Protocol

Dual antibody strategy for high-resolution imaging of murine Purkinje cells and their dendrites across multiple layers

1. Brain mouse perfusion
15-30 minutes

2. Cryostat brain sectioning
1 day

3. Purkinje neurons immunofluorescence
5 days

4. Imaging and 3D-reconstruction
1 day

Salvatore Botta,
Alexei Chemiakine,
Vincenzo A. Gennarino

vag2138@cumc.columbia.edu

Highlights

Adult mouse brain perfusion and sagittal sectioning

Dual-antibody cerebellum immunofluorescence

High-resolution imaging of Purkinje neurons and their dendrites

Three dimensional (3D) imaging reconstruction

Despite being among the largest neurons in the mammalian brain, Purkinje cells are difficult to visualize and trace via immunofluorescence because their dendritic arbors extend through several cerebellar layers. This protocol describes a two-antibody strategy we developed to study Purkinje cell morphology in mice. With it, one can reconstruct three-dimensional images of Purkinje cells at single-neuron resolution across multiple layers. The substantially improved image quality reveals subtle defects, enabling more meaningful morphological analysis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
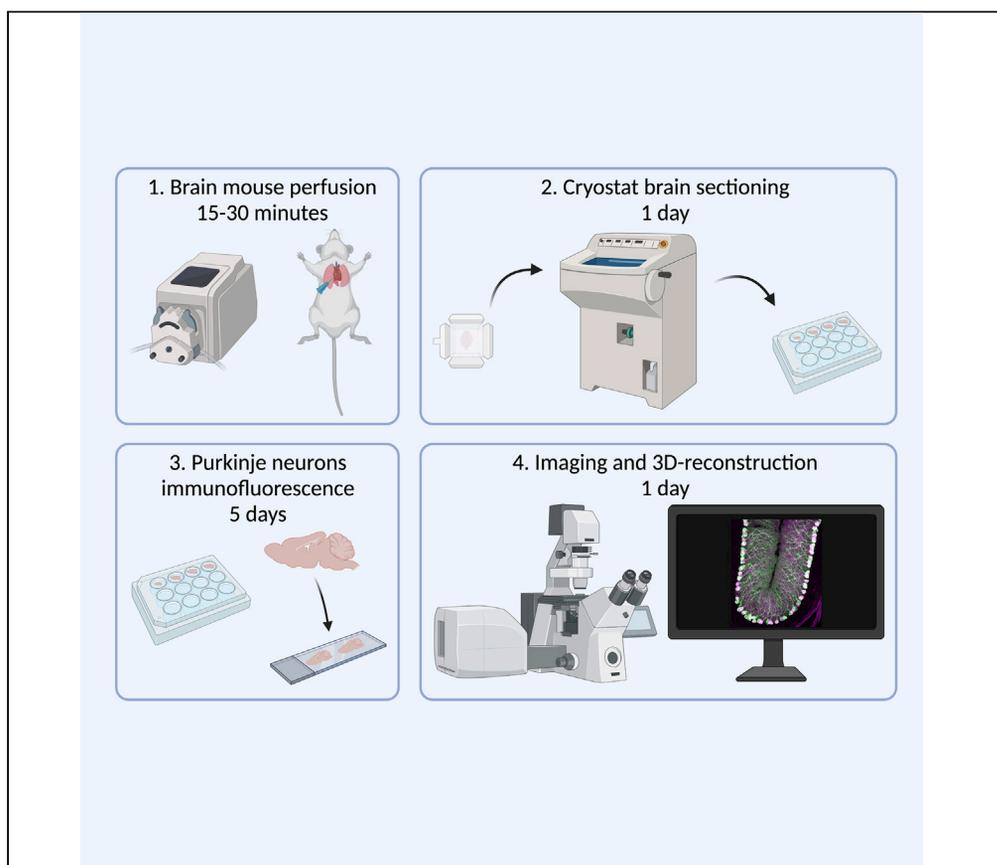
STAR Protocols

Protocol

Dual antibody strategy for high-resolution imaging of murine Purkinje cells and their dendrites across multiple layers

Salvatore Botta,[1,2,6] Alexei Chemiakine,[1] and Vincenzo A. Gennarino[1,3,4,5,7,]*

[1]Department of Genetics and Development, Columbia University Irving Medical Center, New York, NY 10032, USA

[2]Department of Translational Medical Science, University of Campania Luigi Vanvitelli, 80131 Napoli, Italy

[3]Departments of Pediatrics and Neurology, Columbia University Irving Medical Center, New York, NY 10032, USA

[4]Columbia Stem Cell Initiative, Columbia University Irving Medical Center, New York, NY 10032, USA

[5]Initiative for Columbia Ataxia and Tremor, Columbia University Irving Medical Center, New York, NY 10032, USA

[6]Technical contact

[7]Lead contact

*Correspondence: vag2138@cumc.columbia.edu
https://doi.org/10.1016/j.xpro.2022.101427

SUMMARY

Despite being among the largest neurons in the mammalian brain, Purkinje cells are difficult to visualize and trace via immunofluorescence because their dendritic arbors extend through several cerebellar layers. This protocol describes a two-antibody strategy we developed to study Purkinje cell morphology in mice. With it, one can reconstruct three-dimensional images of Purkinje cells at single-neuron resolution across multiple layers. The substantially improved image quality reveals subtle defects, enabling more meaningful morphological analysis.

For complete details on the use and execution of this protocol, please refer to Gennarino et al. (2015).

BEFORE YOU BEGIN

Purkinje cells are output neurons of the cerebellar cortex playing pivotal roles in coordination, control, and learning of movements, and they are particularly vulnerable to insults from toxins, infectious agents, injuries, and a variety of neurological diseases (Baltanas et al., 2021; Gennarino et al., 2015; Hornig et al., 1999; Huang and Verbeek, 2019; Koeppen, 2018; Manto, 2012). To visualize these neurons by immunofluorescence confocal microscopy, the usual protocol is to stain the tissue with an antibody against Calbindin-D-28K (a member of the large EF-hand family of calcium-binding proteins), which is highly expressed in Purkinje cell bodies and can also mark dendrites (Chen et al., 2003; Gennarino et al., 2015; Jafar-Nejad et al., 2011; Kapfhammer and Gugger, 2012; Orr, 2012; Whitney et al., 2008). Unfortunately, the resolution is frequently insufficient to appreciate the stratification of Purkinje neurons layers and dendrites.

We developed the following protocol to achieve better resolution of the whole neuron, across distinct cerebellar layers, in a mouse model of spinocerebellar ataxia type 47 (SCA47) (Gennarino et al., 2015). We envision that a similar dual-antibody strategy could be useful to visualize other neuronal types that have elaborate arbors extending to different depths.

Institutional permissions

Working with mice requires an approved mouse protocol from the IACUC - American Association for Laboratory Animal Science. Mouse colonies were bred and maintained with standard mouse chow

and water ad libitum under a 12 h light/12 h dark cycle in our on-site facility in the Columbia University Medical Center. Mice were group-housed before surgery, up to five per cage, and housed individually with nesting material in the cage after surgery to enable undisturbed recovery.

