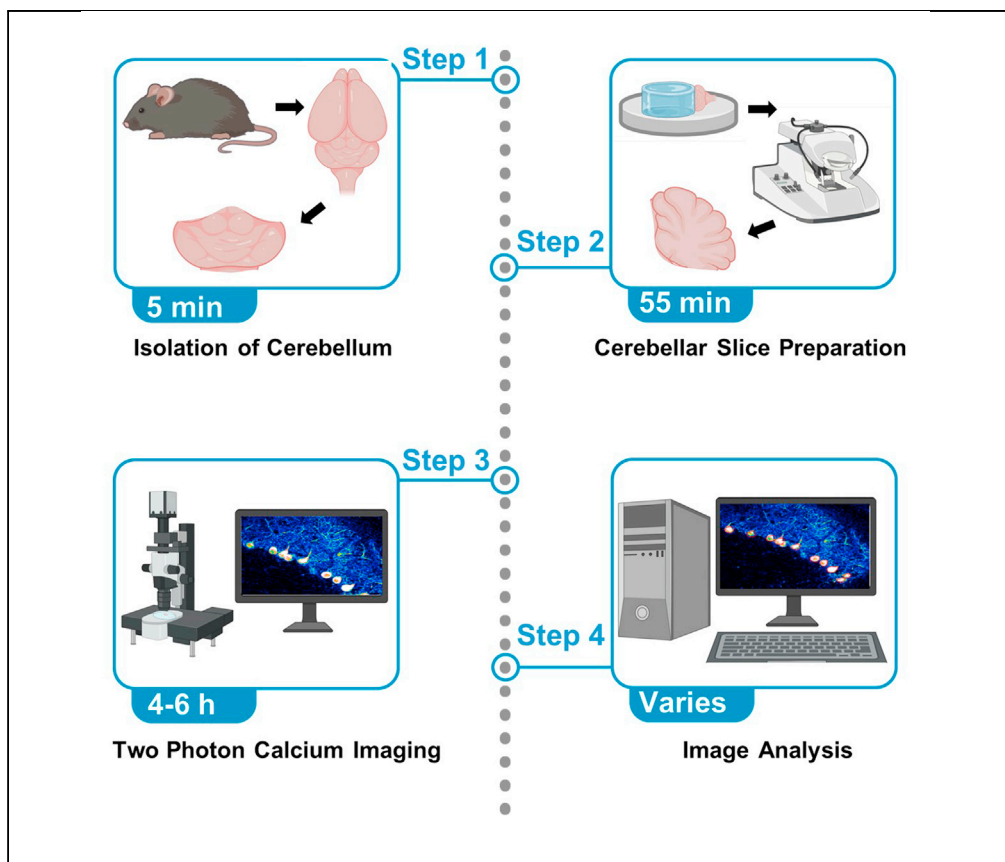


## Protocol

# Two photon imaging of calcium responses in murine Purkinje neurons



Purkinje neurons (PNs) are an important component of the motor learning and coordination circuit and are affected in spino-cerebellar ataxias. Maintaining healthy PNs in cerebellar slices and recording their  $\text{Ca}^{2+}$  transients can be challenging. Here, we describe a protocol for measuring  $\text{Ca}^{2+}$  transients in PNs from adult mice, including problem-solving tips. This protocol can be used to measure neuronal excitability and agonist-mediated  $\text{Ca}^{2+}$  signaling in cerebellar slices expressing a genetic  $\text{Ca}^{2+}$  reporter in all PNs, thus improving yield of data.

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### Highlights

The protocol describes ways of maintaining viable Purkinje neurons in slices for hours

It details measuring neuronal excitability and agonist mediated  $\text{Ca}^{2+}$  signaling in PNs

It describes how to obtain PNs labeled with a genetically encoded  $\text{Ca}^{2+}$  sensor, GCaMP6

It demonstrates altered evoked  $\text{Ca}^{2+}$  transients from PN-specific STIM1 knockout neurons

Dhanya & Hasan, STAR  
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## Protocol

## Two photon imaging of calcium responses in murine Purkinje neurons

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<https://doi.org/10.1016/j.xpro.2021.101105>

## SUMMARY

Purkinje neurons (PNs) are an important component of the motor learning and coordination circuit and are affected in spino-cerebellar ataxias. Maintaining healthy PNs in cerebellar slices and recording their Ca<sup>2+</sup> transients can be challenging. Here, we describe a protocol for measuring Ca<sup>2+</sup> transients in PNs from adult mice, including problem-solving tips. This protocol can be used to measure neuronal excitability and agonist-mediated Ca<sup>2+</sup> signaling in cerebellar slices expressing a genetic Ca<sup>2+</sup> reporter in all PNs, thus improving yield of data. For complete details on the use and execution of this profile, please refer to Dhanya and Hasan (2021).

## BEFORE YOU BEGIN

The novelty of this protocol is that it allows measurement of Ca<sup>2+</sup> responses across multiple Purkinje neurons (PNs) from cerebellar slices maintained in a viable state for hours. The protocol describes how to obtain PNs labeled with a genetically encoded Ca<sup>2+</sup> sensor, such that all PNs express the Ca<sup>2+</sup> sensor. If needed, it can be modified for introducing a Ca<sup>2+</sup> sensor by viral transfection where fewer PNs would be labeled. Most importantly, this protocol describes various critical conditions that need to be followed to maintain healthy PNs in cerebellar slices for several hours that allow the recording of both basal and evoked Ca<sup>2+</sup> transients. This important aspect highlights the strengths and advances of this protocol compared to existing ones (Roome and Kuhn, 2018; Grienberger and Konnerth, 2012; Carrier-Ruiz et al., 2021). As an example of PNs with altered evoked Ca<sup>2+</sup> transients, we show data from STIM1 PN-specific knockout cells. All experiments were performed in accordance with the Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India. Experimental protocols were approved by the Institutional Animal Ethics Committee, National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bangalore. All transgenic mice used for the experiment were bred and maintained in the NCBS Animal Facility, Bangalore, India on a 12-h light dark cycle provided with ad libitum access to food and water. All mice in the study were group housed with 2–6 mice/cage and both male and female mice aged 4 months were used for the experiments.

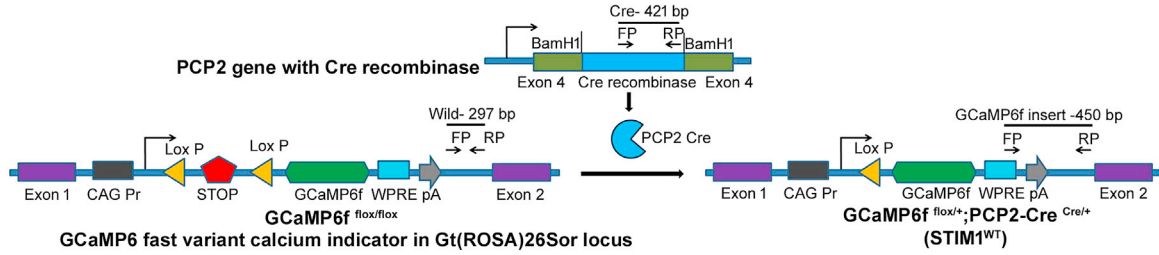
## Generate transgenic mice

⌚ Timing: 4 months

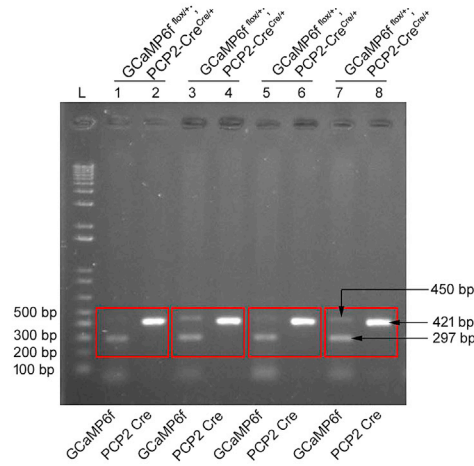
1. Obtain transgenic mice of the genotype *GCaMP6f<sup>fllox/fllox</sup> [B6;129S-Gt(ROSA)26Sor<sup>tm95.1(CAG-GCaMP6f)Hze</sup>/J*, RRID: IMSR\_JAX: 024105 -The Jacksons Laboratory] in which the genetically-encoded fast calcium indicator GCaMP6f can be expressed downstream of a Cre-dependent promoter (Figure 1A).



