How does ‘Y285 Stop Mutation’ affect photoreceptor development in the mouse?

Submitted by
Adam Seidel

Liberal Studies

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Oakland University

Mentor: Dr. Andrew Goldberg
Eye Research Institute
Oakland University

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Abstract

The photoreceptors of our eye's retina are directly responsible for normal vision. Each rod and cone photoreceptor contains a series of continuously regenerating photoreceptor disks. The photoreceptor disks themselves convert photons from the environment to cellular signals, enabling sight. If the photoreceptor disks are unable to form properly or regenerate, the result is retinal degeneration and vision loss.

This Honors College/Liberal Studies thesis project asks whether a Peripherin-2/rds (P/rds) mutation, "Y285 STOP" causes photoreceptor and retinal abnormalities in the mouse, and if so, how. Normal P/rds is 347 amino acids long. In comparison, the mutated variant of P/rds, Y285 STOP, only has 285 amino acids, due to early protein termination. This mutation causes macular pattern dystrophy in humans. Our lab hypothesizes that mice that inherit this mutation this project that Y285 STOP, may have a negative impact on photoreceptor formation compared to normal P/rds.

Using a mouse model, we will measure the thickness of the retina and evaluate whether or not the Y285 STOP mutation causes photoreceptor cell death and retinal degeneration. It is known that the mouse retina is fully developed at about three weeks of age. Previous studies have also demonstrated that a normal retina displays an outer nuclear layer that is about 12 photoreceptor nuclei thick. If the Y285 STOP retina also displays an outer nuclear layer with an identical thickness at three weeks of age, this will suggest that Y285 STOP may not have a significant effect on initial retinal development. In contrast, if the mutant retina shows a thinner outer nuclear layer (fewer photoreceptors) this will suggest that P/rds is important for photoreceptor development. Regardless of the specific outcome, this information will help us use the mouse as a model of late onset human disease.

Background

The retina is an extension of the brain that projects into the back of the eye (**Fig 1**). Light focuses onto the retina's photoreceptors, sending a signal to the brain and enabling vision. The presence, structure, and integrity of photoreceptor outer segments (**Fig 2**) are crucial for human vision.

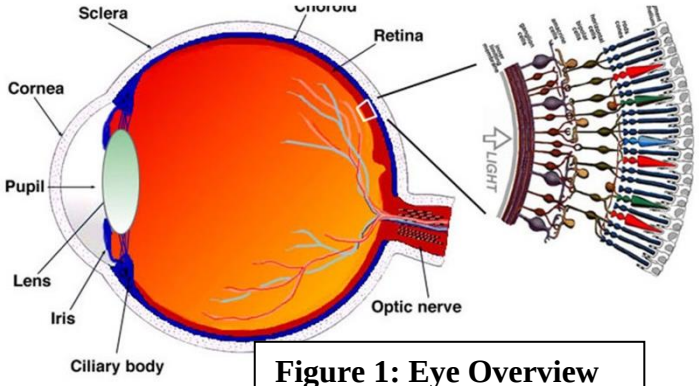


Figure 1: Eye Overview

Without *outer segments*, light cannot be perceived by the photoreceptors, as the signaling process between the eye and the brain is disrupted, leading to impaired vision and/or blindness.

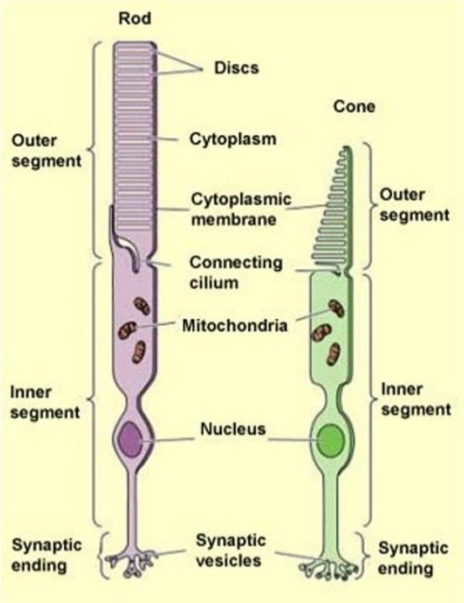
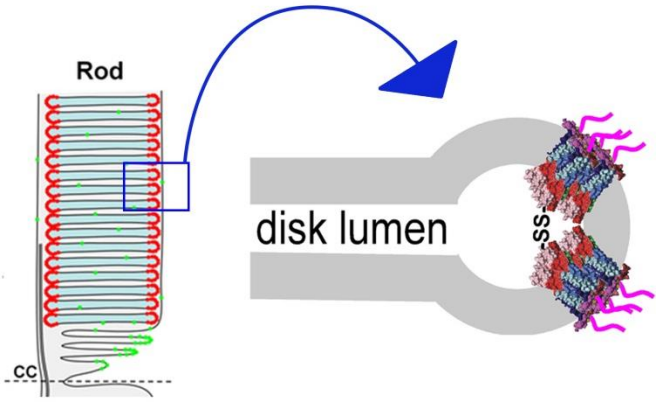


