Measuring microvascular and mitochondrial oxygen tension: novel techniques for studying tissue oxygenation
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Chapter 8

Mitochondrial Oxygen Tension within the Heart


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ABSTRACT
By using a newly developed optical technique which enables non-invasive measurement of mitochondrial oxygenation (mitoPO$_2$) in the intact heart, we addressed three long-standing oxygenation questions in cardiac physiology: 1) what is mitoPO$_2$ within the in vivo heart?, 2) is mitoPO$_2$ heterogeneously distributed?, and 3) how does mitoPO$_2$ of the isolated Langendorff-perfused heart compare with that in the in vivo working heart? Following calibration and validation studies of the optical technique in isolated cardiomyocytes, mitochondria and intact hearts, we show that in the in vivo condition mean mitoPO$_2$ was 35±5 mmHg. The mitoPO$_2$ was highly heterogeneous, with the largest fraction (26%) of mitochondria having a mitoPO$_2$ between 10 and 20 mmHg, and 10% between 0 and 10 mmHg. Hypoxic ventilation (10% oxygen) increased the fraction of mitochondria in the 0–10 mmHg range to 45%, whereas hyperoxic ventilation (100% oxygen) had no major effect on mitoPO$_2$. For Langendorff-perfused rat hearts, mean mitoPO$_2$ was 29±5 mmHg with the largest fraction of mitochondria (30%) having a mitoPO$_2$ between 0 and 10 mmHg. Only in the maximally vasodilated condition, did the isolated heart compare with the in vivo heart (11% of mitochondria between 0 and 10 mmHg). These data indicate 1) that the mean oxygen tension at the level of the mitochondria within the heart in vivo is higher than generally considered, 2) that mitoPO$_2$ is considerably heterogeneous, and 3) that mitoPO$_2$ of the classic buffer-perfused Langendorff heart is shifted to lower values as compared to the in vivo heart.

INTRODUCTION
Mitochondria figure prominently in cardiac physiology by powering virtually all forms of mechanical and chemical work of the heart. These organelles can also play a decisive role in cell death and survival signaling. Oxygen is the underlying, ultimate molecule used by the mitochondria, thus giving it the essential role in dictating life or death. The partial pressure of oxygen within the mitochondria (mitoPO$_2$) is hypothesized to have a regulatory function in important physiological processes such as energy production, radical production, oxygen sensing, and gene expression (1–5). Surprisingly, no direct quantitative data concerning the in vivo mitoPO$_2$ within the intact heart are available in the literature. MitoPO$_2$ can be estimated from PO$_2$ values reported for other compartments such as the cytosolic, tissue or vascular compartments. Interestingly, most studies report rather low PO$_2$ values ranging from 10–17 mmHg for vascular and interstitial compartments (6–8) to 3–7 mmHg for the cytosolic compartment (9–11). Such low values suggest that the oxygen tension at the mitochondria, being at the lowest end of the diffusion pathway which oxygen must travel, is below 5 mmHg, making it likely that oxygen regulates energy production (1,3,5). These values deviate largely from other studies, however, which report cardiac microvascular PO$_2$ values of 50–70 mmHg (12) or interstitial values of 45 mmHg (13). Using these latter, higher values, mitoPO$_2$ is estimated to be between 20 and 40 mmHg. To resolve these conflicting estimated data on such an important (patho)physiological parameter of the heart, our first goal of the present study was to provide a direct determination of mitoPO$_2$ within the intact heart. To this end, we employed a novel optical technique using oxygen-dependent quenching of the delayed fluorescence lifetime from mitochondrial protoporphyrin IX (PpIX) (14,15).
In addition, although spatial heterogeneity of blood flow, oxygen delivery and oxygen metabolism within the heart is well accepted (12,16,17), it is not known whether such heterogeneity extends to the oxygenation status of mitochondria. Aside from heterogeneity dictated by blood flow distribution, heterogeneity may also exist due to the distribution of mitochondria within the cardiomyocyte, i.e., subsarcomemmal and interfibrillar mitochondria (18). When mitoPO$_2$ heterogeneity is present, the determination of a single averaged mitoPO$_2$ for the whole heart is misleading in that a significant, albeit small, part of all mitochondria within the heart may still be oxygen limited. Thus, a second goal of the present study entailed the determination of mitoPO$_2$ heterogeneity using a lifetime-deconvolution
algorithm developed by Golub et al. (19).

