Chapter 16

Sparse Labeling, Rapid Clearing, and Native Fluorescence Light Sheet Imaging in the Developing Rodent Cerebellum

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Abstract

This protocol outlines plasmid delivery via in utero electroporation, rapid tissue clearing using CUBIC, and light sheet microscopy with optimizations for endogenous fluorescent protein imaging to label and image neuronal cells and their projections in their native topographic context of the intact developing rodent cerebellum. This technique enables the study of neuronal migration, circuit architecture, and connectivity of cerebellar neurons, particularly focusing on Purkinje cells.

Key words CUBIC clearing, Plasmid electroporation, Purkinje cell labeling, Projection mapping, Cerebellum, Light sheet imaging

1 Introduction

Purkinje cells are organized in stereotyped microcircuitry within the cerebellar cortex, making topographic connections to output structures as well as receiving topographically organized input from other brain areas [1, 2]. However, determining the precise developmental connectivity and migration trajectories of individual Purkinje cells is difficult with currently available methods. We and others have used sparse labeling of cells via in utero electroporation for tracking of developing neurons and their projections by transfecting cells based on birthdate and anatomical location [3–5]. Here, we describe a method to label subsets of Purkinje cells with plasmids expressing fluorescent proteins and track the three-dimensional formation of the cerebellum by whole brain clearing and imaging with a light sheet microscope [6, 7].

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2 Materials

2.1 In Utero Electroporation

1. E10.5–E12.5 Timed-pregnant mice (e.g., CD1 from Charles River Laboratories) (*see* **Note 1**).
2. Isoflurane.
3. Oxygen tank.
4. 0.1% filtered Fast green FCF in nuclease-free water.
5. Analgesic (e.g., Buprenorphine).
6. Sterile cotton tip applicators.
7. Sterile gauze or Glad Press 'n Seal.
8. Hair removal cream (e.g., Veet).
9. Sterile disposable transfer pipettes.
10. Betadine surgical scrub.
11. Sterile surgical gloves.
12. Sterile bench pads.
13. 1/2 cc syringe with 27 G needle.
14. Parafilm.
15. 50 mL conical tubes.
16. Nylon monofilament reverse cutting 3/8 c 12 mm sutures.
17. Sterile 1× Phosphate Buffered Saline (PBS).
18. Alcohol antiseptic pads.
19. Glass capillary tubes, non-heparinized, diameter 1.1–1.2 mm (e.g., Fisherbrand).
20. Eye lubricant (e.g., Puralube Vet Ointment).
21. Electroporator (e.g., Electro Square Porator ECM 830).
22. Tweezertrodes and independent electrode.
23. Light source.
24. Water heat pad set to 37 °C (e.g., HTP-1500 heat therapy pump).
25. Dry block heater with 50 mL tube block set to 40 °C (e.g., VWR mini block heater).
26. Isoflurane vaporizer.
27. Pipette puller (e.g., Narishige).
28. Pipette beveler (e.g., Narishige).
29. Dissecting microscope.
30. Sterile surgical scissors, medium and micro.
31. Sterile forceps, blunt and sharp.
32. Sterile suturing tools.

33. Aspirator tube assemblies for calibrated microcapillary (e.g., Sigma-Aldrich).
34. Red heat lamp.
35. Fluorescent stereoscope (e.g., Leica).
36. Plasmid DNA (e.g., Addgene 164469).

2.2 Perfusion

1. Surgical scissors.
2. Forceps.
3. Peristaltic pump.
4. Needles (Precision Glide 30G × 1/2″).
5. Isoflurane chamber.
6. PBS.
7. 4% PFA pH 7.0–7.4.

2.3 Tissue Clearing

1. CUBIC-L (Fisher Scientific T3741).
2. CUBIC-R+(M) (Fisher Scientific T3740).
3. Water bath set to 37 °C.
4. Glass scintillation vials (Kimble).
5. 6-well plate.

