Chapter 19
Analysis of Gene Function in the Retina

Takahiko Matsuda and Constance L. Cepko

Abstract The Retina is a good model system for studies of neural development and disease because of its simplicity and accessibility. To analyze gene function rapidly and conveniently, we developed an electroporation technique in mice and rats for use in vivo and in vitro. The efficiency of electroporation into the neonatal retina is quite good, and transgene expression persists for more than a month. With this technique, various types of DNA constructs, including RNA interference (RNAi) vectors, are readily introduced into the retina without DNA size limitation. In addition, more than two different DNA constructs can be introduced into the retina at once, with very high cotransfection efficiency.

In vivo and in vitro electroporation will provide a powerful method to analyze the molecular mechanisms of retinal development and disease.

Keywords: retina, development, progenitors, photoreceptor cells, ganglion cells, electroporation, subretinal space, vitreous chamber, explant, Green Fluorescent Protein (GFP)

1. Introduction

The retina has been an excellent model system for studies of central nervous system (CNS) development and disease, because of its relatively simple structure and accessibility for gene delivery. To deliver genes into the rodent retina, several types of viral vectors, including murine oncoretrovirus (1, 2), lentivirus (3), adenovirus (4–6), and adeno-associated virus (7), have been used. However, there are disadvantages inherent in the use of such vectors. First, it is time-consuming to prepare high titer virus stocks to achieve efficient gene transfer. Second, viral vectors have a size limitation for insert DNA. Third, in general, such vectors do not readily allow introduction of more than two genes into the same cells. Fourth, biosafety is a concern for some viral vectors having broad host ranges.

To bypass these problems, we developed a system to deliver plasmid DNA directly into neonatal mouse and rat retinas by in vivo electroporation ((8, 20),

S. Li (ed.), *Electroporation Protocols: Preclinical and Clinical Gene Medicine.*
From *Methods in Molecular Biology, Vol. 423.*
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Figs. 19.1–19.4). This method is faster and, in some cases, safer than viral gene transfer methods. The efficiency of electroporation into the developing postnatal retina is quite good, and transgene expression persists for more than a month. Moreover, in vivo electroporation has several advantages. First, various types of DNA constructs, including RNA interference (RNAi) vectors as well

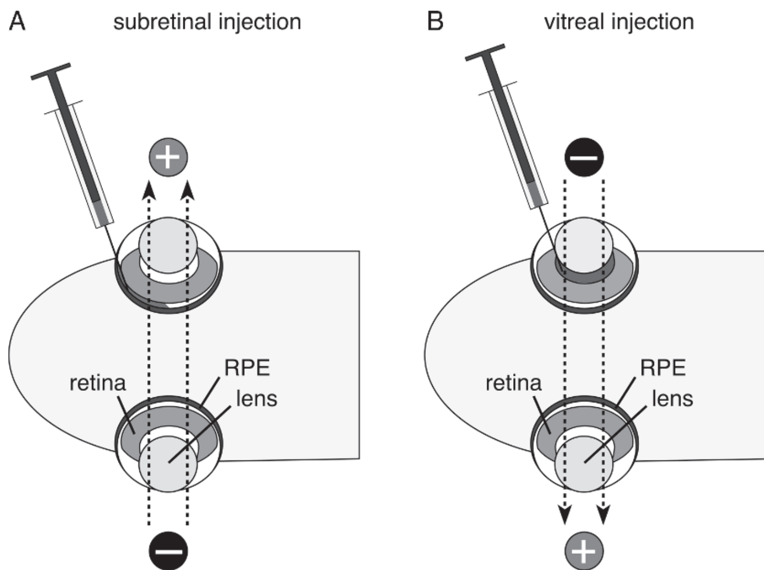
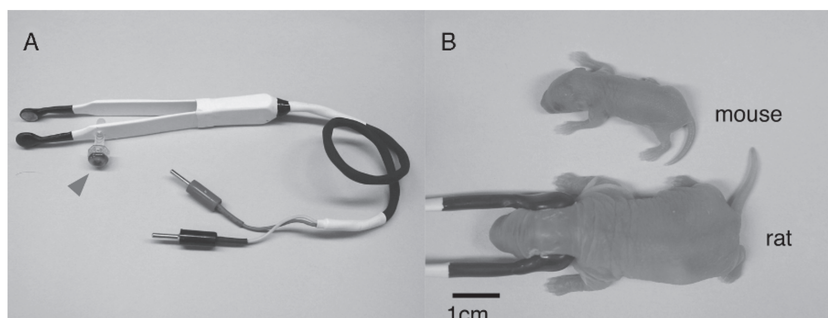


Fig. 19.1 Strategy for in vivo electroporation. (**A**) Electroporation from the scleral (retinal pigment epithelium) side of the retina. (**B**) Electroporation from the vitreal side of the retina



l 19.2 Electrodes and procedure for in vivo electroporation. Tweezer-type electrodes (**A**) are placed to hold the head of newborn (P0) rat or mouse (**B**). *Arrowhead* indicates the plus side of the electrodes

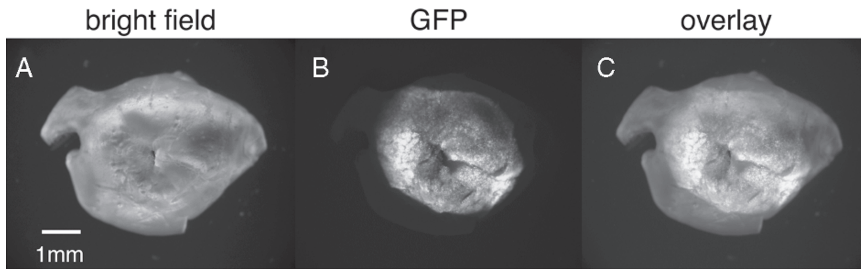


Fig. 19.3 In vivo electroporated retina—whole mount. Whole mount preparation of rat retina in vivo electroporated with pCAG-GFP (GFP expression vector driven by the CAG promoter) at P0 and harvested at P21. Pictures were taken from the scleral side. Bright-field (**A**), GFP (**B**), and merged (**C**) images are shown. (Reprinted from (8), © 2004, National Academy of Science, USA)

as conventional gene expression vectors, are readily introduced into the retina without DNA size limitation. Second, more than two different DNA constructs can be introduced into the retina at once. We found that at least five plasmids can be co-electroporated without a significant reduction in co-electroporation frequency.

We are currently applying this technique to various types of studies, including gain-of-function, loss-of-function, and promoter analyses in the developing retina (8–10, 20).

