Intravitreal injection of AAV for the transduction of mouse retinal ganglion cells

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Abstract

The injection of therapies into the eye is common practice, both clinically and pre-clinically. The most straightforward delivery route is via an intravitreal injection, which introduces the treatment into the largest cavity at the posterior of the eye. This technique is frequently used to deliver gene therapies, including those containing recombinant adeno-associated viral vectors (AAVs), to the back of the eye to enable inner retinal targeting. This chapter provides detailed methodology on how to successfully perform an intravitreal injection in mice. The chapter covers vector preparation considerations, advice on how to minimize vector loss in the injection device, and ways to reduce vector reflux from the eye when administering a therapy. Finally, a protocol is provided on common retinal histology processing techniques to assess vector-mediated expression in retinal ganglion cells. It is hoped that this chapter will enable researchers to carry out effective and consistent intravitreal injections that transduce the inner retinal surface while avoiding common pitfalls.

Key-words Gene therapy, Adeno-associated virus (AAV), Retinal ganglion cells (RGCs), Transduction, Intravitreal delivery, Murine eye, Retina.

1 Introduction

1.1 Retinal Ganglion Cells

Retinal ganglion cells (RGCs) are sensory neurons, vital for the transfer of visual stimuli from the retina to the brain [1]. The axons of RGCs converge at the optic disc to form the optic nerve which extends through the optic chiasm, the optic tract, and the lateral geniculate nucleus (LGN) in the thalamus and superior colliculus (SC) in the midbrain. RGC damage and degeneration underlies several conditions responsible for significant visual loss, including glaucoma, hereditary optic neuropathies, ischemic optic neuropathies, and demyelinating disease [2].

The development of therapeutic strategies to protect and regenerate RGCs is therefore a priority to restore vision, with gene therapies likely to address current unmet clinical needs.

1.2 Recombinant Adeno-Associated Viral Vectors to Transduce RGCs

Recombinant adeno-associated viral vectors (hereafter referred to as AAVs) have become the favored gene therapy carriers for use in ocular treatments [3–8] due to their long-term transgene expression, safety, ease of delivery, and flexibility to target distinct cell populations.

AAVs are small, non-enveloped viral vectors with the ability to transduce post-mitotic neurons such as RGCs. Their low immunogenic response has made them favorable in rodent studies, allowing a high number of viral particles to be injected into the eye without complications [9, 10]. The AAV genome is considered largely non-integrating and persists in the cell as episomal DNA [11].

Like all viral vectors, AAVs consist of two fundamental components: the viral capsid and the viral genome. Both can be altered to optimize gene transfer [3, 8, 12].

The viral capsid is essential for directed cell infectivity, with the proteins on the capsid interacting with cell-surface glycans and receptors required for the cellular uptake [3, 13, 14]. AAV2 is the natural capsid serotype that is most effective in transducing RGCs following intravitreal injection [15–19]. However, various capsid serotypes can be used to transduce RGCs, and new variants are being developed and engineered to further improve cellular tropism.

The viral genome contains the transgene of interest, regulatory elements for its expression, and inverted terminal repeats (ITRs) at both ends. The maximum packaging capacity of AAVs is 4.7 kilobase [20, 21], and therefore, any desired genetic content is restricted to that size. The minimal regulatory elements needed for transgene expression are a promoter and a poly-adenylation signal, whereas a Kozak sequence [22, 23], intron sequence [23–25], and woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) [24, 26, 27] are often included to enhance transgene expression. The choice of promoter is particularly important, and several have been identified that preferentially express in RGCs [22, 28–34].

1.3 Considerations of AAVs for Preclinical Examination

When comparing AAVs, it is important to ensure the quality of the vectors is relatively similar. Vectors should ideally be manufactured using the same protocol and assessed for sterility, purity, and a low endotoxin presence. For instance, whilst both isopycnic cesium chloride (CsCl) and iodixanol gradient ultracentrifugation achieve high purity, the latter harbors more empty viral particles (viral vectors that contain a capsid but are without genetic content) compared to CsCl-purified vectors [35]. Having AAV empty viral particles in the working solution will reduce the efficiency of transduction. The AAV empty viral particles do not contribute to the intended gene transfer, and furthermore, they compete with full AAV viral particles for binding to receptors needed for cellular uptake [36].

AAVs are typically compared titer matched to ensure that the vector solutions contain the same number of genome copies (GC) per mL. GC/mL is commonly determined using real-time PCR. The primers can be directed towards any target sequence in the AAV genome, including the ITRs.

Purity assessments are routinely performed via silver stain analysis to visualize the three AAV capsid protein (VP1, VP2, and VP3) bands and endotoxin levels for murine intraocular studies should fall below 2 endotoxin units (EU) per mL to avoid adverse ocular inflammation [37]. If needed, there are protocols available to reduce endotoxin levels when preparing AAVs [38]. The FDA requires that intraocular pharmaceuticals for human use have endotoxin levels of less than 0.2 EU/mL [39].