Figure 2: Photoreceptor Anatomy

This project focuses upon P/rds and a specific mutation in the protein associated with macular pattern dystrophy in humans (Kohl 1997). P/rds is an integral membrane protein localized to the outer segment disks and, is essential for outer segment (OS) structure (Molday 1987, Conell 1990, 1992, Goldberg 2006) (**Fig 3**). A variety of inherited retinal diseases are caused by defects in P/rds from different mutations. A subset of these pathogenic defects affect the protein's C-terminus; however, the normal functioning of this domain is not yet known, and this prevents a detailed understanding of disease etiology. This project will specifically investigate the functional consequences of Y285 STOP, a pathogenic mutation in P/rds that completely truncates the protein's C-terminal domain while leaving the remainder of the protein intact. Although P/rds is known to be essential for OS structure, it is unknown how P/rds, and specifically,

its C-terminus, promotes OS structure and photoreceptor viability. With CRISPR technology, the Goldberg laboratory recently generated the Y285 STOP mouse (**Fig 4**). As the first animal model for this P/rds-associated disease, this study expects to yield novel insights into the mechanisms underlying the development of this form of retinal degeneration, and better understand P/rds's structure and function in OS biogenesis and stability.



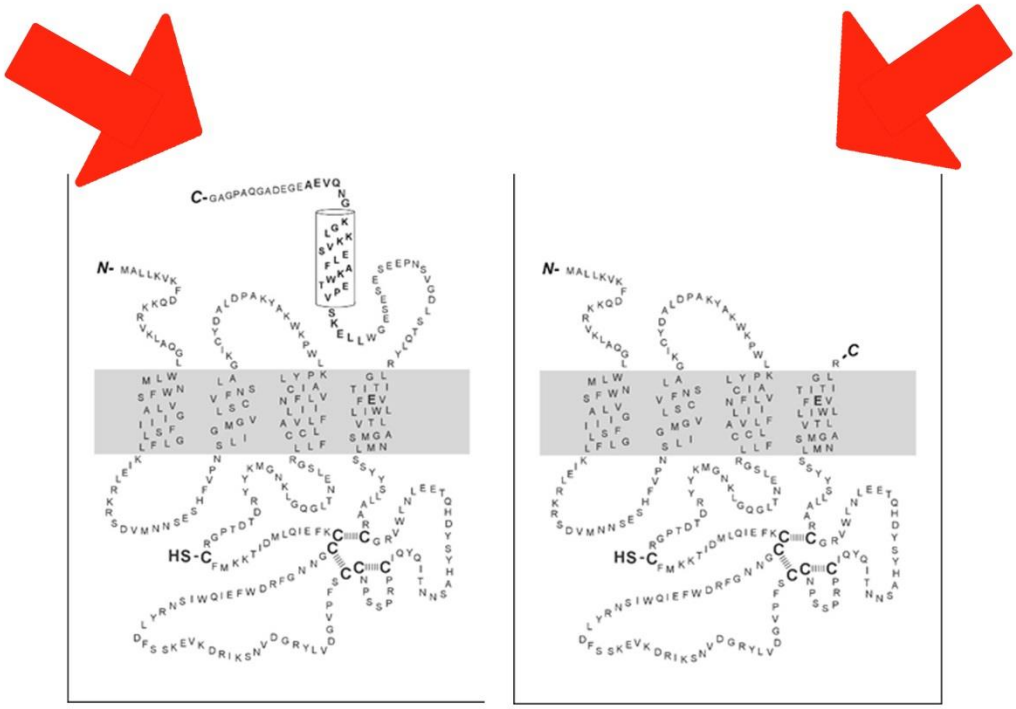


Figure 4 :Wild Type vs Y285 STOP Peripherin: Missing C Terminus Region

Introduction

This study examines the effects of an Y285 STOP mutation on retinal outer nuclear layer (ONL) photoreceptors at three weeks of age. As ONL thickness corresponds to photoreceptor numbers, the ONL thickness provides information regarding retinal integrity. Photoreceptor loss happens naturally over time in both humans and animals; however, inherited mutations can dramatically increases rate of cell loss. RDS, retinal degenerative slow, is an example of a widely studied P/rds mutation. The RDS gene is a viral gene insertion which produces a non-translatable mRNA, preventing protein expression. Heterozygous RDS results in low P/rds levels, preventing proper formation of the OS. Previous studies (Sanyal 1980, Connell 1991) have demonstrated that Wild Type and Heterozygous Retinal Degenerative Slow (RDS heterozygous) mice have comparable ONL thicknesses at one month of age. Thereafter increased retinal degeneration takes place in RDS heterozygous over a series of time points, compared to WT. (Goldberg 2006)

