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TITLE: Development of Nanopharmaceutical Therapy for Combat-Related Proliferative Vitreoretinopathy

PRINCIPAL INVESTIGATOR: Heuy-Ching Wang, PhD

CONTRACTING ORGANIZATION: Metis Foundation
84 NE Loop 410, Suite 325
San Antonio, TX 78216

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Heuy-Ching Wang, PhD

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The Metis Foundation
84 NE Loop 410, Suite 325
San Antonio, TX 78216

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14. ABSTRACT

Objective:
The objective of this study is to develop an anti-fibrotic therapy using pirfenidone-loaded nanostructures to treat proliferative vitreoretinopathy (PVR), a specific type of intraocular fibrosis, in a rabbit model of penetrating eye injury.

Background:
Intraocular fibrosis after penetrating eye injury is a leading cause of vision loss among members of the armed forces, who are vulnerable to penetrating eye injuries due to exposure to high velocity projectiles from detonations of IEDs. Current management of PVR and intraocular fibrosis involves the surgical removal of the intraocular fibrotic tissues. However, despite the 90% anatomic surgical success rate, membranes frequently regrow causing retinal detachment and vision loss, which makes PVR difficult to treat. Pirfenidone is a promising pharmaceutical agent approved for the treatment of other types of fibrosis, including pulmonary and renal fibrosis. Although pirfenidone has been shown recently as a possible treatment for intraocular fibrosis, there are concerns associated with its dosage and number of injections. Thus, to develop pirfenidone as a clinical therapy for PVR, the delivery of the drug must be engineered as to improve its bioavailability and delivery to the posterior segment of the eye. Success of this study will be a significant advancement not only for the treatment of PVR, but also for the treatment of other disorders that are localized to the posterior ocular segment.

Hypothesis:
We hypothesize that intravitreally injectable PLGA nanoparticles and PU nanocapsules will provide an effective platform for delivery of pirfenidone to the posterior ocular segment for the prevention of intraocular fibrosis in rabbits following penetrating eye injury.

Specific Aims:
Specific Aim 1: Development, characterization, and optimization of PLGA nanoparticles and PU nanoparticles for sustained delivery of pirfenidone to the posterior segment of the eye.
Specific Aim 2: Evaluation of pirfenidone-loaded PLGA nanoparticles and PU nanocapsules *in vitro* for determination of biocompatibility and pharmacodynamics of release.
Specific Aim 3:.Assessment of *in vivo* safety, pharmacokinetics, and bioefficacy of intravitreally injected pirfenidone-loaded PLGA nanoparticles and PU nanocapsules.

Study Design:
Aim 1: Pirfenidone will be loaded into PLGA nanoparticles and PU nanocapsules, which will be evaluated for their chemical and physical properties, as well as release kinetics.
Aim 2: Retinal pigment epithelial cells will be treated with pirfenidone-loaded nanoparticles and nanocapsules to evaluate their anti-fibrotic functions and cytotoxicity.
Aim 3: Rabbits with penetrating eye injury will receive intravitreal injections of pirfenidone-loaded nanoparticles and nanocapsules. The nanostructures will be tracked to ensure delivery to the posterior segment. The rabbit eyes will be scored for severity of intraocular fibrosis in treated versus untreated groups.

15. SUBJECT TERMS NONE LISTED

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
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1. INTRODUCTION

Posterior penetrating eye injury is a common battlefield-related ocular injury. Improper retinal wound healing can result in intraocular fibrosis and lead to severe visual impairment. Although there have been numerous technical advances in the surgical management of intraocular fibrosis, the continuous scarring in the eye after surgical intervention can eventually lead to total vision loss or blindness. This suggests that a pharmacological treatment is needed. Pirfenidone is a promising anti-scarring agent that has been approved for the treatment of pulmonary fibrosis and has also shown great potential in the prevention of liver or renal fibrosis. The objective of this study is to develop novel nanostructures for the sustained delivery of pirfenidone to the posterior segment of the eye. The therapeutic effects of pirfenidone-loaded nanoparticles or nanocapsules for the prevention and treatment of retinal scarring will be validated using a rabbit model of posterior penetrating eye injury.

