Measuring microvascular and mitochondrial oxygen tension: novel techniques for studying tissue oxygenation
Mik, E.G.
Chapter 4

Dual-Wavelength Phosphorimetry for Determination of Cortical and Subcortical Microvascular Oxygenation in Rat Kidney

T. Johannes, E.G. Mik and C. Ince

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ABSTRACT
This study presents a dual-wavelength phosphorimeter developed to measure microvascular PO\(_2\) (\(\mu\)PO\(_2\)) in different depths in tissue and demonstrates its use in rat kidney. The used phosphorescent dye is Oxyphor G2 with excitation bands at 440 and 632 nm. The broad spectral gap between the excitation bands combined with a relatively low light absorption of 632 nm light by tissue results in a marked difference in penetration depths of both excitation wavelengths. In rat kidney, we determine the catchments depth of the 440-nm excitation to be 700 \(\mu\)m, whereas the catchments depth of 632 nm is as much as 4 mm. Therefore, the measurements differentiate between cortex and outer medulla, respectively. In vitro, no difference in PO\(_2\) readings between both channels was found. On the rat kidney in vivo, the measured cortical \(\mu\)PO\(_2\) was on average 20 Torr higher than the medullary \(\mu\)PO\(_2\) over a wide PO\(_2\) range induced by variations in inspired oxygen fraction. Examples provided from endotoxemia and resuscitation show differences in responses of mean cortical and medullary PO\(_2\) readings as well as in the shape of the PO\(_2\) histograms. It can be concluded that oxygen-dependent quenching of phosphorescence of Oxyphor G2 allows quantitative measurement of \(\mu\)PO\(_2\) noninvasively in two different depths in vivo. Oxygen levels measured by this technique in the rat renal cortex and outer medulla are consistent with previously published values detected by Clark-type oxygen electrodes. Dualwavelength phosphorimetry is excellently suited for monitoring \(\mu\)PO\(_2\) changes in two different anatomical layers under pathophysiological conditions with the characteristics of providing oxygen histograms from two depths and having a penetration depth of several millimeters.

INTRODUCTION
Due to limitations in available technical methods, not much is known about the distribution of tissue oxygenation within different organs. The oxygen-dependent quenching of Pd-porphyrin phosphorescence first introduced by Wilson and coworkers in 1987 (29) gives the unique possibility to quantitatively determine oxygen pressures within living tissues in a nondestructive way. Oxygen detection by this method includes measurements in intravital microscopy (24) up to fiber-based measurements on organ surfaces like the heart (30, 38), brain (36), gut (25, 28), or kidney (17) in animal models.

Pd-meso-tetra-(4-carboxyphenyl)-tetrabenzoporphyrin (Oxyphor G2) is a relatively new phosphor that is excellently suited for oxygen measurements in vivo (5). It is highly soluble in blood plasma, where it binds to albumin and confines to the circulation, as demonstrated recently for Oxyphor R2 in rat skeletal muscle (18). Oxyphor G2 has its absorptions maxima at 440 and 632 nm and its emission near 800 nm. The excitation wavelength of 632 nm combined with emission in the infrared spectrum allows much deeper tissue penetration than could be achieved with the most widely used phosphor to date, Pd-porphyrin [Pd-meso-tetra-(4-carboxyphenyl)porphine]. A very recent example of the usability of Oxyphor G2 has been the measurement of the distribution of microvascular oxygen pressures in solid tumors by Wilson and coworkers (37).

Although the measurement of the distribution of microvascular oxygen pressures is a powerful means to gain insight into the heterogeneity in oxygen levels within a tissue or organ, we recently argued that the interpretation can be quite difficult (16). Especially in tissues with different anatomical layers, one can never be sure whether the found distribution reflects heterogeneity in microvascular PO\(_2\) (\(\mu\)PO\(_2\)) within a layer or a difference in oxygenation between the two anatomical layers. Additional information could then be retrieved from measurements from different depths. A convenient way to do this is by using different excitation wavelengths (35). Other than the deeper penetration depth, another advantage of Oxyphor G2 is the huge spectral difference in excitation wavelengths. Considering the fact that 440-nm light in contrast to 632-nm light is highly absorbed by tissue, this would allow excellent discrimination between superficial and deeper tissue layers. Although the idea of using different wavelengths for obtaining information from different tissue depths in itself is not new (21, 35), it has not been applied for continuous and simultaneous monitoring of \(\mu\)PO\(_2\) at different tissue depths. If
feasible, it could provide a very useful extension to the already powerful method of oxygen-dependent quenching of phosphorescence.

To profit from the above-mentioned possibilities, we designed and constructed a dual-wavelength phosphorimeter. The hypothesis was that application of this phosphorimeter in combination with Oxyphor G2 would allow the measurement of μPO2 in two different depths. This study was designed to validate and show the feasibility of dual-wavelength phosphorimetry to examine changes in oxygenation in two different anatomical layers: the kidney cortex and medulla. Therefore, four steps were undertaken. First, we developed a dual-wavelength phosphorimeter that allows exciting Oxyphor G2 at 440 and 632 nm near simultaneously. Second, in vitro calibration experiments were done to exclude hardware-induced inter-channel differences. Third, the real penetration depth of both excitation channels was determined by ex vivo experiments in rat kidneys. Fourth, for evaluation of the accuracy of the oxygen measurements in vivo, experiments in rats were carried out in which the fractional inspired oxygen concentration (FiO2) was stepwise reduced. Finally, to demonstrate the usability of this method to detect changes in μPO2 in a pathophysiological relevant setting, we report on cortical and medullar PO2 values and oxygen distributions in rats during endotoxemia and resuscitation.

