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AWARD NUMBER: W81XWH-14-1-0407

TITLE: Discovery of FDA-Approved Drugs that Promote Retinal Cell Survival or Regeneration

PRINCIPAL INVESTIGATOR: Dr. Jeffrey Mumm

RECIPIENT: Johns Hopkins University

REPORT DATE: October 2015

TYPE OF REPORT: Annual Report

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 2015	Annual Report	15 Sep 2014 - 14 Sep 2015

4. TITLE AND SUBTITLE

Discovery of FDA-Approved Drugs that Promote Retinal Cell Survival or Regeneration

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0407

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER
410-502-2210

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

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

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Ocular trauma due to blast or blunt forces affect millions of people worldwide, which often result in an irreversible loss of visual function. However, therapeutic approaches that can reverse this process or promote protection are limited. Recently, research on the damaged zebrafish retina has highlighted the potential of the Muller glia (MG) cells to function as a retinal stem cell to regenerate lost neurons. It is now appreciated that MG cells may retain the potential for retinal repair across vertebrate species, including humans. Other conserved stem cell niches exist for surrounding ocular tissues, e.g., corneal limbal epithelial stem cells. Therefore, to discover chemical compounds that protect retinal cells and/or neighboring ocular tissues from cell death and edema, or that stimulate regenerative outcomes from multiple ocular stem cells, we developed an *in vivo* drug discovery platform named Automated Reporter Quantification *in vivo* (ARQiv). ARQiv quantifies reporter activity in transgenic zebrafish at high-throughput rates. In order to mimic ocular trauma injury in zebrafish, we developed an inducible cell/tissue ablation model; transgenic zebrafish lines in which yellow fluorescent reporter and prodrug converting enzyme nitroreductase (NTR) are exclusively expressed in rod photoreceptors. Here, we show that exposing transgenic lines to the prodrug CB1954 resulted in the robust edema in tissues around the eye as well as loss of NTR-expressing rod photoreceptors and surrounding cells in the retina. In a beta test screen using previously established protective compounds, we identified 10 out of 35 drugs diminished CB1954-induced edema in ~50% of the zebrafish larvae. Further, we identified one compound Panobinostat (LBH589) were able to completely abrogate the CB1954-induced edema and decrease the rod photoreceptor cell loss. Presently, we are screening FDA-approved drugs that can protect ocular cells and tissues following traumatic injuries using visual assay and a robotics integrated ARQiv system. By identifying novel drugs for treating ocular injuries *in vivo* we hope to accelerate the translation of our findings from bench to bedside.

15. SUBJECT TERMS

Zebrafish, highthroughput drug discovery, ARQiv, regeneration, edema

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC, Gay Hayden
a. REPORT	b. ABSTRACT	c. THIS PAGE		23	19b. TELEPHONE NUMBER *(include area code)* 301-619-9883
Unclassified	Unclassified	Unclassified	Unclassified		

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Ocular injuries are the most common injury in the wars and blast exposure can cause damage to the eye, which often result in an irreversible loss of visual function. However, no available therapies promote protection or reverse this process. In this project, we try to identity FDA approved drugs that promote long-term ocular tissue survival and sustain photoreceptor function in zebrafish and mouse models. Drugs that protect the ocular tissues and increase the photoreceptor regeneration across species will have a higher chance of translating to the clinic.

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

Ocular injuries, drug discovery, eye, zebrafish

3. ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?

MONTHS 1-3

Subtask 1 – Optimize assay for HTS primary screen using statistical methods for HTS assay design:

• Determine optimal pigmentation background = 100% Complete
• Establish optimal sample number for HTS ready status = 100% Complete
• Beta test screen using previously established protective compounds = 100% Complete
• Maintain breeding stocks expressing YFP-NTR in rod cells and RFP in ocular tissues = 100% Complete

MONTHS 3-12

Subtask 2 – Perform primary protective screen.

• Perform screens using Johns Hopkins Drug Library (JHDL) = % 7 Complete

MONTHS 4-13

Subtask 3 – Verify hits implicated in primary protective screen.

• Confirm effects by using ARQiv and visual assays = 0% Complete

MONTHS 5-13

Subtask 4 – Establish leads by determining if hits exhibit desirable pharmacokinetic properties.

• Determine LD50/ED50 of confirmed hits = 0% Complete

What was accomplished under these goals?

Our ultimate goal is to discover FDA-approved chemical compounds that promote the survival and/or regeneration of the retina and surrounding ocular tissues following traumatic injuries.

In this report, we successfully completed our initial goals for the high throughput drug screening assay and had begun the **primary protective screen** using a simplified process of assessing survival of photoreceptors only. The specific milestones achieved for this period are:

- Determination of optimal pigmentation background
- Establishment of optimal sample number for HTS ready status
- Maintain breeding stocks expressing YFP-NTR in rod cells and RFP in ocular tissues
- Beta test screen using previously established protective compounds
- Determination of Optimal CB1954 Concentration for Visual Assay

- Calculating sample size for visual assay

*Note: we had begun the **primary protective screen** using a simplified process of assessing survival of photoreceptors only. However, issues with the reproducibility of CB1954-induced rod cell ablation have triggered us to reassess the primary screening methods (see below for full details).*

Specific milestones for this period are detailed below:
*Note: progress was delayed due to time required to establish a new aquaculture facility dedicated to housing drug screening breeding stocks (see previous **W81xWH-14-1-0407** report).*

Determine optimal pigmentation background – 100% completed

We first determined how to achieve the highest possible signal to noise ratios. We previously published that utilizing 'transparent' zebrafish strains during fluorescence based assays facilitates increased signal to noise ratios (Walker et al., 2012); however, concomitant increases in auto-fluorescence background ("noise") are typically observed. To find a solution that would allow quantification of fluorescence signals yet provide relatively low noise levels, we treated transgenic zebrafish (Tg(rho:YFP-NTR)gmc500) with a chemical inhibitor of pigmentation, 1-phenyl 2-thiourea (PTU); is a tyrosinase inhibitor commonly used to block melanization. We observed that the signal to noise ratio was significantly improved in gmc500 fish treated with PTU versus transgenic fish that were raised in a "transparent" genetic background. For the assays proposed here, we have identified that treating gmc500 transgenics with 200 nM PTU at 16 hpf (hour post fertilization) will provide the highest signal to noise ratios, increasing the sensitivity of assay.

Establish optimal sample number for HTS ready status – 100% completed

Initial assessments of data from the CB1954 induced ocular injury assay determined that preprocessing the data using log transformation, a standard HTS practice, would control for higher signal variance observed in the positive control group. Accordingly, a log transformed dataset comprised of 50 positive control (0.1% DMSO) and 50 negative control gmc500 fish (CB1954/0.1% DMSO treated) was used to define an upper SSMD quality measure of $\beta = 1.62$. Using the 'robust' SSMD calculation (aka SSMD*), which account for outliers by replacing mean and variance values with median and median absolute deviation, a value of $\beta = 2.92$ was achieved. This equates to a 'good' moderate control or 'excellent' strong control, respectively. Using power calculation, with a critical value cut-off of $Z\alpha/2 = 3.29$ ($p = 0.001$) and $Z\beta = 1.64$ (power of 95%), we calculated our sample size to be ~13 larvae. To account for HTS instrument error (e.g., 'blanks' during larvae dispensing into multiwell plates), we decided to use an n of 16 for the assay.

