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Direct Injection of a Lentiviral Vector Highlights Multiple Motor Pathways in the Rat Spinal Cord

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Dr. Ronald Myers
Editorial Board
Journal of Visual Education

Dear Dr. Myers,

We are submitting a novel 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. In this manuscript we highlight a protocol demonstrating injection of viral vector into the rat spinal cord for uptake into select populations of neurons, and the use of a retrogradely transportable lentivirus to map lumbar circuits thought to participate in skilled and locomotor behaviors. We also describe the advantages and challenges of working with this particular vector.

All figures in this manuscript are unique and have not been published or under consideration for publication elsewhere. All surgical procedures and animal maintenance complied with the NIH guideline regarding the care and use of experimental animals. All protocols for animal treatment and care were approved by the Institutional Animal Care and Research Advisory Committee. I have also read and have abided by the statement of ethical standards for manuscripts submitted to the Journal of Visual Education.

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.
Sincerely,

George Smith, PhD

TITLE:

Direct Injection of a Lentiviral Vector Highlights Multiple Motor Pathways in the Rat Spinal Cord

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KEYWORDS:

viral vector, retrograde tracing, spinal cord injection, lentivirus, gene therapy, neuroscience

SUMMARY:

This protocol demonstrates injection of a retrogradely transportable viral vector into rat spinal cord tissue. The vector is taken up at the synapse and transported to the cell body of target neurons. This model is suitable for retrograde tracing of important spinal pathways or targeting cells for gene therapy applications.

ABSTRACT:

Introducing proteins of interest into cells in the nervous system is challenging due to innate biological barriers that limit access to most molecules. Injection directly into spinal cord tissue bypasses these barriers, providing access to cell bodies or synapses where molecules can be incorporated. Combining viral vector technology with this method allows for introduction of target genes into nervous tissue for the purpose of gene therapy or tract tracing. Here a virus engineered for highly efficient retrograde transport (HiRet) is introduced at the synapses of propriospinal interneurons (PNs) to encourage specific transport to neurons in the spinal cord and brainstem nuclei. Targeting PNs takes advantage of the numerous connections they receive from motor pathways such as the rubrospinal and reticulospinal tracts, as well as their interconnection with each other throughout spinal cord segments. Representative tracing using the HiRet vector with constitutively active green fluorescent protein (GFP) shows high fidelity details of cell bodies, axons and dendritic arbors in thoracic PNs and in reticulospinal neurons in the pontine reticular formation. HiRet incorporates well into brainstem pathways and PNs but shows age dependent integration into corticospinal tract neurons. In summary, spinal cord injection using viral vectors is a suitable method for introduction of proteins of interest into neurons of targeted tracts.

INTRODUCTION:

Viral vectors are important biological tools that can introduce genetic material into cells in order to compensate for defective genes, upregulate important growth proteins or manufacture marker proteins that highlight the structure and synaptic connections of their targets. This article focuses on direct injection of a highly efficient retrogradely transportable lentiviral vector into the rat spinal cord in order to highlight major motor pathways with fluorescent tracing. This method is also highly appropriate for axonal regeneration and regrowth studies to introduce proteins of interest into diverse populations of neurons and has been used to silence neurons for functional mapping studies[1,2].

Many of the anatomical details of spinal motor pathways were elucidated through direct injection studies with classical tracers such as BDA and fluoro-gold[3–8]. These tracers are considered gold standard but may have certain disadvantages such as uptake by damaged axons, or axons in passage in the white matter surrounding an injection site[9–11]. This could lead to incorrect interpretations of pathway connectivity and may be a drawback in regeneration studies where dye absorption by damaged or severed axons could be mistaken for regenerating fibers during later analysis[12].

Lentiviral vectors are popular in gene therapy studies, as they provide stable, long-term expression in neuronal populations[13–19]. However, traditionally packaged lentiviral vectors can have limited retrograde transport and may trigger immune system response when used *in vivo*[4,20,21]. A highly-efficient retrograde transport vector termed HiRet has been produced by Kato et al. by modifying the viral envelope with a rabies virus glycoprotein to create a hybrid vector that improves retrograde transport[22,23].