Finally, we wanted to compare the mitochondrial oxygenation status in the isolated, Langendorff-perfused heart with that in the in vivo working heart. Investigations with isolated hearts are numerous, and the results obtained have been used to further our understanding of heart physiology, so that the validity of this model is of utmost importance (20). We are not aware of other studies that have directly compared the mitochondrial oxygenation status of the isolated heart with that of the in vivo heart, and as such, this direct comparison will aid in answering questions concerning the validity of the isolated heart model.

In the present study we used a novel optical technique (14,15), which allowed for the first time quantitative and non-invasive measurements of mitoPO$_2$ in the intact heart. Following validation of our technique for determination of mitoPO$_2$ within the heart, our observations demonstrated rather high mean mitoPO$_2$ values of 35 mmHg in the in vivo heart. However, cardiac mitoPO$_2$ was considerably heterogeneous, such that 10% of mitochondria in the in vivo condition and 30% of mitochondria in the isolated Langendorff-perfused condition still had a mitoPO$_2$ between 0 and 10 mmHg, despite the relatively high mean mitoPO$_2$. Only in the maximal vasodilated condition did the mitochondrial oxygenation status of the isolated heart mimic that of the in vivo heart.

MATERIALS AND METHODS

Animals
Male Wistar rats (Charles River, Wilmington, MA), 38 animals in total with body weight of 351±7 g, were anesthetized with 60 mg/kg pentobarbital and received 5-aminolevulinic acid (ALA; 200 mg/kg dissolved in PBS (100 mg/ml)) through the tail vein, 2–5 h before experimentation or saline (controls). The protocol was approved by the Animal Research Committee of the Academic Medical Center at the University of Amsterdam. Animal care and handling were performed in accordance with the guidelines for Institutional and Animal Care and Use Committees (IACUC).

Isolated cardiomyocytes
Hearts (n=4) were excised from pentobarbital-anesthetized animals and cardiomyocytes isolated according to previous reports (21). Cardiomyocytes were kept in albumin-free, low Ca$^{2+}$-containing Krebs-Henseleit solution until measurements.

Fluorescence microscopy
Fluorescence microscopy was performed using a Leica fluorescence microscope (DM RA HC) with a cooled CCD camera (KX1400, Apogee Instruments, Roseville, CA) and CY3 band pass filter set. Detection of protoporphyrin IX (PpIX) and MitoTracker Green was similar to that previously reported (15). Cardiomyocytes were incubated with MitoTracker Green (27 nM for 30 min) and contraction inhibited with butanedione monoxime (BDM; 20 mM). PpIX bleaches rapidly, and we used this property to separate PpIX fluorescence from other sources of autofluorescence (15). Two successive illuminations of 30 s were performed while integrating the fluorescence images on the cooled CCD. The second image was subtracted from the first image using Image-Pro Plus software (Media Cybernetics, Bethesda, MD). The resulting “bleached image” represented the true PpIX signal. MitoTracker Green images were acquired with a 2 s integration time.

Flow cytometry
Cardiomyocytes were isolated from hearts of either ALA-treated or saline-treated animals (control). The PpIX fluorescence was studied in control and ALA cells with a LSRII flowcytometer (LSRII, BDbioscience San Jose, CA). To study PpIX fluorescence, the viable cardiomyocytes were gated from a FSC (forward scatter) and SSC (side scatter) dotplot, and PpIX was excited by the blue laser (488 nm) and fluorescence was detected at 695 nm.
Imaging cryomicrotome
Hearts from ALA-treated and saline-treated animals were rinsed with saline solution and submerged together in a solution of carboxymethylcellulose sodium solvent (Brunschwig Chemie, Amsterdam) and Indian ink (Royal Talens, Apeldoorn) and frozen at −20 °C in an imaging cryomicrotome (22). The frozen hearts were serially sectioned from base to apex into 17 µm slices. After each cut, images were taken from the cutting plane of the remaining bulk using a 4000–4000 pixel camera (ALTA U16, Apogee Instruments, Roseville, CA) equipped with a 70–180 mm Nikon lens, with excitation set at 510/20 nm and fluorescence detected at 635/30 nm.

Calibration of the PpIX delayed fluorescence lifetime with partial oxygen tension in cardiomyocytes
A cardiomyocyte suspension of 2 ml was placed in a custom-made cell oxygenator (15). An adjustable gas-mixture of O₂, N₂ and 5% CO₂ was blown over the cell suspension with %O₂ set at 0, 1, 2, 3, 4 or 5%. Oxygen consumption was inhibited using KCN (10 mM), rotenone (4 µM) and diphenyleneiodonium (DPI; 40 µM).

