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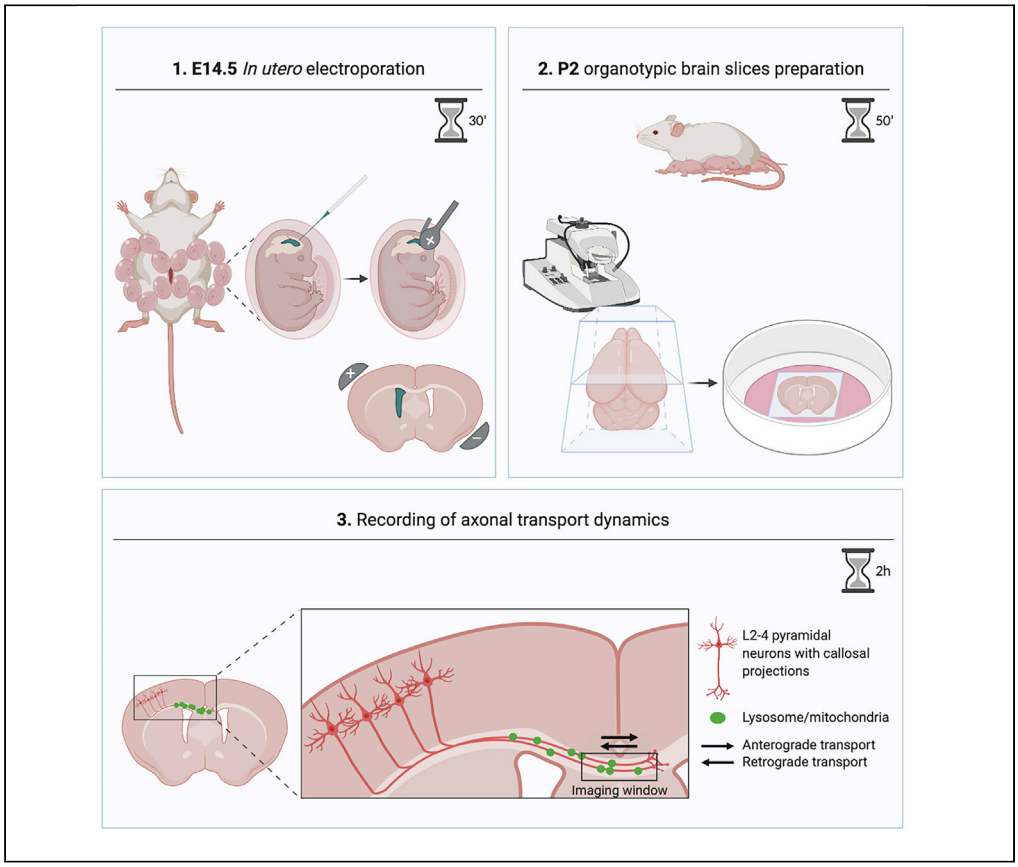
# *Ex Vivo* Recording of Axonal Transport Dynamics on Postnatal Organotypic Cortical Slices




Axonal transport is a physiological process adopted by neurons to transport organelles, proteins, and other molecules along their axonal projections. Here, we describe a step-by-step protocol to record the dynamics of axonal transport along the projections of callosal neurons by combining the *in utero* electroporation technique with the preparation of postnatal organotypic cortical slices. This *ex vivo* protocol has been developed to investigate axonal transport in a physiological setting closely reproducing the *in vivo* environment.

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## HIGHLIGHTS

Descriptive method to electroporate DNA plasmids in the embryonic mouse cortex

Step-by-step procedure to generate and mount organotypic brain slices

Protocol to record and analyze axonal transport in callosal projection neurons

Guidelines for protocol troubleshooting and overview on its limitations

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# *Ex Vivo* Recording of Axonal Transport Dynamics on Postnatal Organotypic Cortical Slices

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## SUMMARY

**Axonal transport is a physiological process adopted by neurons to transport organelles, proteins, and other molecules along their axonal projections. Here, we describe a step-by-step protocol to record the dynamics of axonal transport along the projections of callosal neurons by combining the *in utero* electroporation technique with the preparation of postnatal organotypic cortical slices. This *ex vivo* protocol has been developed to investigate axonal transport in a physiological setting closely reproducing the *in vivo* environment.**

**For complete details on the use and execution of this protocol, please refer to Even et al. (2019).**

## BEFORE YOU BEGIN

Swiss mice are well suited for the described procedure, as noted for their large litters size and good maternal care. However, the strain of choice and/or the number of embryos to electroporate should be chosen in accordance with the duration of the *in utero* electroporation surgery. To enhance the viability rate, a maximum time of 30 min is therefore recommended.

### Plasmid Preparation (for *In Utero* Electroporation; See Step 2 in the Step-By-Step Detailed Protocol)

Timing: 5 min

1. A mix of endotoxin-free plasmids is prepared in sterile nuclease-free water. One ug/μL of each plasmid is diluted in a total volume of 15 μL, with 0.1% (w/v) Fast green at 1/10 volume added to the plasmid solution to visualize the injection site.

   *Note:* to record axonal transport dynamics of membrane-bound organelles such as lysosomes and mitochondria, we combined a reporter gene-expressing plasmid to enable the visualization of the electroporated neurons with another construct coding for a fluorescent organelle-specific protein, such as Lamp1-emerald or DsRed-Mito as markers for lysosomes or mitochondria, respectively (Courchet et al. 2013).

2. Prepare microcapillaries for DNA injection by using a Needle Pipette Puller. To fabricate microcapillaries, we pull glass microcapillaries tubing with an inner diameter of 0.58 mm with a P-97 micropipette under the following conditions: heat, 634; pull, 92; velocity, 115; time, 210.

*Note:* the pulling parameters might need to be adapted when the heating filament is replaced.

⚠ CRITICAL: to ensure harmless perforation of the placenta and optimal injection of the plasmid solution, microcapillaries tip should be cut diagonally to generate an opening with slightly larger diameter; we recommend the use of spring scissors which enable the execution of sharp cuts (Figure 1).

### Preparation for Surgery (for *In Utero* Electroporation; See Step 2 in the Step-By-Step Detailed Protocol)

Timing: 10 min

3. Transfer the pregnant dam into a clean cage filled with a paper towel for nest building.
4. Prepare surgical table as follows: make available autoclaved surgery toolbox and 50 mL syringe filled with warm sterile PBS; cut paper drape to create rhomboidal-shaped incision for the surgical field; turn on the heating pad to 37°C.
5. Fill capillaries with plasmids mix; for this, we recommend to use microloader tips and to cut the tip of the microcapillary filled with the solution just before starting the surgery, to prevent the solution from air drying. If using a microinjector, set the injection pressure that enables a 0.1–0.2 μL droplet of plasmid solution to be ejected from the microcapillary when the foot pedal is pressed.
6. Prepare the electroporation system: make available 3 mm ϕ platinum electrodes and set the electroporator for 5 pulses of 40 V at 50-ms intervals for 950 ms when electroporating E14.5 embryos.

