Growth and Expression of Halorhodopsin for Application in a Protein-Based Artificial Retina

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Growth and Expression of Halorhodopsin for Application in a Protein-Based Artificial Retina

Megan Gillespie

B.S., University of Connecticut, 2012

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A Thesis Submitted in Partial Fulfillment of the Requirements for the University of Connecticut Honors Program in Biological Sciences
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Abstract

For nearly forty years significant research has been carried out on retinylidene proteins for use in device applications. Halorhodopsin (HR), a light-activated chloride ion pump, demonstrates potential for use as the scaffolding in an artificial retina. Retinal implants are needed to restore vision to people afflicted with ophthalmic diseases, such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP). A protein-based chloride ion-patch would be utilized by the eye to create an influx of chloride ions, similar to ion concentrations in healthy retinas during the conversion of light stimuli to electrochemical signals. This protein-based retinal prosthesis will directly stimulate the bipolar cells of the retina, replacing the function of damaged photoreceptor cells. Other alternative treatments for AMD and RP involve invasive surgeries to implant microelectronic devices that are frequently supplemented with external components. Comparatively, the artificial retina under investigation, comprised of a medically inert substrate layered with HR, provides a more practical, less surgically invasive approach to provide higher resolution vision to patients.
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1. Introduction

1.1. Need For Artificial Retina

As the average lifespan continually increases, age-related diseases and ailments are becoming more prevalent. Retinal degenerative diseases, such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP) are especially devastating and commonly found in the elder population. In fact, AMD affects more than one-third of the elderly population in the western world and is the principle cause of irreversible vision loss (1). This statistic is predicted to only increase as the life expectancy of the population increases (1, 2). Age-related macular degeneration is caused by deterioration of the central portion of the retina, leading to a loss of central vision resulting in difficulties with tasks that require high-contrast vision such as reading and driving (3) (Figure 1). Retinitis pigmentosa, the other major cause of vision loss and blindness in the elderly, is caused by a diverse group of genetic mutations in rods and is characterized by a progressive loss of visual acuity resulting from photoreceptor death. This currently incurable disease is in the rod photoreceptor cells. Patients with RP often have difficulty seeing in the dark, which is commonly referred to as night blindness, followed by a reduction of the peripheral vision, and ultimately, blindness (4) (Figure 1). Globally, retinitis pigmentosa affects more than 1 million individuals, a prevalence of about 1 in 4,000 people (4). Visual impairment from AMD and RP significantly reduces the quality of life in patients of older generations and is associated with disability and clinical depression in patients (2).
Retinal prostheses offer a viable treatment option to restoring and improving vision, and therefore, the lives of those afflicted with ophthalmic diseases.

Figure 1: Comparison of vision in a healthy retina and in diseased retinas. Vision as seen in a healthy retina (A) and retinas affected with ocular diseases (B and C). The middle image (B) demonstrates the loss of focused pixels in the central region of vision, which is characteristic of advanced age-related macular degeneration. The bottom image (C) is representative of tunnel vision, typical of a patient afflicted with progressed retinitis pigmentosa.
1.2. **Anatomy of the Eye**

The eye is a complex organ that captures and analyzes light and then sends vast amounts of sensory information to the brain. In the vertebrate eye, light enters through the pupil and is focused on a highly organized array of light sensitive neurons in the retina. The retina, a concave multilayered tissue that lines the inner back of the eye, contains a complex neural circuitry used to convert the graded electrical activity of the photoreceptor cells into a series of action potentials, which travel via axons in the optic nerve to the brain (5) (Figure 2).

Photoreceptors are the light sensitive neurons of the retina. These cells are broadly categorized as either rods or cones, but can be distinguished by shape, type of pigment they contain, distribution across the retina, and pattern of synaptic connections (6) (Figure 3). Rods contain the photopigment, rhodopsin, which is highly sensitive to light allowing these photoreceptor cells to function advantageously under low, or dim, light conditions for night and peripheral vision (7). Cones contain photopigments that are sensitive to bright light and can detect color (7).

Bipolar cells, anterior to the layer of photoreceptors, have two axon extensions that connect the photosensory signaling cascade from the photoreceptors to the ganglion cells, which are found in an adjacent cell layer. Ganglion cells then relay sensory information from the eye to the brain. In a young, healthy eye, reflected light enters the eye and is focused on the retina. Photoreceptors then convert light into electrical signals, or
chemically-mediated nervous impulses, which are relayed to bipolar cells through the process of transduction (8). Bipolar polar cells then synapse onto ganglion cells that come together in bundles to form the optic nerves. The optic nerves then transmit the information gathered from the eye through the nervous system to the brain where it is interpreted as an image. However, with dystrophy due to age and genetic mutations, the rods and cones become less effective at transmitting signals from reflected light.
Figure 2: Anatomy of the Eye and Layers of the Retina. The visual system of humans showing key anatomical components of the eye (a) and the layers of highly organized neural circuitry and light sensitive photoreceptor cells of the retina (b).
Figure 3: Detailed Representation of Rods and Cones. This modified oil painting by German artist Martin Missfeldt, 1993, shows the clear distinction in shape between rod (left) and cone (right) photoreceptor cells. Rods are responsible for dim or low light vision and cones are responsible for bright light and color vision.
1.3. Retinal Degenerative Diseases

The macula is the central, posterior portion of the retina, and contains the densest concentration of photoreceptors. This region of the eye is responsible for allowing a person to read, see fine detail, and to recognize faces (3). Age-related macular degeneration primarily affects the photoreceptors found in the macula, leading to central vision impairment, which hinders the ability for patients to achieve simple day-to-day tasks.