Retrograde tracing introduces a vector into the synaptic space of a target neuron, allowing it to be taken up by that cell's axon and transported to the cell body. Successful transport of HiRet has been demonstrated from neuronal synapses into the brains of mice and primates[23,24] and from the muscle into motor neurons[22]. This protocol demonstrates injection into the lumbar spinal cord, specifically targeting the synaptic terminals of propriospinal interneurons and brainstem neurons. PNs receive connections from many different spinal pathways and can thus be utilized to target a diverse population of neurons in the spinal cord and brainstem. Labeled neurons in this study represent circuits innervating motor neuron pools relating to hindlimb motor function. Robust labeling is seen in the spinal cord and brainstem, including high fidelity details of dendritic arbors and axon terminals. We have also used this method in previous studies within the cervical spinal cord to label propriospinal and brainstem reticulospinal pathways[25].

This protocol demonstrates injection of a viral vector into the lumbar spinal cord of a rat. As seen in **Movie 1,** the incision is targeted by identifying the L1 vertebra located at the last rib. This is used as a caudal landmark for a 3-4 cm incision that exposes musculature over the L1-L4 spinal cord. Laminectomies of the dorsal aspects of the T11-T13 vertebrae are performed and a beveled glass needle is directed 0.8 mm lateral from the midline and lowered 1.5 mm deep into the gray matter to inject virus.

PROTOCOL:

All of the following surgical and animal care procedures have been approved by the Animal Care and Use Committee of Temple University.

1. Pre-surgical preparations

1.1. Prepare pulled glass needles for viral injection a few days before surgery using 3.5 nanoliter glass capillary pipettes designed for nanoliter injectors. Pull each pipette on a two-step needle puller according to the manufacturer's instructions to create two needle templates.

1.2. Refine the tip of the needle templates by cutting off approximately 1-2 mm of excess glass with microscissors. Measure approximate aperture size under a microscope with a microscope calibration slide to isolate needles with 30-40 µm apertures.

1.3. With the needle positioned at 30°, use a micropipette beveller to create a tip with a 30-40 µm aperture and a 45° beveled angle. Verify aperture width with the Vernier scale on the calibration slide. Pass water and ethanol through the glass needle using a syringe with a flexible needle attachment to wash away debris and mark the needle at regular intervals with a black marker.

1.4. Place the needles in a covered Petri dish previously cleaned with 70% ethanol and sterilize for 30 min in a Biosafety hood under UV light.

1.5. Prepare HiRet lentivirus by removing a suitable volume from the freezer immediately prior to the procedure.

NOTE: A suitable volume includes the amount needed for injection (1 µL per injection x number of injections) plus a small amount of extra volume to account for pipetting and loading losses. Transport and store the virus on ice when not in use.

1.6. Prepare the injector by plugging it into the micropump and placing it into a micromanipulator with a Vernier scale.

1.7. To prepare the glass needle, carefully load a colored dye such as red oil with a syringe outfitted with a flexible needle. Ensure that no bubbles remain in the needle.

1.8. Insert the glass needle into the injector, ensuring that the needle is seated correctly into the washers, the injector cap is screwed on tight, and the steel injector needle is extended approximately ¾ the length of the glass needle. Virus can be loaded into the needle in a later step.

2. Anesthesia and surgical site preparation

2.1. Weigh the animal on a digital scale. Record the pre-operative weight to determine the volume of anesthetic required and to allow for monitoring of weight post-surgery. Female

Sprague-Dawley rats approximately 200–250 g were used in this protocol.

2.2. Anesthetize the rat using either isoflorane inhalation or an injected ketamine/xylazine solution (k/x). Here, ketamine is injected intraperitoneally at a 67 mg/kg and xylazine at a 6.7 mg/kg dosage.

