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Ex vivo myelination and remyelination in cerebellar slice cultures as a quantitative model for developmental and disease-relevant manipulations

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Corresponding Author:	Tracy Yuen Genentech, Inc. South San Francisco, CA UNITED STATES
Corresponding Author's Institution:	Genentech, Inc.
Corresponding Author E-Mail:	hugenberger.tracy@gene.com
Order of Authors:	Kimberle Shen
	Tracy Yuen
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Vineeta Bajaj, Ph.D.
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Dear Dr. Bajaj,
Thank you for your letter on 2/6/2020 regarding our manuscript entitled “Myelination and remyelination *ex vivo* as a quantitative model for developmental and disease-relevant manipulations” (JoVE61044R1).

We thank you for reviewing this revision and for your helpful comments. We have addressed each of them in the document with track changes, responding to each of the editorial comments in the manuscript. We also have ensured that the highlighted portion of the protocol is no more than 2.75 pages including headings and spacings. Please feel free to reach out with any additional questions or concerns.

Yours sincerely,

Tracy J. Yuen, Ph.D.
hugenberger.tracy@gene.com

TITLE:

Ex Vivo Myelination and Remyelination in Cerebellar Slice Cultures as a Quantitative Model for Developmental and Disease-Relevant Manipulations

AUTHORS AND AFFILIATIONS:

Kimberle Shen[1], Tracy J. Yuen[1]

[1]Department of Neuroscience, Genentech Inc., South San Francisco, CA

Corresponding Author:

Tracy J. Yuen (hugenberger.tracy@gene.com)

Email Addresses of Co-authors:

Kimberle Shen (shen.kimberle@gene.com)

KEYWORDS:

myelination, remyelination, oligodendrocyte, demyelination, hypoxia, organotypic culture, cerebellum

SUMMARY:

Presented is a protocol for an ex vivo quantitative model of demyelination and remyelination using mouse cerebellar slice cultures. This method closely 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.

ABSTRACT:

Studying myelination in vitro and in vivo poses numerous challenges. The differentiation of oligodendrocyte precursor cells (OPCs) in vitro, although scalable, does not recapitulate axonal myelination. OPC-neuron cocultures and OPC-fiber cultures allow for the examination of in vitro myelination, but they lack additional cell types that are present in vivo, such as astrocytes and microglia. In vivo mouse models, however, are less amenable to chemical, environmental, and genetic manipulation and are much more labor intensive. Described is an ex vivo mouse cerebellar slice culture (CSC) quantitative system that is useful for: 1) studying developmental myelination, 2) modeling demyelination and remyelination, and 3) conducting translational research. Sagittal sections of the cerebellum and hindbrain are isolated from postnatal day (P) 0–2 mice, after which they myelinate ex vivo for 12 days. During this period, slices can be manipulated in various ways, including the addition of compounds to test for an effect on developmental myelination. In addition, tissue can be fixed for electron microscopy to assess myelin ultrastructure and compaction. To model disease, CSC can be subjected to acute hypoxia to induce hypomyelination. Demyelination in these explants can also be induced by lysolecithin, which allows for the identification of factors that promote remyelination. Aside from chemical and environmental modifications, CSC can be isolated from transgenic mice and are responsive to genetic manipulation induced with Ad-Cre adenoviruses and tamoxifen. Thus, cerebellar slice cultures are a fast, reproducible, and quantifiable model for recapitulating myelination.

INTRODUCTION:

Myelination of axons enables the rapid propagation of action potentials, a mechanism known as saltatory conduction[1]. The importance of myelin is underscored by demyelinating diseases, such as multiple sclerosis (MS), which include a wide range of debilitating manifestations, including loss of vision, cognitive issues, and paralysis. There is no cure for MS, and current therapies focus on limiting disease progression by targeting peripheral immune cells. Disability in MS and related diseases is thought to be driven by a failure of remyelination and progressive neurodegeneration. In particular, demyelination, atrophy, and axonal loss are observed in progressive MS[2,3]. Thus, strategies to promote remyelination represent promising therapies that could be carried out in parallel to current treatments and yield additional therapeutic benefits.

In the central nervous system (CNS), myelination is carried out by specialized glial cells known as oligodendrocytes. Oligodendrocyte precursor cells (OPCs) differentiate into mature, myelinating oligodendrocytes through a series of highly orchestrated steps, including the growth of processes that contact axons, increases in morphological complexity, expansion of the myelin membrane, and finally, myelin sheath compaction[4]. Thus, the interaction between oligodendrocytes and neurons is highly intimate. Reciprocal interactions between neurons and oligodendrocytes are also required for the health and maintenance of the CNS[2]. Axonal activity plays a role in stimulating myelination and glial neurotrophic factors support the integrity of neurons. The importance of glia-glia crosstalk in the CNS is also becoming increasingly recognized[5-8]. For instance, astrocytic factors can influence the differentiation of OPCs and the maintenance of white matter tracts. Microglia also play a role in modulating OPC differentiation as well as clearing myelin debris, an important step of the remyelination process. Identifying cell-autonomous factors and understanding the influence of other CNS cell types in demyelination and remyelination will be invaluable for developing therapies for demyelinating and dysmyelinating diseases.

