QUANTIFYING THE CHANGE IN PIGMENT POSITION IN DARK- AND LIGHT-ADAPTED RETINAL PIGMENT EPITHELIUM IN MOUSE RETINAS

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Abstract

Pigment granules change position in the retinal pigment epithelium (RPE) when light conditions change. In the light, pigment granules disperse into the apical extensions of the RPE, which contact photoreceptors. In the dark, pigment granules aggregate to the base of the RPE. The goal of this study is to quantify the position of pigment granules in light-adapted (LA) mice and dark-adapted (DA) mice and compare the pigment positions between the two treatment groups. To accomplish this, eyes from three LA mice and three DA mice were obtained from MD Anderson. The eyes were prepared for transmission electron microscopy (TEM). Thin (70 nm) sections were obtained and viewed using a TEM. The density of pigment granules was quantified by juxtaposing 2 x 2 μm boxes aligned with a feature known as Bruch's membrane and counting granules in each box. Average pigment density (pigment granules/μm^2) was calculated for each mouse. We hypothesized that pigment density would be higher in the DA mice as compared to the LA mice. The average pigment density was 1.4 ± 0.1 pigment granules/μm^2 in DA mice and 0.7 ± 0.1 pigment granules/μm^2 in LA mice. The means were statistically significantly different (p value= 0.02). The higher pigment density calculated is consistent with previously reported observations that pigment granules aggregate towards the basal end of the RPE in the dark and disperse apically in the light. Having a reliable mean allows us to explore the signaling pathways that regulate pigment granule movement.

The morphology in the sub-RPE (area between Bruch's membrane and RPE) appeared less voluminous in DA mice than the sub-RPE in LA mice. This study quantified the sub-RPE on the TEM images to compare the predicted difference in size.

We hypothesized that size of the sub-RPE in LA mouse would be larger. Using PowerPoint tools, the sub-RPE on each TEM image was filled in with solid red and the length of the sub-RPE was measured in inches. Images were imported into Python to get a count of the number of pixels in the sub-RPE. An average pixel count/length of sub-RPE was calculated for each treatment group. The average pixel count was 10,000 ± 2,000 in LA mice and 14,000 ± 6,000 in DA mice. Overall the difference in the means was not significant.

Introduction

Structure of the Eye

The eye (human or mouse) is composed of three layers: sclera, choroid, and retina (Figure 1). Mouse (or murine) models are commonly used for studying normal physiology of the eye as well as diseased because of their quick reproduction and scientists can manipulate their genes (Geng, et al., 2011). Furthermore, using mice to study the eye is less expensive than using other animal models including dogs and primates. The anatomy and physiology of mice are similar enough to humans to make mice a good model for studying origins of human retinal diseases or mechanisms (Chang, 2013).

The retina is a light sensitive layer that is composed of photoreceptor cells that enable an organism to see. The photoreceptor cells include cones, which enable discrimination of different wavelengths of light, thus providing color vision, and rods which function in low light vision. Light enters the eye through the cornea, which in combination with the lens, refracts the light to form an image on the retina. Through a series of intra- and intercellular mechanisms, the retina transduces the light signal and relays the message to the optic nerve. From there, the signal travels to the optic chiasma, through the optic tract, and into the brain where the image is perceived (Purves, et al., 2012). Cones are half the length of the rods, and both cell types are closely neighbored by retinal pigment epithelium (RPE) (Carter-Dawson & LaVail, 1979) (Figure 2). The apical projections of the RPE interdigitate with the rod and cone outer segments. The area between the outer segment and the RPE is known as the sub-retinal space. The RPE is a monolayer of tissue that separates the outer segments from the choroid layer and houses

melanosomes, or pigment granules (Lopes, et al., 2007). A review done by Strauss in 2005 considers the role of the RPE, including maintaining visual functions, controlling the neural retinal environment, and transporting ions and water out of the sub-retinal space and into the choroidal capillaries. RPE cells phagocytose the tips of the rods and cones in the outer segment as part of maintenance and regeneration of visual pigment in the eye (Purves, et al., 2012). The pigment granules found inside the RPE have known functions including absorption of light to minimize light scatter and protect the outer segments from bleaching when under bright light conditions. The pigment granules are also thought to absorb toxins that are generated in the retina (Futter, et al., 2004).

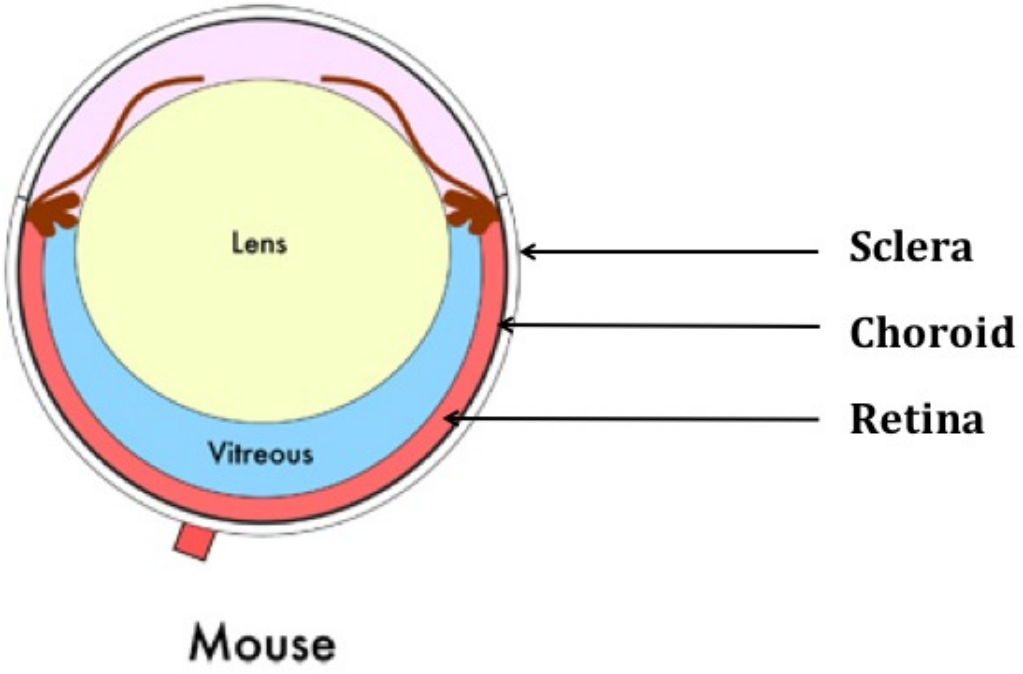

Figure 1. Diagram of the mouse eye illustrating the structure and three layers of the eye. Figure was adapted from Skeie, et al., 2011.

