Video Article

Whole-mount Imaging of Mouse Embryo Sensory Axon Projections

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Abstract

The visualization of full-length neuronal projections in embryos is essential to gain an understanding of how mammalian neuronal networks develop. Here we describe a method to label *in situ* a subset of dorsal root ganglion (DRG) axon projections to assess their phenotypic characteristics using several genetically manipulated mouse lines. The TrkA-positive neurons are nociceptor neurons, dedicated to the transmission of pain signals. We utilize a *TrkA*^{taulacZ} mouse line to label the trajectories of all TrkA-positive peripheral axons in the intact mouse embryo. We further breed the *TrkA*^{taulacZ} line onto a *Bax* null background, which essentially abolishes neuronal apoptosis, in order to assess growth-related questions independently of possible effects of genetic manipulations on neuronal survival. Subsequently, genetically modified mice of interest are bred with the *TrkA*^{taulacZ}/*Bax* null line and are then ready for study using the techniques described herein. This presentation includes detailed information on mouse breeding plans, genotyping at the time of dissection, tissue preparation, staining and clearing to allow for visualization of full-length axonal trajectories in whole-mount preparation.

Video Link

The video component of this article can be found at https://www.jove.com/video/52212/

Introduction

Establishment of precise neuronal networks is a complex developmental process essential for the functionality of the nervous system. Disturbance in this process leads to neuronal dysfunction which has been implicated in human neurological diseases[1-3]. To study the underlying molecular mechanisms of axon growth and target innervation in mammals, we have developed a protocol to visualize the axonal trajectories of TrkA-expressing sensory neurons using a combination of two genetically modified mouse lines.

TrkA is a receptor for nerve growth factor NGF and is a functional marker of nociceptive sensory neurons[4]. TrkA is highly expressed in nociceptive neurons during early development and mediates NGF-dependent neuron survival, axon growth, arborization and target innervation[5-9]. In *TrkA*^{taulacZ} mice, the wild type *TrkA* gene is replaced by a *taulacZ* expression cassette[10], such that the axonal morphology of putative TrkA-positive neurons can be visualized by β-gal (X-gal) staining[11]. Using a heterozygous *TrkA*$^{taulacZ/WT}$ line, we can examine factors that may regulate or interfere with the development of sensory afferent projections *in vivo*.

Moreover, TrkA expression is absent in homozygous *TrkA*$^{taulacZ/taulacZ}$ mice, which can therefore be used to assess the axon growth promoting mechanisms in the absence of NGF/TrkA signaling. Since nociceptive neurons depend on NGF/TrkA signaling not only for axon growth, but also for survival, we employ another mouse line, lacking the pro-apoptotic *Bax* gene, to inhibit apoptosis in embryonic DRG neurons, rescuing them from cell death that is otherwise observed in the absence of TrkA signaling. The *Bax*$^{-/-}$ background[12] thus allows for the molecular dissection of signaling pathways that specifically affect axon growth[7-9,13-15]. In *TrkA*$^{-/-}$ *: Bax*$^{-/-}$ mice, DRG neurons survive, but sensory afferent innervation in the skin is completely abolished[14,15]. We can selectively activate signaling pathways to determine their respective contributions to the development of axon projections. The utility of this method is that it allows the assessment of changes in axonal growth phenotypes when different genetic modifications are bred onto the *TrkA*$^{taulacZ/taulacZ}$ *: Bax*$^{-/-}$ or *TrkA*$^{taulacZ/WT}$ *: Bax*$^{-/-}$ backgrounds.

Protocol

NOTE: All procedures comply with the NIH Guide for the Use and Care of Laboratory Animals. The animal protocol was approved by the IACUC at Weill Cornell Medical College.

1. Tissue Preparation

1. Euthanize timed-pregnancy females by cervical dislocation[15]. Dissect embryonic E16 - E18 embryos from timed-pregnancy females and place embryos individually in the wells of a 6-well dish, filled with cold phosphate buffered saline (PBS).
2. Rinse embryos in cold PBS.

3. Remove a small portion of the amniotic membrane of the embryo and place in a pre-prepared proteinase K solution for subsequent genotyping. Alternatively, if the amniotic membrane is lost, remove a small portion of the tip of the embryo tail and place it in proteinase K solution
4. Poke holes into the skin all around the embryo using insect pins (at least 100 per side).
5. Remove PBS and slowly add fresh 2% paraformaldehyde (PFA), pH 7.4, to the well.
6. Gently shake for 2 hr at 4 °C.
7. Rinse embryos twice in PBS and store in PBS at 4 °C until staining.

