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Retinal Pigment Epithelium Transplantation in a Non-human Primate Model for Degenerative Retinal Diseases

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TITLE:

Retinal Pigment Epithelium Transplantation in a Non-human Primate Model for Degenerative Retinal Diseases

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SUMMARY:

The non-human primate (NHP) is an ideal model for studying human retinal cellular therapeutics due to the anatomical and genetic similarities. This manuscript describes a method for submacular transplantation of retinal pigment epithelial cells in the NHP eye and strategies to prevent intraoperative complications associated with macular manipulation.

ABSTRACT:

Retinal pigment epithelial (RPE) transplantation holds great promise for the treatment of inherited and acquired retinal degenerative diseases. These conditions include retinitis pigmentosa (RP) and advanced forms of age-related macular degeneration (AMD), such as

geographic atrophy (GA). Together, these disorders represent a significant proportion of currently untreatable blindness globally. These unmet medical needs have generated heightened academic interest in developing methods of RPE replacement. Among the animal models commonly utilized for preclinical testing of therapeutics, the non-human primate (NHP) is the only animal model that has a macula. As it shares this anatomical similarity with the human eye, the NHP eye is an important and appropriate preclinical animal model for the development of advanced therapy medicinal products (ATMPs) such as RPE cell therapy.

This manuscript describes a method for the submacular transplantation of an RPE monolayer, cultured on a polyethylene terephthalate (PET) cell carrier, underneath the macula onto a surgically created RPE wound in immunosuppressed NHPs. The fovea—the central avascular portion of the macula—is the site of the greatest mechanical weakness during the transplantation. Foveal trauma will occur if the initial subretinal fluid injection generates an excessive force on the retina. Hence, slow injection under perfluorocarbon liquid (PFCL) vitreous tamponade is recommended with a dual-bore subretinal injection cannula at low intraocular pressure (IOP) settings to create a retinal bleb.

Pretreatment with an intravitreal plasminogen injection to release parafoveal RPE-photoreceptor adhesions is also advised. These combined strategies can reduce the likelihood of foveal tears when compared to conventional techniques. The NHP is a key animal model in the preclinical phase of RPE cell therapy development. This protocol addresses the technical challenges associated with the delivery of RPE cellular therapy in the NHP eye.

INTRODUCTION:

RPE transplantation holds great promise for the treatment of inherited and acquired retinal degenerative diseases. These conditions include retinitis pigmentosa (RP, rod-cone dystrophy) and advanced forms of AMD such as GA. Collectively, these disorders represent a significant proportion of currently untreatable blindness globally[1,2]. The advanced stages of AMD are categorized into neovascular AMD (nAMD) and GA. While there are effective treatment options for nAMD, such as anti-vascular endothelial growth factor (anti-VEGF) injections, patients with GA have limited treatment options. RP is a highly heterogeneous group of inherited retinal disorders characterized by progressive retinal photoreceptor degeneration. In some patients, the causative genetic defect is located within the RPE rather than the photoreceptors; hence, RPE replacement therapy may be an alternative strategy if gene therapy is not feasible.

There is significant interest in developing effective treatments for these conditions. In particular, RPE transplantation has been gaining traction as a potential therapeutic approach[3-8]. Since the first reports on RPE transplantation appeared in the 1980s[9], the field has expanded to include various RPE cell sources, delivery strategies, and experimental models of disease and transplantation[10-14]. Among the various animal models, only the NHP has a 'macula lutea' with a 'fovea centralis,' an anatomic specialization at the posterior pole of the retina shared with humans. The fovea contains a very high density of cone photoreceptors enabling high-resolution central vision[15]. The NHP also has a similar genomic and proteomic make-up[16] when compared to humans. These similarities make it an important and appropriate animal model for the study

of ocular diseases that affect the human retina[17,18].

This manuscript describes a method for the submacular transplantation of an RPE xenograft, supported by a PET cell carrier, in immunosuppressed NHPs. A transvitreal technique for subretinal RPE transplantation in rabbits has been described in a previous manuscript[19]. However, in NHPs, the presence of the fovea requires particular care during intraoperative manipulation[20]. In particular, there is a high risk of a foveal tear if subretinal fluid injection methods generate an excessive force on the retina[20]. The focus of this manuscript is, therefore, on strategies to reduce the risk of inadvertent foveal trauma in NHP.

These include the use of preoperative intravitreal plasminogen injection for the release of parafoveal adhesions and surgical microscope-integrated optical coherence tomography (miOCT) intraoperatively for real-time visualization of the foveal anatomy. A custom-made 25/41 G dual-bore subretinal cannula with intraocular PFCL tamponade under low IOP settings is proposed to allow a more controlled process of foveal detachment. Furthermore, surgical removal of native RPE is recommended before implantation to allow better integration between the transplanted RPE cells and host photoreceptors. Finally, a peri- and postoperative systemic immunosuppression protocol for NHP models is described to improve the survival of the RPE xenograft post-transplantation[11,21].

PROTOCOL:

NOTE: All animal experiments were conducted in accordance with The Association of Research in Vision and Ophthalmology (ARVO) for the Use of Animals in Ophthalmic and Vision Research. Ethics approval was obtained from the Institutional Animal Care and Use Committee, SingHealth, Singapore. Animals were housed at the SingHealth Experimental Medicine Centre approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). This approval highlights that all animal experiments comply with the standards of the National Advisory Committee for Laboratory Animal Research guidelines set out by the Agri-Food and Veterinary Authority of Singapore. The following experimental protocol was established based on experiments conducted in 6 eyes of 6 *Macaca fascicularis* (4 male and 2 female, 4 to 6 years old, 2.8 to 4.0 kg)**.**

1. Achieving immunosuppression in the NHP model

1.1. Start the immunosuppression 7 days before surgery and continue maintaining immunosuppression throughout the follow-up period.

1.2. Weigh the NHP before the administration of systemic immunosuppression to ensure accurate medication dosage.

1.3. Use oral sirolimus, doxycycline, and minocycline to achieve systemic immunosuppression.

1.3.1. Administer a loading dose of 2 mg of oral sirolimus followed by a daily maintenance dosing of 1 mg. Obtain baseline blood sirolimus level before administration and monitor this throughout the follow-up period. Ensure a concentration of at least 5 µg/L for adequate immunosuppression.

NOTE: Sirolimus dosing is **not** weight-adapted.

1.3.2. Based on the body weight (BW) of the NHP, administer a dose of 7.5 mg/kg each of oral doxycycline and minocycline.

1.4. During immunosuppression, monitor all NHPs for adverse systemic effects. Look for significant body weight loss (>10%), decreased appetite and water consumption, ungroomed hair loss, and abnormal behavior such as aggression and sluggishness.

2. Instrument sterilization

2.1. Rinse the surgical instruments using distilled water.

2.2. Place the instruments in an ultrasonic bath filled with 500 mL of distilled water and 2 mL of instrument disinfectant. Clean the instruments using the sweep function of the ultrasonic bath for 15 min.

2.3. Remove the instruments from the ultrasonic bath. Rinse twice—thoroughly—with distilled water for 5 min each rinse. Air-dry the instruments after the rinse.

2.4. Place the instruments in an instrument box. Autoclave the box using the universal program setting (sterilization of instruments at 134 °C for 50 min: 30 min for autoclaving, 20 min for drying).

3. Preparation of preservative-free triamcinolone (40 mg/mL)

3.1. Using a 1 mL syringe, withdraw 1 mL of triamcinolone solution (10 mg/mL). Transfer it to a 15 mL conical tube and mix it with 4 mL of sterile balanced salt solution (BSS).

3.2. Centrifuge the solution at 120 × *g* for 5 min. Ensure all the triamcinolone particles are at the bottom of the conical tube. Discard the supernatant (BSS) from the conical tube.

3.3. Re-suspend the triamcinolone particles with 5 mL of sterile BSS in the conical tube. Centrifuge the solution at 120 × *g* for 5 min. Discard the supernatant again.

3.4. Repeat step 3.3 to complete the washing of the triamcinolone particles with BSS (3x).

3.5. Re-suspend the triamcinolone particles with 0.25 mL of sterile BSS to achieve a concentration of 40 mg/mL.

3.6. Aspirate the re-suspended triamcinolone (40 mg/mL) with a new 1 mL syringe. Attach a 25 G blunt-tip flute needle, and keep the syringe with the triamcinolone solution ready for intraoperative use.

4. Pretreatment of NHP eyes with intravitreal plasminogen (0.25 μg/μL)

4.1. One week before the surgery, administer an intravitreal injection (20 μL) of monkey plasminogen (0.25 μg/μL).

4.2. Sedate the NHP before the procedure with an intramuscular injection of ketamine (10–20 mg/kg BW) and a subcutaneous injection of atropine (0.05 mg/kg BW). Administer tetracaine eyedrops for local anesthesia.

4.3. Before intravitreal injection, disinfect the periorbital region with 10% povidone-iodine. Disinfect the eye by administering 5% povidone-iodine to the conjunctival fornices of the NHP. Ensure the solution stays in the fornices for at least 1 min before rinsing thoroughly with sterile BSS.

4.4. Use a 250 μL syringe to aspirate the prediluted monkey plasminogen (0.25 μg/μL) from the vial. Attach a 30 G needle to the syringe, and keep the monkey plasminogen ready for intravitreal administration.

4.5. Use a pair of calipers to identify the injection site on the eye. Administer the intravitreal injection 3 mm away from the limbus.

4.6. Proceed with the injection with the needle directed towards the center of the globe. Upon removal of the needle from the globe, use a cotton applicator stick to tamponade the injection site and prevent the reflux of the intraocular contents.

