Measuring microvascular and mitochondrial oxygen tension: novel techniques for studying tissue oxygenation

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Chapter 1

General Introduction
OXYGEN

Molecular oxygen, dioxgen, or in short $O_2$ is the prime requisite of aerobic life on earth. Diatomic oxygen gas, mostly spoken of simply as “oxygen”, constitutes approximately 21% of the volume of air. The electron configuration of the oxygen molecule has two unpaired electrons with the same spin in degenerate orbitals. Therefore, oxygen is paramagnetic and, in contrast to many other biochemically relevant molecules, the ground state of oxygen is a triplet state. The amount of oxygen dissolved in a liquid depends on the solubility coefficient of oxygen in the solvent and the partial oxygen pressure (PO$_2$) above the solution. The dissolved amount of oxygen can be expressed as liters O$_2$ per liters solvent, a concentration (mol/l) or as partial oxygen pressure (PO$_2$), which is equivalent to oxygen tension. Since the solubility coefficients in biological compartments are not well known it is common to measure and report PO$_2$ values in kPa, mmHg or Torr (1 kPa = 7.5 mmHg = 7.5 Torr).

The adequate supply of oxygen by inhalation and subsequent transport to tissues via the circulating blood is a conditio sine qua non for mammalian cells to sustain life. Molecular oxygen is the primary oxidant in biological systems and its ultimate destination in vivo is the mitochondria where it is used in oxidative phosphorylation. Besides being indispensable for the energy production of our cells, oxygen is known to play a role in many other biochemical processes and mammalian tissue contains a large number of oxygen consuming enzymes (20). For example, oxygen is used for the production of reactive oxygen species that are important in signal transduction (2, 25).

Because of the importance of adequate oxygen supply, many techniques have been developed for measuring oxygen in vivo (16, 18, 20), in order to gain insight into the mechanisms of oxygen delivery and use. These in vivo measurements of oxygen tension have generally demonstrated that in tissues really low PO$_2$ levels (in the order of a few mmHg) are only observed under extreme pathophysiological circumstances (e.g. circulatory arrest). In the mean time, studies in isolated mitochondria had shown very high affinity of oxidative phosphorylation for oxygen (5, 8, 23), i.e. independency of respiratory rate on PO$_2$ values as low as 0.2-0.5 mmHg. This led to the idea that under physiological conditions oxygen could not be a limiting or regulatory factor for oxygen metabolism in vivo (9). Due to this thinking, and the global trend towards molecular biology, physiological oxygen measurements have been in danger of becoming a superfluous and obsolete art.

However, recent studies have questioned the idea that oxygen only acts as a simple substrate over the physiological range of oxygen tensions, and have identified a mechanism in hepatocytes called “oxygen conformance of metabolism” (14, 17). Here oxygen consumption is downregulated when cells are subjected to moderate oxygen deprivation for extended periods of time (14). What this means for the in vivo situation is still unclear, but recent studies in climbers on the Mount Everest have shown a remarkable adaptation to very low oxygen levels (4). Based on these findings, it has very recently been hypothesized that therapeutic permissive hypoxia might lead to hypoxic cellular adaptation as seen in the fetus in utero (10). We still have a long way to go before doctors at the bedside should even consider to deliberately bringing patients in a state of global hypoxia to protect them from further harm (13), but the idea is exciting. And, while the paradigm seems to shifts in the direction of oxygen being a regulator rather than being a passive bystander, there is again real need to reliably measure oxygen in cells and tissues. We should continue to strive to perfect existing methods and develop novel techniques that allow better, and more detailed, quantification of oxygen levels in biological systems.

PORPHYRINS

Porphyrins belong to the most abundant molecules on Earth and are a group of organic compounds that appear in nature in a large variety. Porphyrins are essential for life as we know it, because of their key role in processes related to oxygen production, oxygen transport and oxygen utilization (6). Porphyrins are cyclic macromolecules composed of four modified pyrrole subunits that form a highly
conjugated system. The parent compound is porphine (fig. 1) and substituted porphines are called porphyrins.

As ligands, porphyrins form easily complexes with metallic ions like iron and magnesium. For example, some iron-porphyrin complexes are called hemes (fig. 2). Hemoglobin is an example of a heme-containing protein that binds oxygen and functions as oxygen carrier in blood (22, 24). Chlorophyll is an example of a magnesium containing porphyrin (Fig. 3). Chlorophyll is the green compound in leaves that absorbs light for photosynthesis, providing us with oxygen and energy (1).