Maintain breeding stocks expressing YFP-NTR in rod cells and RFP in ocular tissues – 100% completed

We finished the process of mating gmc500 fish stocks with a second transgenic line, Tg(atoh7:gap-RFP)cu2 and established the double transgenic breeding stock. Recently, approximately 2000 double transgenic fish have reached breeding age (4-5 months old) in our new facility.

We decided to perform the primary screening in Tg(rho:YFP-NTR)gmc500 line rather than double transgenic line in order to simplify the quantification of the fluorescence reporter. All lead drug candidates will be later validated in double transgenic lines to evaluate the extent of damage / survival of non-rod ocular tissues following induced 'bystander' ablation.

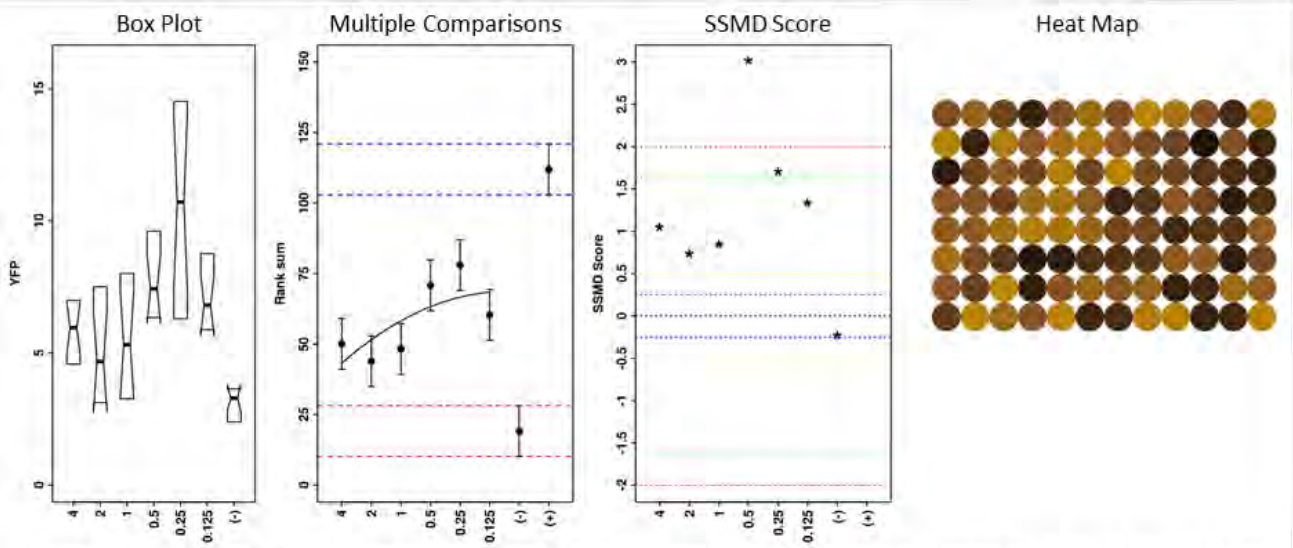
Beta test screen using previously established protective compounds – 100% completed

There has been several months delay in this aspect of the project due to a new aquaculture facility construction for HTS screening, expanding breeding stocks of Tg(rho:YFP-NTR)gmc500 fish and waiting fish to reach

breeding age (3-4 months old) in our new facility.

Accordingly, we initiated the beta testing process with the new gmc500 breeder stocks. We successfully completed the beta screen with the gmc500 line. In keeping with HTS best practices, we assayed each compound across a titration series spanning six different concentrations at a sample size of 16 per condition (~2,000 fish in all including controls). This 'quantitative HTS' (qHTS) primary screening strategy was developed to reduce false call rates in drug discovery. Five-day-old larvae were exposed to drug solutions 4 hours prior to exposure to the rod photoreceptor ablating prodrug CB1954 (150 µM). Fish were then incubated under normal conditions for 2 days, then anesthetized and placed in plate reader for quantification of YFP reporter levels. The level of YFP reporter provides a measure of how many rod photoreceptors are surviving at the termination of the treatment.

Of the 30 compounds screened, we found 4 positive results when we exposed embryos to 150 µM of the prodrug CB1954 to induce rod photoreceptor ablation (example shown below). We retested the 4 positive compounds and found that rod photoreceptor ablation in the negative control (ablated fish in 150 µM CB1954 alone) was not adequate to verify compound effectiveness. These results suggested to us that, despite initial successful results, our method of inducing rod photoreceptor ablation – 150 µM CB1954 for 48 hours – was not consistent /robust enough for the rigor of a full-scale in vivo HTS initiative. Therefore, we tested several alternative approaches outlined below (for full details see, 'Actual Problems or delays and actions to resolve them' section):



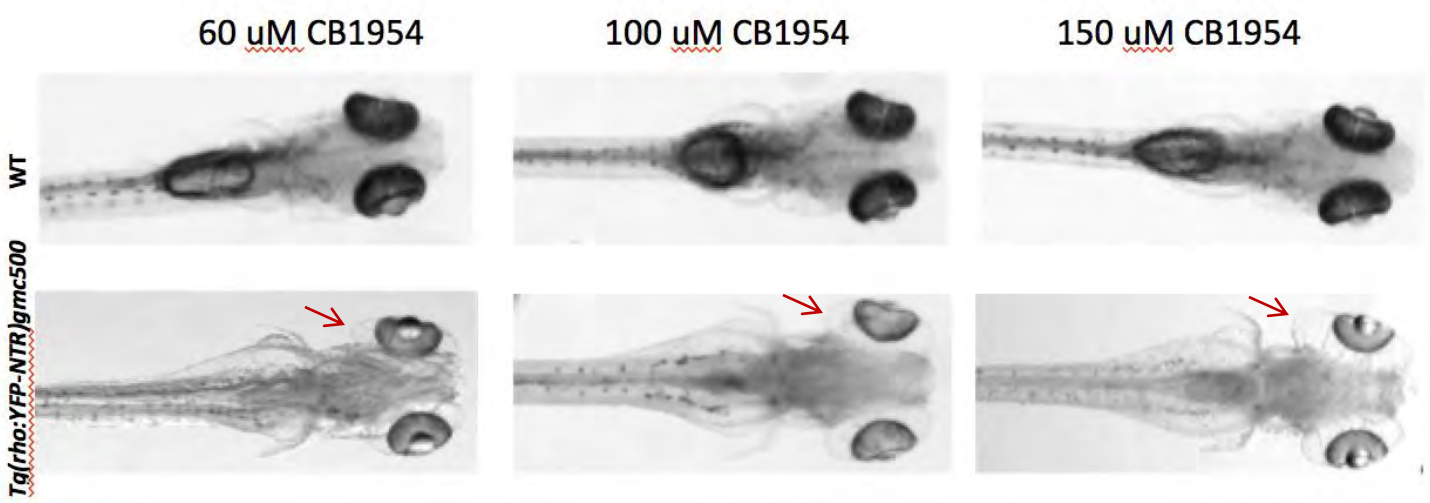
Representative result from 'known' neuroprotective screen. A-D: Graphs/maps produced via R-based plate parsing and extraction algorithm of ARQ*iv* data. Representative assay data were collected from transgenic zebrafish (*Tg(rho:YFP-NTR)gmc500;roy*) exposed to 150µM CB1954 for 48 h, from 5-7 dpf, in the presence of the compound N-tert-butyl--(2-sulfophenyl) nitrone. All compounds are tested across 6 concentrations (2-fold dilution series ranging from 4µM to 0.125µM) for the capacity to protect NTR/CB1954-targeted rod photoreceptors from cell death. ARQ*iv* was used to quantify YFP reporter levels across conditions and the data was downloaded and processed in real-time our R-based code to produce near instant feedback about compound performance. We use four data plot types to highlight different aspects of the results: A) Boxplots – to show dose-response relationships and an indication of variability; B) Multiple comparisons ranking (Kruskal-Wallis) – to indicate conditions significantly different than controls (positive control: non-ablated fish in 0.1%DMSO only, blue dashed bounded range; negative control: ablated fish in CB1954 and 0.1%DMSO, red dashed bounded range); C) SSMD (strictly-standardized mean difference) scores – a relative measure of effect size (blue, yellow, orange, green, and red dashed bounded ranges indicate, very weak, weak, weakly moderate, moderate, fairly strong effects, respectively). D: Heat map of signal intensity in 96-well plate – to facilitate visual follow-up of select conditions to assess toxic/teratological effects, autofluorescence, and/or confirmation of desired effect.