*Note:* the electroporation parameters were set as previously described (Even et al. 2019), adjusted to the developmental stage of the embryos to electroporate. Settings should be arranged to a given experimental design.

### Preparation of Tamoxifen Solution (if Temporal Control of Plasmid Expression Is Desired)

Timing: 10 min

7. Prepare a solution of 2 mg/mL 4-hydroxytamoxifen (4-OHT) containing 1 mg/mL progesterone by dissolving 20 mg of 4-OHT and 10 mg of progesterone in 100 μL ethanol 100% and diluting with 900 μL of corn oil, followed by water bath sonication for 5 min.

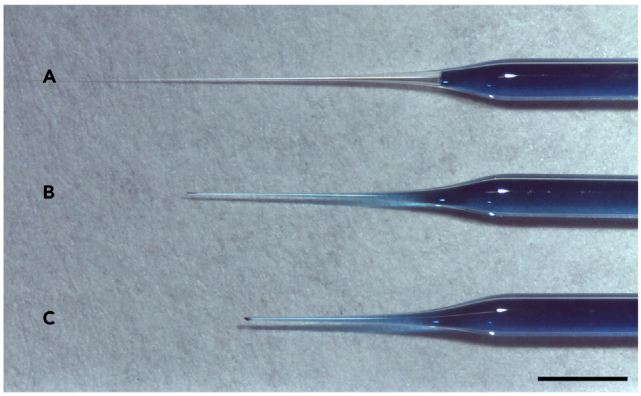


**Figure 1. Microcapillary Cut for Plasmid Injection**
(A) Uncut microcapillary filled with plasmid solution.
(B) Filled microcapillary cut at optimal length to enable ejection of 0.1–0.2 μL droplets of plasmid solution.
(C) Filled microcapillary deeply cut leading to suboptimal injection of plasmid solution. Scale: 0.2 μm.

*Note:* cover solution with foil as progesterone is sensitive to light.

*Note:* solution can be stored at +4° for up to one week.

*Note:* the solution is complemented with progesterone to counteract the mixed estrogen effect of tamoxifen which can cause fetal abortion.

*Note:* the injection paradigm has to be defined in accordance with the plasmid effects and experimental settings.

### Preparation of Embedding Solution (for Brain Embedding; See Step 3 in the Step-By-Step Detailed Protocol)

Timing: 3–5 min

8. Prepare a 4% agarose solution for brain embedding by adding 1 g of low-melting agarose into a 50 mL conical tube containing 25 mL sterile HBSS; Keeping the cap loose, transfer the tube into a microwave and boil until all agarose particles are dissolved and the solution is clear.

   *Note:* monitor the solution to prevent excess boiling and overflow; stop the microwave when the solution starts to boil and mix by whirling the tube before repeating the step until complete melting. Wear heat-resistant gloves when manipulating the tubes to protect your hands from burns.

9. Transfer the solution into a 37°C incubator to allow cooling down.

### Preparation of Slice Culture Medium (for Mounting of Cortical Slices; See Step 4 in the Step-By-Step Detailed Protocol)

Timing: 3–5 min

10. Let Matrigel thaw on ice; prepare culturing media for brain slices by mixing Matrigel with Neurobasal Medium supplemented with 2% B27, 1% penicillin/streptomycin and 1% GlutaMAX at 1:1 ratio; keep the solution on ice to prevent polymerization.

    *Note:* as recommended, Matrigel is stored at −20°C and left thawing in a 4°C refrigerator for 12–16 h to prepare one-time use aliquots. Frozen aliquots can be quickly thawed at 4°C the day of the experiment (~2 h for 1 mL aliquot).

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Anti-RFP antibody | Rockland antibodies & assays | 600-401-379 |
| Anti-GFP antibody | Abcam | Ab6673 |
| DAPI | Sigma | D9542 |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Isoflurane | Abbott Laboratories | N/A |
| Fast green | Sigma-Aldrich | F7252 |
| Nuclease-free water | Schering-Plough | 3098 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Buprecare 0.3 mg/mL injection | Animalcare | N/A |
| Fucithalmic Vet® 10 mg/g | Aventix | N/A |
| Tamoxifen | Sigma-Aldrich | T5648-1G |
| Corn oil | Sigma-Aldrich | C8267 |
| Low melt agarose | Bio-rad | 1613114 |
| HBSS | Lonza | 10-527F |
| Matrigel | Fisher Scientific | 11573560 |
| Progesterone | Sigma-Aldrich | P3972 |
| Neurobasal Medium | Gibco, Invitrogen | 21103049 |
| B27 | Gibco, Invitrogen | 17504044 |
| penicillin/streptomycin | Gibco, Invitrogen | 15140122 |
| GlutaMAX | Gibco, Invitrogen | 35050061 |
| Critical Commercial Assays | | |
| Plasmid DNA, endotoxin-free Midi-prep kit from NucleoBond_Xtra Midi EF | Macherey-Nagel | 740420 |
| Experimental Models: Organisms/Strains | | |
| E14.5 pregnant *RjOrl*:Swiss mice (outbred) | Janvier labs | |
| Recombinant DNA | | |
| pCALNL-DsRed | (Matsuda and Cepko 2007) | Addgene: # 13769 |
| pCAG mEmerald-LAMP1 | (Lewis et al., 2016) | N/A |
| pCX memb-mCherry | (provided by Dr. Xavier Morin) | N/A |
| pCAG-iCreERT2 | (Even et al. 2019) | N/A |
| Software and Algorithms | | |
| Fiji-Image J | (Schindelin et al. 2012) | N/A |
| KymoToolBox - ImageJ | (Hinckelmann, Zala, and Saudou 2013) | N/A |
| Prism | GraphPad Software | N/A |
| Other | | |
| Platinum electrodes | Sonidel | CUY650P3 |
| Electroporator | BTX | ECM 830 |
| Microinjector | Eppendorf | 5252000013 |
| Micropipette puller | Sutter Instrument | P-97 |
| Isoflurane anesthesia station | Harvard Apparatus | 34-1041 |
| Heating pad | Beurer | HK 35 Heat pad |
| Surgical lamp with flexible arm | N/A | N/A |
| Borosilicate glass capillaries | Harvard Apparatus | 30-0016 |
| Sterile PBS | Lonza | 17-512F |
| Syringe 5 mL | Becton Dickinson | SYR005J |
| Syringe 1 mL | Becton Dickinson | 303172 |
| Needle 26GA (0.45 × 10 mm) | Becton Dickinson | 300300 |
| Super GLUE-3 | Loctite | N/A |
| Operating Manual Compact balance | KERN & Sohn GmbH | 1.1 |
| Vibratome Leica VT1000S | Leica Microsystems | VT1000S |
| Culture dish as embedding mold | Thermo Fisher Scientific | 153066 |

