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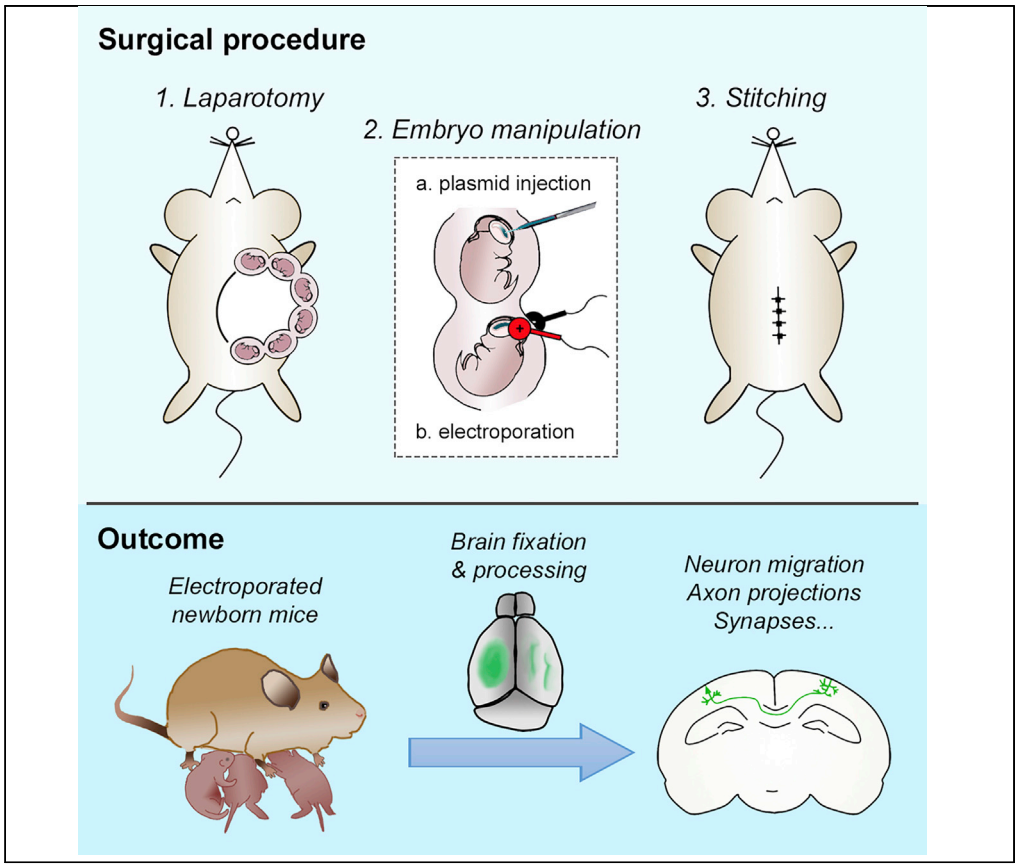
In Utero Cortical Electroporation of Plasmids in the Mouse Embryo



The cerebral cortex is composed of an exquisitely complex network of interconnected neurons supporting the higher cognitive functions of the brain. Here, we provide a fully detailed, step-by-step protocol to perform *in utero* cortical electroporation of plasmids, a simple surgical procedure designed to manipulate gene expression in a subset of glutamatergic pyramidal cortical neurons *in vivo*. This method has been used to visualize defects in neuronal migration, axon projections, terminal axon branching, or dendrite and synapse development.

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HIGHLIGHTS

A detailed protocol to inject and electroporate plasmids into mouse embryonic cortex

Guidelines to ensure aseptia during and after the surgical procedure

Detailed ethical considerations in respect of the '3R' rule in animal experimentation

Description and troubleshooting of the most common pitfall associated to the procedure

Meyer-Dilhet & Courchet, STAR Protocols *1*, 100027 June 19, 2020
https://doi.org/10.1016/j.xpro.2020.100027

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In Utero Cortical Electroporation of Plasmids in the Mouse Embryo

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SUMMARY

The cerebral cortex is composed of an exquisitely complex network of interconnected neurons supporting the higher cognitive functions of the brain. Here, we provide a fully detailed, step-by-step protocol to perform *in utero* cortical electroporation of plasmids, a simple surgical procedure designed to manipulate gene expression in a subset of glutamatergic pyramidal cortical neurons *in vivo*. This method has been used to visualize defects in neuronal migration, axon projections, terminal axon branching, or dendrite and synapse development. For complete details on the use and execution of this protocol, please refer to Courchet et al. (2013), Mairet-Coello et al. (2013) or Shimojo et al. (2015).

BEFORE YOU BEGIN

This protocol describes the material and steps of *in utero* cortical electroporation that we have been performing for more than a decade. Our original protocol has been described in (Hand and Polleux, 2011) and in publications from the laboratory afterwards. For variations, readers may also refer to other published protocols such as (Cancedda et al., 2013; Pacary and Guillemot, 2020; Saito, 2006; Shimogori and Ogawa, 2008).

⚠ CRITICAL: This procedure works well in outbred mice (for example Swiss) that have the benefit of having very large litters. An excellent alternative is using F1 hybrids of C57BL/6 x 129Sv mice that have excellent maternal care and smaller litter size (8-9 embryos on average) which reduces overall surgery time and thus benefits survival.

⚠ CRITICAL: The procedure obeys the rules for veterinary surgical practice and is refined to reduce pain to a minimum by post-procedure monitoring and analgesia. The strict respect of surgical sterility prevents post-surgical complications and alleviates the need for antibiotics.

Plasmid Preparation

Timing: 5 min

1. Dilute selected, endotoxin-free plasmid prep in sterile nuclease-free water. Final concentration range 1μg/μL (single plasmid) to 2μg/μL (plasmid mix). Add Fast green (10% final volume) to visualize injection. Final volume 10-20μL.

