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Preparation and immunostaining of myelinating organotypic cerebellar slice cultures --Manuscript Draft--

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Dear Editor,

You will find attached our manuscript entitled « Myelin immunostaining on organotypic cultures of cerebellar slices», by Thetiot et al., that we wish to submit to Jove following your invitation.

In the nervous system, myelin is a complex membrane structure generated by myelinating glial cells, which ensheaths axons and facilitate fast electrical conduction. Myelin alteration has been shown to occur in various neurological diseases, where it is associated with functional deficits. In order to study the myelination process, we use an *ex vivo* model corresponding to organotypic cultures of mouse cerebellar slices, which can be kept for multiple weeks in culture and further be stained to visualize myelin.

Here, we describe how to detect myelin by immunofluorescence using antibodies directed against epitopes specific to major myelin proteins. We believe that this protocol might help improve the standard of neuroscience research, reduce publication bias and improve reproducibility.

Yours sincerely

Anne Desmazières

TITLE:

Preparation and Immunostaining of Myelinating Organotypic Cerebellar Slice Cultures

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KEYWORDS:

Organotypic cultures, immunostaining, myelin, central nervous system, cerebellum, repair

SUMMARY:

Here we present a method to prepare organotypic slice cultures from mouse cerebellum and myelin sheath staining by immunohistochemistry suitable for investigating mechanisms of myelination and remyelination in the central nervous system.

ABSTRACT:

In the nervous system, myelin is a complex membrane structure generated by myelinating glial cells, which ensheathes axons and facilitates fast electrical conduction. Myelin alteration has been shown to occur in various neurological diseases, where it is associated with functional deficits. Here, we provide a detailed description of an *ex vivo* model consisting of mouse organotypic cerebellar slices, which can be maintained in culture for several weeks and further be labeled to visualize myelin.

INTRODUCTION:

Neurons are highly polarized cells, which comprise a somato-dendritic compartment that receives inputs from its environment and an axon that ensures the generation and propagation of electrical impulses to other cells. Rapid propagation and timely delivery of information is essential for the proper functioning of the nervous system. In vertebrates, it is facilitated by myelination, which allows increasing axonal conduction velocity¹. Myelin is a specialized structure formed by compacted layers of plasma membrane generated by the myelinating glia, namely oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS). Both in the CNS and the PNS, axoglial interactions drive the formation of

specialized axonal domains: the nodes of Ranvier and their surrounding domains, the paranodes, and juxtaparanodes². The axonal segments insulated by myelin, or internodes, alternate with the nodes of Ranvier, which correspond to small unmyelinated domains enriched in voltage-gated sodium channels (Nav). The high concentration and rapid activation of Nav channels at the nodes of Ranvier allow the regeneration of action potentials, and together with the insulating properties of the myelin sheaths, ensure the efficient and fast saltatory conduction of the nerve impulse along the axon³.

In addition to its role in accelerating the conduction velocity of the nerve impulse, myelinating glia provides metabolic support to the axon, preserving its long-term integrity and participating in its survival^{4,5}. Furthermore, it has become clear in recent years that myelin is dynamically modulated throughout life, thus presumably participating in the regulation and plasticity of various nervous system functions. Adjustments of the distribution, number, length, and thickness of myelin sheaths along axons might thus represent a novel way to finely tune various networks⁶⁻⁸. Therefore, the evolutionary acquisition of myelin is a key process for sensory, motor and cognitive functions and the perturbation of the interaction between axons and glia is increasingly considered as contributing to the developmental or acquired neurological diseases⁹.

Myelin composition has been characterized, with the specific feature of a high proportion of lipids (70%) compared to proteins (30%) in contrast to other cellular membranes¹⁰. However, unlike myelin lipids, most of myelin proteins are specific to myelin, including myelin basic protein (MBP), proteolipid protein (PLP), 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), myelin-associated glycoprotein (MAG), myelin-oligodendrocyte glycoprotein (MOG), PMP-22 and P0¹⁰. Various histological methods to stain myelin exist based on its lipid composition, such as Luxol fast blue¹¹, Sudan Black B¹², Baker's acid hematin method¹³, as well as silver staining¹⁴. Nevertheless, these approaches do not always allow for an adequate contrast and resolution to visualize individual fibers. An alternative approach to detect myelin is through immunohistochemistry directed against myelin proteins. Various antibodies target myelin-specific antigens with a high specificity and can be used routinely to detect myelinated structures. The antibody-antigen interaction can be further revealed using a secondary antibody coupled to a fluorophore directed against the primary antibody and visualized with adequate fluorescence microscopy. Here, we describe an immunochemical protocol to stain myelin on *ex vivo* cerebellar slices, a model which allows for a good preservation of the nervous tissue architecture. In addition, the organization and size of the Purkinje cells (the sole myelinated neuron of the cerebellum) make them a classical model for electrophysiological studies and they are similarly ideal to perform fixed or live-imaging studies.

