

NF- κ B Activation as a Biomarker of Light Injury Using a Transgenic Mouse Model

Ginger M. Pocock*^a, Adam Boretzky^b, Heuy-Ching Wang^c, Dallas Golden^c, Praveena Gupta, Gracie Vargas^b, Jeffrey W. Oliver^a, Massoud Motamedi^b

^aAir Force Research Laboratory, AFRL 711 HPW/RHDO, 4141 Petroleum Drive,
Fort Sam Houston, TX 78234;

^bThe University of Texas Medical Branch at Galveston, Department of Biomedical Engineering, 301
University Blvd., Galveston, TX 77555

^cUnited States Army of Institutes of Surgical Research, 3698 Chambers Pass, Fort Sam Houston, TX
78234

ABSTRACT

The spatial and temporal activation of NF- κ B (p65) was monitored in the retina of a transgenic mouse model (cis-NF- κ B-EGFP) *in vivo* after receiving varying grades of laser induced thermal injury in one eye. Baseline retinal images were collected from 26 mice and were reimaged up to five months later after receiving thermal injury using a Heidelberg Spectralis HRA confocal scanning laser ophthalmoscope (cSLO) with a spectral domain optical coherence tomographer (SD-OCT). Injured and control eyes were enucleated at discrete time points following laser exposure for cryosectioning to determine localization of NF- κ B dependent enhanced green fluorescent protein (EGFP) reporter gene expression in the retina using fluorescence microscopy. In addition, EGFP basal expression in brain and retinal tissue from the cis-NF- κ B-EGFP was characterized using two-photon imaging. Regions of the retina exposed to threshold and supra-threshold laser damage evaluated using fluorescence cSLO showed increased EGFP fluorescence localized to the exposed region for a duration that was dependent upon the degree of injury. Fluorescence microscopy of threshold damage revealed EGFP localized to the outer nuclear region and retinal pigment epithelial layer. Basal expression of EGFP imaged using two-photon microscopy was heterogeneously distributed throughout brain tissue and confined to the inner retina. Results validate the cis-NF- κ B-EGFP reporter mouse for *in vivo* studies of laser induced injury to the retina and possibly brain injury.

Keywords: retina, brain, laser, mouse, transgenic, NF- κ B, inflammation, laser damage, spectral domain optical coherence tomography, confocal scanning laser ophthalmoscope, fluorescence microscopy, two-photon imaging

1. INTRODUCTION

Nuclear factor-kappa B (NF- κ B) is a family of heterodimeric transcription factors that regulates stress responses in a variety of experimental stimuli by exerting a modulatory effect on apoptosis, cell survival, and inflammation. Experimental conditions that activate NF- κ B include reactive oxygen species, pathogens, injury, and ischemia. With regards to laser injury, the regime of laser damage to the retina determines the cellular response of tissue at the macro and molecular level. Glutamate released from thermally injured retinal neurons play a role in the destruction of photoreceptor cells¹ and has been shown to activate NF- κ B proteins in the human retina². Intense light stress to the retina in a mouse model activates NF- κ B in photoreceptor cells *in vivo* although its role in photoreceptor degeneration is not well understood³. In addition, NF- κ B is a redox sensitive transcription factor that is believed to be activated upon generation of ROS after exposure to laser fluences incapable of inducing thermal damage to cultured RPE cells³ and skin fibroblasts⁴. Characterization of NF- κ B spatial and temporal activation following laser injury may provide a useful method to assess the degree and type of injury as well as the efficacy of experimental treatments such as stem cell implantation^{5,6}. The activation of NF- κ B in photoreceptors and retinal pigment epithelium (RPE) following light induced stress has been studied *in vitro*^{1,2} and *in vivo*³ but limited to immunohistochemistry, western blots, and histology. These

