FINAL SCRIPT: APPROVED FOR FILMING

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Title: Optimized Minimally Invasive Transscleral Subretinal Injection Technique in Mouse

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

   Can you record movies/images using your own microscope camera?

   No

   If your microscope does not have a camera port, the scope kit will be attached to one of the eyepieces and **you will have to perform the procedure using one eye**.

   Stemi 2000-C

   If a dissection or stereo microscope is required for your protocol, please list all shots from the script that will be visualized using the microscope (shots are indicated with the 3-digit numbers, like 2.1.1, 2.1.2, etc.).

   2.2.1, 2.3.1, 2.6.2, 2.6.3, 2.7.1, 2.7.2, 2.7.3, 2.8.1, 2.8.2, 2.9.1, 2.10.1, 3.1.1, 3.2.1, 3.3.1, 3.4.1, 3.5.1

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length
Number of Steps: 17
Number of Shots: 23

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Oleg Alekseev:** Subretinal injections are an important tool for preclinical investigations of therapies for inherited retinal diseases. In this video, we are demonstrating an optimized minimally invasive subretinal injection technique in mice **[1]**.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.2.1, 3.3.1.*

What are the current experimental challenges?

1.2. **Lindsey Chew:** Transscleral subretinal injections are an excellent way to deliver material to photoreceptors and RPE. However, this technique is technically challenging, which calls for developing an optimized protocol to increase its reproducibility and accessibility **[1]**.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 1B (for the second sentence)*

What advantage does your protocol offer compared to other techniques?

1.3. **Lauren Cao:** Our improvements include the development of a mouse eyelid speculum, preoperative administration of atropine, creation of a pinpoint sclerotomy using a diamond knife, and optimization of needle size and injection approach **[1]**.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.6.1., 2.10.1.*

How will your findings advance research in your field?

1.4. **Lindsey Chew:** We hope that our method's consistency, reproducibility, excellent transduction coverage, and minimal surgical damage will improve the accessibility of subretinal injections to the scientific community **[1]**.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What new scientific questions have your results paved the way for?

1.5. **Lauren Cao:** Our optimizations make it easier and faster to perform subretinal injections in mice, reduce experimental variability, and improve injection success rates. This technique will facilitate future preclinical translational retina research **[1]**.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been performed in accordance with the Duke University Institutional Animal Care and Use Committee and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research

Protocol

Author's Note to Editors: We filmed the whole procedure twice; once, stopping between each shot with the videographer calling out shot numbers before each take, and a second time, where we ran through the entire protocol as we would normally, without stopping. The second take went much more smoothly, so we would like to request that the individual shots be used for steps 2.1.1 – 2.7.3, and for the continuous take to be used for the remainder of the video (2.8.1 – 3.6). Unfortunately, the continuous take doesn't have shot numbers but can be referenced with the individual shots; if it is not convenient to cross-reference the continuous take video to the individual shots, I would be happy to provide timestamp labels of the continuous take for the editors.

2. Animal Preparation and Lateral Canthotomy

Demonstrator: Oleg Alekseev

NOTE: Please use the individual shots for steps 2.1.1 – 2.7.3 per the author's NOTE above

2.1. To begin, administer 1 milligram per kilogram of atropine intraperitoneally in a total volume of 50 microliters, 30 minutes before performing the subretinal injection **[1-TXT]**.

2.1.1. WIDE: Talent administering an intraperitoneal injection of atropine to the mouse. **TXT: This prevents lethal reflexes caused by intraocular pressure elevation**

2.2. After anesthetizing the mouse, apply a drop of 0.5 percent proparacaine to the eye to achieve topical anesthesia **[1-TXT]**.

2.2.1. SCOPE: Talent applying a drop of proparacaine to the eye. **TXT: Anesthesia: 2% inhaled isoflurane in O_2 (2 L/min); Keep the mouse on a warm surface during the entire procedure**

2.3. Apply a drop of a mixture containing 0.2 percent cyclopentolate and 1 percent phenylephrine to the eye to dilate the pupil for postoperative imaging of the subretinal bleb and to reduce intraoperative bleeding **[1]**.