Photoreceptor degeneration due to AMD is caused by both genetic and environmental factors. There are two classifications of advanced AMD: wet macular degeneration, and dry macular degeneration (3). Wet AMD, also known as neovascular or exudative, occurs as a result of the abnormal growth of blood vessels that are directed towards the macula, which in turn causes the retinal pigment epithelium to become detached (9). Wet AMD, while less prevalent comparatively to dry AMD, is often associated with severe vision loss. Dry AMD results from the aging and thinning of the retina, specifically in the macula. The early stages of dry AMD can be diagnosed when drusen, yellow deposits composed of mainly fat and protein, begin to accumulate in and around the macula (3). The advanced, or late form of dry AMD is characterized by gradual geographic atrophy, the degeneration and death of rod and cone cells in the macula, as well as the presence of larger, more obtrusive drusen (10) (Figure 4). To date, there is no cure for AMD (1-3).
Figure 4: Progression of AMD through the view of an ophthalmoscope. This figure from de Jong’s AMD review article (10) provides a view of the retina as AMD progresses. The first four panels (A-D) show drusen present in progressive sizes and quantities (as indicated by the black arrows) in retinas diagnosed with dry age-related macular degeneration. Panels (E) and (F) depict advanced, or late stage, wet AMD with serious detachment of the retinal pigment epithelium as outlined by the arrowheads. Panels (F), (G), and (H) demonstrate late AMD and the remnants of subretinal hemorrhages.
Retinitis pigmentosa is the term for a type of progressive retinal dystrophy in which there are groups of inherited genetic abnormalities of the photoreceptors. While RP involves the accumulation of pigmentation and the gradual loss of both photoreceptor types, typically rods are affected first (Figure 5). Patients afflicted with RP usually experience night blindness and loss of peripheral vision as the initial symptoms because the rods are concentrated in the outer portion of the retina and mediate achromatic vision in dim light (4). Most patients suffering from diagnosis with RP are considered legally blind by age 40 because of tunnel vision, which leads to severely constricted visual fields. As RP continues to advance, the cone cells are affected in a mechanism not yet fully understood, and the patient will ultimately lose remaining eyesight, central vision, by age 60 (4, 11). Ophthalmic diseases such as AMD and RP severely attenuate the outer nuclear layer of the retina, which contains the rod and cone photoreceptors (2, 4).
Figure 5: Fundus photograph of retinitis pigmentosa. This figure from Duane’s Ophthalmology (12) shows the early stages of RP as pigmentation starts to accumulate and blood vessels begin to thin (A). Late stages of RP can be observed as large clumps of pigmentation with attenuated and sparse blood vessels (B).
1.4. **Proposed Treatments for AMD and RP**

Presently, significant research efforts are being made in developing a means to restore vision to those with impairments such as AMD and RP (11, 13-20). To date there are no cures for AMD or RP, however, gene therapy techniques and recently developed medical device technologies offer a viable options for providing some degree of improved vision and an increase in the quality of life for these patients.

Gene therapy, a type of genetic engineering that involves the insertion, deletion, or correction of a gene responsible for specific protein functions, is achieved through the use of a carrier molecule called a vector to deliver the therapeutic gene to the target cells. Currently, the most commonly used vector is a virus. Scientists manipulate the genome of several different classes of viruses, including retroviruses, adenoviruses, adeno-associated viruses, and herpes simplex viruses (21). Recently, scientists of Friedrich Miescher Institute for Biomedical Research (FMI) in Switzerland have introduced HR into the remaining, but nonfunctional, cone receptors in the macula of mice retinas afflicted with RP to restore vision via adeno-associated viruses (11). The protein genetically targeted by the folks at FMI was an enhanced Natronomonas pharaonis halorhodopsin (eNpHR) because similar to healthy photoreceptors, this light-activated chloride pumping protein hyperpolarizes in response to increases in light intensity (11). When testing treated light-insensitive human retinas in vitro, it was discovered that the retinas with HR substituted in for the malfunctioning or
degenerative native phototransduction cascade were capable of responding to light again after treatment, further validating the restoration of vision seen with mice retinas (11).