Previous data generated by the laboratory (**Fig 5**) suggested that Y285 STOP mutant mice may undergo retinal degeneration over time. Before studying retinal degeneration over time however, it is important to ask whether Y285 STOP interferes with initial retinal development. If Y285 STOP heterozygous does not interfere with retinal formation, the ONL thickness of an Y285 STOP heterozygous mouse at one month of age will be comparable to that of a WT and a RDS heterozygous mouse. If the ONL thickness is not comparable, the Y285 STOP heterozygous genotype can be suggested to interfere with retinal formation. For reference, we are also comparing homozygous Y285 STOP (Y285 STOP homozygous) against RDS homozygous (RDS homozygous) in this study.

The information gathered from this study will help us better understand Y285 Stop Codon's effect on the formation and development of the retina, and will help support future studies examining retinal degeneration over time.

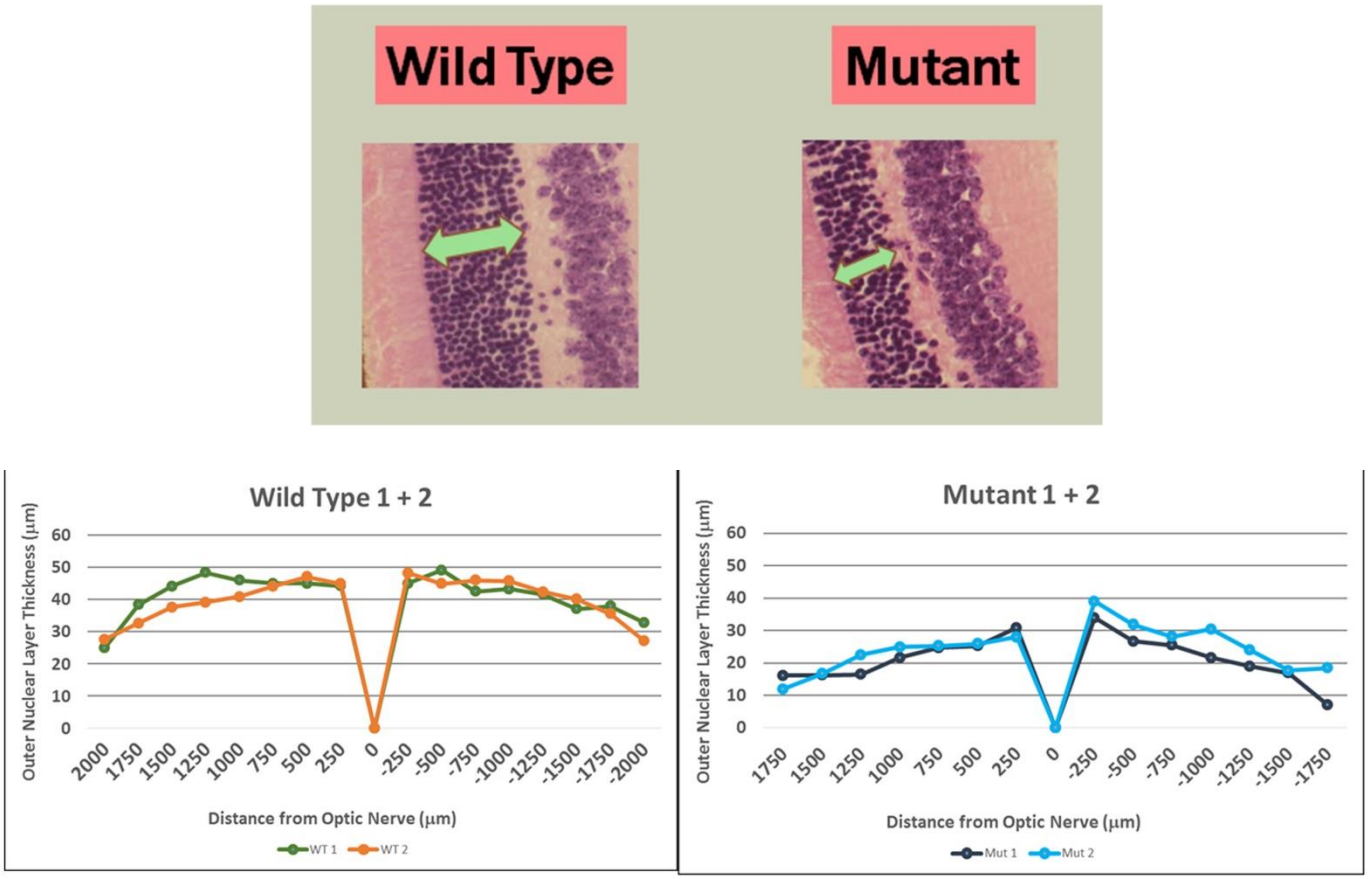


Figure 5: Y285 STOP Retinal Degeneration at 3 months of age.

