Video Article

In utero and *ex vivo* Electroporation for Gene Expression in Mouse Retinal Ganglion Cells

Timothy J Petros[1], Alexandra Rebsam[1], Carol A Mason[1, 2]
[1]Departments of Pathology and Cell Biology, and Neuroscience, Columbia University College of Physicians and Surgeons
[2]Department of Ophthalmology, Columbia University College of Physicians and Surgeons

Correspondence to: Carol A Mason at cam4@columbia.edu

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Abstract

The retina and its sole output neuron, the retinal ganglion cell (RGC), comprise an excellent model in which to examine biological questions such as cell differentiation, axon guidance, retinotopic organization and synapse formation[1]. One drawback is the inability to efficiently and reliably manipulate gene expression in RGCs *in vivo*, especially in the otherwise accessible murine visual pathways. Transgenic mice can be used to manipulate gene expression, but this approach is often expensive, time consuming, and can produce unwanted side effects. In chick, in ovo electroporation is used to manipulate gene expression in RGCs for examining retina and RGC development. Although similar electroporation techniques have been developed in neonatal mouse pups[2], adult rats[3], and embryonic murine retinae *in vitro*[4], none of these strategies allow full characterization of RGC development and axon projections *in vivo*. To this end, we have developed two applications of electroporation, one *in utero* and the other *ex vivo*, to specifically target embryonic murine RGCs[5, 6].

With *in utero* retinal electroporation, we can misexpress or downregulate specific genes in RGCs and follow their axon projections through the visual pathways *in vivo*, allowing examination of guidance decisions at intermediate targets, such as the optic chiasm, or at target regions, such as the lateral geniculate nucleus. Perturbing gene expression in a subset of RGCs in an otherwise wild-type background facilitates an understanding of gene function throughout the retinal pathway. Additionally, we have developed a companion technique for analyzing RGC axon growth *in vitro*. We electroporate embryonic heads ex vivo, collect and incubate the whole retina, then prepare explants from these retinae several days later. Retinal explants can be used in a variety of in vitro assays in order to examine the response of electroporated RGC axons to guidance cues or other factors. In sum, this set of techniques enhances our ability to misexpress or downregulate genes in RGCs and should greatly aid studies examining RGC development and axon projections.

Protocol

Part 1: Setting up for *in utero* and *ex vivo* retinal electroporations

1. For both *in utero* and *ex vivo* retinal electroporations

- Use an electrode puller to prepare fine-tipped micropipettes and break the tip (or bevel the tip) to make an angled point.
- Prepare DNA solutions to desired concentrations and add a small amount of Fast Green Dye (0.05%) to visualize injections. I recommend 5 µl DNA solution per mother for *in utero* retinal electroporations and 1 µl DNA solution per embryonic head for *ex vivo* retinal electroporations. Include a GFP expression construct (or other fluorescent protein) to visualize transfected neurons.
- Sterilize surgical instruments via autoclave.
- Prepare the ketamine-xylazine anesthetic mix with the following ratio (making ~0.2 ml for each pregnant female): a 1.0:0.2:4.6 volumetric ratio of 100 µg/ml ketamine:100 µg/ml xylazine:saline. Other anesthetics may be used.

2. For *in utero* retinal electroporations only

- Turn on the heating pad and cover it with a diaper pad.
- Prepare the antibiotic-antimycotic solution (1:100) in phosphate buffer saline (PBS) and warm on heating pad. Prepare ~2 ml for each dam.
- Place sterile-filtered PBS in warm water bath.