Another modified gene therapy technique capable of restoring vision to those afflicted with AMD or RP is optogenetics. Optogenetics is a new technology that combines genetic and optical methods to control the behavior of cells. This method, developed by Karl Deisseroth and Ed Boyden, has been shown to control neurological events in the cell through the expression of microbial rhodopsins (22). Using recombinant adeno-associated viral vectors (AAVs), the light-activated protein, channelrhodopsin-2 (ChR2), has been expressed in retinal ON bipolar cells (13). ChR2 is a gated algal cation channel activated by blue light. When activated, ChR2 opens and allows an influx of Na+ ions, similar to the influx that causes depolarization of nerve cells and leads to the firing of action potentials. Optogenetics is successful because this technology is capable of selectively targeting cells of interest. Halorhodopsin is often used to inhibit action potentials and ChR2 is often used to trigger a neural response. Inhibition of bipolar cells would not restore sight to those afflicted with AMD or RP. This concept is supported by Busskamp et. al at FMI as they had noted the importance of restricting “the expression of eNpHR to photoreceptors only, because eNpHR in downstream retinal circuit elements, such as ON-bipolar and ON-ganglion cells, may inhibit the flow of information across the retina” (11).
Distinct events in biological systems, including the visual system, can be targeted with precision when using optogenetics. This specificity of optogenetics enables higher resolution vision, in contrast to the electrical stimulation method, which activates all cell types without discrimination. While optogenetics offers vision with higher resolution and greater sensitivity, there are several disadvantages and drawbacks that must be considered. Components of optogenetics, namely ChR2 and HR, require a high light intensity for activation. The laborious and difficult task of designing AAVs with specific promoter to target each cell type is also necessary for the successful implementation of optogenetics in treating retinal degenerative diseases. Lastly, an obvious barrier to overcome when considering the use of optogenetics and other types of gene therapy are the ethical and legislative concerns raised on the issues of genetic engineering (23). Despite major advances in gene therapy and optogenetics, retinal prostheses offer a viable option for vision restoration in patients with late stage AMD or RP.

There are several alternative medical technologies currently being developed and acquiring acceptance with the Food and Drug Administration (FDA) through the FDA drug and biological approval process. In addition to the Birge Group’s protein-based retinal implant, other medical device developments aimed towards treatment of AMD and RP include designs from the following companies: NanoRetina, Boston Retina Implant Project,
Intelligent Medical Implants, Second Sight and the Department of Energy, and Optobionics.

The FDA drug and biological approval process must be understood at a basic level in order to compare the feasibility of these retinal technologies. A medical device must clear five major stages in order to receive FDA approval. The first stage is one of intellectual property and laboratory research in which the concept is patented and initial research is completed. NanoRetina and Boston Retinal Implant Project are currently in this phase. The next phase in achieving FDA approval is pre-clinical research. The Birge Group’s retinal prostheses and technologies of Intelligent Medical Implants is currently in this phase in which the device is being constructed and tested on animals. Obtaining scientific, ethical, and regulatory support for various institutional boards marks the third stage. The fourth stage of FDA approval process is that of clinical trials in which the medical technologies are tested in a controlled manner with human subjects in order to determine if the device is safe and effective. Lastly, devices must be authorized for Premarket Approval (PMA). Currently, Optobionics is at this stage of FDA approval.

Understanding the stage in the FDA approval process a device is currently in is important when considering the relative timing of commercial availability of these alternative approaches for treatment of age-related macular degeneration and retinitis pigmentosa.

NanoRetina, Inc. is developing “an ultra small, easy to implant, artificial retina designed to restore sight” termed Bio-Retina. Bio-Retina is an
implant composed of nanoelectrodes, electronic circuitry, photosensors, and IR recipient circuits, and a pair of eyeglasses that work together to translate the visual input into neural messages via a proprietary alogarithm (14). According to NanoRetina, Inc., the Bio-Retina implant is inserted with a thirty minute, minimally invasive surgery that requires only local anesthesia (15). The implant is attached to the retina and the electrodes of this implant protrude into several layers of the retina and interfaces with the bipolar neurons (14). Since neurons require electrical stimulation, specially designed, rechargeable eyeglasses supply an infrared beam to power the conversion of the eye’s optics to neural stimulation (15). As of now, NanoRetina, Inc. plans to attempt to start trials in 2013, and if trials are successful, they state they hopefully their medical device will be a feasible option for those afflicted with AMD or RP in “two or three more years” (14).