1.1. Basic Strategy

Figure 19.1A shows the basic strategy for in vivo electroporation into newborn mouse and rat pups. DNA constructs are injected into the subretinal space between the retina and retinal pigment epithelium (RPE). Then, electrodes are placed on the heads of pups (Fig. 19.2), and electric pulses are applied to the eyes in the direction shown in Fig. 19.1A. The DNA constructs are transduced from the scleral side of the retina, where undifferentiated mitotic and newly postmitotic cells exist (*see* **Note 1**). In addition to the strategy shown in Fig. 19.1A, it is also theoretically possible to transfect DNAs from the vitreous side of the retina by injecting DNAs into the vitreous chamber, and by applying electric pulses in the direction opposite to that shown in Fig. 19.1A (Fig. 19.1B). Indeed, other groups reported that DNA constructs could be transduced to ganglion cells, which line the surface of the retina facing the vitreous body, by in vivo electroporation using this strategy (11, 12). However, our data show that efficiency of transfection from the vitreal side (ganglion cells) of the neonatal retina, as well as into the adult retina, is much lower than that from the scleral side (progenitor/precursor cells) of the neonatal retina (Fig. 19.7).

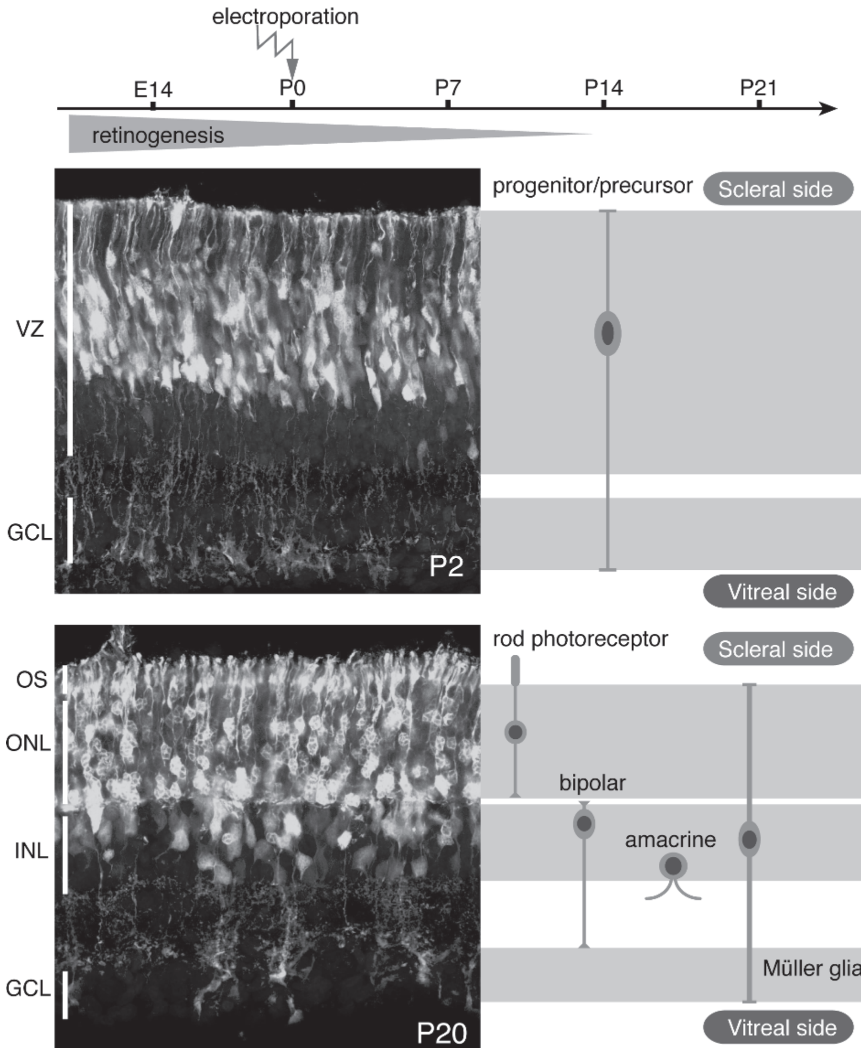


Fig. 19.4 In vivo electroporated retina—section. Rat retinas were in vivo electroporated with pCAG-GFP at P0 and harvested at P2 (*upper* panel) or P20 (*lower* panel). At P2, most of the GFP-positive cells have the morphology of progenitor/precursor cells, suggesting that DNAs are preferentially transfected to progenitor/precursor cells. Retinogenesis is completed within the first 2 weeks after birth. At P20, GFP is observed in four differentiated cell types, including rod photoreceptors, bipolar cells, amacrine cells, and Müller glial cells. *VZ* ventricular zone, *GCL* ganglion cell layer, *OS* outer segment, *ONL* outer nuclear layer, *INL* inner nuclear layer

1.2. In Vivo Electroporation vs. In Vitro Electroporation

In organ cultures of embryonic or neonatal retina, progenitor cells differentiate into neurons and glia and form three layers, mimicking normal development. Taking advantage of this, we also developed a system to electroporate DNAs into isolated

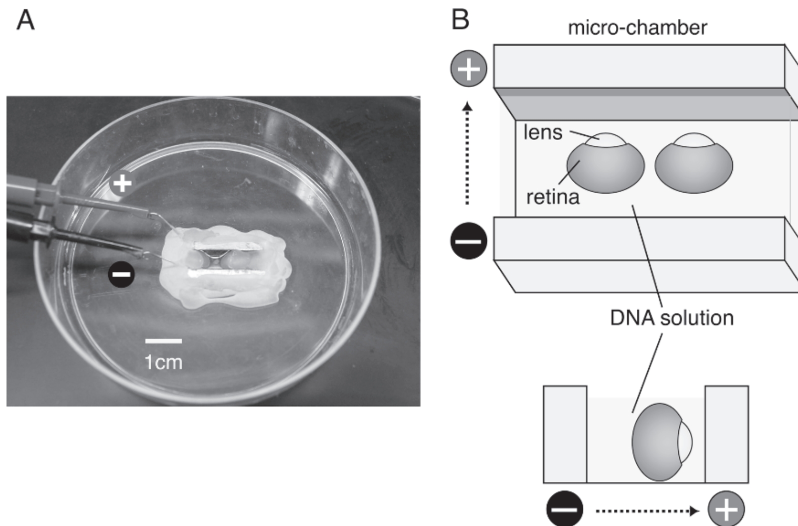


Fig. 19.5 Microchamber for in vitro electroporation. (**A**) Commercially available microchamber. (**B**) Orientation of the retina in the chamber. Maximum transfection efficiency can be obtained when the scleral side is facing the negative electrode

retinas (in vitro electroporation) using a micro electroporation chamber ((8), Figs. 19.5–19.7). Electroporated retinas are cultured for a few days to weeks.