3. For *ex vivo* retinal electroporations only

- Prepare oxygenated serum free medium (SFM) for retina incubation: Bubble 25 ml of DMEM/F12 with 95% oxygen/5% carbon dioxide for ≥ 2 hours. To the 25 ml oxygenated medium, add 0.25 g bovine serum albumin (BSA), 250 µl ITS supplement and 50 µl Penicillin-streptomycin solution. Mix the solution to dissolve the BSA, then sterile filter the solution. This solution should be made fresh for each electroporation experiment.
- Prepare SFM + 0.4% methylcellulose medium for retinal explant incubation: Autoclave 0.20 g of methylcellulose in a 150 ml bottle with a stir magnet. Prepare 50 ml of SFM (as above, without bubbling) and add to autoclaved methylcellulose bottle. Mix this solution at 4°C overnight. Sterile filter this solution the following day and store at 4°C, this solution can be kept for ~1 month.
- On the day of retinal explant harvesting (Part 5), prepare dishes for retinal explant plating: Coat glass bottom culture dishes with 250 µl of poly-L-ornithine for ≥ 2 hours at 37°C. (Alternatively, dishes can be coated with poly-L-ornithine the previous day and incubated overnight at 4°C.) Wash dishes several times with PBS, then coat dishes with 200 µl of 10 µg/ml laminin in DMEM/F12 for ≥ 2 hours at 37°C. Wash dishes several times with PBS at 37°C and leave on PBS until explants are ready for plating.
- For border assays, coat dishes with poly-ornithine as above, then coat half of the dish with protein of interest along with a fluorescent marker to visualize the border (such as Alexa Fluor 555 conjugated to BSA, 1:800) for 2 hours at 37°C. Wash with PBS, then add laminin as described above.

Part 2A: *In utero* retinal electroporation Injecting and electroporating DNA into E14.5 murine retinae *in vivo*

1. Inject 0.15 ml of the anesthetic mix intraperitoneal (i.p.) into an E13.5-E14.5 stage dam. It should take 3-7 minutes until mother has reached a surgical plane of anesthesia. A fully anesthetized mouse should not respond to pinching of the hindpaw.
2. During this time, cut a small piece of parafilm and pipet ~5 ul of DNA solution onto the parafilm. Bend a plunger (metal wire) at a 90° degree angle and insert it into a pulled micropippette. Using the plunger, aspirate the DNA solution into the micropipette (using a dissecting microscope can aid this process).
3. Once the mother has reached a surgical plane of anesthesia, use an electric razor to shave a ~2 inch region of the mother s abdomen, then place her dorsal side down on the heating pad. Prep the shaved area by wiping with an alcohol pad followed by an iodine pad a minimum of two times. Place a drop of ophthalmic Vet ointment on each eye to prevent corneal ulceration of the eyes while the mother is under general anesthesia.

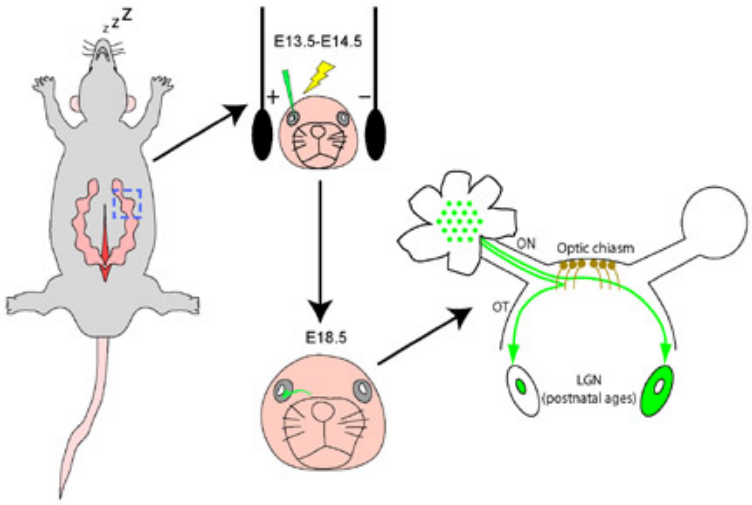

Figure 1. The embryonic chain is removed from an anesthetized mother. DNA solutions with fast green dye are injected into one retina of an embryo. A s eries of current pulses are delivered to the embryo, causing DNA to be taken up into progenitor cells that give rise to RGCs. The embryonic chain is replaced into the mother and allowed to survive for ≥ 4 days. At E18.5, embryos are harvested and retinae are scanned for GFP^+ signal. GFP^+ RGCs are visible in the retina, and GFP^+ axons can be followed through the optic nerve (ON), optic chiasm, optic tract (OT). Alternatively, the mother can give birth and pups can be collected at later ages (i.e. P5-P14) to examine GFP^+ RGC projections into the lateral geniculate nucleus (LGN). Midline radial glia cells are depicted in brown.