Described is an ex vivo system using mouse cerebellar slice cultures (CSC) that allows for the manipulation and quantification of intact CNS tissue. Use of CSC allows myelination, or remyelination after the induction of demyelination by lysolecithin[9,10], to be measured using methods traditionally used with in vivo studies, such as immunostaining and electron microscopy[11-13]. Lysolecithin is a membrane-disrupting chemical that results in a rapid loss of myelin and oligodendrocytes. A potential caveat to note is that lysolecithin may also result in the reduction of other cell types close to the lesion area. Unlike in vivo experiments, however, sagittal slices of the cerebellum can be easily manipulated via the addition of compounds, or genetically altered using Ad-Cre adenoviruses. The method also allows for manipulation of isolated tissue from transgenic mice or mice subjected to environmental insults such as hypoxia[11-14]. The CSC model thus allows the study of developmental myelination, modeling of disease, and identification of factors that promote or inhibit myelination, while integrating the contributions of different CNS cell types to oligodendrocyte function.

PROTOCOL:

All animal studies were authorized and approved by the Genentech Institutional Animal Care and Use Committee.

1. Preparation of media and supplies for dissection (~30–45 min)

1.1. Prepare and sterile filter slice culture medium (SCM) and dissection medium (DM) as detailed in **Table 1**. Store at 4 °C. If any factors are being tested for the myelination assay add them to the SCM just before use.

1.2. Pipette 1 mL of SCM into each well of 6 well plate.

1.3. Using sterile forceps, place an organotypic insert into each well, ensuring no bubbles are trapped under the membrane. Warm the plate with media at 37 °C in 7.5% CO_2 incubator.

2. Preparation of the dissection area (~5–10 min)

2.1. Perform procedures in a sterile laminar flow hood or on the benchtop with adequate aseptic techniques for all steps of the procedure. Wipe all areas clean with 70% ethanol.

2.2. Place a new blade and silicone cutting stage on the tissue slicer. Pipette ~300 µL of sterile water or 70% ethanol under the cutting stage to ensure it stays in place.

2.3. Use a cotton swab to gently wipe the tissue slicer blade and cutting stage with 70% ethanol. Dry before using the tissue slicer.

2.4. Spray all dissection tools with 70% ethanol and dry prior to the dissection.

2.5. Prepare two 10 cm Petri dishes: one with 15–20 mL of DM, one with 10 mL of SCM. Store on ice. Keep all media and Petri dishes on ice when not dissecting.

3. Cerebellar slice culture dissection (~15–20 min per pup)

3.1. Place the Petri dish with DM on a dissecting microscope.

3.2. Euthanize a mouse pup, postnatal day P0–2, by swift decapitation with sharp scissors.

3.3. Place the head in the Petri dish with DM under the dissecting microscope to clear blood.

3.4. Using fine scissors, cut once on each lateral edge at the base of the skull.

3.5. With the head placed upside down in DM, press down firmly on the underside of the skull using #5/45 forceps, forcing the undamaged hindbrain through the hole in the skull.

3.6. Under the dissecting microscope, cut away excess tissue using two #11 scalpels. Ensure the

cerebellum remains attached to the underlying piece of hindbrain. See **Figure 1A** for a detailed diagram.

3.7. Using the spatula, transfer the cerebellum to the cutting stage of the tissue slicer with the tissue resting on the rostral face of the hindbrain and the caudal face of the hindbrain facing the researcher. Ensure that the medial plane of the brain is exactly parallel to the tissue slicer blade.

3.8. Using the P200 pipette, remove excess medium around the tissue, ensuring that it is still moist but not floating or surrounded by liquid.

3.9. Swap the Petri dish containing DM with the Petri dish containing SCM under the dissecting microscope.

3.10. Cut the tissue into 350 µm thick slices using the tissue slicer.

NOTE: The blade speed (e.g., roughly one cut per second) and force need to be optimized to ensure optimal cutting conditions. In rare instances, the tissue will get caught on the blade. To minimize loss of tissue sections, keep the thumb on the power button to quickly turn off the tissue slicer if needed.

3.11. Gently pipette 100 µL of SCM under the tissue, so the slices are floating. Transfer the sliced tissue using the spatula to the Petri dish with SCM under a dissecting microscope.

3.12. Use a cotton swab to gently wipe the tissue slicer blade and cutting stage with 70% ethanol.

3.13. Using the scalpels (blunt side), gently separate the slices under the dissecting microscope. Using a scalpel and spatula, transfer slices to the membrane in the 6 well plate with SCM.

NOTE: The most medial sections will often have the best myelination and tissue integrity. On average, 4–6 slices can be dissected from one mouse brain.

3.14. Incubate slices in SCM at 37 °C in 7.5% CO_2.

4. Culture and media changes (**~15–30 min**)

4.1. Replace the SCM every other day.

4.2. Pipette 1 mL of fresh medium into each well of a new 6 well plate and warm in an incubator. Using sterile forceps, transfer membranes to the new plate, ensuring no bubbles are underneath. Put plates in the incubator.

5. Standard myelination and remyelination

5.1. Standard myelination protocol (**Figure 1B**).

5.1.1. Culture slices dissected in section 3 from 0–12 days in vitro (DIV). If the effect of any factor on myelination is being tested, add it to the medium at the time of culturing (0 DIV) and replenish it with each media change.

