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Purkinje cell survival in organotypic cerebellar slice culture

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Dr. Ronald Myers
Science Editor
JoVE

July 15, 2019

Dear Dr. Myers:

I am pleased to submit our revised manuscript “Purkinje cell survival in organotypic cerebellar slice culture” by Jennifer Rakotomamonjy and Alicia Guemez-Gamboa, for publication in the *Journal of Visualized Experiments*.

We appreciate the interest that the editor and reviewers have taken in our manuscript and the constructive criticism they have provided. We have addressed the major concerns of the reviewers. More specifically, we have added 3 figures to illustrate the protocol: the new figure 1 provides a flow-chart of the whole procedure from the dissection to the quantification step on ImageJ. It also provides a diagram showing the time course of the neuroprotective treatment. Figures 2 and 3 provide screenshots of the settings used during the quantification of Purkinje cells in ImageJ to help readers visualize this part of the protocol. We added a fourth paragraph to the manuscript entitled “Imaging and quantification of cell survival”, to add a detailed protocol of the image acquisition, and quantification steps. We also added higher magnification views of stained cerebellar slices to allow readers to clearly see individual Purkinje cells.

We have included a point-by-point response to the reviewers in addition to making the changes described above. Changes to the text in the manuscript are in red font.

We believe that this manuscript is appropriate for publication by JoVE because this method is a powerful tool to model the development, as well neurological disorders, in the cerebellum. It can be extended to other parts of the central nervous system. It is widely used in the neuroscience field as it can mimic *in vivo* conditions more closely than dissociated cell cultures.

This manuscript has not been published and is not under consideration for publication elsewhere. We have no conflicts of interest to disclose.

Thank you for your consideration.

Sincerely,

Jennifer Rakotomamonjy, PhD

TITLE:

Purkinje Cell Survival in Organotypic Cerebellar Slice Cultures

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KEYWORDS:

neuroscience, cerebellum, organotypic, Purkinje cell, development, neuroprotection

SUMMARY:

Organotypic slice cultures are a powerful tool to study neurodevelopmental or degenerative/regenerative processes. Here, we describe a protocol that models the neurodevelopmental death of Purkinje cells in mouse cerebellar slice cultures. This method may benefit research in neuroprotective drug discovery.

ABSTRACT:

Organotypic slice culture is a powerful in vitro model that mimicks in vivo conditions more closely than dissociated primary cell cultures. In early postnatal development, cerebellar Purkinje cells are known to go through a vulnerable period, during which they undergo programmed cell death. Here, we provide a detailed protocol to perform mouse organotypic cerebellar slice culture during this critical time. The slices are further labeled to assess Purkinje cell survival and the efficacy of neuroprotective treatments. This method can be extremely valuable to screen for new neuroactive molecules.

INTRODUCTION:

In vitro modeling is an essential tool in biomedical research. It allows investigators to study and tightly control specific mechanisms in restricted cell types, or in isolated systems/organs. Organotypic slice culture is a widely used in vitro technique, especially in the field of neuroscience[1]. The method was first established by Gähwiler, who cultured brain slices using the roller tube technique[2], and later modified by Yamamoto et al., who introduced the use of a microporous membrane to perform cortical slice cultures[3]. Compared to primary cell cultures, organotypic slice cultures present the advantage of preserving the cytoarchitecture of the tissue, as well as native cell-cell connections in the plane of the tissue section.

Organotypic slices have been cultured from many parts of the central nervous system, such as

the hippocampus[4], cortex[5], striatum[6], cerebellum[4,7], and spinal cord[8,9], among others. They have been proven to be a powerful tool in drug discovery studies[10]. The effects of neuroactive molecules can be assessed in many ways: survival and neurodegeneration using immunostaining and biochemistry assays, neuronal circuit formation, or disruption using electrophysiology and live-imaging.

The goal of this work is to describe a simple method to perform organotypic cerebellar slice culture, which is known to be a relevant model to mimic cerebellar development in vitro. Particularly, we focused on the study of Purkinje cell developmental death. In vivo, Purkinje cells undergo apoptosis during the first postnatal week, peaking at postnatal day 3 (P3)[11]. The same pattern is observed in cerebellar slice culture, with Purkinje neurons dying by apoptosis when cerebella are taken from animals between P1 and P8, with a peak at P3[4,12]. The use of the organotypic cerebellar slice cultures has allowed to identify several neuroprotective molecules[7,13], as well as understanding part of the mechanisms involved in this programmed cell death[14-16]. Here, we describe a protocol based upon the study of Stoppini et al.[17] in hippocampus, and adapted to cerebellum by Dusart et al.[4] It includes rapid dissection and chopping of postnatal cerebella; slicing culture onto a cell culture insert containing a microporous membrane, with or without neuroprotective treatment; and immunofluorescence staining to assess neuronal survival.

PROTOCOL:

All experiments involving animals were performed in accordance with Northwestern University Animal Studies committee.

1. Preparation prior to organotypic cerebellar slice cultures

1.1. In a cell culture hood sprayed with 70% ethanol beforehand, prepare 200 mL of culture medium in a 250 mL bottle-top vacuum filter attached to a sterile bottle receiver. Add 100 mL of Basal Medium Eagle (BME), 50 mL of Hanks' Balanced Salt Solution (HBSS), 50 mL of heat-inactivated horse serum, 1 mL of 200 mM L-Glutamine (final concentration 1 mM), and 5 mL of 200 g/L glucose (final concentration 5 mg/mL). Vacuum-filter the culture medium and keep it at +4 °C for up to a month.

1.2. In the sterilized biosafety cabinet, take out a 6-well culture plate from its package. Fill each well with 1 mL of culture medium. If a treatment is tested, add the pharmacological agent to the treated well, and the same volume of vehicle solution for the control well. In the present study, we added 1 µL of sterile 3 M KCl solution to the treated well (30 mM final) and 1 µL of sterile water to the control wells.

1.3. Bring inside the cell culture hood the needed number of individually wrapped cell culture inserts with hydrophilic polytetrafluoroethylene (PTFE) membrane (pore size = 0.4 µm). Carefully unwrap them and take out the cell culture inserts with sterile forceps. Drop them one by one in a well of a 6-well plate, avoiding bubbles at the interface between the insert membrane and the cell culture medium. Let the inserts equilibrate in the medium in a 37 °C, 5% CO_2 incubator for at

least 2 h before culture.

NOTE: The tissue chopper permanently resides in the cell culture hood.