⚠ CRITICAL: The cerebellum is readily recognized, mounted, and collected, but it is also extremely fragile when sliced. To ensure that the slice does not fracture during the mounting process, we recommend practicing the steps outlined here with some wild-type (WT) mice before proceeding with the actual experiments. Furthermore, bear in mind that brain tissue is more soft and easily damaged in young mice than in adult mice. Also refer to ''Materials and equipment'' to prepare solutions, materials, and tools prior to the procedure.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER (CAT#)
Antibodies		
Monoclonal Anti-Calbindin-D-28K	Sigma-Aldrich	C9848 RRID: AB_476894 (1:1000)
Rabbit-Polyclonal Inositol 3-phosphate receptor 1 (I3PR1)	Thermo Fisher Scientific	PA1901 RRID: AB_2129984 (1:500)
Goat anti-Mouse IgG1 (γ1) Secondary Antibody, Alexa Fluor Plus 594	Thermo Fisher Scientific	A21125 RRID: AB_2535767 (1:1500)
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488	Thermo Fisher Scientific	A11008 RRID: AB_143165 (1:1500)
Chemicals, Peptides, and Recombinant Proteins		
PBS, Phosphate Buffered Saline, 10× Solution	Fisher Scientific	BP3991
Tween 20	Fisher Scientific	BP337500
TritonX-100	Fisher Scientific	BP151500
Tris Base	Fisher Scientific	BP1525
NaCl	Fisher Scientific	S27110
Sodium Azide	Fisher Scientific	BP922I500
2,2,2 Tribromoethanol	Fisher Scientific	AAA1870622
2-Methyl-2-butanol	Fisher Scientific	AAA18304AP
Paraformaldehyde	MilliporeSigma	158127-500G
Normal Goat serum	Abcam	ab7481
Sucrose	MilliporeSigma	S0389-500G
Bovine Serum Albumin, BSA	Fisher Scientific	BP1600100
Tissue-Plus™ O.C.T. Compound Tissue-Plus™ O.C.T. Compound	Fisher Scientific	23-730-571
ProLong™ Gold Antifade Mountant	Thermo Fisher Scientific	P36930
Experimental models: Organisms/strains		
Female and male Pum1 mutant mice and wild-type littermates in B6/129 mixed background; 3 and 10 weeks of age at sacrifice.	Prof. Haifan Lin's Lab (Yale School of Medicine, New Haven, Connecticut 06520, USA)	(Gennarino et al., 2018)
Software and algorithms		
Fiji is Just ImageJ (Fiji) version 2.0.0-rc/1.5f	https://imagej.net/imagej-wiki-static/Fiji	(Schindelin et al., 2012)
Leica Application Suite Advanced Fluorescence version 3.2.1.9702	Leica Microsystem	n/a
Other		
Tubing for perfusion 1.02 mm in diameter	Fisher Scientific	NC0279757
Corning™ Costar™ Flat Bottom Cell Culture Plates (12-Well)	Corning	07-200-82
Fisherbrand™ Premium Superfrost™ Microscope Slides	Fisher Scientific	12-544-7
Kimwipes	Fisher Scientific	06-666
Embedding mold	Fisher Scientific	50465347
Brush for brain slices: a pointed paint brush, length 16–16.6 cm with nylon tips, size 0.6 cm	Amazon Store	b0776bts5b

STAR Protocols
Protocol

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER (CAT#)
PYREX™ Reusable Petri Dishes: Bottoms Only (diameter 150 mm, height 20 mm)	Fisher Scientific	08-7485
20 mL HDPE Scintillation Vials with Polypropylene Cap	Fisher Scientific	03-337-23B
Double-Ended Micro-Tapered stainless steel spatula	Fisher Scientific	21-401-10
Iris Scissors	VWM	470018-890
MeisterHand LISTER Bandage Scissors, 8" (20.3)	HOSPEQ	MH50550
Fisherbrand™ Standard Dissecting Scissors	Fisher Scientific	0895120
Medium Tip dissecting Forceps	VWR	470001-846
Disposable underpads	Fisher Scientific	23666062
Sterile blunt needles 23G	Fisher Scientific	NC9823755
3P Minipuls III Peristaltic Pump, Fisher Scientific	Fisher Scientific	NC0745002
Leica CM3050S Cryostat	Leica BIOSYSTEMS	958846
Rocker II Platform Rocker	Boekel Scientific™	260350
Leica TCS SP5	Leica Microsystem	n/a
LSM800	Zeiss	RRID: SCR_015963

MATERIALS AND EQUIPMENT

All essential buffers and solutions should be freshly made prior to sample preparation and used the same day to avoid degradation or loss of efficacy. Make sure that there is enough of all solutions that are required for mouse anesthesia, brain slices preparation, incubation, and imagining.

Phosphate-Buffered Saline (PBS)		
Reagent	Final concentration	Amount
PBS 10×	1×	100 mL
Sterile Milli-Q water		Up to 1 L

Note: PBS 1× needs to be filtered before using it with 0.22 μM filter.

Avertin solution		
Reagent	Final concentration	Amount
2,2,2 Tribromoethanol	1.25%	2.5 g
2-methyl-2-butanol 98%		5 mL
Sterile Milli-Q water		Up to 200 mL
Total		200 mL

Note: Protect the solution from light by storing it in a dark glass bottle. Avertin is stable at room temperature for 1 year. Discard the solution if it turns yellow.

⚠ CRITICAL: The 2,2,2 Tribromoethanol is hazardous. It can cause organ (particularly lung) toxicity from inhalation, acute oral toxicity from ingestion, skin corrosion and irritation from contact, eye damage from backsplash. Wear protective gloves, glasses, and avoid breathing fumes/dust. Use the product only under a chemical hood. The 2-methyl-2-butanol 98% is highly flammable and light-sensitive. The chemical is considered hazardous, with inhalation or ingestion causing acute organ toxicity (particularly lungs and central nervous system); contact causes acute dermal toxicity, corrosion, and serious eye damage. Wear protective gloves, glasses, and avoid breathing fumes/dust. Use the product only under a chemical hood.

Paraformaldehyde solution

Reagent	Final concentration	Amount
Paraformaldehyde (PFA)	4%	40 g
PBS (Phosphate buffered saline) 20×	1×	50 mL
HCl 37% and NaOH 5 N	To adjust pH 7.4	
Sterile Milli-Q water		Up to 1 L
Total		1000 mL

Note: Dissolve PFA in 800 mL of sterile Milli-Q H_2O. Stir and heat to ∼60°C. Because PFA powder dissolves very slowly, you need to adjust the pH of the solution to 7.4 by adding 5 N NaOH or 37% HCl drop by drop until a clear solution is formed. Even then some small undissolved white particles may remain, so cool the solution to room temperature and filter it using standard 3 M filter paper to remove all the particles. Add 50 mL of PBS 20×. Make sure the pH is 7.4 and then bring the volume up to 1000 mL with sterile Milli-Q H_2O. Dispense the solution in aliquots of 25–50 mL (usually 25 mL are enough for one brain). You can store the solution at 4°C for 1–2 weeks, or at −20°C for 4 months. Protect the solution from light to avoid degradation. Alternatively, dilute 250 mL of pre-made 16% PFA with 750 mL of milliQ water to obtain 4% PFA.

⚠ CRITICAL: PFA is a confirmed human carcinogen and irritates the skin, eyes, and respiratory tract; prolonged exposure can cause cough, shortness of breath, and lung damage. Prepare under a chemical hood and dispose of it in an appropriate biohazard waste container.

