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A step-by-step guide to mosquito electroantennography

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TITLE:

A step-by-step guide to Mosquito Electroantennography

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Electroantennogram, EAG, GC-EAD, disease vector, electrophysiology, olfaction, mosquito

SUMMARY:

The present article details a step-by-step protocol for successful and low noise electroantennograms in several genera of mosquitoes, including both females and males.

ABSTRACT:

Female mosquitoes are the deadliest animals on earth, claiming the lives of more than 1 million people every year due to pathogens they transmit when acquiring a blood-meal. To locate a host to feed on, mosquitoes rely on a wide range of sensory cues, including visual, mechanical, thermal, and olfactory. The study details a technique, electroantennography (EAG), that allows researchers to assess whether the mosquitoes can detect individual chemicals and blends of chemicals in a concentration-dependent manner. When coupled with gas-chromatography (GC-EAG), this technique allows to expose the antennae to a full headspace/complex mixture and determines which chemicals present in the sample of interest, the mosquito can detect. This is applicable to host body odors as well as plant floral bouquets or other ecologically relevant odors (e.g., oviposition sites odorants). Here, we described a protocol that permits long durations of preparation responsiveness time and is applicable to both female and male mosquitoes from multiple genera, including *Aedes*, *Culex*, *Anopheles*, and *Toxorhynchites* mosquitoes. As olfaction plays a major part in mosquito-host interactions and mosquito biology in general, EAGs and GC-EAG can reveal compounds of interest for the development of new disease vector control strategies (e.g., baits). Complemented with behavioral assays, the valence (e.g., attractant, repellent) of each chemical can be determined.

INTRODUCTION:

Mosquitoes are the deadliest organisms on earth, claiming the lives of more than one million people per year and place more than half the world population at risk of exposure to the pathogens they transmit, while biting[1]. These insects rely on a wide range of cues (i.e., thermal, visual, mechanical, olfactory, auditory) to locate a host to feed on (both plant and animal), for mating and oviposition, as well as to avoid predators at both the larval and adult stages[2,3]. Among

these senses, olfaction plays a critical role in the above mentioned behaviors, in particular for medium to long-range detection of odorant molecules[2,3]. Odors emitted by a host or an oviposition site are detected by various specific olfactory receptors (e.g., GRs, ORs, IRs) located on the mosquito palps proboscis, tarsi, and antennae[2,3].

As olfaction is a key component of their host-seeking (plant and animal), mating and oviposition behaviors, it thus constitutes an ideal target to study to develop new tools for mosquito control[4]. Research on repellents (e.g., DEET, IR3535, picaridin) and baits (e.g., BG sentinel human lure) is extremely prolific[5], but because of the current challenges in mosquito control (e.g., insecticide resistance, invasive species), it is essential to develop new efficient control methods informed by the mosquito biology.

Many techniques (e.g., olfactometer, landing assays, electrophysiology) have been used to assess the bioactivity of compounds or mixtures of compounds in mosquitoes. Among them, electroantennography (or electroantennograms (EAGs)) can be used to determine whether the odorants are detected by the mosquito antennae. This technique was initially developed by Schneider[6] and has been used in many different insect genera since then, including moths[7-9], bumblebees[10,11], honeybees[12,13,] and fruit flies[14,15] to name a few. Electroantennography has also been employed using various protocols, including single or multiple antennae in mosquitoes[16-25].

Mosquitoes are relatively small and delicate insects with rather thin antennae. While performing EAGs on larger insects such as moths or bumblebees is relatively easy because of their larger size and thicker antennae, conducting EAGs in mosquitoes can be challenging. In particular, maintaining a good signal-to-noise ratio and a lasting responsive preparation are two major requirements for data reproducibility and reliability.

The step-by-step guide to low noise EAGs proposed here directly offers solutions to these limitations and make this protocol applicable to several mosquito species from various genera, including *Aedes*, *Anopheles*, *Culex*, and *Toxorhynchites*, and describes the technique for both females and males. Electroantennography offers a quick yet reliable way to screen and determine bioactive compounds that can then be leveraged in bait development after valence has been determined with behavioral assays.

PROTOCOL:

1. Saline solution preparation

1.1. Prepare the saline in advance and store in the fridge.

1.2. Follow Beyenbach and Masia[26] to prepare the solution.

NOTE: Saline recipe in mM: 150.0 NaCl, 25.0 HEPES, 5.0 glucose, 3.4 KCl, 1.8 $NaHCO_3$, 1.7 $CaCl_2$, and 1.0 $MgCl_2$. The pH is adjusted to 7.1 with 1 M NaOH. Do not add glucose or sucrose to the

preparation at this time to increase shelf storage. Add the needed quantity to the saline right before running the EAGs (around 50 mL per experiment).

2. Odor preparation and storage

2.1. Prepare the odorant mixtures or single compound dilutions in advance in 1.5 mL amber vials and store at -20 °C to prevent compound degradation.

NOTE: Concentrations will depend on the test to be conducted. 0.1% or 1% are commonly used to determine whether a compound can be detected or not. For a dose-response curve, prepare serial dilutions of a given chemical and test them from that the lowest to the highest concentrations.

2.2. Prepare the dilutions in water, ethanol, hexane, paraffin oil, or mineral oil, depending on the solubility of the tested chemical.

2.3. Make sure to prepare a solvent control (a vial containing only the solvent) for the experiment.

2.4. Remove the odorants from the freezer 30 min prior to starting the experiments to allow them to thaw. Vortex each vial prior to use to mix well the chemical and the solvent.

2.5. Pipette 10 µL of solution on to a piece of filter paper (0.5 cm x 2 cm) loaded inside a labeled glass syringe or Pasteur pipette.

2.6. Load each compound or mixture in a specific Pasteur pipette or syringe to prevent contamination.

NOTE: Load 10 min before starting the experiment so the odor can diffuse in the syringe but not for longer to prevent degradation. Let the Pasteur pipette or syringe remain capped at this time to allow a good diffusion of the chemical before the experiment starts.

2.7. After each EAG run, dispose the piece of the filter paper and replace it with a new one to prevent the paper from getting oversoaked and risking needle blockage. Replace the needles regularly (every 10 runs).

3. Mosquito separation

3.1. Isolate the mosquitoes on the day of the experiments.

3.2. Use mosquitoes that are at least 6 days old on the day of the experiments to increase the chances that the females are mated to enhance their response to host-related odorants.

NOTE: Adjust mosquito age at the time of the test depending on the project. Check and harmonize the physiological status (e.g., blood-fed, starved, never previously fed, etc.).

3.3. Starve the mosquitoes up to 12 h (i.e., no access to sugar) to increase their motivation and sensitivity.

3.4. Place the mosquito container in the fridge (4 °C) until they stop flying so individuals can easily be delicately transferred to single cups with forceps.

NOTE: Species with a higher tolerance to cold can be put down using a CO_2 fly pad. Ensure that the mosquitoes do not stay on it for a long time to prevent desiccation, which would decrease the responsiveness of the mosquito EAG preparation.

3.5. Store the cups containing single mosquitoes at room temperature before the EAGs are performed and discard any mosquitoes that may not be used during the day.

4. Electrode holder and capillary preparation

4.1. Capillary pulling, preparation, and storage

4.1.1. Use borosilicate capillaries with filaments (I.D: 0.78 mm, O.D: 1 mm). Pull them depending on the equipment[27].

NOTE: Store the pulled capillaries in a Petri dish. Place the Petri dish on to pieces of wax or unscented modeling clay to prevent them from moving and breaking.

4.1.2. Before running the EAG experiment, gently break the tip of 2 capillaries with a pair of forceps under the microscope.

NOTE: Ensure that one is slightly bigger than the other to fit either the neck (larger capillary) or the tips of the antennae (smaller capillary). Ensure that the cut is clean with no crack present on the capillary wall. This requires patience and practice.

4.1.3. If still intact, reuse these capillaries after rinsing with de-ionized (DI) water after the experiment is over. Remove the excess water by gently applying a cleaning wipe against the tip. Place back in storage Petri dish. If the tip is crooked, discard the capillary.

4.2. Electrode holder and capillary mounting

4.2.1. Label the two electrode holders as “recording” and “reference” by using pieces of lab tape of different colors. This will help guide the mounting of the mosquito head and electrodes.

4.2.2. Ensure that the electrode holders are clear inside and that no borosilicate debris are present.

4.2.3. Chloridization: Soak the silver wires of the electrode holders in pure bleach for about 5 min. The wires turn from shiny light grey to matte dark grey.

