Response to editorial and reviewer comments:

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.

We have carefully read through and copy-edited the manuscript.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

We have added a single line space between each step, substep and note in the protocol section, and removed indentations.

3. Please remove the colon from the title and make it a concise.

Also considering a suggestion from Reviewer 1, we have changed the title to "Myelination and remyelination ex vivo as a quantitative model for developmental and disease-relevant manipulations."

4. Please describe all abbreviation during the first-time use.

We have ensured that all abbreviations are described during the first-time use.

5. 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.

We removed some notes and put some notes into steps. We also ensured we consistently used the imperative tense and deleted some phrases to be more concise.

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

We have added more details to our protocol steps and also created a detailed diagram (Figure 1A) to detail the dissection in further detail.

7. 1.1: How is this done… by adding xxx, or as shown in Table 1, etc. Please include the details.

We have made a new Table 1 including details for making slice culture medium, dissection medium, and lysolecithin stock, and refer to this table in the protocol text.

8. 1.2: What are the factors for myelination assay, citations if any? what is the volume and concentration of the added factors?

Experimenters can test any soluble factors alone or in combination using this assay. To illustrate a positive result, we have generated data using Activin-A, previously published to accelerate myelination and oligodendrocyte differentiation. As noted in Figure 2, we tested this at 100ng/ml. We have included new text in the protocol referring to the myelination and remyelination representative results in Figures 2 and 3, respectively. Citations and a more detailed description are also included in the results section.

9. 2: We cannot have two notes following each other. Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step.

We have modified the notes to avoid two notes following each other, and also have moved some notes to steps/sub-steps in the protocol.

10. 2.6: Is just the decapitation approved by the IACUC? Do you need to place the pups on ice?

As noted at the beginning of the protocol, all animal studies and protocols were authorized and approved by the Genentech Institutional Animal Care and Use Committee. We do not need to place the pups on ice. As recommended, we use sharp surgical scissors for swift decapitation of pups to ensure a single, quick, and uninterrupted cut.

11. Step 3 refers to culture and medium change and step 4 talks about variations. Please first bring out how myelination/remyelination is studied and then include variations. Please write exactly how the experiment is performed in your lab.

We have modified the text to reflect this. Step 4 refers to culture and medium changes (as this is part of the standard myelination protocol and variations). Step 5 now details the standard

myelination protocol and then subsequent variations (remyelination, hypoxia-induced hypomyelination and genetic manipulations).

12. All the medium preparations (concentration and volume) can be moved to a table and uploaded as .xls/.xlsx file. Please move the name of the reagent, catalog number, company to the table of materials. We cannot have commercial terms in the manuscript.

We have created a new .xlsx file entitled "JoVE_Table1" and moved all medium preparation information to this file. We refer to Table 1 in the protocol as well.

13. 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.

We have highlighted this portion in yellow.

14. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in Figure Legend, i.e. "This figure has been modified from [citation]."

We have not reused any figures from a previous publication. We have generated all figures ourselves (schematics were done with Biorender and Powerpoint), and all data presented are newly generated data. We have included citations for Figure 1B-E that were adapted or based from previous manuscripts (Yuen TJ, Silbereis J et al., Cell 2014 and Fancy SPJ et al., Nature Neuroscience 2011).

15. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

We have now modified Figure 1A to a diagram (as reviewers suggested the image resolution was not optimal). We have also included a scale bar for the microscope image of the cerebellar slice.

16. Please sort the materials table in alphabetical order.

We have sorted the table in alphabetical order.

Reviewers' comments:
Reviewer #1:
This is a very useful methods paper from Shen & Yuen that details an ex vivo, quantitative method of using mouse cerebellar slice cultures to study developmental myelination as well as de-/re-myelination. Myelination of axons by oligodendrocytes can be studied in culture, but culture phenomena that do not occur in vivo can be introduced and many important cell types are missing from such preparations. Genetic mouse models are highly rigorous, but lack the ability to perform rapid, high-throughput studies. Thus, the method described by Shen & Yuen fills an important gap in the middle of these two approaches.

The protocol is very well-described in the manuscript and allows for highly quantitative measures of myelination as well as the ability to perturb myelination in numerous ways. Data provided are clear and convincing and underscore the utility of this method. Caveats of the approach are openly discussed. This methods paper will be very useful for the myelin field and little needs to be changed.