4.7. Administer a lubricant gel or ointment to reduce immediate postoperative ocular surface irritation.

5. Surgical table and equipment setup

5.1. Establish a sterile field. When in the sterile field, wear surgical scrubs, mask, and hair cover at all times.

5.2. Prepare the preservative-free triamcinolone (40 mg/mL) for intraoperative visualization of vitreous (see section 3). Prepare the sterile BSS in a 10 mL syringe and lubricant in a 5 mL syringe. Place them on a drape.

5.3. Keep other instruments ready on a drape, including 3-0 silk, 7-0 vicryl sutures, cotton applicator sticks, wound closure strips, and chandelier endoillumination fiber wire.

5.4. Connect the vitrectomy set, including the high-speed vitrector, Venturi cassette, and the 25 G chandelier endoilluminator to the vitrectomy machine using sterile technique.

5.5. Open a 500 mL ophthalmic grade BSS bottle and connect it to the Venturi cassette according to the manufacturer's instructions. Proceed with priming the system.

5.6. Switch on the miOCT/surgical microscope. Select preset configurations of the surgical microscope for posterior segment surgery and illumination. Enter the details of the procedure, including ID, gender, laterality of the animal eye, and the name of the procedure.

5.7. Mount a non-contact, wide-angled, 128-degree fundus lens.

5.8. Attach sterile disposable hand-piece covers onto the surgical microscope/miOCT. Adjust the microscope position and focus using the foot pedal. Proceed with surgery.

6. Preparation of anesthesia and positioning of the animal (preferably performed by veterinarian team)

6.1. Ensure the NHP is fasted for at least 8 h before the induction of anesthesia to prevent regurgitation and vomiting. Sedate the NHP before the induction of anesthesia (see step 4.2 for sedation instructions).

6.2. Apply 1% tropicamide and 2.5% phenylephrine eyedrops at least 3x with 5 min intervals to achieve pupil dilation.

6.3. Administer an intramuscular injection of buprenorphine (0.005–0.03 mg/kg BW) 30 min before the surgery to achieve analgesia.

6.4. Intubate the NHP with an endotracheal tube, usually 3–5 mm in size. When attempting intubation, ensure that several sizes are available. Use the largest size that can be passed through the larynx without causing trauma.

6.5. Deliver 2% isofluorane gas via the endotracheal tube to induce general anesthesia. Confirm the state of general anesthesia (lack of response to touch) by assessing the NHP's response to surrounding stimuli, including sounds and touch. Use 0.5–2% isofluorane gas to maintain the state of general anesthesia.

6.6. Continuously monitor the NHP electrocardiogram, respiratory rate, blood pressure, and oxygen saturation during the entire surgery.

6.7. Position the NHP on the surgical table such that the eye is perpendicular to the surgical microscope. Administer a lubricant gel or ointment to the eye, which is not being operated on to reduce ocular surface irritation during anesthesia.

6.8. Cut the eyelashes using scissors to reduce the chance of infections.

6.9. Disinfect the periorbital region with 10% povidone-Iodine. Disinfect the eye by administering 5% povidone-iodine to the conjunctival fornices of the NHP. Ensure the solution stays in the fornices for at least 1 min before rinsing thoroughly with sterile BSS.

6.10. Position a sterile drape such that the pre-cut opening is centered over the eye to undergo surgery. Cover the eye with an adhesive surgical incision drape.

6.11. Perform a lateral canthotomy on the eye to undergo surgery.

6.12. Insert the Lieberman speculum to ensure adequate opening of the eyelids for the visualization of the eye.

7. Vitrectomy

NOTE: To access the subretinal space for the delivery of the PET-scaffold RPE graft, this protocol recommends a 4-port (valved) 25 G vitrectomy to be performed using a standard vitreoretinal surgical setup and a non-contact, wide-angled, 128° fundus lens. The protocol also recommends the use of a surgical microscope equipped with miOCT to guide several critical surgical steps, including the induction of foveal detachment, implantation of the RPE graft, and subretinal fluid drainage.

7.1. Perform a 360° conjunctival peritomy by incising the conjunctiva near the limbus using a pair of vannas scissors. Enlarge the peritomy by performing a blunt dissection.

7.2. Using a 25 G microvitreoretinal blade, perform a sclerotomy at 8 o'clock for the right eye or 4 o'clock for the left eye. Perform the sclerotomy 3 mm from the limbus of the eye.

7.3. Insert and suture a 25 G custom side port-infusion cannula using 7-0 vicryl suture. After confirming the intravitreal location, start the BSS infusion and set the system to maintain an IOP of 20 mmHg.

7.4. Using a 25 G flat head trochar, perform a sclerotomy at 2 o'clock for the right eye or 10 o'clock for the left eye, as in step 7.2.

7.5. Insert the 25 G chandelier light into the flathead trochar and secure it with sticky tape. Adjust the light source to approximately 60%.

7.6. Perform another sclerotomy, similar to step 7.2, at 10 o'clock for the right eye or 2 o'clock for the left eye. Place U-shaped vicryl 7-0 sutures around the sclerotomy without tying the knots. Insert the vitrectomy cutter tip through this sclerotomy.

7.7. Start the vitrectomy around the entry ports, followed by a short core vitrectomy with the following settings: maximum 5000 cuts per minute, maximum aspiration at 400 mmHg.

7.8. Inject 20–50 µL of triamcinolone (40 mg/mL) for better vitreous visualization.

7.9. Induce a posterior vitreous detachment (PVD) by separating the vitreous body from the retina.

7.9.1. Position the vitrector above the optic disc to allow gentle induction of the PVD. Keep the vitrector only on aspiration at the maximum setting of 400 mmHg without any cutting involved.

7.9.2. If required, use 25 G intraocular forceps to manipulate the vitreous at the disc margin to create a tear in the vitreous cortex to facilitate detachment.

NOTE: PVD is considered successful if triamcinolone crystals glide unimpeded over the retinal surface.

7.10. Open the posterior hyaloid membrane with the cutter, and remove the detached vitreous skirt up to the vitreous base (at the retinal equator). Aspirate any remaining triamcinolone on the retinal surface.

8. miOCT-guided foveal detachment

8.1. Inject 1–2 mL of PFCL to cover the posterior pole up to the anterior, mid-peripheral retina.

8.2. Enter the eye with a subretinal injection cannula. Set the IOP to 0–4 mmHg on the vitrectomy machine (ensure perfectly watertight system; if needed, tie sutures around the ports).

8.3. Using either the 25/41 G customized dual-bore subretinal injection cannula or 25/38 G subretinal injection cannula connected to a 250 µL syringe, gently perform a subretinal injection of BSS to induce a localized retinal detachment. Once the bleb just crosses the fovea, stop the injection. Create a second bleb from a separate direction. Merge both the blebs to fully detach the fovea.

8.4. Enable the miOCT function to visualize bleb formation. Ensure the line and cube scans are in HD mode with the settings (512 x 128 pixels, scan width 4 mm) to acquire an image at the fovea. Observe the miOCT image for a complete detachment of the neural retina from the RPE layer at the fovea.

8.5. Enlarge the retinotomy to 1.5 mm with a pair of vertical 25 G vitreoretinal scissors to allow access to the subretinal space for transplantation.

9. Removal of native RPE

9.1. Set the IOP to 50 mmHg on the vitrectomy machine.

9.2. Remove the PFCL via active extrusion using a brushed silicone tip cannula.

9.3. Extend the sclerotomy with a 1.4 mm incision knife to allow the entrance of a 20 G instrument.

9.4. Using a custom 20 G extensible loop instrument, scrape the submacular host RPE for removal. Scrape an area that measures at least 2 x 3 mm.

10. Loading of the shooter for delivery of RPE cell monolayer transplant

10.1. For general instructions on the loading of a bullet-shaped graft cut from RPE cultures on PET cell carriers, refer to a previous publication[22].

11. miOCT-guided graft implantation and position adjustment

11.1. Insert the tip of the shooter device through the sclerotomy at an IOP of 20 mmHg. Inject the implant towards the subretinal space via the retinotomy edge created from the retinal surface.

11.2. Inject the implant with the cell carrier side facing the Bruch's membrane and the RPE xenograft side facing the photoreceptors.

11.3. Enable the miOCT function to visualize the implant location. Ensure the implant is resting flat on the Bruch's membrane in the subretinal space, with an intact overlying retina. Ensure it is located a reasonable distance away from the created retinotomy and not impinged on the retinotomy site.

11.4. Adjust the implant position with the subretinal injection cannula or a 25 G curved intraocular scissor to ensure it is well-positioned under the macula.

12. miOCT-guided drainage of subretinal fluid

12.1. Using a brushed silicone tip cannula, perform a fluid-air exchange and careful subretinal fluid drainage. Attempt gentle subretinal fluid aspiration from the bleb retinal detachment and retinotomy edge apposition.

12.2. Enable the miOCT function for real-time visualization of adequate subretinal fluid drainage until the retina is reattached over the implant.

13. Ending the operation

13.1. Close the working port sclerotomy using the preplaced 7-0 vicryl suture. Remove the 25 G chandelier and 25 G infusion cannula. Close these sclerotomies with 7-0 vicryl sutures.

13.2. Administer 0.05 mL/2 mg of intravitreal preservative-free triamcinolone (40 mg/mL) at the 8 o'clock sclerotomy before suturing.

13.3. Palpate the eye to ensure that the IOP is within the acceptable range. Inject filtered air (or BSS) via a 30 G needle if required.

13.5. Suture the conjunctiva with 7-0 vicryl sutures and canthotomy with 5-0 prolene (remove after 10–14 days).

14. Postoperative animal care

14.1. Position the NHP face down for 1 h post-surgery. Do not leave the animal unattended until consciousness has been regained. Ensure that a veterinarian and animal care technician are available for observation and support during the postoperative process.