MATERIALS AND METHODS

Phosphorescence lifetime measurements. Oxygen-dependent quenching of phosphorescence allows the nondestructive detection of changes in μPO2. Oxyphor G2 [Pd-meso-tetra-(4-carboxyphenyl)-tetrabenzoporphyrin; Oxygen Enterprises, Philadelphia, PA] is a water-soluble phosphorescent dye that binds to albumin. Oxyphor G2 is a large molecule (2.4 kDa), has a strong negative charge, and therefore stays primarily in the microcirculation (18, 37). When excited by a light pulse, the phosphorescence (~800 nm) intensity decreases at a rate dependent on the surrounding oxygen pressure. The relationship between the measured decay time and the PO2 is given by the Stern-Volmer relation:

\[
PO_2 = \frac{1 - \frac{1}{\tau}}{k_q} \tag{1}
\]

where \(\tau\) is the measured decay time, \(\tau_0\) is the decay time at an oxygen pressure of zero and \(k_q\) is the quenching constant.

Equation 1 describes the relationship between the PO2 and the phosphorescence lifetime in case of a homogeneous oxygen distribution. However, large nonuniformities in oxygen pressure exist in vivo, resulting in a phosphorescence signal that in general can be described by an integral over an exponential kernel (6, 32, 33). In this paper, we use the approach published by Golub et al. (6) in which the heterogeneity in oxygen pressure is analyzed by fitting distributions of quencher concentration to the phosphorescence data. According to their work, the fitting function for a simple rectangular distribution with a mean PO2 \(Q_m\) and a PO2 range from \(Q_m - \delta\) to \(Q_m + \delta\) is

\[
y(t) = \int_{0}^{t} \exp(-\lambda t) f(\lambda) \tag{2}
\]
where \( Y_R(t) \) is the normalized phosphorescence data, \( k_0 \) is the first-order rate constant for phosphorescent decay in the absence of quencher, and \( \delta \) is half the width of the rectangular distribution from which the standard deviation (\( \sigma \)) can be calculated from the relationship

\[
\sigma^2 = \frac{\delta^2}{3}
\]

Moreover, Golub et al. (6) showed that a detailed recovery of the oxygen distribution can be obtained by assuming that the phosphorescence signal can be described by a sum of rectangular distributions with adequately small chosen \( \delta \), resulting in the following fit equation:

\[
Y^*(t) = Y(t) \left[ \exp\left( k_q \frac{\delta t}{\sinh(k_q \delta t)} \right) \right] = \sum w_i \exp(-k_q Q_i t)
\]

where \( Y(t) \) is the normalized phosphorescence data and \( w_i \) is the weight factor for the according bin with central \( PO_2 \) \( Q_i \) and width \( 2\delta \) (\( w_i \geq 0 \) and \( \sum w = 1 \)).

The currently developed phosphors for oxygen measurements possess different excitation bands with a wide spread in excitation wavelengths. For example Oxyphor G2 has excitation bands of ~440 and 632 nm (5). Combined with tissue optical properties, this feature can be used for achieving measurements with different penetration depths. Described in the following paragraph is a phosphorimeter that we developed to perform this type of measurements in a near-simultaneous fashion.

