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14. ABSTRACT 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: To compare light-activated bonding of amnion to direct sealing of penetrating cornea wounds, to evaluate photobonding for puncture wounds in sclera, and to evaluate safety of photobonding to the iris. Major findings: Identified the treatment parameters for rapidly and strongly sealing amniotic membrane over scleral puncture wounds. Demonstrated that photobonding amion effectively sealed wounds for simple and complex corneal lacerations. Determined that sealing penetrating cornea wounds using a dye-stained amniotic membrane patch and green light is superior to direct photo-activated bonding of the woundwalls. Established that the green light parameters used to seal amnion over cornea wounds are below the threshold for thermal damage to the iris. Pathological Evaluation pending at the time of this report.					
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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 3 of the studies at Massachusetts General Hospital (MGH) were to evaluate the ability of light-activated bonding to directly seal irregular penetrating wounds in cornea, to evaluate photoactivated bonding for sealing puncture wounds in sclera and to determine if the radiant exposure levels of green light reaching the iris during photoactivated bonding are below the ANSI damage thresholds.

BODY

This research project is a collaboration with Dr. Irene E. Kochevar, MD.. During year 3, Dr. Kochevar and Dr. Johnson continued to hold frequent phone discussions about the studies and discuss results. They also discussed the project status and planned experiments at the Association for Research in Vision and Ophthalmology national meeting in May 2012. This annual report describes the results of studies carried out at MGH by Dr. Kochevar and the results Dr. Johnson obtained at the ISR. A no-cost extension has been requested and a final report will be submitted when the studies are completed.

All of the studies in this annual report employed the same clinical laser that was used in previously reported studies (IRIDEX OcuLight OR KTP green laser, IRIDEX Corp., Mountain View CA), which emits green light (continuous wave, cw) at 532 nm. The amniotic membrane was also prepared, stored and stained with Rose Bengal (RB) as described in our Year 1 annual report.

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

To evaluate the photoactivated bonding over corneal lacerations we have prepared 3 separate experiments. The first arm involves creating a central 3mm laceration in the cornea, removing the central 8mm of epithelium, placing one suture to approximate the stroma, then crosslinking the amniotic membrane to the cornea. This is being compared to suture closure with 4 simple interrupted 10-0 nylon sutures. The second arm of the study was to evaluate a complex laceration. A v shaped central laceration was placed in the cornea, one suture was placed at its apex and the laceration was sealed with rose bengal impregnated amniotic membrane cross linked to the cornea surface. This is being compared to suture closure with 5 simple interrupted 10-0 nylon sutures.

Negative results: Initial closure of the laceration directly without the suture was problematic. First, the brisk aqueous outflow through the laceration prevented sufficient contact time of the impregnated amniotic membrane to the cornea to allow crosslinking. Additional attempts were made to place viscoelastic into the anterior chamber. This likewise was not satisfactory because the laceration was large enough that the viscoelastic either came through the wound displacing the amniotic membrane graft, or aqueous flowed around the viscoelastic preventing complete adhesion. Finally, direct closure of the 3mm laceration (with its significant wound gape would result in a large central scar, and also the gap underneath the amniotic membrane

might place the rabbit as elevated risk for developing a double anterior chamber or epithelial down-growth, both a which would result in significant morbidity.

Positive results: By placing the single suture in the center of the laceration we were able to obtain significant stromal apposition. I was thus able to place air into the anterior chamber, sealing off the aqueous outflow and allowing for crosslinking. (see Photo 1- in the attached appendix which shows the amniotic membrane on the cornea with the laceration approximated with one suture, and air in the anterior chamber.) Additionally, the presence of the suture reduces the wound gap sufficiently to minimize viscoelastic egress, so that a surgeon could use viscoelastic in lieu of air and obtain the same results. This was true for both the simple central corneal laceration and the V shaped complex laceration.

