

Research article

# Assessment of tissue oxygen tension: comparison of dynamic fluorescence quenching and polarographic electrode technique

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## Abstract

**Introduction and methods** Dynamic fluorescence quenching is a technique that may overcome some of the limitations associated with measurement of tissue partial oxygen tension ( $PO_2$ ). We compared this technique with a polarographic Eppendorf needle electrode method using a saline tonometer in which the  $PO_2$  could be controlled. We also tested the fluorescence quenching system in a rodent model of skeletal muscle ischemia-hypoxia.

**Results** Both systems measured  $PO_2$  accurately in the tonometer, and there was excellent correlation between them ( $r^2 = 0.99$ ). The polarographic system exhibited proportional bias that was not evident with the fluorescence method. *In vivo*, the fluorescence quenching technique provided a readily recordable signal that varied as expected.

**Discussion** Measurement of tissue  $PO_2$  using fluorescence quenching is at least as accurate as measurement using the Eppendorf needle electrode *in vitro*, and may prove useful *in vivo* for assessment of tissue oxygenation.

**Keywords** clinical measurement methodology, fiberoptic measurement, fluorescence quenching, ischemia, Stern-Volmer, tissue oxygenation

## Introduction

Accurate measurement of  $PO_2$  in biologic tissues has been of interest to both researchers and clinicians for many years [1]. For basic scientists measurement of  $PO_2$  provides insight into the complexities of oxygen flux at the tissue level, whereas for clinicians it moves the monitoring window a step closer to the cell.  $PO_2$  monitoring has been exploited most effectively by radiation oncologists, who have used intratumoral  $PO_2$  measurements to plan and guide radiotherapy [2]. Many articles in the anesthesia and critical care literature report the application of different technologies designed to measure tissue  $PO_2$  [1,3-14], but the clinical use of  $PO_2$  measurement has largely been limited to assessment of brain tissue [15,16].

Existing technologies for measuring tissue  $PO_2$  are either too expensive for everyday clinical use [14] or are based on polarographic principles [17], meaning that oxygen is consumed in the measurement process. In time this oxygen consumption affects the signal itself, and this effect persists as tissue  $PO_2$  decreases, perhaps making polarographic devices less suitable for detection of tissue hypoxia. We hypothesized that a  $PO_2$  measurement technique based on dynamic fluorescence quenching would provide a way to overcome the limitations of the current polarographic technique. We report here a head-to-head bench comparison of  $PO_2$  measurement using polarography versus measurement using dynamic fluorescence quenching. We also present preliminary data from an animal model of tissue ischemia and hypoxia that provide

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$F_{iO_2}$ =fractional inspired oxygen;  $PO_2$ =partial oxygen tension.

evidence of a potentially useful application of fluorescence quenching as a monitor of tissue integrity.

## Methods

### Tonometry apparatus

We constructed an equilibration tonometer (from a sealed, inverted 50-ml syringe part filled with 0.9% saline and a length of tubing ending in a diffusing stone) and immersed it in a water bath maintained at a constant temperature (37°C). We then connected the tubing to a low pressure oxygen/nitrogen gas mixer, such that gas bubbled through the stone and saline solution. A loose cover maintained the gas mixture above the saline but was not so tight as to cause the pressure to rise above atmospheric pressure. In an earlier experiment we determined that the PO<sub>2</sub> in the saline solution equilibrated within 90 s (unpublished observations). An oxygen fluorescence quenching probe (FOXY; Ocean Optics Inc., Dunedin, FL, USA), electronic thermometer, and polarographic (Eppendorf Instruments, Hamburg, Germany) needle electrode were inserted through the tonometer cover. The oxygen concentration of the inflow gas was measured in-line with a conventional fuel cell oxygen analyzer and this was used to calculate a predicted PO<sub>2</sub> (PO<sub>2 pred</sub>) in the saline according to the following equation:

$$PO_{2 \text{ pred}} = FiO_2 \times (PB - PH_2O) \quad (1)$$

Where FiO<sub>2</sub> is the oxygen concentration in the inflow gas, PB is the atmospheric pressure recorded in the laboratory on the day of the experiment, and PH<sub>2</sub>O is the water vapor pressure.