1.4. Prepare two 200 mL beakers, one filled with 150 mL sterile water, and the other filled with 150 mL 70% ethanol. Dip the surgical tools in the 70% ethanol bath and then in water prior to using them.

NOTE: Do not use surgical tools immediately following contact with ethanol.

1.5. Disinfect the double-edged blade in the 70% ethanol beaker. Place it on the blade holder using sterile forceps and hold it into place using the blade clamp wrench. Let it dry until use.

1.6. Disinfect the plastic disc provided with the tissue chopper in the 70% ethanol beaker. Spray the cutting table with 70% ethanol prior to placing the disc on it. Let dry until use.

2. Dissection and cerebellar organotypic slice cultures

2.1. Bring the mouse pup inside the cell culture hood to perform the dissection.

2.2. Decapitate the pup quickly using straight operating scissors (**Figure 1A**).

2.3. While holding the pup's head by the nose with straight dressing forceps, cut open the scalp using straight eye scissors, starting from the posterior end, and going lateral to midline.

2.4. Proceed identically for the skull.

NOTE: Make sure to point scissor tips outwards to avoid damaging the cerebellum during dissection.

2.5. Take out the brain and transfer it to a 60-mm dish filled with cold HBSS + 5 mg/mL glucose.

2.6. Carefully dissect out the cerebellum using sterile straight fine forceps (**Figure 1B**). Transfer it onto the plastic disc placed on the cutting table of the tissue chopper using sterile curved fine forceps.

2.7. Orient the tissue by rotating the cutting table to perform parasagittal sections.

2.8. Move the cutting table by pulling the table release knob to the right, so the blade is positioned at the edge of the tissue.

2.9. Adjust the slice thickness to 350 µm and the blade speed to medium.

2.10. Start the chopper. Once the whole cerebellum has been sliced (**Figure 1C**), turn off the

chopper.

2.11. Remove the plastic disc with sterile forceps.

2.12. Carefully bring the plastic disc close to a 60-mm dish containing HBSS + 5 mg/mL glucose. Pipette cold HBSS + 5 mg/mL glucose onto the tissue using a sterile transfer pipette to allow harvesting of cerebellar slices in the buffer-filled dish.

2.13. Separate the cerebellar slices from each other using a microprobe. Take out good quality sections with the transfer pipette (it is recommended to use tissue sections that are close to the vermis) and drop them off in a pre-equilibrated cell culture insert (**Figure 1D**).

2.14. Position the cerebellar slices on the cell culture insert using the microprobe, then carefully remove the excess HBSS + 5 mg/mL glucose solution with a Pasteur pipette connected to the cell culture hood vacuum.

NOTE: At this stage the tissue is extremely tender and prone to physical damage. The maximum number of cerebellar slices per cell culture insert depends on the age of the donor animal. 6–8 slices can be cultured for a P0–P4-old animal, and 4–6 slices for animals older than P5.

2.15. Place the culture dish in the incubator, changing the medium completely every 2–3 days.

2.16. To assess Purkinje cell survival, slices can be collected as early as 5 days in vitro. For other purposes, they can be maintained in culture for several weeks (**Figure 1F**).

NOTE: In the case of Purkinje cell survival experiments, there is no need to renew the pharmacological treatment when changing cell culture medium (**Figure 1F**).

3. Immunofluorescence

3.1. Take out the 6-well plate from the incubator. Remove cell culture medium, wash the cell culture insert with 1x PBS, and fix the slices with cold 4% paraformaldehyde solution for 1 h, with 1 mL under the cell culture insert and 500 µL on top of the cell culture insert.

3.2. Wash the inserts 4x 10 min with 1x PBS, with 1 mL under and 500 µL on top of the insert, on an orbital shaker.

3.3. Transfer the cerebellar slices from 1 cell culture insert to 1 well of a 24-well plate with a brush.

3.4. Permeabilize and block the slices by incubation in 1x PBS, 0.25% Triton X-100, and 3% BSA (PBS-TB) for 1 h at room temperature, 500 µL/well.

3.5. Incubate with primary antibodies diluted in the PBS-TB solution, overnight at + 4 °C, on an

orbital shaker, 200 µL/well.

3.6. On the following day, perform four washes of 10 min with 1x PBS, on an orbital shaker, 500 µL/well.

3.7. Incubate with secondary antibodies diluted in the PBS-TB solution, two h at room temperature, on an orbital shaker, and protected from light, 200 µL/well.

3.8. Perform four washes of 10 min with 1x PBS, on an orbital shaker, 500 µL/well.

3.9. Counterstain the slices using Hoechst 33342, diluted in 1x PBS (2 µg/mL), for 10 min at room temperature, protected from light, 500 µL/well.

3.10. Mount the cerebellar slices on a microscopy slide with a brush. Let them air-dry completely, protected from light. Re-humidify them with 1x PBS and apply a coverslip covered with few drops of mounting medium (~80 µL). Avoid making any bubbles.

3.11. Once the mounting medium has cured, cerebellar sections are ready to be imaged.

4. Imaging and quantification of cell survival

4.1. Turn on the microscope and lasers according to the manufacturer and/or the imaging core facility's instructions.

4.2. Start the acquisition software.

4.3. Using a 10X objective, locate non-altered cerebellar slices that present 9–10 lobules (usually cerebellar sections close to the vermis).

4.4. Activate z-stack acquisition. Define the range of the z-stack to include all Purkinje cells present in the cerebellar slice. Set a 2 µm step size and start the acquisition. Save the acquired picture in the format of the acquisition software manufacturer to preserve the associated metadata.

4.5. Open the acquired file in ImageJ using the Bio-formats plugin[18].

4.6. Go to **Image** > **Stacks** > **Z-project…** (**Figure 2A**).

4.7. Choose **Max Intensity** in the Projection type dropdown menu and click **OK** (**Figure 2B**).

4.8. A flattened 2-D image will be generated. Double click on the **Multi-point** tool to let the **Point Tool** window appear. Uncheck the **Label points** option to ease counted cells visualization. Then, click directly on a soma of a Purkinje cell to begin counting (**Figure 1E**). The total number of counted cells will be indicated at the bottom of the **Point Tool** window (**Figure 3**).

NOTE: Usually, at least 3 non-damaged sections containing 9–10 lobules per cell culture insert can be quantified. To assess the neuroprotective effect of a pharmacological agent, at least 3 independent experiments should be performed.