(*Continued on next page*)

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| MatTek glass-bottom dishes | MatTek CORPORATION | P35G-0-20-C |
| FemtoJet microinjector | Eppendorf | 5252000013 |
| Forceps | Dumont 11295-20 | 11295-20 |
| Hair removal cream | Veet | N/A |
| Ethanol | vwr | 20816,298 |
| Scissors | Fine Science Tool | 14094-11 |
| Perforated spoon | Moria | 10370-18 |
| Spring scissors | Aesculap | OC498-R |
| Ring forceps | Fine Science Tool | 11103-09 |
| Olsen-Hegar Needle Holder | Fine Science Tool | 12002-14 |
| Vycril suture 4-0 | Ethicon | V310H |
| Microloader tips | Eppendorf | 5242956003 |

## STEP-BY-STEP METHOD DETAILS

### Induction of Anesthesia

Timing: 3 min

Here we describe the method for induction and maintenance of anesthesia of the pregnant dam.

1. Weigh the mouse to define the amount of analgesic to administer pre- and post-surgery.
2. Prepare the analgesic buprenorphine (Buprecare®): 0.03 mg/mL dilution to administer 0.1 mg/kg.

   *Note:* once the action of the analgesic is over (buprenorphine is effective for 8–12 h), assess post-operative pain by scoring pain behaviors (i.e., by using Grimace Scale; Langford et al. 2010) and evaluate re-administration of analgesic, in accordance with local and national directives.

3. Sedate the pregnant dam in an induction chamber where a mixture of 96% air and 4% isoflurane circulates.

   *Note:* monitor the breathing rate to assess the state of sedation, which takes approximately 60 s.

4. Quickly, to prevent hypothermia, transfer the mouse on the heating pad with the abdomen facing down; apply ophthalmic lubricant (Fucithalmic Vet® 5 mg/g) to both eyes to prevent corneal drying, and inject the analgesic subcutaneously within the loose skin over the interscapular area.
5. With the abdomen facing up, place the nose of mouse inside the mask respirator and adjust the concentration of isoflurane vaporized to 2%.

   ⚠ CRITICAL: given the inter-individual variability in response to anesthesia, anesthesia depth should be closely monitored to prevent insufficient sedation or respiratory/cardiac depression. Parameters that can be clinically monitored are: 1) toe pinch response; 2) respiration rate (approximately 1 breath/s under optimal anesthesia); under too deep or too light anesthesia, flow rate of anesthetic should be adjusted to reach optimal anesthesia depth.

### *In Utero* Cortical Electroporation and Post-op Care

Timing: ~20–25 min; should not exceed 30 min, to ensure optimal viability

The *in utero* electroporation technique consists of the injection of plasmid DNAs into the lateral ventricles of mouse brain embryos and their subsequent incorporation into the apical progenitors lining the ventricle surface by electroporation. By targeting the neuronal progenitors with reporter constructs, it is possible to trace the different steps of development of the derived cortical neurons.

6. Shave the abdomen wall with a depilatory cream and disinfect with 70% alcoholic solution (Figure 2A).
7. Make an incision of approximately 1.5 cm along the skin abdomen midline first, and peritoneum afterwards (Figure 2B).
8. Place a surgical field with a rhomboidal aperture over the abdomen and wet the drape and the open abdomen cavity with warm saline.

   ***Note:*** we suggest to hydrate the drape with warm sterile saline to favor the subsequent step of the procedure, as it would prevent the uterine horns pulled out of the abdomen cavity from attaching to the drape. Make sure to not soak the drape excessively, to avoid mouse hypothermia.

9. By using ring forceps, carefully pull the uterus out from the abdominal cavity by holding it at the gaps between embryos; expose one of the two uterine horns on the surgical drape and, when necessary, apply warm sterile phosphate-buffered saline (PBS) solution to prevent drying of the embryos during the whole surgical procedure (Figure 3A).
10. Gently, hold an embryo with ring forceps or your fingers while inserting the microcapillary through the uterus into the lateral ventricle and inject the DNA plasmid mix (approximately 0.5 μL) by using a mouth-controlled tube or a microinjector activated via the foot pedal. The green dye filling the ventricles indicates the success of the injection. To reduce the lethality rate, a given embryo should not be injected more than two times (Figures 3B–3E).

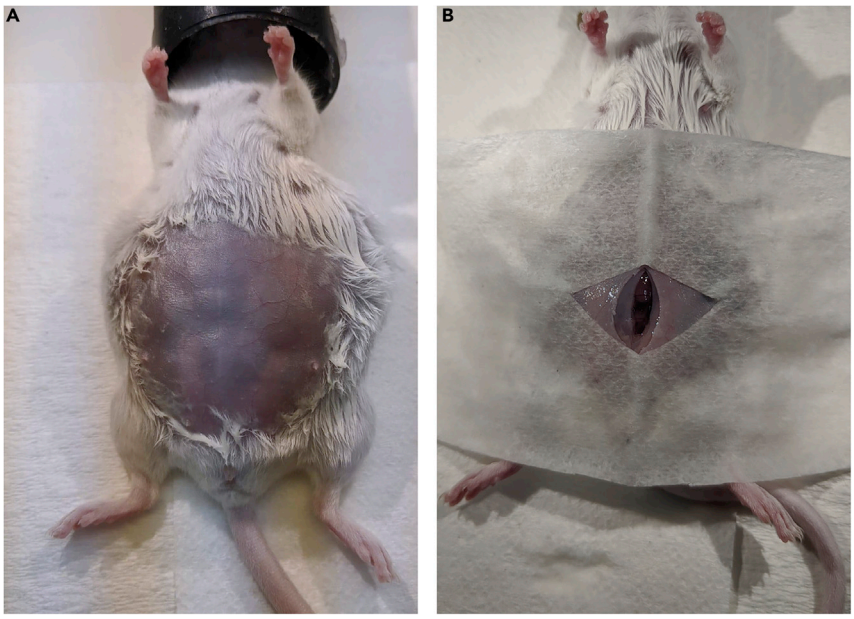


**Figure 2. Preparation for Surgery**

(A) Incision area. Shave the abdomen of the pregnant dam kept under anesthesia and disinfect the skin with an alcoholic solution.

