Optical Scanning of Tissue Oxygen Tension and Hypoxia Imaging in Solid Tumors

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Abstract Immature neovessels in tumors lead to the formation of heterogeneous hypoxic areas within tumors. Such hypoxic conditions not only reduce the effectiveness of radiation treatment but are also related to tumor invasion and metastasis. To better understand tumor-related mechanisms, it is important to quantitate the temporal and spatial changes of tumor hypoxia. However, no useful technique capable of measuring oxygen concentration in vivo has been proposed to date. We aimed to develop a microscope system to measure oxygen metabolism in tumor tissues using a laser-assisted oxygen quenching method. A scanning laser with galvano mirrors was capable of imaging tissue hypoxia and allowed configuration of time and spatial resolution by altering the laser spot size, scan rate, or scan distance. We examined the feasibility of the system by in vitro oxygen measurement, and applied this method to in vivo imaging of tumor oxygenation during oxygen inhalation in tumor-implanted mice. Oxygen tension inside tumors increased soon after oxygen inhalation, but decreased gradually after 20 min in spite of continuous oxygen inhalation, indicating that this model replicates the clinical experience that long-term tumor oxygenation cannot be achieved by oxygen inhalation. Quantitative analysis of tumor oxygenation may help reveal the mechanisms of oxygen metabolism in tumor tissues, leading to the development of more effective radiation therapy.

Keywords: tumor hypoxia, microcirculation, phosphorescence, tumor imaging.

1. Introduction Oxygen molecules diffuse into tissues via microcirculation through vascular branches and are mainly consumed during energy production for the maintenance of cellular functions. Constant microcirculatory blood flow is necessary for continual oxygen transport. However, neovessels in tumors irregularly meander through the tumor, and the vascular structures tend to be fragile, resulting in a reduction or stasis of blood flow and oxygen delivery to tissues [1]. Tumor cells continuously proliferate in a disorderly manner in vivo and maintain their cellular activities by consuming oxygen supplied from the microcirculation [2]. As a result, severely hypoxic conditions develop within tumor tissues, especially in the central part of the tumor or in tissues with low vessel density, leading to necrosis [3]. Such abnormal blood flow reduces drug delivery during chemotherapy, and hypoxic conditions reduce the effectiveness of radiation treatment, thereby necessitating oxygen to kill cancer cells. In clinical practice, oxygen inhalation by cancer patients has been tested in an attempt to improve the therapeutic effect of radiation therapy by increasing the oxygenation of tumor tissues [4, 5]. However, clinical experience has indicated that long-term tumor oxygenation cannot be achieved by oxygen inhalation, since the tumor oxygen level decreases after a certain period of time despite continuous inhalation. For oxygen measurement, oxygen electrodes have been used extensively in biomedical experiments, and some optical oxygen sensing methods have also been developed with the advent of laser technology [6, 7]. We previously reported a microscope system for measuring local blood flow and oxygen tension in vivo. However, the method allowed only pinpoint measurement and not two-dimensional measurement [8]. We also used an X-Y stage for hypoxia imaging, but approximately 15 min was required to obtain an image [9, 10]. Although the system can be used in chronic hypoxia, it has limitations for temporal imaging during oxygen inhalation, in which oxygen tension changes by the minute. Recently, 3-dimensional analysis using a 2-photon microscope has been reported [11, 12], which is able to measure PO2 in deep tissues. However, clinical application may still be far away, because the instruments, including the multi-photon laser, are expensive and quantification of lifetime of emission with a high repetition frequency has not been confirmed. Therefore, although these optical techniques might be applicable to oxygen sensing in tumors in vivo, the methodology has not been well established.

In this study, we aimed to develop a microscope...
system to measure oxygen metabolism in tumor tissues using a laser-assisted oxygen quenching method. In this method, a scanning laser spot with galvano mirrors allows imaging of tissue hypoxia, and time and spatial resolution can be configured by altering the laser spot size, scan rate, and scan distance. We examined the feasibility of the system by in vitro oxygen measurement and used this technique in vivo to measure tumor oxygenation during oxygen inhalation in tumor-implanted mice.