Sucrose 5% in PBS

Reagent	Final concentration	Amount
Sucrose	5%	5 g
PBS 10×	1×	10 mL
Sterile Milli-Q water		up to 100 mL
Total		100 mL

Note: Make it fresh on the same day of the procedure.

Sucrose 10%

Reagent	Final concentration	Amount
Sucrose	10%	10 g
Sterile Milli-Q water		100 mL
Total		100 mL

Note: Make it fresh on the same day of the procedure.

Sucrose 20%

Reagent	Final concentration	Amount
Sucrose	20%	20 g
Sterile Milli-Q water		100 mL
Total		100 mL

Note: Make it fresh on the same day of the procedure.

Sucrose 30%

Reagent	Final concentration	Amount
Sucrose	30%	30 g
Sterile Milli-Q water		100 mL
Total		100 mL

Note: Make it fresh on the same day of the procedure.

Sodium Azide

Reagent	Final concentration	Amount
Sodium Azide	10%	5 g
Sterile Milli-Q water		50 mL
Total		50 mL

Note: Sodium Azide need to be filtered 0.22 μM filter before using it.

⚠ CRITICAL: Sodium Azide is a mutagen. Sodium Azide vapor can irritate the eyes, nose, throat, and lungs; at higher doses it can result in low blood pressure, headache, weakness, and collapse; lower or more chronic exposure can lead to bronchitis. Prepare and use under a chemical hood. Store at 4°C for a maximum of 6 months.

PBS 1× + Sodium Azide

Reagent	Final concentration	Amount
Sodium Azide 10%	0.02%	0.2 mL
PBS 1×		up to 100 mL

⚠ CRITICAL: Sodium Azide is a mutagen. Sodium Azide vapor can irritate the eyes, nose, throat, and lungs; at higher doses it can result in low blood pressure, headache, weakness, and collapse; lower or more chronic exposure can lead to bronchitis. Prepare and use under a chemical hood. It can be stored at 4°C for up to 6 months.

Inactivated BSA solution

Reagent	Final concentration	Amount
Heat-inactivated BSA	2%	1 gr
PBS 1X		50 mL
Total		50 mL

Note: BSA2% needs to be filtered through a 0.22 μM filter before using it. Store at 4°C for a maximum of one week.

Blocking solution in PBS 1×

Reagent	Final concentration	Amount
Normal Goat Serum, NGS	2%	1 mL
Triton-X 100	0.3%	0.15 mL
PBS 1×		up to 50 mL

Note: The serum needs to be from the same species as the secondary antibody. Prepare the blocking solution fresh when needed.

TBST pH 7.5		
Reagent	Final concentration	Amount
Tris Base	50 mM	3.02 g
NaCl	150 mM	89.5 g
Tween 20	0.1%	1 mL
Sterile Milli-Q water		up to 1 L

Note: The solution can be stored for up to 6 months at room temperature.

STEP-BY-STEP METHOD DETAILS

Note: Each perfusion will take between 10 and 20 min. We strongly advise that you transport the mice from the mouse facility to your lab room in their cage at least 30 min before beginning the perfusion. Some labs routinely remove food and water 30 min before anesthesia, but we have not found any scientific justification for this practice for mice that are going to be sacrificed.

Note: Before you begin: i) double-check that the pump is operational. Install all of the tubes in the order specified in the procedure section; and ii) clean your chemical hood with alcohol. If you plan to perfuse your mice on a regular basis, we recommend to have a dedicated chemical hood. Follow the approved mouse protocol from the IACUC - American Association for Laboratory Animal Science.

Brain mouse perfusion

⏱ Timing: 15–30 min per animal

This section will walk you through the steps necessary to ensure appropriate PFA perfusion of the brain.

1. Prepare milliQ water, PBS 1× solution, and 4% PFA. If PFA was previously stored at −20°C, unfreeze the solution at room temperature.
2. Fill the required number of 20 mL HDPE Scintillation vials with polypropylene cap (one per brain) with about 10 mL of 4% PFA in PBS 1× solution.
3. Prepare surgical instruments needed for brain extraction: scissors, forceps, spatula.
4. Fill the tubing with PBS 1× by inserting the free end to the falcon tube (50 mL conical falcon tube) containing PBS 1× solution and turning on the peristaltic pump until the solution starts coming from the needle on the other end (Figure 1A).
5. Under the fume hood, anesthetize the mouse by injecting 240 mg/kg of Avertin. Alternatively, use a IACUC-approved option for anesthesia, e.g., Ketamine cocktail.

   △ CRITICAL: To check if an animal is fully anesthetized, after 10 min pinch the foot with surgical tweezers. A fully anesthetized animal will not respond.

6. Place the animal on its back on a raised flat surface (use a plastic tray or styrofoam) (Figure 1B).
7. Fix paws with tape (or needle if you use a styrofoam) to immobilized them (Figure 1B).
8. Pull the skin on the abdomen and make an incision with standard dissecting scissors (Figure 1C).
9. Insert the tip of the scissors and expand the gap, being careful not to damage the liver.

   △ CRITICAL: Do not damage or touch the liver, or the perfusion will be compromised.
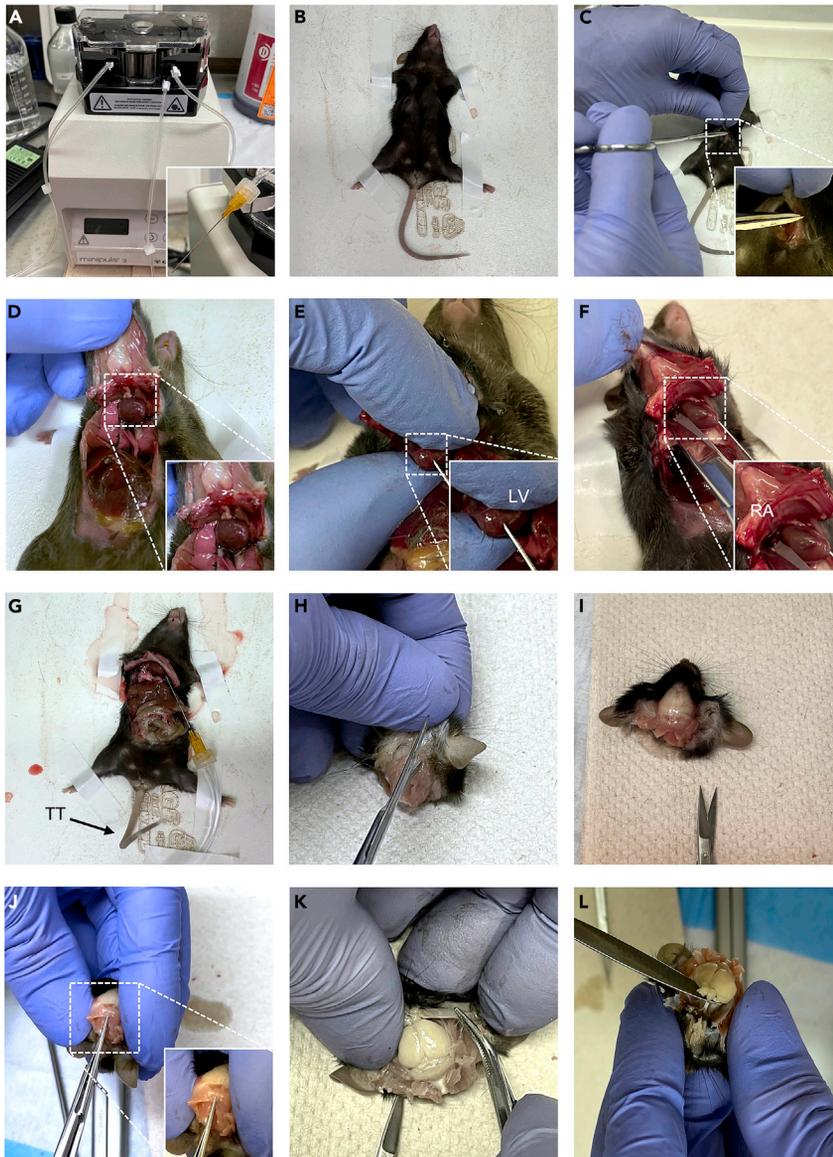
Figure 1. Mouse perfusion and brain dissection