Compared with in vivo electroporation, in vitro electroporation has several advantages. First, in vitro electroporation is easier and less skill-dependent than in vivo electroporation. All retinas subjected to electroporation become Green Fluorescent Protein (GFP) or Red Fluorescent Protein (RFP)-positive when GFP (RFP)-expression vectors are used (*see* **Note 2**). Second, it is relatively easy to handle a large number of retinas in a day. Third, in vitro electroporation can be easily applied not only to postnatal retina, but also to embryonic retina, to which in vivo electroporation (*in utero* electroporation) is hard to apply. Fourth, real-time monitoring of GFP (RFP)-transduced cells is possible under a fluorescence microscope. However, in vitro electroporation has several disadvantages inherent to organ culture. First, the morphology of cultured retina is frequently poor, and photoreceptor outer segments are poorly formed. Second, it is hard to culture retinas for a long period. In our experience, retinas tend to become unhealthy when cultured for more than two weeks (*see* **Note 3**).

1.3. Limitations of Electroporation

Although in vivo and in vitro electroporations are very powerful techniques, they have several limitations. One should take account of these points. First, unlike retroviral vectors that integrate into the host genome and stably express foreign genes for a long time, gene expression from DNA constructs introduced by in vivo electroporation is not so stable. Although we confirmed that the GFP expression is

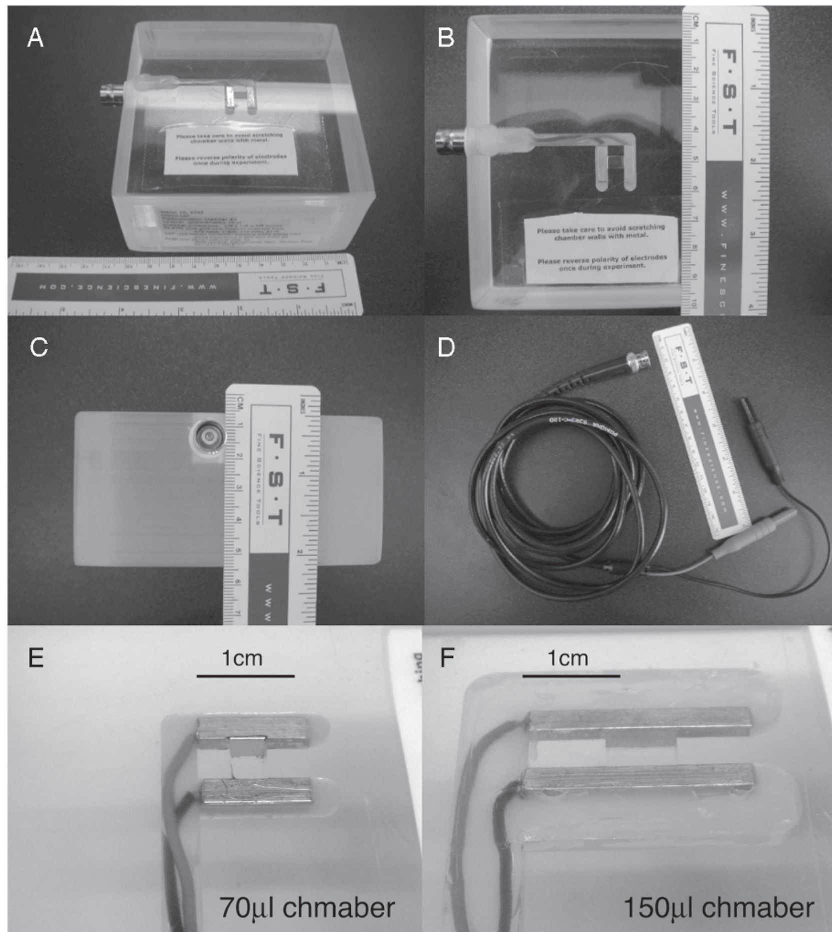


Fig. 19.6 Microchambers for in vitro electroporation—custom made. (**A**, **B**) The electroporation microchamber made in the Machine Shop at Harvard Medical School. (**C**) Side view of the chamber showing a BNC type female connector. (**D**) Cables with a BNC type male connector. (**E**) High-magnification view of a 70-μL chamber. (**F**) High-magnification view of a 150-μL chamber. Pictures were kindly provided by Dr. Douglas Kim

visible for at least 50 days after electroporation, it is unlikely that the gene expression persists for more than several months (*see* **Note 4**). Second, undifferentiated progenitor/precursor cells are highly transfectable, while transfection efficiency of differentiated neurons, including photoreceptors and ganglion cells located at the surface of the retina, is very low for unknown reasons. Thus, electroporation of photoreceptor cells in the adult retina is not practical. Finally, the DNA constructs electroporated to the postnatal retinal progenitor/precursor cells are inherited by late born retinal cell types (rod photoreceptor, bipolar, amacrine, and Müller glial cells) but not by early born cell types (cone photoreceptor, horizontal and

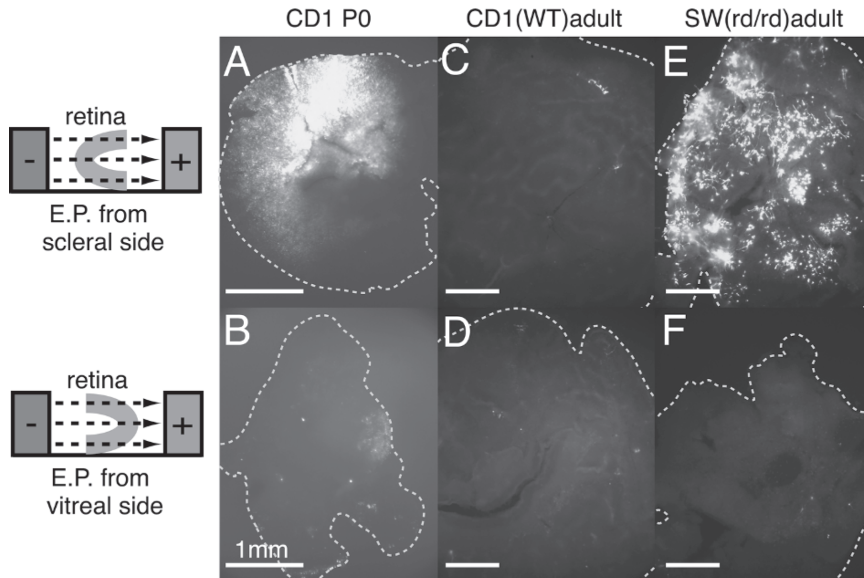


Fig. 19.7 In vitro electroporated retinal explant—whole mount. Mouse retinas of P0 CD1 (**A**, **B**), adult CD1 (**C**, **D**), or adult Swiss Webster having a retinal degeneration mutation (**E**, **F**) were in vitro electroporated with pCAG-GFP from the scleral side (A, C, E) or from the vitreal side (B, D, F), and cultured for 5 days. Pictures a, c, and e were taken from the scleral side, and b, d, and f were taken from the vitreal side. Note that only the scleral side of developing retina or of degenerated retina is highly transfectable. In panel e, most of GFP-positive cells are Müller glial cells. (Reprinted from (8), © 2004, National Academy of Science, USA)

