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Novel method for the rapid isolation of RPE cells specifically for RNA extraction and analysis

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Abstract

RPE cells are involved in the pathogenesis of many retinal diseases. Accurate analysis of RPE gene expression profiles in different scenarios will increase our understanding of disease mechanisms. Our objective in this study was to develop an improved method for the isolation of RPE cells, specifically for RNA analysis. Mouse RPE cells were isolated using different techniques, including mechanical dissociation techniques and a new technique we refer to here as “Simultaneous RPE cell Isolation and RNA Stabilization” (SRIRS method). RNA was extracted from the RPE cells. An RNA bioanalyzer was used to determine the quantity and quality of RNA. qPCR was used to determine contamination with non-RPE-derived RNA. Several parameters with a potential impact on the isolation protocol were studied and optimized. A marked improvement in the quantity and quality of RPE-derived RNA was obtained with the SRIRS technique. We could get the RPE in direct contact with the RNA protecting agent within 1 minute of enucleation, and the RPE isolated within 11 minutes of enucleation. There was no significant contamination with vascular, choroidal or scleral-derived RNA. We have developed a fast, easy and reliable method for the isolation of RPE cells that leads to a high yield of RPE-derived RNA while preserving its quality. We believe this technique will be useful for future studies looking at gene expression profiles of RPE cells and their role in the pathophysiology of retinal diseases.

Keywords

RPE isolation; RNA extraction; gene expression; mouse; novel technique

1. Introduction

The retinal pigment epithelium (RPE) is a versatile monolayer of cells located between the retina and the choroidal vasculature. A wide range of metabolic pathways are constantly active in RPE cells, and are essential to maintaining the delicate homeostasis of the retina. Among its many functions, the RPE is involved in the phagocytosis of shed photoreceptor outer segments, the metabolism of retinol, the formation of the extracellular matrix, the formation of the outer blood-retinal barrier, and the transport of nutrients and debris to and from the retina (Thumann et al., 2006). As a consequence, changes affecting RPE cell

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function or their metabolic activity can have very serious effects on vision. Age-related macular degeneration is just one of many diseases in which changes in RPE function play a major role. Exploring the gene expression profile of these very complex cells under different conditions may hold important clues as to the pathogenesis of retinal diseases. However, there have been significant challenges to analyzing mRNA expression of RPE cells reliably. The first step to achieve such a goal is the isolation of RPE cells from posterior eye cups. This has been done for at least 4 decades using different techniques. These techniques include mechanical disruption techniques (with brushes (Saari et al., 1977) or forceps (Wang et al., 1993; Liu et al., 2010), enzymatic dissociation (Mannagh et al., 1973; Flood et al., 1980; Edwards, 1982; Edwards, 1977; Sakagami et al., 1995; Jaffe et al., 1990) and freeze-thawing followed by fluid flushing (Donita Garland and Eric Pierce - personal communication/ARVO 2010 poster #2590, A379). Clearly, these techniques are very useful for specific purposes including RPE culturing, or studying sub-RPE structures (Garland and Pierce, personal communication). However, there may be some concerns using these techniques when the goal is to isolate RNA for gene expression studies.

Isolating RPE cells from posterior eye cups in a manner that preserves RNA quality, quantity and gene expression profile is challenging. Enzymatic treatments with hyaluronidase, collagenase, trypsin and/or proteinase K involve prolonged incubations. This could in theory affect both the quality of the RNA and also the mRNA expression profile. Mechanical techniques may increase the risk of significant contamination from choroid, and may also affect the mRNA quality and expression pattern. Wang et al. reported that RNA quality from ocular tissues preserved in RNeasy was superior to incubation on ice or snap freezing techniques (Wang et al., 2001). However, as we demonstrate here, RNeasy poses significant problems when isolating RNA from RPE cells. This study reports the development of a new technique for the isolation of RPE cells specifically for the purpose of RNA isolation and analysis. This technique is simple, fast, and results in the isolation of RPE cells with high yield and purity. Importantly, it also preserves the quality of the mRNA.

2. Materials, supplies and detailed methods

2.1. Animals

Adult C57BL/6 wild type mice were used for these experiments. The mice were bred and kept in a barrier animal facility at UT Southwestern Medical Center under normal lighting conditions with 12 h on, 12 h off cycles. All animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision research and approved by the institutional animal care and use committee (IACUC) of the UT Southwestern Medical Center.

