

Award Number: W81XWH-09-2-0050

TITLE: Sealing Penetrating Eye Injuries Using Photo-Activated Bonding

PRINCIPAL INVESTIGATOR: Irene E. Kochevar, Ph.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital  
Boston, MA 02114

REPORT DATE: September 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

<b>REPORT DOCUMENTATION PAGE</b>			<i>Form Approved</i> <i>OMB No. 0704-0188</i>		
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. <b>PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</b>					
<b>1. REPORT DATE</b> September 2011		<b>2. REPORT TYPE</b> Annual		<b>3. DATES COVERED</b> 1 September 2010 – 31 August 2011	
<b>4. TITLE AND SUBTITLE</b>  Sealing Penetrating Eye Injuries Using Photo-Activated Bonding			<b>5a. CONTRACT NUMBER</b>		
			<b>5b. GRANT NUMBER</b> W81XWH-09-2-0050		
			<b>5c. PROGRAM ELEMENT NUMBER</b>		
<b>6. AUTHOR(S)</b>  Irene E. Kochevar  E-Mail: kochevar@helix.mgh.harvard.edu			<b>5d. PROJECT NUMBER</b>		
			<b>5e. TASK NUMBER</b>		
			<b>5f. WORK UNIT NUMBER</b>		
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  Massachusetts General Hospital Boston, MA 02114			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>		
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>		
			<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>		
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited					
<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b>  Purpose: To develop a light-activated technology (called PTB) with the potential to decrease vision loss and ocular complications in warfighters sustaining penetrating eye injuries. Scope: In year 2, the scope was to establish the treatment for direct photo-sealing of corneal lacerations, to identify the best treatment for sealing eyelid skin lacerations, and to optimize and build a prototype light delivery system that is safe for the retina. Major findings: Demonstrated that fibrin glue was not competitive with PTB for sealing is amnion over penetrating cornea injuries, determined that two potential adverse effects (inhibition of epithelial cell migration and keratocyte phototoxicity) are not significant problems, demonstrated that PTB can be used to seal lacerations in thin (e.g., eyelid or periorbital) skin without deep sutures and that this repair requires less time than suturing and stimulates less inflammation than sutures, built and tested a prototype retina-safe optical delivery system that effectively seals amnion to cornea and substantially reduces the treatment time compared to the laboratory optical fiber system.					
<b>15. SUBJECT TERMS</b> cornea, sclera, eyelid skin, penetrating wound, laser, photochemistry					
<b>16. SECURITY CLASSIFICATION OF:</b>			<b>17. LIMITATION OF ABSTRACT</b>	<b>18. NUMBER OF PAGES</b>	<b>19a. NAME OF RESPONSIBLE PERSON</b>
<b>a. REPORT</b>	<b>b. ABSTRACT</b>	<b>c. THIS PAGE</b>			<b>USAMRMC</b>
U	U	U	UU	16	<b>19b. TELEPHONE NUMBER</b> (include area code)

## Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	15
Reportable Outcomes.....	15
Conclusion.....	15
References.....	16
Appendices.....	--

## **INTRODUCTION**

The overall goal of this research is to develop a light-activated technology with the potential to decrease vision loss and ocular complications in warfighters sustaining penetrating eye injuries. Fragments and debris propelled at high velocity by improvised explosive devices (IEDs) have increased the incidence of penetrating eye injuries in the current conflicts compared to earlier wars. Rapid closure of penetrating eye wounds with formation of a water tight seal is critical to preventing infection and stabilizing the eye for further surgery, thus improving vision outcomes. Suturing the cornea, sclera and eyelid skin requires specialized training to precisely place hair-fine sutures and requires long surgery time. Cyanoacrylate glues can complicate further surgery by sticking to sutures and possibly causing additional damage when removed. Our sutureless, glueless method is rapid and uses currently FDA-allowed devices (clinical laser, light-activated dye, amniotic membrane) and thus may move rapidly to the deployment environment. The scope of the research includes evaluating two light-activated approaches to closing penetrating injuries in the cornea and sclera of rabbit eyes. In one method, amniotic membrane is stained with the dye, placed over the wound and treated with green light; in the other, the dye is applied to the wound walls and activated by green light to directly close the wound. The scope also includes developing a light-activated method for rapid closure of eyelid lacerations using hairless mouse skin as a model. Finally, the scope includes designing, constructing and evaluating a green laser light delivery system that meets ANSI standards for retina and iris safety. Major tasks for Year 2 of the studies at Massachusetts General Hospital were to establish the treatment parameters for direct photo-sealing of corneal lacerations, to identify the best treatment parameters for sealing eyelid skin lacerations, and to optimize the design and build a prototype light delivery system that reduces radiant exposure at the retina to below the established damage threshold levels.

## **BODY**

This research project is a collaboration with Col Anthony J. Johnson, MD at Brooke Army Medical Center (BAMC). The Statement of Work includes tasks to be carried at both the Massachusetts General Hospital (MGH) and the BAMC. Dr. Kochevar and Dr. Johnson have discussed results and plans for this project frequently by phone. Dr. Kochevar 's lab assembled and sent to Dr. Johnson the light delivery system used by his group for in vivo studies. Dr. Kochevar visited Dr. Johnson at BAMC in December 2010 to help set up the irradiation system and advise on the first ex vivo studies. These co-PI's also had extensive discussions at the ATACCC meeting in Fort Lauderdale in August 2011 where this work was presented as an oral communication.

