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To: Dr. Wu, Review Editor

JoVE

Dear Dr. Bing Wu,

We would like to thank you for sending out our manuscript for review and providing us a rapid and thorough evaluation of our work. We appreciate the referees' constructive comments and your willingness to further consider our manuscript.

We have made the appropriate changes as requested by all three reviewers and believe that this manuscript has greatly benefited as the result of these changes. We also addressed all editorial issues. The revised manuscript is attached along with our detailed response to the reviewers' questions and comments. We have also included (in a separate file) the revised manuscript in which our changes are marked in red to ease the review.

We believe that the manuscript is ready for further consideration in *JoVE*.

Sincerely,

Ester Segal

Detailed response to editorial comments and reviewers' comments:

Editorial comments:

Changes to be made by the author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Answer: We thank the editor for his careful review and we have thoroughly proofread the manuscript.

2. Please revise lines 45-46, 64-65, and 68-69 to avoid previously published text.

Answer: The mentioned lines have been rewritten and rephrased.

3. Please revise the title to avoid punctuation.

Answer: The title has been changed to avoid punctuation.

4. Please remove all commercial language from your manuscript and use generic terms instead: AZ4533, percoll, etc.

Answer: In line 129, AZ4533 was replaced with "a positive thick". In line 305, percoll was replaced with "silica-based colloidal medium for cell separation by density gradient centrifugation".

5. 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:

2.4: Please ensure that the protocol here can stand alone. As currently written, users must refer to another protocol in order to complete this protocol. Please describe how to determine NGF content.

Answer: Step 2.4 in now thoroughly detailed in a new section that was added to the revised manuscript (section 3. Quantification of NGF loading using NGF ELISA kit), see page 4.

3.4: Please describe how to perform ICP-AES analysis. We need more specific details for filming.

Answer: Step 3.4 is now described in details in a new section that was added to the revised manuscript (section 5. Quantification of in vitro Si erosion by inductively coupled plasma atomic emission spectroscopy (ICP-AES)), see pages 5-6.

4.2.1: Please specify how to isolate DRGs from mice.

Answer: This step is now elaborated and detailed in 5 new steps that were added to the revised manuscript, see pages 7.

4.2.2: How to clean the surrounding connective tissues?

Answer: These details are now specified in step 6.2.6.

6. Line 211: Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Answer: We thank the editor for this comment and we have moved the ethics statement accordingly, before the numbered protocol steps.

7. 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.

Answer: We have highlighted 2.75 pages of the revised protocol to identifie the essential steps of the protocol for the video.

8. 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.

Answer: The highlighted sentences in the revised manuscript follow the instructions above.

9. 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.

Answer: We have carefully followed these instructions in the revised highlighted protocol.

10. Figures 2, 4 and 5: Please define error bars in the figure legend.

Answer: We have added definitions for the error bars to the captions of Figures 2, 4, and 5. In all of them the error bars represent SD, n=3.

11. Figure 3b: Please describe the left and right panels.

Answer: We have added description of the left and right panels to the caption of Figure 3.

Reviewers' comments:

Reviewer #1:

Manuscript summary:

Fabrication of porous silicon (PSi) film as a degradable carrier for nerve growth factor (NGF) release is reported. The PSi carrier was designed to exhibit high loading capacity of NGF, and sustained release was examined in vitro for four weeks. Differentiation of PC12 cells and DRG neuronal cells in NGF-releasing condition was characterized and compared to conventional free NGF administration. The results showed comparable neurite

initiation to the free NGF administration, indicating feasibility of the PSi carrier as a long-term implant for NGF release to treat neurodegenerative diseases. Because of the biocompatibility and biodegradability of PSi, this platform can be a promising delivery system for long-term NGF or other growth factor supply. This report include essential information to fabricate the PSi-based NGF releasing system with step-by-step protocol, therefore the experiment can be readily reproduced by others. Experimental and analytical details are sufficiently described in the present form. This report will be a good reference in the field, and attract much attention to the general readership of the Journal of Visualized Experiment. Therefore, it is highly recommended to be published in the Journal of Visualized Experiment after revision of a few minor concerns as following.