<u>Despite initial successful results, our beta test screening results suggested to us that 150 uM CB1954 for 48 hours was not consistent for the a full-scale in vivo HTS.</u> Contrary to the limiting effects CB1954 has an rod cell ablation (thus diminishing our signal:noise ratios for the plate reader assay), CB1954 does induce a consistent and robust edema in tissues around the eye in our transgenic line targeting rod cells (but not in non-transgenic controls) (e.g., 95% of treated larvae, see micrograph below on 'Actual Problems or delays and

actions to resolve them' section). **Therefore, for the primary screen we have decided to conduct a visual phenotypic screen to more directly assess the phenotype of interest (i.e., ocular edema).** Secondarily, compound efficacy regarding protecting retinal neurons will be assessed.

Determination of Optimal CB1954 Concentration for Visual Assay- 100% Completed

CB1954 does induce a consistent and robust edema in tissues around the eye in our transgenic line targeting rod cells but not in non-transgenic controls (see figure below). To determine the optimal dosage of CB1954 that produce phenotype of our interest (ocular edema) at the lowest concentration, CB1954 was titrated across different concentrations. We treated embryos with 50 uM-, 55 uM -, 60 uM -, 66 uM –, 70 uM CB1954 and 0.1% DMSO (negative control) for two days from 5 dpf to day 7 dpf. The phenotype induced by CB1954 treatment were compared to vehicle only negative controls (0.1%DMSO). This analysis determined that 48 hours exposure with 60 uM CB1954 can achieve 95.6% of phenotype necessary for HTS.



Non-transgenic wt and transgenic Tg(rho:YFP-NTR)gmc500 embryos are treated with 60 uM- 100 uM- and 150 uM CB1954 from day 5 to day 7. CB 1954 induces edema in transgenic fish (red arrow) but not in non-transgenic fish.

Concentration of CB1954	Number of embryo with edema in eye	Number of embryo with normal eye	Percentage of embryo with edema in eye
50 uM CB1954	95	16	85.50%
	52	4	92.80%
	41	9	82%
	38	12	76%
Average			**84.08%**
55 uM CB1954	118	12	90%
	48	6	89%
	56	10	84.80%
	59	1	98.30%
Average			**91%**
60 uM CB1954	97	8	92.30%
	50	2	96.10%
	58	0	100%
	47	3	94%
Average			**95.60%**
66 uM CB1954	133	10	93%
70 uM CB1954	112	7	94%

CB1954 concentration test results Embryos are treated with 50 uM-, 55 uM -, 60 uM -, 66 uM -, 70 uM CB1954 or 0.1%DMSO from day 5 to day 7. Each experiment has been done in different days and averages of percentage of phenotype were calculated.

Calculating sample size for visual assay- 100% Completed

For HTS, the sample size calculation requires stringent significance and power values to minimize false-call rates. We have adjusted our sample size number to the new visual assay using power calculations. First, we treated 100 embryos with 0.1% DMSO (group A) and 100 embryos with 60 uM CB1954 (group B). We performed Fisher's exact test of independence to determine whether the proportion in group A, pA, is different from the proportion in group B, pB. The hypotheses are

$$H_0 : p_A = p_B$$
$$H_1 : p_A < p_B$$

or

$$H_0 : p_A = p_B$$
$$H_1 : p_A > p_B$$

where the ratio between the sample sizes of the two groups is

$$\kappa = \frac{n_A}{n_B}$$

To determine the minimum sample number required to discern compound effects at a given effect size, significance, and power, we used a formula from G* power program for sample size calculation. Formula for sample size calculation:

$$n_A = \kappa n_B \text{ and } n_B = \left(\frac{p_A(1-p_A)}{\kappa} + p_B(1-p_B)\right)\left(\frac{z_{1-\alpha} + z_{1-\beta}}{p_A - p_B}\right)^2$$

$$1 - \beta = \Phi\left(\frac{|p_A - p_B|}{\sqrt{\frac{p_A(1-p_A)}{n_A} + \frac{p_B(1-p_B)}{n_B}}} - z_{1-\alpha}\right)$$

where

- κ=nA/nB is the matching ratio
- Φ is the standard Normal distribution function
- Φ−1 is the standard Normal quantile function
- α is Type I error
- β is Type II error, meaning 1−β is power

The G* power sample size calculator using power and significance values minimizing false-call rates (95% and p = 0.05, respectively) determined that a sample number of **11** was sufficient to detect a 50% effect size. However, to account for occasional automation errors, we elected to screen **12 larvae** per compound concentration.

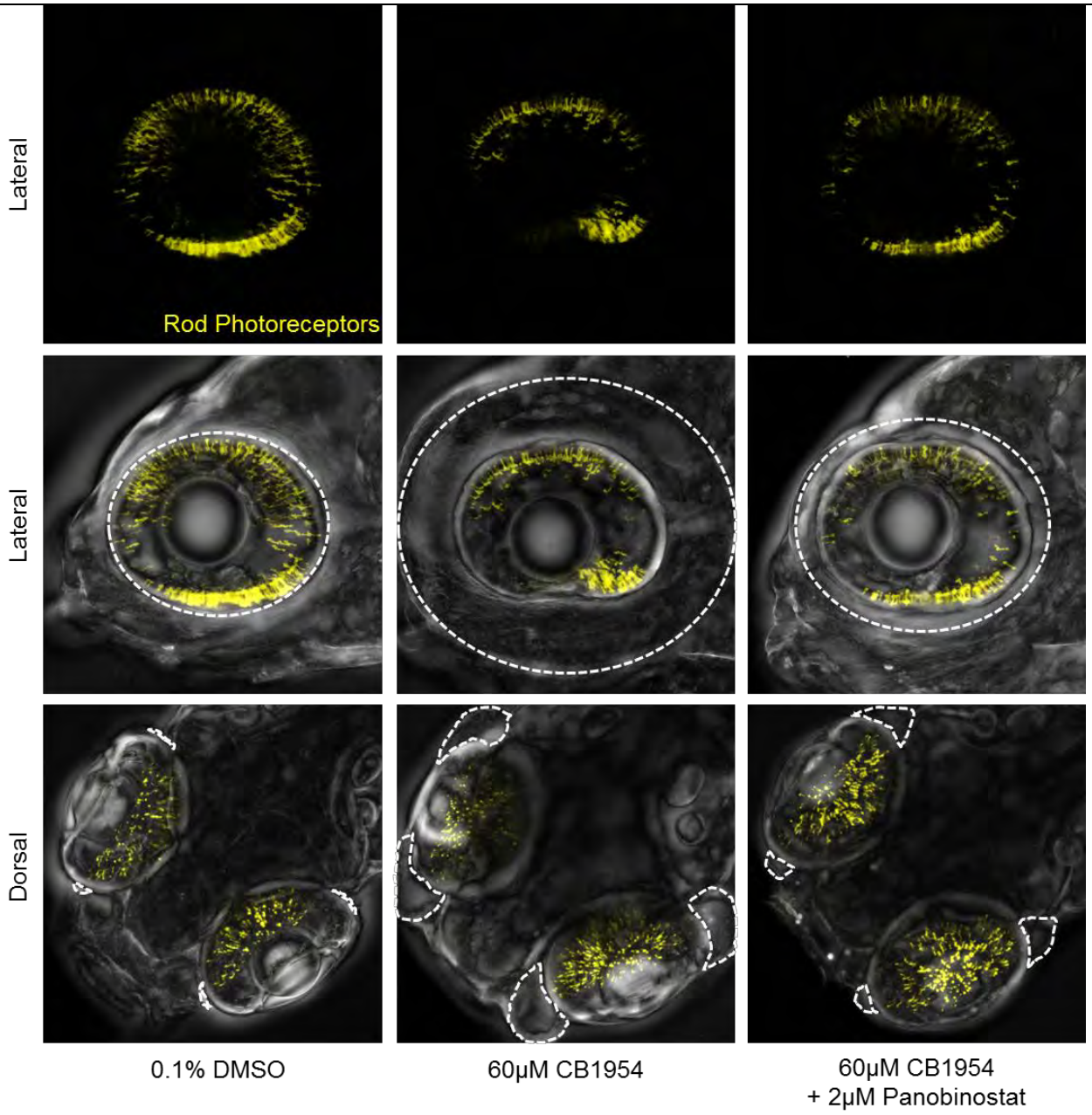
Beta test screen using previously established protective compounds for visual assay- 100% Completed