Please find attached our treatment matrix. You will see that the suture surgical procedures for all three arms of the study have been completed. The laser suture closure for the V shaped laceration has been started, and we are currently awaiting pathologic comparison of the crosslinking vs suture closure. In this area we have been delayed due to the recent retirement of the chief pathologist. Dr. Novak performed some of the analysis for our study, however when Dr. Rose Grimm arrived she is restarting all of the pathological evaluations with a slightly different classification. Thus for this period I will not have pathologic results to report.

Milestone 1. Analyze results and submit a manuscript describing results. A manuscript was submitted in year 2 and was published in Year 3 in *Investigative Ophthalmology and Visual Sciences*, the premier ophthalmology research journal. It is listed in the Reportable Outcomes section. Additionally a poster presentation was made at both the Schepens Eye Conference and the ATACCC conference.

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

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

In addition to sealing amniotic membrane over a penetrating corneal injury with photochemical tissue bonding (PTB)(Task 1), we had proposed to directly bond, with PTB, the edges of wounds with irregular shapes which mimic traumatic wounds. We had previously demonstrated that PTB effectively sealed linear incisional wounds in cornea {Mulroy, 2000 #1082;Proano, 2004 #1090}. For the current studies we used simple linear lacerations,. As noted in our Year 2 report, this approach was challenging for us because the MGH group lacks the appropriate cornea surgical skills and experience- the attempts were unsuccessful. To overcome this problem, Dr. Johnson, a highly skilled corneal surgeon, worked to develop the technique and then would visit Boston to teach. During this year, Dr. Johnson evaluated several approaches to directly sealing simple lacerations, V-shaped incisions in the central cornea, as well as corneal scleral lacerations using photoactivated bonding. What he found was that the v-shaped and other complex wounds of the cornea gape significantly, in contrast to simple lacerations which tend to lie minimally disturbed in anatomic configuration. The gaping of the wound is problematic because it precludes the ability to maintain anatomic alignment during the laser treatment phase. Additionally, isolated corneal lacerations do not traumatize the eye sufficiently to decrease or reduce aqueous production, thus the lacerations have a continuous flow of aqueous through the laceration which effectively washes out any rose Bengal present. Part of the solution involves combining the placement of a few corneal sutures to re-establish anatomic configuration prior to placing the rose bengal. This approach significantly reduces the size of the scar and improves healing when used with the amniotic graft but does not address the aqueous flow issues. For single lacerations involving 4-5 mm or less only one suture was required to obtain adequate approximation. (Of note I additionally tried injecting Rose Bengal directly into the stroma and crosslinking but any wound gap present negated successful closure. For 1-2 mm lacerations I could hydrate the stroma with RoseBengal sufficiently to minimize any wound gap and then cross link the wound but the time involved was not faster than placing one suture. For the complex V shaped laceration Dr. Johnson was able to obtain reproducible bonding with the amniotic membrane graft after only placing one suture at the apex of the V shaped laceration. For the corneal sclera laceration one limbal suture is affective but occasionally there is cornea wound gape necessitating 2 sutures one just posterior to

the limbus to approximate the sclera and one just anterior to minimize corneal wound gape. Ultimately, he reached the conclusion that direct bonding of complex wounds is not feasible unless a more viscous or dense material is used to fill small spaces between wound edges, as the rose Bengal is quickly diluted in the aqueous outflow.

Task 3. Evaluate photoactivated bonding for sealing puncture wounds in sclera.

1.c. Photoactivated bonding for sealing amniotic membrane over penetrating wounds in the sclera.

Sealing amnion over penetrating wounds in the sclera differs from bonding amnion over cornea wounds since the surface composition of the two tissue differs and consequently the photobonding efficiency may differ. The surface of the de-epithelialized cornea is a basement membrane composed mainly of Type IV collagen and laminin. The surface of the sclera is Type I collagen. Similar to the studies for sealing corneal wounds, our studies were designed to evaluate the immediate strength of the seal, first ex vivo and then in vivo, after amnion is bonded over a puncture wound in the sclera. Dr. Johnson is currently studying this via a corneal sclera laceration study.