5.1.2. Fix at 12 DIV (step 7.1), a timepoint optimized for incomplete myelination[11-13]. This allows for the detection of any enhancing or blocking effects on myelination due to the factor added to the medium. A representative positive result with the addition of 100 ng/mL Activin A is pictured in **Figure 2.**

5.2. Remyelination protocol (**Figure 1C**)

5.2.1. Follow the standard myelination protocol described in section 5.1 with no additional factors until 14 DIV, the timepoint when the slices are fully myelinated[11-13].

5.2.2. Prepare lysolecithin stock (125 mg/mL) as detailed in **Table 1**. Pipette 1 mL of 0.5% lysolecithin in SCM into each well of a new 6 well plate and warm in an incubator.

5.2.3. Transfer membranes to a lysolecithin plate and place in an incubator overnight for 16–18 h.

5.2.4. The next day, prepare new plates with fresh SCM, adding any factor(s) being tested for remyelination effects if needed. Warm plates in an incubator.

5.2.5. For the media change, gently tap the membrane on the side of the well to ensure all the lysolecithin medium is removed. Replenish the factor(s) being tested at each media change (section 4).

5.2.6. Fix slices at 28 DIV (step 7.1), a timepoint optimized for incomplete remyelination. This allows for the detection of enhancement or blocking of remyelination due to the factor added to the medium. A representative positive result with the addition of 0.1 µM XAV939 is presented in **Figure 3**.

6. Variations to the myelination and demyelination protocol

6.1. Hypoxia-induced hypomyelination protocol (**Figure 1D**)[11,13].

NOTE: This is presented as a variation of the standard myelination protocol presented in section 5.1.

6.1.1. Plate slices in SCM without factors.

6.1.2. Transfer slice plates to a hypoxic incubator (2% FiO_2) for 24 h between 2–3 DIV.

6.1.3. The next day, prepare new plates with fresh SCM, adding any factor(s) being tested for myelination potential if needed. Warm plates in an incubator.

6.1.4. Following culture in hypoxia, change medium (section 4) and return cultures to a standard culture incubator (37 °C, 7.5% CO_2). Replenish any factors being tested with each subsequent media change (section 4).

6.1.5. Fix at 12 DIV (step 7.1).

6.2. Genetic manipulation protocol (**Figure 1E**)[13]

6.2.1. Isolate tissue from transgenic mice for dissection as in section 3.

6.2.2. With inducible transgenic mouse lines, tamoxifen (100 nM, a dose that does not induce myelination or OPC differentiation) or an Ad-Cre adenovirus can be added to SCM during media changes done at 1 DIV and 3 DIV.

6.2.3. Following the genetic manipulation with tamoxifen or Ad-Cre adenovirus, continue media changes every other day as in section 4 (with factors added if needed).

NOTES: All slices from one mouse will fit on one membrane, allowing for genotyping after dissection, thus making it simple to do experiments with transgenic mice.

7. Tissue processing and analysis

7.1. Fix slices by submerging the membrane gently in 4% paraformaldehyde for 1 h at room temperature (RT).

7.2. Rinse gently by submerging in phosphate buffered saline (PBS). Then, submerge in PBS in a 6 well plate.

NOTE: The protocol can be paused here. Fixed slices can be stored in PBS at 4 °C for up to a month. If not storing slices, rinse 2x in PBS for 5 min before proceeding.

7.3. Use a scalpel to cut out the membrane from the ring and proceed with staining in a 6 well plate. Be sure to keep slices facing up (membrane-side down) for staining and subsequent mounting on slides.

7.4. Block for 1 h at RT in block solution (3% heat-inactivated horse serum, 2% bovine serum albumin, and 0.25% Triton-X 100 diluted in PBS).

NOTE: If antigen retrieval is needed for antibody staining (e.g., CC1/Olig2), this can be done in glass Petri dishes.

7.5. Wrap plates in paraffin film and incubate overnight at 4 °C in primary antibody diluted in blocking solution.

7.6. Wash 3x in blocking solution: 1) brief rinse, 2) 15 min wash, and 3) 1 h wash. For brief rinse, fill a small Petri dish with solution and gently submerge membrane using forceps before transferring to fresh solution. For longer washes (i.e., 15 min and 1 h), submerge membrane in solution in a 6 well plate and place on a gentle rocker.

7.7. Incubate covered from light for 2 h at RT in secondary antibodies diluted 1:500 in PBS.

7.8. Wash 3x in PBS: 1) brief rinse, 2) 15 min wash, and 3) 1 h wash.

7.9. If DAPI staining is needed, submerge in 1:1,000 DAPI solution for 7 min at RT. Rinse briefly and then wash for 15 min in PBS.

7.10. Rinse in double-distilled water.

7.11. Mount membranes on a slide with slices facing up (membrane-side down).

NOTE: Mounted slices can be stored at 4 °C. Imaging on a confocal microscope is best done within 1 week, before the membrane becomes opaque and difficult to image.

