CRB1 gene therapy coming of age: mechanistic insight and rAAV assays on mouse & human retinal organoid models

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Chapter 3

AAV serotype testing on cultured human donor retinal explants

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Abstract

This protocol details on a screening method for infectivity and tropism of different serotypes of Adeno-associated viruses (AAVs) on human retinal explants with cell-type specific or ubiquitous green fluorescent protein (*GFP*) expression vectors. Eyes from deceased adult human donors are enucleated and the retinas are isolated. Each retina is punched into eight to ten 6-mm equal pieces. Whatman™ paper punches are placed on the retinas and the stack is transferred onto 24-well culture inserts with the photoreceptors facing the membrane. AAVs are applied on the retinal explant punches to allow transduction for 48 hours. Retinas are nourished by a serum-free Neurobasal®-A based medium composition that allows extended culturing of explants containing photoreceptor inner and outer segments. The protocols include quality control measurements and histological staining for retina cells. The cost and time effective procedure permits AAV transgene expression assays, RNAi knockdown, and pharmacological intervention on human retinas for 21 days *ex vivo*.

1 Introduction

The first recombinant adeno-associated viral (rAAV) gene rescue therapies have been administered to patients in clinical trials [1, 2]. Preliminary clinical results are promising, but these still have to be converted into medication. Though, for the AAV gene therapy product Glybera (UniQure, Amsterdam, The Netherlands), for lipoprotein lipase deficiency, European market approval has been obtained. The prediction of the cell tropism of different rAAV serotypes and variants on human retinas has been a hurdle. rAAV transduction information of mice has little predictive value for human tissue because the distribution and specificity of receptors hijacked by rAAVs to infect cells differ between species. Yet, information on rAAV serotype infection and both onset and levels of expression in human retinal cell types is limited because adult retinas rapidly lose their morphology during *ex vivo* culturing. Generally, the gene therapy vector protein expression is detectable a week after infection [3, 4] -- provided the AAV particles applied has a sufficient titer -- and peaks at around five weeks [5, 6]. Currently, a fair comparison of cell tropism and infectivity of AAV serotypes and variants with different promoter strengths and onset of expression can only be assessed in monkey studies. Here, we provide a technique to screen rAAVs on cell tropism and infectivity on human retinal explants. This technique can also be employed for neurodegeneration, neuroprotection, and cell transplantation assays.

Many variables influence the success of a retina culture. Differences of the maximum days in culture depends on the species and the age of retina. For example, cultured organotypic neonate mouse retinas last up to 27 days [7,16]. Young adulthood retinas of rats and neonate retinas of chickens have been cultured for up to 7 days [8-10]. However, adult mammalian material, such as human, mouse, and pig retinas have only been cultured for short-term (up to 10 days) [10-14]. This is because many cell, molecular, and morphological changes take place once the retina is cultured *ex vivo*. Changes include the deactivation of the shedding and regeneration of the outer segments (OS) of photoreceptors (PRCs), loss of cells with a successive retinal thinning, collapse of the outer plexiform layer (OPL), and Müller glia stress as identified by the upregulation of glial fibrillary acidic protein (GFAP) [12,13]. The health of the retina needs to be thoroughly monitored and recorded. This can be achieved at the end by immunohistochemistry (retina cells, cell apoptosis, and cell cycling, *see* **Table 1**), morphological description (retinal layer thickness), and comparing it to control samples at the day of dissection; but also by inspecting it for tissue shrinkage and medium usage.

Diverse techniques can be employed to reduce retinal degeneration and culture intervariability, such as to use only fresh tissue (<72 hours post-mortem), to keep the tissue until dissection in Phosphate buffered saline (PBS) or Hanks' Balanced Salt Solution (HBSS) buffers at 4 ºC (<24 hours), to minimally manipulate the retina during dissection, to use serum-free medium compositions with supplements similar to *in vivo* retina environments, to change medium conditionally and frequently, to set the incubator to the optimal temperature

(34 ºC-37 ºC) and air composition (oxygen: ambient or reduced oxygen to 3 %), and to work clean [11–14]. The only two serum-free medium for retina cultures described are R-16 and Neurobasal®-A medium [12,14,15].