Studies were carried out using ex vivo rabbit eyes to establish the size and shape (circular vs rectangular) of the amnion patch to use for in vivo studies. For all studies, the conjunctiva was removed from the scleral area before wounding. The conjunctiva was removed using small surgical scissors and a blunt tip to undermine the conjunctiva, working outwards from the limbus.

The effect of circular patch size (7 mm vs 10 mm circle diameter) was evaluated for sealing 3-mm incisions made perpendicular to the limbus and 3-4 mm away from the limbus. The patch was stained with 0.1% Rose Bengal (RB) for 5 min, rinsed and allowed to partially dry before being placed over the incision. The entire patch was exposed to green laser light using an irradiance of 0.25 W/cm² to deliver 50 J/cm² (200 sec) or 100 J/cm² (400 sec). The control in all experiments was RB-stained amnion that is not irradiated.

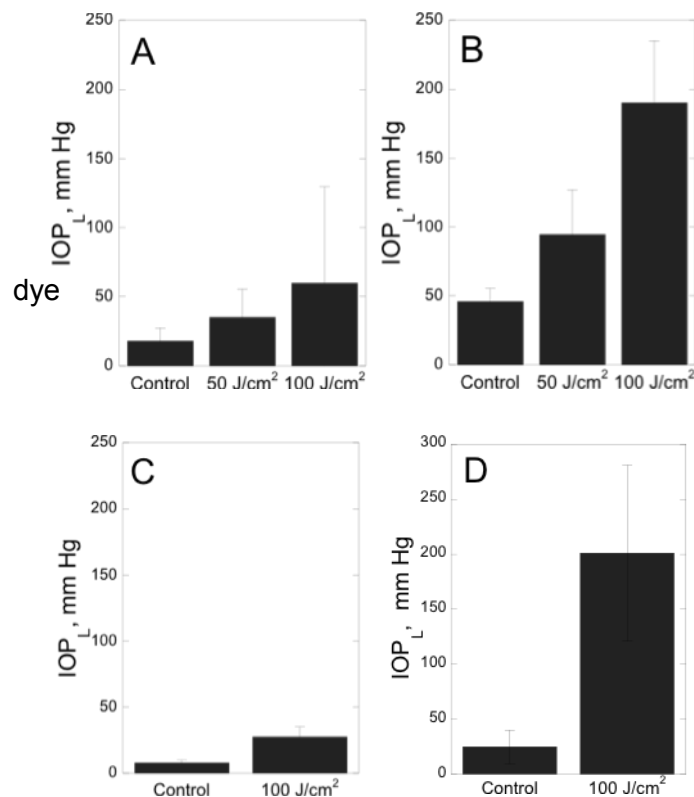


Figure 1. Photobonding amnion patches over linear incision wounds in rabbit sclera ex vivo. (A, B) 3-mm wounds perpendicular to the limbus were covered with Rose Bengal-stained circular amnion patches either 7 mm (A) or 10 mm diameter (B) and irradiated with green laser light. The IOP_L indicates the bonding strength. (C, D) 3.5 mm wounds perpendicular to the limbus were covered with RB-stained amnion either 4 x 8 mm rectangles (C) or 5 x 11 mm rectangles (D) and irradiated with green laser light. * indicates p < 0.01 compared to unirradiated control.

The measurement of the strength of the bonding was somewhat modified from that described previously for bonding amnion over corneal wounds. The patch was moistened with a drop of water before insertion of a 19 gauge needle into the posterior segment ~ 1 cm from the incision site but still within ~ 5 mm of the limbus. The needle point was placed directly behind the incision. An aqueous solution containing a blue (for visualization) was infused into the posterior segment and the pressure measured. The intraocular pressure (IOP) at which leakage from underneath the amnion was detected (the leak intra ocular pressure, IOP_L) indicated the bonding strength.