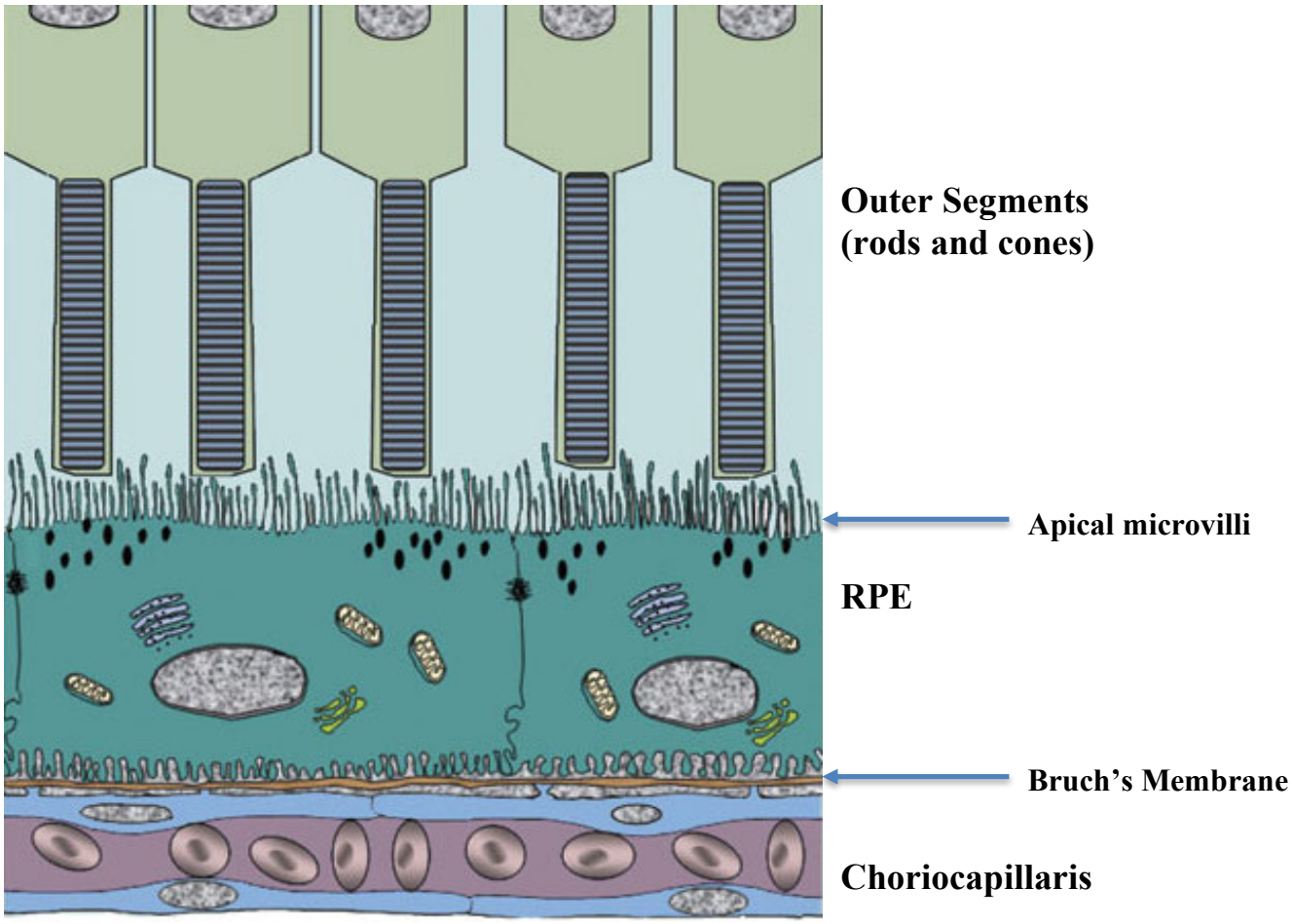

Figure 2. Diagram characterizing the anatomy of the human retina, RPE monolayer separating the choriocapillaris from the outer segments using Bruch's membrane as a barrier. The apical microvilli of the RPE are closely neighboring the tips of the outer segments. Figure was adapted from Sonoda, et al., 2009.

Pigment Granule Movement

In fish, position of pigment granules (or melanosomes), cones, and rods change under changing light conditions. In the light, cones contract, rods elongate, and pigment granules disperse into the tentacle-like extensions of the RPE, which surround the light-sensitive part of the photoreceptors, to expose the photoreceptor cells to optimum light without damaging them (Bruenner, et al., 1986). In the dark, cones elongate, rods contract, and pigment granules aggregate out of the extensions and toward the base of the

RPE, exposing the rods to dim light (Burnside, et al., 1982). These movements are collectively referred to as retinomotor movements.

For a long time, it was thought that mammals did not exhibit changes in retinomotor movements as seen and studied in fish. However, in 2004, Futter, et al., used light microscopy and electron microscopy to demonstrate that melanosomes extended into the apical region of the RPE in wild-type mice at the onset of light. Immediately after onset of light, they observed only a few melanosomes in the apical region, and after an hour and thirty minutes, they observed many phagosomes (most likely caused from disk shedding and phagocytosis) and a continued small number of pigment granules in the apical processes. Then after two hours, they observed a significant increase in the number of pigment granules in the apical RPE extensions and above the adherens junctions (Futter, et al., 2004).

Mammals can reversibly transport their melanosomes in RPE using molecular machinery (Wasmeier, et al., 2008). Figure 3 shows a proposed model of melanosomal movement in mammals. Melanosomal movement in RPE is thought to be facilitated by Myosin VIIa. Myosin VIIa is protein found in retinal tissue that plays a critical role in visual function. Mutations in the myosin VIIa gene disrupt the movement of melanosomes into the apical projections of the RPE (Liu, et al., 1998). This suggests that myosin VIIa may be required for melanosomes to be transported apically in the RPE (Futter, et al., 2006). Another component of the molecular machinery is Rab27a. Rab27a is a protein belonging to the GTPase superfamily and is found to bind melanosomes in the RPE cells (Futter et al., 2006). A mutation in Rab27a (as seen in *ashen* mice) results in melanosomes remaining stationary in the cell body of the RPE (Futter, et al., 2006).

Lastly, a linker protein MyRIP is thought to bind Myosin VIIa and Rab27a (see Figure 3) to attach pigment granules and actin cytoskeleton (Klomp, et al., 2007).

The net pigment granule movement in mouse eyes is less exaggerated than the movement in fish eyes. Experiments show that in a wild-type mouse eye, the melanosomes initially have slow movement and then burst into fast movement in the programmed direction depending on the light conditions (Lopes, et al., 2007).

The motility of pigment granules in the RPE is regulated indirectly by light. The goal of this study is to explore how the retina communicates with other cells in the eye to signal light-adapted and dark-adapted pigment granule movement in the RPE. Previous research suggests RPE cells isolated from fish did not show any change in pigment position *in vitro* under changing light conditions. However, treating the isolated RPE cells with chemicals like cAMP and forskolin provoked pigment granule aggregation, regardless of the light conditions (Bruenner, et al., 1986). This study suggested that pigment granule movement is induced by a chemical signal coming from the retina rather than being elicited directly by light conditions.