7.12. Image slices using confocal microscopy and quantify as described previously[11-13].

REPRESENTATIVE RESULTS:

Cerebellar slice cultures derived from P0-2 mice (**Figure 1A**) were used to study myelination and assess the effects of the addition of various factors from 0–12 DIV (**Figure 1B**). To study remyelination, slice cultures at 14 DIV were first demyelinated with lysolecithin and allowed to remyelinate for 14 additional days in culture with the tested factors (**Figure 1C**), after which remyelination was quantified. The effect of hypoxia on myelination was also studied by placing slice cultures in a 2% FiO_2 hypoxic incubator for 24 h, from 2–3 DIV (**Figure 1D**). Lastly, slice cultures derived from transgenic mice were used to study the effect of gene knockouts on myelination. In this system, Cre recombination was induced by the addition of tamoxifen or Ad-Cre adenovirus at 1 DIV and 3 DIV, and slice cultures fixed for analysis at 12 DIV (**Figure 1E**). After fixation of tissues, quantification of demyelination and remyelination was carried out by immunostaining (**Figure 2**, **Figure 3**) and electron microscopy[11-13].

Representative data shown (**Figure 2**, **Figure 3**) illustrate the dynamic range of myelination and remyelination in slice cultures. The myelination index was quantified by the ratio of Caspr, a paranodal marker that is an indirect readout of compact myelin, to neurofilament protein H (NFH), which stains axons. This myelination index has been validated to represent formation of compact myelin by electron microscopy and sodium channel staining[12]. Lysolecithin-induced demyelination[9,10] led to complete disappearance of Caspr-positive paranodes (myelination index = 0, **Figure 3A**) and loss of compact myelin, which was restored during remyelination[11,12].

To demonstrate a positive result on myelination, slices were cultured with Activin A following the timeline in **Figure 1B**. Activin A engages activin receptors on oligodendrocytes and drives oligodendrocyte differentiation and myelin compaction[15]. The representative data show that treating slices with 100 ng/mL of Activin A during myelination resulted in a higher myelination index (**Figures 2A**,**B**). Consistent with this, Activin A treatment accelerated OPC differentiation, as quantified by the ratio of CC1-positive mature oligodendrocytes to Olig2+ oligodendrocyte lineage cells[11,13] (**Figures 2C**,**D**).

A small molecule inhibitor of Tankyrase, XAV939, has been shown to promote myelination and remyelination by stabilization of Axin2 levels in oligodendrocytes[11]. Following lysolecithin treatment, demyelination was visualized and quantified in slice cultures by fragmented myelin basic protein (MBP) staining and lack of Caspr-positive paranodes (**Figures 3A**). Treatment with 0.01 µM XAV939 during remyelination (15-28 DIV) significantly increased the myelination index compared to vehicle controls, as quantified by the ratio of Caspr to NFH staining (**Figures 3B, C**). The slice culture model can thus be used to examine and quantify effects on OPC differentiation, myelination, and remyelination in intact tissue.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of cerebellar slice cultures showing key steps in the dissection process and timelines for different slice culture protocols. (**A**) Depiction of steps to generate slice cultures: dissection of brains from P0–2 mouse pups, trimming the brain (as shown), cutting the brain with a tissue slicer to generate 350 µm sagittal slices, placing slices on organotypic culture inserts in a 6 well dish, and incubating at 37 °C and 7.5% CO_2. * denotes cerebellum. (**B**) Timeline for myelinating slice cultures. Slice cultures were incubated for 12 days before fixation for analysis. (**C**) Timeline for remyelinating slice cultures. Slices were treated with lysolecithin at 14 DIV for 16–18 h to induce complete demyelination and allowed to remyelinate until 28 DIV before fixation for analysis. (**D**) Timeline for myelination following hypoxic insult. Slices were exposed to an acute hypoxic insult for 24 h between 2–3 DIV, which caused hypomyelination. (**E**) Timeline for CSC from genetically modified mice. Tamoxifen (Tam, 100nM) or Ad-Cre virus was added at 1 DIV and 3 DIV to induce genetic modification, and slices were analyzed at 12 DIV. Timeline diagrams adapted from previous publications[11,13].

Figure 2: Representative data showing Activin A promotes myelination and OPC differentiation. (**A**) Increase in myelination (MBP) and paranodes (Caspr) with 100 ng/mL Activin A treatment, shown by immunostaining of slice cultures. (**B**) Quantification of myelination index (ratio of area stained for Caspr+ paranodes to area stained for NFH+ axons). (**C**) Quantification of OPC differentiation (ratio of CC1+ oligodendrocytes to Olig2+ oligodendrocyte lineage cells). (**D**) Representative images of CC1/Olig2 staining. Values shown are mean + standard deviation. ****$P < 0.0001$; unpaired T-test. Scale bars: A = 25 µm, D = 50 µm.

Figure 3: Representative data showing XAV939 promotes remyelination. (**A**) Images showing demyelination by fragmented MBP staining and lack of Caspr paranodes in slice cultures treated

with lysolecithin. (**B**) Increase of remyelination (MBP) and paranodes (Caspr) with 0.1 μM XAV939 treatment following lysolecithin-induced demyelination shown by immunostaining of slice cultures. (**C**) Quantification of myelination index (ratio of area stained for Caspr+ paranodes to area stained for NFH+ axons). Values shown are mean + standard deviation. ****P < 0.0001; unpaired T-test. Scale bars = 25 μm.

Table 1: List of components and protocol to make slice culture medium (SCM), dissection medium (DM), and lysolecithin solution.