Porphyrins typically are extremely good absorbers of light in the visible range and therefore porphyrin-metal complexes often have intense and dark colors. The absorption of a photon leads to photoexcitation of the porphyrin-metal complex. Photoexcitation can lead to population of an excited triplet state of which the energy can, for example, be used for photosynthesis (19). Oxygen, having a triplet state as ground state, is a very effective quencher of this excited triplet state. In the process of quenching energy is transferred to oxygen leading to the formation of singlet oxygen. The latter is the basis of porphyrin-based photodynamic therapy (12).

**TRIPLET STATE QUENCHING**

A so-called Jablonski diagram can visualize the different electronic states in an atom or molecule. Figure 4 shows the Jablonski diagram of porphyrin and oxygen and their interaction. After excitation of porphyrin to the first singlet state ($S_1$), population of the the triplet state ($T_1$) can occur by intersystem crossing, a process in which the electron is relaxed to the triplet state by changing its spin orientation. This process occurs without emission of a photon. The probability that a porphyrin molecule in the $T_1$ state relaxes to the $S_0$ state by spontaneous relaxation is denoted by rate constant $k_s$. Relaxation to $S_0$ can also occur by collision with an oxygen molecule during which oxygen absorbs the energy from the porphyrin. This process is called “triplet state quenching” and results in relaxation of the porphyrin without emission of a photon.
Quenching of the triplet state is a process that makes the triplet state lifetime dependent on the collision frequency, and thus on oxygen concentration. The collision frequency is determined by the amount of oxygen and the chance that a single oxygen molecule causes a quenching event, defined by the Smoluchowski equation:

$$k_q = 4\pi N \gamma (D_o + D_f)(R_o + R_f)$$

In which $k_q$ is known as the quenching constant, $N$ is Avogadro’s number, $\gamma$ the quenching efficiency, $D_o$ and $D_f$ are the diffusion coefficient of oxygen and the porphyrin respectively and $R_o$ and $R_f$ are the quenching radius of oxygen and the porphyrin respectively.

The rate of relaxation of $T_1$ to $S_0$ is thus determined by both the transition probability by spontaneous relaxation ($k_s$) and the quenching probability ($k_q$). The decay rate of porphyrin molecules in the excited triplet state after excitation with a pulse of light is given by the following differential equation:

$$\frac{d[T_1]}{dt} = -k_s[T_1](t) - k_q PO_2[T_1]$$

Where $[T_1]$ denotes the amount of porphyrin molecules in the excited triplet state and $PO_2$ is the oxygen tension in the surrounding medium. Under ideal circumstances, i.e. when the excitation pulse duration is much shorter than the triplet decay time, the solution of this differential equation yields:

$$[T_1](t) = [T_1]_0 e^{-(k_s + k_q PO_2)t}$$

Where $[T_1]_0$ denotes the amount of porphyrin molecules in the excited triplet state at $t=0$, i.e. immediately after the excitation pulse. Equation 3 can be rewritten in the form:

$$[T_1](t) = [T_1]_0 e^{\left(-\frac{t}{z}\right)}$$

In which
\[
\frac{1}{\tau} = \frac{1}{\tau_0} + k_q P O_2
\]  

This last equation is known as the Stern-Volmer relationship, in which \( \tau \) is the triplet state lifetime and \( \tau_0 = 1/k_q \) is the time constant of the decay of the triplet state in the absence of oxygen, i.e. the decay time of spontaneous relaxation.

**MODES TO MEASURE THE TRIPLET STATE LIFETIME**

From the Stern-Volmer relationship (eq. 5) it is clear that if we are able to measure the triplet state lifetime, by any means, this actually allows measurement of the \( P O_2 \) in the medium containing the porphyrin. Figure 5 shows an overview of optical modalities to measure state transitions that allow direct measurement of the triplet state lifetime. These modalities are phosphorescence, delayed fluorescence and triplet-triplet absorption.

