Utilizing In Vivo Postnatal Electroporation to Study Cerebellar Granule Neuron Morphology and Synapse Development
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1   **TITLE**:
2   Utilizing *In Vivo* Postnatal Electroporation to Study Cerebellar Granule Neuron Morphology and
3   Synapse Development
4
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17   **KEYWORDS:**
18   Neuronal development, cerebellum, dendrite, synapse, granule neuron, electroporation
19
20   **SUMMARY:**
21   Here we describe a method to visualize synaptogenesis of granule neurons in the mouse
22   cerebellum over the time course of postnatal brain development when these cells refine their
23   synaptic structures and form synapses to integrate themselves into the overall brain circuit.
24
25   **ABSTRACT:**
26   Neurons undergo dynamic changes in their structure and function during brain development to
27   form appropriate connections with other cells. The rodent cerebellum is an ideal system to track
28   the development and morphogenesis of a single cell type, the cerebellar granule neuron (CGN),
29   across time. Here, *in vivo* electroporation of granule neuron progenitors in the developing mouse
30   cerebellum was employed to sparsely label cells for subsequent morphological analyses. The
31   efficacy of this technique is demonstrated in its ability to showcase key developmental stages of
32   CGN maturation, with a specific focus on the formation of dendritic claws, which are specialized
33   structures where these cells receive the majority of their synaptic inputs. In addition to providing
34   snapshots of CGN synaptic structures throughout cerebellar development, this technique can be
35   adapted to genetically manipulate granule neurons in a cell-autonomous manner to study the
36   role of any gene of interest and its effect on CGN morphology, claw development, and
37   synaptogenesis.
38
39   **INTRODUCTION:**
40   Brain development is a prolonged process that extends from embryogenesis into postnatal life.
41   During this time, the brain integrates a combination of intrinsic and extrinsic stimuli that sculpt
42   the wiring of synapses between dendrites and axons to ultimately guide behavior. The rodent
43   cerebellum is an ideal model system to study how synapses develop because the development
44   of a single neuron type, the cerebellar granule neuron (CGN), can be tracked as it transitions from

45 a progenitor cell to a mature neuron. This is due, in part, to the fact that a majority of the
46 cerebellar cortex develops postnatally, which allows for easy genetic manipulation and cell
47 labeling after birth[1].
48
49 In mammals, CGN differentiation begins at the end of embryonic development when a subset of
50 proliferative cells in the hindbrain migrates over the rhombic lip to form a secondary germinal
51 zone on the surface of the cerebellum[2-4]. Although they are fully committed to a granule neuron
52 progenitor (GNP) identity, these cells continue to proliferate within the outer portion of the
53 external granule layer (EGL) until postnatal day 14 (P14). Proliferation of this layer results in a
54 massive expansion of the cerebellum as these cells give rise exclusively to CGNs[5]. Once newborn
55 CGNs exit the cell cycle in the EGL, they migrate inwards towards the internal granule layer (IGL),
56 leaving behind an axon that will bifurcate and travel in the molecular layer of the cerebellum,
57 forming parallel fibers that synapse onto Purkinje cells[6]. The position of these fibers within the
58 molecular layer is dependent on the timing of cell-cycle exit.
59
60 CGNs that differentiate first leave their parallel fibers towards the bottom of the molecular layer,
61 whereas the axons of CGNs that differentiate later are clustered at the top[7,8]. Once the CGN cell
62 bodies reach the IGL, they begin to elaborate dendrites and form synapses with nearby inhibitory
63 and excitatory neurons. The mature dendritic tree of a CGN exhibits a stereotyped architecture
64 with four main processes. Over the course of CGN maturation, the structures at the end of these
65 dendrites form a claw that becomes enriched with postsynaptic proteins[9,10]. These specialized
66 structures, called dendritic claws, contain the majority of the synapses onto granule neurons and
67 are important for receiving both excitatory inputs from mossy fiber innervations originating from
68 the pons, as well as inhibitory inputs from local Golgi cells. Once fully configured, the synaptic
69 connections of CGNs allow these cells to relay inputs from pre-cerebellar nuclei to Purkinje cells,
70 which project out of the cerebellar cortex to the deep cerebellar nuclei.
71
72 *In vivo* postnatal electroporation of GNPs is advantageous over other labeling-based methods,
73 such as viral infection and generation of transgenic mouse lines, because the expression of
74 desired constructs can be achieved on a fast timeline, and the method targets a small population
75 of cells, useful in studying cell-autonomous effects. This method has been used in prior studies
76 to study morphological development of CGNs; however, these studies have focused on either a
77 single time point or a short window of time[9-13]. This labeling method was paired with image
78 analysis to document the changes in CGN morphology that occur across the entire time course
79 of CGN differentiation over the first three weeks of postnatal life. These data reveal the dynamics
80 of CGN dendrite development that underlie construction of cerebellar circuits.
81
82 **PROTOCOL:**
83
84 NOTE: All procedures were performed under protocols approved by Duke University Institutional
85 Animal Care and Use Committee (IACUC).
86
87 **1.   DNA preparation for in vivo electroporation or IVE (1 day before surgery)**
88

89 1.1.   Gather the following materials: purified DNA (0.5–25 µg per animal), 3 M sodium acetate,
90 ethanol, Fast Green dye, ultrapure distilled water, phosphate buffer solution (PBS).

91

92 NOTE: For DNA, a construct expressing green fluorescent protein (GFP) under a human ubiquitin
93 promoter was obtained from Addgene (FUGW, https://www.addgene.org/14883/). Any
94 construct expressing GFP or another fluorescent protein under the control of a ubiquitous
95 promoter should work. CGN-specific labeling with this technique is not dependent on the
96 construct, but rather on the electroporation.