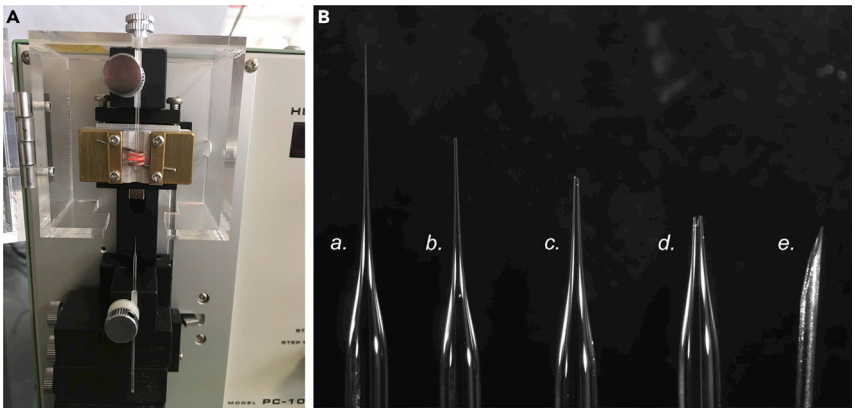

Figure 1. Preparation of Microcapillaries for Plasmid Injection

(A and B) (A) Use of the Narishige PC-10 glass pipette puller. Result can be seen in (B): unbroken pipette after pulling (a.). Pipette tip can be broken with a gentle finger push on the glass tip. Pipette break must be clean and narrow to ensure appropriate plasmid injection with minimum damage to brain structures (b.). In some instances a broader aperture might be better suited when using more viscous DNA solutions (c.). Too wide and/or not sharp breakage should be avoided for they induce too much brain damage (d.). Tip of a 27 gauge needle for scale (e.).

2. Prepare microcapillaries using a puller (Figure 1). We use borosilicated microcapillary and prepare them with a Narishige PC-10 micropipette puller. We recommend the following parameters: one-step pulling, heater level 62 degree with 4 weights blocks. These settings allow to obtain a glass capillary which gets angled gradually, then sharp to the edge.

   Alternatives: For more control over microcapillaries users may prefer to use alternative pipette puller such as the P-97 from Sutter Instruments. This instruments provides better control over variables such as heat, pull strength and delay time between heating and pulling, affecting the length and diameter of the taper.

   Alternatives: Pre-pulled glass pipettes can also be purchased directly and are a good alternative if no puller is available.

3. Break the tip of a pre-pulled microcapillary (Video S1), then place it in the tube containing DNA and let the solution go in by capillarity.

   Note: Break the microcapillary by using the index finger perpendicular to the tip. Gently remove the extremity (approximatively 2mm). The procedure is demonstrated in (Video S1) and the result is illustrated in (Figure 1B). The rise of the plasmid DNA solution by capillarity also provides information on the quality of the microcapillary. In the absence of liquid entering the pipette by capillarity, check if the microcapillary has been properly broken. The microcapillary might also be clogged by debris present in the DNA solution. In this case a slightly larger aperture of the tip of the microcapillary is required (Figure 1B) (Troubleshooting 3).

Prepare Surgical Table and Instruments

Timing: 15 min

4. Connect the anesthesia machine, check that the amount of isoflurane is sufficient for the duration of the surgery and put the anesthetic mask on the surgical table.
5. Turn on the electroporator and microinjector, and check that the settings are set up for the surgery. Connect the cables for the electrodes and the foot pedal.

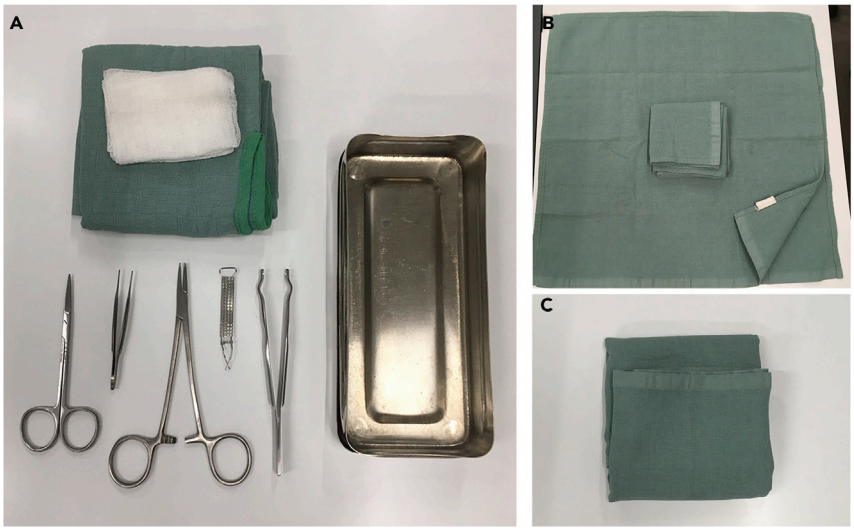

Figure 2. Preparation of Reusable Packs for the Surgery

Composition of surgical packs (A) and surgical drapes packs (B: before folding and C: folded and ready to autoclave). Both must be sterilized in an autoclave before surgery. Sterile, ready-to-use packs can be stored for up to 3 months. It is recommended to use one pack per mouse.

6. Turn on the heating mat (39°C), warm sterile PBS (20mL in a conical tube, using a heating block). Turn on the light.
7. Pick an autoclaved box of surgical tools and surgical drapes (Figure 2).

8. Unfold the surgical drapes pack. Take out the folded drape in the middle of the pack (Figure 2B) and unfold it on the table. This drape will serve to prepare surgical instruments in sterile conditions.

Prepare Animal

Timing: 5 min

9. Place pregnant dam in a clean cage to reduce risk of a post-surgery infection. The cage must contain some enrichment: cotton and paper for nest building, cardboard bio-tunnels and/or plastic hut. Use food adapted to pregnant/lactating mice.