2. Genotyping

1. Immerse amniotic membranes or embryo tails in proteinase K mixture according to the manufacturer's instructions.
2. Incubate at 56 °C for 10 min.
3. Extract DNA using a commercially available DNA purification kit.
4. Take 0.5 µl of the total 200 µl purified genomic DNA solution, combine it with 0.5 µl of each of the gene-specific sense and antisense primers (10 µM), 2 µl dNTP Mix (2.5 mM of dATP, dCTP, dGTP and dTTP), 0.2 µl Taq polymerase (5 U/µl) and 2 µl PCR buffer (10x), add H_2O to a total volume of 20 µl.
   NOTE: Primer sequences are as follows (**Table 1**):
   TrkA: TrkA_F: GCG GGC GCC GCC GCG ATG, TrkA_R: GAA GCC GCC TGC GCG GCT CTG CCA GGG TG; PCR fragment length: 400 basepairs (bp).
   Bax: In5R: GTT GAC CAG AGT GGC GTA GG, Ex5F: TGA TCA GAA CCA TCA TG, NeoR: CCG CTT CCA TTG CTC AGC GG; PCR fragment lengths: wild type, 304 bp; Bax null, 507 bp.
   TauLacZ: lacZup: ACA ACG TCG TGA GTG GGA AAA, lacZdown: ATC AAC ATT AAA TGT GAG CGA G; PCR fragment length: 360 bp.
5. Run a PCR with the following program for TrkA: step 1: 1 min at 95 °C, step 2: 10 sec at 95 °C, step 3: 20 sec at 68 °C, step 4: 30 sec at 72 °C. Repeat steps 2 - 4 for 40 cycles.
   1. For LacZ and Bax, run a PCR with the following program: step 1: 1 min at 95 °C, step 2: 10 sec at 95 °C, step 3: 1 min at 54 °C, step 4: 1 min at 72 °C, repeat steps 2 - 4 for 35 cycles.
6. Run PCR samples on a 2% agarose gel at 100 V in 1x Tris-Acetate-EDTA buffer for 20 min with a lane of DNA ladder to assess fragment lengths.
7. Analyze embryos for the presence of wild type TrkA, TrkA-tauLacZ and Bax null alleles. The genotypes of experimental embryos are $TrkA^{taulacZ/WT} : Bax^{-/-}$ and $TrkA^{taulacZ/taulacZ} : Bax^{-/-}$, depending on the experimental questions. Embryos lacking the tauLacZ reporter will be negative for axonal staining and can therefore serve as negative controls to calibrate the staining process.

3. Embryo Staining

1. Use a commercial kit for lacZ staining with some modifications as described below in **sections 3.2 - 3.8**. Here, use the Millipore kit.
2. Immerse embryos in Buffer A for at least 1 hr, shaking gently at room temperature.
3. Directly immerse embryos in Buffer B for at least 15 min, shaking gently at room temperature.
4. While embryos are in Buffer A/B, pre-warm Tissue Base solution at 37 °C. Use 5 ml of Tissue Base solution per embryo.
5. While embryos are in Buffer A/B, prepare fresh 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside (X-gal) at a concentration of 40 mg/ml in 100% dimethyl sulfoxide (DMSO).
6. Dilute X-gal into the pre-warmed Tissue Base solution at a concentration of 1 mg/ml and add 5 ml of X-gal/Tissue Base mixture to a glass scintillation vial.
7. Transfer embryos to a glass scintillation vial containing X-gal/Tissue Base mixture. Seal lid with Parafilm and incubate at 37 °C overnight, checking for presence of blue stain.
8. Postfix embryos with fresh 4% PFA, pH 7.4. Gently shake for 4 hr at 4 °C.
9. Rinse embryos twice in cold PBS.