ganglion cells) whose progenitor/precursor cells exist only in the embryonic retina (Fig. 19.4). To deliver genes into the early born cell types by electroporation, one needs to target the embryonic retina. Although subretinal injection into the E13/E14 mouse retina is technically possible (13, 14), it is not as easy as that into the postnatal retina.

2. Materials

2.1. Animals

All the animal experiments were approved by the Institutional Animal Care and Use Committee at Harvard University.

1. Timed or untimed pregnant CD1 mice (*see* **Notes 5,6**).
2. Timed or untimed pregnant Sprague-Dawley rats.

2.2. Plasmid DNA

Both ubiquitous and retinal cell type specific promoters can be used. As a ubiquitous promoter, we are using the CAG (chicken β-actin promoter with cytomegalovirus (CMV) enhancer) promoter (15) or human ubiquitin C promoter (16) (*see* **Note 7**). As a reporter, we are mainly using GFP (EGFP) or RFP (DsRed2) purchased from Clontech. Expression vectors used in our laboratory, as well as their detailed sequence information, are available through Addgene (http://www.addgene.org/connie_cepko).

1. Plasmid DNAs are prepared using Qiagen Plasmid Maxi kit (Qiagen). For in vivo injection, plasmids are ethanol precipitated and suspended in phosphate-buffered saline (PBS, Invitrogen) (final, 2.0–6.0 μg/μL). Approximately 10 μL is needed to inject into 10–20 pups. For in vitro injection, plasmids are ethanol precipitated and suspended in Hanks' balanced salt solution (HBSS, Invitrogen) (final, 0.5–2.0 μg/μL). Approximately 100 μL is needed to perform one electroporation. DNA can be stored at −20°C (*see* **Note 8**).
2. 1% Fast Green FCS (J.T. Baker) in H_2O: filter through a 0.45-μm filter and store at room temperature. Add 1/10 volume of 1% Fast Green to DNA solution as a tracer (final, 0.1%).

2.3. Electroporator

1. Square pulse electroporator CUY21 (Nepagene, Japan) or ECM830 (BTX): Both models work well. CUY21 has a function to display the current measurements after electroporation. ECM830 is compact and less expensive, but does not display the current measurements.
2. Foot pedal switch (BTX, model 1250FS): This is optional but facilitates the electroporation procedure, because both hands are occupied holding an animal and electrodes during electroporation.

2.4. In Vivo Electroporation

1. Tweezer-type electrodes (BTX, model 520, 7 mm diameter). Model 522 (10 mm diameter) works as well.
2. Injection syringe with a 33-gauge blunt end needle (Hamilton, no. 0159666) or injection syringe with a 32-gauge blunt end needle (Hamilton, no. 87931) (*see* **Note 9**).
3. Disposable 30-gauge ½ needle (Becton Dickinson, no. 5106).
4. Cotton swab.
5. 70% ethanol.
6. PBS.

7. Slide warmer, heating pad, or heat lamp to warm anesthetized pups.
8. Dissecting microscope.

2.5. In Vitro Electroporation and Explant Culture

1. Microchamber for electroporation: An electroporation chamber is commercially available from Nepagene (model CUY520P5, 3 mm × 8 mm × 5 mm, ~100 µL volume; Fig. 19.5). We made the electroporation chambers with the help of the Machine Shop at Harvard Medical School Neurobiology Department (Fig. 19.6). These chambers (3 mm × 4 mm × 5.5 mm, ~70 µL volume; and 3 mm × 8 mm × 5.5 mm, ~150 µL volume) were made on acrylic blocks (10 cm × 10 cm × 5 cm) with pure gold plate bars (Alfa Aesar, 3 mm × 12 mm × 3 mm for the 70-µL chamber or 3 mm × 25 mm × 3 mm for the 150-µL chamber) as electrodes, and a BNC-type female connector (Allied Electronics no. 885-5369). These chambers were designed by Dr. Douglas Kim.
2. Culture medium: Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum and antibiotics (penicillin (100 U/mL) and streptomycin (100 mg/mL) (*see* **Note 10**).
3. 6-well tissue culture dish.
4. Nucleopore polycarbonate membrane (Whatman, no. 110606, 25 mm diameter, 0.2-µm pore).
5. Dissecting instruments: fine scissors (e.g., F.S.T. no. 14085-08) and fine forceps (e.g., Dumont no. 5) for dissection. Curved forceps (Dumont no. 7) for transfer of dissected retina.
6. HBSS.
7. 10- or 6-cm Petri dish.

2.6. Analysis of Electroporated Retina

2.6.1. Sectioning

1. 4% (w/v) paraformaldehyde in PBS: dissolve 8.0 g of paraformaldehyde (J.T. Baker) in 40 mL of H_2O in a 50-mL centrifuge tube. Add 20 µL of 10 M NaOH, and heat at 65°C, occasionally inverting the tube until it dissolves. Filter through a 0.45-µm filter. Stable at 4°C for up to a week. Before use, make 4% solution in PBS.
2. 30% (w/v) sucrose/PBS: filter through a 0.45-µm filter and store at room temperature.
3. O.T.C. compound.
4. Embedding mold (Ted Pella, no. 106, 12 cavities).
5. Liquid nitrogen or dry ice.

6. Superfrost Plus slide glass (Fisher Scientific).
7. Cryostat.

2.6.2. Dissociation into Single Cells

1. Papain (Worthington, no. LS003126, milky solution). Store at 4°C. Stable for more than a year.
2. 50 mM cysteine in H_2O: filter through a 0.45-µm filter and store at 4°C.
3. 10 mM EDTA in H_2O. Store at room temperature.
4. 60 mM 2-mercaptoethanol in H_2O. Store at 4°C.
5. Poly-D-lysine (10 mg/mL) in H_2O. Store at 4°C. Before use, dilute in H_2O at 1:100 (final, 0.1 mg/mL).
6. HBSS or PBS.
7. DMEM with 10% fetal calf serum.
8. DNaseI (Roche, no. 776785), 10 units/µL.
9. 8-well slide glass (Cell-Line/ERIE Scientific Co. 8 Rect. 11 × 13 mm^2).

