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First experience with a novel luminescence-based optical sensor for measurement of oxygenation in tumors

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Background. *The purpose of this preliminary study was to evaluate a novel luminescence-based fiber-optic sensor (OxyLite system) for the measurement of partial pressure of oxygen (pO_2) in tumors and for the detection of changes in pO_2 as a function of time. The new method was used simultaneously with the laser Doppler flowmetry method for the measurement of relative tissue perfusion.*

Materials and methods. *Blood perfusion and pO_2 were measured continuously via fiber-optic sensors inserted into SA-1 tumors in anesthetized A/J mice. The changes in blood flow and oxygenation of tumors were induced by transient changes of the parameters of anesthesia and by injection of a vasoactive drug hydralazine.*

Results. *Both optical methods used in the study successfully detected the induced changes in blood flow and pO_2. The measurements of pO_2 were well correlated with measurements of microcirculatory blood perfusion. In the majority of pO_2 measurements, we observed an unexpected behavior of the signal during the stabilization process immediately after the insertion of the probe into tumor. This behaviour of the pO_2 signal was most probably caused by local tissue damage induced by the insertion of the probe.*

Conclusion*. The novel luminescence-based optical oximetry can reliably detect local pO_2 changes in tumors as a function of time but some aspects of prolonged pO_2 measurement by this method require further investigation.*

Key words: sarcoma experimental-blood supply; laser-doppler flowmetry; oxygen; luminiscence

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Introduction

Over many years, abundant evidence has been accumulated that the oxygenation status in experimental and clinical tumors can influence the response of these tumors to various therapies, e.g. radiotherapy, hyperthermia, oxygen-dependent chemotherapy, photodynamic therapy and cell-mediated immunotherapy.[1-3] The oxygenation status and hypoxia in particular are also important for the development of malignant growth and for progression and outcome of the disease.[3] The ability to measure oxygenation status in individual tumors would be very valuable for the selection of appropriate therapy, treatment planning, and for prediction of the treatment outcome.

Many different methods have been developed to measure different physiological parameters related to tumor oxygenation or to tumor blood flow.[4,5] In general, tumors are poorly oxygenated in comparison to normal tissues.[1,3,5] The measurement methods should therefore enable an accurate assessment of very low oxygen levels. Extreme intratumoral heterogeneity in oxygenation and blood flow found in many experimental and clinical tumors require an evaluation of oxygenation and blood flow in different regions within the same tumor. Methods suitable for continuous measurement are particularly useful. They enable monitoring of changes in oxygenation at one location. This could be useful for at least two reasons. First, the effectiveness of treatment procedures targeting tumor blood flow and oxygenation in individual tumors could be evaluated. Second, the susceptibility of individual tumors to oxygenation- and blood flow-dependent therapy could be evaluated before the therapy is applied. It is also desirable that the measurement method of choice is noninvasive.

In the present preliminary study, we used two optical measurement methods, which fulfill some of the requirements mentioned earlier in this text. One of them, the time-resolved luminescence-based optical oximetry, presents a new approach for the measurement of oxygen partial pressure in a tissue and is an alternative to the well-established polarographic needle oximetry technique.[6,7] This method has only recently become commercially available (OxyLite instrument, Oxford Optronix, U.K.). There are two major advantages of this new method over standard polarographic method. First, the luminescence-based sensor does not consume oxygen; therefore, to monitor the changes in oxygenation as a function of time, it could be kept in one place in a tissue. Second, its accuracy is inversely proportional to oxygen content in the tissue, which makes this method of particular interest for the measurement of oxygenation in tumors where low oxygen content is typically encountered. The other optical method used in our study, the laser Doppler flowmetry, is not a new technique, but it has not been used extensively for measurements in tumors. Laser Doppler flowmetry enables the monitoring of microcirculatory blood perfusion changes. Although microcirculation and oxygenation in a tissue are related, simultaneous use of both methods can give more information about oxygen supply to a tissue than separate use of a single method. Both methods are minimally invasive and require only thin optical fibers to be inserted into tissue.

Before a new method can be used with confidence in experimental or clinical studies, its characteristics and limitations need to be known and understood. The use of luminescence-based oximetry has been so far documented in very few reports. The main goal of the present study was to get the first experience and to evaluate the usefulness of this new optical method for a continuous measurement of tumor oxygenation and for the detection of short-term oxygenation changes. The measurements were performed on SA-1 tumors in A/J mice under different conditions modifying blood flow- and oxygenation.

Materials and methods

Animals and tumors

The female A/J mice purchased from Rudjer Bošković Institute, Zagreb, Croatia, were used in our study. The mice, 10 to 12 weeks old, were kept in standard animal colony at 22°C and were fed and watered *ad libitum*. The experimental tumor line that we used was SA-1 fibrosarcoma (The Jackson Laboratory, Bar Harbor, USA). Tumor cells for the inoculation of solid tumors were obtained from the ascitic form of SA-1 tumor in A/J mice. Approximately 5×10^5 of viable cells were re-suspended in 0.1 ml NaCl solution (0.9%) and transplanted under the skin. Solid subcutaneous tumors were grown dorsolaterally on the right flank of mice. Experiments were performed 8 to 10 days after the transplantation when the tumors reached the size of approximately 100 mm^3. The size of the tumors was calculated using the ellipsoid formula as $V = \pi abc/6$ where a, b, and c are three mutually perpendicular tumor diameters measured by a vernier caliper. At the end of the experiments, the mice were euthanized under anesthesia by cervical dislocation. The experimentation on mice was conducted in accordance with the pertaining legislation and was approved by the Veterinary Administration of Ministry of Agriculture, Forestry and Food of Slovenia (permit number 323-02-156/99).

