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Oxygen distribution and vascular injury in the mouse eye measured by phosphorescence-lifetime imaging

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Abstract

Maps of the oxygen distribution in the retina of the mouse eye were obtained by phosphorescence-lifetime imaging. Phosphor dissolved in the blood was excited by modulated light and phosphorescence imaged through microscope optics with an intensified-CCD camera. Phosphorescence lifetimes and oxygen pressures were calculated for each pixel of the images. The resolution was sufficient to permit the detection of anomalies that result in reduced oxygen pressures in individual retinal capillaries. High-resolution maps of oxygen distribution in the retina can provide greater understanding of the role of oxygen and vascular function in diseases of the eye.

1. Introduction

Many diseases of the eye, especially those that cause inner retinal neovascularization (diabetic retinopathy, retinopathy of prematurity, sickle cell disease, etc.) and those that involve retinal

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degeneration (retinitis pigmentosa, age-related macular degeneration) have regional hypoxia as either a primary causative or an early contributory factor. Regions of hypoxia are likely to appear before irreversible tissue injury occurs. This is particularly true for diabetic retinopathy, the leading cause of blindness for individuals from 20 to 74 years of age. Multiple structures of the eye are pathologically affected in diabetics by neovascular changes with resultant plasma leakage and tissue disruption.¹ Currently available data are consistent with the hypothesis that hypoxia of the retina is responsible for pathologic growth of new blood vessels in the inner retina, the major cause of blindness associated with diabetes of long duration. Support for this idea includes oxygen electrode measurements by Linsenmeier and co-workers,^{2,3} showing that in diabetic cats the oxygen pressures in the inner half of the retina are approximately one half of those in normal cats, and by Berkowitz *et al.*⁴, showing that there was decreased oxygen response in the retinas of galactosemic rats. Oxygen electrodes are useful in identifying global oxygen deficiency, but they are invasive and not highly effective for assessing hypoxia induced by failure of individual capillaries. Retinal pathology in diabetes, however, is believed to result from progression of intraretinal microangiopathy (nonproliferative diabetic retinopathy) to extraretinal neovascularization.⁵ Similar pathogenic mechanisms are thought to occur in other diseases characterized by inner retinal neovascularization. Small, focal areas of hypoxia resulting from clusters of defective capillaries could be responsible for production of high levels of vascular endothelial growth factor (VEGF). Vitreous samples in human beings with retinopathy have been shown to contain high levels of VEGF.⁶ High levels of VEGF have also been measured in animal models of ocular neovascular disease.^{7–10} VEGF, in turn, has been implicated as a mediator of new blood vessel formation. Recently, treatments for age-related macular degeneration have been proposed based on the premise that increased VEGF in the eye is responsible for the observed vascular proliferation. Clinical trials are under way, for example, to test the efficacy of monthly injections of anti-VEGF aptamer (Eyetechnopharmaceuticals, New York), which binds and inactivates VEGF, in suppressing development of vascular abnormalities.

Oxygen-dependent quenching of phosphorescence^{11–18} appears to be well suited for measuring oxygen in the retinal vessels. It is minimally invasive, requiring only intravenous injection of a phosphor, and phosphors have become available that are water soluble and nontoxic. The lens of the eye readily transmits light of both excitation and emission wavelengths. By use of intensified CCD cameras of sufficient sensitivity, oxygenation of the vessels of the retina can be imaged by phosphorescence with good spatial resolution. However, until recently oxygen maps were obtained only for the relatively large cat or pig eyes.^{13,14,16,19} Recently Shonat and Kight^{17,18} succeeded in obtaining images of oxygen distribution in the mouse eye by using phosphorescence-lifetime imaging. In this paper we demonstrate that phosphorescence imaging can effectively define small vascular lesions, determining both the size and the severity of the local region of hypoxia as well as revealing individual retinal capillaries with abnormally low oxygen levels.

2. Methods and Materials

A. Phosphorescence-Lifetime Imaging

The phosphorescence-lifetime imaging system previously used to image oxygen in the retina of the cat eye^{13,14} was modified to permit imaging of phosphorescence lifetimes in the much smaller mouse eye. Following the lead of Shonat and Kight,^{17,18} we switched to a frequency-domain approach. For clarity, the principles of lifetime imaging in the frequency domain are briefly summarized below.