97

98 1.2.   Prepare DNA for electroporation by mixing the desired amount of DNA, 10% by volume
99 of 3 M sodium acetate and 250% by volume of 100% ice-cold ethanol. Note that the DNA will
100 precipitate out of solution immediately.

101

102 1.3.   Continue to precipitate the DNA mixture overnight at -20 °C or for an hour at -80 °C.

103

104 1.4.   Pellet precipitated DNA in a tabletop centrifuge at >16,000 × g and wash twice with 70%
105 ethanol.

106

107 1.5.   Allow the DNA pellet to dry completely, and reconstitute in a 1x PBS + 0.02% Fast Green
108 solution.

109

110 [Place **Figure 1** here]

111

112 **2. *In vivo* electroporation of granule neuron progenitors in seven-day old postnatal mice**

113

114 NOTE: All electroporation surgeries were performed in a sterile and highly ventilated surgical
115 suite, and all personnel wore complete personal protective equipment including gloves, face
116 mask, hair bonnet, gown, and shoe covers. Alternatively, the surgeries can be performed in a
117 ventilated and sterile hood.

118

119 2.1.   Gather the following materials: DNA for electroporation, small surgical scissors, small
120 surgical tweezers, customized Hamilton syringe, cotton-tip applicator, heating pad, betadine,
121 70% ethanol, 1x PBS, parafilm, tissue adhesive (n-butyl-ester cyanoacrylate), isoflurane,
122 electroporator, and tweezer-type electrodes (see the **Table of Materials**).

123

124 2.2.   Cut a spacer from a loading tip to fit over the Hamilton syringe to limit the injection depth
125 to 1.5 mm (**Figure 1A,B**). Secure the spacer with adhesive or parafilm.

126

127 2.3.   Anesthetize the P7 pup in an isoflurane chamber at a delivery rate of 0.8 L/min. Confirm
128 full anesthesia by monitoring the animal for decreased respiration and a lack of a toe or tail pinch
129 response (**Figure 2A**).

130

131 2.4.   Once the animal is fully anaesthetized, place the pups on a pedestal fitted with a nose
132 cone, delivering constant isoflurane at a delivery rate of 0.8 L/min. Clean the top of the pup's

133 head with betadine and 70% ethanol. Wipe off excess betadine and ethanol with a paper towel.

134

135 2.5.   Using a pair of sterilized scissors, make a small incision with one cut that spans the
136 distance from the top to the base of the ears to reveal the hindbrain (**Figure 2B**).

137

138 2.6.   Locate the cerebellum (**Figure 2C**), insert the exposed tip of the Hamilton syringe through
139 the skull, perpendicular to the brain, and inject 1.5 µL of DNA mixture into the cerebellar
140 parenchyma by slowly pushing the back plunger of the syringe. After delivery of the DNA mixture,
141 slowly pull the needle back to prevent back spills, and allow the DNA solution to diffuse for 30 s.

142

143 2.7.   Turn off the isoflurane, and place the pup on a 37 °C heating pad. Prepare the tweezer-
144 type electrode for electroporation by dipping both ends into 1x PBS.

145

146 NOTE: Wetting the tweezer-type electrode will prevent contact burns on the skin of the pup
147 during administration of the electrical pulses.

148

149 2.8.   Orient the tweezer–electrode above the site of injection with the plus end facing
150 downwards and the negative end above the animal's head (**Figure 2D**). Administer five electrical
151 pulses from the electroporator with the following settings: 50 ms, 130 V, and 950 ms inter-pulse
152 interval.

153

154 NOTE: If needed, perform a test injection to ensure that the injection site is located on the
155 cerebellar vermis (**Figure 2E**).

156

157 2.9.   Pinch the incision closed, and seal the wound with a non-toxic n-butyl-ester cyanoacrylate
158 tissue adhesive. Clean the wound with 70% ethanol as any trace amount of blood increases the
159 likelihood of parental infanticide and cannibalism.

160

161 2.10.   Allow the animal to recover on a 37 °C heating pad before returning the pup to the dam.
162 Monitor the pup(s) every 30 min for at least 2 h after the surgery to ensure full recovery.

163

164 NOTE: Infanticide by either parent is quite common. To prevent cannibalism, house the sire in a
165 different cage before starting the electroporation, and always return cleaned and recovered pups
166 (*i.e.,* no bloodstain, fully mobile) to the original cage on the original bedding. Pups can also be
167 wiped with droppings from the original cage to minimize the smell of blood. The use of a
168 surrogate dam may be necessary if the original dam continues to cannibalize her pups.

169

170 [Place **Figure 2** here]

171

172 **3. Immunohistochemistry of electroporated CGNs**

173

174 3.1.   Gather the following materials: isoflurane, 1x PBS, 4% paraformaldehyde (PFA), 30%
175 sucrose, normal goat serum, non-ionic detergent (**Table of Materials**), glass slides, glass