2 Materials

2.1 Solutions and media

1. Explant medium: 300 µL 50X B-27 Supplement (Invitrogen), 150 µL 100X N-2 Supplement (Invitrogen), 30 µL 50 mM Taurine, 120 µL 200 mM L-glutamine, 150 µL 100 mM sodium pyruvate, 18,45 µL 1 mM N-Acetyl-L-cysteine, 150 µL 100X antibiotic–antimycotic (10,000 units/mL penicillin, 10,000 µg/mL streptomycin, 25 µg/mL Amphotericin B) in a final volume of 15 mL Neurobasal®-A medium (Invitrogen). Aliquot into 5 mL and store at 4 ºC and use up within 5 days. Warm up aliquots only once.
2. Hanks' Balanced Salt Solution (HBSS). HBSS liquid containing Ca^{2+} and Mg^{2+}
3. Sterile distilled water.
4. MilliQ autoclaved water.
5. Phosphate buffered saline (PBS): 2.6 mM KH_2PO_4, 26 mM Na_2HPO_4, 145 mM NaCl, 7.0 - 7.2.
6. Dulbecco's Phosphate buffered saline with Ca^{2+} and Mg^{2+} (DPBS): 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$-6·H_2O, 2.67 mM KCl, 1.47 mM KH_2PO_4, 137.93 mM NaCl, 8.6 mM Na_2HPO_4-7·H2O, pH 7.0 - 7.2
7. 4 % Paraformaldehyde (PFA) in PBS.
8. 5 % Sucrose in PBS.
9. 30 % Sucrose in PBS.
10. Cryo-embedding media, Tissue-Tek® O.C.T. compound.
11. 1% Sodium dodecyl sulfate (SDS).
12. Ethanol absolute 99.99 %.
13. 70 % ethanol in milliQ water (70 % EtOH): 70 mL Ethanol absolute, 30 mL milliQ water.
14. 10 % Poloxamer-188 surfactant solution, sterile.
15. 0.001 % Poloxamer-188 in DPBS. Filter-sterilized. Stored in 1 mL aliquots at -20 ºC.

2.2 Materials and supplies

1. 12 mm filter diameter; 0.4 µm pore Hydrophilic Polytetrafluoroethylene (PTFE) Millicell Cell Culture Inserts (Millipore; catalogue number PICM01250).
2. Whatman™ 3MM Chr Chromatography Paper. Clean a 2-hole punch with warm water and soap, and 70 % ethanol. Punch the Whatman™ 3MM paper. Autoclave the 6-mm punches.

3. 24-well culture plates, flat bottom.
4. Surgical instruments: Lancet. 11 cm Iris Scissor, curved. 18 cm Operating scissor, sharp/blunt, curved. Dressing forceps. Tissue forceps. 6 mm Surgical punch with adapter (*see* **Note 1**).
5. P1000, P200, P20, P10 pipette tips and single-channel pipette set.
6. Standard pipette gun and serological pipettes.
7. 37 ºC 5 % CO_2 incubator.
8. Parafilm.
9. Sucrose.
10. Superfrost Plus microscope slides.
11. 15 mL conical tubes.
12. 9 cm petridish.
13. 10 x 10 x 5 mm Cryomold® biopsy, square.
14. Dry-ice.
15. Cryostat.
16. 70 mm thickness Antiroll glass.
17. Carbon steel microtome blades C-35 pfm.
18. 20 % Bovine Serum Albumin (BSA) dissolved in PBS.
19. Triton X-100.
20. (Goat) Serum.
21. Blocking Buffer: to prepare 10 mL, mix 1 mL normal goat serum, 0.04 mL Triton X-100, 0.5 mL 20 % BSA, and 8.46 mL PBS.
22. First Antibody Buffer: to prepare 10 mL, mix 0.03 mL (goat) serum, 0.04 mL Triton X-100, 0.5 mL 20 % BSA, 9.43 mL PBS, and first Antibody.
23. Second Antibody Buffer: to prepare 10 mL, mix 0.5 mL 20 % BSA, 9.5 mL PBS, and second Antibody.
24. VECTASHIELD HardSet Antifade Mounting Medium with 4′,6-Diamidin-2-phenylindol (DAPI; Vector Labs).
25. Protective equipment: gloves, safety goggles, lab coat, Biosafety level 2 laboratory, and laminar flow hood.