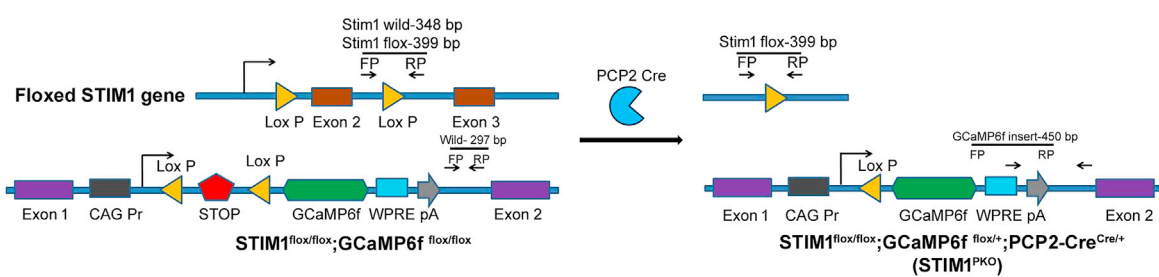
**A Generation of GCaMP6f<sup>lox/+</sup>; PCP2<sup>Cre/+</sup> mouse strain**



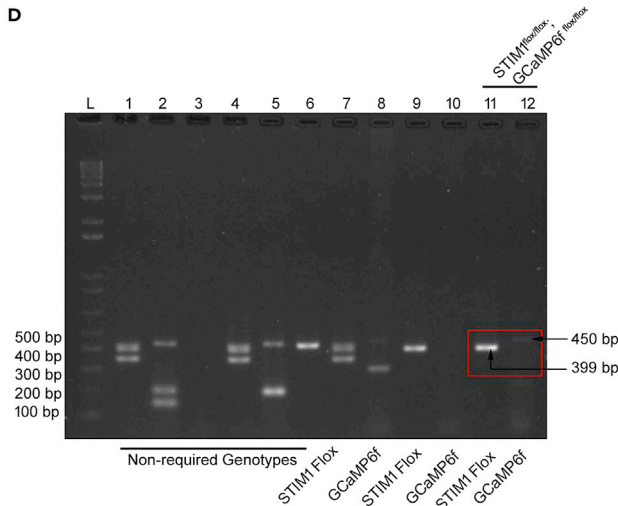
**B**



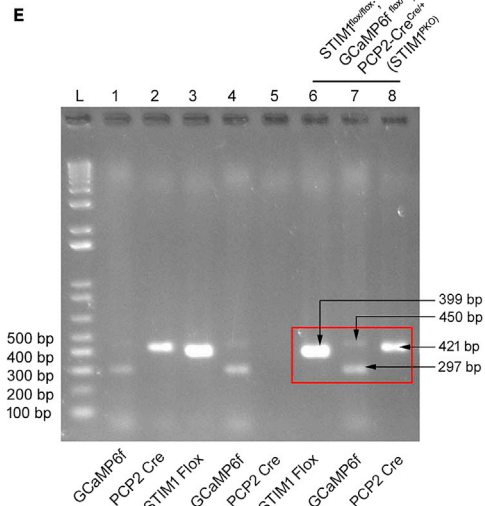
**C Generation of STIM1<sup>lox/flox</sup>; GCaMP6f<sup>lox/+</sup>; PCP2<sup>Cre/+</sup> mouse strain**



**D**



**E**



**Figure 1. Generation and genotyping of GCaMP6f compound strain**

(A) Schematic representation of generation of  $GCaMP6^{flox/+};PCP2-Cre^{cre/+}$  transgenic mouse strain.  $GCaMP6^{flox/flox}$  transgenic mouse contain floxed-STOP cassette inserted upstream of the GCaMP6 fast variant calcium indicator (GCaMP6f) between endogenous exons 1 and 2 of the *Gt(ROSA)26Sor* locus. A GCaMP6f mouse line expressing GCaMP6f exclusively in Purkinje neurons is generated by breeding  $GCaMP6^{flox/flox}$  with  $PCP2-Cre$ . Location of the primers are indicated by arrows (FP- Forward Primer and RP- Reverse Primer).  
 (B) Agarose gel with genotyping of control mice. PCRs from genomic DNA of  $GCaMP6^{flox/+};PCP2-Cre^{cre/+}$  mice are shown (lanes 1–8). Two bands at 297 bp (*Rosa<sup>WT</sup>*) and 450 bp (*GCaMP6f* insert in the *Rosa* locus) (lane 1, 3, 5, 7) and a single band at 421 bp for *PCP2Cre* (lane 2, 4, 6 and 8).  
 (C) Schematic diagram showing the generation of  $GCaMP6^{flox/+};STIM1^{flox/flox};PCP2-Cre^{cre/+}$  transgenic mouse strain.  $STIM1^{PKO}$  mouse line with GCaMP6f expression in Purkinje neurons is generated by breeding homozygous double transgenic  $GCaMP6^{flox/flox};STIM1^{flox/flox}$  with  $STIM1^{flox/+};PCP2-Cre^{cre/+}$ .  
 (D) Genotyping by PCRs of genomic DNA from double transgenic  $GCaMP6^{flox/flox};STIM1^{flox/flox}$  mouse strains are shown in lanes 11 and 12. A single band at 399 bp is from homozygous *STIM1 flox* (lane 11) and band at 450 bp (lane 12) is for the homozygous *GCaMP6f* insert. (E) Genotyping in lanes 6–8 showing PCRs of  $GCaMP6^{flox/+};STIM1^{flox/flox};PCP2-Cre^{cre/+}$  mice. Sizes of DNA bands for all genes are as described above for B and D. 'L' denotes DNA Ladder in panels B, D and E.

**Note:** Upon expression, GCaMP6f exhibits low EGFP fluorescence in absence of a  $Ca^{2+}$  stimulus and high EGFP fluorescence following a stimulus that leads to a rise in intracellular  $Ca^{2+}$  (Madisen et al., 2015).

- Obtain the PCP2 Cre mouse strain [B6.129-Tg(Pcp2-cre)2Mpin/J-The Jacksons Laboratory, RRID: IMSR\_JAX: 004146, (Barski et al., 2000)] that expresses the Cre protein under control of the PN specific PCP2 promoter (Figure 1A).
- Cross  $GCaMP6^{flox/flox}$  mice with PCP2 Cre mice to enable *GCaMP6f* expression exclusively in the Purkinje neurons (Figures 1A and 1B). The double transgenic mouse strain  $GCaMP6^{flox/+};PCP2^{Cre/+}$  was considered as the wild type control for two photon  $Ca^{2+}$  imaging.

**Alternatives:** Instead of transgenic mouse lines, stereotaxic injections of viral vectors can be performed to induce expression of  $Ca^{2+}$  indicators and/or cre recombinase (Dittgen et al., 2004; Inquimbert et al., 2013; McSweeney and Mao, 2015; Levin et al., 2016; Haery et al., 2019). However, several studies have reported neurotoxicity when the virus was delivered via direct injections or systemically into the CNS (Haery et al., 2019; Johnston et al., 2021). Furthermore, low efficiency of gene transfer into adult neurons, late onset of transgene expression and risk of insertional mutations are some of the drawbacks of viral gene delivery systems (Levin et al., 2016).

- The homozygous transgenic strain  $Gcamp6^{flox/flox};STIM1^{flox/flox}$  was crossed with the double transgenic strain  $STIM1^{flox/+};PCP2^{Cre/+}$  to obtain a triple transgenic mouse strain  $Gcamp6^{flox/+};STIM1^{flox/flox};PCP2^{Cre/+}$  that was considered as the *STIM1* knockout strain ( $STIM1^{PKO}$ ) for the  $Ca^{2+}$  imaging experiments (Figures 1C–1E).

**Genotyping of the transgenic mice**

⌚ Timing: 15 h (including an overnight incubation for 12 h)

- Isolate genomic DNA from tail clippings (Age of the mice: 4 months).
  - Cut 0.5–1 cm mouse tail and transfer into 1.5 mL Eppendorf (microcentrifuge) tube.
  - Add 150  $\mu$ L tail lysis buffer (see materials and equipment) containing proteinase K (1.5  $\mu$ L of 20 mg/mL Proteinase K for 150  $\mu$ L of Tail Lysis Buffer) and vortex briefly.

**Note:** Proteinase K should be freshly added to the working tail lysis buffer each time during DNA isolation.

- Incubate at 55°C in a water bath overnight.
- Vortex briefly to mix.

