Quantitative determination of localized tissue oxygen concentration in vivo by two-photon excitation phosphorescence lifetime measurements

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Submitted 29 December 2003; accepted in final form 7 July 2004

Quantitative determination of localized tissue oxygen concentration in vivo by two-photon excitation phosphorescence lifetime measurements. J Appl Physiol 97: 1962–1969, 2004. First published July 9, 2004; doi:10.1152/japplphysiol.01399.2003.—This study describes the use of two-photon excitation phosphorescence lifetime measurements for quantitative oxygen determination in vivo. Doubling the excitation wavelength of Pd-porphyrin from visible light to the infrared allows for deeper tissue penetration and a more precise and confined selection of the excitation volume due to the nonlinear two-photon effect. By using a focused laser beam from a 1,064-nm Q-switched laser, providing 10-ns pulses of 10 mJ, albumin-bound Pd-porphyrin was effectively excited and oxygen-dependent decay of phosphorescence was observed. In vitro calibration of phosphorescence lifetime vs. oxygen tension was performed. The obtained calibration constants were $k_0 = 356$ Torr$^{-1}$s$^{-1}$ (quenching constant) and $\tau_0 = 550$ µs (lifetime at zero-oxygen conditions) at 37°C. The phosphorescence intensity showed a squared dependency to the excitation intensity, typical for two-photon excitation. In vivo demonstration of two-photon excitation phosphorescence lifetime measurements is shown by step-wise PO$_2$ measurements through the cortex of rat kidney. It is concluded that quantitative oxygen measurements can be made, both in vitro and in vivo, using two-photon excitation oxygen-dependent quenching of phosphorescence. The use of two-photon excitation has the potential to lead to new applications of the phosphorescence lifetime technique, e.g., noninvasive oxygen scanning in tissue at high spatial resolution. To our knowledge, this is the first report in which two-photon excitation is used in the setting of oxygen-dependent quenching of phosphorescence lifetime measurements.

phosphorescence quenching; oxygen measurement; palladium porphyrin; tissue oxygenation

OXYGEN-DEPENDENT QUenchING of Pd-porphyrin phosphorescence, originally developed by Wilson and coworkers (38, 42), is a powerful optical technique for quantitative measurement of oxygen concentrations in biological settings (both in vivo and in vitro). One of the attractive features of the technique is its scalability. Its application ranges from single cell microscopy (18) to intravitral microscopy (45, 31) to fiber-based measurements on organs, like the gut (37) and heart (46) to mention only a few examples. Another interesting feature is the ability to detect heterogeneity in oxygen levels in vivo by analysis of the phosphorescence lifetime distribution (14, 41).

Pd-porphyrin can be excited with pulsed or modulated blue (420 nm) as well as green (530 nm) light, and the oxygen-dependent phosphorescence decay can be measured in the time domain or frequency domain, respectively (16, 39). Analysis of the phosphorescence lifetime allows quantitative determination of oxygen concentration, or oxygen pressure (PO$_2$), by application of the Stern Volmer relationship (38). Although the most efficient excitation is in the blue region (Soret maximum), excitation with green is the most popular for in vivo purposes because of greater penetration depth in tissue. Nevertheless, because tissue absorption is relatively high for visible light, Pd-porphyrin measurements are limited to the outer surface of tissues. For a typical tissue like the gut, the catchment depth for the quenching of Pd-porphyrin phosphorescence was estimated to be in the order of 0.5 mm (33) when excited at 520 nm.

The disadvantage of limited penetration depth has been recognized and is partly circumvented by the development of near-infrared phosphors (40). With excitation ~630 nm and emission ~800 nm, these phosphors have a theoretical penetration depth of several centimeters, giving them, in this respect, the edge over Pd-porphyrin. Nevertheless, the emission signal from superficial layers of tissue is likely to overwhelm that from deeper layers (farther away from the detector and more absorption), resulting in a measurement that is still biased toward the surface. In this way, at least part of the advantage of the infrared phosphors over Pd-porphyrin is tempered.