Methodology:

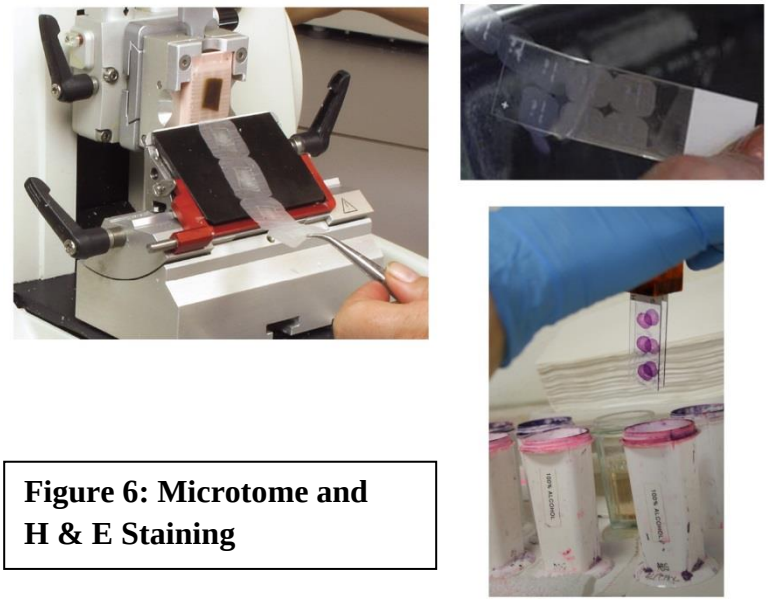


Figure 6: Microtome and H & E Staining

Eyes from humanely euthanized CRISPR mice were collected at 21 days of age by laboratory staff and were then embedded into paraffin blocks. This study compared triplicates of 5 genotypes: Wild Type, Y285 Stop heterozygous, Y285 Stop homozygous RDS homozygous, and RDS homozygous. Paraffin blocks were sectioned into 5 micrometer sections at the optic nerve, roughly 20 per eye, and transferred to slides. These slides were then stained using Hematoxylin and Eosin to become visible underneath a microscope (**Fig 6**).A Nikon microscope equipped with a digital camera was used to acquire micrographs using dedicated software. Micrographs at 40x were then taken, assembled into a montage covering the entire ocular section using Photoshop, and then outer nuclear layer (ONL) thickness was measured using ImageJ software (**Fig 7**). Most of the techniques above were developed and implemented specifically for this project. Authored and co-authored protocols are included in Appendix 1.

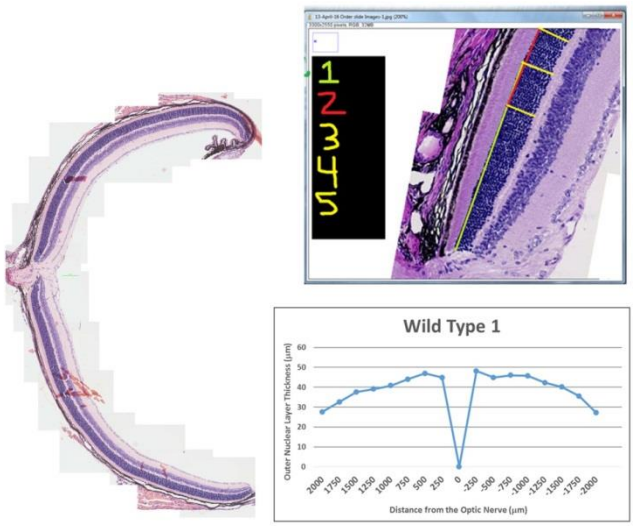


Figure 7: Example analysis of retinal outer nuclear layer thickness

Results:

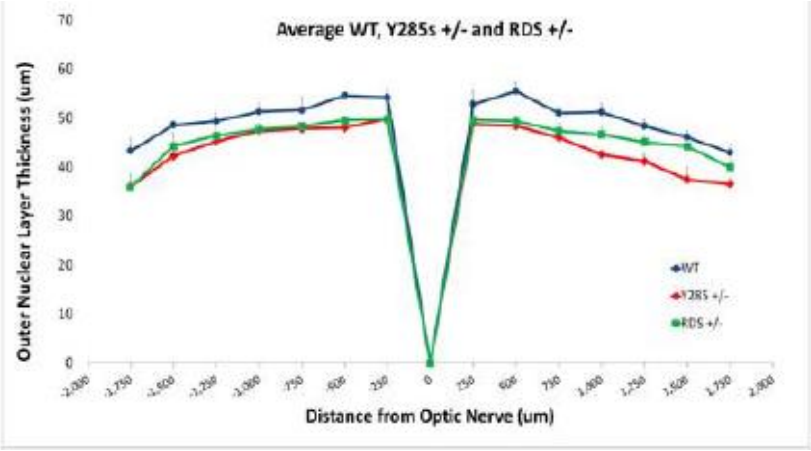


Figure 8: WT vs Y285 STOP heterozygous vs RDS heterozygous

Figure 8 compares WT (blue), Y285 STOP heterozygous (red), and RDS heterozygous (green). **Figure 9** compares WT (blue), Y285 STOP homozygous (green), and RDS homozygous (red). WT and RDS heterozygous, as previously researched by Sanyal et al, remain similar in our own results as well. Y285 STOP heterozygous is not similar to WT. Notably, RDS heterozygous and Y285 STOP heterozygous are similar to each other near the origin of the optic nerve, and RDS heterozygous is more similar to WT at the periphery of the optic nerve. RDS homozygous and Y285 STOP homozygous are both different from WT, RDS heterozygous, and Y285 STOP heterozygous, but the relative similarity of RDS homozygous and Y285 STOP homozygous remains under investigation due to the presence of outliers in the RDS homozygous triplicate set. The significant difference between WT, Y285 STOP heterozygous, and Y285 STOP homozygous at one month of age is the key finding for the discussion.

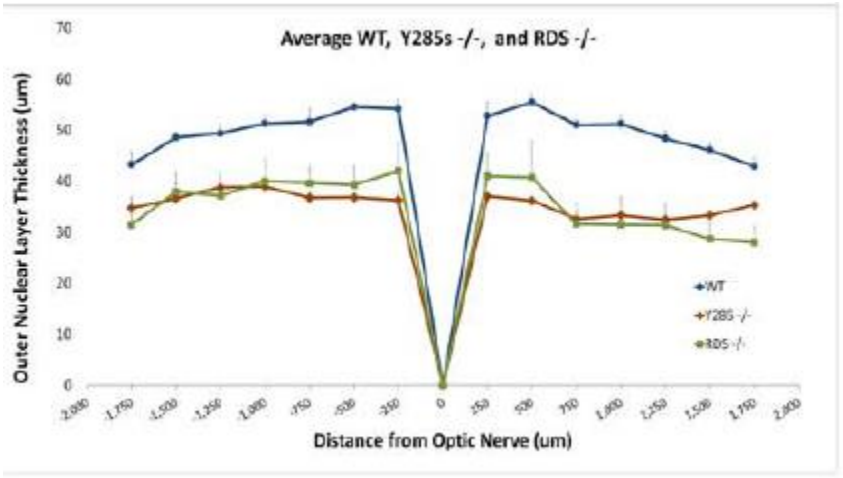


Figure 9: WT vs Y285 STOP homozygous vs RDS homozygous

Discussion:

Beginning this discussion, it should be clearly noted that ONL measurement and comparison at one month of age represents one facet of analyzing Y285 STOP. Firstly, this study does not examine ONL degeneration as a function of time, ONL formation is only analyzed at one month. Secondly, since we are only comparing ONL measurements, this study does not encompass conclusions regarding Y285 Stop's impact on photoreceptor disk formation or structural integrity. Making an overall conclusion upon Y285 Stop's complete effect requires additional studies.

As stated earlier in the results section, a clear difference in ONL thickness exists between WT, Y285 heterozygous, and Y285 homozygous. Knowing that WT and RDS heterozygous are similar, this data tells us that Y285 heterozygous interferes with retinal formation at 1 month of age. Relative to WT and RDS heterozygous, which begin degeneration over time from a similar ONL thickness at 1 month, Y285 heterozygous does not begin degeneration at the same ONL thickness.

With Y285 homozygous having a thinner ONL thickness than WT or Y285 heterozygous, we know that two copies of Y285 STOP are far more destructive to ONL formation at 1 month of age compared to one copy of Y285 STOP. The point which remains under investigation is the dissimilarity or similarity between Y285 STOP homozygous and RDS homozygous. In the event both are similar, we could say that the Y285 STOP gene is as detrimental to ONL formation as is missing P/rds completely. With that said, it will be interesting to analyze ONL thicknesses of Y285 STOP homozygous and RDS homozygous compared to each other as a function of time.