4.2.4. Loosen the rubber stopper and fill the inside of the capillary with 10% saline solution using a 20 G needle.

4.2.5. Fill the borosilicate capillary with the saline solution using a syringe. Ensure that no bubbles are present in neither the electrode holder nor the pulled capillary.

NOTE: To reduce the chances of having bubbles in the capillary, keep pushing saline in the capillary while gently pulling the needle out and use capillaries with a filament. It is possible to load the capillaries with a solution composed of 1:3 electrode gel and saline solution. This can help prevent evaporation of the saline and can be particularly useful when learning and practicing EAGs, as the experimenter will need more time to complete the different steps.

4.2.6. After soaking, rinse the silver wires with DI water and insert them in the two capillaries. Ensure that the tip of the wire is less than 1 mm from the tip of the capillary. Ensure that the capillary passes the rubber ring inside the electrode holder without breaking. Gently tighten the rubber stopper. Verify that no air bubbles are present.

4.2.7. Use the capillary with the wider opening on the reference electrode holder (neck), and the smaller opening on the recording electrode holder (antennae).

4.2.8. Leave the two mounted electrode holders on a wet cleaning wipe to prevent the tip from drying out until ready to mount the head.

5. EAG rig preparation (Figure 1)

5.1. Ensure that the air table is up, no blockage in the airline and is on. Ensure that the tank of medical air is still full to avoid changing it in the middle of the experiment. Make sure that there are bubbles in the humidifier.

5.2. Air and pulse delivery system

5.2.1. Turn on the medical air gas tank.

5.2.2. Check the level of the two flowmeters.

NOTE: The flowmeter controlling the main air current bathing the preparation during the whole experiment should be at 140 mL/min and the other related to the odor pulse should read 15 mL/min.

5.3. If doing GC-EAD, turn on the machine, gas tanks and create/load the file/method.

5.4. Turn on the computers, the software applications, valve power supply, and verify the internet connection for the software application to work.

5.4.1. Software application: A short script can be written to deliver the pulse.

5.4.2. EAG software: use any electrophysiology software.

5.4.3. Implement the parameters in the software (e.g., amplifier, duration of recording, duration of the pulses, etc.).

5.5. Deliver a control pulse to verify that the valve delivering the pulses is functional.

5.6. Set the power supply at 5.2 V. Verify the amplifier parameters.

NOTE: The parameters used for the data presented here are: low cut-off filter of 0.1 Hz; high cut-off filter of 500 Hz; Gain of x100.

6. Mosquito head preparation and mounting (**Figure 2**)

6.1. Place an aluminum plate on ice and place a piece of wet cleaning wipe over it.

6.2. Put a small dollop of electrode gel in a corner.

6.3. Place a mosquito cup on ice and let the mosquito cool down for a couple of minutes, or until it is stops flying.

NOTE: Some species are cold-resistant and might require a quick anesthesia over a CO_2 fly pad to go down. The least the mosquito stays on either, the better.

6.4. Place the mosquito on its back and clip the tip of each antenna (only a small portion of the last segment) with micro scissors.

6.5. Use forceps to drag the mosquito next to the electrode gel dollop and dip each antenna's tip gently in the gel. Avoid dipping more than just the very last segment in the electrode gel.

6.6. Using forceps, pull the mosquito antennae out while maintaining them next to each other. Let them come out together from the gel. Make sure that the antennae do not touch the cleaning wipe's surface, or they might separate.

6.7. Place the mosquito on its side and chop the head using micro scissors or a razor blade.

NOTE: Once the head is chopped, proceed quickly to the next steps and to the EAG rig to start the recordings. The preparation should stay responsive for about 30 min.

6.8. Take the reference electrode and gently deep the tip in the gel. Keep in contact with neck tissues and let the head stick on it.

6.9. Move the electrode holders under the EAG microscope and view through the microscope to place the head (i.e., reference) electrode on a micromanipulator. Ensure that the antennae are at the center.

6.10. Grab the recording electrode, place it in front of the antennae tips. Move and align it as close as possible to the tips using the micromanipulator. Using the microscope, move the tip of the recording electrode towards the antennae.

6.11. Connect both electrode holders to the amplifier before inserting the tips to prevent them from moving after insertion.

6.12. Insert the antennae tips in the recording electrode. Ensure that they just contact the saline and the electrode gel and be visible by transparency through the capillary. The antenna goes in by the "suction effect".

6.13. Adjust the position of the head and tips with forceps under the microscope, if needed.

6.14. Place the airline tubing close to the mosquito head preparation (distance: 1 cm).

NOTE: If the head falls off, return to the dissection station and remount the head or prepare a new one if it has been lost or if it has been more than 5 min since the head was cut off. A good connection between the capillary and the neck/antennae is essential for low noise and reliable recording. Ideally, the antennae tips will be at less than 1 mm from the wire of the recording electrode once inserted.

6.15. Turn off the light source, if used.

6.16. Position the vacuum line near the mosquito head preparation (distance: 20 cm) and align with the main airline.

NOTE: The vacuum will help remove chemicals surrounding the head preparation after the stimulus, which could lead to EAG responses after the pulses were applied.

7. Recordings

7.1. After insertion of the tips of the antennae, turn on the amplifier and the noise reducer. Observe the baseline signal and ensure that it is not noisy.

NOTE: Observe whether large oscillations in the electrical signal are present. Adjust the position of the head and antennae tips as needed until the signal is clean. Use alligator clips to ground

anything that introduces noise to the Faraday cage or air table. A baseline signal of less than 0.01 mV in amplitude is ideal for detecting and discriminating minute EAG responses.

7.2. Once the noise level is satisfying, insert the first odor syringe to test in the airline hole.

7.3. Close the Faraday cage. Do not stay in front of the preparation, to reduce noise.

7.4. Click on **Record** on EAG software.

7.5. Deliver the pulse(s) using the software application.

NOTE: The number and duration of the pulses will vary depending on the experiments. Here, single 1 s. pulses per odorant have been used. Odorants were separated by 45 s.

7.6. Note the response of the mosquito antennae in the lab notebook.

NOTE: If the odorant is detected by the mosquito antennae, a clear deflection in the signal is observed (see **Figure 3A**).

7.7. Proceed with the next odor or concentration. Do not forget to randomize the presentation of odorants unless a dose-response curve is performed.

NOTE: A negative control and a positive control should be used in the experiments. This will ensure that the responses observed are indeed olfactory responses and not due to mechanical or electric noise.

7.8. At the end of the recording, apply a positive control to verify that the antennae are still responsive.

NOTE: Use 0.1% or 1% benzaldehyde as all mosquito species tested so far have been responsive to this compound.

7.9. Proceed with the next mosquito preparation.

8. Cleaning

8.1. Turn off the amplifier, the noise reducer, the airline, and the computer.

8.2. Return the odorants to the freezer.

8.3. Remove the filter papers from the glass syringes and clean with 100% ethanol if residue is visible on the walls. Let dry on a cleaning wipe overnight.

8.4. Clean the electrode holders with DI water to remove any possible trace of salt. Dry by gently applying against a piece of cleaning wipe.

8.5. Place mosquito remains in the freezer and dispose 24 h later.

NOTE: If working with infected mosquitoes, follow safety requirements at the institution.

9. Data analyses

9.1. Measure either manually or automatically the EAG responses.

NOTE: The EAG amplitude (-mV) is measured here. Average if multiple pulses have been applied for each compound. Depending on the software used, EAGs can be automatically detected and measured. However, it is essential to inspect each response individually to verify the shape of the response and assess the possible carry-over, delayed response, etc. The ideal EAG response is aligned with the pulse, shows a clear deflection, and is repeatable between mosquito preparations (**Figure 3**).

9.2. Present the raw data to show minimal variability, low noise signal, and clear responses (**Figure 3B**).

NOTE: The data can also be normalized (e.g., Z-score). The negative control value (e.g., mineral oil) (i.e., the baseline) can be subtracted from the response and, if not, should be presented in the figures. A positive control should also be presented.

9.3. Perform statistical analysis using any statistics software[28].

REPRESENTATIVE RESULTS:

Electroantennography is a powerful tool to determine whether a chemical or blend of chemicals is detected by an insect antenna. It can also be used to determine the detection threshold for a given chemical using a gradual increase of concentration (i.e., dose curve response, **Figure 4B**). Moreover, it is useful to test the effects of repellent on the response to host-related odors[29].

Positive and negative controls should always be used in EAGs. Here, benzaldehyde was used as a positive control (**Figure 3B**, **3C**, **4A**). This compound has been found to elicit an antennal response in all mosquito species tested so far[24,25,29]. A negative control should also be used and can consist of the solvent used for diluting the chemicals (e.g., mineral or paraffin oils, hexane, etc.) and should not elicit a response (**Figure 3B**, **3C**, **4A**).