2.4 Light Sheet Imaging

1. CUBIC-R+(M) as above to fill chamber.
2. Bondic UV liquid plastic welder.
3. Zeiss Light Sheet 7.

3 Methods

3.1 In Utero Electroporation of Purkinje Cells

The Purkinje cell progenitor zone is difficult to target with bipolar electrodes; therefore, the triple electrode approach is utilized [8, 9].

3.1.1 Preparation

1. Either breed or order timed-pregnant mice to be embryonic day 10.5–12.5 on the day of surgery (*see* **Note 2**).
2. Pull glass capillary tubes with a pipette puller, then bevel with constant water flow at a 25–35° angle to create a smooth end. Ensure the appropriate pore size and check with a dissecting microscope that there are no chips in the glass that may damage the embryo. The narrow part of the tip should be at least 6 mm. Push accumulated water out of the tip and onto a Kim wipe with an aspirator.
3. Prepare plasmid DNA that expresses a fluorescent marker to track neurons that were electroporated. Combinations of DNA should stay ≤4 μg/μL total in elution buffer. For visualization

of injection into the ventricle, add fast green at a ratio of 1:10–20 DNA mix for a total volume of 20 μL.

4. Ensure heat pad and heat block come to temperature.
5. Sterile 1× PBS in 50 mL conical tubes are placed in the heat block at 40 °C.
6. The electroporator should be set with the following parameters:
   - Pulse Voltage: 25 V
   - Pulse Length: 50 ms
   - Number of Pulses: 6
   - Interpulse Interval: 1 s
7. Gather a sterile bench pad, sterile gauze, eye lubricant, sterile cotton tip applicators, alcohol antiseptic pads, iodine, parafilm, hair removal cream, sutures, beveled capillaries, aspirator, analgesic in a 1/2 cc syringe with 27 G needle, and sterilized surgical tools, then place within the surgery space.

3.1.2 In Utero Electroporation Surgery

1. Place mouse in the induction chamber with 3% isoflurane until breathing has slowed and anesthesia depth is confirmed through regular observation of respiratory rate, heart rate, and lack of response to toe pinch, or as specified by the lab's animal use protocol.
2. While the mouse is being anesthetized, cover the cap of a 50 mL conical tube with parafilm. Pipette the DNA solution on top of the parafilm, then with the aspirator, suck up the liquid with the prepared capillary needles. All 20 μL can be added to the capillary tube for repeated injections. Ensure there is no breakage of the needle end or bubbles introduced (*see* **Note 3**).
3. Place the mouse prone with its head in the nose cone and ensure deep anesthesia with a toe pinch. With a syringe, inject analgesia subcutaneously (e.g., Buprenorphine) and apply eye lubricant with a cotton tip applicator, which can be reapplied every 20 min as needed. Flip the mouse on its back with its nose to the back of the nose cone. Adjust the isoflurane level to keep breathing slow but without gasps.
4. Apply a small amount of hair removal cream (e.g., Veet) with a cotton tip applicator and spread with small circles on the center of the stomach, ensuring not to apply to the nipple area. Leave the cream on for 1–2 min, then use Kim wipes and PBS to remove all cream from the area.
5. Clean the stomach area with alcohol wipes and apply betadine to the incision area.

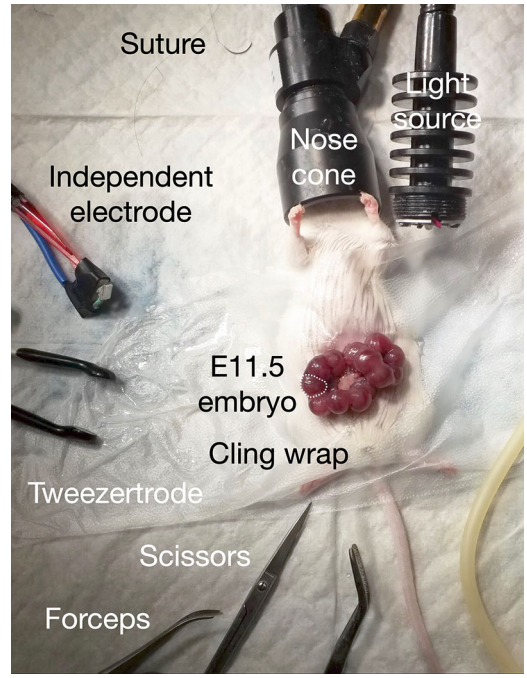

Fig. 1 Exposure of E11.5 embryos within the uterus. Embryos are pulled through the incision in the abdominal wall. Items in surgical field labeled

