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Direct gene knockout of axolotl spinal cord neural stem cells via electroporation of CAS9 protein-gRNA complexes

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Cover Letter

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Date: 12th Feb 2019

Dear Dr. Weldon,

We thank you very much for inviting us to submit this protocol paper. Please find enclosed our manuscript, "Direct gene knockout of axolotl spinal cord neural stem cells via electroporation of CAS9 protein-gRNA complexes" for consideration at JoVE.

In this manuscript, we describe the detailed protocols we have used to efficiently knockout target genes in axolotl, in a spatial- and time-restricted manner, by electroporating the CRISPR/Cas9 system into the spinal cord cells.

We believe our protocol will contribute significantly to the field of axolotl development and regeneration research. It will bring new research possibilities to this important model system, promote and expand the use of axolotls in science.

We thank you again and look forward to your response.

Yours sincerely,

Ji-Feng

TITLE

Direct Gene Knock-out of Axolotl Spinal Cord Neural Stem Cells via Electroporation of CAS9 Protein-gRNA Complexes

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KEYWORDS:

axolotl, spinal cord, regeneration, CRISPR-Cas9, neural stem cells, gene knock-out

SUMMARY:

Presented here is a protocol to perform time- and space-restricted gene knock-out in axolotl spinal cords by injecting CAS9-gRNA complex into the spinal cord central canal followed by electroporation.

ABSTRACT:

The axolotl has the unique ability to fully regenerate its spinal cord. This is largely due to the ependymal cells remaining as neural stem cells (NSCs) throughout life, which proliferate to reform the ependymal tube and differentiate into lost neurons after spinal cord injury. Deciphering how these NSCs retain pluripotency post-development and proliferate upon spinal cord injury to reform the exact pre-injury structure can provide valuable insight into how mammalian spinal cords may regenerate as well as potential treatment options. Performing gene knock-outs in specific subsets of NSCs within a restricted time period will allow study of the molecular mechanisms behind these regenerative processes, without being confounded by development perturbing effects. Described here is a method to perform gene knock-out in axolotl spinal cord NSCs using the CRISPR-Cas9 system. By injecting the CAS9-gRNA complex

into the spinal cord central canal followed by electroporation, target genes are knocked out in NSCs within specific regions of the spinal cord at a desired timepoint, allowing for molecular studies of spinal cord NSCs during regeneration.

INTRODUCTION:

The spinal cord of most vertebrates is unable to regenerate following injury, leading to permanent disability. Several salamanders, such as the axolotl, are notable exceptions. The axolotl can fully regenerate a structurally identical spinal cord and completely restore spinal cord function. Much of the regenerative capability of the axolotl spinal cord is due to ependymal cells. These cells line the central canal, and unlike those in mammals, axolotl ependymal cells remain as neural stem cells (NSCs) post-embryonic development. After spinal cord injury (i.e., from a tail amputation), these NSCs proliferate to regrow the ependymal tube and differentiate to replace lost neurons[1–3]. Uncovering how axolotl spinal cord NSCs remain pluripotent and become activated after injury can provide valuable information on developing new therapeutic strategies for human patients.

Due to advances in the CRISPR-Cas9 gene knock-out technique, performing knock-outs to decipher gene function has become easier and been shown to have broad applicability in various species, including axolotls[4–8]. The recent release of the full axolotl genome and transcriptome now allows any genomic locus to be targeted and for better assess to off-target effects[9–14]. Optimized protocols have been developed for knock-out and knock-in in axolotls using the CRISPR-Cas9 system[15]. Delivery of the CRISPR-Cas9 machinery in the form of CAS9 protein-gRNA ribonucleoprotein (RNP) has been shown to be more efficient than using Cas9 and gRNA-encoding plasmids[4]. This is likely due to the RNP being smaller in size than plasmid vectors, its allowance of DNA breaks to be made immediately, and protecting of the gRNA from RNA degradation. In addition, using RNPs bypasses transcription and translation; thus, it avoids issues such as promoter strength and optimal codon usage when plasmid elements are derived from a different species.

Loss-of-function studies are one of the general approaches to investigating potential functions of genes of interest. In order to study gene function during regeneration, a knock-out should ideally be performed just before an injury to avoid effects on development. In addition, the knock-out should be restricted to both the NSCs and region of regeneration. A knock-out of the target gene in all NSCs (including those in the brain, which is the case in Cre-LoxP systems), may produce effects not related to regeneration that can confound the interpretation of results. Fortunately, the structure of the axolotl spinal cord provides a unique opportunity for time- and space-restricted knock-out in NSCs. Most of the spinal cord NSCs are in contact with the central canal and constitute the vast majority of the cells in contact with the central canal[16,17]. Therefore, an injection of the CAS9-gRNA complex into the central canal, followed by electroporation, allows delivery to spinal cord NSCs to the desired region at a specific time[4,18,19]. This protocol demonstrates how this is performed, leading to highly penetrating knock-out in the targeted spinal cord NSCs. Subsequent analysis are then performed to study the effects on regeneration and NSC behavior.

PROTOCOL:

All animal experiments must be carried out in accordance with local and national regulations on animal experimentation and with approval of the relevant institutional review board.

1. Preparing the CAS9-gRNA RNP mix

1.1. Design and synthesize gRNAs.

NOTE: Refer to other publications for designing and synthesizing gRNAs, including one exclusively concerning axolotls[15,20–22].