Anesthesia

All experimental procedures and measurements were conducted on anesthetized mice in order to eliminate pain and discomfort in mice and to minimize movements of non-restrained mice during long-lasting measurements. Anesthesia was induced and maintained by inhalant anesthetic isoflurane (Flurane-Isoflurane, Abbot Labs, U.K.). The gas mixture of oxygen O_2 and nitrous oxide N_2O (flow of each 0.6 l/min) containing isoflurane at 1.7% concentration was delivered to the mouse via a miniature facemask. While anesthetized, the animals were kept on an automatically regulated heating pad to prevent hypothermia. Rectal temperature was kept as close as possible to 37°C with variations of up to 0.5°C during single measurements and with the contact surface temperature of the heating pad below 39°C.

Oxygenation and blood flow measurement

Partial pressure of oxygen, pO_2, was measured by the OxyLite 2000 instrument (Oxford Optronix Ltd., Oxford, U.K.), a commercially available implementation of a novel time-resolved luminescence-based optical oximetry. The instrument has two independent channels for measuring pO_2 and temperature. The diameter of precalibrated optical probes is 230 μm. A thin wire thermocouple temperature sensor (diameter less than 100 μm) is attached to each pO_2 probe, which allows online temperature correction of pO_2 measurements. The principles of this luminescence-based optical method are described in more detail elsewhere.[6,8] Briefly, pulses of blue light emitted by a LED diode are carried via an optical fiber to ruthenium chloride luminophore, which is incorporated in a silicone rubber that is used to immobilize the tip of the probe. The tip of the probe is placed inside the tissue where oxygenation is to be measured. The incident light pulses induce pulsatile fluorescence of ruthenium molecules. The fluorescence decays in time because of collisions between the oxygen and the ruthenium molecules. The life-time of the excited fluorescence is inversely proportional to pO_2 in the part of the tissue that is in contact with the tip of the probe. The pO_2 can therefore be calculated from the measured life-time of the fluorescence using the so-called Stern-Volmer relation.[8]

Relative blood perfusion was monitored using a two-channel OxyFlo 2000 laser

Doppler instrument (Oxford Optronix Ltd., Oxford, U.K.). Even though laser Doppler flowmetry (LDF) can be applied entirely non-invasively, we used thin invasive probes (diameter 200 μm) in order to assess the perfusion inside the tumor. LDF is an optical method used to monitor local microvascular blood flow in a tissue. Extensive literature exists on its theory and application.[9,10] Briefly, when a tissue is illuminated by a coherent laser light, the light scatters on different tissue structures. When photons are scattered on moving structures, their wavelength is slightly changed. This is the so-called Doppler shift effect, which can be measured. The predominant moving structures in a tissue at rest are red blood cells. Their movement, which results from blood flow, can be detected by means of the Doppler shift effect. The output signal of LDF is proportional to the red blood cell perfusion which is defined as the number of the red blood cells multiplied by the mean velocity of these cells that move in the tissue sampling volume. The constant of proportionality between the perfusion and the detected LDF signal is unfortunately different for each location even within the same tissue. This means that all LDF measurements are intrinsically of relative nature and are quantified in arbitrary blood perfusion units (BPU).

Both instruments OxyLite and OxyFlo were connected to OxyData data acquisition unit (Oxford Optronix Ltd., Oxford, U.K.), which enabled data storage to a PC via a SCSI connector. All signals were sampled and stored at the frequency of 20 Hz.

Measurement protocol

Anesthesia in the mouse was started in the induction chamber at a concentration of isoflurane of 3 %. The mouse was then placed on the heating pad in prone position. No physical restriction was used. The anesthetic gas was delivered via a miniature facemask. The concentration of isoflurane was reduced to 1.7 %, which provided stable anesthesia. Rectal and surface temperature probes were attached for control of the core temperature of the mouse and of the surface temperature of heating pad. Approximately four minutes after the induction of anesthesia, the pO_2 and the LDF probes were inserted into the tumor through small superficial incisions in the skin. The probes were inserted through the incisions, pushed a few millimeters further into the tumor and then slightly withdrawn in order to minimize the pressure of the tip of the probe on the surrounding tissue. Although an exact positioning of the probes was not possible, one pair of pO_2 and LDF probes was inserted in a peripheral region of the tumor and the other in a central region of the tumor. Data recording was started normally about five minutes after the beginning of anesthesia. Special care was taken throughout the measurement not to move the probes or the mouse in order to minimize the movement artifacts in recorded signals.

A typical measurement lasted between one and two hours. In order to evaluate the applicability of both measurement methods for the detection of the changes in the blood perfusion and oxygenation in tumors, different procedures were applied. These procedures to induce changes in blood flow and oxygenation were performed only after the recorded signals had been stable for at least ten minutes. Normally, the stability of all signals (two pO_2 and two LDF) was reached between 20 and 40 minutes after the start of anesthesia.

The effect of hydralazine

An arteriolar vasodilator hydralazine (HYZ) was injected i.v. at a dose of 2.5 mg/kg of mouse weight. The solution for injection was prepared from powdered HYZ (Hydrazinophthalazine, Sigma Chemical Co., U.S.A.) by

dissolving it in sterile physiological saline (0.9 % NaCl). The mice in the control group were injected with sterile physiological saline only.

The effect of anesthetic concentration

Normal anesthesia was maintained by delivering isoflurane at a concentration of 1.7 % in a steady flow of O2 (0.6 l/min) and N2O (0.6 l/min). For the purpose of evaluation of the effect of anesthetic concentration on the blood flow and oxygenation in tumors, the concentration of isoflurane was increased to 3% for three minutes and then returned to the normal level of 1.7 %.

The effect of euthanasic procedure

In order to evaluate the validity of measurements, pO_2 and blood perfusion were also monitored during euthanasia of mice at the end of experiment. First the delivery of O_2 was eliminated from anesthetic gas mixture while maintaining the flow of nitrous oxide and isoflurane. Within two minutes after this procedure, the mice stopped breathing.