A second feature of conventional phosphorescence lifetime measurements in vivo, which needs improvement, is the fact that even though a distribution of PO$_2$ values can be obtained (14, 39, 41), the precise localization of these measurements remains unknown. For example, a bimodal oxygen pressure distribution measured on the gut or kidney could reflect the difference in PO$_2$ in arterioles and venules. However, an alternative explanation of the bimodal distribution, in these cases, could be a difference in mean PO$_2$ within different anatomical compartments, like the serosa and mucosa of the gut (44, 33) and the cortex and medulla of the kidney (24). Therefore, oxygen pressure distributions from phosphorescence lifetime distribution analysis can be quite difficult to interpret.

Finding an alternative method to improve penetration depth and simultaneously increase the in-depth differentiating power of the measurement might provide new possibilities and applications for measurements based on both conventional Pd-porphyrin and the more recently developed near-infrared phosphors. Two-photon excitation is a technique that could be a potential candidate for this purpose. Instead of excitation with a single photon [single-photon excitation (SPE)] having an appropriate energy ($E_{SPE}$), it is possible to achieve excitation by 10.220.33.5 on September 7, 2017 http://jap.physiology.org/ Downloaded from
with two photons having energy of \( \frac{1}{2}E_{\text{SPE}} \), under the restriction that they collide with the molecule simultaneously (7). In the case of Pd-porphyrin, successful application of two-photon excitation would allow excitation at wavelengths \( \sim 840 \) nm and \( 1,060 \) nm. Both wavelengths lie in the so-called “tissue optical window,” which is that part of the spectrum where tissue absorption is relatively low (6, 21, 25, 28). Because the emission wavelength (\( \sim 700 \) nm) also lies in the tissue optical window, the use of two-photon excitation could significantly increase the measurement depth. The fact that the phosphorescent probe emits light in the near-infrared region is very advantageous, as can be seen from the field of near-infrared fluorescence imaging (27). Moreover, because of the nonlinear nature of two-photon excitation, measurements with a high spatial resolution in three-dimensional space in thick tissue are possible (34, 43). Successful two-photon excitation of porphyrins and production of singlet oxygen by interaction of oxygen with the excited triplet state has been reported (3, 4, 8, 15). Because photosensitized production of singlet oxygen and oxygen measurements by triplet-state quenching have much in common, it is likely that quantitative oxygen measurement with two-photon excitation should be possible. If feasible, two-photon excitation of oxygen-dependent quenching of Pd-porphyrin could result in the development of new methods for the study of tissue oxygenation. For example, stepwise oxygen measurements, in a confocal type of manner, would allow axial oxygen measurements to be made in different anatomical compartments.

This study was aimed to investigate the possibility of using two-photon excitation for quantitative oxygen measurements based on oxygen-dependent quenching of Pd-porphyrin. To this end four steps were undertaken. First, it was determined whether Pd-porphyrin could be effectively excited by using a focused laser beam from a pulsed infrared laser (1,064 nm). Second, the oxygen dependence of the phosphorescence lifetime was studied by performing calibration experiments. Third, the nonlinear behavior of the two-photon excitation was investigated by measurements of emission light as a function of excitation intensity. Fourth, the possibility of using two-photon excitation phosphorescence lifetime measurements in vivo was studied by applying it to oxygen measurements in rat kidney, where it was applied for longitudinal oxygen scanning through the cortex of the kidney.

**MATERIALS AND METHODS**

**Principle of oxygen-dependent quenching of phosphorescence.** Molecular oxygen is a well-known quencher of excited triplet states. Under zero-oxygen conditions, excitation of a solution of phosphor molecules, like Pd-porphyrin, results in a transient emission of phosphorescent light with decay time \( \tau_0 \). Collision of excited molecules with oxygen leads to energy transfer without light emission (quenching), resulting in a shortening of the overall phosphorescence decay time. The relationship between oxygen concentration and phosphorescence lifetime is given by the Stern-Volmer relationship:

\[
\frac{1}{\tau} = \frac{1}{\tau_0} + k_q [O_2]
\] (1)

where \( \tau \) is the measured phosphorescence lifetime and \( k_q \) is the so-called quenching constant. Equation 1 can be applied directly to the lifetime of the measured phosphorescence signal if the excitation pulse can be considered as a Dirac delta pulse [\( \delta(t) \)]. Otherwise deconvolution of the excitation pulse shape from the photometric signal is necessary before the lifetime determination (26).

