

Effective Gold Nanoparticle Delivery to the Retina for Plasmonic Retinal Prosthetics

By

Hafithe Mohammed ALGhosain

B.S.E., University of Hartford, 2017

Thesis

Submitted in partial fulfillment of the requirements for the Degree
of Master of Science in the Department of Biomedical Engineering at Brown University

PROVIDENCE, RHODE ISLAND

MAY 2019

This thesis by Hafithe Mohammed ALGhosain is accepted in its present form
by the Department of Biomedical Engineering as satisfying the
thesis requirements for the degree of Master of Science

Date: _____

Jonghwan Lee, PhD, Advisor

Date: _____

Michelle Dawson, PhD, Reader

Date: _____

Marissa Gray, PhD, Reader

Approved by the Graduate Council

Date: _____

Andrew G. Campbell, Dean of the Graduate School

Acknowledgements

First and foremost I would like to thank Dr. Jonghwan Lee for his continued guidance and support from the moment I joined the Lee lab. His input was vital to the completion of this undertaking. Furthermore, Dr. Kyungsik Eom was one of the main resources available to me in lab. He has taught me much of what I know today with regards to procedures, and troubleshooting. I would also like to thank the Berson lab for allowing us to observe their retinal injection procedures, much of our SOPs are based on what we learned from Megan Leyrer.

Megan also taught Dr. Eom and I how to fabricate our micropipette tips for retinal injections. Finally, I would like to thank Gaïa-Marie Gerbaka for conducting retinal dissections, and for teaching me how to conduct dissections myself. If it was not for both her and Dr. Eom I would not be able to conduct my own experiments that are heavily dependent on retinal dissections. I would also like to thank Collin Polucha for conducting retinal dissections whenever his schedule permitted.

My parents have supported me in every way possible, and have always encouraged me to pursue my education. I owe the man I am today to their continued support and guidance, I consider myself very lucky to be the son of Amira and Mohammed. Dr. Mary Arico and Dr. Andrea Kwaczala have encouraged me to pursue my master's degree and were a continued source of inspiration throughout the past two years, I owe them a lot. I would like to thank the Saudi Arabian Cultural Mission for granting me a full scholarship and supporting my academic endeavors. Finally, I would like to thank both Dr. Gray and Dr. Dawson for serving on my defense committee.

Table of Contents

Acknowledgements.....	iii
List of Figures.....	vi
Chapter 1: Visual Impairment.....	1
Section 1.1:Introduction.....	1
Section 1.2: Visual Cascade.....	2
Section 1.3: Mechanisms of Disease Progression	5
1.3.1: Mechanisms of cone death	6
1.3.2: Mechanisms of cell death.....	7
Section 1.4: Genes Implicated in the Disease	10
Chapter 2: Current Treatments and Approaches	11
Section 2.1: Diagnosis.....	11
Section 2.2: Current treatments	12
Chapter 3: Plasmonic Retinal Prosthesis	15
Section 3.1: Neural Stimulation	15
Section 3.2: Infrared Neural Stimulation.....	15
Section 3.3: Biophysical Mechanisms	16
Section 3.4: Gold Nanorods	18
Section 3.5 Infrared Neural Stimulation and AuNRs.....	19
Section 3.6: Gold Nanorod Assisted Near Infrared Neural Stimulation	21
Section 3.6: Proposed Approach.....	22
3.7.1: Retinal Ganglion Cell Targeting	24
3.7.2: Biocompatibility	25
3.7.3: Administration Routes.....	25
3.7.4: Intravitreal Route	27
3.7.5: Sub-ILM route.....	28
3.7.6: Size and Surface Charge	29
Chapter 4: Outline of Current Study.....	31
Section 4.1: Examining the effects of photobleaching on AuNR fluorescence.....	31
Section 4.2: Establishing the efficacy of Sub-ILM injection	31
Section 4.3: Determining optimal injection parameters	32
Chapter 5: Methods	33
Section 5.1:Ex-vivo Studies.....	33
5.1.1: Immunohistochemical procedure	33
5.1.2: Injection Procedure	37
5.1.3: Confocal Imaging.....	40
5.1.4: Bovine eyeball experiments	40

5.1.5: Statistical Analysis	41
Chapter 6: Results	42
Section 6.1: Examining the effects of photobleaching on AuNR fluorescence.....	42
Section 6.2: Efficacy of Sub-ILM injection	44
Section 6.3: Determining optimal injection parameters.....	45
6.3.1: Bovine Eyeball Experiments	45
6.3.2: AuNR S-ILM injections	47
.....	47
Chapter 7: Discussion	52
Section 7.1: Examining the effects of photobleaching on AuNR fluorescence.....	52
Section 7.2: Establishing the efficacy of Sub-ILM injection	53
Section 7.3: Determining optimal injection parameters.....	54
Chapter 8: Conclusion.....	58
9: References.....	60

List of Figures

Figure 1 (A,B) showing the absorption of a photon and subsequent phototransduction cascade. (A) shows typical response of cones in darkness, while (B) shows a cone response in luminated settings. (C) diagram of rods and cones. (A,B) adapted from Klapper et al 2016 [8], (C) adapted from Arshavsky et al 2002 [9].....	3
Figure 2 Hematoxylin and eosin stained histological slides of a healthy retina (left) and a patient with mid-stage RP (right). Adapted from Hartong et al 2006 [3].....	5
Figure 3 Retinal vascular supply, indicating the differences between blood supply to the outer and inner retina. Adapted from Compochiaro et al 2017 [13].	8
Figure 4 Lipid peroxidation in 10-month old Pro347Leu Tg pigs. (Top) A control image obtained from a 10-month old WT pig, (bottom) an image obtained from 10-month old Pro347Leu Tg pigs. (Left) Stained images showing lipid peroxidation, (Right) merged image with DAPI stain. Adapted from Shen et al 2005 [36]	9
Figure 5 Schematic of overall approach utilizing GNRs to induce a neuronal action potential via infrared irradiation. Adapted from Eom et al 2014 [67].	18
Figure 6 TEM image depicting GNR localization to the plasma membrane obtained from rat sciatic nerve tissue sections. Adapted from Eom et al 2014 [81].	20
Figure 7 H&E stained images of nerve tissue. (A) Sham specimen showing no irregularities or damage in tissue. (B) Irradiation of sciatic nerve at 0.956 J/cm ² showing no evidence of tissue damage. (C) Irreversible tissue damage induced by irradiation at 2.23 J/cm ² . Adapted from Eom et al 2014 [81].	20

Figure 8 Schematic of plasmonic retinal prosthetic, a) external view showing device and components; b) NIR laser interaction with eye and retina; c) schematic of NIR laser diffusing to the retina; d) retinal ganglion cell with AuNRs bound to its membrane.....	23
Figure 9 Schematic of the retina showing the BRB. PRS: photoreceptors; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; NFL: nerve fiber layer; oBRB: outer blood retinal barrier; iBRB: inner blood retinal barrier. Adapted from Peynashert et al. 2017 [87]	26
Figure 10 Schematic representation of the eye showing the various delivery routes. Adapted from Weng et al. 2017 [88].....	26
Figure 11 Diagram showing the vitreoretinal (VR) interface, with the structural components of VH and the ILM. Adapted from Le Goff et al. 2008 [94].....	28
Figure 12 Schematic of S-ILM injections, adapted from [95]. ILM: Inner limiting membrane; NFL: Nerve fiber layer; GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer; PR IS: Photoreceptor inner segments; PR OS: Photoreceptor outer segments; RPE: Retinal pigmented epithelium.....	29
Figure 13 Diagram of dissected retina. Incisions are made with straight edged dissection scissors in order to obtain the petal configuration at the locations denoted by an asterisk. The petal configuration is necessary to successfully flatten the retinal specimen	36
Figure 14 Image of dissected retina prior to injection of anti-CD 90.2 antibody, microneedle tip is shown in the top right corner.	36

Figure 15 Image of borosilicate glass microneedles utilized for nano-injector.....	37
Figure 16 Image of nano-injector setup. (Left) Shows typical injection setup under visualization of microscope, (Right) S-ILM injection on retinal-flat mount	38
Figure 17 Results of photobleaching test. T_0 represents the initial timepoint of the streptavidin functionalized Cy5 fluorescently tagged AuNRs. Dark exposed represents the group that was left in darkness for 1 week, while the light exposed group represents the sample that was left in constant ambient light exposure for 1 week. Results are in fluorescent counts (10^3). Error bars represent standard deviation. A Shapiro-Wilk normality test showed that the distribution was non-normal. A Friedman repeated measures ANOVA with Dunn's post-hoc test showed no statistically significant differences between groups ($P > 0.05$) signified by "NS"	42
Figure 18 Retinal confocal images obtained from S-ILM injection validation experiment, showing fluorescence (green) functionalized with streptavidin binding to RGC membranes (RGCs shown in blue), axons are also shown (green). Samples were injected with biotinylated anti-CD 90.2 antibody, and incubated with FITC streptavidin fluorescent dye and a DAPI stain for RGC nuclei . Scale bar represents $10\ \mu\text{m}$	44
Figure 19 Bovine dissection and injection mold. (Left) Shows a top view of the overall dissection mold with the eyeball housing feature. (Middle) shows a bottom view of the top portion of the mold, outlining the hollow design meant to house ice. (Left) Top view of the base, meant to interface with the top to shut the mold when it contains ice.	45
Figure 20 Results of photon correlation spectroscopy experiment. The blue bar represents the relative diffusion of negatively charged streptavidin functionalized	

AuNRs; the red bar represents the relative diffusion of positively charged NH_2 functionalized AuNRs. Student's T-test revealed that results were statistically significant between groups ($P= 1.079 \text{ E}40$). Error bars represent standard deviation.....46

Figure 21 Confocal images obtained from S-ILM injection of large-sized AuNRs (25 X 87 nm, streptavidin functionalized, FITC fluorophore) in naked retinal flat-mounts. Biotinylated anti-CD 90.2 antibodies were pre-incubated with streptavidin functionalized AuNRs (2 μL each, 4 μL total injection volume) and injected via the S-ILM route. RGC nuclei are stained with DAPI (blue) and AuNRs are fluorescently tagged with FITC fluorophores. (Top row) images taken using (1%) laser intensity. (Bottom row) images taken with increased laser strength (2%). Axonal projections are also visible shown in green. Scale bar represents (10 μm).....47

Figure 22 Confocal images obtained from S-ILM injection of large-sized AuNRs (25 X 87 nm, streptavidin functionalized, FITC fluorophore) in naked retinal flat-mounts. Biotinylated anti-CD 90.2 antibodies (2 μL) were injected via the S-ILM route and allowed to incubate before being washed. AuNR injection (2 μL) via the S-ILM route followed, and the sample was washed after incubation. RGC nuclei are stained using DAPI (shown in blue), while AuNRs are fluorescently tagged with FITC (shown in green). Scale bar represents (10 μm)48

Figure 23 Confocal images obtained from S-ILM injection of small-sized AuNRs (10 X 38 nm, streptavidin functionalized, Cy5 fluorophore) in naked retinal flat-mounts. Biotinylated anti-CD 90.2 antibodies (2 μL) were incubated with (2 μL) of AuNRs and allowed to incubate for (1hr) pre-injection. Naked retinal-flat-mounts received (4 μL) of

the antibody-AuNR mixture via the S-ILM route. RGC nuclei are stained using DAPI (shown in blue), while AuNRs are fluorescently tagged with Cy5 (shown in magenta). Scale bar represents (10 μm)	50
Figure 24 Confocal images obtained from S-ILM injection of small-sized AuNRs (10 X 38 nm, streptavidin functionalized, Cy5 fluorophore) in naked retinal flat-mounts. Biotinylated anti-CD 90.2 antibodies (2 μL) were injected via the S-ILM route and allowed to incubate before being washed. AuNR injection (2 μL) via the S-ILM route followed, and the sample was washed after incubation. RGC nuclei are stained using DAPI (shown in blue), while AuNRs are fluorescently tagged with Cy5 (shown in magenta). Scale bar represents (10 μm)	51

Chapter 1: Visual Impairment

Section 1.1: Introduction

The number of people of all ages that suffer from visual impairment is estimated to be 280 million globally, of which 39 million are blind¹. The worldwide prevalence of retinitis pigmentosa (RP) is about 1 in 4000 people, with a total of 1 million people affected. A study conducted in Kuwait, claimed that the leading cause of blindness in individuals aged 21-40 was RP². In most cases, the disease is confined to the eye; 20-30% of the cases have non-ocular related symptoms. These are defined by 30 different syndromes³.

Disease inheritance is also quite complex, with autosomal dominant cases comprising approximately 30-40%, autosomal recessive comprises 50-60% of cases while X-linked traits comprise 5-15% of cases⁴⁻⁶. The gravity of this disease does not lie within the statistics, rather the fact that symptoms differ from case to case and that it is quite difficult to diagnose and track the degree of symptom progression. Some patients may develop symptomatic visual loss during their formative years, while many others will remain asymptomatic until mid-adulthood³.

Patients may also display the classic patterns related to difficulty adapting to darkness, and the development of night blindness during adolescence; then the loss of mid-peripheral visual field in young adulthood. As disease progression occurs, patients may develop tunnel vision after losing peripheral vision. Finally, central vision is lost by about the age of 60³. The patterns of visual loss/symptoms would indicate the gradual

loss of photoreceptor cells. Rods, responsible for dim lit vision and night vision, are the first to degenerate. Cones, responsible for fine daylight acuity and color, are the last to degenerate.

Both rods and cones formulate the photoreceptor layer, an important segment in the outer nuclear layer found in the retina. The hallmark of RP, is the fact that cells in the inner nuclear layer (amacrine cells, bipolar cells, horizontal cell neurons, and the retinal ganglion cell layer) are somewhat preserved; although many do degenerate later in the disease. Most patients are legally blind by the age of 40 due to the severity of constriction in their visual fields. The difficulty of diagnosing this disease, lies within the fact that most patients do not notice any symptom development until their vision has begun to severely decline. Furthermore, photoreceptor cells have the ability to attenuate their sensitivity to account for the loss of photoreceptor cells. Investigators have noticed “tiny islands” of vision that persist when disease progression is severe.

Section 1.2: Visual Cascade

Photoreceptor cells are located in the outer nuclear layer (ONL) within the retina. Photons of light are transduced from the corneal level and make their way to the retina. Within the retina, photoreceptor cells obtain protruding structures, known as disks. Disks comprise the outer segment of the photoreceptor cells, and are responsible for the absorption of a photon and the subsequent activation of the phototransduction cascade⁷. The typical response to a photon of light, and the structures of rods and cones are shown in (**Figure 1**).

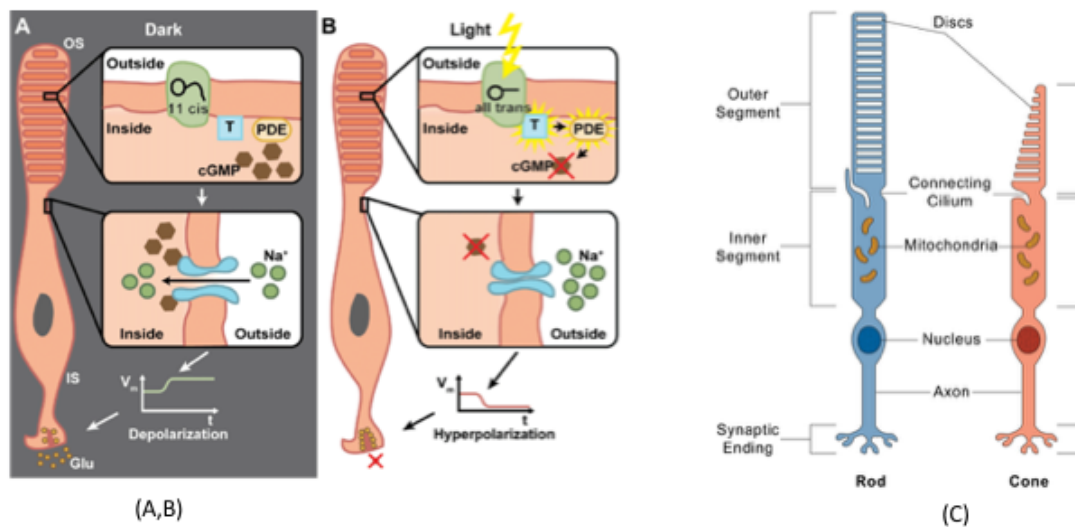


Figure 1 (A,B) showing the absorption of a photon and subsequent phototransduction cascade. **(A)** shows typical response of cones in darkness, while **(B)** shows a cone response in luminated settings. **(C)** diagram of rods and cones. (A,B) adapted from Klapper et al 2016 [8], (C) adapted from Arshavsky et al 2002 [9].

The photon of light is absorbed by a visual pigment molecule that is highly ubiquitous in rods and cones, therefore they are known as the photoreceptor cells. The visual pigment is a G-protein coupled receptor (GPCR) composed of a protein, opsin, and a chromophore, 11-cis-retinal, it is located in the ion-rhodopsin unit. Once a photon is absorbed, a conformational change occurs during which 11-cis-retinal is isomerized into the all-trans conformation. It is this conformational change which induces the pigment to change to its active state, and the activation of the G-protein coupled receptor.

The GPCR in this case is Transducin, and when Transducin is activated it leads to the activation of a phosphodiesterase unit (PDE). The PDE in turn leads to the hydrolysis of endogenous cGMP within the cell. Ultimately, this lowered concentration of cGMP leads to the closure of the non-selective cation channels and the subsequent hyperpolarization of the photoreceptor cell, and the decrease of neurotransmitter that

is released in the synaptic cleft (further downstream). This describes the conduction of an action potential (AP) as a result of photoreceptor activation by a photon of light.