1.2. Obtain CAS9-NLS protein by preparing in-house or purchasing it commercially.

1.3. Prepare CAS9-gRNA mix by mixing 5 µg of CAS9-NLS protein, 4 µg of gRNA, 0.9 µL of 10x CAS9 buffer, and fill up the volume to 10 µL with nuclease-free H_2O. Aliquot the mixture and store at -80 °C if not used immediately.

2. Preparing agarose plates for electroporation

2.1. Prepare 200 mL of 2% (wt/vol) agarose solution in 1x DPBS. Dissolve by heating the solution in a microwave and pour it onto 10 cm Petri dishes to a depth of about 10 mm (deep enough to cover the whole animal).

2.2. Allow the plates to solidify at room temperature (RT). Store plates at 4 °C if not used immediately.

2.3. Using surgical scalpels, cut the agarose plate to make a slit for holding the tail straight, as well as, a well for fitting in the body of the animal, and two extra wells for placing the electrodes (**Figure 1E**).

2.4. Place the plate on ice and fill it up to the brim with ice-cold 1x DPBS. Use the same DPBS solution for making the plates to ensure consistent electrical conductibility in the set-up.

3. Configuring the electroporator

3.1. Configure the electroporator. Set up the poring pulse to consist of one bipolar pulse of 70 V, with a duration of 5 ms, interval of 50 ms, and no voltage decay. Set up the transfer pulse to have four bipolar pulses of 40 V, with a duration of 50 ms, interval of 999 ms, and 10% voltage decay.

3.2. Connect the electrodes to the electroporator and adjust the tweezers to be 7 mm apart. Submerge the electrodes in a beaker of PBS before and in between electroporation.

4. Preparing and loading microinjection glass capillaries

4.1. Perform a ramp test on a flaming/brown micropipette puller as per the manufacturer's instructions to determine the heat value.

4.2. Pull injection capillaries with tapered ends using the following parameters: heat = ramp test value, pull = 100, velocity = 100, time = 100.

4.3. Observe the needle under a stereomicroscope. Using fine forceps, break the capillary at an angle so that it has a slanted tip. Break at a position where it is thin enough to target the central canal, while being strong enough to pierce the skin.

NOTE: The point at which the diameter is at about 20 µm or about 50% of the diameter of the spinal cord is a good starting point. Multiple tries may be needed to obtain optimal breaking.

4.4. Add Fast Green FCF solution to the RNP mix at a ratio of 1:30 and load about 5 µL of injection mix into the capillary with a Microloader pipette tip.

4.5. Mount the capillary on the pneumatic pump, with the slanted tip facing downwards.

5. Configuring the pneumatic pump

5.1. Configure the pneumatic pump. Set the hold to 0.5 psi, eject to 2 psi hold, and duration to gated.

5.2. Test capillary and pneumatic pump settings by dipping the capillary into a drop of water and pressing the foot pedal to inject. Ensure that the injection mix comes out in a slow but steady stream.

5.3. Adjust the hold pressure if the injection mix start leaking out spontaneously or water is sucked up the capillary. Increase the ejection pressure or break more of the capillary if the injection mix is coming out too slow. Decrease the ejection pressure if the injection is coming out too fast.

6. Injecting RNP mix into the spinal cord

6.1. Anesthetize axolotls in 0.03% benzocaine solution for at least 10 min. Check that the animals are not responsive by flipping them upside down in the water.

6.2. Pick up an animal with ring forceps and lay it on a bed of silicon, with the left side of the animal facing up and the tail pointing towards the right. Position the injection site in the middle of the field of view of the stereomicroscope (**Figure 1A**).

6.3. Adjust the micromanipulator so that the capillary is coming in at 60° from the right side of the field of view (**Figure 1B**).

6.4. Identify the spinal cord and central canal (**Figure 1C**).

NOTE: The spinal cord is the tubular structure above the notochord.

6.5. Aiming at the central canal, move the capillary forward to gently pierce the skin and muscle into the central canal of the spinal cord. Limit the movement of the capillary to small steps, as the spinal cord is right beneath the tail muscles.

6.6. Press and hold down the foot pedal to inject the mix into the central canal. Observe whether the RNP mix spreads along the central canal as a blue line in both directions, which shows the capillary is positioned (**Figure 1D**).

6.7. If this does not occur, keep the foot pedal pressed while moving the capillary slightly in and out until the RNP mix starts going in. If the capillary become clogged by tissue, clear it by ejecting into water at higher pressures.

6.8. Adjust the ejection pressure so that it is just enough to cause the RNP mix to spread in the central canal, but not so much that it blows up the structure.

6.9. Continue to hold down the foot pedal so the injection mix spreads further along the central canal, until it reaches the terminal vesicle at the end of the spinal cord and ventricles in the brain.

NOTE: This may take up to 2 min depending on the size of the animal. It may be necessary to increase the pressure after some time to cause the RNP mix to enter the brain ventricles.

6.10. Proceed as soon as possible to electroporation after successful injection.

7. Electroporation

7.1. Transfer the animal with ring forceps onto the prepared agarose electroporation plate (**Figure 1E**) on ice. Place the tail inside the slit so it is sandwiched by agarose (**Figure 1F**).

7.2. Place the electrodes into the wells on both sides of the tail. Ensure that the entire animal and electrodes are covered by ice-cold PBS.

7.3. Press the foot switch to start the electroporation.

7.4. After electroporation is complete, return the animal to water.

7.5. Proceed to inject more animals if necessary.

7.6. Check that the electroporated animals are healthy after anesthesia wears off and that there is no damage done to the tail.