In a conventional frequency-domain lifetime measurement experiment (see, for example, Ref. 20), the excitation source, e.g., a LED, is modulated by a sinusoidal wave of frequency f :

$$Ex(t)=A \sin(2\pi ft)+B, \quad (1)$$

where $Ex(t)$ designates the intensity of the excitation light and A and B are the amplitude and the dc offset of the excitation sine wave, respectively. The phosphorescent response to the sinusoidal excitation [$Em(t)$ is also a sinusoid of the same frequency (f) but delayed in time δt] results in phase shift ϕ :

$$Em(t)=a \sin(2\pi ft - \phi)+b, \quad \phi=2\pi f \times \delta t, \quad (2)$$

where a and b are the amplitude and the dc offset of the phosphorescent signal. The value of the phase shift is related to phosphorescence lifetime τ by a simple relationship:

$$\tan(\phi)=2\pi f\tau. \quad (3)$$

Determination of lifetime τ , therefore, requires collecting signal $Em(t)$ in digital form, finding phase shift ϕ , and converting it to τ by using Eq. (3).

In imaging, it is conventional to use a simplified version of excitation, i.e., to turn the excitation source on and off, producing a sequence of square pulses (Fig. 1). In addition, instead of collecting the entire response $Em(t)$, the detector, i.e., the CCD camera, is also modulated by on-off switching at the same frequency as the excitation but with a delay ΔT . As a result, the integrals of phosphorescent decays that correspond to each pixel are acquired on the CCD array. The excitation square wave [$P(t)$] can be presented as a sum of fundamental frequency f and all its harmonics:

$$P(t)=A \sum_{n=1}^{\infty} \sin(2\pi nft)+B. \quad (4)$$

The resultant phosphorescence is also the sum of sinusoids, each with its own amplitude a_n , phase shift ϕ_n , and time delay δt_n :

$$P(t)=\sum_{n=1}^{\infty} a_n \sin(2\pi nft - \phi_n)+b, \quad \phi=2\pi nf \times \delta t_n. \quad (5)$$

The phosphorescence image [$I(\Delta T)$] formed on the CCD array, modulated with delay ΔT , is given by the integral

$$I(\Delta T)=C \int_{\Delta T}^{1/f} \left(\sum_{n=1}^{\infty} a_n \sin(2\pi nft - \phi_n) \right) dt, \quad (6)$$

where the upper limit of integration ($1/f$) represents the period (360°) of the excitation square wave of fundamental frequency f and constant C is related to the acquisition time. It can be shown that the dependence of image intensity I on delay time ΔT can be closely approximated by the cosine form

$$I(\Delta T) \approx c \times \cos(2\pi f \Delta T - \phi) + d, \quad (7)$$

where ϕ is the phase delay that corresponds to fundamental frequency f used for modulation and c and d are constants. Thus, one can determine phosphorescence lifetime τ in each pixel by collecting a sequence of images $I(\Delta T)$ at different delays ΔT , fitting these images by using Eq. (7) and determining phase shift ϕ for each pixel, and converting phase shift ϕ to lifetime τ in each pixel, using Eq. (3). Obviously, different modulation frequencies will result in different phase delays for the same lifetime τ . As a result of signal-to-noise ratio considerations, optimal phase delays for determination of lifetimes are in the range 20° – 40° . If oxygen pressures and lifetimes in the image vary significantly, it is useful to produce the lifetime maps at several modulation frequencies (see below), making the phase delays that correspond to various lifetimes fit into the optimal range ($\sim 30^\circ$).

In our experimental setup the retina was observed under a microscope with long-working-distance (18 mm) microscope lenses and on-axis illumination that we achieved by placing a dichroic mirror in the optical path. The dichroic mirror reflected the excitation light from a modulated source (LED), positioned at a right angle relative to the optical axis of the microscope, onto the retina while it transmitted the longer-wavelength phosphorescence back along the axis to the CCD camera. Optical filters were used to complete the separation of the excitation from the phosphorescence, which could be done with excellent efficiency because of very large Stokes shifts (more than 150 nm) of the phosphorescence relative to the absorption.