The cerebellar slices are generated from P9-P10 mice, a time corresponding to the early onset of Purkinje cell myelination, a process that is mostly achieved by one week *ex vivo* (6-7 days *in vitro*, DIV)¹⁵. Furthermore, this model is adapted to investigate demyelinating disorders such as multiple sclerosis (MS), as an extensive demyelination can be induced in cerebellar slices using the myelinotoxic compound lysophosphatidylcholine (or lysolecithin, LPC), which is followed by a spontaneous remyelination^{16,17}. Endogenous remyelination takes place from two days after LPC removal from the culture medium and is almost complete a week post treatment.

The completion of this protocol takes approximatively 3 weeks, including half a day for cerebellar slice cultures preparation, a week to obtain fully myelinated slices, followed by 2 days to reach the peak of demyelination and another week for their full remyelination. In addition, immunohistochemistry can be completed in 2 days. The protocol described here is adapted to a standard litter of 6 mice pups and needs to be adapted regarding the number of animals used for the planned experiment.

PROTOCOL:

All work involving animals complied with institutional policies and guidelines established by the UPMC, INSERM and the French and European Community Council Directive 86/609/EEC.

1. Preparation of culture medium and culture inserts (hands-on time ≈ 10-15 min)

NOTE: Perform this step in a flow culture hood under sterile conditions

1.1. Prepare 40 mL of culture medium, which consists of 50% BME, 25% Hank's Balanced Salt Solution (1x), 25% heat-inactivated horse serum, supplemented with 2 mM glutamine derivative, 100 IU/mL penicillin-streptomycin and 4.5 mg/mL D-Glucose. Adjust the pH to 7.4.

1.2. Filter-sterilize the medium through a 0.22 µm filter adapted on a 50 mL plastic syringe.

NOTE: Culture medium can be stored at 4 °C for at least a week.

1.3. For a standard litter of 6 pups, use two sterile 6-well plates. For each plate, remove the cover and add 1 mL of culture medium per well.

1.4. Place one culture insert into each individual well by holding the plastic edge with sterile forceps. Put the cover back on the plate.

NOTE: The cerebellar slices collected from one pup (6-8) can be dispatched on two membranes (3-4 cerebellar slices per membrane insert).

2. Preparation of dissection medium (hands-on time ≈ 5 min)

NOTE: Perform this step in a flow culture hood under sterile conditions

2.1. Prepare 100 mL of dissection medium, which consists of Gey's Balanced Salt Solution supplemented with 4.5 mg/mL D-Glucose and 1x penicillin-streptomycin (100 IU/mL each).

2.2. Filter-sterilize the medium through a 0.22 µm filter unit.

NOTE: The dissection medium can be stored at 4 °C for at least a month.

2.3. Add 5 mL of cold dissection medium into 60-mm cell culture dishes. Prepare one 60-mm cell culture dish for each pup to be dissected.

2.4. Keep the dissection dishes on ice until use.

3. Preparation of dissection material (hands-on time \approx 15 min)

3.1. Clean the bench with 100% ethanol.

3.2. Sterilize all dissection tools, a razor blade and the tissue chopper's plastic platform with 100% ethanol.

3.3. Fix the razor blade and the plastic platform on the tissue chopper and adjust the cutting thickness to 300 μ m.

3.4. Make sure all the ethanol has evaporated before starting the dissection.

4. Cerebellum dissection and sections preparation (hands-on time \approx 15-20 min per animal)

4.1. Place one of the 60-mm cell culture dishes containing the ice-cold dissection medium under the binocular microscope and remove the top plate.

4.2. Swab the head fur with 70% ethanol (carefully avoiding touching the eyes of the animal) and decapitate the animal using large sharp scissors, according to the approved procedure.

NOTE: The cerebellar slices are generated from P9-P10 mice without any specific sex or strain bias.

4.3. Use small scissors to cut the skin along the midline of the head starting from the neck and going up to the mouse muzzle.

4.4. To hold the head and expose the skull, fold back the skin ventrally under the head, and pinch the two folds of skin between one's fingers.

4.5. Insert the small scissors gently into the foramen magnum and cut the skull by making one lateral incision towards the side and then cut all around the head skull. Keep the tip of the scissors as parallel and close to the skull as possible while cutting, to avoid damaging the brain tissue.