4. With the forceps, grasp the skin and use the scissors to make a vertical incision along the midline (~1 inch long) through the skin and muscle of the abdomen, exposing the peritoneum. Lift the peritoneum with the forceps and make a similar vertical incision through this membrane to expose the abdominal cavity (**Figure 1**). Then drape the surgical field with gauze pads to prevent the abdominal contents from contacting and being contaminated by the hair of the animal.
5. Pull back the skin and peritoneum with the forceps and use the dissecting spatula to extract an embryo through the incision (choose the most accessible embryo). Place the spatula between 2 embryos and gently pull the embryonic chain out of the abdominal cavity. You can pull out the embryonic chain with your fingers.
   From this point forward, keep the embryos hydrated with sterile PBS
6. Keep the mother on the heating pad, place her under the dissecting microscope and massage an embryo so that the retina is facing up. I recommend starting with either the most medial or lateral embryo, making it easier to keep track of which embryos were electroporated.
7. Take the micropipette in your dominant hand and stabilize the embryo with your other hand. With the micropipette, pierce the retina through the uterine wall and amniotic sac. Depending on the sharpness of the micropipette, it may take a fair amount of pressure to penetrate the uterine wall, but once through, the micropipette will enter the embryo and ideally the retina. It can be difficult to control and gauge the depth of the micropipette as it enters the embryo. Minimize the amount of micropipette movement once it has pierced the membrane to prevent enlarging the entrance hole, resulting in the leakage of amniotic fluid and embryo death. Avoid piercing blood vessels in the uterine wall, as this will result in bleeding and embryo death.
8. Once you are confident that the micropipette has entered the retina, you are ready to expel the DNA solution into the retina. Slowly depress the plunger and simultaneously withdraw the micropipette, ensuring that DNA is injected throughout the path of the micropipette, as the micropipette often penetrates deep to the retina. If you are in the retina, expel 0.5 µl (can be measured by the 1 µl tick marks on the micropipette) into the extraretinal space between the outer retinal layer and the retinal pigment epithelium (RPE). You should observe green dye filling the retina and leaks out into the amniotic fluid (**Figure 1**).
9. The electroporation paddles should be kept in a PBS-filled Petri dish during the surgery. Gently place the paddles on the sides of the embryo head with the positive (+) paddle on the *same* side as the injected eye. Apply a moderate amount of pressure to stabilize the head, but do not press too hard, as this will pop the amniotic sac and result in embryo death. When the electrodes are in place, deliver 5 40-V, 50 ms square pulses at a frequency of 60 Hz. It is not unusual for the mother to twitch due to current escaping to the skin and muscles.
10. Repeat steps 7-9 for each embryo to be injected and electroporated. I usually electroporate 4-8 embryos per mother, depending on amount of DNA, number of embryos, condition of mother, and the amount of time under anesthetic. Alternatively, multiple embryos can be injected and then electroporated, as opposed to injecting and electroporating each embryo separately.
11. After electroporating the embryos, use the forceps and spatula to replace the embryonic chain back into the abdominal cavity. Pour ~2 ml of the antibiotic-antimycotic solution into the abdominal cavity.
12. Use the hemostat and forceps to suture the peritoneum, starting with a double knot on the anterior portion of the incision and proceed with a simple continuous suture pattern to the posterior portion of the incision. Use the final loop to tie off the suture and trim excess suture.
13. To staple the incision closed, lift the skin at the anterior portion of the incision with blunt forceps, being careful to separate the skin from the peritoneum. Place the teeth of the stapler around the skin and depress the stapler. Continue stapling the incision closed to the posterior end, which usually requires 5-7 staples. Wipe the wound around the staples with an alcohol pad.
14. Let the mother recover on the heating pad (usually takes between 15-90 minutes for her to awaken) and when she begins to roll over, place her in a new cage abdominal side up.