REPRESENTATIVE RESULTS:

As shown in **Figure 4**, this protocol produces organotypic cerebellar slice cultures in which Purkinje cell survival can be assessed following the immunofluorescence and image acquisition steps. Purkinje cells were labeled with a combination of anti-Calbindin D-28K (dilution 1/200) and Alexa594 anti-mouse (dilution 1/300) antibodies. Image stitching was done automatically by the microscope acquisition software (NIS-Elements) to obtain a picture of the whole cerebellar slice. Purkinje cell numbers per slice were entered in the Prism 8 software to generate the chart and perform statistical analysis. At P6, Purkinje cell survival was low in the control, consistent with their known vulnerability window[4] (**Figure 4A,E**). Survival increased as the donor animal grew older and exited this critical period (**Figure 4C,E**). Cerebellar slices were treated with high KCl concentration to successfully induce their depolarization and survival[14] (**Figure 4B,D,E**).

FIGURE AND TABLE LEGENDS:

Figure 1: Illustration of the protocol from dissection to Purkinje cell survival quantification. The mouse pup is quickly decapitated (**A**). Following brain dissection, the cerebellum is isolated (**B**), and then chopped into 350 µm-width slices (**C**). The cerebellar sections are placed onto a control or treated cell culture insert (**D**) and maintained in culture for at least 5 days in vitro. The tissue is then fixed in 4% paraformaldehyde, immunostaining is performed, and pictures are acquired with a confocal microscope. Lastly, Purkinje cell number is quantified in each slice using the Multipoint Tool in ImageJ (**E**). (**F**) Schematic of the time course of KCl treatment during culture.

Figure 2: ImageJ settings used to flatten the 3D-stack prior to quantification.

Figure 3: Settings used to count Purkinje cell number using the Multipoint tool in ImageJ with an example of a cerebellar slice being quantified.

Figure 4: Purkinje cell survival in organotypic cerebellar slice culture following high KCl treatment. Representative image of cerebellar slices taken at postnatal day 6 (**A**, **B**) or 8 (**C**, **C'**, **D**, **D'**), either untreated (**A**, **C**, **C**') or treated with 30 mM KCl (**B**, **D**, **D'**). (**C'** and **D'**) Higher magnification view of the white boxed regions in **C** and **D**. Slices were kept 5 days in vitro prior to fixation. Purkinje cells are labelled with Calbindin D-28K in red. Scale bar = 500 µm (**A**–**D**), and 100 µm (**C'** and **D'**). (**E**) Quantification of Purkinje cell survival from P6 and P8 cultures, in control or treated with 30 mM KCl, shows a higher survival with the treatment (Mann-Whitney test, n = 3 to 5 slices). Data are expressed as mean ± SEM.

DISCUSSION:

Cerebellar slice culture is a powerful tool to study programmed Purkinje cell death during postnatal development. This technique can be used to rapidly screen candidate molecules for their neuroprotective potential. The main advantage is that the setup is simple and very cost

effective, and only requires a modest investment in equipment (a vibratome can cost up to 3 times more than a tissue chopper). Moreover, 10 to 15 healthy slices can be generated from one mouse pup, allowing for different assays to be performed in parallel using only one animal.

In order to obtain consistent and reproducible results, it is critical to perform the culture as efficiently as possible and to choose healthy cerebellar sections to be cultured. This method is also suitable for long-term culture (up to several months). So, it is essential to use good aseptic technique during the dissection and chopping process to avoid contaminations and the need to supplement cultures with antibiotics.

The cerebellar slice culture model preserves existing cell-cell interactions in the plane of the tissue section, and in the region cultured. This comes with a caveat: connections with targets outside of the cultured region might be severed. The supply in neurotrophic factors provided by afferent fibers might be discontinued as well and impair neuronal survival. This can be partially circumvented by performing organotypic slice co-cultures. It has been shown that climbing fibers can penetrate postnatal cerebellar slice cultures when co-cultured with inferior olive slice cultures[14,19]. More interestingly, Purkinje cells contacted by climbing fibers survived[14].

Here, we focused on the use of organotypic slice culture to search for neuroprotective molecules in the developing cerebellum. However, this method is equally suitable for other conditions such as neurodegeneration[20], axonal regeneration[21], and monitoring of neuronal circuit activity[22,23]. The post-culture applications go beyond immunofluorescence. Slices can be used for biochemical or gene expression studies, in order to investigate the biological mechanisms involved in the neuroprotective effect of a given compound. Altogether, this model can lay the groundwork for finding new neuroactive molecules before in vivo testing and be an effective and reliable supplement to in vivo mechanistic studies.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

