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TITLE: Large Extremity Peripheral Nerve Repair in Nonhuman Primate Models

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CONTRACTING ORGANIZATION: Massachusetts General Hospital

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14. ABSTRACT The overarching hypothesis of this proposal is that a rapid, simple, light-activated sealing technology can provide a more secure wound closure and reduce complications leading to improved outcomes for wounded warfighters following traumatic penetrating colon injury. Penetrating bowel wounds can be rapidly sealed and stabilized using biocompatible patches in conjunction with light-activated bonding. Our objective is to determine the optimal implementation strategy for this technology in a large animal model that recapitulates the military trauma scenario and to address a priority research area in the Combat Casualty Care Research Program "to identify and develop medical techniques and materiel for early intervention in life-threatening battle injuries".				
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1. INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The aim of this JWMPR proposal is to employ a relevant large animal model of peripheral nerve injury involving large segmental deficit in the upper limbs that recapitulates human anatomy and is capable of objective functional outcome testing that is not possible in other large animal models. With this model we will determine whether our improved method of restoring continuity to peripheral nerves by photosealing a commercial acellular nerve allograft across the deficit can produce outcomes equivalent to standard of care autologous grafting for wounded warfighters that do not have sufficient autologous donor nerve due to extensive combat trauma. Improving functional recovery for wounded warfighters in this manner would have a major impact on quality of life and well-being of such wounded warfighters.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Peripheral nerve repair, nerve graft, nerve allograft, non-human primate, tissue bonding, photosealing, rose Bengal, PTB, Avance, functional recovery, EMG, muscle mass retention, PSOCT.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goal of this project is to improve functional outcomes following repair of large nerve deficit injuries in wounded warriors that utilize acellular nerve allograft where autologous nerve graft is unavailable following severe trauma.

Milestones for this award are listed below, along with percentage completion to date (**in bold**) where appropriate.

- A. Regulatory approval (MGH IACUC and ACURO) of injury and repair models in Rhesus monkeys (Months 1-4). **100% complete**
- B. Purchase and receipt of laboratory supplies. (Months 1-2) **100% complete**
- C. Harvest of human amniotic membrane (HAM) from placenta obtained from MGH under Discarded Tissue Protocol (Months 1-3). **100% complete**
- D. Crosslinking of HAM with EDC/NHS to make xHAM (Months 2-4). **100% complete**
- E. First meeting of MGH team with surgical advisors at MGH to discuss progress and suggest improvements to the research approach (Month 6). **100% complete**
- F. Purchase and acclimatization of twenty Macaque Monkeys (Months 4-8 at 4 per month). **100% complete.**
- G. Training of monkeys in behavioral task used to evaluate for functional recovery after repair (Months 4-9). **100% complete**
- H. Survival surgeries for peripheral nerve defect and repair in Rhesus monkey model (Months 5-10). **100% complete**
- I. Second meeting of MGH team with military surgeons advisors at MGH to discuss progress and suggest improvements to the research approach (Month 12). **100% complete**

- J. Serial monthly behavioral testing for recovery of function in monkeys. (Months 5-22) **100% complete.**
- K. Serial electrophysiology measurements in monkeys (Months 5-22) **100% complete.**
- L. Third meeting of MGH team with military clinical advisors at MGH to discuss progress and suggest improvements to the research approach (Month 18). **100% complete**
- M. Euthanasia of Monkeys at one-year post-operative time points (Months 17-22) **100% complete.**
- N. Harvest of nerve repair complex at euthanasia and preparation of samples for histology and histomorphometry (Months 17-22) **100% complete.**
- O. Harvest of innervated muscle at euthanasia and determination of retention of muscle mass with respect to unoperated contralateral limb. (Months 17-22) **100% complete.**
- P. Analysis of histomorphometric data from nerve cross-sections. (Months 22-24). **100% complete**
- Q. Final meeting of MGH team with military surgeon advisors at MGH to discuss results and conclusions from the entire project and plan for immediate human trial, if successful. (Month 24). **100% complete**
- R. Preparation of report and manuscript based on outcomes. **50% complete**

What was accomplished under these goals?

In order to be able to accurately test the ability of our photosealing technology to improve nerve regeneration in a true clinically-relevant animal model it was first necessary to develop a model suitable for the purpose that would be sufficiently robust and sensitive for the purpose and involve minimum morbidity to the NHPs. The development is described in the following sections.

Following approval from ACURO the first cohort of 6 Rhesus Macaque monkeys were ordered and arrived at MGH on 09/14/2017 to commence a 6-week quarantine period, from which they were released on 10/23/2017, following successful testing for lack of infectious disease. Acclimation of animals to research staff and facility was commenced, animals were routinely evaluated by research staff who began training animals to reach out of cage through a tube and grasp treats (mimicking the behavioral device which was later constructed) Animals were sedated, weighed, and measurements of upper extremities obtained for construction of wrist extension device (see below.)

Study staff met with MGH veterinary staff and the Environmental Enrichment Program Manager at the MGH Center for Comparative Medicine (CCM) on 9/11/2017 to discuss the equipment to be designed for longitudinal behavioral testing outcome following injury and repair of the radial nerve. As this injury affects the animal's ability to extend the wrist, a prototype was conceived where the animal must reach out the cage with the right arm, bend the elbow and reach through a tube to a box to obtain a food treat, placed on a platform that can be positioned at different heights within the enclosure, thus testing the degree to which the animal can extend the wrist. The concept is shown in Figure 1.

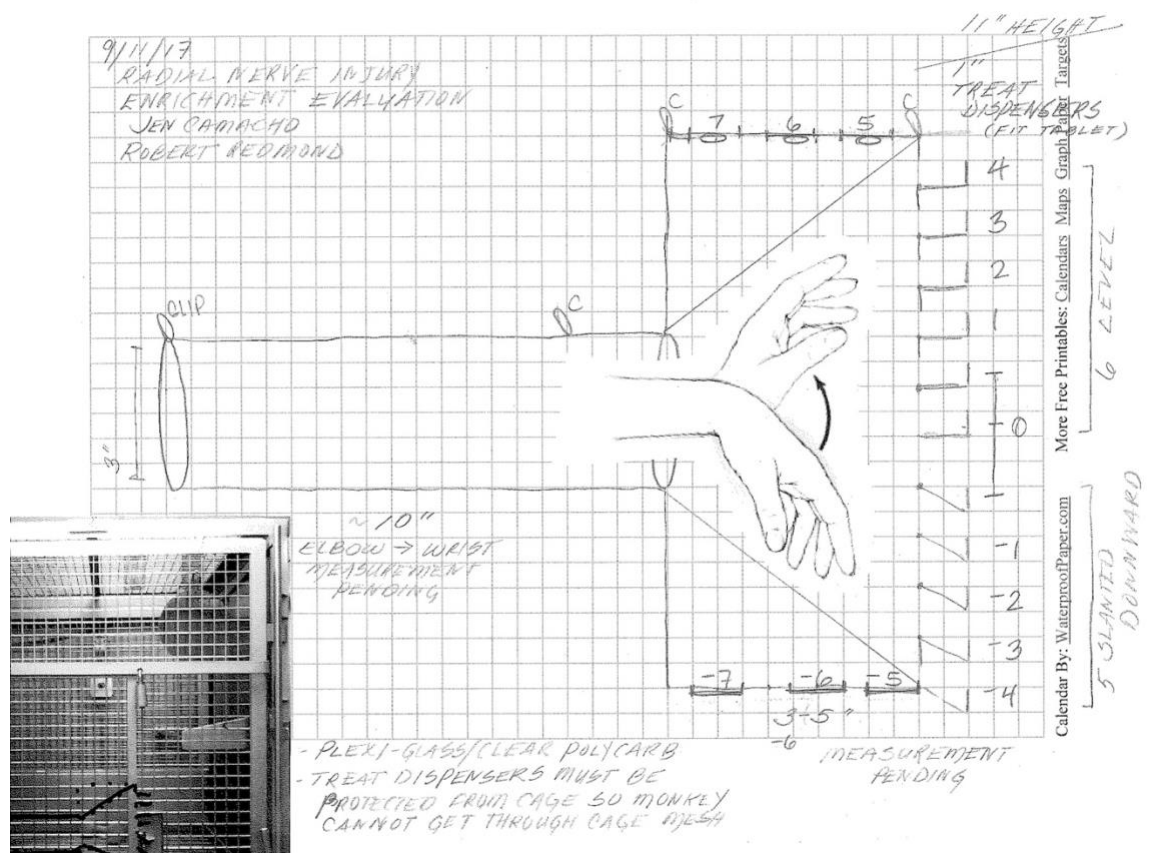


Figure 1: schematic of behavioral testing chamber for recover of function of wrist extension.

Following refinement of the design the behavioral testing device was constructed from clear Plexiglas, allowing good visual coordination for the NHP as is shown in Figure 2. Acclimation of the animals to the final device was performed and testing of wrist extension undertaken. Pictured below is device attached to front of cage with animal reaching through tube and extending wrist in order to grasp a treat placed on a platform. Pre-surgical measurement of extension range for all animals were obtained and monthly serial measurements were performed in this manner for the 12- month postoperative period to euthanasia.

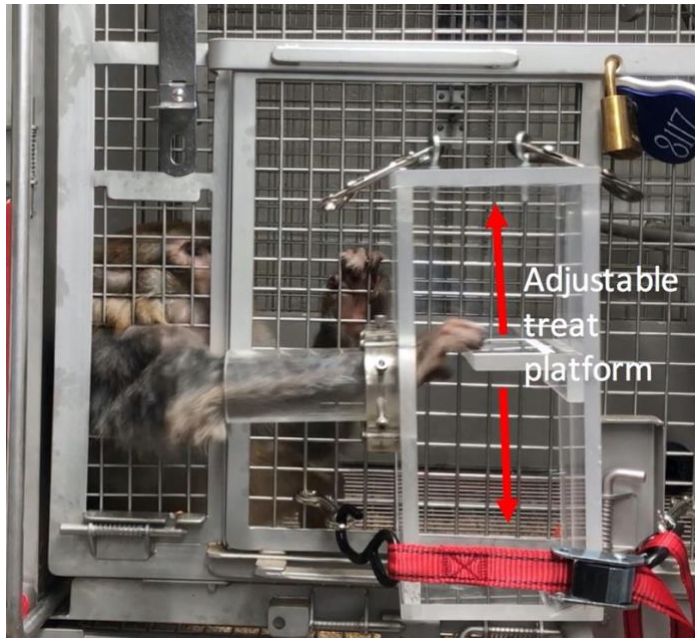


Figure 2: Final device used for functional testing. NHP reaches out the cage with the right arm, through a plexiglass tube that locks arm and extends the wrist and hand into the plexiglass container to access the food treat positioned on a platform that is continuously adjustable vertically. The platform is raised to the point that the animal cannot extend the wrist to reach the treat and the angle measured from freeze frame images of the videography

The large peripheral nerve deficit model chosen was a surgically-created 4 cm gap in the radial nerve of the NHP as shown in Figure 3A. The radial nerve was chosen to limit morbidity of the injury to the NHPs as the nerve controls wrist extension alone in the animal and allows the animal to have full use of the hand and fingers in activities such as feeding and grooming. No cross-talk with other nerves is involved in wrist extension that is solely dependent on the radial nerve. Immediately following injury, “wrist drop” occurs where the animal has lost its ability to extend the wrist. Following repair, the nerve regeneration can be followed through a functional improvement in the NHP’s ability to extend the wrist to obtaining treats with the platform set at increasing height.

Thus, functional recovery was performed as follows: Prior to each radial nerve surgery (Figure 3A) the NHPs were acclimatized to their environment and underwent repeated training to reach out the cage through a cylindrical tube and obtain a food treat from a platform that could be adjusted in height (Figure 3B). Resection of the nerve segment causes loss of extension and ‘wrist drop’, which recovers over time if nerve regeneration occurs. Video recording allowed freeze frame analysis of the wrist extension angle at the maximum platform height that could be reached by the NHP (Figure 3C).

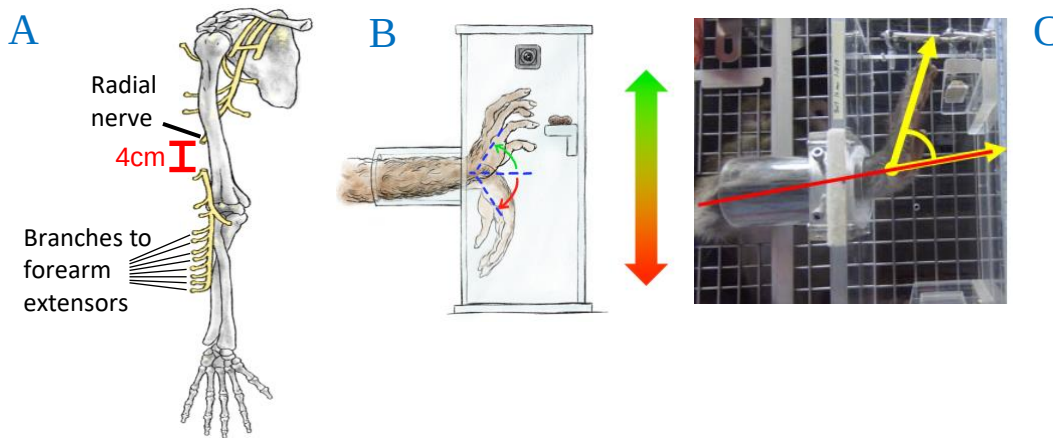


Figure 3: NHP model for nerve deficit and recovery. (A) a 4 cm radial nerve deficit is surgically created to induce loss of wrist extension. (B) Functional recovery is measured by a behavioral test that measures the extent the NHP can extend the wrist to retrieve a food treat from a platform that is adjustable in height. (C) Photo showing evaluation of wrist extension angle from freeze-frame video analysis.

Discussions were also held with Dr. William S. David MD, the Director of the EMG Laboratory and Neuromuscular Diagnostic Center at Massachusetts General Hospital, on the EMG testing which is a component of the outcome measures for nerve regeneration in this study. Dr. David provided valuable advice on the EMG testing and how it should be performed in this NHP model of radial nerve injury and repair and his colleague, Dr. Reiner See, subsequently participated in experimental EMG measurements for all NHPs in the study. EMG measurements were taken prior to injury and then at 4, 8 and 12 months post-operatively.

Following the training period, NHP surgeries were performed to create a large deficit in the radial nerve in the upper arm, followed by repair by one of three methods.

1. standard of care microsurgery using reversed autograft (control);
2. Photosealed acellular nerve allograft (PTB/ANA, Figure 4);
3. Microsurgical attachment of ANA.

The photosealing approach employed a section of processed human amnion that was crosslinked using a biocompatible crosslinker (4mM/1mM EDC/NHS) that was coated with a sterile 0.1% Rose Bengal solution and then circumferentially photosealed over the graft attachment sites by illumination at 532 nm using a KTP laser (0.5 W/cm², 60 J/cm²) as shown in Figure 4.

The ANA used was the clinically-approved Avance acellular nerve allograft graft from Axogen inc. As this human derived product was used in a different species it was necessary to initially test whether an immune response would be developed that could confound the study. A section of Avance was placed under the skin of the first two NHPs that received autograft as a nerve graft (group 1) and then removed after three months at which time histological studies showed evidence of inflammation and a foreign body response. Thus, all animals in groups 2 and 3 were kept on an immune-suppression regimen throughout the course of the 12-month study.

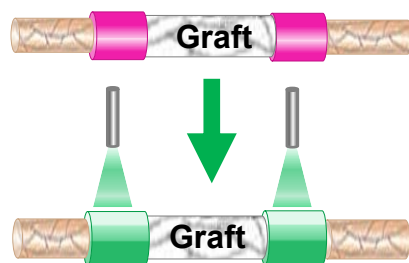


Figure 4: Schematic of photosealing approach to neurorraphy by applying the photoactive dye (pink) rose Bengal to crosslinked amnion and wrapping around coaptation sites of ANA, followed by illumination with green light to seal the graft in place.

In addition to functional recovery and EMG, other outcome metrics included retention of muscle mass in the operative arm compared to the contralateral unoperated arm (measured as % muscle mass retention) and histomorphometric measurements taken from harvested nerve grafts at euthanasia. We also worked in collaboration with Dr. Benjamin Vakoc from the Wellman Center for Photomedicine at MGH to apply a new non-invasive, wide field optical imaging technology (polarization-sensitive optical coherence tomography, PSOCT) to shed more light on the nerve regeneration process through these large-gap grafts.

In summary, we have developed a new clinically-relevant model for measurement of nerve regeneration following peripheral nerve injury and repair that significantly improves on any other model currently in use, that in combination with the other outcome metrics used, provides a comprehensive report on the process of nerve regeneration following injury and can be used to evaluate outcomes following any type of intervention, whether surgical, pharmacological or rehabilitative.

Table 1: Operative Schedules for all NHPs in the Study

NHP #	DOB	Group	OR Date	Euthanasia Date
8117	4/17/13	Control	1/8/2018	1/14/19
8017	5/12/13	Control	1/19/2018	1/22/19
7817	3/25/13	PTB	2/5/2018	2/4/19
7917	3/16/13	PTB	2/13/2018	2/11/19
8217	3/19/13	Control	3/14/2018	3/15/19
8317	5/6/13	Control	3/21/2018	3/21/19
1118	4/11/11	PTB	6/11/2018	5/6/19
1218	3/9/11	Avance/Suture	6/12/2018	5/24/19
818	6/25/11	Avance/Suture	6/18/2018	6/3/19
1018	8/3/11	Avance/Suture	6/19/2018	6/10/19
918	6/10/11	PTB	6/25/2018	5/23/19
1318	8/26/11	PTB	6/26/2018	6/17/19
1918	4/25/11	Control	8/6/2018	8/5/19
2118	2/1/12	Avance/Suture	8/8/2018	8/12/19

1418	6/16/11	Control	8/13/2018	8/19/19
1718	11/25/11	PTB	8/14/2018	8/26/19
1518	5/23/11	PTB	9/17/2018	9/17/19
2018	4/10/11	Avance/Suture	9/25/2018	9/25/19

Results:

There were 3 experimental groups in this study. Six animals have undergone repair using PTB/Avance ANA, 6 animals received microsurgical repair with reversed autologous graft and 5 animals received microsurgical repair with the Avance ANA.

(a) **Functional Recovery** was observed in 5/6 autologous grafts (control), 3/6 PTB/ANA and 3/5 suture/ANA repairs. For those animals that demonstrated recovery the mean extension angle is shown as a function of time in Figure 5 up to the time of euthanasia. As expected, the autologous nerve performs well in terms of magnitude and rate of recovery. Autologous nerve graft has the advantages of Schwann cell support and a pre-existing vasculature that can rapidly be re-established to support nerve regeneration. Both PTB/ANA and suture/ANA repairs are delayed with respect to functional recovery by 3 months in the case of PTB/ANA and 4 months for suture/ANA. The delay is due to the lack of the inherent Schwann cell population and a need for a microvasculature to be generated in the avascular nerve allograft. However, both are capable of full functional recovery as the plateau recovery reached recovers to the pre-operative level.

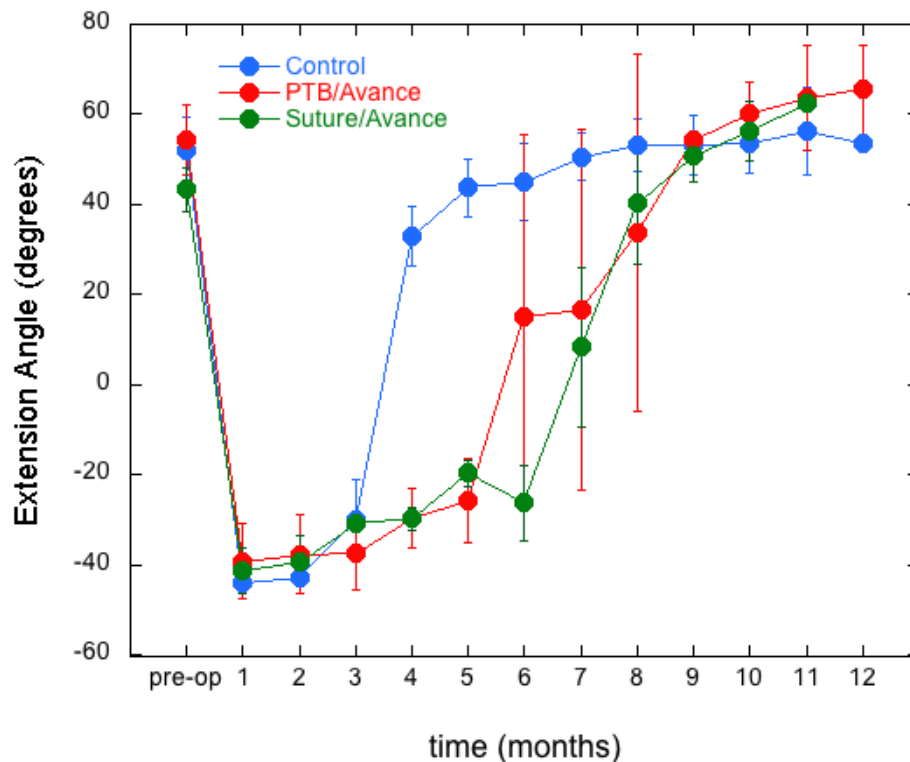


Figure 5: Serial measurement of maximum wrist extension angle as a function of repair procedure (mean +/- standard deviation).

(b) **Muscle mass retention:** The amount of extensor muscle mass retained after surgery and repair is an indirect measure of nerve regeneration efficiency. Lack of reinnervation leads to muscle inactivity

and wastage. Table 3 shows the percentage of the muscle mass on the operative arm (right) on euthanasia at 12 months with respect to the same muscle on the unoperated arm (left). Highlighted in yellow are NHPs that did not show any significant functional recovery in the behavioral test. Those highlighted NHPs have clearly lost the most muscle, in good agreement with the functional testing.

Table 2: Muscle mass (g) retention data for all NHPs at euthanasia expressed as percentage wet weight in comparison to the contralateral unoperated limb.