2. KEYWORDS

Proliferative Vitreoretinopathy (PVR), Intraocular Fibrosis, Pirfenidone, poly lactic-co-glycolic acid (PLGA) Nanoparticles, Polyurethane (PU) Nanocapsules, Posterior Penetrating Eye Injury

3. ACCOMPLISHMENTS

What were the major goals of the project? (Goals to be accomplished and status.)

Navy Medical Research Unit San Antonio/NMRU Updates:

The long-term goal of this project is to develop novel nanostructures for the delivery of therapeutics to treat PVR. The project objectives are 1) to develop biodegradable nanoparticles and nanocapsules to improve the delivery of pirfenidone, an anti-fibrotic drug, in the ocular environment, and 2) to achieve delivery of pirfenidone to the posterior segment of the eye for the treatment of PVR in a penetrating eye injury rabbit model. To achieve the project objectives, the following three specific aims will be completed:

Specific Aim 1: Development, characterization, and optimization of PLGA nanoparticles and PU nanocapsules for the delivery of pirfenidone to the posterior segment of the eye.

STATUS: Synthesis and characterization of PU nanocapsules and PLGA nanoparticles coated with Pluronic F68 and Pluronic F127 have been completed. Fabrication of these nanostructures is ongoing to provide materials for both *in vitro* and *in vivo* studies.

Specific Aim 2: Evaluation of pirfenidone-loaded PLGA nanoparticles and PU nanocapsules *in vitro* for determination of biocompatibility and pharmacodynamics of release.

STATUS: Ongoing. We have obtained some preliminary data on the safety and biocompatibility of pirfenidone-loaded PLGA nanoparticles and PU nanocapsules *in vitro* with a human RPE cell line, ARPE-19. We are currently conducting experiments on the bioefficacy of pirfenidone-loaded nanostructures using RPE cells derived from human iPSCs.

Specific Aim 3: Assessment of *in vivo* safety, pharmacokinetics, and bioefficacy of intravitreally injected pirfenidone-loaded PLGA nanoparticles and PU nanocapsules in a penetrating eye injury rabbit model.

STATUS: Pending. Animal protocol for the *in vivo* study has been drafted. Modification to the protocol will be implemented upon completion of the *in vitro* studies.

What was accomplished under these goals? (Detailed progress and results.)

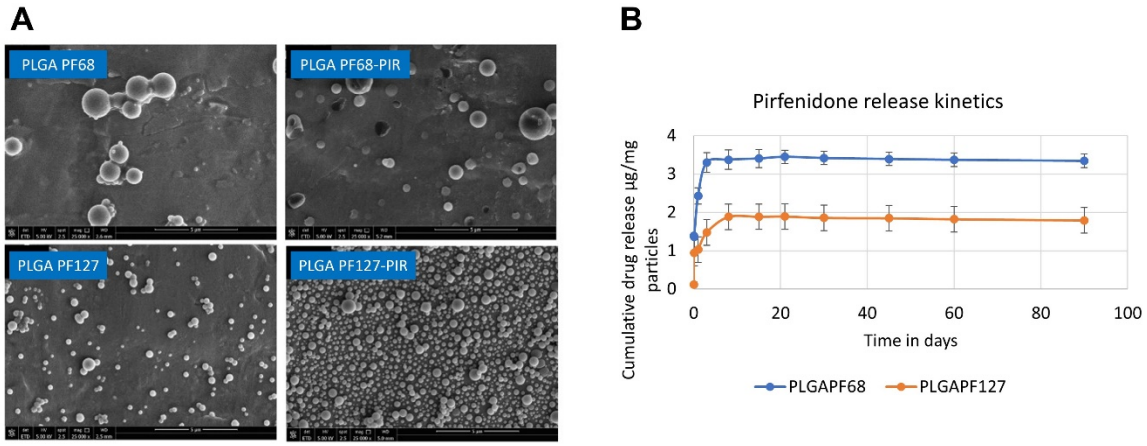
Navy Medical Research Unit San Antonio/NMRU Updates:

Specific Aim 1: Development, characterization, and optimization of PLGA nanoparticles and PU nanocapsules for the delivery of pirfenidone to the posterior segment of the eye.