2.3. Confirm an appropriate anesthetic plane by pinching the foot firmly. If reflexive withdrawal occurs, wait several additional minutes before proceeding.

NOTE: Also observe the whiskers, eyes and breathing rate for signs of consciousness. If the whiskers are twitching, the eye blinks when touched gently, or breathing is rapid and shallow, wait until the anesthetic plane is deeper to proceed with the protocol. Also monitor these signs throughout the laminectomy and injection surgery. If the animal displays a shallow anesthetic plane, administer a booster shot of ketamine-only equal to ½ the original k/x dosage.

2.4. Shave the rat along the dorsal midline from the hips to the inferior angle of the scapulae. Pull the skin of the animal taut for an easier and more precise shave.

2.5. Apply ophthalmic ointment to both eyes.

2.6. Apply antiseptic to the shaved area to sterilize the site. For the first scrub, soak sterile gauze with a 5% iodine solution and wipe away all hair and debris. Follow this with a unidirectional swipe with sterile gauze soaked in 70% ethanol, so that no area is contacted twice. Use this same technique with alternating iodine and ethanol-soaked gauze twice more.

3. Surgical field and instrument preparation

3.1. Prepare a set of autoclaved surgical tools that include a scalpel, rongeurs, rat tooth forceps, spring scissors, hemostats, medium point curved forceps and retractors or weighted hooks by unwrapping the sterile wrap to create a sterile field.

3.2. Open a package of sterile surgical gloves and place the sterile glove wrap on the table. Use this as an additional sterile field for used tools to prevent contamination of the sterile wrap.

3.3. Drop a #10 scalpel blade onto the sterile field. Secure the blade to a handle with hemostats. Position sterile saline, 4.0 chromic catgut suture, and materials to control bleeding such as a cauterizer, sterile gauze, sterile cotton-tipped applicators (for muscle bleeds), or gelfoam or bonewax (for bone bleeds) in an accessible place.

3.4. Retrieve the animal and set it on a sterile cloth. Place gauze underneath the bladder to collect urine. Prop up the target area with a rolled towel under the abdomen. If available, place a surgical heating pad underneath the sterile cloth, especially for longer procedures.

NOTE: Sterility is important during survival surgery. Keep a spray bottle of 70% ethanol on hand

to maintain sterility of gloved hands, and a use bead sterilizer if instrument sterility is compromised, or between individual surgeries.

4. Exposing the vertebral column and identifying the laminectomy site

4.1. Identify the area where a skin incision will be made by pressing the fingers gently at the last rib to locate the L1 vertebra. Using this as a caudal landmark, make a 3-4 cm skin incision with a #10 surgical scalpel blade at the inferior angle of the scapulae to expose the muscle and fatpad. Hold the skin taut by gentle spreading and press firmly with the scalpel blade to ensure a clean incision.

4.2. Cut and spread the superficial fat pad with forceps and scissors.

4.3. Feel for the spinous processes with the flat of the scalpel blade or a finger. Often the midline area will be outlined by a "V" of white fascia on either side. Make a small rostral cut to allow room to grab securely onto an upper process with rat tooth forceps, then make 2 long, deep cuts as close to the processes as possible. At the deepest point of the cut, the dorsal surface of the vertebrae can be felt with the scalpel blade.

4.4. Hold the lateral muscles aside with retractors or weighted hooks to improve visibility. Clear muscle around the processes with a scalpel, spring scissors or rongeurs to determine the shape of their heads. Remove the top of the T11, T12 and T13 vertebrae.

NOTE: Remember that the spinal cord does not extend the full length of the vertebral column, as spinal cord tissue stops growing earlier in development than bone. This means that the target spinal level may be underneath a differently named vertebra.

4.5. Identify the laminectomy site by visualizing the shapes of the heads of the spinous processes. The spaces between the T6-T7, T7-T8 and T8-T9 processes are relatively large in most rats. The gap between T9 and T10 is much narrower, and the head of the T11 process is noticeably longer and flatter than T10. Locate the T11 and the adjoining T12 and T13 processes.