176   coverslips, nail polish, mounting media, Hoechst nuclear dye, and appropriate primary and
177   secondary antibodies.
178
179   3.2.   Anesthetize the experimental animal with isoflurane, and confirm full anesthesia with a
180   toe and tail pinch.
181
182   3.3.   Perform a trans-cardial perfusion by slowly injecting 1x PBS and 4% PFA into the left
183   ventricle of the animal's heart. Allow the blood to drain from the animal by cutting the vena cava.
184
185   3.4.   Fix the brain overnight by submerging it in 4% PFA at 4 °C. On the following day, quickly
186   rinse the brain with 1x PBS, and transfer the brain into 30% sucrose in 1x PBS for cryoprotection
187   for at least 24 h.
188
189   3.5.   If necessary, slice the brain in half along the rostral-caudal axis, and confirm expression
190   of the transfected reporter construct using an upright fluorescent dissecting microscope.
191
192   NOTE: Keep the brain submerged in 1x PBS in a small dish to prevent it from drying out.
193
194   3.6.   Mount the brain on a freezing microtome, slice 25 μm sagittal sections, and allow sections
195   to unfold in a 1:1 mixture of 1x PBS and glycerol.
196
197   NOTE: Sections can be stored in this cryoprotectant solution at -20 °C for long-term storage.
198
199   3.7.   Wash sections three times in 1x PBS for 10 min each to remove cryoprotectant, and block
200   the tissue in 1x PBS + 10% normal goat serum + 0.2% non-ionic detergent on an orbital shaker at
201   room temperature for 1 h.
202
203   3.8.   Prepare primary antibody solution: 1x PBS, 10% normal goat serum, 0.2% non-ionic
204   detergent, and anti-GFP antibody, and centrifuge the solution for 5 min at >16,000 × g. Incubate
205   sections in the antibody solution at 4 °C on an orbital shaker for 48 h.
206
207   3.9.   Wash off primary antibody solution for 15 min five times with 1x PBS + 0.2% non-ionic
208   detergent.
209
210   3.10.   Prepare secondary antibody solution: 1x PBS, 10% normal goat serum, 0.2% non-ionic
211   detergent, and an appropriate secondary antibody to detect GFP; centrifuge the solution at
212   >16,000 × g. Incubate sections in the antibody solution on an orbital shaker at room temperature
213   for 2–3 h. Protect the sections from light exposure to prevent bleaching.
214
215   3.11.   Wash off secondary antibody solution three times with 1x PBS + 0.2% non-ionic detergent
216   for 15 min each time. Incubate sections in 1x PBS + Hoechst for 5 min to stain nuclei.
217

218   3.12.   Wash off Hoechst solution with 1x PBS + 0.2% non-ionic detergent and mount onto glass
219   slides. Cover the sections with mounting media, coverslip the slides, and seal the slide with nail
220   polish to prevent evaporation.
221
222   **4. Morphological analyses of CGNs—three-dimensional (3D) reconstruction and surface area**
223   **and cellular volume**
224
225   4.1.   Image single electroporated CGNs on a confocal microscope at 63x objective with a 2x
226   zoom, taking z-stack images at 0.5 µm per stack. Image one cell per image window to allow for
227   easy image analysis and reconstruction.
228
229   4.2.   Install the Simple Neurite Tracer plug-in for FIJI using the following link
230   (https://imagej.net/Simple_Neurite_Tracer:_Basic_Instructions) to easily and efficiently trace
231   the structure of electroporated CGNs in three-dimensional (3D) space.
232
233   NOTE: There is an updated version of the plug-in (https://imagej.net/SNT).
234
235   4.3.   Analyze neurite length and dendritic claw formation in a blinded manner using Simple
236   Neurite Tracer. Upload single-channel z-stack images of electroporated CGNs onto FIJI, and click
237   on **Plugins | Segmentation | Simple Neurite Tracer** (**Figure 3D**).
238
239   4.4.   Access the drop-down menu, and select **Create New 3D Viewer** (**Figure 3D**).
240
241   4.5.   Scroll to the base of a dendrite, where it connects to the cell soma and start a path by
242   clicking on the junction. Manually trace the path by clicking through the sections where the cell-
243   fill signal is brightest, pressing [**y**] to keep the trace. Trace until the end of the dendrite if it does
244   not contain a claw or until the base of the claw and confirm the path by pressing [**f**] (**Figure 4D**).
245
246   4.6.   Next, trace the claw by starting a path at the base of the structure and tracing until the
247   end of the longest neurite. Trace secondary and tertiary branches by holding down [**ctrl**] on
248   Windows or [**alt**] on a Mac OS and clicking the path. Confirm the path by pressing [**f**].
249
250   4.7.   Observe that measurements for the traces are visible on a separate window; add up all of
251   the measurements of the claw branches (primary, secondary, tertiary) to obtain the total length
252   for each claw.
253
254   4.8.   For analyzing surface area and cellular volume of electroporated CGNs, download Imaris
255   cell analysis software (https://imaris.oxinst.com/).
256
257   NOTE: FIJI can also be used to reconstruct cells in 3D from z-stack images using readily available
258   and free plug-ins. Additionally, there is a volumetric rendering feature in Simple Neurite Tracer
259   but Imaris was used for the reasons outlined below.
260

261   4.9.   Upload z-stack image of an electroporated CGN onto Imaris. Access the 3D-reconstruction
262   toolkit by pressing **Surpass**.

263

264   4.10.   To reconstruct the CGN, press **Surfaces**, and select a region of interest that encompasses
265   the entirety of the cell within the image window. Once finished, press the blue forward arrow at
266   the bottom right corner under **Create**.

267

268   4.11.   If the image contains multiple channels for different signals, select the channel containing
269   the electroporated CGN, and press the blue forward arrow.

270

271   4.12.   Using the slidebar, set a desired threshold that most accurately fits the signal of the
272   electroporated cell. Zoom in closer to the surface of the cell to accurately determine the
273   threshold. Once finished, press the double green arrow to reconstruct the cell and obtain the
274   surface area and volume size from the metadata.

275

276   [Place **Figure 3** here]

277

278   **REPRESENTATIVE RESULTS:**
279   [Place **Figure 4** here]
280   To study the development of granule neuron morphology *in vivo*, a construct expressing GFP
281   under the control of a human ubiquitin promoter (FUGW) was electroporated into the developing
282   cerebellum of CD-1 mice and brains collected 3-, 5-, 7-, 10-, and 14-days post-injection (DPI).
283   Sparse labeling of cells by electroporation in combination with confocal microscopy capture
284   snapshots of CGNs during periods of dendritic pruning, growth, and maturation. To quantitatively
285   analyze and track the growth of CGN synaptic structures, each dendrite was traced using the FIJI
286   plugin Simple Neurite Tracer (SNT). SNT is an easy, fast, efficient, and readily available method to
287   measure neurite and claw length in three-dimensional (3D) space. Conversely, Imaris was used
288   for 3D-reconstruction of CGNs to obtain surface area and volume measurements because the
289   program provides fast and accurate rendering of each cell, and its thresholding capabilities are
290   able to isolate labeled cells from nearby labeled cell debris.