**Description of the phosphorimeter.** The dual-wavelength phosphorimeter had to be able to measure phosphorescence lifetimes with near-simultaneous excitation at two different wavelengths in an automated fashion. There should especially be no need for the operator to physically have to change fiber-optic connections or filters during the experiment. The device and its construction should be simple and easily reproducible. Figure 1 shows schematically the constructed device. The central part of the phosphorimeter is a commercially available randomized trifurcated fiber optic (model no. 77536, Oriel, Stratford, CT). This fiber-optic light guide has a common bundle diameter of 5.5 mm. The fiber-optic light guide has a numerical aperture of 0.56 and an acceptance cone of 68°. The resulting light bundle is slightly diverging and, therefore, does not have spatial discriminating power in itself. Two branches of the fiber-optic light guide are used for the two excitation pathways. Excitation light is provided by two identical flash lamp modules (type C5605, Hamamatsu, Hamamatsu City, Japan), providing pulsed light with a light pulse duration of < 5 \( \mu \)s. The output power at the end of the fiber-optic light guide does not exceed 1 \( \mu \)J per pulse for both wavelengths, as measured with a power meter (Ophir model DGX, Ophir Optics, Jerusalem, Israel). Excitation light is filtered by two band-pass filters (Edmund Optics, York, UK) with central wavelengths of 440 and 632 nm for the respective excitation path. The remaining third branch of the fiber-optic light guide is used for returning the emitted phosphorescence back to the device. The signal is filtered by a 800-nm band-pass filter (Edmund Optics, York, UK). All band-pass filters have a full width at half maximum of 10 nm. The detector is an infrared-sensitive photomultiplier tube fitted in an un gated socket (type PS1250/12F, Thorn EMI Electron Tubes, Ruislip, UK). The current from the photomultiplier tube is voltage converted by a home-built, wide-band (30 MHz) transresistance amplifier. The whole device is under control of a computer containing a 1-MHz multifunction data-acquisition board (DAQ-board, PCI-MIO-16e1,
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National Instruments, Austin, TX). The data-acquisition board controls the triggers to the flashlamps and samples the phosphorescence signal with a sampling frequency of 1 MHz. The trigger pulse that triggers the flashlamp is internally directed to the data-acquisition clock (the used DAQ-board allows for this), meaning that data acquisition is started at the rising flank of this trigger pulse. A short delay of 3 µs exists before the flashlamp is actually fired. The resulting three data points without signal are omitted from the analysis. The used flashlamp units have a very stable time delay and moreover show only a time jitter of < 300 ns between the triggering and the flash occurrence. For our purposes, we regard this as insignificant, and therefore we allowed ourselves to omit the optical trigger feedback system used in our laboratory’s previously described phosphorimeter (15). To improve signal-to-noise ratio, the average signal of 50 flashes is used for analysis. The flashlamps fire with a frequency of 50 Hz, so the duration of one measurement on one wavelength is 1 s. The acquisition of the 632-nm excitation is started immediately after the acquisition of the 440-nm excitation has finished, so no more than a 1-s time interval exists between the two measurements. Before phosphorescence lifetime analysis, the adverse effect of the excitation pulse shape was removed using excitation pulse deconvolution, of which the details can be found in a previous study (15). For the excitation pulse deconvolution, both the blue and red backscattered flashlamp signals were recorded from the kidney before infusion of Oxyphor G2 into the animal. This results in a correction for a difference in flashlamp profile at 440 and 632 nm and also corrects for aging of the flashlamp. The phosphorescence lifetime analysis was performed by nonlinear monoexponential fitting (Eq. 1), nonlinear fitting of the rectangular distribution (Eq. 3), and the \( \text{PO}_2 \) histogram recovery method from Eq. 5. Analysis software for the monoexponential and rectangular distribution fit procedures was written in LabView (version 6.1, National Instruments, Austin, TX). The \( \text{PO}_2 \) histogram recovery was performed with GraphPad Prism (version 4, GraphPad Software, San Diego, CA) using a nonlinear fit procedure with constrained parameters (\( w_i \geq 0 \)).

FIGURE 1 Schematic drawing of the dual-wavelength phosphorimeter. A detailed description can be found in MATERIALS AND METHODS. BP1, BP2, and BP3 are band-pass filters with the shown central wavelength. L1, L2, and L3 are coupling lenses. PMT is an infrared sensitive photomultiplier tube, I/V CONV AMP is a wide-band current-to-voltage converting amplifier, and DAQ-board is a 12-bit data-acquisition board. The optical fiber is a randomly distributed trifurcated fiber-optic light guide.
**In vitro calibration.** In vitro calibrations were performed in a bicarbonate buffer containing 2% bovine serum albumin (Sigma, St. Louis, MO) and a final concentration of 10 µM Oxyphor G2. PO2 values were regulated using a system consisting of gas-flow controllers, an oxygenator, and a closed-loop recirculation system. A detailed description of this system can be found in a previous publication (16). Experiments were performed at 37°C. The pH value of the solution was checked during the experiments and remained at 7.4 ± 0.1.

**Ex vivo penetration depths experiments.** To demonstrate that exciting Oxyphor G2 at 440 and 632 nm results in PO2 reading from different tissue layers in the rat kidney and can ultimately be used to gain information from the cortex and medulla, respectively, experiments were performed ex vivo. The idea was to determine the actual capability of exciting the phosphor at a certain depth for both wavelengths by placing slices of kidney tissue between the fiber-optic light guide and an Oxyphor G2-containing chamber. To this end, the vessels close to the hilus of the rat kidney were ligated, and the kidney was removed directly after the animal died. Either 500- or 1000-µm longitudinal kidney slices were made by using a razor blade-like cutting device. For measurements of the penetration depth of the two different wavelengths (440 and 632 nm), a slice of kidney tissue was placed on top of a chamber filled with Oxyphor G2 (50 µM concentration in a 4% albumin solution). Zero-oxygen conditions within the chamber were created by an enzymatic oxygen removal by using ascorbate-oxidase and ascorbic acid (13). The experiments were performed at room temperature. The kidney slices were placed on top of each other following the anatomy from cortex toward the medulla. The actual thickness of the total reconstructed tissue was always measured using a micrometer. Phosphorescence returning through the kidney tissue after excitation with either 440 or 632 nm was measured for each additional piled-up slice to a total thickness of 5 mm. To take into account the fact that the kidney slices contained no phosphor that would clearly contribute to the overall absorption, the extinction coefficient of Oxyphor G2 at 440, 632, and 800 nm was measured using a Specord UV VIS S10 absorption meter (Carl Zeiss, Oberkochen, Germany). The measured extinction coefficients were used to calculate estimations of additional attenuation of the overall signal due to the presence of the phosphor using Beer's law:

\[
I(d) = I_0 \cdot \exp(-\mu_z d) \quad z = a \cdot d \quad a > 1.0
\]

where \(I(d)\) is the remaining signal at depth \(d\), \(I_0\) is the signal without attenuation, \(\mu_z\) is the extinction coefficient, and \(z\) is the actual path length traveled by the light. Because of multiple scattering in tissue, \(z\) is several times longer than \(d\).