### **Task 1. Evaluate photoactivated bonding for sealing amniotic membrane over corneal lacerations**

#### **1.b. Establish parameters for strong bonding of amniotic membrane to cornea**

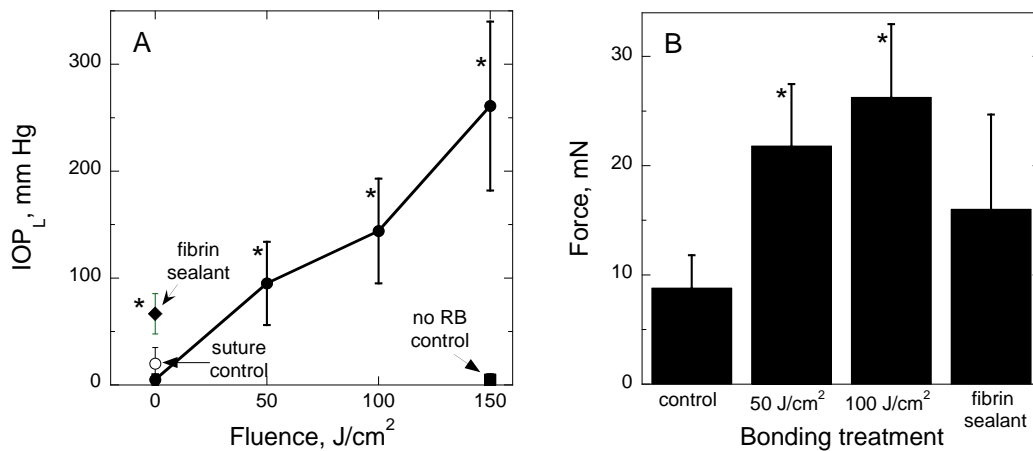
Studies in Year 1 established treatment parameters for sealing amniotic membrane over corneal lacerations using light-activated bonding (called PTB). Detailed studies were carried out using ex vivo rabbit eyes to determine the influence of Rose Bengal dye concentration, light fluence and light irradiance on the bonding strength. Next, an in vivo study in rabbit eyes verified the effectiveness of those photobonding conditions on bonding strength in non-survival surgery.

In Year 2, we carried out additional studies designed to compare the bonding strength of PTB to that produced by fibrin sealant, to identify factors that might influence photobonding strength, and to evaluate potential adverse effects of the photobonding procedure.

### 1.b.i. Comparing the bonding strength of PTB to that produced by fibrin sealant

One of the significant advantages of using PTB to seal amnion over penetrating eye injuries is that the process is sutureless, making it more rapid and providing an excellent seal. Fibrin glues, e.g., Tisseel, have been used to seal amnion to cornea (3, 4). Clinical experience suggests that these glues, while useful when mechanical strength is not needed, may not be strong enough to firmly seal amnion over a penetrating eye wound. Consequently, we tested the bonding and adhesive strength of Tisseel compared to PTB for sealing amnion to cornea using two measurement methods.

In the first method, the procedure described above for sealing amnion to cornea was used. For bonding with Tisseel fibrin sealant (Baxter, Deerfield, IL), equal amounts of thrombin solution and fibrinogen solution were spread on the cornea and amnion, respectively, as described previously (3, 4). The amnion was then placed over the V-shaped wound in the cornea and any wrinkles removed. The eye was allowed to stand at least 15 min before measuring the  $IOP_L$  to assess bonding strength. The results are shown in **Fig. 1A**. In comparison to bonding using three fluences of PTB (50, 100 and 150  $J/cm^2$  reported previously), the bonding strength produced by fibrin sealant was significantly lower. The bonding strength of fibrin glue was slightly lower than that produced by the lowest PTB treatment condition (50  $J/cm^2$ ).



**Figure 1.** Comparison of bonding amnion to cornea with fibrin glue and with PTB. (A) Bonding strength determined by measuring leak pressure ( $IOP_L$ ). For PTB, the fluence was varied using at 0.25  $W/cm^2$ . Amnion was bonded with Tisseel and was also sutured to cornea. Mean  $\pm$  SD.  $n = 6 - 8$ . \* indicates  $p < 0.05$  compared to the unirradiated control. (B) Adhesion was measured as the force (milliNewtons, mN) generated while peeling amnion that had been bonded to cornea with PTB or with Tisseel.  $n = 6 - 8$ . \* indicates  $p < 0.05$  compared to the unirradiated control.

In a second measurement method, adhesion of amnion to cornea was measured using a 180° peel test. This is a standard mechanism testing method for measuring adhesion between two materials. A 5-mm wide, 20 mm long strip of amnion was bonded to a 6-mm wide and 10 mm long strip of cornea using PTB or Tisseel fibrin sealant. The bonded overlap area was 5 mm x 10 mm. The PTB bonding procedure mimicked that used to seal amnion to intact cornea described above. A previously described procedure was followed for bonding amnion to cornea with fibrin sealant (3, 4). Equal volumes of thrombin and fibrinogen solutions were applied to the amnion and cornea surfaces, respectively, which were then placed in tight contact. After at least 1 hr, the force generated while peeling amnion from the cornea was measured using a

NANO UTM™ universal testing system (Surface & Surface Systems & Technology GmbH & Co. KG, Hueckelhoven, Germany) with a separation rate of 0.5 mm/min. The mean force (milliNewtons, mN) generated while peeling amnion from the cornea, after the initial peak, was taken as the adhesion strength.

The results shown in **Fig. 1B** indicate that the force generated while peeling amnion from cornea after bonding with PTB was greater than that for the control when the irradiation was either 100 or 50 J/cm<sup>2</sup> (p<0.0005). In contrast, the adhesion strength when fibrin sealant was used to bond amnion to cornea did not differ significantly from the control (p=0.085).