**Table 1. PCR reaction mixture**

Reagent	Final concentration	Amount
10X Taq Buffer	1X	1.5 $\mu$ L
F. Primer (100 ng/ $\mu$ L)	3.3 ng/ $\mu$ L	0.5 $\mu$ L
R. Primer (100 ng/ $\mu$ L)	3.3 ng/ $\mu$ L	0.5 $\mu$ L
20 mM dNTPs	400 $\mu$ M	0.3 $\mu$ L
Taq Polymerase (3 U/ $\mu$ L)	0.6 units	0.2 $\mu$ L
ddH <sub>2</sub> O	n/a	11 $\mu$ L
Genomic DNA	Crude lysate	1 $\mu$ L
<b>Total</b>	<b>n/a</b>	<b>15 <math>\mu</math>L</b>

**Note:** Once the tail has been completely digested, particulate material such as hair will be observed in the tube.

- e. Incubate at 80°C for 10 min to inactivate Proteinase K.
- f. Centrifuge at 2867 x g (8000 rpm) for 1 min.
- g. Collect the supernatant for PCR.

**Alternatives:** Instead of tail biopsy, samples for genotyping can be collected from ear punching/notching. Ear punching should be performed only after 14 days of age so that the pinnae (ears) are large enough to punch and the biopsy should be no larger than 2 mm.

6. Perform standard PCR for genotyping the offspring generated (Figures 1B, 1D, and 1E).
  - a. Prepare the PCR Reaction mixture as shown in Table 1.
  - b. PCR parameters to run the samples for genotyping are shown in Table 2.
7. PCR products are separated on a 1.5 % agarose gel, stained with ethidium bromide dye, and photographed for documentation (Figures 1B, 1D, and 1E).
  - a. The wild type and *GCaMP6f* insert strains were confirmed using the following primers: wild type forward 5'- AAGGGAGCTGCAGTGGAGTA-3', *GCaMP6f* insert strain forward: 5'-ACGAGTCG GATCTCCCTTG-3' and common reverse: 5'-CCGAAAATCTGTGGGAA GTC-3' (product length for wild type: 297 bp, product length for *GCaMP6f* insert: 450 bp) (Figures 1A–1E).
  - b. The wild-type *Stim1* gene and the floxed *Stim1* gene were confirmed by the following primer pairs: *Stim1*-forward: 5'-CGATGGTCTCACGGTCTCT AGTTTC-3', *Stim1*-reverse: 5'-GGCTCT GCTGACCTGGAATATAGTG-3' (product length for wild-type *Stim1*: 348 bp, product length for floxed *Stim1*: 399 bp) (Oh-hora et al., 2008) (Figures 1C–1E).
  - c. Presence or absence of Cre was confirmed using the following primers Cre-forward: 5'-GCC GAAATTGCCAGGATCAG-3' and Cre-reverse: 5'AGCCACCAGCTTGCATGATC-3', respectively (product length for Cre: 421 bp) (Hartmann et al., 2014) (Figures 1A, 1B, 1D and 1E).

### Preparation of artificial cerebrospinal fluid (ACSF) solution

⌚ Timing: 1 h

**Table 2. PCR cycling conditions for genotyping**

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	30 s	35 cycles
Annealing	<i>GCaMP6f</i> wild- 60°C, <i>GCaMP6f</i> insert- 58°C, <i>STIM1</i> -60°C, Cre-67°C	30 s	
Extension	72°C	45 s	
Final extension	72°C	5 min	1
Hold	4°C	Forever	

8. Prepare two types of artificial cerebrospinal fluid (ACSF) solution: Cutting solution (ACSF-Sucrose) and ACSF solution (see materials Table). Prepare cutting solution (ACSF-Sucrose) one day before the experiment and cool it down to a near-freezing point (0°C–4°C). Cutting solution can also be prepared on the day of the experiment and flash frozen. All solutions should be prepared in distilled water and bubbled with carbogen (95% O<sub>2</sub> + 5% CO<sub>2</sub>) and their pH adjusted to 7.35 ± 0.5.

△ **CRITICAL:** Working ACSF solution should be freshly prepared on the day of the experiment and continuously bubbled with carbogen throughout the experiment. If required a 10× stock of ACSF solution without adding CaCl<sub>2</sub> and MgCl<sub>2</sub> (to prevent precipitation) could be prepared and stored at 4°C for a month.

**Note:** Using the low-sodium cutting solution (ACSF-Sucrose) will help to preserve the viability of neurons in superficial sections of acute slices.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Chemicals, peptides, and recombinant proteins</b>		
Tris HCl	Fisher Scientific	Cat#: 15965
KCl	Fisher Scientific	Cat#: 13305
MgCl <sub>2</sub>	Fisher Scientific	Cat#: M35-500
NaCl	HiMedia	Cat#: GRM853
CaCl <sub>2</sub>	Fisher Scientific	Cat#: 12135
NaH <sub>2</sub> PO <sub>4</sub>	Fisher Scientific	Cat#: M1063461000
NaHCO <sub>3</sub>	Qualigens	Cat#: Q14015
Sucrose	Qualigens	Cat#: Q15925
Glucose	Fisher Scientific	Cat#: D16-500
Gelatin	Sigma-Aldrich	Cat#: G2500
NP-40	Merck Millipore	Cat#: 492016
Tween-20	Sigma-Aldrich	Cat#: P1379
Proteinase K	Invitrogen	Cat#: 25530-015
Isoflurane (Forane Inhalant)	Abbott	Cat#: SKU: MLP4849
Taq Polymerase and 10× Taq Buffer	Genie	Cat#: 06016000317 30
Deoxyribonucleotide triphosphate (dNTP) set	Invitrogen	Cat#: 10297018
DHPG	Tocris Bioscience	Cat#: 0805
CPCCOEt	Tocris Bioscience	Cat#: 1028
<b>Experimental models: Organisms/strains</b>		
<i>STIM1</i> <sup>flox/flox</sup>	Anjana Rao	<a href="#">Oh-hora et al., 2008</a>
<i>PCP2</i> <sup>Cre/Cre</sup> (B6.129-Tg (Pcp2-cre)2Mpin/J)	The Jackson Laboratory	Cat#: JAX:004146, RRID: IMSR_JAX:004146 ( <a href="#">Barski et al., 2000</a> )
<i>GCaMP6</i> <sup>flox/flox</sup> [B6;129S-Gt(ROSA)26Sor <sup>tm95.1(CAG-GCaMP6f)Hze/J</sup> ]	The Jackson Laboratory	Cat#: JAX:024105, RRID: IMSR_JAX:024105
<i>GCaMP6</i> <sup>flox/+</sup> ; <i>PCP2</i> <sup>Cre/+</sup>	<a href="#">(Dhanya and Hasan, 2021)</a>	N/A
<b>Oligonucleotides</b>		
<i>GCaMP6f</i> forward (for wild type): AAGGGAGCTGCAGTGGAGTA	Bioserve ( <a href="mailto:oligodept@bioserveindia.com">oligodept@bioserveindia.com</a> )	<a href="https://www.jax.org/Protocol?stockNumber=024105&amp;protocolID=27076">https://www.jax.org/Protocol?stockNumber=024105&amp;protocolID=27076</a>
<i>GCaMP6f</i> forward (for insert): ACGAGTCGGATCCCCTTG	Bioserve ( <a href="mailto:oligodept@bioserveindia.com">oligodept@bioserveindia.com</a> )	<a href="https://www.jax.org/Protocol?stockNumber=024105&amp;protocolID=27076">https://www.jax.org/Protocol?stockNumber=024105&amp;protocolID=27076</a>
<i>GCaMP6f</i> reverse: CCGA AAATCTGTGGGAAGTC	Bioserve ( <a href="mailto:oligodept@bioserveindia.com">oligodept@bioserveindia.com</a> )	<a href="https://www.jax.org/Protocol?stockNumber=024105&amp;protocolID=27076">https://www.jax.org/Protocol?stockNumber=024105&amp;protocolID=27076</a>
<i>Stim1</i> forward: CGATGGTCTC ACGGTCTCTAGTTTC	Bioserve ( <a href="mailto:oligodept@bioserveindia.com">oligodept@bioserveindia.com</a> )	<a href="#">(Oh-hora et al., 2008)</a>

(Continued on next page)