Evaluation of the calibration of the PpIX delayed fluorescence lifetime in isolated hearts
To test the feasibility of the calibration of PpIX delayed fluorescence lifetime obtained in isolated cardiomyocytes for use in the intact heart, we performed PpIX lifetime measurements in Langendorff-perfused hearts (n=3) perfused with 2 mM potassium cyanide, 25 mM KCl and 0 mM CaCl₂ to prevent contraction and oxygen consumption of the heart. For each heart, the perfusate was equilibrated with two different %O₂ levels between 0 and 7%, balanced with N₂ and 5% CO₂. Oxygen tension was measured in the inflow just above the heart using a needle-type fiber-optic microsensor (World Precision Instruments, Sarasota, FL).

Isolated mitochondria and effects of ALA administration
Mitochondria were isolated from hearts of animals treated with ALA (200 mg/kg) dissolved in PBS (n=3) or with PBS only (n=3), 3–5 h before isolation. Procedures of isolation were as reported previously (23). In short, hearts were placed in isolation buffer (200 mM mannitol, 50 mM sucrose, 5 mM KH₂PO₄, 5 mM MOPS, 1 mM EGTA, and 0.1% BSA) and minced into small pieces; then 5 U/ml protease was added, and the mixture was homogenized. The homogenate was centrifuged at 3220 g for 10 min, and the pellet was resuspended in isolation buffer and centrifuged at 800 g for 10 min. The remaining supernatant was centrifuged at 3220 g for 10 min. The final pellet was resuspended in isolation buffer and kept on ice, and the protein content was determined by the Bradford method.

All procedures were performed at 4 °C. Mitochondrial oxygen consumption was measured polarographically at 37 °C in a respirometer using mitochondria (0.3 mg protein/ml) resuspended in respiration buffer (23). Respiration was initiated with 10 mM succinate + 10 µM rotenone (state 2 respiration), followed by the addition of 200 µM ADP (state 3 respiration). The respiratory control index (RCI) was calculated as the state-3 to state-4 ratio.

In vivo hearts
Animals (n=5) were anesthetized with s-ketamine (150 mg/kg) and diazepam (1.5 mg/kg), and maintenance anesthesia was provided through i.v. administration of α-chloralose (30 mg/kg/h). Mechanical ventilation (70 breaths per min, 0.4 inspiration phase, 5 mm Hg PEEP) was started following intubation, and a left thoracotomy was performed to expose the heart (24). Mean arterial pressure and heart rate were monitored through a fluid-filled catheter positioned in the cannulated carotid artery. Body temperature was maintained at 37 °C. Inspiration oxygen fraction was initially set at 40% and subsequently switched to 100% and finally 10% O₂. The 40% FiO₂ step was deliberately chosen instead of 20% FiO₂ to ensure compensation for ventilation-perfusion defects due to
mechanical ventilation (25) resulting in arterial PO$_2$ values in the physiological range reported for spontaneously air-breathing animals (~120 mm Hg).

**Microvascular PO$_2$ of the in vivo hearts**
To compare the mitoPO$_2$ with other indices of cardiac oxygenation, cardiac microvascular PO$_2$ (µPO$_2$) of the in vivo heart was determined in separate animals (n=4) by the method of oxygen-dependent quenching of the phosphorescence lifetime of intravenously injected Pd-porphyrin (Pd-meso-tetra(4-carboxyphenyl)porphine, Frontier Scientific Inc, Logan, UT). To this end Pd-porphyrin was bound to albumin according to Sinaasappel et al. (26) and intravenously injected (12 mg/kg). Phosphorescence signals were obtained from the in vivo rat heart using a fiber based phosphorimeter (27). These measurements were performed in a similar in vivo heart model as used for the PpIX determinations, at FiO$_2$ of 40%, 100% and 10%, respectively (see description above).

**Isolated Langendorff-perfused hearts**
Hearts were isolated from pentobarbital-anesthetized animals and perfused at constant flow at 80 mm Hg according to previous reports (28). Hearts (n=6) were paced at 300 beats/min, and a balloon was inserted in the left ventricle to record left ventricular pressure. The perfusate contained glucose (10 mM/l), pyruvate (0.1 mM/l), lactate (1 mM/l), and glutamine (0.5 mM/l) as substrates. Venous oxygen tension was measured in the pulmonary artery using a needle-type fiber-optic microsensor (World Precision Instruments, Sarasota, FL). Perfusate was equilibrated with either 95%/0%/5%, 70%/25%/5% or 45%/50%/5% of O$_2$/N$_2$/CO$_2$, respectively, using separate calibrated gas bottles. In a separate series (n=6), the effect of maximal vasodilatation (100 μM adenosine and 1 μM nitroprusside) on the oxygenation status was studied.