With this regard, photoreceptors differ from all neuronal cells in the body because their activation leads to a decrease of the concentration of neurotransmitter released, as opposed to an increase in the conventional mode of operation. There are several differences between rods and cones:

1. Rods are extremely sensitive to light and can detect up to a single photon of light. As such, they are suitable for sight during conditions of dim light¹⁰.
 - a. In contrast cones are at least 100 times less sensitive than rods, rendering them non-functional during darker conditions.
2. Rods saturate in moderately bright conditions and enter a refractory period rendering them non-functional throughout most of the day¹¹.
 - a. In contrast cones possess the ability to attenuate their sensitivity to light, in order to avoid saturation and remain functional in exceedingly bright conditions. This phenomenon is known as light adaptation. Light adaptation is what allows us to see during the day.
3. Rods experience a long refractory time when they are saturated post-light exposure, and may take up to 1 hour for rods to regain sensitivity. Cones however, can regain sensitivity within minutes, known as dark adaptation, preventing them from becoming refractory. Dark adaptation allows us to retain visual perception in conditions of variable brightness⁷.

Section 1.3: Mechanisms of Disease Progression

In RP, loss of night vision occurs first, due to the fact that rod photoreceptor cells are the first to undergo degeneration, over time enough rod cells die and the individual is said to be night-blind³. Patients with RP, retain their cones for some time, and as such their daily tasks can go somewhat un-interrupted. Day-driving, reading, walking outside, etc. are un-affected because we rely on cones to see during these activities. Furthermore, given the fact that technology has become so widespread, lights are almost everywhere and one can sometimes be afflicted with RP and not even know until the onset of cone degeneration. Given that visual acuity still persists with 65-70% photoreceptor loss, this may be a result of the adaptability of photoreceptor cells. Several therapeutics have been shown to provide a decrease in the rate of degeneration, but inevitably the photoreceptor cell line is almost obliterated , as shown in **(Figure 2)**.

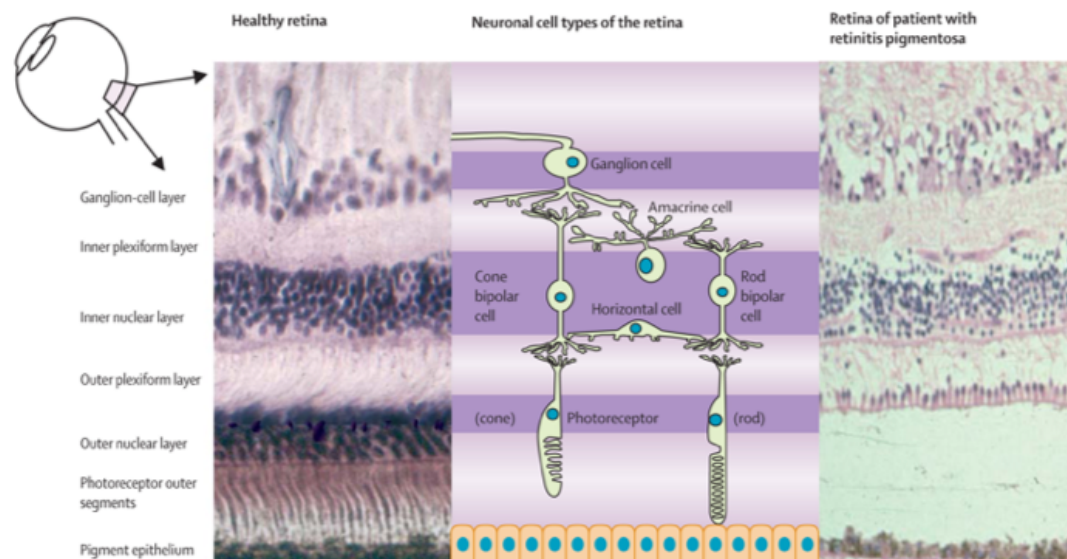


Figure 2 Hematoxylin and eosin stained histological slides of a healthy retina (left) and a patient with mid-stage RP (right). Adapted from Hartong et al 2006 [3]

1.3.1: Mechanisms of cone death

Oddly enough, mutations that lead to RP do not cause cone photoreceptor death. Instead, they focus on the degeneration of rods. Research shows that mutations in rhodopsin, although expressed in both cell types, lead to damage that is focused on rods. It is still unclear what leads to the deterioration of cone cells. RP in humans is fairly similar to that which occurs in animals, previous research has shown that degeneration occurs in rods first, while cones remain intact; until their inevitable degeneration years later¹²⁻¹⁷.

In several animal models, cone degeneration begins centrally and then much of the peripheral cones begin to follow. The detailed patterns of various cell death in human RP are lacking in detail, yet based on the information regarding how vision loss progresses, some inferences can be made. It is said that the visual field is affected first; this is attributed to a loss of cones in the retinal midperiphery. In this region cone density is relatively low.

Degeneration then spreads both medially and laterally; peripheral fields begin first then central vision loss follows. The “islands of vision” usually occur centrally due to this pattern of degeneration¹⁸. Another hypothesis regarding the “islands of vision” relate to cone migration. It is said that cones usually have a set distance between one another. Therefore, their clustering and forming an island may be attributed to this migration phenomenon¹⁸.

1.3.2: Mechanisms of cell death

As noted earlier, rod cell death is followed by cone cell death; it has been hypothesized that this was due to the fact that rods, when degenerating, release toxic substances that affect cones¹⁹, or microglia migration to cones after rod clearance in the retina²⁰, or that rods have a survival factor that helps cones and once degenerated this factor is now eliminated²¹. Yet, cones do not completely degenerate until years after rods die, therefore toxins leading to the death of cone cells is a difficult hypothesis to prove; the same applies to microglia migration. However, a trophic support factor secreted by rods does exist, and a reduction due to rod death seems like a plausible hypothesis.

Rod-derived cone variability factor-short (RdCVF-s) and rod-derived cone variability factor-long (RdCVF-L) have been identified^{22,23}. Their absence however, only resulted in a 20% cone reduction as identified by studies conducted in mice that lack those factors²⁴. It has been hypothesized that the loss of these factors leads to conditions that promote cone death. Making cones more susceptible to hyperoxia conditions, due to oxidative stress²⁵. It is hypothesized that high levels of oxygen in the eye lead to oxidative stress related damage to cones. In the absence of rods, the levels of oxygen within the eye are increased²⁶.

When mice were placed in high oxygen levels for a prolonged time, their photoreceptors degenerated²⁷. Photoreceptor death occurred in regions with the highest O₂ content. Underlying the overall sensitivity of these cells to O₂. When the inner retina contains a large amount of oxygen, the choroidal blood vessels might

constrict to lower the amount of blood flowing, and balance the overall concentration of O_2 . Choroidal vessels however, are not supplying the outer retina; rod cells comprise approximately 90% of the ONL in the retina, as shown in (Figure 3). Therefore, when rods die, overall oxygen consumption is reduced, and there could be an increase in O_2 levels.

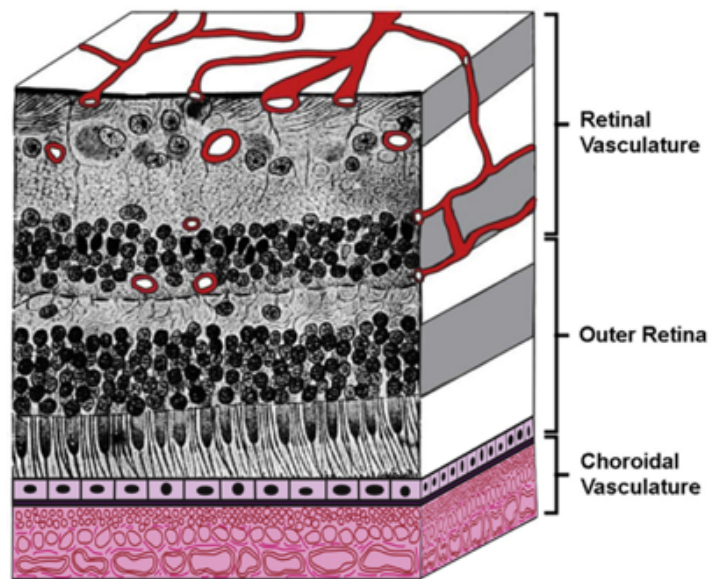


Figure 3 Retinal vascular supply, indicating the differences between blood supply to the outer and inner retina. Adapted from Compochiaro et al 2017 [13].

Constriction of blood vessels in individuals with RP suggests that excess oxygen located in the outer retina is able to diffuse into the inner retina. A study was conducted in Royal College of Surgeons (RCS) rats (retinal degeneration reported as early as 1938²⁸), in which electrodes were placed into the rat retina to measure the levels of oxygen from the choroid to the retinal surface during varying stages of retinal degeneration²⁹. Prior to retinal degeneration, low levels of O_2 were observed in the ONL, these findings were similar to those which were observed in wild-type adult rats. It is known that on the 30th post-natal day (P30), rod degeneration is complete in RCS rats.

$\geq P30$ the low levels of O_2 were no longer present, instead cones were now exposed to O_2 levels that were 4 $\times$ higher. These findings were also confirmed by other studies^{30,31}.

Transgenic (Tg) pigs that expressed a Pro347Leu mutation, Pro347Leu causes autosomal dominant RP³², had their retinas harvested and sectioned for subsequent immunohistochemical (IHC) staining. It is known that rod degeneration is complete at 9 months of age³³, as such retinæ were harvested and sectioned at 10 months of age. Acrolein and 4-hydroxynonenal staining have been utilized in efforts to identify levels of oxidative stress^{34,35}. Retinal sections obtained from 10 month old WT pigs showed very faint staining, whereas those obtained from the Tg pigs (10 months of age) showed intense signals for acrolein and 4-hydroxynonenal in cone inner segments³⁶. This served as an indication of lipid peroxidation, a known indicator for oxidative stress related damage. IHC results are shown in **(Figure 4)**.

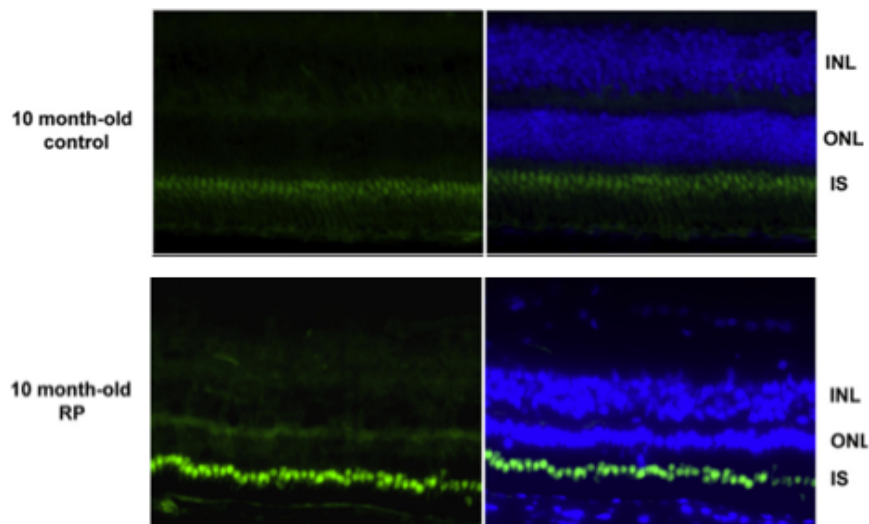


Figure 4 Lipid peroxidation in 10-month old Pro347Leu Tg pigs. **(Top)** A control image obtained from a 10-month old WT pig, **(bottom)** an image obtained from 10-month old Pro347Leu Tg pigs. **(Left)** Stained images showing lipid peroxidation, **(Right)** merged image with DAPI stain. Adapted from Shen et al 2005 [36]

It has been hypothesized that a reduction in oxidative stress, would decrease cone death. Several studies involving different mutations have demonstrated this fact. A study in mice showed that cone density was reduced with increasing oxidative stress damage³⁴. Other studies utilizing different mutational models have also shown that a reduction in oxidative stress via an antioxidant “cocktail” improved cone survival^{38,39}. These findings are relevant to humans, since oxidative stress has been reported in humans with RP.

Section 1.4: Genes Implicated in the Disease

Mutations that cause RP can occur in a wide variety of genes that are responsible for several crucial factors in the cellular functions of the retina, specifically in photoreceptor cells. For example, phototransduction, the visual cascade, photoreceptor cell structure (cilium), and gene transcription within photoreceptor cells can all become affected by mutations. Thus outlining the various pathologies of the disease⁴⁰. Understanding the molecular progression of this disease from a genetic perspective remains quite difficult, and in turn limits the options available for therapeutics that can potentially aid in disease management.

A mutational screening conducted on some of the known causal genes for autosomal dominant inheritance of RP in 200 families, revealed that only 107 families possessed the known DNA sequence (53.5%) thought to be responsible for RP⁴¹. Identifying the DNA sequences that may lead to this disease remains rather difficult. With regards to identifying the other subtypes of RP, such as autosomal recessive and simplex RP, the methods are vastly greater in difficulty. A study suggested the benefits

of identifying the mutational causes of each individual patient separately in order to further the understanding of this elusive disease.

Chapter 2: Current Treatments and Approaches

Section 2.1: Diagnosis

Documentation during the early stages relies on the patient undergoing an electroretinogram (ERG), a diagnostic test that measures the neuronal and non-neuronal response to a light stimulus. It focuses on measuring the electrical activity within these cells, using superficial or implanted electrodes³⁴. What is noticed with patients who suffer from RP, is a reduction in a- and b-wave amplitudes until their inevitable elimination. Visual defects in the mid-periphery of each eye have been documented, but do not seem to affect a patient's activities.

The symptom that follows the reduction of ERG amplitudes and loss of night-vision, is a reduced visual field. Undiagnosed patients at this stage may notice that they are bumping into objects and/or people, signifying a reduction in their visual field. Other reports include visuals that appear into existence yet disappear momentarily afterwards. During this stage, general visual acuity is unhampered; yet the overall field of vision has been reduced.

Ophthalmic examinations often show slightly morphed anatomy. For example, some pigmentary changes in a patient's retina may be observed. Narrowing of blood vessels in the peripheral retina, and in some cases the developments of cataracts have been observed^{3,50}. With the gradual progression of symptoms, fluid cysts spaces may develop

in the fovea. This can sometimes be viewed with fundus examinations, in which the fovea can be observed and analyzed.

It is not until cone loss has progressed into the posterior region of the retina, that it can be clearly imaged. This is done by means of spectral domain-optical coherence tomography (SD-OCT). Several layers of the retina can be imaged, and disease progression can be observed. Specifically a region of interest known as the inner ellipsoid zone (EZ). The EZ represents the border between the inner and outer segments of the photoreceptor layers. One study was able to show a correlation between the decline of this region and a decline in visual acuity⁴³. Visual fields continue to decline until only “small islands” of vision remain. At this point, the patient may be pronounced legally blind, or might be rapidly progressing towards so; as mentioned in a previous section these small islands of vision eventually diminish over time.

Section 2.2: Current treatments

Currently, RP has no treatments other than very simple regimens that hope to mitigate and reduce the rate of photoreceptor degeneration. These treatments usually include vitamin A+E supplements, since several studies have shown that these when used in combination led to a slower rate of photoreceptor degeneration. The studies have provided controversial results, such as variability in the results that depend on the extent of disease progression⁴². Docosahexaenoic acid (DHA), or omega-3 fatty acids, are also included in current treatments provided by clinicians to patients with RP. It is unclear why exactly, but it is speculated that they are important to for photoreceptor cell function, with regards to the formation and maintenance of plasma membranes.

In late or severe RP, amounts of DHA are diminished and when supplemented in the diet, those with the highest amount showed a reduced rate of retinal degeneration. More importantly, the rate was reduced by 40-50% with DHA alone⁴⁴. Therefore, doctors currently supplement with vitamin A, low amounts of vitamin E and omega-3 fatty acids. Studies have estimated that this regimen may add an additional 20 years of visual acuity⁴⁴.

Antioxidant drugs have shown to relieve the degeneration rate of rods, and delay the onset of visible effects in ERG amplitudes (a- and b-waves)³⁴. This leads to the notion that the longer rods can be kept alive, the longer one can protect cones from extensive levels of oxidative stress correlated to rod death. N-acetylcysteine (NAC), a product that is usually converted to cysteine, is a known free radical scavenger (glutathione synthesis substrate). A study in which Rd10 mice were treated with NAC showed a significant difference in oxidative damage, as well as an improved rate of cone survival; this was dose dependent with NAC⁴⁵.

Treatments that target degeneration are not effective in inducing significant change. Apart from prosthetics and genetic approaches (which shall be discussed in this section), treatment mainly revolves around treating secondary symptoms that arise from RP. These symptoms include development of cystoid macular edema (CME), and is estimated to occur in 10-38% of patients^{46,47}. Carbonic anhydrase inhibitors (CAIs) and utilized to treat CME in RP, yet produced varying results in several studies⁴⁸⁻⁵¹. Steroids have also been used to treat CME, as they have the ability to reduce production of inflammatory cytokines^{52,53}. Anti-vascular endothelial growth factor (VEGF) treatments

have been studied in patients with CME as a result of RP. The exact method of action is unclear, yet several studies have monitored its effects. Intravitreal pegaptanib has been shown to be effective in overall central macular thickness reduction⁶²; while intravitreal bevacizumab lead to an improvement of visual acuity and a reduction in macular thickness⁶³.

With regards to retinal implants and prostheses, there are two devices that are either approved or under development. The ARGUS II Retinal Prosthesis System, a device with electrodes implanted in the ganglion side of the retina, and a device known as the Alpha-IMS Retina Implant, a device with electrodes implanted in between the retina and the RPE. The ARGUS II has been approved in Europe during 2011, and received FDA approval in 2013 (humanitarian device). The ARGUS II consists of a video recorder, an image processor, a transmitter coil, a battery for a power source, and the retinal implant that is comprised of 60 electrodes⁶⁴.

29 patients who suffer from RP were involved in the phase II trial of this device. However, the 5 year results were published and have indicated that 12 patients experienced adverse reactions/events. Conjunctival erosion, hypotony, conjunctival dehiscence and presumed endophthalmitis were all reported in several patients⁶⁵. Three of the devices were eventually explanted, yet prior to the side effects patients all showed an improvement in several visual function tests⁶⁵⁻⁶⁷.

The Alpha-IMS implant received approval in Europe during 2013; it consists of a specialized sub-retinal implanted chip (1500 pixels & photo-bodies) that is able to convert light information into an electrical impulse. It contains a postauricular coil, a

power cable and an external coil that is attached to a battery cell⁶⁸. A clinical trial utilizing this implant in 29 patients who were either able to perceive light or had no light perception as a result of outer retinal degeneration, reported improved visual outcomes specifically related to day living, object recognition, etc.⁶⁹ Side effects were reported, such as retinal detachment, a high increase IOP (up to 46 mmHg) in two patients.