2.3 Recombinant AAV with transgene expression cassette

1. Recombinant AAV particles to be tested at a titer of $>10^{12}$ genomic copies, e.g. rAAV2.CMV.*EGFP*.WPRE.pA packaged into serotype AAV9 capsids. The expression vector contains e.g. the two inverted terminal repeats (ITRs) of AAV2, the ubiquitous immediate early CMV promoter (CMV), the cDNA for *EGFP*, the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), and a SV40 polyadenylation sequence (pA).

2. The virus was produced as previously reported [3]. In short, HEK293T cells were triple transfected by Ca-phosphate transfection method with the helper plasmid pAAV9 from the Vector Core University of Pennsylvania, the helper plasmid pAdΔF6 [17], and a pAAV2.CMV.*EGFP*.WPRE.pA transfer plasmid. The cells were lysed and harvest 72 hours post-transfection. AAVs were purified by an iodixanol gradient and concentrated by amplicon-spin columns. AAVs were tested on purity by SDS-PAGE Silver Staining and the genomic copies per mL (titer) were measured against a standard by qPCR.

3 Methods

3.1 Culturing of post-mortem human retina

The tissue was collected in agreement with the guidelines of the ethics committee of the LUMC. Patient anonymity was strictly maintained. All tissue samples were handled in a coded fashion, according to Dutch national ethical guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies).

3.1.1 Dissecting out the eye

1. Within 24 hours after the death of a human donor, removal of the entire globe of the eye and its contents, with preservation of all other periorbital and orbital structures was performed.
2. The optic nerve was cut at 1 cm distance from the eye globe.
3. Eyes were transported and stored in HBSS at 4ºC.
4. A suture was put through an eye muscle to differentiate right and left eyes (*see* **Note 2**).
5. The eye was stored in cold HBSS in the fridge (*see* **Note 3**).

3.1.2 Prepare medium and 24-well culture plate (>1 hour before dissection)

1. Prepare fresh medium and warm it up in a water bath (*see* **Note 4**).
2. Add 15 mL milliQ autoclaved water between 24 wells. Place in incubator (*see* **Note 5**).
3. Add 300 µL medium to the 8 wells in the middle of the plate. Leave the outside wells empty.
4. Add the inserts.
5. Gently shake the plate until all the inserts are wet.
6. Place it back in the incubator for at least 30 min before adding the retina pieces (*see* **Note 6**).

3.1.2 Dissecting out a retina from a post-mortem human eye

1. Place the eye for 30 seconds in 70 % EtOH in a 50 mL tube or 6 cm dish to prevent infections.
2. Rinse off the EtOH with cold HBSS.
3. Place the eye in a 6 cm dish in cold HBSS (*see* **Note 7**).
4. Remove the extraocular tissue (such as muscles, epithelia layers, visible blood vessels) with the operating scissor and the tissue forceps until you only see the white sclera.
5. Fix the eye against the side of the dish with the help of a forceps (*see* **Note 8**).
6. Use the lancet or a surgical blade and slowly make a small cut around 0.5 cm below the lens slightly below the ciliary body (where you can see a colour difference (*see* **Note 9** and **Figure 1.a**)).
7. Subsequently cut around the eye with the Iris Scissor (*see* **Note 10** and **Figure 1.b**).
8. Cut the optic nerve preferably from the inside of the eye (between retinal pigment epithelium (RPE) and retina). If the retina is still attached then you can also cut the optic nerve from the outside (*see* **Note 11** and **Figure 1.c+d**).
9. Remove the lens with the vitreous attached.
10. Make three cuts spaced evenly from the edge to the optic nerve.
11. Carefully flatten the eye by pushing in the sides so they flip upwards. Now, the retina detaches slowly from the RPE (*see* **Figure 1.c+e**).
12. Remove the sclera with the RPE attached to another dish. The retina with the ganglion cell layer (GCL) facing up should float in clean cold HBSS solution (*see* **Note 12** and **Figure 1.e**).
13. Locate the fovea (yellowish dot, avascular zone, ~1.5 mm diameter, ~5 mm from the optic disc). Make punches starting from the fovea going outwards. You should be able to make 8-14 punches (*see* **Figure 1.g+h**).
14. Place the Whatman™ paper punches on each retina (*see* **Note 13** and **Figure 1.g+h**).
15. Take out the previously prepared 24 well plate with inserts and medium from the incubator.
16. Carefully place the retina pieces on the inserts (Whatman™ paper facing upwards, PRCs downwards) and put it back in the incubator (*see* **Note 14**).
17. Take retina pieces for control and process them: Fixate (cold 4 % PFA, 30 min), cryo-protect (cold 5 % sucrose in PBS, 30 min; then cold 30 % sucrose in PBS, 30 min) and freeze in O.C.T Tissue Tek on Dry-Ice (*see* **Note 15**).

