Dear Ms. Jarzylo,

thank you very much for your work on our manuscript ”Recording single neurons’ action potentials from freely moving pigeons across three stages of learning”.

We addressed the reviewers’ comments concerning the protocol and feel that this resulted in a significant improvement of the protocol description. All changes are printed red in the revised manuscript.

We hope that you will now find the manuscript suitable for publication in the Journal of Visualized Experiments.

Yours sincerely,

Sarah Starosta

**Reviewer 1**

Their statement under "Long Abstract" that nobody has looked at single-unit responses in animals trained on both acquisition and extinction in the same session is incorrect. This was done by the below authors who should be cited:

Bingman, V. P., Gasser, B. A., & Colombo, M. (2008). Responses of pigeon wulst neurons during acquisition and reversal of a visual discrimination task. Behavioral Neuroscience, 122, 1139-1147.

**The statement read that “observation periods usually encompass only a single stage of learning” and thus did not imply exclusiveness. Nonetheless, we followed the reviewer’s suggestion and modified the statement as follows:**

**“**But even in these cases, observation periods usually encompass only a single stage of learning, i.e. acquisition or extinction, but not both (exceptions include protocols employing reversal learning; see 1 for an example)”.

**Reviewer 2**

Major Concerns:  
1. It's not clear when the surgery in the animals occurred - before all training? after autoshaping and before training with stimuli? Also, were any sham animals run to ensure that the implant did not interfere with neuronal response in any way?

**This information was indeed lacking from the manuscript. Surgery took place after animals repeatedly (3-4 times) completed the entire acquisition-extinction-reacquisition sequence. We added this note to the protocol.**

**Since there is no significant drop in performance after surgery, one can argue that surgery has no effect on behavioral performance. Whether the implant affects neuronal responses in any way cannot be tested, because the implant is a prerequisite for observing neuronal responses.**

2. Will others be able to replicate the microdrive that is custom built without a picture/diagram? or is this something that is relatively standard among electrophysiology labs?

**We agree that a picture of the microdrive would be very helpful. But since the microdrive was not invented by us and is not the focus of this paper, we refer to the work of Bilkey et al. (1999, 2003) where a detailed description of the microdrive including schematic drawings can be found.**

3. The authors only tested this in one brain area - will this set up work in other areas that require the microdriver to advance through other regions before getting to the area of interest? perhaps note this in limitations.

**In this paper, we only report recording from the nidopallium caudolaterale of the pigeon, a brain region which is extremely well suited for chronic electrode implantations due to its large extent in the dorsal-ventral direction. However, due to the very stable guide cannula of the implant, it is possible to reach deeper brain areas as well. For example, we recorded action potentials from the medial striatum of the pigeon. Other authors (Colombo et al., Brain Research 2001; Bingman et al., Behavioral Neuroscience 2008) used a highly similar implant to record from the entopallium and the visual Wulst of pigeons during behavior. Other recording sites are reported in the Bilkey papers describing the original microdrive. Thus, there is no need to include this issue in limitations.**

4. Because you have to expose pigeons to all stimuli before training (to avoid neophobia), it would be better to refer to stimuli as "untrained" rather than "novel", since the birds had been exposed to them before. However, if the novel stimuli are actually novel, then make this clear in the procedure.

**This issue is indeed confusing. Actually, the birds had not been exposed to the stimuli before. We expose them to a huge set of plain stimuli to avoid neophobia and use all kind of colored patterns as new stimuli in the final task. Therefore, we perceive the term “novel” as adequate, but refined the description of the stimuli in section 1.3.5.**

5. Four months training on this task is a very long time to put in especially if some birds are never able to learn the task. Is this "cost effective" - time and ethics-wise considering the animals have invasive surgery? Could the method be refined to shorten this training time?