### Dynamic fluorescence quenching optode

The optode consists of a 200-μm aluminum-coated, ruthenium-tipped glass fiber with a medical-grade silicone covering on the tip. The response time of the device is about 45 s in a liquid medium. The light source is a dedicated light-emitting diode that emits pure blue light at a wavelength of 470 nm. When excited the ruthenium emits light (fluoresces) with peak intensity at 600 nm that is quenched by the presence of oxygen. The fluorescence signal is then converted to a PO<sub>2</sub> value by specialized software (OOISENSORS; Ocean Optics Inc.).

### Polarographic needle electrode

This system has been described in detail elsewhere [18]. Briefly, it comprises a needle electrode mounted on a stepping motor that sequentially advances and then retracts the needle tip. This allows the system to create a histogram of PO<sub>2</sub> recordings from the tissue of interest. The current produced by the needle electrode is linearly related to the PO<sub>2</sub> value in the medium surrounding the electrode tip.

### Bench comparison experiment

#### Calibration

For the fluorescence quenching system we used a 1% weight/volume solution of sodium sulfite as zero PO<sub>2</sub> for the low calibration standard. This chemical does not affect the

optical sensing system. Sterile water in equilibration with laboratory air was used as a high calibration standard, using equation 1 with FiO<sub>2</sub> set to 0.21. Although it is theoretically reasonable to calibrate the sensor in one medium (e.g. gaseous) and then measure PO<sub>2</sub> in another (e.g. liquid), we have no experimental data to support this.

The needle electrode was calibrated according to the manufacturer's instructions [19]. As described above, our laboratory bench tonometer was kept at a constant temperature of 37 ± 1°C. It was thus not necessary to correct for temperature in this experiment.

#### Measurement protocol

Once both measurement systems had been calibrated and inserted into the saline tonometer, the system was allowed to come to equilibrium for 5 min. We then varied the concentration of oxygen in the inflow gas so that the PO<sub>2</sub> in the saline would rise or fall. After each change, we waited 3 min for the system to equilibrate before recording the PO<sub>2</sub> value from each device and the PO<sub>2 pred</sub> value from the inflow gas. We repeated these steps to record 58 consecutive data triplets. Finally, we re-measured the low and high calibration solutions to assess drift in PO<sub>2</sub> values across the duration of the experiment.

### In vivo application

#### Animal model

The experimental protocol was approved by The University of Texas M.D. Anderson Cancer Center Animal Care and Use Committee. Male outbred Sprague–Dawley rats (*n* = 3) weighing 410–440 g were anesthetized using inhaled isoflurane in a mixture of 35% oxygen and 65% nitrogen. Each animal was placed on a homeothermic blanket (Harvard Apparatus Inc., Holliston, MA, USA); the trachea was then intubated (using a modified neonatal laryngoscope and 14-gauge cannula) and the lungs were mechanically ventilated. A small midline laparotomy was performed to allow for placement of a vascular clip on the infrarenal aorta. Cannulae (PE 20; Harvard Apparatus Inc.) were placed in the left femoral artery and vein to allow measurement of arterial pressure (MLT-1050 transducer; AD Instruments, Mountain View, CA, USA) and administration of 1 ml/100 g per h 0.9% saline. Neuromuscular blockade was achieved using 0.2 mg/kg pancuronium bromide by intravenous bolus, supplemented later as needed. Finally, the dynamic fluorescence quenching optode was attached to a micromanipulator and inserted (via a 19-gauge needle) percutaneously into the right hind limb skeletal muscle bed.

#### Experimental protocol

Once surgery was complete, the animal was allowed to recover for 20 min before the experiment began. Following an initial baseline period of 5 min the aorta was cross-clamped for 30 min, after which the vascular clip was removed. After a 20-min period of reperfusion, the animal was ventilated with

100% nitrogen for 90 s and then the  $\text{FiO}_2$  was returned to 0.35. After a further period of ventilation, the animal was killed by isoflurane overdose and exsanguination via the arterial line.

### Data analysis

To identify relationships between the two measurement techniques and  $\text{PO}_2$  pred, we calculated product-moment correlation statistics. To investigate differences between the two systems and  $\text{PO}_2$  pred, we constructed Bland-Altman plots [20]. For the animal data we performed one-way analysis of variance with Newman-Keuls post-testing to detect differences within groups. Analyses were performed in Excel 2000 (Microsoft Inc., Redmond, WA, USA) using the Analyse-It statistical add-in (Analyse-It Software Ltd, Leeds, UK) and GraphPad Prism 3.02 (GraphPad Software Inc., San Diego, CA, USA).

