Video Article

Gene Transfer to the Developing Mouse Inner Ear by *In Vivo* Electroporation

Lingyan Wang[1], Han Jiang[1], John V. Brigande[1]

[1]Oregon Hearing Research Center, Oregon Health & Science University

Correspondence to: John V. Brigande at brigande@ohsu.edu

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Abstract

The mammalian inner ear has 6 distinct sensory epithelia: 3 cristae in the ampullae of the semicircular canals; maculae in the utricle and saccule; and the organ of Corti in the coiled cochlea. The cristae and maculae contain vestibular hair cells that transduce mechanical stimuli to subserve the special sense of balance, while auditory hair cells in the organ of Corti are the primary transducers for hearing [1]. Cell fate specification in these sensory epithelia and morphogenesis of the semicircular canals and cochlea take place during the second week of gestation in the mouse and are largely completed before birth [2,3]. Developmental studies of the mouse inner ear are routinely conducted by harvesting transgenic embryos at different embryonic or postnatal stages to gain insight into the molecular basis of cellular and/or morphological phenotypes [4,5]. We hypothesize that gene transfer to the developing mouse inner ear *in utero* in the context of gain- and loss-of-function studies represents a complimentary approach to traditional mouse transgenesis for the interrogation of the genetic mechanisms underlying mammalian inner ear development[6].

The experimental paradigm to conduct gene misexpression studies in the developing mouse inner ear demonstrated here resolves into three general steps: 1) ventral laparotomy; 2) transuterine microinjection; and 3) *in vivo* electroporation. Ventral laparotomy is a mouse survival surgical technique that permits externalization of the uterus to gain experimental access to the implanted embryos[7]. Transuterine microinjection is the use of beveled, glass capillary micropipettes to introduce expression plasmid into the lumen of the otic vesicle or otocyst. *In vivo* electroporation is the application of square wave, direct current pulses to drive expression plasmid into progenitor cells[8-10].

We previously described this electroporation-based gene transfer technique and included detailed notes on each step of the protocol[11]. Mouse experimental embryological techniques can be difficult to learn from prose and still images alone. In the present work, we demonstrate the 3 steps in the gene transfer procedure. Most critically, we deploy digital video microscopy to show precisely how to: 1) identify embryo orientation *in utero*; 2) reorient embryos for targeting injections to the otocyst; 3) microinject DNA mixed with tracer dye solution into the otocyst at embryonic days 11.5 and 12.5; 4) electroporate the injected otocyst; and 5) label electroporated embryos for postnatal selection at birth. We provide representative examples of successfully transfected inner ears; a pictorial guide to the most common causes of otocyst mistargeting; discuss how to avoid common methodological errors; and present guidelines for writing an *in utero* gene transfer animal care protocol.

Video Link

The video component of this article can be found at https://www.jove.com/video/3653/

Protocol

1. Ventral Laparotomy

1. Anesthetize a dam whose embryos are at embryonic day 11.5 (E11.5; noon on the day a vaginal plug is detected is day 0.5 of embryonic development) by intraperitoneal injection of sodium pentobarbital anesthetic solution (7.5 µL per gram body weight). Working anesthetic solution: 180 µL of 50 mg/mL pentobarbital sodium solution; 100 µL of absolute ethanol; 320 µL of 65 mg/mL aqueous magnesium sulfate (modulates uterine tone); and 400 µL of propylene glycol (vehicle miscible with aqueous and organic components).
2. Assess completeness of anesthesia by conducting noxious stimuli tests: paw squeeze; tail pinch; and blink response to cheek and vibrissae touch. Apply sterile ophthalmic ointment to the corneas.
3. Shave the abdominal fur from the suprapubic area to the rib cage with shears and a fine blade (Oster #40 blade). Disinfect the abdomen with 70% ethanol, 10% povidone iodine (Betadine), and 70% ethanol, sequentially. Place mouse abdomen-side down on a sterile drape and then set on a heating pad or warm plate (37 °C) for 2-5 mins.
4. Incise the abdominal skin in the ventral midline with ball-tipped scissors. Extend the incision for 10-14 mm. Identify the *linea alba*, an avascular, white connective tissue band along the ventral midline of the abdominal wall. Incise the *linea alba* with ball tipped scissors and extend the incision 10-14 mm. Immediately irrigate the abdomen with prewarmed (37 °C) lactated Ringer's solution.

5. Externalize the two horns of the uterus with ring forceps without applying excessive pressure on the implantation sites. Irrigate the externalized uterine horns with prewarmed, lactated Ringer's solution.