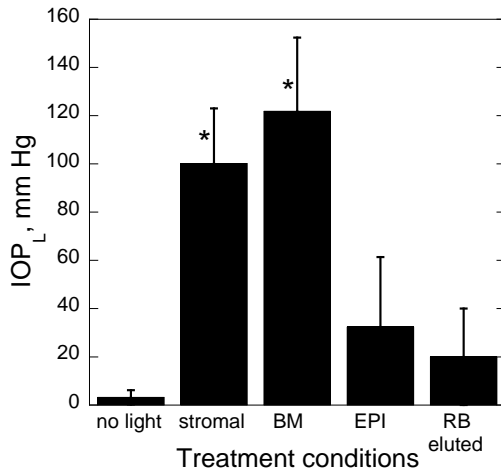
*These results indicate that fibrin glue, an alternative sutureless method for sealing amnion to cornea, did not produce a strong enough bond to be used to seal penetrating eye injuries.*

#### **i.b.ii. Identifying factors that might influence photobonding strength**

The two surfaces of amniotic membrane have different macromolecular compositions, which suggests that they might differ in the ability to photobond to the cornea surface. The amnion is a thin (30-100 µm) membrane consisting of a single layer of epithelial cells that are attached to the thicker stromal layer via a basement membrane. The epithelial layer can be removed from the basement membrane/stromal layer by treatment with trypsin (0.25%, 90 min, 37°C) and light rubbing. We compared the bonding strength to de-epithelialized rabbit cornea ex vivo of: a) stromal surface of amnion, b) basement membrane surface of amnion (epithelium removed) and c) epithelial surface of amnion (epithelium intact).

Since the details of the preparation of the amniotic membrane, the irradiation procedure and the measurement of bonding strength are given in the Year 1 report, only brief experimental procedures are provided here. The amnion disc (13 mm) was stained with 0.1% Rose Bengal for 5 min, then placed on the cornea containing a V-shaped incision. The amnion/cornea surface was irradiated with 100 J/cm<sup>2</sup> of 532 nm (green) light at an irradiance of 0.25 W/cm<sup>2</sup> (n = 5). The bonding strength was determined by infusing an aqueous solution into the anterior chamber and monitoring the intraocular pressure (IOP) as described previously (1, 2). The pressure causing leakage from under the amnion was recorded as the leak pressure (IOP<sub>L</sub>) and identified as the bonding strength. The results shown in **Fig. 2** indicate that bonding the stromal side or basement membrane (BM) side to the corneal produced the same bonding strength (~100-120 mm Hg). However, when the epithelial layer was intact on the amnion (labeled EPI) the bonding strength was much lower and did not differ significantly from the unirradiated (no light) control.

*Thus, from a practical viewpoint, it is not important which surface of the amnion is in contact with the cornea for strong photobonding as long as the epithelium has been removed (as done in commercially available amnion).*



**Figure 2.** Influence on bonding strength of amnion surface that is bonded to cornea and of removal of loosely associated RB. Amnion-covered corneas treated with 100 J/cm<sup>2</sup>, 0.25 W/cm<sup>2</sup> in all groups. stromal = stromal surface bonded, BM = basement membrane surface bonded, EPI = epithelial layer bonded, RB eluted = loosely bound RB removed by soaking in PBS for 1 hr then stromal surface was bonded. Mean ±SD shown, n=5. \* indicates p <0.05 compared to no light group.

We were also interested in learning whether the RB-stained amnion could be kept in an aqueous solution after it was prepared, but before it was placed on the cornea. Our studies indicated that when amnion is stained with Rose Bengal, about 75% of the dye molecules are washed out within a few minutes in phosphate buffered saline (PBS) and about 25% associate strongly with the collagen fibers and cannot be washed out (results not shown). To determine whether the remaining 25% tightly associated RB molecules was adequate for photobonding, amnion was stained with RB for 5 min, then the RB was eluted from the membrane by washing in PBS. This membrane was then photobonded to cornea as described above. The result, shown as the last bar in **Fig. 1**, indicates that the RB that is tightly associated with collagen is not sufficient for the photochemical reactions that bind amnion to cornea. Furthermore, it indicates that the loosely associated RB molecules are responsible for the photobonding.

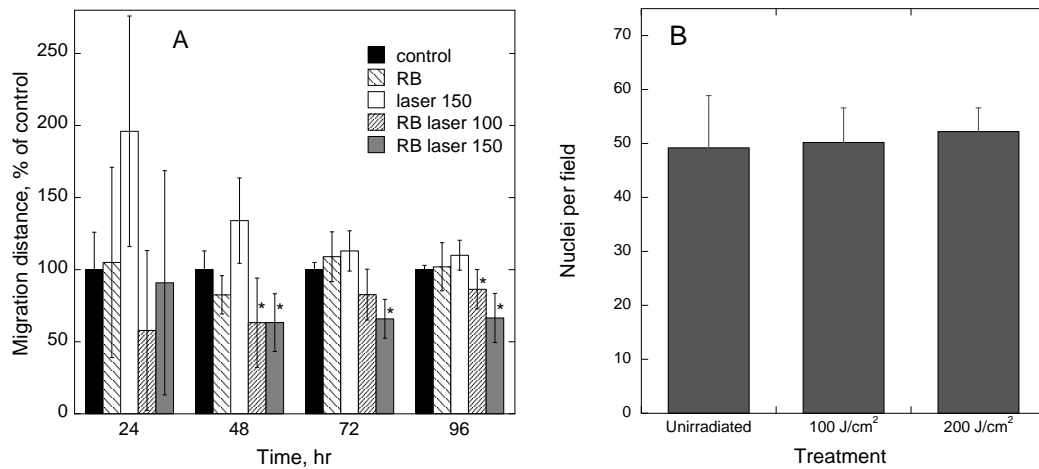
*This result indicates that RB-stained membrane should not be stored in aqueous solution before being used for PTB.*

### 1.b.iii. Evaluating potential adverse effects of the photobonding procedure.

We evaluated two potential side effects of using PTB to seal amnion to cornea, namely, whether the procedure would inhibit the migration of corneal epithelial cells onto the amnion and whether the PTB procedure would affect cornea stromal keratocyte viability.

