Measuring microvascular and mitochondrial oxygen tension: novel techniques for studying tissue oxygenation
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Chapter 9

Summary and Conclusions
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, oxygen and its radicals are playing key roles in many other biochemical processes and signal transduction in our cells (2, 42, 45). Because of the importance of adequate oxygen supply many techniques have been developed for oxygen measurements in vivo (34, 37, 42). Oxygen-dependent quenching of phosphorescence, introduced as a means to measure oxygen in biological systems in the late 80s by Vanderkooi et al. (43), has proven itself to be a particularly flexible and powerful technology. The technique relies on insertion of an oxygen-dependent phosphorescent dye, typically a metallo-porphyrin like Pd-porphyrin (23, 32), into the system of interest. For example, the intravascular injection of albumin-bound Pd-porphyrin has become a standard technique for measurement of microvascular PO$_2$ (μPO$_2$) in vivo. Phosphorescence lifetime measurements are scalable from applications in microscopes on single cells (12, 13) and capillaries (30, 47) to imaging of PO$_2$ in the surface of whole organs (14, 27). Combined with a relatively easy and robust technology these are major ingredients for the success of this technique. The introduction of novel phosphorescent dyes in recent years (3) has even further enhanced its applicability, especially in thick tissue (48).

As with any novel technology, the introduction of phosphorescence lifetime measurements into the realm of scientists interested in PO$_2$ measurements was not without hiccups. Since the phosphorescence lifetime of the used phosphorescent probes is orders of magnitude longer than fluorescence lifetimes, typically tens of microseconds to several milliseconds, the use of short arc xenon flashlamps as excitation source had been advocated in construction of early phosphorimeters (9, 33). The excitation pulse produced by these flashlamps (with a typical pulse width of several microseconds) was considered short enough to be of no influence on phosphorescence lifetime recovery. However, although theory predicted otherwise, the early days showed a considerable variability in calibration constants reported by various laboratories and deviations of calibration lines from the linear Stern-Volmer relationship. For example, reported values for the quenching constant of Pd-porphyrin have ranged from $k_0 = 306 \text{ mmHg}^{-1}\text{s}^{-1}$ (47) to 386 mmHg$^{-1}$s$^{-1}$ (11). In chapter 2 we evaluate the consequences of finite excitation pulse duration and the complex excitation patterns of flashlamps on phosphorescence lifetime recovery. We demonstrate that variations in flashlamp characteristics largely account for the reported differences between laboratories and that finite excitation pulse duration leads to non-linearity of the calibration curves. Furthermore, we present a method for deconvolution of the excitation pulse shape out of the photometric signal that neutralizes adverse effects of the use of flashlamps.

Due to the presence of oxygen gradients, among other things resulting from e.g. cellular respiration and variations in diffusion distances to the capillary network, the PO$_2$ in tissues is highly heterogeneous. Phosphorescence lifetime measurements are very well suited for gaining insight in this heterogeneity. For example intravital microscopy (31, 47) and surface imaging on organs (14, 27) has been used to demonstrate heterogeneity in PO$_2$ in two-dimensional (x-y) direction. However, for some organs, like gut and kidney, it is of interest to gain insight in heterogeneity in the depth because of the anatomical structure of the organs. Multi-photon excitation has become a well known non-linear optical technology used to image fluorescence in three dimensions and has been demonstrated to be useful in vivo (36). In chapter 3 we demonstrate that two-photon excitation can in principle be used for quantitative PO$_2$ measurements using classical Pd-porphyrin. Calibration constants are similar as with normal single photon excitation and the quadratic dependence on excitation intensity is excellent. Two-photon excitation scanning in the z-direction through the renal cortex clearly identifies an abrupt PO$_2$ drop at a depth corresponding to the transition from cortex to outer medulla. Our work represents the first demonstration of the feasibility of measuring PO$_2$ by means of two-photon excitation phosphorescence lifetime measurements. Indeed, others have applied the principle in two-photon microscopy but the low two-photon absorption cross-section of Pd-porphyrin requires impractically high
excitation intensities and scan times (4). Very recently, novel phosphorescent probes with much larger two-photon absorption cross-section have been developed (5). The addition of two-photon absorption antennas to a metallo-porphyrin core has now brought practical implementation of PO\textsubscript{2} measurements based on two-photon microscopy within reach (28).

