5-2018

Migration of Cells During Choroid Fissure Closure

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Migration of cells during Choroid Fissure Closure

A Thesis/Capstone
Submitted in Partial Fulfillment for Graduation with Honors Distinction and the
Degree of Bachelor of Biological Sciences

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RECEIVED BY THE UNIVERSITY THESIS/CAPSTONE PROJECT COMMITTEE ON:

May 5, 2018

Abstract

In the developing embryo, the eyes are formed through an ordered and complex process. A critical facet during the developing eye is the formation of the choroid fissure which forms the optic cup. Failure of the choroid fissure to close during eye development results in ocular Coloboma. Coloboma results an abnormality of the lens, iris and retina. We lack a complete understanding of the migration gene expression required for this process to occur properly. Furthermore, it is unknown if cells within the apposed sides of the CF will maintain their current location within the CF after fusion has occurred. We hypothesize that the cells within the choroid fissure will remain within position post-closure compared to their position pre-closure indicating an inherent positional knowledge. To address positional movement of cells pre- and post-choroid fissure closure, we will utilize UV light activation for UAS Kaede and GAL4 embryos using in vivo imaging.

Acknowledgments

I would first like to express my gratitude to me thesis advisor, Dr. James for teaching me to set and achieve the highest goals. I would also like to thank her for supporting and guiding me through times where I believed the research was impossible. I would also like to thank my fellow James lab members who helped me shape and critique my work.

I would also like to thank the Honors program, without them I never would have thought this would be possible. And finally, to my friends and family who have supported me throughout this whole process. The encouragement and patience has made all the difference.

Dedication

This thesis is dedicated with love to my family and fiancé

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Introduction

During embryonic development the eyes invaginate from the brain. Protein interaction of these cells allows these cells to differentiate into the type of cell they need to be. The optic cup forms from the neural tube and becomes bilayered. When the optic stalk extends back towards the brain the bottom of it closes together like a zipper. This closure is called the choroid fissure closure. The outer layer forms retinal pigment epithelium and the inner layer forms the neural retina which contains ganglion cells, interneurons and photoreceptor cells. When the choroid fissure closes, it forms the optic stalk. The optic stalk houses the vesicles that project toward the brain. The choroid fissure closure starts at the mid-section of the optic stalk and fuses in both directions, proximal and distal from the brain. Failure of the choroid fissure leads to coloboma. Coloboma accounts for 5-10% of childhood blindness worldwide and the cause is still unknown.

Because the zebrafish eye model is so similar to the human eye we studied them in this experiment. They share many genetic similarities with humans and are fairly simple to manipulate. Zebrafish embryos develop very quickly, the closure of the eye is usually complete after 49 hours post fertilization. This quick development allows us to observe the choroid fissure closure in real time using a confocal.

To address the positional movement of cells pre and post choroid fissure closure, we will crossbreed GAL4 with UAS Kaede zebrafish. Upon crossing these strains, the gene of interest is expressed in endogenous and exogenous sequences in the tissue of interest. GAL4 is a transcription factor and it binds to the promoter of the upstream

activating sequence (UAS). This system will be employed for cell tracing during choroid fissure closure and observed through live imaging.