Chapter 3: Plasmonic Retinal Prosthesis

Section 3.1: Neural Stimulation

Neural stimulation is a process that involves an external energy source to initiate action potentials in peripheral and central neurons⁶². The ability to initiate an action potential in neurons provides a great advantage in both clinical and research applications. It is a vital diagnostic and therapeutic tool. The golden standard has been electrical stimulation, and is based on initiating an action potential in neurons by a mechanism of transmembrane ionic exchange. This leads to what has been referred to as the explosive opening of sodium channels. This typically occurs once a certain threshold voltage has been reached.

Section 3.2: Infrared Neural Stimulation

Another option exists, known as optical stimulation, in which lasers or light (either infrared or ultraviolet) can be utilized in order to induce activation of neurons by deposition of energy from light or lasers. Several studies have emerged utilizing this technique, one that relates to stimulating neurons in gerbil cochleae⁶³, one that

stimulated neurons in excised sciatic nerves⁶⁴, and the stimulation of rat sciatic nerves in vivo⁶².

Results from the study involving the stimulation of rat sciatic nerves, provides evidence that suggests optical stimulation of neurons via laser excitation yields artifact-free, contact-free and spatially selective stimulation of neurons⁶². Recordings included those that were obtained from electrical stimulation, and were compared to those obtained from optical stimulation obtained from laser excitation. The data revealed very little difference between both stimulation modalities in nerves and muscles. It was revealed that the stimulation artifact that is usually associated with electrical stimulation was not present in the laser excitation data.

Section 3.3: Biophysical Mechanisms

The efficacy of optical neural stimulation has been demonstrated, but the underlying mechanisms that facilitate it were not studied, until Wells et al. identified them⁶⁴. Three mechanisms exist to facilitate stimulation: 1) photochemical, 2) photothermal and 3) photomechanical. Photochemical effects rely on the ability of absorbed light to catalyze a specific reaction, while photothermal effects result from the transformation of absorbed light to energy to ambient heat; photomechanical effects are usually secondary to tissue heating with laser pulses but then are also able to induce mechanical forces.

Results revealed that neural activation by pulsed light (infrared) is thermally mediated. Data also revealed that the transient temperature change at the nerve level was approximately (6°C-10°C), while at the axonal level the approximate change was

about (3.8°C-6.4°C)⁶⁴. Researchers were able to illustrate that pulse width was an important factor, leading to the notion that longer pulses resulted in an increased time for the onset of CNAP production. This was due to the time required for heat to diffuse across the tissue.

Attempts to optically stimulate cochleae in artificially deafened gerbils revealed contrary results⁶³. Animals were deafened by injecting Ringer's Lactate containing varying concentrations of neomycin in the middle ear cavity. Measured activity revealed that compound action potential (CAP) threshold was elevated, but not statistically significant, the same trend applied to longer pulse durations.

The results can be explained by the fact that optical stimulation was unable to induce synchronous firing of neurons. Authors revealed that the overall number of spiral ganglion cells decreased between animals that received the deafening agent and the control group. It was estimated that this number decreased by 30%⁶³. Furthermore, upon increasing the deafening solution's concentration, no CAPs were detected, and thus the conflicting results could be a result of experimental design.

Based on the work conducted by Wells et al⁶⁴, researchers wanted to identify the molecular mechanisms by which the photothermal effects of laser irradiation can produce an action potential. This would further enhance the understanding with regards to INS, and facilitate the clinical application of this technology. Albert et al investigated the effects of the laser induced response on mouse retinal ganglion cells (RGCs) and rat vestibular ganglion cells (VGCs)⁶⁵. RGCs were isolated from postnatal mice (P2-P4) and VGCs were explanted from P5-P9 rats. The isolated RGCs were irradiated with an IR laser

(1,875 nm), and laser evoked neuronal voltage variations (LEVVs) were recorded for a pulse duration ranging from 7ms-10ms; the power of the laser was set to 230 mW.

LEVVs were initiated repetitively in cells for several minutes without cell damage. In order to identify the specific channels and molecular mechanism of the evoked LEVVs, several blocking agents were applied and patch clamp recordings were collected. TTX a known specific blocker of voltage-gated sodium channels was applied to the bath (10 μ M) and then patch clamp recordings were collected. TTX led to a reversible abolishment of a spike-like component that was visible in the LEVV of both cell types. Thus, this specific component of the LEVV wave trace was attributed to the activation of voltage-gated sodium channels.

Section 3.4: Gold Nanorods

Gold nanorods (GNRs, also referred to as AuNRs) have emerged with a new functionality that provides benefits to many applications. Utilizing a unique optical property referred to as localized surface plasmons (LSPs), particles are illuminated at their resonant frequency. Light is then efficiently absorbed and converted to heat⁶⁶. Nanoparticle plasmons induce electron oscillations that are confined to the nanoscale range. This allows for manipulation of the interaction between the light energy and GNRs. Plasmonically triggered heat can be very local and can activate temperature sensitive ion channels, triggering an influx of calcium which facilitates the stimulation of a neuron with enhanced spatial precision.

The use of GNRS to induce optical neural stimulation provides the advantage of increased neural responsivity, stimulation efficiency and an improvement in the issues

of spatial resolution. GNRs can also reduce the extent of laser exposure and can mitigate the effects of tissue damage that is related to INS⁶⁷. The overall schematic of this approach is shown in the following figure (**Figure 5**).

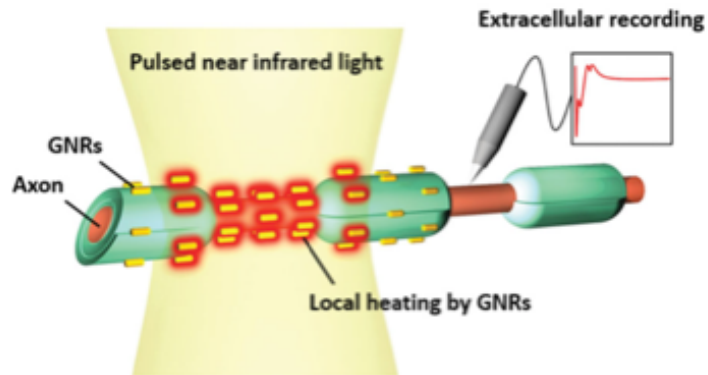


Figure 5 Schematic of overall approach utilizing GNRs to induce a neuronal action potential via infrared irradiation. Adapted from Eom et al 2014 [67].

Section 3.5 Infrared Neural Stimulation and AuNRs

One study utilized LSPs in conjunction with INS in rat sciatic nerves *in-vivo*; researchers were able to monitor and record CNAPs in the exposed sciatic nerve while optically stimulating it with a 2,000 nm IR beam. GNRs were injected into the nerve bundle, and then confirmed via transmission electron microscopy (TEM), as shown in (**Figure 6**) GNRs were localized near the plasma membrane. CNAPs were compared between rats that received a GNR injection in their exposed nerve bundle and those without GNRs. At a laser exposure of (0.641 J/cm^2) recorded CNAPs in the presence of GNRs were larger than their counterpart by more than 6 fold⁶⁷.

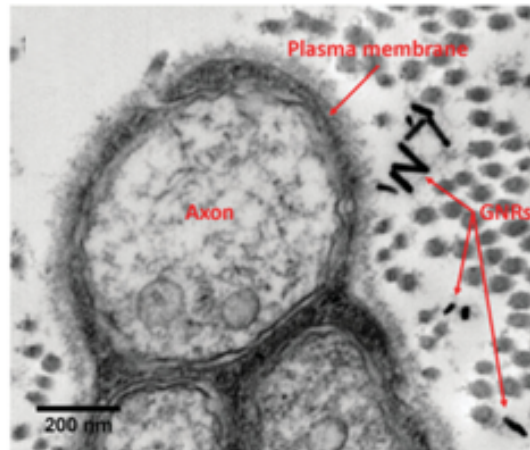


Figure 6 TEM image depicting GNR localization to the plasma membrane obtained from rat sciatic nerve tissue sections. Adapted from Eom et al 2014 [81].

Histological analysis (hematoxylin and eosin staining) revealed that at the exposure levels utilized in this study ($< 1.0 \text{ J/cm}^2$) no irreversible damage was incurred, as shown in **(Figure 7)**. Several studies utilizing INS optically stimulate neurons in close proximity either by direct exposure or by the insertion of a fiber optic cable close to the target site. Surgical manipulation may also be required to expose the target site for the stimulatory effects to be possible; which limits the applications that arise from these studies to implantable devices. In order to penetrate deep tissue, without causing damage, lower wavelengths are necessary.

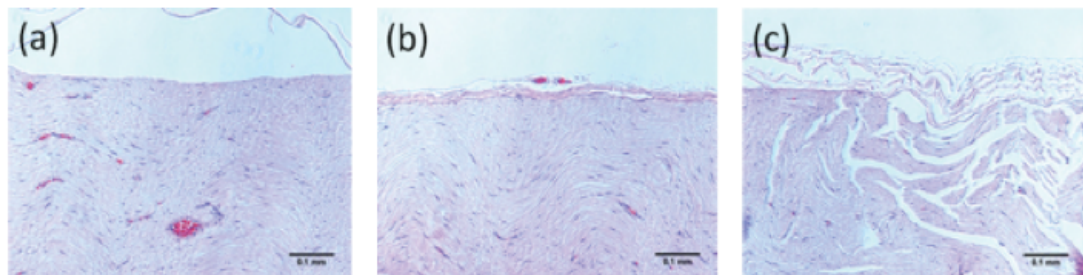


Figure 7 H&E stained images of nerve tissue. **(A)** Sham specimen showing no irregularities or damage in tissue. **(B)** Irradiation of sciatic nerve at 0.956 J/cm^2 showing no evidence of tissue damage. **(C)** Irreversible tissue damage induced by irradiation at 2.23 J/cm^2 . Adapted from Eom et al 2014 [81].

Section 3.6: Gold Nanorod Assisted Near Infrared Neural Stimulation

Near infrared (NIR) light has been proposed as a solution to the shallow tissue penetration of IR light. Since hemoglobin and water are the major absorbers of both visible and infrared light, it is vital to utilize a wavelength that yields a low coefficient of absorption for both mediums. NIR light (650 nm- 900 nm) solves this issue, since both mediums have their lowest coefficients of absorption in this range⁶⁸. Due to water absorbing a high amount of IR light, a higher amount of energy is usually required to successfully optically stimulate a neuron⁶⁹, this may be one of the main culprits of tissue damage.

It is possible to tune the properties of gold to achieve optimal absorption of light at a desired wavelength, in this case the NIR range⁷⁰. One study was successful in stimulating auditory neurons using GNR assisted NIR optical stimulation. Neurons cultured from primary rat auditory neurons were used as an *in-vitro* model⁶⁹. Cultured neurons were able to fire action potentials after being visually identified and stimulated with an NIR beam (780 nm beam). Voltage clamp recordings revealed that the nanorod auditory neurons (NR-ANs) displayed repeatable waveforms as a response to NIR light.

Pulse energy varied between 0 mJ to 0.09 mJ, which resulted in an inward current that was observed at the onset of NIR illumination. Control groups (no GNRs) did not exhibit any response with NIR stimulation. Experiments were also conducted to compare cultured neurons that had GNRs to those that were cultured with gold nanospheres, and results revealed that GNRs produced a greater current⁶⁹. The temperature change in this study was 3.5°C, while Wells et al.⁶⁴ obtained a change of

3.66°C using a pulse duration of 1 ms and an IR beam of 2,120 nm. The power utilized in each study was vastly different. Wells et al. utilized a 1.13 mJ, while this study utilized a max of 0.09 mJ⁶⁹.

These results suggest that GNRs can obtain the same local heating effect at a much lower wavelength and beam power. This may mitigate the effects related to tissue scarring as a result of higher pulse durations and laser power. Interestingly, the effects of 780 nm NIR light is negligible in neurons that do not contain gold ; illustrating the clear advantage provided by GNRs. Finally, the temperature change was highly dependent on pulse duration which causes fluctuations between 0.5°C-6°C.

Section 3.6: Proposed Approach

The proposed to be utilized in this study draws in from the concepts outlined thus far, with further optimization for the targeting of RGCs, and to achieve GNR assisted NINS of RGCs. As mentioned in (**Section 1.3**), in RP the photoreceptor layer is severely degenerated while the remainder of the retina remains fairly intact. RGCs are responsible for transducing visual sensory information from the retina to the visual cortex of the brain via the optic nerve. By injecting optimized GNRs for RGC targeting into the ocular cavity, and utilizing NINS and its enhanced tissue penetration, it may be possible to improve our current standard of implantable devices for the management of visual impairment and diseases like RP.

Several issues related to electrical stimulation, such as biocompatibility of electrodes, immune response due to the implantable device, infections in various endothelial layers, retinal detachment, conjunctival dehiscence, etc. are completely

avoided by the utilization of GNR assisted NINS. GNRs would be the only component of this approach that enters the body, while the remaining components are outside the body on or within a device. A schematic of this potential approach, termed plasmonic retinal prosthetics, is shown in (Figure 8).

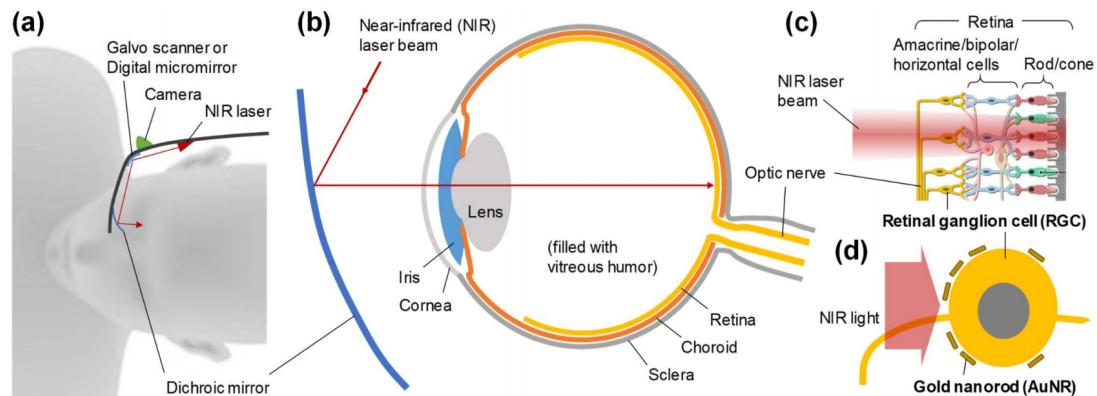


Figure 8 Schematic of plasmonic retinal prosthetic, a) external view showing device and components; b) NIR laser interaction with eye and retina; c) schematic of NIR laser diffusing to the retina; d) retinal ganglion cell with AuNRs bound to its membrane

GNRs will be injected in naked dissected retinæ obtained from wild-type mice, to validate the efficacy of the S-ILM route, and then they shall diffuse to the GCL. Optimization of GNRs for the efficient delivery and circumvention of prominent barriers depends on several critical quality attributes (CQAs)⁷⁰. Particle size, hydrophilicity, surface charge and special membrane protein targets are some of the CQAs that were examined in depth with regards to drug delivery to the deep retina⁷⁰⁻⁷⁸. In the following sections, discussion of CQAs will revolve around optimum GNR delivery and toxicity/biocompatibility.

3.7.1: Retinal Ganglion Cell Targeting

In order to target specific cells, nanoparticles (NPs) can be equipped with special targeting ligands, proteins, antibodies, etc. In the case of RGCs several viable targets have been identified, the Ts1 neurotoxin domain was proposed as a neural target for its efficient binding, antibodies that target the TRPV1 channel are known as well; the P_2X_3 ion channel is also a viable target⁷⁹. Other techniques utilize the ability of the cholera B toxin (CBT) to bind to the S1R receptor (a known ligand-operated chaperone), in hopes of reducing oxidative stress damage and induce neuroprotective effects⁸⁰. Biotinylated anti-Thy 1.1 antibodies have also proven to be promising⁸¹.

Of the options presented, the two most promising avenues for the proposed application are the various neurotoxin routes and the biotinylated anti-Thy 1.1 antibodies. In order to prevent unwanted endocytosis of GNRs and the subsequent toxicity, and to achieve efficient local heating, GNRs need to be either bound to the surface of the target neuron or to reside within close proximity of the target neuron⁶⁷. Both neurotoxin routes possess strong affinity for binding to neurons^{79,80}, yet they both lead to the endocytosis of particles which can contribute to toxicity (**sub-section 3.7.6**)

Thy 1 is highly expressed on the surface of RGCs⁸¹, anti-CD 90 antibodies have been used to efficiently target these entities. As such, anti-CD 90.2 antibodies will be utilized in order to efficiently target Thy 1.2 on RGCs and achieve surface binding, necessary for GNR assisted NINS^{67,81}.

3.7.2: Biocompatibility

Although bulk gold may be biocompatible, many substances become toxic at the nanoscale⁸². Gold nanoparticles (AuNPs) have an increased ability to induce an immune response ; several methods exist relating to surface chemistry that can ameliorate the toxicity and the immune response. NPs have been researched extensively with their application in cancer therapy⁸³, yet their specific toxicity remains unclear. Several trends have been established with regards to size and application.

For example, systemically administered NPs have displayed toxic effects, yet when those same particles are delivered via a different route (e.g.: intraocular) they are non-toxic⁷⁵. Small NPs have been shown to clear the system rapidly, and larger NPs have more resident time and can act as drug reservoirs to release drugs over a long period of time⁷². The following sections will discuss the apparent toxicity as it relates to administration routes.

3.7.3: Administration Routes

One of the main reasons behind the variability of nanotoxicology reports concerning AuNPs is the fact that administration routes differ from study to study due to the wide range of applications. Intravenous (IV) administration, allows systemic distribution of the injected drug, some formulations present toxicity when they accumulate in organs, or when they disrupt cellular function^{75,82-86}. Special consideration needs to be taken in order to prepare particles for intravenous injection⁷⁴.