The results are shown in Figs. 1A and 1B.

Photobonding the 10 mm diameter amnion patch produced a strong seal; the IOP_L was almost 200 mm Hg when irradiated with 100 J/cm². In contrast, photobonding the 7 mm patch with the same fluence only provided an IOP_L of approximately 50 mm Hg. A 7-mm amnion patch only allows a 2-mm rim of

amnion between the end of the 3-mm incision and the edge of the amnion disc. This appears to be an insufficient area to provide a strong seal.

The influence of the distance between the end of the incision to the edge of the amnion was investigated further by photobonding amnion over a 3.5 mm linear wound using rectangular patches, 4 mm x 8 mm or 5 mm x 11 mm. As shown in Fig. 1C, the smaller rectangular patch was not effective; the mean IOP_L was only 30 mm Hg. The larger rectangular patch produced strong bonding (Fig. 1D). The mean IOP_L , ~200 mm Hg, is the same as the value produced when a 10 mm circular disc patch is bonded (Fig. 1B). These results indicate that ~3.5 mm is required from the end of a wound to the edge of the patch (using either circular or rectangular amnion patches) to produce strong sealing of amnion over a wound in the sclera.

The energy of the laser light is absorbed by the RB dye on the amnion, but not all of the energy is used to photochemically link the amnion to the scleral surface. Some is converted to heat, which might damage the sclera if the temperature increase is too great. This could be a problem if the energy is delivered too rapidly, i.e., if the irradiance is too high. Consequently, we measured the temperature of the amnion surface during the irradiation using a non-contact infrared thermometer (model 572, Fluka; Mississauga, ON Canada). The results are shown in Fig. 2A (filled circles). The temperature increase was less than 1°C for an irradiance of 0.25 W/cm².

Decreasing the irradiation time is desirable but might increase the temperature during the irradiation since shortening the irradiation time by one-half requires increasing the irradiance two-fold to deliver 100 J/cm². As shown in Fig. 2B, an irradiance of 0.50 W/cm² produces a 2°C temperature increase. These temperature increases are lower than the heating required to cause damage to tissue, indicating that thermal damage to the sclera does not occur during photobonding even when the higher irradiance is used.

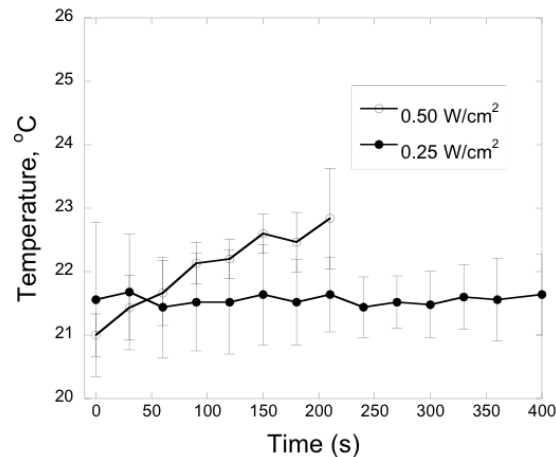


Figure 2. Temperature change during photobonding of RB-stained amnion over scleral wounds. Two irradiances, 0.25 and 0.50 W/cm² were used to deliver 100 J/cm² in 400 and 200 seconds, respectively. N = 4/group

Photobonding amnion over a more challenging type of wound was also evaluated. Our initial motivation for photobonding amnion over penetrating eye injuries was wounds involving both the cornea and the sclera such as shown in Fig. 3A. Incisions (3.5 mm) were made perpendicular and across the limbus, covered with 10-mm RB-stained amnion disc patches and irradiated with 100 J/cm² or not irradiated. As shown in Fig. 3B, reasonably strong bonding was achieved (~130 mm Hg, measured in anterior chamber) indicating that our approach can be used for these severe wounds.