Finally, it is good practice to validate the functionality of AAVs in cell culture conditions prior to performing procedures in animals.

1.4 Intravitreal Injection of AAVs

Intravitreal administration is the optimal delivery route to place the AAVs near RGCs, thereby facilitating cellular uptake. Intravitreal injection of AAVs is relatively simple and easy to perform in all species, including rodents. This chapter provides detailed methodology relating to preparation of the AAVs, precise delivery within the vitreous, and the expected outcome in terms of transgene expression within the murine eye.

2 Materials

2.1 AAV Preparation

- AAV vector
- Low protein binding pipette tips
- Low protein binding tubes
- Single channel pipettes
- Wet ice in a styrofoam box
- 0.001% Pluronic F-68 formulation buffer (*see* **Note 1**)
- 4 °C refrigerator
- -70 °C or below ultra-low temperature freezer

2.2 Anesthetic and Eye Drops

- Mice (typically C57BL/6J mice aged 6-10 weeks)
- 100 mg/mL ketamine (*see* **Note 2**)
- 20 mg/mL xylazine (*see* **Note 2**)
- 5 mg/mL atipamezole (*see* **Note 3**)
- 0.9% w/v sodium chloride (NaCl)
- 1% w/v tetracaine hydrochloride
- 1% w/v tropicamide
- 2.5% w/v phenylephrine
- Optional: 1-2 mg/mL topical dexamethasone, administered once/twice daily for 5 consecutive days starting 1 week after AAV delivery if moderate inflammation is observed (*see* **Note 4**) [40].

2.3 Equipment for AAV Administration

- Acetone
- Animal weighing scale
- Binocular operating microscope with light source and 10X-25X objective magnification
- Colibri ophthalmology forceps (8.5 cm length)
- Cotton swab
- Fluorescein
- Glass coverslips (sterile)
- Hamilton 5µL glass microliter syringe (65 RN)
- Hamilton small hub removable (RN) needle (33G, 10 mm, point style 2)
- Heating pad
- Lab book to score the intravitreal injection procedures and highlight any potential complications (*see* **Note 5**).
- Low protein binding pipette tips
- Single channel pipettes
- Sterile absorbent tissue
- Sterile 1X DPBS
- Sterile water
- ViscoTears polyacrylic acid eye gel carbomer
- 70% (v/v) ethanol (*see* **Note 6**).
- 0.2 µm syringe filter
- 15 mL or 50 mL conical tube
- 27G 13 mm needles

- 30G 13 mm needle
- 1 mL and 5 mL syringes

2.4 Equipment for Ocular Collection

- Cryostat
- Curved dissecting scissors (Vannas spring loaded scissors, 8 cm length, 3-4 mm cutting edge, 0.05 mm tip diameter)
- Dissecting microscope with 10-20X magnification
- Dry ice
- Fluorescence microscope
- Fluorescence mounting medium
- Foil
- Hydrophobic pen
- Microscope slide storage box
- Normal goat serum
- O.C.T compound (*see* **Note 7**)
- Orbital shaker
- Parafilm
- Plastic transfer pipette
- Plastic fine tip transfer pipette
- Plastic dishes (typically 35mm)
- Plastic cryomolds
- Primary and secondary antibodies
- Rectangular cover glass (20 mm x 50 mm) (*see* **Note 8**)

- Sodium azide
- Straight forceps (11 cm length, straight)
- Straight micro-dissecting scissors (Vannas spring loaded scissors, 8 cm length, 2 mm cutting edge, 0.05 mm tip diameter)
- Superfrost slides (25 mm x 75 mm) (positively charged)
- Slide rack
- Slide staining dish
- Tissue paper
- Toothed forceps (12 cm length, 1 into 2 teeth, straight)
- Triton X-100
- 4 °C refrigerator
- -20 °C freezer
- 1.5 mL tubes
- 1X phosphate buffered saline (PBS)
- 30G 13 mm needle
- 4% paraformaldehyde (PFA) w/v in 1X PBS pH 7.4 (*see* **Note 9**)
- 15% sucrose w/v in 1X PBS pH 7.4 (*see* **Note 10**)
- 30% sucrose w/v in 1X PBS pH 7.4 (*see* **Note 10**)

3 Methods

3.1 AAV Working Solution Preparation

1. When preparing AAVs, it is recommended to use low protein binding pipette tips and tubes to minimize AAV loss during dilution. It is also good practice to thaw the AAVs on the experiment day and to avoid multiple freeze and thaw cycles. Once thawed, do not vortex the AAV solution, mix via gentle pipetting prior to dilution.