With regards to the proposed application intraocular injection provides the most advantages. Due to the nature of the tight cellular junctions at the blood-retinal barrier

(BRB), it is difficult to target retinal layers by administration via the IV route⁸⁷ (**Figure 9**).

Furthermore, each administration route provides advantages and disadvantages in terms of toxicity and effective delivery to the target site; several barriers must be circumvented (**sub-section 3.7.8**) in order to achieve targeted delivery.

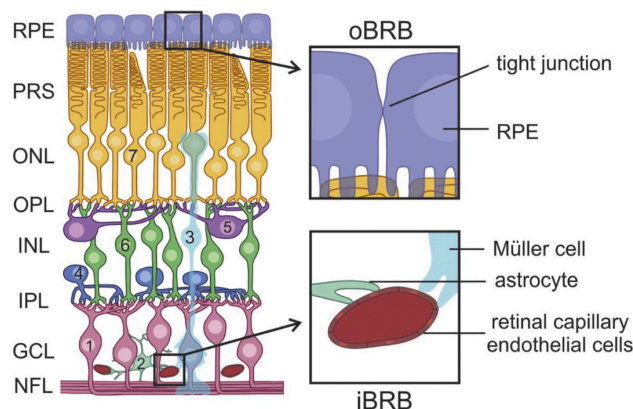


Figure 9 Schematic of the retina showing the BRB. PRS: photoreceptors; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; NFL: nerve fiber layer; oBRB: outer blood retinal barrier; iBRB: inner blood retinal barrier. Adapted from Peynashert et al. 2017 [87]

Several intraocular routes exist (**Figure 10**), in this section the sub-ILM (S-ILM) and intravitreal (IVT) routes will be examined. The S-ILM and IVT routes are among the most widely used routes in the treatment of diseases of the posterior chamber in the eye⁷¹.

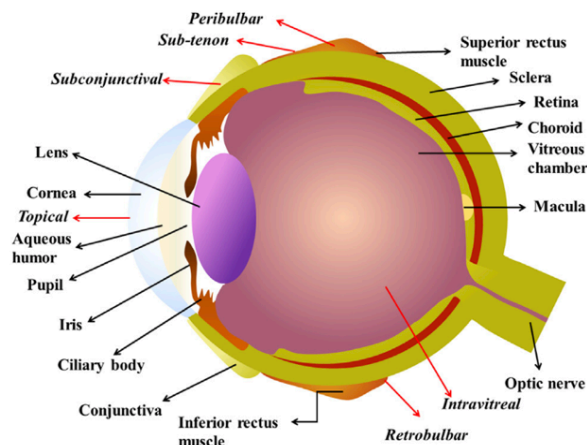


Figure 10 Schematic representation of the eye showing the various delivery routes. Adapted from Weng et al. 2017 [88]

3.7.4: Intravitreal Route

IVT delivery is one of the most commonly used route for intraocular drug delivery. It is shown in (**Figure 10**), this route circumvents all of the anterior barriers and delivers the drug “close” to the target, and is a relatively easy process that is conducted in a relaxed clinical setting⁸⁷. IVT injections are very safe, with a relatively low rate of complications. This method is however, highly dependent on the drug’s ability to migrate towards the retina, and permeate the posterior barriers (**sub-section 3.7.8**).

The two main hampers to successful IVT delivery are: vitreous humor (VH) and the inner limiting membrane (ILM) . Negatively charged particles can distribute successfully across VH, while positively charged particles are blocked by their interaction with components of VH⁸⁸. The ILM is a structural boundary between the vitreous and the retina, it varies in thickness towards the posterior. The structural components of the ILM are shown (**Figure 11**). Studies have shown the accumulation of therapeutics at the ILM in larger animals⁸⁹⁻⁹². Positively charged particles are completely blocked by the ILM, while neutral or negatively charged particles are permeable⁸⁷. A study utilizing bovine eyes was able to show that negatively charged particles (2000 kDa) penetrated the ILM more efficiently than positively charged ones (20 kDa)⁹³.

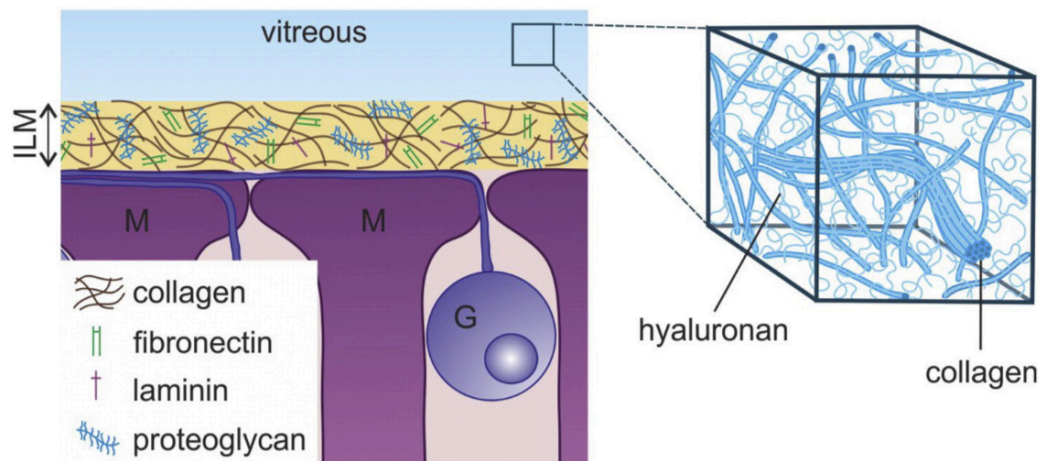


Figure 11 Diagram showing the vitreoretinal (VR) interface, with the structural components of VH and the ILM. Adapted from Le Goff et al. 2008 [94].

3.7.5: Sub-ILM route

The S-ILM space is defined as the space between the ILM and the neural retina.

S-ILM injections, physically permeate the ILM and then inject the desired substance within this space. A study intending to deliver viral vectors to the neural retina of a primate model was successful in demonstrating that S-ILM injections increased overall gene transfection in the GCL⁹⁵. S-ILM injections effectively circumvent all of the barriers of the anterior chamber directly targeting the retina. It is highly recommended for the current approach, due to the fact that it can circumvent the inner limiting membrane, a prominent barrier preventing the diffusion of AuNRs to the retina⁸⁹⁻⁹².

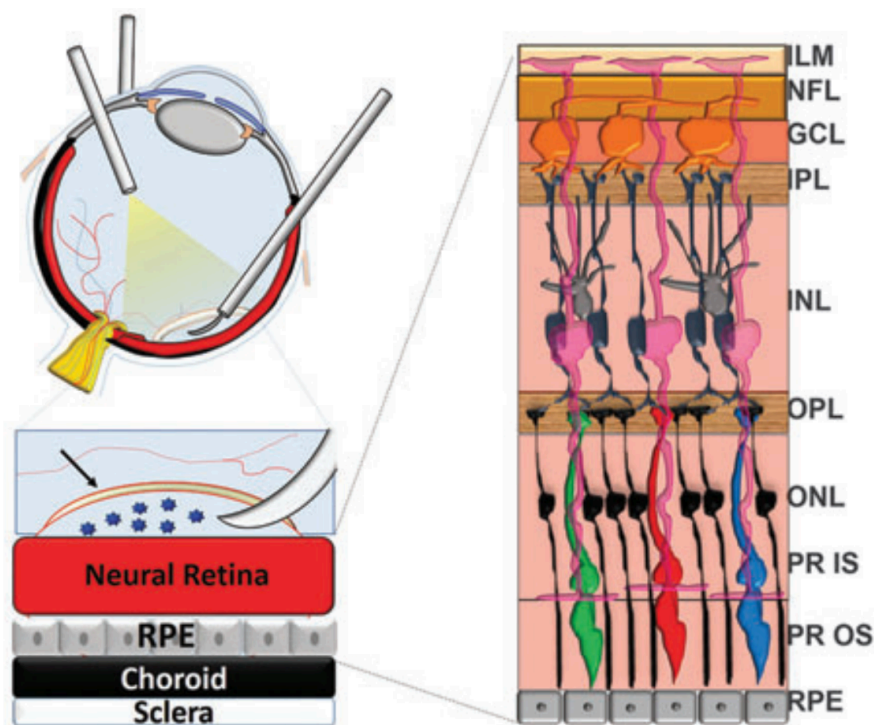


Figure 12 Schematic of S-ILM injections, adapted from [95]. ILM: Inner limiting membrane; NFL: Nerve fiber layer; GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer; PR IS: Photoreceptor inner segments; PR OS: Photoreceptor outer segments; RPE: Retinal pigmented epithelium

3.7.6: Size and Surface Charge

In addition to the importance of selecting an appropriate admin route, surface charge and overall size of NPs are both equally vital CQAs. Many studies attempt to identify overarching guidelines for NP toxicity, yet conflicting results have been presented thus far. In part, due to differences in administration routes, materials used, surface chemistries, etc. One study stated that toxicity is displayed over a range of NP size (8-37 nm). Particles smaller than (8 nm) and particles larger than (37 nm) displayed negligible toxicity⁸³. Another study stated that (2 nm) AuNPs were toxic, but only at high doses⁸⁵.

Concerning size, it seems that smaller NPs (8-25 nm) display the highest levels of toxicity^{75-78,87,96}. This however still depends on the administration route and the material utilized. Small NPs can induce both systemic and local toxicity; disrupting various cellular functions as a result of them being endocytosed. Once endocytosed particles are able to induce mitochondrial defects leading to cell death. Larger particles seem to be confined to local toxicity due to their hampered diffusion⁸³.

Surface charge has been reported as an important factor in both determining penetration and toxicity of an administered NP⁸³. Protein interaction with positively charged particles can hinder their diffusion through various membranes⁹⁷. Furthermore, certain negatively charged particles can induce various adverse effects⁸⁷. Bourges et al concluded that highly negative NPs (150 nm) successfully accumulated at the ILM in a rat model, followed by their diffusion to the retina⁹³.

Furthermore, positively charged particles interact with hyaluronan, a ubiquitous component of VH, leading to aggregation⁸⁸. Negatively charged particles are able to diffuse across VH, the same applies to neutral particles⁸⁷. A study that examined the diffusion of polystyrene beads in an ex-vivo bovine model (40, 100 and 200 nm). Showed that only the (40 nm) particles were found in the retina, and authors believe that their negative charge facilitated their diffusion through the vitreous, increasing their chances of reaching the retina⁹¹.

Authors also provide three important characteristics for retinal delivery⁹¹:

- 1) NP size must be lower than the mesh size of the vitreal collagen network (species dependent)

- 2) Negatively charged particles are necessary to avoid aggregation
- 3) Negatively charged particles have been reported to cross the ILM

Chapter 4: Outline of Current Study

The following section outlines the aims and experimental procedures in brief, with regards to the proposed application. For further details, please refer to *Methods* (Chapter 5).

Section 4.1: Examining the effects of photobleaching on AuNR fluorescence

One of the objectives of the study is to validate the attachment of AuNRs to RGCs *ex-vivo*, and the overall approach to support the feasibility of an *in-vivo* study in wild type mice. IVT injected GNRs will potentially be exposed to a great amount of light while diffusing from VH to the retina, and when they reside within the retina. The proposed *in-vivo* study may run over the course of one week, throughout which the mice will be subjected to a 12h dark/12h bright cycle.

We will prepare GNR samples in order to examine the extent of photobleaching on commercial GNRs. One sample was exposed to constant light for (24hrs/day) for one week, while one was left in complete darkness for the same extent, the control sample was measured immediately after preparing the sample. Emission intensity of the fluorescently tagged GNR samples were compared between groups.

Section 4.2: Establishing the efficacy of Sub-ILM injection

IVT injections have been utilized extensively as discussed in previous sections. Herein, we attempt a sub-ILM injection; the sub-ILM space is defined as the space

between the ILM and the neural retina. Sub-ILM injections, physically permeate the ILM and then inject the desired substance within this space. Dissected retinas with the ILM intact were injected utilizing a pulled glass micropipette tip attached to a nano-injector in order to deliver substances. One group (n=2 retinae) will receive an antibody injection via the sub-ILM route and then will be incubated with fluorescent dye, control samples (n=2) will receive fluorescent dye treatment without antibody. Retinae will be examined via confocal microscopy in order to validate S-ILM injections as an ILM circumvention method.

Section 4.3: Determining optimal injection parameters

In order to develop optimum parameters for *in-vivo* injections, *ex-vivo* experiments were conducted in order to fine tune injection parameters. These experiments determined whether or not GNR and antibody incubation was necessary or if they needed to be separately injected, and whether the needle gauge suggested in literature was suitable for S-ILM delivery. A custom dissection and injection mold was designed to practice various procedures on bovine eyeballs (*see results*). A 30G ½" needle was utilized to deliver brilliant blue G (BBG) dye to stain the ILM to serve as an indication of S-ILM delivery.

VH humor extracted from bovine eyeballs was utilized to examine the relative diffusional behavior of positively and negatively charged AuNRs. The samples (n=5 per group) were measured using a photon correlation spectrometer. Measurements of relative size were obtained and were related to relative diffusion, comparisons were made between samples placed in distilled water and extracted bovine VH. This

information was used to determine the appropriate surface charge for IVT AuNR delivery.

Glass microneedles were fabricated to be used with a nanoinjector for AuNR delivery to naked retinae (see *Methods*). The nanoinjector allows for enhanced control over the rate and the amount of delivered solutions. Control groups will not receive an injection, and will simply be dissected and stained with DAPI. A second group (n=2 retinae) will receive an injection of antibodies incubated with fluorescently tagged GNRs. A third group (n=2 retinae) shall receive two injections, first antibodies will be injected into the sub-ILM space, followed by a fluorescently tagged GNR injection within the same space, allowing the incubation process to occur within the retina. The same experimental setup will be carried out for two GNR sizes (10 X 38 nm, 25 X 87 nm ; *diameter X length*).

Chapter 5: Methods

Section 5.1:Ex-vivo Studies

5.1.1: Immunohistochemical procedure

5.1.1.1: AuNR photobleaching test

To test the effects of photobleaching on AuNR fluorescence, we prepared samples for emission testing. One group was tested immediately after preparation using a spectrofluorometer (Edinburgh Instruments Ltd., Livingston, UK); two groups are left in varying lighting conditions for one week. One group is left in darkness (24hrs/day) for one week, while one sample is placed in light for (24 hrs/day) for one week. After one

week, we tested samples for emission using the spectrofluorometer and their emissions were compared.

Approximately (200 μ L) of AuNR solution (CF-12-10-780-CY5-TS-PBS; 10 X 38 nm) are mixed with (1800 μ L) of distilled water to obtain a final volume of (2 mL) necessary for the experiment. The samples are placed in Eppendorf tubes, for immediate testing or storage using the parameters mentioned earlier. Dark samples are covered with aluminum foil to prevent any light exposure, while light samples are stored in ambient light fully exposed. Measurements for each sample are repeated (n=5) for statistical strength.

5.1.1.2: AuNR-antibody conjugation

In order to target RGCs, AuNRs are incubated with antibodies that are equipped with biotin. The streptavidin functionalized commercial AuNRs can bind to the biotin present on the antibody. We incubated a sample of (2 μ L) of GNRs solution (CF-12-10-780-CY5-TS-PBS or CF-12-25-780-488-TS-PBS, NANOPARTZ, Loveland, CO, USA) with (2 μ L) of anti-CD 90.2 antibody (BDbiosciences, Franklin, NJ, USA). After (1 hr) of incubation time, the antibody-GNR mixture is ready for administration.

5.1.1.3: Retinal Dissection

We euthanized by CO₂ inhalation and then confirmed by cervical dislocation. Eyes are then enucleated and transferred to an petri dish containing AMES media (MilliporeSigma, St. Louis, MO, USA) to ensure that the degradation process does not occur too rapidly. Rigor mortis related tissue stiffening can make the dissection procedure difficult.

Under visualization of a dissection microscope (Olympus, Shinjuku, Tokyo, Japan), the majority of the optic nerve is severed, then connective tissue is grappled by use of dissection forceps (World Precision Instruments, Sarasota, FL, USA). The majority of connective tissue is torn off by use of the forceps, while maintaining initial hold with the other pair. Once the eye has been rid of excess connective tissue, the equator separating the anterior portion of the eye from the posterior is located.

Once located, a pair of straight edged dissection scissors (World Precision Instruments, Sarasota, FL, USA) is used to make an initial puncture, then utilizing a pair of curved edged dissection scissors (World Precision Instruments, Sarasota, FL, USA) an incision is made at the puncture site, and is used to progressively hemi-sect the anterior portion of the eye from the posterior. Once hemi-section is complete, the anterior chamber including the lens are discarded.

Using the forceps, the sclera is torn away exposing the retina, while avoiding any mechanical damage to the retina. Once the retina is exposed, and the sclera is discarded, we cut the different sectors of the retina using a pair of straight edged dissection scissors as shown (**Figure 13**) to resemble a flower petal. The retina is then placed on a piece of filter paper while still submerged in AMES media and placed on the center of a glass microscope slide (Thermo Fisher Scientific, Waltham, MA, USA). The retina is then flattened by use of a fine paint brush (Princeton, Princeton, NJ, USA) with extreme caution as to not to tear the retina. If the retinal sample would dry out throughout the procedure it was re-submerged in AMES media as needed.

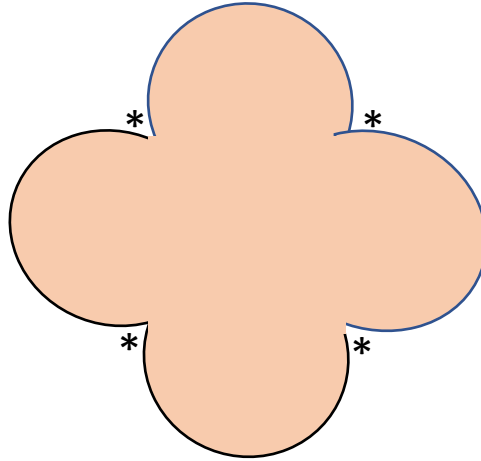


Figure 13 Diagram of dissected retina. Incisions are made with straight edged dissection scissors in order to obtain the petal configuration at the locations denoted by an asterisk. The petal configuration is necessary to successfully flatten the retinal specimen

Once the retinal sample is flattened sufficiently (**Figure 14**), we stained the retinal flat-mount with DAPI mounting media (MilliporeSigma, St. Louis, MO, USA), a glass cover slide is placed on the sample and the edges were sealed using glue.