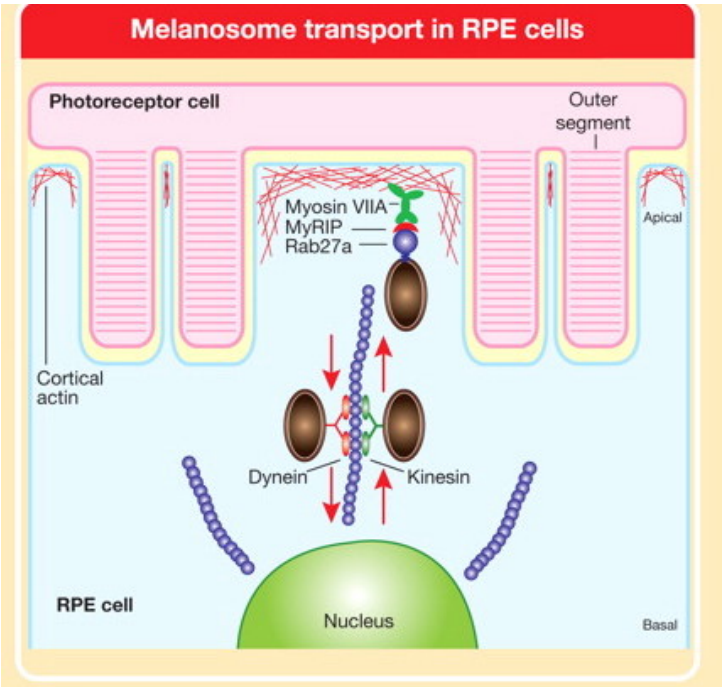

Figure 3. Proposed model of intracellular machinery moving pigment granules apically and basally in the RPE of mouse eyes. Figure was adapted by author from Wasmeier, et al., 2008.

Role of cAMP in Inducing Pigment Granule Aggregation

Cyclic adenosine monophosphate (cAMP) is a well-studied second messenger that plays a role in many biological processes. In regards to the RPE, the idea that a chemical signal from the retina induces dark-adapted pigment granule movements was supported by a study done by García, et al. in 1994. The study showed that regardless of the light conditions, cAMP induced pigment granule aggregation in fish RPE *in vitro.* The study also showed that exogenous cAMP can enter the RPE by means of an organic anion transporter. This research proposed that in the dark in fish, cAMP levels are high; cAMP is exported out of the outer segment and into the sub-retinal space. From there, the cAMP travels into the RPE through an organic anion transporter and induces pigment granule aggregation in the basal RPE (García and Burnside, 1994).

Light- and Dark-adapted Mice

In anticipation of future studies designed to test hypotheses about the mechanism involved in regulating light- and dark-adaptive pigment granule movement, this study aims to quantify the change in pigment granule position in light-adapted (LA) and dark-adapted (DA) mouse retinas. The null hypothesis states that there is no significant difference in pigment position (as estimated by measuring pigment density) in light-adapted versus dark-adapted mice. The alternative hypothesis states that there is a significant difference in pigment granule position in light-adapted and dark-adapted mice; in addition to the change in pigment density, there will be a higher pigment density calculated in dark-adapted mice compared to light-adapted mice.

Materials and Methods

Mouse Eye Tissue

The mice used for this study were graciously provided by the Department of Molecular Carcinogenesis at the University of Texas MD Anderson. The mice were kept in temperature controlled environments and all procedures followed the Public Health Service Guide for the Care and Use of Laboratory Animals. Three mice (two males, one female) were dark-adapted and three mice (two males, one female) were light-adapted.

Eye tissue collection from post mortem mice was carried out following the Instructional Animal Care and Use Committee (IACUC) using an approved protocol 0913_1001_22. There was a 5-minute gap between death and fixation of the eye tissue. The eyes were then brought back to Texas State University to be routinely processed for electron microscopy (Freemen, 2013).

Tissue Sectioning

To section the block of mouse eye tissue, fresh, sharp glass knives were made. Commercial glass strips (6.4mm x 25mm x 400mm) were cut to make the knives using a LKB Bromma glass knife maker. A Leica Reichert Ultracut S ultramicrotome was used to cut 70 nanometer thin sections from mouse eye tissue. Sections were collected on copper grids and stored at room temperature for later use.

Imaging using the Transmission Electron Microscope

Thin sections were examined using a JEOL 1200EXII transmission electron microscope at 120kV. When examining the tissue, if the area showed the landmarks in the retina, i.e., Bruch's membrane, outer segments, choroid, pigment granules, and red blood cells, then an image was obtained and saved for later analysis. Images were acquired using a Gatan digital camera, model 832.J06W0, processed and saved using Gatan digital micrograph software.

Analysis of Images

For each mouse treatment, the images saved were uploaded into PowerPoint. The pigment density was quantified by superimposing 2 x 2 μm boxes aligned along Bruch's membrane. The pigment granules in each box were counted, recorded, and an average pigment density (pigment granules/μm^2) was calculated for each mouse. Student's t-test was used to determine whether pigment density observed in light- and dark-adapted mice were significantly different.

Analysis of Sub-RPE Space

All the LA and DA images acquired from this study were imported into PowerPoint. Using the tools in PowerPoint, a red line was drawn outlining the sub-RPE

region of interest (ROI). Using Python and additional coding, the images with the sub-RPE filled in were imported into the software to measure the pixel count of the ROI for each image (Pesek, 2017). The number of pixels that were inside the sub-RPE were counted and recorded as "membrane," all pixels that were not in the sub-RPE were counted and recorded as "not membrane." Data were imported into an Excel spreadsheet for further analysis. Additionally, using PowerPoint, the length of the sub-RPE space was measured using the line tool. The measurements were recorded and used to normalize the data. The data from dark- and light-adapted mice were compared using a Student's t-test.

Results

Light- and Dark-adapted Mice Results

To test the idea that there is a net movement of pigment granules in mouse eyes under changing light conditions, the images and quantification of pigment granule position in LA and DA are illustrated in Figures 4-7 (TEM). The average pigment density from each block is recorded in Table 1. The average pigment density per treatment group is 1.4 ± 0.1 in DA mice and 0.7 ± 0.1 in LA mice. Student's t-test resulted in a statistically significant difference in the pigment granule density between DA mice and LA mice ($p=0.02$). The light- and dark-adapted results supports the alternative hypothesis that there is a significant difference between the pigment density in LA and DA mouse eyes. The results also support that pigment granule density in the DA treatment group was greater than the pigment granule density in the LA treatment group (Figure 9).