⚠ CRITICAL: Environment enrichment is not only an essential part of protocol refinement following the 3R (Replacement, Reduction and Refinement) practice. It is also essential for the proper development of cortical circuits in newborn mice, and an important element for post-natal survival (see expected results).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Isoflurane (Isoflu-Vet 1000mg/g)	Dechra	Cat#ISO008 (Centravet)
Buprenorphin (Buprecare®) 73422 BUPRECARE 0.3 mg/ml INJ 10 ML	Ecuphar	Cat#73422 (Coveto)

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Povidone-iodine (Betadine) Scrub 4%	Vetoquinol	Cat#VET003 (Centravet)
Povidone-iodine (Vétédine®) Solution	Vetoquinol	Cat#VET002 (Centravet)
Ocry-Gel® 10g	TVM Laboratory	Cat#OCR002 (Centravet)
Experimental Models: Organisms/Strains		
Mice : E15.5 pregnant females - Swiss (outbred) - 129Sv/B6 (F1 hybrid of inbred mice)	Janvier Labs In house	N/A
Critical Commercial Assays		
Plasmid DNA, endotoxin free *Midi-prep kit from NucleoBond® Xtra Midi EF*	Macherey-Nagel	Cat#740420.50
Fast green (1:20 ratio)	Sigma-Aldrich	Cat#F7252
Nuclease Free water	Ambion	Cat#AM9937
Other		
Electroporator ECM 830 BTX and BTX Generator Footswitch (model 1250FS)	Harvard Apparatus	Cat#EC1 45-0002
Micro-injector Picospritzer-III with Footswitcher	Harvard Apparatus	Cat#051-0530-900
Micropipette puller Model PC-10	Narishige	Cat#PC-10
1x Halsey Needle Holder - 13cm	Fine Science Tools	Cat#12001-13
1x Iris Forceps - Serrated Straight 7cm	Fine Science Tools	Cat#11064-07
1x Extra Thin Iris Scissors - Straight 10.5cm	Fine Science Tools	Cat#14088-10
1x Michel Clip Applying Forceps	Fine Science Tools	Cat#12018-12
Michel Suture Clips - 7.5 x 1.75mm	Fine Science Tools	Cat#12040-01
3x Surgical drapes 60x60cm	Alcyon	Cat#8337590
1x Surgical drape 50x50cm with straight aperture (13 cm)	Alcyon	Cat#8037988
Isoflurane anesthesia station Model Mini Hub V3.2	TEM SEGA	N/A
Heating mat 35x40cm	Buster, distributed in France by Alcyon	Cat#8365936
Heating block with 50mL conical tubes adapter	Major Science	MD-MINI-B04
Surgical lamp with flexible arm, attached on the table or wall (Halogen 20W or LED equivalent)	N/A	N/A
Cordless Hair clipper Type 1590	Whal UK	N/A
Borosilicate Glass capillaries O.D.:1mm, I.D.:0.50m, 10cm length, FiMT	World Precision Instruments	Cat#GBF100-50-10
Sterile PBS pH 7,4 (1X)	Gibco	Cat#10010-015
1,5mL conical tube	Dutscher	Cat#72.690001
50mL conical tube	Falcon	Cat#352070
Surgical ethanol	Local pharmacy	N/A
Sterile gauze 5x7,5cm	Coveto	Cat#700099
Syringe 10mL	Terumo	Cat#302188
Syringe 1mL	Terumo	Cat#SS+01T1
AGANI™ Needle 26Gx1/2'' (0,45x13mm) regular bevel	Terumo	Cat#AN*2613R1

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sterile Latex Powdered Surgical Gloves Triflex®	Cardinal Health USA	Cat#2D7254
Coated vicryl™ 5-0, 75cm	Ethicon	Cat#JV389
Weighing scale	KERN	Cat#KB 120-3N

MATERIALS AND EQUIPMENT

All equipments are listed in the table above. The following alternatives may also be considered:

- Electroporator: Nanoject III (Drummond ; Cat# 3-000-207) and other square wave electroporation units such as the NEPA21 (Nepagene) can be used.
- Microinjectors: blow tubes (Merck ; Cat# A5177-5EA) can be used instead of a microinjector if the equipment cannot be set up in the animal facility.
- Micropipette puller: laboratories equipped may use alternative pipette pullers such as model P-97 from Sutter instruments.
- Surgical drapes: Disposable plastic drapes can be used such as Buster surgery cover 30x45cm (Kruuse ; Cat#141765). The main benefit of these is to allow for custom sized aperture by cutting the drape.

STEP-BY-STEP METHOD DETAILS

Induction of Anesthesia

Timing: 5 min

These steps ensure the transition from an awake animal to a state of controlled and stable anesthesia.

1. Weigh mouse - This is important to calculate the proper amount of premedication and to follow recovery and weight gain post-surgery.
2. Prepare premedication/analgesia: Buprenorphin (Buprenex®) diluted at 0.03 mg/mL – Final dose 0.1 mg/kg.

   Note: Analgesia protocols must be adapted to local guidelines and legislation concerning the use of opioid drugs. It is recommended to seek advice from local animal welfare and veterinary committees.

   Note: Keep records of anesthesia/surgery procedures for post-surgical care. See for example Supplementary document 1.

   Alternatives: NSAID are good alternatives to opioids. For example Carprofen (Rimadyl®) diluted at 0.5 mg/mL – Final dose 5 mg/kg.

3. Place pregnant dam in the induction chamber with air flow at 1 L/min and Isoflurane at 5% until the animal is sedated.

   Note: This step takes on average 60 to 90 sec depending on animal's age, weight and body fat. Longer time may indicate a leakage in the circuit or Isoflurane exhaustion from the evaporator.

   ⚠ CRITICAL: This step must be monitored carefully to avoid a potentially lethal Isoflurane overdose.

4. Take the mouse out of the induction chamber and place it face down (ventral decubitus) on the surgical drape.
5. Rapidly inject the premedication mix by subcutaneous injection in the interscapular area.
6. Treat both eyes with moisturizer/corneal protection gel (Ocry-Gel®) immediately before placing the animal's head into the mask.
7. Lay the animal face up (dorsal decubitus), head entirely in the mask, with air flow at 1 L/min and Isoflurane at 2%.
8. Check the absence of reflex (indicating a deep anesthetized state) by pinching the hind paw and the tip of the tail.

Note: Steps 4-7 must be performed quickly (~15 sec) to avoid the animal waking from anesthesia.