**Principle of two-photon excitation.** In contrast to single-photon excitation, two-photon excitation is a nonlinear optical process in which a molecule is excited by two photons instead of a single photon with a double energy (or half the wavelength). By considering the excitation as the rate-limiting step in a chemical reaction consisting of a single-step termolecular process involving one molecule and two photons, one can derive the rate of production of excited-state molecules, \( R_{\text{TPE}} \) (11):

\[
R_{\text{TPE}} = \frac{\delta I}{2A CP^2}
\] (2)

where \( \delta \) is the two-photon cross section, \( I \) is the path-length, \( A \) is the cross-sectional area of the beam (multiplying \( I \) by \( A \) defines the interaction volume), \( C \) is the molar concentration of the excitable molecules, and \( P \) is the power of the excitation beam. In phosphorescence measurements, the intensity of the signal is proportional to \( R_{\text{TPE}} \); therefore Eq. 2 can be rewritten in terms of signal intensity vs. excitation power:

\[
I_0 \propto CP^2
\] (3)

where \( I_0 \) is the measured phosphorescence intensity at time zero, i.e., directly after the excitation pulse. In Eq. 3, constants influencing the absolute value of \( I_0 \), like the molecular constants, excitation geometry, and detection efficiency, are omitted. These constants are intensity independent so that the proportionality sign describes the relation between \( I_0 \) and \( P^2 \). The nonlinear behavior of two-photon excitation provides a means of selective excitation within a three-dimensional space, and the quadratic dependence of emission intensity vs. excitation power is regarded as proof of the two-photon nature of the studied phenomena (4, 12, 17).

**Description of the two-photon setup.** A schematic diagram of the used experimental two-photon setup is given in Fig. 1. Excitation was achieved by using a Q-switched laser operating at 1,064 nm (Laser 1-2-3, Schartz Electro-Optics, Orlando, FL). The laser provided pulses of \( \sim 10\)-ns duration and an energy ranging from 10 mJ per pulse for the in vitro experiments to 100 mJ per pulse in the in vivo

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**Fig. 1.** Schematic diagram of the experimental setup. The laser provided pulses of 10 ns with a wavelength of 1,064 nm at a repetition rate of 10 Hz. F1 is a 1,064-nm laser line band-pass filter. L1 and L2 form a beam expander resulting in a beam width of \( \sim 5 \) mm. Mirror M1 is a standard optical mirror with a central bore hole for passing of the laser beam. Mirror M2 has an enhanced silver surface. L3 is a lens with a focal length of 2 cm. The distance of this lens to the sample can be varied in the z-plane (\( \Delta Z \)) for adjustment of the measurement depth. Filters F2 and F3 are 700-nm band-pass filters. The detector is a red-sensitive photomultiplier tube. Further details are provided in the main text.
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Experiments. The bundle diameter of the laser beam was slightly expanded to a final diameter of 5 mm by a beam expander before being directed to the focusing lens by an optical mirror with an enhanced silver reflection surface (Opto Sigma, Santa Anna, CA). The focusing lens was a single-plane convex lens with a focal length of 2.0 cm. On the basis of Gaussian beam optics, the bundle diameter of 5 mm combined with a lens with a focal length of 2.0 cm results in a focal spot size of 8 μm and a focus length of 94 μm (in air). Assuming a refractive index in tissue of 1.4, the measurement volume is approximately a cylinder with a diameter of 10 μm and a length of 130 μm. The focusing lens was connected to a micrometer screw for manual adjustment of the focal plane, thereby allowing longitudinal measurements to be made. For in vivo application, the reading of the micrometer screw was multiplied by the refractive index of tissue, assumed to be 1.4. Emission light was collected by the same lens and directed toward the photo detector by two mirrors. Selection of the phosphorescence light was achieved by two 700-nm band-pass filters (Oriel, Stratford, CT), positioned in series before the cathode of the photomultiplier tube (PMT, type R928, Hamamatsu, Hamamatsu City, Japan). The output of the PMT was voltage converted by a current-to-voltage converter with subsequent wide-band amplifier (30 MHz) and fed into a digital oscilloscope (Tektronix 2440, Tektronix, Beaverton, OR). The used oscilloscope was featured with 8-bit vertical resolution, with onboard averaging to a microsecond step and provided the averaged data as a 16-bit output on a RS-232 interface. To increase signal-to-noise ratio, phosphorescent traces were averaged on the oscilloscope. Unless stated otherwise, the average of 32 traces was used. The resulting averaged traces were transferred to a computer by serial bus for data collection and analysis by software written in LabView (National Instruments, Austin, TX); see Data analysis.