6. Cut a small circle in gauze or Glad press 'n seal and place the hole over the incision area so that the embryos can be placed on a sterile surface once removed.
7. If the mouse does not respond to a toe pinch, proceed with an incision along the midline of the skin of the stomach, about 3 cm in length. Create another incision along the underlying muscle layer without nicking the internal organs underneath.
8. Once the abdominal cavity is exposed, apply warm PBS with a transfer pipette to keep the embryos and organs moist.
9. Gently move the intestines and find the underlying embryos with blunt forceps. Scoop embryos out through the incision by placing forceps in the space between the embryos and pull up without pinching the forceps forcefully.
10. There will be two uterine horns with embryos. Pull each embryo out until there is slight resistance as the muscle at the end of the uterine horn is gently pulled (Fig. 1). Count the number of embryos and record.
11. Apply PBS to the embryos every few minutes as needed to keep moist and warm.
12. Avoiding the two embryos at the end of each uterine horn nearest the cervix, use gloved fingers to manipulate the embryo to become visible through the uterine wall. Look for the large

dark fourth ventricle, but do not move the embryos excessively as this can cause embryo reabsorption. If no ventricle can be found, move to the next embryo that may be in a better position. Be careful not to rupture the embryonic sac by squeezing too firmly.

3.1.3 Injection and Electroporation

1. Check that blowing through the aspirator pushes the DNA solution dropwise out of the needle. If it is blocked, choose an embryo that will not be injected and push just through the uterine wall back and forth while blowing air until liquid begins to come out.
2. Inject 4–5 μL of the plasmid solution either into the third or fourth ventricle by gently blowing into the aspirator. Injecting into the third ventricle may be less likely to result in embryo death due to the tip impaling underlying structures. Additionally, injecting into the third ventricle until the fourth ventricle is also filled allows electroporation of cells in the superior colliculus, which enables fluorescence screening after birth. Pierce the embryo with the needle just enough to reach the ventricle and not further so as not to damage brain tissue. The entire fourth ventricle should have green dye in the shape of a triangle (Fig. 2).
3. Place the tweezertrode connected to the negative lead on either side of the embryo laterally. The independent positive electrode is placed directly over the filled fourth ventricle but a bit superior so that the third ventricle is also targeted (Fig. 3). The third electrode can be manipulated a small amount left or right to target the Purkinje cell progenitor zone bilaterally or

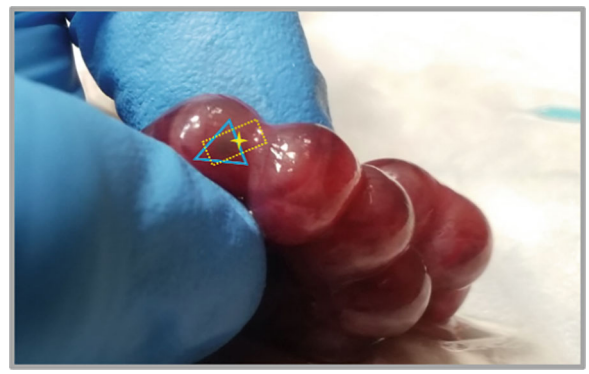

Fig. 2 Injection of DNA solution with fast green into the third/fourth ventricles. Once the entirety of the fourth ventricle is filled (blue triangle with dorsal brain toward the right), place the independent electrode over the ventricles as shown by the yellow dashed rectangle. The center of the electrode should be placed directly over the Purkinje cell progenitor zone, located on either side of the yellow star

