

**George M. Smith, PhD**

9 November 2018 **Professor of Neuroscience**

**Shriners Hospitals Pediatric Research Center**

**Dr. Ronald Myers *(Center for Neural Repair and Rehabilitation)***

Editorial Board **Temple University School of Medicine**

Journal of Visual Education **6th floor Medical Education & Research Building**

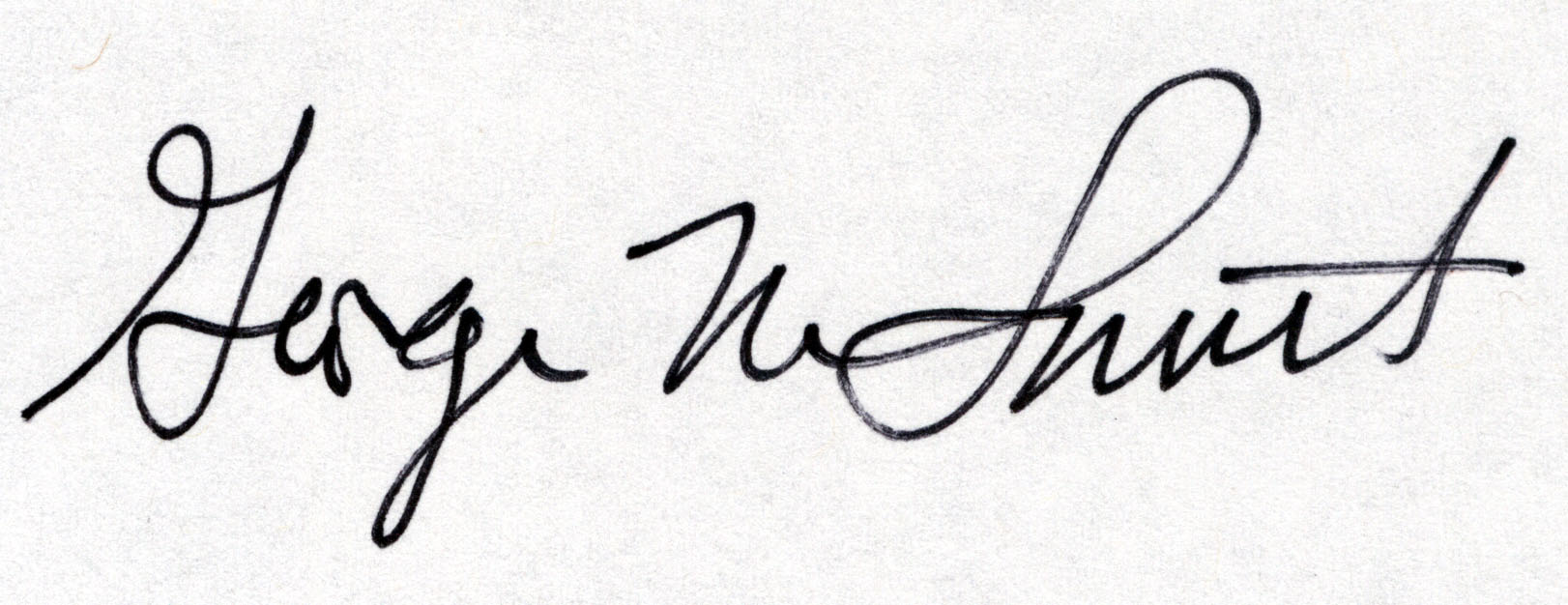
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Dear Dr. Myers,

We are submitting a revision of the manuscript titled “Direction Injection of a Lentiviral Vector Highlights Multiple Motor Pathways in the Rat Spinal Cord” for publication in the Journal of Visual Education. Detailed responses to the comments made by reviewers and the editorial staff are outlined below. Original comments are in black text, and responses are in blue text.

Thank you for your consideration in this matter. If I can be of further assistance please contact me by telephone (215) 926-9359 or email: [george.smith@temple.edu](mailto:george.smith@temple.edu).