2. Transuterine Microinjection

1. Fabricate a thick-walled, borosilicate glass capillary microinjection pipette with the following characteristics: 12-16 µm outer diameter and a 20 degree bevel. On a Sutter P-97 pipette puller with small box filament, use the following program: heat = ramp test plus 3 units; pressure = 200; pull = 0; velocity = 46; time = 100). With the Sutter BV-10 beveler, use the 104C (gold) abrasive disk for large diameter pipettes. Resuspend expression plasmid at 3-4 µg/µL in calcium free phosphate buffered saline (pH 7.2-7.4). Add crystalline fast green to the concentrated DNA, vortex gently for 30 seconds, and spin at 10,000 g for 10 seconds. The minimum amount of fast green required to visually track the efficacy of the injection is determined empirically. Backfill the beveled pipette with the DNA/fast green solution. Connect the loaded microinjection pipette to the pipette holder of the pressure injector (Picospritzer using >99% pure nitrogen as source gas).
2. Transilluminate the uterus with low intensity, halogen light to visualize the embryo within its implantation site. Identify the beating heart, brain vesicles, limb buds, nascent 4th ventricle of the hindbrain, and eye. Irrigate the uterus every 2 minutes with prewarmed, lactated Ringer's solution to maintain hydration. Apply gentle pressure on the uterus to reorient the embryo and identify the anatomical landmarks noted above.
3. Orient the embryo to identify the primary head vein whose anterior and posterior branches flank the mesenchymal territory in which the otocyst resides. The otocyst proper cannot be seen by transillumination of the uterus. The otocyst is located midway between the anterior and posterior branches of the primary head vein which, along with the main trunk of the vein, form the shape of the uprights or goalposts on an American football field. The otocyst is midway between the uprights.
4. Insert the injection pipette through the uterus in a trajectory in line with the presumptive location of the otocyst. Pulse the microinjector once after passing through the uterus to visualize the tracer dye and the approximate the location of the pipette tip. Advance the pipette under micrometer control and pulse again to assess depth. Further advance the pipette into the lateral head mesenchyme and pulse repeatedly. Successful otocyst targeting will reveal the tapered shape of the endolymphatic duct dorsally and the scallop shell-shape of the vestibule. Release pressure on the uterus, remove the pipette from the embryo/uterus in one motion, and immediately irrigate the uterus with prewarmed, lactated Ringer's solution.

3. *In vivo* Electroporation

1. Irrigate the uterus with lactated Ringer's solution. Freshly applied lactated Ringer's solution is necessary to electrically couple the paddle-style electrodes to the uterus. Moisten the tungsten surfaces of the electrodes with lactated Ringer's. Center the injected otocyst in the path of the electrodes. Gently compress the uterus with the electroporation paddles. The cathode is in contact with the uterine wall lateral to the injected otocyst and the anode is in contact with the uterine wall adjacent to the uninjected otocyst. Trigger a square wave pulse train with the foot pedal switch on the electroporator. Electroporation parameters are: 5, 50 msec pulses at 43 volts per pulse and a 950 msec interpulse delay. Immediately irrigate the uterus after the pulse train is delivered. Record the current delivered to the tissue: 60-100 mAmps is sufficient to transfect otic epithelial progenitors. Inject and electroporate 4-6, E11.5 embryos per dam.
2. Perform a second, independent transuterine microinjection of aqueous fluorescent dextran (Alexa Fluor 488 if the expression plasmid encodes a red fluorescent protein or Alex Fluor 594 if the expression plasmid encodes a green fluorescent protein) into the 4th ventricle of those embryos whose inner ears are accurately injected and at least 60 mAmps of current per pulse was delivered during electroporation. The fluorescent dextran will be detectable at birth in the hindbrain and enable selection of pups whose inner ears were manipulated during embryogenesis (see 3.5).
3. Irrigate the uterine horns with lactated Ringer's. Reinsert the uterine horns into the abdominal cavity. Flush the uterine cavity with 2-4 mL of prewarmed, lactated Ringer's solution and allow the overflow to drain out of the incision site onto the sterile drape. Replace the draping with dry, sterile material. Suture the abdominal wall with a non-cutting needle and a 6-0 resorbable suture. We prefer a running stitch, locking every other stitch, for both the abdominal wall and skin.
4. Dry the dams' fur and administer a non-steroidal anti-inflammatory such as Meloxicam by subcutaneous injection. Return the dam to the prewarmed recovery cage on a sterile drape. Monitor and record the dams' respirations, incision site patency, and vagina for bloody discharge. Bleeding is rare but if present and unabated, euthanize the dam while she is still under anesthesia. Note the time she regains consciousness and attempts to ambulate. Return the dam to the mouse colony when she shows signs of eating and drinking and has begun to nest build. Typically, this occurs within 12 hrs.
5. At birth (postnatal day 0), flash the hindbrain region of each pup in the litter to detect the fluorescent dextran using a stereofluorescence dissecting microscope with a GFP or Texas Red filter set as appropriate. Return to the lactating dam only those pups that display hindbrain labeling.