(A) Peristaltic pump setup highlighting the tubing and needle positioning.

(B) After the mouse is anesthetized, immobilize the animal using tapes on a flat surface.

(C) Pull the skin taut to make an incision.

(D) Find the diaphragm, gently cut through it and along the rib cage to reveal the heart.

(E) Insert the needle in the left ventricle (LV) and start pumping with PBS 1×.

(F) Make a small incision on the right atrium (RA).

(G) A good sign of PFA 4% solution entering the system is a twitching tail (TT).

(H) Cut the skin longitudinally.

(I) Expose the skull as much as you can.

(J) Cut the skull longitudinally.

(K) Lift the right and left portion of the skull to reveal the brain (an optimal perfusion will reveal a white brain with no blood).

(L) Delicately lift the brain from the bottom with a spatula to collect the tissue.

10. After visualizing the diaphragm, gently cut through it and along the rib cage to reveal the heart.

11. Cut between the ribs toward the spinal cord, avoiding the lungs, in order to lift the upper part of the rib cage and expose the heart (Figure 1D).

12. Insert the 27G needle into the left ventricle shallowly to avoid damaging the heart (Figure 1E).

13. Turn on the pump at 36 mL/min from the PBS 1× falcon tube (see step 4 above).
14. Quickly make a small incision on the right atrium. Thick blood should be seen (Figure 1F).
15. Continue to perfuse with PBS 1× until the liver changes color from dark to light brown and the body structures (paws, for instance) become white.

   Note: Another way to check the success of the perfusion is to cut the end of the tail and wait until you see clear drops of PBS 1×; by this point the entire body and brain would be perfused.

16. Stop the pump and transfer the free end of tubing into the falcon containing 50 mL of 4% PFA (usually 25 mL are enough) solution and resume the pumping.

   Note: When the PFA solution enters the system, the tail and paws will move or twitch (Figure 1G). When the paws and tail become rigid and inflexible, stop the pump.

17. Remove the needle from the heart, flush the needle and the tubes with water, refill with 1× PBS in preparation for the next animal.
18. Decapitate the mouse with the bandage scissors: cut at the neck and remove the skin longitudinally with standard scissors starting from the neck to the ears (Figure 1H).
19. Using Iris scissors, remove the skull from the occipital region longitudinally to the nasal bones (Figures 1I and 1J).
20. Using forceps, lift the right and left portions of the skull bone in order to reveal the brain (Figure 1K).
21. Use a spatula to remove the brain and place it in the 20 mL vial containing 10 mL of 4% PFA (Figure 1L).
22. Keep brain overnight in 4% PFA solution at 4°C.

Brain saturation in sucrose solution

   ⏱ Timing: 3–4 days

Sucrose prevents the formation of crystal formations within the tissue during the cryo-preservation and -embedding process. Here, we will go over a step-by-step procedure to ensure proper treatment.

23. The next day the brain should have sunk to the bottom of the vial. Replace with 5% sucrose in PBS 1×, gently shake for 1 h at 4°C.
24. Replace the sucrose 5% solution with 10% sucrose water solution, gently shake for 1 h at 4°C.
25. Replace with 20% sucrose water solution and wait until the brain sinks to the bottom of the vial. This can take from 2 h to overnight.
26. When the brain has sunk to the bottom of the vial again, after 2 h or the next day, replace with 30% sucrose water solution and place the vial at 4°C overnight (Figure 2A).

Cryostat brain sectioning

   ⏱ Timing: 1 day

This section will explain how to properly embed tissue for a sagittal section.

27. Carefully remove the brain from 30% sucrose solution with a spatula, place the brain on a Kimwipe and dry it by applying extremely light pressure (do not damage the brain) (Figure 2B).
28. Remove the extra sucrose between the brain hemispheres with the use of a small 10 μL tip and delicately push the Kimwipe between the hemispheres (Figures 2C and 2D).
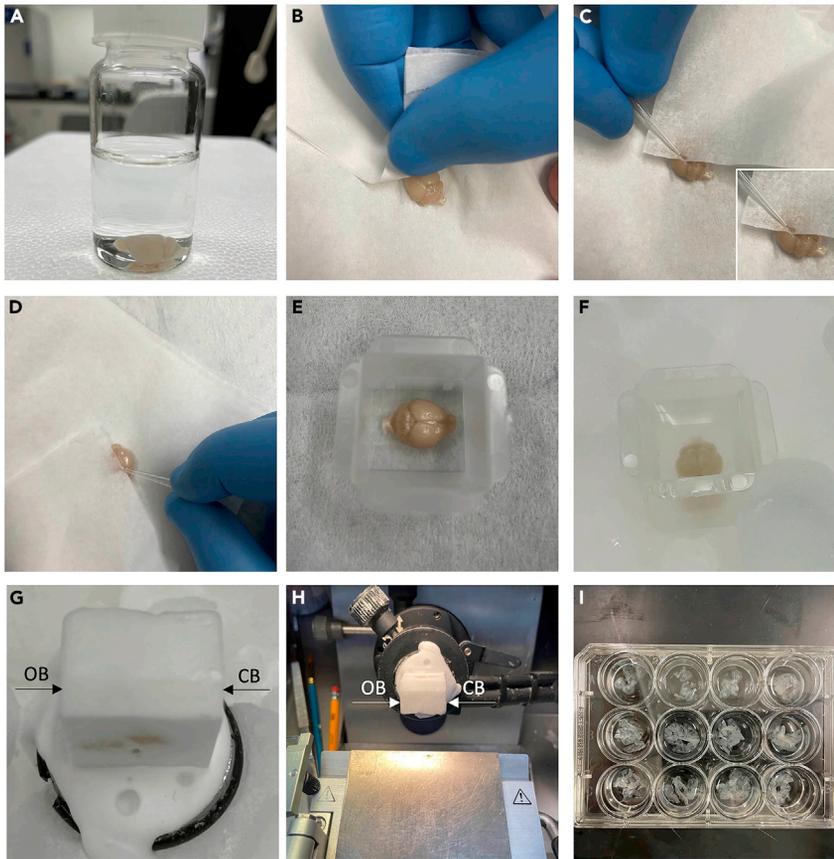
Figure 2. Brain saturation in sucrose and O.C.T. embedding
(A) Brain at the bottom of the vial with 30% sucrose water solution.
(B) Remove the extra sucrose from the brain with a Kimwipe.
(C and D) Remove the extra sucrose from the hemispheres with a Kimwipe and a 10 μm tip.
(E) Place the brain in O.C.T. and align the tissue.
(F) Place the O.C.T. brain in dry ice.
(G) When frozen take out the embedded tissues in O.C.T. and fix it into the cryostat chuck in dry ice.
(H) Place the chuck with the embedded tissue in the cryostat and align it to be sectioned.
(I) Collect the sectioned slices in a 12-well plate. OB, olfactory bulb; CB, cerebellum.