4.6. Retrieve the dorsal part of the skull using fine-straight forceps. While carefully lifting the dorsal part of the skull, cut the adhering meninges (translucent irrigated membrane surrounding the brain tissue) if needed with small scissors to avoid damaging the brain tissue.

4.7. Carefully introduce fine-straight forceps (or alternatively use a spatula) between the ventral skull and the brain, gently flip out the brain and cut the optic and trigeminal nerves with small scissors.

4.8. Help the brain to drop by gravity into the 60-mm cell culture dish containing ice-cold dissection medium by turning the head upside down just above the dish and release the last adhesions with small scissors if needed.

4.9. Using fine forceps, orientate the brain with the dorsal side facing the researcher, and the ventral side lying on the bottom of the dish.

4.10. Under the binocular microscope, use the fine-straight forceps to immobilize the brain on the forebrain side and avoid touching the hindbrain, as it could get damaged. Separate the hindbrain from the rest of the brain (fore- and midbrain, see schematic on **Figure 1Aa**), using fine-straight forceps. To separate the cerebellum from the rest of the hindbrain, use the fine forceps to cut the cerebellar peduncles underneath the cerebellum (see schematic on **Figure 1Aa'**).

4.11. Once the cerebellum is isolated, carefully tear away the meninges using fine-straight forceps (see schematic on **Figure 1Aa'**).

4.12. Hold the cerebellum gently with the fine curved forceps and place it with the dorsal side up onto the plastic platform perpendicularly to the chopper razor blade (see schematic on **Figure 1Bb**).

4.13. Aspirate any excess of dissection medium around the cerebellum using a sterile thin-end pipette tip adapted on a 1 mL pipette (see schematic on **Figure 1Bb**).

4.14. Ensure that the cerebellum lays flat, but is not stretched, once placed onto the platform to get optimal sagittal slices during sectioning (see schematic on **Figure 1Bb**).

4.15. Slice 300 μ m-thick sagittal sections of the cerebellum with the tissue chopper (see schematic on **Figure 1Bb'**). The force and speed of the blade have to be optimized on site.

4.16. Gently add a drop of dissection medium onto the sliced cerebellum. Using a wide-bore pipette tip placed onto a 1 mL pipette, slowly aspirate the sliced cerebellum and transfer it back into the 60-mm cell culture dish containing ice-cold dissection medium (see schematic on **Figure 1Bb''**).

4.17. Clean the tissue chopper's plastic platform with 100% ethanol between each cerebellum slicing. Let the ethanol evaporate before placing the next cerebellum onto the platform.

4.18. Use two fine-straight forceps (or alternatively titanium needles) to separate individual slices and select 6-8 slices from the vermis (see schematic on **Figure 1Cc** and **1Cc'**).

4.19. Take a 6-well plate with culture inserts and transfer up to 4 selected slices onto one culture insert along with some dissection medium, using a wide-bore pipette tip adapted on a 1 mL pipette (see schematic on **Figure 1Cc'**).

4.20. Place the slices in the middle of the culture insert using the dissection medium or forceps to push and pull them gently into the right location, avoiding them to be in contact with each other (see schematic on **Figure 1Cc'**).

4.21. Remove any excess of dissection medium around the slices using a thin-end pipette tip. Ensure that the slices lay flat on the membrane (see schematic on **Figure 1Cc'**).

4.22. Place the 6-well plate into the incubator immediately after having added the slices on the membrane.

5. Slices culture and demyelination (hands-on time ≈ 15-20 min)

NOTE: Perform this step in a flow culture hood under sterile conditions

5.1. Replace the culture medium with 1 mL per well of pre-warmed and buffered fresh culture medium every 3 days after slice preparation.

5.2. For demyelination, remove all culture medium below the culture inserts after 6 days *in vitro* (DIV) and replace it with 1 mL per well of pre-warmed fresh culture medium containing 0.5 mg/mL LPC. Incubate for 15-17 h at 37 °C, 5% CO₂.

5.3. After incubation, wash the inserts by placing them in a 25-mm petri dish containing 1 mL of pre-warmed culture medium.

NOTE: The inserts should be in contact with the medium, but not be covered by it.

5.4. Immediately transfer the culture inserts in a new 6-well plate containing fresh pre-warmed culture medium.

5.5. Replace the culture medium with 1 mL of pre-warmed fresh culture medium every 2-3 days until slices fixation.

6. Immunohistochemistry (hands-on time ≈ 1:30 – 2 h)

NOTE: Carry out the steps 6.1. to 6.3. under a fume hood. Avoid exposition to paraformaldehyde (PFA) and refer to the product safety datasheet for adequate manipulation and protection.

6.1. Use forceps to lift each culture inserts by holding the plastic edge and remove the culture medium beneath the membrane inserts from each well.

