Chapter 23

Quantifying the Activity of *cis*-Regulatory Elements in the Mouse Retina by Explant Electroporation

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Abstract

Transcription factors control gene expression by binding to noncoding regions of DNA known as *cis*-regulatory elements (CREs; i.e., enhancer/promoters). Traditionally, *cis*-regulatory analysis has been carried out via mouse transgenesis which is time-consuming and nonquantitative. Electroporation of DNA reporter constructs into living mouse tissue is a rapid and effective alternative to transgenesis which permits quantitative assessment of *cis*-regulatory activity. Here, we present a simple technique for quantifying the activity of photoreceptor-specific CREs in living explanted mouse retinas.

Key words: Retina, Photoreceptor, *cis*-Regulatory element, Quantification, Electroporation, Mouse

1. Introduction

Transcription factors within cellular gene networks control the spatiotemporal pattern and levels of expression of their target genes by binding to *cis*-regulatory elements (CREs), short (~300–600 bp) stretches of genomic DNA which can lie upstream, downstream, or within the introns of the genes they control. CREs (i.e., enhancers/promoters) typically consist of multiple clustered binding sites for both transcriptional activators and repressors (1–3). They serve as logical integrators of transcriptional input giving a unitary output in the form of spatiotemporally precise and quantitatively exact promoter activity. Most studies of mammalian *cis*-regulation to date have relied on mouse transgenesis as a means of assaying the enhancer function of CREs in vivo (4, 5). This technique is time-consuming, costly and, on account of insertion site effects, largely nonquantitative. Quantitative assays for mammalian CRE function have also been developed in tissue culture systems (e.g., dual luciferase assays), but the in vivo relevance of these results is often uncertain.

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Electroporation offers an excellent alternative to traditional mouse transgenesis in that it permits both spatiotemporal and quantitative assessment of *cis*-regulatory activity in living mammalian tissue. This technique has been particularly useful in the analysis of *cis*-regulation in the central nervous system, especially in the cerebral cortex and the retina (6–8). We recently developed a simple approach to quantify the activity of photoreceptor-specific CREs in electroporated mouse retinas (9). Given that the amount of DNA that is introduced into the retina by electroporation can vary from experiment to experiment, it is necessary to include a co-electroporated "loading control" in all experiments. In this respect, the technique is very similar to the dual luciferase assay used to quantify promoter activity in cultured cells.

When assaying photoreceptor *cis*-regulatory activity, electroporation is usually performed in newborn mice (postnatal day 0, P0) which is the time of peak rod production (10, 11). Once retinal cell types become post-mitotic, electroporation is much less efficient. Given the high rate of rod birth in newborn mice and the fact that rods constitute more than 70% of the cells in the adult mouse retina, the majority of cells that are electroporated at P0 are rods. For this reason, rod photoreceptors are the easiest retinal cell type to study via electroporation. The technique we describe here is primarily useful for quantifying the activity of photoreceptor CREs. Cell-type specific *cis*-regulatory activity can also be quantified in rarer retinal cell types such as bipolar cells (12), but this usually requires selection of areas of interest in vertical cross-sections rather than in flatmount preparations.

2. Materials

2.1. Electroporation Chamber

1. Electroporation microslide model 453 (BTX Harvard Apparatus, catalog #45-0105).
2. 100% Silicone rubber aquarium cement.
3. 3-ml syringe.
4. Plastic microtube rack (Fisher Scientific, catalog #05-541).
5. Dremel tool (for cutting the handle off the plastic microtube rack).
6. Binder clips.
7. Square metal bar, 3–5 mm diameter and about 100 mm long.

2.2. DNA Precipitation

1. 1.5-ml microcentrifuge tubes.
2. DNA, preferably a maxiprep(s) with concentration at least 0.5 μg/μl.

3. 100% Ethanol.
4. 70% Ethanol.
5. 3 M sodium acetate, pH 5.2.
6. Sterile water.
7. Sterile 10× PBS, pH 7.4.
8. Refrigerated microcentrifuge.