Literature Review

Eye Development

There are three layers that form during embryonic development. Endoderm, mesoderm and ectoderm. The lateral plates of the neural plate become elevated and they form the neural folds. The depressed mid region forms the neural groove. The neural groove invaginates to form the neural tube running longitudinal down the dorsal side. After neuralation is completed, the cephalic part forms the brain vesicles while the caudal part forms the spinal cord. The three primary brain vesicles formed are prosencephalon, mesencephalon and rhombencephalon. Prosencephalon, the forebrain, is later divided telencephalon and diencephalon. The eyes arise from the diencephalon. Shallow grooves appear on either side of the forebrain, forming optic grooves. With the closure of the neural tube, the optic grooves become the optic vesicles. The optic vesicles then come into contact with the surface ectoderm which causes the optic vesicles to invaginate which gives rise to the optic cup. "The optic cup consists of two layers: the retinal pigment epithelium and the neural retina. The neural retina develops further into the mature trilaminated retina" (Zagozewski et al. 2014 pg 1). The inner and outer walls of the optic cup are then separated by the intraretinal space. The invagination is not only present in the central portion of the cup but also at the inferior surface, called the choroid fissure. After optic vesicle is formed, it comes in contact with surface ectoderm. The surface ectoderm begins to elongate and forms the lens placode. The lens placode then invaginates and forms the lens vesicle. Cells of the posterior wall of the lens vesicle begin to elongate anteriorly and form long fibrous lumen. The neuroepithelium layer contains photoreceptors known as rods and cons as well as bipolar and ganglion cells. The retinal

fissure will eventually fuse to enclose the hyaloid vessels, the artery and vein. The fusion begins centrally and extends anteriorly and posteriorly. The retina is supplied with blood from the central retinal artery. The neural retina becomes inverted due to invagination which causes the rods and cones to be furthest from the light. The light hits the retina at the posterior portion of the eye, hitting the first line of cells called ganglion cells. The ganglion cells are followed by bipolar cells and then finally the photoreceptors (rods and cones). The ganglion cells and the bipolar cells are transparent which allows the light to interact with the photoreceptors. The pigemented part of the retina then absorbs the scattered light.

Coloboma

Eyes can detect external stimuli, give rise to nerve impulses and sends these impulses to the brain for processing. A coloboma is an absence of tissue and this localized gap appears in the retina. 60-90% of cases are bilateral and inferior to the optic disc (Deml, B. et al. 2015). Coloboma is occurs due to a defective or incomplete closure of the retinal fissure. In normal retinal development the optic cup gives rise to the retina. The optic cup splits to form two different layers, the retinal pigment epithelium and the neural retina. In humans, at the end of the fifth week the two layers of the optic cup become close together but are separated by a thin membrane. The hyaloid artery vascularizes the retina and the neural retina proliferates to form a thick neuroepithelium.

In medical terms, coloboma is defined by an absence of tissue. It is most often caused by a medical disturbance, though surgery or injury to the eye are also causes although far less frequently. In retinal coloboma, the outer thinner layer of the optic cup,

the retinal pigment epithelium, is not fully formed. This leaves a wide or empty zone inferiorly and nasally to the optic disc. Lack of fusion of the retinal fissure is the result coloboma. The optic papilla and stalk are the last regions of the retinal fissure to fuse and thus if closure fails to occur it will be at this site. Both the retinal pigment epithelium and the neural retina (neural epithelium layer) fail to fuse allowing the optic cup to turn outward and a coloboma to form. Another cause of the non-fusion of the retinal fissure is the accelerated development of the neural retina. "Coloboma (gap in the eye structure) (AMC) are a genetically heterogeneous group of disorders affecting 11.9 per 100,000 live births" (Holt, R. et al. 2017, pg 1). There has been extensive research to the cause of coloboma and the predominant finding is that genetics are responsible. There are however a very small number of patients throughout the world who experience coloboma with no evidence of a genetic cause.

GAL4/UAS system

The GAL4/UAS system is a technique for expressing transgenes in specific cells and is employed in a variety of organisms. A gene being turned on means activating the transcription of that gene to mRNA which is then followed my translation of a protein. Various proteins are used in a variety of ways in virtually every cellular process. Every cell possess a complete copy of the organisms' genome and so can theoretically express every gene in that genome. All genes are not expressed in all cells or at all times. Some genes are also regulated over much shorter time spans such as those coding for proteins involved in circadian rhythms such as melatonin. Regulation of genes is a complex system that involves various elements including transcription factors which bind to