A Xybion (now ITT Night Vision, Roanoke, Va.) ISG 750 camera with enhanced red sensitivity was used for detection of the phosphorescence. The intensifier of the camera can be turned on or off (gated) in approximately 100 ns, and the excitation LEDs have response times of less than 1 μ s. Both the camera intensifier and the LEDs could be modulated at frequencies from 100 Hz to 40 kHz. Phosphorescence intensity images were collected at the modulation frequencies that generated 15° – 40° phase delay of the phosphorescence relative to the excitation. For the phosphors used and the oxygen concentrations that are typically present *in vivo*, this collection required modulation frequencies in the range 400–3000 Hz. To calculate phosphorescence lifetimes, we collected phosphorescence-intensity images at 6 to 15 delays and analyzed them as described above.

Quenching of phosphorescence by oxygen follows the Stern–Volmer equation

$$\tau_0/\tau = 1 + k_Q \times \tau_0 \times pO_2, \quad (8)$$

where τ_0 and τ are the phosphorescence lifetimes in the absence of oxygen and at oxygen pressure pO_2 , respectively, and k_Q is a second-order rate constant related to the frequency of collision of excited-state phosphor molecules with oxygen. Equation (8) is used to convert the lifetime image into the oxygen image.

In spite of optical filtering, phosphorescent signals collected from tissue are often mixed with reflected excitation light, endogenous fluorescence, or both. The fluorescence signals have no delay with respect to the excitation (in phase), and when they are added to or mixed with the phosphorescence they shorten the apparent lifetimes. One can remove in-phase signals from the intensity images by collecting additional images at 0° and 180° relative to the excitation using sufficiently high frequencies. At high frequencies, alternating components of signals that correspond to long lifetimes ($\tau > 20 \mu$ s) are greatly suppressed; i.e., the signals become practically constant in time (dc). However, fast signals (fluorescence or reflected light) remain fully modulated. Therefore, collecting the images at 0° (in-phase signal plus phosphorescence)

and 180° (phosphorescence only), using a high-enough frequency (e.g., 36 kHz), and finding their difference allows calculation of the intensity of the in-phase signal and correction of the intensity images used for determination of the phosphorescence-lifetime map. This correction method has been tested with samples containing mixtures of a phosphor and a model fluorophor and shown to effectively remove the in-phase signals (fluorescence), as long as the latter accounted for less than 50% of the total intensity.

B. Resolution

For the measurements of the retina of the mouse eye, the imaged area is approximately 0.92 mm high by 1.2 mm wide and the CCD array is 480×752 pixels. As a result the area per pixel is approximately $1.9 \mu\text{m}$ high $\times$ $1.6 \mu\text{m}$ wide. This area is approximate because no effort has been made to correct for refractive-index changes along the light path between the coverslip and the retina within the eye. Resolution in the oxygen images is limited by movement that occurs during image collection and between images within an image set. There is mechanical coupling between breathing and, to a much lesser extent, systemic blood-pressure changes and position of the retina and elements within the retina. Thus, although $10 \mu\text{m}$ vessels can be observed in most individual phosphorescence-intensity images, they can rarely be resolved in the oxygen maps, and then only if the oxygen pressure is substantially decreased (intensity increased) relative to that of the neighboring vessels.

C. Phosphor

The phosphor used in this study was polyglutamic Pd tetrabenzoporphyrin dendrimer (Oxyphor G2; Oxygen Enterprises, Ltd., Philadelphia, Pa.). Synthesis and properties of this phosphor have been described.^{21–23} Oxyphor G2 was injected intravenously as 0.15 ml of a 1.6 mg/ml solution in physiological saline. Oxyphor G2 (MW 2642) has 16 carboxyl groups on the periphery, giving a net charge of -16 at physiological pH. Because of the large negative charge, it has a low permeability through the vessel walls. Oxyphor G2 has absorption maxima at 450 or 635 nm. We chose to excite this phosphor at 450 nm. The band at 636 nm can also be used, but blue excitation was chosen to minimize the depth to which the excitation light penetrated into the tissue. The phosphorescence (emission maximum at 810 nm) was measured with a 695 nm long-pass Schott glass filter. The values of $255 \mu\text{s}$ and 280 mm Hg s^{-1} ($1 \text{ mm Hg} = 1 \text{ Torr}$) were used for the τ_0 and k_Q , respectively,²¹ at 38 °C.