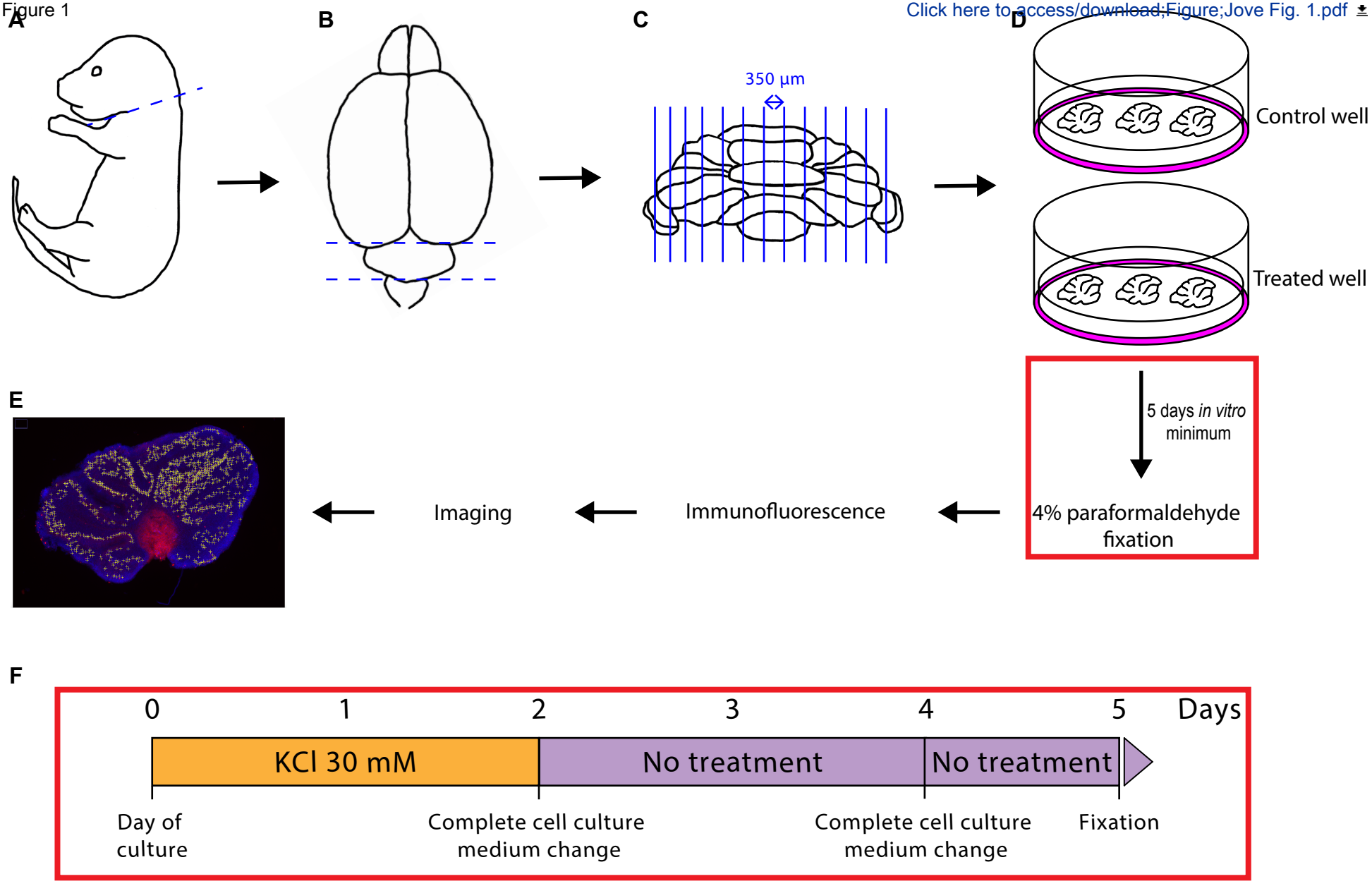
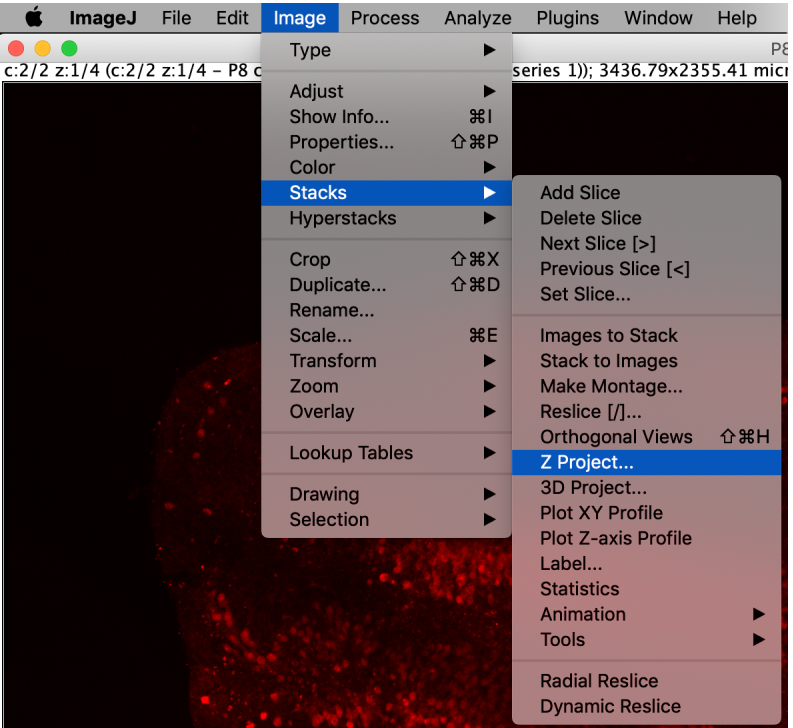