DISCUSSION:

This protocol describes an organotypic cerebellar slice culture model that recapitulates in vivo cellular composition with the simplicity of an in vitro model. This protocol could be further developed to become a more representative model of human pathologies. CSC can be potentially developed as models for disease-specific injuries, such as force-induced tissue injury, injury induced by myelin-specific antibodies, or oligodendrocyte injury in MS with the addition of peripheral immune cells. This protocol can also be optimized for tissue dissected from other parts of the brain and spinal cord[16,17]. While this protocol focuses on the histological quantification of myelination, CSC represent a convenient model for examining various experimental endpoints. These cultures can be used to characterize different cell types via single cell RNA sequencing, assessing the ultrastructure of axons and myelin sheaths via electron microscopy, and studying OPC dynamics by conducting time lapse imaging. Immunostaining can also be bypassed with the use of transgenic mouse reporter lines[18,19]. Finally, different ages of mice pups, time in culture, or thickness of sections may be used to adapt CSC to model different disease mechanisms[10,17-20,23].

Slice cultures are ideal for testing and quantifying factors affecting demyelination and remyelination but are limited in their representation of the CNS in certain contexts. Derived from P 0-2 brains, CSC come from brains that are early in development, and have limited similarities to an aging or neurodegenerative brain. Given an increasing awareness of the importance of myelin in CNS disorders such as Alzheimer's Disease[21] and schizophrenia[22], adult or aging models of quantifying and characterizing myelination are required. Other protocols have been published isolating tissue from older rodents, although still during the developmental stages[10,18,19,23]. In addition, there may be intrinsic and extrinsic differences in oligodendrocytes and OPCs in the cerebellum compared to the spinal cord and other white matter tracts in the brain. CSC are also less suitable than cell-based in vitro systems for large-scale CRISPR or small molecule screens. Due to the initial tissue slicing, there is also an activation of brain-resident innate immune cells (i.e., microglia and astrocytes), an important caveat to the system[24]. Finally, peripheral immune cells play a large role in MS pathology[25]; CSC are devoid of peripheral cells unless these cells are exogenously added, and thus are not an ideal model of the inflammatory CNS milieu.

The dissection portion of this protocol is perhaps the most critical. Dissection steps must be undertaken with the utmost care, so as not to damage the tissue and underlying structure. Proper alignment of the brain along the tissue slicer blade ensures minimal damage to the tissue and cells. In addition, careful dissection and separation of slices, as well as their transfer to the

membranes for culture are important. The timeframes outlined in this protocol have been optimized for the described research but may need to be adjusted for optimal results in different studies. This protocol will be useful for those that would like to study developmental myelination and myelin repair.

Of note, there is no cure for MS. While current therapies are quite effective at dampening the adaptive immune system, no current therapy can halt progression. A failure of remyelination and subsequent neurodegeneration is thought to underlie the progression of MS[2]. The presence of OPCs in chronic MS lesions suggests that the failure of myelin repair might be due to an arrest in OPC differentiation. The use of CSC opens an avenue of discovery for myelin repair therapies that may help to reverse MS progression and restore function. Myelin repair therapy can also aid recovery in patients with spinal cord injury, where demyelinated tracts inhibit locomotor function[26]. Therefore, the significance of CSC lies in its suitability for identifying factors that affect mammalian demyelination and remyelination in a relatively high throughput manner compared to in vivo animal models. While many studies have used primary OPCs for screening compounds affecting differentiation, generating and isolating primary OPCs requires laborious and sequential immunopanning[27]. In addition, cell-based assays do not recapitulate the diversity and interaction of cell types present in vivo. Generating CSC from mice pups is a fast and cost-effective model for studying demyelination and remyelination that does not require expensive equipment or consumables. Cerebellar slice cultures thus represent an invaluable quantitative model for recapitulating myelination in vitro and enable drug discovery and basic science research.

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DISCLOSURES:

The authors are employees of Genentech, Inc., a member of the Roche Group.