Migration of corneal epithelial cells over the amnion is a step in healing process. Since migration of corneal epithelial cells over amnion involves their interaction with amnion basement membrane proteins and because PTB-induced crosslinking of these basement membrane proteins might alter the properties of this surface, we tested whether PTB treatment influenced the migration of these cells on amnion. Amnion was treated with 0.1% RB for 5 min before brief washing and irradiation with fluences of 532 nm between 0 and 150 J/cm<sup>2</sup>. Excess RB was removed by soaking amnion in PBS for 18 hr. Immortalized human corneal-limbal epithelial cells (5) were placed on the basement membrane of de-epithelialized amnion within a 6-mm cloning ring for 3 hr. At 24-96 h after removing the ring, the distance from the ring to the edge of the migrating cells was measured at six evenly separated locations on the circumference of the circle. As shown in **Fig. 3A**, the PTB treatment conditions used for bonding, 100 and 150 J/cm<sup>2</sup>, decreased slightly the extent of migration.

Although RB is initially in the amnion, some RB diffuses from the membrane into the cornea where it might cause phototoxicity to stromal keratocytes. Thus, RB-stained amnion was bonded to freshly harvested rabbit eyes using 100 and 200 J/cm<sup>2</sup>. The central cornea that was protected from light provided the unirradiated control area. After irradiation, the amnion was removed and the corneas were cultured for 24 h. The presence of keratocytes was evaluated on H&E-stained vertical sections 24 h post-treatment. The number of nuclei per 0.25 mm<sup>2</sup> field were counted. As shown in **Fig. 3B**, there were no significant differences between groups indicating that the PTB treatment was not toxic to keratocytes.



**Figure 3.** (A) Migration of corneal epithelial cells on RB and light-treated amniotic membrane. Cells attached to irradiated and control amnions were allowed to migrate away from a circle and the migration distance was measured. Mean  $\pm$  SD, cumulative results from three experiments with duplicate samples. \* indicates  $p < 0.05$ . (B) Viable keratinocytes in corneas 24 h after photo-bonding Rose Bengal-stained amnion to cornea of freshly harvested rabbit eyes.

*These results indicate that two potential adverse effects (inhibition of epithelial cell migration and keratocyte phototoxicity) are not significant problems for sealing amniotic membrane over penetrating eye wounds using PTB.*

**Milestone 3.** A manuscript describing the studies performed in Task 1 during years 1 and 2 was submitted to the journal *Investigative Ophthalmology and Visual Sciences*. It has been reviewed, the reviewers' comments were addressed and a revised manuscript has been submitted.

## **Task 2. Evaluate photoactivated bonding for direct sealing of corneal lacerations**

### **2.a. Establish laser parameters for strong immediate water-tight seals**

An additional approach to sealing full thickness corneal wounds is to use PTB to directly bond the wound edges together. This approach may be challenging for highly irregular or stellate lacerations but has the advantage that amniotic membrane is not needed. In earlier studies, we demonstrated linear corneal incisions were effectively sealed using PTB without amnion and also used PTB for penetrating keratoplasty to seal between sutures (2, 6). To identify the appropriate parameters and methodology for directly sealing corneal wounds, we used the V-

shaped wound in the central cornea of rabbit eyes that we used previously for bonding amnion with PTB. For this approach, a central, pupil blocking disc cannot be used to protect the retina because the light is needed in the central cornea for photobonding. Thus, a retina-sparing light delivery system is being developed (described in Task 5). The strength of the bonding was assessed by IOP measurements, as described previously. The initial bond strength is being measured at MGH and healing studies are being carried out at BAMC to determine the longer term tissue responses.

Sealing V-shaped incisions in the central cornea proved to be highly challenging for our research group at MGH. Maintaining close contact between the walls of the incision and minimizing fluid between the walls is essential for forming covalent protein-protein crosslinks between these surfaces using PTB. After the V-shaped incision was made in ex vivo rabbit eyes, the cornea did not maintain its shape and the incision walls did not align. All attempts, using a variety of techniques, to seal the wound with PTB were unsuccessful. Attempting to stabilize the contact between the incision walls with sutures, either one in each arm of the V or one at the apex of the V also did not lead to successful photobonding. It became apparent that our team, which does not contain a corneal surgeon, did not have the experience and technical expertise to use PTB to directly seal V-shaped corneal wounds. Dr. Johnson, who has extensive experience with suturing irregular corneal wounds, provided excellent advice. However, his skills were not readily transferred to our inexperienced hands. Consequently, he has now initiated studies to establish how PTB can be used (in conjunction with sutures) for direct sealing of full thickness corneal lacerations. When this technique is available, either he will visit MGH or we will visit BAMC in order to transfer the knowledge so that studies to optimize the PTB parameters will be completed.

#### **Task 4. Identify best treatment parameters for sealing eyelid skin lacerations.**

##### **4.a. Identify best PTB parameters for sealing mouse skin incisions.**

Repairing lacerations in eyelid skin and the adjacent periorbita, which are frequently caused by flying debris and fragments, is particularly problematic because the skin is very thin and delicate. Sutures are the current gold standard for closure of these wounds, but are time-consuming, especially for long or multiple lacerations, and require skilled placement of fine sutures. In addition, suture removal is painful. PTB may substantially reduce the time required for closing eyelid skin lacerations compared to sutured closure and has the advantages of allowing normal mobility of this skin compared to the stiffness produced by cyanoacrylate glues or skin tapes.