Sincerely,



George Smith, PhD

**Editorial comments:**  
Changes to be made by the author(s) regarding the manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. We have run spelling and grammar checks on the manuscript.  
2. Please spell out both first name and last name for each author – This has been corrected in the ‘Authors and Affiliations’ section  
3. Please provide an email address for each author – This has been corrected in the ‘Authors and Affiliations’ section  
4. Keywords: Please provide at least 6 keywords or phrases – One keyword was added in the ‘Keywords’ section to bring the number listed to 6.  
5. Line 57: Please note that there is no reference 16. Please number the references in order of appearance – Reference 16 was added to the end of the sentence at line 58.  
6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations – The numbering was revised. Note that the numbering originally submitted was based off of the example article sent to us.   
7. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion. – Text was substantially revised throughout the protocol for greater usage of the imperative tense with fewer notes. Several sentences were removed from the protocol and taken up in the Discussion section. A few ‘notes’ were added where imperative structure was inappropriate but the idea was important to note at that step.  
8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion. – Text in the protocol was restructured throughout to make the steps more concise.  
9. 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. See examples below. Details were added throughout the protocol, including those outlined below.  
10. Lines 78-80: Please provide more specific details, e.g., the diameter of the glass capillary tube used and the diameter of the needles at the tip – More detail was added to protocol steps 1-1.3, including the diameter of the glass capillary tubes used. The needle aperture and angle measurements are seen in step 1.2, which are the important measurements needed to create successful needles.  
11. Line 92: Please specify the virus used in the protocol – ‘HiRet lentivirus’ text was added to protocol step 2.  
12. Line 108: Please specify the animal (age, gender, and strain) used in the protocol – The line ‘Female Sprague-Dawley rats approximately 200-250 g were used in this protocol’ was added to protocol step 4.  
13. Line 118: Please specify the dosage of ketamine/xylazine – The text ‘Ketamine is given at a 67 mg/kg and xylazine at 6.7 mg/kg dosage’ was added to protocol step 5.  
14. Line 172: Please specify the surgical instrument used – The text ‘with a #10 surgical scalpel blade’ was added to the first line of protocol step 13. Details of surgical tools used were also added to several other protocol steps.  
15. Line 203: Please use a superscripted number for the reference – Appropriate superscripts were added to protocol step 17.  
16. After you have made all the recommended changes to your protocol (listed above), 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, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. We have highlighted appropriate protocol steps.  
17. 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. Please do not highlight any steps describing anesthetization and euthanasia. Partial sentence highlighting was corrected and steps involving anesthesia are no longer highlighted.  
18. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted. Sub-steps were highlighted where appropriate.  
19. Please reference the summary animation video in the manuscript. At 00:22, please include a space between numbers and their units (i.e., 0.8 mm, 1.5 mm). Please name this file as Movie 1 and include a title and a description to be placed in the Figure Legends section. – The following text was added to the beginning of the protocol to summarize the video included: ‘This protocol demonstrates injection of a viral vector into the lumbar spinal cord of a rat. As seen in this animation, a 1.5-2 cm incision is made to expose musculature over the L1-L4 spinal cord. The incision is targeted via identification of the L1 vertebra located at the last rib, which is used as a caudal landmark. Laminectomies of the dorsal aspects of the T11-T13 vertebrae are performed to expose the appropriate spinal cord segments. A beveled glass needle is then directed 1.5 mm lateral from the midline and lowered 0.8 mm deep into the gray matter to inject virus, targeting laminae V-VII, an area rich with propriospinal interneurons.’ The suggested changes were made to the movie. A title and description was added to the ‘Figure Legends’ section.  
20. Please reference the Supplemental File in the manuscript, if applicable – The text ‘As seen in the image here’ has been added to the beginning of the second sentence in protocol step 17, which is line 205.  
21. Representative Results: Please describe Figure 1 in more detail, referring to all panels of the figure – The following text was added to the representative results: ‘GFP expression is seen in neurons in the gray matter of the thoracic spinal cord mostly on the side ipsilateral to the injection (Figure 1A, boxed area), though a few neurons are often observed on the contralateral side, especially near the midline. In the white matter, GFP expression is observed in axons in the ipsilateral cord (Figure 1A, arrows and arrowheads), especially in areas typical to propriospinal axons (arrowheads). Figure 1A’ shows a higher magnification of the boxed area in A, demonstrating typical expression in neuronal cell bodies and dendrites. GFP expression in neurons can also be observed in brainstem nuclei such as the pontine reticular formation (Figure 1B, higher magnification of the boxed area in B’).’ (lines 299-307)  
22. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment – The Materials and Equipment sheet has been reattached. It is now listed in alphabetical order.  
23. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. See the example below:  
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998). – Text in the reference section has been edited to follow these guidelines.  
24. References: Please do not abbreviate journal titles – PloS One has been changed to ‘Public Library of Science One’ in two reference entries.  
25. If there are six or more authors, list the first author and then “et al.”. – This has been corrected in the references section.  
  