The two-photon approach did not bring the speed and versatility we needed for monitoring of microvascular PO\textsubscript{2} (\(\mu\text{PO}_{2}\)) in organs of animals during experimental models of (septic) shock and resuscitation. Another potential way to discriminate between layers of tissue is the use of different excitation wavelengths. This idea is based on the principle that longer wavelengths of light tend to penetrate deeper into tissue because of less absorption. Although classical Pd-porphyrin can be excited at two different wavelengths (~420 nm and 530 nm, i.e. blue and green light respectively) both are very well absorbed by hemoglobin, limiting the penetration depth in both cases to very superficial tissue layers. However, the introduction of the near-infrared phosphor Oxyphor G2 (3) allowed us to explore this principle in practice. In chapter 4 we describe the development and use of a dual-wavelength phosphorimeter that alternately excites Oxyphor G2 with blue (440 nm) or red (632 nm) light. In rat kidney we demonstrate that the blue excitation confines the measurement volume to the cortex, while the red excitation extends the measurement volume to the (outer) medulla. Although some overlap between the measurement volumes exists (this single photon approach lacks the true discriminating power of two-photon excitation), in practice the channels behave surprisingly independent. In addition to measuring \(\mu\text{PO}_{2}\) at two different depths we demonstrate that additionally calculating the PO\textsubscript{2} histograms out of the phosphorescence data instead of the average PO\textsubscript{2} provides even better insight in heterogeneity. Dual-wavelength phosphorimetry as described in chapter 4 has now been extensively used in our laboratory and has proven to be a valuable tool for studying microvascular oxygenation in the rat kidney in various pathophysiological models (15-19, 21, 22).

For a more comprehensive understanding of the oxygenation of an organ adding global measurements like oxygen delivery (DO\textsubscript{2}) and oxygen consumption (VO\textsubscript{2}) to the more local \(\mu\text{PO}_{2}\) measurements is required. To this end, one needs to measure the blood flow to the organ and the oxygen content of the arterial and venous blood. The classical way of determining the oxygen content of blood, i.e. by means of drawing blood samples, easily leads to volume depletion in small experimental animals and furthermore the risk of damaging vital blood vessels. To allow near-continuous measurement of renal venous oxygen content we developed a technique based on oxygen-dependent quenching of phosphorescence. In chapter 5 we describe a frequency domain phosphorimeter that allows online monitoring of renal venous PO\textsubscript{2} by extravascular placement of a small fiber-optic assembly. Renal venous oxygen content is calculated with a mathematical model based on hemoglobin saturation curves measured in blood samples from rats and using the hemoglobin concentration determined by arterial blood sampling. This approach allows monitoring of renal venous oxygen content with an error of less than 10% over a wide pH range. However, due to the well-known pH dependency of hemoglobin saturation, the technology cannot completely replace blood sampling under all circumstances. Nevertheless, just as dual-wavelength phosphorimetry, the technique has become a standard in our laboratory. It allows for near continuous monitoring of renal VO\textsubscript{2} and guides for appropriate time points when more invasive measurements are needed.

The previously described techniques are obviously limited to preclinical use in experimental animals because of the need of injection of an exogenous phosphorescent dye. In order to circumvent this drawback we looked into possibilities to use endogenous porphyrins for oxygen measurements. The result of this quest has been quite astonishing and has resulted in the first method to measure mitochondrial oxygen tension (mitoPO\textsubscript{2}) in living cells. In chapter 6 we describe the development of these mitoPO\textsubscript{2} measurements based on oxygen-dependent quenching of the delayed fluorescence of protoporphyrin IX (PpIX), the final precursor in the heme biosynthetic pathway. We demonstrate that 5-aminolevulinic acid (ALA) induced PpIX is located in the mitochondria and exhibits oxygen-dependent delayed fluorescence of which the lifetime is quantitatively related to mitoPO\textsubscript{2}. We calibrate the PpIX
delayed fluorescence signal to $\text{PO}_2$ in various cell lines and provide the first direct measurement of the oxygen difference between the extracellular compartment and the intracellular mitochondria. The latter has been a topic of long standing debate since reported values range from well below one mmHg by indirect means (24, 26) to several mmHg in intracellular oxygen measurements based on injection of phosphorescent dyes (13) or nano-particles (20). We found the $\text{PO}_2$ difference between the mitochondria and the extracellular medium to be dependent on the respiration rate and be typically in the order of several mmHg.

