Measuring Changes in Membrane Structure and Rhodopsin levels in *Drosophila Melanogaster* Retinas using THG Microscopy

By

Abiramy Karunendiran

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Cell and Systems Biology
University of Toronto

© Copyright by Abiramy Karunendiran 2015
Measuring Changes in Membrane Structure and Rhodopsin levels in *Drosophila Melanogaster* Retinas using THG Microscopy

Abiramy Karunendiran

Master of Science
Graduate Department of Cell and Systems Biology
University of Toronto
2015

Abstract:

Third harmonic generation (THG) microscopy is a valuable imaging modality that can be used to reveal structural information in a biological system without staining. THG signal is observed at an interface between refractive indices and is augmented in the presence of conjugated carbon chains. I sought to determine whether THG microscopy could serve as a platform for developmental studies. I used the eye of *Drosophila melanogaster* as a model for its well-known anatomy and availability of genetic tools. In the first series of observations THG microscopy was shown to be an effective tool for monitoring photoreceptor development. In the second series of experiments, using dietary restrictions or genetic alleles, THG microscopy detected rhabdomere degeneration and reduced rhodopsin expression earlier than fluorescence microscopy. In conclusion, THG microscopy presents itself as a new technique to detect retinal degeneration. Further development of this technology could aid in early detection of human retinal degeneration.
Acknowledgements:

This thesis is dedicated to the members of the Stewart and Barzda Groups, for their kindness and support during this past year of my Master’s. My project would not have advanced and developed this far without it.

First and foremost, I would like to thank my supervisors Dr. Bryan Stewart and Dr. Virginijus Barzda for providing me with the opportunity to carry out this project in their labs and for their immense support. Their constant guidance and expertise has helped me to push past my limits and realize my potential as a researcher.

I would also like to thank the fellow graduate students in the Stewart and Barzda Lab for their insight and advice. A special thanks to Richard Cisek and Danielle Tokarz for going out of their way to guide me throughout my project and for their feedback on my data.

Lastly I would like to thank my mom and dad for their ongoing support and patience during this past year.
Table of Contents:

Abstract: .......................................................................................................................... ii

Acknowledgements: .................................................................................................... iii

Table of Contents: ........................................................................................................ iv

List of Tables: .............................................................................................................. vi

List of Figures ............................................................................................................. vii

List of Abbreviations ................................................................................................... ix

Chapter 1: Introduction ................................................................................................. 1

1.1 History of Microscopy in Biology ............................................................................. 1

1.2 Drosophila as a Model Organism .......................................................................... 3

1.3 Drosophila Retina .................................................................................................. 3

1.4 Retinal Development during the Pupal Stage ......................................................... 4

1.4.1 The Early Phase ................................................................................................. 6

1.4.2 The Late Phase .................................................................................................. 7

1.5 Rhodopsin Structure and Function ...................................................................... 8

1.6 Rhodopsin Cycle during Phototransduction .......................................................... 11

1.7 Rh Synthesis and Maturation .............................................................................. 12

1.7.1 Mutations in the ninaE gene ........................................................................... 13

1.8 Chromophore Synthesis ...................................................................................... 15

1.9 Nonlinear Optical Microscopy ............................................................................ 17

1.9.1 Third Harmonic Generation ........................................................................... 19
List of Tables:

**Table 1:** Absorption Spectrum for each rhodopsin found in *Drosophila* .................................................. 9

**Table 2:** Description of the ninaE mutants .................................................................................................. 14

**Table 3:** The ages used for pupal development and their corresponding percentage ................................. 22

**Table 4:** Ingredients used in the vitamin A deficient medium. ................................................................. 23
List of Figures

Figure 1: A simplified schematic of the adult ommatidium cross-section............................................. 4
Figure 2: Timeline of events during pupal development (Cagan 1989). .................................................. 5
Figure 3: a) opsin subunit. b) 11-cis-retinal (from pubchem)................................................................. 9
Figure 4: Emission Spectrum for rhodopsin by UV light........................................................................... 11
Figure 5: Rhodopsin Cycle as adapted by (Wang and Montell 2007). ...................................................... 12
Figure 6: Synthesis pathway for retinaldehyde proposed by (Wang 2007). ............................................. 16
Figure 7: Fluorescence versus nonlinear optical microscopy. ............................................................... 17
Figure 8: Energy state diaram for THG ................................................................................................... 20
Figure 9: An illustration of the microscope. ............................................................................................ 24
Figure 10: THG intensity images of 32% pd retina.................................................................................. 27
Figure 12: Images of THG, SHG and MPF images of a 32% pd w118 pupa............................................. 28
Figure 13: THG intensity image of drosophila eye at 48% pd................................................................. 29
Figure 14: Comparison of different harmonic images of 48% pd w118.................................................. 30
Figure 15: THG intensity images for 64% pd retina............................................................................... 31
Figure 16: Comparison of SHG, THG and fluorescence images of 64% pd retinas............................. 32
Figure 17: THG intensity images for 80% pd retina................................................................................ 33
Figure 18: Comparison of SHG, THG and fluorescence images of 80% pd........................................... 34
Figure 19: THG intensity dependence on the laser power...................................................................... 35
Figure 20: THG images of w118 retina showing the region of interest drawn around pigment cells and rhabdomeres using ImageJ....................................................................................... 36
Figure 21: THG signal intensity changes with age.................................................................................. 37
Figure 22: THG images of 32% pd vitamin A deficient pupal retinas...................................................... 38
Figure 23: Images of THG, SHG and MPF images of a 32% pd w118 pupa........................................... 39
Figure 24: THG image of vitamin A deficient pupa at 48% pd............................................................ 39
Figure 25: THG intensity images for 64% pd vitamin A deficient retina.................................40
Figure 26: Comparison of SHG, THG and fluorescence images of 64% pd retinas..................40
Figure 27: THG intensity images for 80% pd retina.........................................................41
Figure 28: Comparison of SHG, THG and fluorescence images of 80% pd..........................42
Figure 29: Comparison of the intensities measured in the a) pigment cells and b) rhabdomeres between the control and vitamin A deficient pupae. (P < 0.05).................................................................43
Figure 30: THG images of each mutant pupal retina at 32% pd.......................................45
Figure 31: THG images of pigment cells and rhabdomeres for each mutant at 96% and 120% pd..46
Figure 32: Comparison of the intensities measured in the a) pigment cells and b) rhabdomeres for each genotype. (P < 0.05)........................................................................................................48
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADRP</td>
<td>Autosomal Dominant Retinitis Pigmentosa</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>CO</td>
<td>Collection Objective</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>EO</td>
<td>Excitation Objective</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>Gln</td>
<td>Glutamine</td>
</tr>
<tr>
<td>GPRK1</td>
<td>G-protein-coupled kinase 1</td>
</tr>
<tr>
<td>Gyc</td>
<td>Glycerine</td>
</tr>
<tr>
<td>Met</td>
<td>Methionine</td>
</tr>
<tr>
<td>MPF</td>
<td>Multi-Photon Fluorescence</td>
</tr>
<tr>
<td>ninaE</td>
<td>neither inactivation nor afterpotential E</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PC</td>
<td>Pigment Cell</td>
</tr>
<tr>
<td>Pd</td>
<td>Pupal Development</td>
</tr>
<tr>
<td>PH</td>
<td>Pinhole</td>
</tr>
<tr>
<td>PIPO</td>
<td>Polarization In Polarization Out</td>
</tr>
<tr>
<td>PMT</td>
<td>Photon Multiplier Tube</td>
</tr>
<tr>
<td>R</td>
<td>Photoreceptor</td>
</tr>
<tr>
<td>Rh</td>
<td>Rhodopsin</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SHG</td>
<td>Second Harmonic Generation</td>
</tr>
<tr>
<td>THG</td>
<td>Third Harmonic Generation</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>-----</td>
<td>------------</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 History of Microscopy in Biology

When Ernst Abbe stated in his 1873 paper that the resolution of conventional light microscope was limited by diffraction to half of the light source wavelength, various attempts were made to use a light source with a shorter wavelength. After August Köhler’s observation that certain objects emitted light with longer wavelength when illuminated with ultraviolet (UV) light, Oskar Heimstädt constructed the first successful fluorescence microscope in 1911. He concentrated UV light onto the sample using cuvettes and managed to image bacteria. Although he wasn’t convinced that fluorescence microscopy had lasting potential, it quickly became a fundamental tool in biological imaging. It was especially crucial in the 1940s and 1950s when subcellular organelles and mitotic spindle fibers were visualized (Evanko 2009). In order to visualize specific cells in a tissue, fluorescent antibodies were created to attach to the cells of interest. This was accomplished by Albert Coons using fluorescein to detect bacteria in mice (Coons 1942). Although many improvements have been made to improve imaging the basic technique has not changed. In 1957, background signal from fluorescence was eliminated with the creation of the confocal microscope by Marvin Minsky. This microscope was able to reduce unwanted signal by introducing a pinhole at the light source and the objective to restrict the amount of light illuminating the sample. This became a conventional tool that is used in biological imaging even today as it became evident that this technique was able to create clear images from thick tissue samples (Evanko 2009).