Figure 2

A



B

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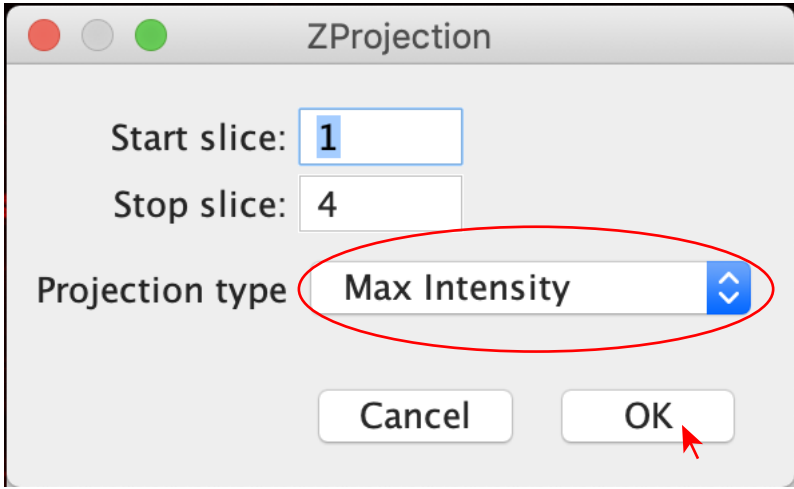
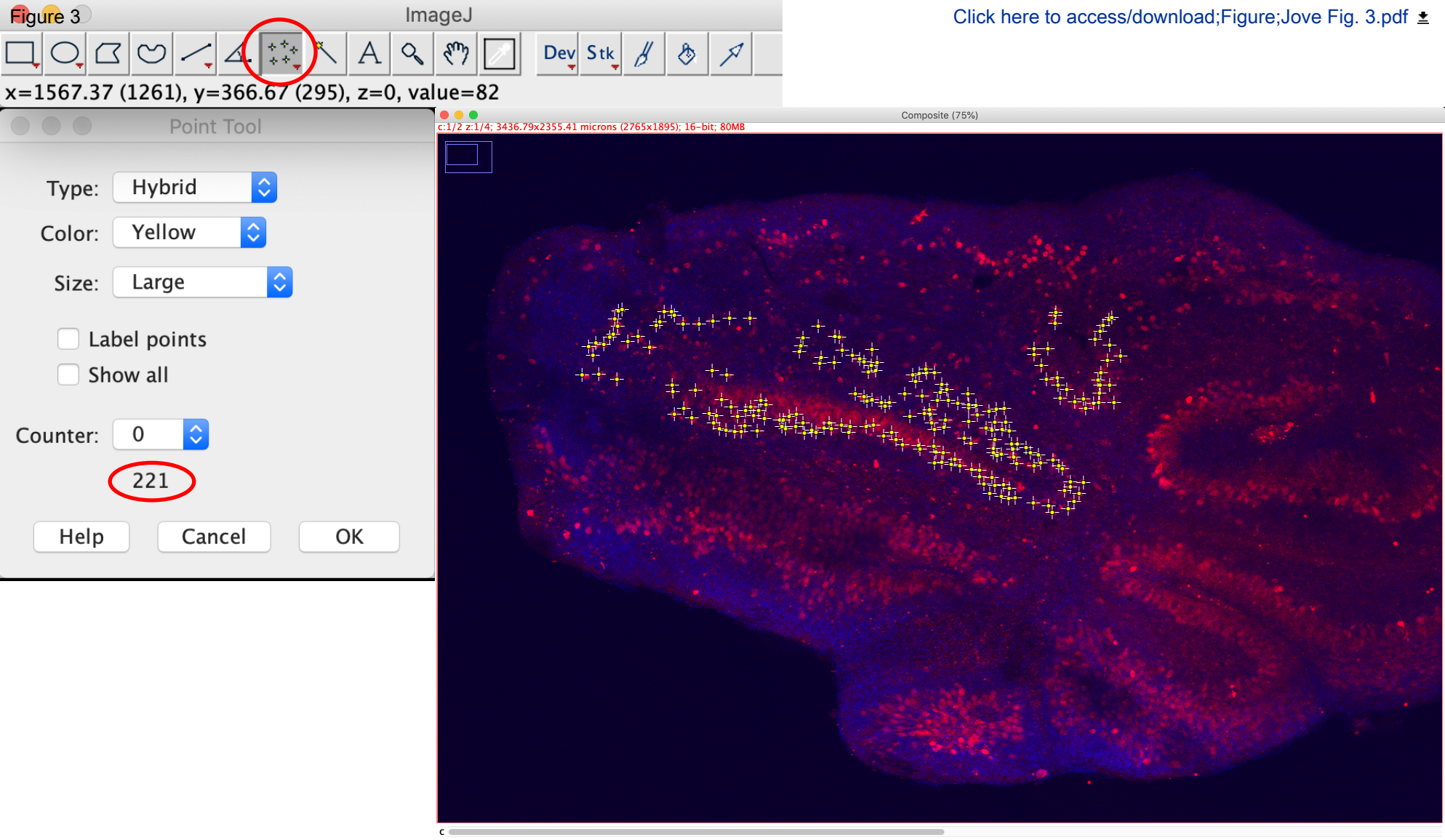
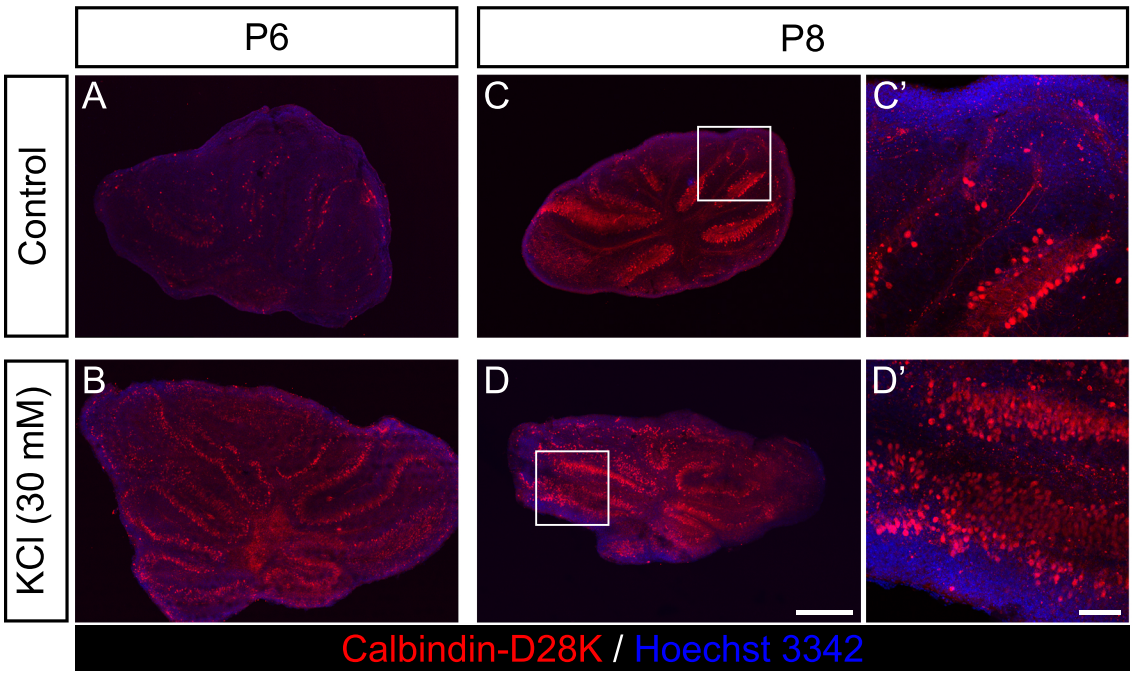
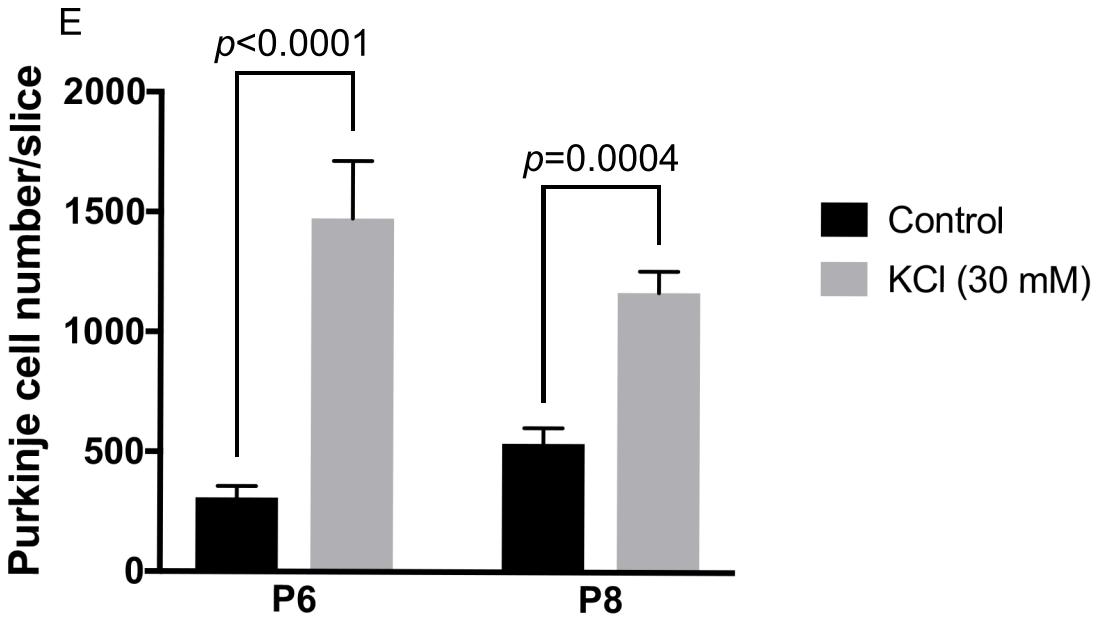