<b>Continued</b>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Stim1 reverse: GGCTCTGCTGA CCTGGAAGTATAGTG	Bioserve ( <a href="mailto:oligodept@bioserveindia.com">oligodept@bioserveindia.com</a> )	(Oh-hora et al., 2008)
Cre-forward: GCCGAAAT TGCCAGGATCAG	Bioserve ( <a href="mailto:oligodept@bioserveindia.com">oligodept@bioserveindia.com</a> )	(Hartmann et al., 2014)
Cre- reverse: AGCCACCA GCTTGCATGATC	Bioserve ( <a href="mailto:oligodept@bioserveindia.com">oligodept@bioserveindia.com</a> )	(Hartmann et al., 2014)
<b>Software and algorithms</b>		
Scan Image	Vidrio Technologies, LLC	r 3.8 <a href="http://scanimage.vidriotechnologies.com/pages/viewpage.action?pageId=17400331">http://scanimage.vidriotechnologies.com/pages/viewpage.action?pageId=17400331</a>
ImageJ	Wayne Rasband, National Institutes of Health, USA	v.1.52p <a href="https://en.softonic.com/download/imagej/windows/post-download/v/1.52p">https://en.softonic.com/download/imagej/windows/post-download/v/1.52p</a>
Origin	Origin Lab Corporation, Northampton, MA, USA	v.8 <a href="https://filehippo.com/download_origin-43d/">https://filehippo.com/download_origin-43d/</a>
Primer 3	<a href="http://bioinfo.ut.ee/primer3-0.4.0/">http://bioinfo.ut.ee/primer3-0.4.0/</a>	v. 0.4.0 <a href="http://bioinfo.ut.ee/primer3-0.4.0/">http://bioinfo.ut.ee/primer3-0.4.0/</a>
<b>Deposited data</b>		
Original data for figures	This paper	<a href="https://data.mendeley.com/datasets/gzb4hf47kc/2">https://data.mendeley.com/datasets/gzb4hf47kc/2</a> (Mendeley Data, V2, <a href="https://doi.org/10.17632/gzb4hf47kc.2">https://doi.org/10.17632/gzb4hf47kc.2</a> )
<b>Other</b>		
Fine scissors	Bangalore Surgicals	NA
Fine forceps	Fischer Scientific	Cat#: 12-000-127
Coarse forceps	Fischer Scientific	Cat#: 12-000-128
Spring forceps	Bangalore Surgicals	NA
Surgical mayo scissors	Bangalore Surgicals	NA
Surgical blade (Scalpel blade No.13)	Bangalore Surgicals	NA
Spatula	Bangalore Surgicals	NA
Spoon micro spatula	Bangalore Surgicals	NA
Glass Pasteur pipette	Bangalore Surgicals	NA
Cyanoacrylate Instant Adhesive (SG0312)	Evobond Super Glue	Cat#: SG0312
Feather Razor Blades, Platinum Coated	Bombay Shaving Company	NA
Slice anchors (SHD-26GH/2)	Warner Instruments	Cat#: 64-0255
Vibratome (Vibrating Blade Microtome)	Leica Biosystems	Cat#: VT1200
Two-photon laser scanning microscope equipped with a Ti-Sapphire laser	Microscope- Custom built Two-photon laser scanning microscope Laser-Coherent Chameleon Ultra II	<a href="https://www.biorxiv.org/content/10.1101/2021.04.28.441782v2">https://www.biorxiv.org/content/10.1101/2021.04.28.441782v2</a> Cat#: 7066 (Laser)
Isoflurane anesthesia system	EZ-7000 Anesthesia Classic System	Cat#: EZ-7000

## MATERIALS AND EQUIPMENT

<b>Tail Lysis Buffer</b>		
Reagent	Final concentration	Amount
Tris HCl pH 8.5	10 mM	1 mL of 1M Tris HCl pH 8.5
KCl	50 mM	2.5 mL of 2M KCl
MgCl <sub>2</sub>	2 mM	400 μL of 0.5M MgCl <sub>2</sub>
Gelatin	0.1 mg/mL	10 mg
NP-40	0.45%	10 mL of 45%
Tween-20	0.45%	10 mL of 45%
Proteinase K	0.2 μg/ μL	1.5 μL of 20 mg/mL Proteinase K for 150 μL of Tail Lysis Buffer
<b>Total</b>	n/a	<b>100 mL</b>

**Note:** Stock solution of Tris HCl pH 8.5, KCl and MgCl<sub>2</sub> should be autoclaved before making the working solution of tail lysis buffer. Proteinase K should be freshly added to the working solution each time during DNA isolation. Tail lysis buffer can be stored at 4°C and used for months.

### Cutting Solution (ACSF-Sucrose)

Reagent	Final concentration (mM)	Amount
NaCl	87	5.08 g
KCl	2.5	0.184 g
MgCl <sub>2</sub>	7	7 mL of 1M solution of MgCl <sub>2</sub>
CaCl <sub>2</sub>	0.5	0.25 mL of 2M solution of CaCl <sub>2</sub>
NaH <sub>2</sub> PO <sub>4</sub>	1.25	0.195 g
NaHCO <sub>3</sub>	26	2.184 g
Sucrose	75	25.6 g
Glucose	10	1.8 g
ddH <sub>2</sub> O	n/a	1000 mL
<b>Total</b>	<b>n/a</b>	<b>1000 mL</b>

**Note:** Cutting solution (ACSF-Sucrose) should be adjusted to pH 7.4 using NaOH and osmolarity adjusted to 300–310 mosM with glucose. Cutting solution should be cooled down to a near-freezing point (0°C–4°C) and bubbled with carbogen (95% O<sub>2</sub> + 5% CO<sub>2</sub>) throughout the experiment.

### ACSF Solution

Reagent	Final concentration (Mm)	Amount
NaCl	125	7.30 g
KCl	2.5	0.184 g
MgCl <sub>2</sub>	1	1 mL of 1M solution of MgCl <sub>2</sub>
CaCl <sub>2</sub>	2	1 mL of 2M solution of CaCl <sub>2</sub>
NaH <sub>2</sub> PO <sub>4</sub>	1.25	0.195 g
NaHCO <sub>3</sub>	26	2.184 g
Glucose	20	3.6 g
ddH <sub>2</sub> O	n/a	1000 mL
<b>Total</b>	<b>n/a</b>	<b>1000 mL</b>

**Note:** ACSF solution should be adjusted to pH 7.4 using NaOH and osmolarity adjusted to 300–310 mosM with glucose. ACSF solution should be freshly prepared at the time of experiment and bubbled with carbogen (95% O<sub>2</sub> + 5% CO<sub>2</sub>) throughout the experiment. The ACSF solution used for recovery (ACSF-Recovery) should be kept in a water bath and maintained at 34°C before the experiment. ACSF Normal solution should be maintained at room temperature (~22°C) throughout the experiment.

### KCl solution (3M stock solution)

Prepare 3M KCl stock solution by dissolving 2.24 g of KCl powder into 10 mL of milliQ water. Add 75 μL of 3M KCl stock solution to 3 mL of ACSF bath solution in the recording chamber during calcium imaging to obtain 75 mM KCl. 3M KCl stock solution can be stored at room temperature for months.

### DHPG (10 mM stock solution)

Prepare 10 mM stock solution of DHPG by dissolving 10 mg of DHPG powder to 5.46 mL of milliQ water. Add 60 μL of 10 mM DHPG stock solution to 3 mL of ACSF bath solution in the recording



chamber during calcium imaging to obtain 200  $\mu$ M DHPG. 10 mM DHPG stock solution should be stored aliquoted in tightly sealed vials at  $-20^{\circ}\text{C}$ .

#### **CPCCOEt (10 mM stock solution)**

Prepare 10 mM stock solution of CPCCOEt by dissolving 10 mg of CPCCOEt powder to 4.04 mL of DMSO. Add 60  $\mu$ L of 10 mM CPCCOEt stock solution to 3 mL of ACSF bath solution in the recording chamber during calcium imaging to obtain 200  $\mu$ M CPCCOEt. 10 mM CPCCOEt stock solution should be stored aliquoted at  $-20^{\circ}\text{C}$  in tightly sealed vials.

## **STEP-BY-STEP METHOD DETAILS**

### **Cerebellar slice preparation**

⌚ **Timing:** 1 h per mouse

This protocol describes the critical steps involved in preparing cerebellar slices from a single mouse brain and preserving the viability of neurons in the acute cerebellar slices for long term imaging purpose of 6–8 h (Age of the mice: 4 months old).

**⚠ CRITICAL:** Steps from Isolation of mouse cerebellum (Step 1) to immersing the mounted cerebellum into the vibratome tray containing oxygenated ice-cold ACSF-Sucrose (Step 4d) should be completed within 5 minutes as it is the critical point of determining the viability of neurons in the slices.

1. Anesthetize the mouse in an induction chamber (Figure 2A) provided with 2 L/min flow of Oxygen and 2% isoflurane to maintain continuous oxygen supply (Jordan, 2021).