The Boston Retinal Implant Project, also in the initial phase of the FDA approval process, is a prototype with a subretinal microchip that has electrodes that directly activate optical nerves. According to Joseph Rizzo III, a principle researcher of the Boston Retinal Implant Project, this retinal implant includes both internal and external components and was designed with six principles in mind: minimal disruption of the anatomy of the eye; minimally invasive surgical methods for implanting the device; minimal sophistication of electronic components implanted into the eye and orbit; use of ultrathin flexible substrate that can bend to match the contour of the ocular tissues; use of wireless technology to provide a functional connection
between the external and the implanted device components; and, finally, use means to individually control and adjust the stimulation parameters to each electrode based on patient feedback (16). The Boston Retinal Implant device consists of a subretinal electrode array with over 200 individually controlled stimulating electrodes, and the rest of the device is outside of the eye, minimizing potential bio-compatibility problems (16). Special glasses with a small camera and primary radio frequency coil around the rim must be worn to collect visual images and wirelessly transmit power and a signal to the radio frequency coil that is sutured around the limbus, or outside of the iris (16). The signal is then propagated from the radio frequency coil around the limbus to a hermetic integrated circuit, or “stimulating chip” that is titanium enclosed to increase the longevity of this retinal prosthesis, which in turn provides the electrical stimulation to the electrodes of the subretinal implant that then transfer the message to the optic nerves (16). The most prominent challenge with the Boston Retinal Implant Project is that with present technology, each of the 200 individually controlled stimulating electrodes requires a hard-wired connection from the hermetic integrated circuit. Currently, these transistors are susceptible to destruction from exposure to water vapor and sodium ions that commonly occurs with the current device design (16). Ideally, looking to improve the quality and resolution of vision provided by this retinal implant in the future, the number of electrodes would need to be increased significantly. Presently, a miniaturized artificial
device with 15 channels instead of 200 electrodes is being tested in Yucatan minipigs (16).

Intelligent Medical Implants is a company based in Germany and Switzerland that has designed a device referred to as the Learning Retinal Implant. At this point, the Birge Group's protein-based retinal prosthesis and the Intelligent Retinal Implant system are both in the pre-clinical research phase of the FDA approval process. The Learning Retinal Implant is an epiretinal device that was developed based on “the transformation of image signals from an extraocular camera into sequences of current pulses applied by implanted microelectrodes” (17). In addition to an implanted microelectrode stimulator, there are two necessary external components of this retinal prosthesis; eyeglasses, termed the Visual Interface by Intelligent Medical Implants, and the Pocket Processor. The digital camera integrated into the eyeglasses of the Visual Interface records visual information and is connected to the Pocket Processor via a cable. The Pocket Processor, which also supplies energy for this entire implant system, then converts these visual recordings into data signals that are transmitted wirelessly to the epiretinal microelectrode stimulator via infrared (IR) transmissions and a high-frequency alternating electromagnetic field generated by metal coils in the frame of the eyeglasses of the Visual Interface (17). The electromagnetic field is received by the high frequency coil sutured to the sclera, the extraocular component of this epiretinal implant, while the IR transmissions are received and translated from optical signals into electrical impulses by
the IR receiver of this epiretinal device (17). Successful animal trials with Gottinger minipigs have been completed with a model implant that is composed of a polyimide circuit board with gold conducting paths and that consists of 49 electrodes (17). Presently, technologies are being fine tuned in terms of the design of the electrodes of the microcontact array, attempting to find an optimal electrode size that balances between charge applied and selectivity. Further studies are being completed to assess biocompatibility in terms of the acute toxicity, subchronic toxicity, genotoxicity, implantation, chronic, toxicity, and carcinogenicity in order to pursue FDA approval (17).

Second Sight and the Department of Energy have also developed a retinal prosthesis referred to as Argus II that is aimed to partially restore vision to blind patients. Argus II is the most advanced implant device in the stages of FDA approval, and it is already approved for commercial use in the European Economic Area and in fact, on October 29, 2011, Argus II became the first successful commercially implanted retinal prosthesis (18). Currently in the late stages of clinical trials in the United States, Argus II is an epiretinal implant that consists of a light-sensitive microphotodiode array, which is connected to an electronics case with an antenna that surrounds the eye ball. The antenna receives electrical signals that were converted in real time from visual images by external equipment of the retinal device. This epiretinal implant, which receives processed electrical signals for the visual images gathered by a camera on glasses via antenna, bypasses degenerative photoreceptors completely and directly stimulates functioning neural cells
such as ganglion and bipolar cells to allow the brain to perceive patterns of light which are ultimately interpreted as vision (24, 25).

The first FDA approved ophthalmic prostheses is the Implantable Telescope Technology of VisionCare Ophthalmic Technologies. Implantable Telescope Technology improves the sight of patients diagnosed with end stage AMD, or bilateral advanced dry AMD by magnifying the image seen by central vision to approximately two and half times the normal size. This magnification allows the image to be projected on a larger region of the retina allowing the remaining healthy photoreceptor cells around degenerate cells in the macula to see the majority of the image (19). The Telescope Implant itself is about the size of a pencil eraser and is implanted into the eye after the removal of the natural lens and cataracts to help improve central vision. Patients with the Telescope Implant wear normal eyeglasses to provide proper focus when viewing objects close up and far away. One eye, without the implant, is used for peripheral vision, and the other eye, with the Telescope Implant is used for detailed central vision (19).