After defining optimal CB1954 concentration and sample number for visual assay, we have initiated another beta test screen to assess the ability of the 30 'known' neuroprotectants to ameliorate CB1954-induced edema. In addition, we tested a panel of known anti-inflammatories as well. We have successfully completed beta test

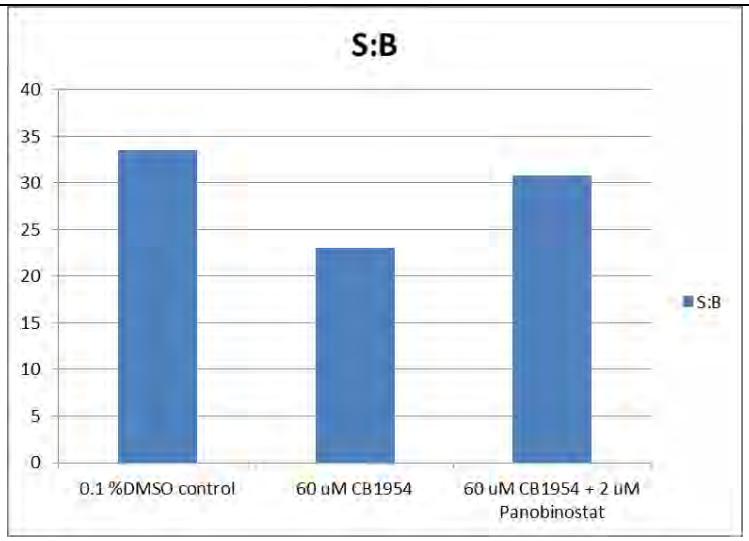
screen with the gmc500 line and identified a robust positive control for a visual phenotype-based primary screen from among the compounds tested. 10 of 35 drugs tested were able to diminish CB1954-induced edema in ~50% of the larvae (see table below). Panobinostat (LBH589) was able to almost completely abrogate the effects of CB1954. We also noticed that Panobinostat was able to protect rod photoreceptors (see figure below- YFP expressing rod photoreceptors). These results validated Panobinostat as a viable positive control compound (see figure below).

Drug Name	Possible function of drug	Concentration of drug	Percentage of normal or diminished eye size phenotype
Panobinostat (LBH589)	HDAC inhibitor	4 uM, 2uM, 1uM, 0.5 uM	99 %
Romidepsin (FK228 ,depsipeptide)	HDAC inhibitor	2 uM	62 %
Vorinostat	HDAC inhibitor	2 uM	43 %
N-acetyl cysteine (NAC)	Antioxidant	2 uM	55 %
Pinacidil	Anti-inflammatory	1 uM	43 %
Niflumic acid	Anti-inflammatory	4 uM	45 %
Chlonidine hyd.	Anti-inflammatory	4 uM	43 %
17-allylamino-17-demethoxygeldanamycin (17-AAG)	Inhibition Hsp90	2 uM	50 %
Oxiconazole nitrate	Infection inhibitor	2 uM	46 %
Alpha-tocopherol	Form of vitamin E	2 uM	46 %

Beta test screen result 10 of 35 drugs tested were able to diminish CB1954-induced edema in ~50% of the larvae.



Confocal images of 0.1%DMSO-, 60 uM CB1954- and 60 uM CB1954+ 2uM Panobinostat - treated larvae at 7dpf. Ocular edema was significantly reduced in Panobinostat treated larvae. Panobinostat also protected YFP-expressing rod photoreceptors from cell loss.



Results from treatment with 0.1% DMSO; 60 uM CB1954; and 60 uM CB1954+ 2uM Panobinostat together. The data shows increases in rod cell loss when larvae were treated with 60 uM CB1954 and decreases in rod cell loss when larvae were treated with 60 uM CB1954+ 2uM Panobinostat together.

Perform primary protective screen- 3% Completed (100 of ~3,300 compounds)

Progress on the primary screen initially has been hindered due to delays in establishing the parameters for a robust HTS-ready fluorescence-based primary screening assay. However, we anticipate being able to recover time lost by virtue of advantages of switching to a visual phenotyping primary assay. The most obvious time savings coming from a reduction in the sample number required to observe compound effects - from 16 to 11 samples per concentration tested - allowing us to perform the primary screen more rapidly.

Pilot Visual Phenotyping Screen

After defining the sample size and positive control for visual screening assay, we initiated a pilot screen of the JHDL. For hit selection, all compounds are compared to negative and positive controls to determine relative effect sizes. In addition, all hit compounds will be secondarily tested for neuroprotective and regenerative effects. To validate neuroprotective effects of hit compounds showing anti-edema effects on rod cells and surrounding ocular tissues, we will perform ARQiv screen and high-resolution confocal imaging. Compounds effective for both abrogating edema and protecting rod cells will be put forward for *in vitro* testing in mammalian cell culture systems.

For the pilot screen, 100 JHDL compounds were screened over six different concentrations – a total of 12,000 fish larvae (with controls). The table below list the five potential hit compounds (an ~5% hit rate) identified thus far.

Drug Name	Indication	Mechanism of Action	FDA approval
Aminocaproic acid	Hemostatic	Aminocaproic acid binds reversibly to the kringle domain of plasminogen and blocks the binding of plasminogen to fibrin and its activation to plasmin.	Y
Apomorphine hydrochloride	Antiparkinsonian	A dopamine D2 agonist. It is a powerful emetic and has been used for that effect in acute poisoning. It has also been used in the diagnosis and treatment of parkinsonism, but its adverse effects limit its use.	USP, BAN

Beta-carotene	Vitamin A precursor	Converted into vitamin A (all-trans retinol). All-trans retinol is further converted into 11-cis-retinal functions in the retina in the transduction of light into the neural signals necessary for vision.	Y
Atropine	Mydriatic	Competitive antagonist of the muscarinic acetylcholine receptors	Y
Cetylpyridinium chloride	Antiseptic	Membrane disturbing agent against microorganisms	Y

Secondary ARQiv screens are ongoing whereby the three most effective concentrations will be re-tested at a higher sample number (required for robust detection of neuroprotective effects by this methodology). The subset of compounds showing efficacy in protecting rod cells will be imaged using confocal microscopy suing double transgenic zebrafish to visualize more specifically the range of protective effects.

