Progress Report for MMPC Pilot & Feasibility Program 5U24DK07616902
Monitoring Diabetic Retinopathy Progression in Mice Using Hyperspectral Imaging
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1. Changes occurred in extending the project deadline to October 2009. The initial period of the grant was from November 1, 2007 to October 31, 2008. A significant portion of the funding was dedicated to the graduate student, Umesh Agarwal. Administratively, it was simpler to initiate Mr. Agarwal at the beginning of the spring 2008 semester resulting in a University Tuition benefit. Meaning the work on the grant started January 15, 2008. In addition the breeding of the mice at Jackson Labs took longer than expected. Thus we requested and received a no cost extension though October 2009. The project goal is to monitor the vascular changes associated with diabetic retinopathy. Our current time line is for continuing data collection through April 2008. Although there are not enough funds to support Umesh past the Fall 2008 semester I have another funding source that can supplement the project.
2. Once data collection started we found our initial optical configuration to be cumbersome. Coupling the liquid crystal tunable filter with the detector and illuminate the retina using a coaxial illuminator also known as a ring light results in simplifying the illumination of the retina and reducing the time it takes for colleting data.
3. There is no significant rebudgeting of funds during the extended budget period.
4. There will be no significant changes in the level of effort for key personnel for the extended budget period.
5. We do not expect any unobligated balance left over at the end of the extension.

A. Specific Aims

The specific aims have not changed from the competing application.

B. Studies and Results

Specific Aim 1: Develop and characterize a hyperspectral imaging system for visualizing vascular dysfunction in the retina of a mouse, in vivo and nonivsively.

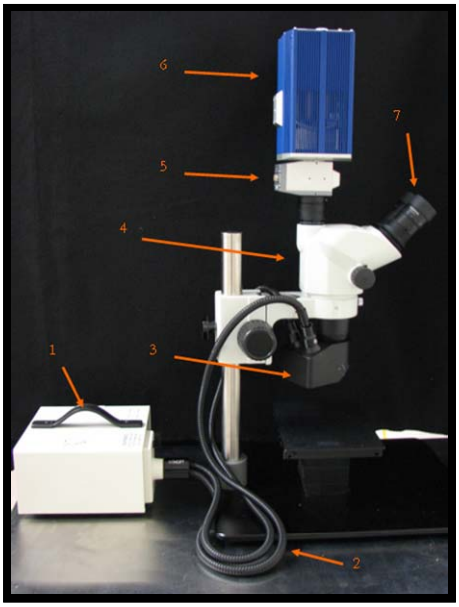

Figure 1 *The in vivo hyperspectral imaging microscope for visualizing vascular dysfunction in the retina of mice. Broadband light is carried from the light source (1) through the fiber optics (2) to the coaxial illuminator (3), from where it illuminates the mouse retina for imaging. The reflected light is then transmitted through the microscope (4) and the LCTF (5), for acquisition by the FPA (6). The eyepieces (7) are used for manual viewing as a backup to the computer software.*

For imaging diabetic retinopathy in a mouse model, the hyperspectral imaging microscope described was developed and characterized, figure 1. This includes a SZ61 Olympus microscope modified to include a focal plane array (FPA), a scientific grade charged coupled device, CCD, (Princeton Instruments, Trenton, NJ) and a liquid crystal tunable filter, LCTF, (Cambridge Research & Instrumentation, Boston, MA). A

ACE® light source (Auburn, NY) illuminator powers a 21-V, 150-W EKE halogen bulb providing stable broadband light to the retina of the mouse via a coaxial ring light.

The light source and coaxial illuminator focus light into the retina of the mouse. Reflected light is magnified through the microscope optics through the LCTF onto the FPA, where the spectroscopic images are digitized and streamed to for processing with specially developed software. The LCTF is a very versatile, electronically controlled, continuously tunable filter with a (150-ms) response time and a 20-mm aperture for rapid, vibration-free spectroscopic image production. Its spectral range is 400 – 720 nm with a bandpass of 6.46-nm at 500-nm wavelength and 16.03-nm at 700-nm wavelength. The FPA consists of a scientific grade CCD with a 1392x1040 imaging array with 6.25-um x 6.25-um pixel size, incorporating a Sony ICX – 285 silicon chip with Interline capabilities and anti-blooming technology and an electronic noiseless shutter. It also uses a 14-bit digitizer with a digitizing rate of 20 MHz and has a quantum efficiency of greater than 60% in the visible range. Image acquisition and LCTF tuning is managed by computer programs written in the laboratory and compiled by V^{++} (Princeton Instruments, Trenton, NJ). All computer programs, image acquisition, processing, and visualization for acquisition preparation are run on a high-end Dell Latitude D630 laptop computer (Austin, TX).