Another alternative device technology is currently in the clinical trial phase of FDA approval. Optobionics has developed an implant device deemed Artificial Silicon Retina (ASR) microchip. ASR is extremely tiny with only a diameter of 2 mm and a thickness of 25 μm (20). This subretinal implant is one-tenth the size of a penny and thinner than a strand of human hair and is classified as a semiconductor microphotodiode array chip. Microphotodiodes are microscopic solar cells each with its own stimulating
electrode, and ASR has approximately 5000 of these microphotodiodes that are powered solely by incident light and intended to “alter the membrane potential on contacting retinal neurons and to stimulate how light would normally activate these cells to form retinotopic visual images (20). After successfully implanting the ASR disc in the cat, pig, and rat models, Optobionics has begun clinical trials with humans. To date, the ASR chip has been implanted in 42 patients with RP, and not only have visual improvements been stabilized, but also, there were unexpected improvements in retinal areas distant from the implant (20). This “neurotrophic rescue effect” has resulted in a degree of vision recovery that has included “visual acuity, contrast and color perception, and expansion of the visual field” (20). One major drawback of ASR, that the ASR microchip relies on the presence of some viable retina for stimulation to occur, is also a concern of the Birge Group’s protein-based retinal prosthesis.

1.5. Design of Birge Group Artificial Retina

The artificial retina proposed by the Birge Group involves the implementation of a multilayered ion-patch layered for the conversion of a light-activated protein to convert light stimulation into an electrical gradient that excites the retinal bipolar cells (Figure 6). In the Birge Artificial Retina, BR and HR will be used to stimulate the retinal bipolar and ganglion cells via an ion gradient.
Figure 6: Protein-Based Chloride Ion-Patch Implant. The layered protein-based patch implant (in purple) uses light activated proteins to replace the function of damaged photoreceptor cells. The Birge Group artificial retina implant is intended to stimulate the retina of patients with AMD and RP by generating an ion gradient, which will activate the bipolar and ganglion cells.
Halorhodopsin is a transmembrane retinylidene protein that naturally pumps a chloride ion in response to light activation. Bacteriorhodopsin, a similar protein to HR that instead natively pumps a proton, can be altered via site-directed mutagenesis into a chloride ion pump, which functions similarly to HR. The retinylidene protein of choice will be layered and oriented on a small piece of Dacron®, a synthetic fabric that is chemically inert in biological systems, such as the retina (26).

The chloride ion gradient-inducing protein-based artificial retina developed by the Birge Group does not require any external equipment such as a camera or power source as necessary for other retinal implants. The Birge Group retinal prostheses implements a subretinal design in order to create an ion gradient close enough in proximity to bipolar cells to stimulate a response (Figure 7). The artificial implant will solely consist of the protein-based chloride ion-patch. Implementation of retinylidene proteins as a light-induced ions requires that the protein be uniformly oriented over the entire multi-layered patch. The protein will be aligned via the electrostatic layer-by-layer method through an automated dipper system, allowing for control of the film thickness and yielding the most uniformly oriented protein layers with a positively charged polymer between each protein layer (27). The longevity of this protein-based chloride ion-patch will depend on whether HR or BR is the protein basis; Bacteriorhodopsin has a stable bench top lifetime of seven years whereas the lifetime of Halorhodopsin is about a year at 4°C (28, 29)
Figure 7: Schematic Diagram of Protein-Based Subretinal Implant. In this design, light enters through the nerve fiber layer and is focused on the retina, stimulating the oriented, multilayered thin films of HR or BR (in purple). The protein-based patch then generates a photochemically induced unidirectional chloride ion gradient to stimulate adjacent bipolar cells.
1.6. Comparison of Halorhodopsin and Bacteriorhodopsin for Use in an Ion Gradient-Inducing Protein-Based Artificial Retina

The seven transmembrane retinal protein Halorhodopsin (HR) uses the energy of green light to act as a chloride ion pump (Figure 8). Halorhodopsin, found in *Halobacterium salinarum*, functions as a light-driven anion pump with an absorption maxima at 578 nm. A chloride ion is pumped from the extracellular matrix into the cytoplasm, forming an ion gradient by HR (30, 31). Bacteriorhodopsin, also a seven transmembrane protein, functions slightly differently in that it is a light-activated hydrogen ion pump (32) (Figure 8). Structurally, HR and BR are considered very similar. The primary amino acid structures of HR and BR are actually homologous (33) (Figure 9). There are just a few key conspicuous residual changes that are representative of the different functional groups and charges needed to transport a chloride ion instead of a proton. A specific example can be found at the eighty-fifth amino acid residue. In BR, the eighty-fifth amino acid is aspartic acid, while in HR the eighty-fifth amino acid is threonine (33). Even though HR is a naturally occurring chloride ion-pump, BR is the ultimate protein of interest for use in an artificial retina because of its inherent biological stability and ultra-fast photovoltaic response (capability of quickly converting light energy into an electrical signal) (28).

This research worked towards exploring and optimizing the methods to express and purify Halorhodopsin in order to create and study the
efficiency of a light-induced chloride gradient from a protein-based artificial retina.