2. AAVs should ideally be prepared in a 0.001% Pluronic F-68 formulation buffer to minimize sticking of AAVs to plastic and thereby reducing vector loss through the injection procedure [41–43].

3. AAV solution concentrations of 1 x 10^8 and 1 x 10^10 GC/eye are optimal for intravitreal delivery and can be injected as a 1-2 µL volume. That is, dilute AAV stock in formulation buffer (Subheading 3.1, step 2) to 5 x 10^12 GC/mL, so that a 2 µL injection delivers 1 x 10^10 GC into the eye.

4. Prepare approximately double the volume of AAVs required. That is, if 20 µL AAV is required for 10 intravitreal injections, prepare 40 µL. This will allow for loss when drawing the AAV solution into the syringe.

5. Transport the prepared aliquot of AAV working solution to the animal unit on wet ice in a styrofoam box and bring to room temperature prior to use.

6. Aliquots of AAV working solution can be stored in low protein binding tubes at 4 °C for up to 1 month. Minimal loss of activity is expected within this timeframe. AAV stock for long-term storage should be kept at -70 °C or lower temperature. Effective AAV transduction should remain for >10 years.

3.2 Preparing the Injection Device

1. Fully prime the Hamilton syringe by aspirating and dispensing 70% ethanol 3-4 times before repeating with 1X DPBS. This will clean any debris from the internal chamber of the syringe and remove trapped air which can cause inaccuracies.

2. Tap away excess solution with sterile tissue and place the empty syringe on absorbent tissue whilst anesthetizing the mouse (Subheading 3.4). Ensure the syringe is dry and empty of DPBS prior to loading the AAV working solution to prevent an adverse dilution.

3. Pipette double the volume of AAVs to be intravitreally injected onto the surface of a glass coverslip.

4. Place the tip of the Hamilton needle into the solution and fill the syringe with the entire content.

5. Lift and hold the syringe vertical, with the needle pointing up to allow any air bubbles to rise into the end of the metal tip. Eject excess sample back onto the coverslip, pushing the syringe plunger to the desired volume to be administered (*see* **Note 11**).

6. When intravitreally injecting multiple mice, it is recommended to repeat Subheading 3.2, step 1 regularly to eliminate sample carryover.

7. If administering multiple AAVs, it can be beneficial to use separate Hamilton syringes to avoid contamination and to reduce needle cleaning requirements during the intravitreal injection procedure.

8. A single Hamilton needle will remain sharp for approximately 200 eye injections. Have a spare Hamilton needle nearby during procedures in case of damage or blockage.

3.3 Hamilton Needle Cleaning and Storage

1. After use, rinse the syringe with 70% ethanol, followed by water, and then finish with acetone.

2. Syringes can be stored in their original packaging (once dry) or stored, fully submerged in 70% ethanol. For storing in ethanol, it is recommended to disassemble the syringe and store in a 50 mL sealed tube (*see* **Note 12**).

3.4 Preparing the Animal for Intravitreal Injection

1. Weigh the mouse and anesthetize with an anesthetic mix of 80 mg/Kg ketamine and 8 mg/Kg xylazine. The volume (μL) required to give the correct dose can be calculated by the weight of the mouse (g) multiplied by 10. That is, a 25 g mouse requires 250 μL, a 30 g mouse requires 300 μL.

2. Deliver the anesthetic through an intraperitoneal (i.p.) injection using a 27G needle and 1 mL syringe into the lower right quadrant of the animal's abdomen. This location minimizes the risk of damage to the urinary bladder, cecum, and other abdominal organs [44].

3. Once anesthetized, place the mouse on a heating pad and administer one drop of topical 1% tetracaine hydrochloride to each eye.

4. After 5 s, wipe away the tetracaine with a cotton swab and add a drop of topical 1% tropicamide and 2.5% phenylephrine to each eye. This will dilate the pupil, allowing better visualization of the needle position within the vitreous.

5. Transfer the anesthetized animal to sterile tissue under an operating microscope.

3.5 Performing the Intravitreal Injection

1. It is recommended that all intravitreal procedures are conducted by the same researcher. This will minimize variability and improve consistency.

2. Place the mouse under the operating microscope and adjust the magnification and focus. A magnification between 10X and 25X is optimal.

3. Angle the mouse so that the eye is fully illuminated, and the ocular anatomy is clearly visible. Use a cotton swab to dry the eye.

4. Use your non-dominant hand to hold the eye with colibri forceps, and gently protrude the eyeball (Fig. 1a).

5. With your dominant hand, pick up the prepared Hamilton syringe (Subheading 3.2, step 5) and place the needle tip, bevel facing upwards approximately 2 mm posterior to the limbus (the divide between the cornea and sclera) in the superior (11-12 o'clock) region of the eye (*see* **Note 13**) (Fig. 1a).