Although confocal microscopy was able to optically section thick samples for three dimensional imaging, it is only able to reveal subcellular structures. Molecular imaging was achieved with the invention of the electron microscope by Ernst Ruska in 1931 (Science 2015). His work on electron optics was used to sidestep the wavelength limitation of light by imaging cells with a beam of
electrons resulting in a significant increase in resolution. Yet despite this major advantage, tissues must be fixed since the conditions that the microscope requires are too harsh for live imaging. This limitation was overcome with the invention of the atomic force microscope (AFM) by Gerd Binnig, Calvin Quate, and Christoph Gerber in 1986. The AFM was able to create three-dimensional images by ‘feeling’ the sample with a mechanical probe (Binnig 1986). However AFM also had its own disadvantages. Firstly, it was only able to scan a maximum area of 150 μm². Secondly, it is unable to cope with highly curved surfaces due to its sensing mechanism (Evanko 2009).

These advancements in microscopy have greatly contributed to our understanding of biological systems at the cellular, subcellular and molecular level. Despite this, the disadvantage of staining protocols still exist in these techniques since it is only able to infer structural features in a biological system. Direct visualization of structures requires the contrast imaging to be endogenously produced, which is possible with nonlinear optical processes in high intensity light (So 2000). The theory of two photon emission was first predicting by Maria Göppert-Mayer in her doctoral dissertation in 1931 (So 2000). Two photo excitation was later proven by Kaiser and Garrett in 1963 when they observed the phenomenon in crystals (Kaiser 1961). This result ushered in the field of multiphoton excitation microscopy, where two or more photons are simultaneously absorbed by a molecule. Additionally, harmonic generation of second and third order were also discovered soon after the invention of a laser. Since then applications of nonlinear microscopy have been explored, especially with second harmonic generation (SHG) microscopy. However, further assessment of this methodology is still required to show its potential in biological and medical research. The purpose of this thesis is to present third harmonic generation (THG) microscopy, which involves three-photon excitation, and its potential to contribute to our understanding of cellular structures via imaging.
1.2 Drosophila as a Model Organism

This thesis aims to utilize THG microscopy as an effective tool to study microlamellar structures in the retinas of *Drosophila melanogaster*. The fruit fly’s short life span makes it practical for studies over many generations. The primary advantage, however, is in its genome. It began with the discovery of the *white eye* mutant by Thomas Hunt Morgan in 1910 (Morgan 1910). Since the discovery, our knowledge in genetics, development and physiology was achieved through the use of the *Drosophila*. Its genome is much smaller than that of mammalian genomes, hence it provides the opportunity to study complex processes in simpler components that usually involve just one gene (Rubin 1988). Approximately 77% of human genes that are related to diseases have a *Drosophila* ortholog (Reiter 2001). Additionally, *Drosophila* brains are architecturally separated into specialized functions such as vision, olfaction and memory, a characteristic that is not present in higher organisms (Rein 2002). This allows for the investigation of changes in one part of the brain without affecting any other function. The fruit fly eye is frequently used in research for developmental biology since its phenotypes are easy to detect and mutations in the eye usually do not affect basic biological functions.

1.3 Drosophila Retina

The adult compound eye of *Drosophila* consists of 750-800 light-collecting units called ommatidium. The phototransduction cascade is carried out by eight photoreceptor neurons located at the center of each ommatidium. Each photoreceptor has a structure called rhabdomeres, which is a series of membrane villi that extends to the full depth of the ommatidium (Ready 1989). This provides a massive membrane surface that can be embedded with high concentrations of the visual pigment rhodopsin (Wang and Montell 2007).
In addition, the ommatidium also has pigment cells that act as a photo-insulator (Figure 1). These help to both increase the ommatidium’s sensitivity to photon absorption, and to protect the rhabdomeres from any side-ways illumination that may occur. The primary pigment cells are functionally equivalent to the mammalian retinal pigment epithelium since they produce chromophore from vitamin A. The secondary and tertiary pigments are shared between either two or three ommatidium respectively and these cells contain colour pigments. Four cone cells rest on top of this arrangement that secrete the lens (Cagan 1989).

![Figure 1: A simplified schematic of the whole adult ommatidium and its cross-section at the lattice layer. Cells and structures visible that are the lens, bristle structures, primary secondary and tertiary pigment cells (PC), photoreceptors, rhabdomeres and axon terminals. Not drawn to scale and adapted from (Cagan 1989).](image)

1.4 Retinal Development during the Pupal Stage

Stages of pupal development were defined by (Cagan 1989) as shown in Figure 2. Development of the retina in the pupal stage can be divided into two phases. The early stage, which occurs in the first
60 hours of pupal development, completes the hexagonal lattice cell pattern. Formation of specialized structures in the retinal cells is done in the late phase, which occurs from 60-160 hours of pupal development.

**Figure 2:** Timeline of events during pupal development (Cagan 1989).
1.4.1 The Early Phase

At the larval stage, a morphogenetic furrow sweeps across the imaginal eye disk from posterior to anterior, temporarily stopping cell division and starting the retinal cell differentiation process. At the pupation event, there are approximately 24 ommatidial columns that have emerged from the furrow and an additional eight columns are produced as the furrow reaches the anterior end of the retina. Cell proliferation during the larval stage also causes the imaginal disc to enlarge to 30 µm thick. However, 10-18 hours after pupation, the retina is everted and stretched, thinning the disc to 12 µm.

During the early phase, the cone cells spread over the top of the photoreceptors, where the anterior and posterior cone cells make contact half-way above the photoreceptors. This contact then extends downward, separating the photoreceptors into two groups, where R1 and R6-8 are near the equatorial side and R2-5 are near the polar side.

Cells that are not specified as either a photoreceptor or a cone cell will assume one of the pigment cell fates. The primary pigment cells, which are added first, are evident at 22 hours and begin to spread out across the anterior and posterior cone cells. The spread starts at the apical surface and then moves basally while following the cone cells. The two primaries will meet midway across the polar and equatorial cone cells, eventually wrapping around them. These cells are usually in place by 48 hours. During their development, the remaining undifferentiated cells that are in contact with the cone cells are pushed to the interommatidial space. The first cells to emerge out of this pool are the oblique secondary pigment cells, which are in contact with only two primary pigment cells. Subsequently, other cells of the interommatidial pool will make either three or four contacts with the primary cells and become the tertiary and horizontal secondary pigment cells respectively, which then arrange themselves into a hexagonal lattice that is visible at 60hrs.
The last to be added to the ommatidium are the mechanosensory bristle groups. Each group is comprised of four cells. These cells first emerge at the center of the ommatidium, and then migrate radially toward the periphery by approximately 24 hours. The neuron loses apical contact and is pushed basally so that it settles 1 µm below the surface. The other three cells enwrap themselves around the neuron one by one before their contacts are extended basally by 40 hours.

At the start of the early phase, the retina will have more cells than it needs, and so the remaining unspecialized cells are eliminated via cell death, which occurs in two stages. The early stage, which last during the ages of 20 to 36 hours, has a high level of cell death and organizes the surviving cells into a hexagonal lattice. The second stage lasts from 36 to 100 hours and has a lower level of cell death, with a purpose to refine the lattice to its adult form. Both stages seem to eliminate cells that fail to establish contact required for a particular cell type. These cells would be released from the surface and degrade into refractive spheres at the base of the retina. They would then be phagocytosed by neighboring cells.