14.2. Apply topical antibiotic (tobramycin), steroid (dexamethasone) ointment, and homatropine eye drops twice a day for 5 days postoperatively.

14.3. Administer another subcutaneous buprenorphine (0.005–0.03 mg/kg BW) injection 6 h after surgery for adequate pain control.

14.4. Return the NHP to the company of other animals only when it has fully regained consciousness.

15. Postoperative monitoring methods for multimodal imaging

15.1. Fast the NHP overnight. Sedate the NHP just before imaging (see step 4.2 for drug and concentration for sedation). If sedation is insufficient to stop eye movement, consider the use of general anesthesia.

15.2. Apply 1% tropicamide and 2.5% phenylephrine eyedrops to achieve pupil dilation before imaging (see step 6.2).

15.3. Perform autofluorescence (AF), fundus fluorescein angiography (FFA), and optical coherence tomography (OCT) using a high-resolution OCT machine with a 55° field lens and 30° field lens.

15.3.1. Administer intravenous 10% fluorescein (0.1 mL/kg BW) for FFA. To obtain an early phase image, capture an image within 30 s of injection. For a late phase image, capture an image 5–10 min after the injection.

15.4. Perform fundus photography using a fundus camera between the early and late phases of FFA.

16. Postoperative monitoring methods for full-field electroretinogram (ERG) studies

16.1. Fast the NHPs overnight. Sedate the NHP before ERG studies (see step 4.2 for drug and concentration for sedation). Throughout the ERG recordings, re-administer sedation when appropriate.

16.2. Separate multimodal imaging and ERG recordings with an interval of at least 2–3 days.

16.3. Once sedated, ensure the NHP is dark-adapted for 30 min before the ERG recording.

16.4. Position the stainless-steel subdermal needle electrodes at the left and right lateral canthi (reference electrodes) and the back of the NHP body (ground electrode). Place the ERG contact lens electrodes on the NHP cornea using vidisic gel to aid contact and adhesion.

16.5. Base all ERG testing on the human protocols recommended by the International Society for Clinical Electrophysiology of Vision (ISCEV)[14]. Commence ERG recording under scotopic conditions and start with the dimmer flashes. Follow the ISCEV recommendations for recommended interstimulus intervals.

16.6. Ensure the NHP is light-adapted for 10 min before photopic testing, again using standard ISCEV recommendations for background strength.

17. Euthanasia of NHP

17.1. To euthanize the NHP for enucleation, administer intravenous sodium pentobarbital (75 mg/kg), as recommended by the Panel on Euthanasia of the American Veterinary Medical Association.

REPRESENTATIVE RESULTS:

The multimodal imaging modalities (fundus photography, fundus autofluorescence imaging (FAF), fundus fluorescein angiography (FFA)—early-phase and late-phase, and optical coherence tomography (OCT)) highlight the features of a successful submacular RPE graft transplant (**Figure 1**). Fundus photography should show the positioning of the RPE graft transplant at the fovea without migration over time. FAF imaging should show minimal changes in hyper-autofluorescence (demonstrated by white, high-intensity areas) overlapping the RPE graft. Early- and late-phase FFA should not show any obvious leakage (demonstrated by white, high-intensity areas that enlarge with time) surrounding the RPE graft. Initial images at Day 3 should show window defect due to the removal of native RPE before graft implantation. Macular OCT images should show the preservation of outer retinal layers (in particular, the photoreceptor layer) over the RPE graft as time progresses. Hematoxylin and eosin staining should show intact retinal layers with no evidence of microtears. The preservation of the outer nuclear layer above the peripheries

of the graft suggests that the RPE cells are performing their physiological functions of maintaining photoreceptor health.

The intraocular and external views of the 25/41 G dual-bore cannula highlight the mechanism by which the IOP is controlled during subretinal injection (**Figure 2**). BSS enters the subretinal space during subretinal fluid injection via the central longer cannula. Significant increases in intraocular pressure cause the BSS within the vitreous cavity to exit the eye via the larger metal bore of the cannula. BSS then travels along the cannula and is eventually ejected from the egress port near the cannula hub. To assess whether the cannula is working as expected, ensure that fluid flows from the egress port near the cannula hub.

The miOCT allows the visualization of the bleb dimensions and a potential foveal tear intraoperatively during foveal detachment (**Figure 3**). **Figure 3A1–A3** highlight a case of bleb with a foveal tear. In **Figure 3A1**, while the inferior bleb is visible under the surgical microscope, visualization of the tear is difficult. **Figure 3A2** shows the longitudinal section of a bleb without any tears. **Figure 3A3** shows a foveal tear when assessing the vertical section of the bleb. **Figure 3B1–B3** show a successfully created bleb without the presence of any tears.

The absence of significant deterioration in the ERG waveforms suggests that the global function of both rod and cone photoreceptors is maintained with subretinal RPE xenografts (**Figure 4**). The ERG waveforms show the overall function of the retina. In particular, attention should be paid to the A-waves to determine any loss of photoreceptor function.

FIGURE AND TABLE LEGENDS:

Figure 1: Postoperative *in vivo* analysis with multimodal imaging. (**A**) *In vivo* imaging of the left eye submacular RPE graft transplant (yellow on fundus photography) on various imaging modalities (left to right columns: fundus photography, autofluorescence, fundus fluorescein angiography—early phase, fundus fluorescein angiography—late phase, optical coherence tomography) for time points up to 3 months (top to bottom rows: Days 3, 14; Months 1, 3). The asterisk on the fundus photograph indicates the site of the retinotomy; the white dashed arrow indicates the direction of the line scan. The yellow drawn shape on fundus autofluorescence imaging highlights the location of the transplant. The white triangles on the OCT images indicate the respective lateral edges of the graft (as per the line scan on the color fundus image). (**B**) Hematoxylin and eosin staining of the transplant under atrophic fovea (due to intraoperative tear) with layers labeled. Scale bars = 1 mm in **A** (autofluorescence and FA images), 200 µm in **A** (OCT images), and 100 µm in **B**. Abbreviations: FA = fundus angiography; OCT = optical coherence tomography; RGC = retinal ganglion cell layer; INL = inner nuclear layer; ONL = outer nuclear layer; RPE = retinal pigment epithelium.

Figure 2: Intraocular and external views of the 25/41 G dual-bore cannula. (**A**) Intraocular view of the 25/41 G dual-bore cannula during subretinal bleb creation. The white arrow points to the longer central cannula for subretinal injection. The dashed arrow points to the opening of the egress cannula through which the BSS passes to exit the eye. (**B**) External view of the 25/41 G

dual-bore cannula. The asterisk marks the egress port near the cannula hub from which the intraocular BSS is drained. Abbreviation: BSS = balanced salt solution.

Figure 3: Intraoperative microscope images and miOCT images of subretinal bleb complicated by a foveal tear. (**A1**) An intraoperative microscope image showing the position of longitudinal (blue) and transverse (red) scans in a bleb with a foveal tear. (**A2**) Longitudinal miOCT scan showing a subretinal bleb at the foveal region (yellow arrow). (**A3**) Transverse miOCT scan capturing a foveal tear (white arrowhead), along with a retinotomy (asterisk and a subretinal bleb (yellow arrow). (**B1**) An intraoperative microscopic image showing the position of longitudinal (blue) and transverse (red) scans in a successfully formed bleb. (**B2**) Longitudinal miOCT scan showing a subretinal bleb at the foveal region (yellow arrow). (**B3**) Transverse miOCT scan showing a successfully created subretinal bleb with an intact fovea superiorly (white diamond). Abbreviation: miOCT = microscope-integrated optical coherence tomography.

Figure 4: ERG of RPE xenograft-transplanted eye. For the functional assessment of the retina, full-field ERG assessments of the RPE-xenografted eye performed at baseline (top row) and 3 months post-transplantation (bottom row) show no significant effect of the RPE xenograft transplantation on any response amplitudes, timing, or waveform under either dark-adapted or light-adapted conditions. Abbreviations: RPE = retinal pigment epithelium; ERG = electroretinogram; DA = dark-adapted; LA = light-adapted.

DISCUSSION:

There are two main approaches being evaluated for submacular RPE transplantation—the injection of an RPE suspension and the transplantation of a monolayer RPE graft. A detailed comparison between the two methods is beyond the scope of this manuscript. However, the transplantation of a monolayer RPE graft may be advantageous as the RPE cells are more organized in a monolayer than in a suspension. RPE cells in the graft are organized in a confluent monolayer, which resembles the organization of the physiological RPE cell layer and enables the transplanted RPE cells to perform their physiological functions. This enables more precise dosing parameters compared to cell suspensions, which is highly relevant for regulatory work and industrial scale-up.

Delivery of the RPE patch graft into the subretinal space requires careful manipulation of the macula and accurate insertion of the graft in the subretinal space. Technological advances in microsurgery, such as miOCT, and a better understanding of intraoperative retinal tissue dynamics have reduced the learning curve of this procedure. In this discussion, the rationales of the following aspects will be explained: i) pre-operative plasminogen injection; ii) the use of intraoperative miOCT; iii) the use of a custom 41 G dual-bore cannula, low IOP settings, and PFCL for subretinal bleb creation; iv) scraping of the native RPE cell layer before transplantation; v) the use of sirolimus, triamcinolone, doxycycline, and minocycline to reduce immunogenic graft rejection.