**Delayed fluorescence setup**
The setup was essentially the same as that described previously (14). In short, the excitation source was a tunable laser providing pulses of 2–4 ns (510 nm, 0.2 mJ/pulse). The detector was a red-sensitive photomultiplier tube combined with a monochromator set at 640 nm. Signal processing was done with a home-built integrator with an integration time of 3.5 μs and a reset time of 0.5 μs. A PC-based data-acquisition system sampled the signal at 250 kHz and averaged 64 laser pulses (repetition rate 20 Hz) prior to analysis. Controller and data-acquisition software was written in LabView (Version 7.1, National Instruments, Austin, TX).

**Recovery of mitoPO$_2$ histograms**
In case of a non-homogenous mitoPO$_2$, the delayed fluorescence signal can be described generally by an integral over an exponential kernel:

$$y(t) = \int_0^t \exp(-\lambda t) f(\lambda)$$

where f(λ) denotes the spectrum of reciprocal lifetimes that should be determined from the finite data set y(t). According to Golub et al. (19), a detailed recovery of the underlying oxygen distribution can be obtained by assuming that the delayed fluorescence signal can be described by a sum of rectangular distributions with adequately small chosen width (2δ), resulting in the following fit equation:

$$Y^*(t) = Y(t) \left[ \exp(k_o t) k_q \delta t / \sinh(k_q \delta t) \right] = \sum_{i} w_i \exp(-k_q Q_i t)$$

where $Y(t)$ is the normalized phosphorescence data, $k_0$ is the first order rate constant for delayed fluorescence decay in the absence of oxygen, $k_q$ is the quenching constant, 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 $\Sigma w_i = 1$). Recently, this approach was
successfully used by our group for the recovery of microvascular PO\textsubscript{2} histograms from phosphorescence lifetime measurements (29) and mitoPO\textsubscript{2} histograms from delayed fluorescence lifetime measurements in rat liver in vivo (14). Recovery of oxygen histograms from the photometric signal was performed with the GraphPad Prism package (Version 4, GraphPad Software Inc, San Diego, CA). Mean mitoPO\textsubscript{2} values were calculated from the retrieved mitoPO\textsubscript{2} histograms.

\[ PO_2 = \frac{1}{\tau} - \frac{1}{\tau_0} \]
\[ k_q \]
\[ 1/\tau \text{ (µs)} \]

\[ k_q = 826 \pm 51 \text{ mmHg}^{-1} \text{ s}^{-1} \]

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\textsuperscript{*} Respiratory rate in nmol O\textsubscript{2} \cdot min\textsuperscript{-1} \cdot mg protein\textsuperscript{-1}

**FIGURE 1** PpIX in cardiomyocytes and mitochondria. (A) Fluorescence microscopy showing (from left to right) PpIX fluorescence, MitoTracker Green fluorescence and colocalization of PpIX and Mitotracker Green fluorescence in isolated cardiomyocyte. (B) In vitro calibration experiments showing two examples of delayed fluorescence traces recorded at two different oxygen concentrations and the reciprocal delayed fluorescence lifetime (1/\(\tau\)) versus PO\textsubscript{2} in suspensions of isolated cardiomyocytes. Inserted is the Stern–Volmer equation in which \(k_q\) is the quenching constant and \(\tau_0\) is the lifetime at zero oxygen. Lifetimes were retrieved from the data by a mono-exponential non-linear Marquart–Levenberg fitting procedure. Shown are the mean and SD. (C) Function of mitochondria isolated from hearts of control or ALA-treated animal showing state 3 and 4 respiration, respiratory control index (RCI) and the P/O ratio.
Chemicals
Mitotracker Green was obtained from Invitrogen (Carlsbad, CA). Pentobarbital was obtained from Ceva (Sante Animale B.V., Maassluis, The Netherlands), Diazepam from Centrafarm (Etten-Leur, The Netherlands), S-ketamine from Pfizer B.V. (Capelle a/d IJssel, The Netherlands) and PBS was obtained from Baxter. ALA, α-chloralose, KCN, rotenone, DPI, BDM, KH$_2$PO$_4$, MOPS, EGTA, BSA, protease, mannitol, succinate, pyruvate, sucrose, lactate, glucose, glutamine, adenosine, nitroprusside and the compounds for the Krebs–Henseleit buffer were all obtained from Sigma (St. Louis, MO).