2.3. Retinal Dissection and Culture

1. Sterile dissection medium: 1:1 ratio of DMEM:F12 (Gibco #11965 and 11765), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.29 mg/ml L-glutamine, 5 μg/ml insulin. The penicillin, streptomycin, and L-glutamine may be purchased as a 100× premixed solution (Gibo #10378-016). Insulin (Sigma-Aldrich) should be reconstituted as a 5 mg/ml solution (1,000×) in 5 mM HCl, filter-sterilized, and stored at –20°C.
2. Sterile culture medium: Dissection medium (see above) plus 10% FBS (Gibco #26140-079).
3. Sterile 1× PBS, pH 7.4.
4. Post-natal day 0 (P0) mouse pups.
5. 70% Ethanol.
6. Dissecting microscope.
7. Dissection instruments: Large scissors, iris scissors, curved forceps, fine forceps.
8. Sterile, disposable transfer pipettes.
9. Sterile Petri dishes: 35, 60, and 100 mm.
10. ECM 830 square-wave electroporator (BTX Harvard Apparatus) with cables and micrograbber adaptors.
11. Sterile 6-well tissue culture plates.
12. Nuclepore filters (25 mm, 0.2 μm) (Whatman #110606).
13. Tissue culture incubator: 37°C, 5% CO_2.

2.4. Retinal Fixation, Imaging, and Quantitation

1. 4% Paraformaldehyde in 1× PBS: Dissolve 2 g paraformaldehyde in 45 ml distilled water. Apply heat and add 1 drop 5 M sodium hydroxide to facilitate the dissolution. Add 5 ml 10× PBS. Filter the final solution through Whatman filter paper. Allow the solution to cool to room temperature prior to use.
2. 1× PBS, pH 7.4.
3. Glass slides.
4. Glass coverslips: 0.16 mm thick, #1.5 (Fisher Scientific #12-544E).
5. Crushed glass coverslips, fragments 3–5 mm in diameter.
6. Fluorescent dissecting microscope.

7. Fluorescent compound microscope equipped with a camera, preferably monochromatic (e.g., ORCA-ER camera by Hamamatsu).
8. ImageJ software: Download from the NIH website http://rsbweb.nih.gov/ij/.
9. 30% Sucrose in 1× PBS, filter-sterilized (store at 4°C) (see Note 8).
10. Tissue-Tek OCT compound (see Note 8).
11. Tissue molds (see Note 8).

3. Methods

Perform all steps at room temperature unless otherwise indicated. Dissection and culture medium should be stored at 4°C but brought to room temperature prior to the beginning of the procedure.

3.1. Construction of the Electroporation Chamber

1. Order a microslide, BTX Model 453 with a 3.2 mm gap (Harvard Apparatus #45-0105) (Fig. 1a). The metal rails should be completely sealed to the bottom of the slide.
2. Use a Dremel tool to cut a handle off a plastic microcentrifuge tube rack. Cut the handle into five small rectangular pieces each with the following dimensions: length 0.8 cm, height 0.6 cm, width 0.3 cm (Fig. 1b). These plastic pieces are reusable spacers that will be used to mold individual wells in the microslide chamber.
3. Insert the plastic spacers between the metal rails of the microslide at even intervals. The spacers should fit snugly (Fig. 1c).
4. Cut the tip off a P200 pipette tip, fit the tip to a 3 ml syringe, and fill the syringe with 100% silicone rubber aquarium sealant. Fill the gaps between the plastic spacers with sealant. Be sure to fill the gaps from the bottom up so that no bubbles form between the sealant plug and the base of the microslide.
5. Place a metal rod atop the plastic spacers and fasten it with binder clips, so that the spacers are held in place while the sealant dries (Fig. 1d, e). Let the sealant dry overnight.
6. Remove the binder clips, metal rod, and plastic spacers (Fig. 1f). Use a scalpel blade to clean the sealant off the top of the metal rails. Use a dissecting microscope to examine the microslide and ensure that no bubbles are present at the bottom of the silicone dams. Remove any sealant film that may have formed in the wells such that bare metal is exposed inside wells. Fill one well with water and make sure that the water does not leak into the adjacent well(s). Repeat for all wells.