Preoperative plasminogen injections release parafoveal retinal adhesions

In the initial experiments, it was challenging to detach the fovea with a single fluid wave. On

assessment with miOCT, the images revealed the presence of parafoveal outer retinal adhesions to the native RPE along with evidence of intraretinal trauma[20]. These adhesions may have led to a vertical expansion of the bleb rather than the subretinal fluid wave spreading across the retinal contour, resulting in foveal trauma. Plasminogen is the inactive precursor of plasmin, a protease targeting fibronectin and laminin. Ocriplasmin is a bioengineered variant of human plasmin, approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of symptomatic vitreomacular traction with or without a concomitant macular hole. However, postapproval reports of cystoid macula edema development after ocriplasmin injection have suggested a more extensive effect of the enzyme on the retina[23].

Although the exact mechanisms have not been identified, it was suggested that plasmin could weaken retinal adhesion through the degradation of the interphotoreceptor matrix elements responsible for photoreceptor–RPE adhesion[24]. In this protocol, NHP eyes were treated with intravitreal plasminogen 1 week before surgery to release the parafoveal outer retinal adhesions. Under the assumption that the photoreceptor–RPE adhesion is weakened, a lower force is required to detach the neurosensory retina, including the distal parafoveal ring, which typically resists the subretinal fluid wave[20]. Thus, the force administered during retinal bleb detachment results in the expansion of the bleb across the retinal contour rather than stretching the retina tangentially. This reduces the risk of foveal tears. However, it should be noted that the effect of plasminogen on long-term graft survival was not studied in this protocol. Future studies should attempt to determine this effect.

miOCT provides anatomical feedback to guide subretinal bleb creation, graft implantation, and subretinal fluid drainage

Intraoperative, atraumatic manipulation of the macula is key to achieving good transplantation outcomes. However, microstructural changes of the macula related to manipulation may not always be evident on the operating microscope. In such procedures, the miOCT is an important tool that provides real-time, three-dimensional, intraoperative feedback of the macular structure. miOCT is especially useful during the steps of foveal detachment, graft implantation, and drainage of the subretinal fluid using a fluid-air exchange. During foveal detachment, miOCT can determine the vertical and horizontal dimensions of the bleb. Foveal microtears, which may not be visualized clearly on the surgical microscope, can be confirmed by miOCT (**Figure 3**). During the graft implantation, miOCT images guide by showing the graft's location or proximity to the fovea, through the often less-transparent, detached retina. miOCT can also highlight possible areas of retinal adhesion during a difficult transplantation process[25]. Finally, in the subretinal fluid drainage process, miOCT can reliably guide subretinal fluid drainage until complete retinal–RPE graft contact is achieved.

The combination of a dual-bore cannula, low IOP settings, and PFCL vitreous tamponade synergistically reduces macular trauma during subretinal bleb creation

Tangential retinal stretching and fluid turbulence can occur during the subretinal BSS injection for foveal detachment leading to unwanted foveal tears. To counteract these phenomena, factors, such as the relative position and distance from the foveal center where the injection is initiated, injection volume and velocity, vitreous tamponade, choice of subretinal

instrumentation, and IOP have all been shown to be relevant[20,26,27]. The subretinal bleb for foveal detachment should be situated at a location adequately distant from the fovea, as retinal stretching may be highest at the bleb initiation site[27]. IOP should also be kept low throughout the creation of the subretinal bleb. When the IOP of the eye is high, a higher vertical increase in bleb size rather than expansion along the contour of the retina is observed, whereas blebs are shallower at lower pressures[20]. Furthermore, although an intravitreal injection of 50 µL will effectively double the IOP in humans[28], given the shorter eye length in NHPs, the IOP rise during subretinal injection will probably be higher and more rapid than in humans. While most vitrectomy machines adjust for IOP fluctuation, the adjustment is not a simultaneous but rather a reactive process that occurs as subretinal injection proceeds. Hence, the higher the IOP, the higher the risk of retinal overstretching and resultant foveal trauma. Thus, it is essential to maintain a stable low IOP during subretinal injection.

A commercial 20/41 G (DORC) or a custom-made 25/41 G dual-bore subretinal cannula is recommended for subretinal injection. The cannula allows fluid to exit the vitreous cavity in exchange for BSS injected into the subretinal space. This ensures the 'simultaneous' regulation of IOP during the subretinal injection. A schematic of the dual-bore cannula is seen in **Figure 2.** Finally, PFCL is utilized to reduce the risk of foveal tears[20,26,27]. As PFCLs, such as octaline, have higher specific gravity, they exert a downward force on the retina during foveal detachment[29]. This further stabilizes the foveal detachment bleb creation process and enhances the expansion of the bleb along the retinal contour. This technique has been successfully used for the subretinal injection of rtPA in the setting of massive submacular hemorrhage due to nAMD[30].

Pretransplantation removal of native RPE allows the restoration of RPE–photoreceptor complex

Host RPE should be removed before graft transplantation. This is because the restoration of the RPE–photoreceptor complex is required to enable the RPE transplant to perform its physiological functions of supporting the photoreceptors[21]. The host RPE, if not removed, may pose as a mechanical barrier, which prevents the restoration of this complex. It can be removed either through the administration of RPE-toxic chemicals or by using physical means of removal. Chemical removal methods include the systemic or subretinal administration of sodium iodate[31,32]. As sodium iodate causes widespread degeneration of photoreceptors, RPE cells, and Choriocapillaris when administered, its retinal and systemic toxicity precludes its use for human trials[32,33]. Hence, physical intraoperative techniques are preferred. Various physical methods have been conceptualized. When physical methods are utilized, it is crucial that the Bruch's membrane remains undamaged. Many *in vitro* studies have demonstrated the dependence of RPE graft survival on an intact Bruch's membrane[34-36].

Attempts at hydraulic debridement were associated with breaks in Bruch's membrane, an increased rate of epiretinal membrane development, and proliferative vitreoretinopathy, resulting in tractional retinal detachment[37]. A diamond-dusted spatula proposed for RPE debridement also led to breaks in the Bruch's membrane, resulting in cellular proliferation from the choroid into the subretinal space[38]. Interestingly, a custom-made extendable loop instrument could remove the overlying RPE with preservation of Bruch's membrane in the eyes of rabbits

and pigs[11,39]. The removal of the underlying RPE is also useful for establishing animal models with RPE and outer retinal atrophy, similar to the advanced atrophic form of AMD. When a focal area of RPE is removed from the macula, the RPE wound closes via the hypertrophy of the remaining RPE cells. However, this wound healing response is associated with atrophy of the outer nuclear layer[40]. While the creation of an animal model is beyond the scope of this manuscript, a similar procedure can create an animal model of an advanced atrophic AMD phenotype for the testing of RPE-derived cell therapeutics.

The use of sirolimus, triamcinolone, doxycycline, and minocycline to reduce immunogenic graft rejection

The subretinal space is thought to be an immune-privileged site, maintained by an intact blood-retinal barrier and other factors[41]. In many studies involving the subretinal transplantation of stem-cell derivatives with an intact blood-retinal barrier, immunosuppressive drugs play a negligible role in graft survival[42]. The outer blood-retinal barrier is thought to be formed by the native RPE layer and the tight junctions between the RPE cells. While native RPE removal allows better integration of the transplanted RPE and host photoreceptors, the blood-retinal barrier is disrupted in the process, increasing the likelihood of an immune rejection. Classically, T-cells are central to the process of transplant rejection of other organs such as the kidney and liver[43]. Hence, initial immunosuppressive regimens for retinal tissue transplantation were targeted towards reducing these adaptive immune responses.

Sirolimus, a mechanistic target of rapamycin inhibitor, and tacrolimus, a calcineurin inhibitor, are examples of immunosuppressive drugs targeting adaptive immune responses. However, despite adequate T-cell suppression, graft survival rates remain low. In addition, RPE cells are known to suppress T-cell activation through the release of inhibitory factors and promote the generation of regulatory T-cells[44]. Hence, it has become increasingly apparent that adaptive immunity may not be the only contributor to graft rejection[42]. Subretinal transplantation of cellular products can result in the accumulation and activation of microglia[45].

Microglia are the macrophages of the retina. They consist of two main populations: 1) the perivascular microglia of the inner retinal vasculature and 2) the microglia within the retinal tissue parenchyma. As microglia are part of the innate immune response, intravitreal glucocorticoids, such as triamcinolone, can suppress cytokine-mediated proliferation[46]. Doxycycline and minocycline can also suppress microglial activation and should be considered[47,48]. Lastly, differences in immune rejection of RPE allografts versus xenografts are incompletely understood[49]. For instance, alloantibodies against induced pluripotent stem cell-derived RPE cells have been reported in the serum of *in vivo* immune rejection models. However, the role of these antibodies and the importance of antibody-mediated rejection in graft survival remains unknown[50]. Hence, a multidrug regimen utilizing sirolimus for the suppression of adaptive immunity and a combination of triamcinolone, doxycycline, and minocycline for innate immunity suppression is proposed. This regimen has been successfully used in rabbits with good graft survival outcomes and minimal systemic effects[11].

Limitations of this surgical technique

This paper describes a possible surgical method to deliver an RPE graft sheet into the subretinal space of NHP; however, this does not mean this is the only optimized way. Different vitreo-retinal surgeons may have other preferences for instrumentation and technique. For example, this implantation device design can only deliver flat implants supported with a stiffer cell carrier and hence may not be suitable for relatively flexible (or rolled) implants. RPE suspension transplants can omit much of this technique. Accordingly, surgical details will require modification based on each delivery strategy.

As interest in cellular therapeutics for the treatment of degenerative retinal diseases continues to grow, the NHP animal model will be essential in preclinical studies for studying the factors affecting RPE graft survival. In this manuscript, strategies are proposed to enable the smoother delivery of a submacular monolayer RPE graft in the NHP eye. Methods for better visualization of intraoperative complications are also recommended. It is anticipated that these methods will continue to improve as the use of cellular therapeutics expands. Future method papers should also consider proposing a comprehensive list of investigations to assess various structural and functional aspects of the graft.