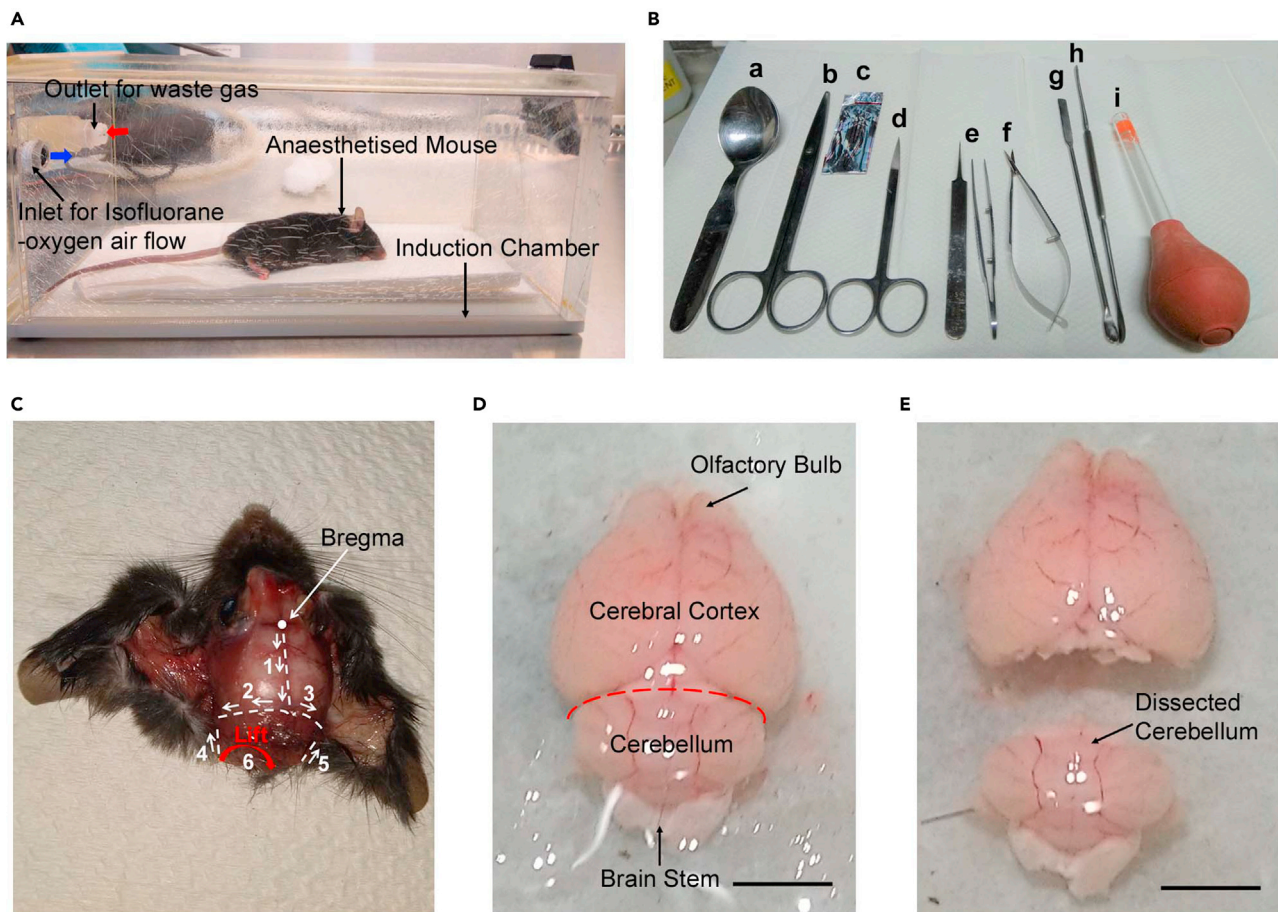
**Note:** The level of anesthesia was monitored throughout the pedal withdrawal reflex to toe pinch. If any pedal reflex is observed while toe pinching the mouse, it indicates that the animal is not fully anesthetized and should remain in the chamber for longer.

**Alternatives:** Mice can be anesthetized using 1 mL of isoflurane applied onto a paper towel kept inside a chamber (Camiré and Topolnik, 2018). Place a grid above the paper towel so as to avoid direct contact of isoflurane onto the skin of mice as Isoflurane can be a skin irritant.

2. Expose the skull by cutting the skin using a pair of scissors from the back of the skull to the top. A small pair of mini bone rongeurs or fine scissors (Figure 2B) can be used to make a hole at the bregma level (Figure 2C). A small pair of scissors can be used to make rostral-to-caudal cut along the sagittal suture starting from bregma level as marked with white vertical dash lines in Figure 2C. After making the incision till the junction between the cortex and the cerebellum, continue cutting the skull laterally towards both sides as indicated with white horizontal dash lines in Figure 2C. Make two small incisions below the lateral most side of cerebellum taking care not to damage the tissue. Use the forceps to grasp the back of skull flap and slowly lift outward and upward (marked with red curled arrow) to remove the skull that covers the cerebellum.

**Note:** It is always advisable to place the severed head directly onto the ice-cold ACSF sucrose solution immediately after decapitation. This will cool the brain, slow down metabolism and thereby reduces action potential in neurons and thus increases the neuron viability.

3. Once the brain is fully exposed, use a small pair of scissors (Figure 2B) to cut the optic nerves and then remove the brain from the skull and place it onto a Petri dish containing oxygenated, cold ACSF-Sucrose that is constantly bubbled with carbogen.
4. Prepare cerebellar slices from the extracted brain.



**Figure 2. Isolation of mouse cerebellum**

(A) Anesthetized mouse placed in the rodent induction chamber provided with controlled flow of oxygen and isoflurane vapors. Blue arrow at the inlet indicates the inflow of isoflurane-oxygen vapors and red arrow at the outlet indicates the outflow of waste gases from the induction chamber to the Charcoal Filters.

(B) Dissection Instruments used for the isolation and preparation of cerebellar slices (a) Spoon, (b) Surgical mayo scissors, (c) surgical blade, (d) fine scissors, (e) fine forceps, (f) spring forceps scissors, (g) spoon micro spatula, (h) spatula and (i) glass Pasteur pipette.

(C) Mouse skull showing the position of Bregma. Dashed white lines indicate the regions for incisions to cut open the skull and expose the whole brain. The white arrows near the dashed lines show the direction of surgical incisions to be made to cut open the mouse skull to expose of the whole mouse brain. Numbers present next to white arrows indicate the order to incisions to be made to expose intact mouse brain. Vertical incisions starting from bregma along the sagittal suture are made till the junction between the cortex and the cerebellum (step 1). The skull is further cut laterally towards both sides as indicated with white horizontal dash lines (step 2 and 3). Make two small incisions below the lateral most side of cerebellum (step 4 and 5) and use forceps to grasp the back of skull flap and slowly lift outward and upward (marked with red curled arrow) to remove the skull that covers the cerebellum (step 6).

(D) Isolated mouse brain showing different regions. Dashed red lines indicate the region for dissection of the cerebellum from the whole mouse brain.

(E) Dissected mouse cerebellum in ice cold cutting ACSF solution. Scale bar in panels D, E indicates 4 mm.

**△ CRITICAL: Steps from 4b to 4d should be completed within a minute to avoid stress to the neurons.**

a. Allow the brain to chill for approximately 2 min in cold ACSF-Sucrose. Transfer the brain onto the petri dish containing ice cold ACSF-Sucrose that is bubbled with carbogen continuously (Figure 2D).

**Note:** Use a spoon (Figure 2B) to carefully transfer the whole brain to the petri dish containing oxygenated ice cold ACSF-Sucrose.

**Note:** Use a piece of filter paper at the bottom of the petri dish containing ice cold ACSF-Sucrose and place the brain on top of the filter paper. Using a filter paper will help the brain to stay in position while dissecting out the cerebellum from the whole brain.

- b. Dissect out the cerebellum from the extracted brain using Scalpel blade No.13 (Figure 2E).
- c. Glue the dissected cerebellum onto the agar block that is stuck to the vibratome table (Vibratome -Leica, VT1200) using cyanoacrylate Instant Adhesive (Evobond Super Glue – SG0312). To obtain sagittal sections of the cerebellum, the orientation of the cerebellum adhered to the agar block is such that the flocculonodular lobe is stuck parallel to the agar block and one of the lateral sides of cerebellum touches the vibratome table as shown in Figure 3A.

△ **CRITICAL:** Glue is added at the bottom of the cerebellum that touches the vibratome table and a small portion of flocculo-nodular lobe so as to ensure that cerebellum remains adhered to the agar block during the vibratome sectioning. Use a flat spatula (Figure 2B) to transfer the cerebellum onto the agar block. Take extra precaution while transferring the cerebellum onto the agar block to avoid the whole tissue falling directly onto the glue. In addition, care should be taken to lightly dry the cerebellum onto a piece of paper to ensure correct gluing as wet brain may tend to detach easily from the agar block and vibratome table.