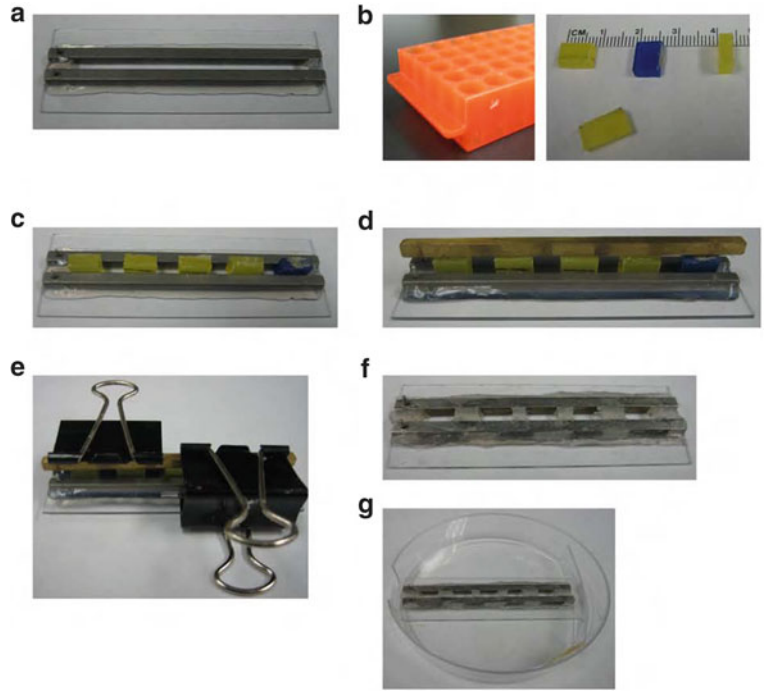

Fig. 1. Construction of the electroporation dish. (**a**) Unmodified microslide chamber from Harvard Apparatus, BTX model 453 (catalog #45-0105). (**b**) A Dremel tool is used to cut the handle off a plastic tube rack. The handle is cut into rectangular spacers with the following dimensions: length 0.8 cm, height 0.6 cm, width 0.3 cm. (**c**) The plastic spacers are fitted into the microslide chamber at equal intervals. Aquarium sealant is injected into the gaps between the spacers (not shown). (**d**) A *metal bar* is placed over the spacers. (**e**) The bar and spacers are clamped onto the slide with binder clips to hold everything in place as the sealant dries overnight. (**f**) The spacers are removed and the wells are tested to ensure that they are watertight. (**g**) The finished slide fits into the plastic dish with the metal bars adjacent to the window in the side of the dish.

7. The finished microslide fits in the plastic dish with the metal poles adjacent to the window in the side of the dish (Fig. 1g); the electrodes will eventually be attached to the metal poles.

3.2. DNA Preparation

1. Add plasmid DNA to a 1.5-ml microcentrifuge tube on ice and bring the volume up to 150 µl with distilled water. Multiple plasmid species may be combined for co-electroporation (see Note 1).
2. Precipitate the DNA by adding 15 µl 3 M sodium acetate (pH 5.2) and 450 µl 100% ethanol. Invert the tube several times to mix.
3. Spin down the DNA at 4°C, 16,000 × *g*, for 30 min. Wash the pellet with 70% ethanol, then spin it down again at 4°C, 16,000 × *g*, for 15 min. Air-dry the pellet until semitranslucent, about 7 min, then resuspend in 54 µl sterile water (vortex well

to mix). Add 6 μl sterile 10× PBS (pH 7.4) and mix by vortexing. The DNA aliquots may be stored at −20°C but should be brought to room temperature prior to electroporation.

3.3. Eye Collection

1. Sterilize the electroporation chamber and all instruments with 70% ethanol (see Note 2).
2. Prepare sterile Petri dishes with dissection medium: Two 35 mm dishes each with 3 ml medium and one 60 mm dish with 6 ml medium.
3. Disinfect the head and neck of a postnatal day 0 (P0) mouse pup with a Kimwipe soaked in 70% ethanol. Quickly decapitate with scissors and transfer the head to a 100 mm dish.
4. Cut away the scalp with small scissors to expose the eyes. Use curved forceps to gently scoop the eye out of the orbit, and place the eye in a 35 mm dish containing dissection medium. It may be helpful to remove the eyes under a dissecting microscope at low power.
5. Repeat steps 3 and 4 until all eyes have been collected. Keep eyes at room temperature while dissecting. You will need 3–4 eyes per DNA aliquot.

3.4. Retinal Dissection

1. Use 70% ethanol to disinfect a razor blade and the plastic wrapper of a sterile transfer pipette. Cut the tip off the pipette with the blade so that it can suck up a whole eye. Store the pipette in the plastic wrapper when not in use.
2. Transfer one eye from the 35 mm dish to the 60 mm dish. Under the dissecting microscope at high power, use fine forceps to remove any tissue from the surface of the eye.
3. Carefully remove the optic nerve, sclera, cornea, and retinal pigmented epithelium. Leave the lens in place (see Note 3).
4. Use the transfer pipette to move the dissected retina into the other 35 mm dish with medium.
5. Repeat steps 2–4 until all eyes have been dissected.
6. Store the retinas in a 37°C tissue culture incubator until ready to electroporate, no longer than 1 h.