Minor comments:
*Title suggestion to have "Myelination" before "remyelination" as this is the temporal order of things. It also better parallels "developmental" and "disease-relevant" in the second half of the sentence.

We have now changed the title to reflect this comment and also to make the title more concise, avoiding the use of a colon (editor suggestion). The new title is now "Myelination and remyelination ex vivo as a quantitative model for developmental and disease-relevant manipulations."

*"Petri" should have an uppercase "P" with every use as Petri dishes are named for Julius Petri.

Thank you - we have changed this throughout the manuscript.

*Line 135, suggest "In rare instances," instead of "Rarely" for clarity

We have changed this wording.

*Line 144, can the authors note why the medial sections have the best integrity?

In our experience, the medial sections tend to have the best tissue integrity and reproducible myelination. We tend to culture only the 4-6 most medial slices from each brain. We hypothesize this is due to the tissue chopper action and how it chops through the tissue. We have made a minor edit in the text.

*Line 245, macrophage M2 polarization is a rather controversial phrasing; even the researcher who coined the M1/M2 nomenclature has called for a reassessment (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3944738/). It may therefore be prudent to avoid such wording as it is not relevant to the method.

We completely agree with this and have removed wording referencing M1/M2, which as the reviewer keenly points out, is now outdated and not relevant to the methodology presented.

Reviewer #2:

Manuscript Summary:

In this manuscript, the authors presented the protocol of demyelination and myelination ex vivo in the CNS. Overall this manuscript is clearly written and provide new insight into the fields of organotypic culture method. In particular, this protocol enabled to induce development, demyelination, and remyelination. The protocol of cult described well, and the author presented the developmental and disease-related model. However, it would be best if you accurately verified the disease model for the appropriate use of this protocol.

Major concerns:

1. Should the authors describe the confirm of the demyelination model more in detail? I did not exactly know whether demyelination was generated without cell death. The authors should present not only cell dedifferentiation of glia cells but also not affect cell death after acute demyelination (lysolecthin) with time by ICC, or Rt-QPCR.

Thank you for this comment. Lysolecithin has been used widely for many years in order to generate a demyelinating insult while avoiding axonal damage. As the effects of lysolecithin have been characterized extensively in previous work, and given the 2-week revision timeframe, we feel that additional experiments to do a detailed and meticulous characterization of the effect of lysolecithin would take considerable time/effort and is outside the scope of this protocol. However, we now include a brief discussion and additional citations (Keough MB et al., JoVE 2015; Birgbauer E et al., Journal of Neuroscience Research, 2004) to bolster this description.

2. Did you check the LPC or hypoxia-induced disease model for a long time for more than one month? To use this protocol for manipulation of developmental and disease conditions, you should show the change of morphology of explants or cells when the LPC or hypoxia were treated in the explants. Furthermore, you should present explant detached in a cell culture dish after treatment with LPC or hypoxia.

We have not cultured cerebellar explants for more than one month. In our experience, one month is the upper limit of these cultures, which also seems to be the consensus in the field as, to our knowledge, no protocol cultures explants past one month. We do not see any gross change of morphology in the slices after treatment or injury. As the explants remain attached to the membrane inserts throughout culturing and staining, we do not feel presenting data of explants detached would be useful.

Minor Concerns:
Please change the Figure 1A images. Especially, the author should change the resolution of each image.

Yes, we agree! We have redone Figure 1A to include higher resolution diagrams to better represent the morphology and anatomy of the dissection.

Reviewer #3:
Manuscript Summary:
This manuscript presents a protocol for an ex vivo quantitative model of re/myelination using mouse cerebellar slice cultures. The methods described recapitulate myelination in vivo in that all CNS cell types are present in the preparation. This model is particularly useful in that it can be modified to include chemical, genetic, and environmental factors that simulated diseased conditions. Overall, the protocol is very thorough and includes many helpful hints throughout.

Major Concerns:
None

Minor Concerns:
*Ensure all company and reagent names are spelled correctly for product look-up. E.g. Millicell is spelled Mllicell.
*Requires minor editing to ensure proper spelling and grammar. Below are a few examples:
*Line 55: Change "Thus, remyelination is an alternative promising therapy…" to "Thus, strategies to promote remyelination present promising alternative therapies…"
*Line 79: Add altered to "genetically altered using Ad-Cre…"
*Line 93: "Store at 4°C."