⚠ CRITICAL: From this point the whole procedure should be finished within 30 minutes to ensure pups viability. Longer surgeries negatively impact litter viability.

Less experienced surgeons should chose to inject/electroporate fewer embryos in order to remain within the 30 minutes optimal timing. This is especially important when using strains with large litter size (such as Swiss mice)

Surgical Preparation

Timing: 5 min

These steps ensure the proper preparation of an aseptic surgical zone and surgeon. If instructions are followed properly, post-surgical antibiotic therapy is not necessary.

9. Surgical hair clipping on a square zone of about 4x4cm centered around the umbilicus (navel). The region corresponds approximatively to the space comprised between the mammary glands 3 (thoracic) and 4 (abdominal) (Figure 3).

⚠ CRITICAL: Stretching the skin of the abdomen avoids skin lesions/micro cuts when shearing. Skin irritation has a strong negative impact on post-surgical well-being and can lead to lack of care of newborn litter.

10. Surgical zone preparation: using surgical gauze, alternate cleaning with 70% surgical ethanol and Povidone-iodine (Betadine®) Scrub 2 times, then one final time with Vetedine® solution (Figure 3).

Note: Follow a circular pattern from umbilic to the outer part of the clipped region without returning to center.

11. Remove the soiled surgical drape by gently lifting the mouse without touching the prepped zone. At this stage the mouse should be in dorsal decubitus, on a clean, sterile drape, above the heating pad.
12. Prepare surgery tools: adjust the light above the surgical site. Open the surgical box and gently drop instruments on the sterile drape (without touching them). Open the electrodes box. Open one suture pack. Open one sterile 10mL syringe. Open the 50mL conical tube containing sterile heated PBS. Gently break the tip of the microcapillary and place it in a tube containing DNA plasmid (Figure 4).

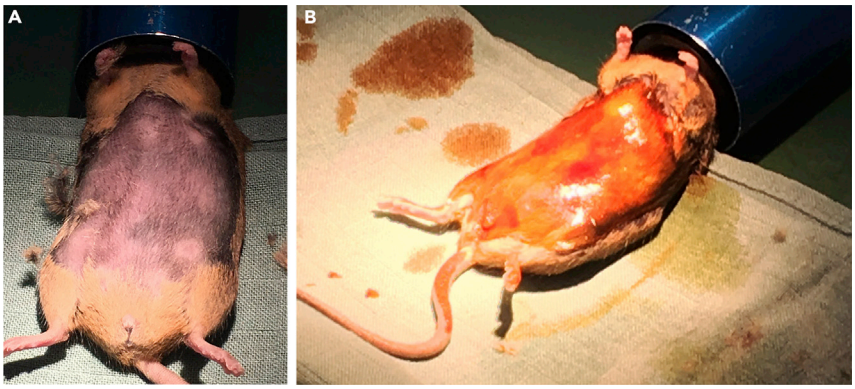

Figure 3. Preparation of the Mouse for the Surgery

(A) Clipping region on the abdomen of the mouse, centered around the umbilic. Anatomical marks are the 3rd and 4th mammary glands.

(B) The region is prepared by alternative Alcohol-Iodine treatment. After the 3rd wash, Iodine solution is left over the skin for the surgery and not washed away.

⚠ CRITICAL: Carefully check that everything 'non sterile' has been prepped for surgery as the next steps will be performed with sterile, surgical gloves.

Surgical Procedure

Timing: ~20 min depending on the number of embryos. Should not be longer than 25 min for optimal viability.

This is the main step of the procedure consisting of a laparotomy to expose the embryos, plasmid micro-injection, electroporation, and suture.

13. Put on surgical (sterile) gloves. You can now touch sterile instruments and drapes but should refrain from touching non sterile parts (animal, lamp…).

Note: The surgical box contains two gauze pads that have been autoclaved with the instruments and can thus be used to touch non sterile parts during the surgery, such as the gene-paddles and injector.

14. Place a sterile surgical drape covering the whole mouse with 4x2cm aperture centered on the umbilic.

Optional: You may use single use sterile plastic drapes. The aperture can be customized by cutting the fabric before placing the drape above the mouse.

15. Use forceps on left hand to raise the skin above umbilic and with scissors on your right hand, gently perform a mid-sagittal ("vertical") incision of about 2.5cm (1cm above umbilic and 1.5cm below). The incision should expose the umbilic scar and the white line joining the two abdominal muscles (Figure 5).

⚠ CRITICAL: Use the needle holder to gently dilacerate adherences between skin and abdominal muscle. To do so, put the tip of the closed needle holder in the wound, in contact with the muscle. Gently open it so its jaws push skin tissue on the side. This step will facilitate embryo mobilization and future suture.

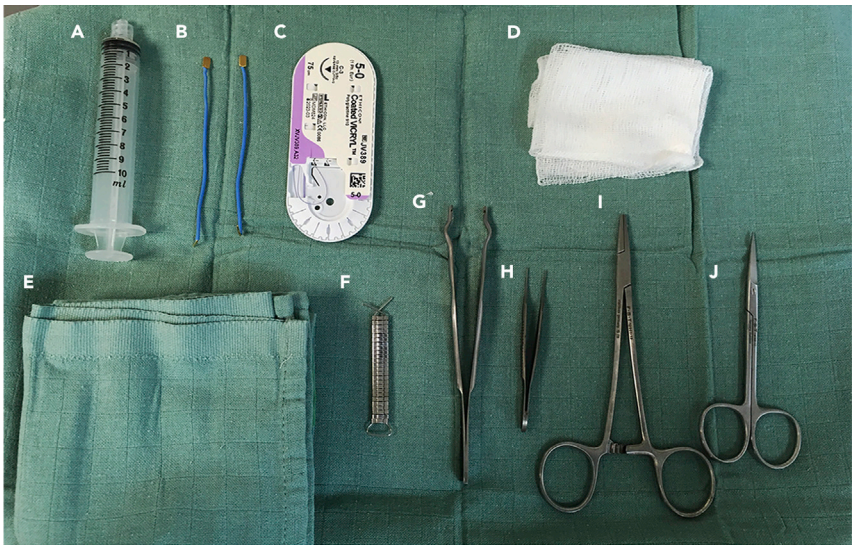

Figure 4. Surgical Instruments Are Disposed on a Sterile Surgical Drape in a Convenient Way for the Surgeon to Avoid Cross-contamination by Touching Non Sterile Material

(A–J) (A) 10mL syringe. (B) Gold paddles. (C) Resorbable suture. (D) Sterile gauze. (E) Surgical drape (to cover mouse during surgery). (F) Suture clips. (G) Michel clip forceps. (H) Iris forceps. (I) Halsey needle holder. (J) Iris scissors.