Figure 3







Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Alexa Fluor 594 Donkey anti-Mouse IgG secondary antibody	ThermoFisher scientific	A21203	
Basal Medium Eagle (BME)	ThermoFisher scientific	21010046	
Biosafety cabinet Class II, Type A2	NuAire	NU-540-400	
Bovine serum albumin	Millipore Sigma	A2153	
Brush			
anti-Calbindin D-28K antibody (CB-955)	Abcam	ab82812	
CO2 Incubator	NuAire	NU-5700	
Corning Costar Flat Bottom 6-well Cell Culture	Fisher Scientific	07-200-83	
Coverslips, 22 x 50 mm	Fisher Scientific	12-545-E	
Dressing forceps, straight	Harvard Apparatus	72-8949	
Double edge blades	Fisher Scientific	50949411	
Ethanol 200 proof	Decon Labs, Inc	2701	
Eye Scissors, straight	Harvard Apparatus	72-8428	
Fine forceps	Fisher Scientific	16-100-127	
L-Glutamine 100X	ThermoFisher scientific	25030149	
Glucose solution	ThermoFisher scientific	A2494001	
Hanks' Balanced Salt Solution	ThermoFisher scientific	14025092	
Hoechst 33342, Trihydrochloride, Trihydrate	Fisher Scientific	H21492	
Horse Serum, heat inactivated, New Zealand	ThermoFisher scientific	26050088	
ImageJ			
McIlwain Tissue Chopper	Fisher Scientific	NC9914528	
Microprobes	Fisher Scientific	08-850	
Millicell Cell Culture Inserts	Millipore Sigma	PICM0RG50	
Nalgene Rapid-Flow Sterile Disposable Filter Units with PES Membrane, 250 mL	ThermoFisher scientific	168-0045	
Nikon A1R confocal laser microscope system	Nikon		
NIS-Elements Imaging Software	Nikon		
Paraformaldehyde	Acros Organics	41678-0010	
Pasteur pipets	Fisher Scientific	13-678-20D	

Potassium Chloride	Fisher Scientific	BP366-500
ProLong Gold Antifade Mountant	ThermoFisher scientific	P10144
Operating Scissors, straight	Harvard Apparatus	72-8403
Orbital shaker Belly Dancer	IBI Scientific	BDRLS0003
Prism 8	GraphPad	
Superfrost Plus Microscope Slides	Fisher Scientific	12-550-15
Tissue Culture Dish, 60 mm w/ grip ring	Fisher Scientific	FB012921
Tissue culture plate, 24 well	Falcon/Corning	353047
Transfer pipettes, sterile	ThermoFisher scientific	13-711-21
Triton X-100	ThermoFisher scientific	BP151-500

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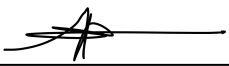
Signature:	
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Re: JoVE60353 "Purkinje cell survival in organotypic cerebellar slice culture"

We would like to thank the editor and reviewers for their time and the useful comments made. We believe it helped substantially improve the manuscript and hope it is now suited for publication in the Journal of Visualized Experiments.

Our response follows. The reviewer's comments are in black and italics font, the authors' response is in black regular font, the corrected excerpts from the revised manuscript are in blue regular font.

In the revised manuscript itself, the revised or added parts are in red.

Editorial comments:

General:
"1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues."

Done.

Protocol:
1. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

The text has been thoroughly reviewed and corrected as instructed.

Specific Protocol steps:
"1. 2.2: How exactly are the skin and skull removed?"

We added steps 2.3 and 2.4 to describe how the skin and skull are removed, page 2, lines 106-111: "2.3 While holding the pup's head by the nose with straight dressing forceps, cut open the scalp using straight eye scissors, starting from the posterior end, and going lateral to midline.
2.4 Proceed identically for the skull.
NOTE: Make sure to point scissor tips outwards to avoid damaging the cerebellum during dissection."

References:
"1. Please do not abbreviate journal titles."

We have reviewed all the references and made corrections accordingly.

Table of Materials:
"1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol."

Done.

"2. Please remove trademark (™) and registered (®) symbols from the Table of Materials."

Done.

Reviewers' comments:
Reviewer #1:
We appreciate the reviewer's careful reading of the manuscript and the helpful comments. It has improved the quality of this manuscript. Our point-by-point responses follow.

Manuscript Summary:
This is a very short method article about the preparation of cerebellar slice cultures. Overall, the instructions are clear and the procedure for setting up the cultures is appropriately described. In contrast, the description of the methods for evaluating Purkinje cell survival in the cultures are virtually absent and need to be added to the manuscript. While this is supposed to be a video article, it would still be helpful if the main procedures, both for setting up the cultures and the evaluation of Purkinje cell survival could be better explained using some graphical illustration, e.g a flow-chart of the procedure or a illustration of some critical steps.

We have added a schematic as figure 1 showing a flow-chart of the procedure, from dissection to image acquisition. As suggested by the reviewer, we also added figures 2 and 3 showing screenshots of the quantification procedure with ImageJ to improve reader understanding of this crucial part of our protocol.

Major Concerns:
Analysis and quantification of Purkinje cell survival is completely missing in the Methods part. Quantification is a crucial part in the analysis of cell survival and cannot be omitted in a manuscript devoted to the analysis of cell survival. In particular, the following aspects need to be much better covered and explained:
Image acquisition: Was the whole thickness of the cultures used for acquisition of stained Purkinje cells. Was the resulting 3D image flattened? There are many important open questions which will have a big impact on quantification.

We agree with the reviewer that the manuscript should include a precise description of the image acquisition step. The pages 3-4, lines 171-186 from the new paragraph entitled "Imaging and quantification of cell survival" address this issue:
"4. Imaging and quantification of cell survival

4.1 Turn on the microscope and lasers according to the manufacturer and/or the imaging core facility's instructions.
4.2 Start the acquisition software.

