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ATTN: Giuseppi Di Giovanni, Ph.D.

Editor-in-Chief

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University of Malta, Msida, Malta

Dear Dr. Di Giovanni,

We thank you and the referees for their time and efforts on our manuscript. Both referees offered addressable, insightful and cogent criticisms of our manuscript, and we have addressed each in turn by adding two new figures, adding new graphical user interfaces, and adding detail in the manuscript where appropriate. Our responses to each remark are written underneath each comment, which are included below in italics.

**Referee #1 (Remarks to the Author):**  
*1. In their submitted manuscript, Romano and colleagues offer useful applications of a Teensy 3.2 board as a cheap, reliable and easy to build tool for behavioural research. The authors present here how this board can be used to implement a rotation encoder and a controller for CS-US learning association. Although the data presented serves the purpose, the manuscript could be somewhat improved.*

*Figure quality can be improved:  
Figure 2: connection schema are appreciated, but may in some cases be confusing. The figure could gain in clarity if the connections were color-coded, making them easier to follow (this might also help understand which of the connections in panel B are actually relevant).*

**Response:** We appreciate the reviewer’s insight, and agree that this figure could be improved by color-coding the connections in this figure. We done so and have also provided additional labels to all of the pins that are utilized by the prop shield as per their board schematics.

*3.* *Figure 3B: The temporal drift reported in the text might be efficiently represented using a smaller line thickness and inserting a magnification making the divergence more evident (e.g. of the first and the last 30us)*

**Response:** We appreciate the reviewer’s note, and agree that our visual display of temporal drift was insufficient. Further, we recognize that we were not sufficiently explicit in describing the contents of Figure 3B. The line described by the reviewer is the best fit of the data, and does not represent a theoretical recording with no temporal drift. We have included a better description of the data shown in Figure 3B, and have augmented this figure with 3 additional panels. 3Ci demonstrates the best-fit line of the measured data versus the theoretical time stamps in red superimposed on the theoretical, zero-drift line. 3Cii demonstrates the magnifications from the beginning and end of the recording session suggested by the reviewer.

*4. Figure 4: Regarding panel A, same observations raised for the panel B of the previous figure applies.*

**Response:** We appreciate the note, and have also included 3 subplots here to better demonstrate the time

delay.

*5. About the eye-blink paradigm, it is mentioned that the amplitude of the CS is set to 0 and then increased when needed; does this happen live via the custom GUI mentioned in the Methods? Once acquired, are the time-stamps in 0dB condition distinguishable from those produced in the 75dB? Moreover, once you plug an Arduino board, the script that is loaded on it starts automatically: does the researcher have a way of controlling start and end of session, pausing and restarting the paradigm?*

**Response:** We appreciate the note, and acknowledge that, in general, we insufficiently described the role of the graphical user interface (GUI). We have clarified the role of the GUI in the *Methods* section. For both implementations of the *Motion Tracking Experiment* and the *Trace Conditioning Eye Blink* experiments, when the Teensy 3.2 board is plugged in, the board automatically initializes a few options by running the Arduino “setup()” function. It is programmed to then wait for serial input from the computer, which it receives when the user presses “Start” on the GUI. This GUI-based MATLAB function saves the Teensy-reported time stamps for each frame. For the *Motion Tracking Experiment*, it saves the displacements obtained by both ADNS-9800 sensors in both the x and y directions, and the amount of time that elapsed during that specific frame. For the *Trace Conditioning Eye Blink* *Experiments*, it reports for each frame the time elapsed in the experiment and in the current trial, the trial number, whether or not the sound was on in that particular frame, and whether or not the puff was active during that particular frame, for example. Thus, the user is able to retrieve the time periods during which either the CS or the UCS are present.

In our original implementation, the timing of the CS and UCS, the durations of both of these, and the frequency of the CS are all hard-coded into the Teensy start-up script. We appreciate after reading your comment that it is more convenient, especially for a novice Arduino programmer, to have the ability to specify within the GUI all of these parameters, so we have created a new GUI and a minimally-modified accompanying Teensy library to allow the user to specify all of these features.

Originally, our GUI only had the ability to control the start of an experiment and specify its length either explicitly (for the *Motion Tracking Experiment*) or by specifying the duration and number of trials (for the *Trace Conditioning Eye Blink* *Experiment*). We have now added a “Stop” feature to both the GUI code and the Teensy code that allows a user to stop an experiment preemptively and then restart it without having to unplug the Teensy and restart MATLAB. This can function as a preemptive experimental termination, or as a pause, as the user is free to press the “Start” button at any time following usage of the “Stop”. The user does have to specify a new filename after pressing “Stop” and before pressing “Start” again, or the computer will by default assume the user wants to overwrite the stopped experiment.

*6. The manuscript could be strengthened by implementing a third experimental condition in which Teensy is used to play 2 sounds (e.g. having not just a single CS that predicts a US as in the eye-blinking paradigm, but having also a "neutral" CS, not associated with any US).*

**Response:** We think that this is an excellent idea, and have designed a new graphical user interface and accompanying Teensy code that allows the user to control two tones, including their timings and frequency, directly from a graphical user interface.

*7. Minor corrections:  
- page 2. The material reference for the Tindie sensors has a link, while other material have not  
- page 5. Typo: "for pre cise image capture"   
- page 5. " eye puff versus the sCMOS camera (Figure 4Bii)."  In this paragraph, Figure 4Biii and 4Biv should also be referred to in the text.  
- page 5. Technical details on the ADNS-9800 sensors would be more suitable for Methods:*

**Response:** We thank the author for these critiques and have implemented the changes suggested.

**Referee #2 (Remarks to the Author):**

*1. This manuscript proposes to solve technical difficulties inherent to integration of image data acquired via an sCMOS camera with animal behavioural data acquired from other sensors.  
  
I find it difficult to understand which specific problem the presented work seeks to address. It would be useful for the authors to elaborate on how exactly it has been" difficult to easily integrate sCMOS cameras and behavioural experiments". Listing a few specific technical difficulties inherent in this process would have been useful.*

**Response:** We thank the reviewer for this criticism and have added more background to both the “Introduction” and “Conclusion and Discussion” to describe why it has been difficult to integrate sCMOS cameras into behavioral experiments.

*2.* *More critically however, while I recognise that this paper is focused upon providing a technical assessment of the teensy microcontroller for neuroscience research applications the authors have performed live animal experiments on a fixed fixed animal. No statement is given regarding the animals genotype, gender, age nor as far as I can see is there any statement regarding ethical approval and licensing for animal experimentation.*

**Response:** We thank the reviewer for bringing to our attention this oversight, and we have added the pertinent information as to our methods.

*3. Additionally while the authors frequently refer to a sCMOS camera as part of the experimental apparatus it is not immediately clear whether this was connected during the aforementioned experiments and if so, what it was recordibng* [sic]

**Response:**  We thank the reviewer for bringing this to our knowledge. While the camera was attached for all of the experiments, it was not turned on and capturing images. To demonstrate that the digital pulses delivered by the Teensy 3.2 were sufficient to elicit an image capture from an sCMOS camera, we added another animal session to our data set during which we were acquiring images at the direction of the Teensy. These results are shown in Figure 5.