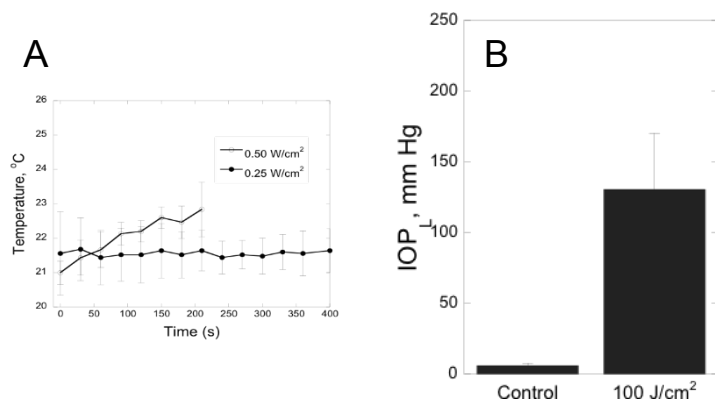


Figure 3. Photobonding amnion over corneal/sclera incisions in rabbit eyes ex vivo. (A) Cornea/sclera penetrating wound from battlefield trauma. (B) Photobonding amnion over 3.5-mm corneal/scleral incisions. 10 mm RB amnion patches and green laser used. The IOP_L indicates the bonding strength. * indicates $p < 0.01$.

Interestingly, the IOP_L values for sealing amnion to sclera under the optimal conditions (Fig. 1A) are very similar to those we measured for bonding amnion to cornea (Year 1 & 2 reports and ref {Verter, 2011 #1097}). Values for IOP_L were in the range 200-250 mm Hg for both. It wasn't apparent whether bonding to sclera would be as strong as to cornea because the molecules available for photo-crosslinking on surface of the two tissues differ, as noted above. This result suggests that the covalent crosslinks between Type I collagen in the amnion and the tissue surface proteins is non-specific, i.e., isn't influenced by type of protein.

The third arm of the live rabbit study is a comparison of a combined corneal scleral laceration with crosslinking vs. suture closure. The suture closure of this aspect of the study has been completed. We were able to successfully close the laceration with crosslinking during our model development phase and we are currently waiting for SAMMC and HJF to update the statement of work so that we can order rabbits to complete the study.

Task 4. Identify best treatment parameters for sealing eyelid skin lacerations. These studies were completed in Year 2.

Milestone 3. The manuscript describing the technique developed for sealing eyelid lacerations with PTB was submitted in Year 2 and was published in Year 3 in *Lasers in Surgery and Medicine*. It is listed in the Reportable Outcomes section.

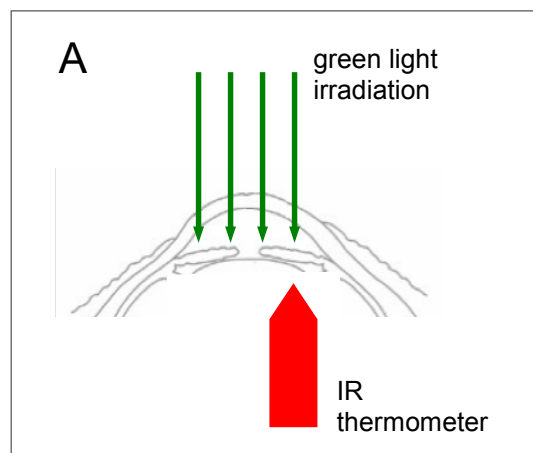
Task 5. Design, construct and test safe light delivery systems for direct bonding of corneal injuries.

In Years 1 and 2 the studies for this task focused on designing and evaluating a light delivery system that would provide sufficient light for photobonding on the cornea surface but would be safe for the retina according to ANSI (American National Standards Institute). In Year 3 our goal was to determine if the radiant exposure levels reaching the iris while photobonding amnion to the cornea are below the ANSI thresholds for damage. This information is needed because the small opaque disc that blocks green light from entering the eye through the pupil does not block this light from reaching the iris.