2. Methods

2.1 \(pO_2\) measurement

The oxygen quenching method has been used for noninvasive measurement of oxygen tension in vivo \cite{8}. Palladium-meso-tetra (4-carboxyphenyl) porphine (Pd-TCPP), an oxygen sensitive dye, is excited by laser irradiation, and Pd-TCPP emits phosphorescence or transfers the energy to oxygen molecules when returning to the ground state. The intensity and lifetime of phosphorescence depend on oxygen concentration, and this relationship is expressed by a Stern-Volmer equation as follows:

\[
\frac{\tau_0}{\tau} = \frac{I_0}{I} = 1 + k_q \Delta \rho O_2
\]

where \(I_0\) and \(\tau_0\) are the phosphorescence intensity and lifetime, respectively, in the absence of oxygen, \(I\) and \(\tau\) are the intensity and lifetime at a given oxygen tension, and \(k_q\) is the rate constant of oxygen quenching.

2.2 Optical setup

Figure 1 shows a schematic representation of the optical system. The second harmonic of a pulsed Nd: YAG laser (wavelength: 532 nm; full width at half maximum: 6 ns; pulse energy: 200 nJ) reflected from mirrors on a galvano scanner (GSI Group Inc., USA) is collimated by a lens and enters the microscope. The pulsed laser is used to irradiate a sample through an objective lens, exciting Pd-TCPP in the sample. The diameter of the laser spot is adjusted to 7 \(\mu\)m by pinholes installed in the microscope. Phosphorescence passing through a long-pass filter of longer than 700 nm is detected using a photomultiplier tube (PMT) on an upright microscope. Signals from the PMT are sampled at a frequency of 500 kHz with data acquisition (DAQ) (NI USB-62290, USA). A single exponential decay is fitted to the data to measure the phosphorescence lifetime.

The angles of the galvano mirrors are controlled by output voltage from a DAQ, and the sequence of rotation timing and data acquisition are programmed using LabVIEW. The sequence consisted of 0.1 ms for mirror rotation, 5 ms for acquisition of the phosphorescence signals from PMT, and 3 ms for real-time processing to calculate \(pO_2\). This process takes approximately 10 ms for 1 pixel. Because the lifetime of Pd-TCPP in the absence of oxygen is less than 1 ms, the acquisition time of 5 ms is sufficient for detection. We set the resolution at 50 \(\times\) 50 pixels for \(pO_2\) imaging, which takes approximately 25 s.

2.3 In vitro model

As a preliminary experiment before in vivo measurement, in vitro model vessels using glass capillaries were used. For the deoxidated sample, sodium dithionite was added to the Pd-TCPP solution to give a \(pO_2\) of 0 mmHg. A second tube was saturated with air to maintain a \(pO_2\) of 150 mmHg. The glass capillaries (internal diameter: 98 \(\mu\)m, external diameter: 500 \(\mu\)m) were sandwiched between cover slips mounted in lens oil to suppress scattering of laser light from the glass slips and capillaries.

To perform calibration, the \(pO_2\) in Pd-TCPP was reduced from 150 mmHg to 0 mmHg in small steps. Glucose oxidase (GOx) and catalase were used to
scavenge oxygen molecules in the sample [13]. In brief, GOx catalyzes the oxidation of β-D-glucose to D-glucono-1, 5-lactone using molecular oxygen, releasing hydrogen peroxide, while catalase converts hydrogen peroxide to water and molecular oxygen according to following reactions:

\[
\begin{align*}
\text{β-D-glucose} + O_2 &\rightarrow \text{D-glucono-1,5-lactone} + H_2O_2 \\
\text{H}_2\text{O}_2 &\rightarrow H_2O + \frac{1}{2}O_2
\end{align*}
\]

Oxygen tension calculated from the lifetime measurements was compared using an oxygen monitor (Microx TX3, PreSens) as control.

### 2.4 Tumor oxygenation in mice

All experimental protocols were approved by the Animal Care Committee of Keio University School of Medicine. Lewis lung carcinoma (LLC), which has high metastatic potential [14], was grown in RPMI medium supplemented with 10% FBS, penicillin, and streptomycin, at 37°C in a humidified atmosphere containing 5% CO_2 in air. C57BL/6N male mice (8–10 weeks old, n = 4) were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg), and Pd-TCPP (10 mg/kg) was administrated via a catheter inserted in the tail vein [15].