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DISCLOSURES:

Boris Stanzel holds a US Patent 9980851 on an instrument (RPE scraper) used in this study. Speaker honoraria from C. Zeiss Meditec and Geuder to Boris Stanzel. The other authors have no conflict of interests to declare.

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Figure 1

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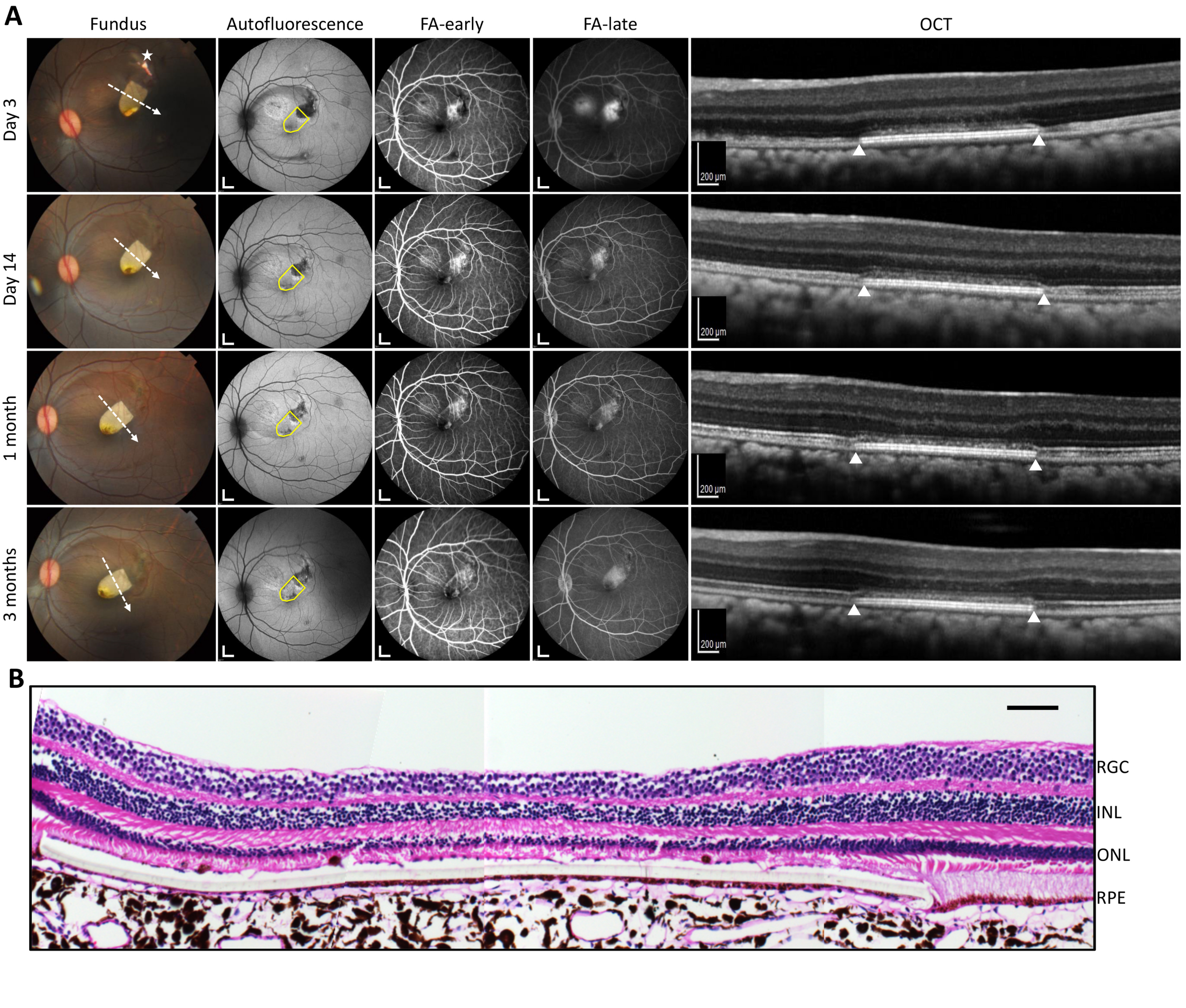