6. Angle the syringe and needle 45° towards the posterior of the eye so that the needle will enter the vitreous between the retina and lens (Fig. 1a). The needle angle and injection position will minimize the risk of hitting larger retinal vessels and keep the central retina void of damage [45].

7. Push the needle firmly into the sclera until the needle penetrates the ocular surface. Once through the sclera, the needle will easily pierce the underlying choroid, retinal pigment epithelium, and retina (Fig. 1b).

8. The needle tip should be inserted approximately 1.5 mm into the vitreous with the tip of the needle visible through the dilated pupil [22, 46]. Avoid pushing the needle too deep or creating excess movement once in the vitreal cavity.

9. Release the eyeball with your forceps and use your non-dominant hand to slowly press the syringe plunger to eject the AAV solution into the vitreous (Fig. 1b, c). An ejection rate of 6 μL/minute (0.1 μL per seconds) is recommended (*see* **Notes 14** and **15**) [47].

10. Hold the needle in position for 30 s to allow the AAVs to disperse throughout the vitreous (Fig. 1d).

11. To minimize reflux and reduce concentrated transgene expression around the AAV needle injection site, it can be beneficial to puncture the cornea with a 30G needle while the Hamilton needle is in the posterior of the eye (Fig. 1e). Releasing a small amount of aqueous helps to reduce AAV reflux through the AAV injection site.

12. Support the needle tip with the forceps as you slowly retract the needle from the vitreous. The mouse eyelid should close over the injection site (Fig. 1f).

13. Score the success of your injection and write down any complications that might cause poor RGC transduction (*see* **Note 5**).

14. Recover the animal with an i.p. injection of 1 mg/Kg atipamezole. The volume (μL) required to give the correct dose can be calculated by the weight of the mouse (g) multiplied by 10. That is, a 25 g mouse requires 250 μL, a 30 g mouse requires 300 μL.

15. Apply ViscoTears eye gel to both eyes to prevent the cornea surface from drying out and carefully transfer the animal to a 37 °C heating pad until fully recovered.

16. It is recommended that animals are recovered in individual cages to avoid unnecessary disturbance/pressure to the eyes.