15. To minimize pain and discomfort, mothers receive buprenorphine (0.05-0.1 mg/kg, s.c.) upon awakening, and at later time points if discomfort continues. To prevent dehydration, inject the mother with 1.0 ml saline subcutaneously (s.c.) ~2-4 hours post-surgery, and again if the evening and following day if needed. 24 hours post-surgery, the mother should regain normal behavior and be drinking and eating regularly.

Part 2B: *In utero* retinal electroporation Retrieval of electroporated retina at E18.5

1. Prepare 4% paraformaldehyde (PFA) in PBS, making ~5 ml per embryo. Perfusions can be done with a gravity-based infusion system or a syringe pump. (Alternatively, E18.5 heads can simply be immersed in 4% PFA overnight.)
2. Inject the mother with 0.2 ml of anesthetic mix i.p. and ensure that she is fully under anesthetic. Use a scissors to cut the skin and peritoneum underneath the staples to expose the abdominal cavity and uterine horns.
3. Remove an electroporated embryo and pin it ventral side up. Cut through the abdominal skin, peritoneum, diaphragm and lateral portions of the ribcage to expose the heart. Pierce the left ventricle with the butterfly needle attached to the perfusion setup, and cut the right atrium with a microscissors. Start the perfusion and allow PFA to flow for ~60 seconds; blood should exit the right atrium and the embryo should become pale if the perfusion is successful. Decapitate the embryo and collect head in 4% PFA in 15-ml conical vial. Repeat this procedure for all electroporated embryos. Keep heads in 4% PFA overnight at 4°C, and then wash in PBS for several days. Euthanize mother via cervical dislocation when all embryos are collected.
   Removing retina
4. After PBS washes, pin the head in a dissection dish with the retina facing up and immerse in PBS. Remove skin from around the retina to reveal the RPE. Using a 26G1/2 needle, puncture the ventral retina at the lens-RPE junction. Insert one edge of a microscissors into this hole and make a vertical incision through the ventral retina to the optic disc. This will allow you to orient the retina when you remove it.
5. Remove the RPE with fine-point forceps. Grab the RPE at the ventral incision with one forceps and use other forceps to peel the RPE away from the retina, specifically at the RPE-lens junction where the RPE is firmly attached. After removing the RPE, grasp the lens with fine forceps and pull up and away to remove the lens. Remove retina by placing forceps around optic nerve head and pinch off retina. Transfer retina to a PBS-filled well, keeping track of which retinae came from which heads.
6. Repeat steps 4-5 for the contralateral retina, and for all other electroporated embryos. The non-injected retina serves as a control for immunostaining.
7. Use a fluorescent dissecting microscope to scan all retinae for GFP+ cells (green retinae). For all GFP+ retinae, the electroporation was successful in this embryo, and these retinae and corresponding brain (with the visual system intact) can be processed for further analysis (i.e. sectioning and immunostaining) (**Figure 1**).

Part 3a: *Ex vivo* retinal electroporation Injecting and electroporating DNA into E14.5 murine retinae *in vitro*