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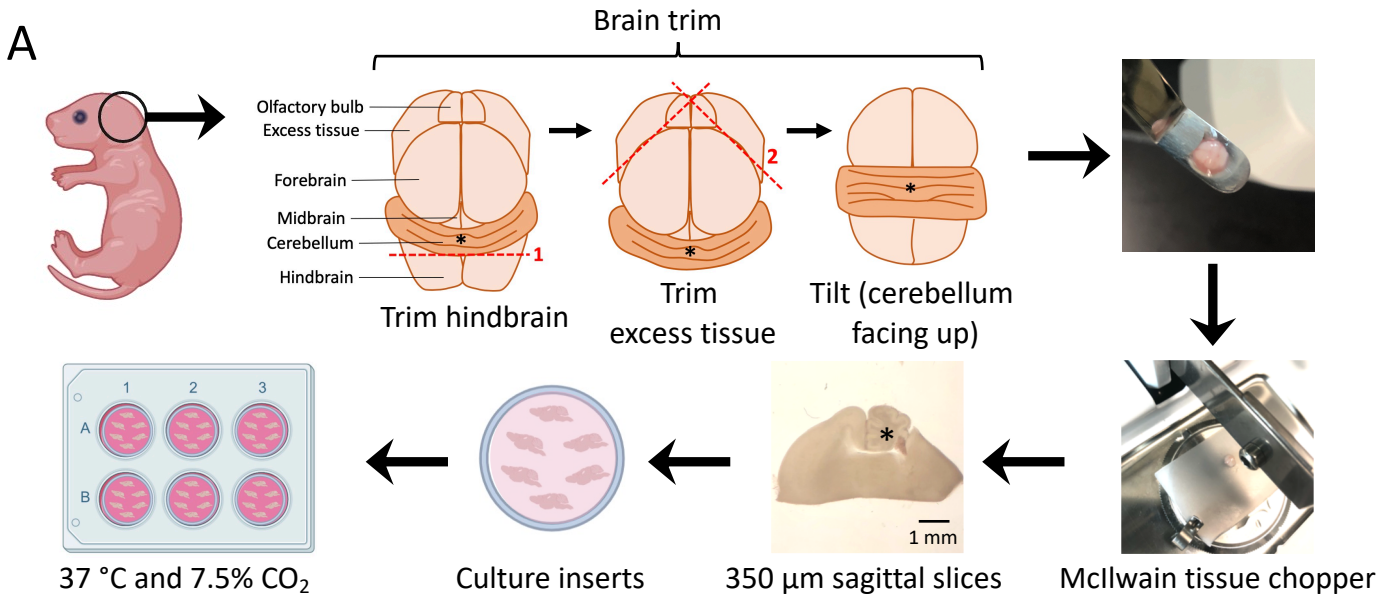
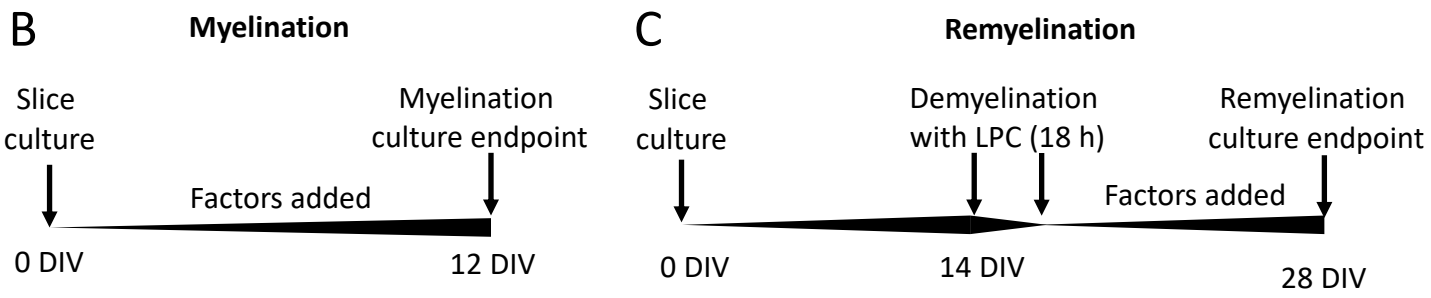
Figure 1