Indeed, when conducting EAGs, a deflection should not be noted when applying the control (**Figure 3B**, **3C**, **4A**). If a response is observed, either the syringe, the mineral oil, and/or the odor line is likely contaminated. If it is the case, a new solution should be prepared, the syringe cleaned with 100% ethanol and dried and/or the airline decontaminated by rinsing with 100% ethanol and dried. If the chosen control elicits a response (e.g., ethanol), the value obtained in -mV for

the control should be subtracted from the value obtained for ethanol and tested chemical combined to assess the impact of the tested chemical on the antennae.

Mosquito species vary in their ability to respond to various compounds as well as in the magnitude of their response. For example, *Toxorhynchites* mosquitoes produce very large EAGs in comparison to *Ae. aegypti*, *An. Stephensi* and *Cx. quinquefasciatus* (**Figure 3C**, **Figure 4A**).

In EAGs, the second pulse and the following usually lead to smaller EAG responses. The presentation of one odorant can also affect the response to the following, so it is important to randomize the odorant order and multiple assays to efficiently test a panel of odorants (unless a dose-response curve is performed). Moreover, separating pulses (e.g., 5 s) and odorants (e.g., 45 s) presentation will help to optimize the EAG responses.

The volatility of the tested chemicals varies and can affect the olfactory response and potentially lead to a delayed response if the tested chemical has very low volatility. Chemical volatility and solubility should be known before conducting EAGs to optimize the assay. The solvent used to prepare the dilutions should also be carefully selected (e.g., ethanol, hexane, mineral, or paraffin oil). Moreover, concentrations should be chosen wisely and should ideally be ecologically relevant. A 1% or 0.1% concentration is often used but is relatively high and not necessarily representative of what the insects can experience in nature. Yet, it is useful to screen compounds with relatively high concentrations in some cases (e.g., for bait development). Repellents can be tested at their commercially available concentration (e.g., DEET is typically sold at a 40% concentration).

If coupled with gas chromatography (i.e., GC-EADs)[25], the compounds eliciting a response can be identified with a GC-MS and then tested individually at various concentrations or in mixtures with EAGs. It is worth mentioning that the valence of the tested chemicals cannot be determined with EAGs. Only a complementary behavioral experiment (e.g., olfactometer, feeding assay) can assess whether the chemical detected by the antennae is attractive, repellent, or neutral to the mosquito. Finally, EAG experiments are only showing responses of the peripheral nervous system.

FIGURE AND TABLE LEGENDS:

Figure 1: Electroantennogram setup comprised of: **A**) Microscope: the microscope used should allow the experimenter to clearly see the preparation to allow the mosquito antennae tips to be inserted in the recording electrodes. **B**) Cold light lamp: the lamp should be turned off when the recordings begin. **C**) Vacuum line: this reduces the risk of accumulation of the odorants around the mosquito head preparation, which could result in antennal responses decoupled from actual stimulation. **D**) Micromanipulators (x2): these will allow for very fine electrode holders movements, which is required for inserting the mosquito antennae in the capillary of the recording electrode. **E**) Recording electrode holder. **F**) Reference electrode holder. **G**) Head stage: both electrodes are plugged in the head stage which is then connected to the amplifier. **H**) Main airline: a constant clean airflow bathed the mosquito head. The flow rate is regulated by a

flowmeter. **I**) Syringe for odor delivery connected to the solenoid valve and flowmeter; **J**) Air table: the air table will reduce noise. **K**) Faraday cage: The Faraday cage will prevent electric noise.

Figure 2: Step-by-step *Aedes* albopictus mosquito head preparation for EAG recordings. **A**) Female mosquito on its back on an icy plate to verify that both antennae are intact. **B**) Last segment of the antennae excision with micro scissors. **C**) Antennae are dipped in electrode gel. **D**) The antennae stick together after pulling them out. Only one segment of each antenna should be in the electrode gel. **E**) Mosquito head excision. **F**) Head mounted on the reference electrode. It should be stable enough to be moved to the EAG rig. **A'-F'**. Same steps as presented above for male EAGs.

Figure 3: Schematic of the mosquito EAG and raw EAG traces. A) EAG schematic (left) and characteristics of the EAG response (right). (Left) The mosquito head is mounted between a reference electrode and a recording electrode connected to an amplifier. The antennae are bathed in a constant airflow in which odorant stimuli are pulsed. Detection of a chemical leads to a deflection (in mV) in the signal. (right) The chemical detection leads to cell depolarization (DPR) followed by cell repolarization (RPR) until return to baseline. The odorant pulse is represented by the gray rectangle. **B**) Screenshot of the WinEDR software highlighting a whole EAG recording trace of a Culex quinquefasciatus female mosquito. Top: unfiltered (i.e., raw) signal. Middle: 1 s odor pulses are indicated by numbers. Bottom: filtered (i.e., 1.5 Hz low pass) signal to 3 odorants and a control (mineral oil). Note the deflections in response to 1% 1-hexanol (1), 1% benzaldehyde (2), and 1% butyric acid (3). Note the absence of response to the negative control, mineral oil (4). **C)** From left to right: Representative EAG responses (in mV) to 1% benzaldehyde (top) and a mineral oil control (bottom) in females *Aedes aegypti*, *Anopheles stephensi, Culex quinquefasciatus*, and *Toxorhynchites rutilus septentrionalis*. The one-second pulse is represented by the colored rectangle above the EAG trace. Note the large deflection in response to benzaldehyde and the lack of response to the mineral oil. Also, note the different scale in *Toxorhynchites rutilus septentrionalis*.

Figure 4: Example representation of EAG results and their statistical analyses. A. Average EAG responses of *Culex quinquefasciatus* (N = 8), *Anopheles stephensi* (N = 10), *Aedes aegypti* (N = 8) and *Toxorhynchites rutilus septentrionalis* (N = 7) females to 1% 1-hexanol (green), 1% butyric acid (orange), 1% benzaldehyde (yellow) and mineral oil (blue). **B.** Culex quinquefasciatus females EAG dose-response curve for 1-hexanol (left) (N = 9) and benzaldehyde (right) (N = 8). Bars represent standard error of the mean. Letters above error bars indicate statistical differences (Pairwise Wilcoxon rank sum test with a Bonferroni correction).

DISCUSSION:

Olfactory mediated behaviors are affected by many factors, including physiological (e.g., age, time of day) and environmental (e.g., temperature, relative humidity)[30]. Thus, when conducting EAGs, it is essential to use insects that are in the same physiological status (i.e., monitoring for age, starving, mating)[31] and to also maintain a warm and humid environment around the preparation to avoid desiccation. A temperature around 25 °C is ideal and 60% to 80% humidity for the main airline. This can be easily achieved by placing a bubbler on the main airline circuit.

Moreover, it is important to consider the ecology of each species to obtain results that are relevant to the insect's biology. For example, if using a nocturnal species, consider inversing their light cycle to test their response during their subjective night. Choosing to conduct EAGs at specific moments of the day (i.e., when the insect is active) is also important. For example, if using *Ae. aegypti* mosquitoes, consider doing the experiments during the peaks of activity of this species (i.e., early in the day and later afternoon). Again, the light cycle can be easily shifted for convenience using climatic chambers or light boxes with an inversed light program using a programmable timer[32]. Eilerts et al.[33] and Krishnan et al.[34], have shown that the sensitivity to specific odorants varies throughout the day. Thus, a good knowledge of the insect's ecology and biology will guaranty more accurate results.

Noise (either electrical or mechanical) can be easily introduced in EAGs. For example, mechanical perturbations can be created by an AC system blowing air towards an EAG preparation. Electrical noise can be reduced with the Humbug, but, if persisting, can be tracked down by plugging elements and grounding them to the Faraday cage using alligator clips (**Figure 3B**). This applies to all the elements present around the preparation (i.e., microscope, lamp, micromanipulators). Some pieces of equipment in the Faraday cage should be unplugged before recording as they might still produce electrical noise (e.g., cold light source) or placed outside the cage. Another type of "noise" is of olfactory nature. The experimenter should avoid wearing perfume or use strongly scented shampoo or detergent. Indeed, many compounds found in these can be detected by mosquitoes (e.g., linalool, citronellol, geraniol, eugenol) and may interfere and affect the results of the experiments. Wearing a lab coat and gloves is also essential to limit unwanted contamination of the airline, syringes, and electrodes.

The presented protocol has the advantage of being easily applicable to all mosquito species, in both males and females, while extending the longevity of the preparation (> 30 min) and with limited variability between preparations. This method leads to very minimal noise in the EAG signal, which allows for testing chemicals at very low concentrations. Once the dissection and mounting steps have been mastered, this technique can produce reliable data in a relatively short amount of time, and straightforward data analyses.

