.9.2 Odorant solution application via the perfusion system: switch off the 1x ND96 buffer (Section 6.1) and flow the odorant solution for 10 seconds. Then record the peak value. To obtain the response of the OR gene to the test odorant, subtract the baseline value from the peak value.

6.9.3 Stop the flow of odorant solution and switch to the 1x ND96 buffer (Section 6.1). Wash the oocyte using the buffer until the current trace recovers from the previous odorant stimulation. The oocyte is then ready for stimulation by another odorant solution. Generally, one oocyte can be used to detect 20-30 chemical stimulations.

NOTE: When testing the concentration-dependent response, the lowest concentration should always be applied first before moving on to the higher concentrations.

REPRESENTATIVE RESULTS

Using the single sensillum recording (SSR) technique, we recently pinpointed human odorants thought to be important for *Ae. aegypti* host-seeking behavior[8]. However, the molecular mechanism driving the process of sensing human odorants in the peripheral sensory system of *Ae. aegypti* remains unknown. ORs play an important role for odorant ligand detection in most insects[10-12]. To perform their function, each OR needs to be co-expressed with Orco to form heteromeric ligand-gated ion channels (**Figure 4**); this is common to most insect species[20,29,39]. Once bound by specific ligands, the heteromeric ion channels expressed on the cell membranes are activated and open, allowing an influx of cations such as Ca^{2+} into the cells[20,35] (**Figure 4**). This produces an inward, or occasionally outward current[30,34] that can be detected by recording electrodes inserted into the cell. These recording electrodes are connected to an amplifier designed for oocyte voltage clamping and the electrical signal acquired is then processed using a digitizer and recorded on the computer. The response of an OR to the ligand perfused can be calculated by subtracting the baseline value from the peak value.

In this study, we examined the function of AaOR55•AaOrco from *Ae. aegypti* using TEVC. Healthy oocytes were harvested at stage V-VII from a *Xenopus* frog and after digestion with Collagenase B, each was injected with 10 ng cRNA of AaOR55, AaOrco, or the premixed AaOR55•AaOrco (1:1). We found that raw cells, cells injected with only AaOR55, and cells injected with only AaOrco showed no response to either two plant-derived chemical compounds

(α-terpinene and citronellal, used as control ligands) or the human odorant (benzaldehyde) (**Figure 5**). However, oocytes injected with both AaOR55 and AaOrco displayed a dose-dependent response to the human odorant benzaldehyde (**Figure 5**), which suggests AaOR55 is at least one of the molecular targets for benzaldehyde in *Ae. aegypti*. This is also consistent with the observation that ORs and Orco need to be co-expressed to form a functional channel[14,20,30,34,35]. Oocytes expressing AaOR55•AaOrco that were bathed in higher concentrations of benzaldehyde elicited stronger responses (**Figure 5**), which indicates more heteromeric ion channels are activated.

The combined AaOR55•AaOrco showed no responses to the two botanical compounds (**Figure 5**), suggesting the distinctive tuning property of each OR/Orco complex[12]. On the other hand, Orco has been reported to respond to a limited number of compounds independent of ORs. For example, the Orco of many Pterygota insect species (including *An. gambiae*, *Culex quinquefasciatus*, *D. melanogaster*, the tobacco budworm *Heliothis virescens*, the Indian jumping ant *Harpegnathos saltator*, and the parasitic fig wasp *Apocrypta bakeri*) can be activated by the agonist VUAA1[34,40,41]. Evidence from evolutionary studies suggests that insect Orco first evolved in the wingless Zygentoma silverfish and the complex ORs/Orco evolved subsequently in the winged Pterygota insects[42], which may explain the conserved role of Orco across different insect species.

Figure 1. A schematic diagram showing the processes involved in isolating oocytes from *Xenopus laevis* and the microinjection of odorant receptors (ORs) and OR Co-receptors (Orco) in *Xenopus* Oocytes. A. *Xenopus laevis;* B. Aseptic surgery; C. Oocytes harvested at stage V-VI with good quality; D. Oocytes after the digestion with 2 mg/mL Collagenase B; E. Oocytes in bad quality that could not be used for study; F. *Xenopus* oocyte arrangement on metal metrix and microinjection of OR and Orco in *Xenopus* oocytes with glass needle.

Figure 2. A diagram illustrates perfusion chamber, wiring, and connection of whole-cell current recording by two-electrode voltage-clamp system.

Figure 3. Two-electrode voltage clamp set up. A. Microscope, perfusion chamber, microelectrodes, magnetic stands, and micromanipulators installed on a TMC vibration isolation table. The perfusion system is suspended on the left of the table. Two microelectrodes are inserted into an oocyte; B. Oocyte clamp system, Digidata digitizer, and monitor.

