Retinal Gene Delivery by rAAV and DNA Electroporation

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ABSTRACT

Ocular gene therapy is a fast-growing area of research. The eye is an ideal organ for gene therapy since it is immune privileged and easily accessible, and direct viral delivery results primarily in local infection. Because the eye is not a vital organ, mutations in eye-specific genes tend to be more common. To date, over 40 eye-specific genes have been identified harboring mutations that lead to blindness. Gene therapy with recombinant adeno-associated virus (rAAV) holds the promise to treat patients with such mutations. However, proof-of-concept and safety evaluation for gene therapy remains to be established for most of these diseases. This unit describes the in vivo delivery of genes to the mouse eye by rAAV-mediated gene transfer and plasmid DNA electroporation. Advantages and limitations of these methods are discussed, and detailed protocols for gene delivery, required materials, and subsequent tissue processing methods are described. *Curr. Protoc. Microbiol.* 28:14D.4.1-14D.4.32. © 2013 by John Wiley & Sons, Inc.

Keywords: retina • eye • gene therapy • gene transfer

INTRODUCTION

Gene therapy of the eye offers the promise of treating many genetic and age-related blinding diseases. The eye is considered a prime target for gene therapy since it is a relatively isolated and immune-privileged organ. The field of ocular gene therapy received its biggest boost in 2001. Dogs, whose eyes are comparable to humans in size, were successfully treated for Leber's congenital amaurosis-2 with a rAAV vector (Acland et al., 2001). The disease is caused by a mutation in the retinal-pigmented epithelium (RPE) protein 65. One of the dogs, named Lancelot, became a media star when he visited Congress.

Delivery of rAAVs to the eye is accomplished by either intravitreal or subretinal injection. Intravitreal injections preferentially infect cells closer to the ganglion cell layer, such as ganglion cells and inner nuclear layer (INL) cells, while subretinal injections tend to infect photoreceptors (PR) and RPE cells. INL cells can also be targeted by subretinal injections depending on the titer and serotype. The protocol describes both the subretinal and intravitreal injection methods for delivery of rAAV to the mouse eye. Methods for injection into both early post-natal (Basic Protocol 1, Alternate Protocol) and adult (Basic Protocol 3) mouse eyes are described. In addition, delivery of plasmid DNA by electroporation at post-natal day 0 is also described (Matsuda and Cepko, 2004; Basic

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Protocol 2). This method targets mainly dividing cells; therefore, only cells born from the time of electroporation onwards will be transduced. This includes rod photoreceptor cells, bipolar cells, Muller glia cells, amacrine cells, and, at very low frequency, horizontal cells. Electroporation of plasmid DNA circumvents viral production and can thus be used as a fast method to test the promoter activity of the viral construct or to test if overexpression of a protein may have a beneficial effect in a retinal degenerative disease model. However, it is not a viable therapeutic approach to treat humans, nor is it useful for testing cell tropism of rAAVs. The protocols presented here discuss advantages and disadvantages of these different methods and describe injection tools that accommodate different budgets (Alternate Protocol). Additionally, tissue preparation (Support Protocols 1 and 2) and processing for immunofluorescence (Basic Protocols 5 and 7) and in situ hybridization analyses (Basic Protocols 6 and 8) on either whole-mount or cryo- and paraffin sections are described. Basic Protocol 4 describes the use of a funduscope to monitor the transduced retinal cells.

CAUTION: Adeno-associated virus is a Biosafety Level 1 (BSL-1) pathogen because neither rAAV nor wild-type AAVs are known to cause disease in humans. BSL-1 status assumes that the rAAV construct does not encode for a gene that is toxic or cancerogenic, and that it is produced without a helper virus. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. The Institutional Biosafety Committee at the institution where the research is being conducted should approve all biosafety protocols.

CAUTION: rAAV-mediated gene transfer to the eye is an Animal Biosafety Level 1 (ABSL-1) procedure. Protocols using live animals must be reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) and must adhere to governmental regulations regarding the use and care of animals. The protocols described here have been approved by the IACUC of the University of Massachusetts Medical School and conform to officially approved procedure for proper care and use of laboratory animals.

BASIC PROTOCOL 1

DELIVERY OF rAAV BY SUBRETINAL AND INTRAVITREAL INJECTION INTO EYES OF NEWBORN MICE

This protocol describes the delivery of rAAVs to the subretinal or intravitreal space of newborn mice. The advantage of subretinal delivery of virus or plasmid DNA at birth is that PR outer segments are not formed yet. This means that the subretinal space, between the RPE and the ONL, is an actual space in which fluid can be injected and can spread. Thus, the biggest advantage of this method is that the entire retinal surface area can be infected. However, at the same time this poses the following disadvantages. First, the eye of the newborn is smaller in size than that of older mice, and targeting the subretinal space correctly may be more challenging. Second, since the retina is still developing, injections that result in too much damage may complicate the interpretation of your results if development is interrupted. For example, injections with phosphate-buffered saline (PBS) in a retinal-degeneration model may result in a protective effect (delay of photoreceptor cell death) from the physical damage to the tissue. Thus, if you study photoreceptor degeneration, it is important to perform enough control injections to account for technical variations of the procedure. An extreme case of neuroprotection occurs when too much fluid is injected into the subretinal space. In such a case, the adult retina can take on the shape of a cone instead of a half sphere. This tends to lead to a more profound protective effect. Both procedures, intravitreal and subretinal, can result in cataracts, and in the worst case in an arrest of eye development.

Intravitreal injections tend to be easier, since the targeting area is larger. Injections can be performed with glass needles or metal needles (Hamilton), and in both cases the

injection route can be either at the intersection of the cornea and sclera or through the sclera. Injections with a glass needle through the sclera directly target the subretinal space. If you prefer that route for vitreal delivery, you need to push the needle through the retina. Injecting at the junction of the cornea and sclera targets the vitreous. However, the same route can also be used for subretinal injections. Basic Protocol 1 will detail injections with glass needles either through the sclera for subretinal injections or through the cornea-scleral margin for subretinal and vitreal injections. This is the most effective route and the one we recommend for delivery. The proper use of fine glass needles results in normal retinal morphology, since it is the least invasive procedure. The alternative protocol will present different tools such as Hamilton syringes and different routes of injection. As you develop your skills, you may prefer one method over the other.

Summary of neonatal injection procedure

Inject naturally delivered neonatal pups subcutaneously with buprenorphine (0.1 mg/kg) 1 hr prior to the procedure. After 1 hr, anesthetize pups by hypothermia by placing the pup onto a dry rubber glove over ice. After 2 to 3 min, place the pup onto a clean paper towel under the dissecting microscope. Clean the skin over the eyelid with Betadine, followed by water and 70% ethanol, using cotton swabs. Cut the skin over the eye with a sterile 30-G needle in the area where the future eyelid develops. If performed properly, the incision will not result in bleeding, as this region is undergoing cell death. Push back the skin gently to the side with a pair of sterile forceps to expose the eyeball. Inject by inserting a beveled glass needle directly into the eyeball. Close the eyelid gently with a cotton swab soaked with Betadine and place the pup onto a warm heating mat until fully recovered, then return it to the mother.

Materials

Naturally delivered newborn mouse pups (0- to 1-day old) of any strain
Buprenorphine
Virus: rAAV can be produced in-house (*UNIT 14D.1*), or obtained through a local vector core or via a commercial manufacturer
0.05% (w/v) Fast Green solution (Sigma, cat. no. F-7252, or equivalent)
Betadine
70% ethanol

Insulin injection needles
Weight trays appropriate for mice
Heating plate/mat
Rubber gloves
Ice bucket
Glass needles (Humagen Custom O from Origio, *http://www.origio.com/*)
Microloader (pipet tips to load the glass needle; Eppendorf, cat. no. 930001007, or equivalent)
Dissecting microscope with appropriate light source
Cotton swabs
Injection pump (FemtoJet from Eppendorf)
30-G disposable needles
Forceps (student Dumont no. 5 work well)

Additional reagents and equipment for injection of mice (Donovan and Brown, 2006a)

1. Remove naturally delivered mouse pups from the mother (all at once) and inject each pup subcutaneously (Donovan and Brown, 2006a) with buprenorphine (0.1 mg/kg) using an insulin injection needle.

   Buprenorphine is an analgesic that alleviates pain during and after the procedure.

2. Place pups in a weight balance tray on a warm (37°C) heating mat for 1 hr.
3. Meanwhile set up your injection station (see Video 14D.4.1 at *http://www.currentprotocols.com/protocol/mc14D04*).
4. Prepare virus mix. Per 10 µl virus solution add 2 µl of 0.05% Fast Green in a microcentrifuge tube.

   The titer should be at least 5×10^{11} genome copies/ml; however, we recommend injecting with a titer of 1×10^{12} to 5×10^{13} genome copies/ml, depending on how many cells you intend to transduce.
5. Microcentrifuge mixture 2 min at maximum speed, room temperature.

   This will remove any small debris that could clog the glass needle.
6. Transfer supernatant into a fresh microcentrifuge tube and place on ice.
7. After 1 hr, anesthetize one pup on ice for 2 to 3 min by placing it on a rubber glove over ice.

   Ice anesthesia has multiple advantages. Pups recover faster, and reducing the body temperature results in vasoconstriction, which reduces potential bleeding from the procedure. Additionally, reducing the body temperature results in an opaque lens, which helps with visualizing the eyeball under the skin.
8. Meanwhile, load the glass needle with a microloader (load 10 µl of virus solution) and mount onto handheld injection device.
9. Once the pup is anesthetized, move it onto a paper towel under the dissecting microscope, placing it on its side with one eye facing up.
10. Clean the skin over the eye with a cotton swabs soaked in Betadine, followed by water and 70% ethanol.
11. With the thumb and the index finger of your left hand (if you are right-handed), stretch the skin over the eye while gently moving a 30-G needle with your right hand over the scar tissue of the future eyelid (see Video 14D.4.1 at *http://www.currentprotocols.com/protocol/mc14D04*). Hold the needle such that the beveled edge functions as a knife (you can also use a scalpel for this step).

    It is not necessary to cut completely through the skin, as stretching the skin with your left hand will open up the cut.

    If you cut too deeply, you may damage the cornea, and if you cut too close to the edge of scar, the tissue may bleed.
12. Once you have a small opening, insert a pair of forceps in a closed position and let it open gently. This will further open the initial incision to the edge of the future eyelid without bleeding (see Video 14D.4.1 at *http://www.currentprotocols.com/protocol/mc14D04*).

    At this point there are two options, depending on the injection route and injection needle. You may pop out the eyeball to better expose the sclera for injections into the subretinal space from the sclera (recommended). You may also choose to use the same procedure to inject into the vitreous by pushing your needle through the retina. To use these procedures, continue with step 13a (see Video 14D.4.1 at http://www.currentprotocols.com/protocol/mc14D04).

    For direct injections into the vitreous without damaging the retina, you do not need to pop out the eyeball; you can just push the skin to the side with your thumb and index finger. You can also use this procedure to inject into the subretinal space. To perform the procedure without popping out the eyeball; continue with step 13b.