Electroantennography only allows the experimenter to assess whether the mosquito can detect a chemical or not. However, to determine the valence of this chemical, complementary behavioral assays, such as olfactometer assays, are critical to determining whether a specific odorant or mixture is attractive, repellent, or neutral in order to develop efficient tools for mosquito control[35].

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DISCLOSURES

The author has nothing to disclose.

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Figure 1

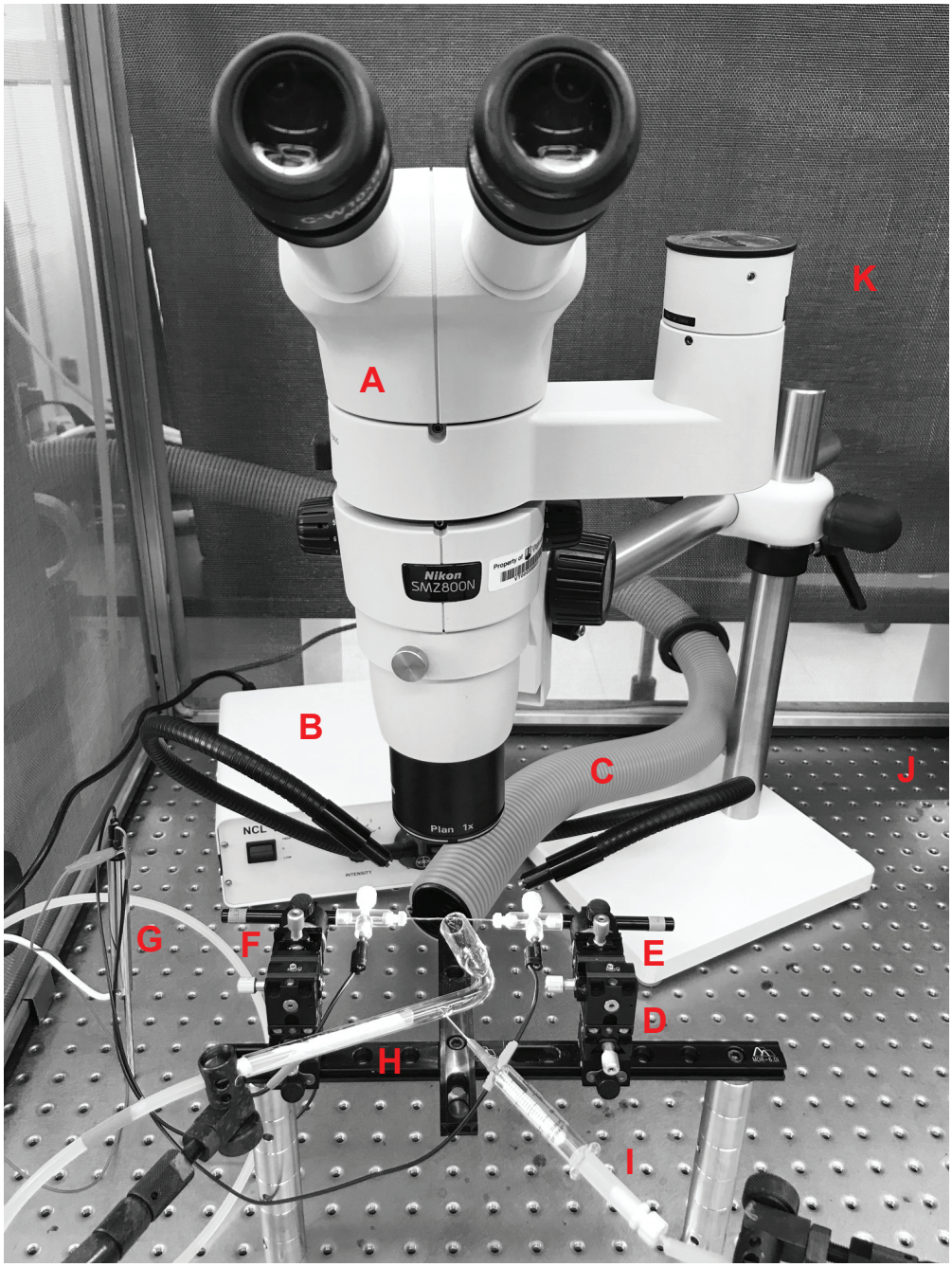


Figure 2

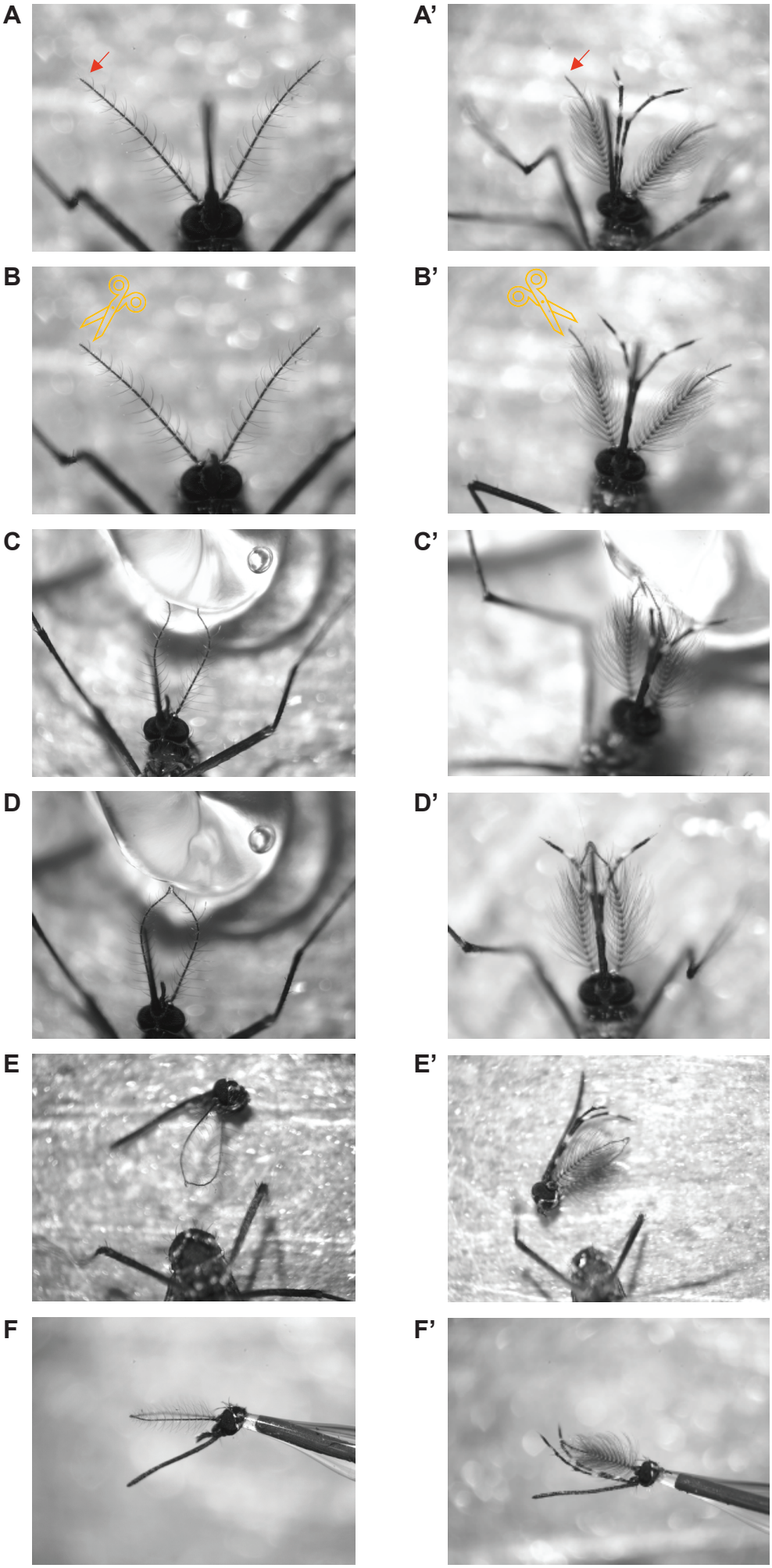
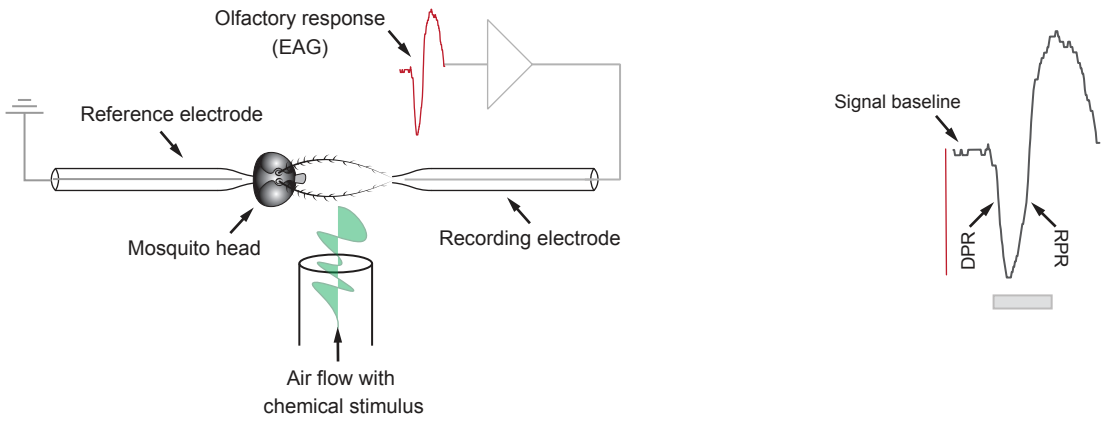
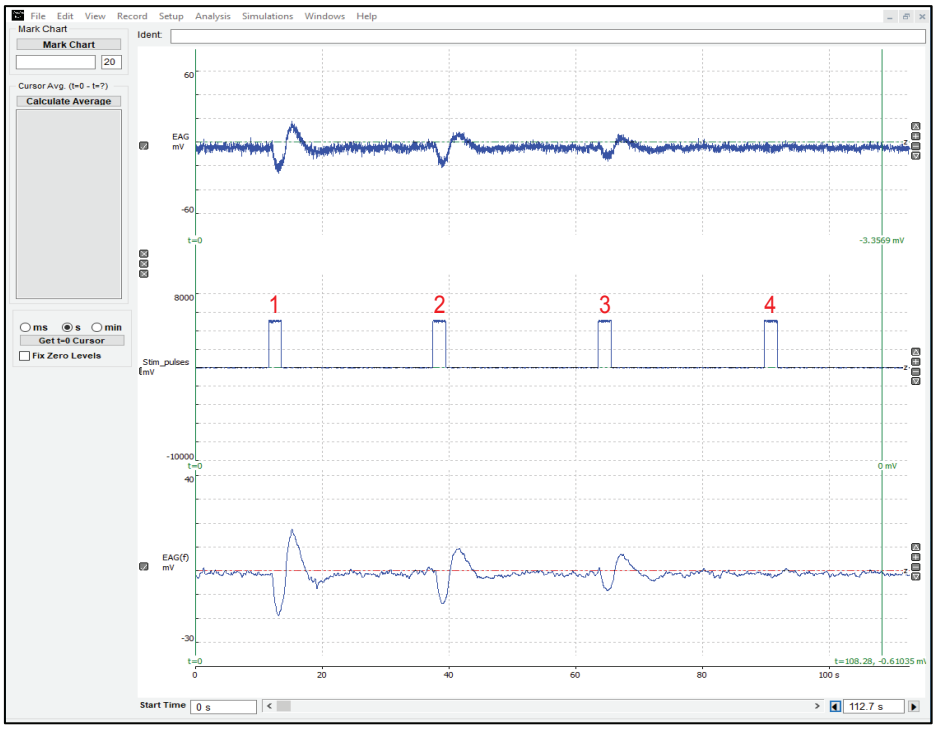