Intriguingly, several of the hits identified in the pilot screen are used in the clinical setting as ophthalmic treatments. For instance, Aminocaproic acid is a topical treatment for hyphema; bleeding in the front part of the eye (called the anterior chamber, between the cornea and the iris) typically caused by traumatic injury. In addition, Atropine is used to relieve pain caused by swelling and inflammation of the eye, whereas beta-carotene (i.e. vitamin A) is used as part of the AREDS formulation to treat age-related macular degeneration (AMD). These results support the core logic of the screen, that the zebrafish eye can be used to identify compounds having potential therapeutic benefit to human patients, and reinforce the possibility of discovering new insights into molecular mechanisms for treating ocular trauma.

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

Dr. Arife Unal Eroglu presented Preliminary Results at the 2015 FASEB Science Research Conference in Big Sky, Montana on June 14-19, 2015

MARZ, Mid-Atlantic Regional Zebrafish Meeting, July 17, 2015 Albert Einstein College of Medicine, Bronx, New York
Poster presentation: Arife Unal Eroglu and Jeff S. Mumm "High-throughput drug screening using zebrafish for traumatic ocular injuries", Poster # 15

How were the results disseminated to communities of interest?

Results were presented during a Poster Session format.

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

We are behind with regard to the primary screen and hit confirmation (Milestone 1, Subtasks 2-4). To make up for lost time we will perform visual screening assays 3 times a week (~120 drugs/per week). Identified hits will immediately be secondarily screened for activity in protecting rod photoreceptor cells using ARQiv and/or high-resolution confocal imaging.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change

in practice or behavior that has come about as a result of the project relative to:

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

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS: The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

Due to the difficulty in establishing an HTS-ready fluorescence assay, we have elected to leverage the robust ocular trauma phenotype induced by CB1954 treatment as a visual screening assay for the primary screen (as noted above). Secondarily, the effects of hit compounds regarding rod cell protection will be assessed. In effect, this reverses the order of the primary and secondary follow-up screens. However, we are confident that this will in no way diminish the power of the project. As evidence of this, we note that our control compound, Panobinostat, is effective at both reducing ocular edema and protecting rod cells from cell death (see figure above). Moreover, in a pilot screen of 100 JHDL compounds we identified several compounds that are currently being used in the clinic to treat ophthalmological disorders. Changes to our screening process, and associated milestone adjustments, are outlined in an updated Quad Chart provided below, and will be detailed in an updated Statement of Work to follow.

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

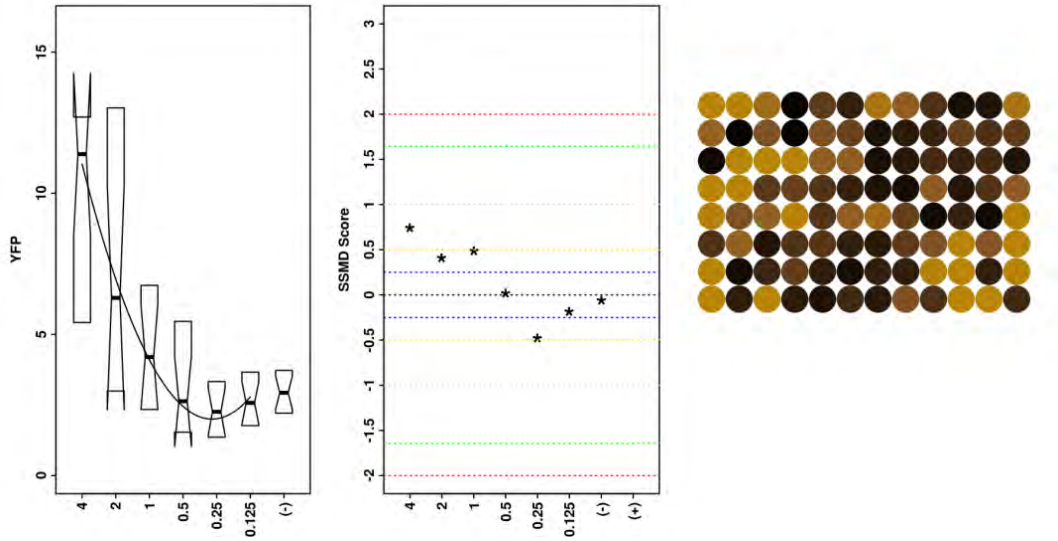
There has been several months delay in the process of the project due to a new aquaculture facility construction for HTS screening, expanding breeding stocks of Tg(rho:YFP-NTR)gmc500 fish and waiting fish to reach breeding age (3-4 months old) in our new facility and establishing the parameters for a robust HTS-ready assay. Beyond these issues, we mainly struggled with getting quantifiable rod cell ablation with the CB1954. To resolve this problem, we tested several approaches outlined below.

1) Alternative CB1954 treatment regimens: In the process of trying to define an optimal concentration and treatment time for CB1954 treatment regimens, we performed dose-response assays and treated embryos at different ages (5 dpf-6dpf) for different time points (4h, 6h, 24h and 48h treatment). Unfortunately, we experienced either embryo viability issues or insufficient rod photoreceptor cell ablation with this approach. It is possible that this arose due to CB9154 lot variation and/or deterioration of existing stocks. Regardless, the degree of variation compromised our ability to reliably detect compound effectiveness. These results drove us to explore additional alternatives.

2) Alternative 'bystander'-inducing prodrugs: In an effort to identify a prodrug with improved performance, we asked Dr. David Ackerley to provide us alternative prodrugs for inducing bystander effects. He sent us two prodrugs (PR104A and A2) he has had success with any mammalian cell culture assays to examine in our zebrafish system. Initial tests of these two prodrugs were performed at concentrations ranging from 25 μM to 600 μM with 50 μM intervals. However, we could not detect adequate rod photoreceptor cell ablation, nor any evidence of bystander effects (i.e., ocular edema) with these concentrations. We then tested these two prodrugs

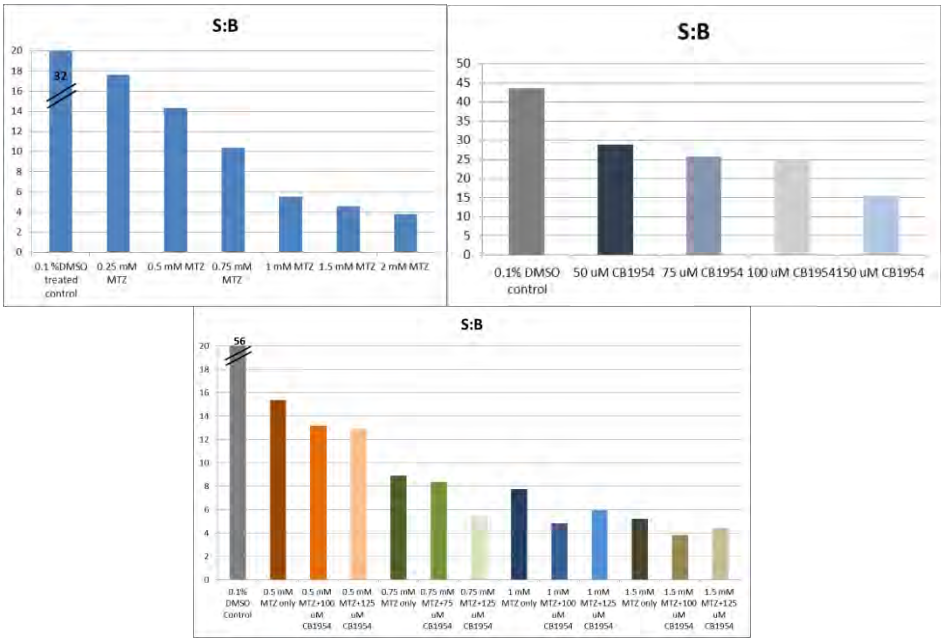
at a highest concentration (4mM). We found that A2 was highly toxic whereas PR104A treated embryos did not show cell ablation or bystander effects. Accordingly, additional alternatives were pursued.