**Animal preparation for in vivo experiments.** The experiments of this study were approved by the ethical committee for animal subjects of the Academic Medical Center at the University of Amsterdam. Care and handling of the animals were in accordance with the guidelines for Institutional and Animal Care and Use Committees. The measurements were performed in seven Wistar male rats (Charles River) with a body weight of 357 ± 35 g (mean ± SD).

Rats were anesthetized, mechanically ventilated, and monitored as described recently by our group (20). In short, induction of anesthesia was performed by intraperitoneal injection of a mixture of ketamine (90 mg/kg, Nimetek, Eurovet, Bladel, The Netherlands), medetomidine (0.5 mg/kg, Domitor, Pfizer, Capelle a/d Ijssel, The Netherlands), and atropine (0.05 mg/kg, Centrafarm, Ettenleur, The Netherlands), and anesthesia was maintained by continuous intravenous infusion of ketamine (50 mg•kg•h-1). To compensate for fluid loss, Ringer lactate (Freeflex, Fresenius Kabi, The Netherlands) was administered continuously at a rate of 15 ml•kg•h-1. Kidney preparation was performed via a 3-cm incision of the left flank. The kidney was exposed, decapsulated, and immobilized in a Lucite kidney cup (K. Effenberger, Geraetebau, Pfaffing, Germany), a perivascular flow probe (type 0.7 mm
V-Series, Transonic Systems, Ithaca, NY) was placed around the left renal artery and connected to a flowmeter (T206; Transonic Systems). Throughout the entire experiment, the operation field was covered with Saran wrap (Downbrands) to prevent evaporation of body fluids. The temperature of the kidney surface was measured and kept at ~37°C.

After surgery, the fiber-optic light guide for phosphorescence lifetime measurements was placed 1 mm above decapsulated kidney surface, and a 15-min intravenous infusion of Oxyphor G2 (1.2 ml/kg of a 5 mg/ml stock solution) was started. After a stabilization time of 50 min, the renal µPO₂ was continuously measured during the entire experiment.

**In vivo feasibility testing.** For testing the reproducibility of renal oxygen measurements over a wide range of different PO₂ in five animals, the FiO₂ was reduced in three steps from 1.0 to 0.1. Changes in FiO₂ have previously been shown to be an effective means to change µPO₂ values (19). Each step comprised an equilibration time of 10 min.

**In vivo experiment during lipopolysaccharide bolus injection.** It is well known that lipopolysaccharide (LPS) infusion leads to marked reduction in blood flow to the kidney and possibly to redistribution of flow from cortex to medulla (8, 9). To test the relevance of simultaneous measurements in two different depths, we studied the response of cortical and medullary PO₂ in a rat receiving an intravenous bolus injection of LPS (20 mg/kg; serotype 0127:B8, Sigma) to induce septic shock.

**In vivo experiment during fluid resuscitation.** For demonstration of the time course of the oxygen measurements under clinically relevant conditions, one rat received fluid resuscitation with 200-kDa hydroxyethyl starch (HAES-steril 6%, 20 ml/h, Fresenius Kabi, The Netherlands) directly after a 1-h intravenous LPS infusion was stopped (15 ml•kg⁻¹•h⁻¹; serotype 0127:B8, Sigma).

**Data analysis.** Labview 6.1 (National Instruments, Austin, TX) was used to develop a software environment to allow data acquisition and analysis of the phosphorescence decay curves. Oxygen histograms were analyzed using the GraphPad Prism package (version 4, GraphPad Software, San Diego, CA). Values are presented as means ± SD, unless otherwise indicated.

![Figure 2](image-url) **Figure 2** Calibration of both channels. Shown is the reciprocal lifetime v.s the applied PO₂. ■ Blue channel exciting at 440 nm ▲ Red channel exciting at 632 nm. The solid line is calculated from the determined quenching constants k_q = 270 mmHg⁻¹ s⁻¹ and b₀ = 240 µs.
RESULTS

Figure 2 shows the results of the in vitro calibrations performed in a 10 μM Oxyphor G2 solution. An excellent correlation between the reciprocal lifetime and PO\textsubscript{2} was found for both channels measuring at different excitation wavelengths. Moreover, over the physiological oxygen range, no significant differences in lifetime readings in both channels were found. The determined calibration constants were \( k = 270 \text{ mmHg}^{-1}\text{s}^{-1} \) and \( \tau_0 = 240 \mu\text{s} \), which are in good agreement with previous reported values (5).

The next step was to get a reasonable estimation of the real measurement depths that could be achieved with our device in the rat kidney. This was performed by measuring the phosphorescence intensity as a function of amount of attenuating kidney tissue between the fiber-optic light guide and an Oxyphor G2 containing chamber. Pulsed excitation of either 440 or 632 nm was able to cause phosphorescence emission while penetrating the different slices of rat kidney tissue (Fig. 3). By using excitation light of 440 nm already, 90% of the phosphorescence signal vanished after placing the first kidney slice between the Oxyphor G2-containing chamber and the fiber optics. No significant phosphorescence could be measured after the tissue reached a thickness of 1000 μm. In contrast, excitation with near-infrared light of 632 nm allowed a much deeper penetration. The phosphorescence derived from those measurements could be easily detected to a depth of 4000 μm of kidney tissue. From the area under the curve, it can be seen that at least 50% of the total signal comes from a depth of more than 800 μm and 25–30% comes from tissue deeper than 1500 μm.