3.5. Preparation for Electroporation

1. Prepare 35 mm dishes of medium. For each DNA aliquot, you will need one dish of dissection medium and one dish of culture medium. Label the dishes appropriately.
2. Use a P200 pipette and sterile 1× PBS to wash out the chambers in the electroporation dish. Each chamber holds a volume of 60–100 μl. Wash out each chamber three times.
3. Fill the chambers with the DNA aliquots. Any unused chambers should be filled with 60 μl 1× PBS. Connect the electrodes to the electroporation dish.

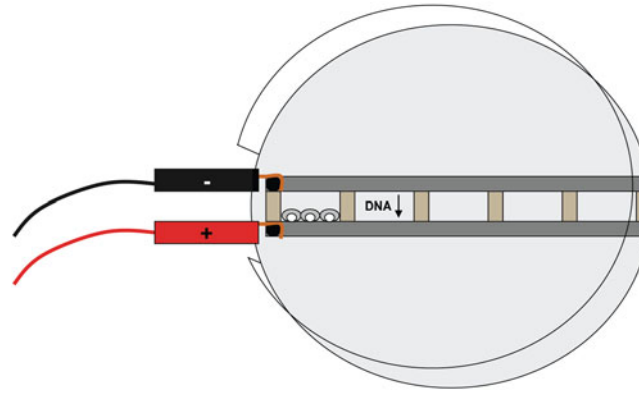

Fig. 2. Diagram of the electroporation dish with retinas. The chambers are filled with DNA solutions (up to five different solutions at a time). Retinas are placed in the chambers and oriented so that the lens is leaning against the metal bar connected to the positive electrode; three or four retinas will fit in each of the five chambers. The electrical current will cause the negatively charged DNA molecules to move into the retinal cells.

4. Use the following settings on the electroporator: Mode, LV; voltage, 30 V; pulse length, 50 ms; number of pulses, 5; interval, 950 ms; polarity, unipolar.

3.6. Electroporation

1. Use fine forceps to grasp the retinas by the lens and transfer them into the electroporation chambers. Each chamber holds up to four mouse retinas (Fig. 2).
2. Use forceps to line up the retinas such that the lens leans against the metal rail attached to the positive electrode. Clean the forceps with a Kimwipe after each transfer to avoid DNA cross-contamination between chambers.
3. Once all retinas are aligned, press "Start" on the electroporator. Tiny bubbles should form on the metal rail attached to the negative electrode.
4. Disconnect the electrodes and turn off the electroporator.
5. Use forceps to gently move the retinas away from the chamber walls.
6. Use a sterile transfer pipette to transfer the retinas from the chambers into the 35 mm dishes containing dissection medium.
7. Wash out each chamber three times with sterile 1× PBS, then rinse with sterile water. Spray dish with 70% ethanol.

3.7. Placing Retinas on Filters for Culture

1. Use a transfer pipette to transfer the retinas into the 35 mm dishes containing culture medium.
2. Label the wells of a sterile 6-well culture plate and fill each well with 3 ml culture medium.

3. Use sterile forceps to place round Whatman Nuclepore filters, shiny side up, atop the medium in each well.
4. Use a sterile transfer pipette to transfer the retinas onto the filter, lens-side-down (see Note 4).
5. Place the culture plate in a 37°C tissue culture incubator (5% CO_2) and grow for the desired amount of time, typically 8 days (see Notes 5–7).

3.8. Harvesting and Flatmounting Fluorescent Retinal Explants

1. Replace the culture medium in each well with 4% paraformaldehyde/1× PBS. If the retinas remain stuck to the filters, use forceps to flip the filters over and gently peel the retinas off the filter. Incubate in paraformaldehyde for 30 min at room temperature. Protect the retinas from light to avoid bleaching the fluorescence.
2. Rinse the retinas twice for 10 min in 1× PBS.
3. Use a disposable pipette to transfer the retinas onto a glass slide in a small drop of PBS. Under a fluorescent dissecting microscope, use forceps to flip the retinas so that they are electroporated-side-up (i.e., lens-side-down).
4. Place glass "feet" made from crushed coverslips at the corners of the slide; these feet prevent flattening of the retina by the coverslip. Place an intact glass coverslip over the slide so that it covers the retinas and rests on the feet. If necessary, use a pipette to add more PBS between the slide and the coverslip.