Figure 3

A



B



C

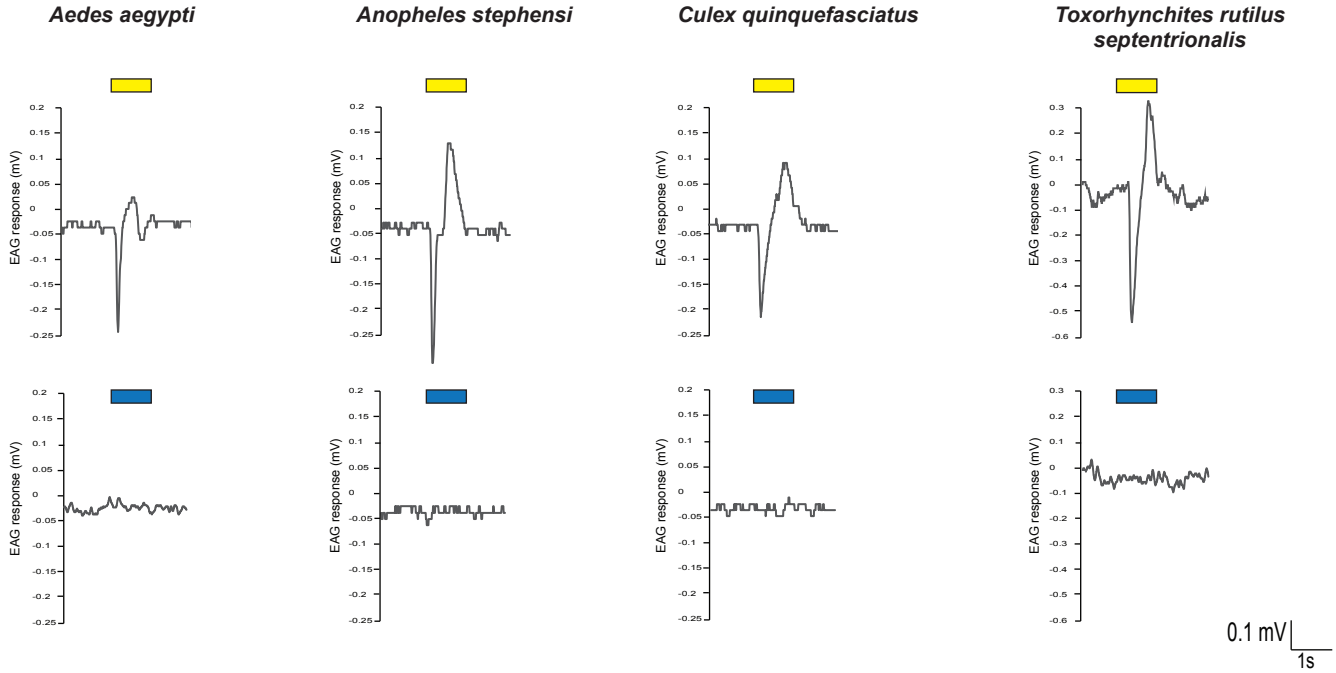


Figure 4

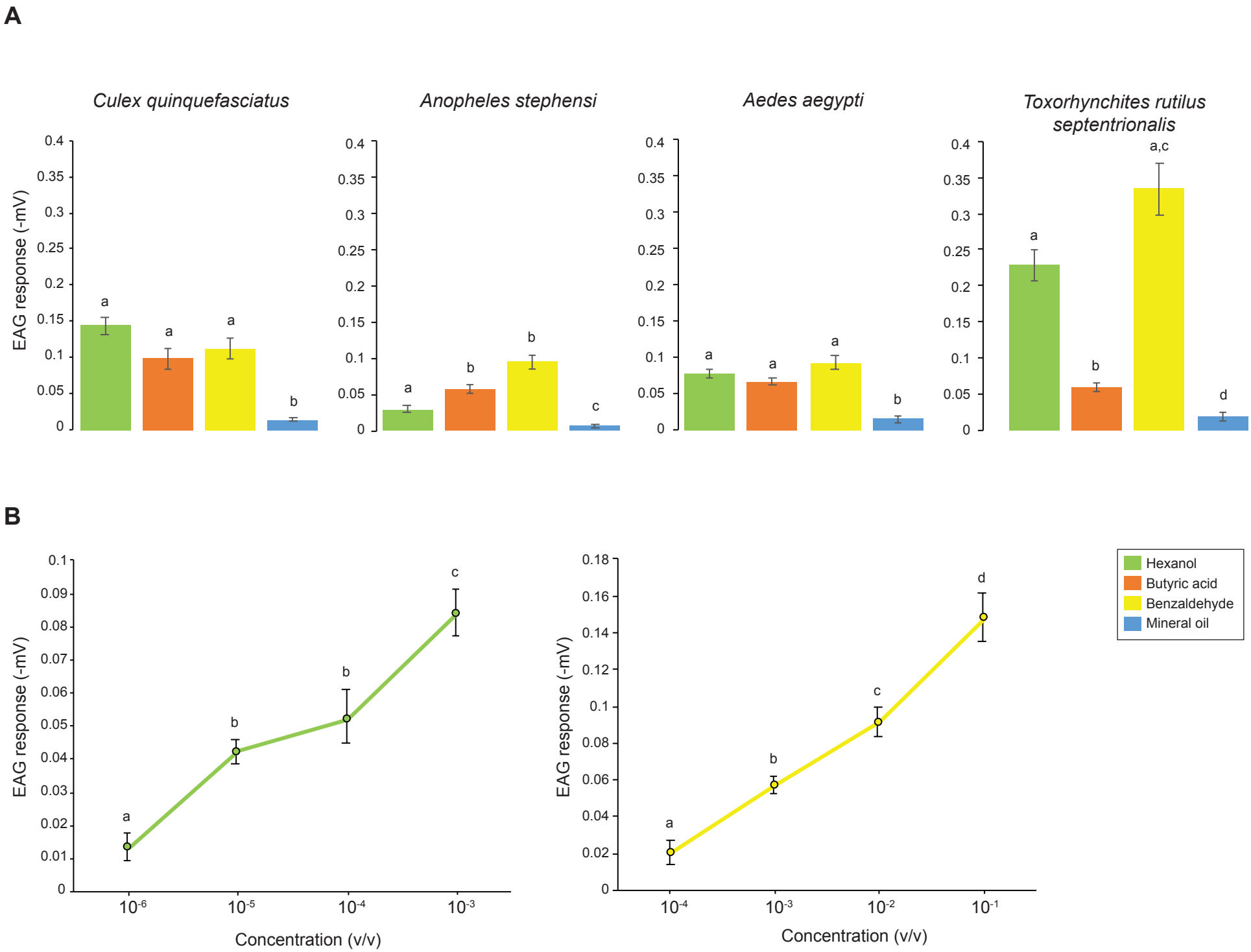


Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_REVISIONS.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Air table Clean Bench	TMC	https://www.techmfg.com/products/labtables/cleanbench63series/accessoriess	Noise reducer
Analog-to-digital board	National Instruments	BNC-2090A	
Benchtop Flowbuddy Complete	Genesee Scientific	59-122BC	To anesthesize mosquitoes
Borosillicate glass capillary	Sutter Instrument	B100-78-10	To make the recording and references capillaries
Chemicals	Sigma Aldrich	Benzaldehyde: 418099-100 mL; Butyric acid: B103500-100mL; 1-Hexanol: 471402-100mL; Mineral oil: M8410-1L	Chemicals used for the experiments presented here
CO2	Airgas or Praxair	N/A	To anesthesize mosquitoes
Cold Light Source	Volpi	NCL-150	
Computer	Variable	Variable	
Disposable syringes	BD	1 mL (309628) / 3 mL (309657)	
Electrode cables	World Precision Instruments	5371	
Electrode gel salt free	Parkerlabs	12-08-Spectra-360	
Faraday cage	TMC	https://www.techmfg.com/products/electric-and-magnetic-field-car	Noise reducer
Flowmeters	Bel-art	65 mm (H40406-0010) / 150 mm (H40407-0075)	One of each
GCMS vials and caps	Thermo-fisher scientific	2-SVWKA8-CPK	To prepare odorant dilutions
Glass syringes (Fortuna)	Sigma Aldrich	Z314307	For odor delivery to the EAG prep
Humbug	Quest Scientific	http://www.quest-sci.com/	Noise reducer
2 mm Jack Holder, Narrow, 90 deg., With Wire	A-M Systems	675748	Electrode holder
Magnetic bases	Kanetec	MB-FX	x 2
MATLAB + Toolboxes	Mathworks	https://www.mathworks.com/products/matlab.html	For delivering the pulses
Medical air	Airgas or Praxair	N/A	For main airline
Microscope	Nikkon	SMZ-800N	
Micromanipulators Three-Axis Coarse/Fine Compact Micromanipulator	Narishige	MHW-3	x 2
Microelectrode amplifier with headstage	A-M Systems	Model 1800	
Mosquito rearing supplies	Bioquip	https://www.bioquip.com/Search/WebCatalog.asp	
Needles	BD	25G (305127) / 21G (305165)	
Pasteur pipettes	Fisher Scientific	13-678-6A	For odor delivery to the EAG prep

PTFE Tubing of different diameters	Mc Master Carr	N/A	To connect solenoid valve, flowmeter, airline ect.
30V/5A DC Power Supply	Dr. Meter	PS-305DM	
R version 3.5.1	R project	https://www.r-project.org/	For data analyses
Relay for solenoid valve	N/A	Custom made	
Silver wire 0.01”	A-M Systems	782500	
Solenoid valve (3-way)	The Lee Company	LHDA0533115H	
WinEDR software	Strathclyde Electrophysiology Software	WinEDR V3.9.1	For EAG recording
Whatman paper	Cole Parmer	UX-06648-03	To load chemical in glass syringe / Pasteur pipette

Reviewer #1:

Manuscript Summary:

This is a very useful protocol provided by Chloé Lahondère. The electroantennography (EAG) recording method will be broadly used for detecting the neuronal response toward the chemicals in insects, particular in disease vectors. Before publication, here are a few minor comments need to be addressed.

A: Thank you to the reviewer for their helpful suggestions to improve this protocol. Please find below responses to specific comments.

Major Concerns:

No.

Minor Concerns:

Line 87, clarify the recipe of saline solution in the manuscript.

A: This has been done (see section 1.2)

Line 208, clarify how long the mosquitoes could be maintained and still could be used for recording after chopped the head.

A: The mosquito preparation can remain responsive for 30 minutes; This has been added to the discussion.

Line 237, clarify the distance between the tip of main air tube for delivering the odor and antenna. And what's the equipment used for controlling the main air and odor pulse?