264
265 6.2. Fix the cerebellar slices for 30 min by adding 2 mL of 4% PFA in 1x PBS pH 7.4 above the
266 membrane inserts.

267
268 NOTE: The time point of fixation depends on the stage of myelination studied: 4 DIV corresponds
269 to the onset of myelination, 7 DIV to fully myelinated slices. In case of LPC treatment, 9 DIV
270 corresponds to the peak of demyelination, 11 DIV to the onset of remyelination and 14 DIV to
271 fully remyelinated slices.

272
273 6.3. Wash the inserts with the slices three times with 2 mL 1x PBS for 10 min.

274
275 6.4. Separate the slices from the membrane inserts under the binocular microscope using a
276 25x to 30x magnification, by gently pushing them using a scalpel or a brush. Alternatively, to
277 avoid the risk of damaging the slices while detaching them from the membrane, use a scalpel to
278 cut the membrane with the slices still attached.

279
280 6.5. Transfer the floating slices into the wells of a 4-well plate containing 1x PBS, using a brush.

281
282 6.6. Aspirate the PBS from each well and incubate the slices in pre-cooled 100% ethanol at -
283 20 °C for 15 to 20 min. This step facilitates antibody penetration in myelinated fibers.

284
285 6.7. Aspirate the 100% ethanol and wash once briefly with 1x PBS, then twice in 1x PBS for 10
286 min at room temperature.

287
288 6.8. Aspirate the PBS and incubate the slices for 30 to 45 min with a solution containing 1x
289 PBS, 5% NGS, 0.3% non-ionic detergent at room temperature to block non-specific antibody
290 fixation sites.

291
292 6.9. Aspirate the blocking solution. No washing is necessary.

293
294 6.10. Incubate the slices overnight with the primary antibodies diluted in blocking solution at 4
295 °C. To visualize the myelin on cerebellar organotypic slices, use MBP (Chicken, 1/150 or Mouse
296 IGg2b, Smi99, 1/200) or PLP (Rat, hybridoma, 1/5 to 1/10) antibodies.

297
298 NOTE: To reveal the expression of more than one antigen in the same slice, perform a double- or
299 triple staining procedure (see **Figure 1** as an example). To perform the incubation simultaneously,
300 ensure primary antibodies are produced in different species. Then, use their corresponding
301 secondary antibodies coupled to different fluorophores (see **Table of Materials** for nodal and
302 paranodal marker¹⁸ antibodies).

303
304 6.11. On the second day, wash the slices three times in 1x PBS for 10 min.

305
306 6.12. Incubate for 3h with the secondary antibodies diluted in blocking solution (1/500 dilution)
307 in the dark at room temperature.

NOTE: Optimization of antibody dilution and incubation time might be required when using other antibodies than the ones described here.

6.13. Wash the slices three times in 1x PBS for 10 min in the dark.

6.14. Under a binocular microscope, mount the slices onto a glass slide. Place 100 μ L of 1x PBS on the slide and transfer the slices into the PBS using a brush. Unfold the slices if needed and flatten them on the slide. Remove any excess of PBS. In case the immunolabeling is performed with the slices still attached to membranes, mount with the slices facing the coverslip, with the membrane on the glass slide.

6.15. Place a drop of mounting medium directly onto the glass coverslip and gently cover the slices. Mounted slides can be stored for several months at 4 °C in the dark.

REPRESENTATIVE RESULTS:

Examples of representative myelin immunostainings in organotypic cerebellar slices obtained from P9-P10 C57black6 wild-type (WT) (**Figure 2A**), as well as PLP-GFP transgenic mice (**Figure 2B**), together with Purkinje cells staining. Cerebellar slices myelinate from the white matter tracks region of the slices towards the periphery of the folia and myelination of the Purkinje cells is mostly achieved after 6 to 7 DIV. At 7 DIV, the induction of a full demyelination is possible through LPC treatment (**Figure 2Ci-ii**). Following demyelination, the slices spontaneously remyelinate and are fully remyelinated 6-7 days after the peak of demyelination (**Figure 2Ciii**).

Figure 1: Illustration of cerebellum slices generation. The dissection is divided in three main steps. (A) The cerebellum is isolated and the meninges removed (steps 4.10 and 4.11). (B) The cerebellum is then transferred to the chopper platform and sliced (steps 4.12 to 4.16). (C) Lastly, the slices are isolated from the vermis and placed on a membrane insert. The dissection medium transferred with the slices is removed and the 6-well plate is placed into the incubator (steps 4.18 and 4.22).