In vitro calibration experiments. To test the hypothesis that two-photon excitation can be used for oxygen measurements by phosphorescence lifetime measurements with Pd-porphyrin, experiments were performed in a clear Pd-porphyrin solution with varying oxygen concentrations. Pd-meso-tetra(4-carboxyphenyl)porphine (Porphyrin Products, Logan, UT) was bound to BSA (Sigma, St. Louis, MO), as is usual for in vivo use of the probe. Therefore, the Pd-porphyrin was dissolved in DMSO (6 mg/ml) before being added to a buffer solution containing 128.3 mM NaCl, 4.7 mM KCl, 1.0 mM MgCl₂, 0.4 mM NaH₂PO₄, 20.2 mM NaHCO₃, 20.2 mM CaCl₂ (chemicals from Merck, Darmstad, Germany) and 2% BSA. The Pd-porphyrin solution. Two typical examples of recorded phosphorescence decays relatively slowly under these conditions (lifetime > 0.6 ms), the average of the first 10 ms of the transient signal was taken as the value for I₀ (in Eq. 3) without introducing significant error.

Testing for local hyperthermia. A direct comparison of two-photon excitation and one-photon excitation was performed in a closed cuvette. One-photon excitation was achieved by a blue diode-laser (Power Technology, PMT-series laser module with a 4-mW 405-nm laser diode) providing 50-μs pulses at a repetition rate of 10 Hz. The diode laser was focused into the same spot as the two-photon laser. Measurements were performed under zero-oxygen conditions at room temperature. Two-photon excitation and one-photon excitation measurements were performed alternately. For each measurement 128 laser pulses were averaged. The measurements were repeated 10 times.

In vivo experimental protocol. Rats were anesthetized with an intraperitoneal injection of a mixture of ketamine (Nimake, Eurovet, Bladel, The Netherlands; 90 mg/kg), medetomidine (Domitor, Pfizer, Capelle a/d Yssel, 0.5 mg/kg) and atropine-sulfate (Centralfarm, Etten- leen, The Netherlands; 0.05 mg/kg). The body temperature of the rat was kept between 36.5 and 37.5°C by using a heating pad that was thermocoupled by a temperature probe placed in the rectum. For mechanical ventilation, a tracheotomy was performed and a 6-Fr polyvinyl chloride tube (Enteral feeding tube, Vycon, Ecouen, France), cut at 3.5 cm, was placed 0.5 cm in the trachea and secured with a suture. A modified infant ventilator MK-78 (Medec, Wormer- veer, The Netherlands) was used to ventilate the animals. To minimize ventilatory fluid loss, a humidity filter (Humid-Vent Micro, Gibeck, Helsingborg, Sweden) was placed before the ventilation tube. Two vessels were cannulated using 0.5 × 0.9 mm polyethylene vein catheter (Braun). Catheters were filled with 0.9% NaCl solution (Baxter, Utrecht, The Netherlands) with 25 IU of heparin (Leo Pharma, Breda, The Netherlands). The right carotid artery was catheterized for continuous monitoring of mean arterial blood pressure and heart rate. In addition, the jugular vein was cannulated for administration of Pd-porphyrin solution. Pd-meso-tetra(4-carboxyphenyl)porphine (Porphyrin Products) coupled to human serum albumin (50 mg in 10 ml 4% albumin solution, 4 mM Pd-porphyrin solution, pH adjusted to 7.4 with HCl) was infused to a final dose of 2 ml solution in 20 min. A midline laparotomy was performed for exposure of the right kidney. To achieve variations in the global oxygen concentration in the kidney, the fraction of inhaled oxygen (FIO₂) was varied from 37 to 100%. All investigations were carried out in accordance with the guidelines of the animal ethical committee of our institution after approval of the experimental protocol. For the in vivo experiments, 64 traces were averaged per measurement at a repetition rate of 10 laser pulses per second. The illumination time was 6.4 s per measurement.