2.2. RPE cell isolation by mechanical dissociation method

Mice were anesthetized with a ketamine-xylazine cocktail (100 mg/kg ketamine, 5 mg/kg xylazine). The first eye was enucleated and was immediately placed on a gauze soaked with PBS. Vannas scissors were used to open the eye just behind the limbus, and the anterior segment was discarded (cornea, iris and lens). The retina was carefully removed, exposing the RPE. The posterior eye cup (sclera-choroid-RPE) was then transferred into a small petri dish and was flash frozen in liquid nitrogen. PBS (200 μ l) was then flushed onto the inside of the posterior eye cup using a pipette with a 200 μ l tip. This forceful flushing was repeated 20 times, reusing the same PBS. The PBS (approximately 200 μ l), containing brown clumps of released material (presumably RPE cells), was transferred into a 2 ml centrifuge tube, and 1.8 ml of RNeasy (Qiagen catalogue # 76104) was added. At this time the second eye was enucleated and the same procedure repeated in order to isolate the RPE cells into a different

2 ml tube. After 10 min in the RNAlater:PBS (9:1), the entire contents of the 2 ml tube were filtered through a Microcon centrifugal filter (Millipore Cat# 42416) according to the manufacturer's protocol. The recovered pigmented material was then used for RNA extraction as described below.

2.3. RPE cell isolation and RNA extraction using SRIRS (simultaneous RPE isolation and RNA stabilization) method

Mice were anesthetized with a ketamine-xylazine cocktail (100 mg/kg ketamine, 5 mg/kg xylazine). After enucleation, the anterior segment and retina were removed as described above. The remaining posterior eye cup was quickly dipped in PBS in order to quickly washout any adherent debris. It was then immediately transferred into a 1.5 ml microcentrifuge tube containing 200 μ l of RNAlater:PBS (Qiagen cat. 76526). It took roughly 1 minute from the time of enucleation to the transfer into the RNAlater-containing microcentrifuge tube. The eye cup was incubated for 10 min. at room temperature. The tube was briefly agitated to ensure most of the RPE cells were released and then the eye cup was removed. Centrifugation was then performed for 5 minutes at 2500 rpm ($685 \times g$) to pellet the RPE cells, which were then subjected to total RNA extraction using the RNeasy Micro Kit (Qiagen cat. 74004) per the manufacturer's instructions. In brief, 75 μ l of lysis buffer (RLT) were added and the sample was homogenized with a disposable pestle grinder (Fisher 03-392-106), and then centrifuged for 2 min. at $16,000 \times g$. The supernatant was transferred to a new tube, and 70% ethanol (1:1 volume) was added. The entire sample was transferred to an RNeasy MinElute spin column. In the final SRIRS protocol a DNA digestion step using DNase I was performed, per manufacturer's protocol, directly on the RNeasy MinElute spin column. We used 30 units of DNase I per sample and incubated for 15 minutes at room temperature. The RNA was eluted with RNase-free water. We used an RNA carrier during our isolation protocol due to the low concentration of RPE RNA isolated from the cells. For the initial experiments (figures 1 and 2) we used a poly-A carrier RNA (included in RNeasy Micro Kit). However, for the last experiments we switched to an E. coli ribosomal carrier RNA (Roche cat 206938) in order to avoid interference with the cRNA generation needed for the Illumina gene expression microarray assay (microarray data is not shown here).

2.4. RNA extraction from posterior eye cups (sclera-choroid-RPE cups)

For RNA isolation from posterior eye cups, we placed the sclera-choroid-RPE cup into RNAlater for 4 hours. The sclera-choroid-RPE cups were then transferred to 1 ml Qiazol (Qiagen cat# 79306) in a 1.5 ml eppendorf tube and homogenized (PRO200 homogenizer Micro) at room temperature. We then added 200 μ l of chloroform (Sigma cat# C2432-500ML) and vortexed for 15 seconds. The contents were then transferred into a PhaseLock Gel tube (5 Prime cat# 2302830), and centrifuged at 13,400 rcf for 15 min at 4°C. We applied the Qiagen miRNeasy Mini kit (Qiagen cat#217004) to the top layer of clear supernatant.