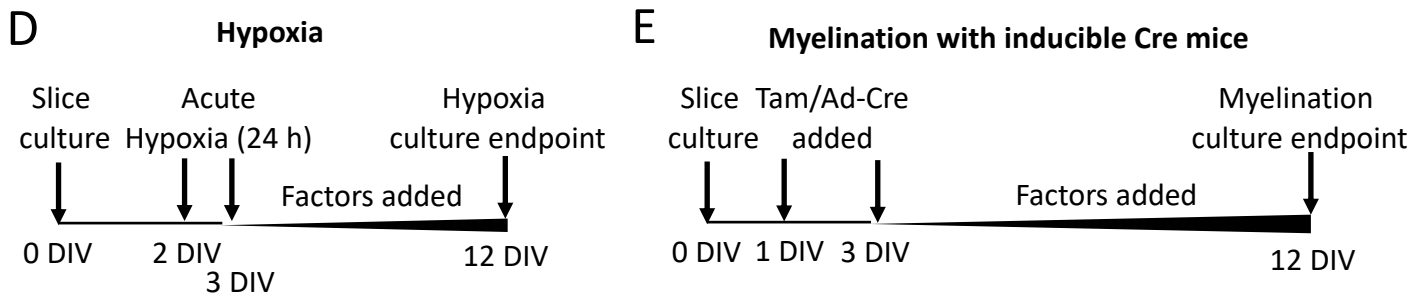


Figure 2

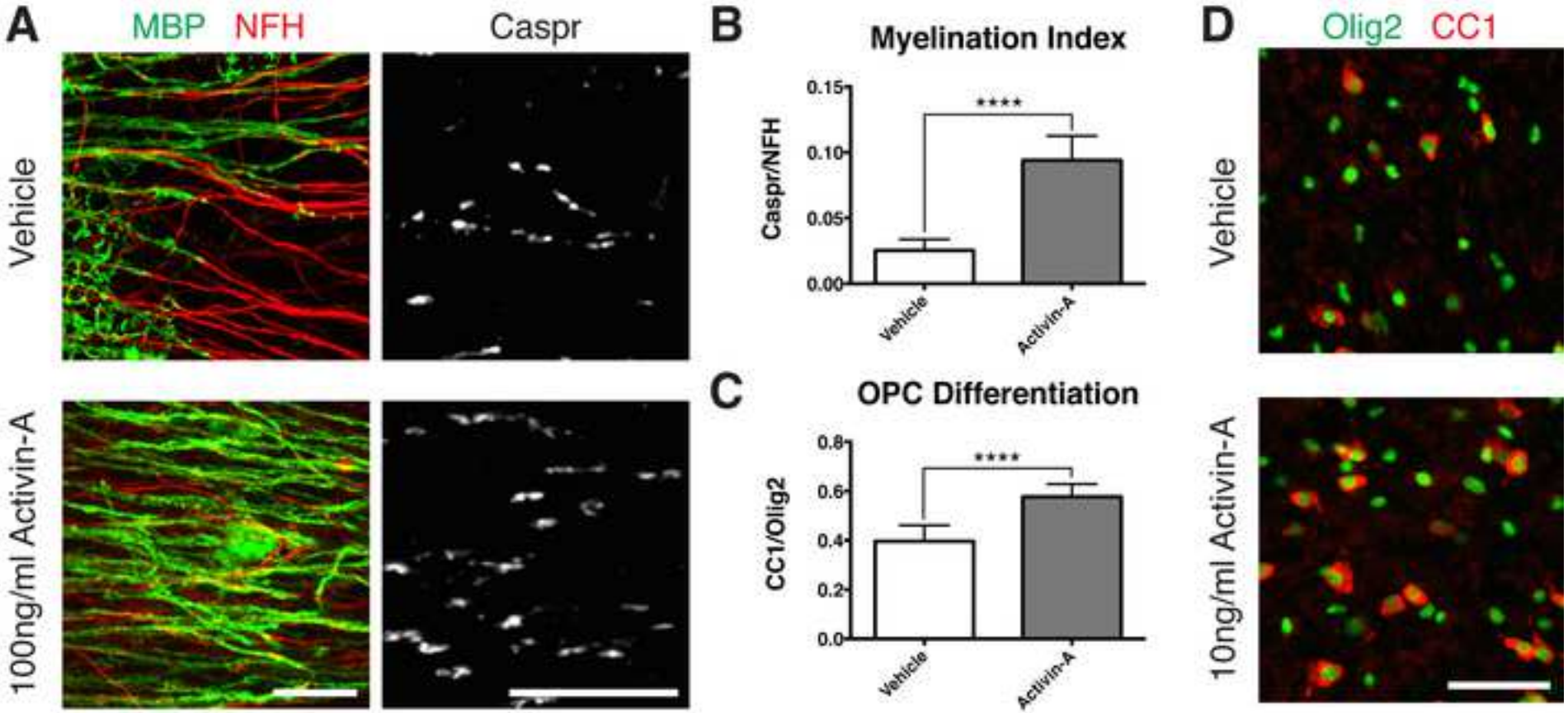
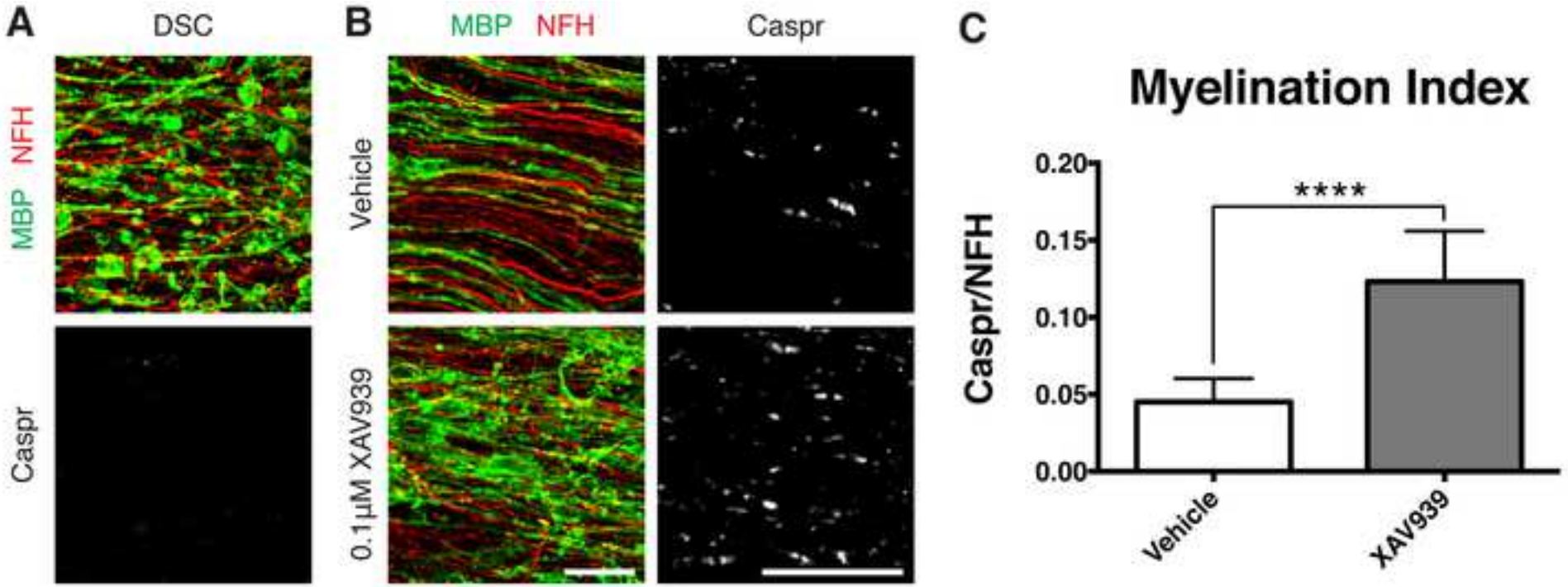


Figure 3



Slice culture media (SCM)

Volume	Reagent
100 mL	Minimum Essential Media (MEM), HEPES, no glutamine
50 mL	Heat-inactivated horse serum
50 mL	Earle's balanced salt solution
2 mL	Penicillin-streptomycin 10,000 U/mL
2 mL	GlutaMAX supplement
2888 µL	45% glucose solution
1 µL	Fungizone

Sterile filter through a 0.22 µm filter and store in 4 °C fridge for up to 2 weeks.