4. Representative Results

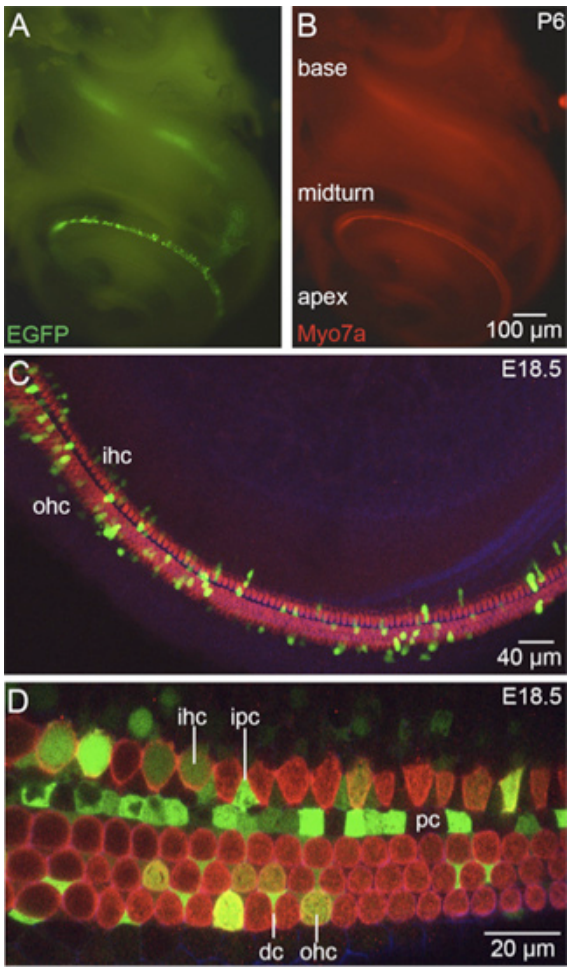

Figure 1. Electroporation-mediated gene transfer to the developing cochlea. The E11.5 otocyst was injected with an expression plasmid encoding enhanced green fluorescent protein (EGFP) and electroporated (pulse train parameters: five, 43 volt pulses at 50 msec/pulse and 950 msec interpulse delay). A) A representative inner ear from a postnatal day 6 (P6) pup whose otocyst was injected and electroporated at E11.5 demonstrating EGFP expression from the base through the middle turn of the cochlea. The lateral wall of the cochlea was removed from the middle turn and apex only. E11.5 progenitors that give rise to the apex were not transfected. B) Whole mount immunostaining of the cochlea in (A) with the hair cell marker, myosin 7a (Myo7a), indicates that EGFP expression follows the trajectory of the organ of Corti and is grossly localized to the hair cell-bearing sensory epithelium. C) A representative inner ear from an E18.5 embryo whose otocyst was injected and electroporated at E11.5. The laser confocal projection demonstrates EGFP expression in Myo7a-positive sensory hair cells. D) Laser confocal projection of the cochlear sensory epithelium from the E18.5 organ of Corti indicating EGFP expression in the inner hair cells (ihc), outer hair cells (ohc), inner phalangial cells (ipc), pillar cells (pc), and Deiters' cells (dc). The scale bar in (B) applies to (A).

Discussion

Gene transfer to the developing mouse inner ear: The mouse inner ear develops from the otic placode during the first week of postimplantation development[12,13]. By embryonic day 9.5 (E9.5), the placode has invaginated and morphed into a fluid-filled vesicle called the otocyst[2]. Otic precursors in the vesicle give rise to the sensory and nonsensory cells within the mature inner ear as well as the neurons that innervate mechanically sensitive hair cells in the vestibular and auditory sensory epithelia. By late embryonic stages, the complex, 3-dimensional morphology of the membranous labyrinth of the inner ear is established[3,12]. Gain- and loss-of-function experimental modalities are typically performed by mouse transgenesis to gain insight into the genetic regulation of developmental processes such as cell fate specification and pattern formation. Dynamic genetic manipulation of the developing mouse inner ear *in utero* represents a complimentary approach to mouse transgenesis that couples gene transfer techniques with mouse experimental embryology[6,11].