We have already demonstrated in porcine skin that PTB is an excellent replacement treatment for superficial interrupted sutures in a layered closure of full thickness surgical wounds in skin (7). However, forming a strong edge-to-edge seal in thin eyelid skin may be a challenge for PTB, because the strength of the bond is dependent on the size of the tissue areas in contact and mouse skin is only 0.4-0.5 mm thick. In this study, we used dorsal skin of the SKH-1 hairless mouse as a model for eyelid skin. This mouse is albino, hairless and immunocompetent. Outcome measures were initial seal strength, adherence at 1, 3 and 7 days, inflammatory infiltrate and procedure time.

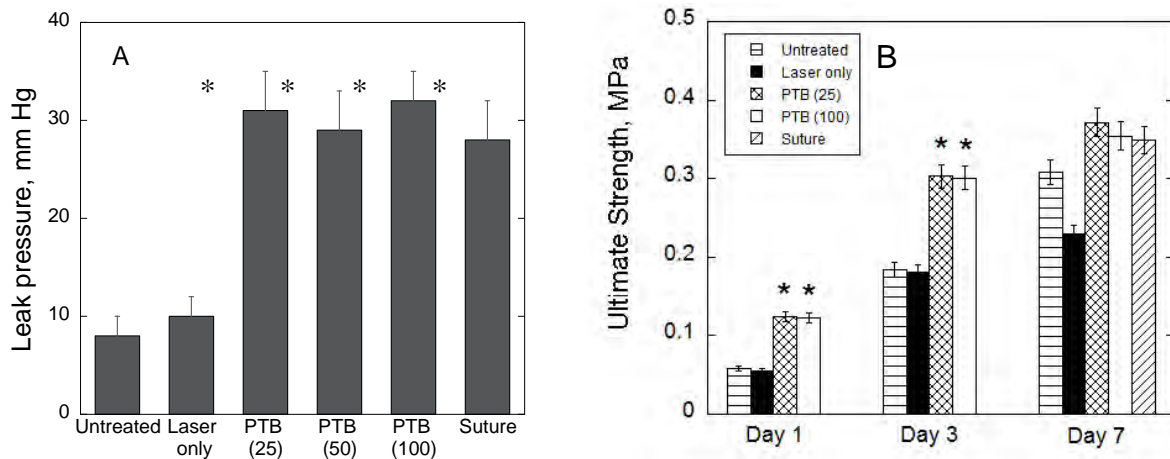
Four full-thickness incisions (1.2 cm long) were made in the skin on the back of each mouse, two on the upper flank of each side. All incisions were made perpendicular to the spine. Rose Bengal (0.1% w/v) was applied to the walls of the incision for 1 min. A cw KTP laser was used to irradiate a 1.13 cm<sup>2</sup> circular area with 532 nm light. The irradiance was 0.25 W/cm<sup>2</sup>. To close the

incisions, PTB was evaluated using laser fluences of 25, 50, or 100 J/cm<sup>2</sup> (100-, 200-, and 400-second exposures, respectively). For the first minute of the irradiation, the wound was very gently held closed with slight eversion of the wound edges.

Incisions in control groups receiving either no treatment or laser only (100 J/cm<sup>2</sup>). were held together with forceps for 400 seconds to allow formation of a natural fibrin seal. In another group the incisions were closed using black monofilament 10-0 nylon. Five sutures, perpendicular to the wound line, were evenly spaced (0.2 cm) and of equal length (0.2 cm) and closed with 2-1-1 knots. The incisions were randomized, using a prescribed order for treatments that did not follow an obvious pattern. All groups were n = 5.

The integrity of the tissue seal was determined at day 0 (immediately after treatment) by infusing saline into a compartment between the dermis and subcutaneous layers and measuring the pressure required to cause leakage of saline through the incision. For this measurement, an angiocath (IV catheter needle, 24 G) was inserted through normal skin 3 mm from one end of the incision and placed between the dermis and subcutaneous layer to the middle-point of the incision before the treatment. The inner metal needle was removed so that the remaining plastic hollow needle did not penetrate into the surrounding tissue. The needle was then connected to both a calibrated blood pressure transducer and a mini-infuser through a T-coupler. The pressure was gradually increased by infusion of saline (0.2 mL/min) through the angiocath. The pressure was increased until either the incision opened or fluid leaked from the incision.

As shown in **Fig. 4A**, significant pressure was required to open all incisions in the PTB groups. Approximately the same pressure was needed to fluid to emerge between the sutures (the sutures remained intact).



**Figure 4.** (A) Relationship between closure methods and leak pressure. Immediately after treatment, saline was infused under incisions and the pressure causing leakage through the incision recorded. (\* indicates  $P < 0.001$  compared to the untreated and laser only groups.) (B) Measurements of ultimate strength after closure with PTB, sutures, laser only or no treatment. Measurements were made on skin strips 0.3 cm wide x 0.8 cm long. N = 3 or 4. (\* indicates  $P < 0.05$  compared with the untreated and laser only groups. \*\* $P < 0.05$  compared with untreated group.)

A second method, mechanical testing, was also used to determine the strength of the bonding between the incision walls on days 1, 3 and 7. After euthanization two strips (0.3 cm wide and ~0.8 cm long) were made across the treated incisions, one for strength measurements and one

for histology. The force needed to break the skin at the incision was measured with a tensiometer coupled to a digital force gauge with a 10 Newton load cell. The peak force for rupture was divided by the cross sectional area of the skin strip to determine the ultimate strength (Mpa). Elongation (%) and Young's Modulus (MPa) were also calculated from the tensiometry results.

The ultimate strength and Young's modulus increased with time after closure in all groups. Ultimate strength for the PTB groups treated with 25 or 100 J/cm<sup>2</sup> on days 1 and 3 were significantly higher than those for the untreated and laser only groups ( $P < 0.05$ )(**Fig. 4B**). The suture group could not be tested on days 1 and 3 because removing the sutures separated the incision. Elongation and Young's modulus were equivalent in all groups at all treatment times except for a difference in elongation between untreated and PTB (25) groups on day 1 ( $P > 0.05$ )(results not shown).