Mouse skin was used as normal tissue sample in the imaging of oxygen tension. The skin tissue was extended on the microscopic stage after median and transverse incision. To measure tumor hypoxia, the dorsal skinfold chamber method was used to observe the tumor microenvironment over a period of up to approximately 4 weeks [16]. To obtain solid tumor for implantation, LLC cells (1 × 10^6) were suspended in PBS and injected into the dorsal skin of mice. After the solid tumor attained a diameter of approximately 8 mm, a 0.5-mm tumor block was prepared and implanted in the dorsal skinfold chamber. After approximately 2 weeks, the solid tumor attained a diameter of 2 mm with characteristic angiogenesis around the tumor, and was used for measurement.

After injecting Pd-TCPP, tumor oxygenation was performed by administrating pure oxygen to mice through a respiration mask at a flow speed of 2 L/min for 50 min. We imaged \( pO_2 \) inside the tumor, around the tumor tissue, and in the normal skin tissue for 50 min from the time of inhalation. All data are presented as means ± standard deviation.

### 3. Results

#### 3.1 Distribution of phosphorescence intensity and its influence on \( pO_2 \) measurement

The Pd-TCPP solution was uniformly filled between 2 glass plates. The phosphorescence intensity, normalized by maximum intensity \( \left( a \right) \), and lifetime \( \left( b \right) \) in the scanning area were obtained as shown in Fig. 2. The average and standard error of the intensity and the lifetime were 1.11 ± 1.24 V (output of PMT) and 332.2 ± 17.2 µs, respectively. The distribution of phosphorescence intensity was higher around the center and decreased toward the edge of the area. Phosphorescence from an excited spot is generally emitted in concentric hemispheres, therefore the intensity of phosphorescence passing through the objective lens can be assumed to decrease with increasing distance from the center of the scanning area. Fluctuation in the phosphorescence intensity leads to measurement errors in \( pO_2 \) as shown in equation (1). However, the advantage of our method is that oxygen tension is calculated from the lifetime of phosphorescence. Theoretically, the lifetime does not depend on the emission intensity, suggesting that accurate measurement can be expected by analyzing the phosphorescence lifetime.

#### 3.2 \( pO_2 \) calibration and imaging in vitro

The Stern-Volmer plot is shown in Fig. 3. Each data point indicates the average and standard deviation of 20 measurements. A linear relationship between oxygen tension and \( \tau_0 / \tau \) was obtained, as predicted theoretically. As the phosphorescence intensity and lifetime decrease with increasing \( pO_2 \) measurement error increased at higher \( pO_2 \). However, in general, oxygen tension in a living body is lower than 110 mmHg. Under these conditions, our setup can measure \( pO_2 \) with high accuracy. From this calibration test, Stern-Volmer constant \( k_s \) and \( \tau_0 \) for our system were 111 Torr^{-1} s^{-1} and 0.35 ms, respectively, at 37°C and pH 7.4.

Glass capillaries filled with Pd-TCPP were used as model vessels, and \( pO_2 \) was imaged as shown in Fig. 4. Figure 4a is a photomicrograph of a capillary, and Fig. 4b shows the phosphorescence intensity in the scanning area and \( pO_2 \) calculated from the lifetime at 0 and 150 mmHg. The phosphorescence intensity varied with scattering on the glass surface and with changes in the amount of Pd-TCPP in the laser spot. In general, phosphorescence decays exponentially; therefore, the lifetime is basically constant even if the emission intensity changes. Actually \( pO_2 \) imaging over the lifetime was constant as expected, suggesting that accurate measurement can be achieved by analyzing the lifetime. The intensity and \( pO_2 \) calculated from the lifetime at 150 mmHg showed some noise outside the glass capillary, which may be due to a decrease in phosphorescence intensity at higher oxygen levels as shown in the Stern-Volmer plot (Fig. 3).
3.3 Tumor hypoxia imaging and temporal changes in tissue oxygenation