SYSTEM CHARACTERIZATION:

Tuning accuracy of the LCTF. Various aspects of the hyperspectral microscope were evaluated for operating procedures and image acquisition of mouse retinas. Various aspects of the HSI microscopy system were evaluated for operating procedures for image acquisition of mouse retinas. The visible LCTF (serial number VIS 51159) was calibrated for its tuning accuracy using a PerkinElmer Spectrometer (PerkinElmer, Wellesley, MA). The LCTF was placed in the collimated optical light path of the spectrometer and was tuned to specific wavelengths. The LCTF was tuned from 490 – 720 nm with a 10 nm increment and passband of the transmitted light intensity at each wavelength was measured. The data was then analyzed in Matlab to determine the tuning accuracy. Theoretically wavelength accuracy is taken to be the actual center wavelength, CW_{actual}, to be correct within the nominal, CW_{nml}.[8]

$$CW_{actual} = CW_{nml} \pm \left(\frac{Bandwidth_{nml}}{8} + 0.5nm \right) \qquad (1)$$

On average this LCTF was found to tune 0.57 nm above the expected wavelength, which is well within the manufacturer's specification of ±1.38 nm. The tuned wavelengths sent electronically to the LCTF electronics module were then plotted against the experimentally measured center wavelengths transmitted by the LCTF. The data was then linearly regressed with $R^2 = 0.99$ and the calibration equation,

$$CW_{actual} = 1.0027 \bullet CW_{expected} - 1.0644\ nm \qquad (2)$$

was incorporated into the V++ software to offset the error and increase the accuracy of the LCTF tuning of the desired center wavelength.

with an $R^2 = 0.99$ and $P_{val} < 0.05$, which is identical to previous studies.[24] Incorporating this calibration equation into the V^{++} program administrating the data acquisition helps to increase the LCTF tuning accuracy.

LCTF Bandwidth Characteristics. Full width at half-maximum (FWHM) is determined as the spectral separation between the two points where the filter's transmission is 50% of the peak value[8]. The bandwidth of the LCTF (Serial # VIS51159) is wavelength dependent and varies from 6.46 nm at

500 nm to 16.03 nm at 710 nm, with an average of 9.63 nm across the range (500-700 nm) of the LCTF. Though the bandwidth is wavelength dependent, increasing with wavelengths, and found to be sufficient to discriminate spectroscopically the peaks of HbO_2 and Hb. **Spatial Resolution.** The spatial resolution characteristics of the system were determined by using a contrast transfer function analysis, which evaluates the ability of an optical system to distinguish between evenly spaced rectangular bars of a resolution target.[26,27] As determined in previous work the percent contrast, *C*, is experimentally calculated, from imaging a standard 1951 USAF resolution target.[20,21,22,24] Utilization of the full chip and binning by 4 provides a 0.06-mm spatial resolution based upon the Rayleigh criterion when the microscope optics are set to a magnification of 4.5. **Data Acquisition Time.** The start and end times of data acquisition are automatically recorded by the acquisition software, however there are many factors which can have an influence on the data acquisition time. This same program tunes the LCTF, which has its own optical response time, depending on many factors such as ambient temperature. Typically, this tuning time is around 80-ms, which comes to 10.08 s for 126 center wavelengths. Once the LCTF is tuned to a center wavelength, the camera shutter opens and closes, exposing the FPA for data acquisition and streaming to the acquisition hard drive over approximately 2.31 s. The remaining time is taken in the binning and additional processing in the computer software.

Specific Aim 2: Monitor retinal vascular changes for 6 months in diabetic mice having a predisposition for developing diabetic retinopathy; using double-knock-out apoE-/- db/db mice.