## Results

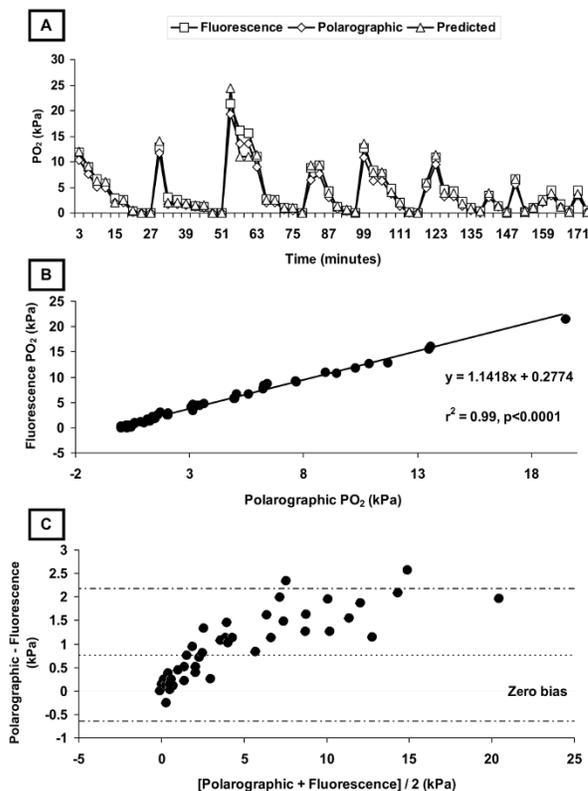
### Bench comparison data

Fig. 1A shows the changes in  $\text{PO}_2$  pred, fluorescence quenching  $\text{PO}_2$ , and polarographic  $\text{PO}_2$  values plotted against time. There was remarkable agreement between the data generated by the quenching technique and that generated by the polarographic technique ( $r^2 = 0.99$ ,  $P < 0.0001$ ; Fig. 1B), but this analysis hides a difference that only becomes apparent on consideration of the Bland-Altman plot of the two measurement techniques (Fig. 1C). Bias proportional to the magnitude of the signal was clearly evident, but it remained unclear which device was responsible for it. Plots of both techniques compared with  $\text{PO}_2$  pred revealed an apparent proportional bias in the polarographic data but not in the quenching data (Figs 2A and 2B). As suggested by Bland and Altman [20], log transformation (Fig. 2C) shows correction of the bias in the polarographic plot, suggesting that the error arose from the polarographic dataset. The fluorescence quenching system showed minimal drift across the course of the experiment (low points were 0.0 and 0.08 kPa and high points were 20.2 and 20.9 kPa at the start and finish, respectively). The polarographic system required recalibrating after approximately 30 data sets, so we were unable to measure the drift of the device.

### In vivo data

A plot of tissue  $\text{PO}_2$  against time is depicted in Fig. 3A. The baseline value of 11.2 kPa reflects the baseline  $\text{FiO}_2$  of 0.35 and is higher than resting values in similar *in vivo* studies that used 0.21 as their baseline  $\text{FiO}_2$  [21]. This is an important concept because arterial  $\text{PO}_2$  has a profound and incompletely understood effect on tissue  $\text{PO}_2$ . The tissue  $\text{PO}_2$  fell very quickly after the aorta was cross-clamped, and it began to rise again when tissue perfusion was re-established. Soon after the animals breathed 100% nitrogen, the tissue  $\text{PO}_2$  fell sharply and rose again when oxygen was reintroduced into the inspired gas mixture. Fig. 3B shows the data presented by intervention. For this graph, the mean values of the last three data points before a change were taken to reflect that intervention.