Key Findings or Accomplishments:

1. We have successfully synthesized blank and pirfenidone-loaded PLGA-pluronic nanoparticles (PLGA-PF68 and PLGA-PF127) based on a modified nanoprecipitation method. The morphology of PLGA nanoparticles was characterized using scanning electron microscopy (SEM) and was found to be spherical (**Figure 1A**). Size distribution and zeta potential analysis were performed using a Zetasizer

Nano ZS90 instrument (Spectris/Malvern Panalytical, Malvern, UK). Particle size of PLGA-pluronic nanoparticles ranged from 308 to 519nm in water, and from 642 to 972nm in DMEM (**Table 1**). Zeta potential (ZP) is a measure of the electric charge on the nanoparticle surface. ZP can be used to predict the long-term stability of particles. Typically, particles with ZP values >25mV or < -25mV have excellent



stability (e.g. PLGA-pluronic nanoparticles in water as shown in **Table 1**). Nanoparticles with lower ZP values can experience aggregation while in solution. A sustained release of pirfenidone has been observed in PLGA-PF68 and PLGA-PF127 nanoparticles for up to 90 days (see **Figure 1B**).

Sample	Size in water (nm)	Zeta in water (mV)	Size in DMEM (nm)	Zeta in DMEM (mV)
PLGA PF68	465.96±3.82	-32.63±0.83	642.43±90.24	-15.06±0.08
PLGA PF68-PIR	519±25.54	-36.33±1.3	823.83±150.65	-22.84±1.8
PLGA PF127	308±11.13	-26 ±1.9	972.8±24.94	-1.29±0.15
PLGA PF127-PIR	397±18.13	-36.22±1.23	833.2±50.9	-2.29±0.99

Table 1: Particle size and zeta potential of blank and pirfenidone-loaded PLGA-pluronic nanoparticles in water or DMEM.

2. We have also fabricated blank and pirfenidone-loaded polyurethane (PU) nanocapsules via interfacial

Figure 1: (A) SEM of blank and pirfenidone-loaded PLGA-pluronic nanoparticles. **(B)** Release kinetics of pirfenidone in PLGA-PF68 and PLGA-PF127 nanoparticles.

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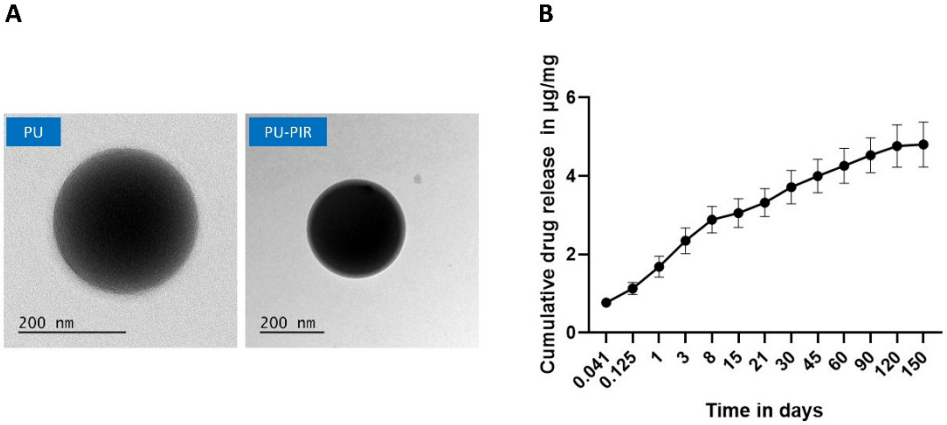
in different solvents such as ethanol, DMEM and water. The nanocapsules appeared to disperse very well in ethanol, but agglomerate in DMEM Furthermore, sustained release of pirfenidone has also been observed in PU nanocapsules for up to 150 days (see **Figure 2B**).



Sample	Size in Ethanol	Size in DMEM	Size in water	Zeta in Kcl	Zeta in DMEM	Zeta in water
PU	344.91±72.67	2352±87.64	445±6.8	-50.9±1.873	-17.6±1.1	-46.4±0.74
PU-PIR	244.666±40.06	836±128	579±54.1	-50.44±11.72	-24.1±1.8	-37.36±0.47

Table 2: Particle size (in nm) and zeta potential (in mV) of blank and pirfenidone-loaded PU nanocapsules in different solvents.