Virus- and electroporation-mediated gene transfer techniques have been developed to manipulate gene expression in the developing inner ear[6,11,14,15]. Adeno-associated viral (AAV) vectors infect otic progenitors that give rise to sensory and nonsensory cell types and have been used to interrogate the molecular basis of calcium-dependent exocytosis in mouse hair cells[16]. AAV vectors produce high titers that enable broad expression in the inner ear and different viral serotypes display unique tropism that may be advantageous for differential cellular expression. Disadvantages include a fixed stuffing capacity that limits the size of the gene that can be misexpressed and the requirement for virological expertise to make, purify, and titer the viruses. Electroporation-mediated gene transfer has been used to evaluate the role of a basic helix-loop-

helix transcription factor in specifying sensory hair cell fate *in vivo*[6]. Expression plasmids adapted for Gateway subcloning technology allow rapid and efficient construction of vectors with unique promoters driving expression of a gene-of-interest and a fluorescent marker protein from an IRES sequence[17]. Genes of interest can be expressed in otic precursors within 24 hrs of electroporation and expression can persist in the postnatal inner ear depending on the promoter used. Plasmid construction, purification, concentration, and quantitation require only standard molecular biological skills. Disadvantages include decreased *in vivo* transfection efficiency as plasmid size increases above ~8kb and a higher rate of embryonic lethality compared to most viral vectors. Both electroporation- and virus-mediated gene transfer techniques require mastery of mouse experimental embryological techniques: survival surgery, *in utero* manipulation of embryos, and transuterine microinjection. The goal of this manuscript is to address the latter issue by demonstrating each critical methodological step by digital video microscopy.

Methodological notes on the video: We deliberately chose not to drape the incision site with sterile field while filming so the viewer could see the position of the ventral midline in relation to the gross anatomy of the dam, i.e., the head (deliberately blurred in most frames but recognizable), limbs, and tail. Draping the incision site effectively blocks this view and can confound efforts to appreciate spatially relevant anatomical relationships during ventral laparotomy and an injection sequence. However, we recommend draping the abdomen of the dam so that the externalized uterine horns rest on a sterile field to maintain surgical asepsis. In addition, we deliberately did not attempt to demonstrate suturing of the surgical incisions in the abdominal wall and skin because these methods must be learned by expert guidance of a veterinarian or qualified surgical technician. Dr. Marcel I. Perret-Gentil (University of Texas at San Antonio), presents an excellent discussion of this topic, complete with pictorial representations, in his handout, "Principles of Veterinary Suturing."[18]

Procedural recommendations: Gene transfer to the developing mouse inner ear is technically challenging. There are a series of best practices that will eliminate a substantial amount of variability and ensure successful outcomes. We make specific recommendations below to facilitate learning this experimental approach.

Consult veterinary staff to develop the mouse survival surgery animal care protocol: The animal care protocol for mammalian survival surgery is demanding to produce since anesthesia, analgesia, and perioperative care must be defined and coordinated. A detailed discussion of animal care protocol development, including language that may form the core of an applicant's protocol, is provided as supplementary information (see "*Animal Care Protocol Development*"). In addition, your home institutions' Animal Care and Use Committee (IACUC) and veterinary staff will undoubtedly provide additional guidance on the formulation of a suitable protocol upon request.

Establish a dedicated mouse survival surgery area for in utero gene transfer: Ventral laparotomy is a classified as a major surgery because it compromises a body cavity. Most IACUCs will not require a sterile, class 100 environment for major mouse survival surgery. Rather, a dedicated area that includes proper lighting, ventilation, supplementary heating, and hard, non-porous, disinfectable surfaces will likely be required. We prefer to conduct our survival surgery in a horizontal laminar flow hood that protects the mouse from infection, reduces physiological stress, and thereby facilities postoperative recovery. We have defined a series of custom modifications to an oscillation-damped, horizontal laminar flow hood (Envirco LF 630) that include directional, low wattage halogen track lighting; expanded electrical circuitry to power gene transfer equipment; and recessed power outlets with bench top cutouts for cord passage. A detailed schematic of these modifications is provided as supplementary information (see "*Laminar Flow Hood In Utero Gene Transfer*"). Consult your IACUC as you develop your animal care protocol to define a suitable location that is dedicated exclusively to mouse survival surgery.