Results

A typical measurement

Both pO_2 and blood perfusion were measured at two locations in each tumor. In most cases the data recording was started immediately after all four probes were inserted into the tissue and, in few cases, the data recording was started just before the probes were inserted. All this occurred within five minutes after the start of anesthesia. The measurement lasted between one and two hours. Figure 1 shows an example of typical signals recorded simultaneously from one tumor during the first 40 minutes of measurement. Several important features characteristic of all pO_2 and LDF measurements can be observed.

After the insertion of the probes, the value of pO_2 in all measurements varied rapidly (within one minute). It decreased from initially high level to zero or close to zero value. We call this a *decrease phase* which cannot be observed in Figure 1 because, in this case, the data recording was started after the insertion of the probes. Following the decrease phase, two different types of pO_2 recordings were observed. An example of the first type, we shall call it a type I pO_2 signal, is shown in Figure 1a. In the type I measurements, the pO_2 value remained at zero or close to zero for a period of time which varied between different measurement locations, but in the majority of measurements, it lasted about five minutes. We call this a *zero pO_2 phase*. Following the zero pO_2 phase, the value of pO_2 in the type I measurements entered the *increase phase* during which the value of pO_2 was slowly increasing. The increase phase lasted on average between 15 and 20 minutes. After the increase phase, pO_2 value stabilized and remained mostly unchanged thereafter unless some oxygenation-modifying procedure was applied. We shall refer to this final stage in type I pO_2 measurements as to a *plateau phase*. In most type I measurements, pO_2 continued to increase very slowly even during the plateau phase but this increase was much slower than the increase during the *increase phase*. The type I pO_2 measurement was found in approximately 70 % of all measurement locations in tumors.

In about 30 % of all pO_2 measurements performed, pO_2 in tumors remained at zero or close to zero level for the entire period of observation after the initial *decrease phase*. An example of this second type of pO_2 recording, we shall call it a type II pO_2 signal, is shown in Figure 1b. In comparison to the type I measurements, the type II measurements are characterized by a complete absence of the *increase* and the *plateau phases* (compare the top two graphs in Figure 1).

It is very important to note that, in some cases, both the type I and the type II pO_2

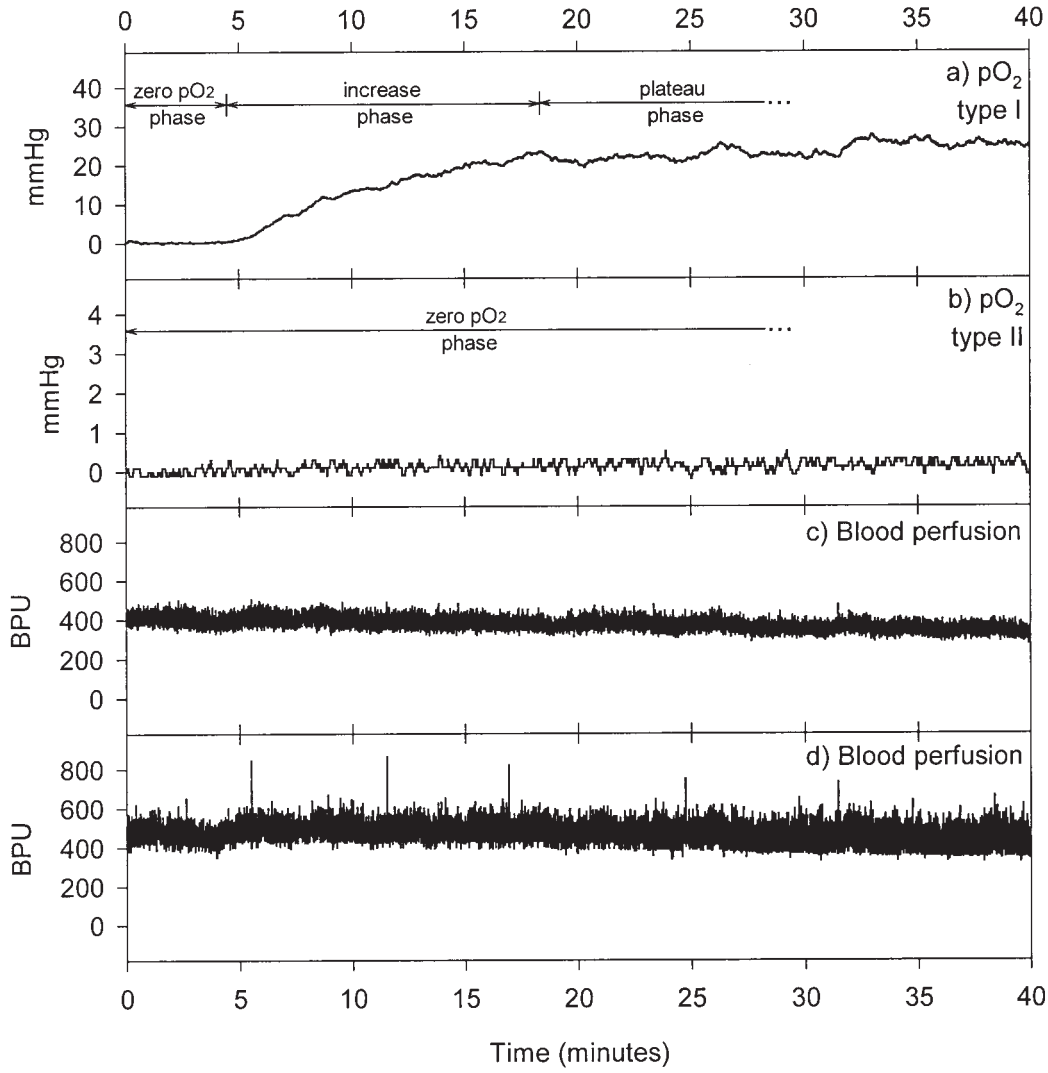

Figure 1. A typical measurement consisting of two pO_2 (a, b) and two LDF measurements (c, d) recorded simultaneously in the same tumor. The two distinct types of pO_2 measurements can be identified (a, b). The first 40 minutes of recording from a single tumor are shown.