4. Tissue Dehydration and Clearing

1. Place embryos in 50% methanol/ 50% PBS mixture for 30 min at room temperature, shaking in the glass vials.
2. Continue to dehydrate in 75% methanol/ 25% PBS for 30 min at room temperature, shaking in glass vials.
3. Continue to dehydrate in 90% methanol/ 10% PBS for 30 min at room temperature, shaking in glass vials.
4. Continue to dehydrate in 100% methanol for 30 min (2x) at room temperature, shaking in glass vials.
5. While embryos are dehydrating, prepare a 1:1 (v:v) mixture of benzoyl alcohol and benzoyl benzoate.
   NOTE: Benzoyl alcohol and benzoyl benzoate is toxic.
6. Immerse embryos in the 1:1 (v:v) mixture of 100% methanol: 100% BABB for 15 min. Only use glass because the BABB will dissolve plastic.
7. Immerse embryos in 100% BABB to completely clear the tissue and to visualize X-gal staining in the cleared embryo.
   NOTE: Image as soon as possible because the X-gal stain will fade over time.

5. Whole Mount Imaging

1. Stabilize the embryo, immersed in 100% BABB, at the appropriate angles for photography using metal forceps. Illuminate using a combination of up-light and under-light light sources.
2. Acquire images with a dissecting microscope outfitted with a digital camera. Take multiple bright field exposures at five successive focal planes 0.5 mm apart along the Z axis. Exposure times and f-stops may vary according to illumination and camera specs and need to be optimized for each setup.

3. Use standard image processing software to process overlay images and generate photomontages.

Representative Results

The genotypes of TrkA$^{WT/taulacZ}$: Bax$^{-/-}$ and TrkA$^{taulacZ/taulacZ}$: Bax$^{-/-}$ embryos can be unambiguously determined by standard PCR genotyping (**Figure 1**). X-gal staining displays detailed peripheral axonal arbors subcutaneously in conventionally stained embryos (**Figures 2, 3a**), and throughout the embryo after tissue clearing (**Figures 3b, 4**).

We have bred the TrkA$^{WT/taulacZ}$: Bax$^{-/-}$ line with mice carrying the constitutively kinase-activated B-RAF (V600E) mutation (LSL-kaBraf[16]) under control of the neuron-specific nestin promoter[17], to obtain LSL-kaBraf : TrkA$^{taulacZ/taulacZ}$: Bax$^{-/-}$ mice. In these embryos, which completely lack TrkA signaling, the morphology of nociceptor peripheral projections nevertheless developed nearly normally (**Figure 4**). This demonstrates a crucial function for B-RAF signaling in peripheral axon growth, and also shows that the survival and axon-growth promoting functions of TrkA signaling can be separated, with the *Bax*$^{-/-}$ genetic background substituting for the loss of TrkA-dependent survival signaling.

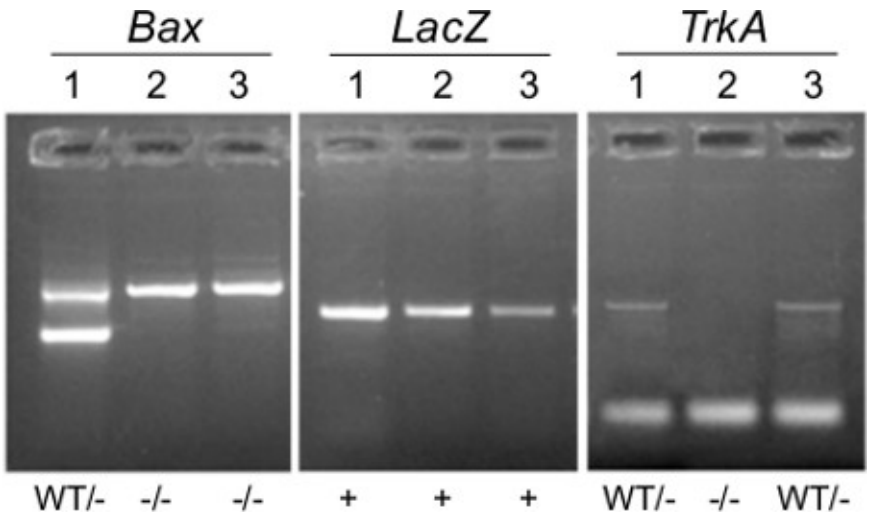

Figure 1: Genotyping of the transgenic mice. Representative agarose gel images. Sample 1: TrkA$^{WT/taulacZ}$: Bax$^{WT/-}$, sample 2: TrkA$^{taulacZ/taulacZ}$: Bax$^{-/-}$, sample 3: TrkA$^{WT/taulacZ}$: Bax$^{-/-}$.