Number	Repair Type	Operative (g)	Control (g)	% Difference
8117	Control	7.061	12.272	-42.463
8017	Control	12.160	16.260	-25.215
7817	PTB	2.044	8.271	-75.287
7917	PTB	11.486	14.536	-20.982
8217	Control	17.840	25.260	-29.375
8317	Control	21.862	21.592	1.250
1218	Avance/Suture	16.570	19.725	-15.995
818	Avance/Suture	2.337	6.441	-63.717
1018	Avance/Suture	6.734	10.090	-33.261
918	PTB	7.774	7.538	3.131
1318	PTB	2.707	12.096	-77.621
1918	Control	5.769	9.916	-41.821
2118	Avance/Suture	6.457	7.618	-15.240
1418	Control	3.846	5.651	-31.941
1718	PTB	7.920	7.343	7.858
1518	PTB	1.638	5.892	-72.200
2018	Avance/Suture	1.410	4.634	-69.573

For the remaining monkeys that did display recovery the percent muscle mass retention as a function of treatment is shown in Figure 6. Interestingly, the PTB/ANA group demonstrated much better muscle mass retention than the autograft or suture/ANA groups.

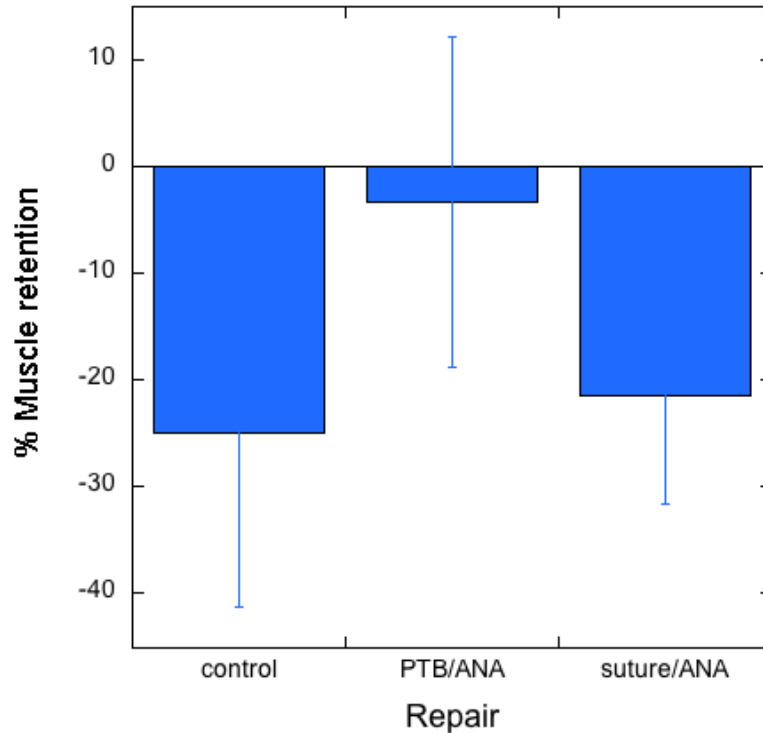


Figure 6: Percentage Muscle mass difference at euthanasia between operative arm and contralateral unoperated arm per repair group (mean +/- standard deviation) for all NHPs with *positive functional regeneration*.

(c) Nerve Histomorphometry. Table 3 shows the correlation between functional recovery and preliminary assessment of axon regeneration that was based on the following scale:

- 0: no visible axons;
- 1: sparse axons lacking organization;
- 2: areas of organized dense axons;
- 3: dense well-organized axons across entire nerve, similar to contralateral unoperated nerve.

Again, a good correlation is observed between nerve histology and functional recovery, giving further credence to the NHP model of functional recovery. Generally, NHPs with poor functional recovery (white cells) also exhibit poor axonal regrowth with values of 0 or 1. One NHP (#8017), highlighted in red, was an outlier as it exhibited full functional recovery but poor distal axonal regrowth. Similarly, NHPs with excellent functional recovery also exhibited high axon scoring of 2 or 3 (#8017 and #7817) highlighted in green. One outlier to this behavior was NHP #7817, highlighted in blue, that had poor functional recovery of only 19% but was scored a 2 in nerve histomorphometry, both mid-graft and distal to the graft. What is also of note is that full functional recovery does not require full axonal density in the regenerating nerve. We estimate that >20% density of contralateral (and by inference, normal uninjured nerve) is sufficient to promote close to 100% functional regeneration. We intend to study this further in the future.

Table 3: Correlation of functional recovery with qualitative assessment of axon regeneration from nerve histomorphometry for each animal at 12-months post-operatively.

NHP	Repair Type	% Functional Recovery	Mid-graft axon density	Distal axon density
8117	autologous	111.49	3	3
8017	autologous	100.0	1	1
7817	PTB/ANA	18.7	2	2
7917	PTB/ANA	102.0	2	2
8217	autologous	105.2	2	3
8317	autologous	100.0	2	2
1118	PTB/ANA	44.1	1	0
1218	suture/ANA	148.8	2	2
818	suture/ANA	38.3	-	-
1018	suture/ANA	134.9	2	2
918	PTB/ANA	129.4	2	2
1318	PTB/ANA	19.3	0	0
1918	suture/ANA	62.0	2	2
2118	suture/ANA	115.3	2	2
1418	autologous	111.4	2	2
1718	PTB/ANA	98.9	2	3
1518	PTB/ANA	39.3	1	1
2018	suture/ANA	27.4	1	1

(d) EMG. In collaboration with Dr. William S. David MD and Reiner See M.D. from the EMG Laboratory and Neuromuscular Diagnostic Center at Massachusetts General Hospital, we performed EMG testing in this NHP model of radial nerve injury and repair. Measurements were performed under anesthesia immediately prior to surgery for each animal to determine baseline electrophysiology. CMAP (compound muscle action potential) values were determined for each and direct nerve recording (DNR) also performed on dissection of the radial nerve and before creation of nerve deficit. Further measurements are made at 4, 8 and 12-month time-points. EMG signals were also obtained from the contralateral unoperated arm immediately prior to euthanasia.

EMG signals have led to interesting outcomes. Figure 7 shows the correlation between percentage functional recovery and CMAP (measured a percentage of unoperated contralateral arm at 12 months. With only two exceptions (NHP #2018, 7817) both with poor functional recovery) the CMAP recovery is significantly lower than functional recovery in magnitude. These are the same two animals that were outliers in the muscle mass retention data. In fact, even at 100% functional recovery, the CMAP values are only in the range of 50-70% of the control value in the contralateral arm. In general, however, we observe reasonable agreement between functional recovery and EMG data,

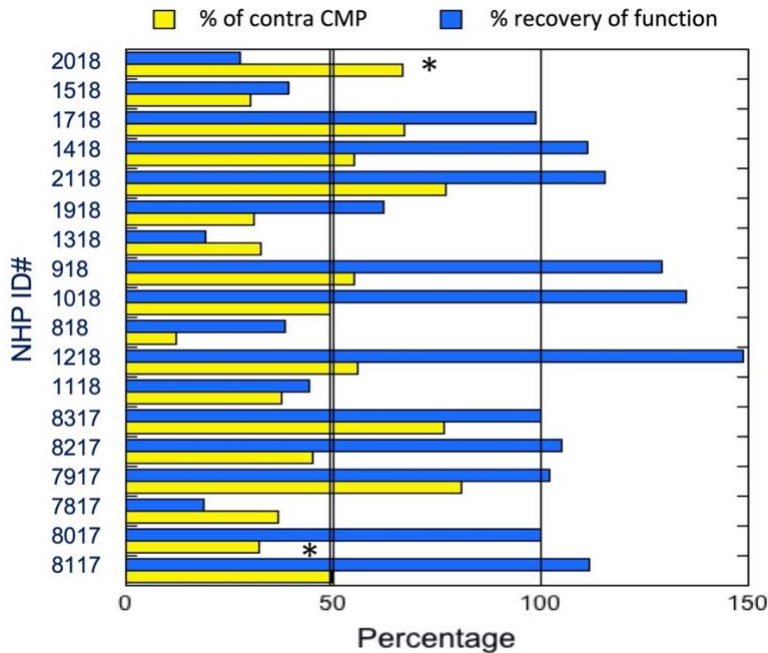


Figure 7: Correlation of functional recovery with compound muscle action potential (CMAP), expressed as the percentage of the value obtained for the contralateral unoperated arm, at 12 months-post-operatively

CONCLUSIONS: This primate model dramatically improves upon existing animal models of peripheral nerve repair and aims to fill the clinical need for a model relevant to the study of peripheral nerve injury, particularly large nerve gaps, by recapitulation of the clinical problem in humans. We report a new method for longitudinal measurement of functional recovery of peripheral nerve regeneration in an objective, quantitative manner by use of a novel testing apparatus that accurately measures the maximal degree of wrist extension following repair of the radial nerve. This model can be utilized to test outcomes of the full range of peripheral nerve injuries and repair techniques with negligible subject morbidity. The close anatomical similarity of the NHP forelimb to humans makes this a valuable quantitative model to mimic human injury and recovery.

Functional recovery is the primary outcome of interest for clinicians following peripheral nerve injury and repair. This model allowed us to evaluate the use of our photosealing technology in potentially improving outcomes of alternatives to autologous graft for large gap peripheral nerve repair. Results showed that the photosealing process could actually provide recovery of the same magnitude as the standard of care autologous graft. The rate of recovery was delayed with respect to autologous graft by a period of 2-3 months, related to the lack of Schwann cells and innate vasculature in the acellular graft but improved compared to microsurgical attachment. In addition, the photosealing process considerably improved the retention of distal muscle mass in the operative limb, even with respect to the autologous graft. These promising outcomes are supportive of the next step of hyamn studies.

NEXT STEPS: On the basis of the positive results obtained in this study, funding was sought for a first in human studies in collaboration with military clinicians. We were successful in obtaining a large grant from the Geneva Foundation to design and perform human studies at both Walter Reed National Military Medical Center (WRNMMC) and the Massachusetts General Hospital under the

direction of Dr. Jason Sousa and Dr. Jonathon Winograd. The IRB for these studies is in the latter stages of development and we expect to commence in the coming months.

What opportunities for training and professional development has the project provided?

Dr. Hansdorfer received advanced microsurgery training from Dr. Winograd and Dr. Randolph including operating under magnification. His mentored training within the Division of Plastic Surgery at MGH resulted in accelerated acceptance into a Plastic Surgery Residency Program at Rush University in Chicago, IL, which he commenced in July 2019. He has also been very active in disseminating the findings of the research at National and International meetings (*vide infra*).

How were the results disseminated to communities of interest?

Results were disseminated at national and international conferences (including MHSRS, 2018-2020) through publications and abstracts, as described in a following section.

What do you plan to do during the next reporting period to accomplish the goals?

The only outstanding item from the Statement of Work is a second publication reporting the comparative results of the overall study with respect to treatment group. This manuscript is in the latter stages of preparation.

4. IMPACT:

We firmly believe that our NHP model is by far the best model in current use for clinically-relevant evaluation of any intervention (surgical, pharmacological or rehab) targeted to improving nerve regeneration and functional recovery in patients. In addition, the use of photosealing of ANA has been shown to be capable of functional recovery equivalent to standard of care autograft in some cases, with improved muscle retention, that extends hope to victims of polytrauma, such as wounded warriors, that have no autologous nerve available for grafting.

What was the impact on other disciplines?

The photosealing approach has a wide capability and is currently being evaluated for sealing of penetrating bowel injury and prevention of post-surgical leaks and adhesions and will be explored in the near future as an augmentation for orthopedic repair of tendon, ligament and cartilage injuries.

What was the impact on technology transfer?

The technology behind the photosealing of nerve grafts was already protected and patented prior to the start of this study. We are very close to launching a start-up company in the vascular repair space and expect nerve repair to be one of the next indications to be commercialized.

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

MGH ran into a problem of shortage of large animal housing that delayed the initiation of the NHP surgeries, resulting in a request for a no-cost extension until 3/1/2020. Since that time the housing shortage has been alleviated and all surgeries were performed and the study concluded within the NCE time-frame.

Changes that had a significant impact on expenditures

None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals

None

Significant changes in use of biohazards and/or select agents

None

Institutional IACUC and ACURO Approvals

- 12/28/2016: Submission of protocol to MGH IACUC (#2016N000627)
- 3/23/2017: Approval of protocol by MGH IACUC
- 3/31/2017: Submission of protocol to ACURO
- 6/26/2017: Approval of protocol by ACURO
- 10/10/2017: Submission of amended protocol to MGH IACUC
- 11/06/2017: Approval of amended protocol by MGH IACUC
- 11/17/2017: Submission of amended protocol to ACURO
- 12/4/2017: Approval of amended protocol by ACURO

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**

Journal publications.

A Clinically Relevant Animal Model to Quantitatively Evaluate Functional Outcomes of Large-Gap Peripheral Nerve Repair. Hansdorfer MA, Runyan GG, Farinelli WA, Randolph MA, Winograd JM, Redmond RW. Submitted to *Plastic and Reconstructive Surgery*.

Medical Applications of Rose Bengal- and Riboflavin-Photosensitized Protein Crosslinking. Redmond RW, Kochevar IE. *Photochem Photobiol.* 2019. doi: 10.1111/php.13126. [Epub ahead of print] PMID: 31111489.

Invited Talks:

Potential Clinical Applications of Protein Photocrosslinking. Redmond RW. *Annual Meeting of the American Society for Photobiology*, April 2018, Tampa, Florida.

Large Gap Peripheral Nerve Repair in a Non-Human Primate Model Utilizing Photochemical Tissue Bonding (PTB). Hansdorfer MA, Tsui JT, Visaggio MC, Runyan GG, Randolph MA, David WS, Winograd JM, Redmond, RW. Presented at 29th Annual Richard J. Smith Conference. 2018 April 27; Boston, MA.

A Clinically Relevant Animal Model to Quantitatively Evaluate Functional Outcomes of Large-Gap Peripheral Nerve Repair. Hansdorfer MA, Tsui JT, Visaggio MC, Runyan GG, Randolph MA, David WS, Winograd JM, Redmond, RW. Accepted for Presentation at *Massachusetts General Hospital Clinical Research Day*. 2018 October 4; Boston, MA

Preclinical Studies of Photocrosslinking Technologies for Tissue Repair and Regeneration. Redmond RW. *17th International Congress on Photobiology*, 2019, August 21, Barcelona, Spain.

Preclinical Studies of Photocrosslinking Technologies for Tissue Repair and Regeneration. Redmond RW. Plenary lecture at the *26th Annual Conference of the Society for Free Radicals in Biology and Medicine*, 2019, November 22, Las Vegas, NV.

Conference Proceedings:

Hansdorfer MA, Tsui JM, Visaggio M, Runyan GG, Randolph MA, David WS, See RS, Valerio IL, Winograd JM, Redmond, RW. **Large Gap Peripheral Nerve Repair in a Non-Human Primate Model: Improving Outcomes Utilizing Photochemical Tissue Bonding (PTB) with Acellular Nerve Allograft (ANA).** 64th Annual Plastic Surgery Research Council (PSRC) Meeting. 2019 May 2-5; Baltimore, MD.

Hansdorfer MA, Tsui JM, Visaggio M, Runyan GG, Randolph MA, David WS, See RS, Valerio IL, Redmond, RW Winograd JM. **Improving Outcomes of Large Gap Peripheral Nerve Repair with Photochemical Tissue Bonding (PTB) and Acellular Nerve Allograft (ANA).** Accepted for Presentation at the 60th Annual New England Society of Plastic and Reconstructive Surgeons. 2019 May 31-June 2; Newport, RI.

Hansdorfer MA, Tsui JM, Runyan GG, Randolph MA, David WS, See RB, Valerio IL, Winograd JM, Redmond RW. ***Large Gap Peripheral Nerve Repair with Photochemical Tissue Bonding (PTB) and Acellular Nerve Allograft (ANA) in a Non-Human Primate Model.*** Military Health System Research Symposium. 2019 August 18; Orlando, FL.

Hansdorfer MA, Tsui JM, Visaggio M, Runyan GG, David WS, See RS, Valerio IL, Randolph MA, Winograd JM, Redmond, RW. ***Improving Outcomes of Large Gap Peripheral Nerve Repair utilizing Photochemical Tissue Bonding (PTB) and Acellular Nerve Allograft (ANA).*** Annual meeting of the American Society of Peripheral Nerve (ASPN), 2020 January 18-20, Fort Lauderdale, FL.

Hansdorfer MA, Tsui JM, Runyan GG, Randolph MA, See RB, Valerio IL, Jonathan M. Winograd JM, Redmond RW. ***Light-Activated Attachment of Acellular Nerve Allograft (ANA) to Repair Large Gap Peripheral Nerve Injury in a Non-Human Primate Model.*** Accepted at the Military Health System Research Symposium. 2020 August 18; Orlando, FL. (Abstract# MHSRS-20-01842).

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.

Nothing to report

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

We have developed a new non-human primate model for the study of any intervention (surgical, pharmacological or rehab) that is far better than any current animal model for the study of nerve regeneration in a clinically-relevant manner. We expect this model to be the standard in future translational peripheral nerve regeneration studies. Manuscript has been completed and submitted for publication.

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Robert W. Redmond PhD
Project Role: PI
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 2
Contribution to Project: Dr. Redmond is responsible for overall coordination of the project

Name: Mark A. Randolph MAS
Project Role: Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Mr. Randolph has been instrumental in designing animal protocols and in the behavioral testing design.

Name: Marek Hansdorfer MD
Project Role: Research Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 6
Contribution to Project: Dr. Hansdorfer has been the lead Fellow on this project and has been involved in all day-to day aspects of regulatory approvals, experimental planning, surgical training and behavioral testing design.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to Report.

Plastic and Reconstructive Surgery

A Clinically Relevant Animal Model to Quantitatively Evaluate Functional Outcomes of Large-Gap Peripheral Nerve Repair

--Manuscript Draft--

Manuscript Number:	
Article Type:	Original Article
Full Title:	A Clinically Relevant Animal Model to Quantitatively Evaluate Functional Outcomes of Large-Gap Peripheral Nerve Repair
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Abstract:	Background Preclinical investigations of peripheral nerve repair techniques and graft materials in animal models have typically utilized the rodent sciatic nerve model, which is not clinically relevant, particularly to large nerve gap injury. Other animal models are hindered by reliance on non-primary and subjective outcome measures. We introduce a non-human primate model with minimal morbidity that recapitulates human anatomy and allows objective quantitative functional outcomes testing, electrophysiology and histomorphometry. Methods Four rhesus macaques underwent 4.0 cm proximal radial nerve defect creation in the right forelimb repaired with autograft and standard-of-care suture neurorrhaphy. The radial nerve was selected as it performs an isolated function with no input from other nerves that could confound recovery data. An objective functional test was conceived using an apparatus that accurately measures wrist extension as a function of time after repair. Results No morbidity was observed. Average loss of extension was $94.0^{\circ} \pm 5.5^{\circ}$ post-operatively. At 5-months subjects recovered $87.3^{\circ} \pm 9.9^{\circ}$ extension, 8-months: $95.3^{\circ} \pm 5.7^{\circ}$, 12-months: $97.8^{\circ} \pm 3.6^{\circ}$, demonstrating recovery which approaches completion at 5-months, with gradual improvement up to 12-months. Conclusion This radial nerve model improves upon existing models by allowing large nerve gap testing in a primate that recapitulates the clinical problem in humans, providing multiple end-points including objective functional assessment. This array of outcomes facilitates the most detailed evaluation of clinically-relevant nerve regeneration described to date, serving as a versatile tool for analysis of the full spectrum of nerve reconstruction techniques or pharmacological interventions in any injury model (transection, crush, gap) for translation to human clinical trials.
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Additional Information:	
Question	Response
Do you feel the manuscript qualifies as an outcomes study?	No
What should be the general public's take away from your research?	A method to accurately assess nerve regeneration
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Rod J. Rohrich, M.D.
Editor-in-Chief
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June 25th, 2019

Dear Dr. Rohrich,

It is with great pleasure and enthusiasm that we submit our study entitled **“A Clinically Relevant Animal Model to Quantitatively Evaluate Functional Outcomes of Large-Gap Peripheral Nerve Repair”** for your consideration for publication in *Plastic and Reconstructive Surgery*.

Our laboratory has developed an improved animal model for the study of peripheral nerve repair and regeneration and this manuscript is a detailed description thereof. We believe that this is the most optimal model for the study of peripheral nerve repair described in the literature to date and anticipate it will be a benchmark model in this area for other researchers. We are confident that this manuscript will be of interest to the readership of the *Plastic and Reconstructive Surgery*.

All authors have read the guide for authors and agree to the terms therein. We have all agreed to the submitted version of the manuscript which has not been previously submitted for publication and is not currently in submission to any other journal.

We thank you sincerely for your consideration and look forward to your favorable response.

Best regards,

Robert W. Redmond, Ph.D.
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A Clinically Relevant Animal Model to Quantitatively Evaluate Functional Outcomes of Large-Gap Peripheral Nerve Repair

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Short Running Head: NHP Model for Recovery of Nerve Function.

Author Contributions:

Marek A. Hansdorfer, MD: Dr. Hansdorfer was the lead Research Fellow on this project. He was involved in the conceptualization of the testing apparatus, training the animals to use the apparatus, surgical operations, post-surgical care and longitudinal functional testing. He also wrote the initial drafts of the manuscript.

Gem G. Runyan, MS: Mr. Runyan worked closely with Dr. Hansdorfer on experimental design and functional training, testing of the animals and video analysis of test results .

William A Farinelli, BA: Mr. Farinelli was responsible for conceptual design, fabrication and modification of the functional testing apparatus.

Mark A. Randolph, MAS: Mr. Randolph was responsible for design of the animal protocol, contributed to the conceptualization of the testing approach and supervised all animal experimentation.

Jonathan M. Winograd, MD: Dr. Winograd provided clinical expertise on peripheral nerve repair and reviewing and revising the draft manuscript.

Robert W. Redmond, PhD: Dr. Redmond had overall responsibility as Principal Investigator for the study, including management of all facets of the study, including writing the research proposal, conceptualization of the animal model and testing approach, submitting reports and final manuscript preparation.

ABSTRACT

Background: Preclinical investigations of peripheral nerve repair techniques and graft materials in animal models have typically utilized the rodent sciatic nerve model, which is not clinically relevant, particularly to large nerve gap injury. Other animal models are hindered by reliance on non-primary and subjective outcome measures. We introduce a non-human primate model with minimal morbidity that recapitulates human anatomy and allows objective quantitative functional outcomes testing, electrophysiology and histomorphometry.