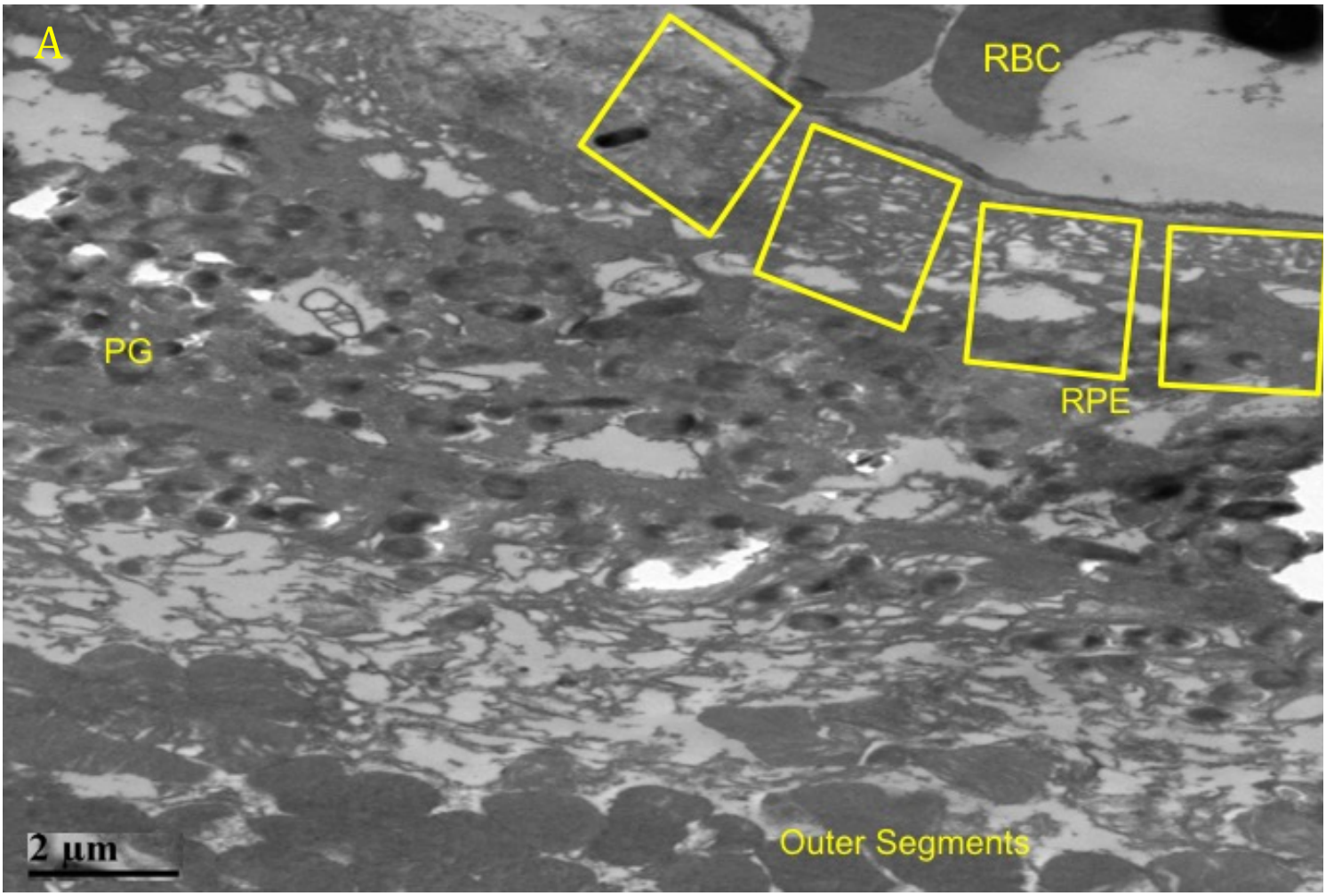

Figure 4. EM image taken from LA mouse retina. Four μm^2 boxes juxtaposed to Bruch's membrane were used to estimate pigment density as describe in Materials and Methods. Images reveals retinal pigment epithelium (RPE), Bruch's membrane (BM), pigment granules (PG), outer segments (OS), and red blood cells (RBC).