Figure 2

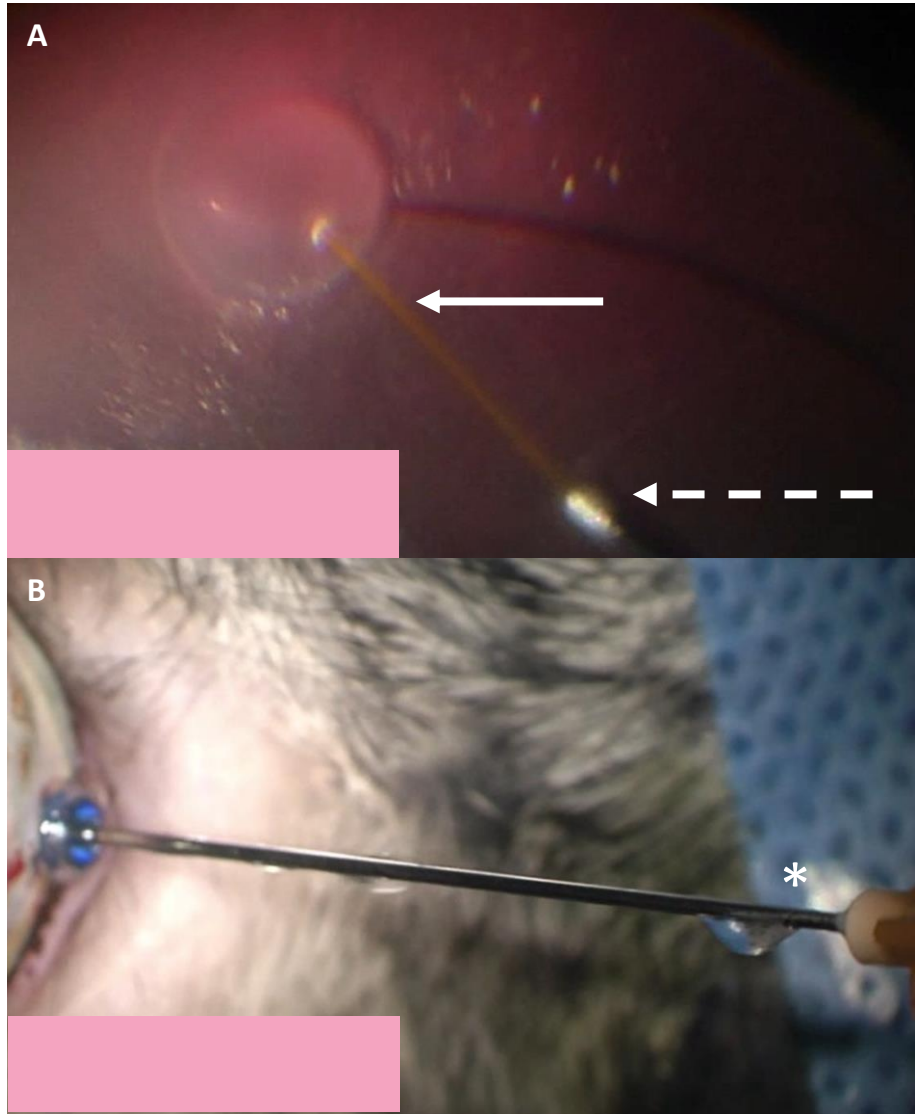

Figure 3

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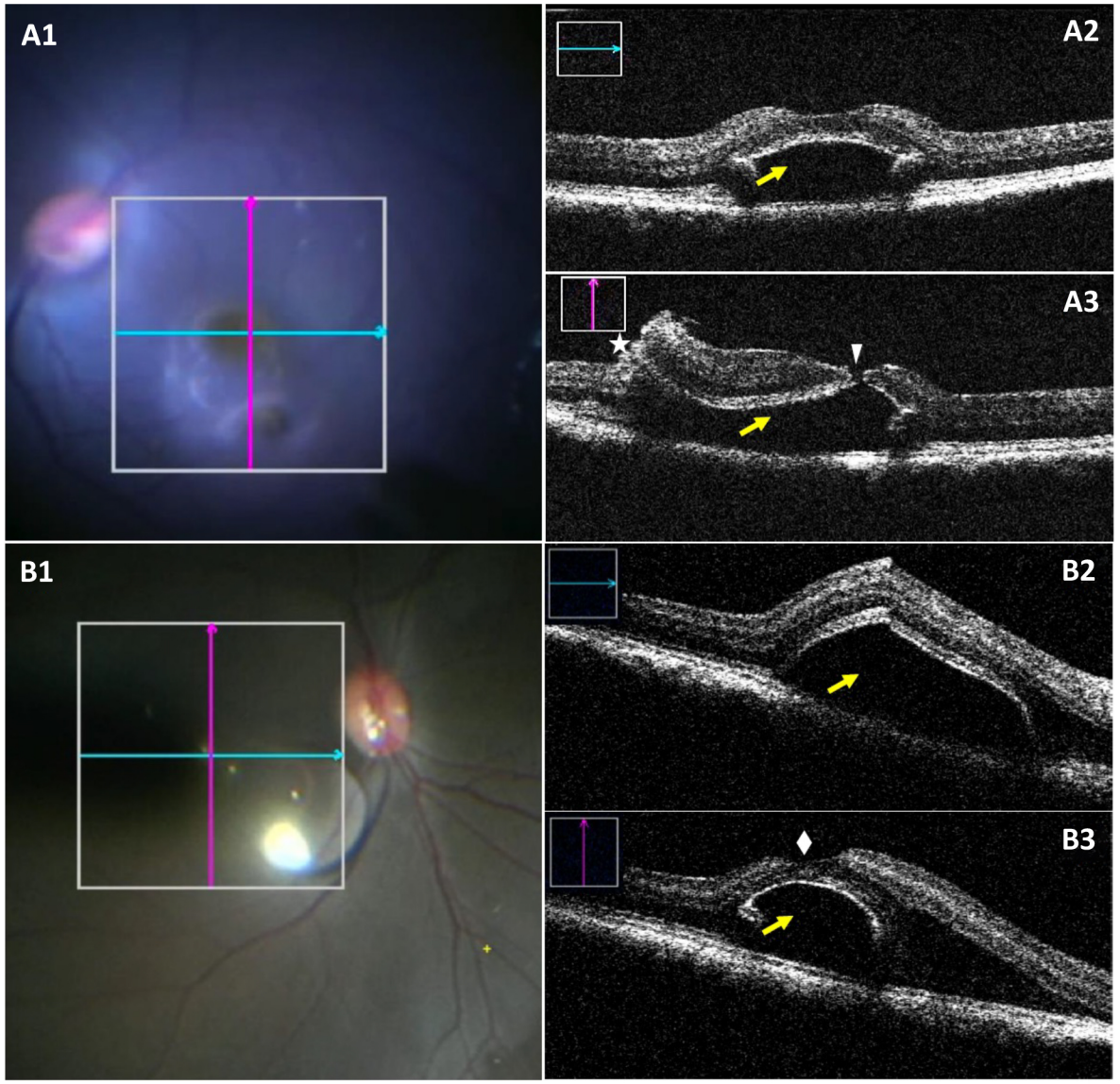
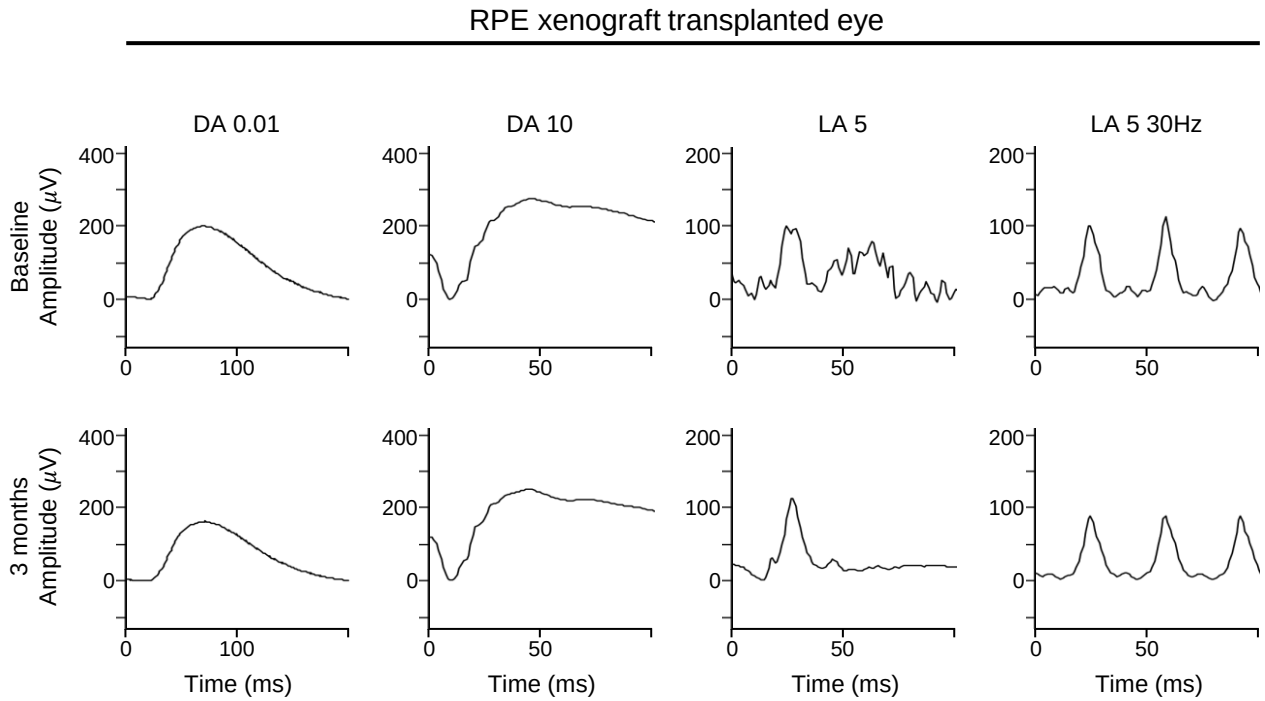


Figure 4



Name of Material/ Equipment	Company	Catalog/Reference Number
1% Mydriacyl (Tropicamide 1.0%) Sterile Ophthalmic preparation	Alcon	SIN 4715P
10% Neutral buffered formalin	Leica	3800598
2.5% Mydfrin (Phenylephrine hydrochloride) Ophthalmic solution	Alcon	No. 01785
25 G AWH Vivid Chandelier	Synergetics	56.54.25P
25 Ga Bi-Blade Vitreous Cutter Combined Wide-Field Stellaris Elite Pack	Bausch & Lomb	SE5525WVB
AMO ENDOSOL Balanced Salt Solution for ophthalmic irrigation	Abbott Medical Optics	15020
Apo-minocycline	Apotex Inc	2084104
AUROVISC - Hypromellose Ophthalmic Solution USP 2% w/v	Aurolab	TN 00002387
Autoclave MELAG, Vacuklav	MELAG	1131-B2300
Autostainer XL (ST5010)	Leica	2433
Balanced Saline Solution	Beaver Visitec	581732
Cotton Bud	WINNER MEDICAL	1NA6-100
Diagnosys Espion E3 Console	Diagnosys	272
Doxycycline	Yung Shin	MAL 19950403AEZ
Eosin Y	Merck Millipore	1.15935.0100
ERG-Jet contact lens electrodes	Fabrinal	F-06
Extendable PolyTip Cannula 25 G/38 G	MedOne	3247
FlexTip Brush (25 g) 1.5 mm	MedOne	3222
Fluoresceine 10% Faure	Curatis AG	5030376
Gauze Swab	WINNER MEDICAL	1NP3275
Hamilton gas tight syringe 250 µL	Hamilton	81101
Heidelberg Spectralis HRA + OCT Computer System	Heidelberg Engineering	N.A.
Hematoxylin Gill II	Merck Millipore	3801520
Inverted microscope eclipse Ti-E main body (100-240V)	Nikon	33131
Ketamin injection	Ceva	37711/58317
Lithium carbonate	Merck Millipore	1.05680.0250
Monkey plasminogen	Molecular Innovations	SKU-CYPLG

Non-contact wide angled 128 degree fundus lens	C. Zeiss Medtech	Resight 700
Non-woven Ophthalmic Drape	Alcon	8065103120
Ophthalmic Corneal/Scleral V-Lance Knife 20 G	Alcon	8065912001
Paraffin Embedding Station	Leica	EG1150 H
Paraplast High Melt Paraffin	Leica	39601095
Phloxin B	Merck Millipore	1.15935.0025
Prepowdered Surgical Gloves	MAXITEX	85-173-2/85-173-3/85-173-4
PRODINE Povidone-Iodine Solution BP	ICM PHARMA	PMLBLP20-01
Righton Slit Lamp Model MW50D (RAA133CB)	Righton-Oph	5200162
Rotary microtome	Leica	RM2255
Safil Polyglycolic acid, braided, coated, absorbable surgical suture 7/0	B.Braun	G1048711
SHINCORT I.M. INJ. Triamcinolone Acetonide 40 mg/mL	Yung Shin	SHI40 SGP-2610015-001
Single-Use Hypodermic Needle 21 G	B.Braun	4657527
Single-Use Hypodermic Needle 23 G	B.Braun	4657667
Sirolimus	Pfizer	SIN12034P
Stainless steel subdermal needle electrode	OcuScience	F-E2
Stellaris Elite vision enhancement system	Bausch & Lomb	BL15455
Sterican Single Use Insulin Needles Long Bevel 27 G 12 mm	B.Braun	4665406
Sterican Single Use Insulin Needles Long Bevel 30 G 12 mm	B.Braun	4656300
Surgical gown + 2 Hand Towels	STERIL	APP10 00 01
Tegaderm Film	3M	1626W
TERUMO Syringe 1 cc/mL Luer SlipTip with needle 26 G	Teruma	SS-01S
TERUMO Syringe 3 cc/mL Luer LockTip	Teruma	SS-03L
TERUMO Syringe 5 cc/mL Luer LockTip	Teruma	SS-05L
TobraDex (Tobramycin, Dexamethasone) Sterile Ophthalmic Ointment	Alcon	No. 01577
Topcon Retinal Camera TRC-50DX	Topcon	948605
Vidisic Gel	Bausch & Lomb	GB41789155517
Xylazil-20	Ilium	38653/50276
Zeiss Opmi Rescan 700	Carl Zeiss Meditec AG	7210

Comments/Description
Surgical procedure
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Response to Editorial Team and Reviewers

Dear Editorial Team and Reviewers of JOVE,

We thank you for your kind comments. Please see below for a point-by-point response of the comments highlighted during the review:

Response to Editorial Comments

1. **Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.**

Response: The manuscript has been proofread to ensure no spelling or grammar issues. All abbreviations have been defined at first use.

2. **Reduce the word count of the summary to be 10 – 50 words.**

Response: The word count has been reduced as advised. It currently stands at 49 words.