Figure 2: Example of myelinated cerebellar slices cultured *ex vivo*. Image stacks (with orthogonal projection) were obtained using an upright confocal microscope following free floating immunolabeling and flat mounting as described in the protocol. The slices are robustly myelinated at 11 DIV (A, PLP; B, GFP in green) and the axonal domains are assembled as observed *in vivo* with nodes of Ranvier enriched in voltage-gated sodium channels (Nav, in red) flanked by the paranodal axoglial junction domain (Caspr, in white) in C57black6 WT (A) as well as PLP-GFP (B) transgenic mice slices. (C) The cerebellar cortex architecture is preserved in the cultured slices, as observed on cerebellar slices from PLP-GFP mice. (i) Purkinje cells axon (Calbindin, Calb, in blue) are robustly myelinated (GFP, in green) after one week in culture. (ii) LPC treatment fully demyelinate the slices, which remyelinate spontaneously and are fully remyelinated 6 days post demyelination (iii, 14 DIV). Scale bars, A, B: 20 μ m; C: 100 μ m.

DISCUSSION:

Here, we detail a protocol to generate an *ex vivo* model corresponding to the mouse cerebellar organotypic slice cultures, adapted from previously published methods^{15,16,19} and the subsequent myelin immunostaining of these preparations. This strategy offers the possibility to visualize myelin components with a high-resolution in both healthy and pathological states.

Cerebellar organotypic slice cultures taken from 10-day-old mice are a well-established experimental model to investigate molecular and cellular mechanisms underlying myelination and remyelination processes, as it allows to reproduce most anatomical and functional features of the corresponding tissue *in vivo* including not only the preservation of a well-defined cellular architecture but also the maturation timeline¹⁷. Furthermore, cerebellar slices have a preserved architectural organization after two weeks *in vitro* (**Figure 1C**). The transgenic PLP-GFP strain is an alternative, allowing, in particular, to observe the myelination status under a fluorescent binocular microscope on live slices, which is particularly convenient for demyelination-remyelination studies (**Figure 1C**).

These preparations are of particular interest as a fast approach to assess myelination rate with the developed automated quantification, including western-blot analysis²⁰, CNPase assay¹⁶ or quantification of PLP/Calbindin (or MBP/Calbindin) staining^{17,21}, thus representing a high-throughput system allowing for the testing of pharmacological drugs in demyelinating disorders^{22,23}.

One of the main critical issues when performing these experiments might be related to the quality of the cerebellar slices. Firstly, the age of mice is of importance for cerebellar organotypic slice cultures. The survival of Purkinje cells, the only myelinated neuron in cerebellar cultures, is compromised if cultures are prepared from P2 to P7 rodents or with donors older than P13¹⁷. More generally, organotypic brain slice cultures are difficult to obtain from adults animals. Secondly, the duration of tissue processing during dissection is critical, as a dissection step of more than 15-20 minutes per animal leads to a decline in the survival rate of the slices. Furthermore, when selecting the slices to be cultured, it is best to avoid using the ones from either end of the cerebellum due to the poor survival of neurons and resultant scarcity of myelinated fibers.

Other parameters such as the stability of temperature and CO₂ concentration while in culture are determining factors to ensure slice health. Lastly, the quality of the slices is influenced by the composition of the horse serum, which cannot be controlled by the users. Therefore, testing several batches of serum is highly recommended.

To obtain an adequate labeling, tissue fixation is a key step. In order to facilitate the penetration of the antibodies to reach myelin antigens, as myelin is a very compact structure, slices pre-treatment with absolute ethanol is advised following fixation. A similar protocol of staining can further be applied to *in vivo* fixed tissues, though Triton concentration may need to be adapted to the thickness of vibratome or cryostat generated *in vivo* sections. The availability of non-commercial antibodies should also be considered. Preparing slices from transgenic mice such as

PLP-GFP²⁴ or CNP-GFP²⁵ thus represents an alternative to visualize myelin and oligodendrocytes processes.

In addition to the immunochemical methods described in this protocol, other approaches are commonly used to study myelin architecture. Complementary techniques also exist to investigate myelin ultrastructure, with electron microscopy being the gold standard²⁶. Other techniques exist, such as label-free approaches including optical coherence tomography^{27,28}, Raman scattering^{28,29}, third-harmonic generation³⁰ or spectral confocal reflectance microscopy³¹; these approaches have the additional advantage of enabling the observation of dynamic cellular processes. However, some of these techniques are complex and further require state-of-the-art microscopy set-ups. For these reasons, myelin study by immunofluorescence labeling remains a standard approach to investigate myelination and its defects in various developmental or acquired diseases and their models, as well as to assess the potential of future therapeutic treatments. It can further be used to study myelination in multiple contexts, such as learning and plasticity, as well as aging, depression, or autism in which myelin is known to be altered³².