3.9. Imaging and Quantification of Fluorescence in Flat-Mounted Retinas

1. Use a fluorescent compound microscope to image the flatmounted retina at low power (4× objective) in the red and green channels. All retinas must be imaged with the same exposure time for a given fluorescent channel to enable comparison of fluorescence intensities. In other words, DsRed must be imaged with exposure time "A" in all retinas, and GFP must be imaged with exposure time "B" in all retinas. Make sure that the pixels are not saturated in any image, or else accurate quantification will be impossible. Export images in grayscale TIFF format. The retinal explants may be saved for sectioning (see Note 8).
2. Open the image set for one retina (i.e., red channel and green channel) in ImageJ software (http://rsbweb.nih.gov/ij/). For the sake of this tutorial, the green fluorescent channel (GFP protein) is the control construct that is constant across all retinas in the experiment. The red fluorescent channel (DsRed protein) is the experimental construct that varies for each set of retinas. The images should be in grayscale.
3. In ImageJ, select the control green image and specify a circle of interest with diameter 100 units (Analyze/Tools/ROI manager/More/Specify). Duplicate this circle (ROI manager/

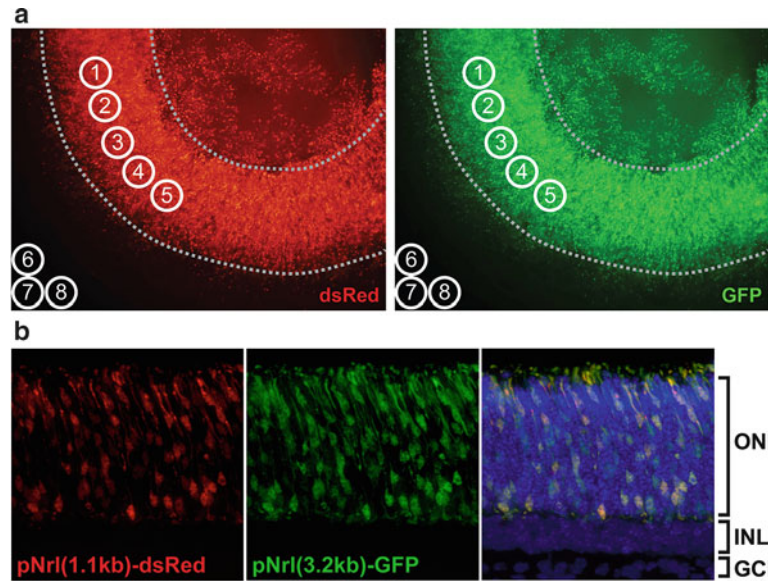

Fig. 3. (**a**) ImageJ measurement of retinal fluorescence levels in flatmount. Grayscale flatmount images in the DsRed (experimental) and GFP (control) channels are opened in ImageJ software. Five measurement circles (1–5) are placed over uniformly electroporated regions, avoiding the edges and lens (*dotted lines*). Three measurement circles (6–8) are placed outside the retina to determine background fluorescence levels. (**b**) Cross-sectional images of an electroporated retinal explant at high power. The explant was fixed at postnatal day 8, cryoprotected in 30% sucrose/1× PBS overnight at 4°C, embedded in OCT, and cryosectioned at 12 μm. The fluorescent constructs pNrl (1.1 kb)-DsRed and pNrl (3.2 kb)-GFP are expressed in photoreceptor cells in the outer nuclear layer (ONL). *INL* inner nuclear layer, *GCL* ganglion cell layer.

Add) to create eight circles total. Move circles 1–5 to select five regions that are uniformly electroporated, avoiding the outer edges of the retina and the region overlying the lens (Fig. 3a). Also, select three regions (circles 6–8) outside the retina/lens to measure background fluorescence. Select the red image, uncheck the "Show all" box in ROI manager, and recheck the box. All eight circles should appear on the red image. Deselect all circle coordinates in ROI manager.

4. With the red image selected, record the mean pixel value for all circles of interest (ROI manager/Measure); measurements 1–8 should appear, where 1–5 are the red retinal measurements and 6–8 are the red background measurements. Select the green image and record the mean pixel value; measurements 9–16 should appear, where 9–13 are the green retinal measurements, and 14–16 are the green background measurements. Copy the measurement data into Excel for analysis.
5. Average the three background measurements in both the red and the green channels. Subtract the red background average

from each of the five retinal measurements in the red channel; repeat for the green channel. For each retinal region-of-interest, divide the background-subtracted red measurement by the background-subtracted green measurement in order to normalize the experimental red level to the control green level.