Figure 4. A diagram illustrates the whole-cell current recording for OR55 and (ORs) and OR Co-receptors (Orco) in *Xenopus* Oocytes. The red bar above each trace indicates a 10 second stimulant application.

Figure 5. Responses of *Xenopus* oocytes to human odorants. *Xenopus* oocytes are injected with deionized distilled water (raw cell), AaOR55 alone, AaOrco alone, or AaOR55/AaOrco. α-terpinene and citronellal are both tested at a concentration of 10^{-4} v/v; Benzaldehyde is tested at serial concentrations, as indicated. The red bar above each trace indicates a 10 second stimulant application.

DISCUSSION

TEVC is a classic technique that is widely used to examine the function of membrane receptors. Although a detailed protocol has already been published[43] that shares considerable similarity with the procedure presented here, the proposed method here introduces some important modifications. For example, here, the cRNA of both OR and ORCO are premixed and aliquoted into small volume samples immediately after synthesis and stored at -80 °C until use rather than mixing them separately on the parafilm immediately before the injection[43]. Moreover, after harvesting the oocytes, we chose to use absorbable sutures to close the wound for both muscle and skin, which is especially useful for closing the muscle because the suture can be absorbed by the frog and does not need to be removed later. In addition, the volume of ovary harvested from each frog is flexible, depending on the needs of each experiment. The protocol in Nakagawa and Touhara (2013) specifically states that one third of the ovary should be removed, which usually results in considerable waste. Our experience suggests that one fifth of the ovary should be sufficient for a single experiment.

The insect ORs, one of the three classes of olfactory receptors responsible for odor sensation, have been extensively tested against different compounds using the TEVC technique[14,18,19,21-34]. Compounds with ecological significance should always be prioritized in functional studies of ORs, including those compounds used by insects to locate food sources, mating partners, and oviposition sites. More than 300 compounds have been isolated from human skin emanations[3], making them a useful reference for chemical panels such as those used for functionally characterizing *Aedes* ORs using TEVC as part of the effort to uncover the molecular basis of mosquito host-seeking behavior.

The successful expression of ORs in oocytes is essential when investigating their function using TEVC. As indicated in previous work in *An. gambiae* and *Cimex lectularius*, 37 out of the 72 ORs in *An. gambiae* and 15 of the ORs in *C. lectularius* have been successfully expressed in *Xenopus* oocytes[14,30]. Several factors could affect the expression of ORs in *Xenopus* oocytes. For example, the quality of oocytes may vary from one frog to another. In this study, we harvested oocytes from *Xenopus* frogs, but it is possible to purchase oocytes with a quality guarantee directly (e.g., Nasco). We were therefore careful to check the oocyte quality after the first 40 min of digestion and continue to check their quality every 10 min until ~80% have been fully defolliculated. Finally, it is important to bear in mind is that some OR genes may be expressed more slowly than others, so the expression should be checked every 24 hours after three days of incubation at 18 °C, with bad oocytes being removed and the buffer changed with fresh modified Barth's saline daily.

The function of ORs can be examined using other experimental methods. The HEK293 cell line, for example, is another in vitro expression system used in insect OR functional studies[35]. Unlike the *Xenopus* oocyte expression system used in the current study, the target *OR* gene needs to be transfected into the HEK293 cells for expression, after which the ion currents are recorded with patch-clamp technology. The *Drosophila* empty neuron system is another in vivo expression system that can be used to investigate insect OR function in a neuron environment[2,11,12], while the empty neuron system is a mutant antennal neuron that lacks any *Drosophila* endogenous OR genes[44]. Exogenous OR genes from other insect species can then be engineered into the mutant antennal neuron using transgenic methods and functionally studied using SSR. Compared to TEVC, these two methods are more complicated and require experienced operators who have completed

a lengthy training process. The *Drosophila* empty neuron system is particularly labor and time-consuming for establishing a stable transgenic UAS line.

A recent study utilized cryo-electron microscopy to identify the structure of Orco in the parasitic fig wasp *A. bakeri*[41]. Future studies that focus specifically on the cryo-EM structure of an insect OR•Orco heterotetramer could help predict the EM structures of other undefined OR•Orco heterotetramers and screen ligands for specific ORs from among the thousands of candidate compounds in a relatively short time via computer modeling. The function of the predicted ligands or ORs could then be confirmed using TEVC.