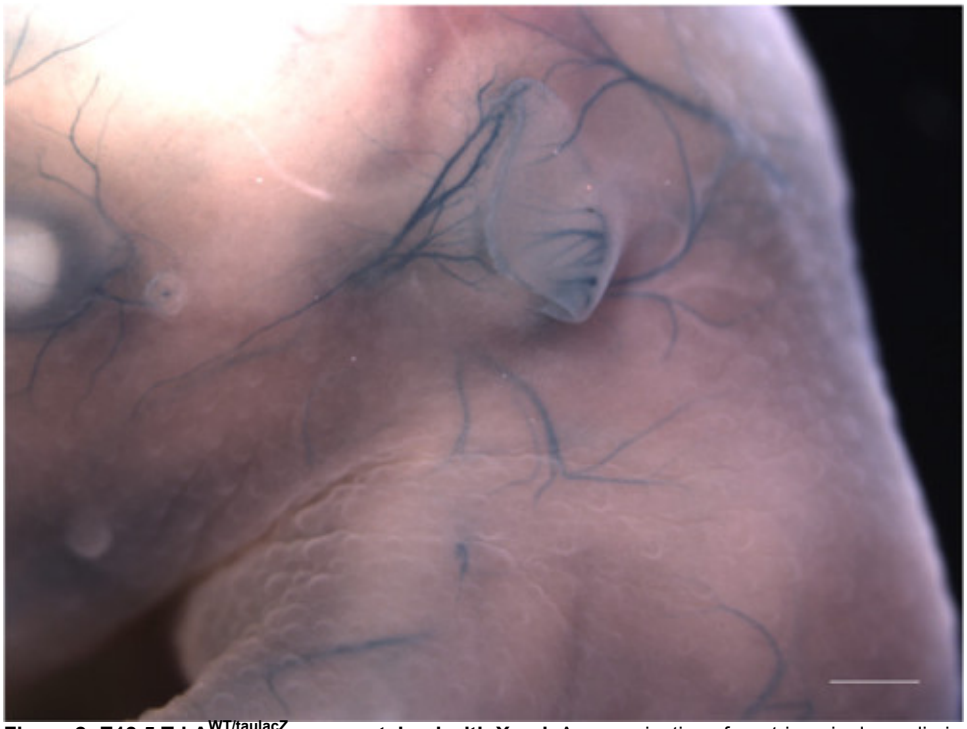

Figure 2: E18.5 TrkA$^{WT/taulacZ}$ mouse stained with X-gal. Axon projections from trigeminal ganglia in torso and head are shown in an uncleared embryo. Scale bar: 1 mm.