2.5. Bioanalyzer

To analyze the quantity and quality of the RNA in each sample, we submitted a small aliquot to the Microarray Core Facility at UT Southwestern Medical Center in order to perform a Bioanalyzer assay using an Agilent RNA 6000 Pico kit. Most samples were diluted 1:6 in RNase-free water before being sent for analysis. The initial samples (figure 2A–2C) were sent without dilution due to low RNA yield (determined by nanodrop analysis; not shown). Analysis of RNA quality (degradation and DNA contamination), a calculation of the RNA integrity number (RIN), and the total amount of RNA in each sample were determined. All samples were within the range of RNA concentration specified by the manufacturer for the Agilent RNA 6000 Pico kit.

The Bioanalyzer uses electrophoresis on micro-chips to separate the RNA samples, which are then analyzed via laser-induced fluorescence detection. The resulting electropherogram and gel-like image provide a detailed visual assessment of the quality of the RNA sample. However, to remove the subjective nature of the visual analysis, the RIN value was developed. It is a calculation based on a computerized evaluation of the entire electropherogram. In brief, it compares the 18S and 28S peaks to any peaks corresponding to degraded nucleic acid material. A sample with high 18S and 28S peaks, and minimal peaks in regions of the electropherogram associated with degradation would have a high RIN value (up to 10). Alternatives to the Bioanalyzer that allow the determination of the quantity and quality of RNA would include the NanoDrop device (based on the spectrophotometric evaluation of absorbance at the 260nm wavelength vs. 280nm wavelength; example in figure 4C), and running a 1.5% denaturing agarose gel (to compare the 18S and 28S bands, vs. bands or smear in other regions).

2.6. Preparation of posterior eye cups and flat mounts for photography and ZO-1 immunohistochemistry

Eyes from C57BL/6 wild type mice were enucleated, fat and connective tissues were removed, the anterior segment was cut out, the lens and retina were removed, and the remaining “posterior eye cups” were photographed or were processed for immunohistochemistry. Photographs were taken pre- and post-RPE cell isolation procedures (sections 2.2 and 2.3) using an Olympus SZH Stereoscope equipped with Optonics Microfire CCD Color Camera and Optonics PictureFrame 2.0 Acquisition Software.

For ZO-1 immunohistochemistry (Kokkinopoulos et al., 2011), the “posterior eye cups” were treated with either freeze-thaw followed by PBS flushing, or by the SRIRS method (see above) and were placed in 4% paraformaldehyde (PFA) for 2 hours at room temperature (RT) followed by two washes for 10 min each in PBS. The posterior eye cups were flattened by making long radial cuts, and the resulting flat mounts were incubated in a blocking buffer of 5% BSA in 1× PBS (w/v) containing 0.3% (v/v) Triton X-100 over night at 4°C. After removing the blocking buffer, the flat mounts were incubated for 2 hours at RT with rabbit anti-ZO1 tight junction protein primary antibody (1:100, Abcam Inc, cat. # ab59720) prepared in a diluted (1:5) blocking buffer. The flat mounts were washed 3×10 min in 1× PBS and incubated with goat anti-rabbit conjugated AF594 secondary antibody (1:200, Invitrogen, Inc.). After 3×10 min washes in 1× PBS, the flat mounts were cover-slipped and mounted with Prolong Gold antifade reagent with DAPI. Images were obtained using a Zeiss AxioObserver motorized wide-field Epifluorescence microscope equipped with a Hamamatsu Orca-BT-1024G monochrome camera, appropriate fluorescence filter sets and Zeiss Axiovision software.

2.7. Reverse transcriptase qPCR (qPCR)

We used qPCR to test the purity of the isolated RPE cell population by analyzing the levels of mRNA for RPE65 (produced in RPE cells) and comparing them to: 1. a vascular endothelium-derived mRNA that would indicate contamination from conjunctival or choroidal vessels (vWF, von Willebrand Factor) (Wu et al., 2005); 2. a choriocapillaries-derived mRNA (collagen VI, COL6A1-3 gene) (Booij et al., 2009; Marshal et al., 1993); and 3. a predominantly scleral-derived mRNA (collagen I, COL1A1 gene) (Keeley et al., 1984; Watson & Young, 2004; Marshal et al., 1993). We used the Superscript III reverse transcriptase kit (Invitrogen cat. 11735-032) to generate cDNA from the extracted total RNA. Singlet qPCR reactions were run in triplicate (iCycler; Bio-Rad Laboratories, Hercules, CA) at 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute with SYBR GreenER qPCR SuperMix (Invitrogen cat. 11735-032). Each reaction contained 2.5 ng cDNA, 200 nM of each primer, and 10 µl qPCR super mix in 20