Presentation of data
The data are presented as mean ± SEM, unless stated otherwise.

RESULTS
Subcellular location of PpIX
Four hours after ALA administration, we observed a similar nonhomogeneous cellular distribution of PpIX fluorescence and MitoTracker Green signals in isolated cardiomyocytes (Fig. 1A). The very high colocalization of both images indicates the mitochondrial localization of the PpIX signal.

Calibration of the mitochondrial signal
Oxygen-dependent delayed fluorescence traces were clearly detectable from cardiomyocyte suspensions subjected to various oxygen levels. The calibration curve of reciprocal lifetime versus partial oxygen pressure for cardiomyocytes demonstrated a quenching constant ($k_q$) that was similar to the $k_q$ obtained in hepatocytes (14), indicating the general applicability of the calibration constant across organs (Fig. 1B). Lifetime under zero oxygen conditions ($\tau_0$) was 0.8 ms.

ALA treatment does not affect mitochondrial oxygen consumption (Fig. 1C)
In order to examine whether ALA-induced mitochondrial accumulation of PpIX affected mitochondrial oxygen consumption, mitochondria were isolated from ALA-treated and vehicle-treated animals. PpIX accumulation had no effect on respiration, coupling ratio or oxidative phosphorylation of the mitochondria, demonstrating that ALA-treatment did not disturb oxygen consumption.

FIGURE 2 Evaluation of the calibration constants in isolated rat heart. (A) Measured delayed fluorescence lifetime versus the PO$_2$ in the perfusate after cessation of mitochondrial oxygen consumption by addition of cyanide. Lifetimes were retrieved from the data by a mono-exponential non-linear Marquart–Levenberg fitting procedure. (B) MitoPO$_2$ versus the PO$_2$ in the perfusate. MitoPO$_2$ was calculated from the delayed fluorescence lifetimes in panel A using the quenching constants obtained in suspensions of cardiomyocytes. The solid line is the line of equality.
Validation of the calibration constants in isolated rat heart
To test the validity of the calibration constants obtained from the isolated cardiomyocytes in intact heart we measured the delayed fluorescence lifetime in isolated Langendorff-perfused hearts from ALA treated animals. Oxygen consumption was blocked by addition of cyanide and measurements were performed at varying oxygen tensions in the perfusate. Blockage of oxygen consumption abolishes the oxygen gradients in the tissue and mitoPO$_2$ should equal the PO$_2$ in the perfusate. Mono-exponential fitting of the delayed fluorescence signals showed clearly oxygen-dependent delayed fluorescence lifetimes (Fig. 2A). Conversion of the lifetimes into mitoPO$_2$ values using the calibration constants $k_q = 826$ mm Hg$^{-1}$s$^{-1}$ and $\tau_0 = 0.8$ ms obtained from cardiomyocyte suspensions resulted in excellent agreement between mitoPO$_2$ and the PO$_2$ in the perfusate (Fig. 2B).

Distribution of ALA enhanced PpIX
Our analysis method assumes that PpIX is homogenously distributed in the measurement volume. For proper interpretation of our data it is essential to know to what extend this assumption holds in practice. Images from the cryomicrotome clearly showed increased, diffuse and near homogeneous red fluorescence throughout the entire myocardium after ALA administration (Fig. 3A). Some local differences in fluorescence intensity were visible, but this comprised relatively large areas also visible in the control heart. Therefore these differences might be due to fixation and cutting artifacts instead of true heterogeneities in the PpIX distribution. This was indeed confirmed by the flow cytometry experiments, which showed a uniform emergence of red fluorescence after administration of ALA (Fig. 3B).