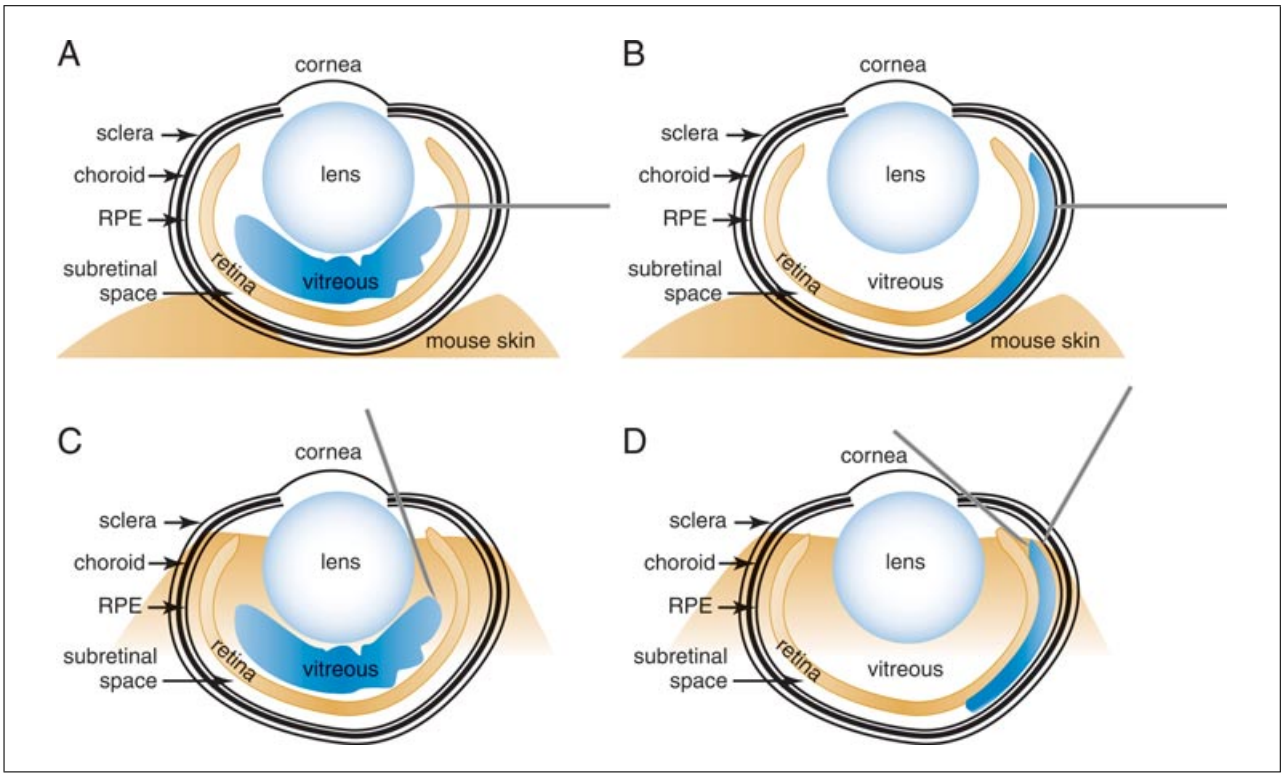

Figure 14D.4.1 Basic Protocol 1. Schematic of injection routes for newborn mouse pups using glass needles. (**A**-**D**) Cartoons of mouse eyes showing sclera, choroid, RPE, cornea, lens, retina, subretinal space, and injected solution (blue). (A, B) Example of injection routes for vitreal (A) and subretinal (B) injections into eyes that are popped out. (C, D) Injection routes for vitreal and subretinal injection if eyes are not popped out. All injection routes shown here can also be used in adult mice depending on how much the eyeball is being exposed. For the color version of this figure, go to *http://www.currentprotocols.com/protocol/mc14D04*.

For injections by popping out the eyeball

13a. Push the skin to the side with your thumb and index finger, and with the forceps in your right hand, push the skin further to the side to expose the eyeball (you can either move your forceps from the left to the right of the eye by pushing down on the skin or open the forceps and push simultaneously left and right). Once the eyeball pops out, hold the skin pushed down with your thumb and index finger (see Video 14D.4.1 at *http://www.currentprotocols.com/protocol/mc14D04*).

14a. Inject into the subretinal space with your right hand. Hold the needle tip perpendicular to the sclera to optimize the force at the tip of the needle. Gently push the needle into the sclera.

If an air pump is used for injections and the back flow pressure is set to 0, after entering the eyeball, the solution in the needle (which appears blue due to the Fast Green) will be slightly pushed back by the intraocular pressure. This indicates that you are in the eye (see Video 14D.4.1 at http://www.currentprotocols.com/protocol/mc14D04).

15a. Slowly inject 0.5 to 1 µl of virus solution.

IMPORTANT NOTE: *Follow the (blue) solution visually when injecting. If the center of the eye immediately turns blue, then your needle entered too far and the needle tip crossed the retina, meaning you are* ***injecting into the vitreous*** *(Fig. 14D.11A). If you see the sclera bulging in a blue color, then your needle tip did not cross the entire sclera and you are not in the subretinal space yet. If the solution is spreading slowly across the eyeball as seen through the lens, then you are in the* ***subretinal space*** *(Fig. 14D.4.1B). If you inject too much volume, once you pull out the needle, the pressure in the eye may push back some of the injected material. This may be the case if you attempt to transduce*

the entire retina. TRICK: Before injecting the virus, poke a hole into the sclera with the glass needle. Afterwards, reposition the needle and perform the injection. The first hole will allow some of the pressure to escape while you are injecting.

16a. After pulling back the needle, close the eyelid with a cotton swab soaked with Betadine.

17a. Place the pup back onto a tray on a heating mat at 37°C.

Mice will start to gasp within 2 to 3 min after removing them from the ice. The anesthetized pup turns purple, and once the pup recovers, it turns pink. It takes less than 1 min to inject one eye. Viral injections can be performed into both eyes. If the pup is waking up while you are attempting to inject the second eye, place it briefly back on ice.

18a. Repeat the procedure with the entire litter. Once all pups are injected and fully recovered return them to the mother all at once.

For injections without popping out the eyeball

13b. Push the skin to the side with your thumb and index finger of your left hand to expose the cornea and part of the sclera.

14b. To inject into the vitreous, insert the needle with your right hand at the margin of the cornea and the sclera (Fig. 14D.4.1C). Inject slowly using titers and volumes as described above—the Fast Green dye should immediately cover the space under the lens rather than spread slowly through the eye. Hold the needle such that it passes by the lens without damaging the lens. After injecting, remove the needle and follow steps 16a to 18a, above.

15b. To inject into the subretinal space, either target the visible area of the sclera or the margin between the cornea and the sclera (Fig. 14D.4.1D). Hold the needle at the appropriate angle to target the subretinal space without damaging the lens (Fig. 14D.4.1D). Inject slowly using titers and volumes as described above—the Fast Green dye should spread slowly across the eyeball. After injecting remove the needle and follow steps 16a to 18a, above.

BASIC PROTOCOL 2

DELIVERY OF PLASMID DNA BY SUBRETINAL INJECTION INTO EYES OF NEWBORN MICE

This protocol describes the transduction of retinal cells by electroporation of plasmid DNA. Since electroporation of plasmid DNA into mitotic cells is more efficient than into post-mitotic cells, the DNA needs to be delivered in proximity to dividing cells before retinal development is completed. Dividing cells in the retina are located close to the subretinal space; thus, the same procedure as described in Basic Protocol 1 can be used. At post-natal day 0, mitotic cells give rise to rods, Muller glia, bipolars, and amacrines. Electroporation at post-natal day 0 will thus result in transduction of only these cell types once retinal development is completed. Because most of the post-natal cells are born within a short window of time after birth, electroporation of plasmid DNA needs to be performed ideally within the first 24 hr after birth. Electroporations at post-natal day 3 will barely yield any transduced cells. This contrasts transduction of cells by rAAV infection, which can be performed at any time.

Materials

Plasmid DNA (concentration, 1 to 5 μg/μl)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Square-filed electroporator (e.g., Harvard Apparatus, cat. no. 450052)
Tweezer electrodes (e.g., Harvard Apparatus, cat. no. 405166)

Additional reagents and equipment for delivery of rAAV by subretinal and intravitreal injection into eyes of newborn mice (Basic Protocol 1)

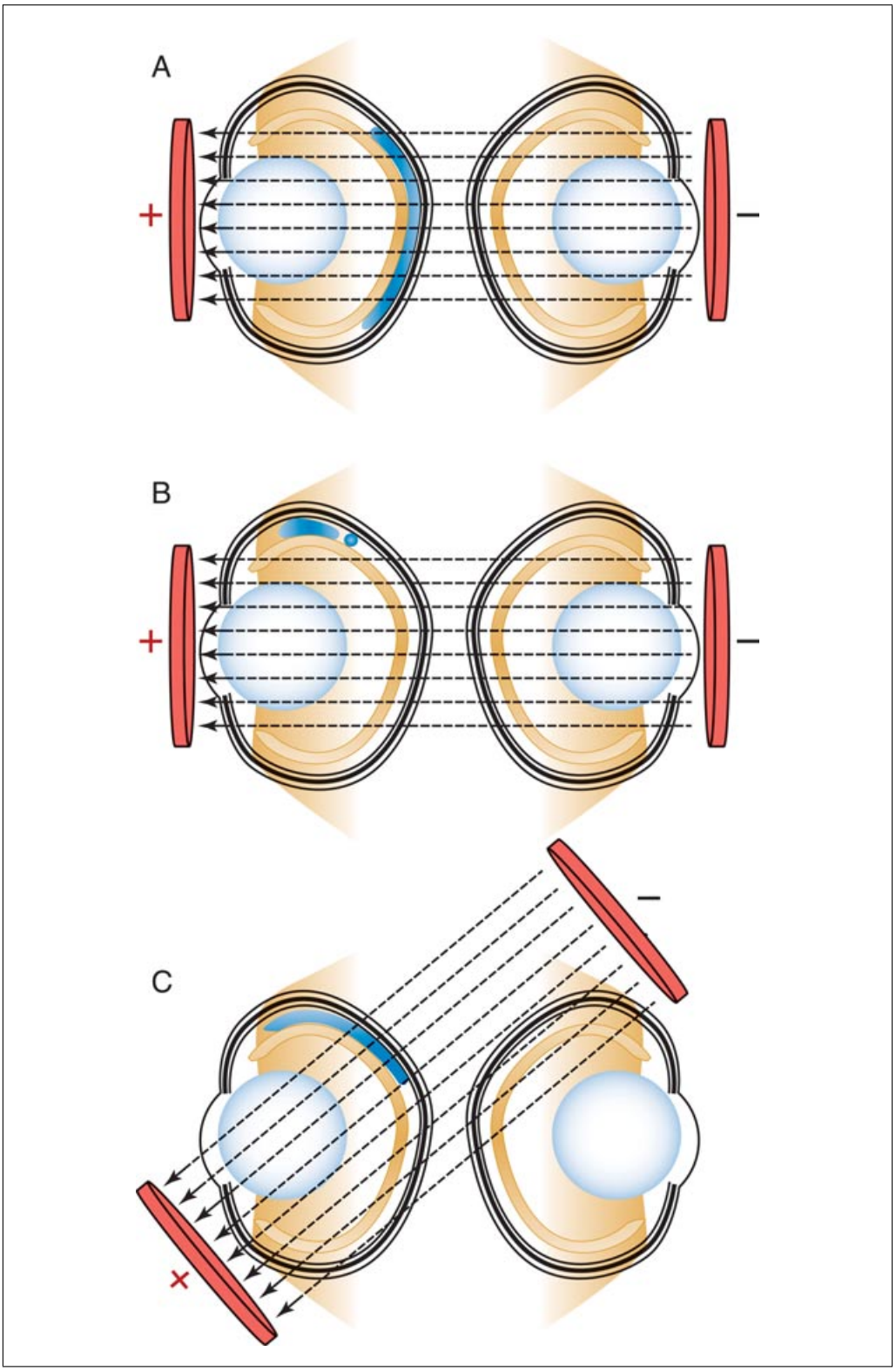

Figure 14D.4.2 Basic Protocol 2. Positioning of tweezer electrodes for DNA electroporation at post-natal day 0. (**A**) Shows correct position of electrode pads when DNA is injected into the central region of the retina. (**B**) Shows wrong position of electrode pads for peripheral DNA injections. The electric field (arrows) generated in (B) will move the DNA along the path of least resistance. Only few cells will be efficiently electroporated and most of the DNA will move along the subretinal space. (**C**) Correct positioning of electrode pads for peripheral DNA injections.

1. Follow Basic Protocol 1 until step 15a or b. Inject a volume of 0.5 to 1 µl of 2 µg/µl plasmid DNA mixed with Fast Green. Use the procedure for subretinal injections. Try to inject the DNA as close as possible to the central region of the retina.
2. Close the eyelid with a cotton swab soaked in PBS.
3. Wet the tweezer electrodes in PBS (PBS increases conductivity between the electrode and the skin) and place them around the head of the pup such that each electrode pad covers one eye. When placing the tweezer electrodes, pay attention to the position of the electrode pads relative to the site of DNA delivery (Fig. 14D.4.2A).

   The DNA will move in the direction of the electrical field that is generated between the electrode pads and move along the path of least resistance. If you injected your DNA in the periphery of the eye, and the electrode pads are placed straight over the eye, then the DNA may move along the subretinal space instead of moving into the retina (Fig. 14D.4.2B). If you inject in the center of the eye, then placing the electrode pads straight over the eye is the correct approach (Fig. 14D.4.2A). If you injected laterally, then rotate the electrode pads around to generate an electrical field that is perpendicular to the retina and the injection site (Fig. 14D.4.2C).

   IMPORTANT NOTE: *The plus pole (+) of the electrode needs to be over the eye you injected with the DNA.*
4. Apply electrical field: 5 pulses at 80 V.