Specific Aim 2: Evaluation of pirfenidone-loaded PLGA nanoparticles and PU nanocapsules *in vitro* for dete

Figure 2: (A) TEM images of PU and pirfenidone-loaded nanocapsules revealed that these nanostructures exhibited spherical morphology. **(B)** Release kinetics of pirfenidone in pirfenidone-loaded PU nanocapsules for a duration of 150 days.

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and PU nanocapsules *in vitro* using ARPE-19 cells, a human RPE cell line. The cell permeant fluorogenic dye 2',7' –dichlorofluorescin diacetate (DCFDA, also known as H_2DCFDA) was used to measure reactive oxygen species (ROS) activity (i.e. oxidative stress assessment) in nanoparticle or nanocapsule-treated ARPE-19 cells. As shown in **Figure 3A-C**, the presence of ROS was not detected in any of the PU nanocapsule-treated groups. The MTS assay was used to quantify the number of viable cells after a 24hr incubation with 0, 250, 500, 1000µg/mL of PU nanocapsules or 300µM of H_2O_2 (see **Figure 3G**). Overall, we have demonstrated that the PU nanocapsules were not cytotoxic and did not affect cell viability.

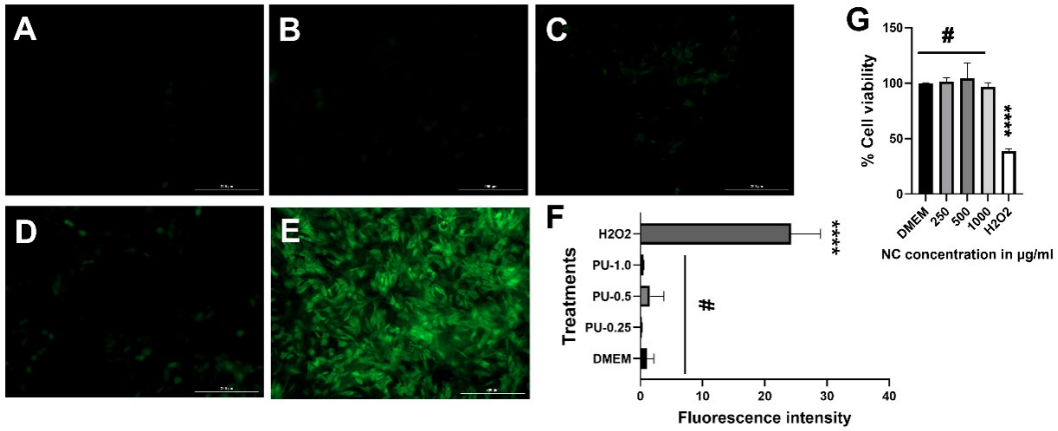

Figure 3: Oxidative stress in PU nanocapsule-treated ARPE-19 cells was measured via DCFDA assay. Representative image of DCFDA staining after incubation with PU nanocapsules for 24hr at **(A)** 250µg/mL, **(B)** 500µg/mL, **(C)** 1000µg/mL, **(D)** no treatment negative control, and **(E)** H_2O_2 treated positive control. **(F)** Quantification of DCFDA fluorescence intensity by Image J (n=4). **(G)** cell viability after incubation with 0, 250, 500 and 1000µg/mL of PU nanocapsules or 300µM H_2O_2. All the results were presented as mean ±SD, one way ANOVA was performed with Turkey's multiple comparisons, # p>0.5,****p<0.0001.

Specific Aim 3: Assessment of *in vivo* safety, pharmacokinetics, and bioefficacy of intravitreally injected pirfenidone-loaded PLGA nanoparticles and PU nanocapsules in a penetrating eye injury rabbit model.

Key Findings or Accomplishments: N/A.

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

Nothing to report for now.

How were the results disseminated to communities of interest?

Navy Medical Research Unit San Antonio/NMRU Updates: We published a manuscript on the animal model that will be used to test the efficacy of pirfenidone-loaded nanostructures and other therapeutics for retinal fibrosis

(Mil Med. 2020 Jan 7; 185 (Suppl 1): 443-447). We also presented relevant work entitled "Automating the Detection of Retinal Tears from Vascular Deformation using Machine Learning" at the Association for Research in Vision and Ophthalmology (ARVO) Annual Meeting on May 1-7, 2021 (poster).