NOTE: Assistance in targeting correct vertebral levels can be found in a rat spinal cord atlas and previous studies outlining landmarks in the mouse, which has a very similar vertebral structure[6,33]. Leave a rostral spinous process such as T9 undisturbed to give a midline landmark.

5. **Performing a laminectomy**

5.1. Once the target area has been correctly identified, perform laminectomies of the dorsal aspects of T11-T13. Gently spread the vertebrae to reveal intervertebral ligaments, which are good sites to insert rongeurs for the initial bite of bone. Hold the rongeurs in a half-closed position to increase fine control.

5.2. Remove the spinous processes and the dorsal aspect of the vertebrae by taking small bites

with the rongeurs. Be careful not to damage the spinal cord or disturb the dura. Lift slightly with the rat tooth forceps to help pull the spinal cord away from the vertebrae and decrease the tendency to hit spinal cord tissue.

5.3. Clear bone away from the midline so that the midline blood vessel can be observed. Leave a window that clearly shows the spinal cord tissue and is free of debris.

5.4. Gently touch the spinal cord with forceps. Some animals may reflexively jump even if their anesthetic plane is deep. Apply a few drops of a numbing agent such as lidocaine directly to the spinal cord to prevent jumping during the injection procedure.

5.5. Secure the animal in a spinal holder by fastening stabilizing forceps to spinous processes rostral and caudal to the laminectomy window. Raise the abdomen of the animal using the spinal holder to negate the effect of breathing movements. This will increase needle stability and ensure appropriate depth of injection.

6. Loading virus and positioning the injector

6.1. Load virus into the injector by pipetting approximately 5 µL onto a piece of parafilm and positioning the needle so that the tip is inside the drop.

6.2. Use the micropump to withdraw up to 4 µL of virus at a rate of 20–100 nL/s.

6.3. Set the controller to inject and release a small amount of virus from the needle to ensure the tip of the needle is not blocked. Wipe off excess virus with a laboratory wipe.

NOTE: A Hamilton syringe with a steel needle may be used as an alternative to pulled glass pipettes.

6.4. Position the micromanipulator so that the Vernier scale is visible and position the needle at the midline of the spinal cord.

NOTE: The midline can sometimes be located by a large blood vessel running on the anterior surface of the spinal cord. However, this can vary in individual rats, and midline targeting should be confirmed by comparison with an intact spinous process.

6.5. Direct the needle laterally by 0.8 mm using the Vernier scale on the micromanipulator.

6.6. Lower the needle to the spinal cord until it is indenting, but not puncturing, the dura. Using a quick twisting motion, puncture the dura with the needle until it has sunk to a depth of 1.5 mm.

7. Injecting virus into the spinal cord

7.1. Once the needle is in place, program the injector to inject at a rate of 400 nL/min. 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. If leakage is observed, this can sometimes be alleviated by reducing the injection speed to 200 nL/min.

7.2. Once the injection is finished, allow the needle to rest in the spinal cord for 2–5 min (depending on volume injected) to facilitate diffusion of the virus.

7.3. Slowly withdraw the needle and move to the next injection site. Inject 1 µL of virus into each of 6 evenly spaced sites approximately 1 mm apart along the length of the L1-L4 spinal tissue. The same needle may be used for each injection as long as it continues to function properly.

8. Wound closure and post-operative care

8.1. Remove the animal from the spinal holder and take out retractors or hooks used to spread lateral muscle. Ensure that the wound is clear of all debris before closing.

8.2. Suture the muscle using a 4.0 chromic catgut suture. Cut suture threads close to the knot to reduce likelihood of internal skin irritation.

8.3. Staple the skin closed using 9 mm wound clips. To allow for optimal healing, line up the edges of the skin before stapling.

8.4. Place the animal on a water convection warming pad and monitor until wakeful.

8.5. Inject 5–10 mL of sterile saline subcutaneously to replenish fluids and an antibiotic such as cefazolin to prevent infection. When the animal is ambulatory, place it back in its home cage and provide analgesics such as carprofen tablets.