- d. Immerse vibratome table containing the cerebellum into the vibratome tray filled with ice-cold ACSF-Sucrose bubbled continuously with carbogen (Figure 3B).
- e. Set the speed and amplitude of the vibratome as 0.16 mm/s and 1.0 mm respectively. Adjust the angle of the blade (Feather Razor Blades, Platinum Coated) such that the cutting edge of blade (white asterisks) is aligned parallel to the top of the mounted cerebellum (red arrow head, Figure 3B). Obtain parasagittal cerebellar slices of ~ 250 μm thickness at a temperature of about ~ 1°C.

△ **CRITICAL:** Use the vibratome cooler or place ice around the vibratome tray to maintain temperature at ~ 1°C during sectioning. The cutting edge of the vibratome blades should be aligned parallel to the cerebellum that has been glued to the vibratome table (Figure 3B).

5. Use a glass Pasteur pipette (Figure 2B) to transfer the cerebellar slices onto a bath containing oxygenated ACSF-Recovery solution maintained at 34°C (Figure 3C).

**Note:** Cerebellar sections will be placed on a nylon mesh fitted to the beaker for adequate oxygenation of the slices (Figure 3C).

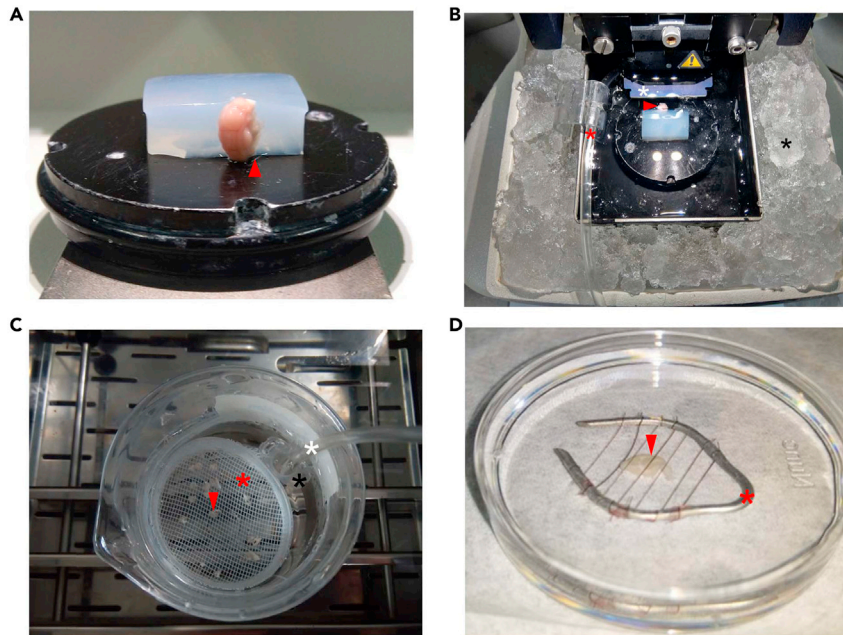
6. Leave the cerebellar slices in oxygenated ACSF-Recovery bath at 34°C for 45 min (Figure 3C).

△ **CRITICAL:** Rate of flow of carbogen gas used to bubble ACSF should be maintained carefully so as to avoid cerebellar slices from floating away in the incubation chamber.

▣ **Pause point:** The experimenter can take a break of 45 min while the slices are kept for recovery.

7. After 45 min of incubation for recovery, the slices are transferred onto a brain slice holding chamber containing oxygenated ACSF-Normal solution maintained at room temperature (~22°C) till imaging.

**Note:** Brain slice holding chamber consists of a 250 mL beaker fitted with nylon netting and a tube attached for the provision of carbogen gas to flow for the adequate oxygenation of the slices. Rate of flow of carbogen gas should be properly controlled to avoid floating of brain slices in the holding chamber (Figure 3C).



**Figure 3. Preparation of cerebellar slices and two-photon calcium imaging**

(A) The dissected cerebellum is glued to the agar block which is stuck to the vibratome table. Red arrow head indicates the region to glue the cerebellum onto the agar block.

(B) vibratome showing the cerebellum (red arrow head) in the vibratome tray filled with ice-cold ACSF-Sucrose and blade placed parallel (white asterisks) to the sagittal region of cerebellum. ACSF is continuously bubbled with carbogen (red asterisks) and ice is placed around the vibratome tray (black asterisks) to maintain temperature at  $\sim 1^{\circ}\text{C}$  during sectioning.

(C) Cerebellar slices (red arrow head) kept in oxygenated ACSF-Recovery solution in water bath maintained at  $34^{\circ}\text{C}$ . Red asterisks indicate the nylon mesh used to hold slices in incubation chamber and black asterisks indicate the portion of a 2.5mL syringe that holds the rubber tubing with carbogen.

(D) Cerebellar slice (red arrow head) with slice anchor (red asterisks) placed in the recording chamber filled with ACSF-normal solution.

### Two-photon calcium imaging

⌚ Timing: 4–6 h per animal

Two-photon calcium imaging is performed to measure  $\text{Ca}^{2+}$  transients in acute cerebellar slices from Purkinje neurons expressing the genetically encoded calcium sensor, GCaMP6f. This protocol is for measuring excitability and ligand-mediated  $\text{Ca}^{2+}$  signals in neurons present within the tissue slices (ex-vivo preparation).

8. Acute cerebellar slices are transferred one at a time to the recording chamber (Figure 3D), which is kept under a custom built two-photon laser scanning microscope equipped with a Ti:Sapphire laser (Coherent Chameleon Ultra II). The microscope is equipped with a  $20\times$  water-immersion objective (Olympus XLUMPLFLN20XW, 1.0 NA).
  - a. Use a glass Pasteur pipette (Figure 2B) to transfer the cerebellar slices onto a recording chamber containing oxygenated ACSF-Normal solution maintained at room temperature.

**Note:** Always use a glass Pasteur pipette to transfer the slices to avoid damage to the tissue slices. [Troubleshooting 5](#)

**⚠ CRITICAL:** Continuously perfuse the bath in recording chamber with oxygenated ACSF-normal at room temperature (Hartmann et al., 2014).

**Note:** Check the flow of carbogen gas in holding chamber frequently to prevent slices from floating and also to avoid air bubbles from getting accumulated near the slices. Oxygenation to the neurons and its viability in the slices gets affected if air bubble forms near the slices.

[Troubleshooting 1](#)

b. Place a slice anchor gently onto the cerebellar slices in the recording chamber ([Figure 3D](#)).

**Note:** Care should be taken to avoid damage to the tissue while placing the slice anchor onto the slice. Slide anchors help prevent excessive motion artifacts while imaging. [Troubleshooting 2](#)

9. Using the image acquisition software Scan Image 3.8, click the “focus” button to locate the cells of interest. Locate Purkinje neurons in the cerebellar sections using the basal green fluorescence signal of GCaMP6 and define a region that includes the PN soma and dendrites for scanning.

**Note:** It is better to avoid neurons from superficial layers as there could be damage caused to the neurons located at the periphery during dissection. Scanning from neurons located deeper than 50  $\mu\text{m}$  is better.

**△ CRITICAL:** Using the laser intensity controllers in the acquisition software, always set the two photon laser (wavelength  $\sim 930$  nm) at a minimal power level so that the baseline green fluorescence is visible minimally. This is in order to avoid phototoxicity during imaging.

[Troubleshooting 4](#)

10. Click the “Grab” button in image acquisition software to acquire the fluorescence of each cell in time lapse mode setting time of acquisition per frame as 1 s and images are acquired with frame size of 512 x 512 pixels.

a. To measure PN excitability and membrane depolarization, 75 mM KCl is added at the 10<sup>th</sup> frame of image acquisition and images are captured for 120 s. This experiment is performed for both control and *STIM1*<sup>PKO</sup> Purkinje neurons to compare depolarization evoked Ca<sup>2+</sup> kinetics ([Figure 4](#), [Methods videos S1](#) and [S2](#)).

b. To assess mGluR1 activation in Purkinje neurons, stimulate the neurons with the mGluR1 agonist Di-Hydroxy Phenyl Glycine (DHPG) ([Hartmann et al., 2014](#)). About 200  $\mu\text{M}$  DHPG can be applied to the cerebellar slices in ACSF solution at the 10<sup>th</sup> frame of the image acquisition and images are captured for 600 s ([Figure 5](#), [Methods videos S3](#) and [S4](#)).

**Note:** Variation in the time taken to respond upon DHPG stimulation is usually seen among PNs of the same slice. One explanation for this is possibly due to the time delay for the DHPG to reach the various PNs present in the area focused under the microscope ([Figure 5B](#)).

c. To test for the specificity of mGluR1 activation, an mGluR1 antagonist, CPCCOEt can be applied to the neurons in ACSF solution before stimulating it with DHPG. CPCCOEt of 200  $\mu\text{M}$  can be applied ([Hartmann et al., 2008](#)) in the bath solution in recording chamber before the image acquisition followed by addition of 200  $\mu\text{M}$  DHPG at the 10<sup>th</sup> frame of the image acquisition ([Figure 5F](#)).