Methods: Four rhesus macaques underwent 4.0 cm proximal radial nerve defect creation in the right forelimb repaired with autograft and standard-of-care suture neurorrhaphy. The radial nerve was selected as it performs an isolated function with no input from other nerves that could confound recovery data. An objective functional test was conceived using an apparatus that accurately measures wrist extension as a function of time after repair.

Results: No morbidity was observed. Average loss of extension was $94.0^{\circ} \pm 5.5^{\circ}$ post-operatively. At 5-months subjects recovered $87.3^{\circ} \pm 9.9^{\circ}$ extension, 8-months: $95.3^{\circ} \pm 5.7^{\circ}$, 12-months: $97.8^{\circ} \pm 3.6^{\circ}$, demonstrating recovery which approaches completion at 5-months, with gradual improvement up to 12-months.

Conclusion: This radial nerve model improves upon existing models by allowing large nerve gap testing in a primate that recapitulates the clinical problem in humans, providing multiple end-points including objective functional assessment. This array of outcomes facilitates the most detailed evaluation of clinically-relevant nerve regeneration described to date, serving as a versatile tool for analysis of the full spectrum of nerve reconstruction techniques or

pharmacological interventions in any injury model (transection, crush, gap) for translation to human clinical trials.

INTRODUCTION

Peripheral nerve injuries occur in 3% of traumatic injuries ¹⁻⁴ with 360,000 people in the United States alone suffering upper extremity paralysis annually ⁵, representing a tremendous social burden in functional disability and cost to the healthcare system ⁶. Peripheral nerve injuries pose unique clinical challenges to surgical repair and are frequently associated with poor functional outcomes. Substantial resources have been directed toward development of improved treatment methods for peripheral nerve injuries in pre-clinical animal models and subsequent translation into humans. Despite progress in understanding of nerve injury pathophysiology and regeneration and advancements in microsurgical techniques, outcomes have remained poor. Particularly challenging, are patients afflicted with large nerve gap injuries (>3 cm) with most suffering permanent disability ^{4, 7}.

Advances in peripheral nerve repair have been possible through pre-clinical investigation of surgical techniques, pharmacotherapies, and bridging materials (acellular nerve grafts, bioengineered grafts, nerve guides, etc.) in animal models. As these models are utilized to evaluate potential for human clinical translation, it is imperative that they mimic anatomic, physiological, and pathologic responses of human conditions ⁷. Table 1 summarizes the outcome measures used in the most commonly employed animal models. The rodent sciatic nerve model (RSNM) remains the most common model of nerve repair ⁷⁻⁹. Relatively low cost, high availability, and comparability to other studies ⁸⁻¹¹ have made the RSNM appealing, particularly for initial proof-of-principle studies. Functional outcome in rodents involves gait observation, sciatic function index (SFI) ^{12, 13}, and examination of sensory response (von Frey test) ¹⁴, along with detailed histomorphometrical and muscle retention analysis. While some investigators have

performed electromyography (EMG) in rodents ¹⁵⁻¹⁷, it is technically challenging and not commonly performed. Rodent extremity anatomy has notable functional, gross, and microscopic anatomical differences compared to humans ^{18, 19}, most evident in the fact that many *successful* studies performed in rodent models fail to be replicated in humans ^{20, 21}.

Quadruped models similarly possess various limitations. The rabbit model focuses on sciatic and peroneal nerve ²²⁻²⁴ injuries, allowing histomorphometry and muscle retention along with the ability to perform EMG ²²⁻²⁴. Like rats, however, rabbit models lack functional outcome measures and do not replicate human anatomy. Swine ^{25, 26}, ovine ⁷, and canine ²⁷ models provide histology, muscle retention, and detailed EMG analysis but suffer from limited functional outcomes. Although quadrupeds and humans share many muscles and nerves, the porcine anatomy, like that of rabbits, sheep and dogs, is distinct, with notable extraneous structures not present in humans ²⁸.

The NHP model of peripheral nerve repair is the only preclinical model that allows the combined analysis of all available outcome measures, histomorphometry, muscle mass retention, and EMG. These are commonly performed in NHP models with the benefit of testing action potentials of the same muscles tested in human clinical practice: radial nerve electrical testing involves radial sensory nerve conduction and muscle action potential, most commonly of the extensor indicis proprius, extensor digitorum communis, or brachioradialis ²⁹. The NHP model also provides functional outcome assessment to a degree and complexity that is not possible in any other species ³⁰. Because humans and NHPs share the same evolutionary origin, they possess discrete anatomic and genetic characteristics ^{30, 31} with a high degree of similarity in extremity anatomy ³². For these reasons, investigation of peripheral nerve repair techniques in NHPs remains a critical step in the translation of novel techniques/materials into humans.

Due to the significant limitations of animal models currently in use, we saw a critical need for an improved forelimb model with the ability to accurately assess functional outcomes and identify only truly effective treatments that merit translation. Inspired by the model described by Wang et al ¹¹, we report here a new radial nerve model incorporating the full gamut of outcomes analysis in addition to a new method of objective quantitative functional nerve recovery testing. The battery of outcomes in this NHP model provides a highly accurate evaluation of true nerve regeneration in a clinically-relevant model to further the overall goal of therapeutic research for improving outcomes for patients with devastating peripheral nerve injuries.

MATERIALS AND METHODS

Animal Use

Procedures were compliant with the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Massachusetts General Hospital's Institutional Animal Care and Use Committee and the United States Army Medical Research and Command's Animal Care and Use Review Office. Four juvenile male rhesus macaques (*Macaca Mulatta*) aged four years were housed at the Massachusetts General Hospital animal facility (Boston, MA). Animals underwent 6-week quarantine prior to clearance for study participation and underwent sequential tuberculosis and viral serology screening for Macacine alphaherpesvirus 1 (Herpes B virus). Animals were pair-housed pre-operatively and following surgery kept in solitary housing for approximately 14 days then returned to social-housing.

Radial Nerve Injury and Repair

The proximal large nerve gap represents the most clinically-challenging peripheral nerve injury and allows full assessment of the utility of any evaluative approach. Therefore, to best assess the benefits of an experimental surgical approach to improve nerve regeneration, we utilized a high severity injury in the upper forelimb: a 4.0 cm gap in the right proximal radial nerve.

Animals received preoperative sedation by injection of ketamine (5-10 mg/kg IM) and atropine (0.04 mg/kg IM). After endotracheal intubation, general anesthesia was administered by 1-5% inhaled isoflurane (titrated to effect) and IV boluses of propofol (1-2 mg/kg) administered as needed. Cefazolin (40 mg/kg IV) was given for prophylaxis. Analgesia was achieved by 0.1-0.2 mg/kg sustained-release buprenorphine (SR-bup) SQ administration 30 minutes prior to first incision, providing 72 hours of analgesia.

The right forelimb was prepped with isopropyl alcohol, chlorhexidine, and povidone-iodine. A 10 cm curvilinear incision was made in the lateral arm over the intermuscular septum (between biceps and triceps brachii muscles) extending toward the antecubital fossa (Figure 1A). The radial nerve was exposed at the base of the septum and 6.5 cm of the nerve was dissected free from surrounding tissues (Figure 2A). External nerve diameters were measured utilizing calipers. A 4.0 cm segment of the radial nerve was then excised by transecting the nerve at its emergence from the humeral spiral groove and distally just-proximal to the branch to the brachioradialis muscle (preserving all forearm extensor branches) (Figure 1B). Nerve branches were identified with intraoperative nerve stimulation to avoid injury to branches supplying forearm extensors. The nerve gap was bridged with the excised autograft (reversed on grafting) and neurorrhaphy performed with six to eight equidistant 9-0 Nylon (Ethicon, Bridgewater, NJ) epineurial sutures at each coaptation site (Figure 2B). Following repair, hemostasis was obtained,

and the wound closed in a layered fashion. The skin was closed with absorbable running subcuticular suture and Vetbond (3M, Maplewood, MN). Animals were then transported back to housing where they were observed until bright, alert and responsive. Subjects were observed twice daily for 72 hours to monitor for signs of postoperative complications and observed daily thereafter.

Functional Outcome Assessment

Improving upon the method described by Wang et al in 2014¹¹ a simple quantitative method of functional outcomes testing was conceived. An apparatus which accurately measures the animal's maximal degree of wrist extension was developed by the study authors (M.A.H., R.W.R, M.A.R). Figure 3 depicts an artist-rendered schematic of the apparatus that was constructed from poly(methyl methacrylate) (plexiglass) and attached to the front of the cage adjacent to an opening through which the animal can pass a limb (Figure 4A). Animals were trained for 2-6 weeks pre-operatively to consecutively: reach out of the cage and (i) grasp a treat from hand; (ii) grasp a treat from a platform, and (iii) reach through the tube of the apparatus and retrieve an easily graspable food treat (e.g. peanut, apple cube etc.) on a vertically-adjustable platform designed to challenge the animal to demonstrate maximal wrist extension. The platform height was sequentially increased (from low to high) until the animal is no longer able to retrieve the treat due to insufficient ability to extend the wrist. Maximal degree of wrist extension is obtained at the maximal platform height that the animal is able to successfully retrieve the treat. The apparatus was designed to only allow treat retrieval with the use of the right forelimb (surgical limb), reinforcing the use of the right forelimb post-operatively despite limited strength and initial wrist-drop.

NHP wrist extension capability was assessed prior to surgery to obtain baseline measurements and then longitudinally at monthly intervals for 12 months (Figure 5). Assessments were video-recorded with a GoPro Hero 6 (GoPro, San Mateo, CA) video camera (1400K resolution, 60 frames per second) that was fixed to and perpendicularly faced the apparatus with an adjustable metal post (Figure 4B). Utilizing QuickTime 7.7.9 (Apple Inc., Cupertino, CA), freeze-frame video was analyzed to calculate the maximal degree of wrist extension defined as the angle between the ulna and the fifth metacarpal (Figure 4C). Longitudinal measurements were compared to preoperative baseline, thereby making each animal its own internal control.

Statistical Analysis

Statistical analysis (monthly wrist extension averages and standard deviation) was performed with Microsoft Excel (Microsoft Corporation, Redmond, WA).

RESULTS

Subject demographics are summarized in Table 2. No morbidity or mortality was observed and animals tolerated the procedure with no wound complications or evidence of pain. No instances of surgical site automutilation were observed, a complication reported in some primate studies³³. Animals could easily groom and feed post-operatively. We stress the minimal morbidity observed with this radial nerve defect model.

Functional Outcomes

All animals successfully completed wrist extension training pre-operatively. Individual longitudinal wrist extension measurements are presented in Figure 6. Baseline wrist extension is displayed at month 0 followed by a steep decline at 1 month with full wrist-drop exhibited at this time-point (Figure 5B). Average loss of wrist extension from pre to post-injury was $94.0^{\circ} \pm 5.5^{\circ}$. No significant recovery was seen until months 3-4 at which time rapid improvement in wrist extension was observed. Recovery plateaued after 5 months, with modest, albeit steady improvements from 5-12 months. NHPs recovered $87.3^{\circ} \pm 9.9^{\circ}$ of extension at 5 months, $95.3^{\circ} \pm 5.7^{\circ}$ at 8 months, and $97.8^{\circ} \pm 3.6^{\circ}$ at 12 months. Functional recovery in this model is predictably expected by 5-months for this nerve gap length, with maximal improvement at 12 months. All subjects recovered a minimum of 100% of baseline extension, averaging $104.2\% \pm 5.5\%$.

DISCUSSION

This study has demonstrated a reliable, objective, and quantitative approach to functional recovery assessment in a clinically-relevant nerve injury model. A cohort of four animals with a 4 cm nerve defect repaired with autologous nerve graft exhibited similar recovery amplitude and timeline (Figure 6). The exhibited curves are sigmoidal, thus one can utilize various parameters to express recovery, as shown in Table 3, Figure 7. A tight distribution was evident in all outcome measurements at all time points across all animals in the study. All subjects showed a rapid recovery of extension between 3 and 4 months, with $>80\%$ recovery at 5 months and maximum recovery at 12 months, by which time animals recovered $97.8^{\circ} \pm 3.6^{\circ}$ corresponding to $104.2\% \pm 5.5\%$.

Following surgery, animals suffered a profound loss of wrist extension of $94.0^{\circ} \pm 5.5^{\circ}$ with minimal to no recovery until 4 months, which demonstrates that functional improvement can only be attributed to downstream reinnervation of wrist extensors by new radial nerve axons and not due to cross-talk from other regional nerves. While functional recovery was seen at 4 months with this nerve gap length repaired with standard-of-care suture neurorrhaphy, we recommend longer follow-up for larger gaps or those repaired with alternative materials (allograft, xenograft, nerve guide etc.). Conversely, shorter gaps or transection injuries repaired primarily are expected to improve with shorter follow-up. While the current study assessed recovery in a challenging large gap injury, this model carries equal clinical relevance to any peripheral nerve injury (transection, crush injuries) with ability to evaluate the full range of reconstruction techniques or pharmacological interventions.

Advantages of this approach for functional assessment include the quantitative nature of measurements, ease of video documentation, and the time frame to recovery (~4 months) for a considerable nerve deficit injury in the most human-relevant preclinical forelimb model described in the literature to date. In comparison, the RSNM^{7,9,34} remains a valuable preclinical tool for proof-of-principle studies, however, outcomes testing is limited and questionable in terms of clinical relevance, particularly to large nerve gaps^{8,9,35,36}. Compared to rodents, human axons regenerate over a much greater distance to reinnervate target muscles resulting in prolonged distal stump denervation, Schwann cell senescence and apoptosis^{8,10,34}. Chronic denervation leads to downstream atrophy and fibrosis of target muscle, preventing recovery³⁷. Furthermore, human axons regenerate 1-2 mm/day³⁶, however, rodent nerve regeneration is more rapid^{10,11,35,38,39} at a rate of 3.0 - 4.4 mm/day^{40,41}.

Another standard outcome measure, electromyography, has been performed in rodents¹⁵⁻¹⁷, but is technically challenging on smaller species and data should be interpreted cautiously⁷. Furthermore, functional outcome testing in rodents relies on footprint analysis by use of walking tracks (sciatic function index, SFI)^{12, 13} which is time-consuming and suffers from limited intra-observer reliability⁴². As a result, some researchers have questioned the overall validity of SFI and conclusions derived from this technique^{9, 43}. Another commonly utilized method to quantify rodent functional recovery involves the von Frey apparatus which measures sensory responsiveness to tactile stimuli¹⁴. This cumbersome test suffers from concerns regarding inter-observer reliability and overall reproducibility of results with a steep learning curve and animal familiarization with the tester required¹⁴. Several authors reported inability to correlate changes in gait parameters and mechanical allodynia as measured by von Frey^{44, 45}. Histomorphometry is also commonly evaluated in rodents, quantifying axon counts, myelin thickness, and axonal diameter. Unfortunately, obtaining statistically significant differences in these parameters requires large sample sizes and time-consuming analysis⁴⁶. These disadvantages lead to a limited ability to extract meaningful conclusions from rodent studies and reasonably extrapolate them to clinical human peripheral nerve repair⁷.

Because of the numerous shortcomings of the RSNM, many researchers prefer translational testing in large animal models such as dogs, sheep, and swine that are better matched to the scale of human anatomy and thought to possess greater clinical relevance¹⁰. Obvious disadvantages of these models include cost and lack of functional outcome testing as these quadrupeds lack a functional limb response, particularly in the more clinically relevant forelimb. Functional testing in quadrupeds is limited to subjective, binary observations of gait/posture^{7, 25-27}.

Primates remain the only model in which detailed functional, electromyographic, and histomorphological end-points are possible in conjunction with recapitulation of human anatomy (Table 1). Furthermore, primate nerve regenerate 1.35 mm/day¹⁰, similarly to the 1-2 mm/day in humans. Primate peripheral nerve models have mainly focused on the median nerve in the forearm^{10, 11, 47}. However, due to cross-talk between the median and ulnar nerves⁴⁸, functional and electromyographic data may be of limited validity^{49, 50}. For these reasons, we developed this radial nerve repair model resulting in a predictable, isolated functional deficit and a means of incorporating all the previously discussed end-points together with a new method of quantitative functional outcomes testing. Although total primate study costs are relatively high, we emphasize the simplicity and low cost of functional analysis in this approach: testing costs are limited to apparatus construction and acquisition of a commercially-available camera. In comparison, the modern rodent model requires construction of large walking tracks and procurement of expensive ultra-high definition cameras.

To our knowledge, this is the first description of a highly objective quantitative method of peripheral nerve functional outcome assessment in a large animal model. Importantly, the functional testing described in this study is a primary end-point. Histology, muscle mass retention, and EMG are secondary end-points and although useful adjuncts to functional outcomes, it is difficult to draw meaningful clinical conclusions based on secondary end-points alone. Previous studies have likely relied on these outcome measures out of necessity and an apparent lack of alternative models.

No morbidity or mortality was observed, along with no signs of pain or distress post-operatively. The lack of morbidity in this model is critically important as other models often result in severe impairment to animals: transection of the sciatic nerve in rodents leads to

difficulty in ambulation, development of sores over insensate areas, and the risk of self-mutilation (a manifestation of underlying neuropathic pain)^{51, 52}.

This study does possess limitations: the relatively small subject number inherent to NHP studies due to high cost associated with acquisition and housing of animals decreases statistical power. However, the reproducibility of the response (Figure 6) overcomes this to a large degree. Primate research can only be performed at highly specialized centers with the pre-requisite accreditation from relevant governing bodies. Rodent research on the other hand is ubiquitous, occurring at most research facilities. Furthermore, like humans, primates are highly social animals and training was observed to be somewhat dependent on who was present in the room at the time of testing and the animal's level of aggression. This variability was minimized by one individual training and evaluating the animals over the course of the study. Our overall experience was that the degree of wrist extension was ultimately extremely reliable and reproducible.

CONCLUSIONS

This primate model dramatically improves upon existing animal models of peripheral nerve repair and aims to fill the clinical need for a model relevant to the study of peripheral nerve injury, particularly large nerve gaps, by recapitulation of the clinical problem in humans. We report a new method for longitudinal measurement of functional recovery of peripheral nerve regeneration in an objective, quantitative manner by use of a novel testing apparatus that accurately measures the maximal degree of wrist extension following repair of the radial nerve. This model can be utilized to test outcomes of the full range of peripheral nerve injuries and repair techniques with negligible subject morbidity. The close anatomical similarity of the NHP

forelimb to humans makes this a valuable quantitative model to mimic human injury and recovery.

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The authors wish to thank Jane M. Tsui, MD, Marco C. Visaggio, MD, Jennifer N. Camacho, LVT, LATG, CMAR, AAB, and Joshua Tam, PhD for technical and surgical assistance.

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FIGURE LEGENDS

Figure 1. (A) Intra-operative photograph of curvilinear surgical incision marking located on the primate's right upper extremity. **(B)** Schematic of radial nerve anatomy with the surgical transection points shown (red lines).

Figure 2. (A) Exposed right radial nerve (black arrow) at the base of the intermuscular septum. The proximal (right yellow arrow) and distal (left yellow arrow) transection points are shown. The branch (white arrow) innervating the triceps muscle (confirmed by intra-operative nerve stimulator) is shown, proximal to the radial nerve transection point. **(B)** Intra-operative photograph after radial nerve gap repair with reversed autograft with the proximal (right yellow arrow) and distal (left yellow arrow) coaptation sites shown.

Figure 3. Artist schematic of the wrist extension testing apparatus. (Acknowledgement: Illustration by Josh Tam, PhD)

Figure 4. (A) Frontal view of testing apparatus fixed to NHP cage with cage opening (red box) shown through which animal reaches into apparatus tube and grasps treat off of the vertically-adjustable platform (arrow). **(B)** Oblique image of testing apparatus with camera attached (arrow) and facing the cage to record wrist extension capability. **(C)** Freeze-frame analysis of NHP utilizing the testing apparatus. The animal has reached through the cage opening with the right forelimb into the plexiglass tube and is retrieving a peanut placed on the vertically-

adjustable shelf. By simply calculating the degree of extension upward from the level (wrist neutral) plane, a positive degree of extension is calculated.

Figure 5. Freeze-frame photograph of video of **(A)** NHP demonstrating baseline wrist extension prior to radial nerve defect surgery and repair. **(B)** NHP 1 month after surgery demonstrating complete loss of wrist extension capability (wrist-drop). **(C)** NHP demonstrating recovery of wrist extension 5 months post-operatively. **(D)** NHP demonstrating maximal recovery of wrist extension 12 months post-operatively.

Figure 6. Wrist extension as a function of time. Individual animal wrist extension capability is plotted in red. The average for the series cohort is represented in green.

Figure 7. Schematic of important time points and variables to the study of wrist extension functional loss and recovery. See table 3 for definitions of each variable.

TABLES

Table 1. Summary of outcome measures and reproducibility of human anatomy by animal model.

Animal Model	Histology	Muscle mass retention	EMG	Functional outcome	Recapitulates human anatomy?
Rodent	+++	+++	+	+	+
Rabbit	+++	+++	++	-	+
Quadruped (swine, sheep, dog)	+++	+++	++	+	+
NHP	+++	+++	+++	+++	+++

Table 2. NHP parameters on day of surgery.