16. Use forceps on left hand to raise the umbilic and with scissors on your right hand, very carefully make a small (1mm) incision. After pneumo-abdomen (entry of air into the abdomen) has been obtained, perform a 2cm incision along the white line (0.8cm above and 1.2cm below umbilicus).

Note: The entry of air into the abdomen should release the abdominal organs from adhering to the abdominal wall and hence facilitate the incision of the muscular plane.

17. Expose one uterine horn with forceps. Carefully pull out all the horn from one side up to the ovary and count embryos.

Note: Assess embryo viability. Healthy embryos have a light pink coloration and clear amniotic liquid. Embryos that are all white, or presenting signs of hemorrhage, or cloudy amniotic liquid, should be skipped as they are unlikely to survive, and injection risks cross-contaminating healthy embryos.

18. Use your left hand to stabilize one embryo between thumb and pointer fingers. Embryos are fairly mobile in the amniotic pouch and with care can be oriented to better suit the injection. It is important to take care not to damage either the placenta or the blood vessels while manipulating/injecting.

⚠ CRITICAL: Refrain to apply too much pressure on the amniotic bag, which could otherwise break as it is punctured by the capillary.

19. Perform plasmid injection: with the microcapillary, gently poke into the lateral ventricle. You may often feel two steps of injection (two hit pass), corresponding to the capillary going through the uterus, and then through the skin and skull. Inject ~0.2-0.3µL of plasmid DNA using the foot pedal from the microinjector (or mouth aspirator tube) (Figure 6) (Video S2).

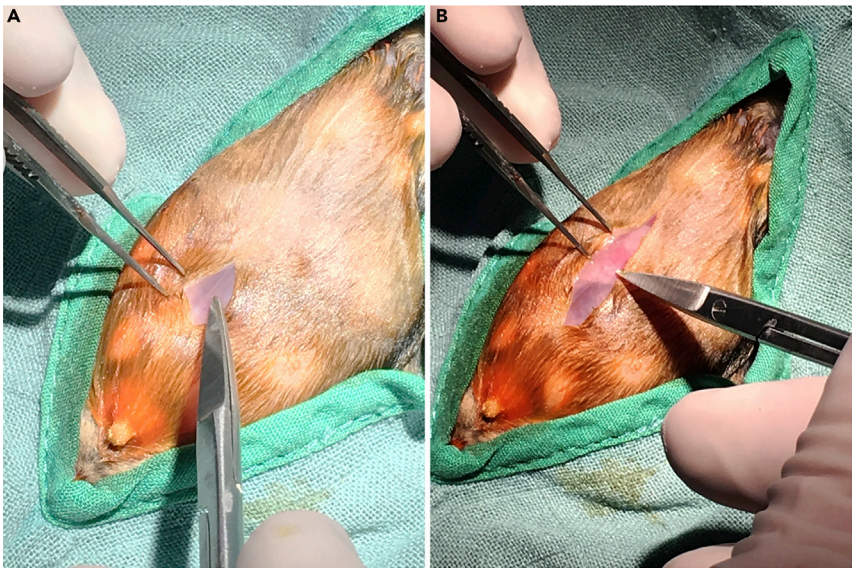

Figure 5. Skin Incision Is Performed by Gently Raising the Skin and Cutting with the Iris Scissors

The white line joining the two abdominal muscles (A) and the umbilical scar (B) are visible under the scissors tip. Subsequent incision of muscle along the white line prevents bleeding during surgery and strongly increases functional recovery and delivery.

Note: The length of the tip inserted in the skull is usually a fairly short length, for the ventricle is not located very deep in the brain. One good estimation would be about 4mm total, 2mm through the uterus and 2mm into the skull (Troubleshooting 3).

⚠ CRITICAL: Injection can be visualized by filling of the ventricle with the blue dye, giving a crescent shape. If ventricle filling is not observed, the injection is most likely unsuccessful owing to a wrong injection angle or depth (Troubleshooting 3). It is not advised to give more than two attempts on a single embryo.

Note: The interval between injection depends on the experience of the surgeon and is approximatively 0.5 min for a trained surgeon.

Note: To save time, experienced surgeons may perform sequentially all injections, then all electroporations. This significantly cuts the procedure time by limiting handling and alternating between injection and electroporation. However sequential injections increase the risk of plasmid diffusion and dilution which would affect the electroporated zone. For these reasons less experienced surgeons should refrain from performing sequential injections.

20. Perform electroporation: place the anode (positively charged electrode) on the side of DNA injection and the cathode on the other side of the embryos head and press foot pedal. Wait until the electroporation has been delivered in full (Figure 7) (Table 1). It is normal to witness slight contractile movements of the embryo during electroporation.

Note: Keep the uterus hydrated at all time during the procedure by dripping warm sterile PBS over the uterus (using the 10mL syringe). This is especially critical to ensure a good electroporation rate. Be careful not to overload with PBS to prevent soaking the anesthetized mouse, since a wet animal is more prone to potentially fatal hypothermia.

21. When all embryos on one side have been electroporated, carefully put back the uterine horn in the abdomen, starting by the extremity attached to the ovary. Pull out the other horn and perform steps 18-20 over again.