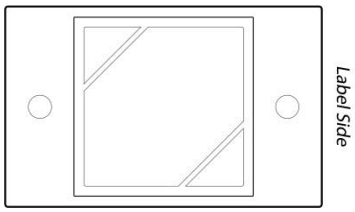
As stated in the first paragraph, the results from this study represent a small piece of fully understanding the effect and role of Y285 Stop in P/rds. Having analyzed ONL formation as a function of genotype, and having found a difference between WT/RDS heterozygous and Y285 STOP heterozygous, the next steps of this project involve analyzing ONL degeneration as a function of time. This next project

involves analyzing these same five genotypes at 3, 6, 9 and 12 months of age, and comparing ONL thickness between genotypes and time. This information will give us a clearer understanding of Y285 STOP's effect on retinal degeneration.

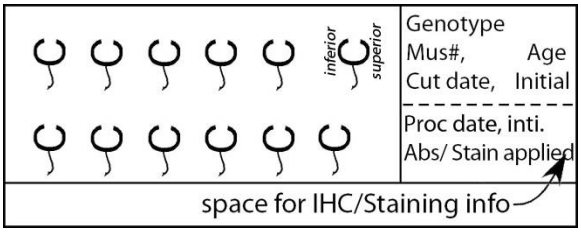
Appendix 1 Lab Protocols:

Cutting Paraffin Wax Sections Using Microtome (June, 2016 ACS)

1. What to bring upstairs (The Lab has plastic bins for one trip)
   A. Paraffin Blocks
   B. Microtome Blades
   C. Slides and Slide Box
   D. Pad of Paper
   E. Sharpie Pen
2. Equilibrate the Instrument/Ready Workspace
   A. Turn on chamber light.
   B. Fill water bath with WARM water and set to ~45°C.
   C. Obtain Small container of cold tap water.
   D. Angle used blade at 5° tilt.
   E. Set aside intended Paraffin block(s) for cutting, rewrap stored Paraffin block(s) in plastic container with fresh ice.
3. Equilibrate tissue blocks



   A. Remove Paraffin block from its mold with razor blade.
   B. Transfer Paraffin blocks from fridge to workspace and secure colored plastic end vertically to microtome. Adjust Microtome distance with left crank.
   C. Set cutting speed to 30 um by adjusting top right front Microtome knob.
   D. Begin cutting at 30um, by turning the right crank clockwise, until you reach sample.
   E. Cut 1mm into mouse eye before collecting slides, see Appendix 1
4. Mounting sections.
   A. Adjust section thickness to 5um.
   B. Replace used blade with new blade carefully.
   C. Remove block from chuck and use razor blade to trim for desired section profile.
   D. Adjust blade stand angle if necessary.
   E. Turn right crank clockwise to begin obtaining sections.
5. Handling cut sections
   A. After roughly 2 cm of cut wax sticks to the blade stand roll a wet brush into the wax edge.
   B. Crank 5-6 more times until strip extends to 4-5 Cm.
   C. Take a second brush, or thin object, and pick up the 2nd end of the strip.
   D. Transport ribbon attached to both brushes to cold water bath.
   E. Repeat once to get two strips in cold water. Juxtapose first strip cut on top of the slide, next to the second on the bottom of the slide.



F. Take slide from box and pickup both wax ribbon strips using slide, try to pick up in slide center. Be careful not to break the ribbon in half while doing this.
G. Dunk slide and ribbon gently into 45°C water bath,
H. Dry slides carefully with paper towel, being careful not to touch the paper towel on the desired tissue.
I. Place slides on slide dryer, file into slide box once dried.
J. For slides with optic nerve, file them flipped horizontally relative to normal slides.

Appendix:

- At Goldberg labs, the optic nerve is the desired section of the eye.
- The **Optic nerve occupies only 400um (0.4 mm) of the 3.5mm (3500um)** mouse eye.
- You want to cut at least 1mm into the eye, or 40 cranks at 30um, and discard the unneeded sections.
- The optic nerve is transparent on slides; the main giveaway of its presence is a gap halfway across the retina, dividing it into two pieces.
- Once you reach the optic nerve**, don't replace or adjust the blade**; because the machine may cut 100 microns with one crank.

H&E Staining for Paraffin Sections (ACS + BC 2016)

Choose tissue sections with adequate morphology.

a. ___ _____ slides, mus #_____, cut _____
b. ___ _____ slides, mus #_____, cut _____
c. ___ _____ slides, mus #_____, cut _____
d. ___ _____ slides, mus #_____, cut _____

