HIGHLIGHTED TOPIC | Oxygen Sensing in Health and Disease

Red blood cell velocity and oxygen tension measurement in cerebral microvessels by double-wavelength photoexcitation

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Tsujioka, Kosuke, Eiichi Sekizuka, Chikara Oshio, Katsuhiko Tsujioka, Haruyuki Minamitani. Red blood cell velocity and oxygen tension measurement in cerebral microvessels by double-wavelength photoexcitation. J Appl Physiol 96: 1561–1568, 2004. First published December 5, 2003; 10.1152/japplphysiol.00764.2003.—Because the regulation of microcirculation in the cerebral cortex cannot be analyzed without measuring the blood flow dynamics and oxygen concentration in cerebral microvessels, we developed a fluorescence and phosphorescence system for estimating red blood cell velocity and oxygen tension in cerebral microcirculation noninvasively and continuously with high spatial resolution. Using red blood cells labeled with fluorescent isothiocyanate to visualize red cell distribution and using the oxygen quenching of Pd-meso-tetra-(4-carboxyphenyl)-porphyrin phosphorescence to measure oxygen tension enabled simultaneous measurement of blood velocity and oxygen tension. We examined how the measurement accuracy was affected by the spatial resolution and by the excitation laser light passing through the targeted microvessel and exciting the oxygen probe dye in the tissue beneath it. Focusing the excitation light into the microvessel stabilized the phosphorescence lifetime at each spatial resolution; moreover, it greatly reduced phosphorescence from the brain tissue. Animal experiments involving acute hemorrhagic shock demonstrated the feasibility of our system by showing that the changes in venular velocity and oxygen tension are synchronized to the change in mean arterial pressure. Our system measures the red cell velocity and oxygen concentration in the cerebral microcirculation by using the differences in luminescence and wavelength between fluorescence and phosphorescence, making it possible to easily acquire information about cerebral microcirculatory distribution and oxygen tension simultaneously.

cerebral microcirculation; erythrocyte velocity; oxygen tension; fluorescence; phosphorescence

THE CEREBRAL NERVE CELLS ARE extremely vulnerable to ischemia because they store hardly any oxygen or substrates. Because higher brain function therefore depends on the microcirculation, the brain is expected to have a strong autoregulation maintaining the local blood flow and volume. Consequently, an extremely effective way to study the regulation of the microcirculation in the cerebral cortex is by measuring the dynamics of the cerebral microcirculation while simultaneously measuring the oxygen concentration in the cerebral microvessels.

When one measures the microcirculatory blood flow in two-dimensional structures such as dorsal skin, the mesentery or cremaster muscle, the cheek pouches of hamsters, and the ears of rabbits, one can observe these structures under transmitted illumination. Although the microcirculation in the brain cortex cannot be observed under transmitted illumination, the dynamics of the blood flow distribution can be visualized by using FITC-labeled red blood cells (RBCs) and an incident-light fluorescence microscope, as described by Ishikawa et al. (3). On the other hand, the oxygen electrodes often used when measuring oxygen tension in vivo cannot be used in microvessels because they physically damage them too much and because their spatial resolution is inadequate. The partial pressure of oxygen in organs must therefore be measured by a noncontact, noninvasive method. The oxygen tension can be measured in vivo by using the changes in the lifetime of phosphorescence emitted from a porphyrin dye, as reported (12, 22), and this technique is being established as an alternative method for measuring the oxygen tension in organs.

In the present study, we used fluorescently labeled RBCs to visualize blood flow and used the phosphorescence decay method to measure oxygen tension. By exciting two dyes with two different wavelengths, both RBC velocity and oxygen tension could be measured continuously, but we had to ensure that the absorption and emission spectra of the fluorescence dye used for visualizing RBC flow and the phosphorescence dye used for measuring oxygen tension did not overlap. In addition, we had to determine how the measurement accuracy was affected by excitation light passing through the targeted microvessel and exciting the oxygen probe dye in the brain tissue beneath it.

The aim of this study was to develop a fluorescence and phosphorescence system that can simultaneously measure the cerebral microcirculatory distribution and the oxygen tension in cerebral microvessels. We examined the emission and absorption spectra of fluorescein and phosphorescence dyes, the spatial resolution, the half-life of probe dye in blood, the extravasation of the dye, and the phosphorescence emission from the tissue lying under the targeted microvessels. To demonstrate the feasibility of our system, we performed animal experiments involving acute hemorrhagic shock.