A: This is shown in figure 1, more details have been added to the protocol for clarity (see section 5 and point 6.14).

Line 239, clarify what's the interval time between different odors delivered.

A: This has been added to the protocol (see section 7.5). Overall, this will depend on the number of odors tested and it will be to the experimenter to ultimately decide.

Line 332, for fig.3, clarify the concentrations of chemicals used in EAG recording and clarify the duration of odor pulse delivered. And clarify the brand and cat. of those chemicals in the table.

A: 1% dilutions were used for Fig. 3 and Fig. 4A. and the information has been added to the new figure legends. Specific concentrations for the dose response curves are indicated in Fig. 4B. Chemical brand and category have been added to the list of material table.

Line 332, for fig.3, clarify the brand and cat. of electrodes holder for E and F.

A: This is indicated in the table of material. A comment has now been added to guide the reader.

Line 332, for fig.3, pulse 3, need to clarify if there is no response of Octenol during recording or it is due to a tiny neuronal response that EAG cannot detect it? A higher concentration of Octenol need to test. It seems like two small currents after odor pulse delivered, are those the signals or contaminations?

A: Two new figures have been provided for clarity along with statistical analyses.

Line 332, for fig.3, two small currents appeared between odor 1 and odor 2 stimulation, it seems like different from the mineral oil control, clarify the possibility.

A: Two new figures have been provided for clarity along with statistical analyses.

Reviewer #2:

Manuscript Summary:

The manuscript describes an improved EAG recording method for mosquitoes, which might also be used for other insects. The protocol is described in detail and the usefulness of the technique is put in a scientific context. In the discussion, the factors to be considered in experiments are mentioned and the limits of the EAG technique are discussed.

A: Thank you to the reviewer for their useful suggestions and comments on this manuscript. Please find my responses below.

Major Concerns:

The title indicating an "improved Electroantennographys method" makes sense, but the main point I am missing in the manuscript is, in how far the described methodology is different (and thus improved) from earlier used techniques. In this context it might be helpful to also mention the results of the improvements: what is the life time of a preparation? How many stimulus cartridges can you test on a preparation?

A: This is a good point and I have now provided more information throughout the manuscript along with several new figures. The title has also been changed as my intent was not to revolutionize the world of EAGs but rather to provide a detailed protocol which is currently unavailable for mosquito EAG.

The second point is the organisation of the protocol: some basic information is missing in the text (such as a brief description of the main elements of the setup and what they are good for), but then small details are mentioned, which seem somewhat trivial or unprecise (e.g. 4.1.1. labeling of "recording" and "reference" electrodes; 4.2.2. They should be fairly pointy). Also some very detailed points of the description depend on the type of experiment which will be conducted (e.g. mosquito separation), so better stay a bit more general in the description.
A: I definitely agree with this comment and I have now improved the protocol, providing the needed details.