μl total volume. The primers used are shown in Table 1. The fold changes in expression of vWF vs. RPE65, collagen VI vs. RPE65 and collagen I vs. RPE65 mRNA in the RPE cell isolates were calculated using the formula $RQ=2^{-\Delta\Delta C_t}$, and using GAPDH as an endogenous reference gene. The RQ values for RPE isolated using the SRIRS method were compared to RQ values for posterior eye cups (again, after removing the periocular tissues, cornea, iris, lens, vitreous and retina).

3. Results and troubleshooting

3.1. Mechanical attempts to isolate RPE cells from posterior eye cups

Our initial attempts to isolate RPE cells from posterior eye cups involved two techniques: 1). mechanical isolation using forceps (data not shown), and 2). mechanical isolation using a flush of PBS to wash out the RPE cells after a freeze-thaw step. We found that the first technique using forceps was difficult to reproduce, and time-consuming. Furthermore, from each eye cup we obtained many pieces (plaques) of brown material that varied in thickness and appearance. Thus we could not discount the possibility that the samples also contained a choroidal contamination (unpublished observations). The second approach involved removing the anterior segment and the retina, freeze-thawing the posterior eye cups and flushing the eye cup with PBS. Using this technique we were able to observe brown clumps of tissue being released from the posterior eye cups.

The use of RNeasy in this procedure in order to protect the RNA, posed two problems: 1. if we treated the enucleated eyes with RNeasy before removing the retina, the RPE became tightly adherent to the retina (figure 1A; compare to the retina isolated in figure 1B in which there is no adherent RPE), and 2. we were unable to properly pellet the RPE cells out of the RNeasy using centrifugation (first two tubes on the left in figures 1D and 1E). Some precipitation is seen in these first two tubes in figures 1D and 1E because, for illustration purposes, we used a 1:1 mix of RNeasy:PBS, rather than the 9:1 mix that we used in the actual protocol. For these reasons we had to modify the mechanical isolation technique to avoid exposure of the eye cups to RNeasy. We only added RNeasy (9:1 by volume) to the RPE cell suspension immediately after flushing the eye cup with PBS. Since it was not possible to obtain a pellet of RPE cells by centrifugation, we filtered out the RPE cells using a Microcon centrifugal filter. However, when we attempted to isolate the RNA, the quality and yield were very poor (figure 2A and 2C). Flushing the RPE with a 1:1 mix of PBS and RNeasy did not improve our results (figure 2B and 2C). Using this technique, the RNA integrity number (RIN) fluctuated between non-detectable and 3.5. The total RPE-derived RNA obtained per posterior eye cup was usually around 50 ng.

3.2. Simultaneous isolation of RPE cells and RNA protection/extraction

An ideal method to generate RPE-derived RNA would allow us to both isolate the RPE cells, while at the same time protecting the RNA from degradation. It should also allow for the easy separation of RPE cells from the RNA protection agent. RNeasy cell reagent, which like RNeasy is designed to prevent RNA degradation, is meant to be used on cell suspensions rather than tissues. It does not contain any enzymes or other proteins. We noticed that, unlike RNeasy, exposing the eyes to RNeasy did not cause attachment of the RPE to the retina (figure 1B). Moreover, we discovered that exposure of the posterior eye cups to RNeasy led to the release of RPE cells from the eye cups (figure 1C, last tube on the right) after a few minutes. Brownish discoloration of the medium became noticeable after approximately 5–8 minutes of exposure, and even earlier if we tapped the tube lightly. Also, in sharp contrast to what was observed when RPE cells were isolated using the RNeasy solution, RPE cells in the RNeasy reagent could be easily pelleted with centrifugation (figure 1D, last tube). Figure 1E shows the same microcentrifuge tubes

as figure 1D, but after a slight tap to the tubes and at a different angle. This released the pellet in the RNeasy Protect sample, which allowed us to photograph it from above. It emphasizes that the size of the pellet obtained using this technique is markedly larger and denser than with the other isolation techniques. We proceeded to extract and analyze the RNA from this pellet as described in section 2.3. Since this method allows for the simultaneous isolation of RPE cells and protection of the RNA we will abbreviate it as “simultaneous RPE cell isolation and RNA stabilization” (SRIRS) method.