Weigh the dam to the nearest 0.1 gram before administering sodium pentobarbital anesthesia: Sodium pentobarbital is an effective anesthetic and will provide at least 105 minutes of working time when the recommended dosing schedule is followed. Determine an accurate body weight for each dam before calculating the dose of the anesthetic mixture to inject. Never estimate dam body weight. Use the Allergy Syringe Tray needle and syringe from Becton Dikison: the human *intradermal* bevel is ideal for atraumatic *intraperitoneal* injections into pregnant mice. Moreover, this syringe has an extremely low dead space volume and is calibrated for the range of anesthetic volumes typically required for dams whose embryos are at E11.5 or E12.5.

Deploy prophylactic analgesia to support embryonic survival: Administer a topical anesthetic on the suture line, such as Bupivicaine, and a systemic, non-steroidal anti-inflammatory drug, such as Meloxicam, prior to placing the dam in the recovery cage so that analgesics are on board by the time the dam recovers from anesthesia. Reducing discomfort in the dam facilitates postoperative recovery and maintenance of a healthy pregnancy.

Bevel the microinjection pipettes and tightly constrain the outer diameter: The single most common cause of embryonic lethality is tissue and/or vascular damage from poorly constructed microinjection pipettes. Transuterine microinjection into the E11.5 mouse otocyst carries different constraints than injection into larger organs in older embryos (i.e., brain vesicles, eye, limb, etc). Unbeveled, poorly beveled, or inappropriately large outer diameter pipettes will cause bleeding from vessels in the uterus, the visceral yolk sac vasculature, and/or the embryo at E11.5, which will decrease embryonic survival. It is possible to hand-break durable pipettes of small diameter that carry an appropriate bevel, but it is difficult to do so routinely. The E11.5 mouse embryo, whose tiny otocyst is the target, will be unforgiving. Consult the P-1000 P-97 Pipette Cookbook 2011 from Sutter Instruments for a full discourse on pipette design and fabrication[19]. Parameters for the pipettes used in this work are noted above and their fabrication has been fully discussed previously[11]. The procedural notes in the owner's manual for the Sutter Instruments BV-10 beveler are also an excellent resource.

Understand why mistargeting the otocyst by transuterine microinjection occurs: The video presents the ideal outcome for transuterine microinjection into the E11.5 and E12.5 otocyst. Mistargeting the otocyst usually results from injecting too superficially, too deeply, or from inadequately fabricated pipettes. Figure 2 demonstrates these conditions. Panel A shows a lateral view of the left side of an E12.5 embryo imaged by transillumination after successful transuterine microinjection of fast green solution into the otocyst. The hindbrain (hb) appears as a wedge-shaped notch dorsal to the otocyst. The eye (e) has an annulus of pigment along its periphery and the left forelimb is prominent. The main trunk of the primary head vein (phv) is just beneath the otocyst. The otocyst is filled with fast green which appears black: the dorsal, endolymphatic duct (ed) and the vestibule of the otocyst (oc) can be discerned. Successful targeting of the E11.5-12.5 otocyst will always present a clearly discernible endolymphatic duct and vestibule. Panel A' shows the same image as in A without the white labeling to facilitate critical review of the injected otocyst.