Plans for the next reporting period to accomplish the goals

Navy Medical Research Unit San Antonio/NMRU Updates: We will continue to synthesize pirfenidone-loaded nanostructures for upcoming *in vitro* and *in vivo* studies. We will finish *in vitro* studies testing the efficacy of pirfenidone-loaded PLGA nanoparticles and PU nanocapsules.

1. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Navy Medical Research Unit San Antonio/NMRU Updates: Upon completion of the proposed research, invaluable data will be generated in the development of novel PLGA nanoparticles and PU nanocapsules that can enhance the bioavailability of pirfenidone, facilitate its delivery to the posterior segment of the eye, and reduce fibrosis in rabbits with penetrating eye injury. The impact of these studies will be significant for several reasons. As of now, an effective treatment for PVR is lacking. Even with surgical intervention, the visual outcomes for patients are poor, leading to severe visual impairment or blindness. Furthermore, an effective system for delivering pharmaceutical agents to the posterior segment is not currently available.

What was the impact on other disciplines?

Nothing to report for now.

What was the impact on technology transfer?

Nothing to report for now.

What was the impact on society beyond science and technology?

Nothing to report for now.

2. CHANGES/PROBLEMS

IMPORTANT REMINDER – Award recipient organization is required to obtain prior written approval from the awarding agency Contracting/Grants Officer whenever there are significant changes in the project or its direction such as significant change in scope or the Statement of Work (e.g. removal, change, or addition of aims/tasks or animal model change), change in PI or key personnel, reduction of 25% FTE, or significant change in budget.

Changes in approach and reasons for change

Navy Medical Research Unit San Antonio/NMRU Updates: We had originally planned to develop functionalized Avidin Fatty Acid (AFA)-PLGA nanoparticles for the delivery of pirfenidone to the posterior segment of the eye. However, after several preliminary trials, it became clear that AFA-PLGA nanoparticles were unstable and may not deliver the drug efficiently.

In order to overcome the issues with instability, we have modified our formulations with pluronics to encapsulate pirfenidone. Pluronics have been shown to possess mucoadhesive, mucopenetrating properties, in addition to other characteristics that are highly favorable for ocular drug delivery. We have fabricated two types of pirfenidone-loaded PLGA-pluronic nanoparticles successfully: PLGA-PF68 and PLGA-PF127. Further, in collaboration with Prof. Lavik, we have developed pirfenidone-loaded PU nanocapsules, which can be triggered multiple times for on-demand drug release.

Therefore, we will be using three different formulations of pirfenidone-loaded structures for this study: 1. PLGA-PF68 nanoparticles, 2. PLGA-PF127 nanoparticles, and 3. PU nanocapsules (two of which can be applied topically, whereas one can be injected intravitreally or may be administered topically for retinal fibrosis in an open-globe blast injury/PVR model).

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

Navy Medical Research Unit San Antonio/NMRU Updates: General delays in experimental data collection and analysis continued to be an issue due to the ongoing COVID-19 crisis. In order to observe social distancing and COVID-19 safety guidelines, laboratory staff members remained partially remote, resulting in some inevitable loss of productivity in the past year. Furthermore, we also lost one of our key staff members in the last quarter. While our new hires gear up with the project, we expect some initial delays in experimental data collection, which should resolve over time.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

Navy Medical Research Unit San Antonio/NMRU Updates:

TOTAL PROTOCOL(S): 1

PROTOCOL (X of Y total):
IACUC Protocol Number: Not Available
ACURO Protocol Number:
Protocol PI:
Protocol Site:
Protocol Title:
Number of Animals Approved for Use: Write Number
IACUC INITIAL APPROVAL DATE: M/D/YYYY (expires M/D/YYYY)
ACURO INITIAL APPROVAL DATE: M/D/YYYY
RENEWAL APPROVAL DATES:
- Due M/D/YYYY

AMENDMENTS:
- None.

ADVERSE EVENTS OR UNANTICIPATED PROBLEMS:
- None.

Significant changes in use of biohazards and/or select agents

Not applicable.