Figure 1



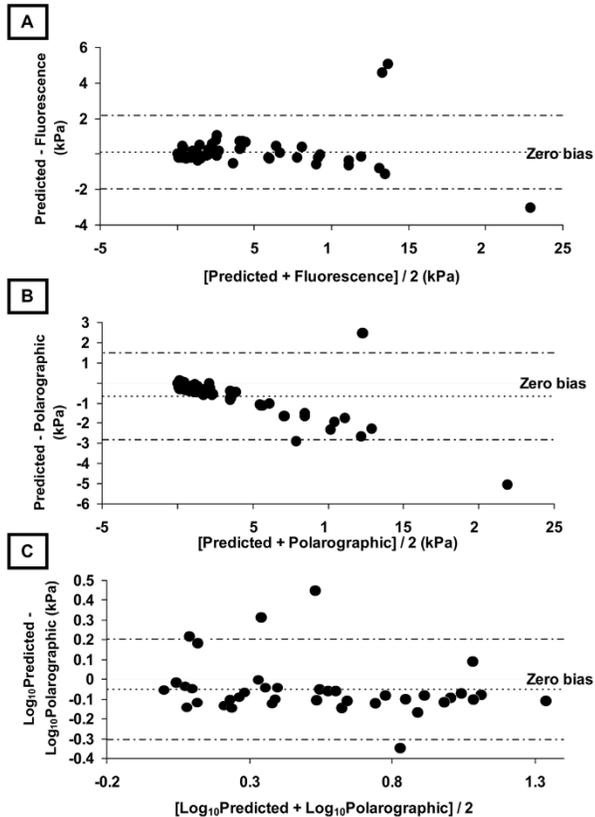
(A) Plot of fluorescence, polarographic and predicted partial oxygen tension ( $\text{PO}_2$ ) against time. (B) Correlation plot of polarographic and fluorescence measurement techniques. (C) Bland-Altman plot of polarographic and fluorescence techniques demonstrating proportional bias arising from one (or both) of the techniques.

## Discussion

There is increasing interest in the use of tissue  $\text{PO}_2$  as a monitor of critical illness [15,16,22-24]. The most favorable tissue in which to record this variable has yet to be determined, and candidates include the gut [25], subdermis [26], skeletal muscle [27], wound margins [11], brain [15], and bladder mucosa [28]. We demonstrated that dynamic fluorescence quenching is at least as accurate as the polarographic system for measuring  $\text{PO}_2$ .

The optical device used in this experiment measures  $\text{PO}_2$  using the principle of dynamic fluorescence quenching. As a triplet molecule, oxygen is able to quench efficiently the phosphorescence and fluorescence of certain luminophores, and it is this concept that underlies the principle used by optical systems such as ours to measure  $\text{PO}_2$ . When an oxygen molecule collides with a fluorophore in its excited state, there is a non-radiative transfer of energy that leads to a reduction in the fluorescence displayed by the fluorophore. The  $\text{PO}_2$  value in the medium that contains the fluorophore is inversely proportional to the intensity of fluorescence exhibited. This relationship is described by the Stern-Volmer equation:

**Figure 2**



(A) Bland–Altman plot of fluorescence technique and predicted partial oxygen tension ( $PO_2$ ), demonstrating close limits of agreement and no systematic or proportional bias over the measurement range. (B) Bland–Altman plot of polarographic technique and predicted  $PO_2$  demonstrating clear proportional bias, which corrects with logarithmic transformation of the data (C).