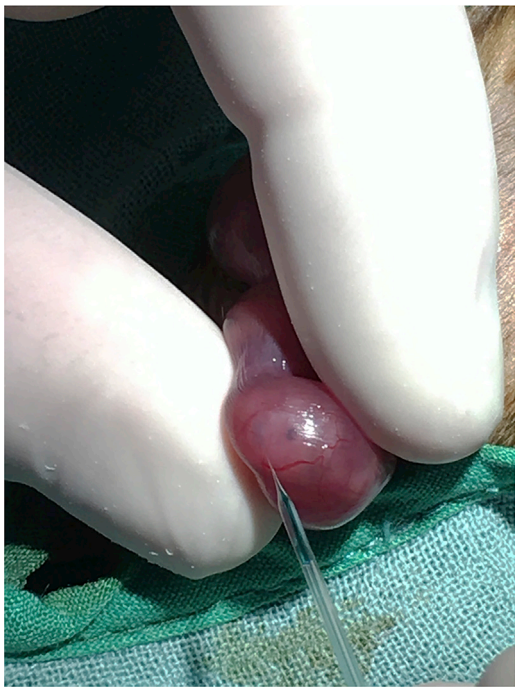

Figure 6. Plasmid Microinjection
Left hand is used to stabilize the embryo.

⚠ CRITICAL: Longer procedure (>30 minutes from induction of anesthesia to arrest of isoflurane) strongly impacts viability of the whole litter. We recommend less experienced surgeons, who are slower at injecting and electroporating, to refrain from injecting and electroporating all the embryos.

Note: Although this is not described in this protocol, it is possible to perform multiple injections in the same embryo. Time between the two injections and electroporations is required for plasmid wash out of the ventricle. See for example (Zhou et al., 2013).

22. Stitching: using the forceps and needle holder, perform sutures using resorbable string. Spacing between two passes of the string should be about the size of forceps tip (closed).
    a) Muscle stitching: running suture, lock with a knot every 3 passes.
    b) Set the isoflurane on 1% to lighten anesthesia and speed recovery.
    c) Skin stitching: running suture, lock with a knot every 3 passes.
    d) Turn off isoflurane. Before the mouse wakes from anesthesia (1 minute), you may place surgical clips (x4) above knots to prevent the mouse removing skin stitches.

Note: Typically the suture size should be 6 passes (with one intermediary knot) for muscle stitching and 9 passes (with two intermediary knots) for skin suture.

Note: Tension on the knot should be limited. Tight knots tend to be less tolerated by the animal and are more likely to be removed by the animal out of irritation/pain.

Post-surgical Care

Timing: 15 min (immediate care) and 2 days (follow-up care)

Post-surgical care includes the immediate aftermath of the surgical procedure (i.e. getting the mouse to awaken completely and be back in the cage), and the follow-up care in the days following surgery.

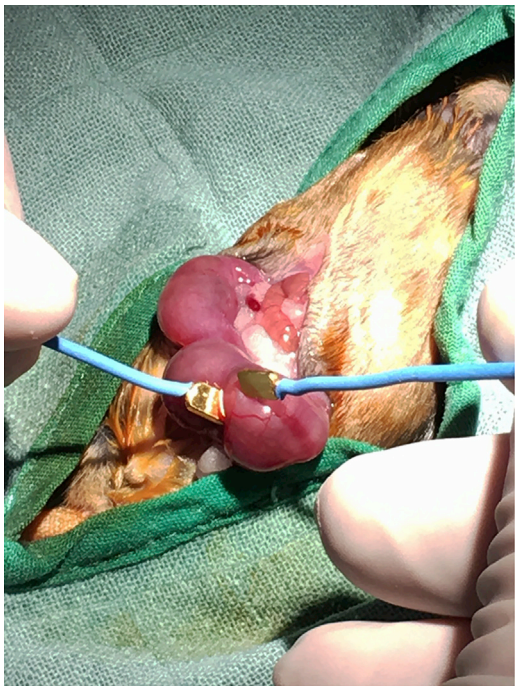

Figure 7. Position of the GenePad Electrodes during the Electroporation Procedure

Use the flat side of the negatively charged electrode to stabilize the embryo. Make contact of the edge of the positively charged electrode close to the injected region ("blue crescent").

23. Immediately after isoflurane has been turned off and/or clips have been placed (step 22), remove the mouse's head from the surgical mask. The mouse should gradually wake up, starting from 30-60 seconds, and be fully awake within 2 to 5 minutes.
24. Heating: place the mouse back in the home cage. A heated block placed under the cage should provide a warm environment. It is important that the heated block does not cover all the surface of the cage so the mouse can move to a part of the cage without heating if it feels more comfortable.

    Alternatives: The cage can be placed instead under a heating lamp. Although this solution offers faster body temperature recovery, it requires extra care since a lamp too close can cause burning and dehydration.

25. Within 5 minutes the mouse should be on its 4 feet and moving upon stimulation. Leave the mouse in shelter and observe regularly for the next 10 minutes. Within 15 minutes following surgery the mouse should be moving spontaneously in the cage and start cleaning.

    Optional: At this stage a pain assessment scoring can be performed. This scoring will serve as a baseline for subsequent recovery and pain evaluation.

    Note: Occasionally vaginal discharge can be observed following surgery, often resulting from breakage of the amniotic bag during surgery. Although this situation should be avoided, this discharge is not systematically predictive of abortion (Troubleshooting 3).

Table 1. Electroporation Parameters for E15.5 Embryos Using the ECM830 Electroporator

ECM830 with 3x5mm GenePad electrodes
Voltage: 45 V
Number of pulses: 4
Pulse duration: 50 msec
Pulse interval: 500 msec
Polarity: unipolar

26. Follow-up care

Inspect the mouse twice a day for 48 hours following surgery. Perform a pain assessment scoring and decide accordingly regarding the use of analgesic medicine (Supplementary document 1). Standardized mouse grimace scales are a good resource to evaluate post-surgical pain (Langford et al., 2010).

On the day following surgery, inspect the wound and be especially attentive to removed stitches. The loss of skin stitches happens rarely (less than 5% of cases) when stitches are performed properly. In most of the cases this problem is detected in the thoracic region when stitches are too tight and is typically well supported by the mouse (Troubleshooting 3).

⚠ CRITICAL: Excessive stress to the mother leads to birthing complications, litter rejection or cannibalism. Stress can disrupt nest building and future care for newborn pups. It is critical to limit mouse handling as much as possible in the few days following surgery and around birth. Mouse cage must be placed in a calm room with little traffic.