   Each pulse should be 50 msec long, with an interval of 950 msec.
5. Follow steps 16a to 18a described in Basic Protocol 1.

   IMPORTANT NOTE: *In contrast to viral injection, it is recommended that for electroporation of plasmid DNA, only one eye be injected. Injecting and electroporating the second eye generates an electrical filed in the opposite direction to the first eye and may reduce the efficiency of electroporation of the first eye.*

BASIC PROTOCOL 3 DELIVERY OF rAAV BY SUBRETINAL AND INTRAVITREAL INJECTION INTO THE EYE OF ADULT MICE

This protocol describes the delivery of rAAVs into the eye of adult mice. The delivery procedure is similar to the one described in Basic Protocol 1. Since the eye is already exposed, popping out the eyeball is not required. Subretinal injections into adult wild-type mice will always lead to retinal detachment, since the PR outer segment and RPE interactions that exist in adult mice are disrupted by the fluid that is injected. This contrasts with injections into newborn mice, as the retina is not attached to the RPE at that age because outer segments are not developed yet. Injecting mice with PR degeneration reduces the amount of retinal detachment caused by the injection, as part of the retina may already be detached due to the disease. However, this increases the efficiency of the viral spread. Targeting the vitreous of adult mice is straightforward. Injecting adult mice has the advantage that development is completed, which reduces some of the undesired procedural effects. Nonetheless, any injection, even PBS, can result in the release of various neuroprotective growth factors. Therefore, when injecting a virus that should result in a neuroprotective effect, enough control injections need to be performed to account for artifacts.

Materials

Adult mouse of any age and strain
Ketamine
Xylazine
Corneal lubricant ointment
Betadine
70% ethanol

Buprenorphine

Dissecting microscope with appropriate light source
Glass needles (Humagen Custom O from Origio, *http://www.origio.com/*)
Heating plate/mat

Additional reagents and equipment for delivery of rAAV by subretinal and intravitreal injection into eyes of newborn mice (Basic Protocol 1)

1. Anesthetize mouse by an intraperitoneal injection (Donovan and Brown, 2006a) of a ketamine/xylazine (100 mg/kg and 10 mg/kg) mixture. Test depth of anesthesia by a sharp tail pinch.
2. Place mouse on a clean paper towel under the dissecting microscope and apply corneal lubricant ointment to protect the cornea. Then, clean the eyelid with Betadine, followed by water and 70% ethanol.
3. Gently push down the skin of the eyelid to better expose the eyeball.
4. Insert the glass needle from the scleral side if you intend to inject into the subretinal space. Inject virus as described in Basic Protocol 1 with the same recommended titer.
5. If you intend to target the vitreous, insert the needle at the margin of the sclera and cornea to inject the virus.
6. After injection clean the eyelid and inject mouse subcutaneously with buprenorphine (0.1 mg/kg) to alleviate pain.
7. Place mouse back into its cage and place cage onto a warm heating plate (37°C) until the mouse is fully recovered.

SUBRETINAL AND INTRAVITREAL INJECTION WITH HAMILTON SYRINGES

ALTERNATE PROTOCOL

This protocol describes an alternate injection tool and route of delivery for injection into newborn mice. The procedure is very similar to the one described in Basic Protocol 1 and can also be used for electroporation of plasmid DNA or for vitreal injections into adult mice. A less costly way to perform the experiment as described in Basic Protocol 1 is to use a handheld Pipetman that allows mounting a glass needle. Alternatively, a Hamilton syringe, which is also less costly, can be used instead of a glass needle with an injection pump. Here we discuss the use of a Hamilton syringe in combination with a blunt metal needle. Pointy (beveled) metal needles are also available for Hamilton syringes. These needles can be used to perform the injection as described in Basic Protocol 1. The advantage of metal needles is that they do not break easily. However, given the larger size of the needle, there is more damage to the tissue. The procedure below explains the use of the Hamilton syringe with a blunt metal needle. This requires a procedure that has been slightly modified from the one explained in Basic Protocol 1.

***Additional Materials** (also see Basic Protocol 1)*

Virus or DNA
Phosphate-buffered saline (PBS; *APPENDIX 2A*; if using DNA)

Hamilton syringe with blunt needle
Square-filed electroporator (e.g., Harvard Apparatus, cat. no. 450052; if using DNA)
Tweezer electrodes (e.g., Harvard Apparatus, cat. no. 405166; if using DNA)
Hamilton syringe with blunt needle

Additional reagents and equipment for delivery of rAAV by subretinal and intravitreal injection into eyes of newborn mice (Basic Protocol 1)

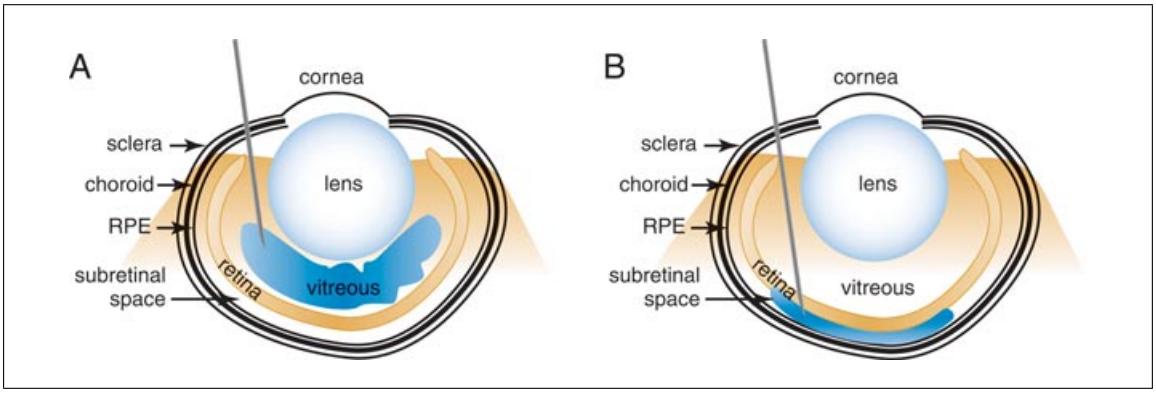

Figure 14D.4.3 Alternate Protocol. Schematic of vitreal and subretinal injection routes into newborn mouse pups using a Hamilton syringe with a blunt needle. (**A, B**) Cartoons of mouse eyes as shown in Figure 14D.4.2. (A) Example of injection route for vitreal injection. (B) Injection route for subretinal injection. The blunt Hamilton needle is pushed through the retina. The needle stops at the sclera if not pushed too hard due to the tougher composition of the sclera. The injection route shown in (A) can be used for adults and neonates, the one shown in (B) is only recommended for neonates.

1. Follow Basic Protocol 1 until step 12.
2. Push the skin to the side with your thumb and index finger of your left hand to expose the cornea of the eye and part of the sclera.
3. With a 30-G needle, poke a small hole at the margin of the sclera and cornea.

   This step is necessary because the Hamilton syringe has a blunt needle tip.
4. Insert the needle loaded with virus or DNA into the hole and move the needle tip past the lens towards to central part of the eye (Fig. 14D.4.3A). Inject virus if the vitreous needs to be targeted. After injecting the virus pull back the needle and continue with Basic Protocol 1 step 16a.
5. If you want to target the subretinal space, push the needle further in until it crosses the retina (Fig. 14D.4.3B).

   Because the needle is blunt it will not cross the sclera. This means that once you hit resistance you can inject the fluid, which will disperse into the subretinal space. This procedure causes more damage as you physically create a hole in the retina through which the virus or DNA is injected.
6. After pulling back the needle continue with Basic Protocol 1, step 16a, if you are injecting a virus, or with Basic Protocol 2, step 2, if you are injecting plasmid DNA.

BASIC PROTOCOL 4

FUNDOSCOPY EXAMINATION TO MONITOR INFECTION AREA

This protocol describes the use of a funduscope to visualize retinal cells that have been transduced with a green fluorescent protein (GFP; Fig. 14D.4.4A). The technique is noninvasive and allows acquiring retinal photographs of mice that are anesthetized with a ketamine/xylazine mixture. Visualizing the area of infection or electroporation is only possible if the expression cassette of your transgene also co-expresses GFP. The advantage of this protocol is that it allows selecting well infected or electroporated animals for further analysis. For example, 30 mice are injected with an rAAV that should delay PR death. Behavioral tests and/or electroretinograms need to be performed to test if the viral transgene leads to improved vision. Preselecting the 10 best-infected mice reduces the overall workload. Additionally, fewer mice need be kept for extended periods of time if long-term effects of the viral transgene are to be tested. However, this procedure is not required for performing histological analyses of GFP-transduced retinas. We do not recommend purchasing such equipment to perform the gene delivery

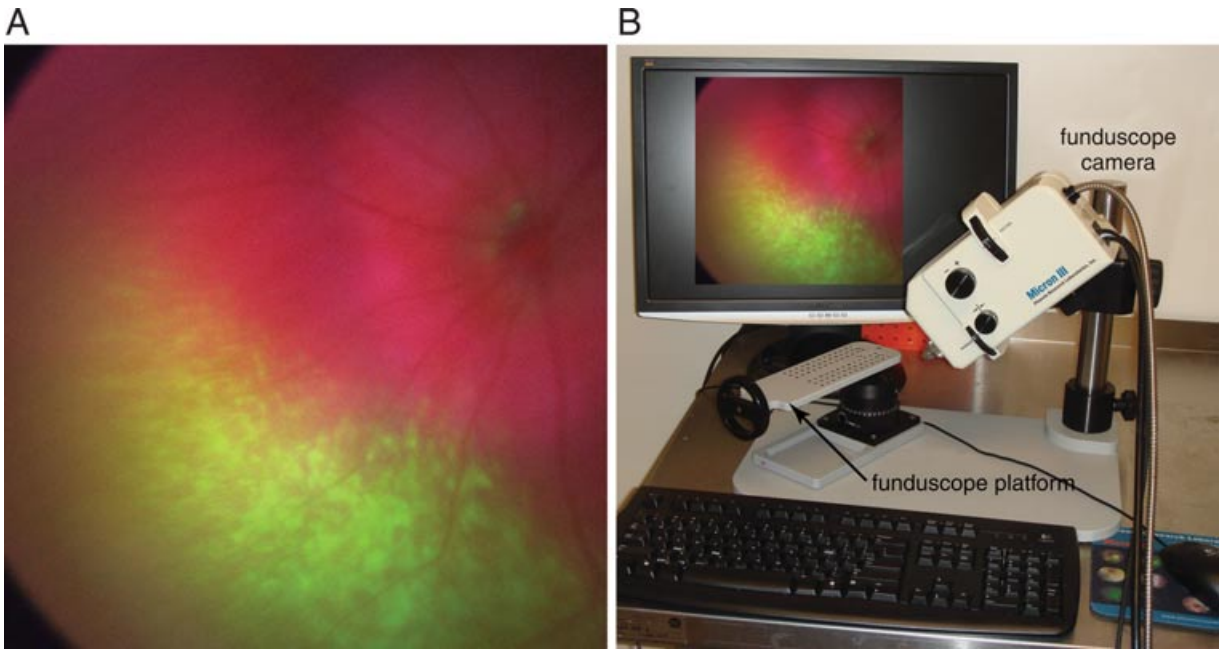

Figure 14D.4.4 Basic Protocol 4. Transduction efficiency as seen by fundoscopy. (**A**) Fundus image showing large area of GFP-positive cells that were infected with a GFP-expressing rAAV(2/5). Similar images can be seen after electroporation of a GFP expressing plasmid. (**B**) Set up of fundoscopy equipment.

protocols described here. Its use is recommended if your institute owns such equipment and if the experimental design benefits from such use.

Materials

Injected (Basic Protocol 1 to 3) mouse of any strain, at least 2 weeks post-injection
Phenylephrine (Akorn, cat. no. NDC: 17478-200-12, *http://www.akorn.com*)
Tropicamide (Akorn, cat. no. NDC: 17478-101-12, *http://www.akorn.com*)
Ketamine
Xylazine

Funduscope equipment (Micron III; Phoenix Research Laboratories Inc., *http://www.phoenixreslabs.com/*):
- Funduscope camera
- Appropriate light source
- Platform to position mouse
- Computer system and software

Heating plate/mat

Additional reagents and equipment for injection of mice (Donovan and Brown, 2006a)

1. Dilate pupils using one drop of phenylephrine (quick and short effect) and one drop of Tropicamide (slow and long effect).

   Since anesthesia decreases the body temperature of the mouse, which can cause the lens to become temporarily opaque, dilating the pupil in advance allows starting immediately after the mouse is anesthetized.