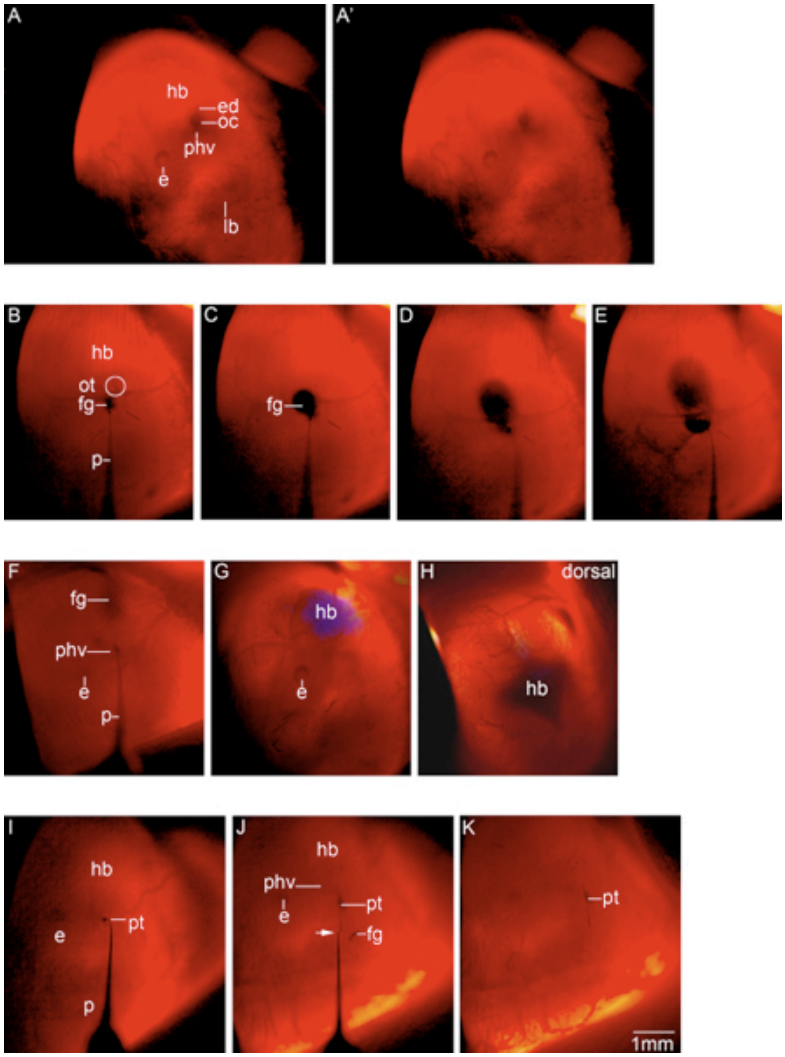

Figure 2. A pictorial guide to mistargeting the otocyst by transuterine microinjection. All panels show the left lateral view of an E12.5 embryo with midbrain up and the forebrain left, except Panel H, which is a dorsal view of the hindbrain-midbrain region. Panels in each row represent images of the same embryo. See the discussion for a detailed explanation of the causes of otocyst mistargeting. Abbreviations: e, left eye; ed, endolymphatic duct; fg, fast green, hb, hindbrain; lb, limb bud; oc, otocyst; ot, otic territory; p, pipette; phv, primary head vein; pt, pipette tip.

Superficial injection is a common cause of mistargeting. Panels B-E demonstrate a superficial injection into the E12.5 conceptus. The hindbrain (hb) notch and the otic territory (ot, circle) are evident in this left, lateral view. The microinjection pipette (p) is in the foreground. Fast green (fg) begins to eject from the pipette tip (B), and with subsequent pulses from the pressure injector (C-D), the dye diffuses into an oval-shape. The distribution of the dye suggests that it was introduced between the uterine wall and the visceral yolk sac, an extraembryonic membrane that envelops the exocoelomic cavity. The experimenter should not attempt to re-target the otocyst in this embryo but should move on to the next embryo. Multiple injections correlate with decreased embryonic survival, an effect that is more acute at E11.5 than E12.5.

Deep injection is a more common cause of mistargeting. Panels F-H demonstrate an injection trajectory that passes through the otic territory into the hindbrain. The primary head vein (phv), left eye (e), and pipette (p) are evident in this lateral view of an E12.5 embryo. The initial ejection of fast green (fg) is displaced dorsally with respect to the primary head vein/otic territory. With subsequent pulses, the wedge-shaped hindbrain (hb) was completely filled with fast green (Panel G). A 90 degree rotation of the injected embryo allows detection of fast green within the hindbrain cavity from the dorsal view (Panel H). Both superficial and deep injections result from the inability to perceive the depth of the microinjection pipette after it makes contact with the uterus. A practical approach that works well is to advance the pipette through the uterus and then immediately pulse once to look for a puff of fast green: the location of the puff indicates the approximate position of the pipette tip. Adjust depth based on this range-finding pulse.

Inadequately fabricated pipettes are the most common cause of mistargeting. The injection attempt shown in Panels I-K was conducted with a pipette tip that was manually broken but not beveled. The lateral view of the E12.5 embryo shows the hindbrain (hb), left eye (e), and the microinjection pipette (p). Note that the pipette tip (pt) is sharply bowed and has failed to penetrate the uterus (panel I). Application of additional force shears the tip of the pipette (Panel J, arrow), leaving the pipette tip embedded in the uterus. A small amount of fast green (fg) was ejected from the fractured pipette and has spotted the surface of the uterus. The embedded pipette tip (Panel K) must be removed with fine, sterile forceps and discarded as sharps waste. If the pipette tip cannot be located or successfully removed, euthanize the dam under anesthesia.

