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IMAGING OXYGEN PRESSURE IN THE RODENT RETINA BY PHOSPHORESCENCE LIFETIME

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1. INTRODUCTION

Many diseases of the eye, especially those causing inner retinal neovascularization (diabetic retinopathy, retinopathy of prematurity, sickle cell disease, etc.) have regional hypoxia as either a primary causative or early contributory factor. This is particularly true for diabetic retinopathy, the leading cause of blindness for individuals between 20 and 74 years of age. In diabetes, multiple structures of the eye are pathologically affected by vascular changes with resultant plasma leakage and tissue disruption[1]. Currently available data suggest that the pathological growth of new vessels results from developing regions of hypoxia in the retina. These new blood vessels in the inner retina are defective and exudate from the vessels and/or bleeding due to vessel rupture are the major cause of the decreased vision/blindness associated with diabetes. Using microelectrodes, Linsenmeier and coworkers[2, 3], reported that in diabetic cats the oxygen pressures in the inner half of the retina are about one half those of normal cats, and Berkowitz et al.[4], reported decreased oxygen response in the retina of galactosemic rats. Oxygen electrodes can very effectively identify global oxygen deficiency but are invasive. Also, since they make point measurements, they are not very effective for measuring focal hypoxia induced through microvessel failure. Retinal pathology in diabeties, for example, is thought to begin with pathological changes in the intraretinal microvessels (nonproliferative diabetic retinopathy) to growth of new vessels in the extraretinal space[5]. Similar pathogenic mechanisms are thought to occur in other diseases characterized by inner retinal neovascularization. Hypoxia resulting from vascular insufficiency could be responsible for production of high levels of vascular endothelial growth factor (VEGF). Vitreous samples in humans with retinopathy have contained high levels of VEGF[5]. High levels of VEGF have also been measured in animal models of ocular neovascular disease [6, 7, 8].

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In the present paper, we report continuing progress[9] in developing a phosphorescence lifetime imaging system capable of obtaining high resolution images of the oxygen distribution in the retina of the rodent eye. These oxygen measurements would make possible critical evaluation of the role of microcirculatory failure in vision loss using a variety of rodent models for human disease.

2. METHODS

2.1. Phosphorescence Lifetime Imaging System

The phosphorescence lifetime imaging system previously used to image oxygen in the retina of the cat eye[10, 11] was modified to allow imaging of phosphorescence lifetimes in the much smaller mouse eye following the lead of Shonat and Kight[12, 13]. A frequency domain approach [14, 15] was used in which the excitation light source was modulated in a square wave while the gate of the intensified CCD camera was similarly modulated but delayed with respect to the excitation. On axis illumination was achieved by placing a dicroic mirror in the optical path and using it to reflect the excitation light into the optical path. Long working distance (18 mm) microscope optics were used between the dicroic mirror and the eye. A Xybion ISG 750 camera (now ITT Night Vision, Roanoke, VA) with enhanced red sensitivity was used for imaging the phosphorescence. The intensifier of this camera can be turned on or off "gated" in approximately 0.10 μsec. The excitation light source is a high power LED with a response time of less than 1 microsecond. Both the camera intensifier and the LED are modulated in square waves with a 50% duty cycle at frequencies from less than 100 Hz to greater than 40,000 Hz. In order to determine the phosphorescence lifetime, phosphorescence intensity images are collected at 7 to 10 different delays over the range from 0 to 360 degrees relative to the excitation light. These are then analyzed by fitting the intensity at each pixel of the image set to a sinusoid of the frequency used for taking the images. The phase of the phosphorescence relative to the excitation is determined and from the phase shift and frequency, the phosphorescence lifetime is calculated. The quenching by oxygen follows the Stern-Volmer relationship:

$$T^o/T = 1 + k_Q * T^o * pO_2 \quad (1)$$

where T^o and T are the phosphorescence lifetimes at oxygen pressures (pO_2) of zero and the experimental value, respectively, and k_Q is a second order rate constant related to the frequency of collision of excited state phosphor molecules with molecular oxygen and the probability that energy transfer will occur in each collision. Equation 1 makes it possible to calculate the oxygen pressure at each pixel of the image array once the phosphorescence lifetime is known.

The imaging software calculates the best fit of the data from each pixel of the image array and generates pixel by pixel maps of: 1. phase delay (phase shift) of the emission relative to the excitation light. 2. goodness of fit to a sinusoid as given by the regression coefficient (r = 1.0 is a perfect fit); 3. phosphorescence lifetime; and 4. oxygen pressure as calculated from Eq. 1. In living tissue, there can be significant tissue auto-fluorescence and/or leakage of excitation light through the optical filters. These signals occur with an effective phase shift of zero relative to the excitation. If no correction is made, they add to the phosphorescence and result in a progressive decrease in phase shift with increasing fraction of the signal. In order to correct for the "in phase" signal, two