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techniques do not allow the study of the NF- κ B activation dynamics over time within the same subject. In addition, a recently published study⁷ cautions on interpreting results from studies of the central nervous system using NF- κ B subunit proteins p65 and p50 antibodies for immunohistochemistry and western blot because of possible non-specificity. Researchers investigating molecular signaling pathways and their effect on the cellular response to neural disease and injury have been afforded the ability to monitor these pathways over several time points without the need for histology using transgenic reporter mouse models. Transgenic reporter mice have been used in studies of light/laser induced injury to the retina⁸, cornea⁹ and skin¹⁰. For the first time to our knowledge, we incorporate a transgenic mouse model (cis-NF- κ B-EGFP)¹¹ that express enhanced green fluorescent protein (EGFP) under the transcriptional control of NF- κ B into laser/light induced retinal damage studies. Monitoring NF- κ B activation could provide insight into understanding the acute or chronic inflammatory response of laser induced injury to the retina.

*ginger.m.pocock.civ@mail.mil; phone 1 210 539-8070; fax 1 210 539-3903;

2. METHODS

2.1 Subject Preparation and Laser-induced Retinal Injury

All transgenic mice used had been backcrossed >7 times to a C57BL/6 background. A cis-NF- κ B-EGFP breeding trio were provided by Christian Jobin (University of North Carolina at Chapel Hill). The cis-NF- κ B-EGFP mice were created using a gene-targeting approach to integrate a single copy of the NF- κ B reporter construct into a single locus of the HPRT gene¹¹. A total of 20 male cis-NF- κ B-EGFP (6-8 months old) and 6 retired breeders were used to monitor changes associated with laser exposure from 4 hours up to five months later.

Prior to retinal lesion placement and imaging, mice were anesthetized with isoflurane (1% - 2%). Once restrained, a topical ocular anesthetic (Proparacaine HCL) eye drops were administered and eyelids were retracted using sterilized retractors. Corneal hydration was maintained throughout imaging by applying 0.5% hypromellose lubricating eye drops (Alcon Laboratories, Ft. Worth, TX) every 2-3 minutes. An adjustable, heated platform was used to maintain body temperature and allow appropriate positioning of the eye for imaging and photocoagulation. A 3.2 mm fundus laser lens (Ocular Instruments) was used to visualize the fundus for lesion placement in the retina. A Coherent Ultima 2000 argon laser operating at 514 nm in conjunction with a Zeiss Model 30 SL-M slit lamp was used to create up to 10 lesion sites surrounding the optic nerve head in one eye per mouse. Power levels of 8.4 mW, 14.6 mW, and 28 mW were used to induce retinal photocoagulation at the sub-threshold, threshold, and supra-threshold level, respectively. Exposure time was set at 0.1 s for all exposures and a laser spot size of either 100 μ m, 200 μ m, or 500 μ m at the cornea.

The animals involved in this study were procured, maintained, and used in accordance with the Federal Animal Welfare Act, "Guide for the Care and Use of Laboratory Animals," prepared by the Institute of Laboratory Animal Resources National Research Council, and DoD Regulation 40-33 Secnavinst 3900.38C AFMAN 40-401(1) DARPAINST 18 USUHSINST 3203 "The Care and Use of Laboratory animals in DOD Programs." All experimental methods were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch. Brooks City-Base, TX has been fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC) since 1967.

2.2 Confocal Scanning Laser Ophthalmoscopy and Spectral Domain-OCT

The Spectralis HRA+OCT (Spectralis; Heidelberg Engineering, Heidelberg, Germany), which contains a confocal scanning laser ophthalmoscope (cSLO) with a spectral domain optical coherence tomographer (SD-OCT), was used to collect retinal cross sections and fundus images. The cSLO features one argon wavelength (488 nm) and two infrared diode lasers (790/820 nm) to collect fundus infrared (IR) reflectance, red free (RF) reflectance, fluorescein angiography (FA), and autofluorescence (AF). A 25 diopter lens was mounted on the front imaging end of the Spectralis to image mice. A small blanket was placed underneath the mouse to dampen breathing artifacts in retinal images. All fundus images were acquired in the high resolution mode (1536 x 1536 pixels) over a 30° x 30° or 20° x 20° (1024 x 1024 pixels) field of view (FOV). The Spectralis eye tracker was used to create images in which the movement artifact is removed or minimized. EGFP fluorescence emanating from the retina was recorded using the cSLO excitation wavelength of 488 nm. To improve the signal to noise ratio of the fluorescence signal, several images were recorded using the eye tracker and aligned to generate a mean image. In addition, reflectance and fluorescence images were collected with varying focus settings to confocally section the mouse retina to investigate the localization of fluorescence