measurements were encountered within the same tumor, as in the case presented in Figure 1. In other cases, both pO_2 measurements in tumor resulted in either of the two types, the type I or the type II measurement. In order to obtain a representative pO_2 value for each type I measurement, we averaged the raw pO_2 signal in the plateau phase over a period of five minutes. It is again important to note that these averaged pO_2 values in the type I measurements varied extremely between tumors and also from one location to another within the same tumor, from as little as 1 mmHg to more than 40 mmHg in a few cases. All tumor pO_2 values at rest including the averaged type II values were pooled to-

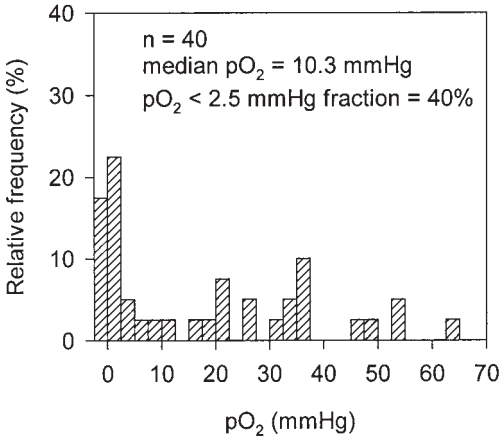

Figure 2. Distribution of all tumor pO_2 values measured at rest (n = number of measurements).

gether and are presented in a histogram in Figure 2. The median pO_2 value of 40 measurements from 28 tumors was 10.3 mmHg. A fraction of pO_2 values below 2.5 mmHg was 40 %.

Contrary to the pO_2 signals, only one type of LDF measurements was identified in tumors (Figure 1c and 1d). All recorded LDF signals became stable soon after the insertion of probes. A zero or close to zero blood perfusion was never encountered, which again is contrary to the pO_2 measurements. Due to the nature of LDF technique, the blood perfusion measurements are extremely sensitive to any kind of movement of probes relative to the surrounding tissue. The "smeared" blood perfusion signals in Figure 1 is a movement artifact caused by a quasi-periodic breathing of the mouse. This is shown in more detail in Figure 3. The spikes in Figure 3 correspond to inhalation and the "valleys" in between the spikes correspond to exhalation. The true blood perfusion is at the lower edge of the "smeared" blood perfusion signals in Figure 1. The amplitude of the quasi-periodic component in LDF signal, which could be even bigger than the true perfusion component of the signal, depended on the position of individual LDF probes relative to the direction of tumor movement caused by respiration. Because of this, substantial differences in the relative amplitude of the quasi-periodic component in comparison to the true perfusion level in different locations within the same tumor were common (compare Figures 1c and 1d).

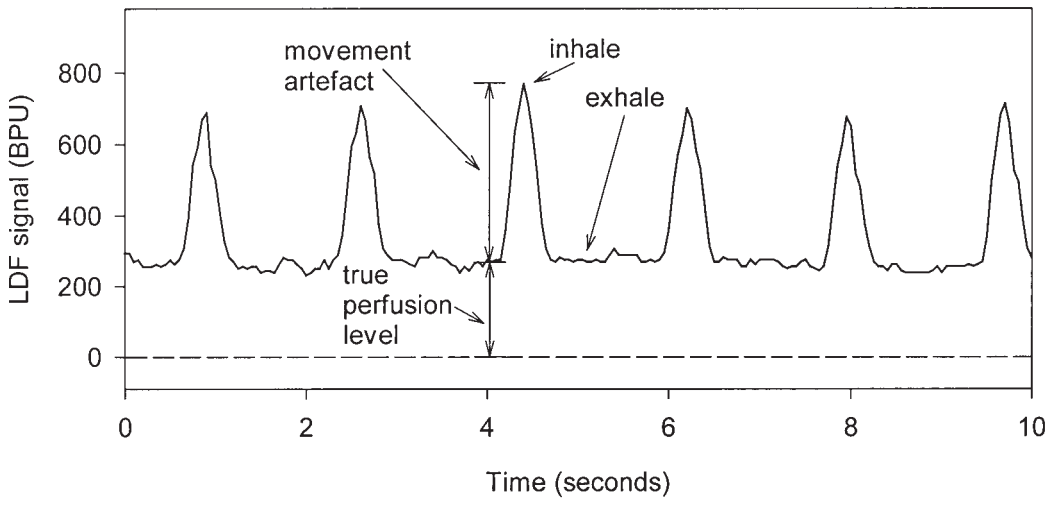

Figure 3. A close-up of a typical blood perfusion signal measured by LDF. The movement artifact caused by quasi-periodic respiration of the mouse is shown.

The effect of hydralazine

Within one minute after the injection of HYZ, all type I pO_2 and all blood perfusion signals in tumor started to decrease (example in Figure 4b, c, d). In five to ten minutes after the injection, these signals reached the lowest level. On average, pO_2 decreased by 80 % (n = 13) and blood perfusion decreased by 50 % (n = 17). HYZ also induced a decrease in respiration rate and an increase in depth of breathing in mice, thus resulting in increased amplitude of the movement artifact in LDF signals. In most tumors treated with HYZ, type I pO_2 signals and blood perfusion signals started to recover very slowly approximately half an hour after the injection of HYZ. In the type II pO_2 signals (Figure 4a) no change was observed after the injection of HYZ. No significant changes were observed either in the type I pO_2 signals or in the blood