If the \( T_1 \) state relaxes directly to the \( S_0 \) state through emission of a photon, this radiation is called phosphorescence. In contrast to fluorescence (emission of a photon due to relaxation of \( S_1 \) to \( S_0 \)), phosphorescence is relatively long lived (typically several orders of magnitude longer than fluorescence) and its spectrum is shifted towards the red. The latter is due to the fact that in case of phosphorescence some energy is lost in the process of intersystem crossing and therefore the energy of the \( T_1 \) state is lower than of the \( S_1 \) state. Because of this red shift, phosphorescence can be optically separated from prompt fluorescence. This makes measurement of phosphorescence the least complicated way to measure the \( T_1 \) lifetime. Unfortunately, not all porphyrins show detectable phosphorescence, but some metallo-porphyrins have high phosphorescence yield which makes them excellent probes for oxygen measurements (21).

If the \( T_1 \) state relaxes to the \( S_0 \) state via the \( S_1 \) state by a process called bi-directional intersystem-crossing and the relaxation of \( S_1 \) to \( S_0 \) leads to emission of a photon, this radiation is called delayed-fluorescence. In contrast to phosphorescence, delayed fluorescence has the same spectrum as prompt fluorescence and cannot be optically separated from fluorescence. Prompt fluorescence tends to overwhelm delayed fluorescence in intensity and in practice this easily leads to saturation of detection systems. Nevertheless, with some precautions is delayed fluorescence a useable mode for measuring the \( T_1 \) lifetime. A practical example is the measurement of mitochondrial \( P O_2 \) by delayed fluorescence of protoporphyrin IX (11).

A method that does not rely on the detection of emitted photons but on transient changes in absorption is triplet-triplet absorption. The idea is that absorption from \( T_1 \) to \( T_2 \) can only take place during population of the \( T_1 \) state. Therefore, an excitation pulse will temporarily increase absorption at
the wavelength corresponding to $T_1$-$T_2$ transition and the transient change in absorption will decay according to the $T_1$ lifetime. A drawback of this technique is that it requires an extra light source, making its application more cumbersome than the emission measurements. Nevertheless, triplet-triplet absorption is useful for non-radiating probes (3).

### MEASURING OXYGEN BY QUENCHING OF PHOSPHORESCENCE

The method of measuring $\text{PO}_2$ in biological systems by means of oxygen-dependent quenching of phosphorescence was introduced by Vanderkooi and co-workers over two decades ago (21). Complexes of porphyrins with certain heavy metals show high phosphorescence yield and are very efficient oxygen probes. Palladium-meso-tetra(4-carboxyphenyl)porphine (Pd-porphyrin, fig. 6), bound to albumin before injection, has become a standard phosphorescent dye for microvascular $\text{PO}_2$ measurements in vivo (7, 15).

![Figure 6](image1.png)

**FIGURE 6** Pd-porphyrin, the standard phosphor.

Oxygen measurements by means of oxygen-dependent quenching of phosphorescence lifetimes are based on the principle that phosphorescence intensity is proportional to the amount of populated triplet states in a porphyrin solution. Therefore, equation 5 can be rewritten as:

$$I(t) = I_0 e^{\left(\frac{-t}{\tau}\right)}$$  \hspace{1cm} (6)

Where $I(t)$ is the phosphorescence intensity over time, $I_0$ is the initial phosphorescence intensity directly after the excitation pulse and $\tau$ now denotes the phosphorescence lifetime. The $\text{PO}_2$ can be calculated from $\tau$ using the Stern-Volmer relationship (eq. 5). From this relationship it is clear that a higher $\text{PO}_2$ results in more quenching and therefore a shorter lifetime (fig. 7)

![Figure 7](image2.png)

**FIGURE 7** Principle of phosphorescence lifetime measurements. Excitation with a pulse of green light in a sample containing Pd-porphyrin induces red phosphorescence of which the lifetime depends on the $\text{PO}_2$ in the sample.
OUTLINE OF THIS THESIS

Chapter 2 Excitation pulse deconvolution
In this chapter we analyze the effects of the shape of the excitation pulse on phosphorescence lifetime analysis in time domain phosphorimetry. The relatively long phosphorescence lifetime of phosphors used for oxygen measurements had favored the standard use of short-arc xenon flashlamps as excitation source. We hypothesized that the side effects of finite excitation pulse duration might be one of the causes of the unexpected variation in reported quenching constants of Pd-porphyrin probes. Besides a computer simulation based analysis also a method for deconvolution of the excitation pulse shape is proposed and tested.