Subject ID	Age (years, months)	Weight (kg)	Baseline wrist extension	Proximal nerve diameter (mm)	Distal nerve diameter (mm)
NHP#1	4,8	7.4	41°	3.6	3.9
NHP#2	4,8	6.1	55°	3.5	3.0
NHP#3	4,11	8.7	50°	2.6	3.2
NHP#4	4,10	8.3	54°	2.6	2.3
Average	4,9.3	7.6 ± 1.1	50.0° ± 6.4°	3.1 ± 0.5	3.1 ± 0.7

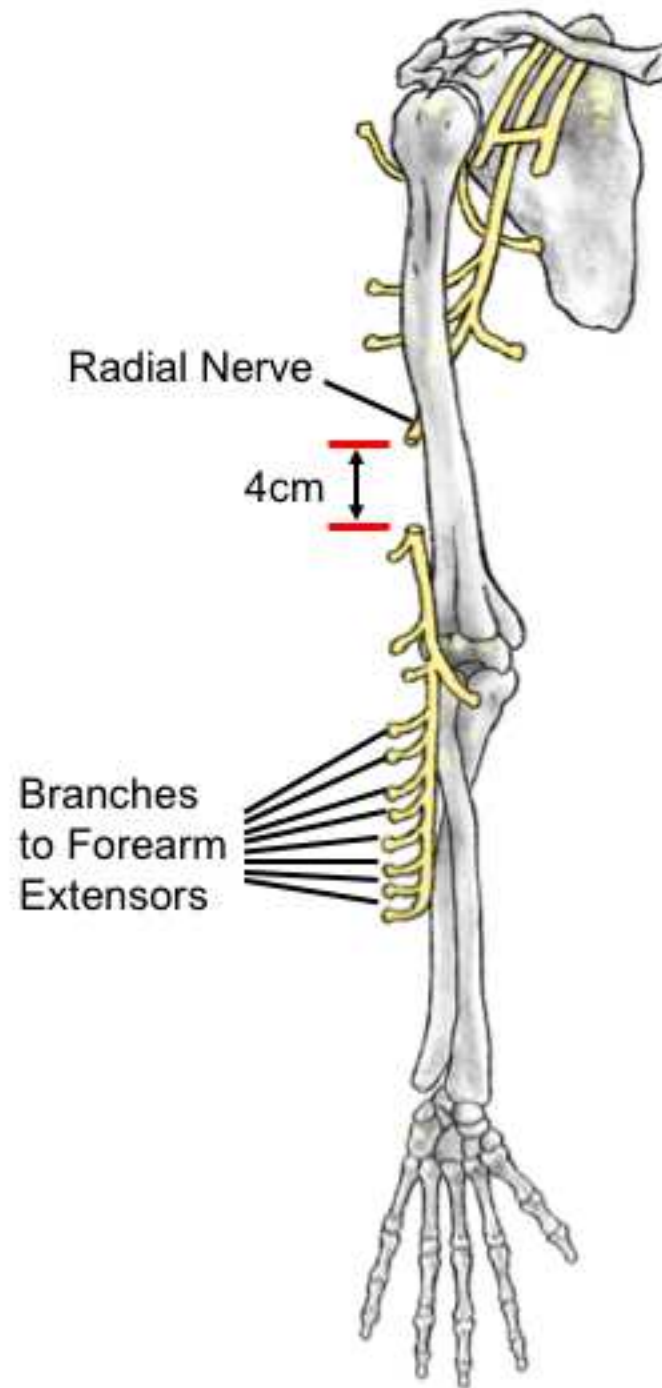
Table 3. Description of variables shown in Figure 7.

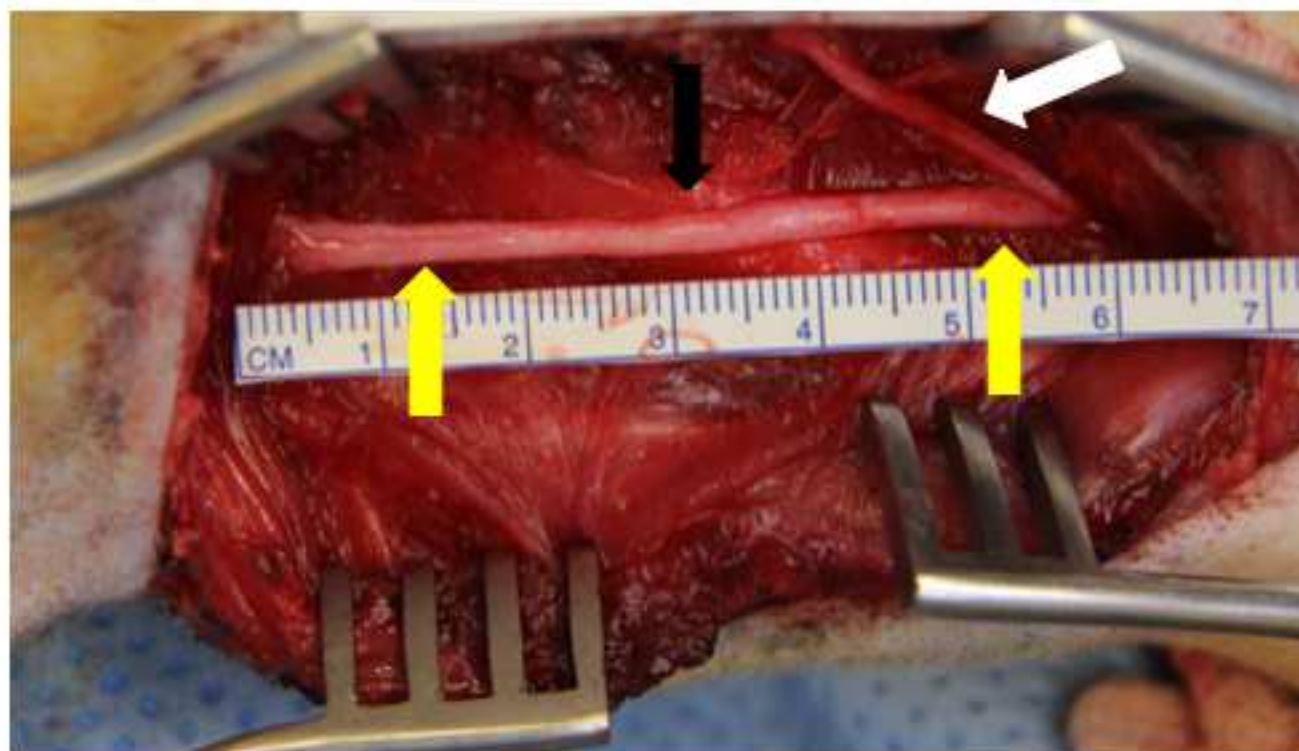
Parameter	Descriptor	Term	Result (Mean +/- SD)
Overall Recovery Amplitude	Final (plateau) extension angle minus angle at initial post-surgical wrist drop.	R_{max} (degrees) (= E_{max} - E_{min})	97.8° ± 3.6°
Time at Initial Response	Month at which extension angle shows significant improvement over angle at initial post-surgical wrist drop.	t_R	3 months ± 0
Half-Life of Recovery	Time of final (plateau) extension angle minus angle at initial post-surgical wrist drop divided by 2.	t_{1/2}	3.5 months ± 0
Maximum Recovery Extension	Final (plateau) extension angle	E_{max}	53.8° ± 1.9°
Percentage Recovery	Overall recovery amplitude divided by pre-op extension minus immediate post-op extension	%R (= R_{max}/(E₀-E_{min}))	104.2% ± 5.5%

Figure 1A

[Click here to access/download;Figure;Figure 1A.tiff](#) 







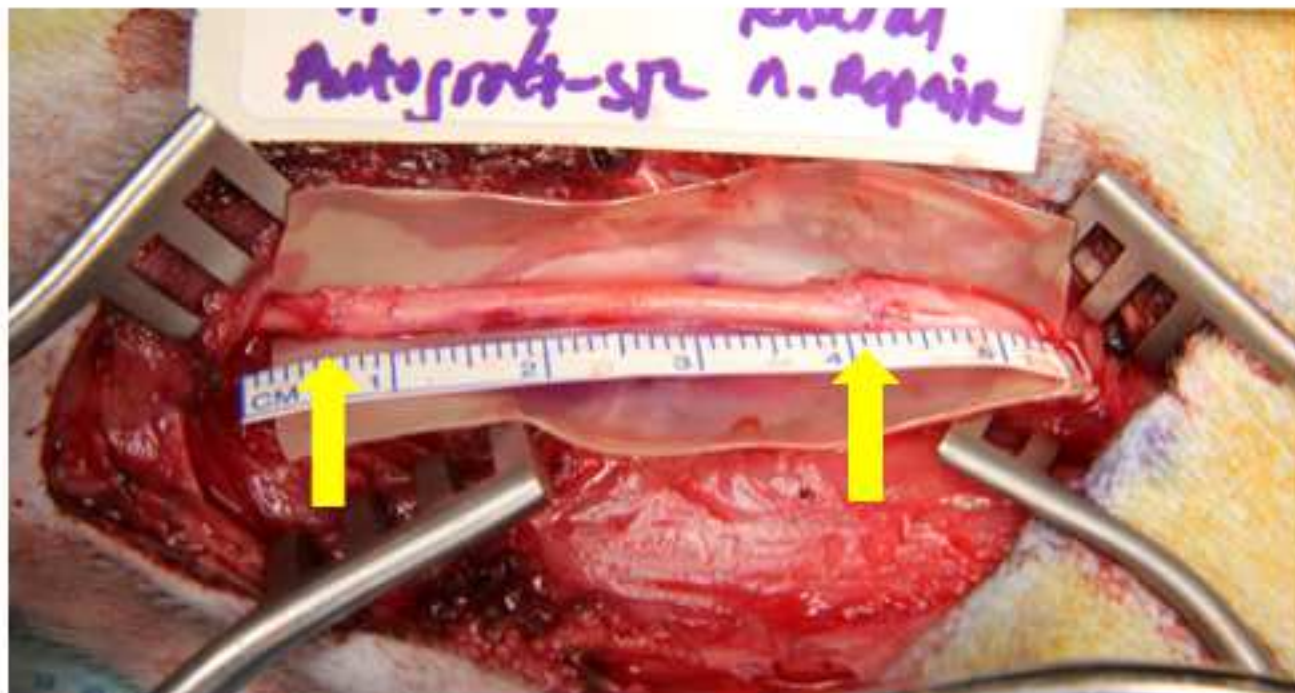
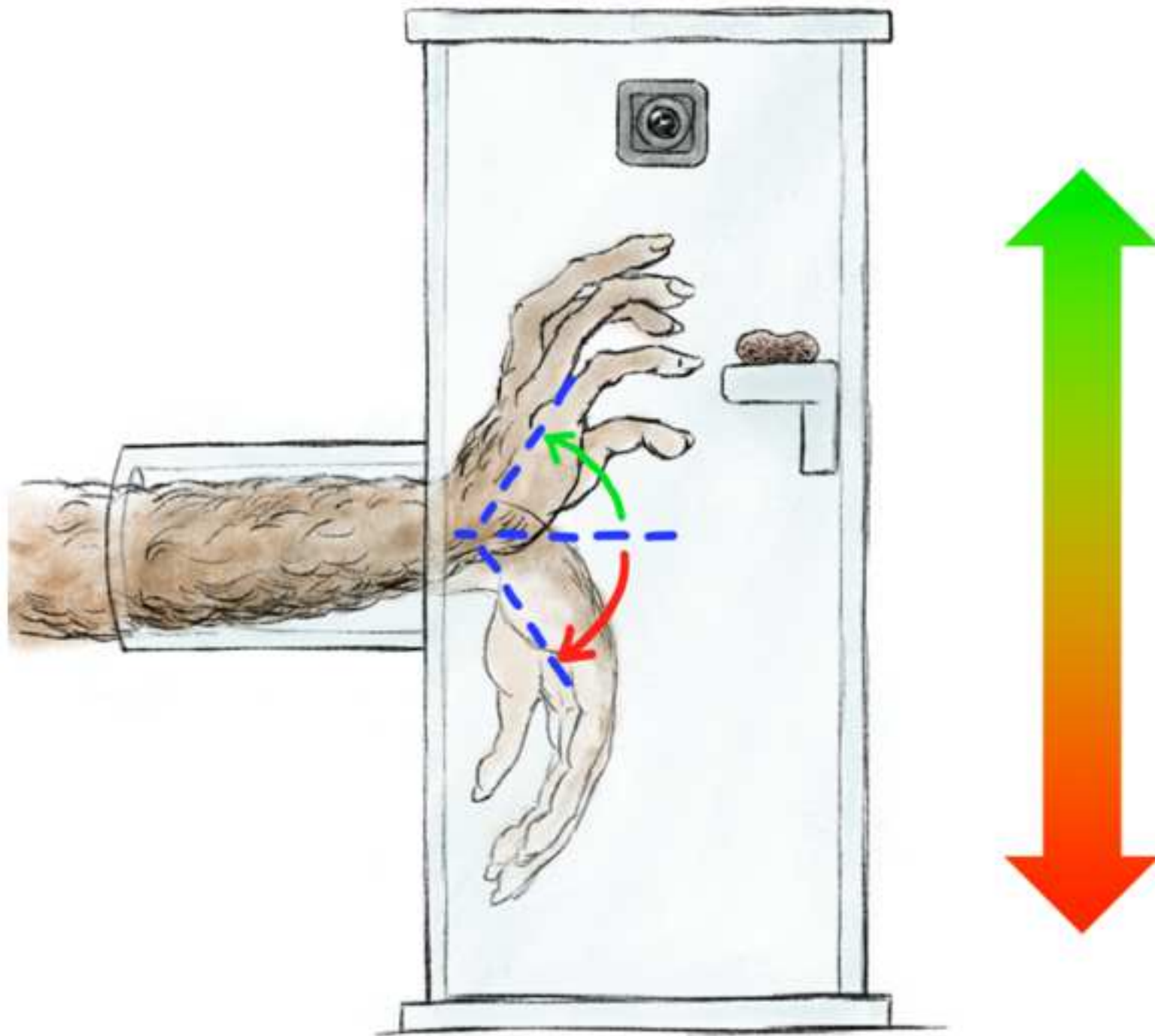


Figure 3

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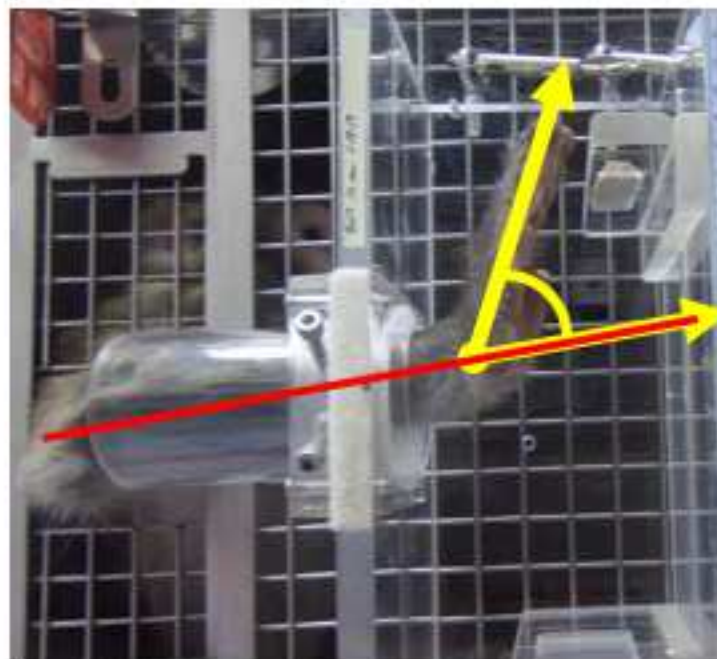










Figure 6

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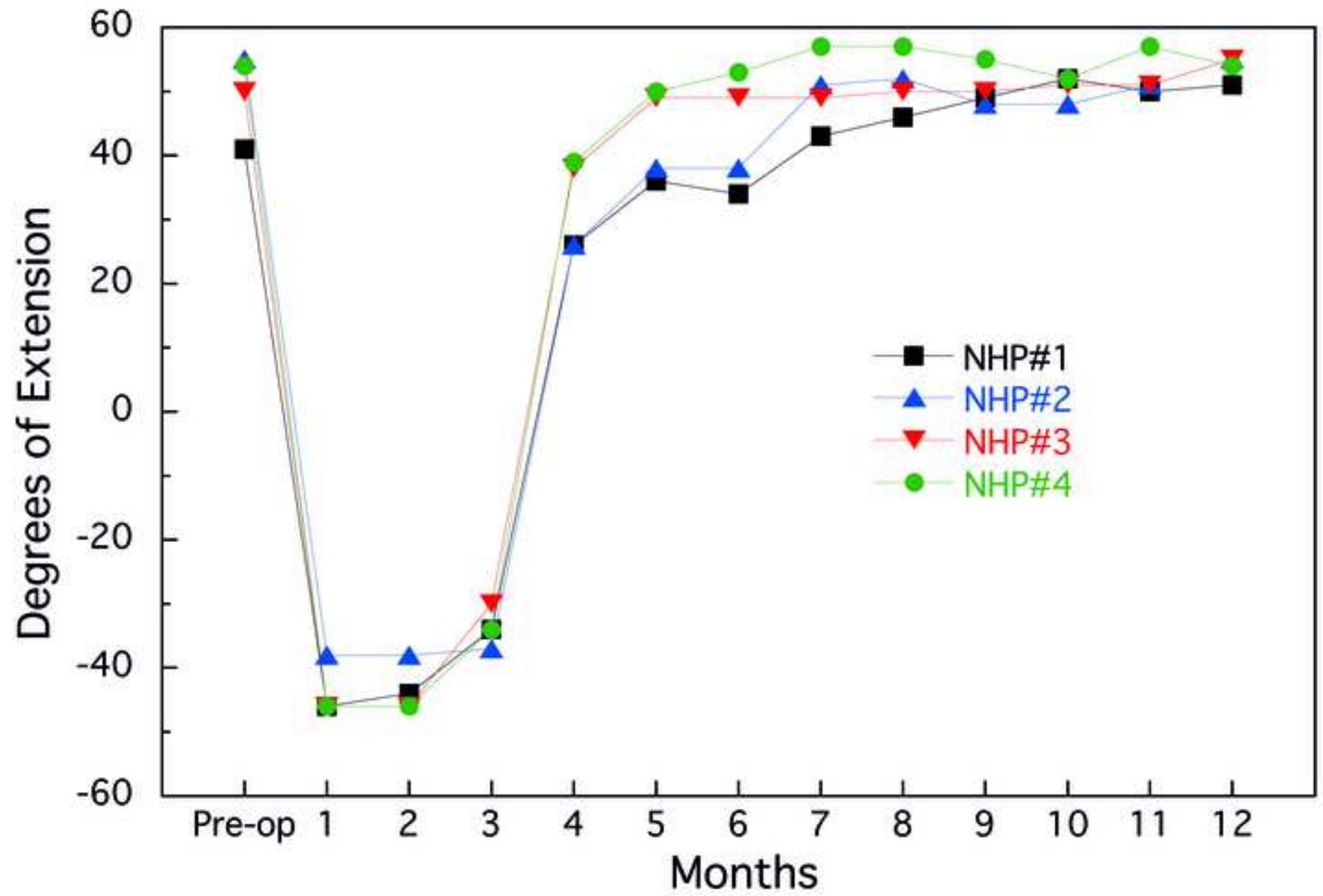
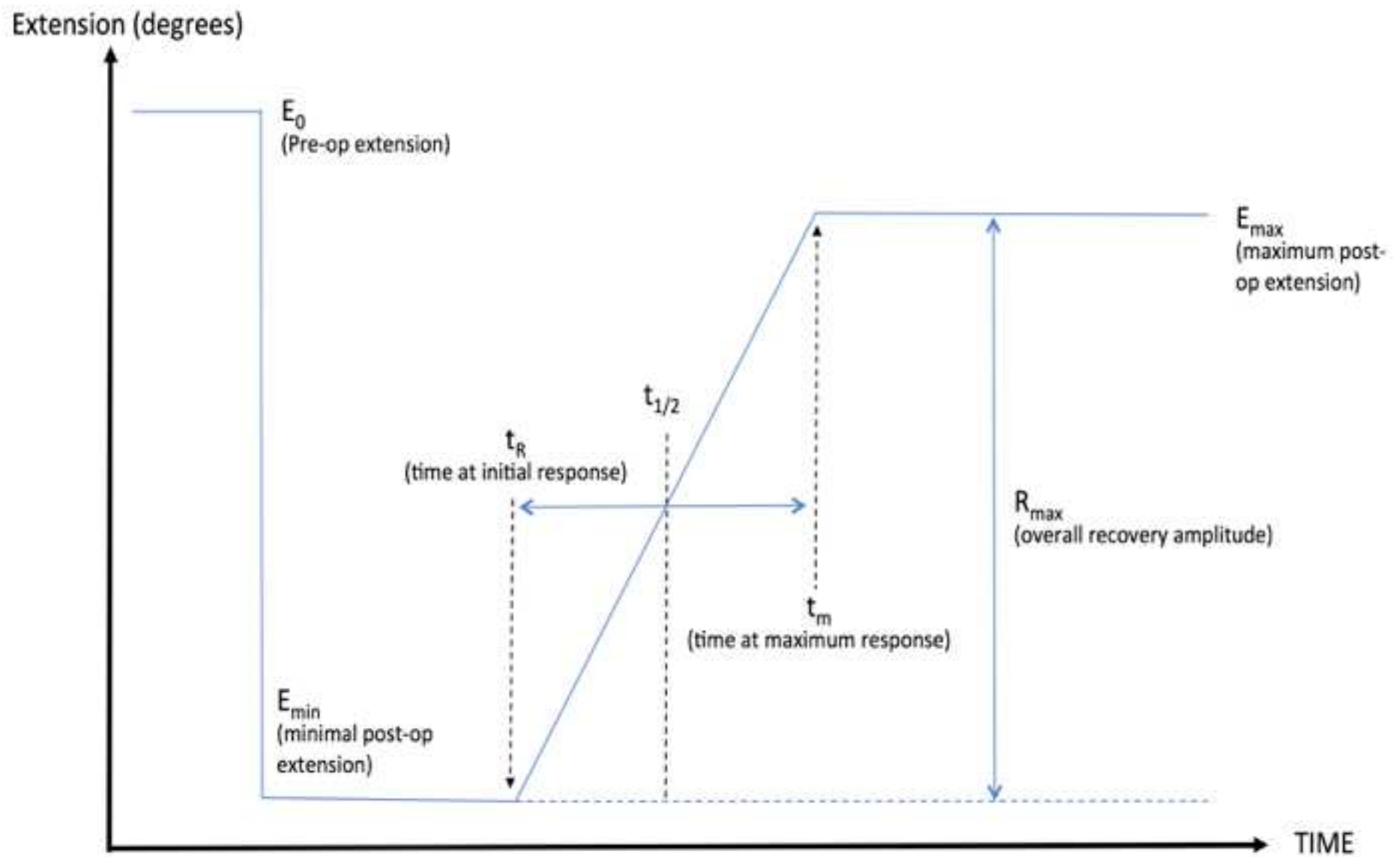


Figure 7



Special Issue Invited Review

Medical Applications of Rose Bengal- and Riboflavin-Photosensitized Protein Crosslinking[†]

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ABSTRACT

This review summarizes research on many of the potential applications of photosensitized crosslinking of tissue proteins in surgery and current knowledge of the photochemical mechanisms underlying formation of the covalent protein–protein crosslinks involved. Initially developed to close wounds or reattach tissues, protein photocrosslinking has also been demonstrated to stiffen and strengthen tissues, decrease inflammatory responses and facilitate tissue bioengineering. These treatments appear to result largely from crosslinks within and between collagen molecules in tissue that typically form by an oxygen-dependent mechanism. Surgical applications discussed include sealing wounds in skin, cornea and bowel; reattaching severed nerves, blood vessels and tendons; strengthening cornea and vein; reducing capsular contracture after breast implants; and regenerating joint cartilage.