*These results indicate that PTB can be used to seal wounds in skin without deep sutures. Since a water-tight seal is formed, this result suggests that the probability of infection may be lowered using PTB to close these wounds.*

Two additional factors that will influence whether PTB is a practical method for closing lacerations in thin skin are the time required for the procedure and the possibility that marked inflammation may occur that could lead to scarring. These two factors were examined.

- **Procedure time**

The time to close the 1.2 mm full thickness skin incisions by PTB or by suturing was compared. For the PTB groups, we recorded the time from applying RB to the wound edges to the time for completion of illumination. For the suture group, the time from opening the suture package to the time for completion of suturing was recorded. For the PTB groups irradiated with 25 and 100 J/cm<sup>2</sup>, the average times required were 160 s and 460 s, respectively. The average time required in the suture group was 311 s. Thus suturing required more time than sealing the wound with 25 J/cm<sup>2</sup> ( $P < 0.05$ ) but less time than sealing with 100 J/cm<sup>2</sup>. Since PTB using 25 and 100 J/cm<sup>2</sup> produced the same wound strength (**Fig. 4**), the shorter time needed for sealing with PTB is significant. Also, the time required for suturing will increase with the length of the wound whereas for PTB, the time can remain the same since the irradiated area can increase (up to the limit of laser power.)

- **Inflammation**

We assessed inflammation by scoring the magnitude of the leukocyte infiltrate in the dermis. Specimens from days 1, 3, 7 and 14 ( $n = 5$ ) were fixed in 10% buffered formalin and embedded in paraffin. Five micrometer vertical sections were stained with H&E. All slides were coded and evaluated in a blinded manner by four researchers. Severity of skin inflammation was semi-quantitatively analyzed and scored on a four step scale: grade 0, normal; grade 1 (infiltrating inflammatory cells were present in <10% in the 200× histology image), grade 2 (10-50%), grade 3 (> 50%). One day after surgery, a greater inflammatory cell infiltrate was present in the group closed by sutures than in the groups treated with PTB, laser only and untreated groups ( $p < 0.05$ ). From day 3 to day 14, there were no significant differences in the amount of infiltrating cells amongst all the groups.

*These results support the use of PTB for sealing lacerations in thin skin because the procedure time is shorter than for sutured closure and the degree of initial inflammation is lower for PTB, suggesting that less scarring might be produced.*

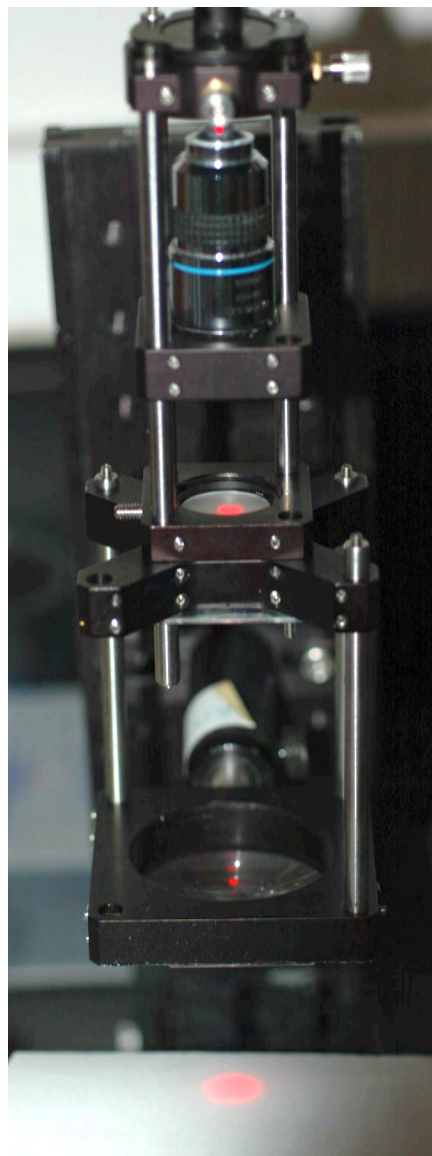
**Milestone 3.** A manuscript describing the studies performed in Task 4 was submitted to the journal *Lasers in Surgery and Medicine*. It has been reviewed and a revised manuscript has been submitted.

**Task 5. Design, construct and test safe light delivery systems for direct bonding of corneal injuries.**

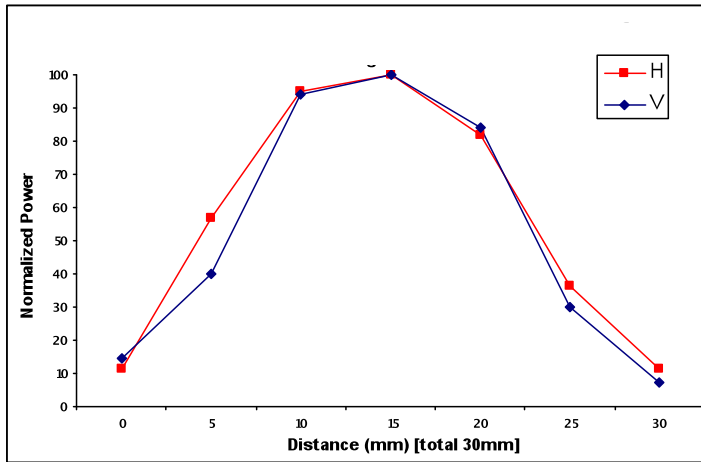
**5.b. Optimize light delivery system.** In Year 1 a light delivery system was designed for safe sealing of cornea wounds (without an opaque pupil-blocking disc) using RB and 532 nm light and the first model of this system was constructed. This system was designed to deliver light fluence to the cornea sufficient for bonding but deliver to the retina fluence and irradiance that was below the threshold for retinal damage according to the ANSI standard for 532 nm (8, 9). Light at 532 nm can cause thermal damage to retinal photoreceptor cells at sufficiently high irradiances and radiant exposures, and can also lead to photochemical damage directly to the retinal photoreceptors. Initial measurements validated that the power delivered to the model retina was well below the ANSI standard for retinal damage.