Figure 8: Comparison of Secondary Structure of HR and BR. Both HR (Top) and BR (Bottom) are integral seven transmembrane alpha-helical proteins with a retinal chromophore.
**Figure 9:** Comparison of key amino acid residues in HR and BR. In this figure, modified from Essen (30), the key amino acids responsible for the functionality of both HR (left) and BR (right) are noted. Halorhodopsin pumps a chloride ion from the extracellular to the cytoplasmic surface. In contrast, BR pumps a proton from the cytoplasmic to the extracellular surface.
2. Materials and Methods

2.1. Expression of Halorhodopsin

Halorhodopsin (HR) was purified and grown from a slant obtained from John Spudich at the Center for Membrane Biology, at the University of Texas Medical School. The expression of HR was measured as a function of the optical density (OD) at 660nm every ten minutes in the UV Spectrometer. The data recorded was then used to create a growth curve for HR.

2.2. Growth of Stock Culture of Halorhodopsin for Plating

Halorhodopsin was scaled up from freezedown by inoculating 5 mL of LB media containing 12 uL of 50 ug/mL ampicillin with 100 uL of freezedown culture. The cultures were allowed to shake overnight at 200 rpm at 37°C. Dilutions of HR (1:50, 1:100, 1:500, and 1:1000) were prepared for plating onto LB-ampicillin plates.

2.3. Inoculation from Plates

Colonies were isolated from the LB-amp and placed in 5 mL of LB broth containing 50 ug/mL ampicillin. After growing overnight in the shaker, a 1:100 dilution was taken from each sample and placed into fresh LB with ampicillin and allowed to shake at 37°C and 200 rpm for three hours.

2.4. Expression and Purification of Halorhodopsin

*Escherichia coli* (E. coli) BL21(DE3) cells transformed with pET21-HR
were grown at 37°C in LB medium supplemented with 50 ug/mL ampicillin. At an OD$_{660}$ of 0.35-0.40, 1 mM IPTG and 1 mM all-trans retinal or 5 mM all-trans retinal were added. After an induction period of three hours at 30°C, the cells were then harvested by centrifugation in JLA-17 for 10 minutes at 5,000 rpm. Harvested cells were washed and resuspended in a HEPES buffer (50 mM HEPES, 500 mM NaCl, pH 7.0) and finally broken up with a sonicator (Fisher Scientific 550 Sonic Dismembrator). Sonicated cells were centrifuged in JA 17 for 15 minutes at 6,000 rpm and to collect the supernatant. Membranes were sedimented at 35,000 rpm for two hours at 4°C (Beckman UltraCentrifuge) and solubilized in buffer S (50 mM HEPES, 500 mM NaCl, 5 mM imidazole, pH 7.0) 10% w/v detergent (n-Dodecyl-β-D-maltoside [DDM]) was added to sample. Pellet was solubilized with a sonicator (Fisher Scientific 550 Sonic Dismembrator) and then centrifuged at 35,000 rpm, 4°C for 20 minutes (Beckman Coulter Optima™ MAX-E Ultracentrifuge, TLA55). Sonication and centrifugation repeated until no strong color from HR remained. Supernatant of the solubilized membranes was combined Ni-NTA agarose his-binding resin (QIAGEN). The Ni-NTA resin was filled into a chromatography column and washed with buffer W (0.1% DDM, 50 mM HEPES, 500 mM NaCl, pH 7.0) with an increasing imidazole concentration ($\leq$30 mM) to remove unspecifically bound proteins. Subsequently, the histidine tagged HR was eluted in buffer F (0.02% DDM, 50 mM HEPES, 500 mM NaCl, 250 mM imidazole, pH 7.0).
2.5. **Halorhodopsin Characterization**

Characterization of induced HR fractions was accomplished using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Protein samples from various time points and dilutions were run on Criterion Precast Gels, 12.5% TRIS-HCL, 1.0 mm, 18 well comb, 30 uL (BioRad, MA) and Coomassie stained. As a standard, protein molecular weight marker (Invitrogen, NY) and a sample of wild type BR were also loaded. SDS-PAGE gels were analyzed for a band at 27 kDa, the molecular weight of HR. Ultraviolet–visible spectroscopy was used to determine the absorption maxima (Cary 50 Scan UV-Visible Spectrophotometer).
3. Results and Discussion

3.1. Growth Curve of Halorhodopsin

HR growth was monitored at 660 nm for three hours after induction with IPTG and all-trans retinal. OD$_{660}$ as listed in Table 1 below were used to construct a growth curve for HR. Analysis of the generated growth curve (Graph 1) determined that the optimal OD$_{660}$ to induce with IPTG and all-trans retinal was between 0.35 and 0.40.

