

Reviewer Assessment

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Fluoro-Ruby as a reliable marker for regenerating fiber tracts

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Reviewers' Comments to Original Submission

Reviewer 1: anonymous

Jul 28, 2016

Reviewer Recommendation Term:	Accept with Minor Revision
Overall Reviewer Manuscript Rating:	N/A
Custom Review Questions	Response
Is the subject area appropriate for you?	4
Does the title clearly reflect the paper's content?	5 - High/Yes
Does the abstract clearly reflect the paper's content?	5 - High/Yes
Do the keywords clearly reflect the paper's content?	5 - High/Yes
Does the introduction present the problem clearly?	5 - High/Yes
Are the results/conclusions justified?	4
How comprehensive and up-to-date is the subject matter presented?	4
How adequate is the data presentation?	4
Are units and terminology used correctly?	4
Is the number of cases adequate?	N/A
Are the experimental methods/clinical studies adequate?	4
Is the length appropriate in relation to the content?	5 - High/Yes
Does the reader get new insights from the article?	3
Please rate the practical significance.	3
Please rate the accuracy of methods.	4
Please rate the statistical evaluation and quality control.	N/A
Please rate the appropriateness of the figures and tables.	4
Please rate the appropriateness of the references.	4
Please evaluate the writing style and use of language.	5 - High/Yes
Please judge the overall scientific quality of the manuscript.	4
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:

The paper is well written, the method is clearly demonstrated. However, the authors have to clarify which approval (country, institution, etc) was given on the animal experiments. They should specify the number of the used animals in all parts of the experiments. What was the pain medication protocol for the animals with transection and transplantation? Why did they use only female rats in the hemitranssection group. Is the cell transplantation group the same as the transection group? If yes, why do they not state this and mention the time in between the experiments.

Is there a histological comparison to FR which shows the same results? It remains unclear how the statement on „no cytotoxicity“ is made only from the FR staining. Is invasion of lymphocytes or macrophages visible with the FR stain?

If bars are put in the figures the measurement has to be stated.

Reviewer 2: anonymous

Aug 25, 2016

Reviewer Recommendation Term:

Accept with Minor Revision

Overall Reviewer Manuscript Rating:

75

Custom Review Questions

	Response
Is the subject area appropriate for you?	5 - High/Yes
Does the title clearly reflect the paper's content?	5 - High/Yes
Does the abstract clearly reflect the paper's content?	5 - High/Yes
Do the keywords clearly reflect the paper's content?	5 - High/Yes
Does the introduction present the problem clearly?	5 - High/Yes
Are the results/conclusions justified?	5 - High/Yes
How comprehensive and up-to-date is the subject matter presented?	4
How adequate is the data presentation?	5 - High/Yes
Are units and terminology used correctly?	5 - High/Yes
Is the number of cases adequate?	N/A
Are the experimental methods/clinical studies adequate?	5 - High/Yes
Is the length appropriate in relation to the content?	4
Does the reader get new insights from the article?	4
Please rate the practical significance.	3
Please rate the accuracy of methods.	5 - High/Yes
Please rate the statistical evaluation and quality control.	5 - High/Yes
Please rate the appropriateness of the figures and tables.	5 - High/Yes
Please rate the appropriateness of the references.	3
Please evaluate the writing style and use of language.	5 - High/Yes
Please judge the overall scientific quality of the manuscript.	5 - High/Yes
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:

The authors describe a clinical problem that is inherent in peripheral nerve surgery. Up to now - with the exception of tedious histological staining methods - there is no really practicable and valuable tool to optimize peripheral nerve coaptation intraoperatively by detecting in vivo which fibers are regenerating and which are not. Histologic staining on the other hand requires surgical biopsy of the regenerating nerve and this leads to additional injury. It also causes an intraoperative time delay.

Based on these clinical hitherto unmet needs the authors describe a technique that may allow a quantitative and objective assessment of nerve regeneration.

