**Reviewer Comments:**  
  
**Reviewer 1**

*This manuscript attempts to use a non-invasive microelectrode to record neural activity in the planarian, Schmidtea mediterranea. However, I do not find sufficient evidence supporting the claim that the recoded signals are related to neural activity.*  
  
We thank the reviewer for the detailed and constructive feedback.

*The major control, dead mosquito larvae, isn’t informative, as many tissue properties are quite different from planarian tissues. The dark control is also problematic as light might induce other electrochemical activities at the planarian surface that are not necessarily related to neural activity.  Have the authors tried recording after decapitation?  This should remove the photoreceptors and render the planarians no longer responsive to visible light.  A cleaner experiment should be using RNAi to remove eyespots without affecting other neural tissues (see Lapan et al. cell reports 2012). Have the authors considered another stimulation, such as UV?  A recent study (Shettigar et al. PNAS 2021) also reported ways to fully block the sensory response to UV stimulation of planarians.  Comparing responsive vs non-responsive conditions for a couple stimulations is essential to determine whether the recorded signal reflects neural activity at all.*    
  
We appreciate the comments regarding the possible confounds in the control condition. To alleviate these concerns, we followed the suggestion to record decapitated planarians, and added the results to the manuscript (see page X, lines XXX). In short, as the reviewer predicted, this abolished the increase in neural activity under light stimulation. In addition, we attempted another experiment, in which we aimed to immobilize the planarians using ethanol. Unfortunately, the planarians did not survive this, and the experimental protocol needs to be fine-tuned in detail before we can safely use this approach in our experiments.

Whereas the suggestion to remove the eye-spots or to block UV-light responsiveness using RNAi sounds very interesting and surely is an excellent path for future experiments, we currently don’t have the technical setup to conduct such an experiment. We have however included the suggestion in the discussion section (see page X, lines XXX).

*Finally, the power spectrum is quite convoluted and hard to interpret.  Why not show individual traces?  How does the waveform look like in the recording?  What about other quantitative measures used in electrophysiological studies?*  
  
In the preparation of the figures, we indeed considered including the individual power spectra as well as exemplary raw data segments. Initially, we decided against this, but we agree with the reviewer that this will convey a much clearer impression of the data. Therefore (and also in line with the suggestions by Reviewer 2), we have included the individual power spectra, and additional figures to illustrate the experimental setup and data along different processing stages.

*Line 91/ Line 115 – Learning and memory are very controversial in this field, one should avoid using this terminology without specific definition.* 

We agree with the reviewer and have clarified this on page X, line XXX.

*Line 114 –Number of neurons in a planarian scales with body size and can change by orders of magnitudes.*

Thank you for catching this inconsistency, we have removed the reference to a fixed number this.

*Are planarians tested at roughly the same time during the day across the two conditions? If not, this could add variance to the data due to potential circadian clock.  
When and how often are planarians fed prior to experiments, was this consistent? It is known that feeding can change behavior.*

All recordings took place between 11 a.m. and 3 p.m., i.e., roughly around noon, without differences between the conditions. Similarly, all animals were treated identically, without differences in feeding or handling prior to the experiments. The animals were fed daily.

We added this information to the methods section (see page X, line XXX).

*What percent gel was used to immobile planarians?* 

The animals were fixated in 2% agarose gel. We added this information to the methods section (see page X, line XXX).

**Reviewer 2**

*In this paper, the authors conducted research on noninvasive EEG recording from planarian, and this paper is kindly like an extension of the work in [6] (reference 6 in the manuscript). It might be an interest work, but the authors did not make it clear and comprehensive, and some important details are missing.*

The reviewer is correct in the assessment, that this is an extension of the pioneering work of Aoki and colleagues. We hope that we could clarify all issues and add all relevant missing information.

*1. Why mosquito larvae were used as a control group? In fact, in this paper, planarian was the main model that stated to be close to human’s CNS. It is unclear why the control group was chosen and the reasonability is unclear.*

We thank the reviewer for pointing out this lack of clarity. Mosquito larvae were used as a control group for several reasons. First, they were readily available in the laboratory. Second, we were looking for an intact but dead animal with approximately the same size as a planarian to test how the recording setup responds to the presence of biological material. We have extended our reasoning in the methods section (see page X, line XXX) to further explain our choice. However, we agree, that this approach is not without problems, and – also in line with the suggestion by the first reviewer – we conducted additional experiments with decapitated planarians.