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MATERIALS AND METHODS

Animal preparations. All animals received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” prepared by the Animal Research Committee of the Kawasaki Medical School. Male Wistar rats weighing 300–350 g were anesthetized with an intraperitoneal injection of α-chloralose (60 mg/kg) and urethane (600 mg/kg). The head of each rat was fixed in a stereotaxic frame, and the left parietal bone was exposed by a longitudinal midline skin incision. After three polyethylene tubes (PE-50) were fixed to the skull with cyanoacrylate, a craniectomy was performed, and a closed cranial window (5 mm in diameter) was made using a cover glass and a rubber O-ring. Artificial cerebrospinal fluid consisting of 147.8 meq/l Na⁺, 3.0 meq/l K⁺, 2.3 meq/l Mg²⁺, 2.3 meq/l Ca²⁺, 135.2 meq/l Cl⁻, 19.6 meq/l HCO₃⁻, 1.7 meq/l lactate, 1.1 mM phosphate, and 3.9 mM glucose was superfused at 0.1 ml/min. A thermistor with a diameter of 2.3 mm was fitted to the cranial window to monitor the temperature, and the cerebral fluid was maintained at 37°C. The left femoral artery was cannulated for monitoring the mean arterial blood pressure (MAP), the right femoral artery was cannulated for withdrawing blood, and the right femoral vein was cannulated for infusing blood or drugs.

Probe administration for RBC flow visualization and oxygen measurement. The RBC flow in cerebral microvessels was visualized by perfusing RBCs labeled with fluorescent isothiocyanate (FITC, Sigma Chemical) as described by Ishikawa et al. (3). Blood was withdrawn from the donor rat, and the RBCs were centrifuged and washed. The FITC was excited by irradiating it at an energy density of 5 mJ/cm² with the light from a mercury lamp and passing it through a band-pass filter (450–490 nm). Each rat received 0.1 ml of FITC-labeled RBC suspension injected into its cannulated femoral vein. (This amount of FITC-labeled RBCs accounted for ~1/50 of all the RBCs in the body.)

Image processing of fluorescent blood flow. Figure 1 shows a block diagram of the system used for measuring RBC velocity, vessel diameter, and oxygen tension in individual microvessels of the cerebral cortex. Microscopic images made with the FITC-emitted light that had passed through a long-pass filter (>520 nm) were recorded with a charge-coupled device camera equipped with an image intensifier (C6653MOD, Hamamatsu Photonics). The images were processed automatically by using a previously developed system (21). Briefly, this system consists of a host computer, a Hi8 video recorder, and an image-data-processing board. One frame of a still image can be viewed on the monitor via the image-processing board, which takes two still 512 × 512-pixel images, and the intensity can be digitized to 256 levels. Automated velocity measurement was made possible by writing C-language sequences to load the images into memory, calculate the correlation, fast-forward through the video frames, and so on.

Oxygen tension measurement using phosphorescence decay. The Pd-meso-tetra-(4-carboxyphenyl)-porphyrin (Pd-TCPP, Porphyrin Products) used as the phosphorescent probe for the O₂-dependent quenching was dissolved (20 mg/ml) in physiological saline containing bovine serum albumin and buffered to a pH of 7.4 with phosphate buffer. Each rat received a bolus of Pd-TCPP (30 mg/kg) through a slow intravenous injection, and the Pd-TCPP in the microvessels was excited by using the second harmonic of a Q-switched Nd:YAG pulse laser (532-nm wavelength, 6-ns pulse width at half maximum, 1-Hz pulse recurrence frequency, 200-nJ/pulse irradiation energy) through the objective lens of a microscope. Phosphorescence passing through a long-pass filter (>620 nm) was detected with a photomultiplier tube (PMT) (R1894, Hamamatsu Photonics). The voltage signal (current signal converted with resistance of 50 kΩ) from the PMT was fed into a personal computer via an analog-to-digital converter (NR-2000, Keyence) with a sampling frequency of 200 kHz and a sampling number of 500 points. When the decay signal was processed for use in calculating the phosphorescence lifetime, the first 10 sampling points (corresponding to 50 μs) from the start of sampling were excluded because they included the intensity of the exciting laser...
light, which was not completely blocked by the long-pass filter. In addition, <1% of the maximum intensity of the PMT signal was rejected to reduce the contribution of dark current generated by the PMT. Phosphorescence lifetime $\tau$ was obtained by least-squares fitting of the remaining data to a single exponential curve. The oxygen tension ($P_{O_2}$) was calculated from the Stern-Volmer equation

$$I_0/I = \tau_0/\tau = 1 + k_q \cdot \tau_0 \cdot P_{O_2}$$

where $I_0$ and $\tau_0$ are phosphorescence intensity and lifetime in the absence of oxygen, $I$ and $\tau$ are the intensity and lifetime at a given oxygen tension, and $k_q$ is the rate constant of oxygen quenching. The $k_q$ and $\tau_0$ values for our system were obtained from a calibration experiment using oxygen electrodes; at 37°C with a pH of 7.4 they were 374 Torr$^{-1}$s$^{-1}$ and 0.74 ms.