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DISCLOSURE:

None

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Figure 1

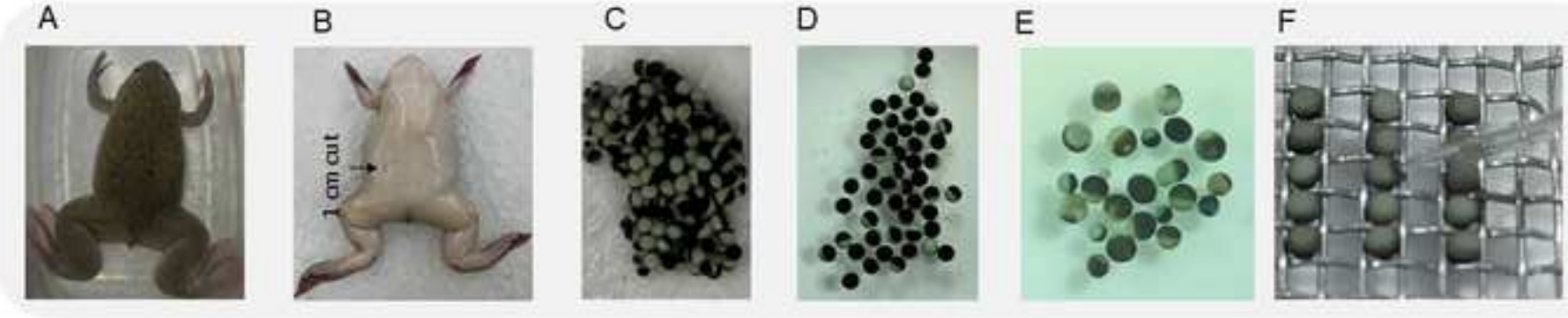


Figure 2