Minor Concerns:
- The nomenclature is unclear at several instances: please clearly state when you talk about electrode holders, and when you talk about electrodes. Also the use of "reference" and "recording" electrode are mixed up in certain parts.
A: I have clarified electrode holders and capillaries across the manuscript. Thank you for pointing out the error for reference and recording in one of the sections.
- It would be useful to know what type of electrode holder you use.
A: This is provided in the table of materials.
- Concerning 4.3.4.: you might want to consider using glass capillaries with a filament, which help to avoid problems caused by bubbles
A: Borosilicate capillaries with a filament were actually used for these experiments (see table of materials) and I have added this information to the protocol.
- An alternative to grounding elements inside the Faraday cage is to place them outside the cage (e.g. the cold light source, just use a hole in the cage to introduce the light-guide)
A: Thanks for the suggestion, I have added it to the protocol.
- There are numerous language issues in the manuscript, which at certain points even make it difficult to understand what the author wants to say.
A: The manuscript has been reviewed by an English native speaker. I wished the reviewer had provided a couple specific examples to improve the quality of the manuscript. I hope the revised version of this manuscript is clearer.

Reviewer #3:
This is such a long title for recordings of the mosquito antennae. This might be suitable for a classroom, but I am not sure such submission is suitable for publication in a scientific journal or video journal, joVE. Then what is the mission implemented in JoVE? Discuss about science, report something new or publish a presentation of a laboratory? There is nothing new and/or improved in this submission.
A: The title has now been changed to reflect the comments of all reviewers. The aim of the present paper is to describe in detail all the steps necessary to perform electroantennograms in mosquitoes. To my knowledge, there is no existing protocol for mosquito EAGs and, by extension, none including a video tutorial. Therefore, the aim of this manuscript is indeed not to revolutionize the field of EAGs but, rather to provide a clear set of guidelines for reproducible EAG recordings in mosquitoes. Finally, for the last comment here, the editor informed me that "novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded".

Figure 1 shows sexual dimorphism in the antennae from mosquitoes, small stick (like ants) in the female, small Christmas tree (like moths) in the male. Then how to mount them for electrophysiological recordings is hardly a breakthrough, especially in this configuration where the functional immobilization of the antenna is not achieved. In addition, I would say that isolated tissue is not a real true phenomenal improvement as whole insect preparations are strongly recommended for recording the average output of the insect antenna to its brain for an olfactory stimulus. 'Isolated head' means recording of the activity of a peripheral organ connected to a central organ deprived of all other peripheral afferents. What is the role of these joint afferents in brain control of odor sensing? What is the impact of surgery and/or anesthetic effects on electrophysiological recordings? How to compare data and multiple test-chemicals with the using of the same air drying tissue over time?
A: I respectfully disagree with the reviewer's description of the mosquito antennae ("small stick" and "Christmas tree like"), as it does not correspond to the standard descriptions of their morphology accepted in the literature. Here again, the aim of the manuscript is not to describe a breakthrough in the methods but to provide a first published protocol. The reviewer brings up some interesting additional questions here (*e.g.*, isolated antennae versus head-mounting), but they are beyond the scope of this protocol article. To test this hypothesis, it would be required to isolate antennae from the head and compare the EAG amplitude between them and *Aedes*, *Anopheles* and *Culex*. However, isolating the mosquito antennae for EAG usually leads to a very low

signal. Finally, the tissues are not "air-drying" as the preparation is bathed in a humidified air current for the duration of the whole experiment.

Figure 2 showing a microscope and the typical display of an electrophysiological laboratory (or bench) is nothing impressive for a scientific article. The novelty on this bench should be more clearly shown.
A: Again, the aim here is to provide a protocol for mosquito EAGs where, according to the JoVE author guidelines, all details must be included.

Also, the author(s) refers to electroantennography coupled to gas-chromatography (see abstract), but we can hardly see a gas chromatogram or a gas chromatograph on this submission. The results presented on Figure 3 are typical responses recorded from the insect antenna, i.e. electroantennogram. While it is clear that there a deflection over stimulus exposure, it is not very clear what value, phase or amplitude is measured to compare two olfactory puffs simultaneously (see Figure 3). The signals are different depending on the chemical stimulus involved in sensory profile. If the amplitude of the signal is depending on the number of receptors on the antenna and/or internalization, then I wonder if 3 is not a "positive" deflection. Antennal responses also depend on the volatility of the ligand stimulus. Therefore, I wonder if mineral oil is a relevant control for this kind of experiment. Not surprisingly, benzaldehyde induced a strong deflection, not hexadecenoic acid. So, how to interpret a no-response for a non-volatile chemical? What is the interpretation for a smaller second deflection in response to hexanol(-1), a compound from guava fruit, compared to other test-chemicals that show no variations? To note that the second pulse evokes a smaller response to each odorant needs a clear measurement of the signal amplitude, repeated experiments and statistical tests. Would the EAG profile (Figure 3A) be the same if the order of test-chemicals would change? Would the responses be similar when using chemical analogues? How to rely approach and reasoning specificity?
A: GC-EAGs are illustrated in Lahondère et al. (2020) for which I used the electrophysiological preparation described in the present manuscript. The mosquito head preparation is identical for EAGS and GC-EAGs, as indicated in the protocol. In response to this reviewer's questions, two new figures have been provided, including dose-response curves and responses to 4 chemicals in 4 different mosquito species. Example traces have been revisited and 2 schematics have been added to guide the reader. Additionally, the reviewers' questions are now addressed in the revised discussion.

On Figure 3B, we note that the EAG signals in response to benzaldehyde are different across species. What is the interpretation behind this observation? What kind of information can be extracted when we extract the characteristic physical qualities of EAG signals? It is supposed to be a 'low noise' recording, which is not really the case for Toxorhynchites rutilis exposed to mineral oil (the amplitude of the signal or voltage deflection is as high as that observed for test-chemical in the other species).
A: The reviewer brings interesting questions here. Regarding the response to benzaldehyde across the tested species, it could be explained by the fact that this chemical is present in many plant scents and could be used as a common chemical signal by mosquitoes to detect nectar food source. Sugar feeding occurs across mosquito species and the response to this chemical seem to be conserved between mosquito genus. One possible explanation regarding the higher amplitude of the *Toxorhynchites* is that they are approximately 3-4 times bigger than all other species tested here and among the biggest existing mosquito species. Finally, the *Toxorhynchites* signal is usually a bit more noisy, probably again due to the mosquito size. Yet, it does not prevent the possibility to analyze the data and compare across species as long as the baseline of the signal is considered in the analyzes and subsequent statistics.

Then, I am surprised that there are two pieces of literature which are missing here: 1) literature on olfactory receptors and binding proteins, 2) literature on the chemical structures that mediate oviposition and/or sex pheromone communication in mosquitoes. The two pieces of information are probably important to understand the true biological relevance of the deflection observed in EAG.
A: Including this information would be relevant for a review on EAGs or a book chapter but not in the context of this methods manuscript.

In summary, I found this submission too naïve for a scientific work with novelty compatible with publication, and too uninformative and/or incomplete for a good documentation about neurophysiology and recordings of antennal neurons. For instance, we are never told that EAG signal is a voltage deflection that can be recorded thanks to an oscilloscope. We are never told how many times we can amplify the electrical signal from such a thin, small and delicate antenna, so that at the end it can be recorded and visualized as an image of a waveform with a specific deflection. We are never told that this deflection corresponds to receptor activation and cell depolarization. That cells that are capable of depolarization, which is described here, are neurons. We are told very few about mosquito ecology and that they are the deadliest animals on earth needs to be rephrased. Not all mosquitoes are a threat to animal

health, and they do not all carry pathogens. Some carry viruses, which makes their saliva a threat for human health. "These insects rely on a wide range of cues (i.e. thermal, visual, mechanical, olfactory) to locate a host to feed on (both plants and animals)"…… Insects are not animals from a zoological point of view. However, like most animals, all insects rely on a wide range of cues, including acoustics (ears, tympanum, flagellum and pendulum, cercal and johnston's organ), for environmental recognition. The threatening originality of mosquitoes may reside in the fact that males are tuned to nectar, but females develop to host blood meal, similarly to ticks and lice that are among the deadliest forms of life on Earth. The whole text should be rewritten accordingly.

A: Some more information has been added about the nature of EAGs and their mechanistic underpinnings, both in the text and in Figure 3. Yet, again, this is not a book chapter, nor a review. The aim of JoVE publications is to provide a reproducible protocol for a given experimental technique. Auditory cues have been added to the introduction. Regarding the epidemiological importance of mosquitoes, I am using words employed in the World Health Organization's reports, where the pathogens vectored by mosquitoes are quantified as responsible for > 700,000 human deaths, annually. I am not sure to follow the reviewer regarding ticks and lice: both sexes do feed on blood, which is not the case in mosquitoes. Finally, I respectfully encourage this reviewer to update their records on the mortality rates associated with ticks and lice borne diseases.