2. Once the pupils are dilated (5 to 10 min later), anesthetize mouse by an intraperitoneal injection (Donovan and Brown, 2006a) of a ketamine/xylazine (100 mg/kg and 10 mg/kg) mixture. Test depth of anesthesia by a sharp tail pinch.

3. Place mouse on funduscope platform (Fig. 14D.4.4B).

   A heating pad can be used to keep the mouse warm, which helps keep the lens transparent. This extends the period of time to perform a better examination. However, with some experience, the procedure is performed within minutes. Therefore, keeping the mouse warm during the procedure is not required.

4. Place camera directly on one eye by moving the platform into the appropriate position
5. Select appropriate wavelength and focus image.
6. Move the platform around to allow you to see different areas of the retina.
7. Acquire movie or individual images.
8. Place mouse back into its cage and place the cage onto a warm heating plate (37°C) until the mouse is fully recovered.

BASIC PROTOCOL 5

WHOLE-MOUNT IMMUNOFLUORESCENCE ANALYSES

This protocol describes the processing of the retina for whole-mount analysis. There are two ways the retina can be dissected and prepared, depending on the cell type that needs to be visualized. Both methods will be introduced at the beginning. Retinal cells can then be visualized either by whole-mount immunofluorescence or immunocytochemistry. These methods will be discussed in this protocol and rely on the use of an antibody that is either cell type specific or directed against the protein that is overexpressed as a result of the transduction of retinal cells. If no antibody is available, whole mount in situ hybridizations can be used to detect either the viral RNA or the mRNA of a cell type specific gene. This procedure will be described in Basic Protocol 6.

Summary of protocol steps

Dissect eye in PBS by removing the retina from the rest of the tissue. If target cells are photoreceptors, leaving the retina attached to the lens allows fixing the retina such that it retains its shape as a cup. The lens can be left on during the entire procedure and removed prior to mounting the retina. This avoids curling of the retina and results in better accessibility of the antibody at the periphery of the tissue. To target inner nuclear layer cells and ganglion cells, the lens has to be removed. If removed after fixation, most of the curling is prevented. Alternatively, if INL cells are targeted, the retina can be left on the lens and the incubation with the primary and secondary antibody can be performed over a period of 2 to 3 days each at 4°C. A longer incubation time allows for better penetration of the antibody to the cell in the center of the tissue. However, the length of time needs to be established for each individual antibody. If not otherwise indicated, all steps are performed at room temperature.

Materials

Injected (Basic Protocol 1 to 3) or noninjected mouse of any strain
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
4% PFA/PBS: 4% paraformaldehyde in PBS, pH 7.4
PBT: PBS with 0.3% (v/v) Triton X-100
PBTB: PBT (see above) with 5% (v/v) bovine serum albumin (BSA)
Primary and secondary antibodies
Appropriate reagents for color development of secondary antibody
Mounting medium: e.g., Gel Mount, Fluoromount (Sigma-Aldrich)
Clear nail polish (optional)

CO_2 chamber for euthanasia (see Donovan and Brown, 2006b)
Forceps (student Dumont no. 5 work well)

6-cm Petri dish
Dissecting scope with light source
Small spring scissor
Cover glasses
Glass slides
Fluorescence upright or inverted microscope

Additional reagents and equipment for euthanasia of mice (Donovan and Brown, 2006b)

1. Euthanize mouse using procedure that has been approved by the IACUC of your institution (Donovan and Brown, 2006b).

2. Remove the eyeball (enucleate) by first pushing down the skin around the eyeball. Then, with a pair of forceps in the open position, slide each arm of the forceps around the eyeball all the way down to the optic nerve. Close the forceps to hold the eye at the bottom (optic nerve) and pull out the eyeball—part of the optic nerve and some muscle and connective tissue that regulate eye movement may be attached to the eyeball. Place eye in a 6-cm petri dish filled with PBS.

   The authors would recommend submerging the eye completely in PBS. At all times during the procedure, try to avoid squeezing the eyeball, as this may damage the retina (see Video 14D.4.2 at http://www.currentprotocols.com/protocol/mc14D04).

Dissect out the retina

Steps 3 to 5 are performed under a dissecting microscope.

3. With your right hand (if you are right-handed), poke a hole at the margin of the sclera and cornea using one arm of the forceps as a needle (you may also use a needle for this step). To hold the eyeball stable, with your left hand, open the forceps and place it behind the optic nerve.

   While you are pushing with your right hand, the eye will not roll away, as the forceps in your left hand will hold it in place (see Video 14D.4.2 at http://www.currentprotocols.com/protocol/mc14D04).

4. Once one arm of the forceps has entered into the eyeball, close the forceps. Insert one arm of the other forceps (left hand) into the hole and close the forceps. Gently move the forceps apart to tear the eyeball along the sclera-corneal margin. Reposition the forceps along the tear and continue until cornea is removed.

5. Insert one arm of each forceps between retina and sclera. Close forceps and gently tear the sclera apart to expose the retina. Once the retina is exposed up to the optic nerve, pinch off the optic nerve without damaging the retina. Continue to remove the sclera until the retina is completely exposed.

   If the procedure worked well your retina will stay attached to the lens (see Video 14D.4.2 at http://www.currentprotocols.com/protocol/mc14D04).

6. Transfer tissue to a 1.5-ml microcentrifuge tube or scintillation vial containing 4% PFA/PBS using transfer pipets and fix for 30 min at room temperature.

   This step can be done in a 1.5-ml centrifuge tube or scintillation vial.

   IMPORTANT NOTE: *For this and all following incubation steps it is recommended that the tube/vial be in gentle motion (rocker, Nutator, etc.).*

7. Wash three times, each time for 10 min, in PBS.

8. If your antibody staining will target photoreceptors, you can leave the lens attached—in this case, proceed with step 10.

9. If you need to target ganglion cells, remove the lens from the retina as follows. Place the tissue back in to a petri dish with PBS. With both forceps, pinch the outer membrane of the lens (lens capsule) and tear open the capsule to remove the lens. Then gently remove the rest of the tissue (lens capsule and ciliary margin) from the retina (see Video 14D.4.2 at *http://www.currentprotocols.com/protocol/mc14D04*). Place the retina back into a centrifuge tube or scintillation vial.
10. Permeabilize tissue for 30 min in PBT.
11. Block for 1 hr in PBTB.
12. Incubate over night at 4°C with primary antibody diluted in PBTB.
13. Wash three times 20 min in PBTB.
14. Incubate for 2 hr with secondary antibody diluted in PBTB.
15. Wash four times 30 min in PBTB.
16. If your secondary antibody requires a colorimetric reaction (e.g., antibody is coupled to alkaline phosphatase or horseradish peroxidase), proceed according to the manufacturer's recommendations for such reaction. Otherwise mount retina as described in step 17.
17. If you have not removed the lens yet, remove the lens as described in step 5. After removing the lens:
    a. Place the retina on a cover glass (photoreceptor side down, ganglion-cell-side-up). Ensure that the retina is covered with enough fluid (large drop).
    b. Under a dissecting microscope, with, a small scissor, make four incisions at an angle of 90° (see Video 14D.4.2 at *http://www.currentprotocols.com/protocol/mc14D04*). The incision should be half the distance from the optic nerve head to the periphery.
    c. To flat mount the tissue, you will need to roll each quadrant outward—for this, first decrease the volume of the drop by holding a Kimwipe into the drop. Remove fluid until retina is barely covered. Then, take two Kimwipes and roll the corner of each sheet between your fingers to generate a pointy tip. Wet the tip with PBT and roll again to remove excess fluid.
    d. Using the two Kimwipe tips, gently roll the retina flat. Once the retina is flat and looks like a clover leaf, add mounting medium to one edge of the cover glass. Do not add it directly on top of the retina, as the quadrants may curl back.
    e. Use a second cover glass and slowly move it downwards starting from the edge where you placed the drop.

       The retina is now mounted between two cover glasses.

    f. Depending on the cell type you need to image, place the cover glass sandwich on a glass slide with the cell type of interest facing up. To prevent the cover glass sandwich to move around, wet the glass slide. Seal the edge of the cover glass if desired with nail polish.

       To prevent the retina from being squeezed too much between the cover glasses you can also break a third cover glass and place a piece of glass left and right of the retina. The glass functions as a spacer between the two cover glasses.
18. Analyze retina with a fluorescent upright or inverted microscope.

    Depending on the intensity of the staining, a minimum magnification of 10× or higher may be required. If the fluorescent staining is intense with a low background, the fluorescent flat mount can also be imaged on a fluorescent dissecting scope.

BASIC PROTOCOL 6

WHOLE-MOUNT IN SITU HYBRIDIZATIONS

This protocol is very similar to Basic Protocol 5; however, gene expression is revealed by in situ hybridization. We recommend removing the lens last after the hybridization step even if PRs are targeted. This avoids background from probe that is trapped between the lens and the retina. Similar to the antibody staining protocol, the incubation steps work best if the tissue is in motion during incubation. Make sure that all your solutions up to the hybridization step are free of RNase. Treat PBS over night with DEPC (diethylpyrocarbonate, dilution 1:1000) and autoclave next day. Make all your solutions for Day 1 in DEPC treated PBS (except 4% PFA/PBS). If not otherwise noted all steps are performed at room temperature.

Materials

10× transcription buffer (supplied with the RNA polymerase)
10× nucleotide labeling mix (Roche; DIG labeling mix, cat. no. 11277073910)
Template DNA: linearized plasmid DNA or PCR product; not shorter than 200 bp, ideally between 500 and 1000 bp
RNase inhibitor (Roche, cat. no. 03335399001)
RNA polymerase (Roche): T7, T3, or SP6, depending on the PCR primers used to generate the PCR product; make sure to take the correct RNA polymerase to synthesize antisense RNA
H_2O, DEPC-treated (see recipe for DEPC treatment)
RNase-free DNase (Roche, cat. no. 04716728001)
TE buffer, pH 8 (*APPENDIX 2A*), DEPC-treated (see recipe for DEPC treatment)
4 M LiCl, prepared with DEPC-treated H_2O (see recipe for DEPC treatment)
100% and 70% ethanol
Hybridization buffer (HB; for whole-mount in situ hybridization; see recipe)
4% PFA/PBS: 4% paraformaldehyde in PBS, pH 7.4
PBS/Tween: 0.1% (v/v) Tween-20 in phosphate buffered saline (PBS; *APPENDIX 2A*) that has been treated with DEPC (see recipe for DEPC treatment)
100% methanol
10 mg/ml proteinase K stock (store at −20°C)
20× SSC (*APPENDIX 2A*)
10 mM PIPES/500 mM NaCl, pH 6.8
MAB: 100 mM maleic acid, pH 7.5/150 mM NaCl
Blocking reagent (Roche, cat. no. 11096176001)
MABT: MAB (see above) containing 0.1% (v/v) Tween-20
Antibody: α-digoxigenin antibody coupled to alkaline phosphatase (α-DIG-AP)
100 mM Tris·Cl, pH 9.5 (*APPENDIX 2A*) containing 0.1% (v/v) Tween-20
Staining solution (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*) containing 10 mM EDTA
Glycerol
Glass scintillation vials

Additional reagents and equipment for agarose gel electrophoresis (Voytas, 2001), for dissecting the retina (see Basic Protocol 5, steps 1 to 5), and for flat mounting of the retina (Basic Protocol 1, step 17)

RNA probe synthesis

Preparation of linearized DNA or PCR product is not described here, nor are the reagents and the equipment needed listed. Purify linearized DNA either by precipitation or by gel electrophoresis. Purify PCR product by gel electrophoresis. For probe synthesis, we recommend using PCR products that have been amplified from a standard cloning vector with a combination of the following primers: T7, T3, SP6.

1. For RNA probe synthesis mix the following reagents and incubate at 37°C for 2 hr:
   a. 2 μl of 10× transcription buffer.
   b. 2μl of 10× nucleotide labeling mix.
   c. *X* μl of template (∼1 μg of linearized plasmid DNA or 300 ng of PCR product; not shorter than 200 bp, ideally between 500 to 1000 bp).
   d. 0.5 μl of RNase inhibitor.
   e. 1 μl of RNA polymerase (Roche: T7, T3, SP6, depending on the PCR primers used to generate the PCR product; make sure to take the correct RNA polymerase to synthesize antisense RNA).
   f. *Y* μl of DEPC-treated water to make up the volume to 20 μl total.
2. After 2 hr, run 1 μl on an agarose gel (Voytas, 2001) to ensure that the synthesis reaction worked.
3. Add 1 μl of DNase (RNase free) to the remaining 19 μl and incubate at 37°C for 15 min.
4. Add 100 μl of DEPC-treated TE buffer, pH 8.
5. Add 10 μl of DEPC-treated 4 M LiCl.
6. Add 300 μl of 100% ethanol, mix well, and incubate overnight at −20°C.
7. Microcentrifuge 15 min at maximum speed (13,500 rpm), 4°C.
8. Wash pellet in 70% ethanol and air-dry pellet for 5 to 10 min.
9. Resuspend pellet in 20 μl of DEPC-treated water.
10. Add 80 μl of warm (65°C) HB, vortex, and store the resulting probe at −80°C. Before each use, warm up probe briefly at 65°C and vortex.