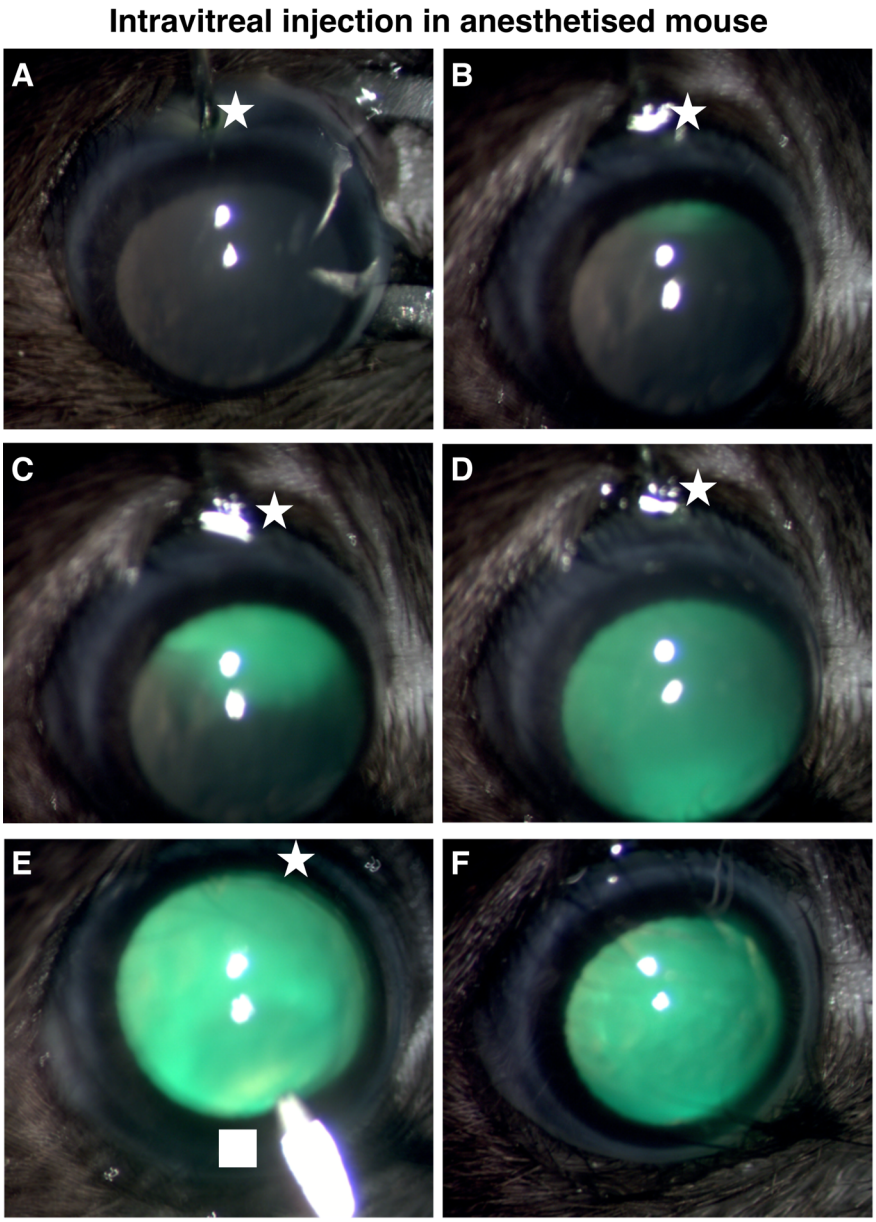

Fig. 1 Frame captures from an intravitreal procedure video delivering 2 µL of AAV mixed with fluorescein. The AAV injection site and corneal puncture site are highlighted by a star and square, respectively. The shown intravitreal injection has minimal leakage of AAVs and fluorescein at both sites.

3.6 Typical Study Duration for Stable Expression

1. AAV gene therapy expression should begin 1 week after intravitreal injection [48, 49] with stable expression from 3 weeks. Expression should remain for at least 6 months [34, 48, 50, 51].

3. 7 Collecting AAV Expressing Mouse Ocular Tissue

1. When ready to terminate the experiment, euthanize the mice with increased carbon dioxide (CO_2) inhalation or terminal/overdose anesthetic.

2. If collecting optic nerves and/or brains, it is recommended to perform a transcardiac perfusion with 1X PBS followed by 4% PFA [52]. A perfusion flow rate of 10-15 mL/min is optimal prior to obtaining tissues from the visual pathway (*see* **Note 16**). Optic nerves and brains can be post-fixed in 4% PFA (2-24 h) prior to further analysis such as longitudinal sectioning [53] or tissue clearing [54].

3. If collecting eyes for the preparation of retinal wholemounts and/or retinal cross-sections, dissect eyeballs from the euthanized mice (Subheading 3.7, step 1) using curved dissecting scissors, gently removing the eyeball by cutting around the eye socket and severing the optic nerve.

4. Remove excess orbital tissue and conjunctiva before rolling the eyeball on tissue paper to remove excess blood.

5. Briefly wash the eyeball in a dish containing 1X PBS and puncture the cornea with a 30G needle to aid the upcoming fixative penetration. Transfer the eyeball to a tube containing 1 mL 4% PFA (*see* **Note 16**) and fix for at least 2 h at room temperature. Overnight fixation at 4 °C is also acceptable for many antibodies (*see* **Note 17**).

3.8 Preparation of Retinal Wholemounts

1. Following PFA fixation (Subheading 3.7, step 5), add 1 mL 1X PBS to the lid of a dish under a dissecting microscope, and using straight forceps, transfer the fixed eyeball to the dish lid (*see* **Note 18**).

2. Carefully cut away excess muscle, conjunctiva and connective tissue using straight micro-dissecting scissors.

3. The corneal puncture prior to fixation (Subheading 3.7, step 5) will enable you to grasp the cornea with toothed forceps, making it easier to hold the eye steady while dissecting.

4. Holding the cornea, insert the tip of the dissecting scissors into the puncture hole, and cut towards the corneoscleral divide.

5. Carefully rotate the eye so that your scissors are parallel to the corneoscleral divide and slowly cut around the circumference of the globe (*see* **Note 19**).

6. Once complete, the cornea should lift away, and the lens will float out with gentle agitation. The inside of the remaining eyecup contains the neural retina, which should look smooth and opaque [55].

7. Applying gentle pressure to the outside of the eyeball with forceps should detach the retina from the retinal pigment epithelium (RPE) at the ciliary body. Once an area of retina is peeling away, carefully insert forceps between the retina and RPE.

8. Slowly rotate the eye with the forceps between the retina and RPE to fully separate the tissues [56]. A single cut may be required at the optic nerve so that the retina floats out of the eyecup.

9. With the retina removed, create a single, straight cut from the retina edge towards the optic nerve head, making sure not to cut into the optic nerve head.

10. Rotate the retinal wholemount and make three further straight incisions from the edge to the center so that the retina flattens with four petals.

11. Transfer the retinal wholemount using a plastic transfer pipette to a 24-well plate containing 500 µL 1X PBS (*see* **Note 20**).