We thank the reviewer for his/her positive evaluation of our manuscript and have carefully revised it based on his/her feedback.

Minor concerns:

1) In the Protocol 1.1, the kind of dopants (e.g., boron) should be noted specifically.

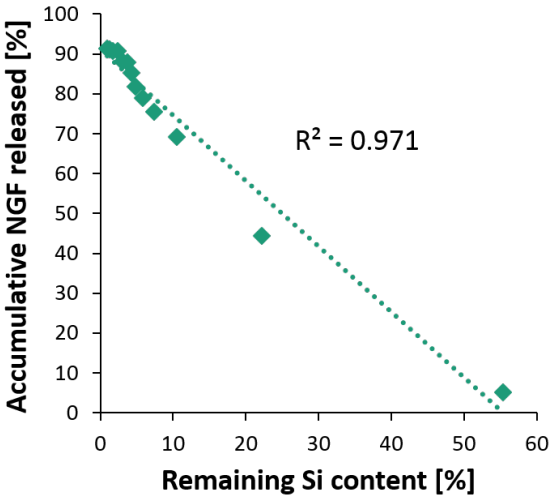
Answer: The dopant type (Boron) is now specified in step 1.1 of the revised protocol.

2) In the Protocol 1.2, 1.9, and 1.10, heating (10 C per min?) and cooling (natural cooling?) rate should be stated.

Answer: We have added the details regarding the heating and cooling rates for step 1.2, 1.9, 1.10 in the revised protocol.

3) It would be helpful to show strong correlation of NGF release with Si degradation profile by plotting NGF release as a function of remaining Si content over time in Figure 2.

Answer: We thank the reviewer for this constructive comment. Please see attached below a graph presenting the excellent correlation between the NGF release and the remaining Si content. We have included this new graph as an inset in Figure 2 and have addressed it in the revised manuscript, see page 10.



4) It would be helpful if additional information about the a few more set of etching condition, pore size, and NGF loading capacity is included.

Answer: In this work we have focused on a specific nanostructured PSi film, which was found to yield the maximal NGF loading efficacy. A prior thorough optimization study, we have investigated the correlation between the anodization conditions, resulting nanostructure characteristics, and NGF loading and release. Please see attached below unpublished data of the prior optimization work performed. Table S1 summarizes the different etching conditions examined and Figure S1 presents NGF release profiles of the corresponding $PSiO_2$ carriers. Condition set no. 4 in Table S1 yielded the highest values of accumulative NGF release (Figure S1) compared to the other $PSiO_2$ carriers examined, and this was one of the reasons for focusing on this specific set of etching conditions. We believe that these results are beyond the scope of this paper, which focuses on the controlled release of NGF and its bioactivity from PSi carriers.

Table S1. Etching parameters

Current density [mA/cm²]	Etching time [sec]
50	101
100	51
150	34
250	20

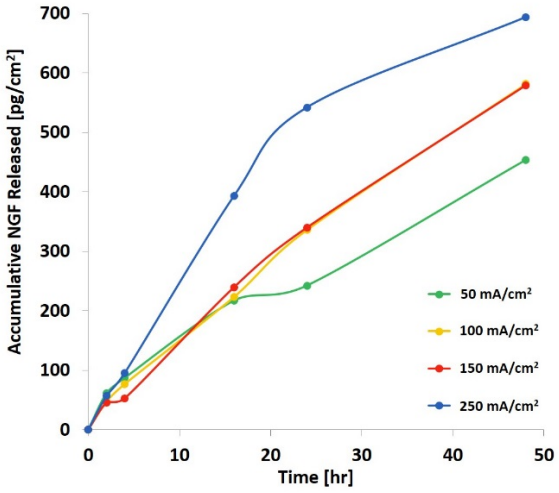


Figure S1: NGF release profiles of the different NGF-loaded $PSiO_2$ carriers

5) As authors mentioned in the manuscript, the release of NGF payload is affected by two

pathway: out-diffusion and silicon scaffold degradation. Therefore, it would be very nice if authors state any information to distinguish each kinetics or suggest any methods for further analyses.