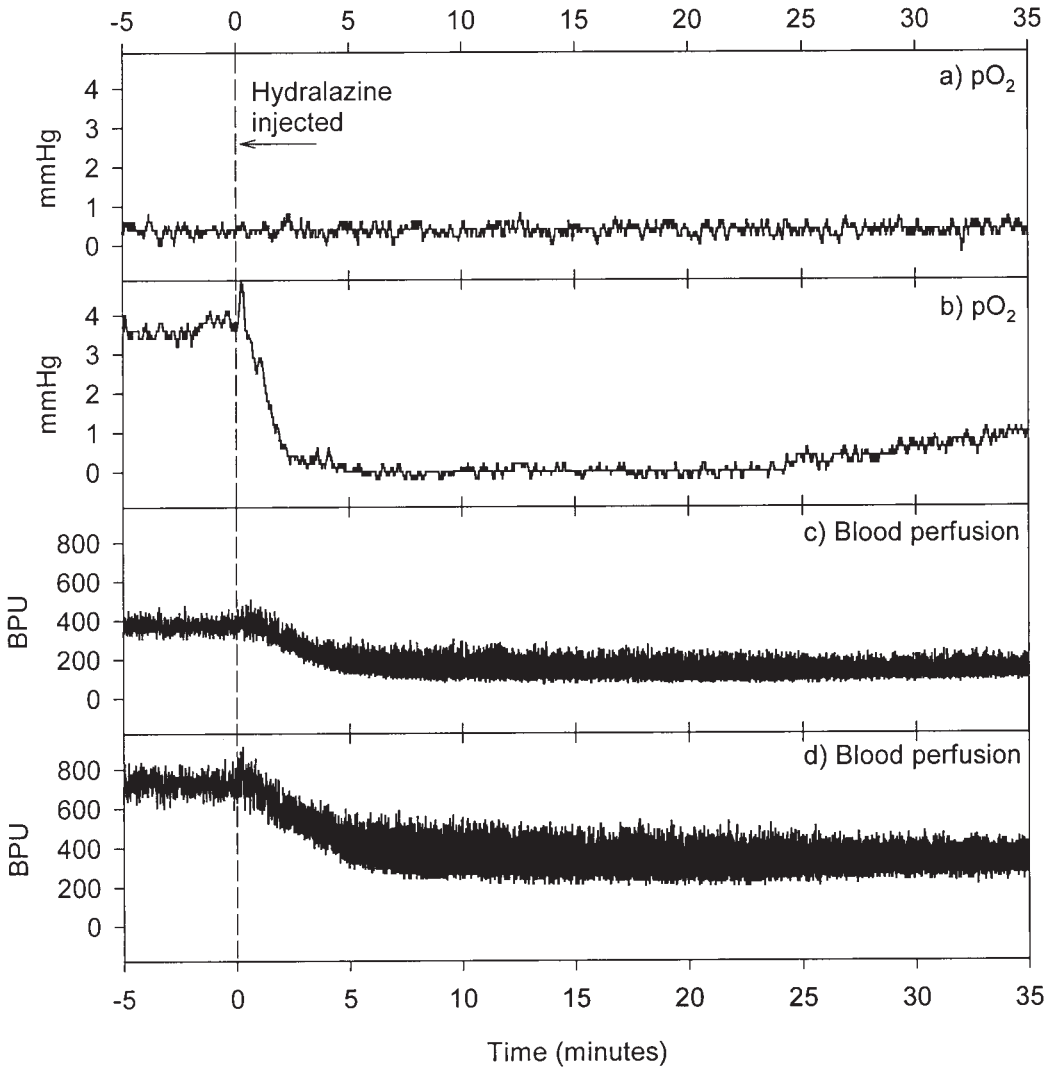

Figure 4. The effect of hydralazine on tumor pO_2 (a, b) and blood perfusion (c, d). The vertical line shows when hydralazine (dose 2.5 mg/kg) was injected. All recordings are from the same tumor.

perfusion signals in control animals which were injected with physiological saline (example not shown). The difference in the decrease of type I pO_2 values in tumors between HYZ-treated and control mice was highly significant ($p < 0.001$; Mann-Whitney rank sum test) as was also the difference in the decrease of blood perfusion between HYZ-treated and control mice ($p < 0.001$; Mann-Whitney rank sum test).

The effect of anesthetic concentration

An attempt was made to modify the blood perfusion and oxygenation in tumors by a transient increase and subsequent decrease of isoflurane concentration in the inhalation mixture. An example of the results in one tumor is shown in Figure 5. About one minute and a half after the increase of isoflurane concentration from 1.7 % to 3 %, the blood perfusion started to decrease, as can be seen in both LDF signals in Figure 5c and d. With the increase of isoflurane concentration the respiration in mice to become slower and jerkier, which resulted in an increased amplitude of the movement artifact in LDF signals. The decrease of blood flow was closely followed by a decrease in the type I pO_2 signals (Figure 5a). When isoflurane concentration was returned to normal level of 1.7 %, the blood perfusion started to increase within a few seconds. After a delay of about 1.5 minutes, the type I pO_2 signal also started to increase. While the LDF signals asymptotically approached the pre-treatment value, the type I pO2 signals usually approached the pre-treatment level after an overshot as can be seen in Figure 5a. No changes at all were seen during the described procedure in the type II pO_2 signals (Figure 5b).

The effect of euthanasic procedure

For the purpose of validation of the measurements, pO_2 and blood perfusion were also monitored during euthanasic procedure. At the end of each measurement, the flow of oxygen to the inhalation mixture was terminated while maintaining the flow of nitrous oxide and isoflurane. A typical example of the effect of this procedure on pO_2 and blood perfusion can be observed in Figure 6 which shows the last four minutes of recorded signals in one tumor. The type I pO_2 signals and blood perfusion signals rapidly decreased. This occurred within one minute after the shutdown of oxygen flow to the vaporizer. The decrease of pO_2 always occurred approximately ten seconds before the decrease of blood perfusion. The bottom level of pO_2 reached after the death was always close to zero in the range of -0.6 to +0.3 mmHg. The type II pO_2 signals (Figure 6b), which were close to zero value during the whole period of observation, remained unchanged during this procedure. In the majority of cases, the bottom level of blood perfusion reached after death was slightly above zero value.