INTRODUCTION

Photosensitized crosslinking of proteins is being developed for medical treatments, and many potential benefits have already been demonstrated in preclinical and clinical studies. Applications include sealing wounds, reattaching severed tissues, stiffening and strengthening tissues, decreasing inflammatory responses and bioengineering tissues. These treatments rely on light-initiated formation of covalent crosslinks between proteins on the surfaces of two tissues or between proteins within a tissue or a biomaterial. This review describes current information on the mechanisms for protein photocrosslinking in tissues and on selected medical applications of this technology with emphasis on results obtained in our laboratories.

Two dyes, rose Bengal (RB^{2-}) and riboflavin-5-phosphate (R5P^{2-}), have been used almost exclusively for medical applications of photosensitized protein crosslinking. Their absorption spectra differ, with RB^{2-} absorbing in the green with a maximum at 550 nm and R5P^{2-} in the UVA and blue with maxima at 380 and 450 nm. However, their photophysical properties are rather similar. Both photosensitizers have high intersystem crossing quantum yields, 0.90 for RB^{2-} and 0.37–0.62 for R5P^{2-} in aqueous solution (1–3). The excited triplet states of RB^{2-} and

R5P^{2-} generate singlet oxygen ($^1\text{O}_2$) by energy transfer to dissolved O_2 with quantum yields of 0.70 and 0.48, respectively (4,5). Electron transfer to triplet states of both dyes occurs from a variety of biological donor molecules to produce semireduced and semi-oxidized radicals and, subsequently, other reactive species that may initiate protein crosslinking (6,7). However, RB^{2-} associates tightly to collagen in tissue, whereas R5P^{2-} freely diffuses, suggesting that photoreactions of these two dyes leading to crosslinks in tissue may differ.

The treatment procedure for photocrosslinking tissue proteins is simple. In fact, the simplicity of this technique is one of its clinical advantages. An aqueous solution of the dye, typically ~1 mM, is applied to the tissue, which is then exposed for a few minutes to wavelengths absorbed by the dye. Crosslinking occurs during the irradiation and is followed by healing processes. The light-induced effects appear to result largely from crosslinks in collagen, the major connective tissue protein, with possible crosslinking to the proteoglycans that surround collagen fibrils and to other proteins.

Although this review focuses on RB^{2-} and R5P^{2-} , other photosensitizers are briefly summarized here. A long wavelength absorbing drug, palladium bacteriopheophorbide, has been shown to stiffen cornea upon irradiation at 750 nm (8). In a different approach, chromophores were designed to form a bridge between proteins. For example, bifunctional 1,8-naphthalimide dyes can react with nucleophilic amino acid residues on two different proteins to form a bridge (9,10). In another example, UVA photoactivated a bifunctional diazopyruvyl agent to form reactive ketene intermediates that linked collagen molecules (11,12). Mixtures of dye and proteins have also been used as solders to attach tissues by photosensitization. For example, R5P^{2-} and chlorin e6 covalently linked to BSA in a solder were studied (13,14). In addition, RB^{2-} incorporated into chitosan films has been demonstrated to act as an adhesive for tissues upon photoactivation (15). More recently, a biomimetic material containing methacrylated collagen and RB^{2-} was used to close wounds in murine skin (16).

It is important to clarify that photosensitized protein crosslinking in this review is distinct from laser tissue welding, which relies on thermal denaturation of tissue proteins and subsequent fusion of the proteins to produce bonding (17). In laser tissue welding, the heat resulting from absorption of photons by endogenous chromophores or added dyes raises the tissue temperature sufficiently to create the bonding effect. In contrast, a temperature rise is not required for photosensitized protein crosslinking. Excellent reviews of laser tissue welding have been published (17–19).

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OVERVIEW OF MECHANISMS FOR PHOTSENSITIZED PROTEIN CROSSLINKING

Multiple reaction pathways are likely to be involved in photosensitized protein crosslinking since proteins contain several amino acids with potentially reactive side chains including tyrosine, tryptophan, histidine, lysine, arginine, methionine and cysteine. In tissues, and particularly in highly structured protein assemblies such as collagen fibers, protein crosslinking is subject to several constraints that are not important for reactions between proteins in solution. For example, neither the reactive amino acid sites in the collagen chains nor the chains themselves can freely diffuse away from the rigid collagen fiber to crosslink with a distant potentially reactive site. Possibly, an initially formed radical might react with an adjacent amino acid forming a new radical but electron delocalization is not feasible in the almost entirely aliphatic collagen molecule. Consequently, covalent linkage only occurs when reactive sites on protein molecules are extremely close. The two reactive sites can be within a protein or between different proteins. In fact, a recent study demonstrated the importance of proximity of reactive amino acids for efficient crosslink formation (20). Also, in protein assemblies some potentially reactive amino acids may be hidden within the structure and not be able to crosslink with other proteins. In addition, at the relatively high irradiances used for crosslinking ($0.2\text{--}0.5\text{ W cm}^{-2}$), oxygen diffusion into a tissue is often slower than its consumption by reactions with tissue molecules, thus leading to oxygen depletion and possibly oxygen-independent mechanisms. Consequently, protein crosslinking mechanisms in tissue and the types of crosslinks formed may differ significantly from those previously established for the proteins in solution.

A general description of potential mechanisms is given in this section, and specific reactions of RB^{2-} and R5P^{2-} are discussed in Sections "Rose Bengal-Photosensitized Protein Crosslinking" and "Riboflavin Photosensitized Protein Crosslinking". Light absorption by a photosensitizer (denoted as PS) produces excited singlet states ($^1\text{PS}^*$ step 1) and subsequently, by intersystem crossing, excited triplet states ($^3\text{PS}^*$, step 2). The $^1\text{PS}^*$ is too short lived to diffuse to a protein and initiate photocrosslinking reactions and, consequently, it decays by fluorescence (step 3) or radiationless processes (step 4). However, if the PS is associated with a protein before light absorption, $^1\text{PS}^*$ might react with the protein and thereby initiate crosslinking (not shown in

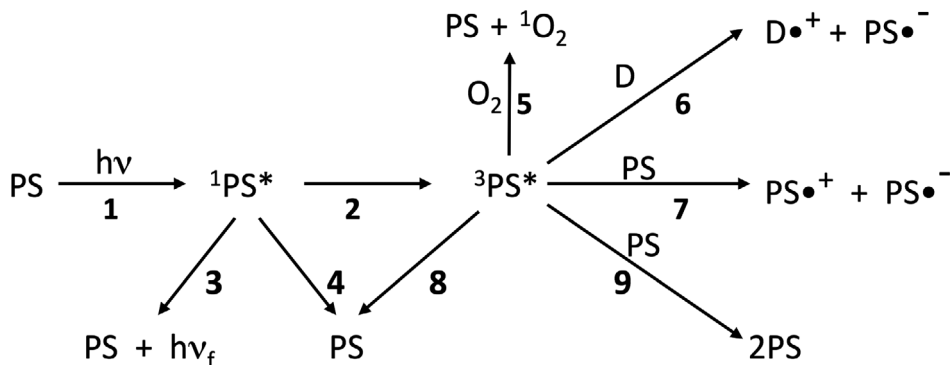
Scheme 1). Despite this possibility, for RB^{2-} , which forms a ground state complex with collagen, the crosslinking is mediated by singlet oxygen via $^3\text{PS}^*$ (see Sections "Association of Rose Bengal with collagen" and "Rose Bengal photoprocesses in aggregates and collagen model systems"). However, it is important to remember that the processes of the excited states can be modified when a photosensitizer is complexed with a protein.

The triplet excited states are longer lived and able to undergo bimolecular reactions that lead to crosslinking. Competing reaction pathways from $^3\text{PS}^*$ produce reactive species that may initiate protein photocrosslinking, namely, energy transfer to dissolved O_2 to form singlet oxygen ($^1\text{O}_2$, step 5) and electron transfer from a donor (D) or from another PS to $^3\text{PS}^*$ to form the PS semireduced radical ($\text{PS}^{\bullet-}$) and semi-oxidized radical from the D or PS ($\text{D}^{\bullet+}$, $\text{PS}^{\bullet+}$; steps 6 and 7). These transient species can undergo further reactions to form additional reactive species (discussed below). $^3\text{PS}^*$ can relax to the ground state (step 8) and also be quenched by ground state PS, another energy wasting pathway (step 9).

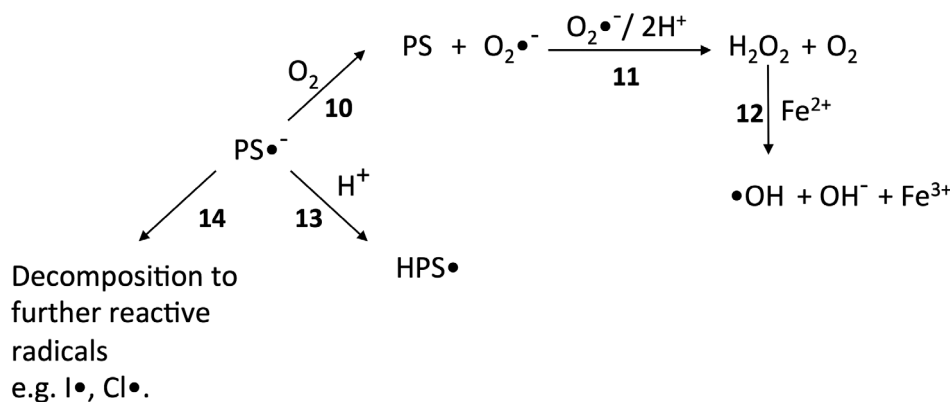
In aqueous solution, early studies suggested a photosensitized protein crosslinking mechanism mediated by $^1\text{O}_2$. In this mechanism, $^1\text{O}_2$ oxidizes histidine to form a product, possibly an imidazolone, that subsequently reacts with a nucleophilic amino acid, such as lysine, to produce a crosslinked protein (21,22). This mechanism was validated in a model polymer system containing pendant histidine and lysine groups and in solutions of ribonuclease A (23,24). Reaction products of $^1\text{O}_2$ with other amino acids initiate formation of other crosslinks. A recent study using ^{18}O labeling and optimized tandem mass spectrometry fragmentation identified the sites of crosslinks (tyrosine–tyrosine, tyrosine–tryptophan, tyrosine–lysine and histidine–lysine) (25).

Oxygen-dependent and oxygen-independent mechanisms may also be initiated by electron transfer to PS. Certain amino acids including cysteine, methionine, tyrosine, tryptophan, arginine and other biomolecules in tissue, for example, ascorbate and NADH, as well as ground state PS, are capable of acting as electron donors to the PS triplet excited states (steps 6 and 7).

$\text{PS}^{\bullet-}$ can react to form reactive radical species (Scheme 2) that subsequently react with amino acids on the collagen chain (26). For example, $\text{PS}^{\bullet-}$ can transfer an electron to O_2 to form superoxide anion radical ($\text{O}_2^{\bullet-}$) that, if a reducing source is available, produces hydrogen peroxide (H_2O_2) and subsequently hydroxyl radical ($\text{HO}^{\bullet}$) (steps 10, 11 and 12). In the absence of oxygen, $\text{PS}^{\bullet-}$ may add a proton to form a neutral radical ($\text{HPS}^{\bullet}$,



Scheme 1. Pathways for formation of reactive intermediates via the triplet state of a photosensitizer (PS) that may be involved in generating protein–protein crosslinks.



Scheme 2. Some of the potential oxygen-dependent (steps 10, 11 and 12) and oxygen-independent (steps 13 and 14) pathways for generating reactive species from the photosensitizer radical anion.

step 13) that can react directly with proteins to generate radicals that couple to form crosslinks or decompose to form species that initiate crosslinking (step 14). The electron donor radical ($D^{\bullet+}$) produced in steps 6 and 7 (Scheme 1) can also react undergo reactions leading to protein crosslinking (not shown).

In summary, several reaction pathways are available for oxygen-dependent protein–protein crosslinking with involvement of oxygen at different steps (27–29) as well as for oxygen-independent pathways.

ROSE BENGAL-PHOTOSENSITIZED PROTEIN CROSSLINKING

Background

Rose Bengal is a halogenated xanthene dye that is frequently used as a photosensitizer for oxidation in biological and nonbiological systems because of its high quantum yields for the excited triplet state and $^1O_2^*$ formation. It is an FDA-allowed diagnostic agent for corneal surface damage and had been used as a diagnostic for liver function, indicating that RB is safe for human use.

The photophysics and photochemistry of RB^{2-} have been extensively studied in solution at low concentrations where it exists as a monomer (30–32). The fluorescence quantum yield is only 0.02 (33) and the triplet quantum yield is very high (>0.9), as is the $^1O_2$ quantum yield (0.76) (3). In addition, in the absence of O_2 , $^3RB^{2-}$ undergoes efficient electron transfer with several biological donor molecules as well as with RB^{2-} ground state at sufficiently high concentrations (Scheme 1, steps 6 and 7 with $PS = RB^{2-}$). These processes produce semi-oxidized radicals ($D^{\bullet+}$ and $RB^{\bullet-}$) and the semireduced radical, $RB^{\bullet-}$. However, direct electron transfer from $^3RB^{2-}$ to O_2 to form superoxide anion radical does not compete with energy transfer to form $^1O_2$ (34,35). The relative rates of energy transfer to form $^1O_2$ and electron transfer from a donor are strongly influenced by the local O_2 and donor concentrations and the redox properties of the electron donor. Since the one electron reduction potential for the couple $RB^{2-}/RB^{\bullet-}$ is -0.78 V vs SCE and the energy of the $^3RB^{2-}$ is 1.77V, reductive quenching is energetically favorable for donors with reduction potentials less than 0.99 V (7). This includes the amino acids tryptophan, tyrosine and cysteine as well as ascorbate and NADH.

In aqueous solution, RB^{2-} photobleaches, losing its strong pink color, without a change in the absorption spectrum. In the absence of O_2 , electron transfer from a donor leads to sequential loss of iodines from RB^{2-} to ultimately produce tetrachloro-fluorescein, which has an absorption maximum at 510 nm (36). For photosensitized crosslinking applications, RB^{2-} is applied to tissues at a high concentration (~ 1 mM) at which RB^{2-} exists as aggregates. Aggregation is detected by a shift of the absorption maximum to 560 nm from 550 nm and a change in the ratio of absorbance at the 550/560 nm maximum to the shorter wavelength shoulder at ~ 525 nm. This ratio for monomeric RB^{2-} is ~ 3 , whereas it decreases to ~ 2 for RB^{2-} in aggregates (37).

Association of Rose Bengal with collagen

Fibrillar collagens are the major protein in the connective tissues typically treated by photosensitized protein crosslinking. Type I collagen, the dominant type in cornea and dermis, consists of three ~ 1000 amino acid long chains twisted into a right-handed triple helix which initially self-assemble into microfibrils and eventually into fibrils that may assemble further into larger structures.

Noncovalent binding of RB^{2-} to collagen molecules redshifts its absorption maximum to ~ 560 nm and broadens the spectrum (Fig. 1) that, along with other evidence, suggests that RB^{2-} binds to triple-helical collagen as RB^{2-} aggregates rather than as individual RB^{2-} monomers (38–40). A more detailed study indicated that RB^{2-} bound equally well to denatured collagen, that is, single collagen chains, as to native triple-helical collagen, indicating that it did not bind in specific sites or “pockets” on collagen as found for other proteins (40). Molecular dynamics simulations at low RB^{2-} concentrations with a 37-amino acid collagen-like triple-helical polypeptide indicated that RB^{2-} bound at positively charged groups (terminal amines and lysines) mainly via hydrogen bonding with RB’s two negative charges (40). Hydrophobic forces in regions of the polypeptide containing proline also contributed to the binding. When the simulation was performed at the higher RB^{2-} concentration that is used for protein crosslinking, dye aggregates were distributed mainly in the positively charged region of the peptide.

In tissues, RB^{2-} also binds as aggregates to collagen since the absorption spectrum in cornea and amniotic membrane (AM) clearly shows the 560 nm broadened peak characteristic of RB^{2-}

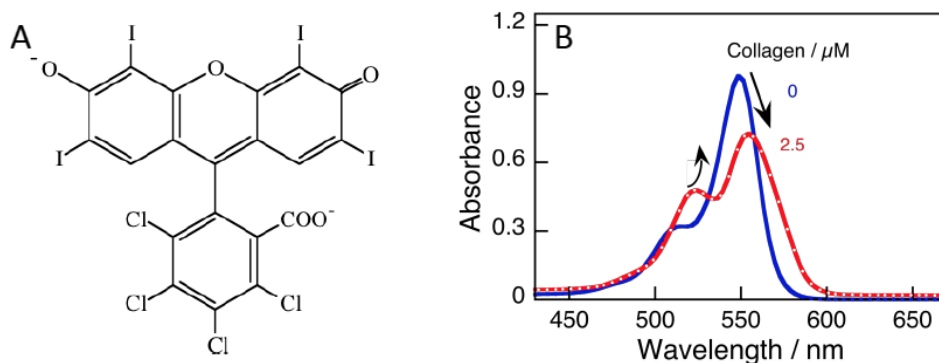


Figure 1. Rose Bengal. (A) Structure at pH 7. (B) Effect of collagen on the absorption spectra of 10 μM RB^{2-} . Blue = no collagen, red = 2.5 μM native type I collagen. Adapted from Ref. (40), <https://pubs.acs.org/doi/full/10.1021/acsomega.7b0067>.

aggregates and the signature ratio of 2 between the main peak and the shoulder (29,41). RB^{2-} binds very tightly with tissue collagen since it cannot be extracted by soaking in PBS for an extended period after application to cornea (29). This association of RB^{2-} with collagen may also explain the observation that RB^{2-} diffuses only about 100 μm into tissues and remains as a surface band over time as shown in cornea (41), dermis (42) and adventitia (43) (Fig. 2).

Rose Bengal photoprocesses in aggregates and collagen model systems

The photochemistry of RB^{2-} aggregates in solution with and without collagen was similar but not identical. In both cases, RB^{2-} photobleaching was enhanced in the presence of O_2 , although binding to collagen decreased the rate of RB^{2-} loss (38). Photobleaching in RB^{2-} aggregates without collagen did not produce a spectral shift, whereas RB^{2-} bound to collagen showed a blueshift of the maximum even in aerated solution, suggesting that amino acids in collagen act as electron donors to produce $\text{RB}^{\cdot 3-}$ (Scheme 1, step 6) and subsequent de-iodination occurs (Scheme 2, step 14) (36). However, a $^1\text{O}_2$ mechanism also contributed to RB^{2-} photobleaching since the rate was

enhanced by D_2O . Formation of de-iodinated RB was enhanced by addition of arginine, which reductively quenches $^3\text{RB}^{2-}$ to form $\text{RB}^{\cdot 3-}$ (40), in RB^{2-} aggregates both alone and bound to collagen. Overall, these results indicate that association of RB^{2-} aggregates with collagen partially inhibits RB^{2-} photochemistry, but both electron transfer to RB^{2-} and energy transfer to oxygen still occur and contribute to RB^{2-} photobleaching.

Consistent with these results, transient absorption measurements carried out on RB^{2-} in collagen matrices showed the presence of the $^3\text{RB}^{2-}$ and $\text{RB}^{\cdot 3-}$ (40). The $^3\text{RB}^{2-}$ lifetime was only 3.5–4.0 μs , significantly shorter than in the collagen-free solutions (~100 μs). Arginine quenched $^3\text{RB}^{2-}$, as shown previously (7), producing $\text{RB}^{\cdot 3-}$, which had a longer lifetime (~100 μs) than the $^3\text{RB}^{2-}$. $\text{RB}^{\cdot 3-}$ was quenched by O_2 generating superoxide anion radical that potentially leads to additional reactive oxygen species (Scheme 2).

Photochemistry of Rose Bengal in tissue

Almost all mechanistic studies on photosensitized protein crosslinking in tissue have used cornea as the model tissue because it is transparent, thus allowing optical monitoring. Human and rabbit corneas are about 450–500 μm and 370 μm

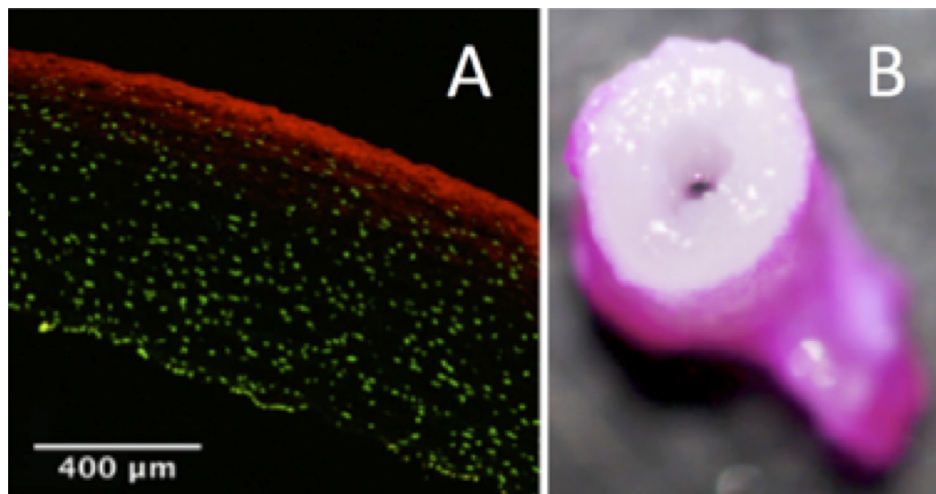


Figure 2. Limited diffusion of RB^{2-} into tissues. (A) Confocal image of a frozen section of rabbit cornea that had been stained on anterior surface with RB^{2-} . Red fluorescence indicates RB^{2-} and green fluorescence represents a nuclear stain (TO-PRO-3). (Adapted from Ref. (41), copyright holder Association for Research in Vision and Ophthalmology). (B) Porcine vein externally coated with 0.1% RB^{2-} (pink). Penetration is limited to outer layers of adventitia (adapted from Ref. (43)).

thick, respectively, and consist of an outer epithelial layer, a collagen-rich stromal layer that accounts for >90% of cornea thickness and a single layer of endothelial cells on the inner surface. Collagen in the stromal layer is highly organized in fibers running mostly parallel to the surface. The limited diffusion of RB^{2-} into the stroma (Fig. 2A) is presumably due to the high molecular mass of the RB^{2-} aggregates and their binding to collagen (see Section “Association of Rose Bengal with collagen”) (41). Preclinical studies of RB^{2-} -photosensitized crosslinking in cornea are described in the sections entitled “Cornea” below.