specific sites on the genome to control the rate of transcription. Activator proteins are a type of transcription factors that bind to enhancer sequences on DNA and triggers transcription by recruiting RNA polymerase to bind the promoter sequence. The GAL4/UAS system makes full use of this molecular mechanism to drive gene expression in a specific way. The GAL4/UAS system is composed of two parts. The GAL4 gene encodes the GAL4 transcription activator from saccharomyces cerevisiae (yeast) and the upstream activating sequence (UAS) which is the enhancer where GAL4 activator binds. GAL4/UAS is particularly useful for artificially controlling gene expression in zebrafish because GAL4 is not usually present and so its expression does not interfere with other processes in the cell. The GAL4 gene itself must be turned on and off so it placed under the control of gene driver. The separate UAS sequence is fused to a target gene of interest known as an effector or responder. Since GAL4 is only expressed in cells where the driver is active the responder will also only be activated in those cells. The UAS responder is present in all cells of the animal but in most cells nothing happens since GAL4 is not present to activate it. In the cells that are expressing GAL4 the UAS is activated, the reporter gene is transcribed, and reporter protein is produced. Zebrafish carrying the UAS responder sequences are crossed with those carrying the GAL4 driver to create a zebrafish that will express the gene of interest in a particular way. It does not matter which chromosomes GAL and UAS are on because translation from mRNA to protein occurs outside of the nucleus. After translation, the GAL4 protein enters the nucleus and can bind to the UAS region on any chromosome and activate transcription of the reporter. In a study done by Pang, S. et al. 2015, they were able to express GAL/UAS

in zebrafish embryos. They determined that the expression dramatically decreased from 4 to 8 days post fertilization.

Green Fluorescent Protein (GFP)

Green fluorescent protein comes from the jellyfish aequoria victoria. GFP can be put onto a desired protein and analyzed inside the living cell under a microscope. GFP is a small protein, consisting of only 238 residues. It has a unique beta can shape. Eleven beta strands containing 9-13 residues each make up a nearly perfectly cylindrical beta barrel. The secondary structure also includes five alpha helices. One of which runs through the middle of the barrel and is covalently bonded to the chromophore. The chromophore consists of three amino residues, serine, tyrosine and glycine. These three amino residues are responsible for the green fluorescence. Zhang, et al. 2016, discovered that this tripeptide sequence is post translational modified in order to link the two pi systems of the rings to form a more conjugated system. This process known as maturation does not require an enzyme and occurs spontaneously. During and after the chromophore maturation the final structure and its intermediates are stabilized Van der Waals interactions as well as hydrogen bonding. The chromophore will not fluoresce unless the GFP is properly folded. The shape of the protein functions to exclude solvent molecules which prevents the energy of the chromophore from being quenched. This allows the protein to fluoresce.

GFP is commonly used as a reporter gene. A reporter gene indicates whether a certain gene is being expressed in a cell or organism population. “A protein of interest can be monitored in cells or even in whole animals without having to purify, label, and

deliver the protein into cells. Thus, it is now possible to label and observe proteins in previously inaccessible environments" (Snapp 2001).

Materials and Methods

Genotyping

PCR strips were prepared to collect the fin clip and the tubes were numbered that correlated with the cups that each fish were kept in. Added 100ul and 50nMNaOH to each tube. The fish bowl was filled with fish water and 10ul of tricaine. After fish was anesthetized it was removed from the tricaine fish water and placed on a dry paper towel. A portion of the tail was removed with a scalpel and the tip was placed in the tube containing 50mMNaOH. The fish was then placed in the cup that corresponded to the same tube number. 5ul of 10% 1M Tris with pH of 8 was added to each sample. The cells were denatured by running the PCR for 20 minutes at 95 degrees Celsius. This genomic DNA extraction was used as a template for the PCR.

Mounting for in vivo imaging

Live embryos were anesthetized with tricaine and mounted in low melt agarose then imaged using a confocal microscopy. During confocal imaging 10% tricaine in fish water was placed over dish.