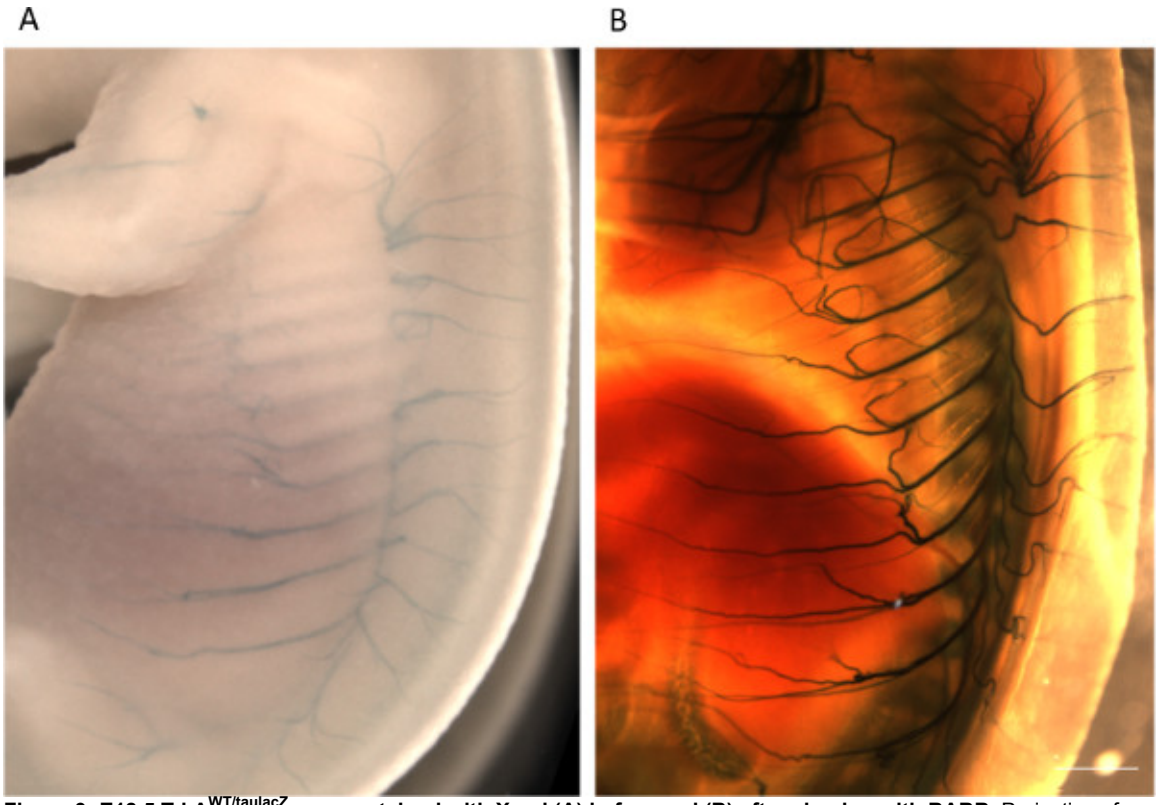

Figure 3: E18.5 TrkA$^{WT/taulacZ}$ mouse stained with X-gal (A) before and (B) after clearing with BABB. Projections from DRGs in mid-torso are shown. Scale bar: 2 mm.