Data analysis. Software for data transfer from the oscilloscope and lifetime analysis of phosphorescence decay curves was written by use of Labview 5.1 (National Instruments, Austin, TX) running on a personal computer (Pentium II, 300 MHz) with Windows NT 4.0 (Microsoft, Bellevue, WA). For calculation of the phosphorescence lifetimes, a monoexponential model, incorporating a baseline, was fitted on the data. The fit was performed by using the nonlinear Marquard-Levenberg fit procedure available in the Labview package, as previously described (26). The data are presented as mean values with standard deviation unless stated otherwise.

RESULTS

Pulsed excitation at 1,064 nm was successful in causing phosphorescence emission from a flow-through cuvette with Pd-porphyrin solution. Two typical examples of recorded traces are shown in Fig. 2. Figure 2A shows the raw data and the result of the fit procedure. Figure 2B shows the residue of
the fit, calculated by subtracting the fitted curve from the raw data. The prolonging of the tail at decreasing oxygen concentration is clearly visible. Moreover, the well-known monoexponential behavior of the phosphorescence decays is preserved under pulsed excitation at 1,064 nm. To prevent overlap of the curves (by the noise), we used the average of 64 laser pulses for this figure. In general, averaging 16–32 traces delivered sufficient signal-to-noise ratio for monoexponential curve fitting without the occurrence of instabilities in the fitting algorithm.

For quantitative measurement of oxygen concentrations by use of two-photon excitation, it is essential to demonstrate good correlation between the oxygen concentration and the reciprocal value of the lifetime, as seen with single-photon excitation. Figure 3 shows the results of the calibration experiments, performed to determine the oxygen dependence of the lifetime with two-photon excitation. An excellent correlation between the reciprocal lifetime and oxygen concentration exists. The calibration constants at 37°C were determined to be $k_q = 356 \text{Torr}^{-1}\text{s}^{-1}$ and $\tau_0 = 550 \mu\text{s}$ (direct measurement under zero-oxygen conditions).

To make sure that the observed phosphorescence was due to two-photon excitation, the squared dependence of intensity vs. excitation power (Eq. 3) was tested. Attenuation of the laser light in small steps was achieved by varying the number of microscope slides put in series in the laser bundle. Light attenuation per slide was determined to be 6.7% (data not shown). The relative initial intensity of phosphorescence as function of the relative laser power is shown in Fig. 4. Both values are relative to the maximum values measured without attenuation. The solid line is the theoretical squared curve. It is clear that the phosphorescence intensity is well described by Eq. 3, proving that we achieved true two-photon excitation.

Possible contributions of leakage of green laser light (532 nm) to the phosphorescence yield were excluded by two additional tests. We started with the direct measurement of spectrum of the laser output, using an Ocean Optics SD2000 spectrograph. No evidence of 532-nm leakage was present (data not shown). In a second test, we compared the phosphorescence yield before and after placing a 720-nm long-pass...
filter (95% transmission for wavelengths >720 nm, <1% transmission for wavelengths < 20 nm) into the laser beam. Measurements were performed at room temperature and under zero-oxygen conditions. Placing the long-pass filter resulted in only a small decrease in phosphorescence yield. The remaining phosphorescence yield was 88 ± 11% (n = 10) of the original signal, proving the hypothesis of two-photon excitation. We therefore have excluded significant contribution of 532-nm leakage as explanation of our apparently high phosphorescence yield.