<table>
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<tr>
<th>Time (Minutes)</th>
<th>Sample 1 OD at 660 nm</th>
<th>Sample 2 OD at 660 nm</th>
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<tr>
<td>0</td>
<td>0.0240</td>
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</tr>
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<tr>
<td>230</td>
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</table>
3.2. SDS-PAGE Induction Assay

Induced HR samples were characterized by SDS-PAGE and the absorption maxima were measured to confirm expression. The SDS-PAGE gels showed a band at 27 kDa, the molecular mass of HR affirming expression (Figure 10). Induced HR samples were found to have an absorbance at 575.9 nm (data not shown), similar to the accepted absorbance of HR is 578 nm, and identical to experimental absorbance of HR (34).
Figure 10: SDS-PAGE Analysis of HR. Assessment of both gels shows an increase in intensity of the HR band (at molecular weight 27 kDa) as time increases from 0 to 180 minutes for induced samples. The top gel (A) shows comparison between a sample induced with 1 mM IPTG and 5 mM all-trans retinal (left) and a control sample (right). The arrow indicates the presence of a 27 kDa band in the HR sample, and the absence of a band at 27 kDa in the control sample. The bottom gel (B) consists of sample induced with 1 mM IPTG and 1 mM all-trans retinal and a 1:10 dilution of that sample. The intensity of the 27 kDa band increases, as indicated by the arrows, from time 0 to 180 minutes after induction. Wells marked with an asterix (*) demonstrate the confirmed expression of HR.
3.3. **Purification of Halorhodopsin**

Protein expression was carried out as described by Spudich et. al. (34). The histidine tagged proteins were not successfully extracted from the solubilized membrane faction by incubation with Ni-NTA agarose. It was expected that after filtering and washing the resin with a step gradient of imidazole the bound HR would have eluted. For future work in purifying HR, alternative buffers, such as a Tris-HCl buffer should be experimented with.

4. **Conclusions**

Halorhodopsin was successfully expressed. It was determined that there was not a significant difference when inducing with 1 mM all-trans retinal versus 5 mM all-trans retinal. For future work, HR purification protocol needs to be optimized. Alternatives to protocol include modifying the buffer from a HEPES buffer to another buffer, such as one with Tris-HCl as the basis. Solubilizing methods could also be altered to include homogenization in addition to sonication. After HR is successfully purified, a protein-based patch with multiple oriented layers of HR should be assembled. Preliminary trials should then be carried out to compare the efficiency and stability of an artificial retina implant based on HR versus chloride ion pumping variants of BR.
5. Literature Cited


Appendix

A: Media Preparation

A.1. Luria Bertani (LB) broth (1L)

10.0 g tryptone
5.0g yeast extract
10.0g sodium chloride
15.0g noble agar
1L ddH₂O

A.2. Luria Bertani (LB) – Ampicillin plates (1L)

10.0 g tryptone
5.0g yeast extract
10.0g sodium chloride
15.0g noble agar
1L ddH₂O

Adjust to pH 7.2 using 5N NaOH
Autoclave (Cycle 3 – Liquid)
Cool before adding 1mL ampicillin (50 ug/mL), pour 25mL media into petri dishes and allow to sit overnight to harden.
A.3. **Ampicillin (50mg/mL) (15 mL)**

750 mg of ampicillin

15 mL ddH$_2$O

Filter sterilize 1 mL aliquots with millex-HA 0.45 um Millipore; store at -20°C.

A.4. **IPTG Stock Solution (1M) (10 mL)**

2.38831 g IPTG

10 mL of RNase free H$_2$O

\[
\text{IPTG: } 238.13 \frac{g}{mol} \times \frac{1}{1 mol} \times 0.01 \ L = 2.38831 g \text{ IPTG}
\]

Filter sterilize 1 M IPTG stock solution in 0.45 um filter.

Aliquot 1 M IPTG into 1 mL microfuge for future use.

Store at -20°C.

A.5. **1 mM all-trans retinal**

50 µL 0.1 M all-trans retinal

5 mL ethanol

\[
0.1 \ M = 100 \ mM = 100,000 \ \mu M
\]

\[
C_1 \cdot V_1 = C_2 \cdot V_2
\]

\[
(100,000 \mu M) \cdot V_1 = (1000 \mu M) \cdot (5mL_{ethanol})
\]

\[
V_1 = 0.05 \text{mL} = 50 \mu L
\]
A.6. Destain Solution (2L)

175 mL methanol
1625 mL H₂O
200 mL acetic acid

Prepared over ice and in hood, slowing adding acid to water.

A.7. 1 M Tris-HCl pH 6.8

78.8g of Tris HCl
0.5 L of ddH₂O

pH to 6.8 using NaOH

A.8. 4x Sample Buffer (10 mL)

4 mL 100% glycerol
2.4 mL 1 M Tris HCl pH 6.8
0.8 g Sodium dodecyl sulfate (SDS)
4 mg bromophenol blue
500 uL 2-mercaptoethanol
3.1 mL ddH₂O

A.9 HEPES Buffer - 50 mM HEPES Buffer, 500 mM NaCl, pH 7.0 (1L)

14.38 g HEPES
7.93 mL 5N NaOH
29.22 g NaCl
1 L dH₂O
A.10  50 mM HEPES Buffer, 500 mM NaCl, 5 mM imidazole, pH 7.0 (0.5L)