2.6.3. Immunostaining

1. Goat serum (Gemini-Bioproducts): Heat-inactivate at 56°C for 30 min, and store at −20°C.
2. PBS.
3. PBST: PBS containing 0.1% (v/v) Triton X-100.
4. Primary antibodies (**Table 19.1**).
5. Secondary antibodies: Cy2-, Cy3-, or Cy5-conjugated anti-mouse, anti-rabbit, or anti-rat IgG. Reconstitute in 50% glycerol and store in dark at −20°C. Use at 1:500.
6. 4', 6-diamidino-2-phenylindole (DAPI) (1 mg/mL; Roche) in H_2O. Store in dark at 4°C. Use at 1:2000.
7. Slide staining dish and holder.
8. Gel/Mount (Biomedia).
9. Cover glass.

3. Methods

3.1. In Vivo Electroporation

3.1.1. DNA Injection

For in vivo electroporation of the retina, DNA injection is the most critical step to achieve good transfection. We are using two injection methods (Figs. 19.8–19.10). One is to inject DNA without opening the eyelid (Method 1). This is a “blind

Table 19.1 Retinal cell type specific antibodies

Antibody	Host	Source	Retinal cell type	Dilution
Anti-rhodopsin (Rho4D2)	Mouse	Dr. R.S. Molday (17)	Rod	1:100
Anti-Gt1α	Rabbit	SantaCruz (no. sc-389)	Rod	1:500
Anti-blue opsin	Rabbit	Chemicon (no. AB5407)	Blue cone	1:500
Anti-green opsin	Rabbit	Chemicon (no. AB5405)	Green cone	1:500
Anti-Gt2α	Rabbit	SantaCruz (no. sc-390)	Cone	1:100
Anti-PKCα	Mouse	Oncogene (no. OP74)	Rod bipolar	1:100
Anti-Chx10	Rabbit	Our laboratory	Bipolar (+progenitor)	1:500
Anti-glutamine synthetase	Mouse	Chemicon (no. MAB302)	Müller glia	1:500
Anti-syntaxin 1a (HPC-1)[a]	Mouse	SantaCruz (no. sc-12736)	Amacrine	1:200
Anti-calbindin D28K[b]	Mouse	Sigma (no. C9848)	Horizontal	1:200
Anti-Thy-1.1[c]	Mouse	SantaCruz (no. sc-19614)	Ganglion	1:200
Anti-Thy-1.2[d]	Rat	PharMingen (no. 553000)	Ganglion	1:200

[a]Syntaxin 1a is also weakly expressed in horizontal cells
[b]Calbindin is also expressed in a small subset of amacrine cells
[c]This antibody works for rat retina, but may not react with most mouse strains (e.g., C57/BL6) expressing Thy-1.2 instead of Thy-1.1
[d]This antibody works for most of the mouse strains expressing Thy-1.2, but does not work for rat retina

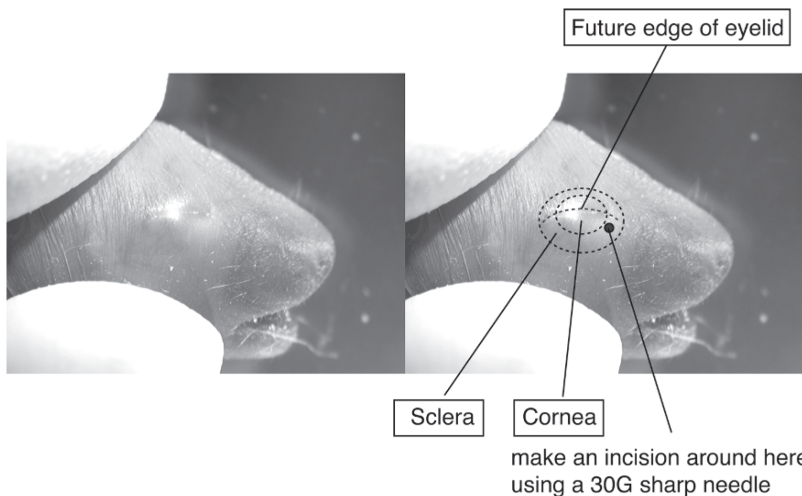


Fig. 19.8 Head of newborn rat

injection," but causes less damage to the animals. This method works well particularly for rat pups. The other one is to inject DNA after cutting the future edge of the eyelid (Method 2). In this method, the tip of an injection needle can be seen through the lens (Fig. 19.10G), and it is easy to check whether DNA was correctly