In situ hybridization

Perform fixation and all following steps in a glass scintillation vial.

Tissue preparation

11. Follow Basic Protocol 5, steps 1 to 5, to dissect the retina. Fix overnight at 4°C or at room temperature in 4% PFA/PBS for 3 hr.
12. Wash three times 10 min in PBS/Tween.
13. Wash three times, each time for 5 min, in 100% methanol (for dehydration of tissue).
14. Store in 100% methanol at −20°C for at least 1 hr (for permeabilization of tissue).

    Better results may be obtained if the tissue is stored for 1 week at −20°C in 100% methanol. Tissue can be stored up to several months in methanol.

Day 1: Pretreatment and hybridization

15. Rehydrate tissue in decreasing concentrations of methanol and increasing concentrations of PBS/Tween (methanol/PBS/Tween concentrations: 75%/25%, 50%/50%, 25%/75%, 0%/100%). Perform each gradient step twice, each time for 5 min and once for 10 min.
16. Treat tissue with proteinase K (10 μg/ml) in PBS/Tween for 20 min.
17. Rinse two times with PBS/Tween.
18. Post-fix tissue for 20 min with 4% PFA/PBS.

19. Wash four times, each time for 5 min, in PBS/Tween.
20. Wash two times 5 min in hybridization buffer (HB, for whole-mount in situ hybridization).
21. Incubate for 2 hr in HB at 65°C.
22. Add 1 μl of probe per 100 μl of pre-warmed HB (65°C), vortex tube, and replace HB solution with HB solution containing the probe.
23. Hybridize overnight at 65°C.

Day 2: Post-hybridization washes:

24. Remove probe with hybridization buffer and store at −80°C.

    The probe can be reused five to six times.

25. Wash two times, each time for 30 min in HB at 65°C.
26. Wash 10 min in 75% HB and 25% 2× SSC at 65°C.
27. Wash 10 min in 50% HB and 50% 2× SSC at 65°C.
28. Wash 10 min in 25% HB and 75% 2× SSC at 65°C.
29. Wash 10 min in 2× SSC at 65°C.
30. Wash 30 min in 0.2× SSC at 65°C.
31. Wash 10 min in 0.2× SSC at room temperature (from here on at room temperature).
32. Wash 10 min in 10 mM PIPES/500 mM NaCl.
33. Wash 10 min in MAB.
34. Block for 2 hr in MAB containing 2% blocking reagent.
35. Incubate overnight at 4°C in MABT with 2% blocking reagent and α-DIG-AP antibody diluted at 1:5000.

Day 3: Post-washes and detection

36. Wash six times, each time for 15 min in MABT.
37. Wash 30 min in 100 mM Tris·Cl pH 9.5, containing 0.1% Tween-20.
38. Incubate in staining solution in the dark.

    Depending on the quality of the probe, the expression level of the gene, and the site that was targeted for hybridization, the in situ hybridization signal can be visible after 30 min or can take several hours. Incubation can be extended overnight at 4°C.

    After signal has been detected, proceed with the following washes to inactivate the enzyme and clear the tissue.

39. Wash for 10 min in PBST.
40. Wash for 5 min in PBS with 10 mM EDTA.
41. Post-fix for 20 min in 4% PFA/PBS.
42. Wash three times 5 min in PBS/Tween.
43. Wash for 15 min in 30% (v/v) glycerol/70% PBS/Tween.
44. Wash for 15 min in 60% (v/v) glycerol/40% PBS/Tween.
45. Wash for 15 min in 90% glycerol/10% PBS/Tween.

46. Flat mount retina as described in Basic Protocol 5, step 17, using glycerol as the mounting medium.

SUPPORT PROTOCOL 1

DISSECTION AND TISSUE PROCESSING FOR CRYOSECTIONING

This protocol describes the processing of the retina for cryosectioning. There are two ways the retina can be dissected and prepared, depending on the need to retain the RPE attached to the retina. If the RPE is not needed, we recommend the dissection method described in Basic Protocol 5, which initially leaves the lens attached. However after fixation, the lens needs to be removed prior to performing the sucrose gradient and the embedding. Leaving the lens attached ensures a nice cup-shaped retina. If you need to retain the RPE attached, follow the dissection method described here. Detection of gene expression by immunofluorescence and in situ hybridization on sections will be presented in Basic Protocols 7 and 8, respectively.

Materials

4% PFA/PBS: 4% paraformaldehyde in PBS, pH 7.4
Phosphate-buffered saline, pH 7.4 (*APPENDIX 2A*)
10%, 20%, and 30% sucrose in PBS
OCT embedding medium (Tissue-Tek)
Dry ice/isopropanol bath

Dissecting microscope with light source
Rocker or Nutator
6-cm Petri dish
Forceps (student Dumont no. 5 work well)
Small spring scissor
Embedding molds
Cryostat

Additional reagents and equipment for removal of the mouse eyeball for whole-mount analysis (Basic Protocol 5, steps 1 and 2) and antibody staining (optional; Basic Protocol 7)

1. Euthanize mouse and remove eyeball as described in steps 1 and 2 of Basic Protocol 5. If RPE is not needed, proceed to step 5 of this protocol. Alternatively, if RPE-PR outer segment interactions need to be preserved, go to step 2.

2. Poke a hole at the margin of the sclera and cornea as described in step 3 of Basic Protocol 5.

   The hole can also be poked within the cornea, which leaves the very peripheral retina intact.

3. Transfer eyeball to 4%PFA/PBS in a 1.5-ml microcentrifuge tube or scintillation vial and fix for 10 min. Place tube on rocker or Nutator.

   Poking a hole before fixing the tissue helps to prevent shrinkage of the eyeball and results in faster exposure of the retina to the fixative.

4. Transfer eyeball back into a Petri dish with PBS and remove cornea and lens under dissecting scope.

   You can either perform the same procedure as described in Basic Protocol 5 by tearing apart gently, with two forceps, the margin between the cornea and the sclera. However, we recommend the following procedure. With a small spring scissor, enter into the hole and cut gently along the margin between the cornea and sclera until the cornea is removed (see Video 14D.4.2 at http://www.currentprotocols.com/protocol/mc14D04). If the very peripheral margin of the retina needs to be preserved, we recommend cutting on the

corneal side along the margin such that a small rim of the cornea remains attached to the sclera. Try not to touch the sclera; rather, hold the eyeball at the optic nerve or any residual tissue that is attached to the eyeball. While cutting with your right hand, hold the eye with a pair of forceps in your left hand. Once the cornea is removed, gently pull out the lens. Prefixing the eyeball ensures that the retina does not detach. Removing the cornea by cutting rather than by tearing the tissue results in less retinal detachment that can occur due to the procedure and therefore in better overall morphology.

5. Fix tissue overnight at 4°C in 4% PFA/PBS.
6. Wash tissue three times, each time for 10 min in PBS.
7. Equilibrate tissue in sucrose gradient by immersing the tissue in the following solutions for the indicated lengths of time.
   a. 10% sucrose in PBS for 2 to 4 hr at 4°C.
   b. 20% sucrose in PBS for 2 to 4 hr at 4°C.
   c. 30% sucrose in PBS overnight at 4°C.
8. Equilibrate tissue for 10 min in a 1:1 mixture of OCT:30% sucrose in PBS.
9. Equilibrate tissue in OCT for 10 min. Make sure the eyecup is filled with OCT by pipetting OCT into the eyecup using a 1000-µl (P-1000) pipet tip to push out the mixture of OCT and 30% sucrose in PBS.

   Frozen OCT is harder than the 1:1 mixture of OCT and 30% sucrose in PBS. The difference in density may result in poor overall morphology when sectioning.

10. Transfer into a mold with OCT.
11. Freeze mold with OCT and tissue on a mixture of dry ice/isopropanol.
12. Store block at −80°C until needed. Make sure to store it air-tight.

    Dehydration of the block causes the OCT to take on a rubber-like consistency over time.

13. Section on a cryostat at desired thickness. Collect sections on glass slides pretreated for cryosections.

    The average diameter of photoreceptors is around 5 to 6 µm; most other retinal cells are slightly larger. We therefore routinely section at a thickness of 14 to 20 µm for frozen sections.

14. Air dry sections for at least 30 min and then either proceed with step 16 or store sections at −80°C for later use.

    If only immunofluorescence analyses are performed sections can be stored −20°C.

15. If sections were frozen, acclimate sections for 20 min at room temperature before use.
16. Rehydrate section by washing three times, each time for 10 min in PBS.
17. If you want to perform an antibody staining follow Basic Protocol 7. Alternatively if you need to detect your gene of interest by in situ hybridization, follow Basic Protocol 8.

    IMPORTANT NOTE: *If the gene of interest will be detected by in situ hybridization, use DEPC-treated PBS for all steps. If, after rehydration of the slides, the tissue has holes, reduce the incubation time with the sucrose gradient. Sucrose leads to swelling and bursting of cells. Adjusting the window of time of fixation and the sucrose gradient will remedy this problem.*

DISSECTION AND TISSUE PROCESSING FOR PARAFFIN SECTIONING

This protocol describes the processing of the retina for paraffin sectioning. Perform dissections as described in Support Protocol 1 according to your needs to retain the RPE attached to the retina. The protocol starts after the initial fixation step of Support Protocol 1 but prior to the overnight fixation for cryosections.

Materials

Tissue (harvested from step 2 or step 5 of Support Protocol 1)
4% PFA/PBS: 4% paraformaldehyde in PBS, pH 7.4
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
25%, 50%, 75%, AND 100% ethanol in PBS
Xylene
Paraffin

Incubator/oven (controllable up to 65°C for paraffin)
Embedding molds
Microtome for paraffin sections
45°C water bath
Glass slides pretreated for paraffin sections
Heating plate

1. Fix tissue harvested from either step 2 or step 5 of Support Protocol 1 for 30 min in 4% PFA/PBS.

   Perform all steps in a glass scintillation vial until tissue is mounted in the embedding mold. When washing and replacing solutions, gently pour out fluid and add fresh solution.

2. Wash three times 10 min in PBS.

3. Dehydrate tissue by increasing concentrations of ethanol up to 100% ethanol in scintillation vials:

   a. 10 min in 25% ethanol/PBS
   b. 10 min at 50% ethanol/PBS
   c. 10 min in 75% ethanol/H_2O
   d. 10 min in 100% ethanol
   e. 10 min in 100% ethanol.

   If needed, tissue can be stored at −20°C for several months in 100% ethanol.

4. Clear tissue in xylene for two times 5 min.

5. Incubate for 30 min in a 1:1 mixture of paraffin/xylene at 60°C (temperature depends on melting point of paraffin used).

6. Wash four times, each time for 30 min with 100% paraffin at 60°C, by gently pouring out the melted paraffin and replacing it with fresh melted paraffin using transfer pipets. Keep transfer pipets at 60°C at all times.

   These washes are important to remove all residual xylene from the tissue. If xylene is not removed properly, when stretching the tissue in a warm water bath, the lower melting point of the xylene will leave holes in your section.

7. Incubate overnight at 60°C with fresh 100% paraffin.

8. Mount tissue in appropriate mold with fresh paraffin.

   Paraffin blocks can be stored for several months at 4°C.

9. Using a microtome appropriate for paraffin sections, section blocks at a thickness of 14 to 20 μm and transfer sections into a water bath at 45°C to allow the paraffin to stretch.
10. Collect section on pretreated glass slides.
11. Dry glass slides with sections overnight at 37°C and store at 4°C until needed.
12. When ready, bake slides at 60°C on a heating plate for 1 hr.
13. Remove from plate and allow slides to cool down to room temperature for 5 min.
14. Dewax slides for two times 5 min in xylene.
15. Rehydrate with decreasing ethanol concentrations to PBS:
    a. Two times, each time for 5 min in 100% ethanol
    b. 5 min in 75% ethanol/H_2O
    c. 5 min in 50% ethanol/PBS
    d. 5 min in 25% ethanol/PBS
    e. Two times, each time for 5 min in PBS

    For antibody stainings follow Basic Protocol 7, for in situ hybridizations follow Basic Protocol 8.