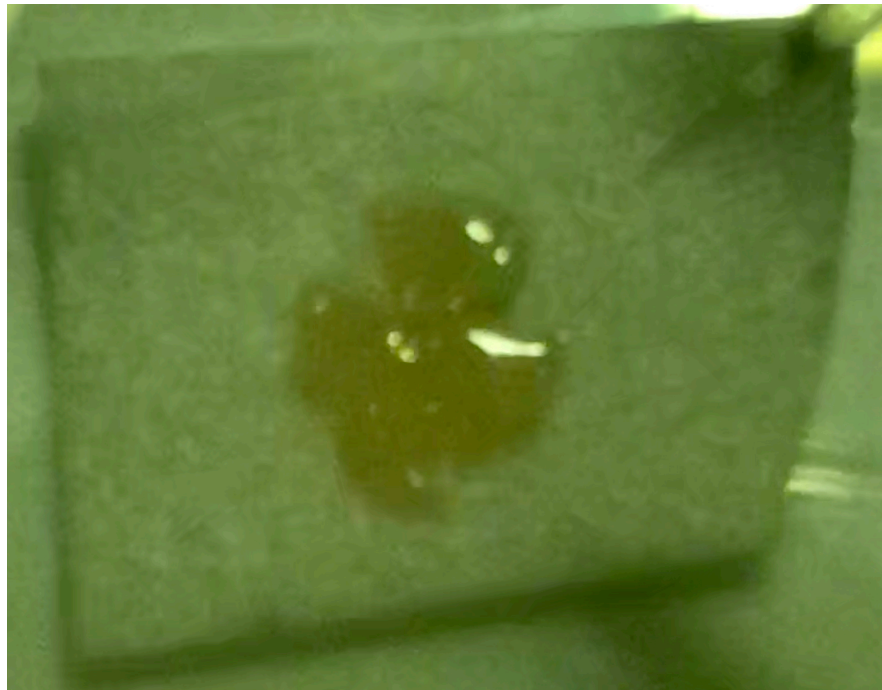


Figure 14 Image of dissected retina prior to injection of anti-CD 90.2 antibody, microneedle tip is shown in the top right corner.

5.1.2: Injection Procedure

The following procedures relate to the *ex-vivo* “naked retina” injections. After dissection and before the DAPI stain is applied, the retinal flat-mount is placed under visualization of the dissection microscope and by use of the nano-injector (NANOJECT, Drummond Scientific, Broomall, PA, USA) the desired substance is delivered.

5.1.2.1: Fabricating borosilicate microneedles

The nano-injector requires the use of pulled glass micropipettes in order to function. We fabricated microneedles by pulling borosilicate glass micropipettes supplied by the manufacturer (NANOJECT, Drummond Scientific, Broomall, PA, USA) in a micropipette puller (Sutter Instruments P97, Novato, CA, USA). Each micropipette was pulled into (n=2) microneedles (**Figure 15**).

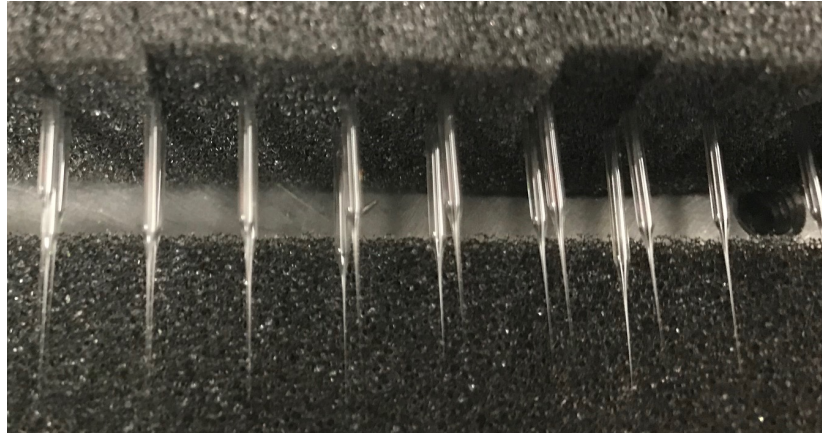


Figure 15 Image of borosilicate glass microneedles utilized for nano-injector

5.1.2.2: Backfilling/front filling the nano-injector

In order to deliver the desired substance, the nano-injector must first be backfilled with silicone oil (MilliporeSigma, St. Louis, MO, USA) as per the manufacturer’s recommendations. Using a 30G tuberculin syringe (Airt-tite, Virginia

Beach, VA, USA) silicone oil is extracted from the vial and inserted in the posterior of the pulled glass micropipette tip , the tip is then attached to the nano-injector and the collet secured (**Figure 16**).



Figure 16 Image of nano-injector setup. (Left) Shows typical injection setup under visualization of microscope, (Right) S-ILM injection on retinal-flat mount .

Front filling was necessary in order to deliver the desired substance. We placed a sample on a section of parafilm and the micropipette tip is directed towards the sample. Filling is made possible by using the “fill” function on the control unit of the nano-injector.

5.1.2.3: Sub-ILM injection

Once the nano-injector is successfully front filled, the retinal flat-mount is placed under visualization of the dissection microscope and the pulled glass micropipette tip is directed slowly towards the retina. Using the fine tune dials, the pipette tip is slowly inserted through the ILM and the injectable is released at a rate of (0.23 nL/s). Once the desired amount is injected (~2 μ L AuNR solution, ~2 μ L Antibody solution) , the pipette

tip is left in place for 30 seconds before slowly being retracted. The sample is left to incubate for 1 hr before washout, fixation and placement of the DAPI stain.

5.1.2.4: Preparation of control and S-ILM validation samples

Groups that did not receive AuNRs or injected materials have a slightly different procedure. One group is not to receive any injections whatsoever, samples in this group are dissected and immediately stained with DAPI and sealed. Another group however, is set to receive an antibody injection with no AuNRs. As such, post-dissection we injected the antibody (~1 µL) and the sample was allowed to incubate for (1 hr), then a drop of fluorescent dye is placed on the retinal sample, and after (1-2) minutes a DAPI stain is added. The sample is left to incubate for (1hr) before washout with AMES media , fixation and a glass slide is placed.

5.1.2.5: Retinal hydration and fixation

The retina, when *in-vivo*, is kept hydrated by ocular fluid and has access to oxygen. In order to prevent unwanted drying out of the retina throughout the various procedures that were outlined, AMES media is progressively added as needed. During fluorescence dye or AuNR incubation, a DAPI stain is used to maintain retinal hydration in order to decrease the overall length of the procedure for tissue viability concerns.

With regards to retinal fixation, we used a solution of (2%) paraformaldehyde and (1.5%) glutaraldehyde. A drop of this solution is added progressively for one hour, after the incubation processes are completed. Once fixation is completed, a DAPI stain is applied.

5.1.3: Confocal Imaging

We acquired images from retinal flat-mounts utilizing a confocal microscope (Zeiss LSM 800, Carl Zeiss AG, Oberkochen, Germany). Samples are irradiated with a laser corresponding to the excitation wavelength of the fluorescently tagged AuNRs. The (10 nm) GNRs are equipped with Cy5 (650 nm) and the (25 nm) GNRs are equipped with Alexa-488 (488 nm). For control samples, only DAPI signals are acquired. The laser intensity is set to (1.5%) and the pinhole size to (52 μm). All remaining settings are left to their default mode. Z-stacks are obtained by manually setting the first, last and center slices. Positions are manually and quantitatively set by examining fluorescence intensity at those respective tissue depths.

5.1.4: Bovine eyeball experiments

5.1.4.1: Injection and dissection mold

We designed a dissection mold using SolidWorks as shown in (**Figure 19**). The main function of this mold is to house a bovine eyeball while practicing dissections and injection procedures. The interior is hollowed to hold ice in order to maintain the viability of tissues while conducting procedures. We printed the mold using a MakerBot replicator (MakerBot, New York, NY, USA).

5.1.4.2: Injection and dissection mold

A 30G $\frac{1}{2}$ " hypodermic needle (Airt-tite, Virginia Beach, VA, USA), BBG dye (MilliporeSigma, St. Louis, MO, USA) was injected in a bovine eyeball via the S-ILM route. BBG is used to stain the ILM, and served as a method of verifying conventional hypodermic needles for S-ILM injections. After injection the eyeball was left to incubate

for 5 minutes before hemi-section and examination of the vitreoretinal (VR) interface to confirm ILM staining.

5.1.4.3: Bovine VH extraction and photon correlation spectroscopy

Bovine VH is extracted from hemisected bovine eyeballs (Rhode Island Beef and Veal Inc., Johnston, RI) using a spatula and is placed in Eppendorf tubes. Samples of AuNR (5 μ L) (positively charged NH₂ AuNRs, and negatively charged streptavidin commercial AuNRs; n=5 each group) are added to VH samples and then placed in a photon correlation spectrometer (Zetasizer Nano Z, Malvern Panalytical Inc. Westborough, MA, USA), control samples placed in distilled water. For samples measuring VH we set the parameters to measure proteins because VH is comprised of collagen, and for distilled water samples we set parameters to distilled water. Measurements of relative size are obtained and compared between groups.

5.1.5: Statistical Analysis

5.1.5.1: AuNR photobleaching test

We ran a normality test (Shapiro-Wilk) to determine distribution within the data set. A Friedman repeated measures analysis of variance on ranks was conducted to determine variance between samples. Data that did not follow parametric distribution is analyzed using non-parametric statistical methods. Furthermore, we ran an all pairwise multiple comparison procedures (Dunn's Method) analysis between groups. We conducted (n=5) measurements per sample in order to increase statistical strength.

5.1.5.2: AuNR diffusion in bovine vitreous humor

We ran a student's T-test between two groups, negatively charged streptavidin functionalized AuNRs and positively charged NH_2 functionalized AuNRs. The sample size was (n=5) per group.

Chapter 6: Results

Section 6.1: Examining the effects of photobleaching on AuNR fluorescence

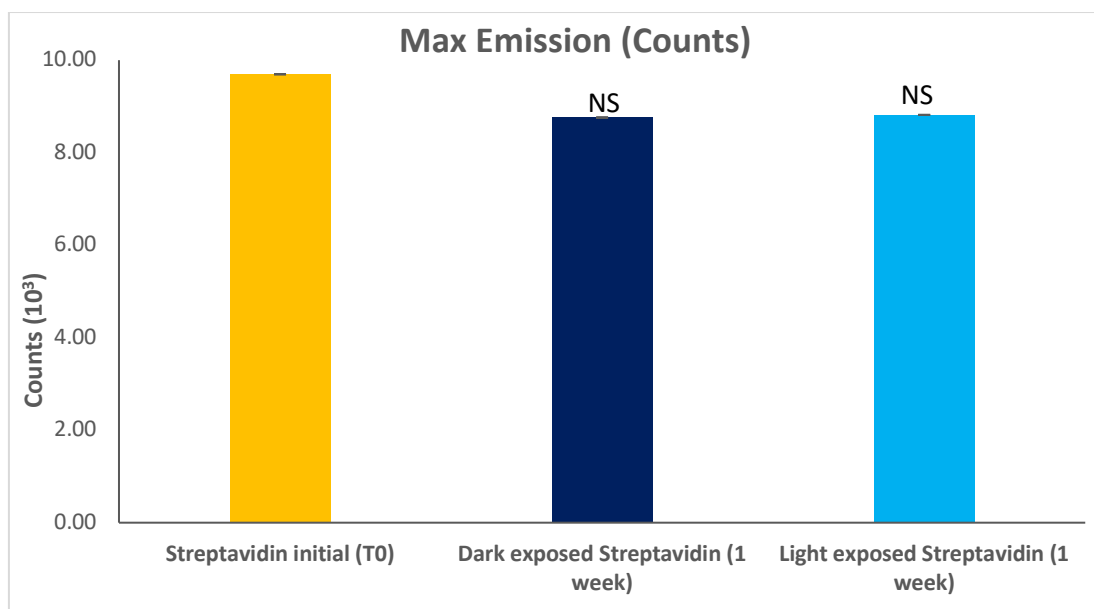


Figure 17 Results of photobleaching test. T_0 represents the initial timepoint of the streptavidin functionalized Cy5 fluorescently tagged AuNRs. Dark exposed represents the group that was left in darkness for 1 week, while the light exposed group represents the sample that was left in constant ambient light exposure for 1 week. Results are in fluorescent counts (10^3). Error bars represent standard deviation. A Shapiro-Wilk normality test showed that the distribution was non-normal. A Friedman repeated measures ANOVA with Dunn's post-hoc test showed no statistically significant differences between groups ($P > 0.05$) signified by "NS".

The photobleaching results obtained from the fluorometry experiment are shown in (**Figure 17**). Samples (n=3) were divided into three groups, one that was tested immediately after preparation (T_0 , Streptavidin initial), one group was left in darkness for one week (Dark exposed, 1 week) and the final group was left in constant ambient

light exposure for one week (Light exposed, 1 week). Average max emission was ($9.16 \text{ E3} \pm 1.65 \text{ E3}$) for the T_0 group, the dark exposure group had an average max emission of ($9.00 \text{ E3} \pm 1.65 \text{ E3}$) for the dark exposure group, the light exposure group had an average max emission of ($7.57 \text{ E3} \pm 1.13 \text{ E3}$). The Shapiro-Wilk test revealed distribution to be non-normal and thus non-parametric analysis was conducted (Friedman Repeated Measures ANOVA on ranks with Dunn's post-hoc test); results showed no statistically significant differences between groups ($P > 0.05$).

Section 6.2: Efficacy of Sub-ILM injection

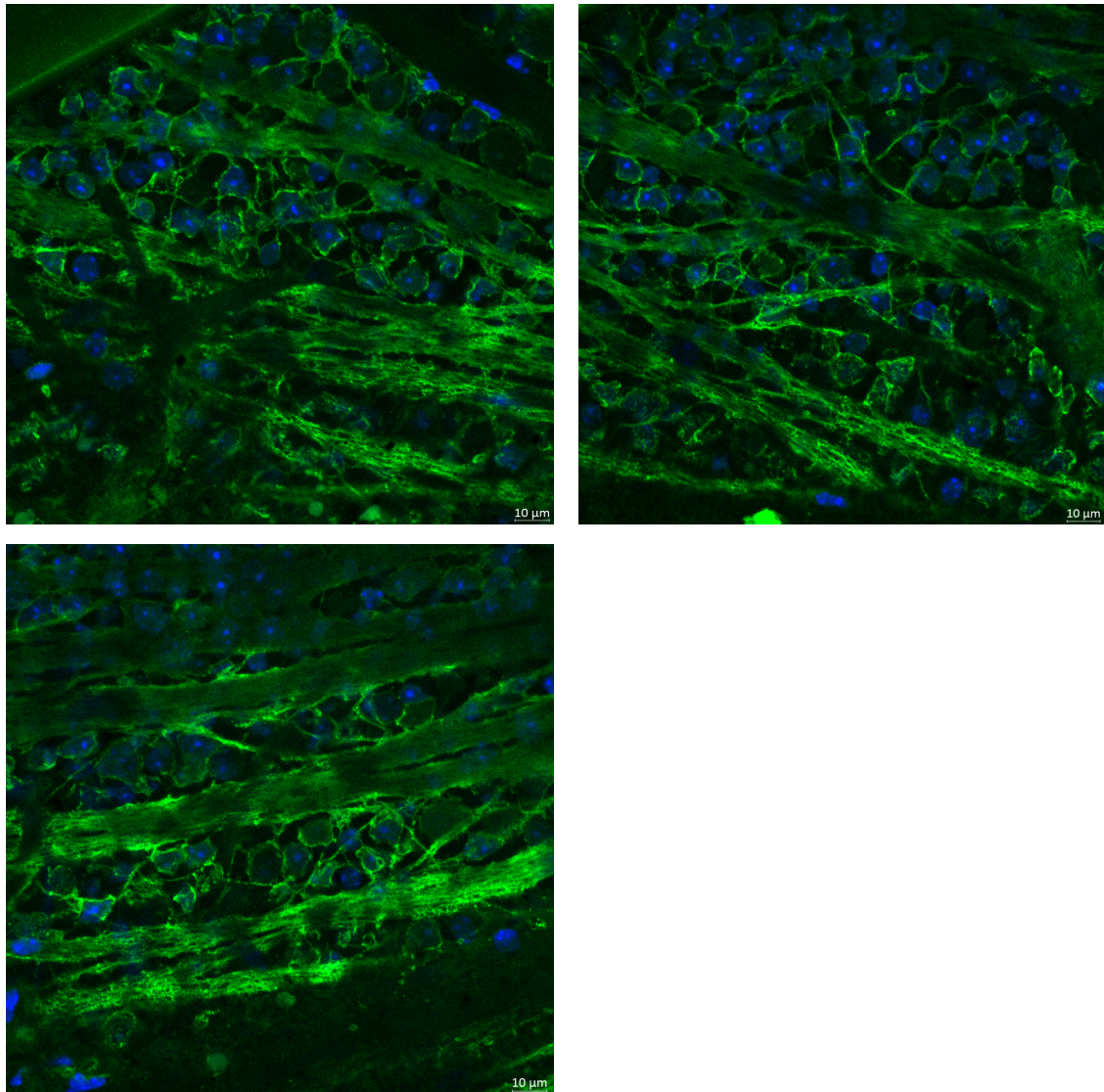


Figure 18 Retinal confocal images obtained from S-ILM injection validation experiment, showing fluorescence (green) functionalized with streptavidin binding to RGC membranes (RGCs shown in blue), axons are also shown (green). Samples were injected with biotinylated anti-CD 90.2 antibody, and incubated with FITC streptavidin fluorescent dye and a DAPI stain for RGC nuclei . Scale bar represents 10 μm .

Retinal confocal images obtained from retinal flat-mounts are shown in **(Figure 18)**, naked retinae were utilized to validate the efficacy of S-ILM injections.