Heat shock protocol

Heat shock GAL4 transgenics were raised and maintenanced in a manner conforming with the animal research facility. Embryos would have then been heat shocked in a PCR thermocyler by raising the temperature to 39.5 degrees Celsius for 30 minutes. After the heat shock, the embryos were returned to 28.5 degrees Celsius for imaging.

Photo switch Kaede

Using 40x objective, took picture of interest at 405nm wavelength laser on one distal side of eye ball 36 hpf. Exposed to 405nm wavelength for 5 seconds and pictures were taken every 15 minutes for 16 hours.

Fish Care

All zebrafish Strains were bred, raised and maintenance in accordance with the animal research facility in ross hall per IACUC protocol. Adult fish were housed at 28 degrees Celsius on a 14 hour light: 10 hour dark cycle. Embryos were housed in a dark incubator at 28 degrees Celsius. Hs:GAL4 and UAS:Kaede transgenic lines were a generous gift from the Gross lab at the University of Pittsburg.

RNA Injection

Kaede RNA sequence contains a poly A tail already attached. The poly A tail is part of the process for translation and protects the transcript for exportation from the nucleus to the cytosol. RNA sequence of Kaede is immediately injected to embryos (less than 16 cells). After injection fish should express UAS Kaede.

Expected Results

GAL4/UAS Kaede Expression in the zebrafish

Fish were pooled and screened by PCR for the presence of GAL4 and UAS Kaede transgene. UAS Kaede-positive fish were then mated to GAL4-positive lines to identify expressors. Analyses of choroid fissure showing kaede expression in retinal pigment epithelium. Kaede would be selectively photoconverted in the cells of the choroid fissure during closure. Photoconversion can be conveniently achieved by scanning with a UV laser. Scan the visual window and the square area of the visual window can be specifically photoconverted. Transgenic embryos analyzed carry 1:GAL3 and UAS:kaede. The fish would then be subjected to green-to-red photoconversion by timed exposure to UV light using DAPI filter under 10x or 20x objectives on the confocal. Images would have been taken every 15 minutes with slices 10um wide for a duration of 10 hours. Viewing of images would have been through Fiji. Cells relocation or stationary positions during choroid fissure closure would have been measured and observed. Cells fate at time of choroid fissure closure would have been determined.

RNA Injection

UAS Kaede embryos would have been injected with double-stranded RNA. Embryos were not past the 16-cell stage. The surface of the chorion would have been pierced, entering the yolk. Injection of the RNA material into the yolk. Several embryos would have been kept uninjected as the controls.

Results

Cell Fate Mapping

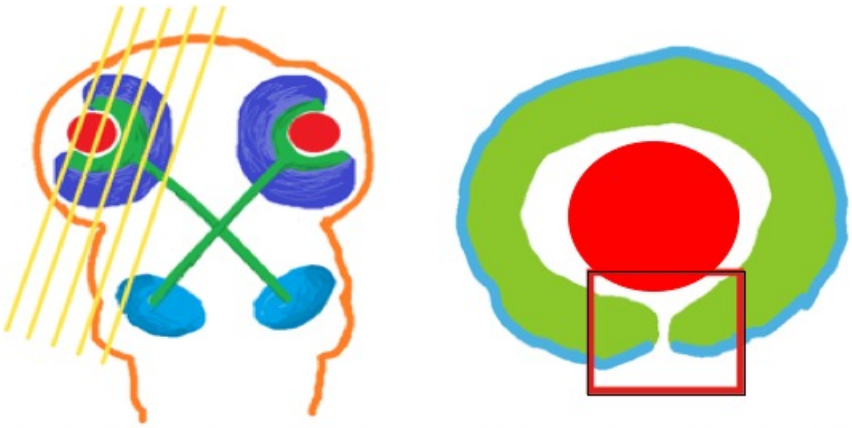

Figure 1. Sagittal views of the developing eye demonstrate a transient structure within the eye known as the choroid fissure. This opening is require for the entrance of the hyaloid vasculature and the exit of the retinal ganglion cells which will subsequently become the optic nerve. Incomplete closure of the choroid fissure in humans will lead to death of localized retinal tissue, possibly via apoptosis.