D. Experimental Protocol

The pigmented mice were anesthetized by intraperitoneal injection of 0.2 ml of a solution of ketamine (25 mg/ml) and xylazine (25 mg/ml) dissolved in phosphate-buffered saline. A drop of 1% tropicamide (Mydracil; Alcon, Ft. Worth, Tex.) was placed on the eyes to dilate the pupils, and Oxyphor G2 (1.6 mg/ml in unbuffered saline, pH 7.5) was given by intravenous injection of 0.15 ml into the tail vein. Approximately 4 min after introduction of the Mydracil, a drop of hydroxypropyl methylcellulose (Goniosol; CIBA Vision Ophthalmics, Atlanta, Ga.) was placed on the eye, and then a small piece of clear plastic sheet was gently placed on the Goniosol. The retina was then imaged through the coverslip. Phosphorescence imaging began as early as 3 min after injection of the phosphor and on occasion continued for as much as 1.5 h.

To test the ability of the oxygen maps to detect local vascular abnormalities that generate local hypoxia, pigmented mice were anesthetized, the pupils were dilated with 1% tropicamide, and indocyanine green (5.0 mg/ml 0.25 ml) was injected in the tail vein. Laser photocoagulation was performed with a diode laser photocoagulator (810 nm; OcuLight Six, IRIS Medical, Mountain View, Calif.) and a slit lamp system with a coverslip as a contact lens. This laser system produces vascular occlusion through photochemical activation of the indocyanine green within the vasculature. The photoinduced radicals cause complete closure of the vessels in the

focal spot of the laser. Two or three spots of laser photocoagulation (90 mW, 75 μm spot size, 0.1 s duration) were applied to the retina, two to three disk diameters from the optic nerve of each eye. These spots were far enough away from the optic nerve head to avoid hitting major vessels in the retina and yet still be readily observed through the pupil. Two days after laser treatment the Oxyphor G2 was injected and the oxygen imaged. This delay was instituted to prevent the local tissue edema and vascular leakage that occur immediately after the treatment. Photocoagulation by use of indo-cyanine green as an intravascular sensitizer was chosen to alter the local retinal blood supply while it minimized direct tissue injury.

3. Results

A. Imaging Phosphorescence Intensity from the Vasculature of the Retina

The camera was focused on the retina, the brightness adjusted, and a sequence of images collected. The sequence of images used delays ranging from 0° to 360° relative to the excitation. A sequence of phosphorescence intensity images, collected by use of 1030 Hz modulation, is shown in Fig. 1. There is a network of small vessels between the major arterioles and veins in the retina that appear to be quite uniformly $\sim 10\ \mu\text{m}$ in diameter. We refer to this network of small vessels as capillaries, although this is likely to be an oversimplification. Observing these small vessels requires that there be little movement of the mouse during acquisition of the image.

As shown in Fig. 1, the individual images of phosphorescence intensity show with good resolution the veins and the arteries that supply blood to the retina. The images are similar to those obtained during fluorescein angiography. In both cases, the luminophors are dissolved in the blood plasma and, therefore, the intensity images show vascular structure. Leaks of either fluorescein or the phosphor through the vessel walls lead to the appearance of small local regions of increased intensity spots in the images, and both the intensity and the size increase with time after phosphor injection. The permeability of fluorescein to the vessel walls is much higher than that of Oxyphor G2 (charge, 1 compared with -16 ; molecular weight, 332 compared with 2,642). The change in the image that is due to dye leakage is greater for the phosphor than for fluorescein. As phosphor leaks out of the vessel, the phosphorescence intensity increases not only because of the increase in the amount of dye near the locus of the leak but also because the oxygen pressure is lower in the extravascular space than in the vessels.

The phosphor is present only in the blood plasma, so the phosphorescence intensity in the images is related to both the blood volume and the oxygen pressures. Thus the lower oxygen pressures in the veins relative to the arterioles result in the veins' being brighter than the arterioles. Larger vessels are brighter than small vessels, and the capillary bed areas, which lack vessels larger than $\sim 10\ \mu\text{m}$ diameter, show much weaker phosphorescence than the larger vessels. Individual capillaries can be seen in the phosphorescence images but are not resolved in the lifetime and oxygen maps unless they are blocked or otherwise depleted in oxygen relative to the surrounding capillaries.