Analysis of the RNA extracted using the SRIRS method provided an RNA yield that was consistently higher than RNA yields from RPE cells obtained using the mechanical methods. This is evident when comparing the 18S and 28S peaks in figure 2D vs. figure 2A. Please note that the samples in figure 2D were diluted 1:6 before being processed in the bioanalyzer and still the 18S and 28S peaks are higher than those in figure 2A (in which the samples were undiluted). We typically obtained 300–400 ng of RPE-derived RNA per eye cup. More importantly, the RNA quality was higher, with RIN values of 5 to 6 when we used the SRIRS method. Addition of a freeze-thaw step to the SRIRS method, did not help increase the yield of RNA any further (figures 2E and 2F).

3.3. Improving the quality of the RNA

Although in our initial experiments using the SRIRS method we incubated the eye cups in the RNeasy Protect cell reagent for 30 min, we noticed that by 10 min it seemed that maximal release of the RPE cells had occurred. Figure 3 shows that increasing the incubation time beyond 10 min did not seem to increase the yield.

One remaining concern was that the Bioanalyzer results were consistently showing a broad peak between the 18S and 28S peaks. This was suggestive of DNA contamination. We decided to apply a 15 min DNase digestion step (30 units of DNase I) to our RNA samples. This treatment completely eliminated the broad peak between the 18S and 28S bands. As can be seen in figures 3D–3F, there was some reduction in the total yield of RNA after the DNase digestion step. Still, we were able to consistently preserve good amounts of this high quality RPE-derived RNA. As seen in figure 4A, in a single experiment we obtained over 100 ng of RPE-derived RNA in 11 out of 12 posterior eye cups, and over 200 ng in 10 out of these 12 eye cups processed using our protocol (10 min incubation in RNeasy Protect followed by RNA isolation, followed by DNase digestion). This was significantly higher than the yield using the mechanical methods even when using the freeze-thaw/flushing method followed by RNA later ($p=0.003$, figure 4A). Furthermore, the DNase treatment led to a significant increase ($p<0.000001$) in the RIN values (to a range of 8.0–9.5; figures 3F and 4B). Finally, we identified several factors that could lead to variability in the total amount of isolated RNA. These included: 1. how far behind the limbus we opened the eyes, 2. some eye cups were partially closed (like a taco) during the RPE isolation step, 3. loss in multiple steps of the RNA isolation procedure (e.g. DNase digestion and column processing), and 4. the intrinsic error of the pipette used to collect the 1 microliter sample that would be used for analysis. We repeated the isolation in six additional eyes, trying to control for all these variables. We then determined the amount of RNA using a NanoDrop device (figure 4C) and found a much lower variability in the total amount of RNA obtained.

3.4. The SRIRS protocol preferentially releases RPE cells

Visual inspection of a posterior eye cup or flat mount before vs. after SRIRS strongly suggests that this technique primarily isolates RPE cells (figure 5A and 5D, vs. figure 5C and 5F). During SRIRS we observe a brownish suspension develop (figure 1C), but the eye cup is for the most part intact (figures 5C and 5F). Only with careful inspection under the microscope can it be noted that the brown color of the inside of the cup is somewhat

decreased. This indicates that the sclera and choroid are still intact. Close inspection of the freeze-thaw/PBS-flushed eyes (figure 5B and 5E) reveals patchy areas of decreased pigmentation. To confirm that we are indeed releasing the RPE cells we used an antibody against zonula occludens antigen ZO-1 to stain flat mounts prepared from eye cups that had undergone either freeze-thaw/PBS flushing (figures 5G & 5H), or SRIRS (figure 5I). The mechanical technique leads to a partial release of RPE cells, leaving large areas with intact RPE cells (figure 5G), while causing release of patches of RPE cells in other areas (figure 5H). On the other hand, there is extensive (almost complete) release of RPE cells with the SRIRS technique (figure 5I).