4.3 Using a 10X Apo Fluor objective, locate non-altered cerebellar slices that present 9-10 lobules (usually cerebellar sections close to the vermis).
4.4 Activate z-stack acquisition. Define the range of the z-stack to include all Purkinje cells present in the cerebellar slice. Set a 2-µm step size and start the acquisition. Save the acquired picture in the format of the acquisition software manufacturer to preserve the associated metadata.
4.5 Open the acquired file in ImageJ using the Bio-formats plugin18.
4.6 Go to "Image < Stacks < Z-project…" (Figure 2A).
4.7 Choose "Max Intensity" in the Projection type dropdown menu and click OK (Figure 2B).
4.8 A flattened 2-D image is generated."

Analysis in Image J: procedures are completely missing; it is not even explained how the data shown in Fig. 1 were obtained.

We agree with the reviewer that the manuscript should include a detailed protocol of the analysis in ImageJ. We have addressed this issue in page 4, lines 182-190 from the new paragraph entitled "Imaging and quantification of cell survival":
"4.5 Open the acquired file in ImageJ using the Bio-formats plugin[18].
4.6 Go to "Image < Stacks < Z-project…" (Figure 2A).
4.7 Choose "Max Intensity" in the Projection type dropdown menu and click OK (Figure 2B).
4.8 A flattened 2-D image is generated. Double click on the "Multi-point" tool to let the "Point Tool" window appear. Uncheck the "Label points" option to ease counted cells visualization. Then, click directly on a soma of a Purkinje cell to begin counting (Figure 1E). The total number of counted cells will be indicated at the bottom of the "Point Tool" window (Figure 3)."
We have added as well Figures 2 and 3 to illustrate the process.

Questions of number of data: In Fig. 1, the authors show the data from 3-5 slices. If the message of this article is that a proper quantification of Purkinje cell survival under different conditions can be done in 3-5 slices in total, it would be a disaster. There needs to be a clear indication of how often an experiment needs to be repeated, how many slices need to be analyzed, how particular slices are included or excluded from the analysis etc.

We thank the reviewer for this helpful comment. We never intended to imply that the efficiency of a treatment on Purkinje cell survival can be accurately assess with just 3-5 slices, and apologize for the confusion. We added a note at the end of the new fourth paragraph to address this issue, page 4, lines 191-193: "NOTE: Usually, at least 3 non-damaged sections containing 9-10 lobules per cell culture insert can be quantified. To assess the neuroprotective effect of a pharmacological agent, at least 3 independent experiments are performed."

It is clearly not appropriate to write a methods paper on the subject of "Purkinje cell survival in organotypic cerebellar slice culture" and then only write a methods paper about setting up cerebellar slice cultures. The entire part about quantification needs to be added to this manuscript.

We agree with the reviewer and have resolved this issue by adding paragraph 4, pages 3-4, lines 171-193, which describes precisely how the quantification was done. We also added information in the Representative results paragraph about the software used to generate the quantified data shown in Figure 4, page 4, lines 201, 202: "Purkinje cell numbers per slice were entered in the Prism 8 software to generate the chart and perform statistical analysis."

Minor Concerns:
117-118: This note is unclear to this reviewer. There is a clear difference between a pharmacological one-time treatment and a repeated or continuous treatment. If the recommendation in the note is considered, this this culture model would be only appropriate for a one-time treatment.

Indeed, our protocol is appropriate for a one-time treatment. We clarified that in the manuscript, page 3, line 142: "NOTE: In the case of Purkinje cell survival experiments, there is no need to renew the pharmacological treatment when changing cell culture medium (Figure 1F)."

127. Chamber slide: missing in the Materials list, no indication how many chamber slides are needed for staining the sections from one culture well.

We apologize for the mistake. We actually used 24-well plates for the immunofluorescence step, and have added the reference in the Material list associated with the manuscript. We also indicated in the manuscript that we use 1 well of the 24-well plate for 1 culture insert in step 3.3, page 3, line 153: "Transfer the cerebellar slices from 1 cell culture insert to 1 well of a 24-well plate with a brush."

128 - 138. No volumes given for antibody solutions.

We have now added this information in the text, page 3, lines 156, 158, 160, 162, 163, and 165.

Reviewer #2:
We would like to thank the reviewer for the constructive suggestions. Our point-by-point responses follow:

Review of Rakotomamonjy and Guemez-Gamboa, "Purkinje cell survival in organotypic cerebellar slice culture"
This is a much needed protocol for the establishment of cerebellar organotypic cultures and for following the process of programmed cell death of Purkinje cells in the early days of the culture. Most of my comments are technical and are related to specific items in the protocol.

"1.6-1.7: Spray the cutting table with 70% ethanol prior to placing the sterile cutting disc on it."
We added the reviewer's suggestion to step 1.7, page 2, line 99.

"Prepare two beakers, one with 70% ethanol and the other with sterile PBS. Dip the surgical

tools in the ethanol and then in the PBS prior to using them. Do not use surgical tools immediately following contact with ethanol."
We revised the text according to the reviewer's suggestion in step 1.5. and the following note, page 2, lines 91-94: "1.5 Prepare two 200-mL beakers, one filled with 150 mL sterile water, the other filled with 150 mL 70% ethanol. Dip the surgical tools in the 70% ethanol bath and then in water prior to using them.
NOTE: Do not use surgical tools immediately following contact with ethanol." However, in our protocol, we use sterile water to rinse off ethanol.

"2.1-2.2: Spray the pup with 70% ethanol prior to the procedure and let dry."

In our protocol approved by the Northwestern University Animal Studies committee, we don't spray the pup with 70% ethanol to the procedure.

"2.5-2.6: Adjust the slice thickness to 350 µm and the blade speed to medium."

We added the sentence as what is now step 2.9, page 2, line 120.

2.8-2.9: Carefully bring the PVC disc close to a Petri dish containing HBSS+5 mg/ml glucose. Gently slide a fine spatula under the tissue slice and move the slice into the buffer.

We modified what is now step 2.12. to adapt the reviewer's suggestions to our existing protocol, page 2, lines 124-126. We don't use a spatula to move the slices from the disc to the HBSS + 5 mg/mL glucose solution. In our hands, chasing out buffer with a transfer pipette to let the slices fall into the buffer-filled dish leads to good quality sections.

2.10-2.11: Separate the tissue slices from each other using two fine spatulas. It is recommended to use tissue sections that are close to the vermis.