The diabetic retinopathy mouse model was provided by Jackson Laboratory (Bar Harbor, ME), as described by previous work,[8] in a newly characterized murine model of nonproliferative diabetic retinopathy, which has been observed that hyperlipidemia accelerates structural vascular changes in diabetic retinas; diabetic retinopathy.[8] Briefly, to generate the apoE$^{-/-}$ *db/db* mice, apoE$^{-/-}$ mice are first back crossed 6 generation into mice heterozygous for diabetes spontaneous mutation (*Lepr^{db}*). Initially crossing male mice heterozygous for the diabetes spontaneous mutation (*Lepr^{db}*) in the leptin receptor gene on chromosome 4 (BKS.C*g-m$^{+/+}$ Lepr^{db}*, former name C57BLK/J-*m$^{+/+}$ Lepr^{db}*, type JAX GEMM TM Strain; spontaneous Mutation Congenic, stock no. 000642); with female mice homozygous for the *Apoe^{tm1Unc}* mutation in chromosome 7 (B6.129P2- *Apoe^{tm1Unc}, former name C57BL/6J- Apoe^{tm1Unc}*, Type JAX GEMM TM Strain; Targeted Mutation congenic, stock no. 002052) at 8 weeks. Heterozygous mice from different parents are crossed again at 8 weeks and mice homozygous for the *Apoe^{tm1Unc}* mutation and heterozygous for the *Lepr^{db}* mutation (apoE$^{-/-}$ *db/m*) are crossed with one another to breed the double-knock-out ApoE$^{-/-}$ *db/db* mice.

In mid September Jackson Labs delivered 5 female and 5 male Homo Apoe/ Wild db, 5 female and 3 male Wild Apoe/ Homo db, 5 female and 4 male Homo Apoe/ Homo db the double-knock-out expected diabetic retinopathy mouse model. All mice were 9 weeks upon arrival giving the mice one week to acclimate to their new environment. So the mice were first imaged when they were 10 weeks old. Unfortunately we didn't receive a full complement due to below average breeding populations; however, JAX performed a second round of breading and we are expecting the remaining and any supplemental mice to arrive within the week so that we will have 5 mice in each group. Each mouse was identified with a tattooing and notching their ears in order to monitor the mouse throughout the experiment; each mouse is selected randomly and imaged once every 2 weeks. At present we have imaged the original complement of mice each 2 times. We expect to monitor the mice over the next 6 months and hypothesize that the hyperspectral imaging microscope and chemometirc deconvolution

will visualize vascular changes while monitoring the development of diabetic retinopathy in the ApoE$^{-/-}$ *db/db* mice that will not be present in the control groups consisting of *Apoe*^{tm1Unc} and *Lepr*^{db} mice. Collecting spectroscopic retinal image data with the visible hyperspectral imaging microscope and deconvoluting the measured spectrum at each pixel detector produces an image visualizing the relative percentage of oxyhemoglobin. Red pixels indicate a high percentage of oxyhemoglobin while yellow, green to blue pixels indicate lower levels of oxyhemoglobin.

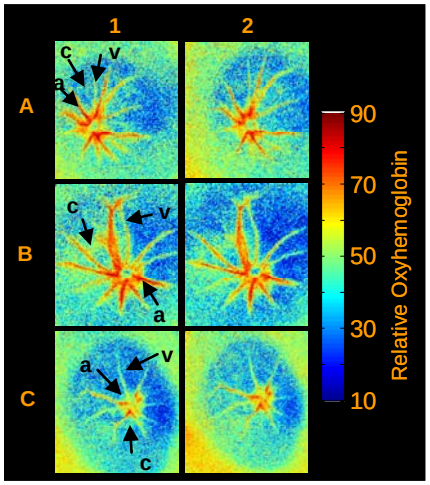

Figure 2 *A montage of images visualizing the relative percentage of oxyhemoglobin. Each row represents the retina of a mouse from differing genetic grouping, (A) diabetic retinopathy in the ApoE$^{-/-}$ db/db mouse, (B) the control Lepr^{db}, and (C) the Apoe^{tm1Unc} mouse. The columns indicate imaging session 1 and 2. For these mice imaging session were approximately 12 days apart. Finally sampling 20 pixels differing structures indicated the possibility of an (a) arterial, (v) venous and (c) capillary structures.*

Currently we have imaged all mice in each genetic group 2 times and plan to continue monitoring the vasculature of the retina. Images will be added to the montage and the same anatomical location will be monitored. In addition we will monitor any anatomical vascular changes that we hypothesize will develop in the double knock out mice, group A, later in the study.