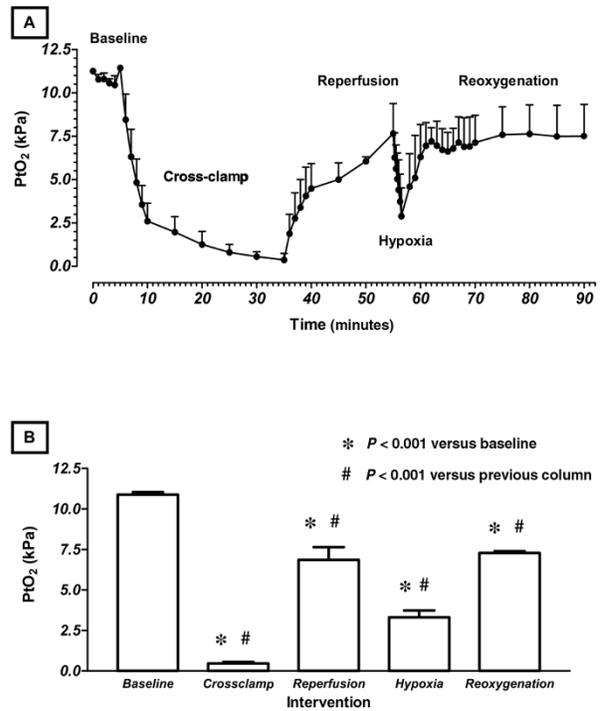
$$I_0/I = 1 + k \cdot PO_2 \quad (2)$$

where  $I_0$  is the fluorescence intensity recorded at zero oxygen tension,  $I$  is the intensity at oxygen tension  $P$ , and  $k$  is the Stern–Volmer constant. According to this equation, the relationship between  $PO_2$  and signal intensity is linear, but this assumption is only valid for lower values of  $PO_2$  (below approximately 20 kPa). For partial pressures greater than 20 kPa, it is necessary to employ a second-order polynomial algorithm:

$$I_0/I = 1 + k_1(PO_2) + k_2(PO_2)^2 \quad (3)$$

where  $I_0$  is the fluorescence intensity recorded at zero oxygen tension,  $I$  is the intensity at oxygen tension  $P$ ,  $k_1$  is the first coefficient and  $k_2$  is the second coefficient. Sinaasappel and Ince [29] pointed out that oxygen is 10% less soluble in serum than in water [30], and thus recommended the use of concentration rather than tension as a unit of measurement for *in vivo* work. The solubility of oxygen in interstitial fluid (in

**Figure 3**



Data are expressed as means  $\pm$  SEM from three animals. (A) Plot of skeletal muscle partial oxygen tension ( $PtO_2$ ) against time, showing clear reduction in signal during both ischemic (cross clamp) and hypoxic epochs. (B)  $PtO_2$  plotted by intervention. Significant differences were found between  $PtO_2$  values for each group and the baseline  $PtO_2$  value, and between each intervention group and the group immediately preceding it.

the tissue milieu) is not known, and it is uncertain whether it differs from that of oxygen in water or serum. Even if it does, it is improbable that the solubility would differ from one type of tissue to the next because that would require a difference in the composition of the extracellular fluid, which is unlikely. Thus, the effect of any unmeasured differences in oxygen solubility would be (at most) to introduce a small systematic bias into the data. The intensity of the fluorescence signal increases as the  $PO_2$  decreases, and this is reflected by the increasing accuracy of optical systems at low  $PO_2$  levels. This feature of dynamic fluorescence quenching, coupled with the fact that it does not consume oxygen in the measurement process, makes it attractive for the detection and monitoring of hypoxia.

According to the manufacturer, the accuracy of the fluorescence quenching technique is affected by the calibration procedure, the resolution (random noise), and deviations from the Stern–Volmer relationship, which occur primarily at higher  $PO_2$  values. It is reasonable to assume that this technique would work *in vivo*, and our representative animal data, although limited, suggest that this approach is feasible and

accurate, at least in skeletal muscle. Predictably, the tissue PO<sub>2</sub> dropped during both the period of presumed ischemia (aortic cross-clamping) and hypoxia (F<sub>IO<sub>2</sub></sub> = 0.0), and we conclude from this that the fluorescence quenching method is able to detect changes in a biologically plausible signal.

The technique of oxygen measurement described here is at least as accurate as the accepted polarographic technique, is cheaper and more wieldy, does not consume oxygen, and may be combined with existing devices (such as a nasogastric tube) more easily. We believe that this approach is a useful addition to the available techniques and that it might allow more widespread use of tissue PO<sub>2</sub> measurement, both as a marker of tissue integrity and an indicator of impending pathology. *In vivo* studies of fluorescence quenching PO<sub>2</sub> measurement under different pathophysiologic conditions are currently underway in our laboratory.

We believe our data illustrate an important concept in the interpretation of method comparison studies. Reliance on the correlation coefficient alone may lead to the erroneous conclusion that there is no difference between the techniques [20]. Close correlation (with a high value for *r*<sup>2</sup>) merely identifies a close relationship between two variables. The method of Bland and Altman reveals differences between the two techniques, and we believe that close limits of agreement with a small overall bias should be characteristic features of a dataset that leads to a conclusion of no difference between the two methods under consideration. Our data revealed a proportional bias in the standard technique, and we were able to demonstrate this by comparing both techniques with a theoretical (but constant for each system) variable.

### Competing interests

None declared.

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