1.4.2 The Late Phase

At this stage, establishment of the hexagonal lattice is complete and the ommatidial cells start to develop specialized structures that is required for their function and the retina thickens from 30µm to 120µm. The cone cells are drawn out into thin fibers. At approximately 70 hours, they start to accumulate pigment granules that contain ommachromes. Secretion of the bristle structure begins at approximately 50 hours.

The late phase is when the photoreceptors start to form rhabdomeres. Significant formation of the rhabdomeres can be detected at around 60 hours. Microvilli first protrude toward the center and then gradually elongate. At 110 hours, the rhabdomeres separate from one another, producing the
interrabdomal space. The retina deepens as the rhabdomeres grow and continue down the length of the retina.

Due to their contact with the cone cells, primary pigment cells lose contact with the floor of the retina as the retina deepens and is only found near the surface. Large brown pigment granules similar to those in cone cells are detected. At the same time secondary and tertiary pigment cells are partitioned to optically insulate the ommatidium. Their feet flatten into plates under the photoreceptors to define the base of the retina, eventually become the fenestrated membrane that supports the photoreceptors. Pigment granules that contain both ommachromes and pteridines start to accumulate in the secondary and tertiary pigment cells.

1.5 Rhodopsin Structure and Function

Rhodopsin is comprised of two subunits. A seven transmembrane protein called opsin is attached to a chromophore called retinaldehyde (retinal) via a Schiff-base linkage on the lysine residue in the seventh transmembrane domain as shown in Figure 3 (Wang and Montell 2007).

The major opsin subunit is encoded by the ninaE gene (O'Tousa 1985), whereas the retinal chromophore is synthesized from vitamin A (this process is described in Section 1.8). The chromophore in Drosophila rhodopsin is highly related to that of vertebrates. The minor difference is that the vertebrate chromophore is 11-cis retinal whereas drosophila chromophore is 11-cis-3-hydroxyretinal (Vogt 1984).
There are six rhodopsins that are expressed in the Drosophila eye, each of which has a characteristic expression pattern and spectral sensitivity (Table 1). Rhodopsin 1 (Rh1) is the predominant isomer, found in photoreceptors R1-R6, which are functionally equivalent to the mammalian rods (Zuker 1985). Rh1 maximally absorbs in the blue range (W. S. Stark 1979b). With the exception of Rh2, the other rhodopsins are found in either photoreceptors R7 or R8 which have similar functions as mammalian cones and maximally absorb in the UV and blue range respectively (Stark 1979a).
**Table 1:** Absorption Spectrum for each rhodopsin found in *Drosophila*

<table>
<thead>
<tr>
<th>Rhodopsin</th>
<th>Photoreceptor</th>
<th>Rhodopsin Absorption Maxima (nm)</th>
<th>Metarhodopsin Absorption Maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh1</td>
<td>R1-R6</td>
<td>486</td>
<td>566</td>
</tr>
<tr>
<td>Rh2</td>
<td>Ocelli</td>
<td>418</td>
<td>506</td>
</tr>
<tr>
<td>Rh3</td>
<td>30% of R7</td>
<td>331</td>
<td>468</td>
</tr>
<tr>
<td>Rh4</td>
<td>70% of R7</td>
<td>355</td>
<td>470</td>
</tr>
<tr>
<td>Rh5</td>
<td>30% of R8</td>
<td>442</td>
<td>494</td>
</tr>
<tr>
<td>Rh6</td>
<td>70% of R8</td>
<td>515</td>
<td>468</td>
</tr>
</tbody>
</table>

Figure 4 shows the emission spectrum for rhodopsin that is excited by UV light in flies reared in high (triangles and dash line) and low (circles with solid line) vitamin A medium as shown in (Miller 1984). It was found that UV light maximally excites blue emission in both groups; however an additional red emission is observed in high vitamin A reared flies. This indicates that vitamin A deprivation results in the elimination of the red emission, which is most likely a mechanism that reduces UV sensitivity in the retina to prevent light-dependent atrophy within the rhabdomeres. Both (Miller 1984) and (W. S. Stark 1979b) observed that changes in emission spectrum between high and low vitamin A reared flies were considerable after 560nm.
Figure 4: Emission Spectrum for rhodopsin by UV light. The upper curve (triangles and dash line) show the emission spectrum for high vitamin A reared flies whereas the lower curve (circles and solid line) show the emission spectrum for low vitamin A reared flies. This figure was taken from (Miller 1984).

1.6 Rhodopsin Cycle during Phototransduction

In both vertebrates and invertebrates, activation of rhodopsin to metarhodopsin is caused by the change in isomerization of 11-cis retinal to all-trans retinal when a photon is absorbed. This, in turn causes the activation of heterotrimeric G-proteins (Emeis 1982). In vertebrate rods and cones, photon absorption causes the all-trans retinal to dissociate from the opsin, initiating the protein to change shape. The rhodopsin regenerates by re-attaching the chromophore to the opsin. Drosophila all-trans retinal does not dissociate from the opsin protein and thus produces a much more stable metarhodopsin.
Photo-transduction amplifies the single photon responses so that the photoreceptor neurons can adapt to a large range of light intensities (Montell 2012). To initiate the phototransduction cascade, photon absorption causes cis- to trans- isomerization of the chromophore and this results in the phosphorylation of metarhodopsin (orange in Figure 5). This phosphorylation is catalyzed by G-protein-coupled kinase 1 (GPRK1). Inactivation of rhodopsin activity occurs when arrestin binds to the GPRK. Regeneration of rhodopsin (blue in Figure 5) requires a second photon of white or orange light to be absorbed (Wang and Montell 2007).

1.7 Rh Synthesis and Maturation

During the pupal stage, the opsin subunit is synthesized and folded in the endoplasmic reticulum (ER) of the photoreceptors (Wang and Montell 2007). It was found by (Kumar 1995) that opsin
begins to be synthesized at 78% pd. Rh1, which is coded by the *ninaE* gene, is folded into its 3D conformation. This requires two chaperon proteins; NinaA and Calnexin, both of which are predominantly localized in the ER. NinaA forms a complex with Rh1 to assist with the folding and transport to the Golgi apparatus (Baker 1994), whereas Calnexin only promotes protein folding (Rosenbaum 2006). Mutations in the either the *ninaA* or *calnexin* genes cause an accumulation of immature Rh1 in the ER and reduced mature Rh1 in the rhabdomeres. However, unlike Calnexin, NinaA mutants do not induce photoreceptor degeneration. This may be due to another response where immature Rh1 is degraded to prevent this.

Before Rh1 is transported to the Golgi, it is glycosylated at Asn20 in the ER (Katanosaka 1998). Glycosylation is required for maturation since it promotes both proper folding and stability. It is also needed for Rh1 to bind to Calnexin (Webel 2000). The sugars are removed once the Rh1 is transported to the Golgi, where it is further processed and matured. The mature Rh1 is then transported to the rhabdomeres where they are linked to the chromophore.

If the chromophore is absent in the rhabdomeres, there is a reduction of Rh1 levels which results in smaller rhabdomeres. However the photoreceptors themselves do not degenerate, indicating that the opsin protein is degraded.

### 1.7.1 Mutations in the *ninaE* gene

Mutation in Rh1 *ninaE* gene leads to changes in the tertiary folding of the opsin protein as well as protein instability. In addition some mutations directly affect the binding site of the retinal chromophore. Rhodopsin expression usually begins at 73% pd and difference between normal and mutant rhabdomere are visible at 90% pd with confocal microscopy (Kumar 1995).
The *nina*E mutants used in this thesis change the conformation of the opsin protein, rendering it unable to bind to retinal (Washburn 1989). Hypomorphic alleles such as *nina*E⁷ and *nina*E⁸ allow for normal microvilli to form in the rhabdomere; however the rhabdomere size is known to decrease (Kumar 1995). This is owing to these alleles still being able to produce a genetic product that is functionally equivalent to the wild-type rhodopsin. However hypomorphic allele produces a small amount of rhodopsin, which ultimately results in light-dependent photoreceptor degeneration after eclosion. Table 2 shows the details of each mutant used in this thesis, as described by (Washburn 1989). Both *nina*E⁷ and *nina*E⁸ exhibit wild-type levels of the *nina*E transcript. The reduced rhodopsin levels are a result of missense mutations that cause changes in the amino acid sequence.