8. Assessing knock-out efficiency

8.1. Fix the tail of the electroporated animal. Perform a cross-section of the tail followed by immunohistochemical staining to assess efficiency of the knock-out at the protein level.

NOTE: If a tail amputation is to be performed after electroporation, the cut off tail could be used for this purpose. Animals electroporated with gRNAs against Gfp or Tyrosinase, for example, can be used as a negative control[4].

8.2. Alternatively, the electroporated spinal cord could be extracted and lysed for DNA. Perform genotyping PCR followed by Sanger sequencing or next-generation sequencing to assess the percentage of edited genetic loci[15].

NOTE: The resulting editing efficiency will underestimate the actual editing efficiency for NSCs, due to the inclusion of non-electroporated cells and neurons that lack apical contact to the central lumen and thus will not receive the RNP mix.

REPRESENTATIVE RESULTS:

Injection and electroporation of CAS9-gRNA complex against *Sox2* into the axolotl spinal cord central canal led to a massive loss of SOX2 immunoreactivity in a majority of spinal cord NSCs, with gRNA against Tyrosinase (Tyr) as a control (**Figure 2A**). TUJ1 (staining for B3-tubulin) is a marker for neurons and was not expressed in NSCs, and SOX2- TUJ1- cells surrounding the central canal were considered to be cells harboring *Sox2* deletions. Quantification showed *Sox2* knock-out in a significant number of NSCs by CAS9-*Sox2*-gRNA electroporation (**Figure 2B**), indicating that this method led to efficient and highly penetrating gene knock-out in spinal cord NSCs.

After simultaneous injection and electroporation, the mixture of two CAS9-gRNA complexes against *Sox2* and *Gfp,* respectively, into transgenic axolotls with ubiquitous GFP expression (CAGGS-GFP), a high percentage of double knock-out NSCs were observed (**Figure 2C,D**), indicating that the protocol can be used to flexibly knock-out multiple genes.

FIGURE AND TABLE LEGENDS:

Figure 1: Axolotl spinal cord injection and electroporation. (**A**) Schematic showing injection of

CAS9-gRNA RNP into axolotl spinal cord central canal. (**B**) Micromanipulator set-up for the injection. (**C**) View through a stereomicroscope of the axolotl tail, with the spinal cord indicated. (**D**) View through a stereomicroscope of the axolotl tail with an injected spinal cord. (**E**) Schematic of the electroporation plate. (**F**) Schematic of the animal and electrodes placed inside the electroporation plate, ready for electroporation. Scale bars = 1 mm. This figure is adapted from Albors and Tanaka[18].

Figure 2: Representative knock-out results in axolotl spinal cord NSCs. (**A**) Spinal cord injection and electroporation of CAS9-*Sox2*-gRNA (n = 21) led to loss of SOX2 in the majority of NSCs at 15 days post-electroporation (dpE) by immunohistochemical staining. CAS9-*Tyr*-gRNA (n = 21) served as a negative control. Scale bars = 100 µm. (**B**) Quantification showed significant reduction in SOX2+ NSCs ($p < 0.001$ by Student's t-test), counting SOX2- TUJ1- cells surrounding the central canal as NSCs harboring a *Sox2* deletion. Error bars = SD. (**C**) Simultaneous injection and electroporation of *Sox2*-gRNA and *Gfp*-gRNA coupled to CAS9 (n = 6) led to loss of SOX2 and GFP in the majority of NSCs by immunohistochemical staining. CAS9-*Tyr*-gRNA (n = 6) serves as negative control. Scale bars = 100 µm. (**D**) Quantification showed that among all targeted NSCs having any deletion, a majority (94%) harbored double deletion (GFP− SOX2−), with only a small proportion (6%) having single deletion (GFP+ SOX- or GFP- SOX2+). This figure is adapted from Fei et al.[4].

DISCUSSION:

The described protocol allows time and space restricted gene knock-out in the NSCs in the axolotl spinal cord. The current protocol allows specific targeting of NSCs at a defined time and location with high penetrance. It avoids potential undesired effects originating from gene knock-out in NSCs in other regions such as the brain that occur when using the Cre-LoxP system. It also avoids developmental effects originating from a persistent knock-out, allowing the study of gene function focused on regeneration. In addition, this protocol can be performed in wild-type animals, avoiding the long waiting time needed to generate additional transgenic axolotls, which is required for the Cre-LoxP system. It also offers the versatility to knock-out multiple genes simultaneously and is highly penetrating, allowing experiments to be performed on F0 animals. Importantly, the procedure is shown to have no observable effect on tail and spinal cord regeneration[4].

The use of CAS9 protein-gRNA RNP complexes also displays much higher efficiency than systems using *Cas9* and gRNA-expressing plasmids[4]. This is likely due to smaller size of the RNP complex compared to plasmids, as well differential codon usage affecting expression of the CAS9 protein from plasmids derived from mammalian systems. In addition, since the CAS9 protein-gRNA RNP complexes can induce breaks immediately, knock-out occurs quickly. 60%–70% of modified genomic loci have been observed by genotyping PCR within 24 h of electroporation (data not shown). There is also a possibility to perform knock-ins using this strategy by co-electroporating a repair template. A number of knock-in strategies are available, the details and considerations of which are out of scope for this publication, but they are described in detail in another publication[15].