Approximately (2 μL) of biotinylated anti-CD 90.2 antibody were injected via the sub-

ILM route followed by FITC streptavidin fluorescent dye and DAPI staining. Images show the FITC streptavidin dye bound to thy 1.2 that is abundant on axonal and RGC membranes (shown in green). RGCs are identifiable by their large nucleus shown in blue as a result of the DAPI stain. Images were taken at the center of the specimen where the fluorescent intensity was highest.

Section 6.3: Determining optimal injection parameters

6.3.1: Bovine Eyeball Experiments

6.3.1.1: Injection and dissection mold

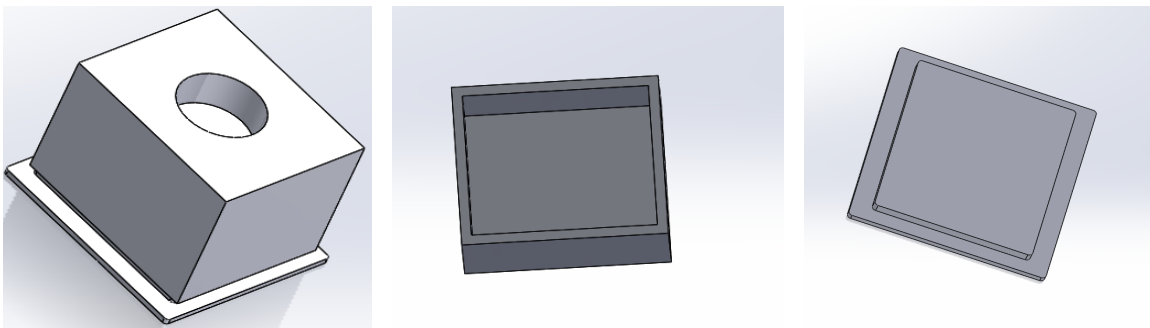


Figure 19 Bovine dissection and injection mold. (Left) Shows a top view of the overall dissection mold with the eyeball housing feature. (Middle) shows a bottom view of the top portion of the mold, outlining the hollow design meant to house ice. (Left) Top view of the base, meant to interface with the top to shut the mold when it contains ice.

The bovine dissection mold shown in (**Figure 19**) was designed to assist with bovine eyeball dissection and determining the appropriate injection method for S-ILM injections. The top of the mold is designed to house the bovine eyeball, and is hollowed in order to contain ice for the preservation of tissue. A 30G ½" needle was used to inject brilliant blue G dye into a bovine eyeball using the S-ILM route (*results not shown*). Staining was only confirmed within the VH of the bovine eyeball specimens.

6.3.1.2: AuNR diffusion in bovine VH

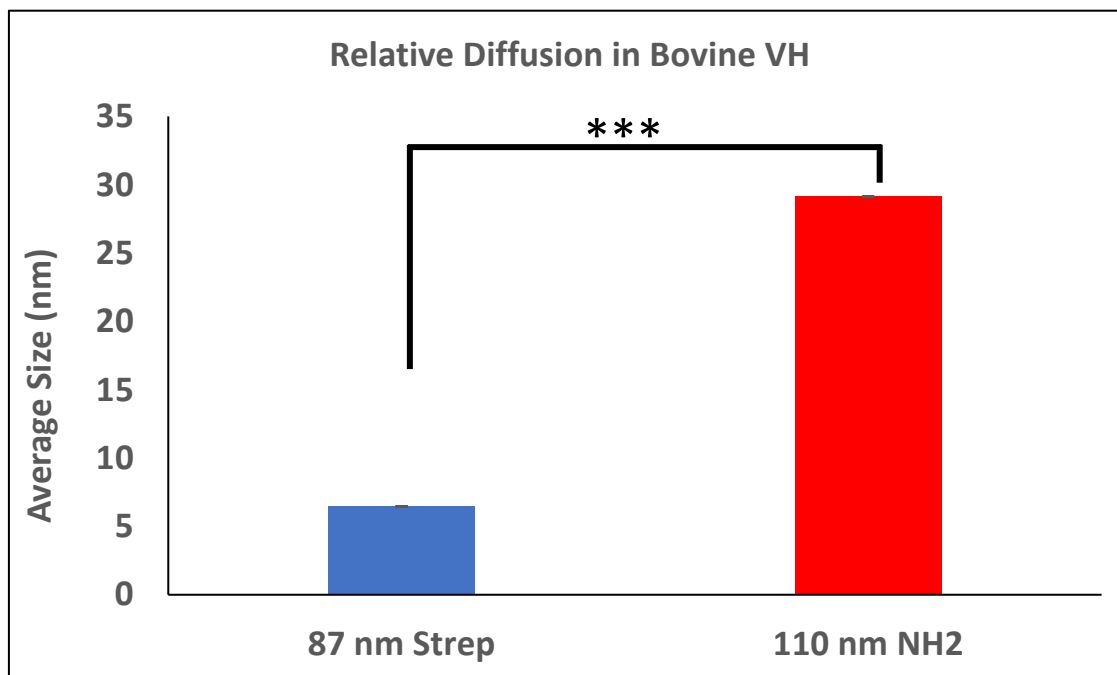


Figure 20 Results of photon correlation spectroscopy experiment. The blue bar represents the relative diffusion of negatively charged streptavidin functionalized AuNRs; the red bar represents the relative diffusion of positively charged NH₂ functionalized AuNRs. Student's T-test revealed that results were statistically significant between groups ($P = 1.079 \times 10^{-40}$). Error bars represent standard deviation.

Photon correlation spectroscopy (PCS) results are shown in (**Figure 20**), AuNR samples were added to extracted bovine VH and measured using PCS. The negatively charged streptavidin functionalized AuNRs had a (92%) difference in their apparent size compared to their actual (87 nm) size. The positively charged NH₂ particles had a (72%) difference in size compared to their actual size (110 nm). A student's T-test revealed that results were statistically significant ($P = 1.079 \times 10^{-40}$).

6.3.2: AuNR S-ILM injections

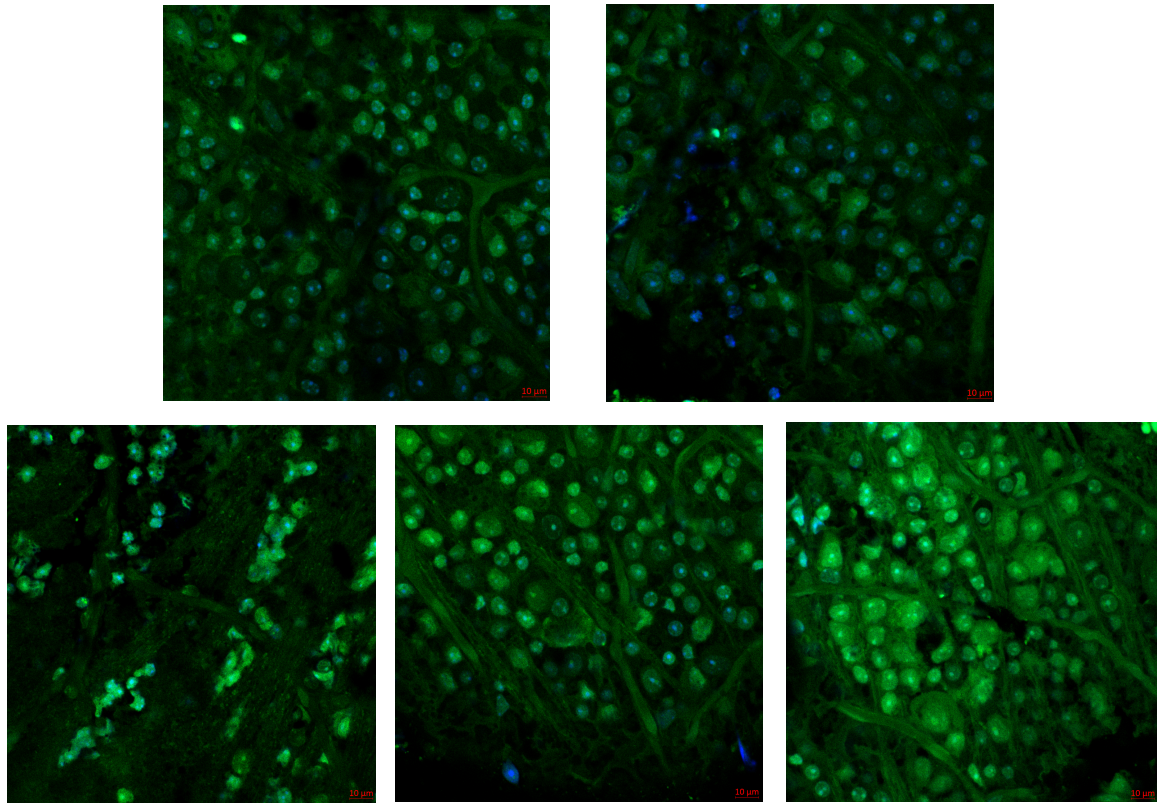


Figure 21 Confocal images obtained from S-ILM injection of large-sized AuNRs (25 X 87 nm, streptavidin functionalized, FITC fluorophore) in naked retinal flat-mounts. Biotinylated anti-CD 90.2 antibodies were pre-incubated with streptavidin functionalized AuNRs (2 μ L each, 4 μ L total injection volume) and injected via the S-ILM route. RGC nuclei are stained with DAPI (blue) and AuNRs are fluorescently tagged with FITC fluorophores. (Top row) images taken using (1%) laser intensity. (Bottom row) images taken with increased laser strength (2%). Axonal projections are also visible shown in green. Scale bar represents (10 μ m).

S-ILM injections were conducted on naked retinal flat-mounts (n=2) with their ILMs intact to deliver FITC fluorescently tagged streptavidin functionalized AuNRs (CF-12-25-780-488) that were pre-incubated with biotinylated anti-CD 90.2 antibodies. A total of (4 μ L) of antibody-AuNR mixture was injected via the S-ILM route and allowed to incubate for (1.5 hrs) before washout and DAPI staining. Confocal images are shown in (**Figure 21**), the top row represents images that were taken at an initial laser intensity of (1%). The bottom row represents images that were taken at an increased laser intensity

of (2%). Axonal projections are shown in both rows. RGCs are shown by circular shapes, stained with DAPI shown in blue, AuNRs are fluorescently tagged with FITC fluorophores shown in green.

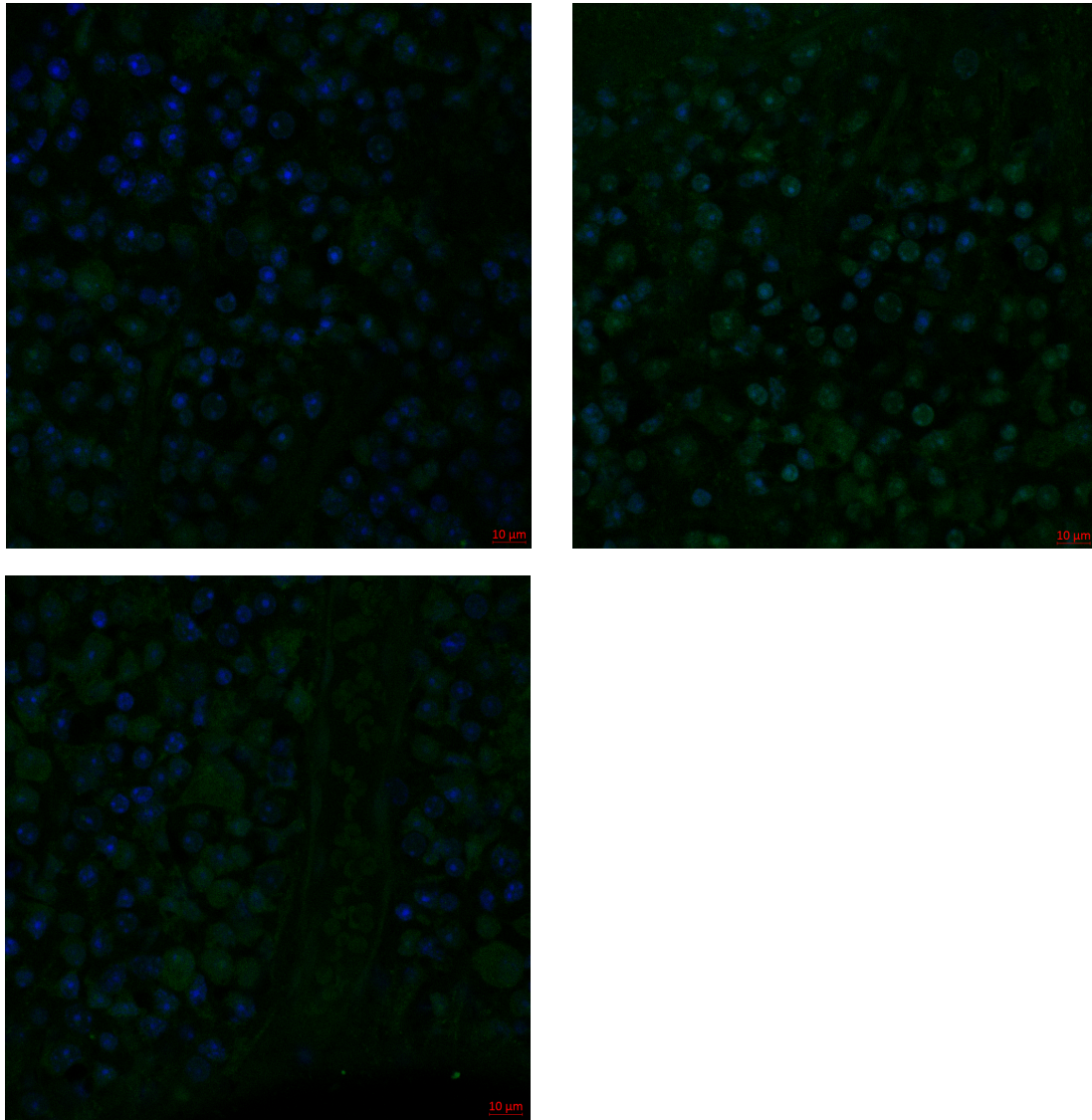


Figure 22 Confocal images obtained from S-ILM injection of large-sized AuNRs (25 X 87 nm, streptavidin functionalized, FITC fluorophore) in naked retinal flat-mounts. Biotinylated anti-CD 90.2 antibodies (2 μ L) were injected via the S-ILM route and allowed to incubate before being washed. AuNR injection (2 μ L) via the S-ILM route followed, and the sample was washed after incubation. RGC nuclei are stained using DAPI (shown in blue), while AuNRs are fluorescently tagged with FITC (shown in green). Scale bar represents (10 μ m)

S-ILM injections were conducted on naked retinal flat-mounts (n=2) with their ILMs intact in order to first deliver biotinylated anti-CD 90.2 antibodies, before

delivering AuNRs via the same route. Approximately (2 μ L) of biotinylated anti-CD 90.2 antibody was injected and allowed to incubate for (1hr) before washout and AuNR injection (2 μ L)(CF-12-25-780-473). AuNRs were allowed to incubate for (1 hr) before washout and DAPI staining. Confocal images are shown in (**Figure 22**). RGC nuclei are shown in blue, and AuNRs fluorescently tagged with FITC fluorophores shown in green.

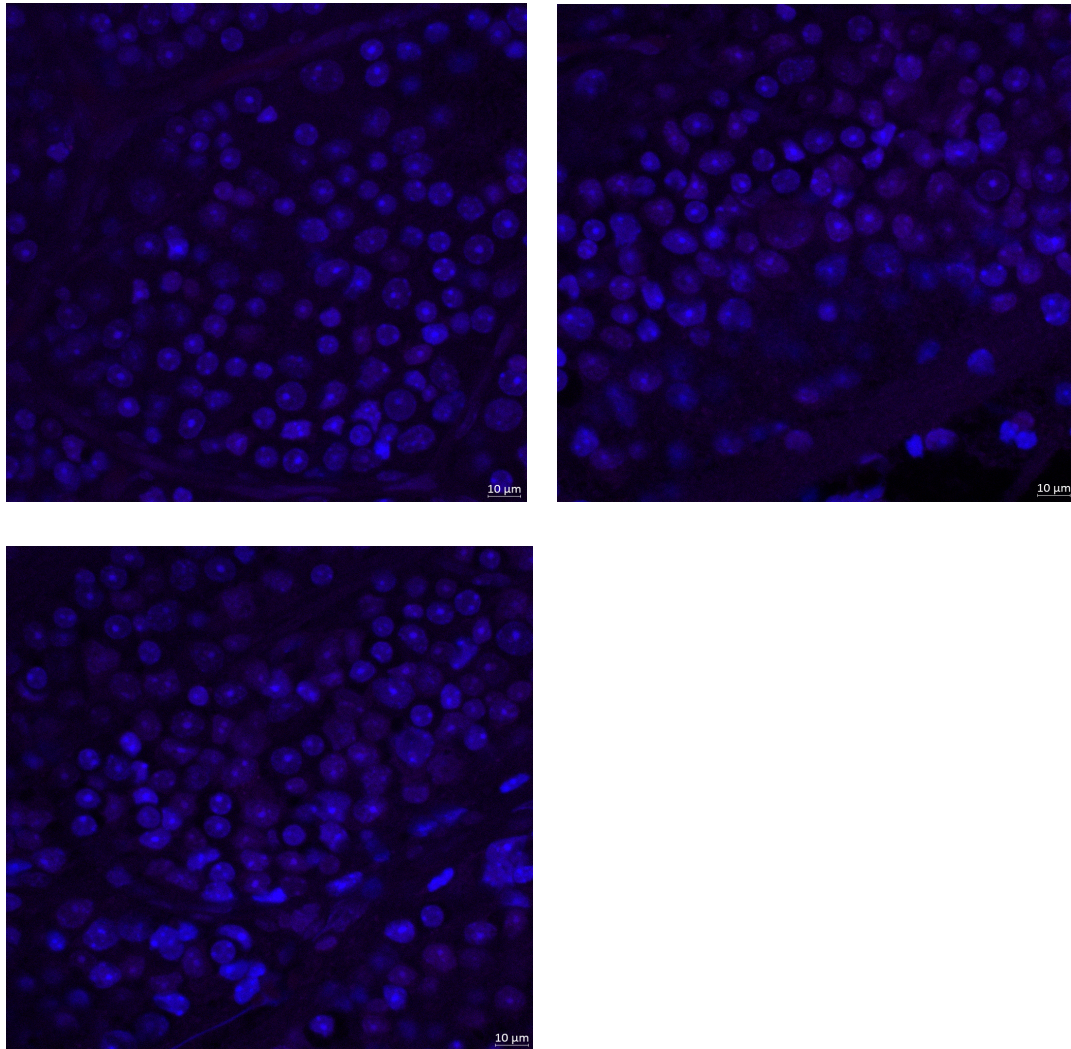


Figure 23 Confocal images obtained from S-ILM injection of small-sized AuNRs (10 X 38 nm, streptavidin functionalized, Cy5 fluorophore) in naked retinal flat-mounts. Biotinylated anti-CD 90.2 antibodies (2 μ L) were incubated with (2 μ L) of AuNRs and allowed to incubate for (1hr) pre-injection. Naked retinal-flat-mounts received (4 μ L) of the antibody-AuNR mixture via the S-ILM route. RGC nuclei are stained using DAPI (shown in blue), while AuNRs are fluorescently tagged with Cy5 (shown in magenta). Scale bar represents (10 μ m)

Biotinylated anti-CD 90.2 antibodies (2 μ L) were incubated with (2 μ L) of streptavidin functionalized Cy5 tagged AuNRs (CF-12-10-780-Cy5) for (1hr) pre-injection. Naked retinal flat-mounts were injected via the S-ILM route with (4 μ L) of the antibody-AuNR mixture; the sample was allowed to incubate for (1.5 hrs) before washout and DAPI stain. Confocal images are shown in (**Figure 23**) , RGC nuclei are stained using DAPI

and are shown in blue, AuNRs are fluorescently tagged with Cy5 fluorophores shown in magenta.

