**TITLE:**

Labeling of Neuronal Morphology using Custom Diolistic Techniques

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Reviewers' comments:

**Reviewer #1:**

Manuscript Summary:

The JoVE article by Hough and Brown describes the protocol for diolistic labeling of neurons from fixed tissue. It is This article will be a valuable resource for other researchers, particularly because it provides information to improve upon the existing equipment so that it is adapted for this specific application. The BioRad gene gun was not intended for diolistic labeling, and the authors explain how their equipment will improve random labeling of neurons. The authors clearly explain the improvements of their system over existing techniques and over Golgi staining. I look forward to watching the video. I think that it will be important to provide visual details for the assembly of the equipment and for the production of the DiI coated bullet cartridges.

Major Concerns:

There are no major concerns.

Minor Concerns:

It will be important to provide sufficient detail in the video to demonstrate how the equipment is assembled and how the bullets are placed in the chamber.

**Author response to Reviewer comments:**

Thank you for your comments and thorough review of our manuscript. We agree that a properly narrated video will facilitate the understanding of equipment set-up and cartridge preparation. To insure this, the authors have…

**Reviewer #2:**  
*Manuscript Summary:*  
In this paper "Labeling of Neuronal Morphology using Custom Diolistic Techniques" Lyon Hough and Michael E. Brown describe a detailed protocol for lipophilic dye delivery transfection (diolistics) using a custom built device. The main purpose of this paper is to present a "low-cost" diolistic protocol with high reliability and reproducibility. The efficacy of this method is supported by referring to recently published work "Effects of developmental hyperserotonemia on the morphology of rat dentate nuclear neurons".  
  
*Major Concerns:*  
1.) Although the authors tackled in principle a very interesting technical issue for neuroscience, there are a number of excellent reviews already available the same very topic.  
2.) The major challenge of this MS is the description of how to utilize an alternative gene-gun apparatus that however has not been developed by these authors and that has already been described by the original authors (Bridgman et al).  
3.) However, in this MS there is no sufficient technical description for building up such device. Moreover, another interesting aspect is the use of pipettes tips as cartridges, however also in this case no sufficient description was given : "Particle carriers (cartridges) are supported by a 108 plastic ring that fits inside a modified Millipore filter holder (cartridge holder)12". How should the modified millipore filter be modified in order to obtain a cartridge holder?  
4.) The description of neuronal imaging after labelling is very well reported but also not novel.  
Maybe the authors could have focused their attention to something new such as for instances (as they mention in the MS) the long-time preservation of DiI fluorescence, something that the community is looking for. Or, combining immunofluorescence for synaptic proteins with DiOlistic.  
5.) Moreover, although the protocol steps are clearly explained in "protocol section", the reproducibility of this methodology might be hampered by inaccurate description of reagents and materials. In fact, some information are missing (i.e. 3.0 um filters, methylene chloride, pipette tips) and should be added in the table of Material/Equipment. Moreover, it is reasonable that pipette tips retention index might affect loading of Dil-/PVP in cartridge and then compromise Diolistic labeling. In the "protocol section" it is unclear if aldehyde are dissolved in PBS and, differently from the above mentioned paper PBS 25mM is used for all procedures.  
6.) Finally, in the "discussion section" data obtained with this protocol should be compared to that obtained using standard gene-gun or other methodology (e.g. Golgi staining) in the same regions.  
  
*Minor Concerns:*  
7.) There are several typos throughout the MS (e.g.: Cartilages should be Cartridges)

**Author response to Reviewer comments:**

1. The authors recognize the availability of reviews and even an available JoVE video covering the topic of Diolistic labeling. However, the goal of this manuscript is to provide an alternative approach to the methods previously reported. We feel the strength of the discussed method comes from its adaptability to a variety of laboratory settings and research aims, utilizing more readily available equipment than that previously covered. Additionally, previously reported conflicting information concerning reagents, concentrations, and techniques have been clearly addressed in this updated protocol.
2. The device previously reported by Bridgeman and Brown was developed, built and tested by Michael E. Brown, coauthor of this manuscript. This same device was modified and protocol optimized by Lyon H. Hough (author) for its use in diolistic labeling and published results in “Effects of developmental hyperserotonemia on the morphology of rat dentate nuclear neurons”. Additional devices have been constructed by Hough utilizing the same basic parameters and available equipment.
3. While the construction of the device was not covered at length in this manuscript, the authors feel sufficient information and description is given in the manuscript, and will be covered in the video, for readers to undertake construction a similar device with available resources. The authors agree that all modifications to products such as the Millipore filter holders and pipet tips need to have sufficient explanation to allow for reproducibility. We feel these modifications can be best described in a narrated video. Therefore we have highlighted sections of the manuscript protocol to be included in the video demonstration which specifically cover these areas.
4. The authors have elaborated the statement on line 449 to read “Experimentation on prolonging tissue labeling utilizing submersion in antifreeze solutions and storage in sub-zero temperatures is currently underway with positive preliminary results.” We feel the novelty of the manuscript comes from its description of the preparation and labeling protocol, and supports pervious descriptions of neuronal labeling. The goal in this section is to demonstrate that a reader can adapt the method presented here and produce neuronal labeling results previously only possible with commercialized equipment and proprietary reagents.
5. Thank you for the suggestion, microfilter inserts, pipette tips, and methylene chloride used in this procedure have been added to the included equipment table. The authors have added the statement “Low-retention pipette tips should not be used in this protocol as it may interfere with particle adherence to the inner wall of the tip. The protocol outlined here has utilized standard polypropylene research grade pipette tips (Fisher Scientific) throughout testing.” To line 159. The statement “prepared with 25mM PBS” has been added to line 192.
6. While the authors agree that the comparison between Golgi, standard Diolistic, and our customized method would be an interesting prospect, we find that to be outside the goals of this particular work. The goal of this manuscript is to inform the reader of a reproducible protocol which has been adapted from previous works and to provide representative results which can be expected if said protocol is followed. The authors feel the representative e results and previously published works provide sufficient evidence as to the efficacy of the method.
7. Thank you for your critical review of our manuscript. Typographical errors have been addressed.