12. Repeat Subheading 3.4, steps 1-11 for all eyeballs prior to further washing and staining (*see* **Note 21**).

3.9 Preparation of Retinal Cross-Sections

1. Following PFA fixation (Subheading 3.7, Step 5), eyeballs are cryo-preserved by immersion in 1 mL 15% sucrose at 4 °C for 6 h, followed by 30% sucrose overnight at 4°C. The gradual dehydration will help to retain the spherical structure of the eye.

2. Dehydrated eyeballs are then placed in individual cryomolds containing O.C.T.

3. Leave the eyeball in the O.C.T for 1 h to equilibrate and sink to the bottom before orientating so that the central part of the cornea and the optic nerve are on the same sectioning plane [57]. The eyeball should face horizontal rather than the cornea facing up.

4. Freeze the cryomold on dry ice ensuring the eye does not inadvertently turn during the freezing. Store the sample at -20 °C or below until ready for sectioning.

5. Using a cryostat, collect 10-15 μm sections through the dorsal–ventral/superior–inferior axis of the eye. This thickness will provide adequate separation of RGCs to assess transduction [22].

6. Sectioning thorough the dorsal–ventral/superior–inferior axis also provides a good indication of global transduction throughout the posterior of the eye.

7. Frozen ocular slices should be collected on appropriately coated slides, such as positively charged superfrost slides. A correct coating will prevent sample loss during wash steps.

8. Slides containing frozen sections can be stored in slide boxes at -20 °C indefinitely.

3.10 Immunohistochemistry

1. A simple immunohistochemistry blocking buffer is suitable for both retinal wholemounts and retinal cross-sections to prevent nonspecific binding of antibodies or other reagents to the tissue. A solution containing 5% normal goat serum (NGS) and 0.3% Triton X-100 in 1X PBS is recommended (*see* **Note 22**) and is suitable for blocking, primary and secondary antibody steps.

2. For retinal wholemounts in a 24-well plate, wash with 500 µL 1X PBS for 15 min, aspirating and applying PBS with a plastic fine tip transfer pipette to avoid damage to the sample. For retinal cross-sections, place the slides in a slide rack (*see* **Note 23**) and carefully lower into a slide staining dish containing 1X PBS. Rock samples on an orbital shaker at a speed of 90 revolutions per minute (RPM).

3. Repeat the wash step three times.

4. Remove the PBS via aspiration for wholemounts or via tapping slides on tissue paper for sections. Ensure samples do not dry out before applying blocking reagent. For wholemounts, blocking in 500 µL per retina is sufficient to cover the retinas. For sections it is recommended to draw around the edge of the slide with a hydrophobic pen to keep the solutions on the sections. A standard 25 mm x 75 mm slide will hold 400-500 µL blocking buffer within the circled barrier and should be applied to the edge of the slide after drying with tissue paper. Submerge the samples in blocking buffer (*see* Subheading 3.10, step 1) for 1 h at room temperature at a slower speed of 75 RPM.

5. Remove the solution by aspiration (wholemounts) or tapping the slide on tissue paper (sections) and incubate the samples in relevant primary antibodies (*see* **Note 24**) diluted in blocking buffer. Again, 500 µL per wholemount/slide is recommended.

6. Seal the 24-well wholemount plate with parafilm and store at 4 °C overnight (*see* **Note 25**). Sections should be placed horizontal on a slide box with adequate humidity to prevent the samples drying out overnight.

7. The next day, wash with 1X PBS three times.

8. Remove the final wash as indicated in Subheading 3.10, step 5 and incubate the samples with relevant fluorescence-labelled secondary antibodies diluted in blocking buffer. Gently rock the samples with the antibody at 75 RPM for 2 h at room temperature. Protect from light by covering the plate/slides with foil.

9. Wash with 1X PBS three times.

10. Transfer the wholemounts with a cut plastic transfer pipette from Subheading 3.8, step 11 to a slide and gently unfold so that the RGC side is facing upwards (*see* **Note 26**). Dry excess solution from around each retina with tissue making sure not to touch the wholemounts. Four retinas can be placed on each slide. For retinal sections, flick or tap away excess solution from the slide.

11. Place several drops of fluorescence mounting medium directly on top the tissue samples and place a rectangular cover glass over the slide, taking care to avoid bubble formation over your samples.

12. Leave slides to dry at room temperature protected from light for 2 h or longer, and then store the slides until ready to image.

3.11 Assessment of RGC Transduction

1. Ideally, retinal wholemounts and retinal cross-sections should be imaged using a fluorescent microscope within 1 month of labeling, although fluorescence should be protected for up to a year.

2. An example of successful RGC transduction is shown for retinal wholemounts in Fig. 2 and retinal cross-section in Fig. 3. Both sets of images show transduction following an intravitreal injection of AAV2.QuadYF with eGFP as a reporter.