Answer: We thank the reviewer for this important comment. The mentioned concept is elaborated in previous works by our group and others and we now refer the readers to relevant references in the discussion section and expand the discussion, see page 12. The two mechanisms, i.e., out-diffusion and degradation of the Si scaffold, occur simultaneously for oxidized PSi and it is difficult to clearly differentiate and identify the amount of drug released due to passive diffusion versus the amount of drug released due to scaffold degradation.

6) The release kinetics of the NGF payload is not a typical curve of 'sustained release'. However, authors claim that a sustained release of NGF without burst release is attained for 1 month. It is recommended to discuss it carefully. The release curve still has a sort of burst release at the very beginning (within 6 days).

Answer: We agree with the reviewer and accordingly have added a clarification and better description of the release kinetics in the revised manuscript, page 10.

Reviewer #2:

Manuscript Summary:

This is a revision of a methods paper to JoVE. I see some highlighted text suggesting that the paper has been revised although I do not see the original reviewer comments. This is suitable for publication after these minor issues are addressed.

1. 2.1. Is there need for BSA as a carrier protein? Might this improve efficacy?

Answer: We thank the reviewer for the constructive comment. Many reports utilize BSA as a carrier protein in order to stabilize the NGF and prevent its denaturation and loss of biological activity during the encapsulation process. In our work, NGF loading into the carriers is carried out at room temperature without using organic solvents and thus the entrapment within the carrier system does not impair the bioactivity of the protein. Moreover, co-loading of NGF and BSA may result in a lower loading efficacy compared to loading of NGF alone as the available volume for accommodation of NGF within the host is reduced when loading both of the proteins. However, adding BSA can be advantageous in terms of sustaining NGF release from the carriers for an extended time period. Numerous studies have demonstrated the use of BSA as a carrier protein to modulate the release rate of NGF; incorporating BSA within the carrier matrix or co-loading of BSA and NGF have been shown to significantly extend the NGF release period[1-3].

1 Valmikinathan, C. M., Defroda, S. & Yu, X. Polycaprolactone and bovine serum albumin based nanofibers for controlled release of nerve growth factor. *Biomacromolecules.* **10** (5), 1084-1089, (2009).
2 Xu, X. *et al.* Peripheral nerve regeneration with sustained release of poly(phosphoester) microencapsulated nerve growth factor within nerve guide conduits. *Biomaterials.* **24** (13), 2405-2412, (2003).
3 Xu, X. *et al.* Polyphosphoester microspheres for sustained release of biologically active nerve growth factor. *Biomaterials.* **23** (17), 3765-3772, (2002).

2. 2.4. Can you recommend a specific manufacturer? Lots of bad ELISA kits.

Answer: The manufacturer of the NGF ELISA kit used in this work is PeproTech, as cited in the table of materials. Detailed information regarding the use of the kit can be found in step 3 in the revised protocol section.

3. 4.1.3. Recommended concentration of cells and seeding density?

Answer: We have added the details regarding recommended concentration of cells and seeding density in step 6.1.3 in the revised protocol.

4. Missing citations to Si-based drug delivery:

a. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4377731/
b. https://pubs.acs.org/doi/full/10.1021/acsami.7b04181

Answer: We thank the reviewer suggesting these references and we now properly cite them is the revised introduction of the manuscript, see references 23 and 25.

Reviewer #3:

The work by Segal's group describes the fabrication of nanostructured PSi films for continuous and prolonged drug release, providing bioactivity of NGF released from the PSi carriers. The studied is well conducted, very well presented and very interesting for the broad audience of this journal. Therefore, I'd recommend acceptance of this work in its present form.

We thank the referee for his positive evaluation of our work and supporting its publication.