291

292   Newborn CGNs undergo an exuberant phase of dendritic growth followed by refinement from
293   P10 to P14 (3- to 7-DPI) that results in the pruning of more than 50% of excess dendrites (**Figure
294   4B**). This event coincides with the gradual lengthening of the remaining arbors (**Figure 4C**) and
295   the formation of claw-like structures at the end of each dendrite (**Figure 4D**), indicating that these
296   developmental processes are happening concurrently. However, while claws are found on
297   roughly 75% of dendrites by P14 (7-DPI), these structures continue to increase in size until P21
298   (14-DPI) (**Figure 4E**).

299

300   The changes in dendrite and claw morphology could reflect either an overall change in total cell
301   size or a redistribution of cell membrane. To address this question, each labeled granule neuron
302   was reconstructed in Imaris to quantify the total somatodendritic surface area and volume. CGN
303   size remained relatively constant across development (**Figure 5A,B**), though at P14, CGNs exhibit
304   a significant 20% decrease in volume compared to P10, P12, and P17 (3-, 5-, and 10-DPI) (**Figure

305 **5B**). These data suggest that membrane recycling from retracted dendrites may be particularly
306 important for permitting the enlargement of dendritic endings into claws and point to P14 (7-
307 DPI) as a key time point in the transition from pruning to synapse development.
308
309 [Place **Figure 5** here]
310
311 **FIGURE AND TABLE LEGENDS:**
312
313 **Figure 1: Limiting injection depth to 1.5 mm using a spacer.** (**A**) A 11.2 mm segment is cut off a
314 loading pipette using a razor blade. (**B**) The spacer is fitted onto the tip of the Hamilton syringe
315 (total length is 1.27 cm or 0.5 in) and secured with either adhesive or parafilm. The exposed tip
316 should be 1.5 mm in length.
317
318 **Figure 2: *In vivo* cerebellar electroporation of granule neuron progenitors in P7 wildtype mouse**
319 **pups.** (**A**) Pups are kept under isoflurane to ensure anesthesia throughout the injection of the
320 DNA solution. Isoflurane is delivered at a rate of 0.8 L/min. (**B**) After sterilizing the animal with
321 betadine and 70% ethanol, an incision is made that spans the distance of the ears, revealing the
322 hindbrain. (**C**) A magnified image of a white demarcation on the cranium, a landmark for the
323 injection site. DNA construct should be injected within 1 mm above the mark; dotted lines outline
324 the demarcation, and black arrow denotes the injection site. The ridges of the cerebellar vermis
325 may be visible and can be useful for finding the injection site. (**D**) Tweezer-type electrode
326 orientation for efficient electroporation. Plus (+) end must be oriented downwards to pull
327 negatively charged DNA into the cerebellar parenchyma prior to administration of electrical
328 pulses. (**E**) Test injection of 1 µL of a 0.02% Fast Green dye shows injection is localized to the
329 middle of the cerebellar vermis between lobules 5–7.
330
331 **Figure 3: Immunohistochemical analysis and three-dimensional reconstruction of**
332 **electroporated granule neurons.** P7 CD-1 mice were electroporated with a construct expressing
333 GFP. Brains were collected and subjected to immunohistochemistry, confocal microscopy, and
334 3D-reconstruction for morphological analysis. (**A**) Timeline from electroporation to image
335 processing of a 10-DPI mouse. (**B**) Maximum projection image of a sagittal cross-section of
336 electroporated cerebellum 10-DPI; white lines demarcate cerebellar layers, and scale bar is 25
337 µm. (**C**) Maximum projection image of a single electroporated granule neuron 10-DPI and the
338 corresponding 3D trace, scale bar is 10 µm. (**D**) 3D-reconstructions were generated using the FIJI
339 plugin Simple Neurite Tracer. All measurements were traced through the z-stack, following the
340 cell-fill signal. Shaft and claw measurements were traced separately for every dendrite; dotted
341 line denotes portion of dendrite within the current plane. Abbreviations: 3D = three-dimensional;
342 GFP = green fluorescent protein; DPI = days post-injection; PSD-95 = postsynaptic density protein
343 95; GNPs = granule neuron progenitors; PFA = paraformaldehyde.
344
345 **Figure 4: Analysis of granule neuron morphology during cerebellar development.** (**A**) Maximum
346 projection images of electroporated CGNs from 3-DPI to 14-DPI (postnatal age P10 to P21), nuclei
347 (blue) and GFP (green); arrowheads indicate individual dendrite, and scale bar is 10 µm. (**B**)
348 Average number of dendrites. (**C**) Average dendrite length measured from the base of the soma