EXPECTED OUTCOMES

Birth of the mouse pups typically happens at E18/E19. For a E15.5 electroporation, pups are typically born 4 days after the procedure (*ie.* on a Monday when electroporation is performed on a Wednesday) (Figure 8). Newborn mice develop normally and can be raised like any regular mice of the colony.

In a typical experiment >80% of injected embryos should be born, out of which most to all should be electroporated (Figure 9). Efficiency and reproducibility (electroporation zone and intensity, survival…) is highly skill-dependent. While practice makes an importance difference, other parameters such as genetics, housing condition and stress… can interfere with the overall success of the method (Troubleshooting 3). Especially environment enrichment is a critical parameter not only for delivery and pups survival, but also for cortical circuits development. Litters raised in a cage with limited stimuli present dramatic alterations of axonal developments.

The *in utero* cortical electroporation procedure has been developed as a way to manipulate gene expression in restricted neuronal populations *in vivo* (Fukuchi-Shimogori and Grove, 2001; Saito and Nakatsuji, 2001). It is important to note that only cortical glutamatergic neurons are targeted by the procedure described. Cortical interneurons born in the subpallium will not be electroporated.

The procedure can also be used to manipulate gene expression through expression of CRE-coding plasmids or shRNA vectors (Matsuda and Cepko, 2007). More recently, it has been adapted to induce single cell knockout or targeted knockin using CRISPR-CAS9 (Shinmyo et al., 2016; Swiech et al., 2015; Tsunekawa et al., 2016).

Plasmid electroporation allows for the expression of fluorescent proteins which is especially suited for morphological and migration analyses of cortical neuronal populations (Hand and Polleux, 2011; Tabata and Nakajima, 2001). As such, a classical post-procedure processing consists in post-mortem histochemical analyses: at selected ages, electroporated mice will be sacrificed by intracardiac perfusion of fixative agent (4% PFA), followed by brain extraction, histological preparations (brain slices) and imaging.

The timing of expression of the transgene depends upon the selected promoter, and can last for several weeks. We have regularly observed fluorescence in neurons up to 90 days after electroporation in the cortex and hippocampus (Courchet et al., 2018; Mairet-Coello et al., 2013).

Figure 8. Home Cage 2 Days after Birth
Plastic biohut has been moved to expose the nest. Cage enrichment is critical for nesting and litter survival, as well as for normal cortical development in the newborn pups.

LIMITATIONS

The population of targeted neurons depend upon two factors: one being the timing of electroporation, the other one being the position of electrodes. For instance electroporation at E15.5 will target superficial neurons (layer 2-3), whereas electroporation at E13.5 will target deeper neurons (layer 5) (Hand and Polleux, 2011).

Targeting of broad cortical regions is easy to achieve, however targeting of other brain regions is often more challenging and results can be more inconsistent. Some alternatives have been developed with modified electrodes to significantly increase plasmid targeting (Cancedda et al., 2013; dal Maschio et al., 2012).

This protocol often results in labeling a large population of neurons, which is well suited to follow patterns of axon projections and terminal branching. However, some applications such as reconstruction of somato-dendritic morphology or dendritic spines analyses may require sparser labelling. One way to achieve sparse labelling is through the use of diluted CRE plasmids (Luo et al., 2016; Young et al., 2008), or through the use of the Thy1 promoter (Ako et al., 2011).

On the other hand, even the most intense electroporations impact a very limited subset of neurons. Therefore, this method is not suitable to observe and induce functional changes in cortical functions (for instance rescue of a given behavior).

TROUBLESHOOTING

Problem 1

Microcapillaries related issues such as clogging or amniotic liquid entry

Potential Solutions

The preparation of the microcapillary is critical to this procedure. Guidelines for microcapillary preparation and aperture can be found in Figure 1.

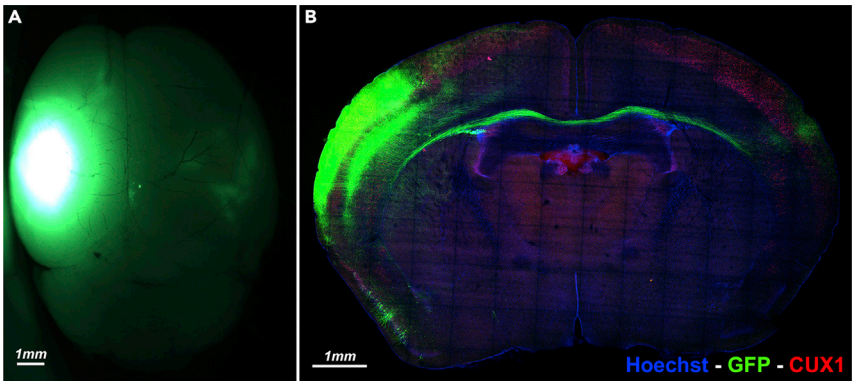

Figure 9. Typical Outcome of *In Utero* Plasmid Electroporation at Postnatal Age 21 (P21)

(A) Dorsal view of a brain electroporated with the fluorescent protein mVenus on the left side (ipsilateral). Long exposure can also reveal patterns of terminal branching of callosal axons on the contralateral hemisphere (right). (B) Following vibratome section and histochemistry it is possible to visualize cortical projection neurons development from neurogenesis and migration, to patterns of axon projection and terminal branching.

- **Microcapillary clogged by DNA debris**: viscous DNA preparations and/or the presence of debris can obstruct the tip of the microcapillary and interfere with filling and injection. We recommend to use high quality plasmid preparations and avoid the presence of debris in the DNA solution. Alternatively a quick pulse of plasmid centrifugation (10 seconds at 10,000 rpm) can pellet the debris.
- **Amniotic liquid leakage through the puncture hole in the uterus:** this can be observed when the tip of the microcapillary is too wide and/or when too much pressure is applied on the embryo while trying to stabilize it for injection. This is typically well tolerated and the puncture hole closes spontaneously. Surgeons should change microcapillary and/or correct finger pressure on the next embryo.
- **Amniotic liquid or LCR entry into the microcapillary:** this problem leads to plasmid dilution and/or microcapillary obstruction. This happens when the pipette tip has too wide an aperture and the solution is to change microcapillary.