Reviewer #4:

Manuscript Summary:

This manuscript seeks to present a step-by-step protocol of an improved method to carry out low noise electroantennograms in different mosquitoes species. I recommend carrying out the following minor and major revisions before publication:

A: Thanks to the reviewer for their helpful and detailed comments and suggestions on the manuscript. Please find below my responses to specific comments.

Major Concerns:

- Protocol: Lines 137-139: Pulling electrodes is really a demanding task. Even more if a sentence like "electrodes should by fairly pointy" is supplied. It is possible to give some more clear recommendations to obtain electrodes enough sharp; for instance: electrodes should be pointy enough to introduce later the antennae in the tip of the electrode (see 6.12).

A: More details have been added as "notes" under the 4.1. section.

- Protocol: To make more clear everything I suggest to consider the following recommendations: 1) Remove the actual 4.1 and 4.1.1. 2) Rename the actual 4.2 as 4.1. 3) Include a new 4.2 call "Electrode holders and pippetes mounting" including first the actual 4.1.1 and then everything included in 4.3.

A: Thank you for this suggestion. Sections have been modified accordingly.

- Protocol: Lines 159 and 164: There is any recommendation to avoid bubbles?

A: Using capillaries with a filament (like in this protocol) can help reduce the chance of having bubbles along with carefully filling the capillary with the needle and keep injecting while the needle is pulled out. This tip has been added to section 4.2.5. as a note.

- Protocol: Line 186: Indicate that the script should consider including two stimuli with a separation time of XX ms or seconds. Also indicate if a series of multiple two pulses should be considered.

A: The information has been added for the experiments presented here (see section 7.5.). However, pulses duration and number may vary depending on the experiments.

- Protocol: Lines 228-230: Noise is always present; but, how much noise is acceptable depends on the magnification of the signal. Do you have any recommendation in order to have an idea of what is to much noise?

A: The reviewer is right, noise will always be present. It is best practice to have a baseline signal less than 0.01 mV in amplitude in order to detect minute responses to some specific chemicals. The HumBug system will remove excessive noise for sure.

- Protocol: Lines 241-243: Only a positive control should be apply at the end of the recording? Why not to have also a positive control at the beginning and end of the recordings? What about the negative control? When should be carried out the negative control? Please revise redaction of the final sentence.

A: Both a negative and a positive controls should be used in EAGs. This has been clarified in the protocol (see section 7.7.)

- Protocol: Lines 257-261: Please indicate clearly what should be measure: Amplitude, latency, duration of the response?

A: Thank you for this suggestion. A schematic has been provided to guide the reader (see Figure 3A).

- Representative results: Lines 293-296: That is right. But, not only randomization is important to avoid reduction in the response of the second pulse. Also separation time between stimuli should be considered. I recommend including some suggestions about time separation between couple of stimuli (see previous comment of line 186).

A: Thank you for this suggestion, which has been added to the protocol and the Representative Results section.

- Representative results: Lines 312-315: I highly recommend that this paragraph should be finished with a sentence highlighting that EAG experiments are only showing responses of the peripheral nervous system.
A: Thank you for this suggestion. This information has been added.
- Figure and table legends: Figure 3: Please highlight the differences in amplitude responses by remarking the differences in y-axis in the displayed recordings. In addition the scale showed on the left lower corner is not showing the correct y-scale for all displayed recordings.
A: Two new figures are now available to the reader.
- Figure and table legends: Figure 3: A clear delayed response to octenol is not visible in the recording. At least for me. There is any other recording showing the mentioned delayed response to octenol?
A: The figure has been replaced to avoid confusion.
- Discussion: Lines 353-355: How long should be inverted the light cycle in a nocturnal mosquito in order to ensure that the animals are in subjective night in daylight hours? Please provide evidence with supporting references.
A: *Aedes aegypti* resynchronizes in 48 hrs when under a Light/Dark regime (See reference: Taylor, B., & Jones, M. D. R. (1969). The circadian rhythm of flight activity in the mosquito *Aedes aegypti* (L.): the phase-setting effects of light-on and light off. Journal of Experimental Biology, 51(1), 59-70). We typically use 72 hrs in the lab.
- Discussion: Line 380: Which evidence is available to conclude that the same technique can be applied to kissing bugs? What do you mean with technique? EAG or the improved EAG presented here?
A: This statement has been changed to avoid confusion.
- Discussion: Something should be mentioned about how long lasts the improved EAG technique suggested here in order to achieve trustable results.
A: That is a good point which has been added to point 6.7.

Minor Concerns:
- Summary: Line 20: step-by-step.
A: This has been changed.
- Abstract: Line 28: Change "we" for "I" because the manuscript has only one author.
A: I will ask the editor to see what the journal policy is for this.
- Introduction: Some references are needed to support sentences between lines 49-51 and 51-53.
A: References have now been added.
- Introduction: Line 78: Change "we" for "I" because the manuscript has only one author.
A: See above.
- Protocol: Line 104: Please indicate which grade of Whatman filter paper is necessary. Also indicate the dimensions of the "piece" of filter paper in order to supply the 10 µl of solution.
A: This is provided in the Table of Materials and the size of the paper has been added to the protocol.
- Protocol: Lines 107-108: To allow the diffusion during 10 minutes of the compound or mixture in the syringe or Pasteur pipette is necessary to put cotton, vinipel or parafilm on both ends? I recommend closing both ends not only to allow diffusion, but also to avoid contamination.
A: The needle remains capped and the end of the syringe is capped. This has been added to the protocol.
- Protocol: Lines 148, 169, 194 and 207: Kimwipe.
A: This has been changed.
- Protocol: Line 154: Please make clear the following: Soak the electrode silver wires of the electrode holders in pure bleach……
A: Thank you for the suggestion, this has been added.
- Protocol: Line 171: Do you recommend any electrode gel? Please include company and reference of the electrode gel.
A: This is in the Table of Materials.
- Protocol: Remove the extra spaces between the numerals (6.10, 6.11, 6.12 and 6.13) and the start of the sentence.
A: This has been changed.
- Protocol: Line 254: What is the meaning of EHS?
A: Environmental Health and Safety. This has been changed (now "Safety") since it is different for every country.
- Representative results: Line 270: Should say: Electroantennography is a powerful tool to determine whether an individual chemical or a blend…….
A: Thank you for the suggestion, this has been added.
- Representative results: Line 277: Please revise redaction of the sentence.

A: The sentence has been revised.
- Representative results: Lines 276-278: References are necessary to support this sentence.
A: References have been added.
- Representative results: Line 291: Cx. quinquefasciatus.
A: This has been corrected.
- Representative results: Lines 302-304: Please complete the sentence. I recommend: ….ecologically relevant. For instance, for positive control a 1% or 0.1% concentration of benzaldehyde (include a reference to support the sentence) is often……..
A: Thank you for the suggestion, this has been added.
- Representative results: Line 309: various concentrations.
A: This has been corrected.
- Discussion: Lines 356-358: Please include references to support the peaks of activity of Ae. aegypti.
A: References have now been added.
- References: A complete revision of the references should be carried out. Many journal names are without capital letters (see lines 412, 416, 436, 439, 442, 444, 451-2, 455, 462-3, 478, 485, 489-90 and 498).
A: Thank you for taking the time to review the references as well. They have now been updated.
- References: Lines 472-473: Remove capital letters from the title of the manuscript.
A: This has been changed.

Reviewer #5:
Manuscript Summary:
Authors have developed what appears to be an effective mosquito preparation that is suitable for Electroantenograms (EAGs). The mosquito EAG signals to odorants are typically far less robust than that of other insects like Drosophila and therefore there is value in this method.
A: Thank you to the reviewer for their suggestions to improve the quality of the manuscript. Please find responses to specific comments below.

Major Concerns:
1. There is little quantification of responses, and little replication, without which it is impossible to judge the utility of this technique. At least N=8-10 per odorant should be reported with error bars.
A: This has been done. Please see Figure 4A.
2. A dose response curve for some odorants should be performed.
A: This has been done. Please see Figure 4B.
3. A quantitative analysis of response strength with the commonly used method(s) for mosquito EAGs should be performed using 1 or 2 odorants.
A: This is not possible because I do not currently own another EAG system (e.g., Syntech) in my laboratory. I have modified the title of the manuscript for clarity.

Minor Concerns:
1. More details about the amplifier and filter settings for recordings should be noted.
A: Thank you for pointing this out, details on this has now been added to the protocol (see point 5.6).