signal. SD-OCT ($\lambda=870$ nm) B-scans were recorded during post-injury image sessions to assess lesion size relative to EGFP signal intensity. OCT images were collected using a line scan and/or raster scan configuration. Each B-scan consists of 1536, 1024, or 768 A-scans acquired at a scan rate of 40,000 A-scans per second to generate OCT cross-sectional images. Raster scans for volumetric representations and measurements consisted of 49 B-scans spaced ~ 3.8 μm apart covering a 548×183 μm area. Baseline retinal images were collected and mice were reimaged after receiving thermal injury for comparison. Subject's retinas were imaged immediately following laser exposure, 4 hours, 1-14 days, and up to 5 months.

2.3 Fluorescence Microscopy of Eye Cryosections

Transgenic were euthanized using cervical dislocation. For cryosectioning, both eyes were enucleated each mouse and were immediately embedded in Tissue-TEK optimal cutting temperature (O.C.T.) compound (Sakura Finetek, Torrance, CA), frozen in liquid nitrogen and stored at -80 $^{\circ}\text{C}$. Microtome sections (6 μm) were cut through the entire retina, along the vertical meridian, on a cryostat at -20 $^{\circ}\text{C}$ and thaw-mounted onto Superfrost Plus glass slides (Fisher Scientific, Pittsburg, PA). Sections were then air-dried, fixed in cold acetone or 4% PFA for 10 minutes, stained with an anti-fade reagent with DAPI (Invitrogen Molecular Probes, Carlsbad, CA), and stored at -80 $^{\circ}\text{C}$ until fluorescence microscopy was performed (filter $\lambda = 350$ and 488 nm; model ; Olympus, Tokyo, Japan).

2.4 Confocal Reflectance, Two-photon Microscopy, and Spectral Imaging

A custom built microscope based on a Zeiss Confocal LSM 410 microscope was used to perform confocal reflectance microscopy and two-photon microscopy to examine localization of EGFP signal within the retina and brain tissue of one transgenic and wild type mouse (non-laser exposed). Excitation source was a Ti:Sapphire laser operating at 800 nm. An emission filter specifically for the EGFP range (525 HQ/50) with a $40\times$, 1.2 N.A. objective was used. Reflected light was collected using a cooled PMT (R6060, Hamamatsu, Japan) along the descanned focus path. Images were acquired up to a depth of 160 μm (2 μm steps, 16 frame average) over a 320×320 μm field of view in both brain and retinal tissue. Additionally, spectral imaging of the brain and retina revealed a distinctive fluorescence peak corresponding with EGFP as well as background autofluorescence. Three dimensional image stacks were created using Image J software (1.43, National Institutes of Health).

3. RESULTS

3.1 Basal Expression of EGFP in the Retina and Brain

Baseline fundus photos collected using cSLO with an excitation wavelength of 488 nm showed basal expression of NF-kB associated with translocation to the nucleus. Fluorescence signal emanating from retinal arterials and veins was a distinguishing characteristic observed in all cis-NF-kB-EGFP mouse retinas (Figure 1). Expression patterns and fluorescence strength associated with NF-kB (p65) was unique to each mouse. Natural biological variability in addition to differences in depth of focus within the retina could account for some these differences. The source of the fluorescence could possibly be associated with either basal NF-kB expression of the blood vessel endothelial cells, pericytes, or basal lamina. In addition, a population blood cells also appear fluorescent and their circulation throughout the retina can be seen in vivo using cSLO. They could be mononuclear blood cells since this cell type is EGFP-positive in the cis-NF-kB-EGFP mouse. Two-photon and reflectance imaging reveal that most of the EGFP signal seen in the retina is mainly co-localized within the inner retinal layers. Two-photon imaging of brain tissue reveals a more heterogeneous distribution of EGFP throughout (Figure 3).