B. Maps of Phosphorescence Lifetime and Oxygen Pressure

Typically, a set of phosphorescence intensity images (see above) with phase delays of 0° to 360° and at 30° intervals was taken. The in-phase correction was obtained by imaging with frequency 36 kHz at 0° and 180° (see above). Subtracting the 180° image from the 0° image yields the intensity image that corresponds to lifetimes of less than 1 μs , i.e., reflected light and fluorescence. This in-phase signal was subtracted from all images in the set to give a set of corrected images for calculation of the phosphorescence lifetimes. Figure 2 shows a plot of the phosphorescence intensity in three regions of interest, corresponding to two veins and an arteriole, as a function of the phase delay used to collect the intensity images. The sets were

fitted to sinusoids in each pixel, and the phase shift was determined for each fit. The phosphorescence-lifetime image was calculated from Eq. (3) and converted to the oxygen image by use of Eq. (8).

The phase delay image, the phosphorescence-lifetime image and, the oxygen pressure image are displayed in Fig. 3. Visualizing the phase delay image is important because the conversion of the lifetime image into the oxygen image can be done most reliably if the lifetimes are determined at frequencies at which the delays are 25° to 35° . Each phosphor is characterized by a distribution of lifetimes and, therefore, changing the phase delay in a frequency-domain measurement can lead to differences in the apparent lifetimes, τ . The narrower the distribution in lifetimes, the less effect the difference in phase delays has on the calculated lifetime. When there were regions in the map with phase shifts that differed substantially ($> 10^\circ$) from the phase (28°) used for phosphor calibration, they were imaged again at a frequency for which the phase shift was nearer 28° .

In the region of the optic nerve's head, the images show relatively large veins and arterioles radiating from a small central region (Fig. 3). As you progress around the disk, the vessels alternate between arterioles and veins, and this is clearly seen in both lifetime and oxygen images. The vessels alternate between low and high oxygen pressures and long and short phosphorescence lifetimes. The oxygen pressure in the veins is typically 30–45 mm Hg, whereas that in the arterioles is 60–80 mm Hg. In regions farther from the optic nerve's head there are substantial areas of capillary bed that exhibit higher oxygen pressures, usually 50–100 mm Hg. This result is consistent with some of the blue (450 nm) excitation light passing through the retina and exciting a small amount of diffuse phosphorescence from the underlying choriocapillaris where the oxygen pressure is high (short lifetime). When this choroidal signal is added to the weak phosphorescence from the retinal capillaries, the resultant mixed signal gives calculated oxygen pressures between those of the retina and of the choroid. The total intensity is very low, such that the oxygen values also become noisy. Where the phosphorescence signal from the retinal vessels is higher, such as for the veins and arterioles, the oxygen pressures in the individual vessels can be accurately determined.

C. Dependence of the Retinal Oxygen Maps on Time after Injection of the Anesthetic

When mice are given anesthetics, the animals often go through a transient period of decreased blood pressure, abnormal breathing patterns, or both. The duration and severity of this period are quite variable among animals owing to individual differences in biochemical and physiological responses to the anesthetic. In the experiment shown in Fig. 4 and Fig 5, oxygen maps were repetitively measured at 1–2 min intervals from the earliest time attainable (3 min) until the mouse began to recover from anesthesia. Representative data are shown in Fig. 4 as the phosphorescence intensity images and oxygen pressure maps at 5 and 35 min after anesthesia, shortly before the mouse awakened. In this animal, the venous oxygen pressures increased with time after anesthesia from ~15 mm Hg at 5 min to ~50 mm Hg at 35 min. In contrast, the arteriolar values were essentially constant and near 75 mm Hg. The time course of the process is shown in Fig. 5, where the oxygen pressures in selected regions of two arterioles and two veins are plotted against the time after anesthesia.

The time course of the oxygen measurements after induction of anesthesia is highly variable among mice, ranging from almost no change over time to failure to attain stable levels before recovery (usually because of an erratic breathing pattern). In most cases, however, there is a transient lowering of the oxygen pressure in the veins, which returns to a nearly stable condition within 5–10 min. In the period when the values are stable, the arteriolar values are 65–85 mm Hg and the venous values 35–55 mm Hg. Oxygen extraction is 25–30 mm Hg. Similar time dependence has been observed for microvascular oxygen levels in normal muscle tissue in mice used as controls for tumor oxygen measurements.²⁴