6. Determine the average and standard deviation of all normalized measurements for a given DsRed construct (e.g., five measurements per retina times three separate retinas). In order to quantitatively compare the results of electroporations carried out on different days, always include a "standard" DsRed/GFP precipitation in each electroporation set. Relative expression values across experiments can be compared by normalizing to the expression level of this "standard."

4. Notes

1. In a typical promoter analysis experiment, two fluorescent constructs are co-electroporated: an experimental promoter driving the fluorescent protein DsRed and a control promoter driving GFP (or vice versa). Ideally, the control construct should be expressed in the same cell type as the experimental construct (e.g., for photoreceptor expression, a good control is the *Nrl* promoter (7) driving GFP which is available through Addgene [http://www.addgene.org/13764/]). When calculating the amount of DNA to add to the tube, keep in mind that the final volume of the DNA aliquot will be 60 μl. Typically, each construct is used at a final concentration of 0.5 μg/μl.
2. It is important to be as sterile as possible throughout the entire procedure, from eye collection and retinal dissection to the placement of electroporated retinas in the culture dish. If sterile conditions are not maintained, fungus and/or bacteria may contaminate the explants as they grow in culture. Be especially vigilant about spraying gloves, surfaces, and dissection instruments with 70% ethanol. Subheading 3.5–3.7 (electroporation and culture prep) should be performed in a sterile laminar flow hood if possible.
3. One dissecting strategy is to poke a small hole in the sclera at the limbus with one pair of forceps. Then, insert one prong from both pairs of forceps into the hole (tangential to the retinal surface) and gently tear open the sclera/RPE. In albino mice, the sclera and RPE appear shiny relative to the retinal tissue, which is a homogeneous matte tan color. It is worthwhile to perform careful dissections, because any gashes in the retinal tissue will cause contortion of the explant as it grows in culture. This will result in nonuniform fluorescence across the

retinal surface in a flat-mount view. It may be impossible to quantify fluorescence levels if the retina is severely distorted. It is not necessary to leave the lens in place, but we have found that it helps to maintain the shape of the retina throughout the procedure and results in more uniform flatmount preparations. In addition, the lens can be utilized as a "handle" for moving the retinas.

4. If the retina lands lens-side-up, pick it up with the pipette and attempt to place it again. It is important to place the retina lens-side-down (i.e., electroporated-side-up) since having the electroporated side of the retina in contact with the filter can adversely affect fluorescence. Do not place more than four retinas on one filter and make sure that the droplets of medium surrounding each retina remain separate from the other droplets. If the filter becomes submerged, remove the retinas from that well, replace the sunken filter with a fresh one, and attempt to place the retinas again.
5. For routine analysis of photoreceptor-specific CREs, 8 days in culture is usually sufficient to detect activity. If desired, retinas may be grown in culture for longer periods. The medium should be changed every 8 days; more frequent medium changes are discouraged because of the increased risk for culture contamination. To change the medium, open the plate in a sterile laminar flow hood, tilt the plate, and remove most—but not all—the medium with a sterile P1000 pipette tip. Do not touch the filter or allow liquid to drip onto the filter. Next, draw up 3 ml fresh medium with a sterile serological pipette and slowly inject the medium into the corner of the well. The filter should slowly lift away from the bottom of the well and float on the surface.
6. If desired, small molecules may be added directly to the culture medium, such as doxycycline and 4-hydroxytamoxifen.
7. The culture plates may be removed from the incubator and examined with a fluorescent dissecting microscope for short periods every day (do not remove the plate lid).
8. To save the retinal explants for histological analysis (e.g., frozen cross-sections) carefully lift off the coverslip and add a few drops 1× PBS to the slide. Use forceps to gently loosen the explants from the slide and transfer them to a vial of sterile 30% sucrose/1× PBS with a disposable transfer pipette. Light-protect the vial with aluminum foil and incubate overnight at 4°C; the retinas should eventually sink when they become permeated by the cryoprotectant. The next day, add an equal volume of Tissue-Tek OCT compound to the vial and gently rock at room temperature for 2–4 h. Remove the sucrose/PBS/OCT solution and replace with 100% OCT, then rock again at

room temperature for 2–4 h. Transfer the retinas and OCT to a plastic tissue mold. Under a fluorescent dissecting microscope, orient the retinas so that they rest on their sides at the bottom of the mold with the fluorescence down. Freeze quickly on dry ice and store at −80°C. Section the blocks with a cryostat at 8–15 μm thickness. The frozen sections may be processed by standard histological techniques including DAPI staining. Figure 3b shows a fluorescent retinal explant that was sectioned, DAPI-stained, and imaged under high power.

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