3. PRODUCTS

Journal publications

1. Navy Medical Research Unit San Antonio/NMRU Updates: Greene W, Burke T, Bramblett G, Wang HC. Detection of Retinal Fibrosis in a Rabbit Model of Penetrating Eye Injury. Mil Med. 2020 Jan 7; 185 (Suppl 1): 443-447. doi: 10.1093/milmed/usz221. Pubmed PMID: 32074329.
    a. Original manuscript
    b. Published
    c. Directly related to SOW, Specific Aim 3
    d. DoD funding acknowledged

Books or other non-periodical, one-time publications

Nothing to Report.

Other publications, conference papers, and presentations

2. Navy Medical Research Unit San Antonio/NMRU Updates: Wang HC, Peitzsch A, Martinez L, Tewolde S, Cardin S. Automating the Detection of Retinal Tears from Vascular Deformation using Machine Learning. Investigative Ophthalmology & Visual Science June 2021, Vol.62, 2126.
    a. Conference proceeding
    b. Published
    c. Related to SOW, Specific Aim 3
    d. DoD funding acknowledged

Website(s) or other Internet site(s)

Nothing to Report.

Technologies or techniques

Nothing to Report.

Inventions, patent applications, and/or licenses

Nothing to Report.

Other Products

Nothing to Report.

4. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Naval Medical Research Unit-San Antonio (NMRU-SA)
Name: Dr. Heuy-Ching Wang
Project Role: Principal Investigator
Nearest person month worked: 2.4 month
Contribution to the project: Dr. Wang has been overseeing the proposed project.

Name: Dr. Zakiya Skeete
Project Role: Investigator
Nearest person month work: 3.6 month
Contribution to the project: Dr. Skeete has written a laboratory protocol for the synthesis of nanoparticles.

Name: Dr. Annette Rodriguez
Project Role: Investigator
Nearest person month work: 1.2 month
Contribution to the project: Dr. Rodriguez has ordered supplies and initiated an *in vitro* PVR assay.

Name: Jenny Mendez
Project Role: Research Associate
Nearest person month work: 3 month
Contribution to the project: Ms. Jenny Mendez has setup the an *in vitro* PVR assay using iPS-RPE

University of Maryland, Baltimore County (UMBC)
Name: Dr. Erin Lavik

Project Role: CO-Investigator (no pay)
Nearest person month work: 1.2 month
Contribution to the project: Dr. Lavik is a biomaterial expert and serves as a co-investigator for this project. She provides laboratory space and serves as a supervisor to Dr. Mahaling.

Metis Foundation
Name: Dr. Christina Rettinger
Project Role: Investigator
Nearest person month work: 7.2 months
Contribution to the project: Dr. Rettinger has written laboratory protocols and technical reports for this project.

Name: Dr. Binapani Mahaling
Project Role: Investigator
Nearest person month work: 9 months
Contribution to the project: Dr. Mahaling has been involved in the synthesis and characterization of pirfenidone-loaded nanostructures. She has also conducted several preliminary studies on the biocompatibility of these nanostructures *in vitro*.

Name: Mr. Shuaishuai Liu
Nearest person month work: 3 months
Contribution to the project: Mr. Liu has been involved synthesis and characterization of pirfenidone-loaded nanostructures. He has also conducted several studies on the biocompatibility of these nanostructures *in vitro*.

Name: Mr. Andrew Peitzsch
Project Role: Investigator
Nearest person month work: 2 months
Contribution to the project: Mr. Peitzsch has developed a machine learning algorithm for automated detection of retinal tears from vascular deformation using fundus images of rabbit eyes with penetrating injury.

Name: Mr. Patrick Hsun
Project Role: Investigator
Nearest person month work: 1.0 months
Contribution to the project: Mr. Hsun refined the machine learning algorithm for automated detection of retinal tears from vascular deformation using fundus images of rabbit eyes with penetrating injury.

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?

Organization Name: University of Maryland, Baltimore Country (UMBC)

Location of Organization: Baltimore, MD

Partner's Contribution to the Project: Supplied equipment/personnel/facility for the project and collaborated on the research.

5. SPECIAL REPORTING REQUIREMENTS

QUAD CHART
Convert this report to a PDF file and append updated quarterly Quad Chart in PDF as an appendix.

6. APPENDICES

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.