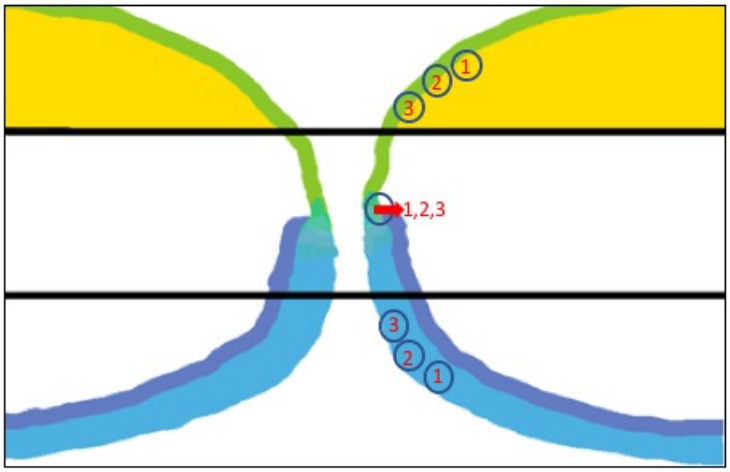

Figure 2. Dorsal and Ventral cells moved towards choroid fissure during closure. Central cells remained in central position during choroid fissure closure.

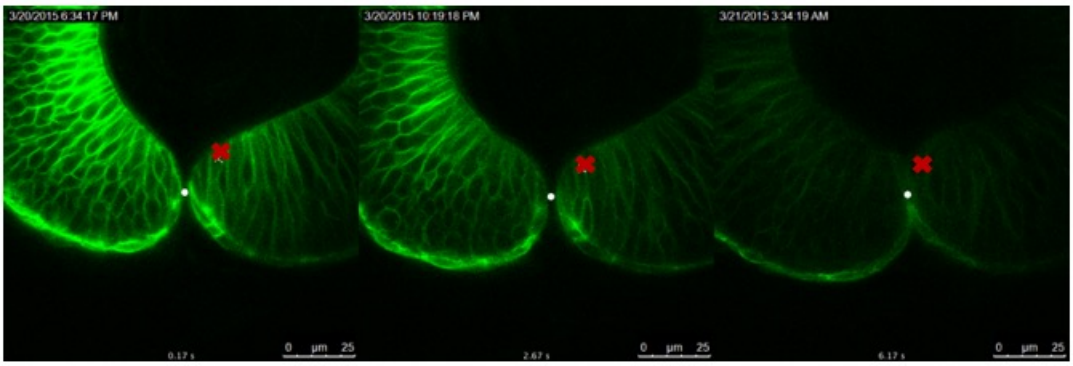

Figure 3. a) At 6:34pm distance from retinal ganglion cell layer to central point of choroid fissure was 15.60 μm. At 10:19pm distance from retinal ganglion cell layer to central point of choroid fissure was 14.59 μm.at 3:34am distance from retinal ganglion cell layer to central point of choroid fissure was 12.19 μm. Distance traveled over 9 hours and 25 minutes was 3.41 μm.