### Table 2: Description of the *nina*E mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Amino Acid Change</th>
<th>Type</th>
<th>% of wild-type levels (Washburn 1989)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>nina</em>E¹</td>
<td>Gln(251)→nonsense</td>
<td>Loss of function</td>
<td>0.0002</td>
</tr>
<tr>
<td><em>nina</em>E⁷</td>
<td>Gly(128)→Arg</td>
<td>Hypomorphic allele</td>
<td>0.08</td>
</tr>
<tr>
<td><em>nina</em>E⁸</td>
<td>Thr(283)→Met</td>
<td>Hypomorphic allele</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>Trp(289)→Arg</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cys(297)→Ser</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The *nina*E¹ gene is a loss of function mutant that contains a nonsense mutation at Glutamine (251). Since this mutation readily decreases the accumulation of its RNA transcript, this strain was found to have only 0.0002% of wild-type rhodopsin levels. In addition, the mutation at this position leads to the loss of the protein’s sixth and seventh transmembrane domain, as well as the binding site of the retinal chromophore (Washburn 1989).

The *nina*E⁷ mutant is a hypomorphic allele, where the protein product has an arginine residue that replaces glycine at the third transmembrane domain (Kumar 1995). Arginine is a charged amino acid causes the domain to be less hydrophobic. In addition, the loss of the glycine residue alters the
structure of the domain, since these regions typically contain more glycine and proline to cause kinks within the helix and create room for the chromophore (Applebury 1986). It was found that this mutant produces 0.08% of wild-type rhodopsin levels (Washburn 1989).

The *ninaE* mutant is also hypomorphic, but this transcript contains three missense mutations in the sixth transmembrane domain (Kumar 1995). Although the effect of each mutant on the overall structure of the domain is not easily distinguished, it has been suggested that the replacement of tryptophan with arginine is the most deleterious mutation. Tryptophan at position 289 is mostly conserved in opsin proteins, as well as some β-adrenergic receptors, and it is thought to play a role in higher level protein folding (Hargrave 1984). The domain becomes less hydrophobic with the charged arginine residue and hence the protein loses its ability to have a sixth transmembrane domain. It is shown by (Washburn 1989), that *ninaE* retains 0.0004% of the wild-type rhodopsin levels.

1.8 Chromophore Synthesis

The retinal chromophore is synthesized from dietary vitamin A (Sarfare 2005). Figure 6 shows the model pathway for the synthesis of vitamin A and retinal using dietary β-carotene as found by (Wang 2007). Vitamin A is only used in *Drosophila* for pigment production and is not needed for its survival. The conversion of carotenoids to the rhodopsin chromophore starts with the transport of dietary β-carotene from the gut to the neurons and glia by the chaperone proteins *ninaD* and *santa maria* (T. J. Wang 2007). Here the β-carotene is metabolized into all-trans-retinol, which is then transported to the primary pigment cells of the retina, where it is converted to the final chromophore.
Figure 6: Synthesis pathway for retinaldehyde proposed by (Wang 2007).

Obstruction of the synthesis pathway in either the opsin protein or the retinal chromophore during pupal development causes membrane atrophy, resulting in photoreceptor degeneration. Here the changes in pigment concentration and localization will be investigated by using unique imaging modality called THG microscopy.
1.9 Nonlinear Optical Microscopy

Fluorescence is the phenomenon in which a photon excites a molecule to a higher energy state and, as it relaxes back to the lower energy state, it emits a photon of light (usually in the visible range). The outcome of this is the emission of a photon with a longer wavelength than the photon used to excite the molecule. For example, the commonly used organic fluorophore fluorescein isothiocyanate (FITC) maximally absorbs 495nm light and emits photons with a wavelength of 519nm (Schauenstein 1978). This difference between the absorption and emission spectra is known as Stokes shift, and this is the principle used in fluorescence-based microscopy (Ishikawa-Ankerhold, 2012).

Although fluorescence has been shown to be a fundamental imaging technique for the last 70 years, there are many downfalls to using this system. The Stokes shift shows that some of the photon energy is absorbed into the molecule. Hence, prolonged exposure to light can cause photo-bleaching. This also denotes that there are limitations in tissue depth and image resolution.

![Figure 7: Fluorescence versus nonlinear optical microscopy. In fluorescence microscopy, some of the photon energy is absorbed into the tissue. In SHG and THG microscopy where two or three photons are interacting with the material, photon energy is conserved.](image-url)
As stated Section 1.1, Göppert-Mayer’s prediction on two-photon excitation began the pursuit of new imaging modalities that don’t require any staining. With the invention of the laser, which has a stronger electric field and a high photon flux, nonlinear optical polarizations were observed from the material and lead to the discovery of various nonlinear optical processes (Carriles 2009). Since these processes require more than one photon simultaneously interacting with the medium, the emission photon was found to have higher energy than the incident photon (Gibson 2011). Nonlinear interactions can be defined by polarization, \( P \), as a function of an intense electric field, \( E \) (Boyd 2008).

\[
\vec{P} = \varepsilon_o \left( \chi^{(1)} \vec{E} + \chi^{(2)} \vec{E}^2 + \chi^{(3)} \vec{E}^3 + \cdots \right)
\] (1.4.1)

Where the \( \chi^{(1)} \), \( \chi^{(2)} \), \( \chi^{(3)} \) terms represent the linear, second and third-order susceptibility tensors respectively.

The most widely used nonlinear contrast mechanism for imaging are second and third harmonic generation (SHG and THG respectively) (Carriles 2009). In addition to the no staining advantage, SHG and THG are also parametric processes. Photon energy is conserved and thus there is not thermal heating in the sample when subjected to high intensity light (Greenhalgh C. P., 2007). SHG microscopy has extensively used in studying collagen arrangement in cancer tissue (Golaraei 2014), and in skeletal muscle dynamics (Nucciotti 2010). THG microscopy has been used to study carotenoids (Tokarz 2014), lipids assemblies (Zimmerley, et al. 2013) and aspects of the human cornea (Gibson 2011). In this thesis, \textit{Drosophila} retinas were imaged and analyzed using THG microscopy.
1.9.1 Third Harmonic Generation

Third order polarization equation can be described by equation (1.4.2) (Boyd 2008).

\[ \vec{P}^{(3)} = \varepsilon_o \chi^{(3)} \vec{E}^3 \]  
(1.4.2)

\[ \vec{P}^{(3)} = \varepsilon_o \chi^{(3)} E^3 \cos^3 \omega t \]
\[ = \frac{1}{4} \varepsilon_o \chi^{(3)} E^3 \cos 3 \omega t + \frac{3}{4} \varepsilon_o \chi^{(3)} E^3 \cos \omega t \]  
(1.4.3)

The first term containing 3\(\omega\) shows the emission of a photon with third harmonic frequency, which occurs as three incident photons of frequency \(\omega\) are nonlinearly scattered. This process is called third harmonic generation and is schematically represented in Figure 8. This process involves virtual energy levels and so absorption at the fundamental laser frequency or the third harmonic frequency does not occur (Boyd 2008).

The second term describes the nonlinear contribution of the refractive index by the incident wave (Boyd 2008). This intensity-dependent refractive index is not used in this thesis. THG is a parametric process, meaning that the initial and final quantum-mechanical states are the same and thus energy is not absorbed by the system.
It was shown by (Ward 1969) that THG disappears in bulk media when using a tightly focused laser beam. This is due to the Gouy phase, where a phase shift of $\pi$ occurs at the focus. This causes destructive interference between the THG signals emitted on either sides of the focal point. Instead, THG can be observed at an interface between to mediums of different refractive indices or third-order susceptibilities since the focal symmetry is broken (Tsang 1995). However, even though THG is observed at interfaces, it is generated from the bulk media on both sides of the interface (Saeta and and Miller 2001). Unlike THG, SHG and MPF do not rely on an interface to generate signal.

1.10 Thesis Objectives

Previous research has utilized confocal fluorescence microscopy to track changes in rhodopsin localization and transport by tagging the opsin protein subunit with a fluorescent dye. It was shown that changes in fluorescence localization were evident at the latter stages of pupal development (Kumar 1995). However, these techniques do not provide information on the transport and binding of the retinal chromophore with respect to retinal development.
Nonlinear microscopy is being increasingly used for biological imaging. THG microscopy particularly, is shown to elucidate multiple structures in a biological system. Since THG is detected both at an interface (membranes) and in the presence of long conjugated carbon chains, Drosophila retinal tissue is likely a good candidate for THG imaging. Hence, the primary goal of this Master’s thesis is to present nonlinear microscopy, especially THG microscopy as a new method to study the changes in chromophore localization and transport in the retina during pupal development. This thesis aims to show that THG is able to show changes in concentrations of rhodopsin as well as elucidate the location and time point that the chromophore binds to the opsin moiety.