3) CB1954 + Metronidazole: In order to achieve significant ablation, we have tested whether treating larvae with another prodrug together with CB1954 improves the level of cell ablation. We decided to combine CB1954 with MTZ prodrug. Our results suggested this strategy is an effective approach for improved photoreceptor ablation and bystander effects. We adjusted the CB1954 and MTZ concentrations for better ablation, phenotype and toxicity. We found that treating larvae with 75-100µM concentration of CB1954 combined with lower concentration of MTZ for 48 h treatment provided desired ablation levels and limited embryo viability issues. Accordingly, we retested the previously established 30 protective compounds using the new protocol (2mM MTZ combined with 75 µM CB1954). Each compound was tested at six different concentrations to maximize our chances of observing protective effects. However, we noticed that the majority of existing neuroprotectants failed to promote rod cell survival in our model due to significantly increased level of photoreceptor ablation. We identified one previously implicated neuroprotectant (XL880), which has the potential to serve as a positive control for the large-scale screen (figure below).



Representative result from 'known' neuroprotective screen. Graphs/heat map produced via R-based plate parsing and extraction algorithm of ARQiv data. Data were collected from transgenic zebrafish (Tg(rho:YFP-NTR)gmc500;roy) exposed to 2 mM MTZ+ 75 uM CB1954 for 48 h, from 5-7 dpf, in the presence of the compound XL880. All compounds are tested across a 2-fold dilution series ranging from 4 uM to 0.125 uM for the capacity to protect rod photoreceptors from cell death. ARQiv was used to quantify YFP reporter levels across conditions and data was downloaded and processed in real-time using our R-based code to produce near instant feedback regarding compound performance. We use three data plot types to highlight different aspects of the results: Left) Boxplots- to show dose-response relationships and indicate variability; Center) SSMD (strictly-standardized mean difference) scores- a relative measure of effect size (blue, yellow, orange, green, and, red dashed bounded ranges indicate very weak, weak, weakly moderate, moderate, fairly strong effects, respectively). Right) heat map of signal intensity in 96-well plate- to facilitate visual follow up of select conditions to assess toxicity/teratological effects, autofluorescence, and/or confirmation of desired effect.

We also failed to detect significant additive effects of CB1954 and MTZ regarding rod photoreceptor ablation (see graphs, below). Furthermore, ARQiv-based *in vivo* HTS for neuroprotectants and/or regeneration-promoting compounds using MTZ-induced ablation alone (a model of retinitis pigmentosa) is already being performed in our laboratory. Therefore, in order to avoid redundancy, we have elected to perform a visual phenotype screen for compounds that ameliorate CB1954-induced ocular edema.



Results from treatment with different concentrations of MTZ; CB1954; and MTZ + CB1954 together. Titrations of MTZ (graph at top left) or CB1954 alone (graph at top right) were performed to identify a concentration range inducing ~50% of rod photoreceptor loss. Assuming the effects of CB1954 and MTZ are additive, combining the two prodrugs should result in a near 100% loss of rod cells. However, the data show only minimal increases in rod cell loss when larvae were treated with both prodrugs (bottom graph). Therefore, any assay using MTZ to augment rod cell loss would likely produce duplicitous results with ongoing screens using MTZ to induce a retinitis pigmentosa-like disease models.

4) Visual phenotypic screen of ocular edema: To more directly assess the phenotype of interest (i.e., ocular edema), we decided to conduct a visual phenotypic screen. The ocular edema phenotype induced by CB9154 alone is highly robust (95% of treated larvae show phenotype, see table above).

Changes that had a significant impact on expenditures

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

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

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**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications.

  Nothing to report

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

  Nothing to report

  Other publications, conference papers and presentations.

  See above

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

  N/A

- **Technologies or techniques**

  N/A

- **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:	Dr. Jeff Mumm
Project Role:	PI
Researcher Identification (JHU)	174706
Nearest person month worked:	2 (10% calendar year)
Contribution to Project:	Dr. Mumm supervises the efforts of Dr. Ergolu in his lab and coordinates efforts with all participating labs.
Name:	Dr. Arife Unal Eroglu
Project Role:	Postdoctoral Fellow

Researcher Identification (JHU)	179225
Nearest person month worked:	6 (50% calendar year)
Contribution to Project:	Dr. Ergolu conducts the HTS assay in zebrafish.
Name:	Dr. Don Zack
Project Role:	PI
Researcher Identification (JHU)	00002707
Nearest person month worked:	1 (5% calendar year)
Contribution to Project:	Dr. Zack supervises the project components in his lab and coordinates efforts with participating labs.
Name:	Dr. Justin Hanes
Project Role:	PI
Researcher Identification (JHU)	00022572
Nearest person month worked:	1 (5% calendar year)
Contribution to Project:	Dr. Hanes supervises the project components in his lab and coordinates efforts with participating labs.
Name:	Dr. Harry Quigley
Project Role:	PI
Researcher Identification (JHU)	00021169
Nearest person month worked:	1 (5% calendar year)
Contribution to Project:	Dr. Quigley supervises the project components in his lab and coordinates efforts with participating labs.

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

Active other support has changed since the last reporting period. Below updated support of all Co-PIs is detailed:

Dr. Mumm

Dates:	**9/01/2013 – 08/31/2018**
Title:	Genetic and chemical screens for factors regulating retinal regeneration
PI:	Mumm (30% effort)
ID number:	R01 EY022810
Sponsor:	NIH, National Eye Institute
Contact:	Lisa Neuhold, lneuhold@mail.nih.gov
Total direct cost:	$1,250,000
Goal:	ID genes & compounds promoting rod photoreceptor regeneration
Specific Aims:	1) Establish rod cell regeneration deficient mutant zebrafish lines, 2) Identify compounds which alter the kinetics of, and/or promote, rod cell regeneration.
Overlap:	None
Dates:	**04/01/2014 – 03/31/2016**
Title:	Discovering compounds promoting rod photoreceptor survival
PI:	Mumm (10% effort)