Although the experiment described above clearly demonstrates the difference in penetration depth of the two wavelengths, it provides only a rough estimation of the penetration depth under measurement circumstances and no true quantitative determination. This is because the kidney slices

![Figure 3](image-url)
themselves contain no phosphor. Because Oxyphor G2 is quite a good absorber of both 440- and 632-nm light, it is likely that our experiment gives somewhat an overestimation of the real penetration depth. To gain insight into the magnitude of this error, we measured the extinction coefficients of Oxyphor G2 at 440, 632, and 800 nm and found them to be 0.156, 0.052, and 0.001 µM⁻¹cm⁻¹, respectively, which is very close to previously reported values (5). Assuming a 300-g rat has a circulating blood volume of 18 ml, the infusion of 1.8 mg of Oxyphor G2 results in a concentration of ~30 µM in full blood. Making the conservative assumption that 25% of the total kidney volume is comprised of blood, the overall concentration of Oxyphor G2 in the kidney is 7.5 µM. Because light in tissue is multiple scattered, the actual traveled path through the tissue at a certain depth is much longer than the depth itself (4, 34). Because for the two excitation wavelengths the optical properties of kidney tissue are not exactly known, we generated results using Eq. 6 with different values for α, creating path lengths ranging from 3 to 10 times the actual depth (Fig. 3B). The extinction coefficient for 800-nm light is sufficiently low to be ignored for our purposes. From Fig. 3B, it can be seen that for 440 nm at a depth of 0.5 mm and the assumption of a five times longer pathlength, the attenuation due to the phosphor is <30%. Even if we assume a 10 times longer pathlength, still 60% of the excitation light will reach a depth of 0.5 mm. And, for example, for 632 nm at 3 mm depth, the additional attenuation due to the phosphor is <50% if we assume the actual pathlength to be five times the penetration depth. These values show that, although there is obviously a nonnegligible contribution of the phosphor to the overall attenuation, the general outcome from the experiment in Fig. 3A is still valid.

Figure 3, inset, shows a 500-µm slice of tissue with a millimeter scale to be able to link the measured penetration depth to an anatomical compartment. The kidney cortex was measured to be 1200 µm in total thickness. The outer medulla started at a depth of 1200 µm. This information allows concluding that the signal after excitation with 440-nm light is solely confined to the cortical compartment and that excitation with 632-nm light indeed gains information from the (outer) medullary compartment.
After the in vitro and ex vivo experiments, the dual-wavelength device was tested in vivo. Figure 4 shows a cartoon of the principle behind the measurement and examples of real phosphorescence traces measured in an anesthetized rat. Clearly, a difference in signal can be seen in lifetime as well as in overall phosphorescence intensity. The solid line is the resulting monoexponential fit. The lifetimes in this example are $\tau = 54$ and 69 $\mu$s for the blue and red channels, respectively. The corresponding PO$_2$ readings are 53 and 38 Torr, indicating that indeed two different compartments were measured. Moreover, although the efficiency of excitation with 632-nm light is much lower than with 440-nm light (5), the phosphorescence yield in vivo appears to be much higher. Although this can partly be explained by the fact that a lower PO$_2$ results in more phosphorescence yield, it is more likely that it indicates a much larger excitation volume in the case of the red channel. The fact that both channels are measuring different physical volumes is also underscored by the observation that, under certain experimental circumstances, the cortical $\mu$PO$_2$ reading drops below the medullary $\mu$PO$_2$. For example, application of the mitochondrial uncoupler carbonyl cyanide trifluoromethoxyphenyl-hydrazone (a single drop of a 10 mM stock solution) to the kidney surface led to a rapid decline in cortical $\mu$PO$_2$ (higher oxygen consumption) and a much slower decline in medullary $\mu$PO$_2$. Over 25 min, the cortical $\mu$PO$_2$ dropped from 60 to 15 Torr, whereas the medullary $\mu$PO$_2$ dropped from 45 to 30 Torr.

To test whether photochemical oxygen consumption influenced the PO$_2$ reading in vivo, we changed the repetition frequency of the flashlamps from 50 to 10 Hz. No significant difference in PO$_2$ reading could be observed, indicating that photochemical oxygen consumption does not interfere with our measurements.