Discussion

The time-resolved luminescence-based optical method used in our study is a new method that has only recently become available on the market. Its use has been reported by few authors so far.[6,7,11-13] Most authors report on evaluation of the new method by comparing this method to other techniques for measuring tissue oxygenation, in particular to the well-established polarographic oximetry. By many researchers, the polarographic oximetry and its implementation in Eppendorf Histograph instrument is considered to be the "golden standard" for pO_2 measurements in experimental and clinical tumors. A generally good correlation between the results of polarographic method and the new optical method was found in tumors,[6,13] but there were also discrepancies between the two methods in certain conditions and in different tissues.[11,13] These discrepancies arose from differences be-

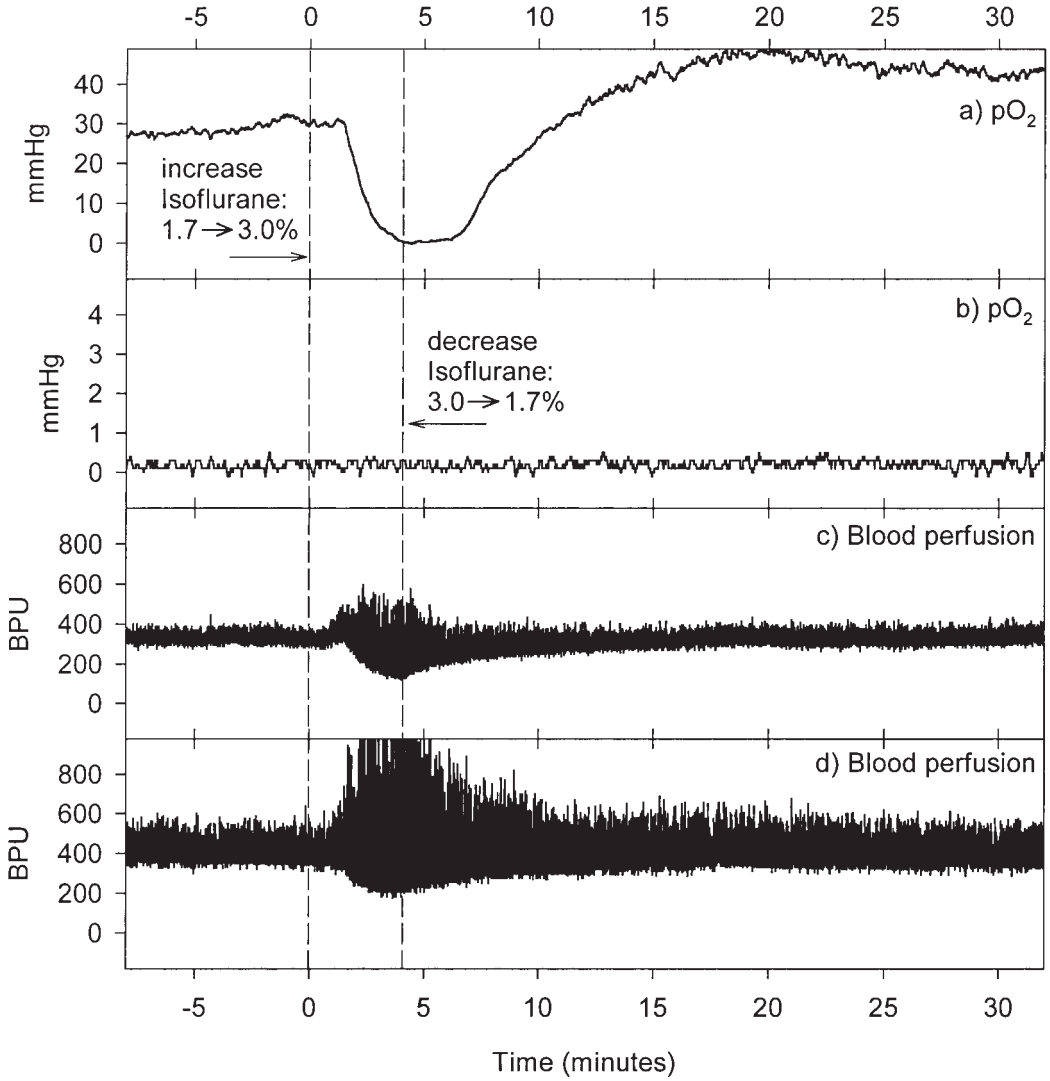

Figure 5. The effect of transient change in anesthetic concentration on tumor pO_2 (a, b) and blood perfusion (c, d). The two vertical lines show when concentration of isoflurane was changed. All recordings are from the same tumor.

tween the two methods such as the underlying physical principle of measurement, dimension of the probe, tissue sampling volume, and consumption of oxygen by the sensor.

A typical measurement

In our study, individual values of pO_2 measured with the novel luminescence-based method at rest prior to any blood perfusion- and oxygenation-modifying procedure were scattered in the range of 0 to above 40 mmHg. The great inter- and intratumoral variability in oxygenation observed in our study is in agreement with the well-documented inter- and intratumoral variability of many experimental and clinical tumors.[14] The histogram of all measured pO_2 values at