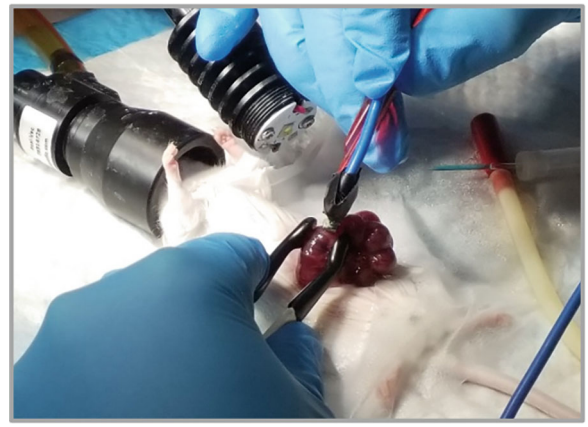

Fig. 3 Placement of triple electrode. Squeeze firmly for contact, but gently to avoid damaging the embryo and apply electrical pulses with foot pedal

both sides can be electroporated simultaneously for whole cerebellar expression.

4. With the electrodes firmly squeezing the embryo for good contact but not enough to rupture the embryonic sac, press the foot pedal to apply the series of electrical currents. To ensure an electrical field is produced at this lower voltage, put all three electrodes close together in PBS and ensure bubbles form before beginning electroporations.
5. Repeat the injection and electrical pulses on 3–6 embryos, depending on litter size.
6. Once all electroporations are complete, return the embryos to the abdominal cavity by gently pushing back through the incision with gloves or forceps and without twisting the uterus. Add a small amount of PBS into the abdominal cavity.
7. Suture the muscle layer with a continuous stitch, then suture the skin layer as well.
8. Place the mouse in its home cage on its side/back with the cage on a heat pad and a red heat lamp over the top of the cage where the mouse is underneath. Monitor to ensure the mouse wakes up and is able to flip over and walk around normally. Monitor the mouse daily for 5 days and administer buprenorphine as needed if the mouse displays signs of pain.
9. After birth, at age E18–20, screen the P0 pups for fluorescence with a dissecting microscope. The cerebellum develops postnatally and is hidden beneath other brain structures, so it is generally easier to screen the superior colliculus (*see* **Note 4**).
10. Return pups to the mother until the desired age. By P14, Purkinje cell migration is mostly complete.

3.2 Perfusion

Adapted from Hoffman and colleagues [10].

1. Prepare tools, dissecting tray, and run ice-cold PBS and 4% PFA through the tubing of the peristaltic pump (*see* **Note 5**).
2. Place the mouse in an isoflurane chamber until breathing is slowed.
3. Place the mouse on a dissecting tray with a microcentrifuge tube containing a Kim wipe and isoflurane over its nose, then if unresponsive to a toe pinch, hold down limbs with pins.
4. Make an incision along the midline of the abdominal skin, then make another incision in the muscle layer underneath without cutting the underlying organs.
5. Cut the diaphragm and sides of the rib cage, which can then be flipped and pinned back to expose the heart. Remove the tube with isoflurane from the nose.
6. Make a small cut in the right atrium to allow blood to drain.
7. Immediately run cold PBS at a speed appropriate for the mouse's age until the liver is pale and fluid leaving the right atrium is clear of blood.
8. Switch to cold 4% PFA and perfuse the mouse for 15–20 min (20–40 mL PFA) to ensure appropriate fixation that will withstand the clearing process. The PFA should cause muscle movements if the needle is placed correctly and fluid is running appropriately through the circulatory system. The animal will be very stiff at the end of 20 min.
9. Dissect the brain out of the skull. To avoid nicking the cerebellum with the scissors, cut first along one lower lateral side of the skull starting at the foramen magnum. Then cut along the midline from the olfactory bulb (place the blade between the two cortical hemispheres) until the tip of the scissors meets the midbrain. Peel one half of the skull away and then the other half, being careful not to pull off the cerebellar flocculus on either side.
10. Post-fix in 4 °C 4% PFA for 12–24 h. Wash in PBS several times, then shake in PBS overnight at 4 °C. Brains should always be kept cold to avoid loss of fluorescent signal.
11. For P0 mice, significantly decrease the perfusion to a slow drip to avoid swelling the ventricles with too much pressure.
12. If looking at embryonic developmental dates, the pregnant dam is kept under anesthesia with a nose cone, an incision is made along the midline, and the embryos are removed from the amniotic sac. The embryo is screened for fluorescence, then drop-fixed in cold 4% PFA.