In order to determine the amount of contaminating RNA (derived from non-RPE sources) in our RNA extracts we performed a qPCR assay using primers for vWF (von Willebrand Factor, expressed on vascular endothelium), collagen VI (expressed in the choriocapillaries), collagen I (expressed predominantly in the sclera, but also choroid), and RPE65 (expressed on RPE cells). We found that the RPE isolates obtained using SRIRS contained a much lower ratio of vWF/RPE65, COL6A1-3/RPE65, and COL1A1/RPE65, when compared to posterior eye cups (figure 6A–6C). Furthermore, when corrected for GAPDH, the RPE isolates had a 2–3 fold higher level of RPE65 than the posterior eye cups (figure 6D). The differences were statistically significant (Student's t test).

4. Discussion

4.1. Source of RNA for gene expression analysis

The first hurdle to confront when trying to analyze the mRNA expression in RPE cells is separating the RPE cells from the rest of the eye. Many studies looking at the expression of genes by RPE cells in mouse eyes have relied on the isolation of RNA from posterior eye cups. However, such analysis of gene expression is really looking at the combination of RPE cells, choroid and sclera. We have shown, by visual inspection, ZO-1 staining, and qPCR, that the SRIRS technique can efficiently and preferentially separate RPE cells from the posterior eye cups. The resulting RPE-derived RNA is highly pure; there is minimal contamination from non-RPE ocular tissues. This allows for the investigation of gene expression specifically by RPE cells.

4.2. Quantity and quality of RNA

The second hurdle to overcome when isolating RNA from RPE cells is maximizing the amount of RNA obtained from each posterior eye cup, while preserving its quality. We showed that using the RNA stabilization reagent RNAlater was not ideal for the isolation of RPE cells or the extraction of RNA from the isolated RPE cells. By contrast, our simple SRIRS technique has the advantage of causing the quick and effective release of RPE cells while at the same time protecting their RNA. We found that 10 minutes of exposure appeared to maximize the amount of RNA recovered. We were able to consistently generate over 200 ng (often over 400 ng) of high quality RNA (RIN values over 8.0 and often around 9.0) from single mouse eye cups. This type of yield is important when dealing with precious samples from genetically-engineered mice, as it will allow for the application of multiple molecular assays to the RPE cells isolated from each eye cup. The quality and quantity of RNA obtained is sufficient for microarray studies, with plenty of residual RNA that can be used for confirmatory qPCR studies. The high quality of RNA should also help generate more accurate and reliable data. The mechanical RPE isolation techniques we tested (forceps removal - data not shown, and freeze-thaw/PBS flush), generated very low amounts of RPE cells and low quantity and quality of RNA.

4.3. Minimizing time after enucleation

It has been shown that the gene expression profile of cells can change markedly within 10 minutes of exposure to some stimuli (deNadal et al., 2011). Although reproducing these findings in our system would be beyond the scope of this work, it seems likely that the process of isolating RPE cells by mechanical and enzymatic approaches (enucleation, followed by opening the eye, followed by exposure to enzymes for at least 1 hour, and or direct mechanical trauma to RPE cells) would lead to stress responses by RPE cells. It seems reasonable that it would be beneficial to minimize the time (and manipulations) after enucleation that it takes to protect the RNA from degradation or change. In our protocol, it takes about 1 minute to get the RPE in direct contact with the RNA protecting reagent. This is significantly lower than the time needed for any of the published protocols using either mechanical-based or enzyme-based techniques. Most of these protocols require at least one hour of lag time between enucleation and RNA stabilization (Saari et al., 1977; Wang et al., 1993; Liu et al., 2010; Mannagh et al., 1973; Flood et al., 1980; Edwards, 1982; Edwards, 1977; Sakagami et al., 1995; Jaffe et al., 1990).

4.4. Limitations

A limitation of this study is that we did not determine the level of choroidal contamination on the RPE samples obtained using the mechanical methods. We suspect, based on our observations, that using the forceps-based protocol there should be some choroidal contamination on the RPE samples. However, we believe that the low quality and quantity of RNA obtained using those techniques are already enough reason to prefer the SRIRS protocol.

A second limitation is that we did not prove that the enzymatic/mechanical methods alter the mRNA expression profile. The time and manipulations involved in these techniques (compared to the SRIRS technique) at least bring this up as a reasonable concern. However, testing this would be beyond the scope of this work.