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**FIGURE 5 A**: stepwise reduction in inspired oxygen fraction (FiO$_2$) resulted in a decline in cortical (solid bars) and medullary (shaded bars) microvascular PO$_2$ ($\mu$PO$_2$). **B**: table showing differences in results of 2 distinct fit models on the same phosphorescence data. Data are presented as means ± SD; $n = 5$. $\sigma$, Standard deviation.
To test the dual-wavelength technique in vivo and to evaluate the different fitting models, the device was used in a simple experimental model in which the oxygenation of the rat kidney tissue was changed by reducing \( \text{FiO}_2 \) stepwise from 1.0 to 0.1. The results of the monoeponential and rectangular distribution fit procedures are given in Fig. 5. Figure 5A shows a bar diagram of the mean cortical and mean medullar \( \text{PO}_2 \) resulting from the rectangular distribution fit. During ventilation with 100% oxygen, the mean \( \mu \text{PO}_2 \) of the cortex was 97 ± 10 Torr and the mean \( \text{PO}_2 \) of the medulla was 68 ± 6 Torr. During ventilation with an \( \text{FiO}_2 \) of 0.4, the average cortical oxygenation was 88 ± 7 Torr and the average medullary \( \text{PO}_2 \) was 59 ± 4 Torr. Ventilation with room air (21% oxygen) resulted in a mean \( \text{PO}_2 \) of 61 ± 9 Torr in the cortex and a mean \( \text{PO}_2 \) of 39 ± 5 Torr in the medulla. During hypoxia (\( \text{FiO}_2 = 0.1 \)), the cortical oxygenation decreased to a mean \( \text{PO}_2 \) of 33 ± 7 Torr and the medullary mean \( \text{PO}_2 \) was 15 ± 2 Torr. The experiments were performed in \( n = 5 \) rats. Oxygen tensions of the rat cortex and medulla during stepwise reduction in \( \text{FiO}_2 \) compare well with reported \( \text{PO}_2 \) values in previous studies using invasive Clark-type oxygen electrodes (1, 14, 22).

Figure 5B shows the existence of a considerable difference in results of the monoeponential fit procedure compared with the fit of the rectangular distribution. This denotes the presence of significant heterogeneity in oxygen pressure and also shows the tendency of the monoeponential fit procedure to bias toward low oxygen pressures, i.e., long lifetimes (6). This heterogeneity is also reflected in the values for the standard deviation (\( \sigma \)). It is very interesting to see how the mean \( \text{PO}_2 \) values and estimated standard deviations determined by the rectangular distribution fit correspond to the \( \text{PO}_2 \) histograms recovered using Eq. 5. Figure 6 shows the \( \text{PO}_2 \) histograms of \( \text{FiO}_2 \) variations in one rat. The width of the bins was chosen to be 15 Torr so that 10 bins covered the \( \text{PO}_2 \) region from 0 to 150 Torr. From each stable period after an \( \text{FiO}_2 \) step, seven histograms were calculated. Each presented histogram contains the means and standard deviations of these seven histograms. The signal-to-noise ratio of both channels was well above 20, and the histogram recovery was remarkably reproducible, corresponding very well to the results obtained in computer simulations (6). From direct comparison with Fig. 5B, it is clear that the rectangular distribution fit gives adequate mean \( \text{PO}_2 \) values and that the values for \( \sigma \) give a good indication for the underlying heterogeneity. The monoeponential fit indeed has the tendency to report too-low values for the average \( \text{PO}_2 \) and moreover gives no indication of heterogeneity at all. The histogram recovery, however, gives most detailed information. For example, the rectangular distribution fit gives a mean cortical \( \text{PO}_2 \) of 33 Torr when the \( \text{FiO}_2 \) is 0.1, whereas from the \( \text{PO}_2 \) histogram it is clear that a large part of the measurement volume has a \( \text{PO}_2 \) below 15 Torr.

**FIGURE 6** Histograms showing oxygen distributions in the cortex (top) and outer medulla (bottom) of the rat kidney for 4 different \( \text{FiO}_2 \).
The changes in FiO$_2$ caused a more or less parallel drop in PO$_2$ values for both cortex and medulla, as was expected. Therefore, the true value of the technique is better demonstrated in a model in which the response is less predictable. Figure 7A shows the response of a rat to a bolus injection of 20 mg/kg LPS. The solid line is a running average of the measurement values. Both cortical and medullary PO$_2$ values decline, but the time course of the events is completely different. The mean medullary PO$_2$ drops acutely after the bolus LPS, whereas the mean cortical PO$_2$ shows a much slower decline. Figure 7C shows PO$_2$ histograms calculated at the time points corresponding to numbers 1–3 in Fig. 7A. From the PO$_2$ histograms in Fig. 7C, it can be seen that the acute drop in mean PO$_2$ at the medullary level is due to a homogeneous low PO$_2$ value since 90% of the signal corresponds to lifetimes from PO$_2$ values below 15 Torr. At the same time point after LPS infusion, the cortical PO$_2$ is highly heterogeneous with a large fraction of low PO$_2$ values that was not existent before the bolus LPS. The shown rat is a typical example of a small study performed in six rats that was reported elsewhere (12).

Another example of a different pattern in cortical and medullary PO$_2$ responses to an intervention is given in Fig. 7, B and D. This example shows the response during fluid resuscitation with a starch solution (HES 200; rate: 20 ml/h) after stop of a 1-h continuous infusion of 15 mg/kg LPS. Resuscitation has an initial beneficial effect on both mean cortical and medullary PO$_2$ values (Fig. 7B). However, after a short time, the mean cortical PO$_2$ value declines rapidly to become almost equal to the medullary PO$_2$. The PO$_2$ histograms in Fig. 7D show us in more detail what happens. In the medulla, the infusion of LPS induced a fraction with very low PO$_2$ values that is substantially reduced following resuscitation. In the cortex, however, resuscitation appears to have the opposite effect. Here, the fluid resuscitation actually induces a fraction with very low PO$_2$ values, explaining the decline in

