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Spatiotemporal measurement of tumor oxygenation reveals repeat hypoxic phenomenon in mice

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Abstract—**Tumor hypoxia is considered a potential therapeutic problem because it reduces the effects of radiation therapy. Clinical experience has shown that long-term tumor oxygenation cannot be achieved with oxygen inhalation, but the mechanisms behind this phenomenon remain unknown. In this study, we designed an optical system for evaluating spatiotemporal changes in tissue oxygen tension (pO_2) by phosphorescence quenching. The system can measure continuous changes in pO_2 at a fixed point and can also perform two-dimensional mapping of pO_2 in any part of the tumor tissue. We implanted tumor tissue in a dorsal skinfold chamber of C57BL/6 mice and observed tumor growth. After the tumor attained a diameter of 2 mm, the mice received oxygen inhalation and pO_2 was measured. Tumor pO_2 increased after inhalation but the oxygen level was not maintained despite continuous inhalation of pure oxygen; the tumor returned to a hypoxic state. These results mimic the clinical experience of oxygen inhalation treatment in radiation therapy. Our system reproduces the repeat hypoxic phenomenon in a murine tumor model and can be used to determine the mechanisms of oxygen metabolism in tumors.**

I. Introduction

TUMOR tissues require more abundant oxygen and nourishment than normal tissues because of their continuous growth. However, neo blood vessels in tumors have non-homogeneous vessel structures, stagnant blood flows and immature vessel walls, resulting in abnormal oxygenation conditions [1]. Thus, tumor vessels fail to efficiently supply sufficient oxygen and nourishment and the balance of supply and demand of oxygen collapses. This leads to the formation of a hypoxic area, particularly in the center of the tumor or between vessels; this anoxic condition ultimately results in tissue necrosis [2]. Hypoxic conditions decrease the effects of radiation therapy, which requires 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 oxygenation of tumor tissues. However, clinical experience has indicated that long-term tumor oxygenation cannot be achieved by oxygen inhalation. The mechanisms responsible for this phenomenon remain unknown because of the lack of suitable techniques for determining tumor oxygen metabolism *in vivo* [2].

We aimed to investigate oxygen metabolism in tumors. Therefore, we developed and evaluated a laser-assisted optical system for measuring continuous changes ,different from other methods, in pO_2 non-invasively at a fixed point and mapping two-dimensional (2D) pO_2 distribution in tumor tissues by a phosphorescence technique. With this system, we observed a repeat hypoxic phenomenon in murine tumor tissues after inhalation of pure oxygen in tumor tissue implanted in dorsal skinfold chamber.

II. Materials and Methods

A. Oxygen probe

We employed a phosphorescence quenching method to measure pO_2 in tumor tissue with meso-tetra (4-carboxy phenyl) porphine (Pd-TCPP) as the oxygen probe. The lifetime of the phosphorescence emission is dependent on pO_2 and follows a Stern–Volmer relationship, as shown in equation (1). Here τ_0 is the phosphorescence lifetime in the absence of oxygen and k_q is the Stern–Volmer constant [4].

$$\frac{\tau_0}{\tau} = 1 + \tau_0 k_q [pO_2] \quad (1)$$

B. Optical System

Figure 1 shows a schematic diagram of our system. Nd:YAG laser (wavelength: 532 nm; full width at half maximum: 6 ns; pulse energy: 200 nJ) was used because Pd-TCPP is absorbed at approximately 530 nm. A photomultiplier tube (PMT, Hamamatsu Photonics) with a long-pass filter more than 700 nm was used to detect phosphorescence, and was attached to an upright microscope. The signal was digitized with a 500 kHz sampling rate. A pinhole of 100 μm in the microscope resulted in a laser spot 20 μm in diameter. We controlled the X-Y stage for 2D pO_2 image mapping of the tumor with a resolution of 2500 pixels/mm^2. It is required 15 min. for 2D scan. The trigger signal for stage control, laser irradiation, and data acquisition was programed in C language (Figure 2).

C. Glass Capillary model

To determine the feasibility of oxygen mapping *in vitro*, Pd-TCPP solutions with different pO_2 were filled in two glass capillaries (inside diameter: 98 μm, outside diameter: 500 μm). For the deoxidation sample, sodium sulfite was added to the Pd-TCPP solution to give a pO_2 of 0 mmHg; the remaining tube was saturated with air to maintain a pO_2 of 150 mmHg. The glass capillaries were sandwiched between cover

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slips with lens oil to suppress scattering of laser light from the glass slips and capillaries.