ID number:	NA
Sponsor:	Foundation Fighting Blindness/Wynn Gund TRAP award
Contact:	Stephen M. Rose, Ph.D., Chief Research Officer, srose@fightblindness.org
Total direct cost:	$450,000
Goal:	ID compounds promoting rod photoreceptor neuroprotection
Specific Aims:	1) Promote photoreceptor survival in retinitis pigmentosa vertebrate models, 2) Promote photoreceptor regeneration in retinitis pigmentosa vertebrate models.
Overlap:	None
Dates:	**08/01/14 - 07/31/15 (in no cost extension)**
Title:	Robotic whole organism HTS platform for drug discovery and development
PI:	Mumm (5% effort)
ID number:	R41 PR000945
Sponsor:	NIH, National Center for Advancing Translational Science
Contact:	Christine Colvis, ccolvis@mail.nih.gov
Total direct cost:	$208,000
Goal:	Develop commercializable HTS in vivo drug discovery system based on ARQiv platform
Specific aims:	Validate two robotics-integrated ARQiv prototypes by achieving three milestones designed to test specific strengths the ARQiv platform brings to whole-organism screening.
Overlap:	None
Dates:	**10/15/14 - 09/30/17**
Title:	Discovery of FDA-approved drugs that promote retinal cell survival or regeneration
PI:	Mumm (10% effort)
ID number:	MR 130301 / GRANT11576494 / PD52904
Sponsor:	Department of Defense
Contact:	Marc L. Mitchell, marc.l.mitchell.ctr@mail.mil
Total direct cost:	$617,000
Goal:	Identify drugs that protect and/or regenerate ocular tissues following traumatic ocular injury.
Specific aims:	1) Promote eye tissue/cell survival in fish & mouse models of traumatic ocular injury. 2) Promote eye tissue/cell regeneration in fish & mouse models of traumatic ocular injury. 3) Optimize & validate regenerative leads in mammalian ocular injury models.
Overlap:	None
Dates:	**01/01/15 - 12/31/16**
Title:	Optimized Human iPS Cell-Derived Mini-Retina System for Improved Degenerative Disease Modeling, Biomarker Discovery and HTS Drug Development
PI:	Canto-Soler
Role:	Co-Investigator (20% effort)
ID number:	N/A
Sponsor:	Falk Medical Research Trust – 2015 Catalyst Research Program
Contact:	Lauren Macdonald, lauren.k.macdonald@ustrust.com
Total direct cost:	$500,000
Goal:	To develop inducible models of degenerative photoreceptor disease in human

	iPSC-derived eyecups.
Specific aims:	1) To develop the first human iPSC-derived retinal model consisting of a fully mature neural retina and associated RPE, and 2) To establish an inducible degenerative disease modeling system suited for high-throughput screening (HTS) drug discovery using our hiPSC-derived mini-retinas..
Overlap:	None
Dates:	**07/01/15 - 06/30/17**
Title:	Novel drug discovery platform for identifying choroideremia therapeutics
PI:	Mumm (20% effort)
ID number:	N/A
Sponsor:	Choroideremia Research Foundation
Contact:	Christopher Moen, chrismoen@curechm.org
Total direct cost:	$300,000
Goal:	Test previously identified neuroprotectants in a zebrafish model of choroideremia.
Specific aims:	1) Identify cytoprotective / regenerative drugs in the chmru848 zebrafish model of choroideremia. 2) Create an improved HTS-ready CHM model by inserting human rep2 at the zebrafish rep1 locus.
Overlap:	None

Dr. Zack

R43EY023495 (Zack) 05/01/14-04/30/15 (NCE to 04/30/16) 0.6 cal month
NIH/NEI
"Sustained-Release of a Novel Neuroprotective Agent for Improved Glaucoma Therapy"
Goal: to work with a small drug delivery biotechnology company, GrayBug, to optimize the use of slow release nanotechnology-based particles for the neuroprotective treatment of glaucoma and other optic nerve neurodegenerations.

P30EY001765 (Zack) 07/01/14-06/30/19 0.12 cal month
NIH/NEI $500,000
"Core Grant Visual Research"
Goal: to support five service modules (imaging, animal models, bioinformatics, biostatistics, and machine shop) that in turn are designed to support NIH supported vision research projects at Johns Hopkins.

T32EY07143 (Zack) 08/01/15-02/29/20 0.12 cal month
NIH/NEI $245,398 no salary support
"Visual Neuroscience Training Grant"
Goal: to support the training of pre- and post-doctoral trainees in visual neuroscience research.

NA (Zack) 05/01/14-04/30/16 0.36 cal month
KKESH $75,000
"Serum Biomarkers for Retinitis Pigmentosa"
Goal: to use RNA-seq to define molecular biomarkers for retinitis pigmentosa patients.

RFA-MD-14-1 (Mao) 07/01/14-06/30/17 1.5 cal month
Maryland Stem Cell Res. Fund $173, 913
"Nanofiber Matrix to Enhance Retinal Ganglion Cell Generation"
Goal: to develop nanofibers to promote the differentiation and directionality of axons from human stem cell-derived retinal ganglion cells.
Role: Co-Investigator

NA (Mumm) 07/01/14-06/30/16 0.6 cal month
Foundation Fighting Blindness $271,801
"Novel Drug Discovery Platform for Identifying Compounds Promoting Rod Photoreceptor Survival"
Goal: to identify drugs that promote rod photoreceptor survival in fish and mouse models of retinitis pigmentosa.
Role: Co-Investigator

R01EY024580 (Qian) 09/01/14-08/31/19 0.27 cal month
NIH/NEI $250,000
"Differential Regulatory Networks in Disease: Application to Macular Degeneration"
We propose several computational algorithms to construct regulatory networks and will apply the approaches to age-related macular degeneration.
Role: Co-Investigator

Macular Degeneration Research (Zack) 03/31/15-03/30/17 0.6 cal month
The Medical Foundation (Thome Found.) $250,000
"Development of a Small Molecule only Protocol for the Directed Differentiation of RPE from hPSC for the Treatment of AMD"
Goal: to use high-content and high throughput screening technologies to identify small molecules that promote retinal pigmented epithelial cell differentiation.

R01EY024249 (Berlinicke) 12/01/14-11/30/19 0.6 cal month
NIH/NEI $250,000
"A High Content Screening Approach for the Retinal Degenerative Diseases"
The goal of this proposal is to utilize a high content screening approach to identify photoreceptor neuroprotective compounds and compounds that promote retinal cell survival and function.
Role: Co-Investigator

MR130166 (Welsbie) 09/01/14-08/31/17 0.24 cal month
DOD/CDMRP $202,528
"Targeting Dual Leucine Zipper Kinase as a Therapeutic Strategy for Traumatic Optic Neruropathy and Brain Injury"
Goal: to test the effectiveness of DLK inhibition in promoting retinal ganglion cell survival in a model of optic nerve traumatic injury.
Role: Co-Investigator

MR130301 (Mumm) 10/01/14-09/30/17 0.6 cal month
DOD/CDMRP $192,251
"Discovery of FDA-Approved Drugs that Promote Retinal Cell Survival or Regeneration"
Goal: to use the zebrafish model to screen for candidate, already FDA approved drugs that promote photoreceptor survival and/or regeneration following ocular trauma, and to then test the identified "hits" in mouse models of retinal degeneration.
Role: Co-Investigator

MR141331 (Benowitz) 10/01/14-09/30/17 0.96 cal month
DOD/CDMRP $87,145 (subcontract)
"Molecular Control of Optic Nerve Regeneration"
Goal: to use gene expression profiling, and gain-of-function and loss-of-function studies, to identify to identify the pathways involved in RGC injury, cell death, and neuroprotection.
Role: Co-Investigator

Dr. Hanes

U19AI113127 (Hendrix - PD/Hanes - Project 2) 07/01/14 – 06/30/19 1.2 calendar
NIH/NIAID $786,865
Development of Rectal Enema As Microbicide (DREAM)
Project 2 – Pre-Clinical Progressive Selection of a Tenofovir Analog Enema
The goal is to develop novel enema vehicles and drug/nanotechnology combinations for rectal microbicide delivery.