Irradiation timing of two light sources. To obtain two physiological values, the cerebral microcirculatory blood flow distribution and oxygen tension, we had to alternately irradiate each of two dyes in a targeted microvessel with the light from two excitation light sources. To measure the RBC velocity and oxygen tension at 1 Hz, we used a mechanical shutter to control the output of a mercury lamp and used the signal from a limit switch to trigger an Nd:YAG laser when the shutter was closed. The irradiation times of the laser and mercury lamp were, respectively, 6 ms and 300 ms.

Estimation of Pd-TCPP concentration in blood plasma. After five rats received 30 mg/kg of Pd-TCPP in solution (20 mg/ml) by slow injection into the femoral vein, blood samples were removed from the postcaval vein with a heparinized syringe at 5 and 30 min and at 1, 3, and 6 h. The plasma was extracted by centrifuging the samples at 1,500 rpm for 15 min; the skimming fluid taken as a measurement sample was obtained by additional centrifugation at 1,000 rpm for 5 min. The Pd-TCPP concentration in the plasma sample was quantified by absorption spectrophotometry, using the relation between absorbance and concentration previously calibrated at Pd-TCPP concentrations of 0.50, 0.25, 0.13, and 0.06 mg/ml (data not shown).

Narrow capillary model. Because the spatial resolution depends on the diameter of the laser spot, the effect of the spot size on lifetime was determined by changing the objective lens of the microscope. A narrow glass capillary with a 410-μm outer diameter and a 350-μm inner diameter was used as a microvessel model; it was set in a cranial window made by using the same materials and conditions as for the in vivo experiments. A solution of Pd-TCPP deoxygenized with sodium sulfite was kept at 37°C and a pH of 7.4 and perfused through the glass capillary. The phosphorescence intensity and lifetime were measured at different resolutions (i.e., spot sizes) by changing the magnification of the objective lens ($\times 20$ or $\times 50$).

Flushing blood from microvessels. To photoexcite Pd-TCPP diffused into extravascular cerebral tissue (i.e., not in microvessels), we inserted a catheter tube 0.8 mm in diameter into the left common carotid artery in the direction of the middle cerebral artery, and we fixed the edge of the tube at the internal carotid artery. After preparing the closed cranial window on the left parietal bone and administering the Pd-TCPP, we left the rat for 1 h so that the Pd-TCPP had sufficient time to leak from the microvessels into the extravascular tissue. Then, while applying continuous laser irradiation to a venule, we gave the rat an overdose of pentobarbital sodium, reducing both the MAP and heart rate to 0. Physiological saline was immediately infused via a carotid artery, flushing the blood and Pd-TCPP from the microvessels, and the targeted microvessel was observed with a microscope to verify that it was filled with saline. The phosphorescence emission from the Pd-TCPP that had leaked into the tissue was measured at a constant laser power before and after the flush.

Acute hemorrhagic shock model. Four rats were put into acute hemorrhagic shock by letting them bleed from a catheter in the femoral artery. They were bled, at 7–8 ml/min, to a MAP of 40 mmHg in <1 min. The shed blood was collected in heparinized syringes and kept at 37°C. The MAP was maintained at 40 ± 3 mmHg for 5 min by intermittently withdrawing 0.3–0.4 ml of blood. After the period of shock, the rats were resuscitated by infusing autologus whole blood.

**RESULTS**

Absorption and emission spectrum of FITC and Pd-TCPP. The absorption and emission spectra of the FITC used for labeling the RBCs and Pd-TCPP used as the oxygen-sensitive probe are shown in Fig. 2. For RBC velocity and oxygen tension to be measured simultaneously, the Pd-TCPP absorption must be extremely small in the wavelength band used to excite the FITC (from 460 to 490 nm). The Q-band of the Pd-TCPP was observed near 532 nm, which is the wavelength of the second harmonic of the Nd:YAG laser.