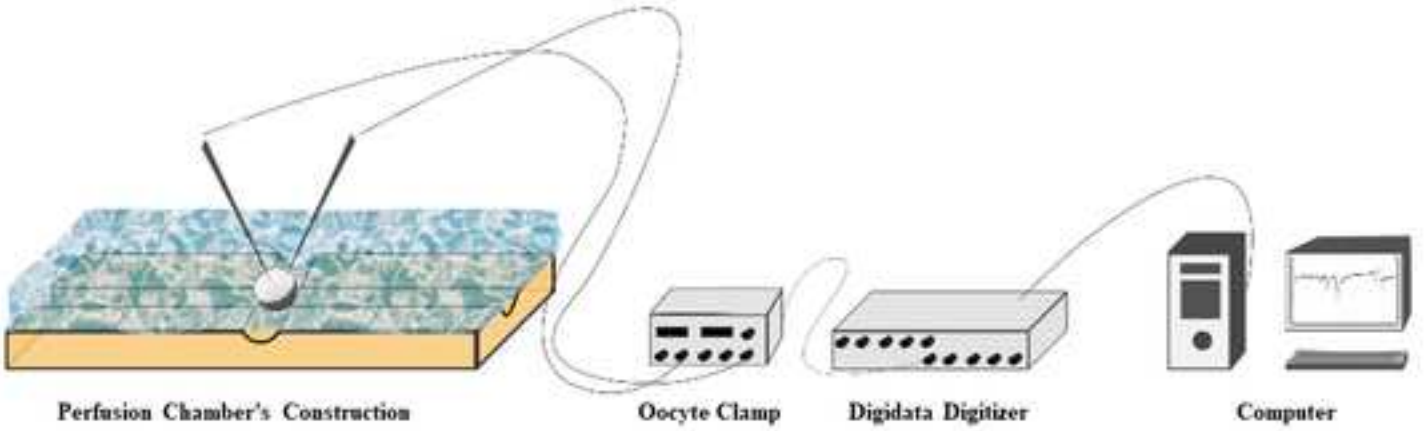


Figure 3

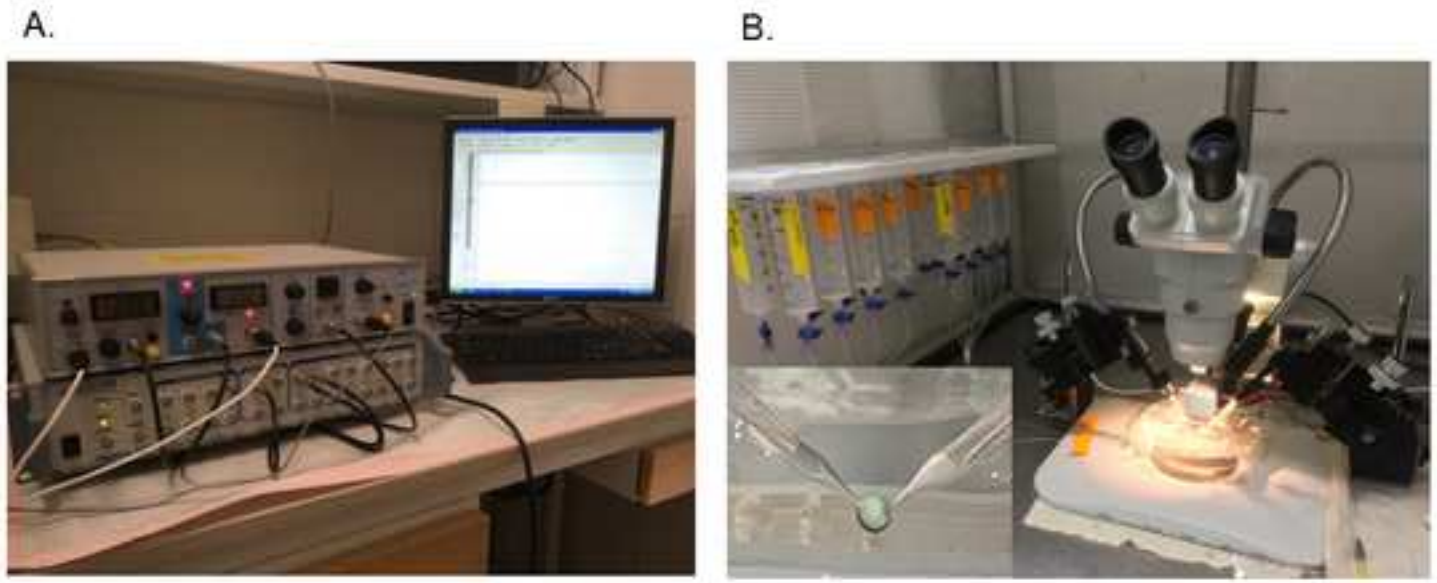


Figure 4

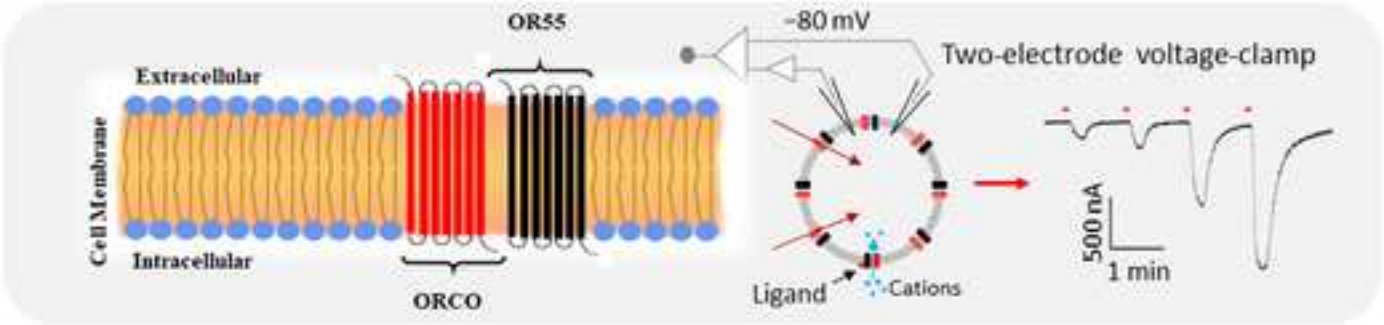
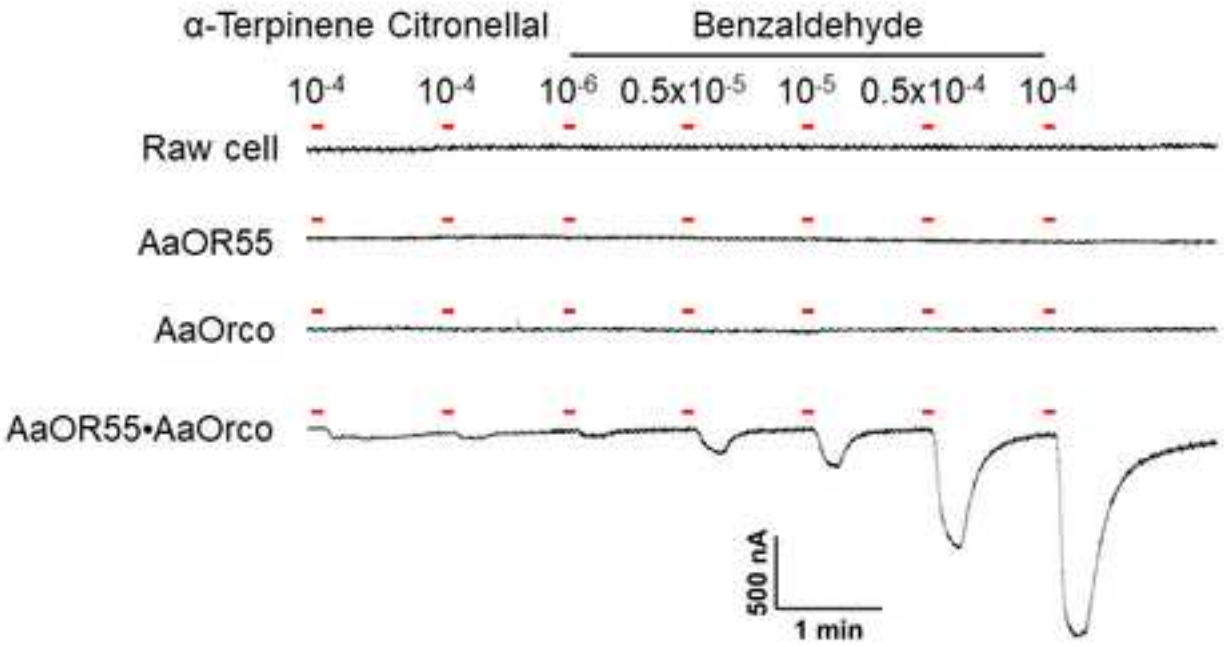