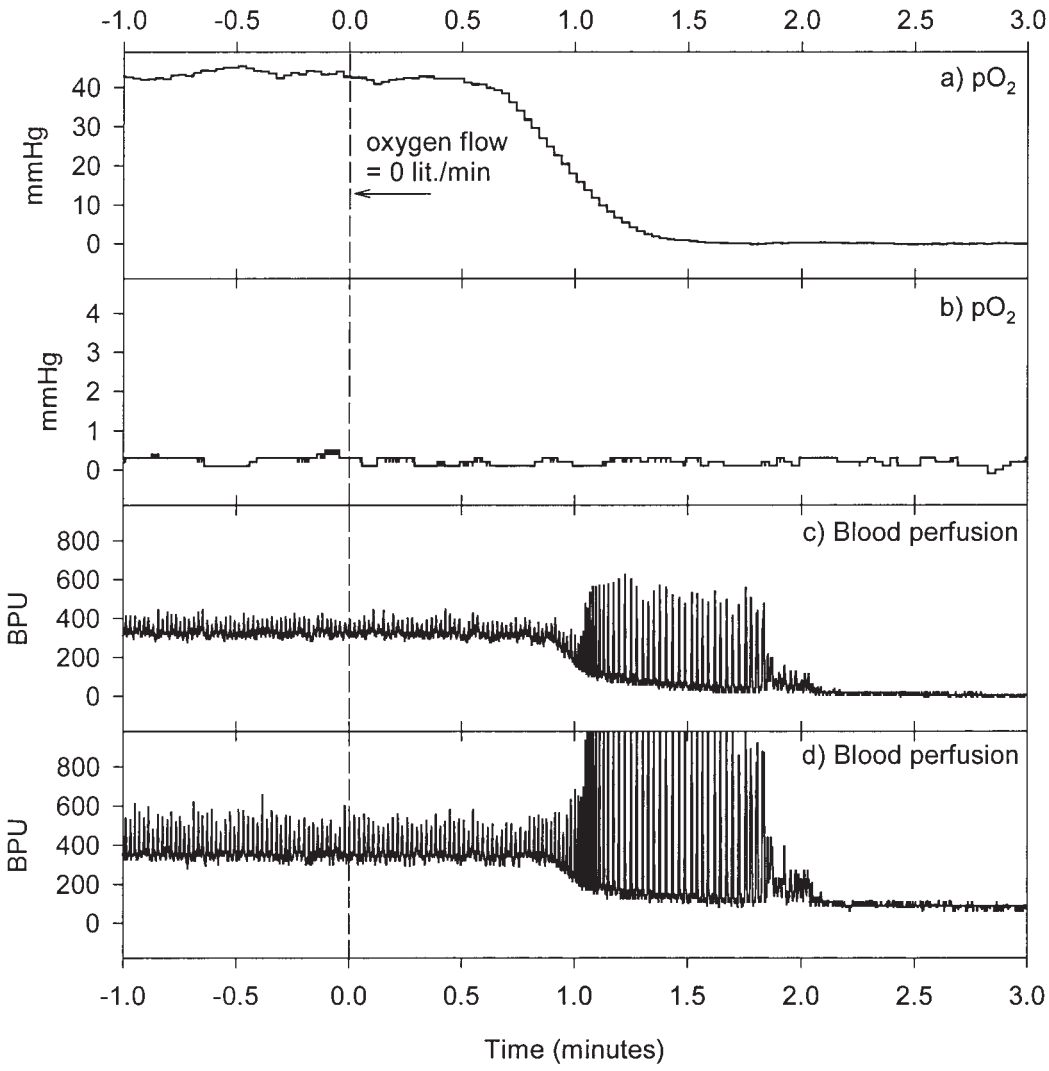

Figure 6. The effect of euthanasic procedure on tumor pO_2 (a, b) and blood perfusion (c, d). The vertical line shows when oxygen supply to inhalation mixture was terminated. All recordings are from the same tumor.

rest (Figure 2) shows a commonly encountered distribution of pO_2 values in tumors with the majority of pO_2 values close to zero. However, we did not expect the multiphase behavior found in the type I pO_2 measurements (Figure 1a).

The most probable reason for the observed phenomenon is the direct effect of the insertion of the probe on the tissue oxygenation. Steinberg *et al*. evaluated the injury caused by the insertion of polarographic pO_2 probe into tumor tissue in different tumor models.[15] They found clear histological evidence of tissue destruction and extravasation of erythrocytes around the insertion channel in tumor tissue caused by the insertion of probe. On the other hand, Schramm *et al*. provided histological evidence for compressed microves-

sels in the vicinity of the tip of polarographic sensor inserted into the rat muscle tissue.[16] The capillary compression increases the perfusion resistance of the passing erythrocytes, decreases the oxygen-carrying capacity of capillaries and, thereby also the oxygenation of the tissue in direct contact with the probe. It is also possible that the insertion of the probe into the tissue causes the vasoconstrictive reaction and subsequent deoxygenation of the tissue.[13]

The following hypothesis may explain the type I pO_2 signals. The insertion of the probe undisputedly injures the tissue in the immediate vicinity of the insertion channel. Some capillaries are ruptured and others can be occluded; a vasoconstrictive reaction in the tissue can occur. All these physiological changes can decrease the oxygen delivery to the tissue in the immediate vicinity of the tip of the probe. This tissue might therefore be severely deoxygenated immediately after the insertion of the probe. This deoxygenated state probably corresponds to the *zero pO_2 phase* in the type I pO_2 measurements (Figure 1a). In the period that follows, this tissue could be re-oxygenated due to gradual restoration of microcirculation. Gradual reoxygenation is probably reflected in the *increase phase* in the type I pO_2 measurements. In case of the type II pO_2 measurements, pO_2 of the tissue is most probably so low that no further deoxygenation and subsequent reoxygenation could be observed after the insertion of the probe.

The multiphase behavior of the type I pO_2 signals was never experienced in our blood perfusion measurements. This difference between generally well correlated LDF signals and type I pO_2 signals can be explained by a much larger tissue sampling volume in case of LDF measurement than in pO_2 measurement. LDF samples a tissue volume of the order of a cubic millimeter. The actual tissue sampling volume for the new time-resolved luminescence-based method is unknown, but is much smaller than the tissue sampling volume of LDF. The measured pO_2 values reflect local oxygenation in a very small part of tissue surrounding the tip of the probe. It is believed that this method samples pO_2 in the cells and intercellular space in direct contact with the tip of the probe, which means a volume of about 400 cells only.[7] The tissue farther away from the insertion channel, which is not affected by the insertion itself, should therefore contribute significantly to the LDF signal but not to the pO_2 signal.