(B) Skin incision. Cover the incision area with a surgical drape and incise the skin and peritoneum along the abdomen midline.

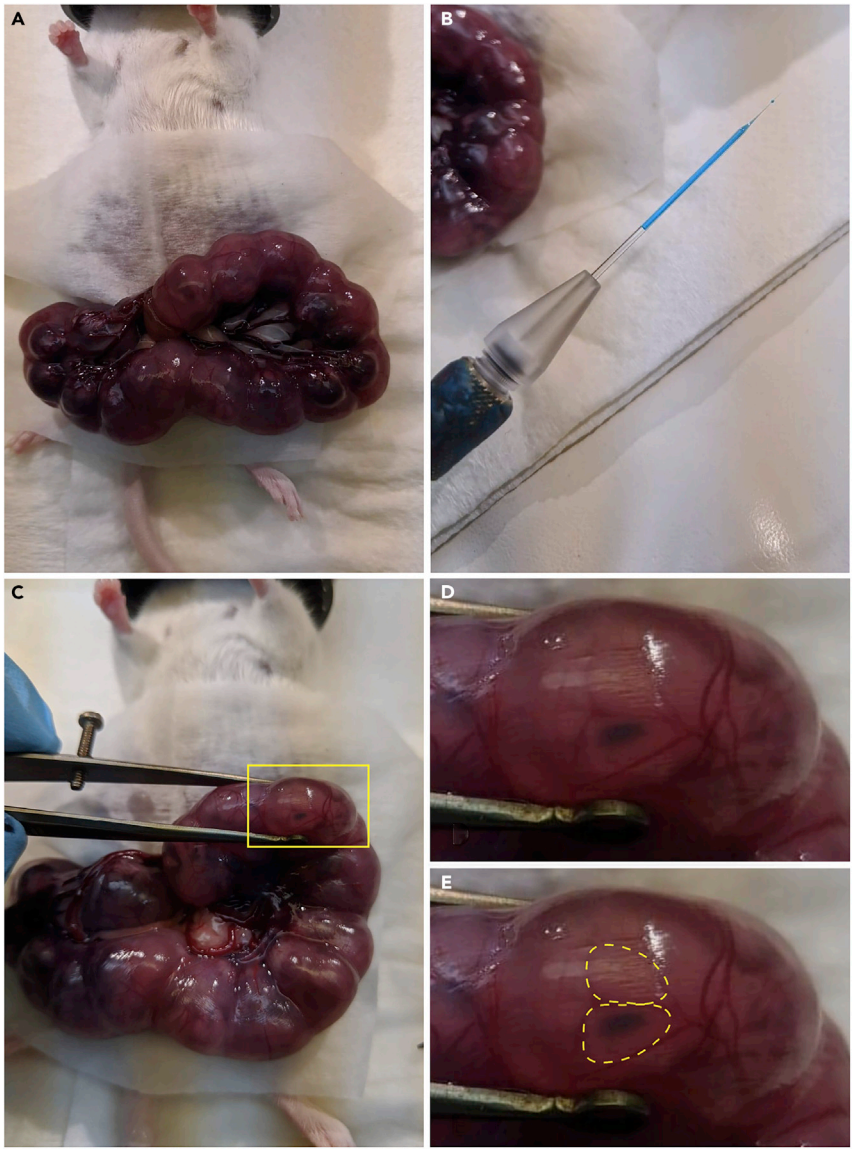


**Figure 3. Microinjection of E14.5 Embryos**

(A) Exposition of the uterine horns. Pull uterus horns out the abdomen cavity by holding it at the gaps between embryos and expose the uterine horns. Keep the uterus hydrated with warm sterile PBS during the entire procedure. (B–E) Embryos microinjection. (B) Cut diagonally the tip of a previously pulled microcapillary to enable an optimal ejection of the plasmid solution. (C–E) Inject the plasmid solution into one of the lateral ventricles of the embryo (yellow dotted line in E).

⚠ CRITICAL: make an oblique cut of the microcapillary with optimal width (see also the Trouble-shooting section). This is a critical factor for the success of the electroporation. Microcapillaries shortened sub-optimally could compromise the integrity of the perforated tissues as well as lead to undesired dispersion of the plasmid solution from the injected to the other interconnected ventricles. The tip of the capillary can be cut progressively, to identify the minimal cut which enables the ejection of 0.1–0.2 μL droplets of plasmid solution.

⚠ CRITICAL: If the fast green is observed in other regions than the ventricles or cannot be released from the microcapillary, change the position of the microcapillary by slowly pulling it back until the fast green can be injected and observed in the ventricle.

11. Place the anode on the injection site and the cathode on the opposite side of the brain, and deliver the electrical impulses by pressing the foot pedal of the electroporator; the correct delivery of the electric impulses is indicated by the formation of bubbles around the electrodes (Figure 4).

*Note:* electroporation at E14.5 was chosen for axonal transport recordings to target a sparse population of upper layer II-IV neurons, enabling the visualization of isolated fluorescent callosal projections.

*Note:* in order to target the callosal projection neurons, the electroporation is performed by placing the anode at 45° with respect to the intra-hemispherical plane.

⚠ CRITICAL: warm PBS should be made available during the entire procedure, to keep embryos and electrodes wet.

12. Once the plasmid injection and the electroporation have been performed on the remaining embryos, hydrate the uterine horn and, gently, slide it back into the abdomen cavity; repeat the same procedure on the second uterine horn.
13. Suture abdomen wall and skin; transfer the mouse from the operating table to a cage placed under a warming light and containing paper towel to monitor eventual bleeding. Monitor the mouse closely until recovery from anesthesia.