We modified what is now step 2.13 to adapt the reviewer's suggestions to our existing protocol, page 2, lines 127-129: "Separate the cerebellar slices from each other using a microprobe. Take out good quality sections with the transfer pipette (It is recommended to use tissue sections that are close to the vermis) and drop them off in a pre-equilibrated cell culture insert."

Using a wide spatula, lift and position the tissue slices onto the cell culture insert. Note that at this stage the tissue is extremely tender and prone to physical damage. Place no more than four tissue slices per insert.

We completed the protocol to describe in more details the positioning of the cerebellar slices in the culture insert, page 2, line 131: "Position the cerebellar slices on the cell culture insert using the microprobe".
We added a note about the extreme fragility of the cerebellar sections, page 3, line 134.
In the manuscript first version, we specified the number of slices per insert in the note following now step 2.14, page 3, lines 134-136. In our hands, "The maximum number of

cerebellar slices per cell culture insert depends on the age of the donor animal. 6-8 slices can be cultured for a P0-P4-old animal, and 4-6 slices for animals older than P5."

3.1: Remove the medium and wash the tissue slice with 100 mM PBS. Add 1 ml of 4% PFA beneath the insert and 1 ml above it, and leave for 1 hr at room temperature.

We modified the manuscript to add the PBS wash to step 3.1, page 3, lines 147, 148: "Remove cell culture medium, wash the cell culture insert with 1X PBS, …"
In our protocol, we use low-height cell culture inserts. Thus, when performing the fixation and washes, we can't put more than 500 µL above the insert membrane.

Reviewer #3:
We would like to thank the reviewer for the constructive suggestions that helped improve the representative results figure and the accuracy of the overall manuscript. Our point-by-point responses follow:

In this article, the authors demonstrate a method of organotypic cerebellar slice culture. The method is described compactly but adequately. However, the description of the background is insufficient, and some important aspects are lack in the protocol and the representative result.
1. The organotypic slice culture method was first demonstrated by Gahwiler (1981), and an organotypic culture technique with hydrophilic membrane was demonstrated in cortical slice culture by Yamamoto et al (1989). These papers should be cited with an appropriate description.

We thank the reviewer for this comment, We have now added these two citations in the introduction, page 1, line 39-41: "The method was first established by Gähwiler who cultured brain slices using the roller tube technique[2], and later modified by Yamamoto et al. who introduced the use of a microporous membrane to perform cortical slice cultures[3]."
These two references can also be found in the References list, page 6, lines 273-276:
"Gahwiler, B. H. Organotypic monolayer cultures of nervous tissue. *Journal of Neuroscience Methods.* **4** (4), 329-342, (1981).
Yamamoto, N., Kurotani, T. & Toyama, K. Neural connections between the lateral geniculate nucleus and visual cortex in vitro. *Science.* **245** (4914), 192-194, (1989)."

2. Is 1 ml of the culture medium enough for each well (line 77)?

In our protocol, we use low-height cell culture inserts, because it allows us to perform live imaging experiments with a multiphoton microscope. Putting more than 1 mL of cell culture medium in the well will cause the low-height cell culture inserts to float, and prevent the maintenance of an air-free contact between the membrane bottom and the medium.

3. Both descriptions, "HBSS + 5 mg/ml glucose" and "HBSS-glucose solution" are mixed in the text (line 94-111).

We thank the reviewer for noticing the discrepancy. We have made sure that every reference to this buffer is now *"HBSS + 5 mg/ml glucose"* in the manuscript.

4. How much medium is exchanged (line 115)?

We change the entirety of the medium every 2/3 days. We have added this information in page 3, line 138, 139: "Place the culture dish in the incubator, changing the medium completely every 2/3 days."

5. Any pharmacological treatment is not descried (line 117). In particular, the detail of KCl treatment should be shown.

We have added the detail of KCl treatment. We now describe when the treatment is performed in step 1.2, page 1 lines 79-82: "If a treatment is tested, add the pharmacological agent to the treated well, and the same volume of vehicle solution for the control well. In the present study, we added 1 µL of sterile 3 M KCl solution to the treated well (30 mM final), and 1 µL of sterile water in the control wells." In addition, we have added a schematic in figure 1F where the timeline of the treatment is shown.

6. Is it easy to remove the slices from the culture insert (line 127)? No damage?

In our hands, the slices either come off when adding 1X PBS after the fixation step, or we just need to carefully lift them with a fine brush, without resulting in any tissue damage.

7. The amount of each solution should be described in anti-body staining (line 122-137).

We have modified the manuscript to add the amount of each solution in the "Immunofluorescence" paragraph. Page 3, lines 156, 158, 160, 162, 163, and 165.

8. How long were the cerebellar slices cultured in Figure 1?

We cultured the slices for 5 days. We added the information in the manuscript in step 2.16, page 3, lines 140, 141: "To assess Purkinje cell survival, slices can be collected as early as 5 days in vitro. For other purposes, they can be maintained in culture for several weeks." We have also mentioned it in figure 4's legend, page 5, lines 224, 225: "Slices were kept 5 days *in vitro* prior to fixation."

9. The time course of KCl treatment should be described.

We have added a schematic in figure 1F where the timeline of the treatment is shown.

10. A high power view should be added to Figure 1, as it is hard to see individual Purkinje cells in the present pictures.

We have added panels 4C' and 4D' to show higher magnification views of the cerebellar slices, and modified the figure legend accordingly, page 5, lines 221-225.

11. It would also be better to add counterstained pictures.

Our original figure showed counterstained pictures, but we agree that the low magnification makes it hard to see it. The two new panels added as Figures 4C' and 4D' allow a better visualization of individual Purkinje cells, as well as the nuclear staining.

12. Some interesting results using organotypic slice cultures have been demonstrated in cerebellum from physiological and developmental points of views (for example, Audiat et al, 1990; Uesaka et al, 2012). These papers should be cited in the discussion section.

We have added the Uesaka's citation as reference 19, page 5, line 250, when discussing the use of the co-culture model. We have added the Audinat's citation as reference 23, page 5, line 254, when discussing contexts other than cell survival where slice culture could be an appropriate model. These two references can also be found in the References list, page 7, lines 317-319, and lines 327-329:

"Uesaka, N. et al. Organotypic coculture preparation for the study of developmental synapse elimination in mammalian brain. *Journal of Neuroscience*. **32** (34), 11657-11670, (2012)."

"Audinat, E., Knopfel, T. & Gahwiler, B. H. Responses to excitatory amino acids of Purkinje cells' and neurones of the deep nuclei in cerebellar slice cultures. *Journal of Physiology*. **430** 297-313, (1990)."