Rose Bengal-photosensitized protein crosslinking in tissue is mainly, if not entirely, oxygen dependent. For example, photobonding AM over incisions in the cornea was very effective when carried out under oxygen but little, if any, bonding occurred in a nitrogen atmosphere (Section “LASIK”) (29). Since O_2 depletion was expected at the irradiance used (0.25 W cm^{-2}), the thin AM ($\sim 40 \mu\text{m}$) may have allowed sufficient O_2 to diffuse to the bonding interface to support an O_2 -dependent mechanism. When the photobonding was carried out using D_2O -based RB^{2-} solution, a medium that extends the lifetime of $^1\text{O}_2$, the bonding strength was enhanced, indicating $^1\text{O}_2$ participation in the photobonding mechanism.

To assess the O_2 dependence for photocrosslinking at a greater depth in tissue compared with amnion, RB^{2-} photobleaching and photosensitization were studied in rabbit corneas *ex vivo* (44). After application of RB^{2-} to the de-epithelialized stroma surface, the absorption spectrum indicated that aggregated RB was present. RB^{2-} photobleached more rapidly in the presence than in the absence of O_2 without a shift in the absorption maximum. In the presence of oxygen, RB^{2-} photobleaching was enhanced by D_2O and inhibited by azide consistent with a major contribution of $^1\text{O}_2$ to RB^{2-} photobleaching in tissue. In the absence of O_2 , the spectrum blueshifted slightly during photobleaching. Arginine greatly enhanced RB photobleaching and the absorption maximum blueshifted to that of tetra-chlorofluorescein similar to that found in solution (Fig. 3). These results indicate that in cornea, $^3\text{RB}^{2-}$ generates $^1\text{O}_2$ by energy transfer and, in the absence of O_2 , accepts an electron from arginine and subsequently undergoes de-iodination reactions (36,40).

Cornea stiffness measured by tensiometry after RB_2 photosensitization showed that O_2 was required for a two-fold increase in Young's modulus (44). Sodium azide, although not a specific

$^1\text{O}_2$ quencher, blocked this stiffness increase. In addition, significant enhancement of the photosensitized stiffness increase was observed when D_2O was the solvent, a signature of $^1\text{O}_2$ involvement in the rate-determining step. In the absence of O_2 , RB photosensitization in the presence of arginine, but not in its absence, increased Young's modulus of the cornea by two-fold, indicating that electron donation to $^3\text{RB}^{2-}$ can initiate crosslinking. Indeed, this O_2 -independent crosslinking was as efficient as O_2 -dependent crosslinking. Overall, these results indicate that in tissue, RB-photosensitized crosslinking is largely O_2 -dependent via a $^1\text{O}_2$ mechanism but that with an appropriate electron donor in the absence of O_2 , an O_2 -independent mechanism can mediate protein crosslinking.

RIBOFLAVIN-PHOTOSENSITIZED PROTEIN CROSSLINKING

Background

Increasing cornea stiffness with riboflavin photosensitization is currently the only approved clinical treatment based on photosensitized protein crosslinking. This treatment is approved worldwide for keratoconus, a condition in which the cornea thins and weakens leading to protrusion of the cornea in response to the normal intraocular pressure, and for ectasia after LASIK. The photosensitizer used is riboflavin-5-phosphate (R5P^{2-} , Fig. 4A), which has greater water solubility than riboflavin, although it is generally referred to simply as “riboflavin.” The treatment protocol involves applying a R5P^{2-} solution to the cornea and exposure to UVA light, 365–370 nm, and is described in Section “Cornea”.

Riboflavin-5-phosphate absorbs in the UVA with a maximum at 380 nm and in the blue with a maximum at 450 nm (Fig. 4B). The fluorescence quantum yield for riboflavin in aqueous solution is 0.30, and the triplet quantum yield is 0.61 (2,45). Riboflavin is quenched by O_2 to form $^1\text{O}_2$ with a quantum yield of 0.49 (4) although electron transfer O_2 to form superoxide anion radical is inefficient (quantum yield = 0.01) (35). Depending on the conditions, riboflavin undergoes a variety of photochemical reactions leading to products and transients that might be involved in protein crosslinking. For example, several amino acids (tyrosine, tryptophan, methionine and cysteine) and other biological molecules quench triplet riboflavin by electron transfer to form radical ion pairs (4,6,46). Subsequent reactions of these species with oxygen can produce reactive radicals (Scheme 2). In addition, the isoalloxazine ring of riboflavin can undergo intramolecular photoreduction with the ribityl side chain as the electron donor. The chain-cleaved products formed include lumiflavin, which is also a photosensitizer. The photochemistry of riboflavin is complex, and other pathways may also contribute to protein crosslinking. In contrast to RB^{2-} , R5P^{2-} does not associate with collagen and instead freely diffuses into and through tissues, consistent with showing the same absorption spectrum in solution and in cornea (Fig. 4B).

Riboflavin-photosensitized protein crosslinking in solution

Protein crosslinking after R5P^{2-} photosensitization in solution is generally detected by the appearance of higher molecular weight species by methods such as gel electrophoresis. In most studies, the crosslinks were not identified except for dityrosine that can be detected by its fluorescence around 410 nm. Dityrosine and

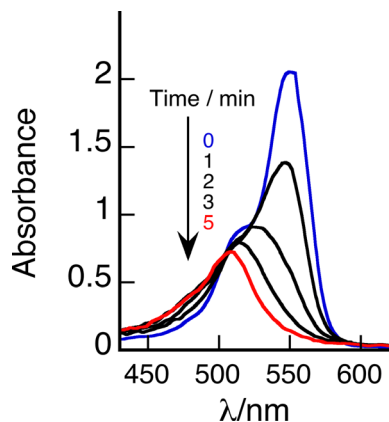


Figure 3. Photobleaching of RB^{2-} ($125 \mu\text{M}$) in the presence of arginine (25 mM) in nitrogen-saturated solution irradiated at 532 nm. Adapted from Ref. (40), <https://pubs.acs.org/doi/full/10.1021/acsomega.7b0067>.

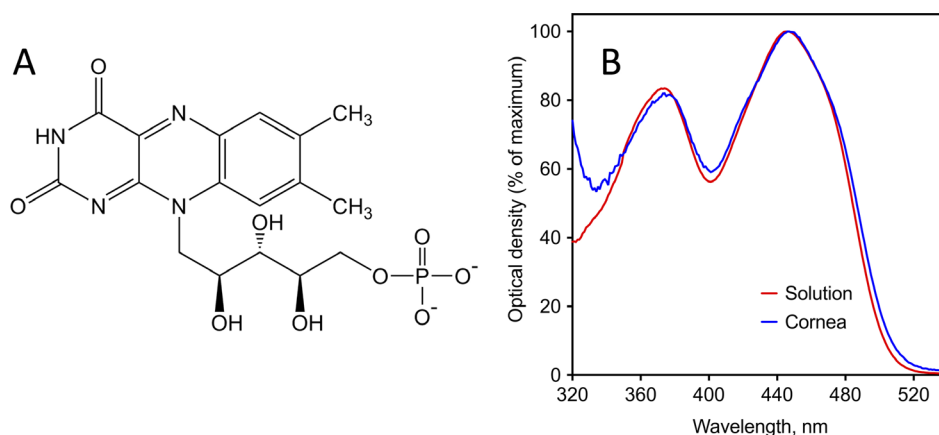


Figure 4. Riboflavin-5-phosphate. (A) Structure at pH 7, and (B) absorption spectrum in PBS and on cornea.

dityryptophan appear to be the dominant crosslinks produced by $R5P^{2-}$ photosensitization although the use of more sophisticated mass spectrometric methods indicates that many other types of crosslinks are formed (47,48). Collagen type I lacks tryptophan, and, consequently, tryptophan-containing crosslinks are not formed.

A mechanism for dityrosine formation consists of electron transfer from tyrosine (Tyr) to the $^3R5P^{2-}$ (Scheme 1, step 6), followed by proton loss from the Tyr cation radical to produce a Tyr phenoxyl radical (49). Coupling of two Tyr phenoxyl radicals via a carbon-carbon bond produces dityrosine. Results of two studies of $R5P^{2-}$ -photosensitized protein crosslinking in solution are consistent with this mechanism. In both, irradiation of $R5P^{2-}$ solutions containing proteins (collagen or silk fibroin) produced protein aggregates and dityrosine as detected by fluorescence or amino acid analysis (50,51). The dityrosine fluorescence and aggregation were enhanced by addition of superoxide dismutase (SOD). Removal by SOD of superoxide anion radical ($O_2^{\cdot-}$) (formed in step 10, Scheme 2) is consistent with the increase in protein crosslinking observed in both these studies since the tyrosyl radical reacts rapidly with $O_2^{\cdot-}$ (52). Other pathways may contribute to dityrosine formation because in these two studies, the yields were decreased by O_2 , histidine and azide. In a simpler system, a polymer with only pendant tyrosines, dityrosine formation by photosensitization with a riboflavin derivative did not involve $^1O_2$, H_2O_2 or superoxide anion radical and dityrosine formation was oxygen dependent (23). Thus, the $R5P^{2-}$ photosensitization mechanism for protein crosslinking in solution seems to vary with the conditions used.

Riboflavin-photosensitized protein crosslinking in tissues

Tissues and tissue mimetics have been crosslinked by $R5P^{2-}$ photosensitization to increase their stiffness or reduce enzymatic degradation. The results of an X-ray scattering study of corneal stroma after $R5P^{2-}$ photosensitization indicate that crosslinks are formed predominantly on the collagen fibril surface, both within and between collagen molecules, within and between the proteins of the proteoglycans and between these proteins and collagen molecules (53). However, the distance between collagen fibrils is too great to actually allow fibril-fibril crosslinking.

Oxygen appears to play a role in the mechanism for protein crosslinking with $R5P^{2-}$ in cornea although the molecular mechanism is not entirely clear. Cornea stiffness increased, as

measured by extension tensometry, after irradiation in the presence of oxygen but not in its absence (28). Also, studies of the irradiance-dependence of cornea stiffening are consistent with an oxygen-dependent mechanism (54–56). In one study, the increase in stiffness inversely correlated with irradiance using 3, 9 and 18 $mW\ cm^{-2}$ and the same fluence, 5.4 $J\ cm^{-2}$ (57). Since O_2 is depleted in the cornea during the irradiation (see below), it appears that higher irradiances did not allow sufficient time for oxygen diffusion into the cornea during the crosslinking. These results are consistent with oxygen-dependent crosslinking, and an early study in which the breaking strength of cornea was used to assess protein crosslinks, azide inhibited and D_2O enhanced the breaking strength, suggesting that $^1O_2$ mediated crosslink formation (27).

Irradiation produced very rapid O_2 depletion at 100 μm below the cornea stroma surface with oxygen reaching undetectable levels within 5 s and restoration of initial O_2 level by diffusion requiring 3–4 min (58). Thus, it appeared that crosslinking was occurring in an anoxic environment, which contrasted with the experimental results indicating an O_2 -dependent process. A hypothesis connecting these contrasting observations postulates that photocrosslinking occurs initially only near the stroma surface where there is sufficient O_2 . Subsequently, as all the potential oxidizable sites on collagen have reacted, O_2 diffuses deeper and crosslinking occurs at a greater depth in the tissue. Consequently, the zone of active photocrosslinking that utilizes O_2 moves deeper into the cornea stroma throughout the irradiation.

Additional photocrosslinking processes may contribute to the increased cornea stiffness after $R5P^{2-}$ /UVA treatment. One study identified tissue ketone and aldehyde carbonyls as components in crosslink formation because chemically blocking these groups almost completely inhibited the increase in cornea strength (27). Ketones and aldehydes are normally produced by oxidation in tissues, but how they participate in RF-CXL is not clear.

COMPARISON OF RB AND R5P PHOTSENSITIZATION MECHANISMS IN TISSUE

Rose Bengal and $R5P^{2-}$ -photosensitized protein crosslinking in tissue share certain features. Most notable is that crosslinking requires O_2 although an exception has been found for $^3RB^{2-}$ when an electron donating species is present that facilitates O_2 -independent crosslinking (44). Sodium azide, an efficient

although not entirely specific quencher of $^1\text{O}_2$, blocked the increases in cornea stiffness produced by photosensitization using either RB^{2-} or R5P^{2-} (27,44). Although quenching of dye triplet states could also account for these results, the rate constant for quenching $^3\text{RB}^{2-}$ is low ($\sim 5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) and our recent study of RB^{2-} in collagen gels showed that azide completely quenched $^1\text{O}_2$ but did not reduce the $^3\text{RB}^{2-}$ lifetime (40). D_2O enhanced the RB^{2-} photosensitized increase in cornea stiffness (44) as well as enhancing the breaking strength of cornea after R5P^{2-} photosensitization, indicating that $^1\text{O}_2$ mediates crosslink formation (27).

An alternative, oxygen-mediated pathway for photosensitization involving superoxide anion radical and subsequent reactive oxygen species has also been considered. In this mechanism, electron transfer to $^3\text{RB}^{2-}$ or $^3\text{R5P}^{2-}$ from donors or ground state dye molecules produce $\text{RB}^{\cdot 3-}$ and $\text{R5P}^{\cdot 3-}$, respectively (Scheme 1, steps 6 and 7), which transfer an electron to O_2 forming superoxide anion radical (Scheme 2, step 10). Formation of $\text{RB}^{\cdot 3-}$ in aggregates by electron transfer may be efficient from RB^{2-} to $^3\text{RB}^{2-}$ whereas this process is much less probable for $^3\text{R5P}$ because R5P^{2-} is not aggregated. However, for $^3\text{RB}^{2-}$ this oxygen-mediated mechanism is unlikely because azide, which blocks crosslinking, did not quench $\text{RB}^{\cdot 3-}$ at least in collagen gels (40).

The efficiency of RB^{2-} and R5P^{2-} -photosensitized crosslinking in tissue differs substantially. The fluence required for approximately the same level of cornea stiffness increase is 150 J cm^{-2} for RB^{2-} but only 5.4 J cm^{-2} for R5P^{2-} (41). A probable reason is that in RB aggregates, each RB molecule is adjacent to at least one other RB, favoring step 9 (Scheme 1) whereby $^3\text{RB}^*$ returns to the ground state without forming potentially reactive species.

PHOTOSENSITIZED PROTEIN CROSSLINKING VERSUS PHOTODYNAMIC EFFECT

Rose Bengal and R5P^{2-} have been widely used as photodynamic agents for killing or damaging cultured cells and for studying cellular responses to reactive oxygen species. Photodynamic therapy (PDT) is an application of the photodynamic effect in medicine that is used to treat certain cancers, macular degeneration, acne and other conditions. Usually, the intent of PDT treatment is to kill cells. However, in early studies of photosensitized protein crosslinking using RB^{2-} in animal models *in vivo* there was no evidence of phototoxicity such as an inflammatory response to dying cells (42,59–63). To directly evaluate whether photocrosslinking initiated cytotoxicity in tissue, RB^{2-} was used to seal incisional wounds on swine skin *ex vivo* and in rabbit skin *in vivo* (42). No significant difference in the number of dead cells within $100 \mu\text{m}$ of the wound surface (where RB^{2-} localized) was observed between treated and untreated controls. However, these treatment conditions cause 100% lethality when applied to cultured dermal fibroblasts. Very low RB^{2-} phototoxicity was also observed *in vivo* in rabbit skin when biopsies were examined from tissue taken 2 h, 1 day and 7 days post-PTB treatment. In addition, less granulation tissue appeared in the treated wounds, suggesting lower inflammation than in sutured wounds.

In order to understand the greater RB^{2-} phototoxicity to cultured cells than to cells in skin, dermal fibroblasts were cultured in collagen gels that mimic the extracellular matrix of tissue

(64). Direct comparison showed >200-fold greater RB^{2-} phototoxicity for cells in monolayer culture compared with those cultured in a collagen gel matrix. The results of this study indicated that several factors contributed to the difference between cells cultured on plastic *versus* a more tissue-like environment including upregulation of the PI3K-Akt cell-survival signaling pathway in cells attached to the collagen matrix. In addition, the high irradiances used, similar to those used for photocrosslinking tissues, caused oxygen depletion and consequent decrease in phototoxicity. Another factor may be that binding of RB^{2-} to collagen in the matrix (see Section “Association of Rose Bengal with collagen”) reduces the amount that enters the cells.

In more recent studies, RB^{2-} phototoxicity to keratocytes in the corneal stroma (65) has been observed and studied (66,67), which was consistent with earlier studies showing that R5P^{2-} photosensitization causes phototoxicity in the same tissue (68). The reason for greater photocytotoxicity in corneal keratocytes compared with dermal fibroblasts is not clear.

CLINICAL APPLICATIONS OF PHOTOCHEMICAL CROSSLINKING

There have been a number of uses of photochemical crosslinking of tissues that have been investigated for clinical application. These fall into four categories, although some applications can be considered as a combination of categories.

- 1 Wound closure: Photocrosslinking occurs between two tissue surfaces (*e.g.*, a wound) resulting in wound closure or sealing.
- 2 Biomechanical stiffening: Photocrosslinking occurs within a tissue leading to a change in the mechanical properties of the tissue.
- 3 Photochemical tissue passivation (PTP): Photocrosslinking occurs within a tissue and leads to a change in the biological response of the tissue.
- 4 Tissue engineering: Photocrosslinking is applied to modify a gel matrix or biomaterial to render it more suitable for cellular activity to provide a regenerated tissue.

Table 1 summarizes the different clinical applications that have been studied in our laboratory in preclinical and clinical models, specifically using RB^{2-} as the photoactive agent, differentiated by crosslinking category.

Wound closure—photochemical tissue bonding (PTB)

The oldest and most extensively investigated application of photosensitized protein crosslinking is for closing and sealing wounds. This general application is called photochemical tissue bonding and referred to as PTB.

Cornea. Our first application of PTB was for sealing surgical incisions in cornea, and these early results demonstrated some of the advantages of PTB over other wound closure modalities such as suture, tissue glues or laser welding (69).

Cataract surgery. During cataract surgery, a short linear incision is made in the cornea near the limbus to remove lens material and implant an intraocular lens. These short full-thickness incisions were initially used to test the ability of PTB to seal wounds. *Ex vivo* studies to seal incisions in rabbit eyes evaluated bonding strength as a function of fluence (J cm^{-2}) and irradiance (W cm^{-2}) by instilling PBS into the anterior chamber and

Table 1. Potential clinical applications of photosensitized protein crosslinking demonstrated in preclinical and clinical studies

Specialty	Wound closure	Biomechanical modification	Tissue passivation	Tissue engineering
Ophthalmology	Corneal wound closure (29,69,70,73) Corneal transplants (71) LASIK flap Attachment (72)	Cornea strengthening (41,44,66,67,93,98) Scleral strengthening (119)		Limbal cell grafting (111) Amnion graft (29,73) Intraocular lens attachment (112)
Neurology	Neurorrhaphy (62,63) Nerve graft fixation (81,82,105,113)	Nerve wraps (62,63,81)	Nerve wraps (83,62,81)	Nerve conduits (82,105)
Vascular surgery	Vessel anastomosis (61,87)	Vein grafts (43,79) Arterio-venous fistula (AVF) (101)	Vein grafts (43,79) Arterio-venous fistula (AVF) (101)	
Orthopedics	Tendon repair (78,84)		Tendon repair (78)	Tendon repair (78) Cartilage regeneration (106–109)
Gastroenterology	Colon anastomosis (80) Bowel puncture*	Esophageal wall strengthening*		
Plastic Surgery/ Dermatology	Skin incisions and excisions (42,59,83,114,115) Skin grafting (116)		Wound contracture (117) Capsular contracture (77)	
Otolaryngology	Vocal fold repair (118)			

*Unpublished study.

measuring the intraocular pressure required to rupture the seal and cause leakage (IOP_L) (69). For RB^{2-} and $R5P^{2-}$, a fluence-dependent increase in IOP_L was observed with a maximum of >300 mm Hg compared with <20 mm Hg for controls. Higher irradiances were less effective and deformed the cornea, probably due to photothermal effects. Photochemical O_2 depletion using RB^{2-} at the highest irradiance could also be inferred by the color change from pink to orange, indicative of formation of the de-iodinated products (Section “Rose Bengal photoprocesses in aggregates and collagen model systems”).

Evaluating PTB to seal the same incisions *in vivo* produced even higher wound closure strengths (70). Using 1.5 mm RB^{2-} and 514 nm with an irradiance of 0.64 W cm^{-2} , IOP_L values of >500 mm Hg were obtained at 192 J cm^{-2} in comparison with ~ 50 mm Hg in controls.

The wound strength was retained over the 14-day study and complications were not observed, demonstrating the potential of PTB for rapid and lasting closure of corneal wounds.

Corneal transplant. The more challenging clinical problem of sealing corneal transplants (penetrating keratoplasty, PKP) was then investigated (71). Sutures for fixation of the corneal graft act as a nidus for infection that leads to rejection, produces inappropriate suture tension (too loose or too tight) that can lead to astigmatism and causes irritation leading to patient discomfort. Rabbits underwent bilateral PKP, and the excised corneal buttons were then switched between eyes and sutured in place with 16 sutures. Control eyes received no further treatment and the experimental eyes received 1.5 mm RB, applied to the radial wound interface, and were treated with a ring-illumination system (532 nm, 80 J cm^{-2}). The mean IOP_L in PTB-augmented repairs was 410 mm Hg, a statistically significant increase over control repair values of 250 mm Hg. Because effective sealing with PTB requires full contact interface between host and graft, sutures would be needed clinically during the irradiation but possibly could be removed early to avoid suture-initiated side effects.