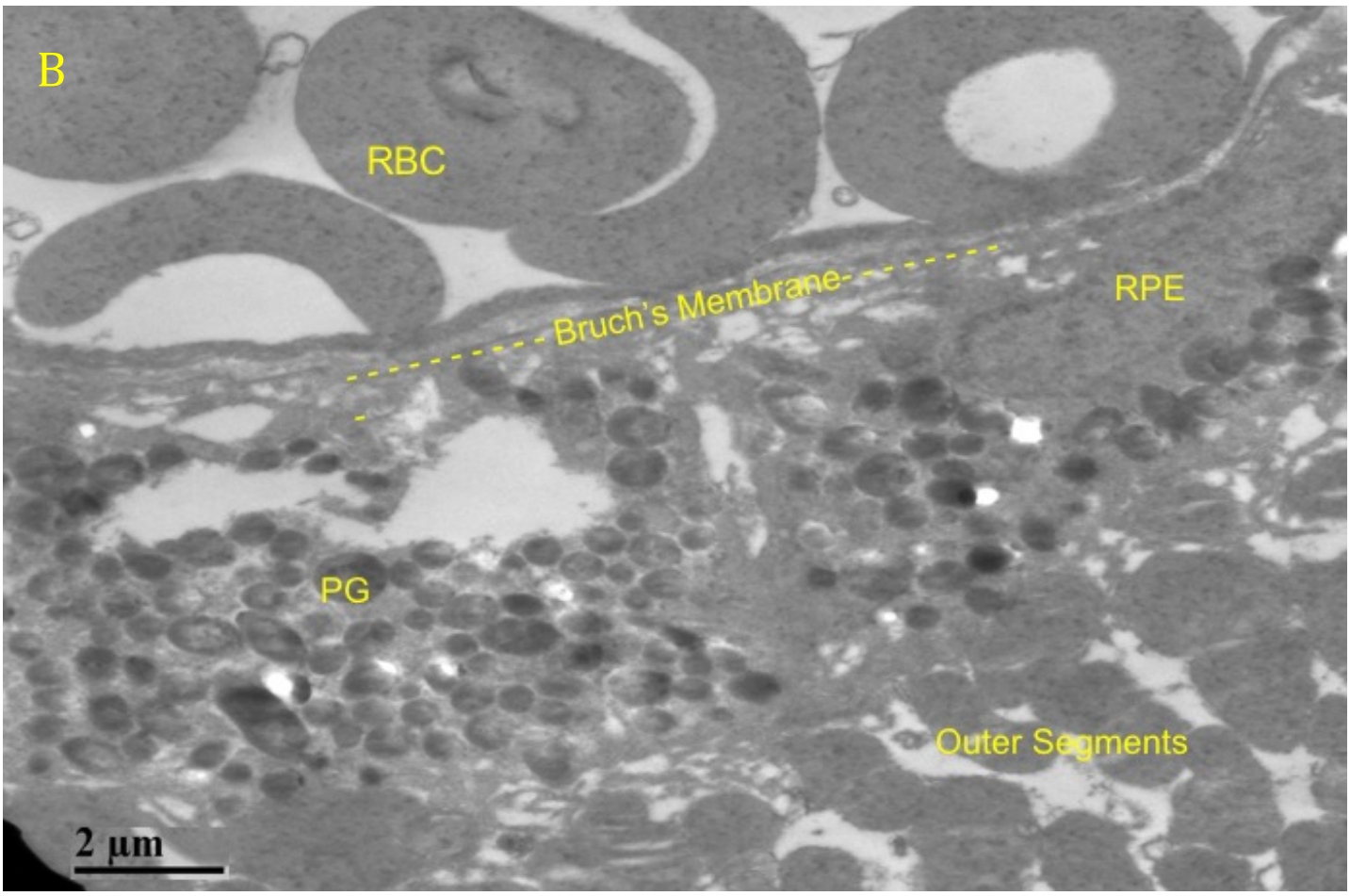

Figure 5. Electron microscopy (EM) image taken from a LA mouse retina

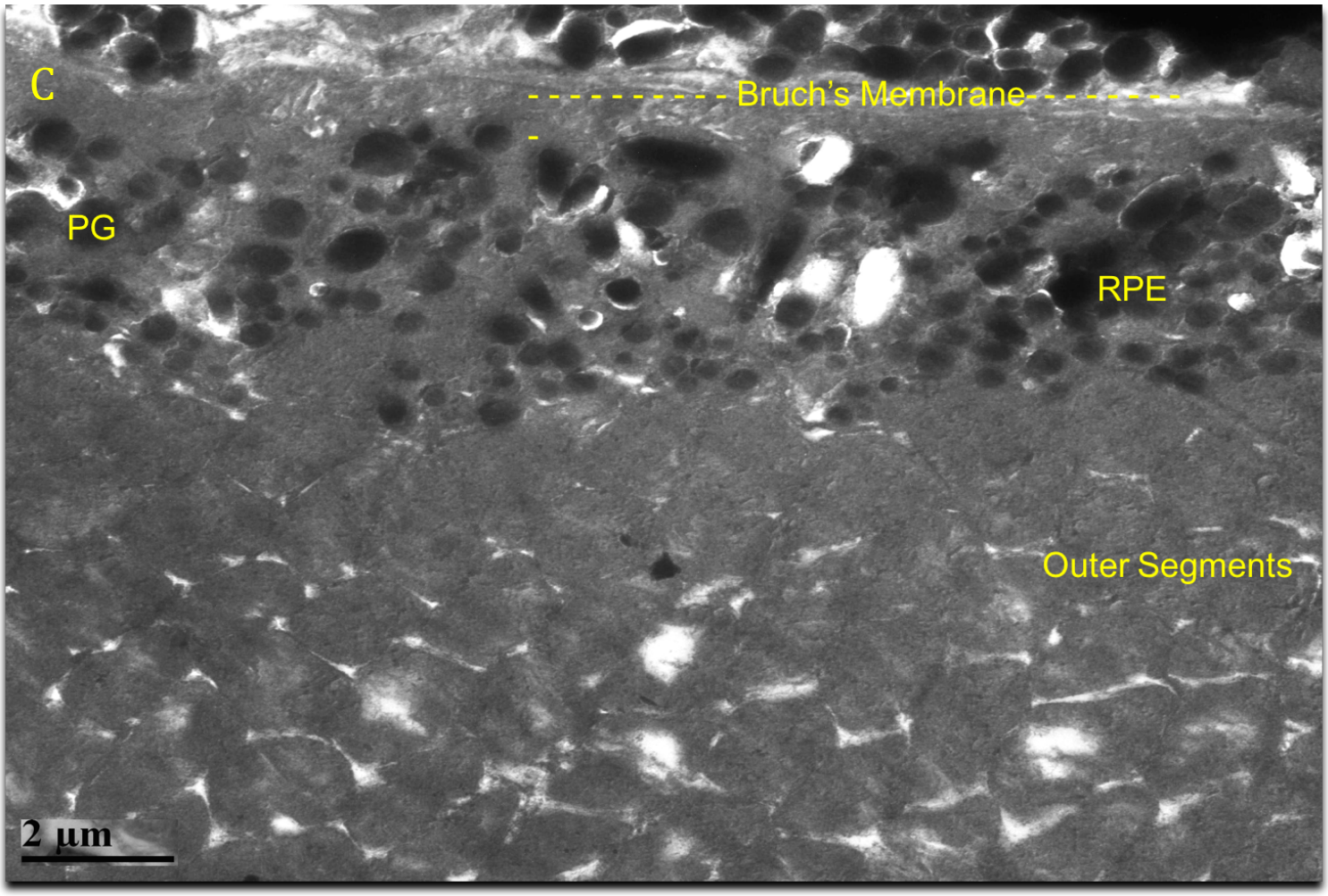

Figure 6. EM image taken from a DA mouse retina.

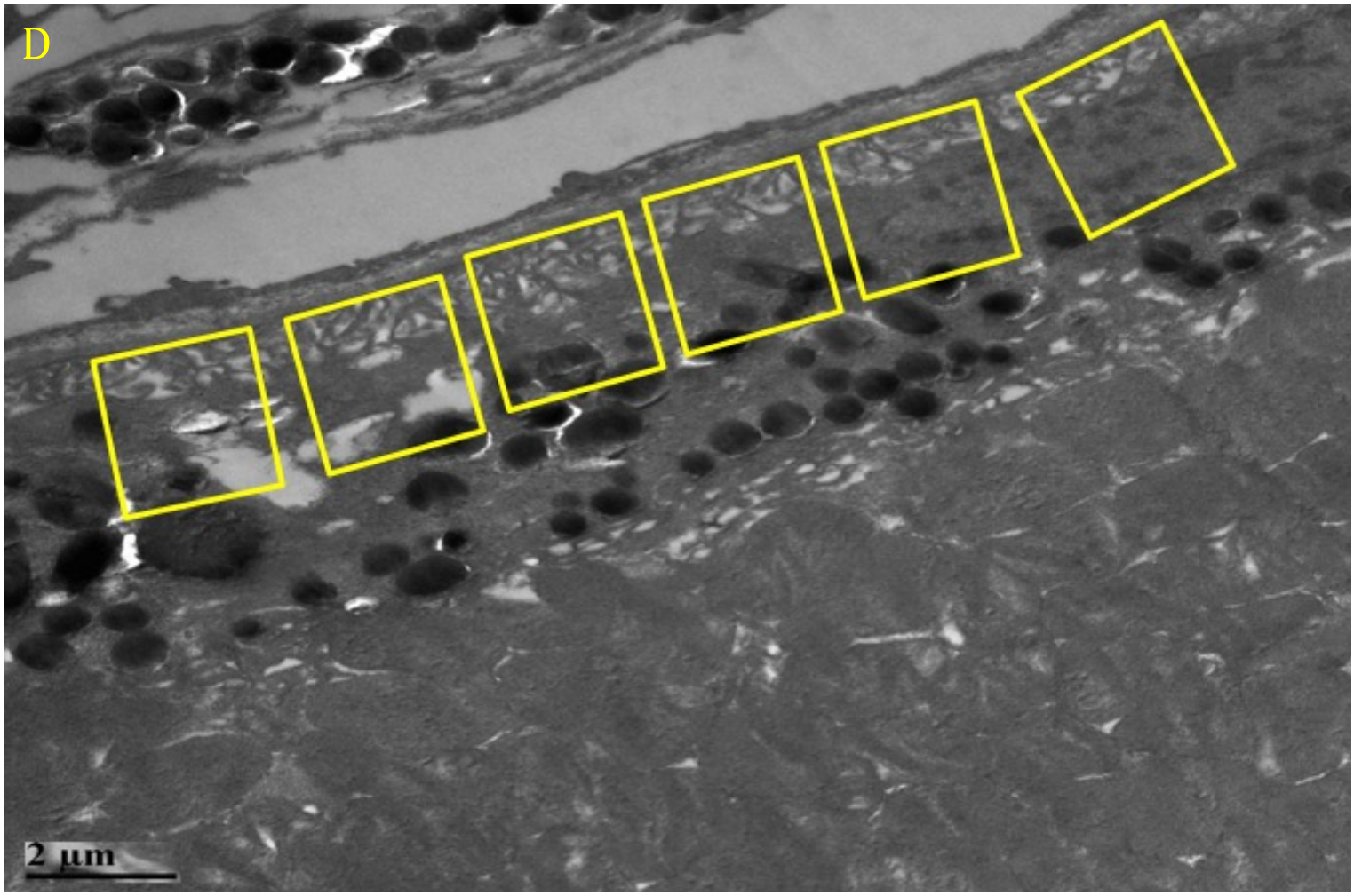

Figure 7. EM image taken from DA mouse retina.