**Due to the high complexity of the task, training time is definitely extensive compared to average training durations in small animals. However, in comparison to training procedure of non-human primates, four months is relatively short. We believe that the complexity of the task and the resulting insights into neuronal mechanisms of learning and cognition are worth the effort. Also, animals which are not able to learn this complex task do not undergo surgery and can be allocated to other projects**.

6. Is it realistic to expect that this could be applied to other animals (e.g. rats/mice) if you must require lots of responding for little reward? You state in your introduction that pigeons are "especially suitable" for this task.

**We do not know whether rats or mice would be capable of performing this task, but we see no reason why they should not be. Reversal learning is certainly possible in these species (see e.g. Roesch et al., Nature Neuroscience 2007). However, pigeons are especially suitable for this task, because they are willing to work for extended periods of time at lean schedules of reinforcement and can be trained on a wide set of easy-to-produce visual stimuli.**  
  
Minor Concerns:  
P6 step 1.2.3 - define "reliable"

**We define reliable responding as >85% responses in average. We added this information to step 1.2.3.**

P6 step 1.2.4 - when repeating steps for L and R response keys, do you include trials with previously trained keys?

**Yes, we include trials with the trained middle key. We added this point to the description.**

What happens on a trial when the pigeon pecks the centre key but then does not make a R or L choice?

**This information was indeed lacking and is now included in the description of the behavioral paradigm (figure 2, step 1.3.2.). If an animal did not give a choice response within three seconds, the trial was aborted and the ITI started.**

P7 step 1.4.1 - Let the subject perform 50 "SIFC" trials? How do you decide what reward probability to use?

**We do not give a clear instruction what reward probability to choose, because it depends highly on the individual. However, this issue is addressed already in the discussion part. The reward probability should be chosen high enough to keep the animal motivated through the time course of the session and low enough to avoid premature saturation. Therefore, the optimal reward probability has to be found out for every animal individually by trial and error. However, reward probabilities between 0.5 and 0.8 have been proven to work best.**

Providing approximate or average number of sessions for each step may help others decide whether a pigeon is "getting it" or should be discarded.

**This is a very useful suggestion. We added this information for some of the critical steps.**

Provide full term Spike Density Function on first use in paper.

**We now introduce the abbreviation in the section “representative results”.**

Can you advance the microdrive mid session if not getting any clear neuronal response?

**We advance the electrodes before every session. If we do not get any clear neuronal response, we do not start the session but wait until the next day and advance the electrodes further. We expanded the paragraph describing this procedure to illustrate it more clearly.**

**Reviewer 3**

1. Perhaps the authors could expand the introduction slightly to elaborate on why they developed this particular task. For example, some readers might wonder if a simpler task could works as well. For example, simple train a pigeon to peck the key to receive food, then extinguish it, and then retrain it. One could use a new stimulus each session. Is there a reason that a procedure like this was not adapted? This is not a criticism, because the new procedure is interesting in its own right, but I think the reader might benefit from a slightly more detailed explanation of why this task was developed.

**The reviewer addresses an important issue. We discuss this issue in the 5th paragraph of the discussion section, describing why a Go/NoGo task (which is what the reviewer suggests) is suboptimal for concurrent single-unit recordings and how the developed task circumvents problems associated with such a simpler task.**

2. My main comment/concern is with Figure 5A. In the text, the authors state that "response modulation during presentation of the NS designated for extinction is shown in Figure 5A". From my interpretation, Figure 5A shows both NS1 and NS2, and only one of these stimuli should subsequently undergo extinction (from my read of the methods, only 1 of the stimuli should subsequently undergo extinction). Is this correct? So, I think the authors can be a more clear about what is being shown in Figure 5A.

**We added the information to the description of Figure 5 and changed the paragraph “Representative results; 2. Neural data” as follows:**

“Response modulation during presentation of the NS ~~designated for extinction~~ is shown in Figure 5A. In the acquisition phase, the unit responds strongly to NS 2 (designated for extinction), with responses declining towards the end of the acquisition phase and little change in firing during the other two stages of learning.

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