3.1.3 Culturing retina punches

1. Conditionally change medium daily. Remove 150 µL and replace it with fresh 150 µL pre-warmed CO_2 equilibrated medium.
2. End the culture at desired endpoint (7-21 days) as described under **3.1.2.17.**

3.2 AAV infection of post-mortem donor retinas

3.2.1 Preparation of the virus (1 hour before infection)

1. Warm up 50 µL medium per punch to be infected.
2. Centrifuge the concentrated virus suspension shortly and store on ice.
3. Prime the pipette tips in 0.001 % poloxamer-188 in PBS solution (*see* **Note 16**).
4. Prepare the 50 µL infection mix: Add $3.4x10^{11}$ genome copies of AAV2/9.CMV.*GFP* to a final volume of 50 µL pre-warmed medium (*see* **Note 17**).

3.2.2 AAV infection of post-mortem donor retinas (3 h after dissection)

1. Remove 150 µL of each 24 well.
2. Pipette the 50 µL infection mix on top of the Whatman™ paper in the inserts.
3. Incubate the retina with the AAVs for 48 hours.
4. Remove medium and add 300 µL fresh pre-warmed equilibrated medium
5. Conditionally change the medium every day as described at **3.1.3.1.** and end the culture at the desired endpoint as described at **3.1.2.17.**

3.3 Workflow: Tissue processing of post-mortem donor retinas

1. Prepare a fresh 24-well plate. Add 300 µL of PFA, PBS, 5 % sucrose, and 30 % of sucrose to the wells (horizontally).
2. Lift off the retinas with the Whatman™ paper attached from the insert (*see* **Note 18**).
3. Wash/dip the insert in the PBS well.
4. Move it to the 4 % PFA in PBS well for 30 min (continue as described in **3.1.2.17** and in **Figure 2**; *see* **Note 19**). Then quickly wash it in PBS.
5. Set the cryostat to -18/20 ºC. Temperate the blade, antiroll glass (70 mm), the sample(s) at least for 1 hour before cutting in the cutting chamber to -18/20 ºC. And set the cutting thickness to 8 µm.
6. In the meantime, label 1-15 Superfrost slides per 2 samples. (Initials, date, experiment, slide number).
7. Freeze the sample on a freezing block on the thin edge as depicted (*see* **Figure 2**)
8. Orientate the block horizontally (*see* **Figure 2**).
9. Cut as described e.g. in Fischer et al. [18]. In short: cut one section and move in on slide #1. Cut one section and move it on slide #2. (*see* **Figure 2**).
10. Dry sections between 1 to 18 hours before storing them at -20 ºC or -80 ºC (*see* **Note 20**).
11. Staining guideline: air dry glasses for 1 hour.
12. Wash 1x in PBS.
13. Incubate in a blocking buffer with a serum of the second antibody (but not raised against it) for 30-60 min. Dip off medium. Incubate with 150-200 µL / slide of first

antibody buffer with the appropriate first antibody dilution (*see* **Table 1**). Incubate in a humid chamber at 4ºC overnight. Wash 3x for 15 min in PBS. Add 200-300 µL of the second Antibody buffer with the appropriate second antibody dilution for 40-60 min (*see* **Note 21**). Wash 3x for 15 min in PBS. Dip off excess PBS and mount with 1 drop (30 µL) of VECTASHIELD HardSet Antifade Mounting Medium with DAPI. Harden at room temperature for 3 hours or at 4 ºC for >48 hours (storage condition).