The human iris contains melanin in the stromal layer and in a pigmented epithelial layer on the posterior surface. When the melanin absorbs green light, the light (electromagnetic) energy is converted into thermal energy. If the rate of light energy absorption is greater than the rate of dissipation of the thermal energy, the temperature will rise. However, the temperature increase will be limited by blood flow in the iris, which will remove heat.

A threshold for thermal damage to the iris is not well defined. It is sometimes taken to be the same threshold as for skin, but because skin pigmentation varies the standard is not clear. The distribution and amount of melanin and vasculature in the iris differs from that of the retina; consequently, the damage threshold values determined for retina are not applicable to iris. Consequently, we decided to directly measure the iris surface temperature increase and compare this value to the information about thermal damage to tissue.

We measured the change in iris temperature under the conditions used for bonding amnion to the cornea (irradiance = 0.25 W/cm^2 , fluence = 100 J/cm^2 .) Freshly obtained swine eyes were used for these measurements because the iris is darkly pigmented (high melanin content) whereas the albino rabbit eyes used in our studies lack melanin in their iris. The cornea, iris and a rim of sclera were removed and placed in a specially constructed holder that allowed irradiation of the cornea from above and measurement of the iris temperature from below (Fig. 4A).



B. being repeated with better set up;
about 3 degree temperature increase

Figure 4. Measuring temperature of iris while irradiating cornea with green light. (A) Schematic diagram of experimental set up. (B) Temperature of posterior surface of iris measured with non-contact infrared thermometer during bonding amnion to cornea using green light.

The temperature increased and then reached a plateau approximately 3°C higher than the starting temperature (Fig. 4B). This is an upper limit because the ex vivo eye does not have blood circulation in the iris to remove heat. Thus, our measurements indicate that photobonding amnion onto cornea will not cause thermal damage to the iris.

KEY RESEARCH ACCOMPLISHMENTS

- Identified the treatment parameters for strongly sealing amniotic membrane over a puncture wound in sclera using light-activated bonding without a damaging increase in temperature. The amnion patch size and shape, and light fluence were established for subsequent in vivo study.
- Successfully photobonded amnion over wounds extending over the cornea and sclera ex-vivo and in live animals.
- Determined that directly bonding the wound edges of complex cornea wounds with PTB is not feasible unless small gaps are filled with an additional material.
- Established that the irradiation parameters used to bond amnion to cornea are below the threshold for thermal damage to the iris.

REPORTABLE OUTCOMES

Verter EE, Gisel TE, Yang P, Johnson AJ, Redmond RW, Kochevar IE. Light-initiated bonding of amniotic membrane to cornea. Invest Ophthalmol Vis Sci. 2011 Dec 9;52(13):9470-7.

Yang P, Yao M, DeMartelaere SL, Redmond RW, Kochevar IE. Light-activated sutureless closure of wounds in thin skin. Lasers Surg Med. 2012 Feb;44(2):163-167.

Posters

SEALING PENETRATING CORNEAL WOUNDS WITH A LIGHT-ACTIVATED PROCESS

E. Verter, M. Yao, A. Blandon, Y. Wang, A.J. Johnson, R.W. Redmond and I.E. Kochevar, Wellman Center for Photomedicine, Massachusetts General Hospital, Boston MA and Brook Army Medical Center, San Antonio, Tx, Schepens Research Conference, May 2009

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 4th 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 further extended our development of a simple and rapid light-initiated technology for bonding tissue surfaces together that can be used to seal penetrating eye injuries. The goal of applying this technology is to decrease vision loss and ocular complications after traumatic ocular injuries. Rapid closure of these wounds is critical to stabilizing the eye for further surgery and preventing infection. Our approach seals penetrating wounds by sutureless, glueless attachment of a biological membrane over the wound or by directly bonding the wound edges together.