Document procedural observations on a mouse survival surgery data sheet: A crucial element to learning these methods is to document observations and make adjustments based on those observations. Produce a mouse survival surgery data sheet and note preoperative, operative, and postoperative information. Preoperative data include dam weight, dose of anesthetic delivered, plasmid injected, etc. Operative data include the embryo injected (i.e., R2 is the second embryo from the ovary in the right uterine horn), the quality of the injection (i.e., no endolymphatic duct detected, dye in 4th ventricle), the current delivered during electroporation (i.e., 60mAmps, heartbeat present post-electroporation), and overall impressions (i.e., pipettes were too flexible and pipette trajectory was difficult to maintain). Postoperative data include the time ambulation is first attempted, patency of the incision site, the presence of vaginal bleeding, the appearance of grooming behaviors and nest building, etc. A template for producing a survival surgery data sheet is provided in the supplementary information (see "*Mouse Survival Surgery Data Sheet*"). Quality notes form the basis for making productive methodological refinements that will lead to successful outcomes.

Use current delivered per pulse to optimize transfection efficiency: The square wave pulse train consists of 5, 43volt, 50msec pulses with a 950msec interpulse delay. The 5mm platinum, paddle style electrodes ensure that the entire otic territory is contained within the electric field. Similar transfection efficiencies can be achieved with 3mm paddles, though they are more difficult to accurately position. The pulse paradigm was optimized by evaluating the current transferred to the embryo during the final pulse, not by sweeping through voltages and assessing transfection efficiency. The rationale is that the current delivered per pulse varies at constant voltage depending on the patency of electrical coupling between the uterus and the platinum electrodes. Assuming that the uterus is freshly irrigated with lactated Ringer's solution immediately prior to placing the paddles and charge carrier is present in excess, the chief variable is the amount of "coupling" pressure applied to the uterus with the paddles. Gentle pressure at 43volts results in <60mAmps of current delivered and sporadic transfection at best. Moderate pressure at 43volts results in 60-100mAmps delivered and the most efficient transfection. Heavy pressure at 43volts results in >100mAmps delivered and correlates with persistently altered heart rate and increased embryonic lethality. Excessive pressure ruptures the visceral yolk sac (a "pop" may be felt through the paddles) and causes embryonic lethality. It is likely that the variable pressure applied alters the impedance between the electrodes which affects the current delivered. The pulse frequency of 1Hz (one 50 msec pulse per second) was defined as the least disruptive to the embryo's cardiac cycle, which is a positive correlate for embryonic survival. We conclude that the most crucial parameter to monitor in establishing an efficient *in vivo* electroporation paradigm is the current delivered per pulse. Modification of any pulse paradigm should include careful monitoring of the current delivered per pulse.

Adopt a modular approach to learning the technique: Module 1: Practice a sham surgery with uterine externalization, but do not inject or electroporate. Dams tolerate ventral laparotomy extremely well and should never experience postsurgical complications related to surgical technique. Mouse survival surgical skills are undoubtedly resident in the home institution so pursue adequate training. Achieve mastery of surgical skills before proceeding. Module 2: Practice transuterine microinjection into the otocyst on an anesthetized dam without the expectation of completing the survival surgery: euthanize the dam under anesthesia, isolate the injected embryos, and evaluate the quality of the otocyst injections. Module 3: Target only 2 otocysts in 2 different embryos with fast green, do not electroporate, complete the surgery, and validate downstream embryonic survival. Module 4: Target only 2 embryos with DNA/fast green solution, electroporate, validate downstream embryonic survival, and ascertain the transfection efficiency. Module 5: Target only 2 embryos with DNA/fast green solution, electroporate, inject Alexa Fluor into the 4th ventricle to label the manipulated embryo, select labeled embryos at P0, and ascertain the transfection efficiency.

Procedural learning curve: Mastery of the mouse survival surgery techniques, with appropriate guidance from the veterinary and surgical staff of one's home institution, is fairly rapid and should only require 3-5 dams-worth of practice for the novice to gain expertise. Transuterine microinjection and *in vivo* electroporation to generate usefully transfected inner ears will require 10-50 dams-worth of effort, assuming there are 6 workable embryos per pregnancy and the modular approach is followed. Those students who enjoy activities that require fine motor skills (i.e., playing a musical instrument, sewing, model-making, or competitive athletics) have shorter training periods than those that do not. The critical correlates for successful mastery of these methods are scrupulous attention to detail (i.e., microinjection pipette fabrication, vascular anatomy, explicit note-taking) and a reasonably calm demeanor.