The method of measuring mito$\text{PO}_2$ by means of delayed fluorescence of ALA induced protoporphyrin IX, as described in chapter 6, would come to full potential if it would not only be useful in cultured cells but also in tissues and organs in vitro and in vivo. Indeed, in chapter 7 we describe the use of this method for the first in vivo measurement of mito$\text{PO}_2$. To this end the delayed fluorescence lifetime of PpIX is calibrated in isolated hepatocytes harvested from ALA treated rats. We report a quenching constant $k_\tau = 832 \pm 25$ mmHg$^{-1}$s$^{-1}$ and a lifetime under zero oxygen conditions $\tau_0 = 0.8 \pm 0.1$ ms. The validity of the found calibration constants for intact tissue has been verified in isolated perfused liver, also obtained from ALA treated rats. Ultimately we report mito$\text{PO}_2$ values and mito$\text{PO}_2$ histograms measured on rat liver in vivo at different inspired oxygen fractions and in ischemia-reperfusion. The reported mito$\text{PO}_2$ values are typically an order of magnitude larger than generally anticipated (8). Average mito$\text{PO}_2$ is around 30-40 mmHg and differs not much from tissue $\text{PO}_2$ measured by micro oxygen electrodes (39, 41). Under baseline conditions hypoxic fractions of mitochondria (mito$\text{PO}_2$ in the range 0-10 mmHg) are not present in the mito$\text{PO}_2$ histograms. They become evident during breathing of gas mixtures with low oxygen content and during ischemia-reperfusion. Interestingly, we found that mito$\text{PO}_2$ values in vivo are within the $\text{PO}_2$ range that is of interest in the concept of oxygen conformance of metabolism (29, 35).

The heart is a very organ that has been classically of prime interest concerning matters of oxygenation. Both in situ and as isolated perfused organ, the rat heart is widely used for studying oxygenation of the myocardium and phenomena like ischemia-reperfusion injury and preconditioning. In chapter 8 we report mito$\text{PO}_2$ histograms measured in isolated perfused and in vivo rat hearts by delayed fluorescence of PpIX. Here calibration of the technique is performed in isolated cardiomyocytes from rats pre-treated with ALA. The quenching constants $k_\tau = 826 \pm 51$ mmHg$^{-1}$s$^{-1}$ and $\tau_0 = 0.8$ ms are similar to the values found for rat liver and are shown to be valid for the intact heart. The mean mito$\text{PO}_2$ value of 35 ± 5 mmHg found in vivo is in accordance with tissue $\text{PO}_2$ values of 45 ± 8 mm Hg (1) and microvascular $\text{PO}_2$ values of 50–70 mmHg reported in the present and past studies (49). However, we report mito$\text{PO}_2$ values that are much higher than anticipated from estimates derived from cytosolic/interstitial/vascular measurements of 3–17 mmHg (6, 7, 25, 38, 44, 46). In addition, the mito$\text{PO}_2$ heterogeneity indicates that approximately 10% of the mitochondria in the in vivo heart are exposed to a $\text{PO}_2$ between 0 and 10 mmHg. This implies that some fraction of the mitochondria within the in vivo heart may be partially controlled by the prevailing oxygen tension. This fraction of mitochondria with a mito$\text{PO}_2$ between 0 and 10 mmHg is as large as 30% in the isolated perfused rat heart and decreased to 10% by vasodilation. Our data suggest that the normally perfused isolated heart is oxygen limited and this is in agreement with older literature suggesting that the isolated heart is partially hypoxic (10, 40).

Overall, this thesis describes the implementation of novel approaches for measuring tissue oxygenation based on oxygen-dependent quenching of the triplet-state lifetime of porphyrins. Ultimately we created a tool for assessment of mitochondrial oxygen tension in vivo. The first measurements indicate much higher mito$\text{PO}_2$ values than generally expected. These findings come in an era of new insights in cellular oxygen sensing and oxygen-dependence of gene expression and metabolism. The described methods are expected to be a valuable addition to the arsenal of tools available to scientist for unraveling the mechanisms of oxygen delivery and consumption under various pathophysiological circumstances. Oxygen is again important, as it has always been...
REFERENCES