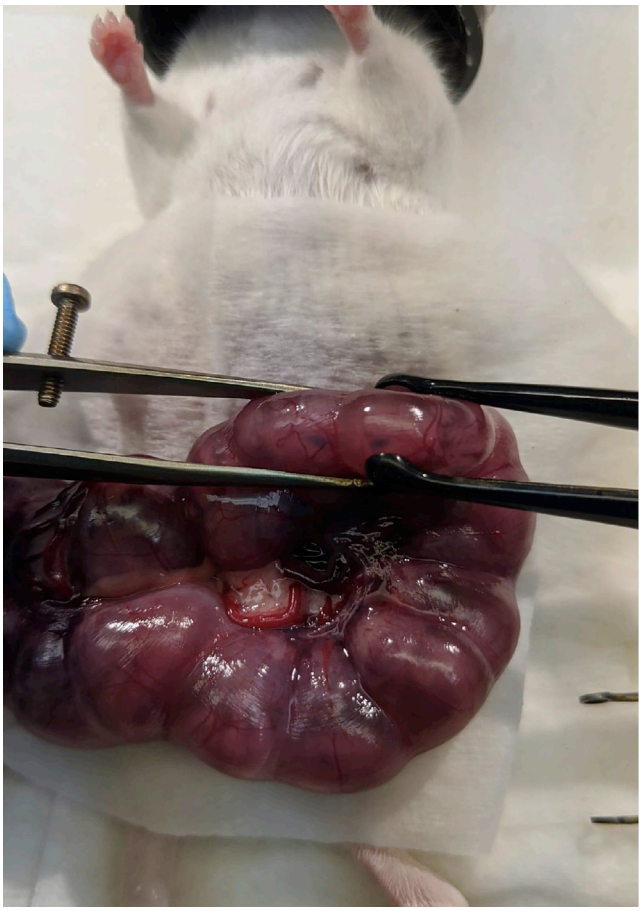

**Figure 4. *In Utero* Electroporation of E14.5 Embryos**
Hold the injected embryo with ring forceps to stabilize its position while placing the electrodes over the target site for electroporation.

⚠ CRITICAL: skin and muscle layer should be sutured separately: this measure would prevent damage of the underneath muscle suture and potentially lethal bleeding if the mouse would bite and remove the external skin suture.

14. After surgery, monitor and document the mouse well-being on a scoring sheet with daily observations by checking and evaluating the surgical wound, outer appearance, and spontaneous behavior. If properly performed, laparotomy for in utero electroporation is likely to not correlate with post-surgery symptoms of discomfort or pain.

### Dissection and Embedding of P2 Brains

Timing: 30 min

Brain dissection of electroporated pups is performed at postnatal day two (P2), a stage at which the electroporated callosal projection neurons have crossed the interhemispheric midline. In order to provide mechanical support prior to slicing, dissected brains are embedded into an agarose matrix.

15. Decapitate the P2 pup with surgical scissors, carefully dissect out the full brain including olfactory bulbs, forebrain, and hindbrain as elsewhere described (Beaudoin et al. 2012), and collect into a petri dish containing ice-cold HBSS.

*Note:* we recommend to dissect and perform the protocol described below on one pup at a time, given the strict time window of some of the steps and the importance of their timing for a successful execution of the protocol.

*Note:* If a reporter plasmid was electroporated, dissected brains can be checked for fluorescence under a binocular microscope equipped with fluorescent illumination.

16. Place embedding molds on ice and pour the 4% agarose solution that was previously brought to 37°C

*Note:* we generally prefer petri-dishes over conventional embedding molds as their larger diameter facilitates the brain rotation within the polymerizing agarose solution and its orientation in the position of interest.

17. Quickly, remove the excess HBSS and transfer the brain into the agarose solution; with the forceps, rotate the brain gently and repeatedly while agarose polymerizes, until steady in the final position with the olfactory bulbs facing upwards and the coronal axis parallel to the mold bottom on an ice bucket.

⚠ CRITICAL: a gentle and repeated rotation of the brain within the agarose solution is important to remove the HBSS at the interface between the brain and the agarose solution; this precaution would prevent the detachment of the brain slice from the surrounding agarose layer while being sliced from the agarose block.

⚠ CRITICAL: orienting the brain within the agarose solution undergoing polymerization would simplify the downstream manipulation of the agarose block; By positioning the brain with the sectioning plane parallel to the mold bottom, the bottom side of the agarose block would not require further cutting adjustment and could be directly glued on the vibratome tray platform.

18. Let the agarose block solidify on ice for about 15 min.

### Slicing and Mounting of Cortical Slices

Timing: 20 min

Organotypic brain slices are generated and mounted on coated glass-bottom dishes, to be used for recordings of axonal transport along the projections of callosal neurons.

19. Set the vibratome: fill the vibratome tray with cold HBSS and the vibratome bath with ice; adjust the sectioning parameters as follows (adjusted for a vibratome Leica VT100S): section thickness: 300 μm; cutting speed: 3; cutting frequency: 3.
20. Coat the glass-bottom dish with a thin layer of culture medium containing Matrigel, by adding with a pipette 1 mL solution and aspiring back most of the volume, leaving a thin pellicle; keep it at RT (below the polymerizing temperature of 37°C).
21. Once the agarose block is polymerized, shape it around the brain by making a cut at the rostral and ventral sides of the brain, parallel to each other and to the sectioning plane, at an approximate distance of 0.5 cm from the brain surface; apply the same procedure to remove the excess agarose also at the ventral and dorsal sides of the brain. We recommend cutting the block with a truncated pyramid shape making sure the olfactory bulbs of the brain face the minor base of the pyramid. This shape will provide a greater adhesion surface and will increase the stability of the agarose block glued onto the vibratome tray platform (Figures 5A–5C).
22. Glue the agarose block with the olfactory bulbs facing upwards onto the vibratome tray platform.
23. Fix the platform with the glued agarose block into the vibratome tray filled with cold HBSS.
24. Section the agarose block to generate coronal slices and collect with a spatula the brain sections containing the callosal projections crossing the midline in a dish filled with ice-cold HBSS; check