    IMPORTANT NOTE: *If the gene of interest will be detected by in situ hybridization, use DEPC-treated PBS for all steps.*

IMMUNOFLUORESCENCE ANALYSIS ON CRYO- OR PARAFFIN SECTIONS

BASIC PROTOCOL 7

This protocol describes immunofluorescence analysis on retinal cross-section. The protocol works equally well for cryo- and paraffin sections.

Materials

Tissue: cryosections (Support Protocol 1) or paraffin sections (Support Protocol 2)
PBT: Phosphate-buffered saline PBS with 0.3% (v/v) Triton X-100
PBTB: PBT (see above) with 5% (v/v) bovine serum albumin (BSA)
Primary and secondary antibodies
Mounting medium: e.g., Gel Mount, Fluoromount (Sigma-Aldrich)

Nuclear DAPI stain (Sigma-Aldrich, cat. no. D-9542)
Mounting media: Gel mount, Fluoromount, etc.
Humidified incubation chamber: slide box with wet paper towels on the bottom; seal box with plastic tape
Cover glasses

1. Permeabilize tissue with PBT for 30 min.

   This and all subsequent steps should be performed in a humidified incubation chamber.

   In this and all subsequent steps, the buffer/antibody is added to cover the area of the slide containing the section.
2. Block with PBTB for 30 min.
3. Incubate with primary antibody diluted in PBTB either overnight at 4°C or for 2 hr at room temperature.
4. Wash three times, each time for 20 min, with PBTB.

5. Incubate with secondary antibody diluted in PBTB either overnight at 4°C or for 2 hr at room temperature.
6. Wash three times, each time for 20 min with PBTB. Add nuclear DAPI stain in the first wash if desired.

   Alternatively, DAPI can also be added with the secondary antibody.

7. Apply mounting medium and add cover glass to slide.

BASIC PROTOCOL 8

IN SITU HYBRIDIZATION ANALYSIS ON CRYO- OR PARAFFIN SECTIONS

This protocol describes in situ hybridizations on sections. It differs from Basic Protocol 6 since it is optimized for section in situ hybridizations. The same RNA probe synthesis procedure as described in Basic Protocol 6 can be used for section in situ hybridizations. The recipes for solutions that are the same between both protocols are not described here.

NOTE: The hybridization buffer (HB) for section in situ hybridizations differs from the whole mount in situ hybridization buffer.

Materials

Tissue: cryosections (Support Protocol 1) or paraffin sections (Support Protocol 2)
4% PFA/PBS: 4% paraformaldehyde in PBS, pH 7.4
PBS/Tween: 0.1% (v/v) Tween-20 in phosphate buffered saline (PBS; *APPENDIX 2A*)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
10 mg/ml proteinase K stock (store at −20°C)
Acetic acid anhydride
1 M triethanolamine (TEA) stock, pH 8.0)
Hybridization buffer (HB; for section in situ hybridization; see recipe)
RNA probe (Basic Protocol 6)
20× SSC (*APPENDIX 2A*)
Formamide (molecular biology grade, 99% pure)
TNE (see recipe)
RNase A (Sigma-Aldrich, cat. no. R-4875 or equivalent)
MAB: 100 mM maleic acid, pH 7.5/150 mM NaCl
MABT: MAB (see above) containing 0.1% (v/v) Tween-20
HISS (heat-inactivated sheep serum)
Antibody: α-digoxigenin antibody coupled to alkaline phosphatase (α-DIG-AP)
NTMT, pH 9.5 (see recipe)
Staining solution (see recipe)
Mounting medium (e.g., Gelvatol; see recipe)

Cover glasses or home-made coverslips from polyethylene bags: cut plastic bags with ruler and razor blade into small pieces the size of a cover glass and store them in a petri dish
Humidified incubation chamber: slide box with wet paper towels on the bottom; seal box with plastic tape
65°C hybridization oven

Day 1: Pretreatment and hybridization

1. Post-fix tissue for 10 min in 4% PFA/PBS.
2. Wash three times 5 min in PBS/Tween.
3. Treat tissue for 10 min with 1 μg/ml proteinase K in PBS.
4. Wash two times 5 min in PBS/Tween.
5. Post-fix in 4% PFA/PBS for 5 min (re-use PFA from first post-fix)

6. Wash three times 5 min in PBS/Tween.
7. Prepare acetylation mix (add 625 µl of acetic acid anhydride to 250 ml of 100 mM triethanolamine). Immerse the section in this mixture for 10 min to perform acetylation.

   Use solution immediately after mixing. Do not prepare in advance!

8. Wash three times 5 min in PBS/Tween.
9. Air dry slides for 10 min.
10. Meanwhile pre-warm probe and hybridization buffer to 70°C.
11. Mix 1 to 3 µl of RNA probe with 120 µl of HB (for section in situ hybridization; see Reagents and Solutions), vortex, and add to slide.
12. Cover glass slides either with cover glasses or with home-made coverslips from polyethylene bags.

    Plastic cover slides are easier to remove and do not shear the sections.

13. Place slides in humidified slide box (use paper towels soaked with water and place them at the bottom of the box. Seal box with plastic tape. Hybridize in oven overnight at 65°C.

Day 2: Post-hybridization washes & detection

Use pre-warmed solutions for washes at 65°C.

14. Remove cover slides by immersing slides in 5× SSC at room temperature.
15. Wash for 30 min in a 1:1 mixture of 1× SSC and formamide at 65°C.
16. Wash for 10 min in TNE at 37°C.
17. Treat for 30 min with RNase A (20 µg/ml in TNE) at 37°C.
18. Wash for 10 min in TNE at 37°C.
19. Wash for 20 min in 2× SSC at 65°C.
20. Wash two times, each time for 20 min, in 0.2× SSC at 65°C.
21. Wash two times, each time for 5 min, in MABT.
22. Block for 30 min in MABT containing 20% (v/v) HISS.
23. Incubate in humidified chamber overnight at 4°C or for 2 hr at room temperature with MABT containing 20% HISS and α-DIG-AP antibody diluted at 1:2500.
24. Wash four times 15 min in MABT.
25. Wash for 10 min in NTMT, pH 9.5.
26. Add staining solution to slides and incubate in the dark.

    Signal may take 30 min to several hours to develop. If necessary, continue incubation overnight at 4°C or incubate from the beginning overnight at 4°C.

27. Rinse with NTMT, pH 9.5
28. Post-fix for 30 min in 4% PFA/PBS to inactivate AP.
29. Wash two times, each time for 5 min, in PBS.
30. Mount in desired mounting medium (e.g., Gelvatol).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BCIP

Prepare 50× 5-bromo-4-chloro-3-indolyl phosphate (BCIP) stock (25 mg/ml in water). Store up to 1 year at −20°C.

DEPC treatment of solutions

Treat solution overnight with diethylpyrocarbonate (DEPC) at a dilution of 1:1000, and autoclave the next day.

Gelvatol

Gelvatol is a water-soluble mounting medium made from polyvinyl alcohol (PVA) and glycerol. The following recipe is a simplification of the original method: Add 5 g PVA 2000 (Sigma-Aldrich, cat. no. P-8136) to 100 ml phosphate-buffered saline (PBS; *APPENDIX 2A*) every hour for 4 hr (total 20 g) while stirring constantly. Keep covered and stir overnight at 4°C. Add 3 more grams PVA 2000 and stir until dissolved. Add a single sodium azide crystal and 50 ml glycerol. Mix thoroughly, aliquot, and store up to 2 years at 4°C in sealed storage vials.

Alternatively, Gelvatol can be bought ready to use from multiple vendors.

Hybridization buffer (HB) for whole-mount in situ hybridization

In a 50 ml conical polypropylene centrifuge tube (e.g., BD Falcon), combine the following reagents:
3.25 ml of 20× SSC (*APPENDIX 2A*)
1.25 ml of 200 mM EDTA, pH 8 (*APPENDIX 2A*)
17.5 ml of DEPC-treated H_2O (see recipe)
2.5 mg of yeast tRNA
5 mg of heparin
Vortex to dissolve
2.5 ml of 10% (w/v) CHAPS
500 µl of 20% (v/v) Tween-20
25 ml of formamide
Mix gently and heat to 65°C if necessary to dissolve tRNA and heparin
Store up to 2 years at −20°C; heat to 65°C and mix well before each use

Hybridization buffer (HB) for section in situ hybridization

In a 50-ml conical polypropylene centrifuge tube (e.g., BD Falcon), combine the following reagents:
500 µl of 1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
6 ml of 5 M NaCl
250 µl of 200 mM EDTA, pH 8 (*APPENDIX 2A*)
1 ml of 50× Denhardt's solution (see *APPENDIX 2A* for 100×)
625 µl of 20% (w/v) SDS (*APPENDIX 2A*)
10 ml of 50% (w/v) dextran sulfate stock
1 ml of DEPC-treated H_2O (see recipe)
10 mg of yeast tRNA
Mix gently to dissolve the tRNA
25 ml of formamide
Mix gently and heat to 65°C if necessary to dissolve tRNA
Store up to 2 years at −20°C; heat to 65°C and mix well before each use

MAB, 10×

To 800 ml distilled water, add the following reagents:
116.1 g of maleic acid
Adjust pH to 7.5 with 10 N NaOH (until pH is adjusted, malic acid will not enter well into solution)
Add 30 ml of 5 M NaCl
Adjust volume to 1 liter, and confirm pH
Store up to 2 years at room temperature

NBT

Prepare 50× 4-nitro blue tetrazolium chloride (NBT) stock (50 mg/ml in 70% N,N′-dimethylformamide; DMF). Store up to 1 year at −20°C.

NTMT, pH 9.5

100 mM NaCl
100 mM Tris·Cl, pH 9.5 (*APPENDIX 2A*)
50 mM $MgCl_2$
0.1% (v/v) Tween-20

Prepare solution fresh each time from stock solutions: 5 M NaCl; 2 M Tris·Cl, pH 9.5 (APPENDIX 2A); 1 M MgCl; 20% (v/v) Tween 20.

TNE

10 mM Tris·Cl pH 7.5 (*APPENDIX 2A*)
500 mM NaCl
1 mM EDTA

Prepare solution fresh each time from stock solutions: 5 M NaCl; 1 M Tris·Cl, pH 7.5 (APPENDIX 2A); 0.5 M EDTA (APPENDIX 2A).

Staining solution

NTMT (see recipe) containing:
1× BCIP (see recipe for 50×)
1× NBT (see recipe for 50×)
Prepare fresh

COMMENTARY

Background Information

Gene therapy has long been viewed as one of the tools of modern molecular medicine to treat many human disease conditions. However, less than a decade after the first clinical trials that began in 1990, the field suffered a major setback. In 1999, an 18-year-old boy died only 4 days after receiving an injection of a therapeutic adenovirus. His death was likely caused by a severe innate immune response to the virus. The rAAVs used today elicit a minimal immune response and are thus much safer. Compared to the first generation of rAAVs, which were based on rAAV2, the ones used today achieve sustained and efficient gene expression. This new generation of rAAVs and their successful use to treat blind dogs (Acland et al., 2001) has paved the road for the future ocular gene therapy in humans.