**Note:** The length of scan can be varied depending on the evoked Ca<sup>2+</sup> kinetics. Care should be taken to minimize scan length to prevent photodamage. [Troubleshooting 4](#)

**Note:** Always make sure to select healthy neurons that are 50  $\mu\text{m}$  below the superficial surface as injury during sectioning can affect the viability of neurons and no response will be observed on application of stimulus to the neurons during calcium imaging. [Troubleshooting 3](#)

△ **CRITICAL:** Continuously perfuse the bath in recording chamber with oxygenated ACSF-normal at room temperature (Hartmann et al., 2014). Image acquisition should be stopped when the neurons show signs of deteriorating health such as enlarged soma, increase in the basal GCaMP6f fluorescence suggesting enhanced  $\text{Ca}^{2+}$  levels, blebbing and fragmentation of dendrites. Signs of deterioration might be observed if the cells are imaged for longer durations (i.e. after 20 minutes in recording chamber) and also if the slices are kept in the incubation chamber for prolonged periods. [Troubleshooting 1](#)

### EXPECTED OUTCOMES

Two photon calcium imaging of Purkinje neurons present within cerebellar slices generate ex vivo data of neuronal  $\text{Ca}^{2+}$  responses upon various stimulations. GCaMP6f fluorescence intensity for each cell at various time points can be measured and  $\text{Ca}^{2+}$  transients are measured as the relative changes in fluorescence  $\Delta F/F_{\text{basal}}$  [ $\Delta F/F_{\text{basal}}=(F_t-F_{\text{basal}})/F_{\text{basal}}$ ], where  $F_t$  is the fluorescence of the cell at a particular time point and  $F_{\text{basal}}$  is the fluorescence of the cell at the start of the experiment. Rate of  $\text{Ca}^{2+}$  entry ( $\Delta F/t$ ) during various stimulations can be calculated by analyzing the average rate of change in fluorescence intensity ( $\Delta F$ ) between the time at which  $F_{\text{max}}$  ( $\Delta F/F$ ) occurs and the time point of addition of various stimulant. Peak values of  $\Delta F/F$  and area under the curve can also be calculated for every cell. From each animal, about 6–8 healthy slices can be obtained. At least two slices from each mouse can be used to record responses of each stimulus. From each slice at least 5–8 viable Purkinje neurons can be imaged.

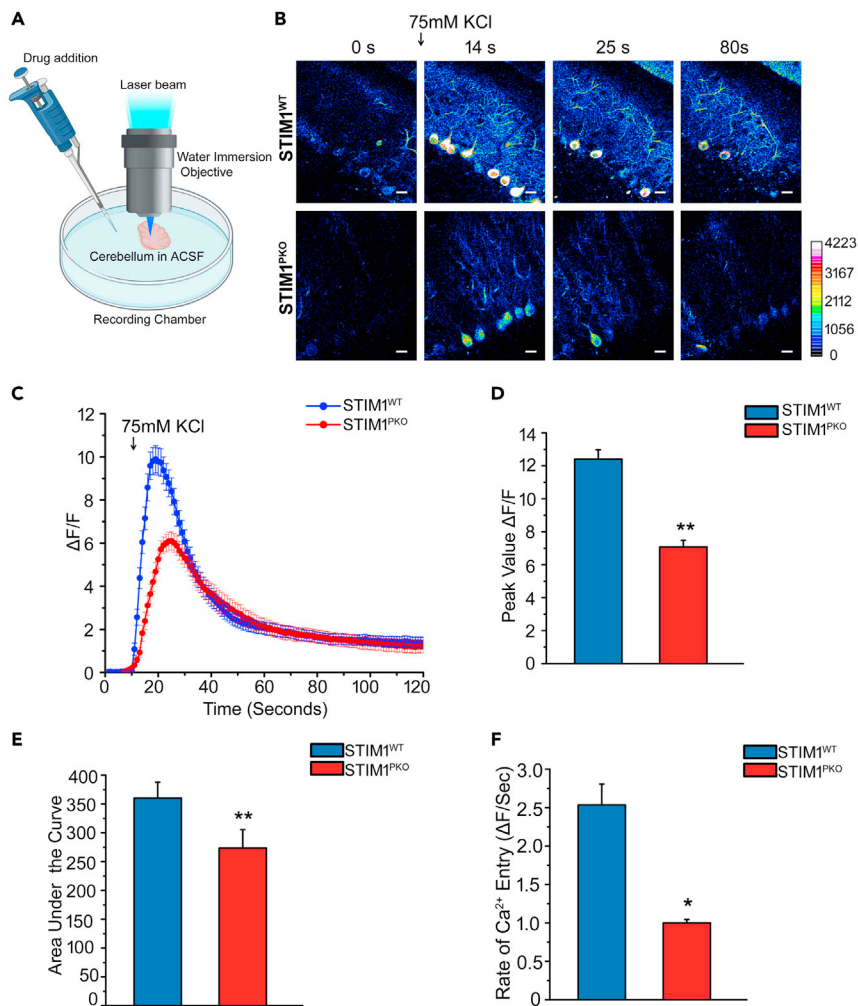
As an example, changes in excitability and membrane depolarization between control and *STIM1*<sup>PKO</sup> Purkinje neurons can be measured by depolarizing the neurons with 75 mM KCl. Wild type Purkinje neurons show robust cytosolic  $\text{Ca}^{2+}$  elevations upon KCl stimulation whereas calcium responses from *STIM1*<sup>PKO</sup> Purkinje neurons were significantly reduced when compared to controls (Figure 4). This protocol also assesses mGluR1 activation of the Purkinje neurons of both control and *STIM1*<sup>PKO</sup> mice. DHPG stimulation in control Purkinje neurons evoked large  $\text{Ca}^{2+}$  transients whereas DHPG application in *STIM1*<sup>PKO</sup> Purkinje neurons failed to evoke measurable calcium transients (Figure 5). An mGluR1 antagonist, CPCCOEt, was added to check for the specificity of mGluR1 activation. Application of CPCCOEt abolished the DHPG-induced  $\text{Ca}^{2+}$  kinetics otherwise observed in control Purkinje neurons (Figure 5).

### QUANTIFICATION AND STATISTICAL ANALYSIS

Images captured can be further analyzed using ImageJ software. Mean intensity can be measured from the somatic region of interest. For the analysis of GCaMP6f-related fluorescence transients for each cell, region of interest (ROI) is drawn around each cell and the fluorescence intensity is determined at each time point.  $\text{Ca}^{2+}$  transients are measured as the relative changes in fluorescence  $\Delta F/F_{\text{basal}}$  [ $\Delta F/F_{\text{basal}}=(F_t-F_{\text{basal}})/F_{\text{basal}}$ ], where  $F_t$  is the fluorescence of the cell at a particular time point and  $F_{\text{basal}}$  is the fluorescence of the cell at the start of the experiment. Data can be plotted using the Origin 8.0 software. Peak values of  $\Delta F/F$  and area under the curve can be calculated for every cell and the data can be plotted as a histogram. Rate of  $\text{Ca}^{2+}$  entry during stimulation can be calculated by computing the average rate of change in fluorescence intensity ( $\Delta F$ ) between the time at which  $F_{\text{max}}$  ( $\Delta F/F$ ) occurs and 11<sup>th</sup> sec time points and expressed as  $\Delta F/t$ .