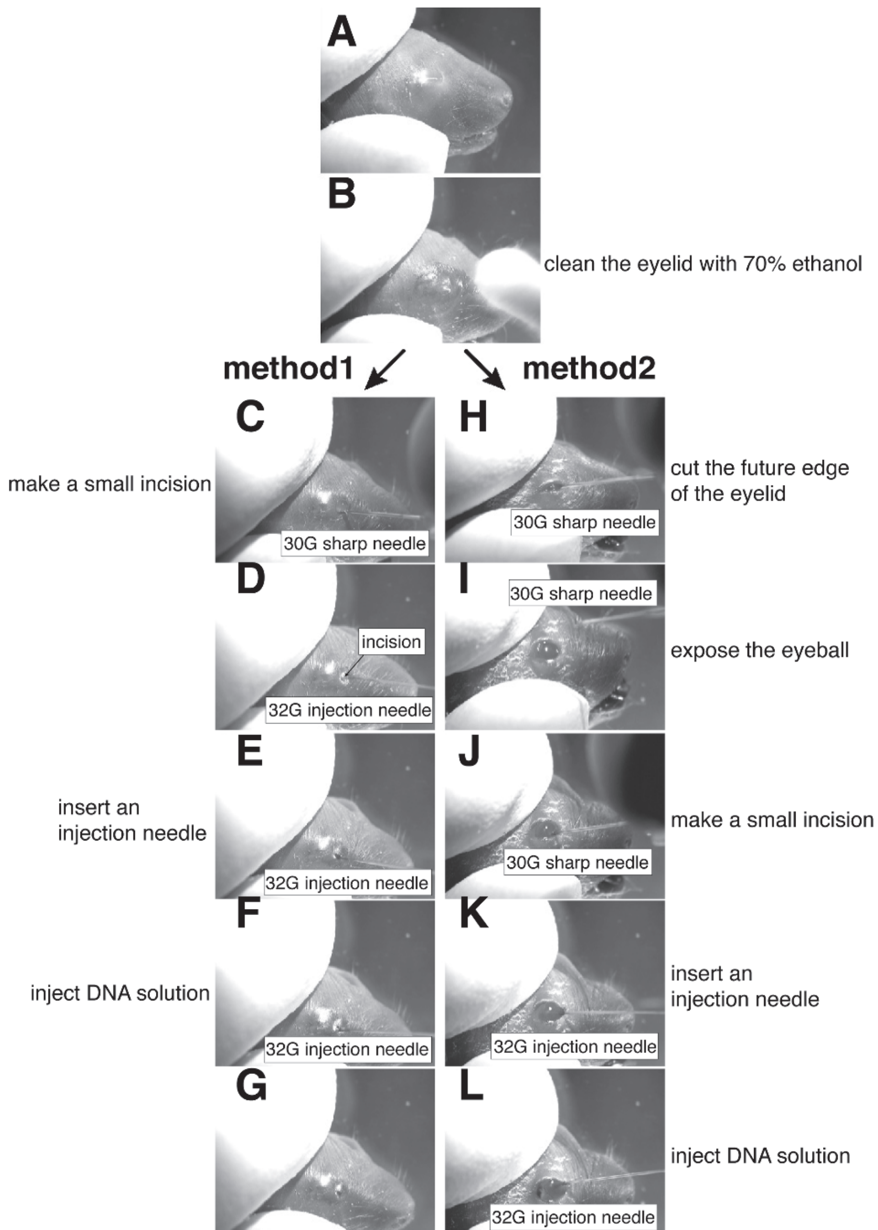


Fig. 19.9 Procedure for subretinal injection—pictures. Two injection methods, injection without opening the eyelid (**A**, **B**, **C**–**G**) and injection after opening the eyelid (**A**, **B**, **H**–**L**), are shown. See text for detailed description of the procedure

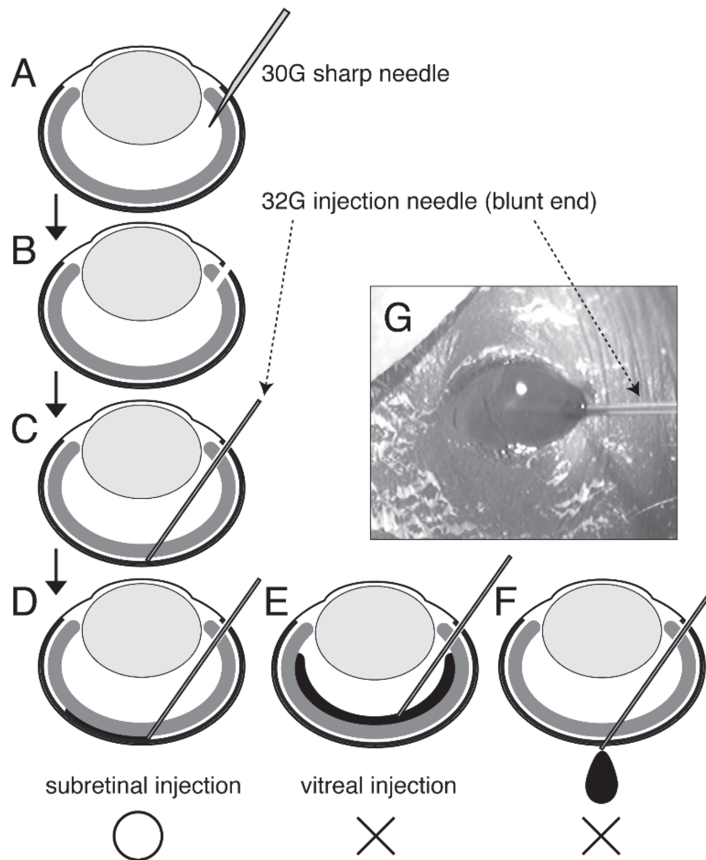


Fig. 19.10 Procedure for subretinal injection—illustration. (**A**–**D**) Schematic drawing of the subretinal injection procedure. See text for detailed description the procedure. (**E**, **F**) Examples of bad injection. (**G**) Injection needle can be seen through the lens if the eyelid is opened before injection

injected into the subretinal space. DNA injection is done under a dissecting microscope.

3.1.1.1. Method 1 (Injection Without Opening the Eyelid)

1. Anesthetize newborn pups on ice for several minutes until they stop moving. It takes several minutes (*see* **Note 11**).
2. Clean the eyelid with 70% ethanol using a cotton swab (Fig. 19.9B). By wetting the eyelid, the shape of the eyeball and the future edge of the eyelid can be easily recognized.
3. Make a small incision in the sclera near the cornea through the skin using the tip of a sharp 30-gauge needle (Figs. 19.9C and 19.10A, B).

4. Carefully insert an injection needle (Hamilton syringe, 32 or 33 gauge) into the eyeball through the incision until you feel a slight resistance (Figs. 19.9D, E and 19.10C (*see* **Note 12**)). When you feel resistance, the tip of the injection needle is located at the subretinal space between RPE and retina.
5. Slowly inject DNA solution containing 0.1% Fast Green into the subretinal space (Figs. 19.9F and 19.10D (*see* **Note 13**)). For rat newborn pups, we inject 0.3–0.5 µL of DNA, and for mouse newborn pups, we inject 0.2–0.3 µL of DNA. If DNA is injected correctly, the eyeball becomes green (Fig. 19.9G).

3.1.1.2. Method 2 (Injection After Opening the Eyelid)

1. Anesthetize newborn pups on ice for several minutes.
2. Clean the eyelid with 70% ethanol using a cotton swab (Fig. 19.9B).
3. Carefully cut the future edge of the eyelid using the tip of a sharp 30-gauge needle (Fig. 19.9H (*see* **Notes 14,15**)).
4. Expose the eyeball by pulling down the skin (Fig. 19.9I).
5. Make a small incision in the sclera near the cornea using the tip of a sharp 30-gauge needle (Figs. 19.8, 19.9J, and 19.10A).
6. Insert an injection needle (Hamilton syringe, 32 or 33 gauge) into the eyeball through the incision until you feel resistance (Figs. 19.9K and 19.10C). The inserted needle can be seen through the lens (Fig. 19.10G).
7. Slowly inject DNA into the subretinal space (Figs. 19.9L and 19.10D). If you correctly inject DNA into the subretinal space, the dye spreads within a relatively small area (usually not in the entire retina), and you can see “green” and “nongreen” areas in the retina by slightly rotating the injected animal. If you inject DNA into the vitreous chamber, the dye spreads more rapidly and uniformly in the eyeball (Fig. 19.10E). If you inject DNA outside the eyeball, the eyeball does not become green (Fig. 19.10F).