*2. What kind of electrodes were used to acquire EEG signals? The device and its photo should be shown. Also, the photo of the constructed recording environment is also suggested to be added in the manuscript.*

The electrodes used in the current setup were off-the-shelf electroretinography electrodes, similar to these: https://www.spesmedica.com/en/eeg/erg-electroretinography/

We have added this information to the methods section (see page X, line XXX). Following the reviewer’s suggestion, we added detailed photographs of the recording setup.

*3. Based on my knowledge, the power-line frequency of German should be 50 Hz. It is hard to understand “The 23 Hz low-pass filter was chosen due to the high contamination with environmental noise at harmonic frequencies of the power line frequency.”.*

It is correct that the power line frequency is 50 Hz in Germany. To our surprise, the EEG did not only show the expected peak at 50 Hz, but also at the subharmonic frequency 25 Hz. To get rid of this contamination, we chose a low-pass filter below the subharmonic. Sharper notch-filters unfortunately were not effective. To illustrate the data quality, we now include examples of data segments along the different processing steps.

*4. The results in the paper could not sufficiently prove the hypothesis. The authors have to provide more results to further supporting their statement, such as spectrogram and other methods that could support the results. Also, it is suggested to added more stimulation such as vibration or others. There is only one stimulation condition (Light).*

We are sorry that our results could not convince the reviewer. However, we are not sure, how exactly our results are insufficient to prove our hypothesis. As we state in the introduction, “[…] we hypothesized that the ongoing neural activity is characterized by a 1/fx power spectrum with an exponent ‘x’ close to 1, and that changes in lighting will induce changes in neural activity due to the reported photophobia”. Both hypotheses could be answered by the results of the statistical analysis and can be seen in the illustration of the power spectrum. However, to further corroborate our findings, we conducted additional experiments with decapitated planarians, and revised the illustration of the power spectra (please see page X, line XXX).

*5. It is stated that the study will help with the understanding or improvement of AI. However, how they help and what they help are not clarified.*

We thank the reviewer for pointing out this lack of clarity. We have revised the references to artificial intelligence in the introduction and the conclusion (see page X, line XX and page X, lines XXX).

*6. The writing should be improved since there are some errors on both spelling and grammar. Please revise the whole manuscript carefully.*

We carefully revised the manuscript for spelling and grammar.

*7. Non-invasive electrode has its pros and cons. It is understood that for the invasive electrode, it could accurately acquire signals from the location where electrodes were put. But for non-invasive electrodes, the signals acquired is the sum of the electrophysiological signal in the surface. How can prove that the non-invasive signals were EEG signals? A control study for invasive study might be needed.*

The reviewer is correct that in the current experiments, we can’t definitively say whether the recorded signal originate from neural tissue, or for example from muscles. However, Aoki et al., successfully recorded from the cephalic ganglia using invasive electrodes, and the signal we recorded strongly resembles these data. In order to make a more direct argument in support of our interpretation of the current data as originating from neural tissue, we conducted additional experiments with decapitated planarians.

*8. 10 min light stimulation will produce heat. Are there any effects on the temperature of the study? Also, in reference [6], they used cooling anesthesia to avoid myogenic potential, and it was clear they without cooling, the signals not only with spikes, but also with fluctuation. Therefore, I am wondering whether it is sufficient to just eliminate signals based on the amplitude, as stated in the manuscript.*

We thank the reviewer for the pointer to changes in temperature, which we hadn’t considered before. We have double checked the change in temperature due to light stimulation and report the results in the methods section (see page X, line XXX). In short, the light stimulation did not strongly change the planarians’ surface temperature (18.97 °C before the experiment and 19.95 °C after the experiment). With respect to the effect of the different data analysis steps, we hope that the additional figures will clarify how the signal changed after each step.

*Overall, this paper has its merit in some contents. However, this is an immature paper which should be major revised to be accepted for the publication.*

We hope that our revision could address all concerns of the reviewer.  
  
  
**Reviewer 3**

*The aim of present report is to show the feasibility of in vivo and non-invasively recording of the EEG of the planaria during some simple behavioral activities. The results showed a typical 1/fx EEG, with clear differences with a control dead mosquito larvae, and differences in spectral content in light and darkness. As the Ms. is basically a methodological one, only a few clarification of the methods are needed.*

We thank the reviewer for the detailed and constructive feedback.

*Introduction.  
The intro provide all the needed information and clearly expose the objectives of the report.*

Thank you for this positive assessment.