2.3.1. SCOPE: Talent applying a drop of a mixture containing cyclopentolate and phenylephrine to the eye.

Author's NOTE: Move steps 2.4 and 2.5 before 2.2 (so the order would be 2.1, 2.4, 2.5, 2.2, 2.3). This allows for having all the scope shots together instead of moving back and forth between perspectives

2.4. Load a glass microneedle with the desired volume of the injection solution **[1]**. Attach the needle to the microinjector and set the compensation pressure to 5 hectopascals to counteract capillary action **[2]**.

2.4.1. Talent loading a glass microneedle with solution. NOTE: Shots 2.4.1 and 2.4.2 were combined.

2.4.2. Talent connecting the needle to a microinjector ~~and adjusting the compensation pressure~~.

2.5. Place the mouse under a dissecting stereomicroscope **[1-TXT]**.

2.5.1. Talent positioning the anesthetized mouse under the stereomicroscope. **TXT Confirm anesthesia by performing a toe-pinch**

2.6. Using a custom miniature wire-speculum **[1]**, expose the superior fornix **[2-TXT]** and immobilize the speculum to enable hands-free upper eyelid retraction **[3]**.

2.6.1. ~~A shot of the custom miniature wire-speculum~~. LAB MEDIA: Figure 1C Author's NOTE: This was not filmed as a picture of the custom speculum is already in the text as a figure.

2.6.2. SCOPE: Talent inserting the wire-speculum to retract the upper eyelid, revealing the superior fornix. **TXT: For young mice, perform lateral canthotomy prior to the procedure** Author's NOTE: Shots 2.6.2 and 2.6.3 were combined.

2.6.3. SCOPE: Talent securing the speculum to enable hands-free upper eyelid retraction.

2.7. Using angled Vannas scissors, make a paralimbal incision approximately 1 millimeter posterior to the limbus in the superior conjunctiva and Tenon's capsule **[1]** to expose the superior sclera **[2]**. Carefully remove any remnants of Tenon's capsule over the injection site **[3]**.

2.7.1. SCOPE: Talent making a paralimbal incision posterior to the limbus in the superior conjunctiva and Tenon's capsule using angled Vannas scissors. Author's NOTE: Shots 2.7.1 – 2.7.3 were combined

2.7.2. SCOPE: A shot of the exposed superior sclera.

2.7.3. SCOPE: Talent clearing residual Tenon's capsule tissue.

NOTE: Please start using the continuous take from this step onwards. Refer to the individual shots to identify which shot is performed. The author is ready to help if needed.

2.8. Now, grasp the anterior edge of the conjunctival peritomy and tenotomy with locking toothed forceps **[1]**, then gently inferoduct the globe to expose the superior sclera **[2]**.

2.8.1. SCOPE: Talent grasping the anterior edge of the conjunctival peritomy and tenotomy with locking toothed forceps. Please start using the continuous take from this step onwards. Author's NOTE: Shots 2.8.1 and 2.8.2 were combined.

2.8.2. SCOPE: Talent gently rotating the eye downward using forceps.

2.9. Immediately apply a drop of balanced buffered saline to the eye to keep the bare sclera hydrated and to achieve greater optical magnification **[1]**.

2.9.1. SCOPE: Talent applying a drop of balanced buffered saline to the eye.

2.10. Using a diamond knife, make a pinpoint sclerotomy at approximately 12 o'clock and 1 to 2 millimeters from the limbus, positioning the blade tangentially to the sclera to ensure that the sclerotomy is small and shallow **[1-TXT]**.

2.10.1. SCOPE: A shot of the diamond knife making a precise incision at the 12 o'clock position, held in a tangential plane to the sclera to ensure a shallow, narrow entry. **TXT: Slight choroidal bleeding at the sclerotomy site doesn't affect the outcome**

3. Transscleral Subretinal Injection

3.1. Use a surgical sponge to gently remove all balanced buffered saline from the surface of the eye **[1]**.

3.1.1. SCOPE: Talent dabbing the eye surface with a surgical sponge to absorb the saline.

3.2. Hold the needle bevel-down at a shallow angle to the sclera and slowly insert the tip into the sclerotomy, advancing just 0.5 to 1 millimeter to access the subretinal space without penetrating the retina **[1]**.

3.2.1. SCOPE: Talent positioning the needle bevel-down, approaching the sclerotomy at a shallow angle, and then inserting the tip into the sclerotomy to access the subretinal space. Author's NOTE: Shots 3.2.1 – 3.4.1 were combined.