**Reviewers' comments:**  
  
**Reviewer #1:**  
Manuscript Summary:  
This is a methodological paper by Keefe and colleagues demonstrating injection of a viral vector into the rat spinal cord. The paper is well written and clear, and important to the field. Some details in the paper are required if the goal is set for laboratories to actually replicate the protocol. Some examples include:  
- A video of the setup (rather than cartoon) when injecting one versus five injections (Line 268). – Text has been added to step 28 of the protocol to talk about multiple injections, and this can be covered during filming. The following text has also been added to the Discussion: ‘It should be noted that the majority of this protocol remains the same whether one injection or six are made, the difference is only in the number of spinal segments exposed via laminectomy, and the fact that you will need to reload the glass needle with additional virus if injecting more than 4µl.’  
- Are there any signs of cord bulge or dye leakage on the surface? The following text was added to step 26 of the protocol: ‘Confirm that virus is entering the spinal cord by observing the progress of the dye front. There should be no obvious leakage or bulging of spinal cord tissue.’  
- Does injection into a subsequent site need new needles? The text ‘The same needle may be used for each injection as long as it continues to function properly.’ has been added to step 28 of the protocol.  
- Picture of the setup (nano-injector and capillary needles) – This is important and will definitely be covered while filming.  
- How long should rats be kept prior to sacrificing them so that the tracer transport is efficient? Is this duration different for pathways they demonstrate here in the paper? The following text is present in the fourth paragraph of the Discussion section: ‘In this experiment, brain and spinal cord tissue were processed and probed after four weeks to ensure abundant time for amplification and transport from the lumbar cord to all brain areas. HiRet is transported via fast retrograde axonal transport, and thus a shorter experimental period may be appropriate if the distance traveled is lesser, such as if the injection area is in the cervical spinal cord.’  
- Line 350-353 (referring to the line ‘HiRet-GFP was unable to label spinal motor neurons or dorsal root ganglion neurons when injected into the transiently demyelinated sciatic nerve’, which is now around line 377):, the authors make this statement based off experiments that seemed to have performed in their lab, but provide no evidence for this. Showing preliminary data or a reference of previous work will help support this statement. – Text in this paragraph was reworded and referenced to show supporting evidence from our previous HiRet paper, and the line about the sciatic nerve was credited as an unpublished observation.  
  
**Reviewer #2:**  
Commenting or showing surgeries in a mouse would provide additional benefit with the large number of transgenic mice available. The following text was added to the Discussion to address the adaptability of this protocol to the mouse and to use the first two references below to direct the reader to the fine details of mouse surgery: ‘The protocol can also be easily adapted for the mouse. The main differences being to adjust downward the volume of virus injected and the measurements needed to hit the correct spinal laminae because of the smaller size. Several videos of similar surgeries on the mouse can be found at the Journal of Visual Education34,35.’

It is somewhat surprising the difference in CSMN labeling between species (see Schoderboeck et al. 2015) and this should be mentioned. We have found that HiRet doesn’t label the corticospinal tract well in any of our rat experiments and would not recommend it for investigations into that tract. Though there could be some difference in mice, this would need to be looked into. Due to this, we believe that a mention of the difference between species here might be confusing for the reader.

A comment on where to get these novel pseudotyped lentiviruses or a reference (either JOVE or other) to packaging these vectors would be helpful too. Many labs set up for surgery are not proficient molecular biologists and vice versa. – A reference to the Hirano paper describing packaging of the HiRet vector was added with the text ‘(for details of construction of the HiRet vector see Hirano et al.)20. In the Discussion section. We received the original plasmids as a gift.  
  
Minor Concerns:  
Introduction:  
-Cite papers that use LV in spinal cord inj? for example:  
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2862356/> - This reference was added to the Introduction when discussing lentiviral vector expression studies.  
  
Protocol:  
-Include setup of surgical area (sterile) – Notes and sterile setup are included in protocol steps 9-12. This will be further demonstrated when filmed.  
-Suggest as an alternative to pulled glass pipettes, the use of a 36 gauge needle/Hamilton syringe as this is easier for researchers without a pipette puller. The text ‘Note: a Hamilton syringe with a steel needle may be used as an alternative to pulled glass pipettes.’  
-Are other means of anesthesia an option? also IP not ideal way of administration and not recommended by some animal ethics committees. Text was added to step 5 of the protocol to offer isoflurane inhalation as an alternative. Considering the different methods of anesthesia practiced in different labs, steps outlining IP injections were cut from the protocol.  
-Include comments on where to obtain virus/reference to packaging protocols – Text containing details about the pseudotyping of the HiRet virus and where to find the vector construction protocol was added to the Discussion section   
-In several places transfection is confused with transduction (this should be used when referring to viral vector injections) – This has been corrected in lines 302 and 336.  
  