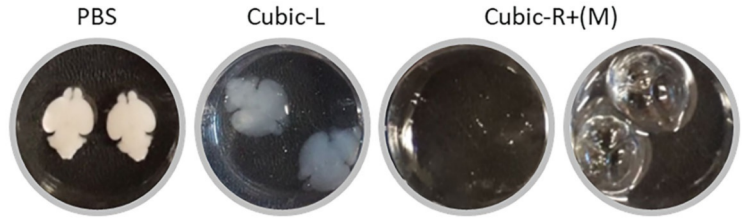

Fig. 4 P7 brains in the solution indicated within a six-well plate. After several days in Cubic-L, the brains become translucent. In Cubic-R+(M), the brains are completely transparent when covered in solution (left) but can be identified when the liquid is removed gently with a transfer pipette (right). It is normal to see some expansion of the brain. At P7, the white matter tracts are not visible, but will remain if the animals are older

3.3 Clearing with CUBIC Reagents

Modified from Matsumoto and colleagues [11].

1. With a blade, cut off the prefrontal cortex to provide a flat side for gluing to the light sheet specimen holder.
2. Place the fixed brain into fresh CUBIC-L and shake at 37 °C. Every 2 days, replace the solution with fresh CUBIC-L until the brain is uniformly opaque and white matter tracts can no longer be seen (Fig. 4). Mice under the age of P7 clear within a few days, while older mice take longer to clear. Mice several months old may not clear to the extent that younger mouse brains will, especially the white matter tracts.
3. At this stage, brains can be checked for fluorescence and imaged on a dissecting microscope if desired.
4. Remove brains from CUBIC-L with a spoon and place them in a small amount of CUBIC-R+(M). Gently shake the brains to remove the majority of CUBIC-L, then scoop brains into a new well with fresh CUBIC-R+(M).
5. Shake in CUBIC-R+(M) at room temperature for 2 days or until the brain is transparent.

3.4 Light Sheet Imaging and Analysis

1. Fill the chamber with CUBIC-R+(M).
2. Apply Bondic glue to the edges of the flat side of the cortex and use UV light to dry glue on the brain and sample holder. Apply additional glue until steadily held (Fig. 5).
3. Place the sample holder and glued brain into the chamber to equilibrate, usually a few hours, but can be left overnight. If the brains have not had enough time to equilibrate in the chamber, there may be high background and poor resolution upon imaging.
4. Click the ZEN icon to start ZEN for light sheet.

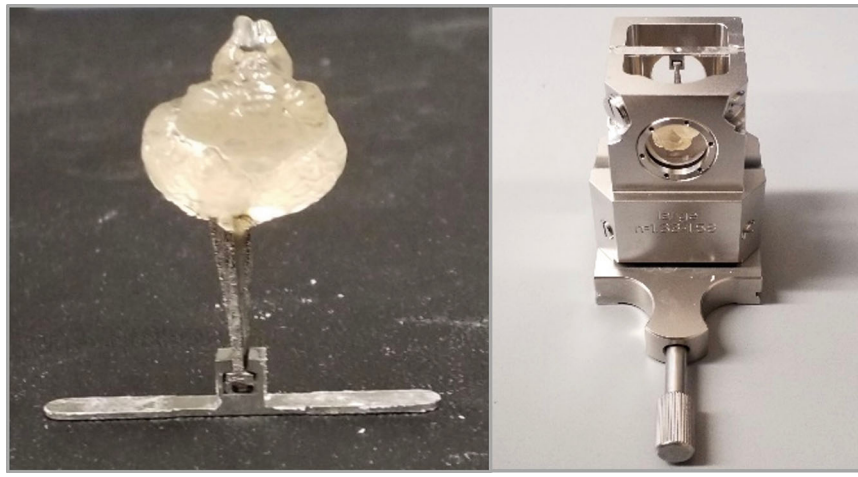

Fig. 5 P14 brains require some balancing and extra glue on the small sample holder (left). Use the cortex to glue onto the end of the metal piece. Once sturdy, can flip and place in chamber (right)