On the other hand, this protocol does have a few limitations. Staining or genotyping PCR is generally needed to assess the extent of knock-out in each experimental animal, which can be laborious. While gene knock-out from this method is largely restricted to the NSCs surrounding the central canal, it cannot be ruled out that other cell types could also be targeted, because 1) they are also in contact with the central canal, or 2) the RNP mix has leaked out of the central canal before electroporation. These factors should be considered when interpreting the results.

gRNA design

With the recently published axolotl genome and transcriptome, identifying axolotl genes and their sequences has become much easier[9–13]. Detailed guides in designing gRNAs has been described elsewhere, including one that deals exclusively with axolotls[15]. It is advised to design and test at least three gRNAs for editing efficiency beforehand and to choose the optimal design for the actual experiment. Efficiency of the gRNA can be tested by injecting the CAS9-gRNA mix into freshly laid axolotl eggs at the one-cell stage[23]. Editing efficiency can be then assessed by genotyping the hatched larvae.

Injection considerations

Since the injection site of the spinal cord suffers from physical damage caused by the needle and spreading of the RNP mix to tissues outside the central canal, it is important to perform the injection at positions away from the region of interest to be analyzed. For example, if a tail amputation is performed after electroporation to study the effects of a targeted gene in spinal cord regeneration, it is necessary to perform the injection where the site will be removed by amputation.

Entering the central canal with an injection capillary is the most critical step of this protocol, and new users are advised to practice before the actual experiment. Injection is best performed on smaller animals (less than 2.5 cm long) at the posterior end of the spinal cord, where it is easiest to position the capillary tip into the central canal. It is advised to observe the initial spread of the blue-colored RNP mix closely. There is the possibility that the RNP mix may be injected into the meningeal space around the spinal cord. A sharp and quick spread as a line along the middle of the spinal cord indicates successful injection into the central canal[18]. If nothing exits or the spread stops prematurely, it is recommended to keep the foot pedal pressed while moving the capillary slightly in and out. If this fails, it may be necessary to re-position the capillary or check for a clogged opening. For the RNP mix to spread into the brain ventricles, it is often necessary to increase the ejection pressure towards the end. Having the brain ventricles filled up is not necessary for electroporation of the spinal cord, but it is the best indication that the RNP mix has been injected into the central canal.

Electroporation considerations

It is advised to optimize the electroporation parameters beforehand to achieve sufficient delivery of the RNP to a majority of NSCs without causing extensive damage to other tissues or killing the animal. This protocol has been optimized for animals 2.0–2.5 cm long in size[18]. A reduction in transfer pulse voltage to 35 V is likely necessary when working with animals

smaller than this. On the other hand, larger animals will require higher voltage and/or more pulses. In addition, the distance between the electrodes affects the efficiency. A reduction of the distance to 6 mm may be necessary for larger animals.

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DISCLOSURES:

The authors declare no conflicts of interest.

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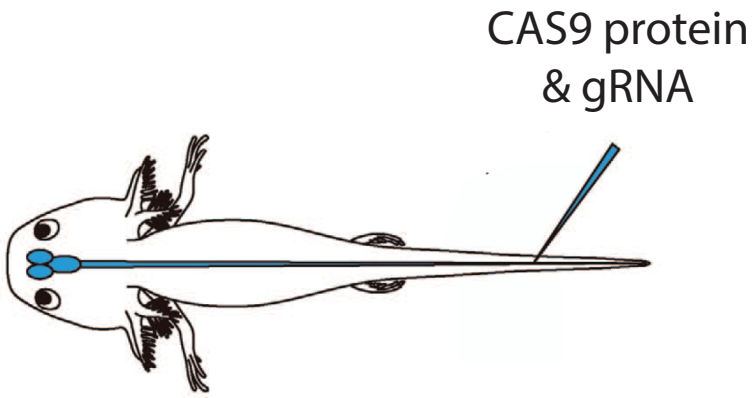
Figure 1

A)



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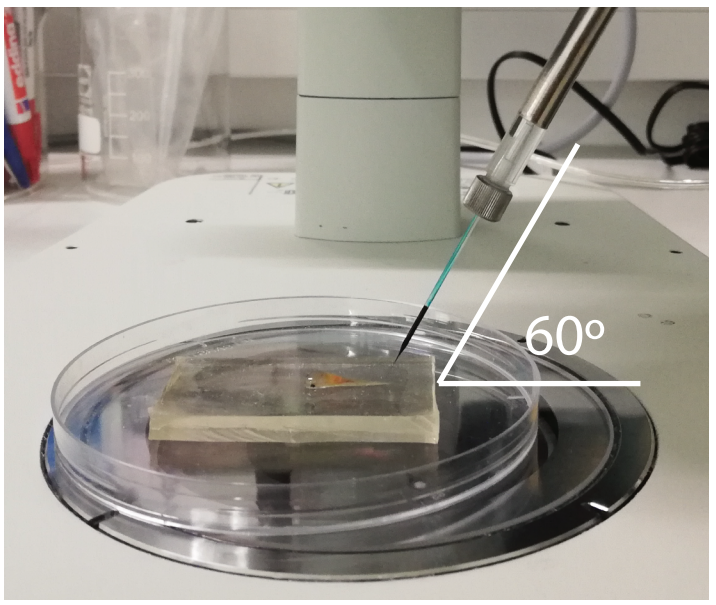