During Year 2, the light delivery system has been refined; a prototype was constructed and tested. This system still operates on the novel concept of using a diffusing plate and collimating Fresnel lens system to form an image of the diffuser on the cornea (for bonding) but prevents the cornea from focusing the light to a point on the retina. This allows sufficient light to bond amnion to cornea at the anterior surface while being safe for the retina. The prototype device is shown in Figure 5. Light is delivered via an optical fiber to the microscope objective at the top of the device and the image (of the aiming beam) is shown at the bottom.

Figure 5. Prototype of the light delivery system for photobonding on the cornea that reduces the laser power at the retina to below the threshold for damage according to ANSI 136.1 standards.

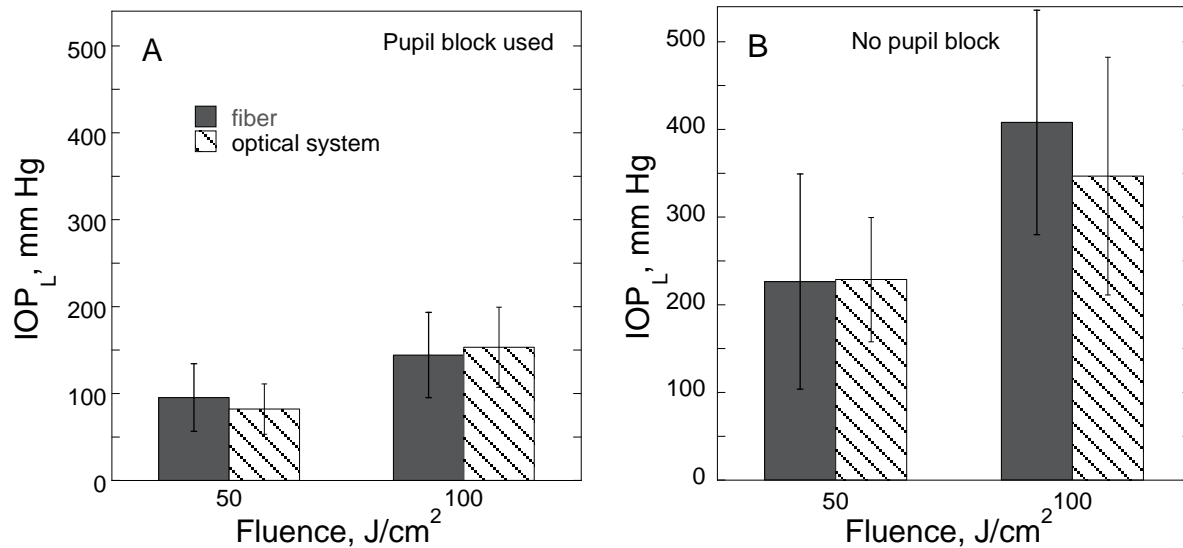


A spectroradiometer (Luzchem SPR-01) was used to measure the power distribution in the beam. An Iridex Oculight OR KTP 532 nm green laser was used with a 600  $\mu\text{m}$  optical fiber. A 1 mm iris placed over the spectroradiometer sensor was translated along two perpendicular axes in a 30 mm image of the beam. Measurements were made every 5 mm. Figure 6 shows the light distribution normalized to the highest reading measured along horizontal (H) and vertical (V) axes. Power measurements indicated that there was about a 40% loss of power through this optical system.



**Figure 6.** Power distribution of 532 nm radiation emerging from the prototype device along two perpendicular axes (horizontal, H; vertical, V). The image of the beam was 30 mm diameter.

The ability of light delivered by this system to bond amnion to cornea was compared to that produced by our conventional system using ex vivo rabbit eyes and the protocol described in 1.b.i. The irradiance was  $0.25 \text{ W/cm}^2$ . The bonding strength produced by two fluences, 50 and  $100 \text{ J/cm}^2$ , was determined by measuring the leak pressure ( $\text{IOP}_L$ ) immediately after bonding as described in 1.b.i. One comparison was made in which the central cornea was covered with an external pupil block (4 mm diameter) as was done in our previous studies (Figs. 1 & 2). The results shown in **Figure 7A** indicate that the light delivery system (stripped bars) produced equivalent bonding strength as our conventional laboratory fiber irradiation system (black bars) when either 50 or  $100 \text{ J/cm}^2$  were delivered.



**Figure 7.** Comparison of bonding amnion to rabbit eye cornea with PTB using either the laboratory optical fiber system or the prototype retina-sparing light delivery system. (A) A 4-mm diameter pupil block was placed on the central cornea before irradiation. (B) No pupil block was used.

This same comparison was also made without the pupil block during the irradiation. This configuration allows light in the center of the beam to photoactive the Rose Bengal on the amnion and bond it to the cornea. The results shown in **Fig. 7B** indicate that the two light delivery systems produced the same strong bonding. In addition, comparison of the results in **Figs 7A and 7B** indicate that bonding strength without the pupil block is significantly higher than with the pupil block. In fact, a fluence of 50 J/cm<sup>2</sup> without the pupil block produced a higher IOP<sub>L</sub> than 100 J/cm<sup>2</sup> with the pupil block. This result is significant because it means that very low fluences can be used to seal the amnion onto cornea in the absence of the pupil block when the new prototype system is used. This will significantly decrease the treatment time. Thus, even though this system was designed to allow direct bonding of corneal lacerations with PTB without causing retinal damage, it will be very useful for shortening the time required for bonding amnion over penetrating eye wounds.