Mouse A	Session 1	Session 2
a	79.12±5.69	79.563±5.32
v	50.55±8.31	56.21±7.80
c	57.92±7.91	57.82±10.76
Mouse B		
a	80.50±6.25	82.88±3.38
v	48.24±5.10	50.49±6.76
c	52±.23±10.57	54.04±8.76
Mouse C		
a	73.21±6.29	73.19±5.39
v	46.03±9.56	46.64±8.13
c	50.71±5.33	54.82±5.30

Table 1 *Presents the average of the sampled pixels from figure 2 in areas a,c, and v, which are presented as mean ± the standard deviation.*

We are pleased to see the instrument has the ability to image the vasculature of mice retinas repeatedly and we look forward to monitoring vascular changes over the next 5 to 6 months.

C. Significance

As proposed in specific aim 1, we successfully developed and characterized a hyperspectral imaging system and are in the process of visualizing, in vivo and noninvasively, the vasculature of mice retinas. We are in the process of monitoring the retinal vasculature changes for the next 5 to 6 months. At the end of the period we look forward to having enough empirical evidence to either support or refute our hypothesize that the hyperspectral imaging microscope and chemometirc deconvolution will visualize vascular changes while monitoring the development of diabetic retinopathy in the apoE$^{-/-}$ *db/db* mice that will not be present in the control groups consisting of *Apoe*^{tm1Unc} and *Lepr*^{db} mice.

Plans

The grant funding was available in the middle of our semester and in order for the graduate student to receive a tuition benefit we started the work on this a few months behind. In addition the breeding of the mice took a bit longer than first expected and therefore we requested and were granted a no cost extension. Since we are showing positive progress we are interested in continuing to monitor the mice for the next 5 to 6 months and funding for the graduate student to continue monitoring will come from other funding sources. In addition during the breeding process we were made aware of certain characteristics that may develop in these special genetic mice and are interested in collecting the preliminary data toward that end. We are interested in examining the foot pad skin of the mice to see if there are any correlation with PNS damage and any skin lesions. Our protocol would be to

1. Imaging the oxygenation in the paw skin and/or ears of the mice ultimately we would image try to image the sciatic nerve but we may not be able to see through the muscle and our special resolution may be too poor.
2. At death, we will collect footpad skin and sciatic nerve, fix and ship to Nigel Calcutt at UCSD. Dr. Calcutt is willing to work with us he is willing to show us the protocols for collecting and fixing the footpad skin and sciatic nerve and he has a video for experimental details, which we can view.
3. Skin: epidermal C fibers, dermal fibers, dermal blood vessels sciatic: endoneurial blood vessels, myelinated fiber axonal diameter and number will be measured by Dr. Calcutt's group.
4. Later we will correlate the percentage of oxyhemoglobin measured in vivo with the above histology.

REFERENCES FOR SKIN AND NERVE METHODS AND PURPOSE:

- Beiswenger KK, Calcutt NA, Mizisin AP.Dissociation of thermal hypoalgesia and epidermal denervation in streptozotocin-diabetic mice. Neurosci Lett. 2008 Sep 19;442(3):267-72.
- Beiswenger KK, Calcutt NA, Mizisin AP.Epidermal nerve fiber quantification in the assessment of diabetic neuropathy. Acta Histochem. 2008;110(5):351-62.
- Calcutt NA, Allendoerfer KL, Mizisin AP, Middlemas A, Freshwater JD, Burgers M, Ranciato R, Delcroix JD, Taylor FR, Shapiro R, Strauch K, Dudek H, Engber TM, Galdes A, Rubin LL, Tomlinson DR.Therapeutic efficacy of sonic hedgehog protein in experimental diabetic neuropathy. J Clin Invest. 2003 Feb;111(4):507-14

E. Publications supported by 5U24DK07616902

- Agarwal UC, Ufret-Vincenty R, Tang L, Hawkins D. Burgess S., Behbehani K, Wehner EF, and Zuzak KJ, Characterization of a Non-Invasive Hyperspectral Microscopy Imaging System for Monitoring In-Vivo Vasculature in Mouse Retinas. In the submission process to Analytical Chemistry.
- As soon as we have collected and analyzed the hyperspectral data we plan to publish our results on hyperspectral imaging visualizing the vascular development of diabetic retinopathy in the double-knock-out apoE$^{-/-}$ *db/db* mouse.
- Finally we expect a Master of Science Thesis, "Characterization of a Non-Invasive Hyperspectral Microscopy Imaging System and visualizing the vascular development of diabetic retinopathy in the double-knock-out apoE$^{-/-}$ *db/db* mouse." to be written and defended by Mr. Agarwal

F. Project-Generated Resources

None.