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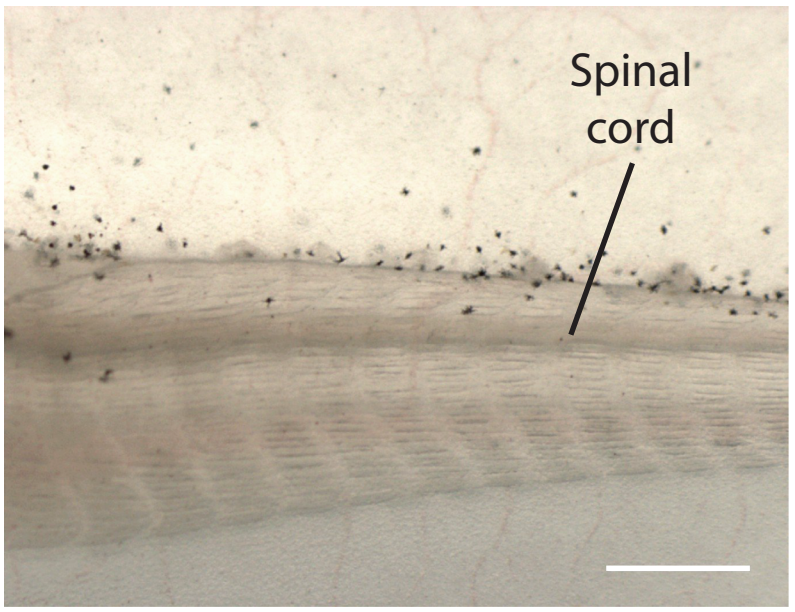

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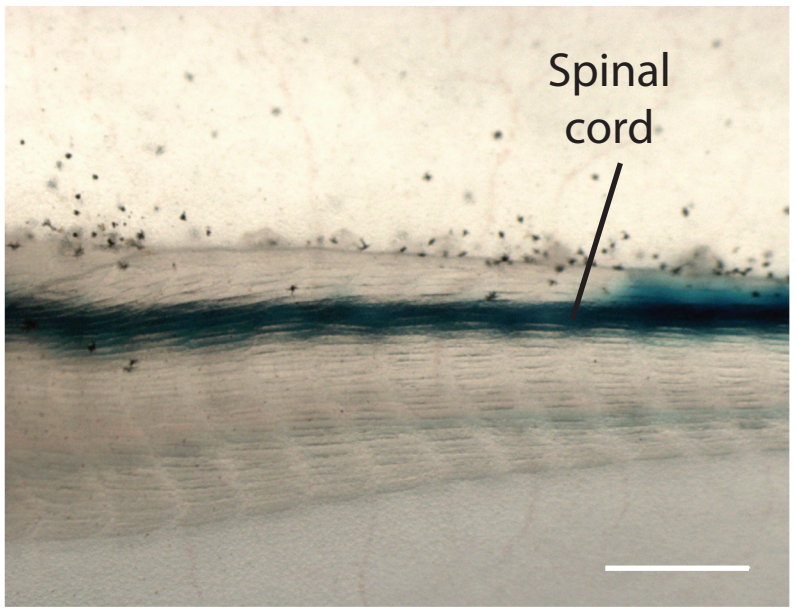

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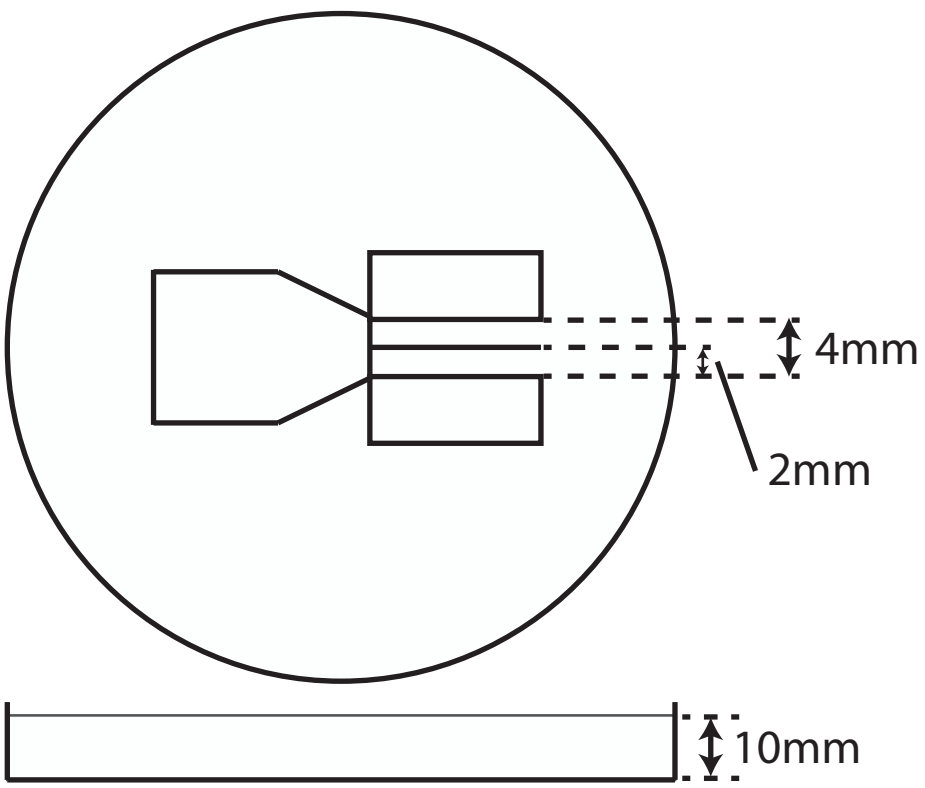


F)