1. Anesthetize an E13.5-E14.5 stage mother with 0.2 ml of the anesthetic mix and ensure that she is fully under anesthetic. Use scissors to cut through skin and abdominal cavity to expose embryos. Lift up embryonic chain with forceps and cut through connective tissue to fully remove entire embryonic chain from the mother. Place embryonic chain in a Petri dish with DMEM/F12 on ice. Euthanize mother via cervical dislocation.
2. Cut through the entire uterine wall with a microscissors. Then use forceps to remove each embryo from the amniotic sac and pinch off placenta. Decapitate each embryo and collect heads in another Petri dish with DMEM/F12 on ice.
3. Using mouth pipet or plunger pipet, fill micropipette with desired amount of DNA solution (see Part 1).
4. In this protocol, DNA will be injected into the peripheral dorsal retina. One can target other retinal regions by adjusting the DNA injection site and the positioning of the electrode paddles.
5. Transfer one head to dissecting dish with PBS and pin the head dorsal side up. Use forceps to remove skin above retina.
6. Holding the micropipette ~10° from horizontal position (essentially parallel to the curvature of the dorsal retina), push micropipette through the RPE so that the tip is between the RPE and outer retinal layer. Expel ~0.1-0.4 µl DNA solution into this space; green liquid should be observable filling entire space and leaking out through hole in RPE. Repeat injection for the contralateral retina.
7. Carefully but quickly remove the pins and (keeping head immersed in PBS) place electrode paddles around the head, with the positive (+) paddle on the *ventral* side of the head. Deliver 4 40-V, 50 ms pulses at 60 Hz, then place the head back into a dish with DMEM/F12 on ice (**Figure 2**).

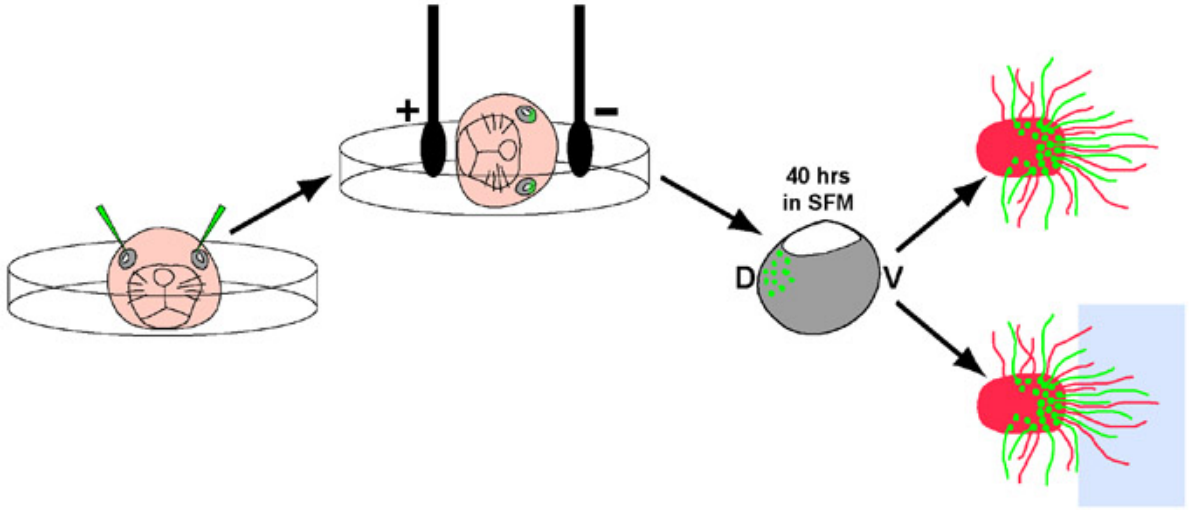

Figure 2. E13.5-E14.5 heads are harvested and placed in a dish with PBS. DNA solutions are injected into the peripheral dorsal region of both retinae. Electrode paddles are then placed around the head (positive paddle on the ventral side) and heads are electroporated. Retina are removed and cultured in oxygenated serum free medium (SFM) for ~40-48 hours. Retinal explants are prepared from GFP$^+$ retinal regions and plated on laminin coated dishes, or adjacent to border substrates (blue region).