29. Place the brain into a freezing mold, cover with Tissue-Plus™ O.C.T. Compound (an embedding medium for frozen tissues specimens to ensure optimal cutting temperature) and with the spatula flip over itself a couple of times to coat the brain with O.C.T.
30. Gently transfer the brain at the bottom of a new freezing mold and gently push it down to make it as flat as possible (Figure 2E).

⚠ CRITICAL: When covering with O.C.T. do it first at room temperature very slowly to avoid bubbles. Too many bubbles, in particular close to the tissue, will affect the sectioning process.

Note: In this protocol we use sagittal sections. Be sure the brain is flat when placed at the bottom of the mold and annotate the front (olfactory bulbs), rear (cerebellum), left, and right side of the brain outside the mold with a permanent marker in order to preserve the orientation. Alternatively, you can first cut the brain in half along the sagittal axis and include the two half separately in order to process them in two different phases.

31. Surround the freezing mold containing the brain and O.C.T. with dry ice in order to slowly solidify the O.C.T (Figure 2F).
32. Take the frozen O.C.T. embedded brain out from the mold and fix it into the cryostat chuck attaching the frontal-occipital part of the brain with a small amount of O.C.T. in dry ice (Figure 2G); in this way the brain is ready to be sectioned sagittally.
33. Mount the frozen O.C.T. embedded tissue on Leica CM3050S Cryostat and start sectioning sagittally at 40 μm thickness (Figure 2H).

   Note: Brain tissues embedded in O.C.T. can be stored at -80°C for a long time. Before sectioning the tissue, place the O.C.T.-embedded tissues at -20°C in the cryostat for two hours to equilibrate the tissue. Do not leave the O.C.T.-embedded tissues in the cryostat overnight because the cryostat usually is set to defrost every 24 h. Alternatively, you may take out the O.C.T.-embedded tissue out of -80°C and leave it in the -20°C the night before sectioning.

34. Collect the slices in a 12-well plate filled with 1 mL of PBS 1× with 0.02% of Sodium Azide.

   Note: The wells can accommodate four slices from adult mice without crowding. While the number of slices you can accommodate for wells depends on your genotype (e.g., smaller brain), we nevertheless recommend that you don't go over four.

35. Keep track of how many slices you suspend in each well and store them at 4°C (Figure 2I) this information will help you estimate where the middle of the brain is (for the next step).
36. After all the slices have been collected, we recommend mounting them beginning with the middle sections of the brain, which will be the largest sagittal sections (and also contain the largest sections of the cerebellum).

   Note: If you are dissecting a wild-type adult mouse, and you do not lose any slices, you will likely place four slices in each well and use six 12-well plates. 6 × 12 = 72 wells, so your 36th well will contain slices from the middle of the brain. Start the mounting from this well and move one well at a time toward either side of the brain.

   ⚠ CRITICAL: One adult mouse brain sectioned into 40 μm slices can be accommodated in five to six 12-well plates. Considering the large number of sections, we recommend the use of Sodium Azide at 0.02% to avoid bacterial growth over longer storage periods.

Purkinje cells immunofluorescence

   ⏱ Timing: 5 days

The immunofluorescence method and imaging of Purkinje neurons will be described in this section.

37. Use the disposable 2 mL plastic transfer pipet (cut off the thin tip first for this passage) to transfer the sections from the 12 well plate where they were collected during the sectioning to a new 12 well plate already filled with PBS 1×.
38. Rinse for 10′ on a Rocker II at room temperature.
39. Remove the PBS 1× using a transfer pipette and block the sections with 0.5 mL–1 mL blocking solution. Gently shake the sections for 1 h at 4°C on a Rocker II.

   Note: Select 4–5 sections per brain from the middle of the brain to cover the entire structure of the cerebellum.

   ⚠ CRITICAL: We find that 0.5 mL of blocking solution seems to work best with the Leica microscope, but 1 mL seems to give a better signal with other confocal microscopes.
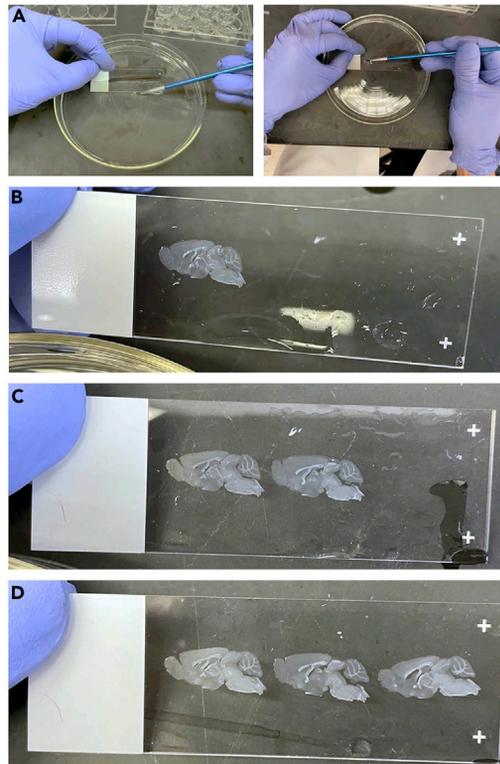
Figure 3. Transferring the O.C.T. brain sections to slides
(A) Handling brain sections in a PIREX reusable Petri dish bottom filled in half with PBS 1×.
(B–D) Gently spread the sections on the slides using the brush to flatten them. One to three sections can easily fit onto one microscope slide.