3. **Please revise the following lines to avoid overlap with previously published work: 54-55; 83-84; 299-300; 342-344; 364-365; 394-395; 409-411**

Lines 53-54 & 82-83: “Retinal pigment epithelial (RPE) cell transplantation is a promising therapeutic option for the treatment of both inherited and acquired degenerative retinal diseases”.

Response: We have edited this to “Retinal pigment epithelial (RPE) transplantation holds great promise for the treatment of inherited and acquired retinal degenerative diseases.” (Lines 51 – 52, 79 – 80)

Lines 299-300: “Place a sterile drape with pre-cut opening in the middle over the operation eye and cover with sticky surgical incision drape”

Response: We have edited this to “Position a sterile drape such that the pre-cut opening is centered over the operation eye. Cover the eye with sticky surgical incision drape.” (Lines 301 – 302)

Lines 342 – 344: “Induce a posterior vitreous detachment (PVD) by separating the vitreous body from the retina. Hold the vitrector just over the optic disc to allow gentle separation”

Response: We have edited this to “Induce a posterior vitreous detachment (PVD) by separating the vitreous body from the retina. Position the vitrector above the optic disc to allow gentle induction of the PVD.” (Lines 346 – 348)

Line 364 – 365: “Gently perform a subretinal injection of ophthalmic grade BSS to create a localized bleb detachment”

Response: We have edited this to “gently perform a subretinal injection of BSS to induce a localized retinal detachment.” (Lines 369 – 370)

Lines 394 – 395: “Approach the created retinotomy edge and inject the implant towards the subretinal space from an epiretinal position”

Response: We have edited this to “Inject the implant towards the subretinal space via the created retinotomy edge from the retinal surface”. (Lines 398 – 400).

Lines 409 – 411: “Perform fluid-air exchange through active extrusion using a brushed silicone tip cannula. Attempt gentle subretinal fluid aspiration from the bleb retinal detachment and retinotomy edge apposition”

Response: We have edited this to “Using a brushed silicone tip cannula, perform a fluid-air exchange and careful subretinal fluid drainage.” (Line 416 – 417)

4. **JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Falcon tube etc**

Response: All trademark symbols, registered symbols and company names have been removed from the manuscript. These include Lines 178 (Falcon tube), 214 (Hamilton syringe), 368 (Hamilton syringe), 484 (ERG-Jet contact lens electrodes).

5. **Being a video-based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:**
   a) **Please mention how proper anesthetization is confirmed, and specify the use of vet ointment on eyes to prevent dryness while under anesthesia.**

Response: The confirmatory process of anesthetization is added in Lines 281 – 283. “The state of general anesthesia is confirmed by assessing the NHP’s response to surrounding stimuli including sounds and touch. No response to touch confirms the general anesthesia state.” While under anesthesia, the eye to be operated should not have any ointment as it will reduce the view of intraocular structures (which is required during the surgery). The use of vet ointment on the eye which is not being operated has been added in Lines 291 – 292. “Administer a lubricant gel or ointment to the eye which is not being operated to reduce ocular surface irritation during anesthesia.”

   b) **For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.**

Response: The post-surgical treatment of the NHP has already been described in Lines 439 – 452. This includes the recovery conditions and treatment for post-surgical pain. We have added in an additional statement in Lines 441 – 443. “Ensure a

veterinarian and animal care technician are available for observation and support during the post-operative process."

c) **Discuss maintenance of sterile conditions during survival surgery.**

Response: The establishment and maintenance of a sterile field has already been elaborated in Lines 231 – 232, 258 – 260, 296 – 299.

d) **Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.**

Response: This has been mentioned in Lines 441 – 443.

e) **Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.**

Response: This has been added in Lines 451 – 452 as recommended.

f) **What happened to the animals after the study? Please specify the euthanasia method without highlighting any steps describing euthanasia.**

Response: This has been added in Lines 495 – 498 as recommended.

6. **Please use SI units and replace ml with mL, hrs with h etc.**

Response: This has been updated as recommended.

7. **Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.**

Response: Line 430 – 431 has been edited to "Give 0.05 mL/2mg of intravitreal preservative free triamcinolone (40 mg/mL) at the 8 o'clock sclerotomy prior to suturing". Apart from the above statement, all the other instructions have been written in the imperative tense. There is no more usage of the terms "could be", "should be", "would be" in the protocol.

8. **Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.**

Response: There is adequate detail in each step to supplement the actions seen in the video to be made.

9. **After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.**

Response: The 3 pages of protocol text for filming have been highlighted in yellow. This includes the text between Lines 310 – 437.

10. **Please rewrite your representative results so that it doesn't look like the legends section. Please discuss all figures in the Representative Results, and include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.**

Response: Results from Figure 1 has been written to explain the representative results in the context of the technique that we have described. We have also added further descriptions to identify imaging findings such as hyper-autofluorescence (Line 508 – 509) and leakage on fundus angiogram (Line 509 – 600). Results from Figure 2 has also been written to explain how to see if the dual-bore cannula is working as per its design (Lines 525 – 526). Results from Figure 3 were detailed in the original submission, to show the difference between a successful and a sub-optimal experiment (highlighting the presence of subfoveal tears). Finally, results from Figure 4 have been rewritten to highlight the importance of the A-wave in determining any loss of photoreceptor function post-transplantation.

11. **In Figure 3, if a1, b1 etc are actually representative of panels, please use uppercase letters.**

Response: The panel labels have been changed to uppercase letters (see edited Figure 3) as recommended. This is also reflected in the legend (Lines 568 to 575) and results (Lines 530 to 535) segment.

12. **Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.**

Response: Scale bars have been added to Figure 1 for the fundus photography images, autofluorescence and fundus angiography images (see edited Figure 1). As the operative images were taken using a surgical microscope intraoperatively, there are no scale bars available for Figures 2 and 3.

13. **As we are a methods journal and your discussion is already quite long, please add a few sentences about any limitations of this technique.**

Response: We added the following paragraph in lines 742-748: "Here we described a possible surgical method to deliver a RPE graft sheet into the subretinal space of NHP,

but it does not mean this is the only optimized way. Different vitreo-retinal surgeons may have other preferences for instrumentation and technique. For example, our implantation device design used in this protocol can only deliver flat implants supported with a stiffer cell carrier and hence may not be suitable for rather flexible (or rolled) implants. RPE suspension transplants can possibly omit much of our technique. Accordingly, surgical details will require modification based on each delivery strategy."

14. **Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ital.). Volume (bold) (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate journal names.**

Response: The references are already in the stated format.

Response to Reviewer Comments

Reviewer #1

This is a well written and performed work. The problem is well presented, and the results are very interesting and promising. The experimental model is well chosen and imply both high costs and managing difficulties. The experiment and protocols are well designed and adequate to arrive at the conclusions the authors discuss. In all I think this manuscript is well suitable to be published in this journal in its present form.

Response: We thank the reviewer for the above comments.

Major Concerns: N/A

Minor Concerns:

As a future suggestion, I would advise the authors that Electrooculogram (EOG) could be used to evaluate the retinal pigment epithelium (RPE) functionality, and multifocal electroretinogram (mfERG) could be used to evaluate different retinal regions regarding functionality. The use of these techniques could give a sectorial idea of the functional outcomes of the surgery and the integration of RPE and the rest of the retina. Moreover, if there was a chance of studying the focalized choroid blood flow, it would add to the wider landscape of RPE functionality.

Response: We agree with the reviewer's suggestion that the investigations proposed (EOG, mfERG, focalized choroid blood flow studies) can provide more information regarding the functionality of the graft and its relationship with the host retina. However, the main focus for this manuscript is the surgical technique of transplantation. While we have provided the protocol for post-transplantation graft assessment (eg. multi-modal imaging and ERG), it is not meant to be a comprehensive list of modalities but adequate to assess the basic structural integrity and functionality of the graft. We included this in the discussion section (Lines 755 – 757).

Reviewer #2

This manuscript described step by step submacular RPE transplantation method in non-human primates. The manuscript is well written and protocol well described.
However, I have some concerns before publication.

Response: We thank the reviewer for the above comments.

Major Concerns:

In the introduction authors do not quote other studies using NHP for RPE implantation. They should do so (Kamao, 2014; Ben M'Barek, 2020; Fujii, 2021) and then in the discussion and should compare the advantage of their methods vs the one already published. Mainly, discussing the advantages of RPE scrapping (not done in Ben M'Barek, 2020) and the immunosuppression regimen (Fujii, 2021, Sugita, 2020).

Response: We would like to thank the reviewer's suggestion. The above-mentioned studies have been included in both the introduction and discussion segment (Kamao, 2014 Line 94; Ben M'Barek 2020 Line 94; Fujii 2021, Line 94). The scientific rationale and proposed benefits of RPE scraping and the proposed immunosuppression regimen were discussed in the discussion segment titled "Pre-transplantation Removal of Native RPE Allows Restoration of RPE-Photoreceptor Complex" and "The Use of Sirolimus, Triamcinolone, Doxycycline and Minocycline to Reduce Immunogenic Graft Rejection".

Moreover, in their introduction authors quote only two studies of RPE clinical trial. They should explain this choice or quote the other studies (da Cruz, 2018; Mehat, 2018; Mandai, 2017; Sugita, 2020).

Response: The above-mentioned studies have been included in the introduction of the manuscript (da Cruz 2018, Mehat 2018, Mandai 2017, Sugita 2020 Line 91).

In results, authors should show immunohistochemistry pictures showing interaction between grafted RPE and photoreceptors as they claim that removing endogenous RPE improve connection between these cells.

Response: The primary focus of the current manuscript is to provide detailed instructions in order to perform the RPE graft transplantation procedure in a non-human primate. The immunohistochemistry images showing phagocytosis between transplanted RPE and photoreceptors after removing endogenous RPE has been previously published by our group in Liu Z et al, Stem Cell Rep. 2021 (PMID: 33450191). The citation has been added to Line 684. An additional large manuscript currently under revision detailing RPE xenograft-photoreceptor/outer retinal interaction with immunohistochemistry is available on a pre-print server: https://www.researchsquare.com/article/rs-396167/v1.