**FIGURE 7** Examples of cortical and medullary PO$_2$ (cµPO$_2$ and mµPO$_2$, respectively) readings in different pathophysiological circumstances. A: effect of a bolus of 20 mg/kg LPS on the mean cµPO$_2$ and mµPO$_2$ values. The time point of injection is denoted by the arrow. B: differing responses of mean cµPO$_2$ and mµPO$_2$ on resuscitation after a 1-h continuous LPS infusion (15 mg/kg) was stopped. Start of resuscitation and termination of the experiment by injection of 1 ml of 3 M KCl are denoted by the arrows. C: oxygen histograms corresponding to A. Histogram 1c corresponds to the cortical signal at indicated time point 1, histogram 1m to the medullary signal, and so on. Data were calculated for 3 different time points (1–3). D: oxygen histograms corresponding to B. The labeling is according to the same systematic as in C.
mean cortical PO$_2$. These last two examples clearly demonstrate the additional information that can be gained from PO$_2$ readings at different depths.

**DISCUSSION**

This study shows for the first time the use of a dual-wavelength phosphorimeter to detect changes in oxygenation in two different depths in the rat kidney by oxygen-dependent quenching of phosphorescence of Oxyphor G2. The main findings of this study are that 1) excitation of Oxyphor G2 in vitro at 440 and 632 nm results in the same calibration constants and the same PO$_2$ reading in each channel; 2) excitation light of 440 nm penetrates the kidney tissue 700 µm, whereas excitation light of 632 nm penetrates up to 4000 µm of tissue; 3) in contrast to the in vitro situation, placing the detection fiber-optic light guide on a kidney in vivo results in different PO$_2$ readings, with the deeper PO$_2$ value generally being less than the superficial PO$_2$; 4) the dual-wavelength device is able to record PO$_2$ values over a wide oxygen range in vivo, giving values similar to those reported by oxygen electrode measurements; and 5) this method is suitable to detect differences in responses in the cortical and outer medullary microvascular oxygenation in different pathophysiological relevant models.

To detect reliable heterogeneity in PO$_2$ levels in the depth, it is obvious that in a homogeneous in vitro situation no differences in PO$_2$ readings over the physiological relevant PO$_2$ range should exist. It is already known that different excitation wavelengths per se do not cause differences in phosphorescence lifetime readings in Oxyphor G2 (5). Also, the excitation pulse deconvolution algorithm (15) should cancel out differences in measured lifetimes caused by variation in flashlamp decays. Our calibration experiment shows indeed that over a wide oxygen range no differences in PO$_2$ readings in both channels exist. For the in vivo situation with heterogeneous µPO$_2$ values, the rectangular distribution fit is clearly preferable over the monoexponential fit procedure because it lacks the tendency to bias toward low PO$_2$ values and provides an indication of the level of heterogeneity. Furthermore, the PO$_2$ histogram recovery method suggested by Golub et al. (6) appeared to be easy to implement and very robust with the signal-to-noise ratios achieved with our phosphorimeter. An alternative method would have been the maximum entropy method (33). The latter method allows the recovery of even more detailed PO$_2$ histograms, but this comes at the expense of longer computation times (especially in the time domain), and it requires fairly high signal-to-noise ratios (33). The PO$_2$ histogram recovery method reveals details that otherwise remain unseen. For example, in Fig. 6, some small contributions of high PO$_2$ regions can be seen even at an FiO$_2$ of 0.1. Because some PO$_2$ values are higher than the possible arterial PO$_2$, these values probably are the result of oxygen diffusion from air into the outer surface of the kidney or caused by small contamination of the kidney surface with Oxyphor G2-containing blood.

In a previous study (25), our laboratory calculated, using Monte-Carlo simulations, that green (520 nm) light results in a catchments depth of ~0.5 mm in gut tissue. For near-infrared Oxyphor G2, a general penetration depth of several centimeters has been claimed (31). In the present study, we made an attempt to determine the catchments depth of the 440 and 632 nm in a direct manner since no sound estimations of the needed tissue optical properties of kidney (i.e., absorption coefficient, scatter coefficient, and anisotropy factor) at the specific wavelengths could be found, preventing Monte-Carlo simulations. We show that 440 nm is limited to a penetration depth of 700 µm and is therefore confined to the kidney cortex. In contrast, the 632-nm light easily reaches 3-mm depth and even excites phosphorescence at a depth of 4 mm, and therefore catchments volume includes the outer medullary region of the rat kidney. A drawback of our kidney-slice experiment is that the kidney slices contained no phosphor that would contribute to a higher overall absorbance and thus lower penetration depth. However, even very conservative assumptions about phosphor concentration and actual pathlength calculations show that the results of the kidney-slice experiment are a very reasonable estimation. This finding is confirmed by the fact that in vivo the red channel reports substantially lower PO$_2$ values than the blue channel. The latter observation is known for the kidney as
due to its anatomy; within the kidney, there is a diffusion of oxygen from arterial to venous vasa recta, thus leading to a relatively low PO$_2$ within the renal medulla (1). In the superficial kidney cortex, PO$_2$ ranges from 50 to 70 Torr (2), whereas the medullary PO$_2$ from 10 to 20 Torr (3). These reported values, measured by Clark-type oxygen electrodes in rats (where penetration of the kidney is needed), are similar to our findings with the two-wavelength technique. Deviations (especially in the medullary PO$_2$) may be explained by differences in FlO$_2$, deeper penetration with oxygen electrodes, or even tissue destruction by oxygen electrodes.