1. Melt paraffin off slides @ 65° C for 20 minutes.
   Warning: Xylene is an dangerous aromatic hydrocarbon solvent. Inhaling xylene vapor depresses the central nervous system. Skin exposure dissolves natural protective oils and can cause irritation and dermatitis. Perform ALL staining steps in a Fume Hood with Appropriate PPE.
2. Treat with Xylene twice for 10 minutes each.
3. Treat with 100% EtOH twice for 5 minutes each.
4. Air dry slides
5. Stain with filtered Gill III Hematoxylin for 2 minutes in a Coplin jar.
6. Rinse in cool running tap for 5 minutes
7. Stain with Eosin (pH: 4.6-5.0) 1 minute.
8. Dip in distilled H2O until the eosin stops streaking.—Don't overwash!
9. Dip in 50% EtOH 10 times
10. Dip in 70% EtOH 10 times
11. Equilibrate in 95% EtOH for 30 seconds.
12. Equilibrate in 100% EtOH for 1 minute.
13. Dip in Xylene several times.
14. Clean slide off with a kimwipe; mount and coverslip with Cytoseal XYL
15. Cover/Seal Coplin for future use or Dispose of Chemicals
16. Leave Hood one to allow of Xylenes have fully evaporated.

Materials

Appropriate Xylene Safe PPE
10-13 Coplin Jar Staining Set Up (See Attached)
Five-Slide Grippers
Access to Running Water
Cytoseal 60 mountant
Cover glass
Tweezers

Solutions

120-180 ml	Xylene
120-180 ml	100% EtOH (200 proof)
60 ml	95% EtOH (200 proof)
60 ml	70% EtOH (200 proof)
60 ml	50% EtOH (200 proof)
60 ml	Gill III Hematoxylin
60 ml	Eosin Y (Aqueous)
60 ml	ddH20

H&E Staining for Paraffin Sections		
Deparaffinize	Melt Paraffin off slides @ 65° C	20 minutes
	***Xylenes are Dangerous**. Perform Staining in Fume Hood with proper PPE*	
	xylene	10 minutes
	xylene	10 minutes
	absolute alcohol	5 minutes
	absolute alcohol	5 minutes
	Air Dry Slides	
Stain	hematoxylin	2 minutes
	running tap H2O water	5 minutes
	***Do not let the water run directly on the slides*	
Counterstain	eosin	1 minutes
	tap water	Dip until the eosin stops streaking
Dehydrate	50% alcohol	Dip 10 Times
	70% alcohol	Dip 10 Times
	95% alcohol	30 seconds
	absolute alcohol	1 minutes
Clear	xylene	Dip Several Times Leave here until cover slipped
Coverslip	xylene based mounting medium	

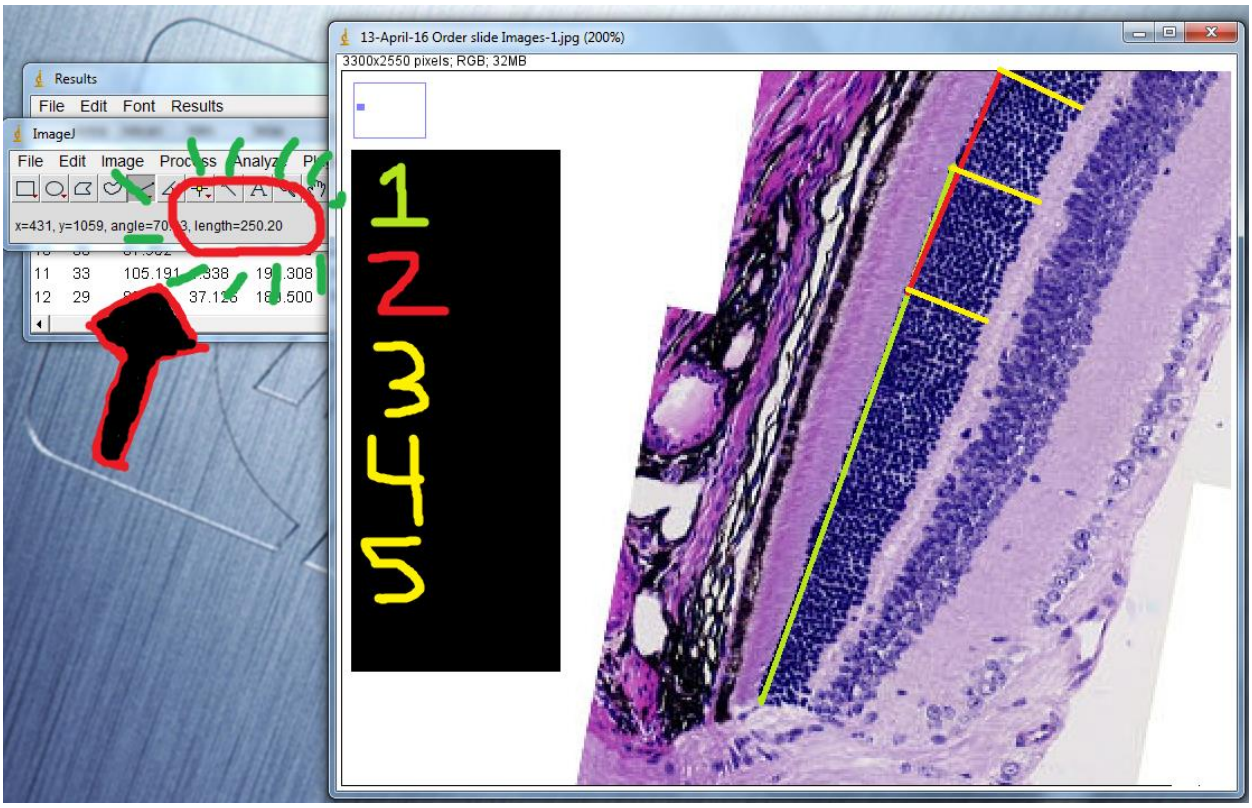
Measuring ONL Length of Mouse Retinas using ImageJ (ACS, JUL 2016)