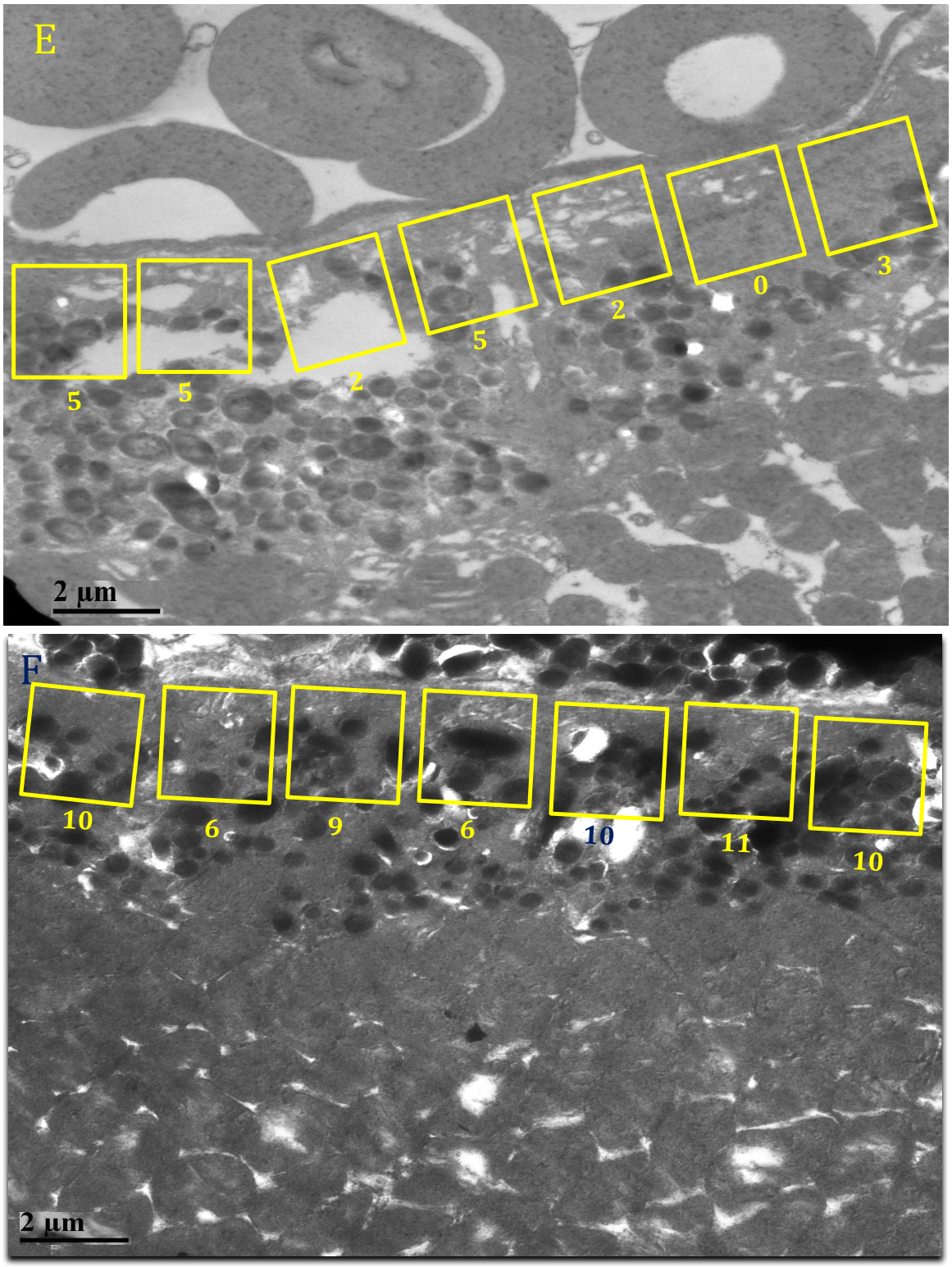

Figure 8. EM image taken from LA mouse (E) and DA mouse (F) retinas. Estimated quantification (using 4 μm^2 boxes) of pigment granules can be compared on the LA image and DA image. E shows less pigment granules towards Bruch's membrane compared to F showing more pigment granules towards Bruch's membrane.

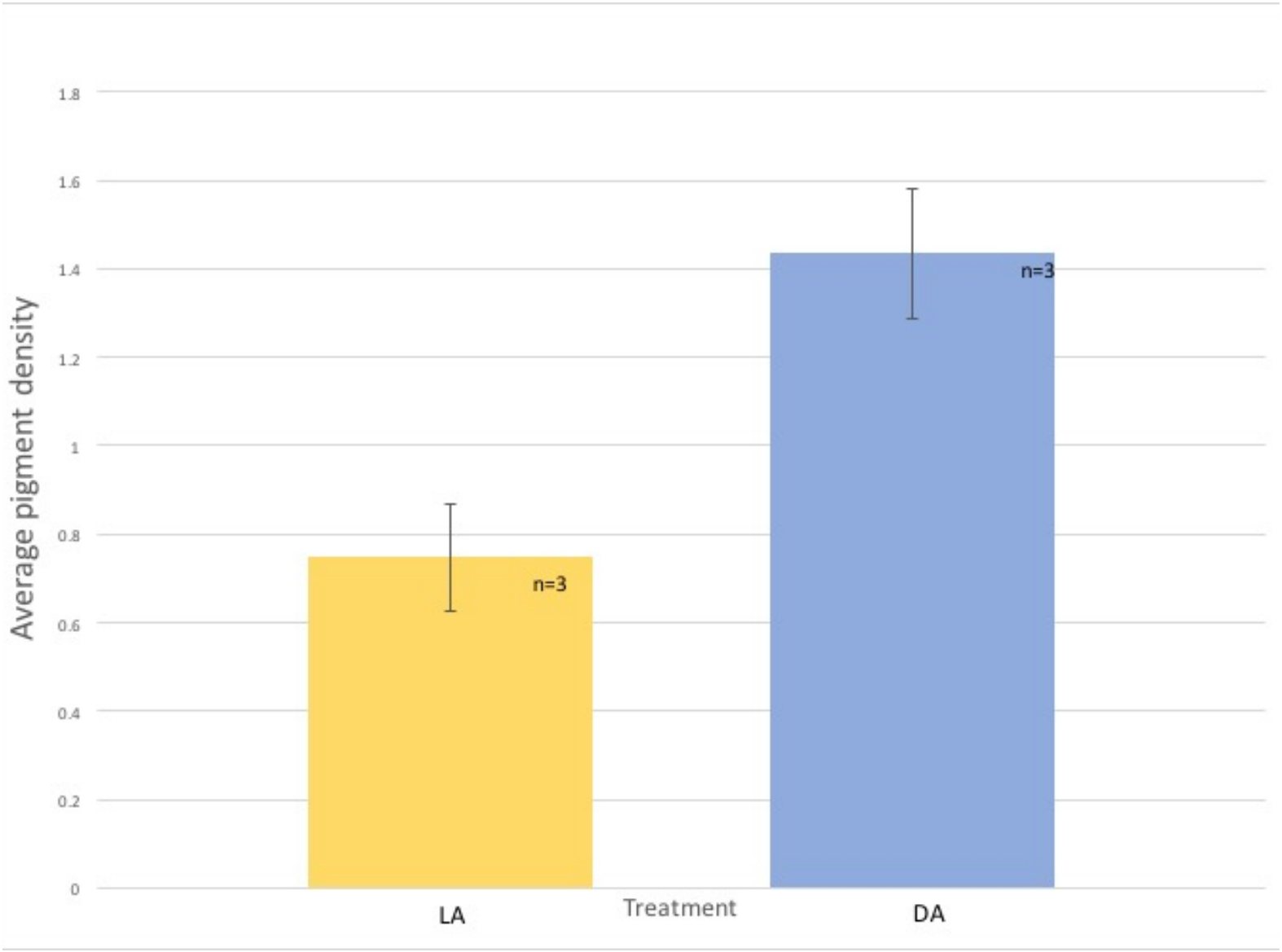

Figure 9. Average pigment density (pigment granule/μm^2) in LA and DA. There is a significant difference between the pigment granule density between DA and LA ($p=0.02$).