When the parameters for direct bonding of corneal lacerations are established (Task 2), we will be able to compare the effectiveness of our conventional fiber system to this optical delivery system. However, based on the results obtained so far (Fig. 7), we anticipate that the newly designed system will be equally effective for bonding without the potential for retinal damage.

*These results indicate that use of the prototype optical delivery system, which is designed to spare the retina, decreases the treatment time required for sealing amnion over penetrating cornea lacerations.*

## KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that fibrin glue, an alternative sutureless method for sealing amnion to cornea, does not produce a strong enough bond to be used to seal penetrating eye injuries.
- Practical results were obtained showing that either surface of amniotic membrane strongly photobonds to cornea, that Rose Bengal-stained amnion should not be stored in aqueous solution before being used for PTB and that two potential adverse effects (inhibition of epithelial cell migration and keratocyte phototoxicity) are not significant problems.
- Demonstrated that PTB can be used to seal lacerations in thin (e.g., eyelid or periorbital) skin without deep sutures and that the repair requires less time than suturing and stimulates less inflammation than sutures.
- The prototype retina-safe optical delivery system effectively seals amnion to cornea and substantially reduces the treatment time compared to our conventional fiber system.

## REPORTABLE OUTCOMES

*Photoactivated technology for repair of penetrating eye injuries.* E. Verter, A.J. Johnson, M. Yao, R.W. Redmond and I.E. Kochevar. Poster presented at the 4<sup>th</sup> Military Vision Symposium, Schepens Eye Research Institute, Boston, MA, September 27-29, 2010,

Light-initiated bonding of amniotic membrane to cornea. E. Verter, A.J. Johnson, T. Gisel, P. Yang, R.W. Redmond and I.E. Kochevar. Presented at the 2011 ATACCC Conference, Fort Lauderdale FL, August 15-18, 2011.

## CONCLUSIONS

We extended the development of a simple and rapid light-initiated tissue bonding technology to decrease vision loss and ocular complications after penetrating eye injuries. Rapid closure of these wounds is critical to preventing infection and stabilizing the eye for further surgery. Current methods have substantial drawbacks: suturing is tedious, time-consuming and can damage the tissue; fibrin glues are not strong enough; and cyanoacrylate glues can cause damage upon removal and interfere with subsequent surgery.

In Year 2 we established that lacerations in thin eyelid and periorbital skin are effectively sealed using our sutureless, glueless technology in an animal model system. The light-activated sealing procedure was more rapid than suturing, generated less inflammation and provided an immediate water-tight seal. This method eliminates painful suture removal while not using stiff repair materials that inhibit blinking during recovery.

We characterized practical aspects of our method for sealing a biological membrane over penetrating corneal wounds by examining storage and preparation aspects of the biological membrane, by showing that the seal produced was stronger than a fibrin glue seal and by demonstrating that two potential adverse responses are not important.

The prototype light delivery system, designed to be safe for the retina, was shown to be highly efficient for repair of penetrating cornea injuries by sealing a biological membrane, thus shortening the treatment time.

These results indicate that significant problems are not expected in the translation of this light-activated repair technique to clinical use. Of importance for translation are safety studies, which are not included in this project. This should be addressed.

## REFERENCES

1. Mulroy L, Kim J, Wu I, Scharper P, Melki SA, Azar DT, et al. Photochemical keratodesmos for repair of lamellar corneal incisions. *Investigative ophthalmology & visual science*. 2000 Oct;41(11):3335-40.
2. Proano CE, Azar DT, Mocan MC, Redmond RW, Kochevar IE. Photochemical keratodesmos as an adjunct to sutures for bonding penetrating keratoplasty corneal incisions. *Journal of cataract and refractive surgery*. 2004 Nov;30(11):2420-4.
3. Kheirkhah A, Casas V, Blanco G, Li W, Hayashida Y, Chen YT, et al. Amniotic membrane transplantation with fibrin glue for conjunctivochalasis. *American journal of ophthalmology*. 2007 Aug;144(2):311-3.
4. Kheirkhah A, Casas V, Raju VK, Tseng SC. Sutureless amniotic membrane transplantation for partial limbal stem cell deficiency. *American journal of ophthalmology*. 2008 May;145(5):787-94.
5. Gipson IK, Spurr-Michaud S, Argueso P, Tisdale A, Ng TF, Russo CL. Mucin gene expression in immortalized human corneal-limbal and conjunctival epithelial cell lines. *Investigative ophthalmology & visual science*. 2003 Jun;44(6):2496-506.
6. Proano CE, Mulroy L, Jones E, Azar DT, Redmond RW, Kochevar IE. Photochemical keratodesmos for bonding corneal incisions. *Investigative ophthalmology & visual science*. 2004 Jul;45(7):2177-81.
7. Kamegaya Y, Farinelli WA, Vila Echague AV, Akita H, Gallagher J, Flotte TJ, et al. Evaluation of photochemical tissue bonding for closure of skin incisions and excisions. *Lasers in surgery and medicine*. 2005 Oct;37(4):264-70.
8. American National Standard for Safe Use of Lasers (ANSI 136.1-2000). The Laser Institute of America Orlando FL. 2000.
9. Delori F, Webb R, Sliney D. Maximum permissible exposures for ocular safety (ANSI 2000), with emphasis on ophthalmic devices. *J Opt Soc Am A Opt Image Sci Vis*. 2007 2007;24(5):1250-65.