349   to the tip of the dendrite. (**D**) Fraction of dendrites that contain a claw; a value of 1.00 is 100%,
350   i.e., all dendrites have claw. (**E**) Total length of dendritic claw. N > 30 cells per condition, collected
351   from at least 4 animals per condition; all measurements were analyzed by one-way ANOVA and
352   either a Dunnett's multiple comparison test (**B, C**, and **D**) or a Tukey's multiple comparison test
353   (**E**), **** denotes significance with $p < 0.0001$ across time; errors bars are S.E.M. Abbreviations:
354   GFP = green fluorescent protein; DPI = days post-injection; PSD-95 = postsynaptic density protein
355   95; CGNs = cerebellar granule neurons; ANOVA = analysis of variance; S.E.M. = standard error of
356   the mean.
357
358   **Figure 5: Analysis of granule neuron size during cerebellar development.** Electroporated CGNs
359   were reconstructed in Imaris to determine cellular size. (**A–B**) Surface area and volumetric
360   analysis of granule neurons (*i.e.*, cell soma and dendrites) during cerebellar development. N > 30
361   cells per condition, collected from at least 4 animals per condition. All measurements were
362   analyzed by one-way ANOVA and a Dunnett's multiple comparison test, ** denotes significance
363   with $p < 0.005$; errors bars are S.E.M. Abbreviations: DPI = days post-injection; CGNs = cerebellar
364   granule neurons; ANOVA = analysis of variance; S.E.M. = standard error of the mean.
365
366   **DISCUSSION:**
367   Cerebellar granule neurons are the most abundant neurons in the mammalian brain, making up
368   almost 60–70% of the total neuron population in the rodent brain[1,14]. The cerebellum has been
369   extensively utilized to elucidate mechanisms of cellular proliferation, migration, dendrite
370   formation, and synapse development[6,9-11,15-20]. Moreover, decades of electrophysiological
371   studies have helped to establish the role of granule neurons in long-term cerebellar plasticity,
372   which underlies the circuit's involvement in learned motor behaviors[21-23]. Thus, granule neurons
373   are an excellent model system to interrogate key questions regarding synapse and circuit
374   development.
375
376   The goal of this study is to highlight a technique that takes advantage of the cerebellum to track
377   the morphology of a single neuron type *in vivo*. Electroporation of the cerebellum targets dividing
378   cells; thus, it is technically capable of labeling both granule neuron progenitors and glial cells,
379   although because granule neuron progenitors are so numerous, the electroporated population
380   is largely neuronal even without the need for cell-type specific promoters. Additionally, this
381   technique can be adapted to genetically manipulate genes *in vivo* to study their roles in CGN
382   development. This can be achieved by transfection of a plasmid expressing either short-hairpin
383   or small-interfering RNAs to knock down genes or a plasmid expressing Cre recombinase into a
384   transgenic mouse strain to excise regions flanked by LoxP sites and knock out the intended gene
385   of interest[10,24].
386
387   Electroporation has both strengths and weaknesses for genetic studies. It offers a faster method
388   to manipulate genes compared to traditional transgenic strategies, although the spareness of the
389   electroporation limits its utility for behavioral studies, which require large numbers of genetically
390   modified cells to see effects. A second limitation is that it only reliably targets granule neurons
391   between lobules 5 and 7, and this is due, in part, to how the cerebellum is oriented at the site of
392   injection. However, within the electroporated region, this method yields a relatively large

393 number of individually labeled cells, giving the potential for statistically robust measurements.
394 For example, not only was it possible to follow dendrite development over time, but also subtle
395 redistributions of membrane between the dendrites and claws could be measured, extending
396 previously published data[20]. CGN claws are particularly interesting to study as enlargement of
397 these dendritic structures provides additional real estate for granule neurons to form both
398 excitatory connections with mossy fiber terminals and unipolar brush cells as well as inhibitory
399 connections with nearby Golgi cells. Therefore, combining these dendritic measurements with
400 immunohistochemical labeling of specific pre- and postsynaptic proteins could be useful to
401 advance the study of cerebellar circuit formation and synapse maturation.
402

406

407 **DISCLOSURES:**
408 The authors declare no conflicts of interest.
409

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435 cerebellar granule neurons in vitro and in vivo to study neuronal morphology and migration.
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442   gene family, regulates the trafficking of ASTN1 during glial-guided neuronal migration. *Journal of*
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447   cerebellar development and foliation in BDNF-/- mice reveals a role for neurotrophins in CNS
448   patterning. *Neuron.* **19** (2), 269–281 (1997).

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450   differential expression of BDNF and NT3 receptors in developing cerebellar granule cells. *Journal*
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453   cerebellar precursors. *Neuron.* **55** (1), 53–68 (2007).

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455   dendritic survival in cerebellar granule cells in vivo. *Scientific Reports.* **8** (1), 17561 (2018).
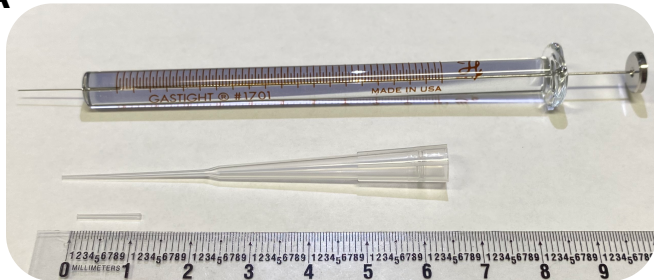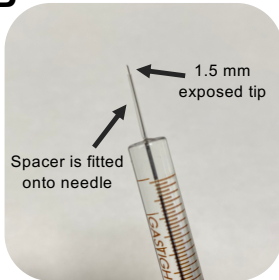
456   21   Ito, M. Synaptic plasticity in the cerebellar cortex and its role in motor learning. *Canadian*
457   *Journal of Neurological Sciences.* **20** (Suppl 3), S70–74 (1993).

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459   cerebellar parallel fiber-Purkinje cell synapses. *Neuron.* **52** (2), 227–238 (2006).

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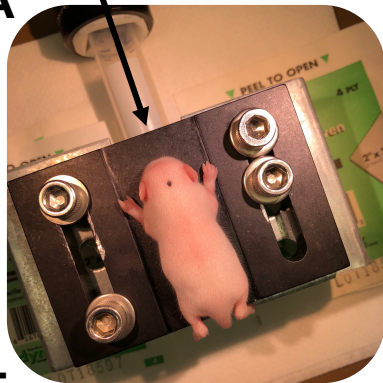461   *Neuroscience.* **162** (3), 723–731 (2009).