14. Image on a confocal (or fluorescent) microscope at >20x magnification (*see* **Figure 3** and **Note 22**).

Notes

1. Clean surgical tools with warm water, soap and then 70 % EtOH.
2. Eye should be enucleated within 24 hours after death, to obtain the best results.
3. Retina should be dissected out within 36 hours after dissection.
4. You can also warm up the medium by placing it in the incubator with unscrewed cap. This allows activation of the sodium bicarbonate buffer in a 5 % CO_2 environment.
5. This ensures that the plate is an evenly humid chamber for all retinas.
6. If the dissection is in another room with incubator than your standard culture room then take the prepared 24-well plate wrapped in parafilm to the dissection room and place it there in the incubator. Don't move a dissected retina in a 6 cm plate. It will shear off all the OS of the retinas.
7. HBSS medium provides the glucose to cells while keeping the pH in check under atmospheric conditions.
8. A second person can support the fixation with an additional forceps.
9. Cutting below the ciliary body helps to remove the vitreous body and the RPE from the retina.
10. Make large cuts to minimize the physical shearing on the retina during dissection.
11. The older the material and human donor, the stickier the retina. Cold HBSS (without Magnesium, Calcium) can help detaching the retina from the RPE.
12. You can conditionally replace the medium to keep the temperature low.
13. The punches should easily attach to each piece. Autoclaving the punches can cause punches to stick to each other. Use forceps to take only one punch at a time.
14. Try to minimize pressure and area touched with the forceps. Work quick (<5 min) so the medium stays warm and the pH of the medium stays intact (*see* **Figure 1**).
15. See Figure 1 for detailed tissue processing steps.
16. This helps to prevent AAV attachment to the plastic tip.
17. Different AAVs are differently effective in infecting retinal cells. Other AAVs can infect as low as 10^7 genome copies per infection mix. Limit the amount added to the medium to 15 µL added not to diluting the medium to much.

18. The retinas never attached to the inserts so cutting out the inserts is not necessary. When handling retinas, minimize the contact (touch only sides). You can also select Whatman™ punches where not the complete retina is attached to so you can manipulate on areas where no retina is attached to it.
19. After the cryopreserving step in 30 % sucrose and the tissue dropped to the bottom, then you can store it for a few days in the fridge. We always continued immediately by freezing it. At this step you can also peel off the Whatman™ paper. Yet, we did not find a difference in staining if it was carefully peeled off or not. We decided not to peel it off anymore to have less artefacts introduced by the peeling off.
20. It is a good practice to stain one section directly after cutting with 1 % toluidine blue in milliQ water or anti-fade mounting medium with DAPI to quickly assess the tissue morphology under a light or fluorescent microscope.
21. If not enough liquid is on the slide then you can place parafilm or a cover slide on top of it. It helps to get even distribution of the second antibody on the slide.
22. Compare the OS of PRCs after dissection and at the time of harvest with e.g. PNA and Rhodopsin staining (*see* **Figure 3**). We observed that OS seem to be relevant in the AAV infection pathway to photoreceptors (unpublished).

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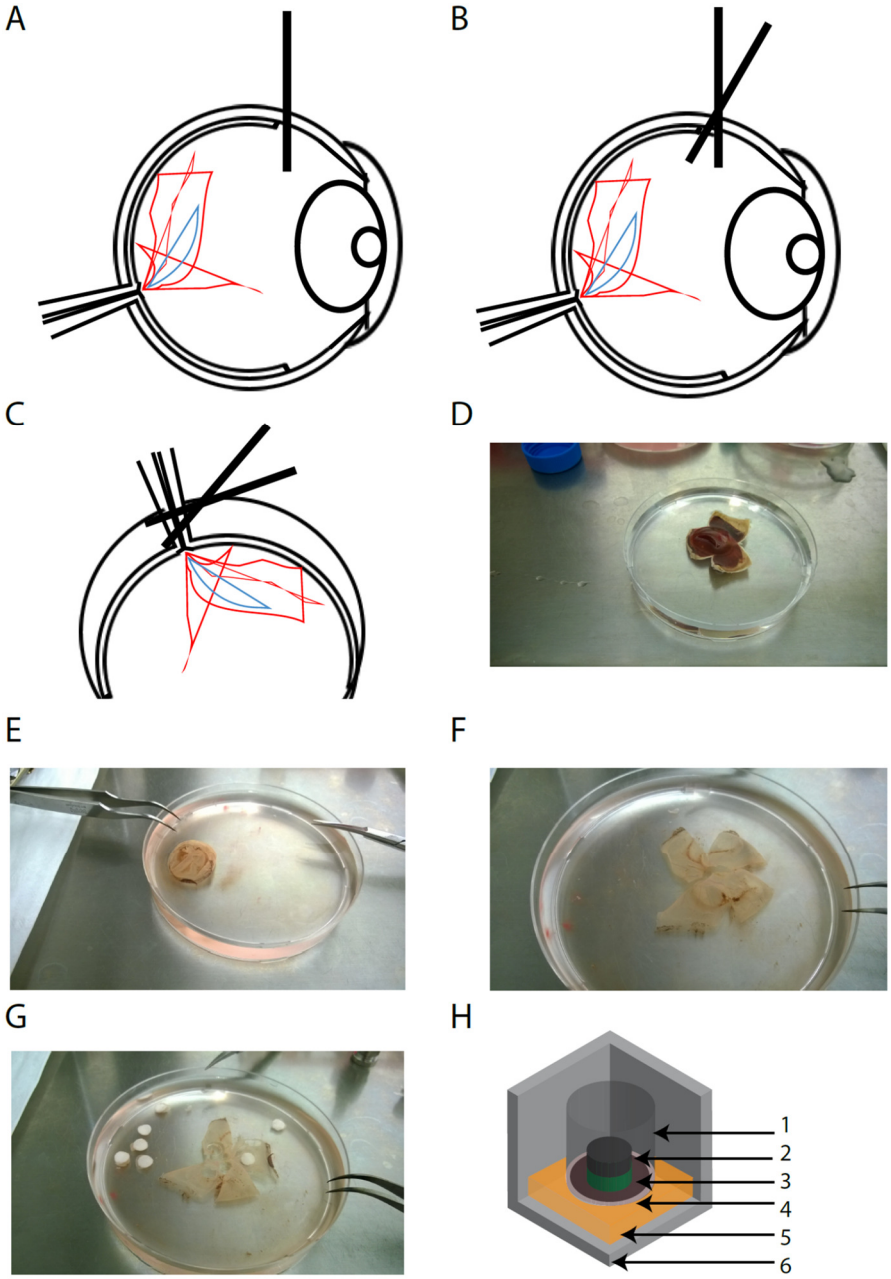