Treatment	Block Number	Average PG density
LA	02A13	0.51
LA	04A13	0.80
LA	05A13	0.93
DA	07A13	1.21
DA	10A13	1.71
DA	12A13	1.38

Table 1. Pigment granules found in each 4 μm^2 box divided by four and recorded as pigment density per mouse eye. Average pigment density for each mouse eye was recorded.

Treatment	Treatment Average PG density	Standard Deviation	Standard Error	Variance
LAWT	0.7	0.2	0.12	0.05
DAWT	1.4	0.3	0.14	0.06

Table 2. The average pigment granule density was averaged from three LA eyes and three DA eyes. Standard deviation, standard error, and variance were calculated.

T-test	0.02
F-stat	1.44
F-crit	19
F stat< F crit	Sample variances are equal

Table 3. Calculated results from the F-test and T-test ran to test statistical significance of average pigment granule movement between LA and DA mice. Unpaired, type 2, t-test ran using Excel.

Quantification of Sub-RPE Area

In the course of examining sections from LA and DA mice, I observed that the sub-RPE appeared more voluminous in LA sections and less voluminous in DA sections. One objective of this study is to quantify the sub-RPE and compare the two treatment groups. The null hypothesis states that there is no significant difference in size of the sub-RPE between LA and DA mouse eyes. The alternative hypothesis states that there is a significant difference in the size of the sub-RPE, and the LA mouse sub-RPE area will be larger. To determine if the space in the sub-RPE is larger in LA mice and smaller in DA mice, the pixels in sub-RPE were counted for each TEM image imported into Python (Figure 10). The average pixel count/length of sub-RPE for LA mice is 10,000 ±2,000 and DA average is 14,000 ± 6,000 Student's t-test revealed no significant difference in the means of the two treatment groups (Figure 11).

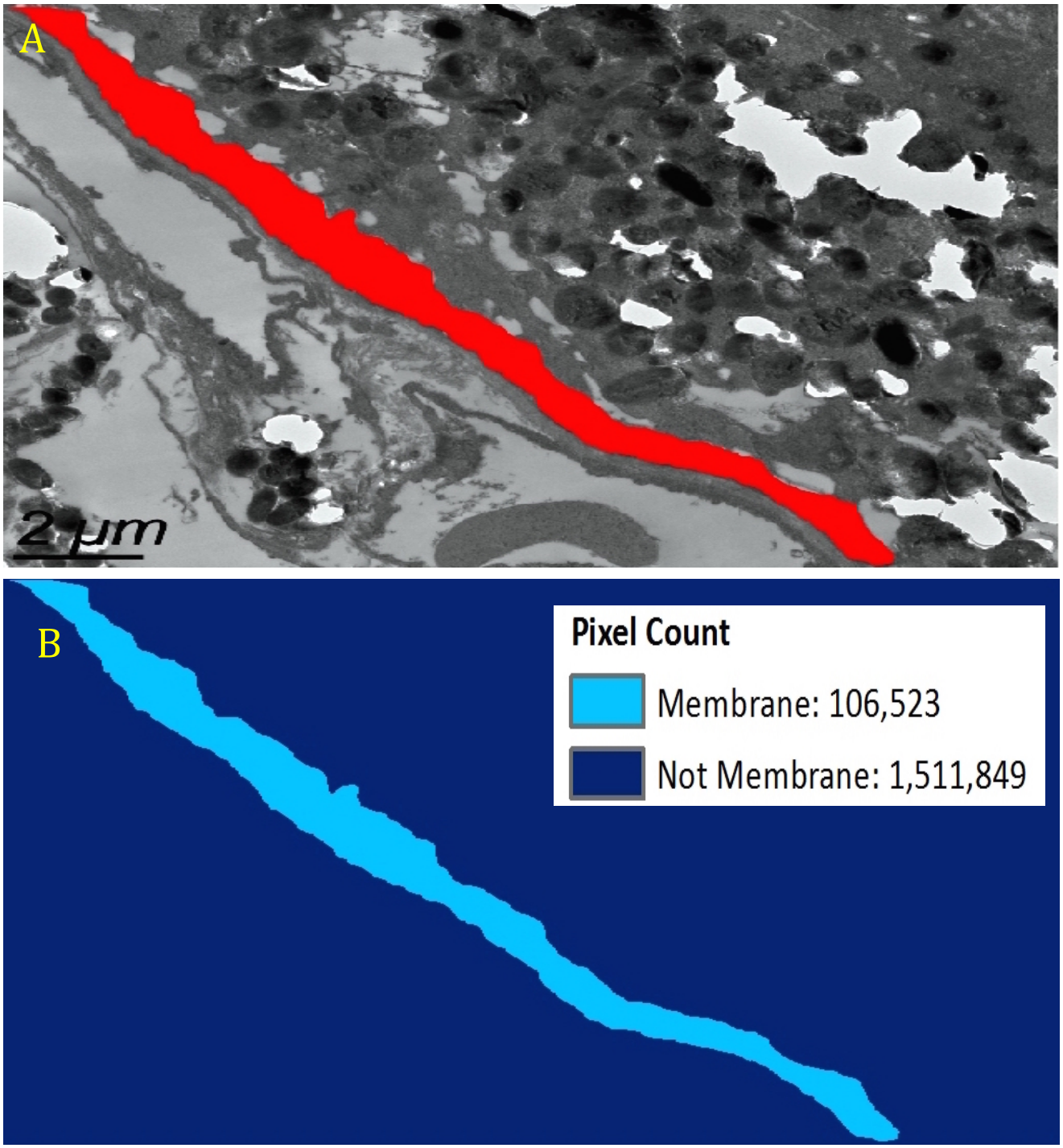

Figure 10. (A) EM section of mouse retina highlighting the sub-RPE with red. (B) Python software isolated and counted the number of pixels in the "membrane," meaning sub-RPE, and the number of pixels in the "not membrane," representing pixels outside of sub-RPE. The number of pixels in the sub-RPE was divided by the length of the sub-RPE. The pixel count/length was all averaged for each treatment group.