Because of the high power density in the measurement volume (on the order of TW/cm²) and significant one-photon absorption at 1,064 nm (7), a concern about the possible occurrence of local hyperthermia inside the measurement volume exists. Therefore, direct comparison between lifetimes at two-photon and one-photon excitation was performed. The hypothesis was that a significant temperature jump would cause disturbance of the phosphorescence signal and a decrease in lifetime. Both two-photon and one-photon excitation resulted in visibly undistorted single-exponential phosphorescence traces. Two-photon excitation resulted in a phosphorescence lifetime of 699.1 ± 8.7 μs. One-photon excitation resulted in a lifetime of 705.4 ± 6.6 μs. Therefore, a small, but nonsignificant (P = 0.08), difference in τ₀ exists. The shortening of τ₀ under two-photon excitation could be caused by a small thermal effect. If the difference of ~6 μs is assumed to be real, this would indicate a temperature elevation of ~1.5°C (32).

As an example of the use of two-photon excitation phosphorescence lifetime measurements for in vivo applications, we tested our setup on rat kidney in vivo. On a fixed focus depth of 280 μm, lowering the FiO₂ from 37 to 10% demonstrated the existence of two-photon excited oxygen-dependent phosphorescence. Examples of obtained phosphorescence traces under these circumstances are shown in Fig. 5A. While the rat breathed 37% oxygen, the measured lifetime was 58 μs, corresponding to a PO₂ value of 43 Torr. Lowering the FiO₂ to 10% results in an extreme PO₂ drop to 3.6 Torr, as can be derived from the prolonging of the lifetime to 325 μs. Once it was clear that two-photon excitation was feasible in the rat kidney, a longitudinal oxygen scan was performed. The focusing depth was manually adjusted in small steps from the organ surface to a measurement depth of ~2.2 mm. Starting with the focus point above the organ surface, the surface was easily identified by a sudden appearance of phosphorescence at the moment the focus point entered the tissue. Although at a depth of 2.2 mm still more than sufficient phosphorescence could be detected, deeper measurements were not possible with our specific setup because of collision of the focusing arm with anatomical structures. The resultant PO₂ measurements as function of measurement depth are shown in Fig. 5B. The first 600 μm is a zone with marked variations in oxygen pressure around an average of 50 Torr. At a depth of more than 600 μm, there is a zone with a lower average PO₂ of 35 Torr and less variation in oxygen pressure.

DISCUSSION

This study shows, to our knowledge, the first use of two-photon excitation for oxygen measurements based on oxygen-dependent quenching of phosphorescence. The main findings in this study can be summarized as follows: 1) Pd-porphyrin can be effectively excited at 1,064 nm, 2) the correlation between the reciprocal phosphorescence lifetime and oxygen concentration is preserved after excitation in the infrared, 3) excitation of Pd-porphyrin at 1,064 nm shows an excellent nonlinear optical behavior, consistent with the theory of two-photon excitation, 4) two-photon excitation phosphorescence lifetime measurements can be successfully applied in vivo, and 5) longitudinal oxygen scanning with two-photon excitation is able to detect axial heterogeneity in PO₂ levels in rat kidney.