462   24   Chang, C. H. et al. Atoh1 controls primary cilia formation to allow for SHH-triggered
463   granule neuron progenitor proliferation. *Developmental Cell.* **48** (2), 184–199 e185 (2019).
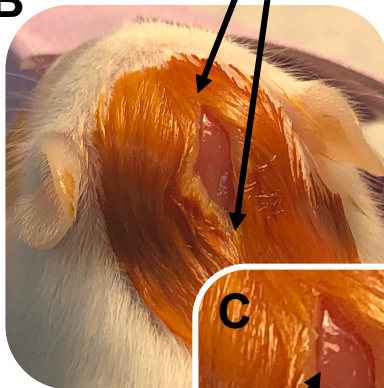
464

A

GASTIGHT ® #1701   MADE IN USA

MILLIMETERS
0 1 2 3 4 5 6 7 8 9

B

1.5 mm
exposed tip

Spacer is fitted
onto needle

A Constant delivery of isoflurane to ensure anesthesia

B Surgical incision stretches from the top to the base of the ears

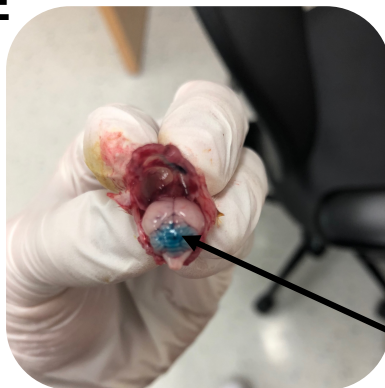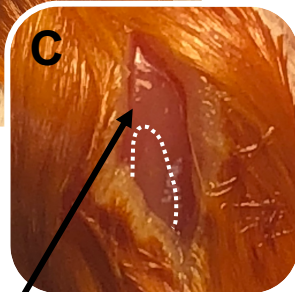**C** Injection site should be within 1 mm above white demarcation

D Tweezer-type electrode right above the incision
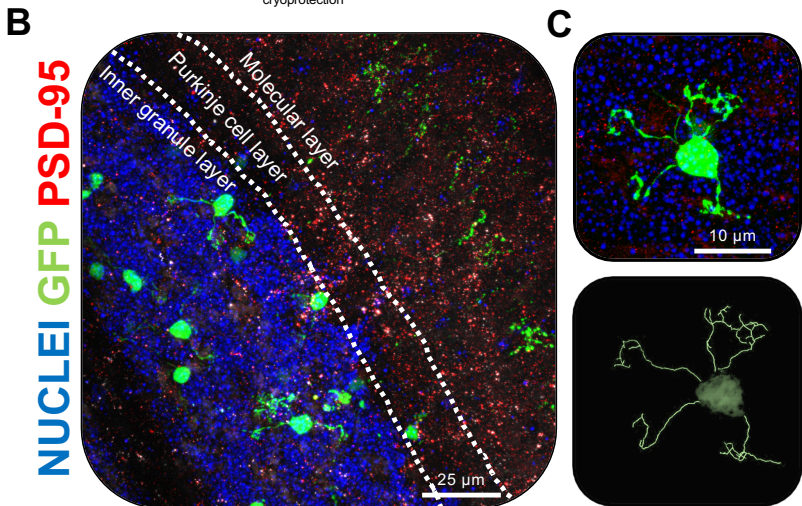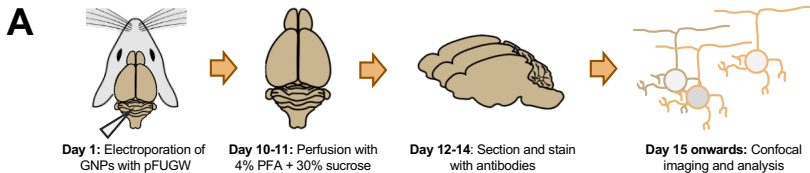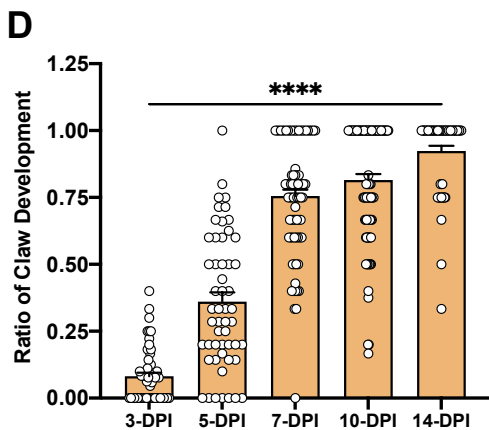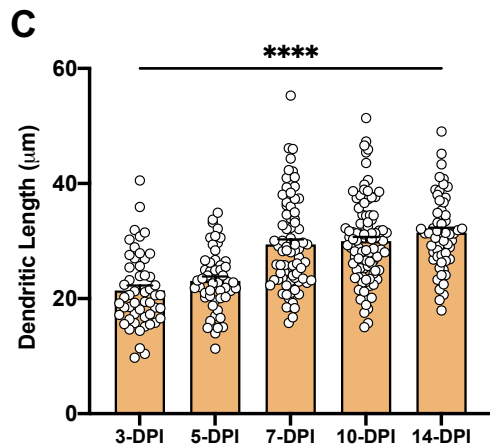
(+) end oriented downwards

E Fast Green dye is localized to the vermis

A Day 1: Electroporation of GNPs with pFUGW · Day 10-11: Perfusion with 4% PFA + 30% sucrose cryoprotection · Day 12-14: Section and stain with antibodies · Day 15 onwards: Confocal imaging and analysis

B NUCLEI GFP PSD-95 — Molecular layer · Purkinje cell layer · Inner granule layer — 25 μm

C 10 μm

D Single channel z-stack image

Simple Neurite Tracer (v3.1.6)

Tracing of C2-SIVE_63X_GFP_PSD95_C183P1.lsm (3D):

☐ Enforce non-inverted grayscale LUT
☐ Use three pane view? (will use an extra: 351MiB of memory)
☐ Look for Tubeness ".tubes.tif" pre-processed file?
☐ Look for Tubular Geodesics ".oof.ext" pre-processed file?
☐ Look for previously traced data (".traces" file)?