5. Click on the arrows to expand boot status and hardware configuration database and choose the clearing database under "recent." Choose "start system."
6. Once start-up is complete, ensure that the detection objective and each illumination collar is set at 1.52 to match the refractive index of the CUBIC solutions.
7. Place the chamber into the light sheet and dock the sample onto the sample holder.
8. Bring the sample above the solution so it does not interfere with alignment. Align the light sheet so that the waist is as thin and in focus as possible by turning the illumination collars.
9. Adjust the brain to be in line with the detection objective, configure the light path and laser, then press continuous to find the region of interest. It is easiest to start at a zoom of 0.4 to have a wider view and brighter fluorescence. Scroll through the z-plane to get an overview of the desired area. Decreasing the maximum range of the lookup tables so that the background is visible can help to orient within the brain when fluorescence is not visible (*see* **Note 6**).
10. While at a 0.4 zoom, set the four outer corners of the region of interest in the bounding grid tab (Fig. 6) and set the z-stack range. Setting an overlap of 15% and reducing the frame size will avoid a dark gradient at the edges of each tile. Using one side of light sheet illumination will reduce acquisition time; however, dual-side illumination can be used, if necessary, with larger brains (*see* **Note 7**).

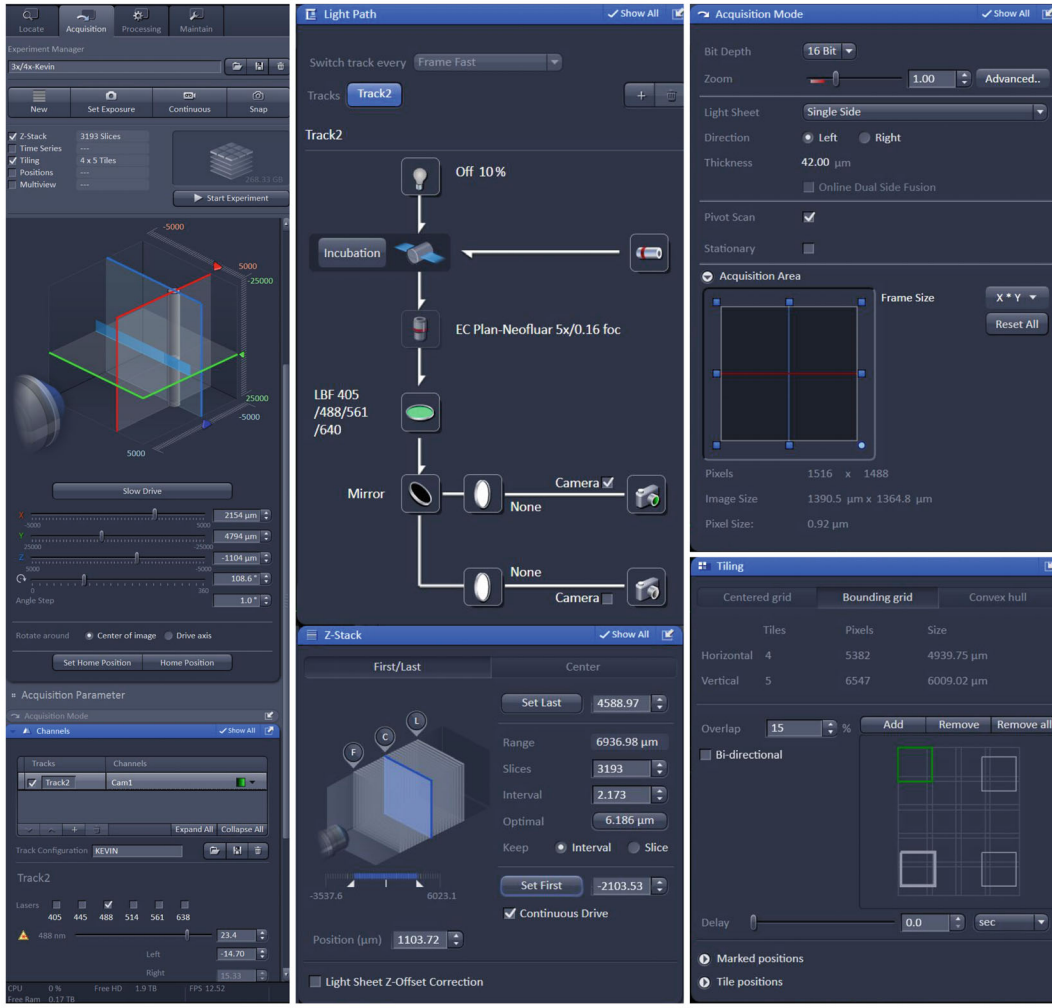

Fig. 6 Typical settings for light sheet imaging

11. Imaging will take less time if kept at a wider zoom, so can be imaged as is. Alternatively, adjust the zoom to 1, focus on a typical area, and adjust the laser power and exposure to give the desired dynamic range for imaging. Check pivot scan, then start experiment to save the file and begin imaging.