REPRESENTATIVE RESULTS:

Successful injection and transport of the viral vector should result in transduction of a robust population of unilateral neurons in the spinal cord and in certain brainstem nuclei. **Figure 1** demonstrates stereotypical labeling of neurons and axons in the thoracic spinal cord and in the pontine reticular formation of the brainstem at four weeks post-injection. Significant GFP expression is seen in neurons in the gray matter of the thoracic spinal cord on the side ipsilateral to the injection (**Figure 1A**, boxed area). A few neurons are also 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 **Figure 1B'**).

FIGURE AND TABLE LEGENDS:

Figure 1: Transduction of Neurons in the Spinal Cord and Brainstem. (**A**) GFP expression in neurons (boxed area), axons of propriospinal neurons (arrowheads) and axons of other tracts (arrows) in the thoracic spinal cord. (**A'**) Higher magnification of neuronal expression in the boxed area of A. (**B**) The pontine reticular formation in the brainstem expressing GFP-labeled neurons and dendrites. (**B'**) Higher magnification of the boxed area in **B**. Scale bars: (**A**) = 500 μm; (**B**) = 1 mm; (**A'**), (**B'**) = 50 μm.

Movie 1: Targeting the injection into the lumbar spinal cord of the rat. This video summaries the basics of the targeting and injection of a viral vector into the rat spinal cord.

DISCUSSION:

Genetic manipulation of neurons in the brain and spinal cord has served to highlight sensory, motor and autonomic pathways via fluorescent tracing and to explore regrowth potential of neuronal tracts after injury[27-33]. Direct injection of a retrogradely transportable viral vector into the spinal cord can target neuronal populations via their synaptic connections, making this method an excellent choice for mapping pathways in the central nervous system. The HiRet vector specifically shows selective uptake at the neuronal synapse[22,24,25], and previous tracing with similarly structured vectors showed no spread into injured axons or unrelated cells[34,35], which is consistent with results featuring the HiRet construct[22,23,25]. Thus, direct injection of HiRet as a retrograde tracer is an ideal method for exploring interneuronal circuits that undergo plasticity after injury.

There are two critical concepts involved in successful injection of a viral vector into the spinal cord. The first is establishing the correct target with the glass needle, and the second is ensuring adequate flow of the virus from the needle into the tissue. As this protocol involves retrograde tracing, targeting the correct area necessitates a thorough understanding of the synaptic connections of the neuronal population of interest. Propriospinal and reticulospinal neurons connecting to the lumbar spinal cord are targeted here. These neurons are spread throughout the brainstem and cervical and thoracic spinal cord segments, with the majority of PNs localized to laminae V-VII within the gray matter. To ensure transduction of an adequate population of neurons, six viral injections are made over an area spanning four spinal segments. The L1-L4 segments are chosen due to the presence of the central pattern generators, and their known connections to other areas of the spinal cord. Once the correct spinal segment(s) and laminae are known, physical location of these targets via anatomical landmarks is crucial. Atlases showing details of anatomical structure and vertebral shape can be helpful to confirm targeting landmarks[8,36]. 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 4 μL. The protocol can also be easily adapted for the mouse. Due to its smaller size, the volume of virus injected into the cord should be adjusted downward and the measurements needed to hit the correct spinal laminae adjusted. Several videos of similar surgeries on the mouse have previously been published[37,38].

The next consideration is adequate diffusion into the tissue. Careful preparation of the needle is necessary to ensure that the aperture is sufficient for the viral suspension to flow outward and that debris does not block the tip, and visualization of the flow from the needle into the spinal cord via a front of colored dye is helpful to confirm that the suspension is penetrating the tissue. Once the suspension has been distributed into the tissue, maintaining the needle within the spinal cord for 2-5 minutes will ensure adequate diffusion.