3.3. While holding the needle steady, initiate injection at 500 hectopascals using the foot

pedal and maintain continuous pressure for 15 seconds **[1]**.

3.3.1. SCOPE: A shot of the needle being held steadily in place within the sclerotomy while the injection begins using the foot pedal, with visible fluid displacement.

3.4. Remove the needle and observe a partial reflux of the injected solution through the sclerotomy site, indicating successful delivery, as the subretinal space cannot accommodate the full volume of the solution injected **[1-TXT]**.

3.4.1. SCOPE: Talent removing the needle and a partial reflux of the injected solution through the sclerotomy site is being observed. **TXT: Maintain pressure until the needle is fully removed to prevent reflux into the needle**

3.5. Carefully remove the locking forceps and the eyelid speculum without applying pressure to the globe to avoid additional reflux **[1]**.

3.5.1. SCOPE: Talent removing the speculum and forceps while stabilizing the eye region gently.

3.6. Immediately after injection, apply a generous amount of lubricant eye ointment to the eye to assist with subretinal bleb visualization ~~by Optical coherence tomography or OCT (O C T)~~ **[1]**.

3.6.1. Talent applying a thick line of lubricant ointment over the eye. Author's NOTE: OCT is not demonstrated.

~~3.7. Finally, apply topical erythromycin ointment to the eye before performing general post-operative care for the animal **[1].**~~

3.7.1. ~~Talent applying erythromycin ointment to the treated eye.~~ Author's NOTE: This shot was not filmed—it is visually redundant with 3.6.1, which also shows application of a gel ointment.

Results

4. Results

4.1. A subretinal bleb was clearly visible on OCT immediately after transscleral injection, confirming successful delivery **[1].**

4.1.1. LAB MEDIA: Figure 2A. *Video Editor: Highlight the dome-like structure.*

4.2. To evaluate retinal pigment epithelium or RPE *(R-P-E)* targeting, AAV8-Bestrophin1-GFP *(A-A-V-eight bestrophin-one G-F-P)* was injected, resulting in widespread green fluorescence across the epithelium layer, indicating efficient and broad RPE transduction using the transscleral approach **[1]**.

4.2.1. LAB MEDIA: Figure 2B.

4.3. AAV8-Rhodopsin-GFP *(A-A-V-eight rhodopsin G-F-P)* led to a strong green fluorescent signal in rod photoreceptors, visible in both cross-sections and fundus views, indicating successful targeting and expression in photoreceptor cells **[1]**.

4.3.1. LAB MEDIA: Figure 2C, 2D. *Video Editor: Highlight 2C when the VO says "cross sections", and 2D when the VO says "and fundus views".*

4.4. AAV8-CAG-mCherry *(A-A-V-eight kag M-cherry)* produced broad red fluorescence across retinal layers, demonstrating the ability of this technique to support generalized transgene expression beyond specific cell types **[1]**.

4.4.1. LAB MEDIA: Figure 2E, 2F.

4.5. Western blotting of retinal lysates also showed strong green fluorescent protein expression with the highest levels corresponding to the center of the injection bleb **[1]**, validating photoreceptor transduction with AAV8-Rhodopsin-GFP **[2]**.

4.5.1. LAB MEDIA: Figure 2G. *Video editor: Highlight the two black bands in the top row grey boxes (GFP).*

4.5.2. LAB MEDIA: Figure 2G.

4.6. AAV titer optimization using immunofluorescence analysis of retinal sections allows for the selection of the viral titer that achieves the transduction of approximately 90

percent of target cells. For example, in this case **[1],** a 1:10 *(one to ten)* dilution of AAV8-Rhodopsin-GFP viral stock produced transgene expression in almost all photoreceptors **[2]**. By contrast, a 1:100 *(one to hundred)* dilution resulted in an insufficient transduction rate **[3]**.

4.6.1. LAB MEDIA: Figure 3.

4.6.2. LAB MEDIA: Figure 3. *Video Editor: Highlight the middle row (1:10).*

4.6.3. LAB MEDIA: Figure 3. *Video Editor: Highlight the bottom row (1:100).*

4.7. Electroretinograms showed that transscleral injections preserved normal retinal function **[1]**, while transretinal injections led to significantly reduced a-wave and b-wave responses **[2]**, indicating retinal damage **[3]**.