Dissection media (DM)

Volume	Reagent
100 mL	MEM, HEPES, with Earle's salts
1 mL	Penicillin-streptomycin 10,000 U/mL

Sterile filter through a 0.22 µm filter and store in 4 °C fridge for up to 2 months.

Lysolecithin stock (125 mg/mL)

Amount	Reagent
100 mg	Lysolecithin
0.8 mL	Sterile PBS

Dissolve 100 mg of lysolecithin in 0.8 mL of sterile PBS. Store 80 µL aliquots of stock solution at -20
Before use, thaw 80 µL aliquot and dissolve in 20 mL of SCM (0.5% lysolecithin in SCM), warmed in

°C.
37 °C, 7.5% CO2 incubator. If needed, vortex to dissolve lysolecithin into SCM.

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number
#5/45 Forceps	Fine Science Tools	11251-35
32% Paraformaldehyde	Electron Microscopy Sciences	50-980-495
45% glucose solution	Sigma	G8769
6-well tissue culture plates	Corning	3516
Alexa Fluor Goat anti-chicken 647	Thermo Fisher Scientific	A21449
Alexa Fluor Goat anti-mouse 647	Thermo Fisher Scientific	A21236
Alexa Fluor Goat anti-rabbit 555	Thermo Fisher Scientific	A11006
Alexa Fluor Goat anti-rat 488	Thermo Fisher Scientific	A21428
Blades, Double-edge, PTFE coated Stainless steel	Ted Pella	121-6
Bovine Serum Albumin (BSA)	Sigma	A9418
Chicken anti-NFH antibody	Encor Biotech	CPCA-NF-H
Confocal Microscope	Zeiss	LSM780
Corning 250 mL vacuum filter/storage bottle system, 0.22 µm pore	Corning	431096
Earl's balanced salt solution	Sigma	E2888
Feather surgical blade	Feather	2976#11
Fungizone	Thermo Fisher Scientific	15290-18
GlutaMAX supplement	Thermo Fisher Scientific	35050-061
Heat-inactivated horse serum	Thermo Fisher Scientific	26050-88
Lysolecithin	Sigma	L4129
Lysophosphatidylcholine from egg yolk	Sigma	L4129
McIlwain Tissue Chopper	Ted Pella	10180
MEM, Hepes, no glutamine	Thermo Fisher Scientific	12360-038
MEM, Hepes, with Earle's salts	Sigma	M7278
Metal spatula	Fisher Scientific	470149-442
Millicell Culture Plate Insert 30mm Organotypic PTFI	Fisher	PICM0RG50
Mouse anti-CC1 antibody	Millipore	OP80-100UG
Noyes spring scissors	Fine Science Tools	15012-12
PBS pH 7.4	Gibco	10010023
Penicillin-streptomycin 10,000U/mL	Thermo Fisher Scientific	15140-122
Rabbit anti-Caspr antibody	Abcam	ab34151

Rabbit anti-Olig2 antibody	Millipore	AB9610
Rat anti-MBP antibody	Serotec	MCA409S
Scalpel handle #3	Fine Science Tools	10003-12
Silicone rubber foam sheet	MSC Industrial Direct Co	31939176
Stereo Microscope	Olympus Life Sciences	SZ61
Student surgical scissors	Fine Science Tools	91401-12

Comments/Description
Dissection tools
Fixative for staining
Slice culture media component
Plates for cultures
Secondary staining antibody
Secondary staining antibody
Secondary staining antibody
Secondary staining antibody
Chopping blades
Blocking solution component
Staining antibody
Confocal for imaging
For media preparation
Slice culture media component
Dissection tools
Dissection media component
Slice culture media component
Slice culture media component
To induce demyelination
Tissue chopper
Slice culture media component
Dissection media component
Dissection tools - bend flat end at angle for use
Culture insert
Staining antibody
Dissection tools
For dilution of fixative
Slice culture media component
Staining antibody

Staining antibody
Staining antibody
Dissection tools
Cutting base
Microscope for dissection
Dissection tools

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.

SPRINGER NATURE

Axin2 as regulatory and therapeutic target in newborn brain injury and remyelination

Author: Stephen P J Fancy et al
Publication: Nature Neuroscience
Publisher: Springer Nature
Date: Jun 26, 2011

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Number of figures/tables/illustrations	1
Format	electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Title of new article	Ex vivo myelination and remyelination in cerebellar slice cultures as a quantitative model for developmental and disease-relevant manipulations
Lead author	Tracy Yuen
Title of targeted journal	JoVE
Publisher	JoVE
Expected publication date	May 2020
Portions	Figure 2A (hypoxia slice culture timeline)
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