LASIK. LASIK treatment for correction of refractive errors is currently the most frequently performed cornea surgery. Post-

LASIK photocrosslinking using $R5P^{2-}$ as the photosensitizer is offered in some clinics to strengthen the treated cornea. This treatment also appeared to bond the LASIK flap to the stroma. A recent study tested the hypothesis that immediate bonding is produced between the LASIK flap and stroma by photosensitized protein crosslinking and identified optimized treatment parameters (72). In an *ex vivo* study, both photosensitizers increased by two-fold the adhesion between flap and stroma as determined by shear stress testing. Further research is needed to determine the clinical viability of this approach for enhancing LASIK.

Sealing traumatic wounds. The jagged and irregularly shaped corneal wounds resulting from flying debris, such as shrapnel, are extremely difficult to seal with sutures and require hours of tedious skilled surgery. Rapid sealing is often required but must allow subsequent reconstructive surgery. In collaboration with the US Army Institute of Scientific Research, PTB was used to bond AM to the cornea surface over the wound. AM is a thin, transparent biological material that is often attached by sutures in ophthalmologic surgery as a temporary patch or reconstructive graft.

Bonding RB^{2-} -stained AM over full-thickness corneal wounds of varying shapes effectively sealed the wounds in rabbit eyes both *ex vivo* and *in vivo*. Irradiation with 532-nm light produced IOP_L values 15- to 50-fold greater than the normal IOP of ~ 15 mm Hg *ex vivo* and *in vivo*, respectively (29,73). Adhesion *ex vivo*, measured by a peel test, was much greater for AM bonded to cornea using PTB than by fibrin glue, and PTB was not toxic to keratocytes. Further studies comparing sutures and PTB for sealing three types of complex corneal lacerations followed the rabbits for 4 weeks (73). Photobonding AM patches over each type of laceration was sufficient to provide a water-tight seal, although a single suture was required to stabilize the wounds prior to PTB/AM treatment. All corneas were clear by 4 weeks, which would allow further reconstructive surgery. No significant differences were observed between PTB and sutured corneas on a range of histological measures. No photochemical or thermal damage to the iris and no leakage from retinal capillaries were found. Thus, PTB sealing of AM over serious

penetrating corneal injuries appears to be a safe and effective technique that requires much less time than suture repair (SR).

Peripheral nerve repair. Peripheral nerve injuries present a complex clinical challenge to plastic surgeons. Peripheral nerves have an innate ability to regenerate but recovery of sensory or motor function is often unsatisfactory. Suture creates additional trauma to the nerve via needle passage and, like many tissue glues (74,75), is associated with foreign body response, inflammation and scarring that inhibits axonal regeneration (76). The endoneurial environment may also be compromised due to an incomplete seal allowing escape of regenerating axons that decreases recovery of function and/or form a painful neuroma, and leakage of neurotrophic and neurotrophic factors that are essential for nerve regeneration. PTB is therefore an attractive alternative to microsurgical repair, with the added benefit of minimizing skill-intensive microsurgery.

Nerve transection. Initial studies in a rat model of sciatic nerve transection demonstrated equivalent functional outcomes and superior histological outcomes using PTB compared with suture for repair involving an epineurial cuff (60). Next, PTB sealing of biocompatible nerve wraps that isolate the endoneurial environment was evaluated (63). Neurorraphy with suture was compared to AM wraps that were sealed over the repair sites using PTB (using RB) or to PTB without a nerve wrap. The PTB/AM group exhibited statistically significant improvements at 12 weeks in functional recovery and in axon diameter and myelin thickness compared with standard neurorraphy. An observation that sparked the future development of the tissue passivation approach (77–80) was a marked reduction in perineural adhesions between the repaired nerve and surrounding tissue in the PTB/AM repairs (63). The AM wrap did not provoke immunological response or inflammation and additionally acts as a physical barrier to adhesion between tissues. The PTB/AM wrap approach was also evaluated electrophysiologically in common peroneal nerve repair in larger rabbit models. Standard neurorraphy was compared to augmentation with an AM wrap either affixed using suture or photosealed using PTB (62). Electroconduction measurements were performed pre- and immediately post-surgery and out to 120 days. The recovery of the compound muscle action potential (CMAP) at 120 days in the PTB/AM group demonstrated a statistically significant improvement compared with standard neurorraphy. Suturing AM was the same as SR control, showing the crucial value of photosealing the regenerating neural environment.

Nerve deficit injury (nerve grafts). More complex injuries that involve significant nerve deficit are more challenging. Nerve grafting across a deficit requires two sites of attachment with PTB/AM used to photoseal both proximal and distal attachments. Pretreating AM with the biocompatible chemical crosslinking system of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride)/NHS (N-hydroxysuccinimide) greatly increased its resistance to proteolytic degradation *in vivo*, retaining the seal until axonal regeneration across the entire graft was complete (81). EDC/NHS treatment did not affect photobonding of the nerve wrap to nerve, as different reactive sites are involved for the chemical and photochemical processes.

A nerve graft study in isogenic Lewis rats allowed transposition of nerve segments between animal pairs as isografts (81). A 15-mm deficit was surgically created in the sciatic nerve and isografts inserted using crosslinked (xAM) and uncrosslinked AM

and single layer swine intestinal submucosa (SIS) that were affixed by suture, fibrin glue or PTB. The PTB/xAM group showed greater functional recovery than all other groups and exhibited statistically significant improvement in gastrocnemius muscle mass retention and in nerve fiber diameter, axon diameter and myelin thickness at the end of the study (150 days) compared with sutured isograft (81).

Acellular nerve allografts (ANA). More challenging again is a method to improve nerve regeneration after *severe* trauma (e.g., amputation) where autologous nerve graft is not available and alternatives must be sought. In a subsequent study (82), two further treatments were compared to those described in “Nerve deficit injury (nerve grafts)” for sealing a 15-mm deficit, namely, microsurgical attachment of ANA and PTB/xAM photosealing of ANA. Both isograft groups showed statistically significant improvement over ANA counterparts but of major significance was that no statistically significant difference in functional recovery or gastrocnemius muscle mass retention was observed between standard repair using isograft + suture and ANA + PTB. Photosealed xAM wraps isolated the regenerative environment and improved regenerative outcomes using ANA to the extent that there is no significant difference between ANA + PTB/xAM and standard of care autologous grafting.

Skin. While effective and inexpensive, sutures and staples generate inflammatory responses that may result in very noticeable scars. Sutureless methods such as glues and strips are also used in certain situations but all of these methods simply hold the tissues together to allow healing to take place whereas PTB joins collagen fibers between tissues through covalent crosslinking to form a continuous molecular-scale seal.

Human study of skin wound sealing. Prior to initiating a clinical study, the response to PTB closure of skin wounds was evaluated in swine skin *in vivo* (59). No wound dehiscence was observed, and no significant differences in cosmetic outcomes were observed compared with controls. A split-lesion comparative study of PTB *versus* standard epidermal suturing for wound closure was then performed on 31 skin excisions in patients that underwent standard elliptical excision of skin lesions (83). PTB seals the epidermis and reticular dermis, but not deeper layers, because of the limited penetration of 532 nm into skin (~50% decrease at 350 μ m). Thus, PTB sealing was compared only to epidermal suturing. After deep layer closure with absorbable sutures, the upper layer of one half of each wound was closed with nonabsorbable suture and the other half was sealed using PTB (0.1% RB²⁻, 532 nm, 0.5 W cm⁻², 100 J cm⁻²).

Scars were rated at 2 weeks and 6 months by three dermatologists viewing photographs, by two onsite physicians and by patients. Scar appearance at 6 months post-surgery was the primary outcome measure, and scar appearance 2 weeks after surgery and patient satisfaction at 2 weeks and 6 months were secondary outcome measures. At 2 weeks, the PTB-sealed half of the wound showed little erythema (Fig. 5A). At 6 months, the clinical evaluators judged PTB wound closure to improve scar appearance, with sutured wounds displaying a significantly wider scar. Patients were more satisfied with their PTB scar appearance at both 2 weeks and 6 months. These results indicate in humans that PTB has the ability to immediately seal skin wounds leading to less fibrosis and superior scar appearance compared with suture.

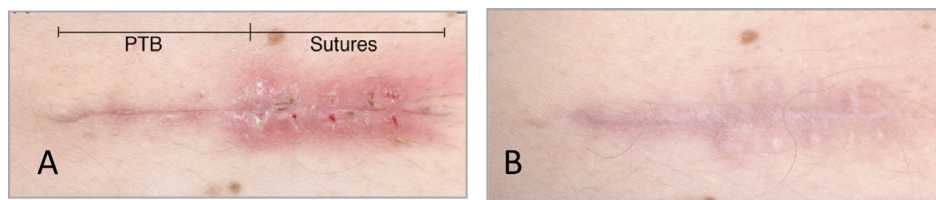


Figure 5. Epidermal closure of an excisional wound using PTB to seal the left half and interrupted sutures to appose the wound edges of the right half. (A) The excision site 2 weeks postsurgery after removal of sutures and (B) 6 months postsurgery. (Adapted from Ref. (83)).

Tendon repair. Sutureless repair in orthopedics using PTB may improve healing and shorten the usually lengthy time for recovery of function. For example, a ruptured Achilles tendon is sutured together and the joint requires weeks of immobilization for sufficient wound healing and scarring of the tissue to provide enough strength to prevent rerupture on remobilization. Suture causes complications due to adhesions, basically strands of scar tissue, forming between the healing tendon and surrounding tissues that cause stiffness and reduce the range of motion of the ankle. Earlier strength in the repair could allow for earlier mobilization, with reduced adhesions and improved functional recovery.

Direct bonding with PTB of tendons, ligaments and cartilage is limited by the poor light penetration into these tissues. In an initial *ex vivo* study of bovine tendon, the fluence and irradiance dependences were nonlinear and higher irradiances (1.5 W cm^{-2}) caused thermal damage (84). A subsequent *in vivo* repair of transected rat Achilles tendons (78) looked to provide increased strength and reduce postrepair adhesions by photosealing a mechanically strong, biocompatible wrap, fabricated from electrospun silk (ES), around the repair site (78). Following transection of the Achilles tendon in a rabbit model, SR by modified Kessler and epitendonous suture was compared with PTB plus ES wrap and with SR augmented by PTB/ES. PTB/ES repair alone did not provide as strong an acute repair as the sutured repair but did significantly augment early strength (7 days) when combined with SR. ES also acted as an adhesion barrier with a dramatic reduction in postsurgical adhesions. Thus, a thin wrap of ES bonded over the Achilles tendon repair complex could provide additional strength and considerably reduce the adhesions complication.

Vascular anastomosis. The fluid-tight seal created by PTB is important in luminal structures like blood vessels. Suturing, especially in microsurgery, is associated with complications due to needle trauma, foreign body reaction, inflammation and scarring at the repair site than increases the risk of stenosis and occlusion (85,86).

After a successful *ex vivo* proof of principle study (61), PTB was tested *in vivo* following transection of the femoral artery of Sprague-Dawley rats using a proximal-in-distal vascular cuff approach where 0.1% RB was applied to 5 mm of the outer surface of the proximal segment and 5 mm of the inner surface of the distal segment and the cuff was then approximated and illuminated (532 nm , 0.5 W cm^{-2} , 150 J cm^{-2}). A 24 g angi catheter was inserted into the vessel to support the lumen during PTB (532 nm , 0.5 W cm^{-2} for 30 s on each side) and then immediately retracted. All repaired vessels in the PTB group or suture group (10-0 nylon) were patent immediately and at 6 h. At 8 weeks, eight

of 10 vessels in each group were patent and histology of the PTB vessels harvested at 8 weeks showed minimal disruption of the normal vessel architecture while SR vessels showed evidence of intimal hyperplasia and structural disruption of all layers of the vessel wall.

An improved approach investigated hollow biocompatible intravascular stents for luminal support using permanent or dissolving materials. Polyvinyl alcohol (PVA) stents (87) were prepared that were microscopically smooth and semi-rigid ($750 \mu\text{m}$ outer diameter and $105 \mu\text{m}$ wall thickness, Fig. 6). PTB repair over the PVA stent was compared to primary SR and SR over the inserted stent (Fig. 6). All primary SR vessels were patent at 7 days, whereas only five of 14 and two of eight repairs in the PTB + stent and suture + stent groups, respectively, were patent. The permanent stent causes turbulent flow that results in thrombosis. What is required to realize the clinical potential of PTB for microvascular repair is a stent that supports PTB but immediately dissolves following re-establishment of blood flow. Inorganic glass materials that dissolve in minutes to component physiologic ions (K^+ , Ca^{2+} and PO_3^-) are currently under investigation in large animal models.

Bowel repair. Leakage of bowel contents following incomplete bowel anastomosis after surgical resection is a dreaded complication that leads to infection, sepsis and mortality (88). Abdominal surgery is also complicated by the high probability of postsurgical adhesions between the repair and the surrounding viscera that can result in pain, stricture and bowel occlusion, requiring further surgeries. Photosealing an AM wrap over the anastomotic line could increase resistance to leak. A rat colon transection study compared standard SR with an AM wrap, either sutured in place (SR + AM) or photosealed (SR + AM/PTB) (80). The ability of PTB/AM alone to seal the bowel anastomosis was also investigated using only three sutures to approximate the anastomotic site for photosealing (3 + AM/PTB). Anastomotic burst pressure (BP) was measured at 3 and 7 days on a 6-cm colon segment with the anastomosis at mid-point. Fig. 7 shows that both PTB groups exhibited a statistically significant increase in BP over the standard SR group. At 7 days, the BPs measured for all repair groups were significantly >3 days due to wound healing, except for the 3 + AM/PTB group where no significant change occurred, possibly due to proteolytic degradation of the AM exposed to bowel contents. At both day 3 and day 7, the SR group exhibited a statistically significant increase in adhesions compared with all other repair groups (Fig. 7). Adhesion score was significantly lower in the SR + AM/PTB group than all other groups at day 7. These results suggest that augmenting SR with AM/PTB may improve outcomes after bowel anastomosis.

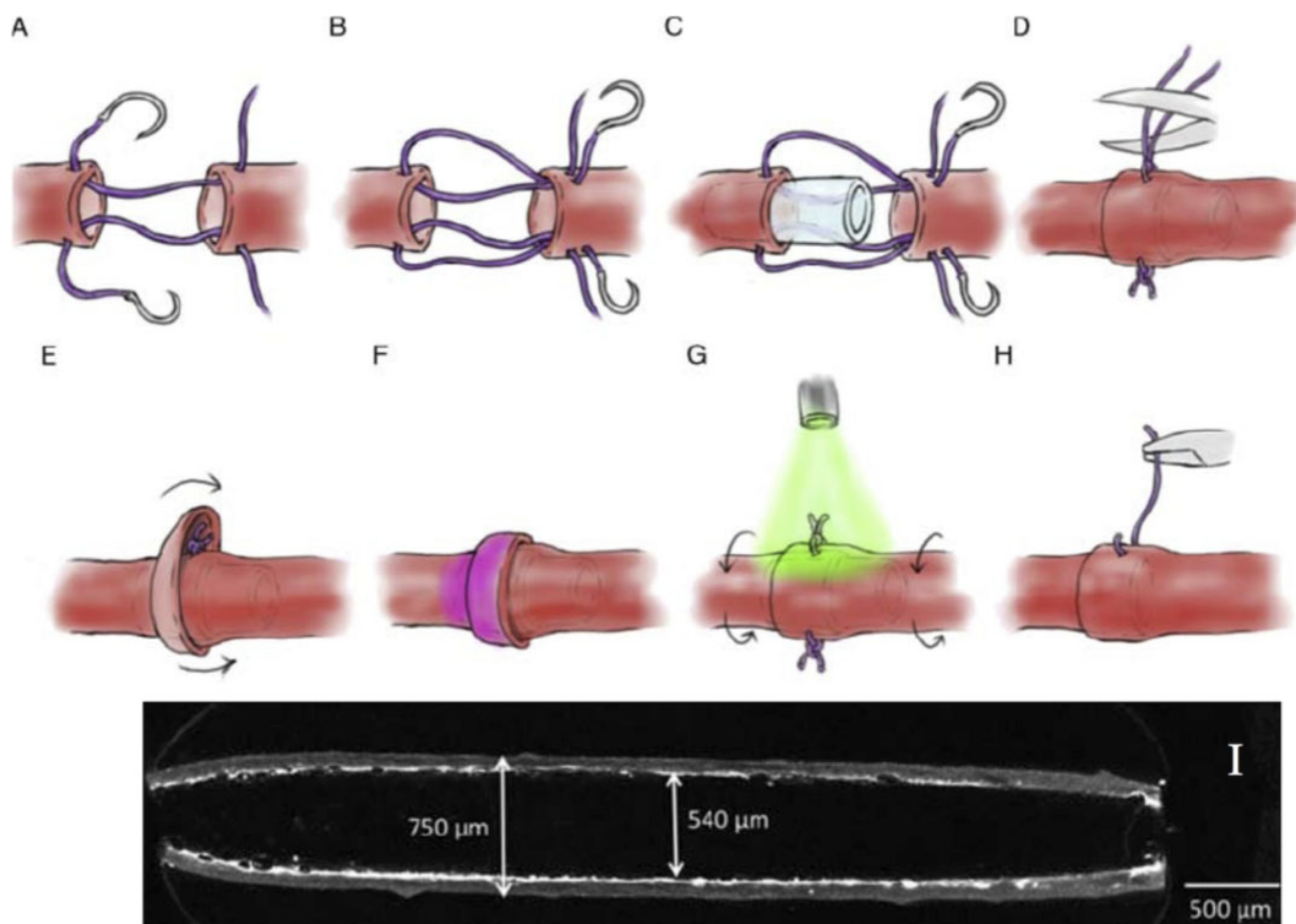


Figure 6. (A–H) schematic of light-activated anastomosis over a hollow PVA stent. Suture is passed from distal to proximal vessel and returned (A) and repeated (B) at 180°. Stent is inserted into proximal and then distal vessel (C) and sutures tightened (D) to form a proximal-in-distal cuff of around 2 mm. Overlapped segment was everted (E) and stained with RB solution (F). The everted portion was reversed and illuminated at 532 nm (G) for 30 s and rotated 180° and repeated. Suture was then removed prior to clamp removal (H). (I) Confocal microscopy image of hollow PVA stent used for light-activated vascular anastomosis showing smooth, thin outer wall (Adapted from Ref. (87)).

Photochemical tissue passivation (PTP)

PTB typically exhibits a marked decrease in inflammatory response and/or scarring in comparison with control suture groups (42,63,78,83). While a contribution is clearly due to foreign body response, PTB augmentation of SR also reduced inflammation and scarring (Figs. 7 and 8). This reduced biological response was termed photochemical tissue passivation (PTP), and applications were evaluated where tissue surfaces that were at risk for robust inflammatory responses and exuberant scarring were treated with RB and green light.

Capsular contracture. A capsular contracture model of breast augmentation (77), a common complication of breast implant surgery (89), was used as the first test for PTP. The synthetic implant is placed in a surgically created pocket and a thick collagenous capsule forms around the implant in a foreign body response. The capsule then undergoes fibroblast-induced contraction resulting in a painful and deforming contracture that requires surgical revision, with a high potential for recurrence. In a rabbit model, incisions were made on the dorsum and implant pockets dissected. PTP treatment was performed by coating the pocket with 1% RB and inserting a modified tissue expander filled with 10% intralipid with

a centrally located optical fiber, connected to a 532 nm KTP laser, to homogeneously illuminate the entire surface of the pocket. One treatment group received PTP and then 3 mL of pro-inflammatory fibrin glue, one pocket received fibrin glue only, and the other pocket received neither PTP or fibrin glue. All pockets then received 6 mL saline-filled tissue expanders as implant models.

At 8 weeks, the pockets with fibrin glue showed a statistically significant increase in capsule thickness compared with untreated pockets while the pocket that was pretreated with PTP reduced the capsule thickness by 52% compared with fibrin glue treatment alone, to a level even lower than untreated controls. Histology showed that PTP-treated pockets had less inflammatory invasion, fewer fibrohistiocytic and giant cells and reduced synovial metaplasia with less smooth muscle actin, a marker for myofibroblast activity, in the pocket. PTP pretreatment blunted the pro-inflammatory effect of fibrin in the pocket, giving promise to this treatment for procedures to reduce inflammation, scarring and subsequent scar contracture in a variety of susceptible tissues.