Small solid tumors with angiogenesis were observed in dorsal skinfold chambers approximately 1 week after implantation, as shown in Fig. 5a. We microscopically observed a few microvessels on the surface and inside the tumor. We studied the edge of the solid tumor to determine the difference in oxygen concentration between normal and tumor tissues, as indicated by the dashed square in Fig. 5a. Tumor pO2 images were obtained every 30 s during experiments, including during a 50-min period of oxygen inhalation. Tumor oxygenation is shown as pseudo-color images in Fig. 5b, and the time courses of changes every 3 min at three points of measurement are shown in Fig. 5c. The time course of changes were measured at the following three points: (1) inside the tumor, (2) at the margin of the tumor, and (3) in normal tissue, as shown in Fig. 5b. Inside the tumor at point (1), pO2 was approximately 15 mmHg before oxygen inhalation, which was the lowest of the 3 measurements. Oxygen tension inside the tumor increased soon after oxygen inhalation and reached 60 mmHg, but declined gradually after 20 min and returned to the initial value after 50 min in spite of continuous oxygen inhalation. In the marginal region, pO2 increased transiently over 100 mmHg upon inhalation but subsequently decreased to the same level (initial pO2 ± 10 mmHg) as before inhalation. In normal skin tissue, contrary to tumor tissue, a hyperoxygenated state was maintained; even though pO2 also decreased during oxygen inhalation, the pressure difference was only approximately 20 mmHg. The results of our in vivo experiments demonstrate the phenomenon that only tumor tissue returns to a hypoxic state despite continuous oxygen inhalation (re-hypoxia phenomenon), as is observed in clinical therapy.

4. Discussion

4.1 Image resolution and scanning speed

Immature tumor vessels exhibit irregular branching.
resulting in severely hypoxic regions distributed heterogeneously within tumor tissue [17]. The spatial resolution required for hypoxia imaging is considered to be 10 µm, which corresponds to the capillary diameter in vivo. The imaging target in this experiment was an early cancer measuring a few millimeters in diameter. Therefore, we set the scanning area at 1 mm² to compare the oxygen level between normal and tumor tissues. The spot diameter of the focused laser in our apparatus was 7 µm, and we set the scanning step at 50 × 50 pixels for a 1 mm square; thus, the laser spot was within 1 pixel. The spot size was set to be smaller than the resolution (20 µm) because excessive expansion of the laser spot increases the probability of tissue damage or spatial overlapping with the next pixel. The spatial resolution can be easily varied by changing the power of the objective lens or the angular resolution of the galvano mirror. However, increasing the spatial resolution increases the imaging time. Acquisition time for each pixel is 10 ms, and image acquisition takes approximately 25 s for 50 × 50 pixels.

The image resolution and acquisition time should be determined based on each target of measurement. Oxygen inhalation causes an acute change in tissue oxygenation. Hence, we minimized the spatial resolution so as to achieve fast imaging. For example, chronic hypoxia caused by daily changes in tumor growth [18] should be imaged at a higher resolution with time. In addition to on-line measurement, off-line measurement is also possible. In such case, all the phosphorescence decays at each pixel are stored in the PC, and can be processed afterwards for pO₂ calculation and imaging. Processing images cannot be observed during a measurement. However, faster acquisition and extra analysis of phosphorescence decay are expected.

Averaging multiple decays in the pixel improves accuracy. However, in principle, laser-excited Pd-TCPP molecules transfer the energy to oxygen molecules, and this generates toxic singlet oxygen. Repeated irradiation of the same site consumes oxygen molecules in the tissue, and leads to a change in baseline oxygen concentration. Faster scanning decreases the generation of singlet oxygen and maintains measurement accuracy.

### 4.2 Measurement accuracy

As shown in Fig. 2, the phosphorescence intensity in the scanning area is not constant. The intensity decreases especially at the edges and corners of the area observed. Faster scanning can be expected when the pO₂ is quantified from the phosphorescence intensity. However, for accurate measurement, pO₂ should be quantified from the lifetime, rather than from the intensity, because the phosphorescence lifetime remains constant over the area. In fact, as shown in Fig. 4b, the phosphorescence lifetime in the glass capillary is constant in spite of the uneven phosphorescence intensity due to light reflection from the glass capillary or other factors. In animal experiments, the Pd-TCPP density in vessels and tissues is very heterogeneous. In addition, the target tissue is not always flat under the objective lens. Taken together, accurate measurement based on the lifetime is recommended for in vivo measurements.