Figure 5



Name	Company
24-well cell culture plate	CytoOne
African clawed frog	Nasco
Ag/AgCl wire electrode	Warner Instruments
Clampex 10.3	Axon
Clampfit 10.3	Axon Instruments Inc.
Collagenase B	Sigma
Digidata Digitizer	Axon CNS
E.Z.N.A. Plasmid DNA Mini kit	Omega
Ethyl-M-aminobenzoate methanesulfonate salt	Sigma
Glass capillary	FHC
Glass capillary	Warner Instruments
GyroMini Nutating Mixer	Labnet
Insect Growth Chambers	Caron Products
Leica Microscope	Leica
Light Source	Schott
Magnetic stand	Narishige
Micromanipulator	Leica
Micropipe puller	Sutter
Micropipette beveler	Sutter
mMESSAGE mMACHINE T7 kit	Invitrogen
Nanoject II Auto-Nanoliter Injector	Drummond
Oligo d(T)20-primed SuperScript IV First-Strand Synthesis System	Invitrogen
Olympus Microscope	Olympus
One Shot TOP10 Chemically Competent *E. coli* cells	Invitrogen
Oocyte clamp amplifier	Warner Instruments
QIAquick gel extraction kit	Qiagen

TMC Vibration Isolation Table	TMC
TURBO DNA-free kit	Invitrogen

Catalog Number
CC7682-7524
LM00535
64-1282
N.A.
N.A.
11088815001
Digidata 1440A
D6942-01
886-86-2
30-30-1
64-0801
S0500
model 6025
S6 D
A20500
GJ-1
115378
model P-97
model BV-10
AM1344
3-000-204
18091050
SZ61 C404003
model OC-725C
28704

63-500

AM1907

Comments
Used for oocyte culture
Used to harvest *Xenopus* oocytes
Used for microelectrodes
Used for signal recording
Used for data analysis
Used for oocyte digestion
Used for data acquisition
Used for plasmid preparation
Used for anesthetizing frogs
Used for microinjection
Used for preparing microelectrodes
Used for oocyte digestion
Used for oocyte incubation
Used for cutting mosquito antennae
Providing light sources for observation
Used to hold the reference electrode
Used for minor movement of electrode
Used to pull capillaries
Used to sharpen capillaries
Used for synthesizing cRNA
Used for microinjection
Used for synthesizing cDNA
Used for microinjection
Used for transformation
Used for TEVC recording
Used for gel purification

Used for isolating the vibration from the equipment
Used to remove DNase and other ions in RNA

August 31, 2020

Dr. Lyndsay Troye
Science Editor
JoVE

Dear Dr. Troye:

Enclosed is the revised version of SREP-19-26401 with a revised title of "Heterologous Expression and Functional Analysis of *Aedes aegypti* Odorant Receptors to human odors in *Xenopus* Oocytes" (title has been revised ccording to the editor's and reviewers' comments). Point-by-point responses to the reviewers' and editor's comments are attached. I believe that the revised manuscript is more informative, clear, and compelling. It will be very interest to your readers. However, we will be very happy to consider any changes you feel are necessary.

The reviewers' and the editor's thoughtful, considerate, and valuable comments have been very much appreciated. Thank you very much.

Sincerely yours,

Nannna

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