3. Retinal wholemounts are used to demonstrate global transduction across the entire retinal surface and are an informative method to assess AAV efficacy [58].

4. Retinal cross-sections provide information to the depth of AAV expression within the retina and have the advantage that numerous slides can be collected, and separately probed, from a single eye.

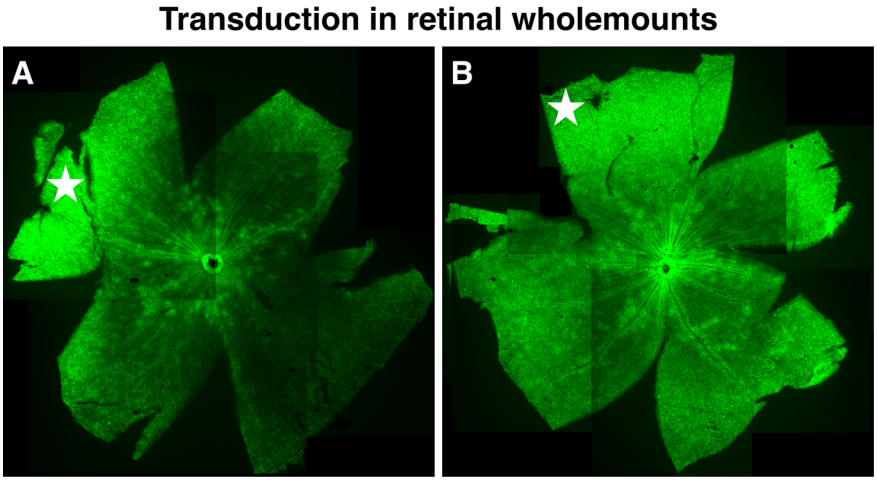

Fig. 2 Mouse retinal wholemounts showing transduction of RGCs after 3 weeks following intravitreal delivery of 2 µL AAV2.QuadYF-CAG-eGFP-WPRE at a titer of 5 x 10^9 genome copies per eye. Stars indicate the intravitreal injection site and eGFP transgene expression is visible in green. (**a**) Retinal wholemount from an eye without a corneal puncture which highlights concentrated transduction around the injection site. (**b**) Retinal wholemount from an eye that did undergo a corneal puncture (Subheading 3.5, step 11) and displays improved global transduction across the retina.

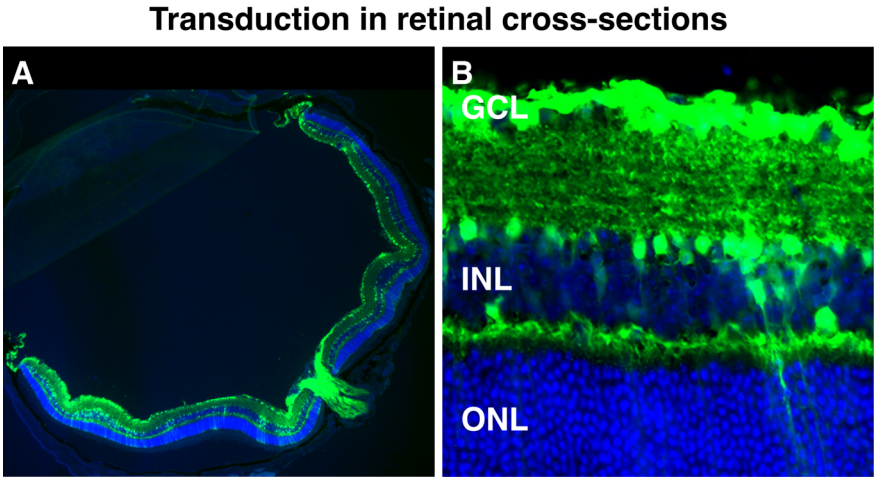

Fig. 3 Mouse retinal cross-section showing transduction of RGCs and cells in deeper retinal layers after 3 weeks following intravitreal delivery of 2 µL AAV2.QuadYF-CAG-eGFP-WPRE at a titer of 5 x 10^9 genome copies per eye. Retinal cross-section at 4X (**a**) and 40X (**b**) objective-magnification. The section is stained for DAPI in blue and eGFP transgene expression is visible in green. Abbreviations: *GCL* ganglion cell layer, *INL* inner nuclear layer, *ONL* outer nuclear layer.

4 Notes

1. Protocol for 0.001% Pluronic F-68 formulation buffer. Firstly, create a 0.1% Pluronic F-68 stock: 49.5 mL 1X DPBS + 500 µL 10% Pluronic F-68 under sterile conditions. Then dilute this stock 100X: 49.5 mL 1X DPBS + 500 µL 0.1% Pluronic F-68, to provide a working formulation buffer. 0.001% Pluronic F-68 can be stored at 4 °C for several weeks.