40. Remove the blocking solution and incubate the sections in 0.5 mL blocking solution containing the primary antibody Rabbit α-IP3R1 (1:500, Pierce) and mouse α-Calbindin (1:1000) for 48 h at 4°C gently shaking on a Rocker II.
41. Wash the sections 4 times with TBS-T 1× gently shaking, 20 min per wash.
42. Wrap the 12-well plate with an aluminum foil to avoid the bleach of the fluorescent signal.
43. Incubate the sections in 0.5 mL of blocking solution containing 1:1500 of secondary antibody conjugate to a fluorophore for 48 h at 4°C, gently shaking.
44. Wash the sections 4 times with TBS-T, gently shaking, 20 min per wash.
45. Rinse off TBS-T 1× with PBS 1× before mounting the sections.
46. Cut off the thin tip of the plastic transfer pipette, treat the inside of the pipette with 2% BSA in PBS 1×. Use this treated pipette for transferring the sections to the slides.
47. Fill in half the PIREX reusable Petri dish bottom with PBS 1×.
48. Transfer the sections to the Petri dish using a transfer pipet (we suggest to cut the tip by 1–2 cm to make it blunt).
49. Gently arrange the sections to the slides using the brush without folds or wrinkles. Be careful to not damage the tissue sections (Figures 3A and 3B).
50. Remove the extra PBS 1× with vacuum or Kimwipe, 1–3 sections can be mounted on one slide with one 25 mm × 25 mm cover glass (Figures 3C and 3D).
51. Use one drop of PROLONG-GOLD antifade mounting medium for one 25 mm × 25 mm cover glass.
52. Use the vacuum with a 200 µL tip to remove the excess mounting medium around the cover glass and between the slide and cover glass.
53. Leave the slides in the dark and let them dry overnight. Seal the slides the following day with clear nail polish, let dry, and store at 4°C.
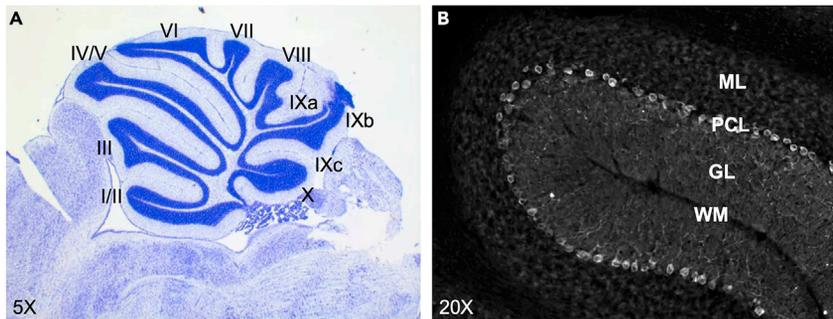
Figure 4. Murine cerebellar 2D structure
(A) Nissl staining and brightfield imaging of mouse cerebellum at 8 weeks of age. Cardinal numbers indicate the ten cerebellar folia.
(B) 20× magnification of DAPI staining of adult mouse folium showing the molecular layer (ML), Purkinje cells layer (PCL), granule cell layer (GL) and white matter (WM).

⚠ CRITICAL: From step 42 onward the 12-well plates and the mounted sections need to be protected from light as much as possible.

54. Images for immunohistochemistry are taken from anterior lobules with confocal microscopy.
55. Acquire images of the cerebella stained with IP3R1 and Calbindin at 20× and 63× magnification using the Z-Stack imaging function and acquiring at least 40 images at 1 μm thickness.
56. Using Leica Application Suite Advanced Fluorescence make 3D-reconstruction of the Z-Stack acquired, using the pre-standard and automatic "Volume reconstruction" function.
57. Save all the images in single channel and in 3D.

Note: The fully developed cerebellum is composed of ten folia (Figure 4A), each of which contains three layers (Figure 4B). Because Purkinje cells degenerate at different rates in different folia, we recommend that you scan the entire cerebellum first with a 10× or 20× objective, then focus on the larger/middle folia (V to VIII) that include more aligned Purkinje cells.

Note: Here we used two antibodies, Calbindin and IP3R1. Both can, in principle, mark both soma and dendrites. We find, however, that Calbindin is best for visualizing Purkinje cell bodies, while IP3R1, which is a large-conductance cation channel mediating Ca^{2+} release, is best for marking the dendrites.

Note: The more images acquired by Z-stack, the higher the 3D resolution will be. Since acquiring more images takes more time and increases the likelihood of signal bleaching, we recommend the acquisition to 40 images (1 μm thick).

EXPECTED OUTCOMES

The combined IP3R1-Calbindin staining allows us to see the whole Purkinje cell and assess cerebellar morphology. Calbindin is more highly expressed in the soma, while IP3R1 is found in both the soma and dendrites (Laure-Kamionowska and Maslinska, 2009; Storey and Gardner, 2012). If successful, with this protocol, we can achieve enough resolution to count every single Purkinje neuron across all cerebellar layers (Figure 5).

To compare a healthy cerebellum with a degenerated one, Figure 5 shows the same staining in wild-type and $Pum1^{-/-}$ mice (also known as SCA47 mice) at 3 and 10 weeks of age. The latter show frank Purkinje cell degeneration (Figure 5). Interestingly, in comparison to wild-type animals, the SCA47 mice can be seen to have defects in their dendritic arbors starting at 3 weeks of age (Figures 5A and 5B). However, the 3D reconstruction staining reveals that at 10 weeks of age Purkinje cell loss occurs in the middle layers,
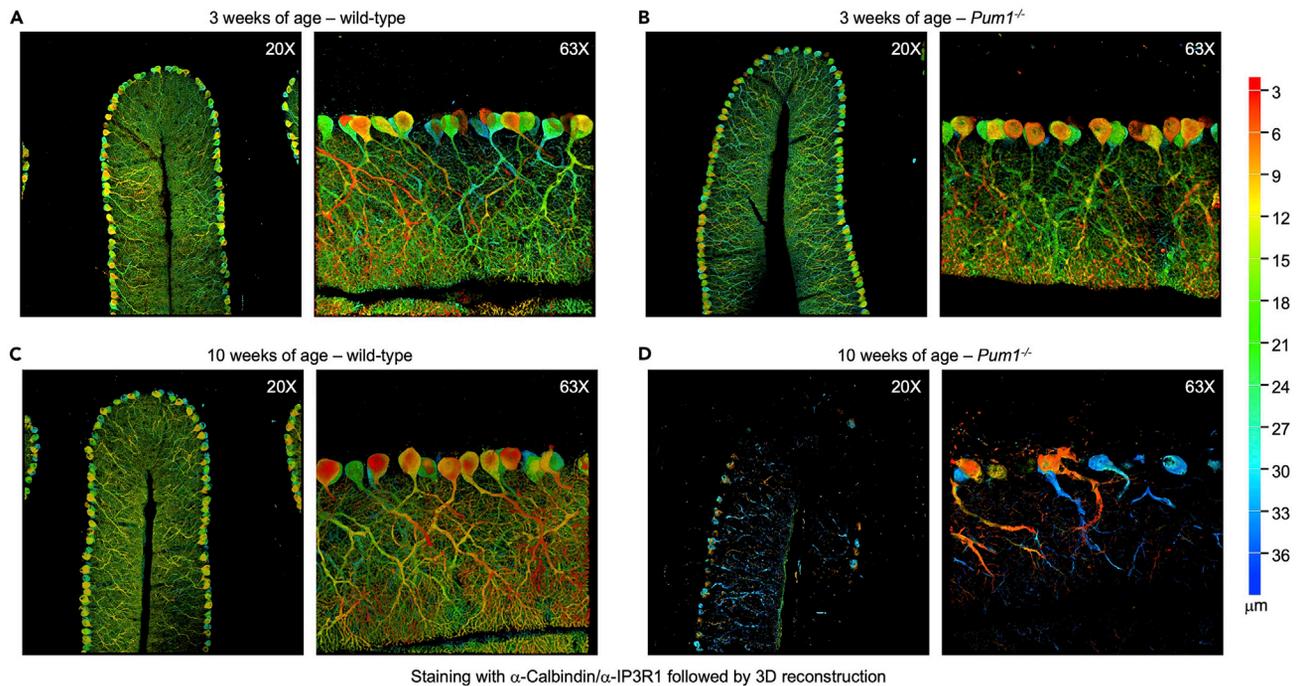
Figure 5. Expected outcome of Purkinje cell imaging in 3D reconstruction with IP3R1/Calbindin

(A and B) 3D image reconstruction of Purkinje neurons acquired at 20× (*left*) and 63× (*right*) in (A) wild-type mice and (B) *Pum1*$^{-/-}$ mice at 3 weeks of age. (C and D) 3D image reconstruction of Purkinje neurons acquired at 20× (*left*) and 63× (*right*) in (C) wild-type mice and (D) *Pum1*$^{-/-}$ mice at 10 weeks of age. Color scale representing the depth across cerebellar layers is automatically generated from the software and is shown on the right from red (1 μm) to blue (40 μm). Staining was performed with antibodies against IP3R1 and Calbindin.

where the color range between the yellow and green has disappeared, leaving some neurons in blue and red, indicating the lower and upper layers, respectively (Figures 5C and 5D).