4.7.1. LAB MEDIA: Figure 4A, 4B. *Video Editor: Highlight the green plots in both A and B.*

4.7.2. LAB MEDIA: Figure 4A, 4B. *Video Editor: Highlight the blue plots in both A and B.*

4.7.3. LAB MEDIA: Figure 4A, 4B.

Pronunciation Guide:

1. **atropine**
   Pronunciation link: https://www.merriam-webster.com/dictionary/atropine howtopronounce.com+8merriam-webster.com+8collinsdictionary.com+8
   IPA: /ˈæt.rə.pɪn/
   Phonetic: AT-ruh-pin
2. **proparacaine**
   Pronunciation link: https://www.merriam-webster.com/medical/proparacaine merriam-webster.commerriam-webster.com+5merriam-webster.com+5merriam-webster.com+5merriam-webster.com+8merriam-webster.com+8merriam-webster.com+8
   IPA: /proʊˈpær.əˌkeɪn/
   Phonetic: proh-PAR-uh-kayn
3. **cyclopentolate**
   Pronunciation link: https://www.merriam-webster.com/medical/cyclopentolate youtube.com+8merriam-webster.com+8collinsdictionary.com+8
   IPA: /ˌsaɪ.kloʊˈpɛn.təl.eɪt/
   Phonetic: SY-kloh-PEN-tuh-layt
4. **phenylephrine**
   Pronunciation link: https://www.merriam-webster.com/dictionary/phenylephrine youtube.com+10merriam-webster.com+10merriam-webster.com+10merriam-webster.com+13merriam-webster.com+13collinsdictionary.com+13
   IPA: /ˌfɛn.əˈlɛf.rɪn/
   Phonetic: FEN-uh-LEF-rin
5. **sclerotomy**
   Pronunciation link: https://www.merriam-webster.com/medical/sclerotomy merriam-webster.com+3merriam-webster.com+3merriam-webster.com+3en.wikipedia.org+8merriam-webster.com+8howtopronounce.com+8
   IPA: /skləˈrɑːtəmi/
   Phonetic: sklah-RAH-tuh-mee
6. **Tenon** (as in Tenon's capsule)
   Pronunciation link: https://www.merriam-webster.com/dictionary/tenon
   IPA: /ˈtɛn.ən/
   Phonetic: TEN-uhn
7. **conjunctiva**
   Pronunciation link: https://www.merriam-webster.com/dictionary/conjunctiva youtube.com+15merriam-webster.com+15merriam-webster.com+15oed.com+4dictionary.cambridge.org+4oed.com+4
   IPA: /kənˈdʒʌŋk.tɪ.və/
   Phonetic: kun-JUNK-tuh-vuh

8. **bleb**
   Pronunciation link: https://www.merriam-webster.com/dictionary/bleb en.wiktionary.org+1merriam-webster.com+1merriam-webster.com+6merriam-webster.com+6merriam-webster.com+6
   IPA: /blɛb/
   Phonetic: bleb

9. **microinjector**
   No Merriam-Webster page; generic formation.
   IPA: /ˌmaɪ.kroʊ.ɪnˈdʒɛk.tər/
   Phonetic: MY-kroh-in-JEK-ter

10. **intraperitoneally**
    No direct entry; break into familiar elements.
    IPA: /ˌɪn.trəˌpɛr.ɪˈtoʊ.ni.əli/
    Phonetic: IN-truh-PER-uh-TOH-nee-uh-lee

11. **subretinal**
    Constructed term.
    IPA: /ˌsʌbˈriː.tɪ.nəl/
    Phonetic: sub-REE-tih-nul

12. **transscleral**
    Constructed.
    IPA: /ˌtrænzˈsklɪər.əl/
    Phonetic: tranz-SKLEER-uhl

13. **tortuosity** (common in retinal surgery context)
    Pronunciation link: not found.
    IPA: /ˌtɔr.tʃuˈɒs.ə.ti/
    Phonetic: tor-choo-OSS-uh-tee

14. **hectopascal**
    Constructed term based on metric naming.
    IPA: /ˈhɛk.toʊˌpæz.kəl/
    Phonetic: HEK-toh-PAZ-kul