D. Identifying Vascular Lesions Induced by Alterations in Tissue Oxygenation

In initial experiments, a laser was used to block one of the larger retinal blood vessels and the phosphorescence images were collected 48 h later. The measured phosphorescence was well defined, with no evidence of leakage from the vessels (spreading of intensity, increase in intensity with time, or both). The phosphorescence from the large damaged area was high owing to the low oxygen levels, and this obscured the emission from the surrounding normal tissue. As a result, the oxygen maps showed only large areas with oxygen pressures near zero, confirming the presence of a massive injury. This model was not further pursued because such large injuries can be readily detected by simple ophthalmoscopy. It was more interesting to test for the ability to detect injury that is due to the pathology involving microvessel failure, where blockage of capillaries would result only in small hypoxic regions. To this end, a model of local vascular failure was generated. Two or three 75 μm focal spots of laser photocoagulation with indocyanine green as an intravascular sensitizer were made in regions that spared observable major blood vessels. This procedure restricted injury to the capillary bed within the focal area. Oxygen measurements were made 48 h later to limit the contribution of immediate local edema and vascular leakage. An oxygen pressure map of a region with two laser-induced lesions is shown in Fig. 6.

The lesions appear in phosphorescence intensity images as bright spots (not shown). In the oxygen map they appear as nearly round areas with central core oxygen values well below those of the surrounding tissue. The absence of significant leakage of the phosphor from the vessels was confirmed by measurements made over periods of 1 h following phosphor injection, during which time the size and the oxygen profile of the region of hypoxia remained constant. Figure 6 presents an oxygen map of one of the two lesions reimaged at a lower frequency (800 Hz) to yield a better estimate of the core oxygen pressure. The laser-induced lesion consists of a central region of acute hypoxia surrounded by a region of graded oxygen deficit that extends outward to approximately 150–200 μm from the center. The mean oxygen pressure in the core was less than 7 mm Hg.

E. Naturally Occurring Vascular Anomalies

Images of the retina in mice, particularly old obese mice, occasionally show anomalous hypoxic vessels within the capillary bed of the retina. An example of such vessels is shown in Fig. 7. The phosphorescence intensity image [Fig. 7(a)] does not show any obvious vascular anomalies, whereas when the oxygen pressure map is calculated there are vessels in three regions with retinal capillaries that resemble, on a microscale, varicose veins. The oxygen pressures in these vessels are well below that in the surrounding capillaries. It is likely that they have greatly diminished blood flow and that, as a result, much more oxygen has been extracted from the blood within these vessels.

4. Discussion

Oxygen measurements by quenching of phosphorescence originating from phosphors dissolved in the blood plasma *in vivo* can be compared with tissue oxygen measurements made with micro-oxygen electrodes. This has been done for the cat eye (see Shonat *et al.*¹⁴ for comparison with Linsenmeier²), for skeletal muscle,²⁵ and for tumors growing in window chambers.²⁶ In each case, oxygen electrodes were inserted into the tissue, whereas the phosphor was in the blood serum. The values measured by the oxygen electrodes are generally slightly lower than those measured by phosphorescence lifetime. The difference indicated that there is a small decrease in the oxygen pressure from the microcirculation to the extracellular space, a decrease consistent with that predicted by oxygen diffusion. Several investigators, including Linsenmeier and co-workers^{2,3} and Yu *et al.*,^{27–29} have used oxygen-sensitive microelectrodes to measure oxygen tension in the retina. These studies have concluded that tissue hypoxia may

contribute to the development of pathology in the retina of a diabetic animal but not in the Royal College of Surgeons model for retinitis pigmentosa. The data, however, apply only to models of global hypoxia, in which all or at least a large fraction of the tissue becomes hypoxic. In most diseases of the eye, however, the pathology is likely to involve functional failure at the level of individual capillaries. This would initially result in localized regions of focal hypoxia, but as the numbers of defective vessels within a given region accumulated the region with an oxygen deficit would increase. Oxygen electrodes make point measurements and are statistically unlikely to hit a region of focal hypoxia. If one were hit, slight movement of the electrode tip would lead to a widely different value, and the measurement would likely be considered an artifact. Thus oxygen electrodes can effectively measure oxygen in macroscopic areas but are less able to detect or evaluate small regions of hypoxia interspersed among much larger volumes of normoxia. As a result, they are not suitable for evaluating early stages of pathologies in which the failures occur in individual capillaries and therefore cause only small focal regions of tissue hypoxia.