Simultaneous measurement of RBC velocity and oxygen tension. Figure 3A shows the microcirculatory vessel structure in the cerebral cortex observed in the closed cranial window. Figure 3B shows a high-magnification blood flow image with FITC-labeled RBCs. Figure 3C shows an image of laser light irradiating a targeted microvessel to measure the oxygen tension in the vessel. The RBC velocity and oxygen tension were
measured alternately at 1 Hz by using the shutter installed in the microscope to change between the two light sources (Nd:YAG laser and mercury lamp). After a microvessel was chosen for measurement, the microscope stage was positioned so as to direct the laser into the targeted vessel.

**Spatial resolution.** The phosphorescence intensity and lifetime at various locations in a glass capillary under different laser spots are shown in Fig. 4, A and B. Values are expressed as means ± SD for 20 laser irradiations. The phosphorescence lifetimes at the center were 0.72 ± 0.03 and 0.74 ± 0.05 ms measured alternately at 1 Hz by using the shutter installed in the microscope to change between the two light sources (Nd:YAG laser and mercury lamp). After a microvessel was chosen for measurement, the microscope stage was positioned so as to direct the laser into the targeted vessel.

Even when the phosphorescence intensity was lower for spots near the wall, the lifetime was not affected at either resolution, indicating that stable oxygen measurement can be obtained by irradiating the inside of the microvessel. Values are means ± SD for 20 laser irradiations.

Fig. 3. Microscopic images of cerebral microvessel structure observed in closed cranial window (A), high-magnification blood flow of cerebral microcirculation visualized with FITC-labeled RBCs (estimated to be ~2% of all circulating RBCs) (B), and image of laser light irradiating a targeted microvessel to measure oxygen tension (C). Irradiation times of mercury lamp in B and laser in C were, respectively, 300 ms and 6 ns.

Fig. 4. Effect of laser spot size on measurement accuracy was small. Laser spot diameter, 52 (A) and 27 µm (B), i.e., the spatial resolution, was determined by magnification of objective lens: ×20 (A) and ×50 (B). A narrow glass capillary tube with a 410-µm outer diameter and a 350-µm inner diameter was filled with Pd-TCPP solution and used as a microvessel model. Even when the phosphorescence intensity was lower for spots near the wall, the lifetime was not affected at either resolution, indicating that stable oxygen measurement can be obtained by irradiating the inside of the microvessel. Values are means ± SD for 20 laser irradiations.
with ×20 and ×50 magnification, which was almost equivalent to \( \tau_0 \). The laser spot, as measured from the irradiated images, was \(~52\) and \(27\ \mu m\) in diameter with objective lenses with ×20 and ×50 magnification, respectively. The phosphorescence lifetime remained about the same, even though the intensity decreased as the spot approached the wall because the amount of irradiated Pd-TCPP near the capillary wall was less than at the center in the Z-axial direction.

**Pd-TCPP half-life in blood.** Figure 5 shows the blood concentration after bolus injection of Pd-TCPP. Five minutes after the injection, the plasma concentration of Pd-TCPP was 624.7 \(\mu g/ml\). At 0.5, 1, 3, and 6 h, the concentrations were, respectively, 366.2, 302.5, 222.2, and 155.7 \(\mu g/ml\). Fitting this data to an exponential decay curve yielded 2.23 h for the half-life of Pd-TCPP in blood.

**Phosphorescence emission from cerebral tissue after flushing blood from microvessels.** Figure 6, A and B, shows, respectively, the decay curves of the phosphorescence before and after flushing the blood from the microvessels, and Fig. 6, C and D, shows, respectively, the log-intensity plots of the data in A and B. After the subject rat was killed, the oxygen tension calculated from the phosphorescence decay curves, obtained both before and after flushing, was 0.9 Torr. Correlation coefficient \( r^2 \) for the data in the valid area could be fitted to a single exponential function (0.998), suggesting that phosphorescence decay can be detected with high sensitivity and that lifetime can be calculated with high accuracy. In contrast, the phosphorescence intensity from the microvessel from which Pd-TCPP had been flushed with physiological saline was

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**Figure 5.** Blood concentration curve obtained after bolus injection of Pd-TCPP. Leakage of Pd-TCPP from microvessels to extravascular tissue was observed. Fitting this data to an exponential decay curve yielded the equation \( y = 0.52 \exp(-0.31x) \), \( r = 0.885 \), and 2.23 h for the half-life of Pd-TCPP in blood.