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DISCLOSURES:

None of the authors have competing interests or conflicting interests.

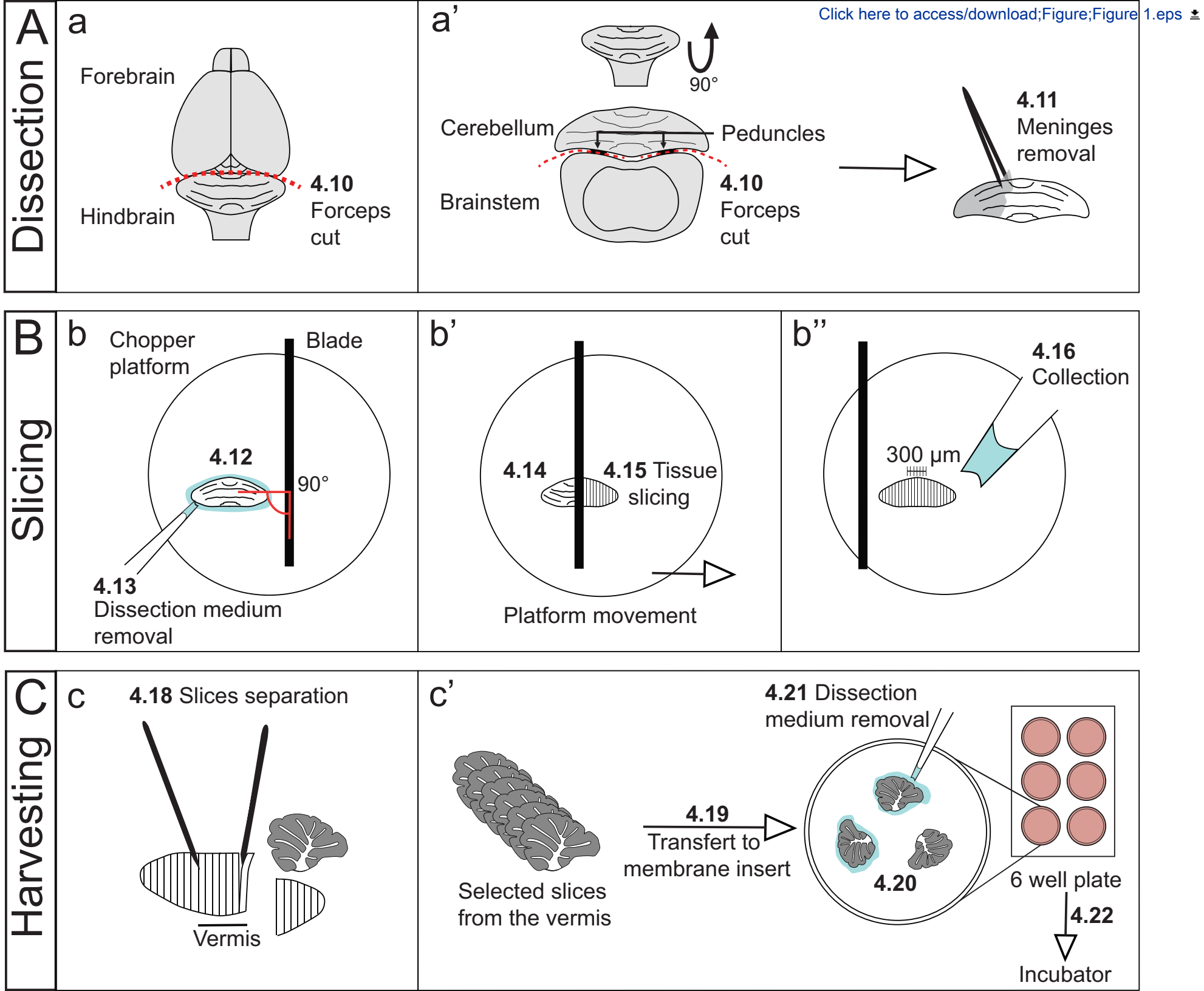