I hypothesized that the THG signal is generated by the visual pigment rhodopsin, due to the retinal chromophore. To test this hypothesis, pupal retinas were imaged using THG microscopy at various stages of development to identify the earliest point in time that the visual pigment can be detected. The pupal retinas were also observed for any second harmonic response or fluorescence. The THG signal intensity localized at both the rhabdomeres and the pigment cells were measured to attain an age-dependent curve. Then, changes in THG signal were observed in the absence of visual pigment. This was done for both the absence of the retinal chromophore (vitamin A deficiency) and for the absence of the opsin protein subunit (mutations in the ninaE gene).
Chapter 2: Materials and Methods

2.1 Drosophila Rearing and Strains

All flies were grown in 20°C on a standard cornmeal diet by following the recipe provided by the Bloomington Stock Center (Bloomington Drosophila Stock Center 2014). For the control, the w^{1118} strain was used, so that colour pigments usually found in the secondary and tertiary pigments would not interfere with the signal generation. In addition, the following stocks were provided by the Bloomington stock center: ninaE^l, ninaE^7, ninaE^a, which are mutations in the Rh1 ninaE gene. Pupal development at this temperature lasts approximately 150 hours. The stage at which the pupas were dissected is indicated by % pupal development (% pd), which is simply the hours the fly has spent in the pupal stage divided by 150 hours. Table 3 show the developmental percentages for the ages used in this theses.

Table 3: The ages used for pupal development and their corresponding percentage

<table>
<thead>
<tr>
<th>Hours Spent in Pupal Stage</th>
<th>Percentage of pupal development (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>32</td>
</tr>
<tr>
<td>72</td>
<td>48</td>
</tr>
<tr>
<td>96</td>
<td>64</td>
</tr>
<tr>
<td>120</td>
<td>80</td>
</tr>
</tbody>
</table>

2.1.1 Vitamin A Deficient Media

In order to investigate the changes in THG signal in the absence of the retinal chromophore, w^{1118} flies were also raised on Vitamin A deficient media. Table 4 shows the components used to make the medium, which was adapted from (Elgin 1978). The grape juice replaces cornmeal in order to eliminate β-carotene, which can be used to produce vitamin A.
Table 4: Ingredients used in the vitamin A deficient medium.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grape juice</td>
<td>230 mL</td>
</tr>
<tr>
<td>Water</td>
<td>270 mL</td>
</tr>
<tr>
<td>Sucrose</td>
<td>40 g</td>
</tr>
<tr>
<td>Bacto-agar</td>
<td>11 g</td>
</tr>
<tr>
<td>Yeast</td>
<td>10 g</td>
</tr>
<tr>
<td>NaOH</td>
<td>10 mL</td>
</tr>
<tr>
<td>Propanoic Acid</td>
<td>2 mL</td>
</tr>
</tbody>
</table>

Flies were transferred into these vials for a day to lay eggs. Lack of vitamin A did not affect viability, so the newly hatched larvae were allowed to grow in this medium until pupation. For the entire duration, the vials were maintained in a humidified environment, since this medium dries out quicker than our standard medium.

2.2 Dissection and Sample Preparation

The pupal stages are defined by (Cagan 1989). White pre-pupa on the sides of the vial were marked so that their development could be accurately recorded. When pupae had reached the appropriate age they were collected with small forceps and pinned down onto a Sylgard-filled Petri dish. A pupa was submerged in a 1X Phosphate Buffered Saline (PBS) before the operculum at anterior end was removed and the sac was pierced. The brain with the attached retinas were extracted using a 20μL pipette and fixed in 24% formaldehyde for 20 minutes. After washing the specimens with 1X PBS solution, the retinas were cleaved off the optic lobes of the brain using dissections needles. The retinas were place on a 22×60 mm cover-slide in 1X PBS solution, and a 22×22 cover-slide is placed on top (both cover-slides are approximately 0.15 mm thick). Parafilm spacers were used in between the cover-slides to ensure the retina was not flattened and for optimal 3D-imaging.
2.3 Nonlinear Contrast Microscope

All imaging was done using a custom-built nonlinear optical contrast microscope (Sandkuijl 2010). A simplified schematic of the laser beam path and microscope setup is shown in Figure 9. The homebuilt laser used for the microscope is a ytterbium-doped potassium gadolinium tungstate (Yb:KGd(WO$_4$)$_2$) laser to provide 430 fs pulses at 1028 nm with a frequency of 14.3 MHz (Major 2006). The laser was mode-locked using a semiconductor saturable absorber mirror. Since high power levels are required to detect the harmonic responses, continuous excitation would severely damage the tissue. Hence, relaxation time in between high powered pulses ensures that the tissue is not overly excited (Tuer 2013).

![Figure 9: An illustration of the microscope.](image)
The single-photon counting multi-contrast nonlinear microscope (SPC-MNM, or ZAP) microscope was built by the members of the Barzda Group (Sandkuijl 2010). As shown, the beam passes through a pinhole (PH), which is placed at the focus of Telescope 1 to achieve a Gaussian profile. Telescope 1 magnifies the beam so that it fits the size of the aperture in the scanning mirrors which achieve a raster scan of 10 frames per second for a 128 × 128 pixel area. The beam then passes through Telescope 2 so that it can match the size of the aperture of the excitation objective (EO), and is focused onto the sample. The third harmonic generation signal is then collected in the forward direction by the collection objective (CO). This is detected by the photon multiplier tube (PMT) in a single-photon counting methods since the harmonic generation produced by the biological sample are at low intensity levels. The signal is filtered for 310 ± 40 nm with a band pass interference filter (THG filter).

2.4 Imaging and Intensity measurements:

For imaging, the sample was placed on a translation stage which moves in the x, y, and z direction and was controlled using custom software in LabView. The THG response was observed at full laser power since the retinas produce very low signal intensities. Once a region of interest (ROI) was selected, THG intensity images were taken as the focus of the laser was moved deeper into the tissue in 1 µm increments. The area and number of frames were set to 40×40 µm and 100 frames respectively for all imaging to maintain consistency.

The THG intensities were measured using ImageJ and Origin. A region of interest (ROI) was selected around either the pigment cells or the rhabdomeres and a histogram of the photon counts for each pixel in the ROI was created. The histogram was then fitted with a Gaussian to find the average counts for that region. For each condition nine intensity measurements were performed.
Chapter 3: THG Intensity Images and Analysis of Pupal Retina

This chapter will discuss the main results found for THG microscopy in drosophila retina. THG intensity images are compared both quantitatively and qualitatively.

3.1 Nonlinear optical properties of developing *Drosophila* retinas

Pupal retinas from various developmental stages (as indicated in Table 3 of Chapter 2) were imaged with THG microscopy. Z-stacks of images were collected by moving focal plane of the laser through the tissue in 1 or 2 µm increments, for a total depth of approximately 30µm. Comparison of the THG images of Oregon-R and w^118^ pupa revealed that w^118^ was more suitable for imaging since Oregon-R retinas did not produce high contrast images. In addition to THG, second harmonic generation (SHG) and multi-photon fluorescence (MPF) were also used to image the retinas, at the wavelengths 510 and 530nm respectively.

First, retinas from 32% pd w^118^ pupae were imaged using THG microscopy. Figure 10 shows representative images of the pupal retina at 1 µm focal depth increments. The high contrast THG images produced by cellular membranes highlights the hexagonal arrangement of each ommatidium, marking this age as the earliest at which this characteristic lattice can be detected. However, the pigment cells and the bristle groups were not clearly defined in these images. Also, as the focus of the laser was moved below the lattice layer, the THG signal became more disorganized, which rendered it difficult to detect the photoreceptors. This corresponds with previous findings that these cells were still not fully developed at this age (Cagan 1989).
Figure 10: THG intensity images of 32% pd retina as the laser focus was moved down in 1μm increments. Scale bar represents 10μm.