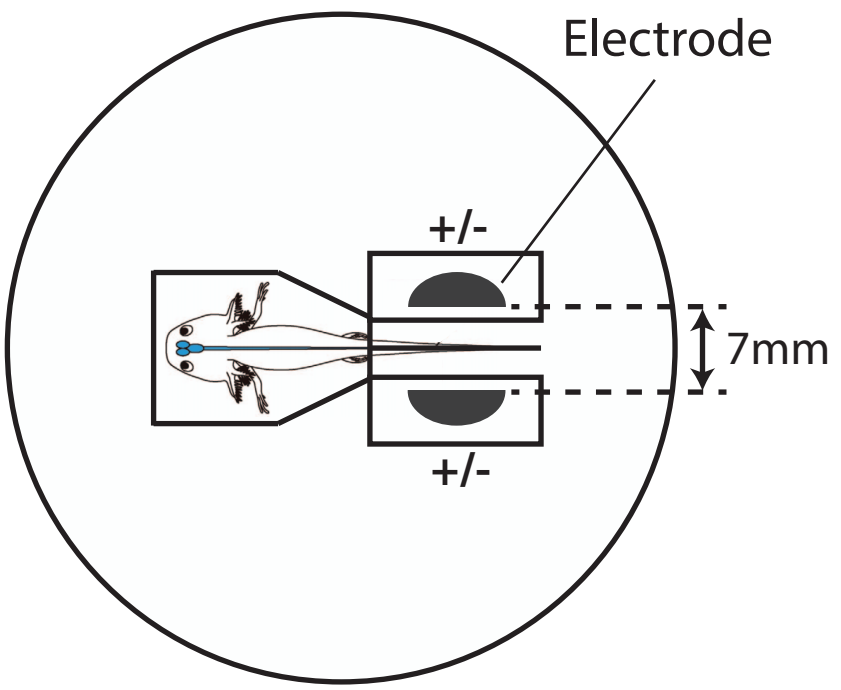
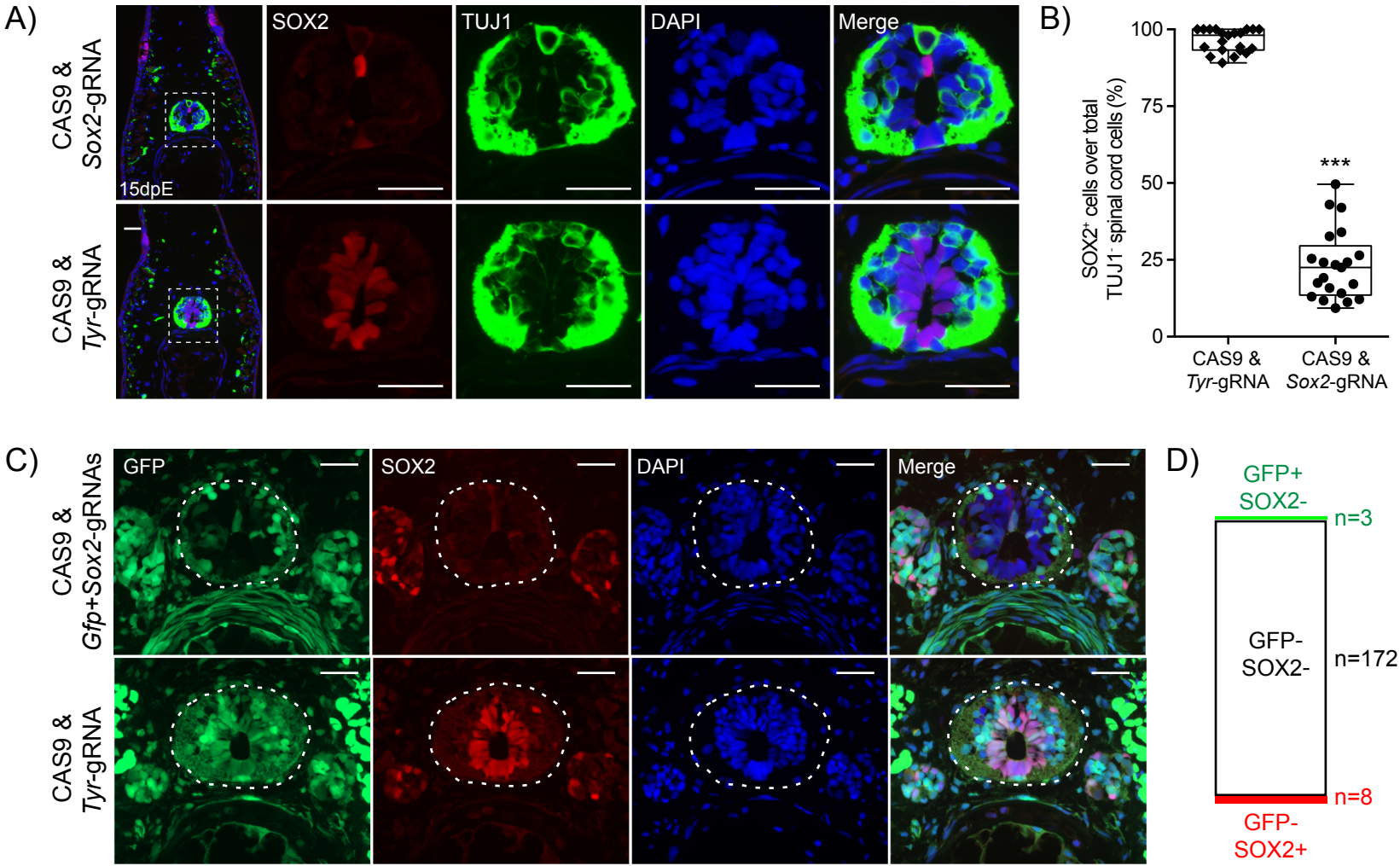


Figure 2

Click here to access/download;Figure;fig2_ko eff_v3b.pdf



Name of Material/ Equipment

Agarose

Benzocaine

Benzocaine 0.03 % (wt/vol)

Benzocaine 10 % (wt/vol)

Borosilicate glass capillaries 1.2 mm O.D., 0.94 mm I.D.