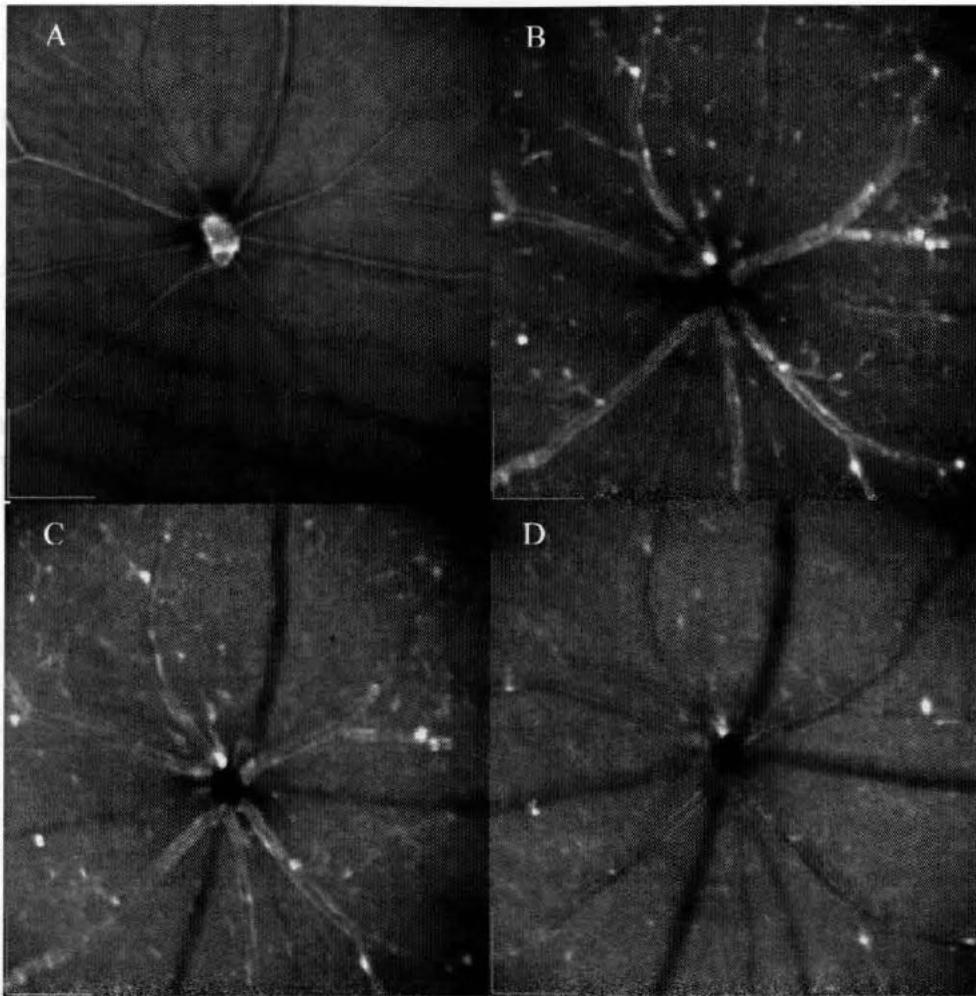


Figure 1: Fluorescence and infrared SLO images of a single cis-NF-kB-EGFP mouse retina (A) Infrared image with focus setting at -5 Diopters (30 FOV). (B) Fluorescence image with focus held at the same setting to show EGFP signal association with anatomical structures (30 FOV). (C) Fluorescence image with focus setting at zero Diopters (D) Fluorescence image with focus setting at +5 Diopters.