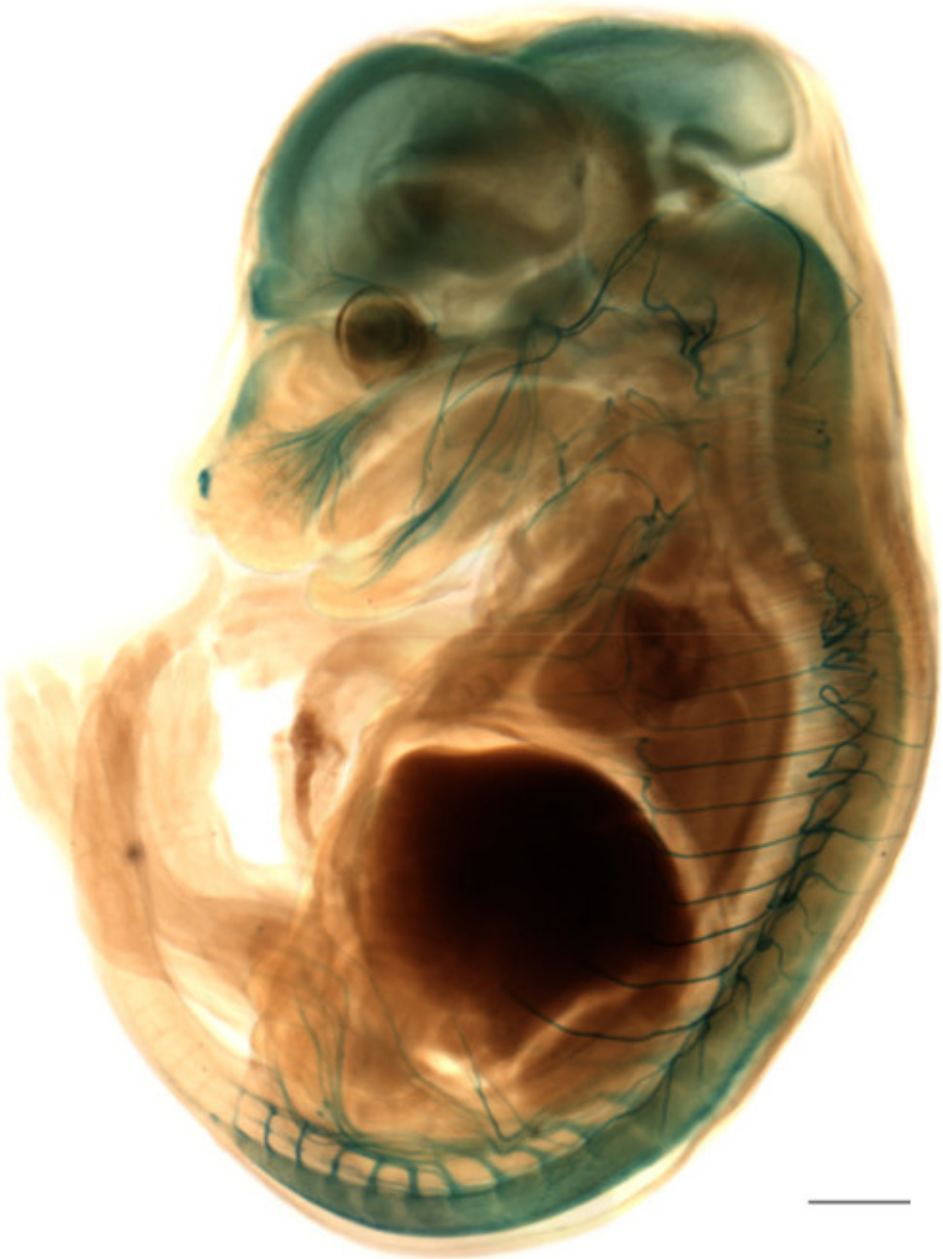

Figure 4: An E16.5 LSL-kaBraf : TrkA$^{taulacZ/taulacZ}$: Bax$^{-/-}$: nestin-Cre mouse embryo stained with X-gal and cleared with BABB. *kaBraf* encodes a kinase activated B-RAF kinase protein, B-RAF^{V600E}. Expression of kaB-RAF in DRG neurons resulted in full-length, close to normal axon extension in the absence of TrkA signaling. Scale bar: 1 mm. For more images including controls, see ref. 15, **Figure 2**.

	Primers used for PCR genotyping	
1	TrkA	DNA fragment size: 400 bp
	TrkA_F	GCG GGC GCC GCC GCG ATG
	TrkA_R	GAA GCC GCC TGC GCG GCT CTG CCA GGG TG
2	Bax	DNA fragment size: 304 bp (wild type), 507 bp (Bax null)
	In5R	GTT GAC CAG AGT GGC GTA GG
	Ex5F	TGA TCA GAA CCA TCA TG
	NeoR	CCG CTT CCA TTG CTC AGC GG
3	TauLacZ	DNA fragment size: 360 bp

	lacZup	ACA ACG TCG TGA GTG GGA AAA
	lacZdown	ATC AAC ATT AAA TGT GAG CGA G

Table 1: Primers used for PCR genotyping.

Discussion

The above-described X-gal staining procedure of embryonic *TrkA*^{taulacZ} mice allows for the rapid and detailed visualization of long distance axon projections in the intact fixed embryo. Because of the *Bax* null background these mice allow for the probing of signaling mechanisms that may contribute to both axon growth and neuronal survival. Mating with transgenic or knockout mice of interest allows for comprehensive assessment of axonal phenotypes and can serve as useful guide for future experiments to examine axon growth signaling in a more detailed fashion. A major challenge for *in vivo* axon growth studies is the time to prepare tissue sections and evaluate the complex phenotypes of growing axons that may be affected in multiple ways by any one genetic manipulation. The *TrkA*$^{taulacZ/taulacZ}$ *: Bax*$^{-/-}$ line enables evaluation of the full effects of a gene of interest on peripheral nociceptor axon development.

The technique is limited to the visualization of nociceptor axonal trajectories that would normally express TrkA during the early developmental stages. In addition to the peripheral nociceptors, it may be possible to visualize sympathetic and basal forebrain cholinergic projections; however this would require specific optimization of staining techniques

Tissue clearing is a key step of the protocol described here. It enables detailed assessment of deeper lying projections in whole-mount preparations and thus the potential identification of distinct phenotypes. Fluorescent reporters, such as TdTomato or GFP reporters[18,19], may offer visual clarity under certain conditions, however it is difficult to acquire whole-mount image because we have found that in contrast to the X-gal staining, fluorescent signals fade substantially during the tissue clearing procedure. DiI staining is also widely used for axon labeling[20]; its shortcomings are that it cannot selectively label a specific subtype of axons such as the nociceptor fibers here, and it is difficult to fully label long trajectories in the periphery.

In summary, we offer a protocol that uses a combination of two genetically modified mouse lines to allow routine visualization of the full arbors of nociceptive axons in the mouse embryo.

Disclosures

The authors declare that there are no competing interests.

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