Thank you – we have changed these and also carefully copy-edited and corrected typos throughout the manuscript.

*In Section 3, what medium is the slice cultured in long-term?

We have edited the text to clarify that the slices are cultured in SCM long-term.

o3.1.1: Should it read "transfer membranes to the new plate"?

We have changed this.

*Since DIV seems to be an essential unit, please define it.

We have defined DIV (days in vitro) and also carefully gone through the manuscript to ensure that all abbreviations are defined the first time they are mentioned.

*4.1: Are the factors mentioned experimental compounds/factors?

We have clarified this in the protocol and text to explicitly describe our data examining Activin-A in the myelination slice culture and XAV939 in the remyelination slice culture. These commercially-available factors have been previously shown to modulate myelination (Dillenburg A et al., Acta Neuropathologica 2018) and remyelination (Fancy SPJ et al., Nature Neuroscience 2011). We have also cited these references in the text.

Reviewer #4:

Manuscript Summary:

This is an interesting manuscript, with a detailed protocol for ex vivo cerebellar explants culture, which should be useful to the scientific community. However, some limitations and previously published protocols for cerebellar slice cultures should be discussed.

Major Concerns:

Protocol, line 102: (…) "minimize the time cultures are exposed". An approximative maximum duration should be given regarding dissection. More generally, a timing of the different steps of the procedure would be helpful.

We have not had any issues with a maximum duration of dissection, and the note was simply to remind experimenters to minimize the time cultures were exposed (as with any dissection). However, we have modified the text (also incorporating editor and other reviewer comments) and have provided an approximate timing of the various steps.

Figure 1, part A: Better quality image and/or schematics would help to distinguish the different structures of the brain.

Thank you, we completely agree. We have redone Figure 1A to include higher quality schematics with more extensive labeling.

Discussion: The authors present a technique using tamoxifen treatment prior to myelination mechanisms assessment, though tamoxifen is known to modulate myelination. This should be discussed by the authors.

The reviewer is completely correct that tamoxifen is known to modulate myelination. The dose of tamoxifen that we use in our cultures (100nM) has been previously optimized to induce robust Cre recombination without inducing OPC differentiation/myelination (used in Yuen TJ, Silbereis J et al., Cell 2014). We have included a brief discussion of this in the protocol. In addition, we have also used an AdCre virus for genetic modification experiments, as now noted in the text.

Discussion: Here the authors present a technique using animals at P0-2. In these animals the the cerebellum is not yet properly organised (Dusart el al, 1997), unlike the protocols previously published (Birgbauer et al, 2004; Thetiot et al, 2019), which use mice at P9-10. This is a major point and a limitation of the P0-2 organotypic culture model, that should definitely be discussed by the authors, with the previously published protocols being cited.

Thank you for this astute point. We have included text discussing this limitation and added additional citations. We do believe that this method represents a useful resource for the field, allowing for multiple disease-relevant manipulations, particularly given the early postnatal myelination that takes place during development.

Minor Concerns:
Introduction, line 66-69: References regarding glia-glia crosstalk in myelination and the role of astrocytes and microglia should be added.

We have included several references: Nave KA & Werner HB, Annual Rev. Cell Dev. Biol 2014; Franklin RJM & ffrench-Constant, Nat. Rev. Neurosci. 2017; and Molina-Gonzalez I & Miron VE, Neurosci. Lett 2019.

Protocol, line 126-130: step 2.10 and 2.11, the cerebellum is transferred onto the chopper platform without meninges removal, though their removal is requiered with older animals. Is this step skipped deliberately? Could the authors explain why it is not needed?

We have not found that removing the meninges is required given the age of pups. As the tissue is sliced and then transferred from plate to plate prior to culturing on the membrane, any residual meninges (post-slicing) is likely washed away.

Protocol : The thickness of the slicing with the chopper is given only in the figure 1, and never mentioned in the text, which would be helpful.

We have added this to the protocol (step 3.10).

Protocol, line 153-156: step 3.1.2, an alternative solution is given in which the medium is not buffered by CO2 prior to slice transfer. Does this alternative solution have any side effect?