8. Repeat steps 5-7 for all heads and for all DNA solutions. After completing electroporations, add ~1.5 ml of oxygenated SFM solution to a 4-well dish and keep on ice (one well for each different DNA solution injected).
9. Transfer one head to a dissecting dish with DMEM/F12, with one retina facing up. On ventral retina (or side opposite target region), use fine forceps to pinch and tear a hole in the RPE. Insert one end of the forceps through this hole to peel the RPE away from the retina, specifically at the lens-retina junction. Do not cut or damage the retina because this will cause retina to collapse onto itself during incubation. After removing RPE, use forceps to pop out retina by pinching optic nerve at the base of the retina (leave the lens intact). Transfer retinae to oxygenated SFM. Flip head over and repeat for contralateral eye.
10. Complete step 9 for all electroporated heads. Incubate retina in oxygenated SFM at 37°C for 40-48 hours.

Part 3b: *Ex vivo* retinal electroporation Harvesting and plating of GFP+ retinal explants (2 days post-electroporation)

1. Transfer all retinae from one well of oxygenated SFM into a Petri dish lid with DMEM/F12. Select one retina and use fluorescent dissecting scope to visualize GFP+ (green) portion of retina.
2. Using a microscalpel and forceps, dissect and discard the non-GFP+ portion of retina so that only a GFP+ chunk of retina remains. It is helpful to continually switch between fluorescent and regular light throughout the dissection to specifically select the GFP+ retina. Transfer the GFP+ retina to a well with DMEM/F12.
3. Repeat step 2 for all retina from one well. For each DNA condition (each different well), use a new Petri dish and DMEM/F12 medium.
4. These GFP+ retinae must now be dissected into smaller retinal explants for plating. Under a dissecting scope, cut large piece of GFP+ retina into smaller explants with a microscalpel. Explants should be rectangular, ~200-300 µm in length and width. Collect all explants in a well with DMEM/F12. One GFP+ chunk of retina should produce ~4-10 GFP+ retinal explants depending on tissue size. Repeat this step for all GFP+ retinal regions that were harvested.
5. After preparing all GFP+ retinal explants, add 250 µl of SFM + 0.4% methylcellulose (kept at 4°C) to laminin-coated culture dishes (see Part 1). Transfer 4-8 GFP+ explants to the culture dish and use the forceps to gently arrange the explants into a polygon, with explants located halfway between the center and the edge of the dish.
6. Determine which side of the explant is the RGC layer. Sometimes RPE crystals remain attached to the outer retinal layer, indicating that the opposite side is the RGC layer. In addition, the RGC side usually has some cellular debris that can aid in the proper explant orientation.
7. With the RGC layer down, use forceps to firmly depress the center of the explant so that it will adhere to the glass coverslip. An indentation in the explant from the forceps may be visible; this is normal.
8. After plating all explants on a culture dish, transfer to a 37°C incubator. Retinal axons are first observed 4-6 hours after plating and explants remain healthy for several days, but significant overgrowth can occur after 24 hours. Explants can be used for numerous *in vitro* assays, fixed with 4% PFA for 30 minutes and immunostained (**Figure 2**).

Border Assay

9. Alternatively, transfer 4 GFP+ explants to a dish that has been prepared for the border assay (see Part 1). Arrange the explants in a vertical line in the center of the dish, approximating the location of the border.
10. Under a fluorescent dissecting microscope, plate the explants so that they are ~50-150 mm from the fluorescent border. Incubate dishes at 37°C for 24 hours, allowing ample time for RGC axons to reach border substrate. Explants can be fixed in 4% PFA for 30 minutes, followed by immunostaining (**Figure 2**).