Here the authors use Fluoro-Ruby (FR) which is a bidirectional tracer, but especially in tracing of anterograde fibers remarkable results are described. Among various other markers Fluoro-Ruby has been described as a nerve fiber marker previously, and is here investigated in adult wistar rats with direct transplantation of OECs into the transected spinal cord. They show that doing so resulted in anatomical regeneration of the transected fibers 21 days after the lesions. One important finding is that in addition to anterograde labeling of the ascending fibers, retrograde labeling of dorsal root peripheral nerve fibers was observed.

This is of interest in further evaluating this marker. The clinical prospectus is also important, since the technique presented in this paper would allow in vivo tracing of regenerating axons by simple injection into the peripheral nerve. Injection may be performed proximally or distally of the lesion, the retro- and anterograde transport of FR could be detected easily by use of a fluorescent light.

The experimental set up is straight forward and well done and described. The results are clearly reported and discussed before the background of current literature.

Given the difficulties of axonal regeneration, FR tracing may provide an important marking technique to better distinguish and establish the regeneration of neuronal circuits.

References should be worked over:

The authors need to quote and discuss previous literature with a comparison of various markers a little more, such as for instance:

Fluorescent retrograde neuronal tracers that label the rat facial nucleus: a comparison of Fast Blue, Fluoro-ruby, Fluoro-emerald, Fluoro-Gold and Dil. *Journal of Neuroscience Methods*; Volume 117, Issue 2, 30 June 2002, Pages 167-172 or:

Efficacy of fluorescent tracers in retrograde labeling of cutaneous afferent neurons in the rat; *Journal of Neuroscience Methods* Volume 191, Issue 2, 30 August 2010, Pages 208-2

Authors' Response to Reviewer Comments

Dec 05, 2016

Reviewer #1:

The paper is well written, the method is clearly demonstrated. However, the authors have to clarify which approval (country, institution, etc) was given on the animal experiments. They should specify the number of the used animals in all parts of the experiments.

Response: We now clearly indicate that the experiments were carried out in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and the U.S. VA Connecticut Healthcare System Institutional Animal Care and Use Committee (IACUC) approved all animal protocols.

What was the pain medication protocol for the animals with transection and transplantation.

Response: We added the Methods: Pain relief with buprenorphine (0.05 mg/kg/day SQ) for 48 h and ibuprofen (0.15 mg/ml = 5 mg/kg/day PO) for 72 h was provided for all animals.

Why did they use only female rats in the hemitranssection group.

Response: We have an historic data base with female rats and for our long term survival studies the females do not get as large as males and are easier to handle.

Is the cell transplantation group the same as the transection group? If yes, why do they not state this and mention the time in between the experiments.

Response: The cell transplantation groups and the transection groups is the same and the spinal cord was transected and teh cells were injected immediately after hemitranssection. We apologize for not mentioning it clearly enough and this information is now added in detail.

Is there a histological comparison to FR which shows the same results?

Response: We regret that there is no histological comparison to FR and we fully agree that this should be included in consecutive studies.

It remains unclear how the statement on "no cytotoxicity" is made only from the FR staining. Is invasion of lymphocytes or macrophages visible with the FR stain?

Response: We have now clarified in the text that is „no obvious cytotoxicity“. This observation is from no increased neurological deficits in the animals or visible cell infiltration.

If bars are put in the figures the measurement has to be stated.

Response: The measurement not stated.

Reviewer #2:

The authors describe a clinical problem that is inherent in peripheral nerve surgery. Up to now - with the exception of tedious histological staining methods - there is no really practicable and valuable tool to optimize peripheral nerve coaptation intraoperatively by detecting in vivo which fibers are regenerating and which are not. Histologic staining on the other hand requires surgical biopsy of the regenerating