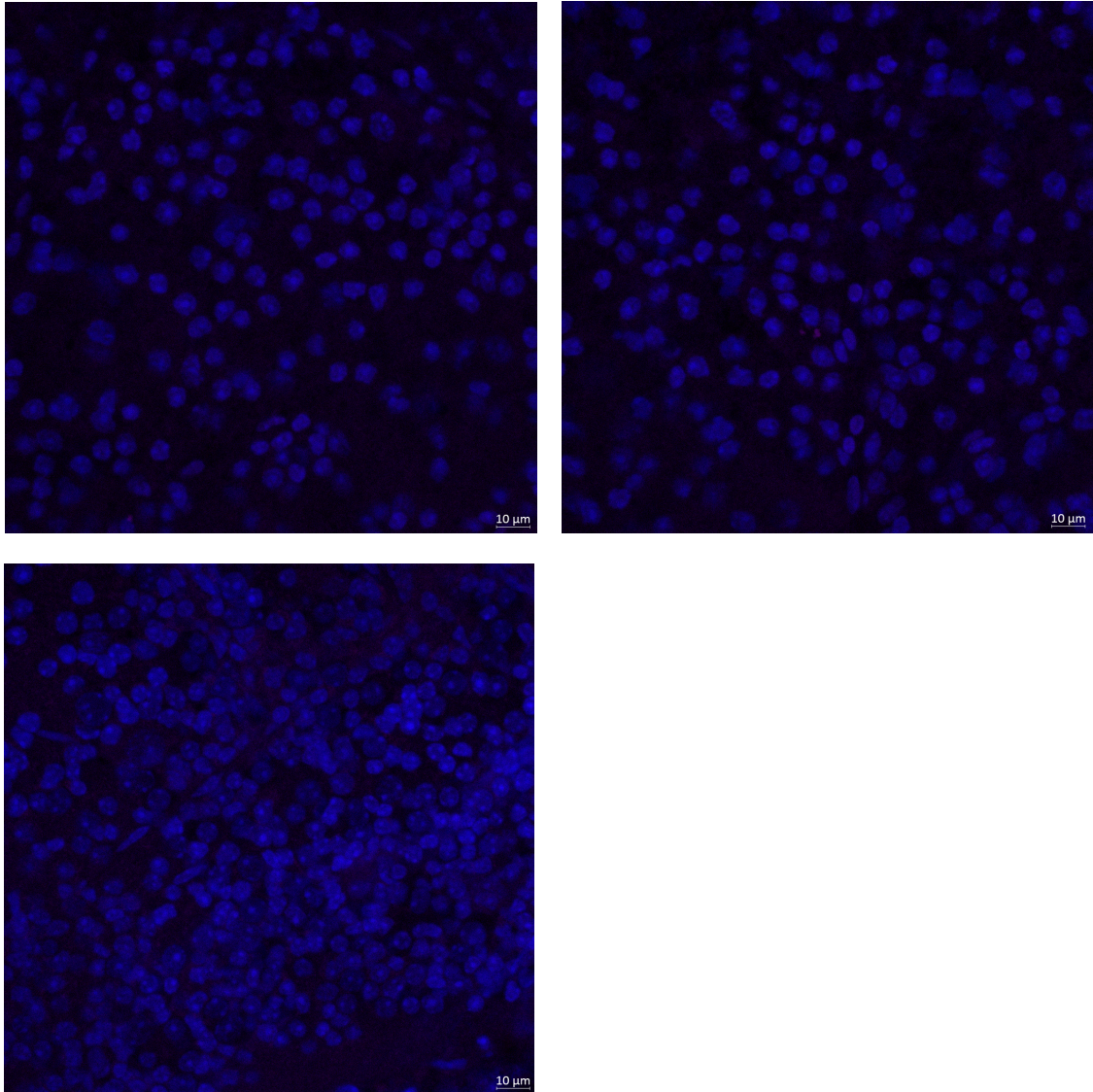


Figure 24 Confocal images obtained from S-ILM injection of small-sized AuNRs (10 X 38 nm, streptavidin functionalized, Cy5 fluorophore) in naked retinal flat-mounts. Biotinylated anti-CD 90.2 antibodies (2 μ L) were injected via the S-ILM route and allowed to incubate before being washed. AuNR injection (2 μ L) via the S-ILM route followed, and the sample was washed after incubation. RGC nuclei are stained using DAPI (shown in blue), while AuNRs are fluorescently tagged with Cy5 (shown in magenta). Scale bar represents (10 μ m)

S-ILM injections were conducted on naked retinal flat-mounts (n=2) with their ILMs intact in order to first deliver biotinylated anti-CD 90.2 antibodies, before delivering smaller-sized AuNRs (10 X 38 nm) via the same route. Approximately (2 μ L) of biotinylated anti-CD 90.2 antibody was injected and allowed to incubate for (1hr) before washout and AuNR injection (2 μ L)(CF-12-25-780-488). AuNRs were allowed to incubate for (1 hr) before washout and DAPI staining. Confocal images are shown in (**Figure 24**). RGC nuclei are shown in blue, and AuNRs are fluorescently tagged with Cy5 shown in magenta.

Chapter 7: Discussion

Section 7.1: Examining the effects of photobleaching on AuNR fluorescence

The effects of photobleaching on fluorescently tagged AuNRs was examined utilizing a spectrofluorometer after varying the conditions of light exposure on samples. One group was left in complete darkness for 1 week, one group was left in constant ambient lighting for (24 hrs/day) for 1 week, and one group was tested immediately after sample preparation. This was done in order to validate the feasibility of a terminal in-vivo study utilizing AuNRs, in which AuNRs may be subjected to ambient light exposure during a typical (12h light/ 12h dark) cycle.

The graph shown in (**Figure 17**) shows the extent of photobleaching on these various samples, and they are very similar with regards to max emission. The difference between light exposed and dark exposed samples was (4.5%), and the difference between initial measurements and the one week light exposure group was (12%). A

student's T-test revealed that the differences between all groups was not statistically significant ($P > 0.05$). Furthermore, a difference of (12%) can be accounted for by increasing the size of the pinhole or increasing laser strength during confocal imaging procedures.

Distribution was non-normal as a result of the nature of spectrofluorometric measurements. The wavelength of the excitation laser is progressively increased in a pre-defined range. This range was defined based on the excitation wavelength of the Cy5 functionalized AuNRs (646 nm), as such the detector picks up noise signals as a result of the excitation laser. Max emissions were obtained from the specified max emission wavelength specified by the manufacturer. Statistical analysis would suggest that fluorescently tagged AuNR photobleaching effects are negligible, and the suggested approach for in-vivo experimentation has been validated with regards to photobleaching.

Section 7.2: Establishing the efficacy of Sub-ILM injection

In hopes of validating S-ILM injections, we conducted experiments to examine the attachment of fluorescent dye to RGCs. In brief, we injected (2 μ L) of biotinylated anti-CD 90.2 antibody via the S-ILM route and allowed to bind to RGCs before washout and fluorescent incubation with FITC streptavidin dye. The results of these experiments are shown in (**Figure 18**), where FITC-streptavidin is shown in green, and RGC nuclei are shown in blue. Since Thy 1.2 is also abundant on axons, we observed that FITC-streptavidin was also binding to axons as shown in the figure. FITC-streptavidin was able to bind to RGC membranes and was resistant to washout (3X with AMES media).

S-ILM injection is effective for delivering antibodies to the RGC layer, further testing was required to determine if AuNRs would be able to effectively bind in a manner similar to FITC-streptavidin. AuNRs are functionalized with streptavidin, and thus may bind to RGC cells; it is worth noting that FITC-streptavidin dye has a higher concentration of streptavidin than AuNRs due to their size and ability of materials to bind to AuNRs. FITC-streptavidin dye was able to diffuse from the ILM level to RGC since the fluorescent dye was applied using a pipette directly on to the ILM. Furthermore, the resistance to washout further outlines the ability of the antibodies to effectively bind to Thy 1.2 on RGCs. AuNRs were injected via the S-ILM route similar to the antibody injection.

Section 7.3: Determining optimal injection parameters

The 30G ½" needle that was suggested for use in literature for IVT injections was not suitable for S-ILM injections. In part, due to the length of the needle, it is not able to reach the neural retina in the location specified in (**Figure 12**). Obtaining a longer needle would be feasible for ex-vivo injections but it does not allow for precise control of the amount required, even when utilizing specialized syringes. As such, the nano-injector approach was utilized in order to obtain a controlled rate of (0.23 nL/s) and to be able to handle minute amounts of fluid in the (μ L) range.

PCS experiment were conducted in order to determine the relative diffusion of negatively charged and positively charged AuNRs (**Figure 20**). The measurements of particle size were related to the diffusion of particles in samples of bovine VH: since the device detects scattering light from particles present in aqueous samples. Samples that

report sizes larger than their relative size could suggest potential aggregation, or interaction with components of VH. If particles were reported smaller than their actual size, it could suggest that they are achieving the desired diffusion through VH.

Particle sizes from both positively and negatively charged particles differed from their actual manufactured sizes. This was most likely due to the fact that VH interfered with the readings, yet the extent of the difference between both groups varied. Both groups reported sizes that were less than their actual sizes, yet the negatively charged streptavidin AuNRs resulted in a (93%) difference, while the positively charged NH₂ AuNRs resulted in a (73% difference). A student's T-test revealed that the differences between groups was highly significant ($P = 1.079 \times 10^{-40}$). These findings lead to the notion that negatively charged AuNRs experience better diffusion than positively charged AuNRs, and are recommended for in-vivo experimentation and for IVT injections that heavily rely on a particle's ability to diffuse through VH.

With regards to particles that are injected via the S-ILM route, there are conflicting reports. The ILM completely blocks positively charged particles, while allowing neutral to negatively charged particles through to the neural retina. For S-ILM injections, positively charged particles may be well tolerated, but until the S-ILM injection method is completely optimized it is recommended that negatively charged particles be utilized in order to increase their chances of diffusion to the neural retina. As such, we utilized streptavidin functionalized AuNRs for S-ILM experiments.

With regards to AuNR S-ILM delivery, we conducted experiments to validate the delivery route for AuNR delivery and to examine the effects of different sizes on RGC

targeting. Furthermore, we attempted to optimize injection parameters with regards to antibody incubation. We injected large-sized AuNRs (25 X 87 nm, streptavidin functionalized, FITC tagged) via the S-ILM route in naked retinal flat-mounts (n=4). We varied antibody incubation conditions, in one group (n=2) we injected biotinylated anti-CD 90.2 antibodies via the S-ILM route and allowed them to incubate for (1 hr) before washing and injecting AuNRs (**Figure 21**). Another group (n=2) received an S-ILM injection containing an antibody-AuNR mixture. We pre-incubated streptavidin functionalized AuNRs with biotinylated anti-CD 90.2 antibodies for (1hr) before injecting via the S-ILM route. Results are shown in (**Figure 22**).

We injected small-sized AuNRs (10 X 38 nm, streptavidin functionalized, Cy5 tagged) via the S-ILM route in naked retinal flat-mount (n=4). We varied the antibody incubation conditions similar to what was conducted with large-sized AuNRs. Results from the pre-incubation group are shown in (**Figure 23**); results from the dual S-ILM injection group are shown in (**Figure 24**). We hypothesized that AuNR binding would be similar to that which was observed in (**Figure 18**), with AuNRs binding to RGC membranes and outlining the structures of axons. As observed in the S-ILM AuNR trials (**Figures 21-24**), fluorescence emission other than DAPI was not sufficiently detected. Increasing laser strength did not improve the outcome (**Figure 21**). This trend was common for both small sized AuNRs and large-sized AuNRs, yet large AuNRs had better axonal labeling than small-sized AuNRs.

Larger-sized AuNRs (CF-12-25-780-488) possess twice as many streptavidin molecules per nanorod than small-sized AuNRs (CF-12-10-780-Cy5). Furthermore, the

difference in size may also account for differences in fluorescence intensity. Smaller-sized AuNRs might possess less fluorophores per molecule than large-sized AuNRs, the exact number of fluorophores per molecule is not known; nor was it provided by the manufacturer. FITC-streptavidin molecules are approximately (5 nm) in size, and antibodies are much smaller than both AuNRs and FITC-streptavidin, whereas large-sized AuNRs were (25 X 87 nm) and small-sized AuNRs were (10 X 38 nm). If the S-ILM injection that was conducted was not sufficient to deliver antibodies past the ILM, it is possible that they could diffuse to the retina on their own. AuNRs may not possess the same ability to diffuse to the retina due to the difference in size. Further experimentation is necessary to determine the validity of S-ILM injections for AuNR delivery to the retina; antibodies would be incubated with retinal samples as opposed to injected via the S-ILM route. This would confirm the validity of S-ILM injections and their necessity for antibody delivery.

In order to optimize the AuNR delivery parameters, experiments need to be conducted that vary the ratio of antibody solution to AuNR solution. In current experiments, we adopted a 1:1 ratio, future work could increase the volume of AuNR solution to obtain a ratio of 2:1, or 4:1. It is hypothesized that the number of molecules in the biotinylated antibody solution differ from AuNRs, as such there could be less AuNRs available to bind to biotinylated antibodies that are bound to Thy 1.2 on RGCs and axonal projections. Varying the ratio may improve the results due to discrepancies between the number of fluorophores present on AuNRs and the number of FITC fluorophores bound to the streptavidin molecules of FITC-strep.

Chapter 8: Conclusion

We were able to characterize the effects of photobleaching on commercial fluorescently tagged AuNRs. We found that the effects of photobleaching are negligible and AuNRs can withstand ambient light exposure up to 1 week. This supports the feasibility of an in-vivo experiment in which AuNRs are injected and reside in the retina for 1 week, allowing for stimulation studies to take place before euthanasia and dissection of retinæ for confocal analysis. Furthermore, we validated the efficacy of S-ILM injections for antibody delivery ex-vivo ; supporting the feasibility of utilizing S-ILM injections for in-vivo studies relevant to NINS. We also validated the overall biotin-streptavidin approach for RGC targeting. As shown in confocal images biotinylated antibodies were bound to RGC membranes and streptavidin functionalized fluorescent dye was able to bind to the antibodies.

We determined that the most efficient method for S-ILM injections and IVT injections for ex-vivo experiments was the nano-injector approach. The nano-injector allows for increased control over the rate of injection of the desired substance and enhanced control over the amount within the (μ L) range. With regards to surface charge and interactions with components of VH, we were able to demonstrate via PCS that the relative diffusion of negatively charged AuNRs in VH is less hampered than the diffusion of positively charged particles. PCS results suggest that negatively charged particles may improve the chances of AuNRs diffusing to the neural retina when injected via the IVT route, as well as the S-ILM route.

The efficacy of S-ILM injections for the delivery of AuNRs to the retina still remains unclear. Delivery of antibodies seems to have been successful, but due to their small-size compared to AuNRs it is not enough evidence to reach a conclusion with regards to the efficacy of this delivery route. Lower intensity signals observed in AuNR figures suggest that further optimization is required to determine efficacy of AuNR delivery to the retina. Parameters such as antibody and AuNR ratios, size of AuNRs and type of fluorophore utilized are all valid routes to troubleshoot AuNR delivery. Future work will focus on optimizing these parameters in order to reach a conclusion with regards to S-ILM delivery of AuNRs to the neural retina.