Successful transduction of a target population can also depend on intrinsic properties of the virus injected such as titer, serotype and infection efficiency. We find a genomic copy titer of at least 10^{10} GC/mL for HiRet lentivirus to work well for in vivo experimentation. Higher titers or injection volumes might show higher labeling index, but care needs to be taken since virus could diffuse out of the intended area or into the contralateral spinal cord. Consideration should also be given to the type of vector appropriate for labeling cells of interest based on their tropism. Certain viral serotypes may have better infection and transduction rates in different populations of neurons[39]. Traditional lentiviral vectors are known to infect a broad range of cell types due to the VSV-G envelope protein, however modifications to this vector may influence its tropism[40]. The HiRet construct modifies the envelope by pseudotyping with a fusion glycoprotein (FuG-B) of rabies virus, which allows for highly-efficient retrograde transport[22,23] and is effective in transducing propriospinal and reticulospinal tracts, with neuronal numbers comparable to tract tracing via methods such as fluorogold and microruby[4,41] (for details of construction of the HiRet vector see Hirano et al.)[22]. However, it did not sufficiently label the adult corticospinal tract in these experiments, though it does label the CST in neonates with high efficiency in other studies[1]. It is possible that receptors necessary for HiRet uptake at the targeted synapses, such as NCAM or $p75^{NTR}$, are only weakly expressed on adult CST neurons[42,43], though this is still being investigated. In any case, this demonstrates the importance of determining whether the vector being used is appropriate for the targeted cells. 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.

Direct injection surgery is a useful tool for introduction of viral vector technology into the spinal cord. Use of HiRet for genetic experimentation is advantageous as it permits stable, long lasting transgene expression, and is non-toxic to neurons. 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 injury[25]. Additionally, HiRet-GFP was unable to label spinal motor neurons or dorsal root ganglion neurons when injected into the transiently demyelinated sciatic nerve (unpublished observations). Together, these data suggest that HiRet does not readily enter through axons and efficiently transduces neurons by uptake of the vector at synapses, providing a more detailed and high-fidelity map of neuronal connections of the targeted population. This is an advantage over other retrogradely transportable viral vectors such as retrograde adeno-associated virus (rAAV-retro), which is known to be taken up by axons in passage[44] and makes HiRet especially useful in studies mapping regenerating and reconnecting circuitry in the injured spinal cord. HiRet's advantages may also allow for specific