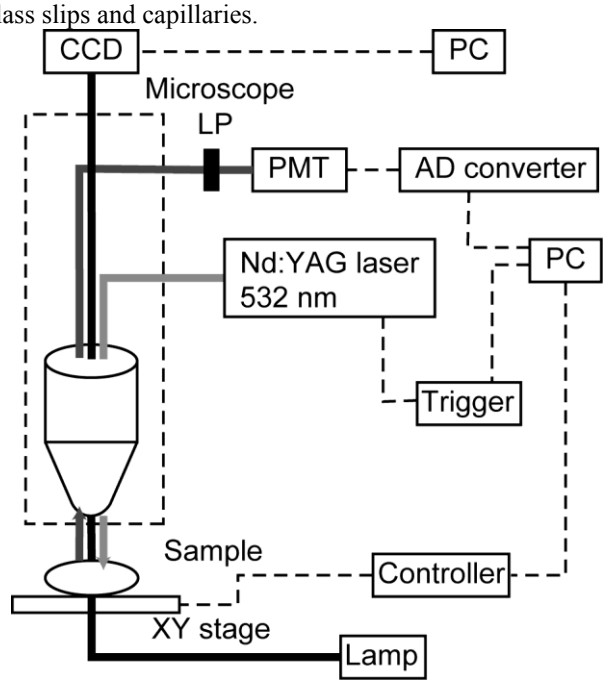

Fig. 1 Schematic diagram of the optical system. Nd:YAG pulse laser was irradiated to sample through the microscope, and phosphorescence signals were detected with PMT.

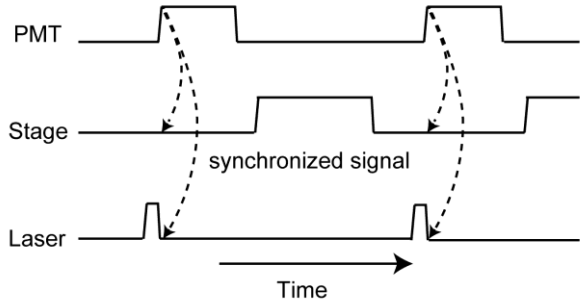

Fig. 2 Time course of synchronized signals for continuous measurement. Nd:YAG laser irradiation, phosphorescence sampling and stage control were controlled by PC.

D. Cancer Cells

Cells from the wild-type murine lung cancer cell line, Lewis lung carcinoma (LLC), were cultured in RPMI medium with 10% FBS (v/v), penicillin, and streptomycin, and the cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air.

E. Animals

Male C57BL/6 mice (8–10 weeks) were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) during surgery, tumor implantation, and microscopic observation. The oxygen probe, Pd-TCPP dissolved in PBS, was injected through a catheter in the tail vein. For pO_2 measurements in the femoral artery and vein, the femoral skin was incised and the artery and vein exposed on the microscope stage.

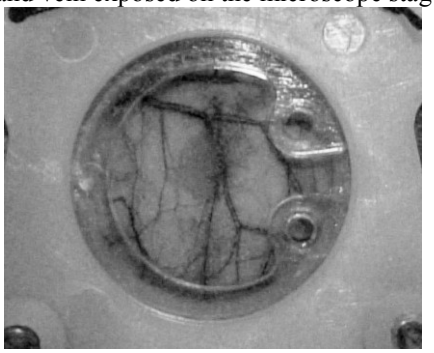

Fig. 3 Solid tumor about 2 mm in diameter in the dorsal skinfold chamber was observed and pO_2 was measured.

F. Tumor implantation and pO_2 measurement

The dorsal skinfold chamber method was used to observe the tumor microenvironment over a period of up to approximately 4 weeks. 1×10^6 LLC cells were suspended in PBS and injected into the dorsal skin of the mice to generate a source tumor [4]. After the solid tumor attained a diameter of approximately 1 cm, we made 0.5 mm-tumor chunk and implanted it in the dorsal skinfold chamber. Approximately 2 weeks thereafter, the solid tumor attained a diameter of 2 mm with characteristic angiogenesis (Figure 3).

Tumor oxygenation was performed by administration of pure oxygen to mice through a respiration mask. We measured pO_2 in tumor tissue without necrosis and in normal tissue from 150 μm outside the tumor for 35 minutes from the time of inhalation. We also profiled pO_2 across the tumor by 2D image mapping before and after oxygen inhalation.