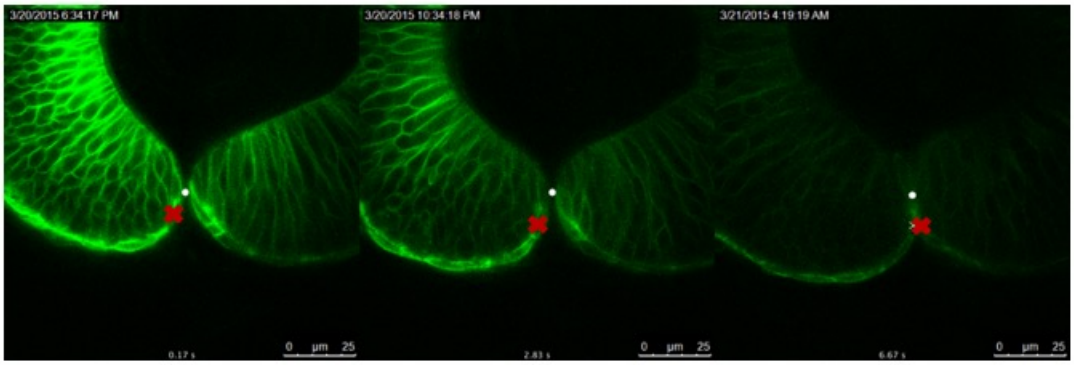

Figure 3. b) At 06:34pm distance of RPE to central point of choroid fissure was 9.754 μm. At 10:34pm distance of RPE to central of choroid fissure point was 10.256 μm. And at 4:19am distance of RPE to central point of choroid fissure was 9.827 μm. Average distance covered over a 10 hours and 25 minutes was 0.466 μm.

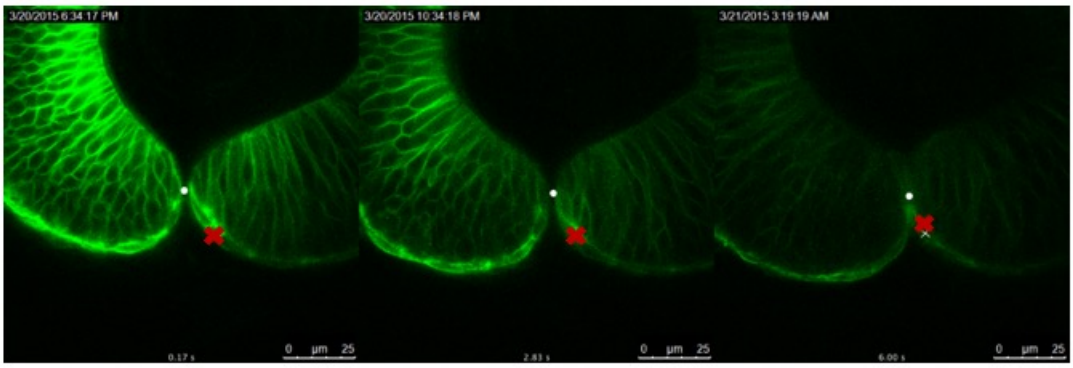

Figure 3. c) At 06:34pm distance of RPE from central point was 19.028μm. At 10:34 pm distance of RPE cell was 16.000 μm. And at time 3:19am distance of RPE from central point was 12.876 μm. Distance traveled of RPE cell was 6.152 μm during the duration of 8 hours and 45 minutes.

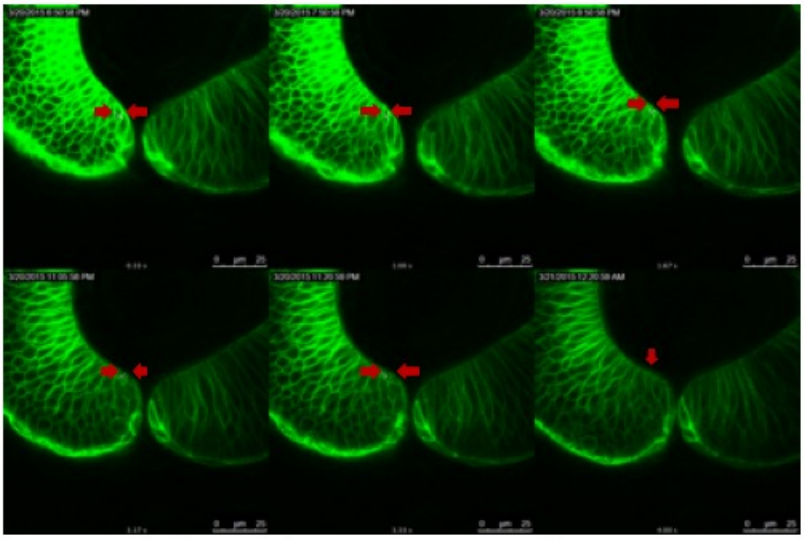

Figure 4. Dorsal cell layer rolls deeper into eye moving out of frame.