targeting of neuronal populations for silencing or ablation studies[25,44–47].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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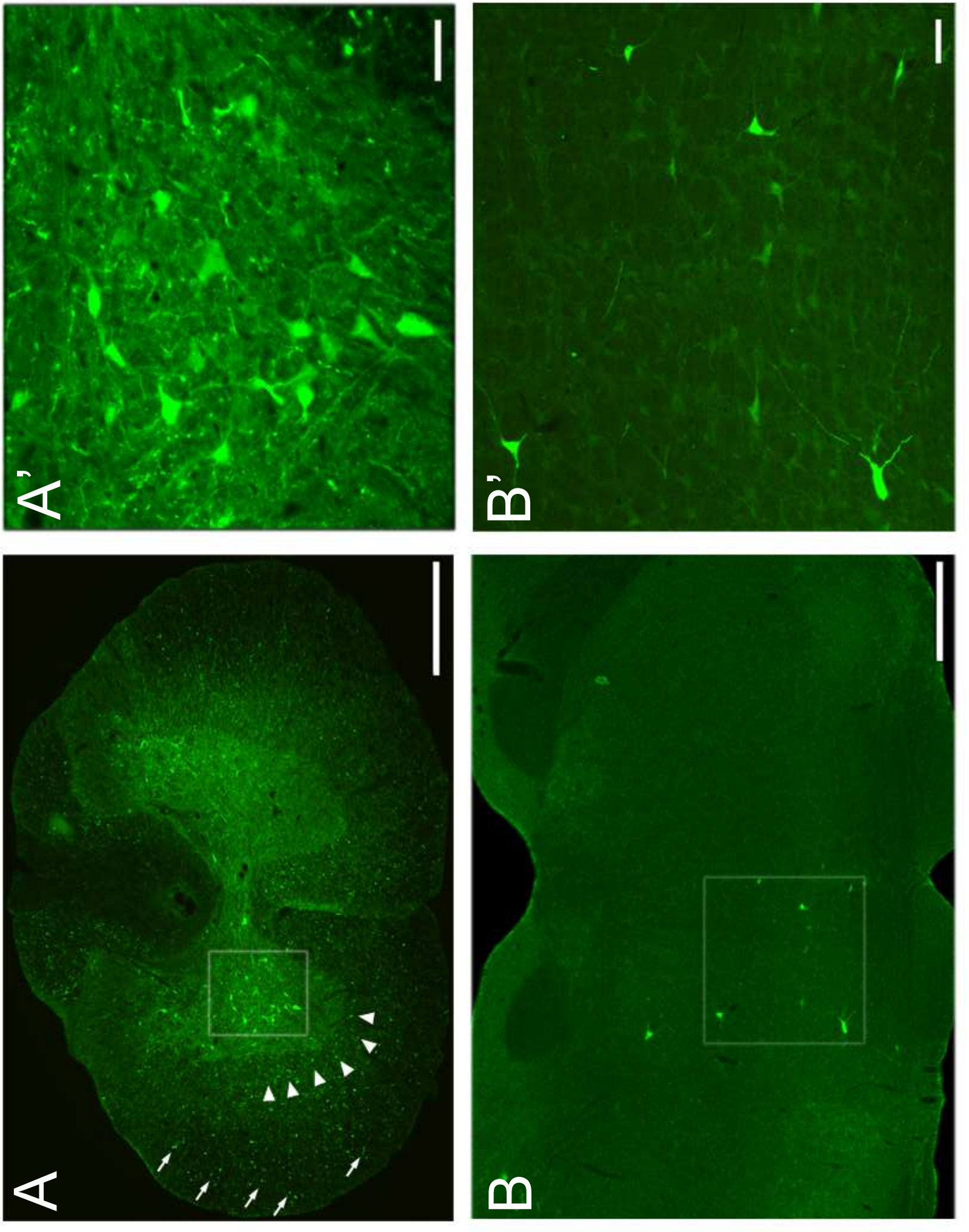

Movie 1

Click here to access/download

Video or Animated Figure

Movie 1.mp4

Table of Materials

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Name of Material/ Equipment	Company
#10 Scalpel Blades	Roboz
1 mL Syringes	Becton, Dickinson and Company
10mL Syringes	Becton, Dickinson and Company
4.0 Chromic Catgut Suture	DemeTECH
48000 Micropipette Beveler	World Precision Instruments
5% Iodine Solution	Purdue Products L.P.
70% Ethanol	N/A
Anesthetic (Ketamine/Xylazine Solution)	Zoetis
Antibiotic (Cefazolin)	West-Ward Pharmaceuticals
Bead Sterilizer	CellPoint
Bonewax	Fine Science Tools
Cauterizer	Fine Science Tools
Digital Scale	Okaus
Flexible Needle Attachment	World Precision Instruments
Gelfoam	Pfizer
Glass Capillary Tubes	World Precision Instruments
Hair Clippers	Oster
Hemostats	Roboz
Kimwipes	Kimtech
Medium Point Curved Forceps	Roboz
Micromanipulator with a Vernier Scale	Kanetec
Microscissors	Roboz
Microscope with Light and Vernier Scale Ocular	Leitz Wetzlar
MicroSyringe Pump Controller	World Precision Instruments
Nanoliter 2000 Pump Head Injector	World Precision Instruments
Needle Puller	Narishige
Ophthalamic Ointment	Dechra Veterinary Products
Parafilm	Bemis
PrecisionGlide Needles (25G x 5/8)	Becton, Dickinson and Company
Rat Tooth Forceps	Roboz
Red Oil	N/A
Retractors	Roboz
Rimadyl Tablets	Bio Serv
Rongeurs	Roboz
Scalpel Blade Handle	Roboz