R01HL105847 (Rowe/Hanes - sub) 02/15/14 – 01/31/16 0.6 calendar
NIH/NHLBI/University of Alabama $36,445
Molecular Pathogenesis and Phenotype of Acquired CFTR dysfunction in COPD
The Rheology Core will analyze the effects of COPD stimuli, cigarette smoke, and modulators of CFTR dependent pathways on the properties of airway mucus. Characterize mucus microstructure and micro- to bulk-rheology a key component in mucociliary clearance.

R24MH106083 (Wong) 09/26/14 – 06/30/17 0.24 calendar
NIH/NIMH $311,658
Imaging In Vivo Neurotransmitter Modulation of Brain Network Activity in Realtime
The goal of this project is to develop a transformative brain imaging technique which will allow minimally invasive/non-invasive imaging of neuronal depolarization and related neurotransmitter release ultimately in the living human brain.

R03CA182679 (Resar) 07/01/14 – 06/30/16 0.36 calendar
NIH/NCI $43,500
Developing Nanotechnology to Target HMGA1 in Pancreatic Cancer
The major goal of this project is to develop nanoparticles with short hairpin RNA as a potential therapy in pancreatic cancer.

W81XWH-14-1-0239 (Lee) 08/01/14 – 07/31/16 0.24 calendar
DOD/CDMRP $180,000
Role of TRAIL signaling through the development of carcinogen-induced colorectal cancer
The goal of this project is to discover roles of TRAIL signaling across the different stages of colorectal cancer (CAC) development induced by chemical carcinogens.

W81XWH-14-1-0407 (Mumm) 10/01/14 – 09/30/17 0.6 calendar
DOD/CDMRP $311,448
Discovery of FDA-Approved Drugs that Promote Retinal Cell Survival or Regeneration
The goal of this project is to use a novel drug discovery approach to identify drugs that promote improved survival or repair of eye cells and tissues.

KKESHJHU/03-13 (Ensign) 04/24/14 – 04/30/18 0.6 calendar
KKESH $91,976
Preparation/Characterization of Bioabsorbable, antibiotic-eluting sutures
Our goal is to develop biodegradable, polymeric sutures of the appropriate size and strength for ophthalmic surgery that locally release antibiotics to prevent post-surgical infections.

P30EY001765 09/01/14 – 06/30/19 0.12 calendar
The goal of this grant is to support five service modules (imaging, animal models, bioinformatics, biostatistics, and machine shop) that in turn are designed to support NIH supported vision research projects at Johns Hopkins.

HANES15G0 (Hanes) 04/01/15 – 03/31/16 0.6 calendar
Cystic Fibrosis Foundation $100,000
Hypotonic Delivery of Mucus Penetrating Nanoparticles for CF
The goal of this project is to enhance efficacy of inhaled gene therapy of cystic fibrosis, using mucus-penetrating DNA nanoparticles in conjunction with optimized delivery vehicle.

R01HL127413 (Hanes/Suk) 04/01/15 – 02/28/19 0.6 calendar
NIH/NHLBI $263,000
Biodegradable Mucus Penetrating DNA Nanoparticle for Gene Therapy of CF
The goal of this project is to test novel gene vectors in combination with a novel aqueous vehicle that we hypothesize will greatly enhance the effectiveness of gene transfer in the lungs.

R01EB020147 (Price/ Hanes) 05/01/15 – 02/28/19 0.6 calendar
NIH/NCI/NIBIB/University of Virginia $482,437
MRI-Guided miRNA Nanoparticle Delivery to Glioblastoma with Focused Ultrasound
The goal of this project is to utilize MR image-guided focused ultrasound, in conjunction with microbubbles, to open the blood-brain barrier in and around glioblastomas to both target the delivery of specially-engineered miRNA/anti-miRNA nanoparticles and determine the experimental therapeutic efficacy of this approach.

R01EB016121 (Hanes/Campochiaro) 07/01/15 – 06/30/20 0.6 calendar
NIH/NEI/NIBIB $289,767
Biomaterial Inhibitor of HIF-1 for Prolonged Anti-Angiogenesis in Eye
The goal of this project is to develop a new longer-lasting therapy for neovascular AMD that not only controls angiogenesis, but also causes unwanted new blood vessels to regress, potentially leading to improved efficacy and safety.

R01CA197111 (Price/Hanes) 06/01/15 – 05/31/2020 1.2 calendar
NIH $392,191
Immunotherapeutic Nanoparticle Delivery to Melanoma with MR-Guided Focused Ultrasound
The goal of this project is to utilize brain-penetrating DNA nanoparticles designed to stimulate anti-tumor immune responses, in conjunction with focused ultrasound and microbubbules, to treat melanoma metastases in the brain.

(Ensign) 06/01/15 – 05/31/19 0.48 calendar
Burroughs Wellcome Preterm Birth Initiative $150,000
The role of vaginal progesterone delivery in cervical remodeling and preterm birth

W81XWH-15-1-0301 (Lee) 09/01/15 – 08/31/18 0.6 calendar
DOD $316,971
Molecularly-targeted Therapy for Pancreatitis
The goal of this project is to explore the role TRAIL signaling in pancreatic fibrogenesis and anti-nociceptive effects in suppressing pain in chronic pancreatitis (CP).

Dr. Quigley

Title	Sponsor	Period
EY 024952 (P.I. Ian Pitha)	NEI-NIH (K08)	9/1/2014-8/31/2020

This **Clinician—Scientist Mentored Training program** will assist Dr Pitha in becoming an independent investigator in the area of sustained delivery of therapeutic agents to the eye using microparticles. The Wilmer Nanomedicine Center is the site of the project and Dr. Quigley is a primary mentor.

EY01765 (P.I. Don Zack) NEI-NIH (P30) 7/1/2014 -- 6/30/2019
Core Grant for Vision Research, Wilmer Institute: The Core Facilities Grant for the Wilmer Institute provides key support for microscopy/Imaging, computer, animal quarters, nanomedicine, biostatistics and bioinformatics. Dr. Quigley is Module Director for Imaging/Microscopy.

MR130301 (P.I. Jeff Mumm) CDMRP 10/01/2014 -- 9/30/2017
Discovery of FDA-Approved Drugs that Promote Retinal Cell Survival or Regeneration: This Department of Defense sponsored project investigates protective agents in traumatic injury to the eye, retina and optic nerve in Zebrafish and mouse models. Dr. Quigley is co-investigator.

(P.I. Harry Quigley) Wilmer KKESH (Saudi Arabia) Consortium 2014-2016
Improving drop and nanoparticle bioavailability with a tight junction modulating peptide: This program will develop pressure lowering drugs to treat glaucoma and investigate what the barriers to this new approach will be among patients with glaucoma in both the United States and Saudi Arabia. Dr. Quigley directs the team of investigators including Justin Hanes and Ian Pitha.

What other organizations were involved as partners?

N/A

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

Year 1	Year 2	Year 3
M1: 1º *in vivo* HTS fish (Mumm)	M3: 1º *in vivo* HTS fish (Mumm)	
	M2-1: 2º *in vitro* screen (Zack)	M4-1: 2º *in vitro* screen (Zack)
	M2-2: Optimization of leads (Hanes)	M4-2 Optimization of leads (Hanes)
	M2-3 3º *in vivo* screen (Quigley)	M4-3 3º *in vivo* screen (Quigley)

9. 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.

N/A