3.1.2. Electroporation

1. Soak the tweezer-type electrodes in PBS (optional). This step is to increase the contact between the pup and the electrodes.
2. Place the tweezer-type electrodes to hold the head of the pup, and slightly squeeze them (Fig. 19.2B). The positive electrode, marked by a plastic screw (Fig. 19.2A, arrowhead), should be at the DNA-injected side, if you transfect DNA from the subretinal space into the retina.
3. Apply five square pulses of 50-ms duration with 950-ms intervals using a pulse generator. For newborn rat pups, we apply 100-V pulses, and for newborn mouse pups, we apply 80-V pulses. We usually apply electric pulses right after DNA injection. The measured current is 0.10–0.20 mA (*see* **Notes 16,17**).
4. Warm the operated pups (e.g., on a 37°C slide warmer) until they recover from anesthetic, and then return them to their mother (*see* **Notes 18,19**).

3.2. In Vitro Electroporation

In the neonatal or fetal retina, the scleral side is highly transfectable, whereas the vitreal side is not (Fig. 19.7). Therefore, good transfection can be achieved when the scleral side is facing the negative electrode in an electroporation chamber (Fig. 19.5B). Multiple retinas can be electroporated at once, and the DNA solution can be used several times, although transfection efficiency gradually decreases. All the procedures are done at room temperature. We are not using a tissue culture hood for dissection and electroporation.

1. Put polycarbonate filters on culture medium (2 mL/well) in 6-well dishes, and keep the plates in a CO_2 incubator. Do not sink the filters into the medium.
2. Dissect eyeballs in HBSS in a Petri dish under a dissecting microscope, and carefully take out the retina with lens. RPE is usually removed with sclera (*see* **Note 20**).
3. Collect the retinas in a new Petri dish. The dissected retina can be kept in HBSS for 1 h at room temperature.
4. Transfer the retina(s) to a micro electroporation chamber filled with a DNA solution.
5. Set the position and orientation of the retina(s) in the chamber. When the vitreal side (lens) is facing the positive electrode, transfection efficiency becomes maximum (Figs. 19.5B and 19.7)
6. Apply five square pulses (30 V) of 50-ms duration with 950-ms intervals using a pulse generator. Air bubbles are generated only from the negative electrode (*see* **Note 21**).
7. When electroporation is repeated using the same DNA solution, gently stir the DNA solution by pipetting several times. Do not make air bubbles. If necessary, DNA solution can be recovered and stored at −20°C after removing cell debris by centrifugation for 3 min at 13,000 rpm.
8. Transfer the electroporated retina(s) into HBSS in a Petri dish to wash out the DNA.
9. Remove the lens from the retina, and flatten the retina. If necessary, carefully make several incisions to facilitate flattening.
10. Using curved forceps, carefully transfer the retina onto polycarbonate filters in 6-well dishes with the scleral side down.
11. Apply 20 µL of the culture medium onto the retina.
12. Culture the retina at 37°C in a CO_2 incubator.
13. Change half of the culture medium every 3 days.

3.3. Analysis of Electroporated Retina

Electroporated retinas are harvested 2 days to several weeks after electroporation and dissected under a fluorescent dissecting microscope (Leica, MZFL III) to select

GFP (RFP)-positive retinas (*see* **Note 22**). Dissected retinas are analyzed by making sections to examine the morphology of GFP (RFP)-positive cells. Alternatively, retinas may be dissociated into single cells. These single cells can be used for immunocytochemistry or for FACS sorting of GFP (RFP)-positive cells to analyze gene expression profiles (Reverse Transcription-PCR (RT-PCR) and microarray analyses).

3.3.1 Sectioning

1. Dissect eyeballs in HBSS or PBS in a Petri dish, and carefully take out the retina with lens (*see* **Note 23**).
2. Fix the harvested retina with 4% paraformaldehyde in PBS for 30–60 min at room temperature (*see* **Note 24**).
3. Incubate the retina in 30% sucrose in PBS for several hours to overnight at 4°C (*see* **Note 25**).
4. Embed the retinas in O.C.T. compound using an embedding mold.
5. Snap-freeze the retinas in the O.C.T. compound with liquid nitrogen or dry ice. Frozen retina can be stored at −80°C for more than a year.
6. Cut cryosections (20 µm thickness) on a cryostat.
7. Air-dry the slides at room temperature for 30 min. Dried slides can be stored in a tightly sealed slide box with several pieces of dry silica gel (Drierite) at −20°C for several months.

3.3.2. Dissociation into Single Cells

1. Prepare a papain activation solution by mixing H_2O (7 mL) with 50 mM cysteine (1 mL), 10 mM EDTA (1 mL), and 60 mM 2-mercaptoethanol (100 µL).
2. Activate papain by diluting 5 µL (× tube numbers) of papain in 200 µL (× tube numbers) of the activation solution. When the color of this solution changes from white to clear, the papain solution is ready to use. Usually it takes a few minutes.
3. Put retina(s) into a 1.5-mL microtube containing 200 µL of HBSS.
4. Add 200 µL of the activated papain solution into the tube.
5. Incubate at 37°C for ~10 min. During incubation, tap the tubes several times by finger to promote digestion (*see* **Note 26**).
6. Add 600 µL of DMEM/10%FCS to stop the digestion (*see* **Note 27**).
7. Add 50 u (5 µL) DNase, and incubate at 37°C for ~5 min.
8. Suspend the dissociated retinal cells by gently pipetting up and down using a P1000 pipetman (10–15 times).
9. Centrifuge the tubes for 30 s at 4,000 rpm.
10. Remove the supernatant (but not completely), and then break the cell pellet by finger tapping (*see* **Note 28**).

11. Add 600 µL of DMEM/10%FCS, and suspend by gently pipetting up and down using a P1000 pipetman (5–10 times). Dissociated retinal cells can be kept on ice for 1 h.
12. Coat 8-well glass slides with poly-D-lysine (0.1 mg/mL) for more than 30 min at room temperature.
13. Aspirate the poly-D-lysine solution, and air-dry the slides at room temperature. Coated slides can be stored at room temperature.
14. Plate the dissociated retinal cells (50 µL/well) on coated 8-well slides.
15. Incubate the slides at 37°C for 40–60 min in a CO_2 incubator.
16. Aspirate the medium, and fix the cells with 4% paraformaldehyde in PBS at room temperature for 5 min.
17. Wash the slides twice with PBS.
18. Aspirate PBS and air-dry the slides at room temperature for 30 min. Dried slides can be stored in a tightly sealed slide box with several pieces of dry silica gel (Drierite) at −20°C for several months.