Workflow: Once expertise is gained, one can anticipate injecting and electroporating 4-6 embryos per dam; 3-5 embryos will survive; and 2-4 embryos will have usefully transfected inner ears for analysis. The hindbrain Alexa Fluor labeling technique to label embryos for sorting at birth requires the additional hindbrain injection in each electroporated embryo, but this is well tolerated and does not adversely affect procedural efficiency. Thus, the recovery rate of usefully transfected inner ears from *in vivo* electroporation compares favorably to the recovery rate of homozygous mutant mice from a heterogygous breeding paradigm. With experience, 2-3 dams per experimenter per day is a reasonable workflow: 1.5 hrs per dam for surgery and a total of 1.5-2 hrs for post-operative monitoring.

Disclosures

No conflicts of interest declared.

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References

1. Gillespie, P.G. & Muller, U. Mechanotransduction by hair cells: models, molecules, and mechanisms. *Cell*. **139**, 33-44, doi:10.1016/j.cell.2009.09.010 (2009).
2. Bok, J., Chang, W., & Wu, D.K. Patterning and morphogenesis of the vertebrate inner ear. *Int. J. Dev. Biol.* **51**, 521-533, doi:10.1387/ijdb.072381jb (2007).
3. Kelley, M.W. Regulation of cell fate in the sensory epithelia of the inner ear. *Nat. Rev. Neurosci.* **7**, 837-849, doi:10.1038/nrn1987 (2006).
4. Ohyama, T., *et al.* BMP signaling is necessary for patterning the sensory and nonsensory regions of the developing mammalian cochlea. *J. Neurosci.* **30**, 15044-15051, doi:10.1523/JNEUROSCI.3547-10.2010 (2010).
5. Pan, W., Jin, Y., Stanger, B., & Kiernan, A.E. Notch signaling is required for the generation of hair cells and supporting cells in the mammalian inner ear. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 15798-15803, doi:10.1073/pnas.1003089107 (2010).
6. Gubbels, S.P., Woessner, D.W., Mitchell, J.C., Ricci, A.J., & Brigande, J.V. Functional auditory hair cells produced in the mammalian cochlea by *in utero* gene transfer. *Nature*. **455**, 537-541, doi:10.1038/nature07265 (2008).
7. Guide for the Care and Use of Laboratory Animals. 8th edn., The National Academy Press, (2010).
8. Matsuda, T. & Cepko, C.L. Controlled expression of transgenes introduced by *in vivo* electroporation. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 1027-1032, doi:10.1073/pnas.0610155104 (2007).
9. Chen, C., Smye, S.W., Robinson, M.P., & Evans, J.A. Membrane electroporation theories: a review. *Med. Biol. Eng. Comput*. **44**, 5-14, doi:10.1007/s11517-005-0020-2 (2006).
10. Saito, T. *In vivo* electroporation in the embryonic mouse central nervous system. *Nat. Protoc.* **1**, 1552-1558, doi:10.1038/nprot.2006.276 (2006).
11. Brigande, J.V., Gubbels, S.P., Woessner, D.W., Jungwirth, J.J., & Bresee, C.S. Electroporation-mediated gene transfer to the developing mouse inner ear. *Methods Mol. Biol.* **493**, 125-139, doi:10.1007/978-1-59745-523-7_8 (2009).
12. Morsli, H., Choo, D., Ryan, A., Johnson, R., & Wu, D.K. Development of the mouse inner ear and origin of its sensory organs. *J. Neurosci*. **18**, 3327-3335 (1998).
13. Sher, A.E. The embryonic and postnatal development of the inner ear of the mouse. *Acta. Otolaryngol.* **Suppl. 285**, 1-77 (1971).
14. Sheffield, A.M., *et al*. Viral vector tropism for supporting cells in the developing murine cochlea. *Hear Res*. **277**, 28-36, doi:10.1016/j.heares.2011.03.016 (2011).
15. Bedrosian, J.C., *et al*. *In vivo* delivery of recombinant viruses to the fetal murine cochlea: transduction characteristics and long-term effects on auditory function. *Mol. Ther.* **14**, 328-335, doi:10.1016/j.ymthe.2006.04.003 (2006).
16. Reisinger, E., *et al*. Probing the functional equivalence of otoferlin and synaptotagmin 1 in exocytosis. *J. Neurosci.* **31**, 4886-4895, doi:10.1523/JNEUROSCI.5122-10.2011 (2011).
17. Magnani, E., Bartling, L., & Hake, S. From Gateway to MultiSite Gateway in one recombination event. *BMC Mol. Biol*. **7**, 46, doi:10.1186/1471-2199-7-46 (2006).
18. Perret-Gentil, M. Principles of Veterinary Suturing.
19. Oesterle, A. P-1000 & P-97 Pipette Cookbook, (2011).