Discussion  
-Comment on GC required for LV are a bit arbitrary. This depends on titering details which are not referenced or indicated here. Perhaps change to 'higher the better" type comment – The text was changed here to make this more specific for HiRet lentivirus.  
-Comment on applicability to other species including mice and larger animal models. The following text was added to the Discussion to address the adaptability of this protocol to the mouse: ‘The protocol can also be easily adapted for the mouse. The main differences being to adjust downward the volume of virus injected and the measurements needed to hit the correct spinal laminae because of the smaller size. Several videos of similar surgeries on the mouse can be found at the Journal of Visual Education34,35.’ Although this protocol may be adaptable to larger animal models, we do not have experience with this and so are cautious in commenting on it.  
  
  
**Reviewer #3:**  
  
Minor Concerns:  
It is not clear why some of the text is highlighted in yellow colour – This is required by JoVE to demonstrate text that forms the basis of the narrative spoken during the filmed segments.  
Section 3.2 - is the glass needle secured to the steel injector needle somehow? (clear nail polish / glue?) – I originally used the phrase ‘affix the needle to the injector’. This was poor wording as it created the impression that glue, etc. was needed. This text was revised and expanded in protocol step 3.2 to reflect that the glass needle is held on with washers and a screw on cap.  
Section 2 - Were male / female rats used? Weight of rat? Strain? what dose of Ketamine / Xylazine was used? – The line ‘Female Sprague-Dawley rats approximately 200-250 g were used in this protocol’ was added to protocol step 4 and the text ‘Here ketamine is injected intraperitoneally at a 67 mg/kg and xylazine at 6.7 mg/kg dosage.’ was added to protocol step 5.  
I feel the pace of the video recording could be slowed down a little – The pace of the video was slowed.  
  
**Reviewer #4:**  
  
Major Concerns:  
1) While the authors do report on the efficacy of the HiRet vector and potential limitations, there is a fairly limited discussion on the particular eccentricities and applications of HiRet labeling. It would be nice to see a more detailed explanation of the potential uses and efficacies of HiRet labeling through spinal injection. – Text was added in the Introduction and Discussion sections about the usefulness of HiRet in injury studies and in looking at the brainstem (especially the reticulospinal tract). References were added to our previous paper.  
2) There is limited information provided on methods for analysis of transynaptic labeling. A description of how the virus might be employed and methods to examine connectivity and structure would be a welcome addition. A second figure demonstrating such potential applications of the method would heighten the impact of the paper. – The HiRet virus does not show signs of traveling transynaptically in these experiments or in our previous studies on injured animals. This is mentioned briefly in the Discussion section in the text ‘In this experiment, no GFP labeling was seen in neurons that do not make direct synaptic connections to the injection area. This was also true in previous studies in animals with a thoracic contusion injury40’  
  
Minor Concerns:  
1) The example histology is fairly low resolution. The downloaded photoshop file is set to 90 DPI. If the authors have higher magnification and higher pixel density images, it would be preferable to visualize structure of the labeled cells. Still the supplied figure is sufficient to demonstrate the efficacy of the applied technique – The supplied Figure is now at 300dpi.  
2) The JOVE archive already has protocols detailing spinal injections and spinal transfections[1][2]. There are also descriptions of methods to create the vector and employ it through muscular and tongue injections to label cells in the brain and spinal cord [3]. - The following text was added to the Discussion to address the adaptability of this protocol to the mouse and to use the first two references below to direct the reader to the fine details of mouse surgery. The Hirano article is also referenced several times in the manuscript: ‘The protocol can also be easily adapted for the mouse. The main differences being to adjust downward the volume of virus injected and the measurements needed to hit the correct spinal laminae because of the smaller size. Several videos of similar surgeries on the mouse can be found at the Journal of Visual Education34,35.’  
  
Nevertheless, I do not find a detailed methods paper for stereotactic spinal injection of a retrograde transynaptic vector to label brainstem nuclei and motor associated circuitry. The only papers I can find detail methods to generate the virus, inject it intramuscularly, or to pre-transfect neural grafts with HiRet before deploying them in the brain and spinal cord. As such, a method for direct transfection of circuitry in the spinal cord is important to describe and report.  
[1] P. Inquimbert, M. Moll, T. Kohno, and J. Scholz, "Stereotaxic injection of a viral vector for conditional gene manipulation in the mouse spinal cord," J. Vis. Exp. JoVE, no. 73, 2013.  
[2] K. S. Carbajal, J. G. Weinger, L. M. Whitman, C. S. Schaumburg, and T. E. Lane, "Surgical transplantation of mouse neural stem cells into the spinal cords of mice infected with neurotropic mouse hepatitis virus," J. Vis. Exp. JoVE, no. 53, 2011.  
[3] M. Hirano, S. Kato, K. Kobayashi, T. Okada, H. Yaginuma, and K. Kobayashi, "Highly efficient retrograde gene transfer into motor neurons by a lentiviral vector pseudotyped with fusion glycoprotein," PLoS One, vol. 8, no. 9, p. e75896, 2013.