In Year 3 we addressed the application of light-activated bonding to additional types of penetrating eye wounds.

We identified the amount of light, amount of dye, and size of the repair patch that strongly seal amniotic membrane over a puncture wound in the sclera. Temperature measurements indicated that thermal damage will not result from this repair procedure. A similar study indicated that wounds involving both the cornea and sclera are successfully sealed using light-activated patch technology.

Our studies indicated that light-activated bonding of amnion over a wound is superior to directly bonding together the edges of a penetrating wound in cornea. The amnion patch approach does not require exact approximation of the wound edges, however when the surgeon can approximate the wound edges prior to the light-activated bonding the ensuing wound is equal or superior to suture closure as there is no scar present from the sutures, while at the same time significantly reducing surgical time for the complex lacerations because the water tight seal is quickly obtained. Direct bonding of complex cornea wounds was shown to be ineffective because of small gaps that would require additional sealing material. This was identified as a limitation of the light-activated tissue sealing technology for penetrating eye wounds.

We demonstrated that the green light used for tissue bonding of corneal wounds, even though it is absorbed by melanin in the iris, does not cause thermal damage to this structure. Thus, a potential safety concern has been eliminated.

Together with our results of previous years' studies, we now have a good understanding of the light treatment parameters for sealing penetrating eye wounds and have demonstrated that these repair procedures are safe to ocular tissues. Longer term animal model follow up studies are needed before translation of this technology to the clinic.

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Laceration+laser	Model develop	POD#28	1057	1058	1063	1064	1077	1080						
							"							
Laceration+suture	Exp-2: Group 3	POD#3	2122	2984	2985	2986	2987							
Laceration+suture	Exp-2: Group 3	POD#7	2106	2107	2108	2109	2110	2993	2202	2203	2245			
Laceration+suture	Exp-2: Group 3	POD#28	2105	2125	2126	2127	3090							
Laceration+laser	Exp-2: Group 1	POD#3	2214	2215	2216	3089	3091							
Laceration+laser	Exp-2: Group 1	POD#7	2217	2218	2219	2243	2244							
Laceration+laser	Exp-2: Group 1	POD#28	1082	1138	2240	2241	2242							
Laceration+suture(V)	Exp-2: Group 4	POD#3	3382	3383	3392	3393	3402							
Laceration+suture(V)	Exp-2: Group 4	POD#7	2988	2989	2993	2202	3310							
Laceration+suture(V)	Exp-2: Group 4	POD#28	3311	3312	3384	3385	3409							
Laceration+laser(V)	Exp-2: Group 2	POD#3												
Laceration+laser(V)	Exp-2: Group 2	POD#7	2203	3374	3304	3405	3406							
Laceration+laser(V)	Exp-2: Group 2	POD#28	1134	3394	3396	3403	3404	3386, 3395(convert to suture)						
scleral+suture	Exp-3: Group 1	POD#3	2206	2208	2209	3410	3411							
scleral+suture	Exp-3: Group 1	POD#7	2204	2205	2210	2212	2213							
scleral+suture	Exp-3: Group 1	POD#28	3092	3093	3094	3407	3408							
scleral+laser	Exp-3: Group 2	POD#3												
scleral+laser	Exp-3: Group 2	POD#7												

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scleral+laser	Exp-3: Group 2	POD#28												