*Methodology.  
-“ from dead mosquito larvae as a control group.” Please justify this type of control. The following sentence did not clarified the purpose: “As a control group to test how the recording setup responds to environmental noise, we recorded data from six red mosquito larvae purchased as food for our planarians.” Why not dead planarias?*

We appreciate that our justification of the control group was not sufficient. Reviewer 2 posed a similar question. Mosquito larvae were used as a control group for several reasons. First, they were readily available in the laboratory. Second, we were looking for an intact but dead animal with approximately the same size as a planarian to test how the recording setup responds to the presence of biological material. We have extended our reasoning in the methods section (see page X, line XXX) to further explain our choice. However, we agree, that this approach is not without problems, and – also in line with the suggestion by the first reviewer – we conducted additional experiments with decapitated planarians. Dead planarians unfortunately dissolve rather quickly so it would be necessary to preserve them, which would again change the electrical properties.

*- “Because we were usure how …”, is that a typo?*

Yes, we fixed this, thank you for catching this.

*- “we used wire electroretinography electrodes” Why were used these electrodes?*

Our goal was to use readily available materials, so off-the-shelf electrodes used to record from other soft tissue (in this case electroretinography electrodes usually placed on the eyeball) were our first choice. These electrodes have the great advantage, that they don’t cover the entire animal as for example cup or ring electrodes would. We have added this to the discussion section (see page X, line XX).

*- Which level of viscosity presented the agarose gel. In order other scientists to reproduce this type of experiment is crucial to know the conditions that permitted the animal to survive in these conditions. Humidity, viscosity, temperature. Where the planaria completely covered by the agarose gel?*

Yes, planarians were completely covered by the agarose gel. We have added further information regarding the composition and condition of the gel to facilitate replications (see page X, line XXX).

*- Which level of mobility did the animal have?*

The animals were completely covered in agarose gel and were thus unable to move. We have added this information to the methods section (see page X, line XXX).

*-Did the light changed the agarose temperature?*

We thank the reviewer for the pointer to changes in temperature, which we hadn’t considered before. We have double checked the change in temperature due to light stimulation and report the results in the methods section (see page X, line XXX). In short, the light stimulation did not strongly change the planarians’ surface temperature (18.97 °C before the experiment and 19.95 °C after the experiment).

*- How far were the electrodes from the animal?*

The animals were placed on top of the electrodes and thus had direct contact to the wires. We have added this information to the methods section (see page X, line XXX).

*- If exponent are computed with fieldtrip please indicated the used functions, similarly FFT and permutation comparisons. Same if they were computed from matlab functions.*

We thank the reviewer for pointing out this lack of precision. We have added the references to the specific fieldtrip functions in the methods section (see page X, line XX). Please also note that we provide all analysis scripts in the github repository (https://github.com/juliankeil/Planarian) and will add this to an OSF repository when the manuscript is accepted.

*- There were qualitative or quantitative differences in movements level in the two experimental conditions?*

As mentioned above, the animals were completely covered in agarose gel and were thus unable to move. We have added this information to the methods section (see page X, line XXX).

*Results, limitations and discussion are clearly exposed.*

Thank you for this positive assessment.

**Reviewer 4**

*In this well-written paper, the authors replicate and extend prior findings (that the planarian EEG is characterized by a 1/f^x power spectrum and that the exponent ‘x’ of the power spectrum is close to 1) to show that changes in lighting induce changes in neural activity likely due to the planarian photophobia. They also establish a non-invasive recording protocol to measure neural activity from planarians under different lighting conditions. This is an important technique and will be of interest to a number of subfields.  
  
There's not much to complain about here, the paper is well done.*

We thank the reviewer for the very positive assessment of our manuscript.

*My only suggestions are:  
- Fig. 2 - I would add some shaded areas around each line to indicate the variability among individuals.  It's important, to understand how different the light and dark datasets really are, compared to the differences between individuals.*

In the preparation of the figures, we indeed considered including the individual power spectra as well as exemplary raw data segments. Initially, we decided against this, but we agree with the reviewer that this will convey a much clearer impression of the data. Therefore (and also in line with the suggestions by Reviewers 1 and 2), we have included the individual power spectra, and additional figures to illustrate the experimental setup and data along different processing staged.

*- Why do the curves in Fig. 2 have the same shape, including the dead larvae? In general, the similarities of the dead larvae response suggest that there are significant improvements that need to be made here, which the authors discuss and acknowledge.*

We agree with the reviewer that this is a curious finding. The shape is highlighted by the log-log display of the power spectra, and this suggests that substantial low-frequency noise is present in the recording. We are actively working on improving the setup for future experiments.