**Figure 6.** Phosphorescence emission from Pd-TCPP in tissue was estimated by irradiating microvessels with a laser beam after flushing the blood from them. A and B: decay curves of phosphorescence before and after flushing. C and D: log-intensity plots of the data shown in A and B. Decay signals used for calculating oxygen tension were taken from “valid data areas” in C and D.
greatly reduced. As described above, the valid data for calculating the phosphorescence lifetime are the data gathered starting 50 μs after data acquisition. The greatest phosphorescence intensity (voltage signal from PMT) in this range was 15.4 mV in blood (Fig. 6A) and 1.9 mV in tissue (Fig. 6B). And shown in Fig. 6D, the sampling signal in the valid data area was extremely low, suggesting that there was very little phosphorescence emission from the extravascular tissue.

**Hemorrhagic shock and resuscitation.** Typical changes in the MAP, RBC velocity, and oxygen tension in venules are shown in Fig. 7A, where we can see that the changes in the venular velocity and oxygen tension were synchronized to the change in MAP. The average values of these variables (n = 4) before bleeding (baseline), during shock, and for 10 min after resuscitation are shown in Fig. 7B. MAP fell from 117.3 ± 9.2 to 38.2 ± 1.4 mmHg during shock and then increased to 119.3 ± 11.9 mmHg after resuscitation. The venular velocity dropped to 43 ± 4% of its baseline value and then increased to 110 ± 12% of its baseline value. The venular oxygen tension fell from 41.9 ± 5.8 to 24.0 ± 9.0 Torr and then increased to 40.0 ± 7.2 Torr.

**DISCUSSION**

We set up and evaluated the effectiveness of our optical system for measuring the RBC velocity and oxygen tension in cerebral microcirculation simultaneously and continuously by using the difference in wavelength and luminescence between fluorescence and phosphorescence. We also examined factors affecting the accuracy of our phosphorescence photometry measurements through in vitro experiments.

**Simultaneous measurement of RBC velocity and oxygen tension.** Because brain cells store hardly any oxygen or substrates, brain function depends on microcirculation. An extremely efficient way to analyze cerebral microcirculatory regulation would thus be to simultaneously measure the RBC velocity and oxygen concentration in microregions. However, there is no convenient equipment suitable for measuring these two parameters continuously and simultaneously. Liss and Liss (8) monitored tissue oxygen pressure in flap microcirculation with electrodes and measured blood flow velocity by using the laser-Doppler method. In macroscopic measurement of tissue oxygenation, these techniques are probably easy to use. With regard to measurement in microcirculation, Parthasarathi and
Lipowsky (9) used oxygen electrodes to measure the oxygen in rat cremaster muscle and used the two-slit technique to measure blood flow in the muscle. Although oxygen electrodes can be used to measure the oxygen tension in various types of tissue, they cannot be used in blood vessels (especially microvessels) because their use requires physical invasion. The phosphorescence decay method using the photochemical reaction between porphyrin dye and oxygen molecules makes it possible to measure oxygen tension noninvasively, to obtain the local oxygen concentration with high spatial resolution, and to obtain the absolute partial pressure instead of a relative value.

Systems based on phosphorescence decay can be roughly divided into two types: those that can be used to draw oxygen mappings and those that can provide one-point measurements suitable for microcirculation study. The former type has been applied to kidney (11), liver (25), the carotid body (7), cells (10), and brain (24) and has also been used in ophthalmology (16) and in cancer studies (23). Shonat et al. (15) imaged hemoglobin saturation and oxygen tension in the cerebral cortex of mice. The latter type can be used to measure the local oxygen tension in tissue or in microvessels by focusing excitation light through the objective lens of a microscope (1, 2, 6, 13, 18). Kerger, Tsai, and colleagues (5, 19, 20) applied it and used the photodiode correlation method to measure blood velocity in dorsal skin microcirculation while using originally developed equipment to measure the diameter of the microvessels. As explained in the introduction, however, when researchers needed to measure both RBC velocity and oxygen tension, they were restricted to two-dimensional tissue samples.