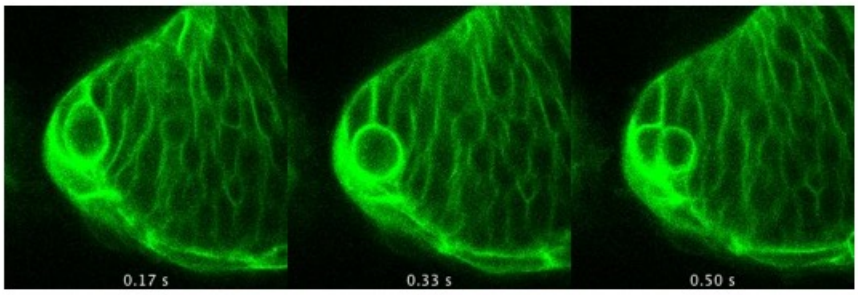

Figure 5. Cells centrally located constantly going though mitosis during choroid fissure closure

Images were taken in sectional slices called z stacks. During the duration of 9 hours, cell movement was observed. We hypothesized cells would not relocate during choroid fissure closure. Cells would already recognize what type of cell they are and were not needed to facilitate during choroid fissure closure. Therefore the cell would not relocate to choroid fissure closure to differentiate into retinal pigment epithelium. Cells located centrally were filmed from 6:34pm and the distance from the retinal ganglion cell layer to the central point of the choroid fissure was 15.60 micrometers. At 3:34am the distance from the retinal ganglion cell layer to the central point of the choroid fissure was 12.19 micrometers. The distance traveled by this cell over the duration of 9 hours and 25 minutes was 3.41 micrometers. Cells centrally did relocate to a distance closer to the choroid fissure closure. Cells located at the central point of choroid fissure traveled 0.466 micrometers over the duration of 10 hours and 25 minutes. Cells located on ventral side of choroid fissure traveled a distance of 6.152 micrometers toward the central point of the choroid fissure during the duration of 8 hours and 45 minutes.

We were able to observe centrally located cells to roll back and out of the frame. We were also able to observe cells located centrally going through mitosis before both sides of the choroid fissure came into contact.

Discussion

One of the strengths of zebrafish model is that certain specific gene products can be added to the embryos by injection. During the development of the eye, the RNA is occurs throughout the organism. The green fluorescent protein, as seen in the figures, allows us to watch cell movement under confocal microscopy.

The cells that migrate out of the image could be because of cell migration through maturation process. During this time the axons would be forming in the choroid fissure which would be caused by cells preparing to extend the axons. However, 10 microns behind this image these cells do not appear. This could be due to apoptosis. To determine whether or not these cells are going through apoptosis, an antibody staining could be performed using amino fluorescent with an apoptosis marker. However, these cells do not look as though they are going through apoptosis because of their cell shape and size. Apoptosis occurs normally during development and aging and is a highly regulated process. The dorsal cells relocate but still remain in the dorsal area. These cells are the retinal ganglion cells and do no facilitate choroid fissure closure. The centrally located cells had very little movement and suggest that these cells have their identity and the main priority is to facilitate choroid fissure closure. Cells located ventrally did remain ventral, however, relocation closer to the choroid fissure occurred. This might suggest that these cells facilitate in same during choroid fissure closure but also know their identity as ventrally located retina which contains the rods and cones.

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