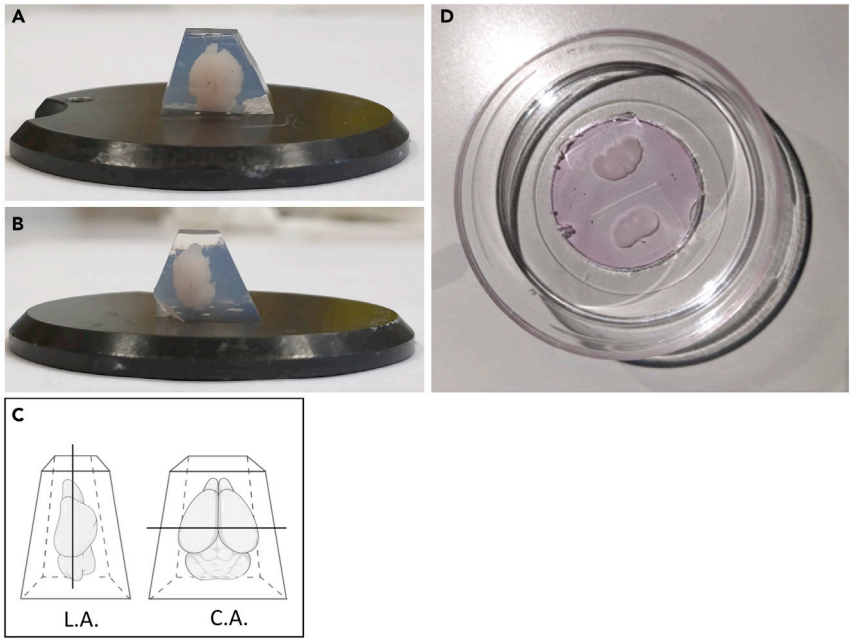


**Figure 5. Cortical Slices Preparation**

(A–C) Brain embedding. (A and B) Embed the brain in a 4% agarose solution ensuring the optimal positioning of the brain until agarose polymerization. Shape the agarose block to ensure a thickness of 0.5 cm from the brain surface, and glue the agarose block onto the vibratome tray platform. (C) Scheme for brain orientation within the agarose block. L.A., longitudinal axis; C.A., coronal axis.

(D) Mounting of cortical slices. Slice the embedded brain to generate coronal slices and transfer them onto coated glass-bottom dishes.

the collected slices for fluorescence and transfer onto coated MatTek dishes only the slices with the electroporated region of interest (Figure 5D).

*Note:* depending on the thickness of the agarose block, two or three sectioned slices can be placed per MatTek dish; it is very important to not overlap the edges of the agarose slices for an optimal live-imaging acquisition.

*CAREFUL:* gently manipulate the brain slices in order to avoid the separation of the brain slices from the surrounding agarose. Take care that the embedded brains stay at 4°C during the whole sectioning procedure by changing the HBSS regularly and refilling the vibratome bath with ice.

25. With a pipette, cover each section slice with a drop of culture medium containing Matrigel and place the glass-bottom dishes in the incubator at 37°C and 5% $CO_2$ for 20 min, to allow the Matrigel to polymerize and slices to recover.

*CAREFUL:* due to the short survival time of postnatal cultured organotypic brain slices (approximately 4 h), incubation times of all steps following brain dissection should be reduced to the minimum. Additionally, all steps should be performed at 4°C, by using ice-cold reagents and performing all the manipulations on ice. Altogether these measures would enable a longer recording time of axonal transport dynamics.

### Transport Recordings

Timing: 2 h

After recovery in the incubator (at least 20 min), the transport dynamics of fluorescently labeled organelles/vesicles can be recorded along the projections of callosal neurons.

26. To record axonal transport dynamics, use a 63× oil lens with a working distance of 0.14 mm and position the bottom-glass dish within a recording chamber controlled at 37°C and 5% $CO_2$.
27. Acquire time-lapse images using a confocal microscope (here, Zeiss LSM 880) for a desired capturing time at the corpus callosum midline to discriminate the directionality of the transport. As the movement of mitochondria and lysosomes relies on fast axonal transport, it is recommended to image at least three frames per second for 60 s. The number of acquisitions per second can be adapted depending on the speed of the cargos.

*Note:* to locate the corpus callosum midline, we recommend to first use the 10× lens to identify the fluorescently labeled neurons in the electroporated hemisphere of the brain slice and follow the neuronal projections to the midline.

⚠ CRITICAL: set the microscope lasers at a power low enough to visualize the cargos for the entire acquisition time; as the neurons are sensitive to photodamage, this measure would limit the loss of fluorescent signal over time.

⚠ CRITICAL: given the critical survival time of the mounted brain slices, it is important to acquire the transport recording in the shortest time possible. For this, we recommend processing one brain at a time.

### Optional Step of the Protocol: Tamoxifen Injection

Timing: 5 min

Tamoxifen is administered to activate a given Cre recombinase to temporally regulate the expression of a plasmid of interest. Tamoxifen is injected intra-peritoneally to the pregnant dam for a vertical diffusion via the blood system to the electroporated embryos, and after delivery to modulate the tamoxifen transmission to the pups via mother's milk.

28. To perform an IP injection, restrain the animal with the ventral abdomen facing up and the head slightly tilted downward. Insert the syringe at 4–5 mm depth into the lower right quadrant of the abdomen toward the mouse head, by keeping a 20°–30° angle to the abdominal surface; aspirate by pulling back syringe plunger to check if vital organs were punctured and, and if any fluid is aspirated inject slowly the tamoxifen solution. If aspirate is observed, withdraw and discard the needle, and gently perform the procedure again.

⚠ CRITICAL: injection volume should be the lowest possible (ideally less than 0.2 mL and needle size should be less than 21G). A new needle should be used for each animal, to reduce the risk of infection.

29. Observe the animal to check for signs of bleedings at injection site or sign of pain or distress due to complication of injection. If complications persist 24 h after the injection, the animal is sacrificed.

*Note:* misplaced intraperitoneal injections can lead to peritonitis, lacerations of abdominal organs, bleeding, or infections. All these complications are associated with pain or distress whose severity can be clinically evaluated (i.e., by using Grimace Scale; Langford et al. 2010).

*Note:* for optimal plasmids effect at postnatal stage P2, we routinely perform injections on pregnant dams E17.5 and E18.5, and one day after delivery. Frequency and number of doses administered justify the time needed for cre-driven recombination and expression of the plasmids of interest; adaptations might be necessary depending on the timing for tissue harvesting and biological effects of the plasmid/plasmids of interest.

## EXPECTED OUTCOMES

This protocol has been developed to record the transport dynamics of motile vesicles and/or organelles along the axonal projection of layer II-V pyramidal neurons *ex vivo* on cortical slices. This procedure relies on the *in utero* electroporation of plasmids expressing fluorescently labeled membrane proteins targeting species-specific organelles. In opposition to transgenic animal models used for studies of axonal transport, the electroporation of fluorescent plasmids enables the selective visualization of a restricted population of neurons, to ultimately facilitate the tracking of vesicles trajectories (Mattedi and Vagnoni 2019).

Here, we report the use of the Lamp1:emerald or DsRed-mito expressing plasmids to selectively label and track lysosomes or mitochondria, respectively. A cortical slice prepared at postnatal day 2 (P2) following electroporation at E14.5 in the somatosensory cortex would typically manifest fluorescent callosal neurons projecting from the upper cortical layers to the contralateral hemisphere through the interhemispheric midline (Figure 6). At the interhemispheric midline, dotted fluorescent signals corresponding to Lamp1-expressing lysosomes or DsRed-expressing mitochondria is expected to move bidirectionally along the callosal projection of electroporated neurons (Figure 7C and Methods Video S1).