The retina is a thin neuronal tissue at the back of the eye, which initiates the process of vision. Three distinct nuclear layers characterize it. Each nuclear layer is composed of a subset of specialized neurons (Fig. 14D.4.5; Masland, 2001, 2011). The outer nuclear layer (ONL) harbors rod and cone photoreceptors (PR), the cells that absorb photons. Rods are 1000 times more sensitive to light than cones and function primarily in dim light, while cones are used for daylight, color, and high-acuity vision. Although humans are diurnal and mice are nocturnal, in both species rods outnumber cones 20:1 (Masland, 2001), with the exception of a small region in the human eye referred to as the fovea. The fovea is composed only of cones and is the center for high-acuity vision in humans. Outside the fovea the mouse and human retina are alike. Upon

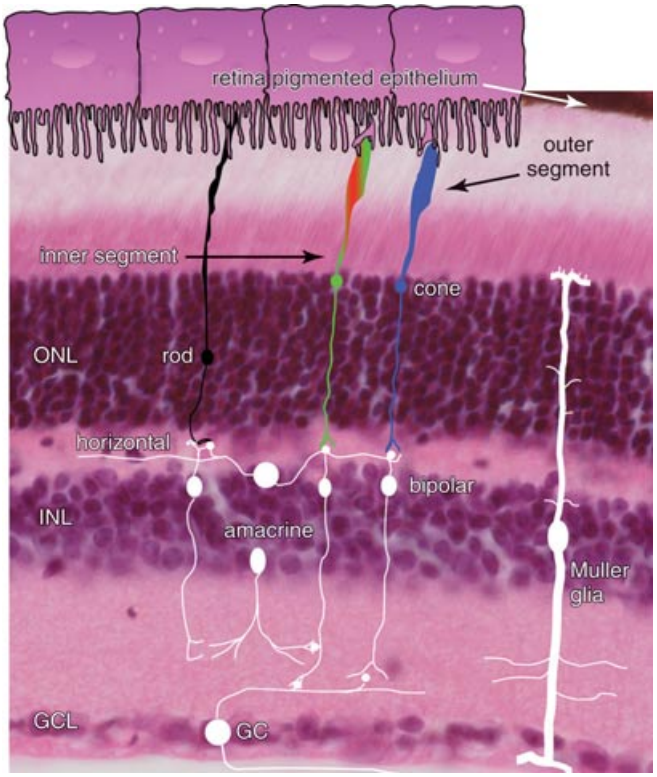

Figure 14D.4.5 Retinal morphology. Cross-section stained with H&E showing cartoons of different retinal cell types. Cells located in the inner nuclear layer (INL) and ganglion cell layer (GCL) are shown in white, while cones and rods located in the outer nuclear layer (ONL) are shown in color or black respectively. On top, cartoon of RPE cells and their interactions with PR outer segments. For the color version of this figure, go to *http://www.currentprotocols.com/mc14D04*.

absorption of a photon, PRs hyperpolarize and signal to bipolar cells, which reside in the inner nuclear layer (INL). Bipolar cells connect to ganglion cells in the third nuclear layer, which send their axons through the optic nerve to the visual centers of the brain (Masland, 2001). In addition to bipolar cells, the INL is also populated by amacrine and horizontal cells (Masland, 2001), which modulate the signal, and Muller glia cells, which are the only non-neuronal cell type in the retina and form the retinal-blood barrier. Nutrition for retinal cells is provided by the retinal vasculature and the retinal-pigmented epithelium (RPE). The RPE is in intimate contact with PR outer segments. It provides nutrients and oxygen for PRs and is involved in the visual cycle (Parker and Crouch, 2010; Wang and Kefalov, 2011).

Retinal degeneration is a major cause of blindness in the industrialized world. The degeneration affects either ganglion cells or PRs. Loss of ganglion cells results in glaucoma, while loss of PRs is associated with a variety of retinal degenerative diseases such as dry and wet age-related macular degeneration, diabetic retinopathy, retinitis pigmentosa (RP), Leber's congenital amaurosis (LCA), etc. Retinitis pigmentosa and LCA are inherited retinal degenerative diseases. In such cases, rAAV-mediated gene therapy entails either the replacement of a non-functional gene or the knockdown of a dominant allele. In contrast, age-related macular degeneration, diabetic retinopathy, and glaucoma are caused by a combination of environmental and genetic factors. rAAV mediated gene therapy is still possible for these diseases; however, it requires an understanding of the molecular mechanisms that lead to the disease pathology. For example, in wet age-related macular degeneration and diabetic retinopathy, neovascularization of either the choroidal or the retinal vasculature, respectively, causes leakage of fluid into the retinal proper, which then results in PR death. This neovascularization is stimulated by the vascular endothelium growth factor (VEGF), and overexpressing the soluble form of the VEGF receptor-1 (sFLT-1) reduces the incidence of new blood vessel formation (Lai et al., 2005). Retinal gene therapy can

thus be applied to a wide range of genetic and nongenetic eye diseases. Recently a new treatment strategy for PR degenerative diseases has emerged, referred to as optogenetics. This strategy uses the endogenous remaining retinal circuit after PRs have died to reactivate vision. rAAVs are engineered to overexpress light-sensitive ion channels such as channel rhodopsin2 in bipolar cells and/or ganglion cells. Such an approach is independent of the initial insult that resulted in PR death and can thus in principle be applied to almost all PR degenerative diseases. Finally, the most successful rAAV mediated gene therapy in the eye of humans thus far targeted the RPE cells. LCA-2 is an early-onset disease caused by a mutation in the RPE protein RPE65 (Gu et al., 1997; Marlhens et al., 1997). Delivery of the RPE65 gene by rAAV to the RPE of individuals suffering from LCA-2 has restored vision in blind people (Bainbridge et al., 2008; Maguire et al., 2008), giving hope to many others suffering from vision loss. Since every retinal cell type, including the RPE, is a potential target for gene therapy, a large arsenal of rAAVs capable of infecting all different cell types is needed. Cell tropism of rAAV serotypes in the eye is only known for the most commonly used serotypes (Stieger et al., 2011). Knowing the tropism is particularly important, since serotypes that preferentially infect only a subset of cells can reduce unwanted side effects. In combination with cell-type-specific promoters or microRNA regulation (Xie et al., 2011), such rAAVs can potentially restrict transgene expression to a specific cell type.

The procedure for subretinal delivery of viruses in rodents was initially described by the Cepko laboratory (Price et al., 1987) using a replication-incompetent retrovirus for lineage tracing. The same laboratory also published the DNA transduction technique of retinal cells in newborn rodents by electroporation (Matsuda and Cepko, 2004) and the embryonic transduction of retinal cells (Turner et al., 1990; Punzo and Cepko, 2008). Since then, many laboratories have used these procedures successfully and modified them according to their needs.

Critical Parameters and Troubleshooting

Viral injections yield, in general, successful infections even for beginners. The simple act of inserting a needle into the eye and injecting virus will result in infection. The amount of infected target cells will increase with experience. The most important variable regarding viral injections is the viral titer and the infectivity of the viral preparation. rAAV titers are generally determined by genome copies. While this number reflects the actual amount of virus particles, the number of infectious particles can be quite different and as much as 100 to 1000 times lower. If little infection is seen with a high-titer virus, then most likely the infectivity of the viral preparation is low. A rough assessment of infectivity can be performed in cell culture by adding 5 µl of the viral preparation to one well of a 6-well culture plate with HEK239 cells. This is only possible if (a) the viral cassette uses a broad expressing promoter such as CMV, (b) if the cassette overexpresses an easy detectable marker such as GFP, and (c) if the serotype used is capable of infecting HEK293 cells. In such a case, if 2 days post infection many cells are GFP positive, the infectivity of the viral preparation should be sufficient to successfully transduce many retinal cells. However, not all rAAV serotypes will lead to such a fast expression. This method is not meant to replace the standard quantification method. It relies on the fact that super-infection of cells with more than 100 virus particles per cell will lead to a fast expression. While a high enough viral titer is usually the most important concern regarding expression in a tissue, a too-high titer can lead to unwanted effects in the retina. For example, we have observed that injections with a high titer (5×10^{13}) virus preparation, which shows also good infectivity, can lead to PR death. However, it remains unclear if this is solely due to a high number of virus particles per PR cell. If PR death occurs due to a high titer, diluting the virus or repurifying it over a CsCl gradient or specialized exchange columns may help mitigate the problem.

Electroporation of plasmid DNA tends to be more complicated than viral injections. Simply inserting a needle into the eye and injecting DNA will not lead to GFP-positive cells. Although the procedure is technically the same, the biggest hurdle is targeting the subretinal space properly, delivering enough DNA into that space, and placing the electrode pads adequately. It may take a couple of mouse litters to master the technique. When dissecting the retina, if GFP is seen only on top of the PR outer segments but not in the PRs themselves, it suggests that the DNA was targeted correctly to the subretinal space but not electroporated efficiently. Macrophages that enter the retina and clean up the remaining plasmid

DNA will be GFP positive due to the plasmid they took up. In such a case, either the electroporator is not delivering enough current or the electrode pads were not placed properly. The plus pole of the tweezer electrodes tends to oxidize over time, which will result in a reduction of the electric field. The electrode pads need to be kept clean and the tweezer electrode needs to be replaced every so often. The most critical parameters for this procedure are the quality and concentration of the DNA and the proper targeting of the subretinal space, the electroporator, and the tweezer electrodes. We recommend practicing subretinal injections into newborn mice with the CD1 mouse strain. CD1 mice have large litters and are albino, which helps to initially visualize the spread of the injection solution.

In situ hybridizations on retinal whole mounts or sections can be quite challenging. In general, the quality of the probe and the target sequence that was chosen for hybridization are the most critical parameters. If the whole-mount in situ hybridization is not working, we recommend testing the probe on sections. After resuspending the probe with water, we recommend adding 80 µl of HB (for section in situ hybridization; see recipe in Reagents and Solutions). Diluting the probe afterwards in the whole-mount HB for the actual hybridization does not affect the whole mount in situ hybridization, while the probe can still be used for section in situ hybridizations. This allows testing the probe on sections if whole mounts are not working. If the probe does not work on sections, we recommend either that the probe be resynthesized or that a different target region of the gene be chosen. As a positive control, we recommend starting with a gene that is expressed at high levels, such as one of the PR-specific opsins. If there is too much background, increasing the number of washes and the wash temperature may mitigate the problem. Incubation with the antibody alone will determine if the background stems primarily from the antibody being trapped in the tissue or from unspecific trapping of the probe. A sense probe can be used as a control for the hybridization. However, many genes have coding sequence of another gene running in the antisense direction. Thus, it is important to determine on the UCSC Genome Browser (*http://genome.ucsc.edu/*) if there is a gene running in the opposite direction prior to designing and synthesizing a sense control probe. We also recommend using the UCSC Genome Browser to determine if there are alternative splice isoforms of your gene of interest. In such a case, designing a probe against the most common region may be a good starting point.

Antibody staining tends to be straightforward when it works and difficult to troubleshoot when it does not work. The antibody and the epitope that is recognized by the antibody are the most important factors, which are unfortunately difficult to control. Here are some tips if the staining does not work. In general, more antibodies tend to work better on cryosections than on paraffin sections. However, there are exceptions, and some antibodies work better on paraffin sections. If your antibody does not work, adding SDS at a final concentration of 0.01% to 0.03% in PBTB may help. Alternatively an antigen-retrieval protocol can help. There are many different protocols for antigen retrieval, and it is difficult to predict which ones work best for a specific antibody. If the signal is weak by immunofluorescence, then immunocytochemistry may yield a better result. In such a case we would recommend to use a secondary antibody coupled to horseradish peroxidase. If such an approach is used, the tissue needs to be pretreated with H_2O_2 to inactivate the endogenous peroxidase in order to reduce background.

Anticipated Results

Subretinal injections at post-natal day 0 take some time to master. Viral injections tend to yield positive results sooner than electroporations, as injecting a virus into the eye will result in infection even if only few of the desired target cells are hit. Most people tend to have positive transduction with both methods after injecting 3 litters. With some practice, 50% to 70% of electroporated retinas will be successfully transduced. In such cases, the retinal surface area that is positively transduced ranges anywhere between 5% and 50%, with 25% being the norm for most people (Fig. 14D.4.6). Viral injections yield similar results; however, it is easier to transduce a larger area of the retina. With some experience, it is possible to even transduce the entire retinal surface area with a single injection (Fig. 14D.4.6). This may take quite some practice, and even with a lot of experience only 10% to 20% of virally infected retinas will show infection across the entire retinal surface area.