4.5. “Simultaneous RPE cell isolation and RNA stabilization” (SRIRS) method summary

After inducing deep anesthesia on a mouse, enucleate the first eye and place it on an ice cold PBS-soaked gauze. Sacrifice the mouse only after enucleating the second eye, approximately 2 minutes later. Under a dissecting microscope, quickly remove all the periocular tissues and open the eye posterior to the limbus using Vannas scissors. Pay close attention to the distance posterior to the limbus at which the incision is made, and keep it constant for the entire eye, and from eye to eye. Remove the anterior segment and the retina. Quickly dip the resulting “posterior eye cup” into a PBS-containing microcentrifuge tube to remove any loosely adherent debris. Transfer the eye cup immediately (ideally within 60–90 seconds from enucleation) into a microcentrifuge tube containing 200 μ l of ice cold RNeasy Protect cell reagent. Ensure that the eye cups remain open within the microcentrifuge tube. This can be done by gently tapping on the tube, and sometimes by using the forceps on the outside of the cup to get it to open. Gently tap on the tube every 1–2 minutes to aid in the release of the RPE cells. After 8–10 minutes, tap again on the tube, and then remove the eye cup (by now it is only sclera and choroid) with forceps. The brownish suspension in the tube can now be centrifuged (5 minutes at $685 \times g$) to pellet the RPE cells, which are then subjected to total RNA extraction using the RNeasy Micro Kit. DNA digestion using 30 units of DNase I for 15 min should be performed, per manufacturer's protocol, directly on the RNeasy MinElute spin column. We recommend using *E. coli* ribosomal carrier RNA.

4.6. Conclusion

Isolation of RPE cells has traditionally been performed by mechanical and enzymatic methods that are very useful for the generation of RPE cell cultures. However, if the main objective of a mouse-based experiment is to try to understand the expression of genes by RPE cells, the SRIRS method we present here would be the simplest approach to isolate the RPE-derived RNA. It leads to high RNA yield and quality. It also minimizes the potential issues of: 1. contamination from other ocular tissues, 2. RNA degradation, and 3. RNA expression changes.

We believe that the novel method described here will make a significant impact in the quality of data that can be obtained when analyzing gene expression profiles (or changes in expression of specific genes) in RPE cells. We hope that this will be a helpful tool in the quest for a better understanding of retinal diseases.

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Highlights

- We describe a new method to isolate RPE cells for gene expression analysis.
- The new method simultaneously isolates the RPE cells and stabilizes the RNA.
- RNA is protected from degradation within 1 minute of enucleation.
- There is no significant contamination from non-RPE tissues.
- This method is fast, simple, reproducible and leads to high yield and quality of RNA.

Figure 1. Photographic documentation of steps in the different RPE isolation protocols
 (A) Exposure of the eye cup to RNAlater before removing the retina leads to the tight adherence of RPE cells to the retina. (B) There is no detectable adherence of RPE cells to the retina when the eye is exposed to RNAprotect (as part of the SRIRS protocol) instead of RNAlater prior to the removal of the retina. (C) Posterior eye cups (after removing the retina) were used for RPE isolation. There is a marked increase in the release of RPE cells using the SRIRS protocol (last tube on the right) vs. mechanical isolation using PBS flushing (first two tubes) or simple exposure to RNAlater (third tube). RNA later was added to the first two tubes (1:1 by volume) after the PBS flushing. (D) Centrifugation leads to a well-formed pellet only in the SRIRS tube. (E) Another view of the tubes (tubes are lying down) after a light tapping has released the pellet in the last tube, demonstrating the size/density of the pellet in the SRIRS sample.

Figure 2. Comparison of RPE-derived RNA generated using mechanical vs. SRIRS method Representative individual samples are shown. RPE cells were initially isolated using a mechanical-based method (A–C). After removing the retina, the posterior eye cups were freeze-thawed, followed by vigorous flushing of the RPE surface with either (A) PBS, or (B) PBS:RNA later (1:1 by volume). Bioanalyzer results of the undiluted RNA extracts are shown. The RNA integrity number (RIN) and the total RNA yield are summarized in (C). There was a significant improvement in the quality and yield of RNA using the SRIRS protocol (D,E,F). Bioanalyzer results of RNA extracts from RPE cells that were isolated using the SRIRS protocol (including a 30 min exposure of the eye cups to RNaprotect), either immediately after removing the retina (D), or after a quick freeze-thaw step in liquid nitrogen (E) are shown. The samples were diluted 1:6 using RNase-free water prior to analysis. The RIN values and total RNA yields are summarized (F).