FIGURE 3 Distribution of ALA enhanced PpIX in the myocardium. (A) Typical example of an image obtained in the cryomicrotome showing PpIX fluorescence in hearts from a control (saline treated) and an ALA treated animal. Excitation at 510 nm, emission at 630 nm. (B) Flow cytometry in cardiomyocytes isolated from a control and ALA treated animal. Excitation at 488 nm, emission at 695 nm. The histograms are normalized to the maximal counted number of cells having equal fluorescence intensity (% of Max), i.e. the peaks of the individual histograms.
Mitochondrial PO$_2$ in the in vivo heart

FiO$_2$-dependent delayed fluorescence signals were obtained from in vivo rat heart (Fig. 4A). At 40% FiO$_2$, the largest fraction (26%) of mitochondria was found in the range of 10–20 mmHg, with a mean mitoPO$_2$ of 35±5 mmHg (Fig. 4B). Increasing FiO$_2$ to 100% had no major effect on the mitoPO$_2$ distribution; however, reducing FiO$_2$ to 10% resulted in large changes: 46% of the mitochondria were in the lowest oxygen range of 0–10 mmHg (Fig. 4B). The imposed FiO$_2$ changes were accompanied by alterations in arterial oxygen tension and blood pressure, without changes in arterial CO$_2$ tension (Table 1). In separate experiments, we measured microvascular PO$_2$ (µPO$_2$) using the exogenous dye Pd-porphyrin for the three different FiO$_2$. µPO$_2$ amounted to 67±3, 108±9 and 22±4 mmHg, at 40%, 100% and 10% FiO$_2$, respectively.

FIGURE 4 MitoPO$_2$ in the in vivo heart. (A) Delayed fluorescence traces from the heart of an animal ventilated with 10% oxygen (left panel) and 40% oxygen (right panel). (B) Distributions of mitoPO$_2$ at different FiO$_2$ (n=5). The grey lines denote the mean mitoPO$_2$.

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<th>MAP mm Hg</th>
<th>Heart rate Beats/min</th>
<th>PaO$_2$ mm Hg</th>
<th>PaCO$_2$ mm Hg</th>
<th>tHb mmol/l</th>
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<td>40</td>
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TABLE 1 Hemodynamics and blood gas values of rats in vivo (n = 5). FiO$_2$ = inspiration oxygen %; MAP = mean arterial pressure; PaO$_2$ = partial arterial oxygen tension; PaCO$_2$ = partial arterial carbon dioxide tension; tHb = total hemoglobin. Values are given as mean ± SEM.
MitoPO$_2$ in the Langendorff-perfused heart

Delayed fluorescence signals were obtained from isolated hearts, showing decreasing decay times (shorter $\tau$) with increasing percentage of oxygen (%O$_2$) in the perfusate or with increased flow (going from the normal vasodilated to the maximal vasodilated condition) (Fig. 5A). The recovery of mitoPO$_2$ histograms provides a detailed view of the effects of %O$_2$ and vasodilatation on mitochondrial oxygenation (Fig. 5B). Only at maximal oxygenation (95%) and maximal vasodilatation did mitoPO$_2$ start to approach a normal distribution in the isolated heart, with a mean mitoPO$_2$ of 40±3 mmHg. The normally perfused Langendorff-condition had a mean mitoPO$_2$ of 29±5 mmHg (at 95% oxygen). A dichotomous distribution was observed, with the largest fraction of mitochondria falling into the lowest oxygen range (0–10 mm Hg). The fraction of mitochondria in the lowest oxygen range increased further as the % O$_2$ was decreased to 70% and 45%.

FIGURE 5 MitoPO$_2$ in the isolated Langendorff-perfused heart. (A) Examples of delayed fluorescence traces for 95%, 45%, and 6% oxygen saturated perfusate in a vasodilated heart (left panel) and delayed fluorescence traces of a normal and maximally vasodilated heart (right panel). (B) Distributions of mitoPO$_2$ at different %O$_2$ in perfusate for normal (n=6) and maximally vasodilated hearts (n=6). The grey lines denote the mean mitoPO$_2$. 

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TABLE 2 Functional characteristics of isolated Langendorff-perfused rat hearts. Values are given for hearts (n=6) perfused at normal vasodilatation (normal flow) and at maximal vasodilatation (n=6; high flow) for different % oxygen in the perfusate. Gww = gram, wet weight; Pperf = perfusion pressure; Psys = systolic pressure; Pdia = diastolic pressure; VPO2 = venous oxygen tension; MVO2 = oxygen consumption, RPP = rate pressure product (Psys × heart rate). Values are given as mean ± SEM.

Decreasing %O2 was associated with decreased mechanical performance and oxygen consumption of the hearts, whereas maximal vasodilatation increased mechanical performance and oxygen consumption (Table 2). It should be noted that oxygen consumption increased with vasodilation due to the large coronary flow increases, despite the increase in venous PO2 with vasodilation for each level of %O2 perfusate. Increasing the heart rate from 300 to 400 BPM did not change the mitoPO2 distribution in hearts without vasodilation (Fig. 6). In hearts with maximal vasodilation there appeared to be a tendency towards lower mitoPO2 values at higher heart rate without reaching significance in any of the bins (paired non-parametric testing).