To demonstrate the benefit of using the two antibodies simultaneously, Figure 6 shows cells with staining against only calbindin or IP3R1. The 3D reconstruction still improves signal quality, staining against Calbindin highlights the Purkinje bodies, while the staining against IP3R1 is better for dendrite resolution (Figure 6). This protocol, therefore, improves the visualization of Purkinje cells at single-neuron resolution and across layers; it also provides sufficient resolution of the granule cell layer that you can trace the ramifications of individual dendrites.

## QUANTIFICATION AND STATISTICAL ANALYSIS
Alternative 3D reconstruction with Fiji

⏱ Timing: 1 day

In this paragraph, we will explain how to use Fiji to obtain a 3D reconstruction of confocal images.

Any confocal microscope with 3D software can be used with this protocol. While the automatic setup of Leica 3D software does not require any optimization, other software from other microscopes may. Therefore, as proof-of-principle, we re-ran the same protocol using Zeiss LSM 800 and re-created the 3D reconstruction in Fiji (Figure 7). Please find a step-by-step guide using Fiji below.

1. Open the experiment file (.czi for Zeiss microscopy or .lif for Leica microscopy) using FIJI software (Figure 7A).
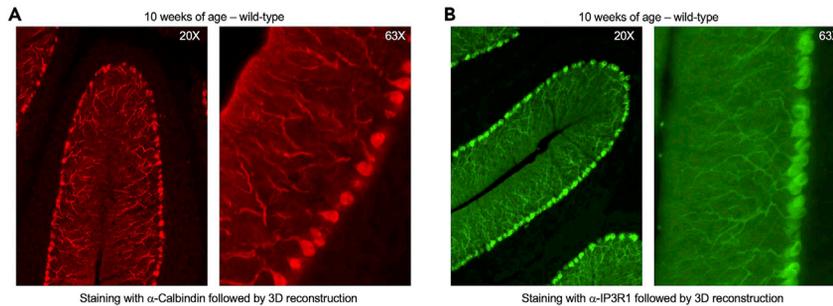
Figure 6. Typical outcome of Purkinje cell imaging in 3D reconstruction with a single antibody
(A and B) 3D image reconstruction of Purkinje neurons acquired at 20× (*left*) and 63× (*right*) in wild-type mice at
10 weeks of age with staining against Calbindin (A, red) or IP3R1 (B, green).

2. Step 1 will open a "Bio-formats import Options" window. Select "Hyperstack" in the "View Stack with:" under "Stack viewing" and press "OK" (Figure 7B).
3. Separate the channels by selecting "Image", then "Color", and finally "Split Channel" (Figure 7C).
4. Under "Image" click "Color" and then "Merge Channels" (Figure 7D).
5. Under the "Merge Channels" tab we used C1 in red (for calbindin), and C2 in green (for IP3R1). Note that to avoid using red and green in the same figure, you can choose other colors that suit your purpose, e.g., red and blue. After choosing the color select "Create composite" (Figure 7E).
6. Go under "Image", "Stack" and then "Z Project…" (Figure 7F).
7. Under the "ZProjection" tab choose the number of slices you want to reconstruct (in this example we use 10), and select "Average Intensity" from "Projection type" menu (Figure 7G). You may choose "Max Intensity" if you think your signal is low.
8. Under "Image" click "Color" and then "Stack to RGB" (Figure 7H). This will convert your file in RGB.
9. Export the image in TIFF. Under "File" click "Save as" and then choose the tiff format to obtain the best quality photo (Figure 7I).

LIMITATIONS

The success of this protocol depends on several factors that can be grouped into two classes: quantities and signal-to-noise ratio. In the first category: a) the entire brain must be mounted, not just the cerebellum, in order to avoid any damage and maintain the correct orientation; b) the quantities of antibodies used for the staining and the time of incubation must be optimized based on the size of the section you choose to image; c) it is important to image different cerebellar folia to be sure to capture the full Purkinje cell and perform a sufficient number of different biological replicates to ensure the significance of morphological changes detected. In the second category, to maximize the signal-to-noise ratio: d) the perfusion must be extremely thorough, leaving no blood in the mouse brain; e) the post-perfusion sucrose steps must also be well executed; f) adjusting the intensity of the signal with reference to DAPI helps avoid either high background noise or very low signal, which depend on antibody penetrance and confocal bleaching.
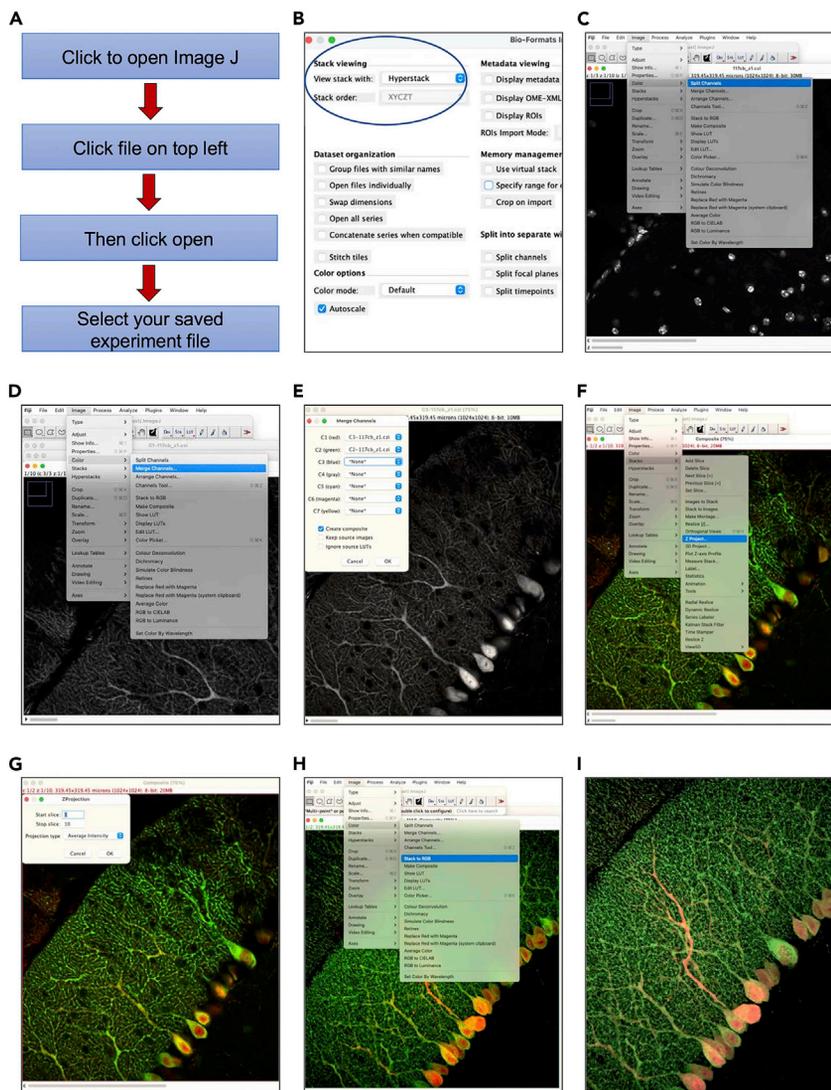
TROUBLESHOOTING

Problem 1
During the buffer changes (steps 41–44), the cerebellum detaches from the rest of the brain.