Choice of 3D Viewer: Create New 3D Viewer

Resampling factor: 2 (can be left at the default)

Cancel   OK

Cell body · Claw · Shaft

A

NUCLEI GFP PSD-95

10 μm

Surgery age: 3DPI --- 5DPI --- 7DPI --- 10DPI --- 14DPI

Postnatal age: P10 — P12 — P14 — P17 — P21

B

# of Dendrites

****

3-DPI 5-DPI 7-DPI 10-DPI 14-DPI

C

Dendritic Length (μm)

****

3-DPI 5-DPI 7-DPI 10-DPI 14-DPI

D

Ratio of Claw Development

****

3-DPI 5-DPI 7-DPI 10-DPI 14-DPI

E

Claw Length (μm)

****

7-DPI 10-DPI 14-DPI

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Betadine	Purdue Production	67618-150-17
Cemented 10 μL needle	Hamilton	1701SN (80008)
Chicken anti-GFP	Millipore Sigma	AB16901
Cotton-tip applicator		
Donkey anti-chicken Cy2	Jackson ImmunoResearch	703-225-155
Ethanol (200 proof)	Koptec	V1016
Electroporator ECM 830	BTX Harvard Apparatus	45-0052
Fast Green FCF	Sigma	F7252-5G
FUGW plasmid	Addgene	14883
Glass slides	VWR	48311-703
Glycerol	Sigma-Aldrich	G5516
Heating pad	Softheat	
Hoescht 33342 fluorescent dye	Invitrogen	62249
Imaris	Bitplane	
Isoflurane	Patterson Veterinary	07-893-1389
Micro cover glass	VWR	48382-138
Nail polish	Sally Hansen	Color 109
Normal goat serum	Gibco	16210064
O.C.T. embedding compound	Tissue-Tek	4583
Olympus MVX10 Dissecting Scope	Olympus	MVX10
P200 pipette reach tip	Fisherbrand	02-707-138
Parafilm	Bemis	PM-996
PBS pH 7.4 (10x)	Gibco	70011-044
Simple Neurite Tracer	FIJI	
Sucrose	Sigma	S0389
Surgical tools	RWD Life Science	
Triton X-100	Roche	11332481001
Tweezertrodes	BTX Harvard Apparatus	45-0489
Ultrapure distilled water	Invitrogen	10977-015
Vectashield mounting media	Vectashield	H1000
Vetbond tissue adhesive	3M	1469SB

Zeiss 780 Upright Confocal   Zeiss   780

Comments/Description

33 gauge, 1.27 cm (0.5 in), 4 point style

Our lab uses this antibody at a 1:1000 concentration

Our lab uses this antibody at a 1:500 concentration

Superfrost plus

Used for needle spacer

https://imagej.net/Simple_Neurite_Tracer:_Basic_Instructions

Small scissors and tweezers
non-ionic detergent
5 mm, platinum plated tweezer-type electrodes

Hello,

We thank the editor and the peer reviewers for careful reading of our submitted manuscript to JOVE. We have addressed all concerns and comments as outlined in blue text below:

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have proofread this manuscript to ensure there is no spelling or grammatical errors.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points
We have changed the font style of the manuscript to Calibri 12-pt and a space has been added between each step, substep, and note in the protocol section.

3. What kind of animal DNA is used in the study?
We elaborated on the type of DNA that can be used with this technique under step 1.1 (lines 87-94) of the protocol.

4. 2.6: How do you inject DNA in the cerebellum? Details on the needle used for injection.
We added more detail describing the injection of the construct into the cerebellum in step 2.7 (lines 136-140) of the protocol. Additional detail regarding the needle and preparation of the needle can be found in the Table of Materials as well as step 2.3 (lines 121-122) of the protocol.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."
All steps of protocol are written in the imperative tense.

6. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
More detail has been added to the protocol describing the "how" of each step.

7. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identify the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
We have highlighted two sections of the protocol step in yellow as filmable content.

_____

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The authors described a technique that uses the advantage of the cerebellum to track the morphology of a single neuron type in vivo. The method is simple and effective to visualize granule neurons in the mouse cerebellum over the time course.
The authors performed paired labeling method (GFP transfection + IHC) with image analysis in Imaris to document the changes in cerebellar granule cell morphology. This allowed quantification of total somatodendritic surface area and volume of the cells, which revealed that CGN size remains relatively constant across development but at P14 exhibits a 20% decrease in volume.

Major Concerns:
None

Minor Concerns:
The small picture panel visualizing tweezertrode positioning will be useful. However this is not an obligatory point.
We added a new panel to Figure 2 (Figure 2D), which shows the position of the tweezertrode along the head of the animal.

Reviewer #2:

Manuscript Summary:
This method article by Chan and coworkers carefully describes the procedures for electroporation of cerebellar granule neurons (CGNs) and monitoring their morphological development. The step-by-step procedures of the protocol are clearly explained and detailed. This method article will be useful for those who will start using this experimental approach. I only have minor comments or remarks.