We employed galvano mirrors for laser scanning. Other reports have described the use of high speed scanning with a polygon mirror for microvessel imaging [19, 20]. As described above, measurement of phosphorescence lifetime is required for accurate pO₂ measurement, and at least 5 ms is required to obtain phosphorescence decay for each pixel. A polygon mirror allows faster movement of the mirror to the next pixel. However, extremely fast scanning cannot be expected because the 5-ms required for lifetime measurement is the major determinant of imaging time. As a result, lifetime measurement using a polygon mirror has little advantage in our system.

### 4.3 Tumor oxygenation and metabolism

To study hypoxia in a tumor in vivo, we obtained tissue samples from mice and stained the hypoxic area in histological sections with pimonidazole [21]. Despite growth of the tumor over time, we were only able to sample the tissue at 1 time point; in other words, the method did not allow continuous imaging of tumor hypoxia in a living body. In the present study, we imaged the time course of changes in tumor hypoxia during oxygen inhalation in the same individual mouse. As shown in Fig. 5c, oxygen tension in the tumor increased after oxygen inhalation was started. However, the oxygen tension gradually returned to hypoxic levels despite continuous inhalation. This phenomenon appears to replicate the clinical experience that long-term tumor oxygenation cannot be achieved by oxygen inhalation. The "normal tissue" with a higher pO₂ level during oxygen inhalation was next to a tumor tissue. A more appropriate control sample should have been collected from intact skin without tumor implantation and measured under oxygenation. Moreover, systemic blood pressure, arterial oxygen saturation, and rectal temperature during oxygen inhalation are important parameters to study the re-hypoxia phenomena. Hypoxic conditions within tumors not only reduce the effectiveness of radiation treatment but are also related to tumor invasion and metastasis [22]. Imaging of hypoxic conditions, especially determining the time course of changes in the hypoxic areas of a tumor, will help to elucidate cancer mechanisms. Hypoxic areas distribute heterogeneously in the tumor, depending on the type of tumor, size, neovascular density, and blood flow distribution. Laser scanning using the galvano mirror allows faster imaging than using an X-Y stage as in our previous studies [19, 10]. Quick imaging during oxygen and metabolic changes could reveal the heterogeneous hypoxic responses related to cellular distribution and enzymatic activity in cells. The response to oxygen inhalation probably varies depending on the type and size of the tumor.

Hypoxia inducible factor-1 (HIF-1), which regulates metabolic enzymes, is thought to be a major factor in tumor biology [23]. Hypoxic conditions in tumors cause the switching of ATP production from oxidative phos-
phorylation to glycolysis, and this process differs in aerobic and anaerobic respiration. We hypothesize that the re-hypoxia phenomenon might be related to the modification of glucose metabolism in tumors. Inhalation of oxygen would change the metabolism in the tumor from anaerobic respiration to aerobic respiration. Recently, imaging mass spectrometry has been developed, which can visualize the spatial distribution of metabolites [24]. Study of the relationship between hypoxia and distribution of metabolites may lead to the development of more effective radiation therapy. Long-term oxygenation in a tumor would reduce the radiation dose, which is expected to reduce the adverse effects of radiotherapy. HIF-1 expression in hypoxic tissues depends on the neovascular network and blood flow. Previous report described that normalization of abnormal neovessels in the tumor improves tissue oxygenation, which leads to an increase in the radiation effect [25]. Our method allows observation of blood flow distribution simultaneously with hypoxia imaging, which is useful to study hypoxic mechanisms in the tumor.

5. Conclusion

We developed an optical system for oxygen imaging in tissues using a laser-assisted oxygen quenching method, and used the system for tumor hypoxia imaging. Quantitative analysis of tumor oxygenation may reveal the mechanisms of oxygen metabolism in tumor tissues, and lead to the establishment of more effective radiation therapy. This method can be used to measure different types of hypoxia; acute (quick change) and chronic (slow change) hypoxia, by changing the time and spatial resolution of measurement, and this method is potentially useful in the research of hypoxia caused by cerebral infarct, thrombus, or other pathological conditions.

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References


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Ryo Yamada received the master’s degree in engineering in 2013 from Keio University, Japan. He studied imaging method for tumor hypoxia with laser-assisted phosphorescence measurement.

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