As shown in Fig. 7, both venular velocity and oxygen tension in microvessels were synchronized to changes in blood pressure. The phosphorescence decay method has been used to evaluate the physiological reactions in certain organs during hemorrhagic shock (4, 14). Song et al. (17) reported deterioration in the oxygen tension in cortical tissue caused by hemorrhagic hypotension and recovery with PEG solution in piglet brain, and Yonetani et al. (26) reported a decrease in the hemorrhagic hypotension and recovery with PEG solution in the oxygen tension in cortical tissue caused by the blood. Also, as shown in Fig. 4, the spatial resolution increased when the magnification was increased, meaning that the measurement of the true capillaries is theoretically possible (27). However, increasing the magnification reduces the volume of phosphorescent light received, and, in the case of true capillaries, the volume of Pd-TCPP in the vessels also decreases, so measurement accuracy drops noticeably, meaning that the noise and the error rate will increase.

The time resolution of our measurements was 1 s because the laser irradiation frequency was set at 1 Hz (although the maximum was 110 Hz). Increasing the irradiation frequency and adding the decay curves to smooth them reduces the noise in the phosphorescence signals. However, as shown in Fig. 6, clear decay curves with little noise were obtained by one-shot irradiation, indicating that 200 nJ of pulse energy is sufficient for measurement. Moreover, increasing the pulse irradiation would create excessive singlet oxygen production. Measurement in milliseconds is more than sufficient for a vital reaction. For these reasons, a time resolution of 1 s is sufficient for continuous measurement of oxygen tension.

Reactive oxygen caused by double-wavelength photexcitation. We monitored RBC velocity and oxygen tension in rat brain microcirculation simultaneously by injecting RBCs labeled with FITC and oxygen probe Pd-TCPP and then exciting these probe dyes by irradiation from a mercury lamp and an Nd:YAG laser, respectively. Exciting photosensitive substances like porphyrin dyes inevitably generates reactive oxygen in microvessels, and reactive oxygen, especially singlet oxygen, causes thrombus formation, which can affect blood flow. We tested continual irradiation of capillaries in intestine for 10 min but did not observe any change in flow velocity (data not shown). This result depends on not much light energy being needed to measure phosphorescence and the irradiation time being extremely short. In contrast, the excitation light for visualizing the FITC-labeled RBCs came from a mercury lamp and passed through a band-pass filter (450–490 nm) while the shutter was open. The stronger the excitation light, the sharper the images we can create for measuring RBC velocity. When the microvessels were irradiated with high power, however, flow stasis in the vessels was observed within a few minutes. Singlet oxygen must be suppressed to reduce thrombus formation and stasis as much as possible, so we decreased the irradiation power to <5 mW/cm² and equipped the recording camera with an image intensifier. We observed no stasis and obtained clear blood flow images.

Phosphorescence emission from tissue under microvessels. When the oxygen tension in a single microvessel in the cerebral cortex is measured, the laser light irradiated through the microscope’s objective lens should pass through the microvessel. The measured value may thus be affected by the excitation of Pd-TCPP in the tissue under the target microvessel. The oxygen concentration in brain tissue is considerably lower than that of blood in a microvessel, so any mixing of the phosphorescence emitted from intravascular Pd-TCPP with that emitted from interstitial Pd-TCPP would artifactualy reduce the measured oxygen concentration in a blood vessel. The effect of this mixing on the measured concentration needs to be investigated. Moreover, the effects of a laser light irradiated not from a confocal system but from a conventional incident-light system also need to be investigated. The main reason that the phosphorescence from the tissue irradiated though a microvessel was extremely weak is attributed to the focusing of the laser light by the objective lens. The irradiation angle of laser light depends on the numerical aperture. The microvessel was irradiated at an angle, not vertically, and the profile of the laser intensity was Gaussian. Consequently, the excitation energy is thought to have been focused in the laser spot area and at the focal depth of the objective lens. For instance, at a wavelength of 532 nm, the focal depth of the objective lens we used, which had a numerical aperture of 0.4, is theoretically 1.7 μm. Therefore, even when scattering by blood is taken into consideration, when the light was focused in a microvessel almost 50 μm in diameter, little excitation light...
could have reached the underlying extravascular tissue by
passing through the vessel.

In summary, we measured the RBC velocity and oxygen
tension in cerebral microcirculation by using the differences in
luminescence and wavelength between fluorescence and phos-
phorescence. With this system, we can acquire information
about cerebral microcirculatory distribution and oxygen ten-
sion simultaneously and easily. We evaluated our method by
examining the factors causing measurement error. Because
blood flow and oxygen concentration are associated in organs
using large amounts of oxygen, particularly the brain, the heart,
and the kidney, simultaneous measurement of the microcircu-
larly red cell velocity and local oxygen tension should be
widely applicable to research.

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