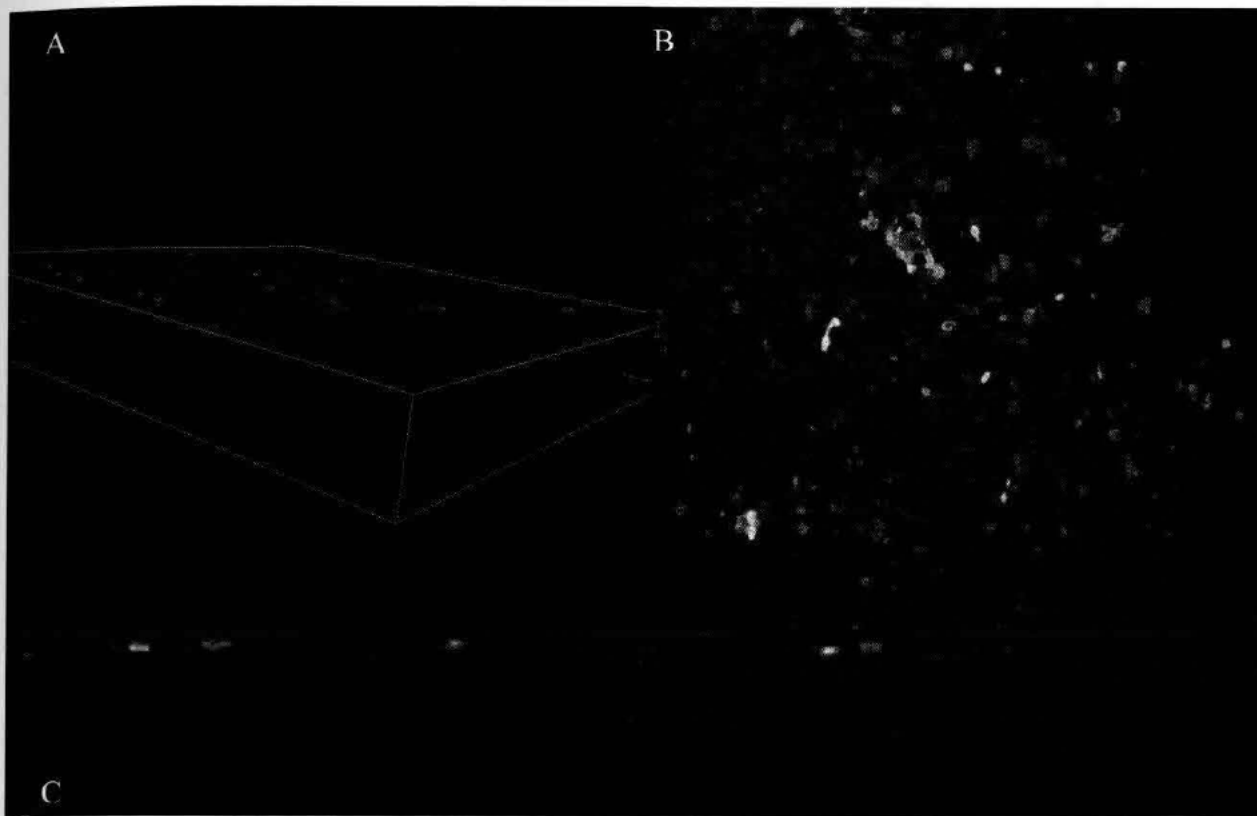


Figure 2: Two-photon imaging of cis-NF-kB-EGFP mouse retina *ex-vivo*. (A) Three dimensional reconstruction showing hyperfluorescent regions associated with EGFP signal. (B) Anterior two-dimensional view of the same retina shown in (A). (C) Cross-sectional slice of three dimensional volume demonstrating hyperfluorescent regions in the inner retina.



Figure 3: Two-photon imaging of cis-NF-kB-EGFP mouse brain *ex-vivo*. Cross-sectional slice of three dimensional volume demonstrating hyperfluorescent regions in the brain.

