**Response to Editors/Reviewers: JoVE58873**

Arvin *et al*. Probing nicotinic acetylcholine receptor function in mouse brain slices via laser flash photolysis of photoactivatable nicotine

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| **Reviewer 1 Comment** |  |
| While many of the steps are applicable to anyone regardless of the system/software used, the manuscript is based upon PrairieView software. Would the authors consider mentioning this in the abstract? | **Response**: While we appreciate the reviewer’s point and agree that our approach is specific to PrairieView, we have been instructed by JoVE that commercial language cannot be published. |
| It may be useful to provide some description of the optics of the point-photoactivation / fiber input module. Presumably this is a narrow beam that underfills the back aperture of the objective? Some discussion of the advantages / disadvantages of a focal spot as is often done in 2P uncaging by overfilling the back aperture versus a column of light associated with a narrow beam could be useful. Mentioning the caveat that nicotine would be uncaged above and below the focal point would be helpful for a general audience. | **Response**: Thank you to the reviewer for this point. Our system utilizes a strategy involving filling/over-filling the back aperture of the objective with the 405 nm photostimulation beam. This results in a focal spot, similar to 2P imaging or 2P uncaging. However, our system is capable of a narrow beam too. We have included the requested discussion in various places (Protocol, Results, Discussion) in the revised text. |
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| **Reviewer 2 Comment** |  |
| The authors should demonstrate that the inward currents in Figure 8 are in fact nicotinic currents by reversibly inhibiting the currents with a nicotinic receptor antagonist. | **Response**: Thank you for bringing up this important control. We have demonstrated this already in our recent publication (Banala *et al*.). The traces shown in the present JoVE submission are from MHb neurons, which are the same neuron type that we demonstrated nAChR antagonist-mediated block of nicotine uncaging inward currents in Banala *et al*. |
| In the introduction the authors write "Cholinergic signaling modulates numerous brain processes, including attentional control, volitional movement, and reward." However, no references are provided. | **Response**: References have been added. |
| Similarly they write "Drugs that enhance acetylcholine (ACh) transmission are used to treat cognitive impairment associated with Alzheimer's disease, implying an important role for cholinergic systems in cognition." Again no references are provided. | **Response**: A reference has been added. |
| In the introduction the authors wrote "In most respects, photolysis of PA-Nic is superior to other methods of delivering nAChR ligands to receptors within brain slices. Such approaches include bath application and local drug delivery via a puffer pipette." No references are provided to these other two approaches. | **Response**: References have been added. |
| The authors also omitted another major approach and that is to use channelrhodopsin to evoke ACh release. At least this other approach should be mentioned in the introduction. | **Response**: We have inserted text and references acknowledging this approach, although we have also mentioned the drawbacks associated with usage of problematic ChAT-ChR2-EYFP transgenic mice that have perturbed cholinergic transmission. |
| In the Protocol, the authors mention about the 60X/1.0 NA objective but should also mention the working distance and objective type. | **Response**: The revision now indicates the working distance in the protocol. However, we list the specific objective Olympus model number only in the materials table. |
| The authors report "The pipette holder should be connected via appropriate tubing to a pressure ejection system capable of sustained low-pressure application (1−2psi)." The authors should also mention the specific model and manufacturer of the pressure ejection system that was used. | **Response**: This information is listed in the materials table/excel sheet. |
| In section 3.1.3 the authors refer to Alexa Fluor 488, 568 or 594 but should refer to the full name Alexa Fluor 488, 568 or 594 hydrazide. | **Response**: The full vendor name is specified in the materials table/excel sheet. |
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| **Editorial Comment** |  |
| Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. | **Response**: This has been done. |
| Please provide an email address for each author. | **Response**: Email addresses are as follows:  Matthew C. Arvin: [matthew.arvin@northwestern.edu](mailto:matthew.arvin@northwestern.edu)  David L Wokosin: [scopedoc@northwestern.edu](mailto:scopedoc@northwestern.edu)  Sambashiva Banala: [banalas@janelia.hhmi.org](mailto:banalas@janelia.hhmi.org)  Luke D. Lavis: [lavisl@janelia.hhmi.org](mailto:lavisl@janelia.hhmi.org)  Ryan M. Drenan: [drenan@northwestern.edu](mailto:drenan@northwestern.edu) |
| Please rephrase the Introduction to include a clear statement of the overall goal of this method. | **Response**: The following sentence in the Introduction addresses this point: “Here, the key methodological steps involved in this approach are described, with the overall goal of enhancing the ability to connect nAChR function with neuronal structure.” |
| Please include a space between all numerical values and their corresponding units: 405 nm, 15 mL, 37 °C, 60 s; etc. | **Response**: This has been corrected in the revision. |
| Please remove all commercial language from your manuscript and use generic terms instead. | **Response**: We have removed commercial language from the revised manuscript. |
| Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution. | **Response**: Our IACUC protocol statement was placed before the relevant protocol steps. |
| Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). | **Response**: Personal pronouns have been removed in the revised text. |
| Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). | **Response**: All protocol steps have been updated or checked to have appropriate and unambiguous action items. |
| Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. | **Response**: Protocol steps now include sufficient detail. |
| 1.1.3: How to zero meter? | **Response**: In the revision, we have specifically mentioned that zeroing the meter involves depressing a button. |
| 1.1.6: Please describe how to modulate the laser output. Is there a knob to turn or is it controlled by computer software? | **Response**: In the revision, we have specified that software controls are used to modulate the laser power. |
| 1.1.7, 3.1.3, etc.: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion. | **Response**: These paragraphs have been moved to the Results section. |
| 1.2.4: Please break up into sub-steps. | **Response**: The revised text includes this change. |
| 2.2.2: Please provide the composition of recording solution. How is the solution filtered? | **Response**: We have referenced our recent papers with recording solution recipes. |
| 2.2.5: Please specify the source of tissue used in this step. | **Response**: In the revision, we have specified that the tissue is mouse brain tissue. |
| 2.2.7: How to turn on low pressure application? | **Response**: In the revision, we have clarified that a manual switch needs to be activated. |
| 2.2.8: Please describe how to verify PA-Nic application using 2PLSM. | **Response**: We have removed this step as we feel it is optional and is fully described in the results section. Moving 2PLSM imaging text into 2.2.8. would require a large re-organization of the protocol, which we would like to avoid. |
| 3.1.4, 3.2, and sub-steps: Please describe in imperative tense the specific actions being performed in these steps. Add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps. | **Response**: This sequence has been adjusted as requested with imperative tense phrases and additional details/directions. 3.2 has been collapsed for clarity. |
| 4.7: Please point the specific steps that are repeated here. | **Response**: The specific steps to repeat have been indicated in the revised text. |
| Please include single-line spaces between all paragraphs, headings, steps, etc. | **Response**: Spacing has been adjusted as requested in the revised text. |
| please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video. | **Response**: Sections for the video (about 2.5 pages) have been highlighted with grey highlighting. |
| Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. | **Response**: Action items in complete sentences are included in the revised text. |
| Please include all relevant details that are required to perform the step in the highlighting. | **Response**: We have verified this. |
| References: Please do not abbreviate journal titles. | **Response**: All journal titles have been spelled out completely in the revised text. |
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