$CaCl_2 \cdot 2H_2O$

CAS9 buffer, 10x

CAS9-NLS protein

Cell culture dishes, 10cm

Dumont #5 - Fine Forceps

Electroporator

Fast Green FCF

Fast Green FCF Solution, 5x

Flaming/Brown Micropipette Puller

Holtfreter's solution 400% (wt/vol)

KCl
$MgSO_4 \cdot 7H_2O$
Microloader pipette tips
Micromanipulator
NaCl
Pneumatic PicoPump
Ring Forceps
Stereomicroscope
Tris base

Tris-buffered saline, 10x

Tweezers w/Variable Gap 2 Round Platinum Plate Electrode,
10mm diameter

Company	Catalog Number
Sigma-Aldrich	A9539
Sigma-Aldrich	E1501-100G
Mix 500 ml of 10× TBS, 500 ml of 400% (wt/vol) Holtfreter's solution and 30 ml of 10% (wt/vol) benzocaine stock solution. Fill up the volume to 10 L with dH2O. The solution can be stored at room temperature for up to 6 months.	
Mix 50 g of benzocaine in 500 ml of 100% (vol/vol) ethanol. The solution can be stored at room temperature for up to 12 months.	
Stutter Instrument	BF120-94-8
Merck	102382
Mix 200 mM HEPES and 1.5 M KCl in RNase-free water. Adjust pH to 7.5. Filter sterilize, aliquot and store at −20 °C for up to 24 months	
PNA Bio	CP03
Falcon	351029
Fine Scientific Instruments	11254-20
Nepa Gene	NEPA21
BEX	Pulse Generator CUY21EDIT II
Sigma-Aldrich	F7252-5G

Dissolve 12.5 mg of Fast Green FCF powder in 10 mL of 1× PBS.	
Stutter Instrument	P-97
Dissolve 11.125 g of $MgSO_4 \cdot 7H2O$, 5.36 g of $CaCl_2 \cdot 2H2O$, 158.4 g of NaCl and 2.875 g of KCl in 10 L of dH2O. The solution can be stored at room temperature for up to 6 months.	
Merck	104936
Merck	105886
Eppendorf	5242956003
Narishige	MN-153
Merck	106404
World Precision Instruments	SYS-PV830
Fine Scientific Instruments	11103-09
Olympus	SZX10
Sigma-Aldrich	T6066
Dissolve 24.2 g of Tris base and 90 g of NaCl in 990 ml of dH2O. Adjust pH to 8.0 by adding 10 ml of 37% (vol/vol) HCl. The solution can be stored at room temperature for up to 6 months.	
Nepa Gene	CUY650P10

Comments/Description

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Title of Article:	Direct gene knockout of axolotl spinal cord neural stem cells via electroporation of CAS9 protein-gRNA complexes
Author(s):	Wilson Pak-Kin Lou, Liqun Wang, Lei Liu, Ji-Feng Fei

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CORRESPONDING AUTHOR

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Department:	Institute for Brain Research and Rehabilitation
Institution:	South China Normal University
Title:	Professor
Signature:	
Date:	14.2.2019

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Dear Dr. Bajaj,

Thank you very much the constructive comments from you and the reviewers. We have addressed all the issues raised, please find the changes described below and also marked in blue in the revised manuscript, as well as the updated figures and tables in the submission system. We look forward to any further feedback and the shooting of the video!

Best regards,

Ji-Feng Fei

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Done

2. Please define all abbreviations during the first-time use.

Done

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "We present here a protocol to …"

We have rephrased the summary.

4. Please rephrase the Long Abstract to more clearly state the goal of the protocol in 100-300 words.

Abstract has been expanded slightly to improve clarity.

5. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: NEPA electroporator, FAST green FCF, etc.

Done. We have retained the use of Fast green FCF since it is not a commercial name and is widely used in other articles.

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.

We have modified the protocol to conform to this format.

7. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Our protocol has no centrifugation steps.

8. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

Done

9. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

The table has been removed and the information is rewritten as text (line 107-109).

10. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have modified the entire protocol to conform to this standard.

11. The Protocol should contain only action items that direct the reader to do something.

We have modified the protocol to adhere to this.

12. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

Done

13. Line 98: the note mentions many literatures but cites only one.

Missing references have been added.

14. Step 2: Please provide volume and concentrations all the solutions used.

Done

15. Step 3 and 5: Please write in complete sentences using imperative tense throughout.

We have modified the protocol to adhere to this.

16. 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.

We have modified the protocol to adhere to this.

17. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. 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. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next.

Our whole protocol is within 2.75 pages, and we would like to film it all.
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19. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
The Discussion has been made into 6 paragraphs, in which we have include all the above mentioned points.
20. Please expand the journal titles in the reference section.
Done
21. Figures: Please include a scale bar for all the figures panels having images from a microscope.
The missing scale bars have been added.
22. Please alphabetically sort the materials table.
Done

Reviewers' comments:

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Reviewer #1:

Manuscript Summary:
The methods articule by Lou, Wang and colleagues thoroughly describes a very timely and straightforward approach to efficiently knock-out genes in the ependymal cells of the axolotl spinal cord. Ependymal cells are the main drivers of spinal cord regeneration in the axolotl and, even though their

mammalian counterparts seem to also retain neural stem cell potential, they fail to resolve spinal cord injuries. As the authors point out, having the tools to investigate what makes axolotl ependymal cells unique is thus of great importance. This protocol nicely complements a protocol and a research paper (PMID: 25241743) previously published by the authors (PMID: 30429597) - and it will do more so when accompanied by a video! I have no doubts this paper will become a reference in every axolotl lab, and beyond. The authors have also done a good job gathering relevant references from the literature. I much recommend publication in JoVE (and I am looking forward to watch the video!), but will appreciate if the authors could address my minor comments below.
Thank you very much for the positive comments.