Problem 2

Inconsistent or lack of electroporation

Potential Solutions

Typically for an experienced surgeon, >80% electroporated animals will give satisfying electroporation in the cortex (success rate might be lower in other brain regions such as hippocampus). Despite survival, experimenters may face poor electroporation rates. Electroporation inconsistencies can become a frustrating issue for troubleshooting, because the result is seen several weeks after the procedure and it is not possible to track individual embryo/injection. The main reasons for poor electroporation are:

- **Injection out of the cortical ventricle**: the untrained surgeon often misinterprets the injection angle and depth resulting in plasmid being injected in sub-cortical parenchyma. In this case electroporated neurons are usually found in subcortical regions such as the striatum. Another classical mistake is injection in the amniotic cavity or under the skin, when the surgeon did not feel the "two-hit" pass of the micropipette. This results in a complete absence of electroporation.

Note: Lateral ventricle filling gives a typical "blue crescent" image that indicates a proper injection.

- **Absence or low electroporation**: position and polarity of the electrodes are critical to this procedure. Verify that 1) the electrical circuit is not interrupted (cables plugged properly), 2) the calibration of the electroporator is correct (pulse intensity and duration are key parameters), and 3) the position of electrodes is respected.

Note: Small movements of the embryo, and a light, temporary mark on the uterus are signs that electrical current is passing through the electrodes.

Problem 3

Amniotic fluid discharge and peri-partum complications

Potential Solutions

- **Amniotic fluid discharge:** vaginal discharge of amniotic fluid can occasionally occur at the end of surgery. The liquid is viscus, clear or lightly tainted with blood, and low in quantity, and most likely results from breakage of one or several amniotic bags during surgery. This complication likely indicates a difficulty while handling the embryos, such as excessive pressure exerted during injection (common with beginner surgeons). Although this should be corrected with practice, it is often well tolerated by the mouse and seldom leads to abortion. Blood or an abundance of liquid are signs of poor prognosis.
- **Peri-partum complications:** severe conditions including dystocia (arrested labor caused by abnormally large fetus) can arise from poor surgical procedure such as an abnormally large incision, or muscle incision not following the white line and resulting in poor abdominal muscle tonus. Dystocia is a life-threatening condition causing exhaustion, hypotension and hypothermia that in most cases is detected too late and lead to euthanasia.

Note: Perinatal stress is strongly associated to dystocia. It is advised to limit handling of mice before and after birth to avoid interrupting the birth process. Observation through the cage is very often sufficient to detect potential birth complications.

Problem 4

Surgery-related complications such as bleeding or stitches removal.

Potential Solutions

When performed properly, this rapid surgical procedure is well tolerated by the animal. Potential surgery-related complications are as follow:

- **Bleeding:** when done properly, the surgery does not cause bleeding. The breakage of a vein can occur if the incision is not performed on the midline. Typically limited in volume, the blood loss may cause pain and delayed wound healing. The solution is increased post-surgical surveillance.
- **Stitch removal and open wound**: disruption of stitches can sometimes happen, usually when stitches are over tightened resulting in local tissue necrosis and pain, or when the skin incision is too long toward the thorax, resulting in increased skin tension (the skin is more loose on the abdomen). This is well tolerated by the animal and often does not require any treatment. Upon signs of local inflammation (exudation, red and swollen wound), local topic application of antibiotic or antiseptic over 3-5 days is sufficient.

Problem 5

Poor survival or efficiency

Potential Solutions

One major pitfall from the strategy is lack of survival of the embryos following electroporation or at birth. In most cases the experimenter would find the female no more pregnant but no live pups in the cage. In our experience the most frequent causes are summarized below:

- **Embryo death/resorption following electroporation:** The main cause in our experience is a prolonged procedure, but can also result from traumatic handling during surgery, poor electroporation parameters, bad quality DNA or high level of stress in the animal facility.
- **Lack of maternal care:** The leading cause is maternal stress around/following delivery. It is important to limit handling around birth (1 day prior/3-4 days after). Litter change should not be necessary if the mouse has been placed in a clean cage immediately before the procedure.

The cause can often be identified by finding newborns dead in the cage (-> lack of maternal care); however, the absence of newborn can also result from cannibalism. In case of persisting trouble, it is advised to check embryo survival 48 hours following electroporation.

⚠ CRITICAL: Not all mouse strains give similar results. C57BL/6 background especially are prone to stress and rejecting litters. For transgenic lines on a C57BL/6 background, it is advised to either introduce some outbreeding to the genetic background (introduce some 129/Sv for example), or resort to using a foster mother from another background. The latter however leaves the risk of pup rejection by the foster mother, and the use of more mice is an ethical challenge that must be reserved for when no alternative solution is available.

⚠ CRITICAL: As a general rule, and especially when using strains with poor maternal care, it is recommended to use mice that had a litter before, for primipara (first gestation) mice have a higher chance of neglecting litter.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100027.

ACKNOWLEDGMENTS

This protocol has been developed by applying the standards of veterinary practice to laboratory mouse surgery and refined with time and interaction with esteemed colleagues. We are especially grateful to Randal Hand, Santos Franco, Tommy Lewis, Olivier Raineteau, Franck Polleux, and members of the Polleux laboratory for technical advice and discussions. The use of F1 hybrid mice was a recommendation of Santos Franco for which we are extremely grateful. We wish to thank Virginie Courchet, DVM, for technical and veterinary advice. We thank the scientists and laboratories who published protocols on similar procedures and apologies for not citing them all in the references. Finally, we thank the personnel at the Service Commun des Animaleries Rockefeller for mouse care. Equipment and our current work is funded by an ERC Starting Grant (678302-NEUROMET).

AUTHOR CONTRIBUTIONS

This protocol has been developed by J.C. and is routinely exerted by G.M.-D. The detailed procedure was written by G.M.-D. and J.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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