The 32% pd retinas were also imaged using SHG and MPF microscopy at the corresponding focal depth of the lattice arrangement. Figure 11 shows SHG, THG and MPF images of the retina with normalized intensity. The MPF signal predominantly highlighted the same structures as THG at a significantly lower intensity. The SHG signal was not detected in any part of the lattice that was similar to THG and MPF. It elucidated circular structures with high intensity. The origin of the SHG signal is not known.
Figure 11: Images of THG, SHG and MPF images of a 32% pd w^{118} pupa at the same focal depth. Scale bar represents 10 µm.

Next, retinas from 48% pd pupa were then imaged with THG at 1 µm increments as shown in Figure 12. The THG signal emphasized a much more defined hexagonal lattice, at this stage in comparison to the 32% pd retina; the THG signal clearly outlined the pigment cells. THG signal organization changed with varying focal depth, which revealed more than one cell/structure due to the ommatidium’s multi-membrane arrangement. Superficial to the hexagonal lattice, THG is generated by the bristles as well as the cornea (shown in the first 3 images of Figure 12) as they create an interface at the focal plane. The THG contrast images also reveal a fully developed lattice, where the THG is generated by both the pigment cells as well as the base of the bristle structures. Similar to the 32% pd retinas, the signal organization became more disordered below the lattice. Hence, the photoreceptors and their developing rhabdomeres structures were not detected at this stage.
Figure 12: THG intensity image of drosophila eye at 48% pd. The images were taken in 1μm increments and scale bar represents 10μm.

Additionally, the 48% pd retinas were imaged with all three filters at various focal depths. Figure 13 shows SHG, THG and MPF images at focal depths of 0, 3, 6 and 9 μm from the top of the retina. All three signals are generated from the bristle structures. The MPF signal was generated at a higher intensity, whereas SHG and THG signal were generated at much lower intensities. SHG and MPF also elucidated some portion of the lattice arrangement at varying intensities. However SHG and MPF images displayed a much lower resolution when compared to THG for both bristle structures and the lattice. Also, SHG and MPF did not clearly show the pigment cells like THG.
Figure 13: Comparison of different harmonic images of 48% pd w\textsuperscript{118} pupal retina at various depths. a) THG, b) SHG, c) MPF. Scale bar represents 10 µm.

Retinas from pupae were imaged for a total of 25 µm in depth. Figure 14 shows THG images at 2 µm increments for 16 µm, focusing on images from the mid-range of the Z-stack stack. At 64% pd, secondary pigment cells are observed for a larger depth. The THG signal emitted from these cells showed an increase in cell thickness 3-4 µm below the lattice layer, which corresponded to the upper portion of the secondary pigment cell structure shown in the illustration in Figure 1.

Approximately 8 µm below the lattice layer, THG signal was more organized than seen in the earlier developmental stages: the photoreceptors (i) and their rhabdomeres (ii) were both evident, marking this stage as the earliest stage that the rhabdomeres can be detected. THG signal generated by the
photoreceptor cellular membrane had a considerably lower intensity compared to THG emitted from the rhabdomeres. Signal localization at the rhabdomeres revealed a distinct crescent shape with a dark region in its center. This shape was repeated in an array that was positioned at the center of each ommatidium.

**Figure 14:** THG intensity images for 64% pd retina. The images were taken in 2μm increments and scale bar represents 5μm. At this stage, photoreceptors (i) and rhabdomeres (ii) are visible.

The 64% pd retinas were then imaged with all three filters at two focal planes. As shown in Figure 15, all three filters were applied to the lattice first. Similar to the 48% pd retinas, SHG and MPF signals were partially detected at the hexagonal outlines of the ommatidia at significantly lower intensities. The focal plane was then moved 10 μm below the lattice layer to image the rhabdomeres. SHG and MPF signal localization corresponds the THG signal generated from the rhabdomeres.
However, SHG and MPF were not observed in the photoreceptors themselves. As with the pigment layer, SGH and THG signals were significantly lower in intensity. In both the pigment cells and the rhabdomeres, SHG emission had the lowest signal intensity.

![Comparison of SHG, THG and fluorescence images of 64% pd retinas at the pigment cell layer and at the rhabdomeres layer. Scale bar represents 10 µm.](image)

**Figure 15:** Comparison of SHG, THG and fluorescence images of 64% pd retinas at the pigment cell layer and at the rhabdomeres layer. Scale bar represents 10 µm.

Lastly, retinas from 80% pd pupae were imaged. Figure 16 shows the THG images taken at 2 µm increments for a total of 14 µm. The cellular structure of the bristles and pigment cells remain similar to that of the 64% pd pupa. At the rhabdomere plane, however, THG signal localization was notably different. Photoreceptor cellular membranes were not observed. THG signal generated by the rhabdomeres exhibited a different shape that lacked a dark region in its center. The crescent-shaped structures previously observed at 64% pd appear to have come together into closer contact, representative of rhabdomere maturation.
Figure 16: THG intensity images for 80% pd retina. The images were taken in 2μm increments and scale bar represents 5μm.

SHG and MPF images were also taken at the pigment cell layer as well as the rhabdomeres (Figure 17). These retinas did not emit any SHG signal at either focal plane. MPF signal barely highlighted the hexagonal lattice and the rhabdomere and was emitted at lower intensity.
Figure 17: Comparison of SHG, THG and fluorescence images of 80% pd retinas at the pigment cell layer and at the rhabdomeres layer. Scale bar represents 10 µm.

Lastly, as a control measurement to ensure the harmonic response were dependent on laser power, *Drosophila* retinas were imaged using the nonlinear optical microscope to measure the intensity of the signal at different laser powers. As shown in Figure 18, the signal was found to be cubically dependent on the laser power, thus proving that the signal is generated by a third-order nonlinear process.
Figure 18: THG intensity dependence on the laser power. The slopes for the edges and the centers of the ommatidium were found to be $2.9 \pm 0.3$ and $3.0 \pm 0.1$ respectively. This proves that the signal is generated is due to the third-order non-linear process.

In brief, these results show that Drosophila retinas are a good candidate for THG imaging due to its multi-membranous arrangement. Multiple structures were revealed as the THG signal organization changed when the laser focus was moved deeper into the tissue. THG was emitted from the retina at a higher signal intensity compared to SHG and MPF images.

3.1.1 Age dependent THG intensity curve

Following on the morphological characterization of nonlinear signals arising from Drosophila retina, I carried out quantification of the signal intensity. THG signal intensity from the pigment cells and from the rhabdomeres was measured at each developmental stage, by measuring the average THG
intensity from those regions using ImageJ (Figure 19). Such values were normalized to the intensity measured from polystyrene beads that were imaged at the same time as the tissue sample.

**Figure 19:** THG images of $w^{118}$ retina showing the region of interest drawn around pigment cells and rhabdomeres using ImageJ.

Figure 20 shows the normalized intensity found in pigment cells and rhabdomeres plotted against pupal development. The THG signal localized in the pigment cells was found to steadily increase from 32 to 64% pd, after which the intensity decreased.

At the center of the ommatidium, where the rhabdomere develop, the THG signal intensity was also quantified. In the 32 and 48% pd retinas intensity values were measured from images collected 10µm below the lattice layer, at the focal plan where the rhabdomeres were observed in later developmental stages. THG intensities were lower in this region when compared to the pigment cells. The difference in intensity between the pigment cells and rhabdomeres was significantly larger in 48% pd retinas when compared to 32% pd pupas. This difference decreased in 64% pd retinas since there is a sharp increase in the rhabdomere THG intensity. In 80% pd retinas, the THG intensity in the rhabdomeres
was found to be higher than that of the pigment cells. The difference in intensities was also observed to be similar to that found at 48% pd retinas.

![AGE DEPENDENT THG INTENSITY](image)

**Figure 20:** THG signal intensity changes with age.

This result showed that THG microscopy has the ability to observe changes in membrane structure with respect to cellular development in a quantitative manner. This technique can be applied to in different pupal retinas to observe changes in THG intensities.

### 3.2 Effects of Vitamin A deficiency on Pupal Retinas

We suspect that THG signals in retina may arise from two sources, cellular membrane and photopigments. In order to determine if the THG signals are dependent in the retinal-based
photopigments, flies were reared on Vitamin A deficient media. Such flies should lack the chemical precursor to synthesize retinal. Thus, any changes in THG signal from retinas can be assigned to changes in photopigment synthesis. THG images were obtained from the retinas of Vitamin A deficient flies at the four developmental stages as described above.