Scissors	Roboz
Stainless Steal Wound Clips	CellPoint
Staple Removing Forceps	Kent Scientific
Sterile Cloth	Phenix Research Products
Sterile Cotton-Tipped Applicators	Puritan
Sterile Gauze	Covidien
Sterile Saline	Baxter Healthcare Corporation
Surgical Gloves	N/A
Surgical Heating Pad	N/A
Surgical Microscope	N/A
Surgical Stapler	Kent Scientific
T/Pump Heat Therapy Water Pump	Gaymar
Water Convection Warming Pad	Baxter Healthcare Corporation
Weighted Hooks	N/A

Catalog Number

RS-9801-10

309659

309604

NN374-16

32416

L01020-08

N/A

240048

NPC 0143-9924-90

5-1450

19009-00

18010-00

REV.005

MF34G-5

H68079

4878

111038-060-000

RS-7231

34155

RS-5136

N/A

RS-5621

N/A

62403

500150

PC-100

RAC 0119

PM-996

305122

RS-5152

N/A

RS-6510

MP275-050

RS-8300

RS-9843

RS-5980
201-1000
INS750347
BP-989
806-WC
2146
281324
N/A
N/A
N/A
INS750546
TP500C
L1K018
N/A

Comments/Description

For use with the scalpel.

For anesthetic IP injection, potential anesthetic booster shots, and antibiotic injections.

For injecting saline into the animal, post-surgery.

To re-bind muscle during closing.

Used to bevel the tips of the pulled glass capillary tubes to form functional glass needles.

For use in sterilzation of the surgical site.

For sterilization of newly prepared glass needles, animal models during surgical preparation, and surgeon's hand

For keeping the animal in the correct plane of consciousness during surgery.

To be injected subcutaneously to prevent infection post-surgery.

To heat sterilize surgical instruments.

To seal up bone in the case of bone bleeding.

To seal any arteries or veins severed during surgery to prevent excessive blood loss.

For weighing the animal during surgical preparation.

For cleaning glass needles and loading red oil into glass needles.

To seal up bone in the case of bone bleeding.

For pulled glass needles - should be designed for nanoliter injectors.

For clearing the surgical site of hair.

For general use in surgery.
For general use in surgery.
For general use in surgery.
For precise targeting during surgery.
For cutting glass whisps off of freshly pulled glass capillary tubes.
Used to visualize and measure beveling of pulled glass capillary tubes into functional glass needles.
To control the rate of injection.
To load and inject virus in a controlled fashion.
To heat and pull apart glass capillary tubes to form glass needles.
To protect the animal's eyes during surgery.
To assist with loading virus into the nanoinjector.
For use with the 1mL and 10 mL syringes to allow injection of the animal model.
For griping spinous processes.
To provide a front for visualization of virus entering tissue during injection.
To hold open the surgical wound.
For pain management post-surgery.
To remove muscle from the spinal column during surgery.
To slice open skin and fat pad of animal model during surgery.

For general use in surgery.
To bind the skin of the surgical wound during closing.
To remove the staples, should they be applied incorrectly.
To provide a sterile surface for the operation.
To soak up blood in the surgical wound while maintaining sterility.
To clean the surgical area and surgical tools while maintaining sterility.
For use in blood clearing, and for replacing fluids post-surgery.
For use by the surgeon to maintain sterile field during surgery.
For maintaining the body temperature of the animal model during surgery.
For enhanced visualization of the surgical wound.
To apply the staples.
To pump warm water into the water convection warming pad.
For use in the post-operational recovery area to maintain the body temperature of the unconscious
To hold open the surgical wound.

s during surgery, as well as all other minor maintainances of sterility.

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Author(s): KeeFe, K.M., Junker, I., Sheikh, I.S., Smith, G.

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9 November 2018

Dr. Ronald Myers
Editorial Board
Journal of Visual Education

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.
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 Education[34,35].'

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 Education[34,35].' 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 injury[40]'

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 Education[34,35].'

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.