The time point of electroporation determines the cell types that can be transduced efficiently. To broaden the range of cell types by electroporation, the procedure needs to be performed at embryonic time points. Such a procedure has been described previously (Punzo

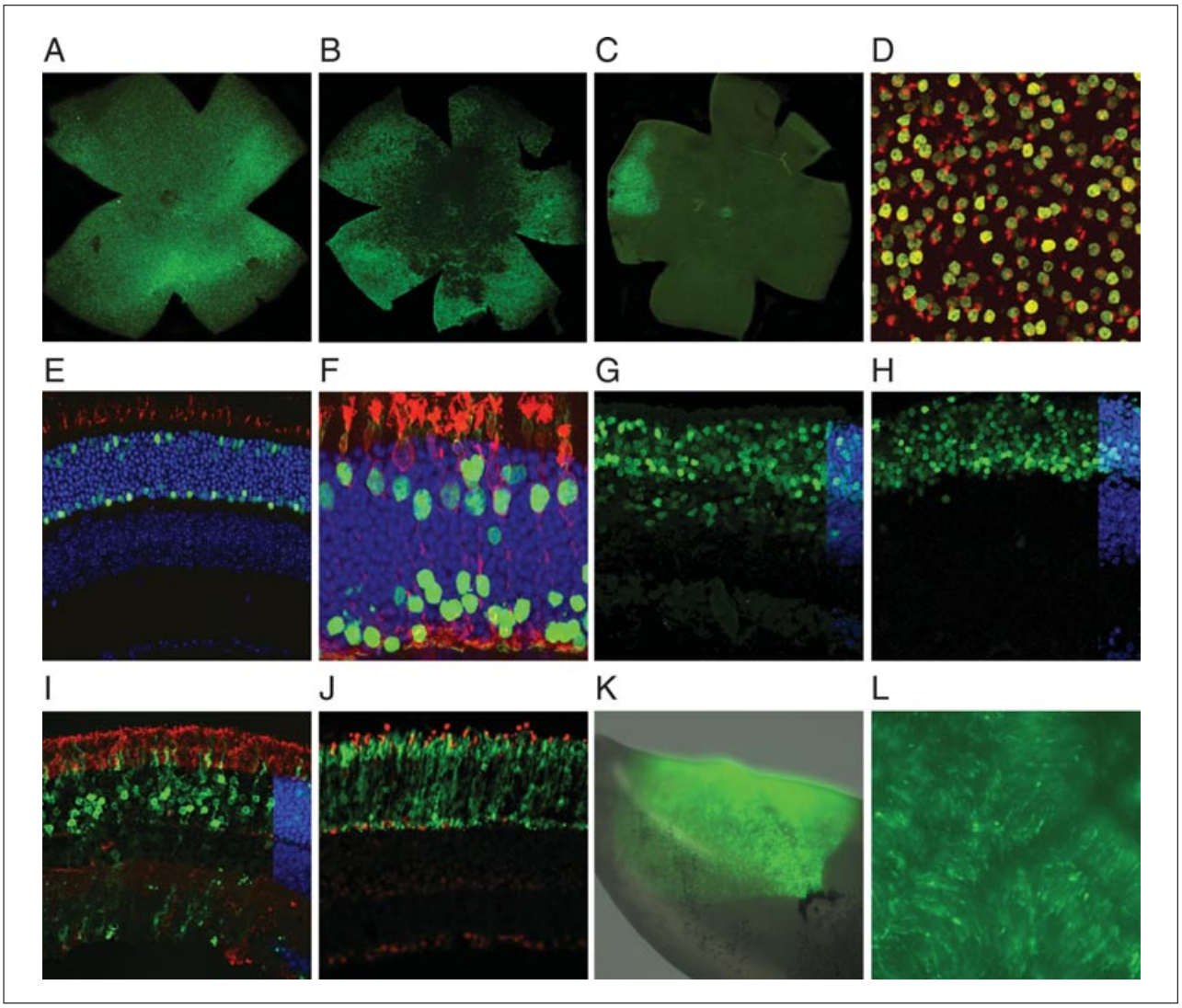

Figure 14D.4.6 Expected results after viral infection or electroporation of plasmid DNA at post-natal day 0. Panels (**A-D, K, L**) show retinal whole mounts and panels (**E-J**) show retinal cross-sections of GFP-transduced cells. Panels (A-H) show transduction of retinal cells by rAAV(2/5)-expressing nuclear GFP and (I-L) show transduction by electroporation of plasmid DNA expressing GFP. (A-D) Injection with a viral titer of 5×10^{12} genome copies/ml showing either uniform infection across the entire retinal surface area (A, B) or local infection (C) at 5 weeks of age. (B,C) Retinas from retinal degeneration mice (*rd*[1]) that harbor a mutation in a rod-specific gene (Bowes et al., 1990). The lack of GFP expression in the center (B) reflects the loss of central cone photoreceptors at 5 weeks of age. (D) High magnification of an immunofluorescence on a wild-type retina that was uniformly infected (see A). Medium-wavelength opsin expression in cones (red) was used to determine how many cones are infected by the virus. (E-H) Expansion of cell types infected with increasing titer. (E) Infection with a viral titer of 10^{12} genome copies/ml shows some rods and cones infected. (F) Infection with a titer of 5×10^{12} genome copies/ml shows most cones infected and an increasing number of rods. (G) Infection with a titer of $>10^{13}$ genome copies/ml shows that almost all photoreceptors are infected and a large number of INL cells. (H) Same titer as in (G); however, expression is restricted to photoreceptors by use of a cone-specific promoter that shows also expression in rods. (E) Electroporation of GFP expressing plasmid shows many GFP-positive rods and some GFP-positive INL cells. GFP processes that extend to the GCL mark Muller glia cells. (J) Electroporation as shown in (I) with a rod-specific promoter restricting GFP expression to rod photoreceptors. (K) Region of a freshly dissected unfixed retina electroporated with a GFP-expressing plasmid. (L) High magnification of region in (K) showing rod inner segments that are GFP positive. GFP-positive inner segments can also be seen in (I, J). [green, transduced cell either by rAAV or electroporation expressing GFP; red, cone opsin expression (D, E, F) or rhodopsin expression (i); blue: nuclear DAPI]. For the color version of this figure, go to *http://www.currentprotocols.com/mc14D04*.

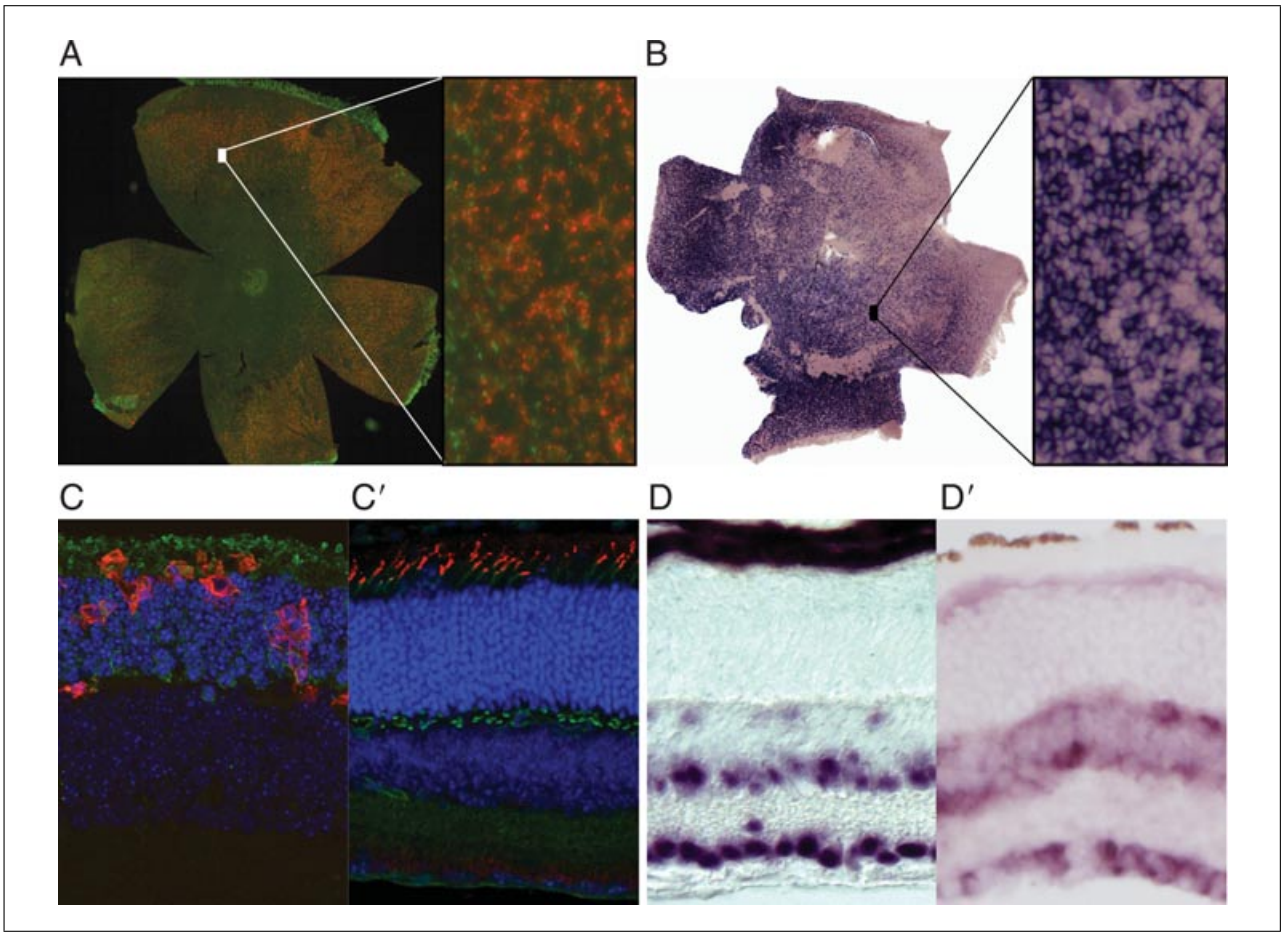

Figure 14D.4.7 Expected results for immunofluorescence and in situ hybridization analyses. Compilation of immunofluorescence stainings (**A, C**, C′) or in situ hybridizations (**B, D**, D′) on retinal whole mounts (A, B) or retinal cross-sections (C-D′), respectively. Both types of retinal cross-section preparations are shown—paraffin (C, D) and cryosections (C′, D′). (A, B) Retinas from retinal-degeneration mice (*rd*[1]) (Bowes et al., 1990) at 6 weeks and 3.5 weeks of age, respectively, showing cones marked by cone arrestin antibody and peanut agglutinin lectin (A; red and green respectively) and by cone opsin in situ hybridization (B: purple). Regions to the right show higher magnification. (C) Retina from *rd*[1] mice during the peak of rod cell death at post-natal day 12. Macrophages (red: F4/80 staining) move into the ONL to remove dying rods (green: rhodopsin staining). Retinal cross-section of wild-type mice at 5 weeks of age marking cones by cone opsin expression (red) and by peanut agglutinin lectin (green). In situ hybridizations (purple-pink) for Ubiquitin-like 3 (Ubl3) (D) and Down's syndrome cell adhesion molecule (Dscam) (D′) on wild-type sections of 5-week-old mice. For the color version of this figure, go to *http://www.currentprotocols.com/mc14D04*.

and Cepko, 2008). In contrast, viral injections with rAAVs can target all retinal cell types at any age. While the cell type that is efficiently infected is dependent on the serotype, the viral titer and the infectivity of the viral preparation can influence the perceived tropism. Additionally, the route of delivery may also influence the cell types that are preferentially infected. Figure 14D.4.6 shows examples of subretinal injections with a rAAV(2/5) that carries nuclear GFP. At a low titer and low infectivity, cone PRs and some rods are preferentially infected. With increasing titer and infectivity, INL cells appear infected as well. It is thus important to determine if the tropism of a virus has been tested to its maximum potential before concluding which cells can be infected with a specific serotype. Performing both subretinal and intravitreal injections with a high-titer virus that shows good infectivity should yield a comprehensive picture of the cell tropism of a specific rAAV serotype. Of note, the tropism observed in the mouse retina with a specific serotype may change when performing injections with the same viral preparation in a different organism.

Immunofluorescence or in situ hybridization analyses depend on the antibody and probe used. A compilation of immunofluorescence and in situ hybridization data is shown in Figure 14D.4.7.

Time Considerations

Viral injections or electroporations at postnatal day 0 take roughly 1½ to 2 hr per litter depending on your experience. This includes the 1-hr lag time, in which pups are injected with buprenorphine. The individual injection of a pup takes only 2 to 3 min. Viral injections into adults take a similar amount of time. Transduction of retinal cells either by rAAV or by electroporation differs mainly in the time it takes for gene expression to occur. Gene expression upon electroporation can be detected as early as 2 days post electroporation and stays stable for several months. In contrast, gene expression from rAAV vectors may take 1 to 3 weeks; however, expression tends to persist for years. Two variables determine the speed of expression for rAAVs. One is the viral serotype and the other is the multiplicity of infection (MOI), which depends on the amount of virus that is injected in a particular area and the actual infectious titer of the virus. In general, when more than one virus particle enters a cell, expression tends to start earlier. In our hands, the rAAV(2/5) shows robust GFP expression within 10 days post infection. Preparation of the virus is not discussed here but takes generally 1 to 2 weeks, including cell line expansion, purification and virus titering.

Retinal analysis by immunofluorescence or in situ hybridization may take 2 days to 2 weeks depending on the protocol and whether sections or whole mounts are processed. The specific time windows for the different procedures are indicated in the individual protocols. When performing an antibody staining, we recommend incubating with the primary antibody overnight until you have determined that the antibody works well, at which point that step can be shortened to 2 hr.

Acknowledgments

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