How:

Overview: Measure ONL 250um intervals from the optic nerve, for thickness At each interval, Two additional measurements, 50uM from each anchor point, and graph the mean of the three measurements for each anchor point. Do this for both the superior and inferior hemispheres of the Retina Section. ImageJ provides tools to record each line length directly into an Excel sheet format.

ImageJ:

1. Open ImageJ and file of interest – Should be acquired using 20x objective.
2. Double-Click "Line" tool
    a. Set the line width to "10" and close the dialog box.
3. Use the "Line" tool to set the scale, this must be done for every new image.
    a. Hold down the shift key to constrain to a horizontal line.
    b. Draw a line to match the scale bar at the bottom of the micrograph.
    c. Select Analyze>Set Scale
    d. Enter the micrograph info. IE –"known distance"and "UnitsofMeasurement"(um).
4. Select the "Hand" tool and move the image to ONL start location near base of Optic nerve, you can also hold the spacebar and click to move. Zoom in and out with + and -.
5. Using the "Line" tool
    a. Draw a 250um line from the origin of the ONL, superior or inferior, beginning from ONH edge.
    b. Press D to make the first line Permanent (**Green**)
    c. Readjust the first line with the line tool to be 50uM away from the edge of the first line, using the information from the length tool, adjust the line to be an additional 50uM in the opposite direction, you should have a line 100uM long by the end of this step, press D to make the 2nd line permanent (**Red**)
    d. Now, draw three lines from the 3 anchor point edges set by lines one and two, after drawing each individual line, press "D" to make the line permanent, and then press "M" to record the measurement. You have to record the measurement immediately after pressing D. (**Yellow**)
    e. Repeat steps 5a-5d for additional lengths on top and bottom halves of image.



6. Save the image (with the added lines) using a NEW filename, and save the results.
    a. You will have to save the results separately from the image.
    b. The results will be saved as an Excel file automatically.

NOTES

a. You can only manipulate one line at a time with ImageJ, pressing D draws a black/colored line that cannot be changed.
b. If you zoom into the image, you'll have greater precision of line length.
c. In ImageJ, Photoshop-style Layers are nonexistent, you only have one.
d. ImageJ authors strongly discourage using JPEG file format.
e. Relax and turn on some music in the background. ☺

A Short Primer on Epi-fluorescence Microscopy –Nikon Optiphot (ACS & BLC, 12/15)

1) **Sign** the logbook noting the Date, Department, User's Name, and Lab.
2) Turn on the **LED UV Light source** (Little Yellow Switch).
3) Adjust the **eyepiece diopters** to their neutral positions
4) Position a sample on the stage and choose an **objective** (5x or 10x).
5) Turn on lamp and use **Brightfield illumination** to find section: Switch to 20x objective.
6) Adjust the **coarse/fine focus knob** to bring the specimen into focus.
7) Use 20x or 40x objective to **evaluate** and document as needed.
8) When you are finished, **turn off** the LED light source and brightfield lamp.
9) Note any issues/problems in the logbook.
10) **COVER** the microscope.

For Image Acquisition Only

1) Turn on Computer

2) Login: AFXG Lab Password: vizsla

3) Turn on camera power supply/interface

4) Open NIS Element's and Login (same as above)

5) Press "play" button to open a live image in the acquisition menu bar

6) Use stage to select R.O.I.

7) Rotate binocular head ~90° CW to shunt image to camera

8) Make sure no epifluorescent filter is selected for the filter block slider on the microscope

9) In the Menu bar, click desired Brightfield Filter and objective used (10x/20x/40x)

10) Adjust microscope focus and camera exposure as necessary

11) Maintain a consistent exposure for all images

12) Click "capture" (camera image) in the acquisition menu bar

13) Save As jpg: “Filename convention”

14) Click “play” button to return to live view and repeat steps 8-13 for each image

Annotated Bibliography

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