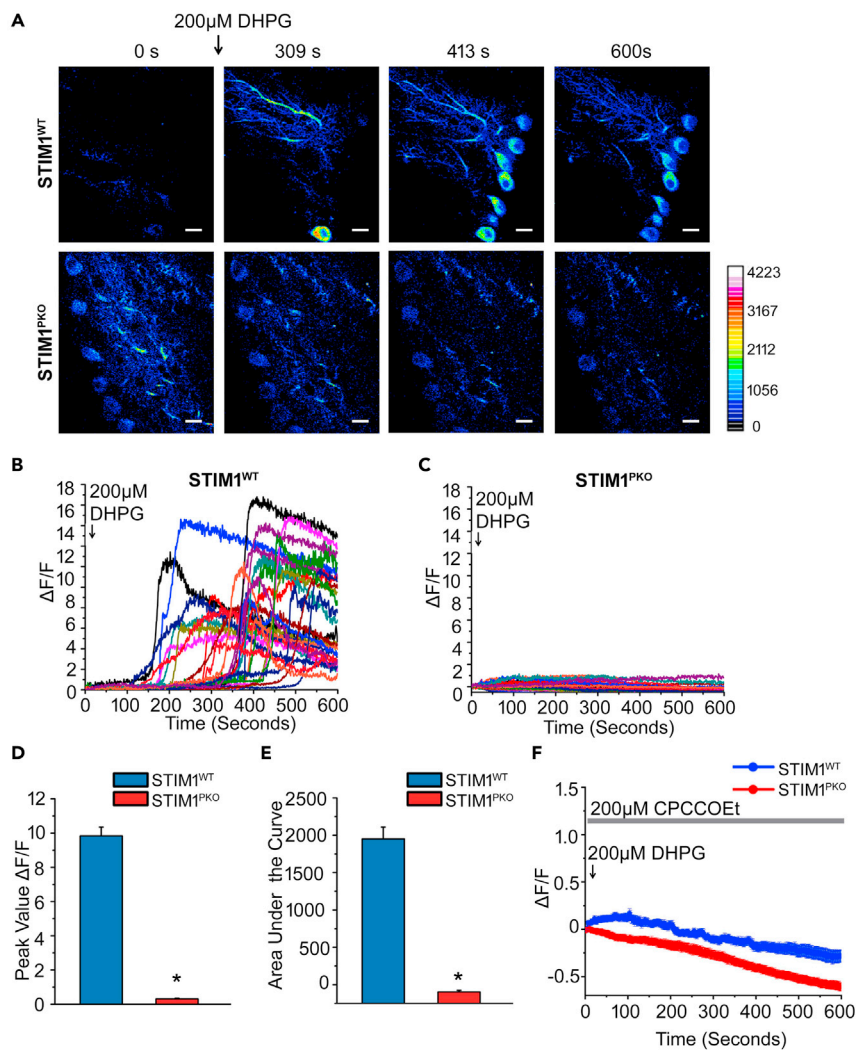
### LIMITATIONS

Limitations of this protocol are usually a consequence of damage to the cerebellum that occurs most often during the isolation and sectioning steps. Maintaining viability of Purkinje neurons in cerebellar sections is another challenging factor that needs to be critically monitored throughout the experiments. Clean bottles (alkali free) and double distilled water should be used for preparing ACSF solutions. ACSF should be continuously bubbled with carbogen throughout the experiment. Extra caution needs to be taken to maintain the flow rate of the carbogen in the incubation chamber.



This is required to maintain proper oxygenation of slices. However, if the cerebellar slices move in the chamber due to the carbogen flow, this can also affect neuronal health.

Movement of slices in the recording chamber while imaging for prolonged periods is another significant limitation, due to which the images appear out of focus. Always ensure that the slice anchors are fixed appropriately without damaging the tissue slice. Motion artifacts, while adding of drugs of interest can also bring down image quality. Care should be taken to avoid air bubbles and water turbulence during the addition of drugs. Adopting microfluidics or perfusion system to apply drugs to the slices in the recording chamber is a better option to overcome air bubbles and excessive motion artifacts (Chen et al., 2016; Babic et al., 2018). Tips to overcome these limitations are provided in the troubleshooting section.



**Figure 5. Monitoring  $\text{Ca}^{2+}$  kinetics evoked upon mGluR1 activation**

(A) Snap shots of GCaMP6f responses from Purkinje neuron soma at indicated time points upon DHPG stimulation. Scale bars - 20  $\mu\text{m}$ .

(B and C) Line plots showing GCaMP6f fluorescence responses ( $\Delta\text{F}/\text{F}$ ) in Purkinje neuron soma of control (27 PN, 4 mice) and (C)  $\text{STIM1}^{\text{PKO}}$  (55 PN, 4 mice) upon addition of the mGluR1 agonist, 200 $\mu\text{M}$  DHPG.

(D and E) Bar graphs with Peak  $\Delta\text{F}/\text{F}$  and (E) Area under the curve quantified from (B and C). Each bar is compared to control (\* $p < 0.0001$ ; Two-tailed Student's t-test).

(F) Line plot of the mean traces ( $\pm$  SEM) of normalized GCaMP6f fluorescence responses ( $\Delta\text{F}/\text{F}$ ) in Purkinje neuron soma upon DHPG stimulation in slices incubated with 200 $\mu\text{M}$  CPCCOEt, a mGluR1 antagonist ( $\text{STIM1}^{\text{WT}}$  - 39 PN, 4 mice;  $\text{STIM1}^{\text{PKO}}$  - 43 PN, 4 mice). Panels 5A to 5C and 5F are from our recent paper (Dhanya and Hasan, 2021).

## TROUBLESHOOTING

### Problem 1

Neurons might show signs of deteriorating health like increase in the baseline  $\text{Ca}^{2+}$  levels, enlarged soma, blebbing and fragmentation of dendrites (steps 8 and 10).

### Potential solution

To maintain healthy slices for imaging, proper oxygenation should be provided through carbogen bubbling into ACSF solution throughout the experiment in the incubation and recording chamber.



Flow of carbogen should be regulated to avoid slices from floating in the recovery and incubation chamber. Using freshly prepared ACSF is highly recommended to maintain healthy slices. The pH of the ACSF solution should be accurately adjusted to 7.4 using NaOH and osmolarity should be adjusted to 300–310 mosM with glucose.

### **Problem 2**

Excessive motion artifacts observed while capturing the images (step 8b).

#### **Potential solution**

Before imaging, make sure that the slices are strongly fixed in place by the ‘slice anchors’. Care should be taken not to disturb the slice or the recording chamber while adding drugs during image acquisition. Perfusion or microfluidics system can be adopted to apply drugs to the slices in the recording chamber to avoid air bubbles and excessive motion artifacts while imaging the cells.

### **Problem 3**

No response is detected on application of stimulus to the cerebellar slices in bath solution during Ca<sup>2+</sup> imaging (step 10).

#### **Potential solution**

Neuronal conditions do affect the Ca<sup>2+</sup> response to various stimuli. Healthy neurons should be selected and used for Ca<sup>2+</sup> imaging experiments. Ensure that the cells being focused are 50 μm below the superficial surface as injury during sectioning can affect the viability of neurons. Always make sure that proper oxygenation is maintained continuously in the incubation and recording chamber. Before testing a new stimulus, it is advisable to test a slice by delivering an acute depolarization stimulus (75 mM KCl) and observing the expected change in fluorescence kinetics of the Ca<sup>2+</sup> indicator.

### **Problem 4**

Photobleaching and reduced fluorescence signal might be observed during imaging (step 10).

#### **Potential solution**

High photon flux can cause irreversible photodamage to the neurons (Gautam et al., 2015). To prevent photo damage extra care should be taken to limit the use of laser power and to keep the laser power as low as possible for imaging. This can be done by decreasing the duration of scans and by increasing the scan speed during image acquisition (Camiré and Topolnik, 2018).

### **Problem 5**

Fluorescence signals may not show dynamic changes in intensity during Ca<sup>2+</sup> imaging experiments (step 8a).

#### **Potential solution**

Sometimes “False” fluorescence signals that are usually derived from auto-fluorescent materials like damaged tissue and not from GCaMP6f might be observed in the area being focused. To prevent these, ensure that the cells are viable and the slices are maintained in oxygenated ACSF solution throughout the experiment. Proper care should also be taken not to damage the tissue while isolating the cerebellum and mounting onto the vibratome table for sectioning. Always use glass Pasteur pipette to transfer the cerebellar slices onto the incubation and recording chamber.

## **RESOURCE AVAILABILITY**

### **Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, [Gaiti Hasan] ([gaiti@ncbs.res.in](mailto:gaiti@ncbs.res.in)).

### Materials availability

Transgenic mouse lines used for the study are available in the Jackson Laboratory. *PCP2<sup>Cre/Cre</sup>* (JAX:004146) and *GCaMP6f<sup>fllox/fllox</sup>* (JAX:024105) can be obtained from the Jackson Laboratory. *STIM1<sup>fllox/fllox</sup>* can be obtained from Prof. Anjana Rao from La Jolla Institute for Allergy and Immunology upon completion of a material transfer agreement.

### Data and code availability

Original data for figures in this paper is available in Mendeley Data <https://data.mendeley.com/datasets/gzb4hf47kc/2> (Mendeley Data, V2, <https://doi.org/10.17632/gzb4hf47kc.2>).

### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2021.101105>.

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### AUTHOR CONTRIBUTIONS

S.K.D. contributed with conceptualization, performing and analyzing the experiments, developed methodology, data curation, writing of the original manuscript. G.H. contributed with conceptualization, supervision, funding acquisition, writing and editing of manuscript.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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