In vitro, emission of phosphorescent light could be readily detected after pulsed excitation at 1,064 nm. This wavelength was imposed by the available laser system, but 1,064 nm is likely to be not the most optimal excitation wavelength (see discussion in the next paragraph). Nevertheless, the phosphorescence yield was adequate to be detected by analog detection, whereas in two-photon microscopy photon counting methods are mandatory (7). The relatively high phosphorescence yield in our study is most likely due to the relatively large measurement volume in our study. We calculated our measurement...
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volume to be more than 3,000 times larger than would be the case in a diffraction limited setup in which the laser bundle is focused into a spot of ~1 μm diameter. There is an excellent correlation between PO₂ and reciprocal lifetime, as predicted by the Stern-Volmer relationship. Moreover, the determined quenching constants at 37°C (k₉ = 356 Torr⁻¹ s⁻¹ and τ₀ = 550 μs) with the described setup correspond very well to previously reported values measured with a pulsed laser system operating at 535 nm (29). The true two-photon nature of the excitation at 1,064 nm is clearly demonstrated by the quadratic dependence of phosphorescence intensity against the excitation intensity, a criterion generally used to prove two-photon excitation (2, 4, 12, 17, 22). Contributions to the phosphorescence yield by leakage of visible light from the laser were excluded by additional tests. These results show the feasibility of quantitative oxygen measurements with two-photon excitation phosphorescence lifetime measurements.

In the mid 1980s, Bodaness and King (4) demonstrated that hematoporphyrin derivative could be effectively two-photon excited with a 20-ns Q-switched Nd:YAG laser. However, one year later they demonstrated a 100-fold increase in two-photon efficiency in hematoporphyrin derivative with excitation at 750 nm (3). They attributed this 100-fold enhancement in the effective two-photon excitation cross section to a resonant enhancement contribution from the S₁ state. Recently, Karotki et al. (19) also showed that tuning the excitation wavelength from λₑₓ = 1,100–1,400 nm to λₑₓ = 700–800 nm enhances the two-photon cross section by almost two orders of magnitude as a result of near-resonant Q transition. Our laser system was limited to 1,064 nm output, but it appears that probably a major improvement in performance can be achieved by choosing a more effective excitation wavelength. For our application of in vivo oxygen measurements, it is relevant to note that a change of wavelength could indeed improve performance while keeping the advantage of making use of the tissue optical window (6, 21, 25, 28).

Another gain in performance can be expected by using shorter laser pulses to increase photon flux per unit time. Although several reports on two-photon excitation in porphyrins by nanosecond pulsed laser sources exist (3, 4, 22), more recent studies make use of femtosecond pulsed laser systems (see, e.g., Refs. 7–9, 15), providing effective two-photon excitation with a reduced average laser power. This is of importance for reduction of hyperthermia caused by high power densities and local light absorption, a factor that is attributed to at least a part of the reported tissue damage in two-photon excitation photodynamic therapy (15). For our application of oxygen measurements, occurrence of hyperthermia could have a second important consequence. Temperature is known to have a significant influence on phosphorescence quenching, leading to temperature dependency of the calibration constants (32). A short local temperature jump could therefore disturb the phosphorescence signal and lead to erroneous oxygen measurements. In our study, hyperthermia appears to be a factor of limited importance (no significant difference between τ₀ with two-photon excitation vs. one-photon excitation). From our results we calculated an increase in local temperature of at most 1.5°C. This allows oxygen measurements to be made within the accepted 5% overall error of phosphorescence quenching measurements (32). Also, it is not likely that this mild increase in temperature will cause tissue destruction with our relatively short (compared to photodynamic therapy) illumination of tissue.

In our study, Pd-meso-tetra(4-carboxyphenyl)porphine was used because to date it is the most widely used porphyrin for in vivo oxygen measurements. However, it is a metalloporphyrin with a centrosymmetrical chemical structure. Centrosymmetrical molecules have in general low two-photon cross sections (see, e.g., Ref. 19). Two-photon excitation is of relevance not only for biomedical applications, such as two-photon fluorescence microscopy (7), but also for other applications in which an increase in three-dimensional spatial selectivity is advantageous, like high-density optical data storage and microfabrication (5). Recent reports on designing molecules with increased two-photon cross sections were published (1, 9). Efforts are also being made to increase the two-photon cross section of porphyrins by changing the chemical structure to increase its asymmetry (19). These efforts could lead to phosphorescent porphyrins with large two-photon cross sections, leading to a major performance gain in our application of in vivo oxygen measurements.