Potential solutions
Try to replace the 2 mL plastic transfer pipette with P200 pipette using a 200 μL tip. Restore the integrity of the cerebellar sections when they are transferred on the slides to be mounted.

Figure 7. Purkinje cells 3D reconstruction using FIJI software
(A-I) Diagram illustrating how to reconstruct Z-stack images of slices dual-stained with antibodies against IP3R1 and Calbindin as described in the main text.

Problem 2
From step 42. Fluorescence bleaching.

Potential solutions
Switch off the laser by switching off the light source when the image acquisition is complete. To avoid any light exposure in between image acquisition and sample storage, store all the slides in a dark slides box. Also, protect each step from light.

Problem 3
During the mounting steps (step 49), when aligning the brain on the slides, the sections move on the slide.

Potential solutions
After transferring the brain slices into the slide, try to dry the surrounding solution to avoid to move the sections from the slides. When applying the mounting medium, 1 or 2 drops is usually enough.

Problem 4

High background noise in immunofluorescence (IF) experiment (step 54).

Potential solutions

Titrate the amount of NGS in blocking solution from 2% up to 10%. Also, adjust the timing and the amount of secondary antibody used.

Problem 5

Only one antibody is working (step 54). This can happen when one of the antibodies does not penetrate across the sections.

Potential solutions

Add a permeabilization step using a solution of 1× PBS with 0.1% Triton-X for 15' at room temperature. Alternatively, instead of adding a permeabilization step, add an antigen-reveal step using citrate buffer pH7.4.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Vincenzo A. Gennarino (vag2138@cumc.columbia.edu).

Materials availability

This study did not generate unique reagents.

Data and code availability

No software was generated for this project. All software used in this study is publicly available and links are provided as appropriate in different sections of the methods and key resources table.

AUTHOR CONTRIBUTIONS

S.B. collected the data and drafted the technical sections of the manuscript. A.C. contributed to the draft and generated the figures for the perfusion section. V.A.G. designed the protocol, drafted, and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Baltanas, F.C., Berciano, M.T., Santos, E., and Lafarga, M. (2021). The childhood-onset neurodegeneration with cerebellar atrophy (CONDCA) disease caused by AGTPBP1 gene mutations: the Purkinje cell degeneration mouse as an animal model for the study of this human disease. Biomedicines 9, 1157. https://doi.org/10.3390/biomedicines9091157.

Chen, H.K., Fernandez-Funez, P., Acevedo, S.F., Lam, Y.C., Kaytor, M.D., Fernandez, M.H., Aitken,

A., Skoulakis, E.M., Orr, H.T., Botas, J., and Zoghbi, H.Y. (2003). Interaction of Akt-phosphorylated ataxin-1 with 14-3-3 mediates neurodegeneration in spinocerebellar ataxia type 1. Cell 113, 457–468. https://doi.org/10.1016/s0092-8674(03)00349-0.

Gennarino, V.A., Palmer, E.E., McDonell, L.M., Wang, L., Adamski, C.J., Koire, A., See, L., Chen, C.A., Schaaf, C.P., Rosenfeld, J.A., et al. (2018). A mild PUM1 mutation is associated with adult-onset ataxia, whereas haploinsufficiency causes

developmental delay and seizures. Cell 172, 924–936.e11. https://doi.org/10.1016/j.cell.2018.02.006.

Gennarino, V.A., Singh, R.K., White, J.J., De Maio, A., Han, K., Kim, J.Y., Jafar-Nejad, P., di Ronza, A., Kang, H., Sayegh, L.S., et al. (2015). Pumilio1 haploinsufficiency leads to SCA1-like neurodegeneration by increasing wild-type Ataxin1 levels. Cell 160, 1087–1098. https://doi.org/10.1016/j.cell.2015.02.012.

Hornig, M., Weissenbock, H., Horscroft, N., and Lipkin, W.I. (1999). An infection-based model of neurodevelopmental damage. Proc. Natl. Acad. Sci. U S A 96, 12102–12107. https://doi.org/10.1073/pnas.96.21.12102.

Huang, M., and Verbeek, D.S. (2019). Why do so many genetic insults lead to Purkinje Cell degeneration and spinocerebellar ataxia? Neurosci. Lett. *688*, 49–57. https://doi.org/10.1016/j.neulet.2018.02.004.

Jafar-Nejad, P., Ward, C.S., Richman, R., Orr, H.T., and Zoghbi, H.Y. (2011). Regional rescue of spinocerebellar ataxia type 1 phenotypes by 14-3-3epsilon haploinsufficiency in mice underscores complex pathogenicity in neurodegeneration. Proc. Natl. Acad. Sci. U S A *108*, 2142–2147. https://doi.org/10.1073/pnas.1018748108.

Kapfhammer, J.P., and Gugger, O.S. (2012). The analysis of purkinje cell dendritic morphology in organotypic slice cultures. J. Vis. Exp. 3637. https://doi.org/10.3791/3637.

Koeppen, A.H. (2018). The neuropathology of the adult cerebellum. Handb. Clin. Neurol. *154*, 129–149. https://doi.org/10.1016/b978-0-444-63956-1.00008-4.

Laure-Kamionowska, M., and Maslinska, D. (2009). Calbindin positive Purkinje cells in the pathology of human cerebellum occurring at the time of its development. Folia Neuropathol. *47*, 300–305.

Manto, M. (2012). Toxic agents causing cerebellar ataxias. Handb Clin. Neurol. *103*, 201–213. https://doi.org/10.1016/b978-0-444-51892-7.00012-7.

Orr, H.T. (2012). SCA1-phosphorylation, a regulator of Ataxin-1 function and pathogenesis. Prog. Neurobiol. *99*, 179–185. https://doi.org/10.1016/j.pneurobio.2012.04.003.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682. https://doi.org/10.1038/nmeth.2019.

Storey, E., and Gardner, R.J. (2012). Spinocerebellar ataxia type 15. Handb Clin. Neurol. *103*, 561–565. https://doi.org/10.1016/b978-0-444-51892-7.00037-1.

Whitney, E.R., Kemper, T.L., Rosene, D.L., Bauman, M.L., and Blatt, G.J. (2008). Calbindin-D28k is a more reliable marker of human Purkinje cells than standard Nissl stains: a stereological experiment. J. Neurosci. Methods *168*, 42–47. https://doi.org/10.1016/j.jneumeth.2007.09.009.