3.3.3. Immunostaining

1. Wash slides in PBS for 5 min at room temperature.
2. Block the slides with 10% goat serum in PBST for 1 h at room temperature.
3. Remove the blocking solution, and apply primary antibody diluted in the blocking solution.
4. Incubate overnight at 4°C in the dark.
5. Remove the antibody solution, and wash the slides in PBS thrice for 5 min each at room temperature.
6. Apply secondary antibody diluted in the blocking solution containing 0.0005% DAPI.
7. Incubate for several hours (more than 2 h) at room temperature in the dark.
8. Wash the slides in PBS thrice for 5 min each at room temperature.
9. Air-dry the slides at room temperature for 30 min.
10. Mount the slides using Gel/Mount.

Acknowledgments We thank Dr. Douglas Kim for providing the pictures. This work was supported by NIH EYO 8064 and 9676 and Howard Hughes Medical Institute.

4. Notes

1. It is possible to transfect DNA into the RPE in the adult mouse and rat by injecting DNA into the subretinal space, and by applying electric pulses in the direction opposite to that shown in Fig. 19.1A (12, 18).
2. After in vitro electroporation with GFP (RFP)-expression vectors, 5–20% of cells become GFP (RFP)-positive in the retina, depending upon the DNA concentration, type of promoters in the expression vectors, and orientation of the retina in the electroporation microchamber.

3. Another group reported that neonatal mouse retinal explants could be maintained for more than 4 weeks using improved culture conditions (19).
4. This problem might be partly overcome by the use of ΦC31 integrase, which stably integrates the plasmid DNA containing bacterial attB site into the mammalian genome (18).
5. We examined several outbred mouse strains maintained by Charles River and Taconic, and found that CD1 mice from Charles River have normal retinal morphology. Most other outbread mouse strains, including Swiss Webster (Taconic, Charles River), ICR (Taconic), and Black Swiss (Taconic), have a retinal degeneration (*rd1*) mutation and are not suitable for the study of retinal development.
6. Inbred mouse strains, such as C57BL/6, can also be used. However, inbred mice sometimes do not take care of their pups after the pups are subjected to surgery. Careful monitoring is needed when inbred mouse pups are returned to their mothers after surgery.
7. We compared several ubiquitous promoters, including CAG, ubiquitin C, cytomegalovirus (CMV), and human elongation factor 1α (EF-1α) promoters, in the developing rat retina and found that the CAG and ubiquitin C promoters work very well. The ubiquitin promoter is stronger than the CAG promoter in the developing rat retina. The CMV and the EF-1α promoters also work but appear to be silenced in photoreceptor cells (8).
8. Repeated freeze-thawing of DNA solution sometimes generates an insoluble precipitate, which is probably an aggregate of DNA. To prevent an injection needle from clogging, such an aggregateh has to be removed by centrifugation at 13,000 rpm for 3 min.
9. We are using a blunt end needle (point style 3) instead of a sharp beveled needle. Use of a blunt end needle facilitates subretinal injection. Pulled sharp glass needles also can be used, but practice is needed for successful injection.
10. We tested several culture media, and found that the serum-free medium (Neurobasal medium (Invitrogen) with 1× B-27 serum-free supplement (Invitrogen)) also works well. In the serum-free culture, however, the number of surviving ganglion cells is lower than that in the serum-containing medium. Other cell types are largely not affected.
11. If newborn pups are left on ice for too long (more than 10 min), some pups do not recover.
12. Note that even a blunt end needle can easily penetrate the sclera of newborn pups when the needle is pushed strongly. Careful and slow needle insertion is a key point.
13. If the same injection syringe is used for different DNA solutions, wash the syringe by filling and ejecting water several times until the dye disappears from the syringe.
14. You may cut the entire edge of the eyelid (Fig. 19.8). But if you correctly make a small cut in the edge line of the eyelid (just above the lens), and pull down the skin, the eyelid will open like a "zipper" with little damage.
15. After cutting eyelids of ~10 pups, needles appear to become dull. Change the needle to a new one, or clean the needle tips with a kimwipe and 70% ethanol.
16. We usually transfect DNA into only one eye per animal. It is possible to electroporate DNA into both eyes, but there must be more than 1-h interval between electroporations in the same animals. This is particularly true when newborn mouse pups are used. Shorter interval electroporations in the same animal occasionally damage the operated pups.
17. When electric pulses are correctly applied, slight twitching of the muscles can be observed.
18. Almost all operated pups survive and are apparently healthy after electroporation. In our hands, an average of more than 80% rat retinas and more than 50% mouse retinas express GFP, when GFP expression vector is used. In a good transfection, GFP expression is observed in a wide area of the retina (Fig. 19.3).
19. When a heat lamp is used to warm the pups, the temperature has to be carefully monitored. If the distance between the lamp and the pups is too close, the pups can die because of high temperature.
20. The lens may be removed from the retina at this step. But if the lens is attached to the retina, scleral side and vitreal side can be easily distinguished. In the flattened retina, it is often hard to distinguish these two sides. In addition, the lens prevents the retina from

directly touching the electrode if the orientation of the retina in the chamber is as shown in Fig. 19.5B.

21. When the retina touches the electrode during electroporation, the contacted area may be only slightly (but not seriously) damaged.
22. When the CAG promoter is used to express GFP, GFP can be visible 24 h after electroporation, but the expression is low at this time point. Maximum expression is observed a few days after electroporation.
23. Lens may be removed at this step. But if the lens is removed before fixation, the retina tends to curl up during the following procedures. Good shape (cup-like structure) is maintained when the lens is removed from the retina after fixation.
24. Fluorescence of GFP (RFP) diminishes by fixation but recovers during the cryoprotection procedure. Overnight fixation with 4% paraformaldehyde significantly reduces the brightness of GFP (RFP) and is not recommended.
25. Long cryoprotection (more than 12 h) increases the level of autofluorescence, particularly in the photoreceptor outer segments.
26. Do not suspend the retina by pipetting at this step. Pipetting at this step significantly decreases the viability of the dissociated retinal cells.
27. If serum is not desired in the experiments, 10% FCS can be substituted with bovine serum albumin (1 mg/mL; BSA).
28. If medium is added without finger tapping, resuspension of the cell pellet becomes hard.

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