Major Concerns:
None.

Minor Concerns:
1) Figure 2 shows the effect of SOX2 knock-out 15 days post-electroporation. But, could the authors comment on how fast does the knock-out occur? Animals grow in size and the waiting time until the gene is effectively knocked out might be important for researchers interested, for example, in imaging ependymal cells live.
Thank you for raising this point. To address this issue, we have isolated spinal cord tissue from the CAS9-gRNA electroporated region within 24 hours of electroporation, extracted the genomic DNA and determined the genomic modification efficiency at the gRNA targeted loci via genotyping PCR. From this we observed a 60-70% genomic editing rate. This is likely an underestimation of gene editing efficiency, as this piece of dissected spinal cord includes non-electroporated cells, as well as neurons which will not receive CAS9-gRNA complexes due to lack of apical contact to the central canal. The fast acting is likely due to the CAS9 protein-gRNA RNP being able to induce cutting immediately upon entry into the cell. However, the disappearance of the protein coded by the targeted genomic loci varies with regards to factors such as protein decay rate. In the case of GFP, we can still observe the presence of GFP protein for many days post CAS9-gRNA electroporation. We have added this to the discussion (line 212-215, 273-275).

2) Have the authors considered including their gene knock-in strategy in this protocol?
Thank you for your suggestion. We have previously published another protocol paper which describes detailed axolotl knock-in strategies (Fei et al., 2018). According to the policy of that journal, it is not allowed to publish again the knock-in protocol, even at the video format. Therefore we have instead

referred the readers to this article instead. We have added this point in the discussion (line 275-278).

3) Legend Figure 2. The difference in the percentage of SOX2 cells between control and CAS9-Sox2gRNA are striking, and very clear! But please write in the figure legend which statistical test was used to calculate the p-value, it is good practice.
Student's t-test was used, and this has been added to the figure legend (line 247).

Reviewer #2:

Manuscript Summary:
The manuscript describes a method to inject gRNAs complexed with cas9 protein into the spinal cord of a salamander, the axolotl

Major Concerns:

This method has previously been published as NPJ Regen. Methods paper in 2016, the figure showing knockdown of Sox2 where published in Stem cell Reports paper in 2014 but the figure shown here is not referenced as being taken from that paper in 2014. The figure for showing how the electroporation is set up is taken from a book chapter but is again not credited.
Thank you for your valuable comment. In the current protocol, figure 2 is taken from the 2016 paper in NPJ Regenerative Medicine, which describes a temporal and spatial controlled target gene knockout approach via electroporating CAS9 protein-gRNA complexes. The method published in the 2014 Stem Cell Reports paper is different from the current protocol, and describes the global knockout/knockdown of the Sox2 gene in entire animals via injection of Cas9 mRNA and gRNA into single stage fertilized eggs. We have credited the above-mentioned book chapter, which shows how the electroporation is set up in the legend of figure 1 in the revised manuscript (line 241).

A crucial part of carrying out knockout experiments is actually showing that the gene is knocked out, the protocol should give clear detailed information on how to carry out this part of the analysis.
Thank you very much for your great suggestion, we have expanded the protocol to describe this crucial step (line 202-215).

Reviewer #3:

Manuscript Summary:

This is a nice paper describing methodology for local gene perturbation of mature tissues. I have only minor comments listed below
Thank you very much for your positive comments.

Major Concerns:

Minor Concerns:

Protocol section 1.2. Give reference to commercial source
We have provided the reference in the Table of Materials.
Protocol section 2.2. One could add that the distance between electrodes affects the efficiency
This has been elaborated more in the discussion (line 326-328).
Protocol section 3.1 Give reference for NEPA
We have provided the reference in the Table of Materials. We also included another equivalent electroporator from the company BEX in the Table of Materials.
Protocol section 4.1 Give reference for capillaries and puller
We have provided the reference in the Table of Materials.
Protocol section 4.3. Give reference for FCF solution
We have provided the reference in the Table of Materials.
Protocol section 4.4. An image showing this would be great
Thank you for this point. A panel has been added to show this (Fig 1B).
Protocol section 5.1 Which model of the pump is used?
We have provided the reference in the Table of Materials.
Protocol section 6.4. An image showing this would be great
Thank you very much for your valuable suggestion. Two panels have been added to show this (Fig 1C and D).

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Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Will you be translating?	no
Circulation/distribution	501 to 1000
Author of this Springer Nature content	no
Title of new article	Direct gene knockout of axolotl spinal cord neural stem cells via electroporation of CAS9 protein-gRNA complexes
Lead author	Pak Kin Lou
Title of targeted journal	Journal of Visualized Experiments
Publisher	MyJove Corp.
Expected publication date	Jun 2019
Portions	Figure 1
Requestor Location	Pak Kin Lou P.O.Box 84309 Hung Hom Bay Post Office Hong Kong, 0000 Hong Kong Attn: Pak Kin Lou
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