Black: Before Aug 2013

Red: After Aug 2013

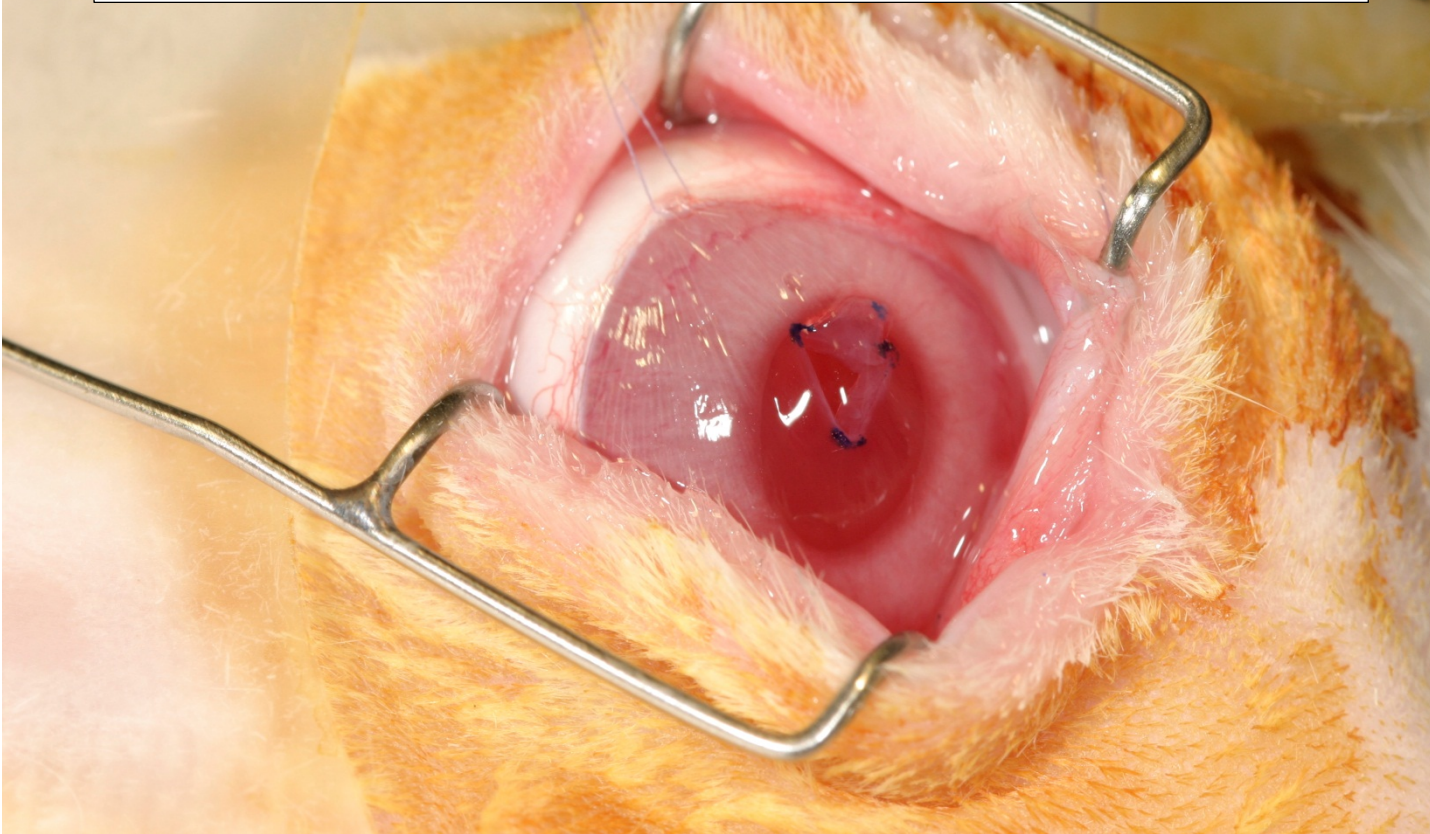
yellow box: We have to fill.





Shown here is the laser apparatus used to apply a broad beam 532nm laser spot to the ocular surface of the rabbit.

Shown here is the large wound gape that is possible from the central V shaped laceration. The traction suture is placed to facilitate corneal centration.



Shown here is a V shaped Central corneal laceration, with amniotic membrane attaché to the cornea with air in the anterior chamber to prevent aqueous outflow.