3.2 In vivo visualization of NF-kB activation in laser injured retina

Increased EGFP signal correlated with the grade of laser damage although this was not experimentally quantified. Regions of the retina exposed to threshold and supra-threshold laser damage evaluated using fluorescence cSLO showed increased EGFP fluorescence localized to the exposed region. No change or increase in EGFP was observed for sub-threshold damage. It may be that the increases in production of EGFP associated with NF-kB activation was too small to be observed within the resolution limits of the cSLO. Another possibility is that an increase in NF-kB translocation to the nucleus does not occur with exposure to sub-threshold laser stimulus. Time point at which increases in fluorescence was observed was approximately 6 hours and seemed to be dependent upon grade of damage. Overall, EGFP increases

indicative of NF-kB translocation to the nucleus occurs sooner (2 hours – 1 day) for supra-threshold damage in comparison to threshold damage. EGFP expression associated with one type of grade of laser injury was variable between mice and also variable within the same mouse. For example, a mouse receiving multiple threshold lesions within one retina could express EGFP within a day for some lesions while other lesions expressed EGFP within two days. Overall, the EGFP signal was greatest in the region anterior to the RPE since the EGFP signal appeared diminished at the point at which the natural autofluorescence of the retina became visible.

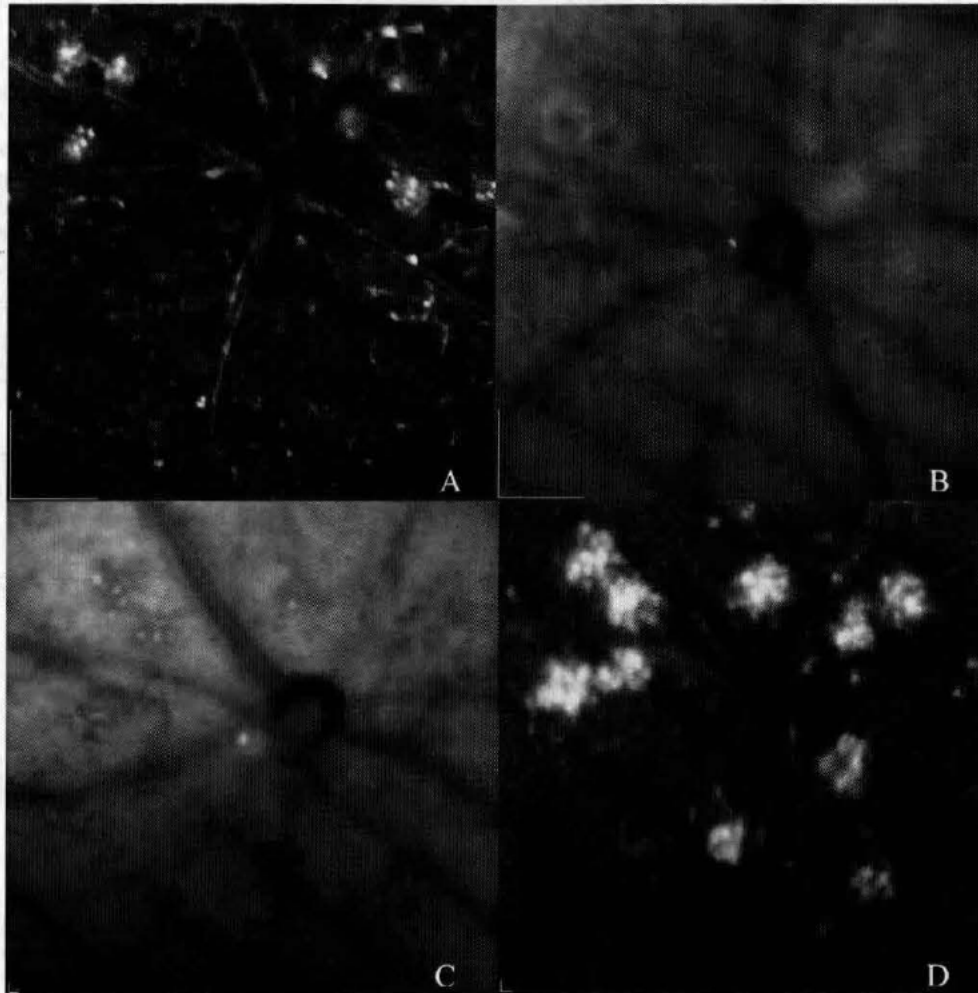


Figure 4: (A)Fluorescence and (B)infrared SLO images of a single cis-NF-kB-EGFP mouse retina one week post threshold laser exposure (30 FOV). (C) Infrared and (D) fluorescence SLO images of a single cis-NF-kB-EGFP mouse retina 48 hours post supra-threshold laser exposure (30 FOV).

3.3 NF-kB activity co-localization within the retina following laser exposure

Light microscopy of retinal cross-sections revealed a damage zone localized to the retinal pigment epithelium (RPE) and spreading into the neural retina. Fluorescence microscopy revealed increased EGFP production associated with NF-kB promoter activity in the choroid, RPE, and outer nuclear layer. A DAPI stained cross-sectional view of a cis-NF-kB-EGFP mouse receiving a threshold lesion is shown in Figure 5. OCT cross-sectional images reveal areas of hyporeflection extending from the RPE into the neural retina at the 24 hour time point and later (Figure 6).

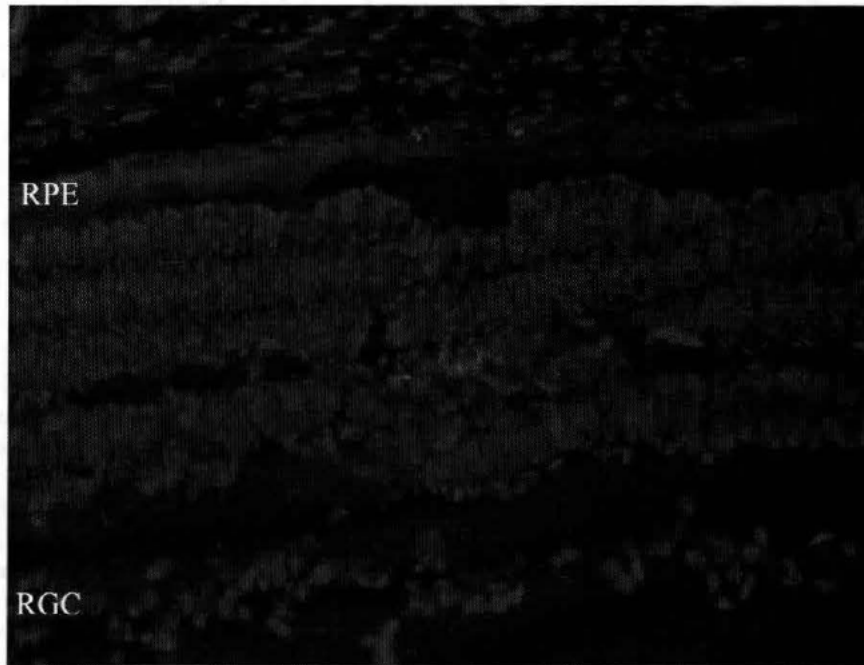


Figure 5: DAPI stained retina cross-section of a threshold laser exposure in the cis-NF-kB-EGPF mouse. Increased EGFP expression can be seen in the RPE and outer nuclear layer.