nerve and this leads to additional injury. It also causes an intraoperative time delay. Based on these clinical hitherto unmet needs the authors describe a technique that may allow a quantitative and objective assessment of nerve regeneration. Here the authors use Fluoro-Ruby (FR) which is a bidirectional tracer, but especially in tracing of anterograde fibers remarkable results are described. Among various other markers Fluoro-Ruby has been described as a nerve fiber marker previously, and is here investigated in adult wistar rats with direct transplantation of OECs into the transected spinal cord. They show that doing so resulted in anatomical regeneration of the transected fibers 21 days after the lesions. One important finding is that in addition to anterograde labeling of the ascending fibers, retrograde labeling of dorsal root peripheral nerve fibers was observed. This is of interest in further evaluating this marker. The clinical prospectus is also important, since the technique presented in this paper would allow in vivo tracing of regenerating axons by simple injection into the peripheral nerve. Injection may be performed proximally or distally of the lesion, the retro- and anterograde transport of FR could be detected easily by use of a fluorescent light. The experimental set up is straight forward and well done and described. The results are clearly reported and discussed before the background of current literature. Given the difficulties of axonal regeneration, FR tracing may provide an important marking technique to better distinguish and establish the regeneration of neuronal circuits.

Response: We thank the reviewer for the comments.

References should be worked over: The authors need to quote and discuss previous literature with a comparison of various markers a little more, such as for instance: Fluorescent retrograde neuronal tracers that label the rat facial nucleus: a comparison of Fast Blue, Fluoro-ruby, Fluoro-emerald, Fluoro-Gold and Dil. Journal of Neuroscience Methods; Volume 117, Issue 2, 30 June 2002, Pages 167-172.

or:

Efficacy of fluorescent tracers in retrograde labeling of cutaneous afferent neurons in the rat; Journal of Neuroscience Methods Volume 191, Issue 2, 30 August 2010, Pages 208-2.

Response: We thank the reviewer for this comment and the references are worked over and the mentioned study with regard to fluorescent tracers in retrograde labeling is included in the discussion.

Reviewers' Comments to Revision

Reviewer 1: anonymous

Jan 03, 2017

Reviewer Recommendation Term:	Accept
Overall Reviewer Manuscript Rating:	N/A
Custom Review Questions	Response
Is the subject area appropriate for you?	5 - High/Yes
Does the title clearly reflect the paper's content?	5 - High/Yes
Does the abstract clearly reflect the paper's content?	5 - High/Yes
Do the keywords clearly reflect the paper's content?	5 - High/Yes
Does the introduction present the problem clearly?	4
Are the results/conclusions justified?	4
How comprehensive and up-to-date is the subject matter presented?	4
How adequate is the data presentation?	4
Are units and terminology used correctly?	4
Is the number of cases adequate?	N/A
Are the experimental methods/clinical studies adequate?	4
Is the length appropriate in relation to the content?	4
Does the reader get new insights from the article?	4
Please rate the practical significance.	3
Please rate the accuracy of methods.	4
Please rate the statistical evaluation and quality control.	N/A

Please rate the appropriateness of the figures and tables.	3
Please rate the appropriateness of the references.	3
Please evaluate the writing style and use of language.	3
Please judge the overall scientific quality of the manuscript.	4
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:

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Reviewer 2: anonymous

Dec 12, 2016

Reviewer Recommendation Term:	Accept
Overall Reviewer Manuscript Rating:	70

Custom Review Questions

	Response
Is the subject area appropriate for you?	4
Does the title clearly reflect the paper's content?	5 - High/Yes
Does the abstract clearly reflect the paper's content?	5 - High/Yes
Do the keywords clearly reflect the paper's content?	5 - High/Yes
Does the introduction present the problem clearly?	5 - High/Yes
Are the results/conclusions justified?	4
How comprehensive and up-to-date is the subject matter presented?	4
How adequate is the data presentation?	5 - High/Yes
Are units and terminology used correctly?	5 - High/Yes
Is the number of cases adequate?	N/A
Are the experimental methods/clinical studies adequate?	5 - High/Yes
Is the length appropriate in relation to the content?	4
Does the reader get new insights from the article?	4
Please rate the practical significance.	4
Please rate the accuracy of methods.	5 - High/Yes
Please rate the statistical evaluation and quality control.	4
Please rate the appropriateness of the figures and tables.	4
Please rate the appropriateness of the references.	4
Please evaluate the writing style and use of language.	5 - High/Yes
Please judge the overall scientific quality of the manuscript.	4
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:

The authors have replied appropriately to the queries and comments.