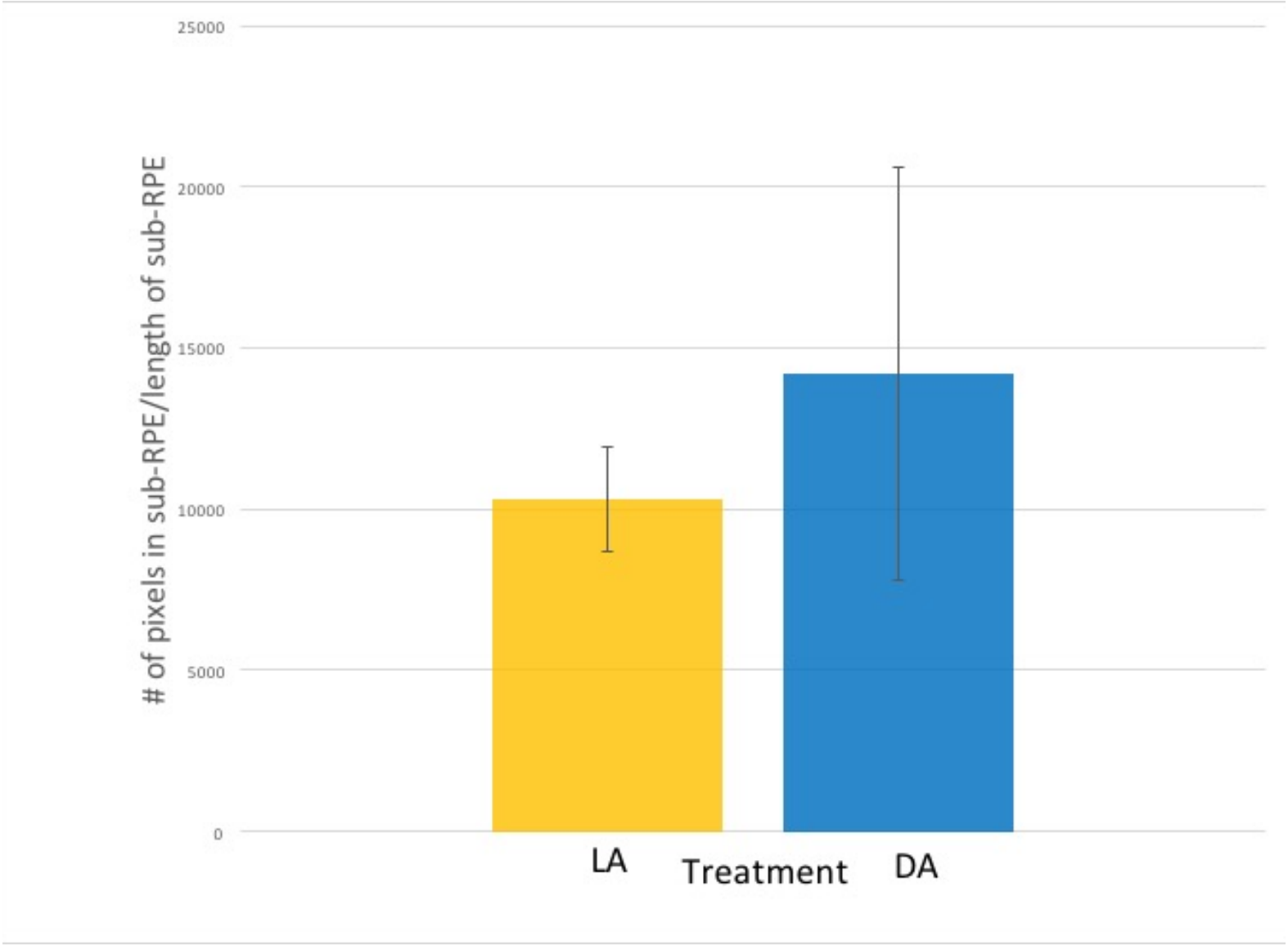

Figure 11. Evaluation using a graph showed LA average is 10,000 ± 2,000 and DA average is 14,000 ± 6,000. Overall the difference in the means was not significant.

Discussion

Pigment Granule Movement in LA and DA Mice

The results presented here demonstrate that mouse retinas do undergo a change in pigment position under changing light conditions. The higher calculated pigment density in the DA mice reflects our prediction that pigment granules aggregate towards the basal end of the RPE in the dark and disperse towards the apical end in the light. These results are consistent with earlier reports of pigment aggregation in the dark (Futter, et al., 2004). The results from this study improve upon the earlier reports by testing a quantification method to back the qualitative change in pigment position in the two treatment groups with data.

Sub-RPE in LA and DA Mice

Observations of the images taken from the LA and DA mouse eyes suggested an apparent difference in the morphology between the two treatment groups in a specific area of the eye. The area of interest is located between Bruch's membrane and the basal RPE membrane. In DA images obtained for this study, this space appears to be less voluminous with less elaborate microvilli compared to the LA images, which show more volume and elaboration. The area of interest is formally known as the sub-RPE (Castaño, 2013). This region of the retina has high interest because studies have linked the act of smoking cigarettes, specifically the high exposure to a potent oxidant hydroquinone, to age-related macular degeneration (AMD) (Espinosa-Heidmann, et al, 2006). Hydroquinone is thought to stimulate injury to the RPE by promoting the sub-retinal deposits; a hallmark sign for early stages of AMD (Marin-Castaño, 2013).

Based on the results from the quantification of the sub-RPE, I concluded that there is no significant difference in the sub-RPE area between LA and DA mice. Initially, I predicted that the LA mice, on average, would have a larger sub-RPE than the DA mice. Although the difference between treatment groups was not significant, the DA mice showed a larger sub-RPE than LA mice which is opposite of what I predicted. Also, the standard error in the DA mice data was variable.

Future Work

Future mechanistic studies can be done using the results highlighted in this study. Further research can be conducted to investigate how the retina communicates with the other cells in the eye to induce pigment granule aggregation in the dark and dispersion in the light. This retinal-choroidal interface in the eye is an area of interest for research and development due to its crucial physiological role in maintaining visual function. Studies show that melanosomes inability to migrate into the apical projections of the RPE cells might add to retinal degeneration in humans (Futter, et al., 2004). Failure to any of the interworking in the retinal-choroidal interface of supplying photoreceptors with nutrients, absorbing or displacing excess light that enters the retina, or regulating ion balance could lead to retinal degeneration, and eventual blindness from diseases like retinitis pigmentosa or age-related macular degeneration (AMD) (Bennis, et al., 2015). Currently, AMD is the leading cause for blindness globally and its origin is still not well understood.

Exploring MRP4

Further research will use this pigment position data to investigate a possible model for pigment granule movement. As stated in the introduction, there is a proposed model explaining how cAMP signals pigment granule aggregation in the dark in the RPE.

The missing link in this proposed model is to identify the transporter protein that is exporting the cAMP out of the retina and into the sub-retinal space. García, et al., in 2015 suggested and provided evidence for the transporter protein MRP4 as a candidate mechanism for export of cAMP from retinal cells. MRP4 is a multidrug resistant protein that is embedded either apically or basolaterally in cell membranes (Sodani, et al., 2012). MRP4 is responsible for regulating cyclic nucleotides like cAMP or cGMP at a microdomain level (Sodani, et al., 2012). Observations have been made that cAMP is exported out of cells in the proximal tubule of nephrons and gut epithelium via MRP4 transporter proteins (Sodani et al., 2012; Abel et al., 2002). The next step in this research is to analyze mouse eye tissue from six knockout mice that lack the *MRP4* gene (three light-adapted mice and three dark-adapted mice) using the same data collection and quantification method from this study. In conclusion, an overall comparison between the four treatment groups can be analyzed to assess whether MRP4 is necessary for dark-adaptive pigment aggregation.

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