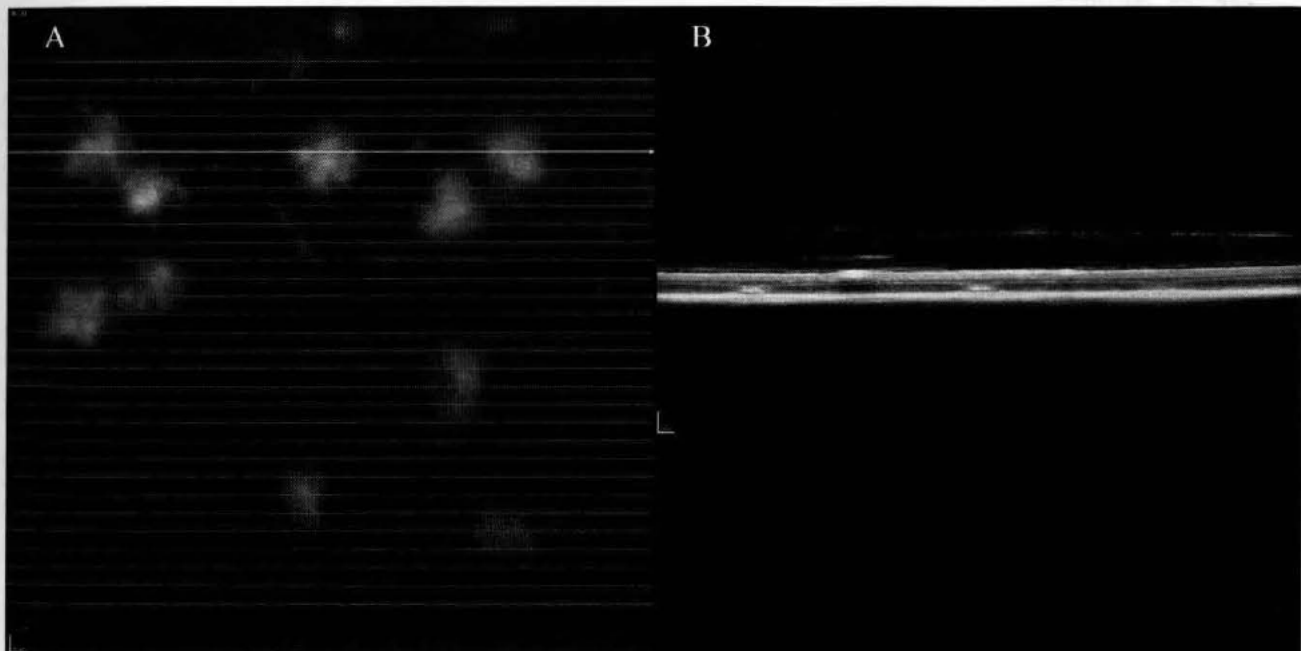


Figure 6: (A) Fluorescence (30° FOV) and (B) OCT image of mouse retina one week post supra-threshold laser exposure.

4. CONCLUSION

Basal levels of EGFP expression was observed in the brain and retinal tissues using various microscopy methods. Regions of the retina exposed to threshold and supra-threshold laser damage evaluated using fluorescence cSLO showed increased EGFP fluorescence localized to the exposed region for a duration that was dependent upon the degree of injury. EGFP expression persisted for up to 3 months in one subject receiving supra-threshold laser damage to the retina

demonstrating a possible prolonged inflammatory response. Fluorescence microscopy of threshold damage revealed EGFP localized to the retina outer nuclear region and retinal pigment epithelial layer. Future work will further characterize express levels of EGFP with NF-kB binding activity using electrophoretic mobility shift assay (EMSA) as well as elucidate the source of NF-kB within the retinal vasculature. Overall, the cis-NF-kB-EGFP reporter mouse could be used for in vivo studies of NF-kB (p65) activation in relation to injury and healing in the retina. Future research will seek to understand the NF-kB signaling pathways and the variability between expression patterns of NF-kB regulated genes based on regimes of laser damage. Inhibition of these inflammatory pathways may represent a feasible treatment strategy for laser retinal injury. In addition, future studies of retinal inflammation associated with stem cell implantation into the retina could be possible using this mouse model. Basal EGFP expression can be detected in the brain using two-photon imaging possibly enabling in vivo studies of brain inflammation following injury¹².

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