**Advantages and applications of this technique.** The presented method has as its main advantage that it allows continuous and nondisruption measurements of the cortical and outer medullary microvascular oxygenation in the rat kidney. In addition to measurement of lifetime distributions (35, 37), it gives information about the in-depth heterogeneity of µPO$_2$ levels, although not to such a detailed extent as two-photon excitation (16). The given examples in this paper demonstrate that the combination of dual-wavelength phosphorimetry and lifetime deconvolution (retrieval of PO$_2$ histograms) provides a very powerful tool for in vivo oxygen measurements. Compared with oxygen electrodes (1, 14, 22), the obvious advantage is in its nondisruptiveness, which allows monitoring changes in time. In this study, the method allowed us to monitor different behavior in oxygenation in cortex and medulla. Oxygen measurements in the kidney in superficial and deep tissue using a method based on the same principal of using different excitation wavelengths have been reported by Rumsey et al. (21). For imaging of oxygen distributions, they had to inject two different oxygen probes in different rats. Although their PO$_2$ readings were similar to our findings, our technique has the clear advantage of continuous and simultaneous measurements in two different depths. Furthermore, ease of handling and cost effectiveness are advantages of our method over the application of blood oxygenation level-dependent magnetic resonance imaging for the noninvasive detection of changes in intrarenal oxygenation in vivo, reviewed recently by Swartz and Dunn (27). The same counts for electron paramagnetic resonance with magnetic resonance imaging (11) where an additional advantage of our technique is that no injection of relatively large particles is needed (7).

Regarding the above-mentioned advantages, our method can be applied for PO$_2$ readings not only in the kidney but also on different organs like the gut, heart, or skin. This is of special relevance as, for example, in the gut it has been shown that the mucosa responds differently to shock and resuscitation than the serosa (23). The calculation of PO$_2$ histograms adds a certain complexity to the results, and straightforward interpretation of the distribution in terms of anatomical or microcirculatory compartments is not possible. However, the example of resuscitation after a period of endotoxemia clearly shows the additional value compared with only an average µPO$_2$ reading. The increase in medullary µPO$_2$ following resuscitation appears to be the result of only disappearance of very hypoxic regions instead of a right shift of the total histogram, probably indicating recruitment. In contrast, the drop in cortical µPO$_2$ seems to be the result of the appearance of a hypoxic fraction that is actually induced by the treatment. In research concerning optimization of treatment strategies in septic shock, this could prove to be valuable information, since microcirculatory dysfunction is considered to be a major contributor to the pathophysiology of sepsis (10, 25).

**Methodological limitations.** The use of the near-infrared phosphor Oxyphor G2 allows a deeper penetration into the tissue than the conventional Pd-porphyrin dye. In this study, we could measure oxygenation at a depth of 4 mm in the outer medulla of the rat kidney. Although this is an improvement, this is much less than the originally acclaimed penetration depth of centimeters (31). Apparently in kidney with increasing depth of the measurement, a rapid decline of excitation and emission intensity occurs. For our current application, this means that, although readings of the outer medullary µPO$_2$ can be performed, the inner medulla cannot be reached. Furthermore, in the recording of the deep signal, a substantial part of the total signal is superficial in origin. Nevertheless, our measured values for cortical and medullary PO$_2$ compare well with previously reported values by other groups using oxygen electrodes (14) and are very comparable to the values we found using two-
photon excitation phosphorescence lifetime measurements (16). Moreover, we demonstrate differences in the behavior of cortical and medullary PO2, indicating that the deeper signal is not overwhelmed by the superficial signal. This can also be seen from Fig. 3, where a substantially higher amount of phosphorescence is measured in the red channel, although excitation at 632 nm has lower efficiency than excitation at 440 nm. This also indicates a much larger catchment volume in the case of red excitation. However, since the contribution of the cortex to the medullary signal could be a potential source of error, we will investigate possibilities to improve the presented method either by changing the fiber geometry or by mathematical deconvolution algorithms.

One other general point of concern regarding phosphorescence lifetime measurements in vivo is the issue of phototoxicity. The collision of oxygen with an excited phosphor molecule results in the production of oxygen radicals that can potentially harm the tissue under investigation (26). Therefore, the light dose and phosphor concentration used for oxygen measurements should be kept as low as possible. Although we cannot exclude phototoxicity in our application, no macroscopic alterations to the kidney surface are observable after hours of measurement. Moreover, in long-lasting time control experiments, no changes in μPO2 values are observed. Furthermore, it is very unlikely that the excitation light itself influenced the physiological function of the tissue because of the relatively low excitation energy used (<1 µJ per flash). The power density is <0.06 µJ/mm2, and this is in orders of magnitude less than the power density used in, for example, two-photon approaches (16).

Our results demonstrate that oxygen-dependent quenching of phosphorescence of Oxyphor G2 is excellently suited for quantitative μPO2 measurements in two different depths in vivo. Oxygen levels measured in the renal cortex and outer medullary region are consistent with previous reported values detected by oxygen electrodes in the rat kidney. This technique provides a new tool for quantitative measurements of oxygen levels under physiological and pathophysiological conditions with the characteristics of providing spatial information and a penetration depth of several millimeters.

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