The *ex vivo* protocol described here was set up to study axonal transport dynamics in a new in vitro setting closer to the in vivo situation. In comparison to existing microfluidic devices, that are commonly used to physically separate axons from dendrites to facilitate the study of axonal transport, organotypic slices better recapitulate the cellular and chemical cortical environment of neurons

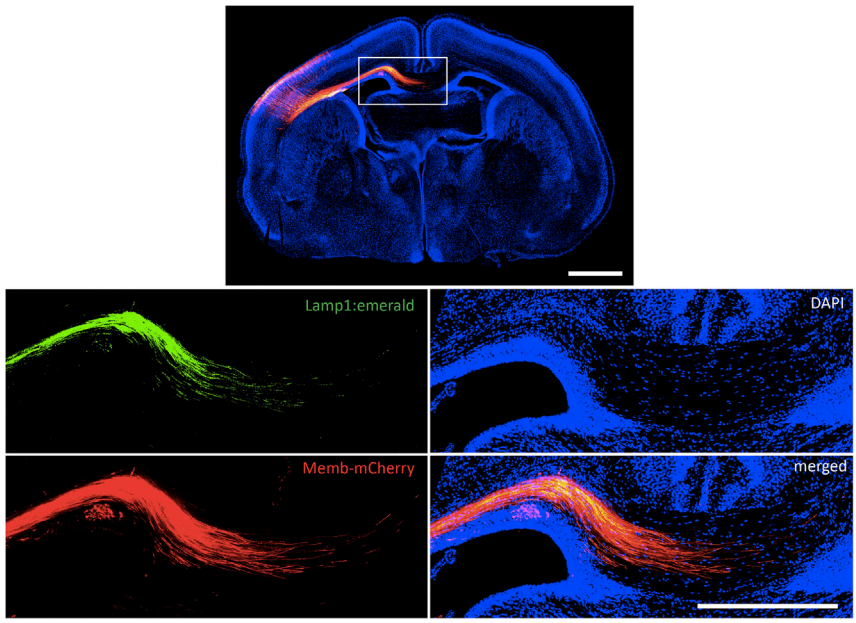


**Figure 6. Representative Image of a Cortical Slice Collected at P2 following Electroporation at E14.5 of Lamp1:Emerald and Memb-mCherry**

Electroporated L2–4 pyramidal neurons with callosal projections (red); Lamp1:emerald (green); nuclei (blue). Scale bar, 500 μm.

that plays an importante role in defining their metabolism and morphology (Polleux, Morrow, and Ghosh 2000; Villarin et al. 2016).

## QUANTIFICATION AND STATISTICAL ANALYSIS

1. Download the FIJI/ImageJ plugin Kymotoolbox.
2. Open an image series in FIJI/ImageJ.
3. If necessary, separate stacked channels [Image | Color | Split channels] (Figure 7A).
4. Adjust the image proprieties [Image | Proprieties].
5. Select the Segmented Line Tool [right-click on the Line icon | Segmented Line] and set the width value that has to be used for the complete data analysis [double-click on the Segmented Line Icon].
6. Track the axon by drawing a segmented line along its length and add the defined line region of interest (ROI) to the ROI Manager [click t]. For subsequent calculations of vesicles run length, segmented lines of the same length should be traced. Export the obtained data set into excel (Figure 7B).
7. Generate a kymograph and corresponding video of the tracked axon [Plugin | KymoToolBox | Draw Kymo] by setting the width previously defined (see step 5) and ticking [Get Kymo] and [Get KymoStack] (Figures 7C and 7D and Methods Video S1).
8. Track the particles trajectories by drawing a segmented line along their length and add the defined ROIs to the ROI Manager, as previously described.
9. Analyze the particles displacement over time along the tracked axon [Plugin | KymoToolBox | Analyse Kymo] by defining the directionality of the motion [outward], setting the minimum speed for movement and selecting [Log all data]. Select [Show colored kymo] to generate a color-coded kymograph of the trajectories analyzed. We commonly consider vesicles as stationary when speed is lower than 0.1 μm/s (Figure 7E).
10. Export the Summary data set from the Result panel into Excel. We routinely analyze Average velocity (μm/s), Run length (μm), Pausing time (%).

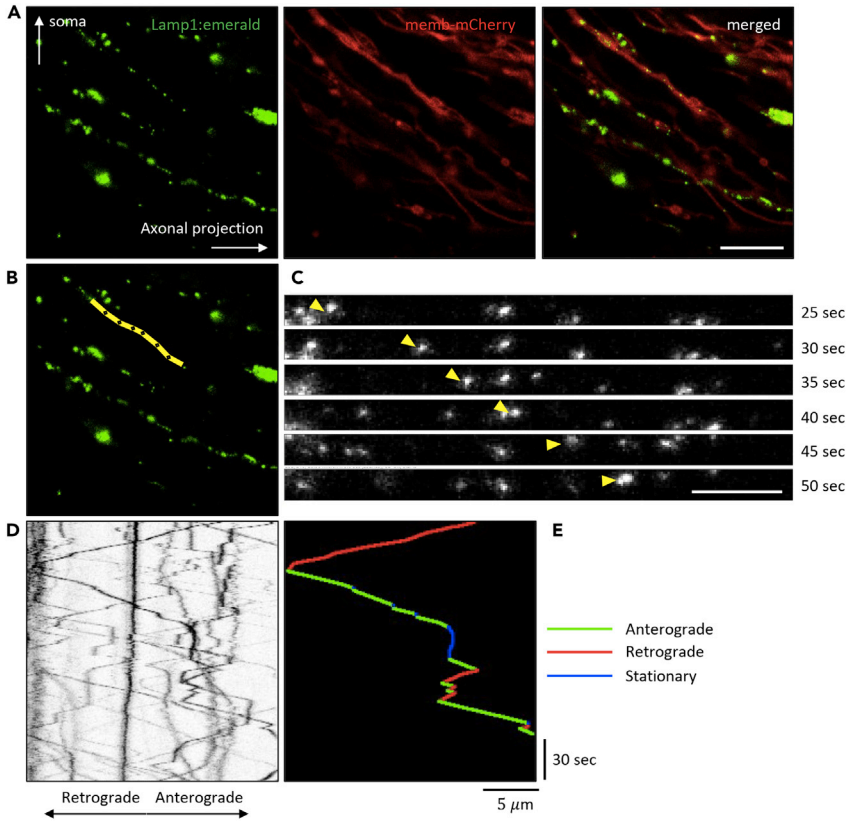


**Figure 7. Axonal Transport Analysis of Lamp1-Positive Lysosomes in Cortical Projection Neurons of P2 Cortical Slices**

(A) Representative image of memb-mCherry callosal projection neurons with Lamp1-expressing lysosomes (emerald) crossing the interhemispheric midline of P2 cortical slices. Scale bar, 10 μm.