Representative results

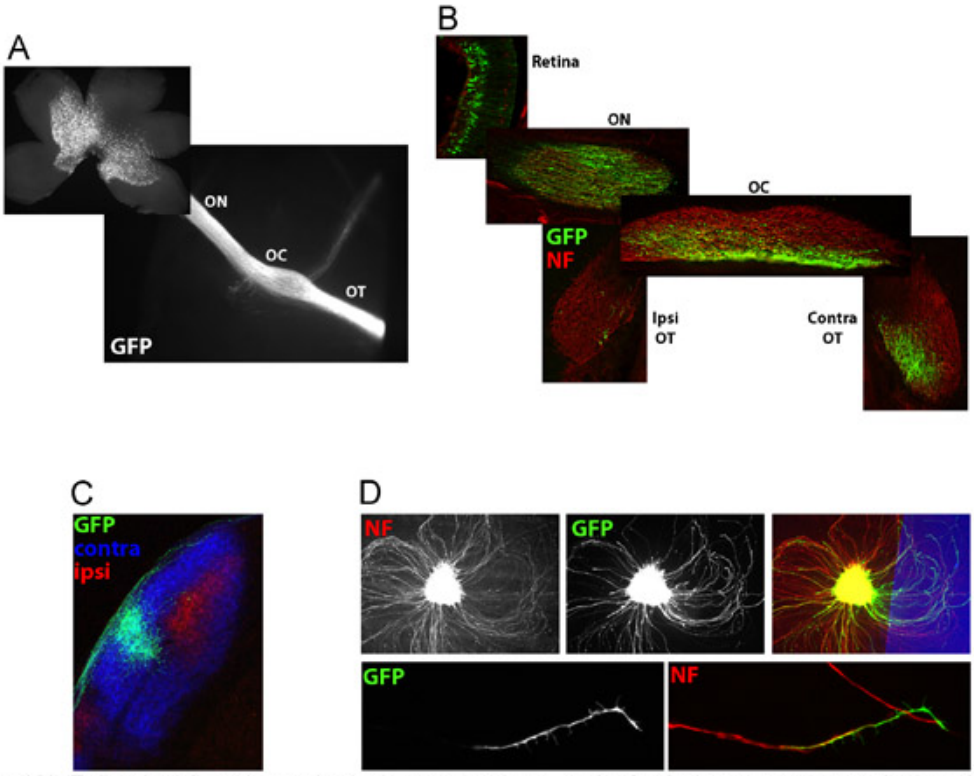

Figure 3: (**A**) Following *in utero* retinal electroporations, retinal whole mounts reveal abundant GFP$^+$ RGCs in the retina, and semi-intact visual system preparations permit visualization of GFP$^+$ RGC axons throughout the optic nerve (ON), optic chiasm (OC) and optic tract (OT). (**B**) Alternatively, frontal cryosections can be made through the retina and RGC pathway to visualize and analyze GFP$^+$ RGC cell bodies in the retina and axons in the ON, OC and OT. All retinal axons are visualized with neurofilament staining. (**C**) In addition, *in utero* electroporation can be used to study RGC axon targeting in the lateral geniculate nucleus (LGN) by harvesting pups at P4-P10. At P8, GFP$^+$ axons are visible in the LGN, with CTB-Alexa Fluor 594 (red channel) injected into the ipsilateral retina and CTB-Alexa Fluor 647 (blue channel) into the contralateral retina to label the entire RGC projection. (**D**) Following *ex vivo* retinal electroporations and plating of retinal explants, one can clearly visualize a subset of RGC axons that are GFP$^+$, both at lower (top) and higher (bottom) power. Response of GFP$^+$ axons to various substrates can be analyzed by plating explants adjacent to borders of various substrates (blue).

Discussion

In this video, we have demonstrated electroporation techniques, both *in utero* and *ex vivo*, for gene delivery into embryonic murine RGCs. *In utero* retinal electroporation provides a mechanism for manipulating gene expression in RGCs and visualize their axon projections *in vivo*. Allowing the embryos to survive postnatally permits examination of these GFP+ RGC projections into their target, the lateral geniculate nucleus (LGN). *Ex vivo* retinal electroporation provides a more controlled ability to target specific retinal regions for use with *in vitro* assays. Combining *in vivo* and *in vitro* analyses derived from these techniques allows thorough characterization of RGC development and axon projections in a subset of RGCs in an otherwise normal background. While our demonstration focuses on RGC axon guidance at the optic chiasm, both *in utero* and *ex vivo* retinal electroporations could be beneficial for studying how perturbations in gene expression effect RGC differentiation, dendritic morphology, electrophysiological properties, synapse formation and proper targeting in termination zones such as the LGN and superior colliculus.

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