![FIGURE 6 Response of mitoPO2 to a heart rate step in the Langendorff-perfused heart. Distributions of mitoPO2 at 300 and 400 beats per minute (BPM) with and without maximal vasodilation and with 95% oxygen saturated perfusate.](image-url)
DISCUSSION
This study is, to our knowledge, the first to describe direct measurements of mitochondrial oxygen tension within the intact heart. The main findings provide evidence that mean cardiac mitoPO$_2$ in vivo has a relatively high value of 35 mmHg. Importantly, we found that mitoPO$_2$ was heterogeneously distributed within the rat heart in vivo, such that even when mean mitoPO$_2$ was 35 mmHg, 10% of mitochondria still had a low PO$_2$ between 0 and 10 mmHg. In addition, the frequently used isolated heart preparation operates at a lower mitochondrial oxygenation status than that of the in vivo heart.

In vivo myocardial PO$_2$ and mitoPO$_2$ heterogeneity
Because oxygen transport to the mitochondria is driven by a concentration gradient determined by consumption in the mitochondria, the oxygen tension in the mitochondrial compartment should be at the lowest end of oxygen tensions present compared to other (4) compartments (vascular, interstitial, cytosolic) of the intact heart. The mean mitoPO$_2$ value of 35±5 mmHg reported here is in accordance with tissue PO$_2$ values of 45±8 mmHg (13) and microvascular PO$_2$ values of 50–70 mmHg found in the present and past studies (12), but is much higher than anticipated from estimates derived from cytosolic/interstitial/vascular measurements of 3–17 mmHg (6–11). Interestingly, this observation coincides with recent findings of oxygen pressure in tissues other than heart (30). Myoglobin saturation studies in in vivo pig hearts, using either optical reflectance spectroscopy (31) or $^1$H NMR (32), were unable to detect desaturation of myoglobin. Knowing that these techniques can detect myoglobin desaturation only when intracellular PO$_2$ falls below 22 mmHg (32), and that only an average myoglobin saturation is detected, these studies indicate an average cytosolic PO$_2$ above 22 mmHg. Thus, the values obtained with myoglobin saturation techniques are commensurate with our averaged mitoPO$_2$ of 35 mmHg. Only recently has consensus been reached that the levels of oxygen in the tissue are much higher than originally thought of, due to the development of new techniques with increased accuracy (30). The present study with the use of a novel technique clearly indicates that this new consensus may also hold for the heart.

Although the averaged mitoPO$_2$ observed in vivo was higher than anticipated, it is important to take into account the heterogeneity of mitoPO$_2$ within the heart. The observed heterogeneity in mitoPO$_2$ in the present study extends previously observed heterogeneities in, for example, blood flow and oxygen consumption down to the level of the mitochondria (12,16,17). In addition, the mitoPO$_2$ heterogeneity indicates that approximately 10% of the mitochondria in the in vivo heart are exposed to a PO$_2$ between 0 and 10 mmHg. Isolated mitochondria studies have demonstrated that mitochondrial oxidative phosphorylation is dependent on oxygen concentrations at PO$_2$ < 12–15 mmHg (1,3,5). This implies that some fraction of the mitochondria within the in vivo heart may well be partially controlled by the prevailing oxygen tension. That myocardial oxygen may be limiting is also supported by studies in myoglobin-knockout mice. Although the first studies in these mice concluded that myoglobin is of no physiological consequence for cardiac function (33), subsequent studies demonstrated that multiple compensatory mechanism in these mice were present (34), indicating an important role for myoglobin in oxygen delivery. Considering the major role that mitochondria play in cellular oxygen sensing (4), the quantitative determination of mitoPO$_2$ in the in vivo condition provides important information for the design of oxygen critical experiments.

Isolated versus in vivo hearts
In non-vasodilated Langendorff-perfused isolated hearts, the fraction of mitochondria with oxygen tensions between 0 and 10 mmHg increased to 30%, as compared to the 10% observed in the in vivo condition. Interestingly, studies using three-dimensional microvascular modeling together with myoglobin saturation information (35,36) have also concluded that approximately 15–30% of the myocardium in the Langendorff-perfused heart was hypoxic. Alleviating the 30% “hypoxic mitochondrial area” to 10% by increasing perfusate flow was indeed accompanied by increased
myocardial oxygen consumption and mechanical performance, suggesting at least that the normal perfused isolated heart was oxygen limited. This data agree with older literature suggesting that the isolated heart is partially hypoxic (37,38). Our data indicate that the oxygenation status of the isolated Langendorff-perfused heart model is comparable to that of the in vivo heart only in the maximally vasodilated condition.