Figure 1. Dissecting out a human retina and placement of the retina on the insert. A) Stance at the border of the retina and the ciliary body. You can see color change from black to white. 2) Make long cuts with a scissor. 3) Cut off the optic nerve to free the retina. 4) Eye with the retina removed. You can see the retinal pigmented epithelium in black. 5) The retina without retinal pigmented epithelium. F) Flattened out retina. G) Punches made from the Ganglion cell layer side and Whatman paper placed on the top. H) Culture system. AAVs are applied on the Whatman™ paper from the top and the PRCs face on the membrane.

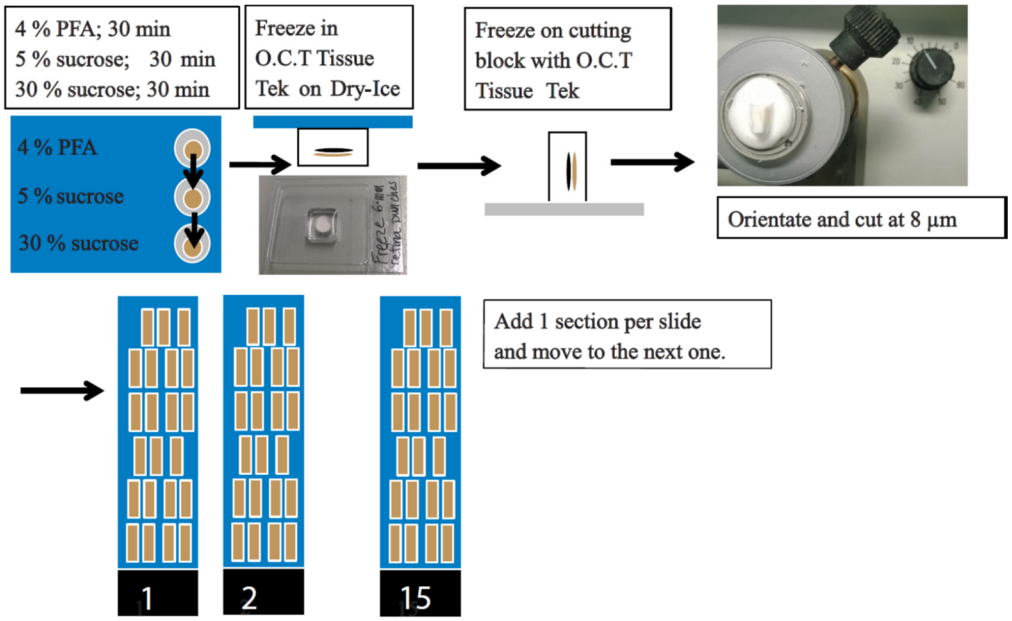

Figure 2. Workflow for tissue processing. A) Fixing process and cryopreservation. B) Freeze in a cryomold for better orientation. C) Flip the block and then freeze it on the cutting block (see image) to get all retina layers on one section. D) Add one section per slide and then move to the next slide. You can add two samples per slide to have an internal control for immunohistochemistry staining.