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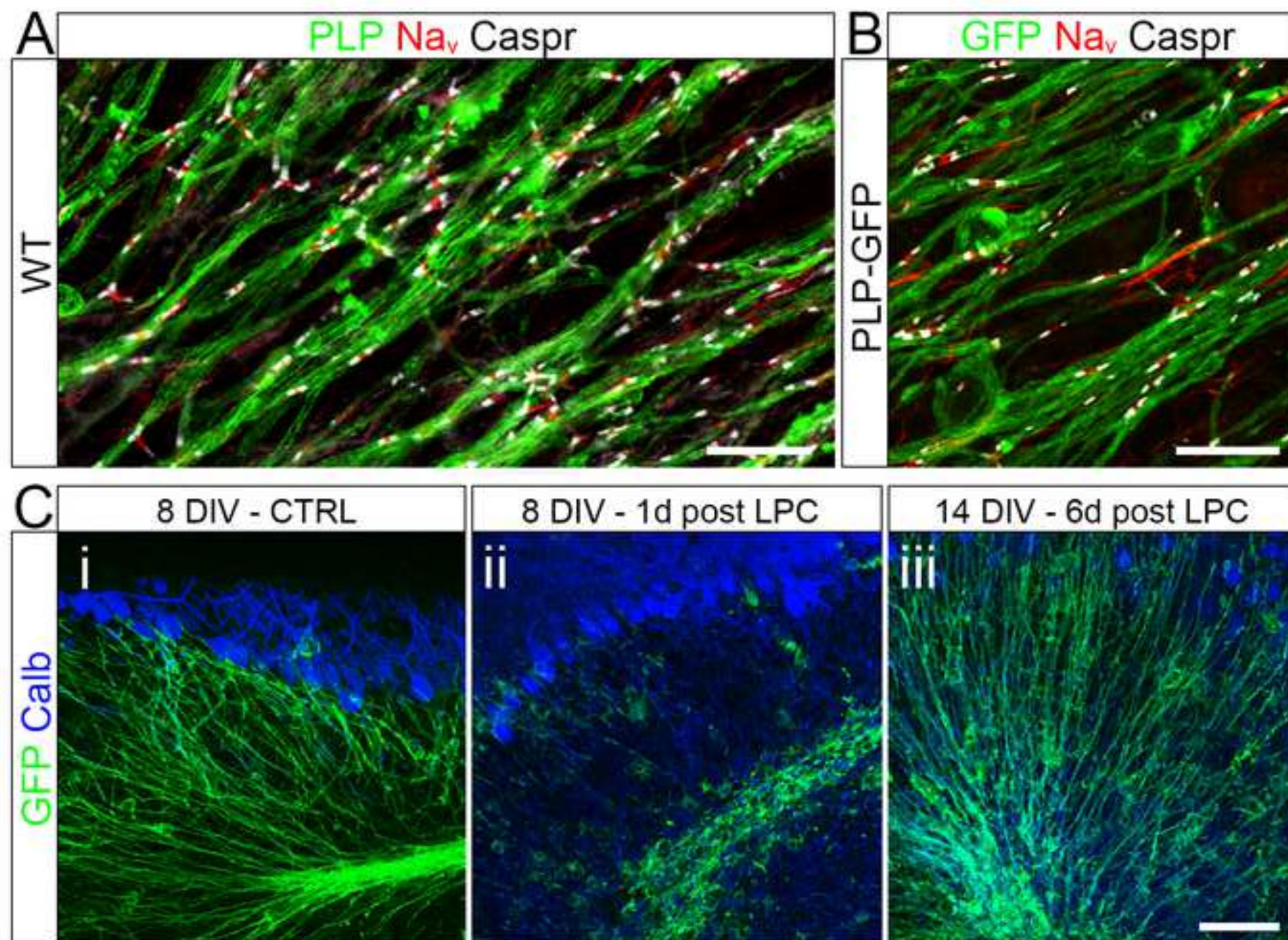
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- 489