Figure 21 shows images of 32% pd retinas a 1 µm increments with the cornmeal reared retinas for comparison. There were no notable changes in the developing lattice structure when compared to the control. Photoreceptors were not detected below this focal plane. An additional bright area was observed at the center of each ommatidium.

![THG Images](image)

**Figure 21:** THG images of a) 32% pd vitamin A deficient pupal retinas along with b) cornmeal reared 32% pupa for comparison. The images were taken in 1µm increments and scale bar represents 10µm.

SHG and MPF signal was also collected from these retinas (Figure 22). SHG signals were generated in the regions that corresponded to the bright areas found in the THG images, suggesting that these areas may have a non-centrosymmetric components. Further, SHG and MPF signals did not highlight any part of the lattice, as was seen previously in the raised on normal media.
Figure 22: Images of THG, SHG and MPF images of a 32% pd w^{1118} pupa at the same focal depth. Scale bar represents 10 μm.

Figure 23: THG image of vitamin A deficient pupa at 48% pd. Each image was sub sequentially take with a focal plane difference of 1 μm. Scale bar represents 10 μm.

Figure 23 shows the THG images obtained from 48% Vitamin A deficient retinas. The THG signals generated by the lattice revealed that the pigment cells had fully developed, without morphological changes. Similar results were found in 64% pd retinas (Figure 24). In the rhabdomere region, a THG signal was present, but at much lower intensity in comparison to flies reared on normal diet.
Figure 24: THG intensity images for a) 64% pd vitamin A deficient retina along with b) cornmeal reared pupal retinas. The images were taken in 2μm increments and scale bar represents 5μm.

Imaging 64% pd retinas with all three filters (Figure 25), revealed that little SHG and MPF signal is generated by the pigment cells. The bright regions seen in the SHG and MPF images correspond to the position of the bristles. Similarly, minimal SHG and MPF signal was generated by the rhabdomeres.

Figure 25: Comparison of SHG, THG and fluorescence images of 64% pd retinas at the pigment cell layer and at the rhabdomeres layer. Scale bar represents 10 μm.
Figure 26 shows THG images of 80% pd retinas. At this stage THG generated by the rhabdomeres revealed more disorder in THG signal organization when compared to the control. The distinct bright regions seen in the cornmeal reared pupas were replaced by low intensity circular structures.

\textbf{Figure 26:} THG intensity images for a) 80% pd vitamin A deficient retina along with b) cornmeal reared pupal retinas. The images were taken in 2\textmu m increments and scale bar represents 10\textmu m.

SHG and MPF images were also taken at the pigment cell layer as well as the rhabdomeres (Figure 27). SHG was generated at both focal planes at very low intensities compared to the MPF images. MPF was detected at the rhabdomeres with low intensity and poor resolution.
Figure 27: Comparison of SHG, THG and fluorescence images of 80% pd retinas at the pigment cell layer and at the rhabdomeres layer. Scale bar represents 10 µm.

As with flies reared on normal media, the THG signal intensity was quantified for the pigment cells and the rhabdomeres in pupae raised on Vitamin A deficient media. Figure 28 shows the THG intensities localized in the a) pigment cells and in the b) rhabdomeres against pupal development for both control and vitamin A deficient pupae. Again these data were normalized to signal intensity measured from polystyrene beads. The average THG intensity measured in 32% pd Vitamin A deficient retina was similar to the value found in the control in the pigment cells (Figure 28a). Only a slight increase in the intensity occurred between 32% and 48% pd, much lower compared to the control, as predicted. However an increase the signal intensity occurred in the pigment cells between 48% and 64% pd. This resulted in the THG signal intensities at 64% and 80% pd to be much higher than the control, which was not as predicted.

A different trend was observed with the rhabdomeres (Figure 28b). The average THG intensities measured in all four developmental stages were found to be lower than the intensity obtained for
pupae raised on normal media. A notable increase in the THG intensity was observed between 48% and 64% pd, which is mostly due to the development of rhabdomere membranes. A decrease in the average intensity measured in 80% pd resulted in a large difference in intensity when compared to the control. This can be attributed to the degradation of the rhabdomeres observed in the THG intensity images of 80% pd retinas (Figure 26).

**Figure 28:** Comparison of the intensities measured in the a) pigment cells and b) rhabdomeres between the control and vitamin A deficient pupae. (P < 0.05)
These results confirm that the chromophore subunit of visual pigment augments THG intensities in pupal retinas. It also shows that, even with low intensities, THG microscopy has the ability to demonstrate membrane atrophy in rhabdomeres.

### 3.3 Absence of opsin protein

In order to observe the changes in rhabdomeres structure due to reduced levels of opsin, three mutations for the Rh1 *ninaE* gene were considered. A loss of function mutant strain (*ninaE*\(^1\)) and two hypomorphic mutant strains (*ninaE*\(^7\) and *ninaE*\(^8\)) were used to study their age dependent effects on the retina’s cellular structures as well as the THG intensities. It is predicted in this thesis that reduced levels of opsin would alter both the THG image and THG intensities throughout development. Specifically, it is expected that THG intensities localized at the pigment cells would increase due to the accumulation of retinal and THG intensities at the rhabdomeres to decrease.

Figure 29 shows the THG images of the 32% pd pupal retina at 1 μm focal depth increments for each mutant. It was observed that the ommatidial cells and its hexagonal lattice arrangement are developing at the same rate as the control with no notable structural variations. This trend was also observed in 48% pd retinas.
Figure 29: THG images of each mutant pupal retina at 32% pd. The images for each row were taken in 1μm increments and the scale bar represent 10μm.

However, I began to note structural changes in 64% and 80% pd retinas, as shown in Figure 30. Although the pigment cells are unaffected by the reduced levels of Rh1, the rhabdomeres of 64% pd retinas are not structurally similar to the control. Further, the rhabdomeres are not as visible in 80% pd. These results indicate that rhabdomeres degradation started as early as 64% pd and become less structurally intact with pupal development.

The mutant retinas were also imaged with SHG and MPF filters. Similar to the vitamin A deficient retinas, the ninaE mutants did not generate a lot of SHG with exception of the bristles. Both MPF and SHG were unable to reveal pigment cells or rhabdomeres.
As with the vitamin A experiment, the THG intensity for each \textit{ninaE} mutant was measured and compared to the controls. Figure 31 a) and b) show these intensity values found in pigment cells and rhabdomeres respectively. The THG intensities measured in 32\% pd retinas for each mutant was found to be similar to that of the control in the pigment cells (about 0.25±0.05 for the pigment cells and 0.11±0.03 in the rhabdomeres). A significant increase in the intensity measured in the pigment
cells (with the exception of \textit{ninaE}^8) was observed in 48% pd. This resulted in the \textit{ninaE}^1 and \textit{ninaE}^7 intensities to be higher than the control, where the highest value was measured in \textit{ninaE}^1 pigment cells. A decrease in the intensity was seen in all of the \textit{ninaE} mutants at 64% pd, where their values were found to be lower than the control. A similar trend was seen in 80% pd.

Unlike the pigment cells, the THG intensities measured in the rhabdomeres of each mutant was found to be lower than the control at each developmental stage. The intensities were found to increase from 32% to 64% pd in all of the mutants, which corresponded to the partial development of the rhabdomeres seen in the THG images (Figure 30). However, there is a decrease in the intensities at 80% pd.

In brief, reduced levels of opsin result in changes in THG signal organization and THG intensity for both null and hypomorphic alleles of the \textit{ninaE} gene. However, these changes were found have a different profile compared to vitamin A deficient retina. It was noted that the \textit{ninaE}^8 strain showed a different intensity pattern compared to the other two.
Figure 31: Comparison of the intensities in \textit{ninaE} mutant flies (raised on cornmeal diet) measured in the a) pigment cells and b) rhabdomeres for each genotype. (P < 0.05)
Chapter 4: Discussion and Future Directions

The primary goal of this thesis was to present THG microscopy as a new and effective technique in the study of developmental biology. This was done using the Drosophila eye as a model to investigate if THG is able to detect the changes in membrane structure and rhodopsin localization. This was accomplished in two ways. First, changes in ommatidial morphology were observed in the absence of rhodopsin. Developing retinas from w^{1118} pupae were imaged with SHG, THG and MPF to observe its nonlinear properties. Due to the requirement of an interface to observe THG, the ommatidium’s multi-membrane arrangement was detected as the laser focus as moved deeper into the structure, elucidating more than one cell/structure with high resolution. This advantage was not as predominant in SHG or MPF. As the pupae developed, the establishment of the hexagonal arrangement of cells and the maturation of the rhabdomeres were clearly seen. Changes in rhabdomere structure and development was detected in Vitamin A deficient pupae and in ninaE mutants were detected. This data proved that THG microscopy is a useful imaging modality to visualize structural development in the retina.