500 mL HEPES Buffer (A.9)
0.1702 g imidazole

A.11  1.0% DDM, 50 mM HEPES Buffer, 500 mM NaCl, 5 mM imidazole, pH 7.0 (80 mL)

80 mL HEPES Buffer (A.9)
0.027 g imidazole
0.8 g n-Dodecyl-β-D-maltoside (DDM)

Appendix B: Halorhodopsin Growth and Expression

B.1 Halorhodopsin Expression

1.  5 mL LB media
2.  12 uL of 50 mg/mL ampicillin
3.  Add 50 uL of HR culture
   a.  Freezedown stock
   b.  Active culture
4.  Place tubes in shaker at 200 rpm at 37°C overnight (~8-10 hrs.)
5.  Pass the freezedown culture into new, fresh LB media contain 12 uL of 50 mg/mL ampicillin
   a.  5 mL of fresh LB using aseptic technique into autoclaved test tube
   b.  12 uL of stock amp into each
   c.  50 uL of previous culture; place in shaker 37°C
6.  After approximately 2 or 2.5 hours, remove tubes from 37°C shaker
7.  Create 3 dilutions of culture in fresh LB
   a.  1:100
b. 1:500  
c. 1:1,000.

8. Warm LB-Amp plates in incubator

9. After a half hour, of warming, remove the plates and plate 50 uL of  
each dilution onto separate LB-ampicillin plates.
   a. Be sure to evenly spread 50 uL dilution across the whole plate  
      with a plate spreader

10. Place plates in 37°C incubator for 6-8 hours until colonies grow
    a. Note: Monitor growth to avoid overgrowing

11. Remove plates and store upside down in 4°C up

B.2 Inoculation of HR from LB-ampicillin plates for growth

1. Add 3 mL LB into tube using aseptic technique

2. Add 12 uL of amp to each tube

3. Take one healthy colony (large and isolated) from the plate with
   inoculation tip or pipet tip into autoclaved test tubes

B.3 Halorhodopsin Freezedown Preparation

1. Combine 500 uL of sample and 500 uL of 80% glycerol

B.4 Induction of Halorhodopsin

1. Samples were passed into fresh LB and amp and allowed to shake for
   approximately 3 hours at 37°C, 200 rpm

2. Induce cultures to 1 mM IPTG and 1 mM all-trans retinal OR to 1 mM
   IPTG and 5 mM all-trans retinal.

3. Incubate in shaker at 30°C, 200 rpm
B.5 Preparation for SDS-PAGE Induction Assay

1. Induce samples to 1 mM IPTG and either 1 mM all-trans retinal or 5 mM all-trans retinal
2. Take 500 uL from sample every 30 minutes after induction starting at time 0 (time of induction). Keep samples over ice.
3. Determine the OD$_{660}$ at each time point.
4. Add 200 uL dH$_2$O and 200 uL 10% TCA (to lyse cells) to each 400 uL portion of sample.
5. Pellet in centrifuge at 12,000rpm for 10 minutes. Aspirate/remove all TCA waste.
6. Resuspend pellet in 25 uL of 4x Sample Buffer and 75 uL RNAse-free water.
7. Insert 25 uL samples into each well.
8. Run gels at 200 V for approximately an hour
9. Place gels in coomasie stain and gently shake overnight.
10. Soak gels in destain.
11. Transfer gels to water; proceed to dry gels if desired.

B.6 Purification of His tagged HR

1. Pellet the E. coli cells by centrifugation at 5,000 rpm for 15 minutes.
2. Resuspend pellets in 50 mM HEPES, 500 mM NaCl, pH 7.0 buffer.
3. Disrupt the cells with a sonicator.
4. Sonicated samples were collected by low-speed centrifugation at 6,000 rpm for 15 minutes.
5. Supernatant was collected and spun at 35,000 rpm for two hours.
6. Pellet was resuspended in 50 mM HEPES, 500 mM NaCl, 5 mM imidazole, pH 7.
7. 10% w/v detergent (n-Dodecyl-β-D-maltoside [DDM]) was added to resuspended pellet.
8. Sample was solubilized using a sonicator and then ultracentrifuged at 35,000 rpm for 20 minutes. This step was repeated until no strong color due to HR remained in pellet.

9. Solubilized fraction was combined with QIAGEN Ni-NTA agarose his-binding resin with an anticipated binding efficiency of about 3.5 mg/mL (protein/resin).

10. Resin was packed in column and washed with 4x bed-volume of 1.0% DDM, 50 mM HEPES, 500 mM NaCl, 5 mM imidazole, pH 7.0.

11. Resin was washed with 2x bed-volume of 0.1% DDM, 50 mM HEPES, 500 mM NaCl, 5 mM imidazole, pH 7.0.

12. Resin was washed with more than 4x bed-volume of 0.02% DDM, 50 mM HEPES, 500 mM NaCl, 20 mM imidazole, pH 7.0.

13. Sample was eluted with 10 mL 0.02% DDM, 50 mM HEPES, 500 mM NaCl, 250 mM imidazole, pH 7.0.