Figure 3. Optimization of incubation time on SRIRS protocol, and removal of DNA contamination

Representative individual samples are shown. RPE-derived RNA was obtained from posterior eye cups (without retina) using the SRIRS method including different exposure times to RNAprotect cell reagent: 10 min (A and D), 1 hour (B and E) or overnight (C). A DNase digestion step was added to the samples in D and E. Bioanalyzer results of the RNA extracts are shown. The samples were diluted 1:6 using RNase-free water prior to analysis. The RIN values and total RNA yields are summarized (F).

Figure 4. Marked improvement in the quality and quantity of RNA isolated using the SRIRS protocol vs. optimized mechanical-based protocol

Three eye cups were processed with the optimized mechanical-based isolation protocol using freeze-thawing and PBS flushing, followed by RNA protection with RNAlater. Twelve eye cups were processed using the SRIRS protocol (including 15 min of DNase digestion). The total RNA obtained for each eye cup was tested with a Bioanalyzer and the values for total RNA yield (A), and RNA integrity number (B) were graphed. After identifying some of the factors that could be responsible for the variability on total RNA obtained (see section 3.3), we applied the SRIRS method to another six eyes (C). The total amount of RPE-derived RNA obtained from each eye was determined using a NanoDrop device.

Figure 5. SRIRS protocol leads to the efficient release of RPE cells from posterior eye cups
 Photographs of posterior eye cups, after removal of the retina (A–C), and subsequent flat mounts (D–F), pre- and post-RPE isolation are shown. In the pre-RP isolation images (A and D) the RPE cell layer with evenly distributed darker tone is noticeable compared to the post-RPE isolation images (B and E underwent freeze/thaw/PBS flushing, while C and F underwent the SRIRS protocol). In the SRIRS samples the choroidal vasculature becomes more visible indicating the complete removal of the RPE cell layer. Immunostaining for ZO-1 clearly shows the complete removal of RPE cell layer (I) with SRIRS method compared to partial disruptions of RPE cell layer in the freeze/thaw mechanical method (G and H).

Figure 6. SRIRS-derived RPE isolates have negligible levels of choroid and sclera-specific gene expression

RNA was isolated from either posterior eye cups (sclera/choroid/RPE complex, after removing the retina, n=3 eyes), or from RPE cells obtained after 8 minutes of SRIRS (n=6 eyes), or from RPE cells obtained after 1 hour of SRIRS (n=3 eyes). qPCR was applied to examine the expression of (A) a vascular endothelium gene (vWF), (B) a choroidal gene (collagen VI), and (C) a scleral gene (collagen I). For A–C, RQ values for each gene in relation to RPE65, and after normalizing for GAPDH, were calculated for each of the 12 processed eyes. We also determined (D) RPE65 expression, normalized to GAPDH. In all cases, mean and SEM were calculated and expressed as a fraction of the results for the posterior sclera/choroid/RPE cups. The Student's t test was used for statistical analysis.

Table 1

List of primer pairs used for qPCR analysis

Gene	Primer pairs (f- forward, r- reverse)	Size(bp)	Reference
RPE65	f- 5' TGACAAGGTCGACACAGGCAGAAA 3' r- 5' AAATTCAAAGGCTTGACGAGGCC 3'	133	NM_029987.2
vWF	f- 5' AGGGAGGGATCTGGTTTCT 3' r- 5' GTACCACGAGGTCATCAACG 3'	137	NM_011708.3
COL1A1	f- 5' TCGGCGAGAGCATGACCGATGGAT 3' r- 5' GACGCTGTAGGTGAAGCGGCTGT 3'	254	NM_007742.3 (Ponsioen et al., 2008)
COL6A1-3	f- 5' TAGCAGAGATAGCTGGCTTG 3' r- 5' AGAGGCTTAGCTTGACAGT 3'	174	NM_009933.4
GAPDH	f- 5' TGTGTCCGTCGTGGATCTGA 3' r- 5' CCTGCTTCACCACCTTCTTGA 3'	77	NM_008084.2