12. Once complete, the file can be opened in Zen Blue for processing or an alternative software such as Arivis. When imaging with a zoom factor, large tile artifacts are often produced so that Zen Blue does not automatically align the tiles properly. Arivis has an option to manually move tiles to a larger degree, then automatically stitch the rest together. For example, in the Tile Sorter window in the "manual" tab (Fig. 7), the left column of tiles is selected (white borders) and dragged over

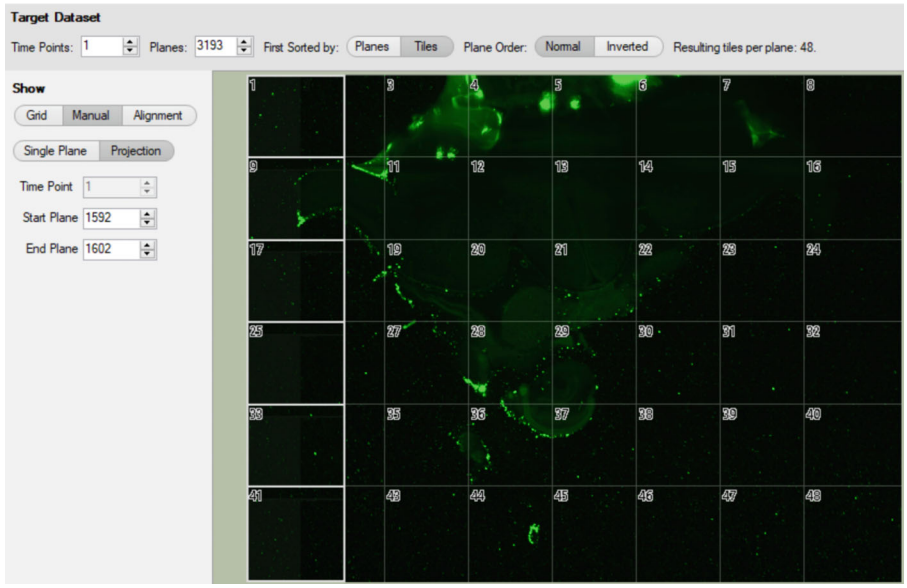

Fig. 7 Stitching tiles in Arivis

the top of the underlying tiles in which the region was imaged with a large degree of overlap (this typically occurs at each side of the image set). This manually positions the overlapping regions closer to the correct alignment so that automatic stitching can be appropriately calculated when running the "create stitched planes" operation. It is helpful to use the "projection" tab to collapse multiple z planes of each tile and use the transparency slider to correctly overlay one tile on top of another.

13. Once stitched, an Arivis pipeline can be created for standard imaging processes such as background correction and denoising as required.
14. Typically, background from brain tissue can be seen when adjusting the lookup tables. This can be helpful to orient within the brain and determine how the electroporation field is oriented in a 3D space. The 4D view control is helpful for 3D movement, and strategically placed 4D clipping planes allow movement through the image stack to visualize regions of interest more clearly (Fig. 8). Depending on the goal, downstream processes can be incorporated to quantify cell body location and map projections to output structures (*see* **Note 8**).