As a second method, changes in THG intensity localized in the pigment cells were measured for quantitative analysis. THG intensity localized in the pigment cells and in the rhabdomeres showed a distinct age-dependent curve. In the absence of vitamin A or the opsin subunit, changes were observed in the THG intensities as well as the age dependent curve. These findings highlight another advantage of THG microscopy to quantitatively measure development in different areas of the ommatidium.

Morphology

Ommatidial morphology during pupal development was observed by collecting z-stacks of THG images as the focal plane of the laser was moved through the structure. In earlier stages of
development (32% and 48% pd), only the hexagonal lattice was detected, corresponding to previous findings that the pigment cells fully develop in the early phase (Cagan 1989). The THG signal becomes more disorganized as the focal plane is moved deeper into the structure. Again this confirms that the rhabdomeres do not start to form until the late phase. Although previous literature using electron microscopy shows that photoreceptors also develop in the early phase of development (Cagan 1989), its cellular membrane is not observed in the THG images until 64% pd in w1118 pupae. The photoreceptors were not visible at any stage of pupal development in vitamin A deficient pupae or ninaE mutant pupae. This suggests that the accumulation of rhodopsin in the photoreceptors is required for THG signal generation. At 64% pd, THG signal localization also revealed crescent like structures at the center of the ommatidial structures, suggesting that this was the earliest stages that developing rhabdomeres would be detected with THG. In the Vitamin A experiment, THG images of the rhabdomeres at this stage indicate that they are only partially developed and are not as visible. In ninaE mutant pupae, THG signal is disorganized at the rhabdomeres, suggesting that membrane degradation has already begun. These results indicate that rhabdomeres are visible at 64% pd due to the presence of rhodopsin, suggesting that Rh1 expression begins around 64% pd, which is much earlier compared to previous work using confocal microscopy that stained for Rh1 antibody (Kumar 1995).

Morphological comparison shows that degradation of the rhabdomeres in the ninaE pupae occur at different rates. The THG images indicate that ninaE1 and ninaE8 rhabdomeres degrade faster than ninaE7 rhabdomeres. It was previously found by (Washburn 1989) that the mutation in ninaE1 flies causes the loss of the sixth and seventh transmembrane domain. This also causes the loss of the chromophore binding site and as a result causes the protein levels to decrease and the rhabdomeres to degrade. The ninaE8 flies contain a point mutation that causes a less hydrophobic sixth transmembrane domain. It was suggested by (Washburn 1989) that this causes the rhodopsin to lose this domain. The mutation in ninaE7 flies, on the other hand, only affects the third transmembrane
domain. This along with the data from THG images in this thesis suggest that mutations affecting the sixth and seventh transmembrane domain of rhodopsin cause faster degradation of the rhabdomeres. Similarly, vitamin A deficient pupae showed rhabdomeres degradation at a similar rate to *ninaE* retinas. This suggest that decrease in chromophore concentrations eventually leads to protein instability and is rapidly lost from the photoreceptors, leading to membrane atrophy. These findings over all emphasize the importance of chromophore binding in maintaining the rhabdomeres’ structural integrity.

Although the term ‘degradation’ is liberally used in this thesis, it is still not clear-cut whether what is seen is truly degeneration of the rhabdomere membranes or if it is actually a failure to develop.

SHG and MPF microscopy was also used to image the retinas. In all of the experiments both channels detected bristle structures, pigment cells and rhabdomeres but with much lower intensity and resolution. SHG and MPF images emphasized similar structures in all of the experiments. This is most likely due to both processes having similar wavelengths.

**THG intensity**

The THG intensity localized in the pigment cells and rhabdomeres were measured and normalized for each age to attain an age-dependent intensity curve. The intensities localized in the rhabdomeres in Vitamin A reared flies and *ninaE* flies had similar curves, suggesting that the rhabdomeres developed similarly to the control until 64% pd. In *w*1118 pupae, the intensity localized at the rhabdomeres was generally found to increase throughout pupal development whereas a decrease in intensity was seen in pigment cells after 64% pd. At 80% pd the THG intensity localized in the rhabdomeres is higher than that of the pigment cells. At the earlier stages of pupal development, retinal mostly localized in the pigment cells. The changes in intensity at the pigment cells suggests
that retinal begins to be transported to the photoreceptors after 48% pd. This is further supported by the sudden increase in intensity at the rhabdomeres.

The intensity curves attained for ninaE pupae looked very different compared to the control. Intensity localized in the pigment cells were found to decrease after 48% pd, unlike w1118. By 80% pd, ninaE pigments cells had similar THG intensities, ninaE7 pigment cells had higher intensities and ninaE8 pigment cells had lower intensities compared to the control. THG signal localized in the rhabdomeres shows a similar curvature to w1118 from 32% to 64% pd, after which the intensity decreases. At 64% pd, all three ninaE mutants had lower intensities than the control. These results supports the morphology data and suggests that Rh1 begins at 64% pd. The decrease in chromophore binding due to changes in the opsin globular structure causes a decrease intensity due to rhabdomere degradation.

Similarly, a THG intensity curve was attained for vitamin A deficient pupae. The intensity curve at the rhabdomeres is very similar to the w1118 intensity curve from 32% and 64%, suggesting that rhabdomeres develop normally in the earlier stage even with the absence of retinal. As with the ninaE pupae, there is a decrease in the THG intensity after 64% pd. Moreover, the rhabdomere intensity were found to be lower than w1118 at all for stages of pupal development. This suggests that reduced levels of retinal causes the decrease in THG intensity. The intensity localized at the pigment cells was found to sharply increase between 48% and 64% pd, which is not expected. Further work is required to determine the cause of this increase. Overall the intensity results suggest that change in the THG intensity at both pigment cells and the rhabdomeres is linked to the concentration of rhodopsin.
Future Directions

This thesis proves that THG microscopy is a valuable imaging tool to observe changes in rhodopsin concentration and localization and its effect on rhabdomere morphology. THG microscopy has the potential to be used for early detection for macular degeneration. It can also be developed as a new technique to track movement and localization of carotenoids, a feature that is not offered in the more readily available fluorescence microscopy. However, a question did arise from the results. Firstly, although it was found that THG signal varies with changes in rhodopsin concentration and localization, it was not confirmed that the signal was augmented by the retinal portion of the visual pigment. THG intensities measured in pigment cells of vitamin A deficient pupae showed a sharp increase after 48% pd, contrary to what was expected. Further work is required determine why this intensity increase occurs in the pigment cells. In addition, the morphological and THG intensity differences seen in ninaE mutant retinas are due to the reduced levels of Rh1 protein. This opsin protein was chosen since it is the major pigment found in the retina. However, the remaining THG intensity seen could be due to the other rhodopsins (Rh3-6) present in the ommatidia. Therefore further experiments with multiple mutations affect more than one opsin must be performed to observe the changes in morphology and THG intensity with a complete absence of opsins.

Mutant rhodopsin in the rods of human retinas that has defective protein folding similar to some ninaE mutants causes a loss of rods, the primary characteristic of autosomal dominant retinitis pigmentosa (ADRP) (Mendes 2005). Since structural changes are detect in Drosophila photoreceptors with THG, future research can determine if THG microscopy can be to be used as a new method to study the pathology of retinal degeneration via rhodopsin localization and concentration.
On a broader scale, *Drosophila* retinas can also be used with THG microscopy to study the pathology of various neurodegenerative diseases. Rhabdomere degeneration has been found to be a characteristic of glutamine repeat disorders (Jackson 1998), such as Huntington’s disease. Since its mutations don’t usually affect basic functions, the *Drosophila* eye can be used as a model system with THG microscopy to gain a new understanding of neurodegenerative disorders during development.
References


58
Tuer, A. 2013. "Nonlinear Microscope of Histology." PhD, University of Toronto.