To our knowledge, the unexpected multiphase behavior of pO_2 has been so far reported only in a recent paper by Seddon *et al*..[13] Our results are in excellent agreement with their results even though Seddon *et al.* performed their measurements on non-anesthetized and physically restrained mice. Other authors using OxyLite system have not reported this phenomenon.[6,7,11,12]

The effect of hydralazine

The results of our study showed that hydralazine at a dose of 2.5 mg/kg significantly reduced blood flow and oxygenation of SA-1 fibrosarcoma tumors in A/J mice. The effect was seen in all tumors in case of LDF measurements and in case of all type I pO_2 measurements (Figure 4). The amplitude and dynamics of the decrease in blood perfusion after the injection of HYZ obtained in our study are in direct agreement with the results obtained with LDF by other authors in various mouse tumor models after the injection of HYZ.[17-20] Our measurements of pO_2 by means of the novel time-resolved luminescence-based optical oximetry showed a pronounced decrease of pO_2 after the injection of HYZ. On average, pO_2 decreased by 80 % from the pretreatment level. This decrease was well correlated to the decrease in blood flow measured by LDF. Our results are also in agreement with the results of Okunieff *et al*.[21] who showed that metabolic rate in experi-

mental mouse tumors as measured by ^{31}P-NMR spectroscopy was significantly decreased by HYZ at a dose similar to ours. In their study the decreased metabolism caused by the lack of oxygen was demonstrated by the decrease of organic phosphates and increase of inorganic phosphates. Hydralazine is an effective peripheral vasodilator that has been used in the treatment of hypertension in humans.[22] It relaxes arteriolar smooth muscle, thereby effectively reducing the peripheral vascular resistance and decreasing blood pressure. In these conditions, the organism is trying to maintain normal blood flow in vital organs and tissues by "stealing" the blood flow in less vital tissues.[21] This "steal phenomenon" is responsible for the demonstrated decrease in blood perfusion in tumors. In our preliminary study we provide direct evidence of markedly decreased tumor oxygenation caused by the decrease in blood perfusion after the injection of hydralazine.

The effect of anesthetic concentration

Anesthetics undoubtedly affect a number of parameters of physiological conditions in mice. Isoflurane used in our study is a recommended anesthetic for small animals due to its minimum side effects, stable anesthesia, and wide safety margin. Isoflurane produces little or no depressant effect on cardiovascular system but it causes some respiratory depression.[23] Nitrous oxide, which was used together with oxygen to deliver isoflurane to anesthetized mice, has no significant effects neither on cardiovascular nor respiratory system.[23] Despite these facts, it was shown that a transient increase in concentration of isoflurane from 1.7 % (concentration used for maintenance of long-term stable anesthesia) to 3 % (concentration used for induction of anesthesia) produced a significant decrease both in the blood perfusion and in oxygenation of tumors (Figure 5). Both variables decreased with similar dynamics. When isoflurane concentration was returned to normal level, there was a delay between the increase of blood perfusion increase and that of pO_2. This can be explained by the delivery-limited oxygen consumption in low pO_2 conditions. All additional oxygen delivered by the increasing blood flow was readily consumed until oxygen delivery became abundant. When this happened, pO_2 also started to increase. The demonstrated effect of the change of anesthetic concentration indicates that: i) anesthetic conditions should be kept as constant as possible during the prolonged measurements of tumor blood perfusion and oxygenation; and ii) the values of tumor pO_2 measured under anesthesia are probably not entirely representative of the pO_2 in non-anesthetized conditions.

The effect of euthanasic procedure

Tumor pO_2 and blood perfusion were monitored during euthanasic procedure in order to verify if the measurements were valid. The results were as expected: the blood perfusion and type I pO_2 signals both decreased when oxygen supply to inhalation mixture was terminated (Figure 6). It is noteworthy to mention that the decrease in pO_2 preceded the decrease in blood perfusion by several seconds. This is a consequence of the decreased oxygen delivery in the presence of the still functioning blood flow. The value of type I pO_2 signals in all measurements dropped to zero level (as it should at death). However, in the majority of LDF measurements, there was still some residual blood perfusion signal present after death. This fake "blood perfusion" signal experienced in the absence of true blood flow is the so-called *biological zero* signal which is usually observed in laser Doppler measurements. The principle of laser Doppler flowmetry is based on the measurement of movement of red blood cells. In case of biological zero, the movement detected is predominantly Brownian motion

(thermally induced random motion) of various structures in the tissue.[9] In the laser Doppler signal picked up from the living tissue, the ever-present biological zero component is outweighed by a much stronger component originating from the true blood flow. In our measurements in tumors, the biological zero level detected after the death of the mice was normally less than 5 % of the total signal level detected in tumor at rest.

Conclusions

The first very important finding of the present study is that two distinct types of pO_2 signals were encountered in tumors. Only the type I measurements resulted in pO_2 values different from zero and, only in the type I measurements, the effect of oxygenation-modifying procedure could be seen. It is however possible that a procedure, which increases oxygenation, might convert some of the type II pO_2 measurements to the type I measurements. The second very important finding is that it takes a considerable amount of time before the type I pO_2 signals stabilize in the *plateau phase*. Reliable measurements of pO_2 changes can only be performed after the signal has entered the plateau phase. But it remains to be seen whether and to what extent the pO_2 value measured in the plateau phase represents the true pO_2 as it was before insertion of the sensor.

In our preliminary study using a novel time-resolved luminescence-based method for measuring the tissue oxygenation in combination with a well-established laser Doppler flowmetry, we have shown that both methods can be effective in the detection of local oxygenation and blood perfusion changes in tumors. Good correlation between the signals of both methods was found as it should be found since oxygenation in tissue depends on tissue microcirculation. It is important to note that these two methods are essentially showing different things and that their respective tissue sampling volumes are very different. Therefore, the results of one method can only supplement, but not replace the results of the other. Based on our results, we conclude that the interpretation of some aspects of pO_2 measurements with the novel luminescence-based method requires further investigation.

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