Thank you for this comment. We have removed this alternative approach, as we rarely use this.

Protocol, line 164-165: could the authors better define "incomplete myelination", maybe by giving an approximative % compared to total myelination.

Thank you for this comment. It is difficult to define this, but we have quantified the amount of "incomplete myelination" at this timepoint in the vehicle condition. As shown in the graphs (Figure 2), we see a myelination index value of 0.02533 +/- 0.0022 and a CC1/Olig2 ratio of 0.3960 +/- 0.02.

Protocol, line 171 : Fix at 28 DIV should be detailed (which rate of remyelination is seen at this timepoint?)

We have detailed this further in the protocol to explicitly mention that 28 DIV is a timepoint in which remyelination is not complete, allowing for the experimenter to observe an enhancing or blocking effect on remyelination.

Protocol, line 183-185: (…) "Fixed slices can be stored in PBS at 4 °C for several weeks". The tissue, even fixed still degrades with time. The authors should suggest a maximum duration of storage.

We have now included a maximum duration of storage (up to a month).

Protocol, line 207: given the thickness of the slice, do the authors use a special mounting technique to avoid crushing the tissue?

This will be best shown in the video protocol, but the slices do flatten out over time during culture. We have not had any issues with crushing the tissue during mounting after staining.

Discussion, line 303-304 (…) "or with the addition of peripheral immune cells to mimic oligodendrocyte injury in MS " and 325-326 "CSCs are devoid of peripheral cells and thus cannot be used to model the inflammatory and inhibitory CNS milieu". These two sentences seem contradictory.

Thank you for pointing this out – we have clarified the text in the discussion.

Discussion, line 311-312: (…) "different ages of mice pups, time in culture, or thickness of

sections may be used to adapt CSCs to different model", references should be added by the authors.

We have included several references here.

Discussion, line 320-322: (…) "ex vivo model, behavioral studies (e.g., rotarod), and imaging modalities used in human patients (e.g., MRI) cannot be performed". This sentence does not appear to be necessary.

We have removed this sentence.

Reviewer #5:

Manuscript Summary:

The work by Shen & Yuen details a protocol to generate organotypic cultures of cerebellum slices from P0-P2 rodent pups. This culture allow in vitro manipulations, including demyelination. Overall, the manuscript is properly written and describes a methods that has been extensively used by many groups in the World, with minimal variations. My main concern (see below) suggests that it should not be published.

Major Concerns:

Although novelty is not required by JoVE, in the opinion of this reviewer, the current manuscript does not represent any improvement in the protocol to be considered enough for its publication in a prestigious journal, like this. When compared to previous works published by different groups, this procedure does not provide with better results (but similar), nor it is easier, faster, offer an opportunity to the R-R-R recommendation on the use of animals in research, or more cheap. In this sense, the authors describe the use of newborn rodents, and the better results were obtained with cerebellar slices from P2 animals. The method is widely used by the international community for tissue (cerebellar slices) of P0-P7 animals with no problem (wider time window than this), the thickness of the slices is similar to the used by many other researchers and, although the authors proposed the use of hypoxic conditions, figures are not significantly better than published results by others, including those referred in their bibliography. Altogether, the manuscript does not have interest to be published in a prestigious journal like JoVE.

We thank this reviewer for this concern. As we have detailed in the manuscript (also emphasized by the other reviewers), this method is valuable as it recapitulates an in vivo model with its full complement of CNS cell types in intact tissue, while maintaining the chemical, genetic, and environmental amenability of an in vitro system. JoVE has previously published protocols based on widely used methodology (e.g. Experimental demyelination and remyelination of the murine spinal cord by focal injection of lysolecithin, 2015 – example in the myelin field). This protocol and others have been an invaluable asset to the field, as researchers can visually learn how to execute a new protocol in their laboratories. The slice culture methodology is quite detailed and requires careful dissection and separation of slices, which will be best captured in a video

protocol. Given the multiple disease-relevant manipulations possible with this protocol, it is our hope that this protocol will be useful to those that would like to study developmental myelination and myelin repair, and serve as a useful resource to the field.

Minor Concerns:
-The authors did not precisely describe which animal species they used (or if they method is useful for different species).

We have included more explicit descriptions of the animal species we use (mice). We have not examined the use of this method for different species.