Minor Concerns:
Introduction line 52: what exactly means '"distal arbors of Purkinje cells". The position of the parallel fiber on the dendritic tree depends on the timing of CGN migration. As shown by Espinosa and Luo (J. Neurosci 2008) or Markwalter et al (J. Neurosci 2019), CGNs that migrate early (~P5) establish contact on the lower part of the molecular layer (that is bottom part of the dendritic arbors of Purkinje cells) while those that migrate late make contact near the pia (distal part of the dendritic arbors). This needs to be clarified.
We have corrected this error and added additional information, detailing the findings of the two papers mentioned here (Espinosa and Luo, J. Neuroscience 2008 and Markwalter *et al.,* J. Neuroscience 2019) in lines 55-58.

Introduction line 61:
Purkinje cells are the sole (not the main) output of the cerebellar cortex. The main outputs of the cerebellum are neurons of the deep cerebellar nuclei. This needs to be corrected.
We have corrected this error as reflected in lines 65-67.

Protocol:
Suggestions
1- specific syringes for cerebral injections with a 33 gauge needle and built-in spacer are available from Hamilton company (Neuro syringe 700/1700 series).
Indeed, this protocol uses a customized syringe from the 1700 series and the information regarding the syringe can be found in the Table of Materials.

2-we have noticed that the survival rate of pups just immediately after electroporation is increased if the delivery of isoflurane is stopped 1-2 minutes before the delivery of electrical pulses.
We have updated our protocol to reflect this change in step 2.8 of the protocol (lines 142-144) – delivery of isoflurane is stopped prior to electroporation and the animal is moved to a heating pad prior to electroporation.

One of the main problems after doing surgery on pups is their cannibalism by the mother. The procedure to minimize cannibalism needs to more detailed:
-careful cleaning of the site of incision (the smell of blood increases the risk of cannibalism)
-pups can be kept isolated (1 pup alone in a small box place below an IR lamp -27/30°C) for several hours before returning them to the dam (thus the wound has time to heal and the smell of blood decreases or disappears) … in this case, pups should receive an intra-peritoneal injection of warm NaCl 0.9% (ideally + glucose 5%).
-pups can be wiped with the litter and droppings from the cage where the dam is.
We agree that parental infanticide and cannibalism are common problems associated with electroporations and we have incorporated more detail into step 2.11 of the protocol (lines 161-166) on how to handle the issue. We incorporated all but one of the reviewer's comments as we have never tried delivering an intra-peritoneal injection of warm 0.9% NaCl + 5% glucose solution.

Fig2 : a schematic showing the position of the 2 electrodes may be useful.
We added a new panel to Figure 2 (Figure 2D), which shows the position of the tweezertrode along the head of the animal.

Reviewer #3:
Manuscript Summary:
A potentially useful technique for examining CGN after n vivo labeling is described.
I consider JOVE a journal where innovative methodology is featured and explained with sufficient detail to educate researchers for use in their research. With this in mind, my concerns are below:

Major Concerns:
1. The title is very misleading. This protocol does not describe in vivo tracking, but rather an ex vivo snapshots. This is still potentially useful technique but it is a big letdown. Another more descriptive and less misleading title should be chosen. This should likewise be corrected in the manuscript body.
The title has been changed to reflect the technique more accurately and it has been corrected in the manuscript body.

2. No effort is taken to explain the label used outside the figure legends. What will work as a label? What won't work?

Are there alterations used to make it compatible with this methodology? This manuscript should state these useful guidelines and it does not. Although creation of the label might be outside the scope of this manuscript, these considerations must be included in the manuscript.

We elaborated on the type of DNA label that can be used with this technique under step 1.1 (lines 87-94) of the protocol. We also described additional ways to achieve specificity with certain DNA labels in the discussion in lines 369-372.

3. A lot more effort needs to be devoted to explaining the DNA label, and the image acquisition and image processing steps.

In addition to adding more details about the DNA label, we have added additional steps to the image analysis and processing portion of the protocol in lines 219-272.

4. Better explanation of why this technique is valuable and its potential applications needs to be conveyed in the introduction. (The last two sentences of the introduction just do not suffice.) Advantages over other techniques need to be clearly stated upfront.

We further explained the benefits of this technique, detailing the advantages over other techniques in the introduction of this manuscript in lines 69-77.

Minor Concerns:
1. Line 76. The acronym "IVE" needs explanation.
The acronym IVE has been changed to its full name, "*in vivo* electroporation."

2. Line 195 is the first mention of Imaris, and this software is not explained.
Imaging analysis using Imaris has been further explained in steps 4.8-4.12 of the protocol (lines 252-272).

3. Even though, use of FIJI is common, some effort must be taken to introduce FIJI and it plugin. As a suggestion begin this section with required software package and explain the rationale behind selection of this software.
We added more detail about FIJI and the plug-in used in this protocol in step 4.2 (lines 226-230). We also explained the advantages of using FIJI and this particular plug-in in the discussion in lines 282-285.

4. There is heavy reliance on proprietary software (eg. Imaris) and its capabilities are not explained. Why should anyone use Imaris? Can one use something else for image processing and what capabilities should it have?
While we used Imaris for our image analysis, other programs can also be used, and we have added more information about this in step 4.8 of the protocol (lines 255-257). Furthermore, we detailed the advantages of using Imaris in the discussion in lines 285-288.

5. Tissue adhesives? Are you referring to cyanoacrylates? Explain.
Yes, vetbond used in the protocol is a non-toxic n-butyl-ester cyanoacrylate and that has been updated in the protocol in step 2.10 (lines 154).

6. Why are some sections of the manuscript high-lighted?
Highlighted sections of the manuscript are to be filmed by JOVE.

7. Are these procedures performed in a hood? What are the minimum hood requirements?
The minimum hood requirement has been in step 2.1 of the protocol (lines 111-114).