2. Protocol for a working anesthetic solution. Combine 0.8 mL ketamine (100 mg/mL) with 0.4 mL xylazine (20 mg/mL) and 8.8 mL NaCl (0.9%). This solution is suitable for approximately 30 mice and can be stored at 4 °C for a week.

3. Protocol for a working anesthetic reversal solution. Combine 0.2 mL atipamezole (5 mg/mL) with 9.8 mL NaCl (0.9%). The working reversal solution is suitable for approximately 30 mice and can be stored at 4 °C for a week.

4. The use of topical dexamethasone following intravitreal injection is more common in larger species.

5. Eyes displaying intraocular bleeds or cataracts will still show signs of transduction but may negatively impact transgene expression and retinal function.

6. Protocol for 70% ethanol. Dilute 35 mL of 100% ethanol with 15 mL of distilled water. The solution can be stored at room temperature for up to 1 year.

7. Peel-A-Way disposable embedding molds or 8 mm flat end polyethylene embedding capsules are ideal for murine eyes.

8. A #1.5 cover glass thickness (0.17 mm) is recommended for most microscope objectives.

9. Protocol for 4% PFA. Dissolve 40 g of PFA in 1 liter of 1X PBS at 60 °C while stirring. Alternatively, 37% w/v pre-bought formaldehyde can be diluted 10 times in 1X PBS to create a suitable fixing reagent. 4% PFA can be stored in aliquots at −20 °C for up to 1 year.

10. Protocols for sucrose in 1X PBS. 30% sucrose: dissolve 30 g of sucrose in 100 mL 1X PBS. 15% sucrose: dissolve 15 g of sucrose in 100 mL 1X PBS or dilute 20 mL 30% sucrose in 20 mL 1X PBS. Store at 4 °C for up to 3 months.

11. Do not dry the needle with tissue which could inadvertently draw out AAV from the needle tip.

12. Further details about syringe cleaning and storing can be found in Ref. [59].

13. Having the bevel facing upwards aids with the penetration of the sclera and reduces the risk of scratching the lens.

14. It is common when using volumes over 1 µL to see the eyeball increase in size and the cornea to become temporarily opaque with a transient spike in intraocular pressure. An example with photographs is shown in supplementary figure 3 of Ref. [22].

15. Addition of the green dye fluorescein (0.1-0.5 mg/mL) to the viral suspension can help to visualize the diffusion inside the vitreous [45]. To create a 0.5 mg/mL fluorescein solution, firstly dissolve 250 mg of fluorescein in 10 mL of 0.9% NaCl creating a 25 mg/mL stock. Once dissolved, filter sterilize the solution and dilute 50-fold with sterile 1X DPBS (1 mL stock + 49 mL DPBS). Mix 1:5 with your viral prep prior to intravitreal injection.

16. Photographs of the collection of mouse eyes, optic chiasm, and brain can be found in supplementary figure 4 of Ref. [22].

17. A longer fixation time will reduce the fragility of the retinal wholemount making dissection, detachment from the RPE layer, and handling easier.

18. Eyes should be dissected submerged in 1X PBS so that excess ocular tissues float away from the eyeball and to prevent intraocular tissues from sticking to plastic. Keeping the eyeball in a suspension also maintains the eyes spherical shape, allowing you to work with this shape rather than against it [55, 60].

19. The intention is to cut just below the iris, so that the retina is detached from the ciliary body as you cut around the eyeball.

20. Cutting 2 cm off the end of a plastic transfer pipette creates an opening wide enough to transfer wholemounts between solutions.

21. Retinal wholemounts can be stored at 4 °C in 1X PBS for several months prior to staining. For long-term storage (years) it is recommended to store tissues in 1X PBS + 0.05% sodium azide to inhibit bacterial growth [61]. That is, firstly create a 10% stock by dissolving 10 g sodium azide in 100 mL 1X PBS. Dissolve this 10% stock 200X by adding 5 µL per 1 mL 1X PBS to create a 0.05% working sodium azide stock. Store at 4 °C for up to a year.

22. Protocol for 5% normal goat serum (NGS) and 0.3% Triton X-100 in 1X PBS. Firstly, create a 0.3% Triton X-100 stock in PBS by adding 300 µL Triton X-100 into 100 mL 1X PBS. Store at room temperature for 1 year. Take 19 mL 0.3% Triton X-100 and add 1 mL normal goat serum to create a working blocking solution. Store at 4 °C for up to 1 week.

23. If removing slides from a freezer prior to staining, air dry the slides at room temperature for 30 min before initiating washes.

24. Recommended RGC-specific antibodies can be found in the following Refs. [62–66] including details of concentrations and blocking requirements.

25. Improved antibody penetration and staining is obtained with gentle rocking overnight in a cold room.

26. It is easier to mount samples using a dissecting microscope.

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