Several reports of in vivo use of two-photon excitation are available in literature. A recent example is cortical imaging in a mouse through the intact skull (43). In an earlier study, studying the feasibility of two-photon excitation in photodynamic therapy, Lenz (22) demonstrates two-photon excited fluorescence from hematoporphyrin derivative in a rat ear. In his study, Lenz used a relatively simple setup and Q-switched Nd:YAG laser providing pulses of 10 ns at 1,064 nm (i.e., comparable to our laser system). Therefore, we felt confident that successful use of our two-photon excitation setup in vivo should be possible. Indeed, the experiments performed on rat kidneys clearly demonstrated the feasibility of two-photon excitation phosphorescence lifetime measurements in vivo. After intravenous administration of Pd-porphyrin, phosphorescence could be detected in the kidney. Variations in FI O₂ prove the oxygen-measuring capability of our technique, because lowering the FI O₂ from 37 to 10% results in prolonging of the phosphorescence lifetime. The typical two-photon excitation phenomenon of selective excitation within a three-dimensional space is shown by the longitudinal oxygen scan through the cortex. The two-photon excitation scan shows significant heterogeneity in PO₂ values, of which the most striking is the sudden decrease in PO₂ at a depth of more than 600 μm. Both the absolute PO₂ values as the heterogeneity pattern compare very well to previously reported results, measured with micro oxygen electrodes (24).

Methodological limitations. Two-photon excitation is generally used for its deeper penetration in tissue, and, indeed, in this study we readily achieved oxygen measurements at a depth of 2 mm in kidney. Signal intensity and the quality of focused laser light rapidly decrease with increasing depth of measurement because of disturbances in vivo. Scattering and absorption lead to a decrease of two-photon-generated emission. Furthermore, the nonideal optical conditions in vivo are likely to have their impact on the resolution of the measurement in deeper layers of tissue. We therefore expect the method to be limited to a measurement depth of several millimeters. Further research is needed to explore the possibilities and limitations of this technique, e.g., by means of Monte Carlo simulations (10).

Advantages and applications of this technique. Several techniques have been described in the literature to measure quan-
titative tissue oxygen levels in vivo. These techniques vary from the polarographic oxygen electrode to more recently developed techniques like electron paramagnetic resonance, recently reviewed by Swartz (36). Each technique has its own advantages and disadvantages. Although oxygen electrodes can be miniaturized and are capable of measuring tissue oxygen levels at high spatial resolution and penetration depths of centimeters (24), some tissue destruction is inevitable. Electron paramagnetic resonance on the other hand is relatively noninvasive but is limited in spatial resolution to the size of the paramagnetic particles, typically one hundred to several hundred micrometers (35, 36). Oxygen-dependent quenching of phosphorescence has its strength in its scalability, ranging from single-cell measurements in vitro (18, 20) to measurements in single microvessels in intravitral microscopy (13, 30, 31, 45) to macroscopic oxygen measurements on organ surfaces (33, 37, 42). Its main limitations are that 1) the lack of discriminating power in the z-plane in intravitral microscopy leads to complications in the interpretation of PO2 measurements due to contributions to the signal of out of focus regions, as discussed by Golub and Pittman (13), and 2) the limited penetration depth of the excitation light leads to a bias toward the outer surface in macroscopic measurements (33). The proposed two-photon excitation phosphorescence lifetime technique essentially overcomes these limitations. Combined with intravitral microscopy it potentially provides a tool for high-resolution measurements (34), allowing true three-dimensional measurements of oxygen profiles around (and inside) microvessels. On a more macroscopic scale, improved penetration depth and discrimination power allow for an oxygen electrode-like capability of oxygen profile measurements (as demonstrated), without the disadvantage of tissue damage. This can be applied, e.g., for PO2 measurements in a selective part of an organ like the kidney or the gut. This is of relevance because, e.g., in the kidney or the gut. This is of relevance because, e.g., in the

ACKNOWLEDGMENTS

The authors would like to thank Anneke Koeman of the department of Physiology of the Academic Medical Center in Amsterdam, The Netherlands for help with the animal experiments.

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