Phosphorescence-lifetime imaging is suited to evaluation of microcirculatory functions for the following reasons: The measurements are noninvasive, i.e., do not involve physically entering the eye or inserting an object into the retina; repetitive measurements can be made in the same animal over time; and the oxygen maps have excellent spatial resolution and can even identify and quantify vascular failure at the level of individual retinal capillaries.

Oxygen-dependent quenching of phosphorescence has been shown to produce good maps of the distribution of oxygen in the retina of the mouse eye. This opens a wide range of applications to experimental models of diseases of the eye, many of which are in mice and rats. It is not yet possible to discuss with certainty the concentration of oxygen in normal retinal tissue because it is necessary to immobilize the animals with anesthetics. Anesthetics can alter the cardiopulmonary function, changing blood pressure, cardiac output, breathing rate, etc., with subsequent increases or decreases in tissue oxygenation. The measurements are selective for the retina, as the choroidal vasculature lies behind the highly absorbing and scattering pigmented layer. The choroidal vascular bed, therefore, contributes only a weak, highly scattered background phosphorescence with a short lifetime. Although emission from the choroid can interfere with the oxygen pressure measurements in the retinal capillaries during normoxic conditions, hypoxic retinal capillaries have brighter phosphorescence and can be readily observed and the oxygen level estimated.

5. Summary

The phosphorescence-lifetime imaging method is a useful tool for studying eye physiology and pathology. It has been shown to be able to identify and characterize vascular anomalies at the level of individual retinal capillaries. As a result, phosphorescence quenching is well suited for critical evaluation of the hypothesized role of local hypoxia in diseases such as diabetic retinopathy and age-related macular degeneration. Where as local hypoxia has a causative role in, or accompanies, early development of disease, such measurements can contribute significantly to both early diagnosis of the disease and monitoring progression of the pathology. Oxygen measurements may also provide a basis for selecting pharmaceutical agents that delay or prevent the development of tissue hypoxia and thereby delay or prevent loss of vision.

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Fig. 1.

Images of the phosphorescence intensity from the retina of the mouse eye. The mouse was treated as described in Section 2, and the phosphorescence intensity images were collected at 1030 Hz. The number on each image indicates the delay (in degrees) relative to the excitation for which the image was collected. The retinal area imaged is approximately 0.92 mm high by 1.2 mm wide.

Fig. 2. Phosphorescence intensity versus phase delay (in degrees) for three regions of interest in the image, two veins and one arteriole. Least-squares fits to a sinusoid yielded phase shifts of 43.6° and 40.4° for the veins and 35.1° for the arteriole. (Graphed in Origin.)

Fig. 3.

Maps of (a) the phosphorescence intensity at 30° phase shift, (b) the phase shift between excitation and phosphorescence, (c) the phosphorescence lifetime, and (d) the oxygen pressure calculated from the set of images in Fig. 1. The maps correspond to a region whose physical dimensions are 0.92 mm high by 1.2 mm wide. The oxygen maps were smoothed with a running-median 5×5 filter.

Fig. 4.

Dependence of the retinal oxygen pressures on time after induction of anesthesia. The mouse was given an intraperitoneal injection of anesthetic, the pupil was dilated, and retinal imaging of the phosphorescence lifetimes began ~3 min later. The measurements shown were made at 5 and 35 min after induction of anesthesia. The area of the retina that was imaged was approximately 0.92 mm high by 1.2 mm wide.

Fig. 5. Time course of the oxygen pressures in the retinal veins and arterioles following anesthesia. Oxygen pressure maps of the retina were repetitively measured; a total of 19 measurements was made over the period of 3–35 minutes following anesthesia. Regions of interest were selected within two arterioles and two veins, and the average oxygen pressures for each region were determined at every time. The resultant values are plotted as a function of time after anesthesia.

Fig. 6. Oxygen pressure maps of (a) the region containing two laser photocoagulation spots and (b) a region centered on one of the two spots. The measurements were made with modulation frequencies of 3030 and 800 Hz, respectively. The area of retina covered by the oxygen maps is approximately 0.92 mm high by 1.2 mm wide.

Fig. 7. Phosphorescence intensity image (left) and oxygen pressure map (right) of the eye of a 2-year-old mouse. The measurements were made at 1000 Hz. The retinal area imaged was approximately 0.92 mm high by 1.2 mm wide.