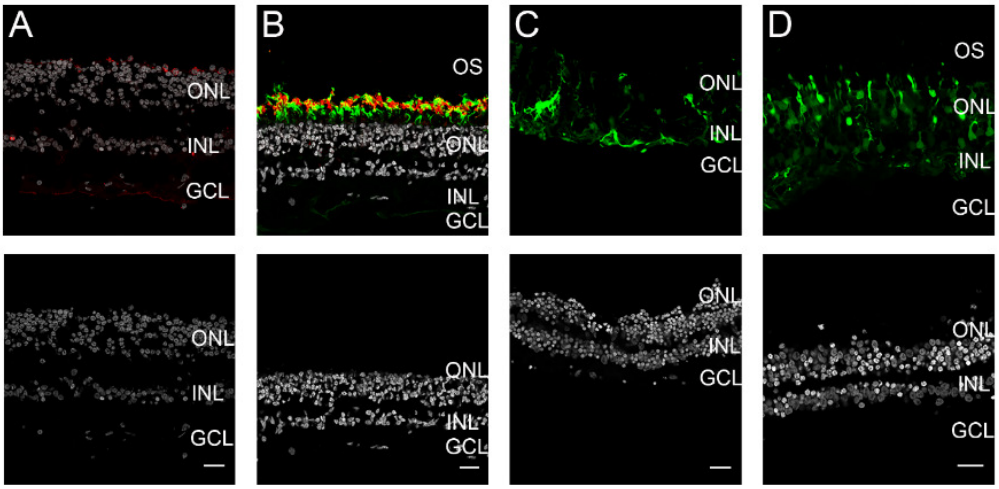

Figure 3. Expected results. A) Poor dissection or tissue without outer segments present (stained with PNA in red). B) Good dissection and tissue with outer segments present (stained with Rhodopsin in red and PNA in green). C) Retinal explant infected by AAV2/9.CMV.*EGFP*, 21 days after infection (GFP in green). D) Retinal explant infected with AAV2/9.CMV.*EGFP*, 14 days after infection (GFP in green). Retinal layers: Outer Nuclear Layer (ONL), Inner Nuclear Layer (INL), Ganglion Cell Layer (GCL). Nuclear staining (DAPI) in gray. Scale bar: 25 µm.

Table 1. List of antibodies for immunohistochemistry.

Antibody	anti-	dilution	Company	Staining property
Calbindin	rabbit	1/250	AnaSpec	Strongly horizontal cells/INL-OPL; lower amacrine/INL
Calretinin	rabbit	1/500	Chemicon	Amacrine/INL; displaced amacrine and ganglion cells/GCL
CRB1	rabbit	1/250	Home made	Subapical region
Cone arrestin	rabbit	1/500	Millipore	Cone photoreceptors
PKCα	mouse	1/250	BD Biosciences	Rod bipolar cells
GFAP	rabbit	1/200	DAKO	Stressed Müller glial cells and gliosis
Glutamine Synthetase	mouse	1/200	BD Biosciences	Müller glial cells
$p27^{kip1}$	rabbit	1/150	Millipore	Müller glial cells and cell cycle inhibitor protein
SOX9	rabbit	1/250	Millipore	Müller glial nuclei/INL; astrocyte nuclei/GCL; RPE nuclei
Recoverin	rabbit	1/500	Chemicon	Rod and cone somas and segments
Rhodopsin	mouse	1/250	Millipore	Outer segment and weakly soma of rods
CD45	mouse	1/250	Emelca Bioscience	Activated microglias or macrophage/monocyte lineage cells
Rhodamine PNA	N/A	1/200	Vector	Outer segments. Added with second Antibody Buffer.
Caspase 3 (cleaved)	rabbit	1/250	Cell Signaling	Cell apoptosis
Phospho-Histone H3	rabbit	1/100	Millipore	Mitosis Marker