Chapter 3 Two-photon excitation
In chapter three we describe a method for multidimensional oxygen scanning in tissues based on a non-linear optical technique. We hypothesized that two-photon excitation might be applicable to phosphorescence lifetime measurements in vivo. In analogy of multi-photon fluorescence microscopy this would allow PO2 measurements in a confined tissue volume and enable detailed measurement of PO2 heterogeneity. The concept of oxygen measurements based on two-photon excitation of Pd-porphyrin is tested and applied in rat kidney in vivo.

Chapter 4 Dual wavelength phosphorimetry
In chapter four we report the development of a technique for measurement of microvascular PO2 histograms in two different depths in tissue. The penetration depth of light in tissues depends on its wavelength and longer wavelengths tend to penetrate deeper. Most phosphorescent dyes can be excited at several wavelengths. We hypothesized that the use of multiple wavelengths could be a means to retrieve phosphorescence from distinctive tissue layers. A dual wavelength phosphorimeter for use with the Pd-porphyrin derivative Oxyphor G2 (excitation with blue and red light) is described, validated and used in rat kidney for retrieval of PO2 histograms from cortex and outer medulla.

Chapter 5 Venous PO2 measurement
In this chapter we explore an optical method for monitoring PO2 and oxygen content in full blood within large vessels of rats. The classical phosphor Pd-porphyrin can be excited with blue (~420 nm) and green (~530 nm) light and therefore cannot be used for PO2 measurements in full blood. This is because hemoglobin has very high absorption at these wavelengths. We hypothesized that the more recently introduced near-infrared phosphor Oxyphor G2 would be useful for this purpose. Oxyphor G2 has excitation and emission wavelengths in the red (~630 nm) and the infrared (~800 nm) respectively. A small frequency domain phosphorimeter is described and calibrated. The feasibility of venous PO2 measurements with subsequent calculation of venous oxygen content is tested in the renal vein of rats.

Chapter 6 Mitochondrial PO2 Measurements
Here we report the development of the first method to measure mitochondrial PO2 (mitoPO2) in intact living cells. The need for injection of exogenous phosphors obviously limits the use of phosphorescence lifetime measurements to the laboratory. Therefore we researched the feasibility of using endogenous porphyrins for oxygen measurements and hypothesized that protoporphyrin IX (PpIX) would be a good candidate. We searched for useful oxygen-dependent optical properties of PpIX, performed calibration experiments in solutions and cell suspensions, and researched the intracellular PpIX localization. The chapter ends with the description of an experiment aimed at determining the PO2 gradients between the extracellular milieu and the mitochondria of respiring cells.
Chapter 7 Mitochondrial PO$_2$ in vivo

In this chapter we report the results of our testing whether delayed fluorescence of protoporphyrin IX can be used to measure mitoPO$_2$ in vivo. We hypothesized that intravenous administration of δ-aminolevulinic acid would induce enough mitochondrial PpIX to observe delayed fluorescence in vivo, and that the delayed fluorescence lifetime would be quantitatively related to the PO$_2$. The results of calibration experiments in freshly isolated rat liver cells and isolated perfused rat livers are shown. Based on these calibrations, the technique is used to measure mitoPO$_2$ histograms in rat liver in vivo.

Chapter 8 Mitochondrial PO$_2$ in the heart

In this chapter we provide the first measurements of mitoPO$_2$ in rat heart, both in vivo and in vitro. The heart has always been an organ of prime interest concerning matters of oxygenation. The rat heart, both in situ and as isolated perfused organ, is widely used for studying oxygenation of the myocardium and phenomena like ischemia-reperfusion injury and preconditioning. We measured mitoPO$_2$ histograms in isolated perfused rat hearts and in rat hearts in vivo by delayed fluorescence of PpIX.

Chapter 9 Summary and conclusions

The research presented in this thesis has been performed over an extended period of time. During this time our laboratory has applied the techniques described in this thesis in several pathophysiological models. Furthermore, other research groups have further developed PO$_2$ measurement techniques based on ideas described in this thesis. In chapter 9 we look back at the presented results, discuss the usability of the described techniques based on recently performed studies, and review our work in the light of current international developments.

REFERENCES