Moreover, they should precise the duration of the experiment (and so

immunosuppression treatment) and show results about the immunosuppression levels in animals and regimen adjustment.

Response: The primary focus of the current manuscript is to provide detailed instructions in order to perform the RPE graft transplantation procedure in a non-human primate. The purpose of discussing the immunosuppression protocol at the end of the manuscript is to provide the reader with a working immunosuppression protocol and the purported mechanisms of these immunosuppressants. To date, the longest follow-up with this immunosuppression protocol in our group is 3 months. We acknowledge that there are multiple alternative immunosuppression protocols. However, the discussion with regard to the efficacy of each immunosuppression protocol is complex and thus, in our view, well beyond the scope of this manuscript.

Reviewer #3

Manuscript Summary:

The paper by Ivan Seah et al. describes the subretinal implantation of an RPE sheet in non-human primate eyes. As primate eye and retina resemble the human counterparts in terms of number and distribution of photoreceptors, macula, and function, this model is the most suitable for studying the RPE cell therapy.
The article is well explained and the procedures are well described. Discussion covers the most important issues of the analyzed topic of the study. The paper has a strong impact on preclinical and clinical practices since the transplantation of RPE sheets an invasive intervention and reducing and optimizing the surgical technique is of interest.

Response: We thank the reviewer for the above comments.

Major Concerns: None

Minor Concerns:

There are some comments addressed to the manuscript that need to be explained

General comments:
This paper focused on the description of microsurgery however, it would be interesting to have information about the RPE differentiation procedure and sheet (maybe an image as well). Reviewer noticed that there is a reference from the same group where they described this issue, but still, it would be interesting to visualize the graft to be transplanted to compare with the HE staining.

Response: We thank the reviewer for the comments. From the color fundus images in Figure 1, an image of the implanted graft is shown. The surgical video will also show the visualization of the graft during transplantation. With regards to the RPE differentiation procedure, there are various RPE differentiation protocols depending on the type of cell resource. Our group has published several different manuscripts including fetal human RPE (Stanzel BV et al. Invest Ophthalmology Vis Sci. 2012, PMID 22167099); human RPE stem-cell derived RPE (Liu Z et al, Stem Cell Rep. 2021, PMID 33450191) and has one manuscript under revision describing hES-RPE differentiation and transplantation, which is available on a pre-print server (https://www.researchsquare.com/article/rs-396167/v1). However, as the primary focus of this manuscript is to provide a detailed NHP surgical protocol for the viewers to transplant their own cell resource, we believe that the differentiation protocol is out of the scope of this manuscript.

Protocol:
Point 3. they produce triamcinolone without preservative but they remove it afterward. There are commercial products ready to use taken into account that all triamcinolone is removed, so preservatives are not causing damage in this short time.

Response: Intravitreal triamcinolone is utilised for the visualization of vitreous and aids the surgeon's separation of the posterior hyaloid membrane from the retina. This is useful during the step of posterior vitreous detachment. We are aware of commercial triamcinolone products suitable for intravitreal use (e.g. Triesence), but also cognizant of their (unnecessary) cost for the sake of these experiments, too. Anyhow, the triamcinolone removal process is not intended be complete, as the same preservative-free triamcinolone is also used as part of the immunosuppression protocol at the end of the surgery, as also elegantly shown by the group of Dr. Sugita at the RIKEN Institute, Kobe/ Japan.

Point 4. Justify the use plasminogen during 1 week. Did the authors tried without plasminogen in the same experiment? In humans, plasminogen is only used in some children because of the attachment of the hyaloid.

Response: We have previously a manuscript detailing what happens to the NHP fovea, if detached with BSS without prior plasminogen pretreatment. We kindly refer the reviewer to reference 20 (Tan GSW et al. TVST 2021). There we described significant (physiologic) parafoveal vitreoretinal adhesions during the subretinal injection process, which made the bleb retinal detachment technically challenging. Such control, without plasminogen pre-treatment is also included in this manuscript, pls see Figure 3 A1-A3.

Point 7. Why 4 ports instead of 3? The usage of a chandelier is necessary? If they use valved cannulas, there is no need for conjunctival peritomy and the posterior suture (with silk, that is not reabsorbed)

Response: 4 ports were used in order to perform the subretinal injection step (1 for chandelier, 1 for infusion, 2 for subretinal injection) as the subretinal injection had to be performed both from nasal and temporal to fully detach the fovea. The chandelier allows the surgeon to have adequate illumination hands-free; this is very helpful for stabilization of the injecting hand with the other (non-injecting) hand in these non-standardized experimental settings. The forehead orbit of the NHP is much smaller than in human and the eye is located deeper in the orbit. We have had instances where the valved cannulas were dislocated resulting in conjunctival chemosis if peritomy was not performed. We thank the reviewer for highlighting the error regarding the silk suture. This is supposed to be vicryl (now changed in Line 326). The vicryl sutures are pre-placed at the sclerotomy sites to enable the efficient closure of the sclerotomy post-RPE graft delivery.

Point 8. why the PFCL is injected before the subretinal bleb? Did you tried without PFCL and perform the procedure slower in the same experiments?

Response: As explained in Line 673 - 675, PFCL is utilized to reduce the risk of foveal tears during the foveal detachment process. In our previous experiments, micro-sized foveal tears were observed with the intraoperative optical coherence tomography when creating a bleb at the fovea. The PFCL can provide additional downward vector forces on the subretinal bleb to allow a gentler bleb formation process. This method has been investigated by our group in a previous manuscript (Tan GSW et al. Transl Vis Sci Technol. 2021; 10 (1):10 PMID 33510949).

Point 12. Why authors don't use the laser to attach the retina?

Response: The posterior vitreous was already completely detached from the retinotomy. Hence, there should not be any traction on the retinotomy edge. Furthermore, while the use of endolaser can seal the bleb and retinotomy edge, the inflammatory and eventual fibrotic reaction from the laser may affect the outcomes of the graft. As such, the protocol recommends for gentle subretinal fluid drainage with the aim of apposing the retinotomy edges.

Figures:
In the OCTs, RPE sheets are transplanted near the fovea but not under the macula. In contrast, in the HE staining, the sheet appears under the macula. Does this image correspond to any OCT in figure 3? Do you have a HE staining of a successful graft? And regarding the RPE graft, it seems that endogenous RPE partially remains, and the RPE in the graft is patchy and aggregated, justify.

Responds: Figure 1 has been revised as suggested by the reviewer. A new image of HE staining from the same case has been provided. This corresponds to the location of OCT images in Figure 1.

Reviewer #4

Manuscript Summary:

The authors described a detailed protocol for RPE graft transplantation in NHP eyes. As it shares similar anatomy structure with human retina and the most importantly, it has a macula which dominates visual acuity and color vision. The procedures contain every steps of the surgery and treatment of pharmaceutic is also included. There's still some concerns.

Response: We thank the reviewer for the above comments.

Major Concerns:
1. Dose the plasminogen injection have further effect beyond 3 months?

Response: The role of plasminogen prior to surgery is to facilitate the release of parafoveal vitreous adhesions during the surgery for easy detachment of the fovea. The effects of plasminogen on the graft survival were not closely studied. We have stated this in Lines 618 – 620.

2. Full field ERG assessments may not evaluate effect of the RPE transplantation accurately since the the area of RPE xenograft only take a small proportion of the retina. Visual acuity, visual function assessments or focal ERG may be a better choice for this circumstances.

Response: We agree that full field ERG assessments may not be the most accurate modality to evaluate the effect of RPE transplantation since the graft only takes a small proportion of the retina. However, the main focus for this manuscript is the surgical technique of transplantation. While we have provided the protocol for post-transplantation graft assessment (eg. multi-modal imaging and ERG), it is not meant to be a comprehensive list of modalities but enough to assess the basic structural integrity and functionality of the graft. Nevertheless, we have also proposed more comprehensive assessments for future experiments (Line 755 – 757).

3. The transplantation surgery is performed immediately after native RPE removal and the RPE cells around the damage site are healthy, the authors should demonstrate that the healthy native RPE have not proliferate and affect the recovery of RPE damage.

Response: We thank the reviewer for raising this issue. As mentioned in Lines 683 – 686, it has been previously shown that healthy, native RPE can migrate to the RPE wound and hypertrophy to enable wound closure after RPE damage. However, despite this wound healing response, an atrophy of the outer nuclear layer is observed, suggesting that these re-grown RPE cells are suboptimal in terms of their ability to maintain photoreceptor health. This is possibly due to the lack of polarization in the correct orientation with respect to photoreceptor cells. As such, it is unlikely to confound the effect of the graft on the overall functional recovery of the retina. However, as shown in our recently published manuscript (Reference 21, Liu Z et al. Stem Cell Reports 2021), anti-human staining labels all RPE cells on scaffold, with some signal alterations at the edge of the transplant.

4. The authors do not explain the reason performing vitrectomy.

Response: A pars plana vitrectomy has to be done in order to reach the subretinal space for the delivery of the graft. While there are subretinal implantation methods available that do not require a vitrectomy, these methods are more suitable for small rodents or cell suspension injections. Suprachoroidal delivery of carrier-supported is known to induce significant subretinal fibrosis. The PET-scaffold graft unfortunately is not flexible or small enough to be injected into the subretinal space using these methods. We have stated the reason in Line 311.

We hope that the above responses do address the comments provided and look forward to your journal's favourable reply.

With best regards,

Dr. Boris Stanzel, on behalf of the authors