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).