Figure 1





MATERIALS

Name	Company	Catalog Number	Comments
BME medium	ThermoFisher Scientific	41010026	
Hank's Balanced Salt Solution (10X HBSS)	ThermoFisher Scientific	14180046	
GlutaMAX (100X)	ThermoFisher Scientific	35050038	
Heat-inactivated Horse Serum	ThermoFisher Scientific	26050088	
Penicillin–Streptomycin (10.000 IU/mL)	ThermoFisher Scientific	15140122	
Gey's Balanced Salt Solution	Sigma Aldrich	G9779-500ML	
D-Glucose Solution (45%)	Sigma Aldrich	G8769-100ML	
Lysophosphatidylcholine (LPC)	Sigma Aldrich	L4129-100MG	
Paraformaldehyde (PFA)	Electron Microscopy Sciences	15714	
Absolute ethanol (100% ethanol)	VWR Chemicals	20821.330	Cooled at -20°C
Triton® X-100	Sigma Aldrich	X100-500ML	
10% Normal Goat Serum (NGS)	ThermoFisher Scientific	500622	
Phosphate Buffer Solution	EuroMEDEX	ET330-A	pH 7.4
Anti-GFP Antibody (Polyclonal, Chicken)	Merck Millipore	06-896	Dilution 1/300
Anti-Myelin Basic Protein (MBP) Antibody (Polyclonal, Chicken)	Merck Millipore	AB9348	Dilution 1/150
Anti-Myelin Basic Protein (MBP) Antibody (Monoclonal, Mouse IgG2b)	Merck Millipore	NE1019	Dilution 1/200
Anti-PLP Antibody (Rat, Hybridoma)	Gift from Dr. K. Ikenaka; Okasaki, Japan		Dilution 1/5 to 1/10
Anti-Sodium Channel, Pan Antibody (Monoclonal, Mouse IgG1, clone K58/35)	Sigma Aldrich	S8809	Dilution 1/150
Anti-Caspr Antibody (Polyclonal, Rabbit)	Abcam	ab34151	Dilution 1/500
Goat Secondary Antibodies conjugated to Alexa Fluor 488, 594, 647 or 405	ThermoFisher Scientific		Dilution 1/500
Fluoromount	SouthernBiotech	0100-20	
Tissue chopper	McIlwain		
Razor blades			
Large scissors	F.S.T	14101-14	
Small scissors	F.S.T	91500-09	
Fine-straight forceps	F.S.T	91150-20	
Curved-fine forceps	F.S.T	11297-00	
Cell culture dishes (60-mm and 100-mm)	TPP		
4-, 6-well culture plates	TPP		
Millicell culture inserts (0,4 µm, 30-mm diameter)	Merck Millipore	PICM0RG50	
Cell culture incubator			37°C, 5% CO2
Fine-end pipette tips	Dutscher	134000CL	
Wide-bore pipette tips	ThermoFisher Scientific	2079G	
Sterile syringe	Terumo Europe		20 or 50 mL
Sterile syringe filters	Terumo Europe		0.22 µm
Scalpel	Swann-Morton	0510	
Brush			
Microscope slides	RS France		76 x 26 x 1.1 mm
Glass coverslips	RS France		22 x 22 mm
Kimtech Sciences Tissue Wipers	Kimberly-Clark Professional	5511	
Binocular microscope			



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Myelin immunostaining on organotypic cultures of cerebellar slices

Author(s):

M. Thetiot, R. Ronzano, M-S. Aigrot, C. Lubetzki, A. Desmazières

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Dear Editor,

We thank you again for your insightful comments. You will find attached the revised version of our manuscript by Thetiot et al., with the modifications made to address the editorial comments detailed below.

Yours sincerely,

Anne Desmazières

Editorial comments:

1. *Lines 81-83: Citation?*

We have added a citation.

2. *Line 93: What kind of animal- mouse/rat?*

We have specified that we generate slices from mice in this protocol however rats may also be used for cerebellar organotypic slices preparation.

3. *Lines 89-94: We cannot have paragraph of texts in the protocol section. So, moved this paragraph here. Please check.*

We validated this modification.

4. *Please highlight 2.75 pages of the protocol including heading and spacings for filming purpose. This should form the most cohesive story. However, steps involving cervical dislocation cannot be filmed.*

We have highlighted approximatively 2.75 pages of the protocol for filming. In case these steps do not seem adequate for filming, we could alternatively cover steps 5 and 6 of the protocol (culture and immunohistochemistry).

5. *We cannot have non-numbered sentences in the protocol section. Converted to a note instead throughout the protocol. Please check.*

We validated this modification.

6. *We cannot have commercial terms in the manuscript. Please move the commercial term to the Table of Materials and use generic term instead.*

We have removed this commercial term from the manuscript and inserted it to the Table of Materials, as suggested. It has been replaced by the generic term “tissue chopper” in the protocol section.

7. *Is there any age, sex, or strain specific bias?*

Bias related to age is discussed lines 377-381. To our knowledge, no sex or strain bias has been specifically described in the case of cerebellar organotypic slice cultures.

8. *Included here from the introduction.*

We validated this modification.

9. *Converted to a note. Please check.*

We validated this modification.

10. *Moved here. Please check.*

We validated this modification.

11. *Wash the inserts with the slices? Please be as specific as you can wrt to the protocol.*

We have now added a sentence regarding this point.

12. *Magnification?*

We have now suggested a magnification (lines 276-277).

13. *Non-specific?*

We have corrected this error.

14. *Citation will help.*

We have added a citation.

15. *Made a different step. Please check.*

We have moved the alternative approach of immunolabeling the slices while still attached to membranes in the step 6.14 and made a common step (6.15) for mounting the slices.

16. *It will be nice to provide result figure for these statements as well.*

Unfortunately, we can't provide a result figure for these statements, as we do not have cerebellar organotypic slices from PLP-GFP mice in culture at this time. In addition, images obtained with a fluorescent binocular microscope while the slices are still cultured on the inserts would be of low resolution and would not be adequate for publication.