Methodological considerations
The PpIX delayed fluorescence technique provides a powerful tool for monitoring mitoPO$_2$ in cardiac muscle. The underlying lifetime technology is highly robust in that it needs no recalibration and is immune to changes in tissue optical properties and tissue geometry, important features in a beating heart. A practical advantage is also that the technique does not need a physical contact with the heart, like oxygen electrodes, and is not locally destructive. As an optical technique it is scalable from microscopic to macroscopic implementations providing extensive opportunities for study of the heterogeneity of mitoPO$_2$ and its cause. Because of its resemblance to phosphorescence lifetime measurements further technological developments could provide e.g. imaging (39), 3-dimensional scanning by two-photon excitation (40) and multi-wavelength excitation (29). The main disadvantage is the relatively small penetration depth of light in tissues. Therefore the technique is not non-invasive for the whole organism (like e.g. MRI), but one always needs to make the organ or tissue accessible to the optical device.

In the present study we converted lifetime distribution to oxygen histograms, based on the assumption that the heterogeneity in delayed fluorescence lifetimes arises from a volume with homogeneous distribution of PpIX and heterogeneous concentration of oxygen (19). Such an assumption is generally relied on when converting phosphorescence lifetime distributions into quencher distributions (19,41,42). In the present study, fluorescence microscopy of freshly isolated cardiomyocytes from ALA-treated animals showed the typical mitochondrial fluorescence patterns well known from NADH autofluorescence microscopy (43,44), indicating that PpIX synthesis is not sequestered within a cardiomyocyte. On a more macroscopic level, ALA administration induces a diffuse and near homogenous PpIX fluorescence throughout the heart as observed by the cryomicrotome and flow cytometry experiments.

Based on the work of Gandjbakhche et al. (45) the measurement depth with excitation at 510 nm is estimated to be between 400 and 500 µm, due to absorption and scatter of the excitation light. Sinaasappel et al. (46) estimated a catchment depth of 500 µm, using Monte-Carlo simulations on the penetration depth of 520 nm excitation light in phosphorescence lifetime measurements in the intestine. Assuming a left ventricular free wall thickness of 1.5 mm during diastole and 2.5–3.0 mm during systole, our mitoPO$_2$ measurements mainly reflect the oxygenation of the epicardium. Therefore, the mitoPO$_2$ distributions are unlikely to be caused by differences in endocardial and epicardial oxygenation. We previously demonstrated in rat liver that diffusion of ambient oxygen into the tissue has limited influence on the shape of mitoPO$_2$ histograms (14). Oxygen in the ambient atmosphere marginally contributes to the highest PO$_2$ bins without altering the overall shape of the histograms. Exposure of epicardial surface to air is therefore very likely not the cause of the observed heterogeneity in mitoPO$_2$.

The delayed fluorescence lifetime in vivo may also be affected by bound versus unbound PpIX and pH. Theoretically, unbound PpIX could mimic a very high PO$_2$ and distort the mitoPO$_2$ histograms. Unbound PpIX has a lifetime which is too short to be resolved with our current setup and therefore will not contribute to our signal. Furthermore, binding of PpIX with different environments could affect the lifetime measurements by introducing additional lifetimes at a homogenous PO$_2$. However, the linear relationship between reciprocal delayed fluorescence lifetime and PO$_2$ after abolishing oxygen gradients by addition of cyanide, as predicted by the Stern–Volmer equation (26,47) and obtained by monoexponential fitting on the data, argues against such influence. Physiological changes in pH have
in general only minor effects on the quenching constants of phosphorescence (26,48,49) and delayed fluorescence (15). Overall, despite the assumptions made in the current analysis and the possible caveats this introduces into the interpretation of the data, we regard PpIX delayed fluorescence as a valuable addition to the arsenal of techniques that allows new insight into myocardial oxygenation.

In summary, the data of the present study revealed that the amount of oxygen present within the mitochondria of the intact heart is higher than previously anticipated, is heterogeneously distributed and is lower in the often-used tyrode perfused isolated heart model as compared to the in vivo heart. Together, these results provide a first quantitative characterization of mitochondrial oxygenation within the intact heart in both the in vivo and isolated condition.

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