(B and C) Kymograph generation and analysis. (B) Drawing of a segmented line along axon length. (C) 5-s interval snapshots of Lamp1:emerald lysosome displacement along tracked axon. Scale bar, 5 μm.

(D) Kymograph generated from tracked axon, used to characterize the trajectories of moving vesicles.

(E) Representative tracking of a vesicle moving with anterograde and retrograde motion; the vesicle is considered stationary when speed is lower than 0.1 μm/s. Scale bar, 5 μm.

## LIMITATIONS

The *ex vivo* protocol described here was developed for recording intracellular trafficking within axonal projections of layer II-V pyramidal neurons. These neurons have an organized interhemispheric distribution that enables the visualization of all their subcellular compartments, from the cell soma to the axonal projections, within a coronal slice. For this reason, the transfection of this neuronal population via *in utero* electroporation is an ideal tool to visualize their spatial distribution and study their intracellular dynamics on a cortical slice. Accordingly, by recording axonal transport along the callosal projections at the interhemispheric midline, it is possible to precisely define the directionality of the organelles motility (i.e., whether it is moving with anterograde or retrograde motion) and standardize the location of the recording window with regard to the distance from the cell soma. If transport directionality and distance from the cell soma are parameters of interest in the analysis, recordings of axonal transport in other neuronal population with a more complex and not clearly organized spatial distribution might be associated with increasing technical challenges.

The ability to record transport dynamics in organotypic cortical slices is limited by the age of pups and the time of culture. In comparison to P2 slices that can be kept up to 4 h, cultures at later stages are expected to have a shorter survival rate and lower organelle-associated fluorescent signal.

The *in utero* electroporation technique enables the co-transfection of multiple plasmids, thus allowing multi-level genetic alterations. By co-electroporating plasmids that simultaneously enable the labeling of organelles and regulation of a given protein expression, it is possible to study the dynamics of axonal transport applied to a gene-dependent phenotype. If the expression level of a given protein has to be genetically regulated, i.e., via RNA interference technology, it is important to evaluate its physiological role during brain development and assess whether changes in its expression could affect key processes such as neuronal proliferation and migration. In these circumstances, it is recommended to control the timing of gene expression by using inducible plasmid systems that can be activated after a defined developmental stage.

If the *in utero* electroporation technique is used to regulate the expression level of a given protein, axonal transport dynamics cannot be investigated by using fluorescent dyes for organelles such as mitochondria (MitoTracker) and lysosomes (LysoTracker). These dyes would ubiquitously label every neuronal population of the cortical slices, preventing the unique visualization of motile organelles/vesicles along the electroporated neurons.

## TROUBLESHOOTING

### Problem 1

Suboptimal shortening of the microcapillary leading to a problem with injection.

### Potential Solution

For a targeted injection of the plasmid solution, it is important to control the quality of the microcapillary tip. A microcapillary tip with an optimal internal diameter would enable a precise and harmless injection into cortical ventricles. A tip excessively shortened would present a too large internal diameter, which could potentially lead to difficulties in penetrating the uterine wall or damage of the amniotic sac with following leakage of amniotic fluid. On the other hand, a tip with a too narrow internal diameter due to insufficient shortening would prevent an efficient penetration of the uterine wall, as it could bend over the uterus surface and break. Lastly, if the injection is optimal but too much volume is released into a given ventricle leading to filling of the interconnected ventricles, the ejected volume can be reduced by controlling the pressure delivered by the injector. This measure would prevent the need for replacing the microcapillary. Overloading of the ventricles with plasmid solution could also cause hydrocephalus and ultimately lead to embryonic lethality. Therefore, to ensure optimal injection of the plasmid solution into the ventricle it is important to cut the capillary at the appropriate length ensuring the best compromise between penetrance and plasmid flow (Figure 1).

### Problem 2

Low number of electroporated embryos or survival rate.

### Potential Solution

If low electroporation rate is experienced, the surgeon should pay attention to: 1) concentration or quality of the plasmid solution (endotoxin-free plasmid solution is highly recommended); if the expression of the plasmid is unknown to the experimenter, *in vitro* transfection tests should be performed to assess the expression of fluorescence; 2) the site of injection; a crescent shape corresponding to the lateral ventricle should progressively fill and be visualized with fast green while injecting; 3) the correct positioning of the electrodes with respect to the neuronal population to electroporate; an imprecise orientation of the positively and negatively charged electrodes might lead to missed electroporation or electroporation of undesired neuronal population. 4) Electroporation settings should be determined according to the embryonic stage. Commonly, the electroporation voltage is increased at later embryonic stages in comparison to early stages while other parameters remain constant (E14.5: 40 V, E15.5: 45 V). Electrodes with a larger diameter (5 mm) can be used to target a larger area of the brain with the same pulse conditions than the 3 mm electrodes.

To improve the low survival rate of electroporated embryos: 1) keep the surgery time within 30 min; 2) do not electroporate the first embryos of each horn adjacent to the cervix, in order to minimize the chances of abortion; 3) manipulate the embryos with care making sure the uterus is hydrated for the whole procedure; keep the mouse warm with the heating pad while being under anesthesia and by using a warming lamp during post-operative recovery; 4) reintroduce the uterine horns into the abdomen respecting their original position, to prevent twisting and tangling of the associated vasculature system which could lead to resorption; 5) preferentially use mice aged two to three months as they show better recovery; 6) make sure the plasmids are produced with an endotoxin-free DNA isolation kit. 7) do not try to inject the same embryo more than twice.

### Problem 3

Problem in the detection of organelles/vesicles transport dynamics.

### Potential Solution

If no movement of the organelles/vesicles is recorded, this may result from poor slice fitness. Post-natal cortical slices have a short survival rate (about 4 h) and therefore every measure that could endorse it should be followed. We highly recommend performing all steps of the procedure in the shortest time possible, to maintain all reagents and perform all manipulations at 4°C.

## RESOURCE AVAILABILITY

### Lead Contact

Further information and requests should be addressed to Laurent Nguyen (lnguyen@uliege.be).

### Materials Availability

This study did not generate new unique reagents.

### Data and Code Availability

This protocol includes all datasets generated or analyzed during this study.

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100131.

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## AUTHOR CONTRIBUTIONS

This protocol relies on experiments done by L.B. and S.T. and has been written by S.T., L.B., and L.N.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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