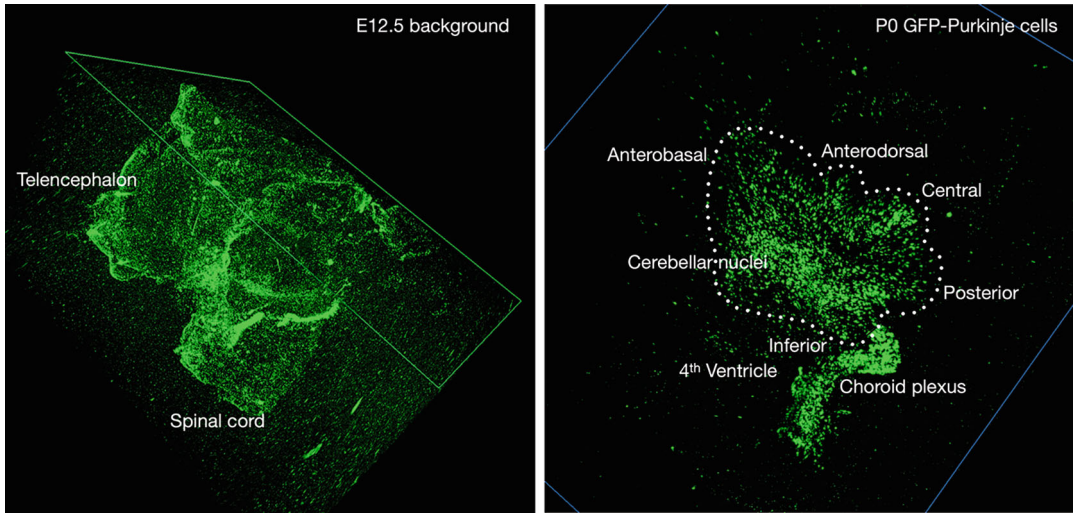

Fig. 8 Light sheet imaging examples. Left: An E12.5 brain with background fluorescence enhanced to orient the electroporation field within the whole brain. A 4D clipping plane is utilized to find the electroporation field within the stack. Right: A P0 brain Purkinje cell electroporation field with background removed, illuminating the emerging 3D structure of the cerebellum

4 Notes

1. E10.5 mice are very difficult to see through the uterine wall. Pressing LED lights directly against the back of the embryo may be required to target the ventricles. E11.5 and E12.5 are more routinely used, with 11.5 giving the largest number of electroporated cells.
2. Purkinje cells are not born outside of E10.5–E12.5.
3. After targeting a few embryos, the tip may become compromised. If there is resistance when targeting the next embryo, break the end of the tip and push out the remaining DNA solution onto the parafilm cap, then load a new needle without creating bubbles.
4. Survival rate of positive embryos is 40–50%. Important factors to increase chances of survival are using the lowest possible voltage, having a smooth end tip of small diameter and without bubbles in solution, keeping the injection depth shallow to avoid damaging underlying structures, and manipulating the embryos gently without squeezing too hard with the electrodes.
5. Keeping the brains cold at all times is important for endogenous fluorescence surviving through to the end of the clearing process.

6. The key factor in retaining enough fluorescence signal to image with light sheet is the quality of the electroporation. A strong electrical field and small plasmid sizes are needed to have a high transfection efficiency in the Purkinje cell progenitor zone. If the field is weak due to poor contact and the transfection is low, much of the endogenous fluorescence will be lost in the clearing process and will be difficult to separate from the background.
7. High laser power does not typically quench the fluorophores, so it is preferred to increase the laser power (100% is routinely used without bleaching) and decrease the exposure time for faster imaging. It is recommended to take whole brain images zoomed out since the signal is brighter for light sheet and then zoom in if specific regions are needed at higher resolution.
8. If projection mapping is required, a GPI-anchored fluorescent marker is recommended. Projections are typically brighter and better retained after clearing, although high laser power is recommended. Arivis Vision 4D accompanied with a VR headset may be useful to aid in tracing projections.

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