Biomechanical stiffening

Crosslinking of materials typically imparts increased mechanical strength, seen as an increase in the Young's modulus of

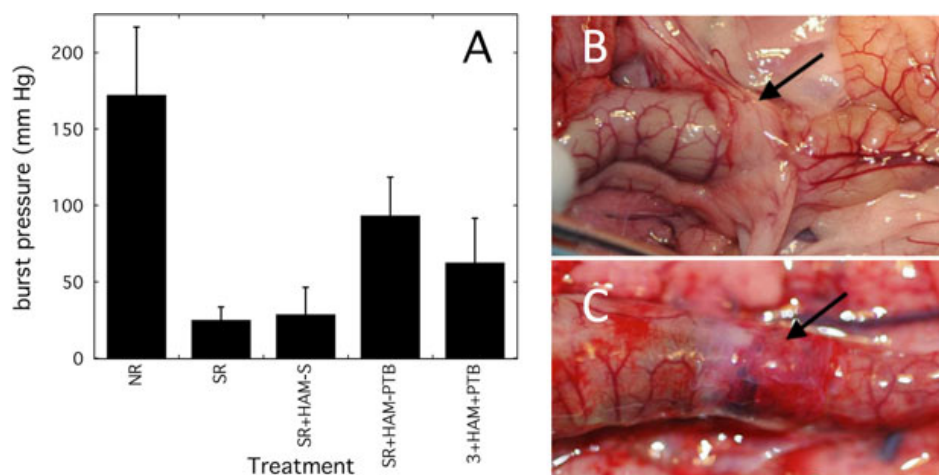


Figure 7. (A) Anastomotic burst pressure measured as a function of repair method. Augmentation of sutured anastomosis with photosealed AM wrap (SR + HAM + PTB) increased burst pressure by a factor of 4 over SR alone. Gross appearance of repair sites (arrow) at 7 days postrepair using SR (B) and SR + HAM + PTB (C). Note the presence of adhesions in the SR group and absence of adhesions in the photosealed group (Adapted from Ref. (80))

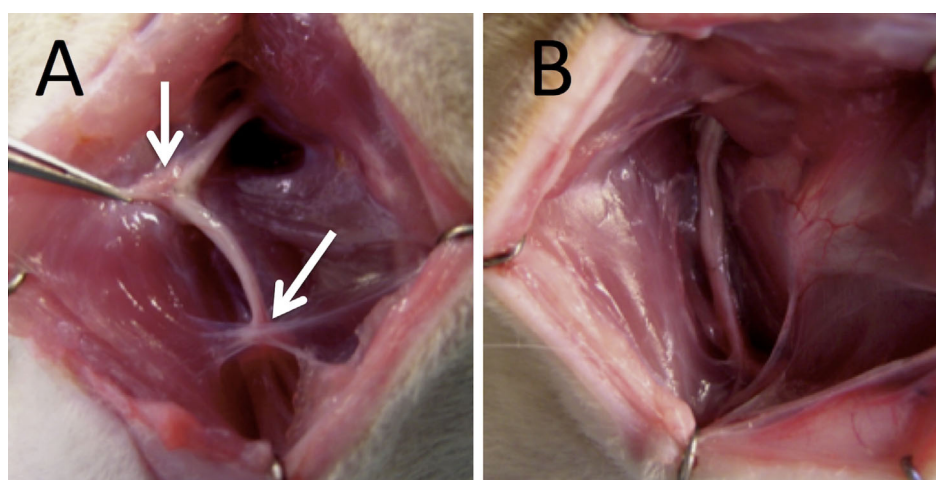


Figure 8. Gross appearance of rat sciatic nerve at 12 weeks postrepair using (A) standard neurorraphy with sutures and (B) photosealing via PTB and an AM wrap. Note postsurgical adhesions in sutured repair (arrows) that are absent in repair using PTB/AM (Adapted from Ref. (63)).

elasticity, due to the addition of large numbers of chemical bonds within the material. Photochemical crosslinking has been used in a variety of preclinical studies aimed at strengthening or stiffening tissues to prevent disease progression.

Cornea. Keratoconus is a corneal disease in which the stroma gradually thins and weakens and the cornea begins to bulge forward in response to the normal intraocular pressure, producing severe astigmatism and poor visual acuity. A major treatment goal is to stabilize the corneal shape to prevent further visual deterioration. Clinical studies have shown that photosensitized crosslinking using R5P²⁻ as the photosensitizer and UVA at 365–370 nm (RF-CXL) halted the progressive bulging in keratoconus patients and led to long-term stability of the cornea (90). Use of RF-CXL for keratoconus and other corneal conditions is a clinically approved treatment.

The classical RF-CXL treatment involves removal of the corneal epithelium, application of a 0.1% R5P solution in 20% dextran every 5 min for 30 min, then irradiation with 5.4 J cm⁻² UVA delivered over a 30-min period at

3 mW cm⁻² with continued application of R5P²⁻ (68). In order to protect the critical corneal endothelium from phototoxicity, sufficient R5P must be present in the stroma to absorb the UVA radiation before it reaches the endothelium. In order to reduce the treatment time, the irradiance can be increased (91) although very high irradiances may not be effective due to oxygen depletion (57). To avoid the pain associated with epithelium removal, which allows greater R5P uptake, transepithelial application of R5P²⁻ is being clinically evaluated (91).

RF-CXL is toxic to stromal cells and corneal nerves although an *in vivo* confocal microscopy study confirmed that both the cell density and the nerve density returned to baseline levels after 1 year (92). In animal models, RF-CXL increased corneal stiffness as determined by air puff corneal deformation imaging with finite element modeling and by optical coherence elastography (93,94), consistent with previous studies in *ex vivo*-treated corneas that indicated ~2-fold increase in Young's modulus (95–99). Responses to RF-CXL in rabbit corneas included downregulation of genes for ECM components and upregulation of glycan

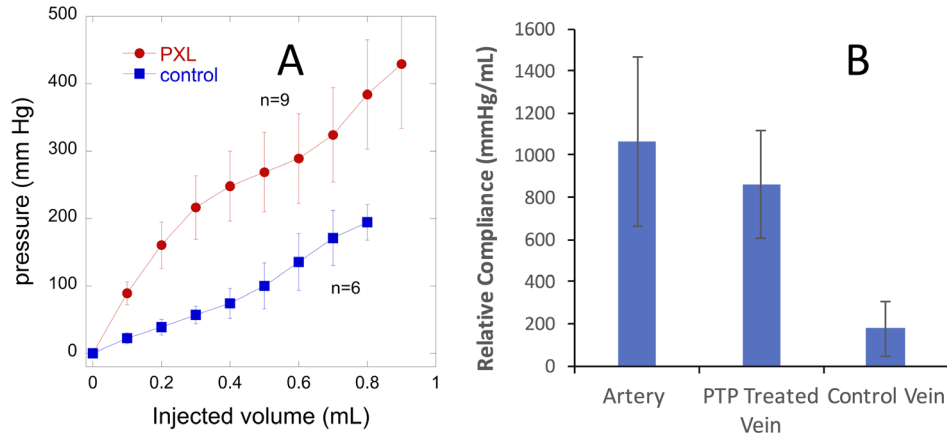


Figure 9. (A) Increase in measured intravascular pressure on instillation of saline into clamped swine veins *ex vivo*. Photocrosslinking treatment of the external vein wall increases compliance as seen as an increase in pressure with injected volume compared with native vein. (B) Relative compliance (from slope of pressure/volume plots) in native artery, native vein and photocrosslinked vein. (Adapted from Ref. (43)).

and proteoglycan glycosylation (100) as well as stromal cell death throughout the anterior and middle stroma (65).

Rose Bengal-photosensitized protein crosslinking also stiffens cornea and has potential advantages over RF-CXL. The treatment involves de-epithelialization followed by a 2-min application of 0.1% RB²⁻ in PBS and irradiation at 532 nm for 10 min (0.25 W cm⁻², 150 J cm⁻²) (67). RB²⁻ penetrates <120 μ m into rabbit cornea (Fig. 2A) such that only cells in a relatively narrow band near the anterior stromal surface experience cytotoxicity (66,67). Since RB²⁻ does not reach the endothelium, photocytotoxicity to endothelial cells is prevented. *Ex vivo* PTP studies of rabbit cornea showed a dose-dependent increase in stiffness with a two-fold increase at 150 J cm⁻² (41). This response was further dissected by Brillouin microscopy, which showed that the increase in elastic modulus was limited to the most anterior layer of the stroma, consistent with the location of RB²⁻ (41).

In vivo, RB²⁻-photosensitized crosslinking increased cornea stiffness 1.9-fold after 1 day and 2.8-fold after 1 month, as measured by tensiometry (67) and by larger factors when estimated from air puff corneal deformation imaging (93). Significantly, the same level and depth of stromal cell death was found after RB²⁻-photosensitized crosslinking as for control eyes that were only de-epithelialized (66). When the potential problem of green light damage to the retina was assessed in rabbit eyes, RB²⁻-photosensitized crosslinking did not cause breakdown of the blood-retina barrier as assessed by fluorescein angiography and the retina appeared normal histologically (67).

Thus, protein crosslinking photosensitized by R5P²⁻ and RB²⁻ increases cornea stiffness to approximately the extent although greater cellular toxicity is produced by R5P²⁻, the established clinical treatment.

Biomechanical stiffening + photochemical tissue passivation

In some photocrosslinking treatments, passivation occurs along with stiffening or wound closure. For example, treatment of veins for use as vascular grafts or arterio-venous fistulas (AVF) stiffens vein to better match the compliance of the arterial system and also passivates the vein to produce less intimal hyperplasia (43,79,101). Also wound closure in nerve (63), tendon (78) and bowel (80) produced markedly less fibrosis, indicating a passivation effect.

Vascular Grafts. Cardiovascular disease is the leading cause of death in the United States. Both cardiac and peripheral arterial disease patients benefit from autologous vein grafts to revascularize tissue when significant occlusion has developed. However, vein grafts are still associated with a high failure rate (25–50% at 5 years, (102)) that is related to intimal hyperplasia (IH), medial thickening and accelerated atherosclerosis, triggered initially by endothelial stretch injury due to the compliance mismatch between veins and arteries (103). When placed in an arterial system, the vein graft suffers acute dilatation causing endothelial injury, starting an inflammatory cascade that leads to IH. The hypothesis for these studies was that preventing the acute dilatation after grafting could prevent endothelial stretch injury and the ensuing complications that lead to graft failure.

In vascular surgery, it is important to avoid endothelial disruption; thus, the photochemical stiffening treatment was applied to the *external* (adventitial) surface of the graft. RB penetrates only around 100 μ m from the surface (Fig. 2), and the photocrosslinking only affects this outer layer. In a pilot study, epigastric vein interposition grafts were placed in rat femoral arteries (79). Photochemical crosslinking of the adventitia prevented acute venous dilatation of the graft on re-establishment of blood flow, while a statistically significant doubling of the graft diameter in untreated controls was observed. All grafts remained patent at 4 weeks. Histologic examination at 4 weeks demonstrated a >2-fold increase in intimal thickness in controls compared with PTP-treated grafts with the normal vessel architecture only preserved in the PTP-treated graft.

To test photochemical crosslinking to stiffen autologous vein grafts in a clinically relevant model, an interpositional vein graft study was performed in swine (43). *Ex vivo* PTP on swine saphenous vein grafts increased Young's Modulus of elasticity from 99 to 954 kPa. Acute compliance was investigated by clamping both ends of the graft and measuring the increase in intraluminal pressure as a function of volume of saline injected into the vessel (Fig. 9A). The slope of the pressure vs volume graph was five-fold higher for PTP-treated vessels and close to that of swine artery (Fig. 9B). Thus, PTP treatment was able to significantly reduce the mismatch in compliance between vein graft and host artery.

In vivo, 4.5 cm interpositional saphenous vein grafts were placed in the carotid artery of swine. In a bilateral study, PTP

treatment of one graft using (550 nm , 300 mW cm^{-2} , 90 J cm^{-2}) was compared to untreated graft in each animal. The vein segments underwent proximal and distal anastomosis with suture. After 4 weeks, untreated grafts had expanded radially and longitudinally, whereas the PTP-treated grafts were far less affected (Fig. 10). PTP treatment reduced the mean intimal area in the graft from 3.9 to 1.3 mm^2 and the intimal thickness from 0.74 to 0.38 mm , compared with control. Medial area and medial thickness were also reduced in a statistically significant manner. Histology showed controls with complete loss of internal elastic lamina and a large disruption of the external elastic lamina while these were preserved in the PTP-treated grafts. Immunohistochemistry also showed a greater degree of staining for smooth muscle actin in the control grafts.

Arterio-venous fistula. Arterio-venous fistulas, the standard for vascular access for hemodialysis in end-stage renal disease (ESRD) patients, suffers from a 40–60% failure rate within 1 year due to luminal stenosis caused by intimal hyperplasia (104). Thus, it was hypothesized that PTP treatment could prevent the acute stretch injury and subsequent cascade to intimal hyperplasia in AVF (101). Sprague-Dawley rats underwent epigastric vein-femoral artery AVF. In the PTP-treated group, the epigastric vein was isolated and treated as for the vascular graft (see “Vascular grafts”). An end-to-end anastomosis was created between the distal femoral artery and proximal epigastric vein. External vein diameter increased by 78% in the untreated AVF compared with only 10% in PTP-treated AVFs, immediately after restoration of blood flow. At 4 weeks, the control AVFs had dilated by 241% compared with 69% in the PTP-treated AVFs. IH measured at the crucial juxta-anastomotic region in controls was $3\times$ higher than PTP-treated AVF ($P < 0.05$) and $2\times$ higher over the entire AVF ($P < 0.03$). Thus, PTP may

significantly prevent AVF failure through reducing venous stretch injury and IH development after AVF creation.

Tissue engineering/biomaterials

Photocrosslinking has been used to prepare biomaterials for use as implants in medical applications. In some cases, it modifies the properties of a material to better suit the application (*e.g.*, crosslinked AM nerve wraps for greater strength and persistence *in vivo*) or to provide an environment that facilitates tissue regeneration.

Nerve guides. Nerve deficit injury requires a graft to bridge the gap, and alternative nerve guides including collagen, silicone and autologous tissues have been evaluated. Following the early success of photosealing nerve transection injuries with PTB/AM (Section “Nerve transection” Ref. (63)), a novel nerve guide was synthesized to both photoseal the nerve stumps to the conduit and permit axonal regeneration through the guide (105). Amnion sections of $2 \times 3\text{ cm}$ were stained with RB in the central $1 \times 3\text{ cm}$ area and wrapped around an angiocatheter. The dye-treated area was illuminated at 0.5 W cm^{-2} , resulting in a semi-rigid, internally crosslinked amnion tube with uncrosslinked ends that can be subsequently photosealed to the nerve stumps (Fig. 11).

Experiments were then performed using a 1 cm sciatic nerve deficit model in Sprague-Dawley rats with three experimental groups; (1) autologous nerve graft (excised segment reversed and sutured in place); (2) crosslinked amnion conduit (conduit secured in place using stay sutures); and (3) PTB/amnion conduit (uncrosslinked ends received RB solution and nerve stumps inserted and photosealed using 532 nm light (0.5 W cm^{-2} , 30 J cm^{-2}). After 12 weeks, there was no significant difference

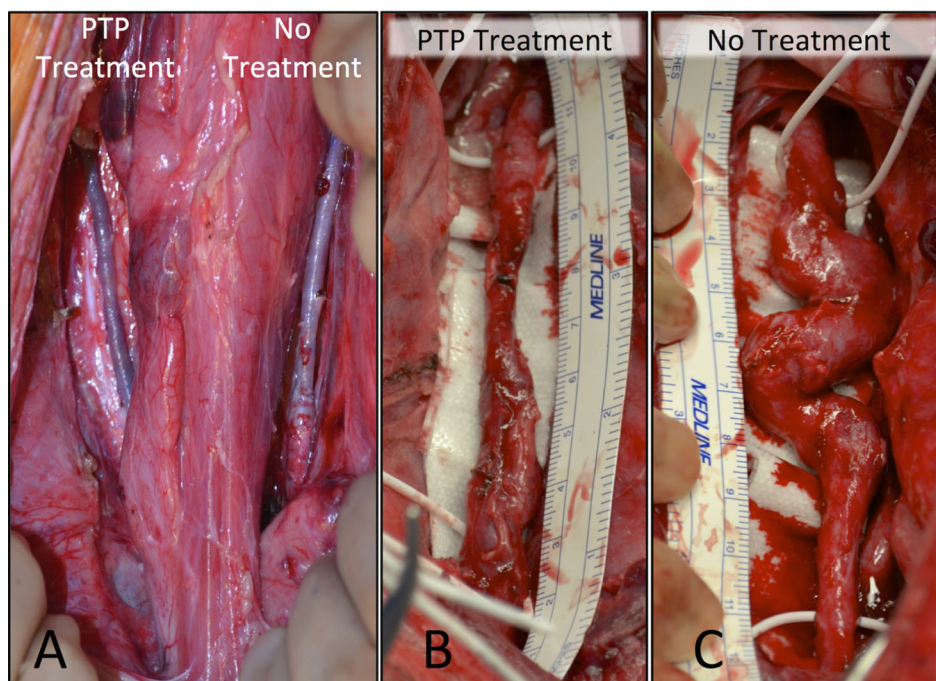


Figure 10. (A) Bilateral saphenous vein interpositional grafts placed in left and right carotid artery immediately after surgery. Left graft has been treated with PTP, whereas right graft is native vein. Note that native vein graft has already undergone dilatation compared with PTP-treated graft. (B) PTP-treated graft at 4 weeks postrepair. (C) Native vein graft at 4 weeks postrepair showing excessive dilatation in all dimensions with resultant tortuous appearance.

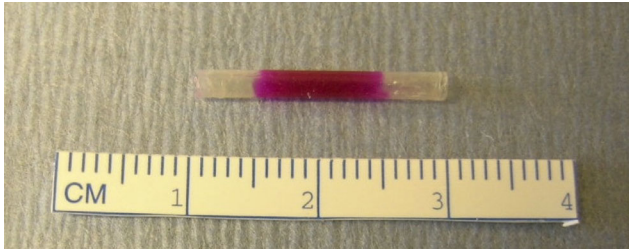


Figure 11. Amnion conduit formed by crosslinking central 1 cm area of a 2 × 3 cm amnion segment (shown in red). Transparent ends are uncrosslinked and used to photoseal to nerve stumps.

in functional recovery or gastrocnemius muscle mass retention between autologous nerve graft and PTB/amnion conduit but both were significantly improved compared with sutured amnion conduit. Histomorphometry showed statistically significant increase in nerve fiber count, fiber diameter and myelin thickness for the PTB/amnion conduit with respect to sutured amnion conduit. This simple nerve guide clearly seals and isolates the regenerating nerve environment and facilitates nerve regeneration that is equivalent to autologous nerve graft.

Cartilage regeneration. Collagen gels offer a biocompatible matrix for many tissue engineering applications but can be limited by the excessive contractile forces exerted by cells such as fibroblasts that deforms the matrix and produces volume mismatch for implant applications. Crosslinking collagen gels can lead to improved mechanical stability and a potential resistance to cell-induced contraction but many chemical crosslinking agents (e.g., glutaraldehyde) are toxic to cells and unsuitable for use *in vivo*. A biocompatible method for crosslinking collagen gels would be of benefit, particularly for articular cartilage defects, using a cell-containing collagen solution that conforms to the defect shape in liquid form and can be photocrosslinked *in situ* to form a robust gel implant that is resistant to contraction *in vivo*.

In a pilot study, chondrocytes or human dermal fibroblasts were photoencapsulated in photocrosslinked collagen gels *in vitro* and tested for cell viability and gel contraction as a function of photocrosslinker (R5P²⁻ versus RB²⁻) and light fluence (106). The best parameters were used to create constructs that were implanted subcutaneously in a nude mouse model to study cartilage generation *in vivo* (107). Constructs of 0.6% collagen containing 4 million swine articular, auricular or costal chondrocytes and 0.25 mm R5P were prepared and photocrosslinked using 475 nm light (0.5 W cm⁻², 20 J cm⁻²). Photocrosslinking prevented gel shrinkage in articular chondrocyte constructs, and in each chondrocyte type, the construct size remained greater after photocrosslinking. Biochemical analysis showed generation of new type II collagen matrix containing glycosaminoglycan (GAG), resembling native articular cartilage.

Next, a dual crosslinking paradigm was investigated to increase mechanical strength and resistance to digestion, while retaining the ability to generate new articular cartilage. (108). Type I collagen gel constructs with 2% collagen containing swine articular chondrocytes and RB were prepared and allowed to spontaneously gel. Gels were either photocrosslinked (532 nm, 20 J cm⁻²) or photocrosslinked plus chemically crosslinked by treating with EDC/NHS. Viscoelastic storage modulus increased by a factor of 5 using the dual crosslinking paradigm compared with photocrosslinking alone, which was mirrored by

a considerable increase in resistance to degradation on incubation with 0.08% collagenase. Dual crosslinked constructs that were cultured for 10 days retained 96% cell viability, showing that the increase in gel stiffness and persistence *in vitro* produces no adverse effects on the encapsulated chondrocytes, allowing more practical handling of potential gel implants for articular cartilage repair.

Although some neocartilage was regenerated in the vicinity of each cell, it was far from contiguous in nature. Greater stimulation of new matrix formation is required to remodel the crosslinked Type I collagen gel into a new hyaline cartilage matrix of predominantly Type II collagen. A further hybrid technology was investigated to combine an *in vitro* dynamic self-regenerating cartilage (dSRC) procedure with photochemical crosslinking to fix the dSRC in place in focal defects *in vivo* (109). Formation of dSRC is achieved by 14-day incubation of articular chondrocytes in suspension under reciprocal motion. A mouse model was employed *in vivo* using rings punched out of devitalized swine articular cartilage, and the dSRC formed *in vitro* was placed in the center of the ring. Hydrogel (Type I collagen or fibrin) was then added over the dSRC and the ring implanted subcutaneously in dorsal pockets of nude mice. After 6 weeks *in vivo*, the hydrogel capped dSRC had generated contiguous neocartilage within the ring with far greater proteoglycan and GAG content than simple encapsulated chondrocyte preparations. Immunohistochemical staining showed predominantly type II collagen in the neomatrix, typical of hyaline cartilage and good integration of neocartilage, and devitalized cartilage ring was observed.

These promising results led to a pilot study in a load-bearing swine articular defect model. Defects of 2 mm diameter and 5 mm depth were created in the patellar groove. After 6 weeks *in vivo*, repair with dSRC capped with collagen gel generated a smooth contiguous articular cartilage surface with generation of a Type II collagen matrix containing GAGs. Defects filled with collagen alone or left empty did not produce a positive outcome. These defects were quite small and ongoing research looks to expand this experience into larger and more complex defects that typically lead to development of arthritis if unrepaired.

CHALLENGES AND LIMITATIONS

Light delivery to the desired site for photosensitized protein crosslinking can be a challenge for many applications. In some tissues, a natural pigment such as blood, melanin in skin or myoglobin in muscles absorbs the green radiation used for RB photosensitization. This factor limits the penetration of light into the tissue and, at the irradiances typically used (0.2–0.5 W cm⁻²), produces an unacceptable temperature increase and possible thermal damage. Sealing tissue surfaces together, as in wound closure or reattachment of structures, requires that virtually no fluid remains between the surfaces since it would block the intimate contact needed for covalent bond formation between proteins. Optically, all tissues, except cornea, are turbid and scatter the incident green light, severely limiting its penetration. An approach to overcoming this limitation may be bioabsorbable optical waveguides that were shown to seal skin incisions to a depth of ~1 mm, a 10-fold greater depth that achievable by delivering the light to the skin surface (110). Development of alternative photosensitizers absorbing at longer wavelengths would also allow deeper tissue delivery of activating light for

crosslinking. Different types of challenges, namely, translation of the preclinical results to clinical studies and commercialization to bring photosensitized protein crosslinking to patients, are now being actively pursued.

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