9: References

- [1] Pascolini, D. & Mariotti, S. Global estimates of visual impairment: 2010. *British Journal of Ophthalmology* 96, 2011, 614-618.
- [2] Al Merjan JI, et al. Registered blindness and low vision in Kuwait. *Ophthalmic Epidemiol* 12, 2005, 251–57.
- [3] Hartong, D. et al. Retinitis pigmentosa. *The Lancet* 368, 2006,1795-1809.
- [4] Bunker CH, et al. Prevalence of retinitis pigmentosa in Maine. *Am J Ophthalmol* 97, 1984.,357–65
- [5] Grondahl J. Estimation of prognosis and prevalence of retinitis pigmentosa and Usher syndrome in Norway. *Clin Genet* 31, 1987., 255–64
- [6] Novak-Lauš K, et al. Primary tapetoretinal dystrophies as the cause of blindness and impaired vision in the republic of Croatia. *Acta Clin Croat* 41, 2002 ,23–27
- [7] Kefalov, Vladimir, et al. “Role of Visual Pigment Properties in Rod and Cone Phototransduction.” *Nature*, vol. 425, no. 6957, 2003, pp. 526–531.
- [8] Klapper, Simon D., et al. “Biophysical Properties of Optogenetic Tools and Their Application for Vision Restoration Approaches.” *Frontiers in Systems Neuroscience*, vol. 10, Feb. 2016, doi:10.3389/fnsys.2016.00074.
- [9] Arshavsky, VY, et al. “G Proteins and Phototransduction.” *G Proteins and Phototransduction / Annual Review of Physiology*, 2002,
- [10] Witkovsky P et al. A microspectrophotometric study of normal and artificial visual pigments in the photoreceptors of *Xenopus laevis*. *Vision Res* 1981;21:867–873
- [11] Moritz O. L. et al. A functional rhodopsin–green fluorescent protein fusion protein localizes correctly in transgenic *Xenopus laevis* retinal rods and is expressed in a time-dependent pattern. *J Biol Chem* 2001;276:28242–28251
- [12] Gargini, C. et al. Retinal organization in the retinal degeneration 10 (rd10) mutant mouse: A morphological and ERG study. *The Journal of Comparative Neurology* 500, 2006,222-238

- [13] Sung, C.-H. et al. A rhodopsin gene mutation responsible for autosomal dominant retinitis pigmentosa results in a protein that is defective in localization to the photoreceptor outer segment. *J. Neurosci.* 14, 1994.,5818-5833
- [14] Dryja, T.P. et al. A point mutation of the rhodopsin gene in one form of retinitis pigmentosa. *Nature* 343, 1990, 364-366
- [15] Campochiaro, P et al. The mechanism of cone cell death in Retinitis Pigmentosa. *Progress in Retinal and Eye Research* (2017).
- [16] Petters, R. et al. Genetically engineered large animal model for studying cone photoreceptor survival and degeneration in retinitis pigmentosa. *Nature Biotechnology* 15, 1997, 965-970
- [17] Kondo, Mineo, et al. "Generation of a Transgenic Rabbit Model of Retinal Degeneration." *Investigative Ophthalmology & Visual Science*, vol. 50, no. 3, Jan. 2009, p. 1371.
- [18] Lin, B. et al. Remodeling of cone photoreceptor cells after rod degeneration in rd mice. *Exp. Eye Res.* 88 (3), 2009,589-599
- [19] Ripps, H et al. Cell Death in Retinitis Pigmentosa: Gap Junctions and the 'Bystander' Effect. *Experimental Eye Research* 74,2002,327-336
- [20] Peng, B. et al. Suppression of Microglial Activation Is Neuroprotective in a Mouse Model of Human Retinitis Pigmentosa. *Journal of Neuroscience* 34, 2014,8139-8150
- [21] L veillard, T. et al. Identification and characterization of rod-derived cone viability factor. *Nature Genetics* 36, 2004,755-759
- [22] Byrne, L. et al. Viral-mediated RdCVF and RdCVFL expression protects cone and rod photoreceptors in retinal degeneration. *Journal of Clinical Investigation* 125, 2014 ,105-116
- [23] Mei, X. et al. The thioredoxin encoded by the rod-derived cone viability factor gene protects cone photoreceptors against oxidative stress. *Antioxid. Redox. Signal.* 24 (16), 2016, 909-923.
- [24] Cronin, T. et al. The disruption of the rod-derived cone viability gene leads to photoreceptor dysfunction and susceptibility to oxidative stress. *Cell Death Differ.* 17, 2010 ,1199-1210.
- [25] Ait-Ali, N. et al. Rod-derived cone viability factor promotes cone survival by stimulating aerobic glycolysis. *Cell* 161, 2015 ,817-832.

- [26] Elachouri, G. et al. Thioredoxin rod-derived cone viability factor protects against photooxidative retinal damage. *Free Radic. Biol. Med.* 2015 (4), 22-29.
- [27] Yamada, H. et al. FGF2 decreases hyperoxia-induced cell death in mice. *J. Am. Pathol* 159, 2001, 1113-1120.
- [28] Bourne, M.C. et al. Cataract associated with an hereditary retinal lesion in rats. *Br. J. Ophthalmol.* 22 (10), 1938 ,608-613.
- [29] Yu, D.Y. et al Intraretinal oxygen levels before and after photoreceptor loss in the RCS rat. *Invest. Ophthalmol. Vis. Sci.* 41, 2000 ,3999-4006.
- [30] Yu, D.Y. et al. Photoreceptor death, trophic factor expression, retinal oxygen status, and photoreceptor function in the P23H rat. *Invest. Ophthalmol. Vis. Sci.* 45, 2004 ,2013-2019.
- [31] Padnick-Silver, L. et al. Retinal oxygenation and oxygen metabolism in abyssinian cats with a hereditary retinal degeneration. *Invest. Ophthalmol. Vis. Sci.* 47, 2006, 3683-3689.
- [32] Berson, E.L. et al. Ocular findings in patients with autosomal dominant retinitis pigmentosa and rhodopsin, proline-347-leucine. *Am. J. Ophthalmol.* 111, 1991, 614-623.
- [33] Li, Z.-Y. et al. Rhodopsin transgenic pigs as a model for human retinitis pigmentosa. *Invest. Ophthalmol. Vis. Sci.* 39, 1998, 808-819.
- [34] Yoritaka, A. et al. Immunohistochemical detection of 4-hydroxynonenal-protein-adducts in Parkinson's disease. *Proc. Natl. Acad. Sci. U. S. A.* 93, 1996, 2696-2701.
- [35] Uchida, K. et al. Protein-bound acrolein: potential markers for oxidative stress. *Proc. Natl. Acad. Sci. U. S. A.* 95, 1998, 4882-4887.
- [36] Shen J, Yang X, Dong A, et al. Oxidative damage is a potential cause of cone cell death in retinitis pigmentosa. *J Cell Physiol* 2005; 203(3): 457-464
- [37] Komeima, K. et al. Antioxidants reduce cone cell death in a model of retinitis pigmentosa. *Proc. Natl. Acad. Sci. U. S. A.* 103, 2006, 11300-11305.
- [38] Chang, B. et al. Retinal degeneration mutants in the mouse. *Vis. Res.* 42, 2002 ,517-525.
- [39] Gargini, C. Retinal organization in the retinal degeneration 10 (rd10) mutant mouse: a morphological and ERG study. *J. Comp. Neurol.* 500, 2007 ,222-238.

- [40] Wright, A. et al. Photoreceptor degeneration: genetic and mechanistic dissection of a complex trait. *Nature Reviews Genetics* 11,2010, 273-284
- [41] Sullivan, L. et al. Prevalence of Disease-Causing Mutations in Families with Autosomal Dominant Retinitis Pigmentosa: A Screen of Known Genes in 200 Families. *Investigative Ophthalmology & Visual Science* 47, 2006,3052
- [42] Wang, Angeline, et al. "Retinitis Pigmentosa: Review of Current Treatment." *INTERNATIONAL OPHTHALMOLOGY CLINICS*, vol. 59, no. 1, 2019, 263–280.
- [43] Rangaswamy, N. et al. A Comparison of Visual Field Sensitivity to Photoreceptor Thickness in Retinitis Pigmentosa. *Investigative Ophthalmology & Visual Science* 51, 2010,4213
- [44] Berson E.L. et al. Further evaluation of docosahexaenoic acid in patients with retinitis pigmentosa receiving vitamin A treatment: subgroup analyses. *Arch Ophthalmol* 122, 2004, 1306–14
- [45] Lee, S. et al. N-acetylcysteine promotes long-term survival of cones in a model of retinitis pigmentosa. *Journal of Cellular Physiology* 226, 2011 ,1843-1849
- [46] Hajali M. et al. The prevalence of cystoid macular oedema in retinitis pigmentosa patients determined by optical coherence tomography. *Br J Ophthalmol.* ,92, 2008, 1065–1068.
- [47] Ozdemir H. et al. Intravitreal triamcinolone acetonide for treatment of cystoid macular oedema in patients with retinitis pigmentosa. *Acta Ophthalmol Scand.*83, 2005, 248–251.
- [48] Cox S. N. et al. Treatment of chronic macular edema with acetazolamide. *Arch Ophthalmol.* 106, 1988, 1190–1195.
- [49] Fishman G.A. et al. Acetazolamide for treatment of chronic macular edema in retinitis pigmentosa. *Arch Ophthalmol.* 107, 1989, 1445–1452.
- [50] Moldow B. et al. The effect of acetazolamide on passive and active transport of fluorescein across the blood-retina barrier in retinitis pigmentosa complicated by macular oedema. *Graefes Arch Clin Exp Ophthalmol.* 236, 1998, 881–889.
- [51] Chung H. et al. Optical coherence tomography in the diagnosis and monitoring of cystoid macular edema in patients with retinitis pigmentosa. *Retina* 26, 2006, 922–927.

- [52] Wielders L.H. et al. Prevention of cystoid macular edema after cataract surgery in nondiabetic and diabetic patients: a systematic review and meta-analysis. *Am J Ophthalmol.* 160, 2015, 968–981.
- [53] Scorolli L. et al. Treatment of cystoid macular edema in retinitis pigmentosa with intravitreal triamcinolone. *Arch Ophthalmol.* 125, 2007, 759–764.
- [54] Querques G. et al. Intravitreal pegaptanib sodium (Macugen) for refractory cystoid macular edema in pericentral retinitis pigmentosa. *Int Ophthalmol.*, 29, 2009, 103–107.
- [55] Yuzbasioglu E. et al. Intravitreal bevacizumab (Avastin) injection in retinitis pigmentosa. *Curr Eye Res.*, 34, 2009, 231–237.
- [56] Humayun M.S. et al. Interim results from the international trial of second sight's visual prosthesis. *Ophthalmology.* 119, 2012, 779–788.
- [57] da Cruz L. et al. Five-year safety and performance results from the Argus II retinal prosthesis system clinical trial. *Ophthalmology.*, 123, 2016, 2248–2254.
- [58] Ahuja A.K. et al. Blind subjects implanted with the Argus II retinal prosthesis are able to improve performance in a spatial-motor task. *Br J Ophthalmol.* 95, 2011, 539–543.
- [59] Dorn J.D. et al. The detection of motion by blind subjects with the epiretinal 60-electrode (Argus II) retinal prosthesis. *JAMA Ophthalmol.*, 131, 2013, 183–189.
- [60] Koitschev A. et al. Extraocular surgical approach for placement of subretinal implants in blind patients: lessons from cochlear-implants. *J Ophthalmol.* 2015;2015:842518.
- [61] Stingl K. et al. Subretinal visual implant Alpha IMS – clinical trial interim report. *Vis Res.*, 111, 2015, 149–160.
- [62] Wells, Jonathon, et al. “Application of Infrared Light for in Vivo Neural Stimulation.” *Journal of Biomedical Optics*, 10, no. 6, 2005, 064003-1 – 0064033-12.
- [63] Richter, Claus-Peter, et al. “Optical Stimulation of Auditory Neurons: Effects of Acute and Chronic Deafening.” *Hearing Research*, 242, 1-2, 2008, 42–51
- [64] Wells, Jonathon, et al. “Biophysical Mechanisms of Transient Optical Stimulation of Peripheral Nerve.” *Biophysical Journal*, 93, 7, 2007, 2567–2580.

- [65] Albert, E. S., et al. "TRPV4 Channels Mediate the Infrared Laser-Evoked Response in Sensory Neurons." *Journal of Neurophysiology*, 107, 12, 2012, 3227–3234
- [66] Hutter, E., and J. H. Fendler. "Exploitation of Localized Surface Plasmon Resonance." *Advanced Materials*, 16, 19, 2004, pp. 1685–1706.
- [67] Eom, Kyungsik, et al. "Enhanced Infrared Neural Stimulation Using Localized Surface Plasmon Resonance of Gold Nanorods." *Small*, 10, 19, 2014, 3853–3857.
- [68] Weissleder, Ralph. "A Clearer Vision for in Vivo Imaging." *Nature Biotechnology*, 19, 4, 2001, 316–317.
- [69] Yong, Jiawey, et al. "Gold-Nanorod-Assisted Near-Infrared Stimulation of Primary Auditory Neurons." *Advanced Healthcare Materials*, 3, 11, 2014, 1862–1868
- [70] Lee, Kyeong-Seok, and Mostafa A. El-Sayed. "Dependence of the Enhanced Optical Scattering Efficiency Relative to That of Absorption for Gold Metal Nanorods on Aspect Ratio, Size, End-Cap Shape, and Medium Refractive Index." *The Journal of Physical Chemistry B*, 109, 43, 2005, 20331–20338.
- [71] Saraiva, Sofia M. et al. "Synthetic Nanocarriers for the Delivery of Polynucleotides to the Eye." *European Journal of Pharmaceutical Sciences*, 103, 2017, 5-18
- [72] Simpson, Carrie A, et al. "In Vivo Toxicity, Biodistribution, and Clearance of Glutathione-coated Gold Nanoparticles." *Nanomedicine: Nanotechnology, Biology and Medicine*, 9, 2013, 257-263
- [73] Hayashi, Atsushi, et al.,. "Subretinal Delivery of Immunoglobulin G with Gold Nanoparticles in the Rabbit Eye." *Jpn J Ophthalmol*, 53, 2009, 249-256
- [74] Bakri, Sophie J., et al. "Absence Of Histologic Retinal Toxicity Of Intravitreal Nanogold In A Rabbit Model." *Retina*, 28, 1, 2008, 147–149.
- [75] Zhou, Jie, et al. "Functionalized Gold Nanorods for Nanomedicine: Past, Present and Future." *Coordination Chemistry Reviews*, 352, 2017, 15-66
- [76] Zhang, Xiao-Dong, et al. "Size-Dependent in Vivo Toxicity of PEG-Coated Gold Nanoparticles." *International Journal of Nanomedicine*, 6, 2011, 2071-2081
- [77] Prow, Tarl W., et al. "Ocular Nanoparticle Toxicity and Transfection of the Retina and Retinal Pigment Epithelium." *Nanomedicine: Nanotechnology, Biology and Medicine*, 4, 4, 2008, 340–349.

- [78] Jo, Dong Hyun, Tae Geol Lee, and Jeong Hun Kim. "Nanotechnology and Nanotoxicology in Retinopathy." *Int. J. Mol. Sci.*, 12, 2011, 8288-8301.
- [79] Zhao, Lei, et al. "An Intraocular Drug Delivery System Using Targeted Nanocarriers Attenuates Retinal Ganglion Cell Degeneration." *Journal of Controlled Release*, 247, 2017, 153–166.
- [80] Carvalho-De-Souza, João L., et al. "Photosensitivity of Neurons Enabled by Cell-Targeted Gold Nanoparticles." *Neuron*, 86, 1, 2015, 207–217.
- [81] Eom, Kyungsik, et al. "Synergistic Combination of near-Infrared Irradiation and Targeted Gold Nanoheaters for Enhanced Photothermal Neural Stimulation." *Biomedical Optics Express*, 7, 4, 2016, 1614.
- [82] Chen, Yu-Shiun, Yao-Ching Hung, Ian Liao, and G. Steve Huang. "Assessment of the In Vivo Toxicity of Gold Nanoparticles." *Nanoscale Research Letters* 4,8 ,2009, 858-864.
- [83] Alkilany, Alaaldin M., and Catherine J. Murphy. "Toxicity and Cellular Uptake of Gold Nanoparticles: What We Have Learned so Far?" *Journal of Nanoparticle Research* 12, 7, 2010, 2313-2333.
- [84] Jo, Dong Hyon, et al. "Nanoparticles in the Treatment of Angiogenesis-Related Blindness." *Journal Of Ocular Pharmacology and Therapeutics*.29, 2, 2013, 135-142
- [85] Jia, Yan-Peng, Bu-Yun Mab, Xia-Wei Weia, and Zhi-Yong Qiana. "The in Vitro and in Vivo Toxicity of Gold Nanoparticles." *Chinese Chemical Letters*, 28, 2017, 691-702.
- [86] Zhu, Xiao-Ming, et al. "Cellular Uptake Behaviour, Photothermal Therapy Performance, and Cytotoxicity of Gold Nanorods with Various Coatings." *Nanoscale*, 6, 19, 2014, 11462–11472.
- [87] Peynshaert, Karen, et al. "In Vitro and Ex Vivo Models to Study Drug Delivery Barriers in the Posterior Segment of the Eye." *Advanced Drug Delivery Reviews*,2017.
- [88] Weng, Yuhua, et al. "Nanotechnology-Based Strategies for Treatment of Ocular Disease." *Acta Pharmaceutica Sinica B.* ,7, 3, 2017, 281–291.
- [89] F.M. Mowat, et al. , "Tyrosine capsid-mutant AAV vectors for gene delivery to the canine retina from a subretinal or intravitreal approach", *Gene Ther.* 21, 2014, 96–105
- [90] M. Hellström, et al. , "Cellular tropism and transduction properties of seven adeno-associated viral vector serotypes in adult retina after intravitreal injection", *Gene Ther.* 16, 2009, 521–532.

- [91] K. Takahashi, et al. , " Improved intra- vitreal AAV-mediated inner retinal gene transduction after surgical internal limiting membrane peeling in cynomolgus monkeys", *Mol. Ther.* 25, 2017, 296–302.
- [92] R.F. Boyd, et al., "Photoreceptor-targeted gene delivery using intravitreally administered AAV vectors in dogs", *Gene Ther.* 23, 2016, 223–230.
- [93] L. Bourges, et al. , "Ocular drug delivery targeting the retina and retinal pigment epithelium using polylactide nanoparticles", *Investig. Ophthalmol. Vis. Sci.*, 44, 2003, 3562–3569.
- [94] M.M. Le Goff, et al. " Adult vitreous structure and postnatal changes", *Eye (Lond)*, 22, 2008, 1214–1222.
- [95] Boye, Shannon E., et al. "Highly Efficient Delivery of Adeno-Associated Viral Vectors to the Primate Retina." *Human Gene Therapy*, 27, 8, 2016, 580–597.
- [96] del Pozo-Rodríguez, A., et al. "Solid lipid nanoparticles for retinal gene therapy: transfection and intracellular trafficking in RPE cells". *Int. J. Pharm.* 360, 2008, 177–183.
- [97] Huang, Di, et al. "Overcoming Ocular Drug Delivery Barriers through the Use of Physical Forces." *Advanced Drug Delivery Reviews*, 2017.