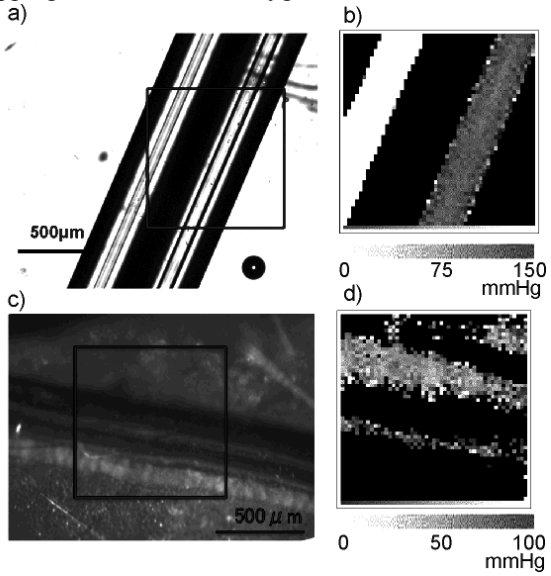

Fig. 4 pO_2 imaging in glass capillaries in different pO_2 resolution for *in vitro* model and femoral vessels for *in vivo* model. Each panel shows transparent image of the capillaries (a) and femoral artery and vein (c), and the imaging of pO_2 level (b, d), respectively.

III. Results

A. In vitro evaluation using glass capillaries

pO_2 imaging of the Pd-TCPP solutions in the glass capillaries was performed for validation. Figure 4(b) shows the pO_2 readings for the capillaries and the known pO_2 values. Laser irradiation indicated that only the Pd-TCPP solution, and not, for instance, the cover slip or glass capillaries, showed a signal. This indicated that 2D imaging of pO_2 was successful *in vitro*.

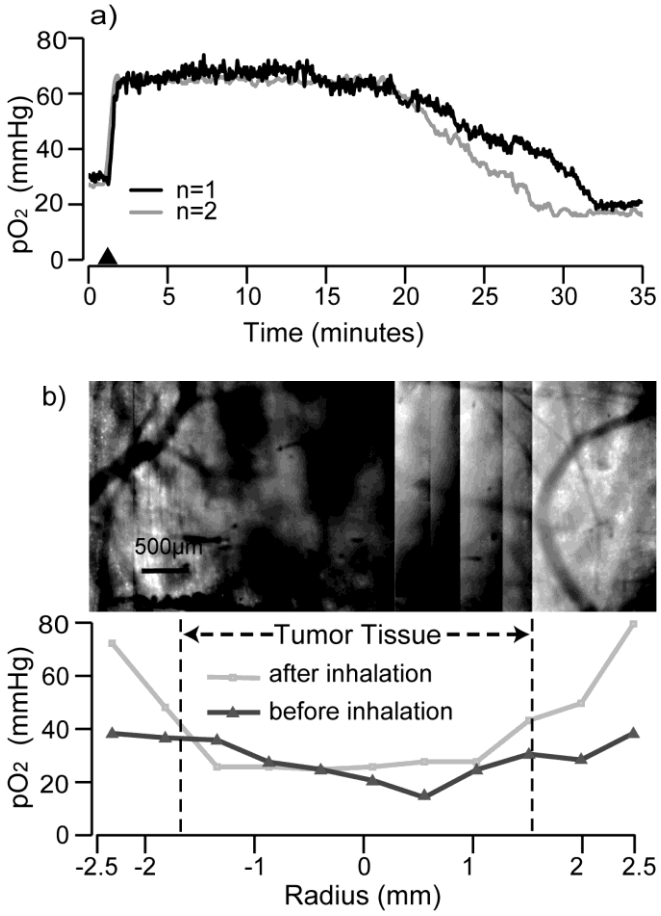

Fig. 5 Time course changes of pO_2 in the tumor during oxygen inhalation (a) and pO_2 profile across the tumor tissue (b) before and after oxygen inhalation. Triangle indicates start of pure oxygen breathing in (a).

B. In vivo measurement of pO_2

Figure 4(d) shows a pO_2 image of the femoral artery and vein of a mouse after Pd-TCPP injection, and Figure 4(c) is an image of the same region taken under transparent light. We were able to identify the artery and vein running alongside each other in the 1 mm^2 area. In addition, we were able to determine a difference of 20–30 mmHg in pO_2 and a difference in diameter between the artery and vein. The oxygen probe, Pd-TCPP, is believed to circulate in vessels for approximately 15 minutes and to permeate tissue after 30 minutes. Therefore, intravascular pO_2 can be measured immediately after probe injection and tissue pO_2 can be measured at a suitable time thereafter. In figure 4(c), no signal from the tissue was observed, indicating effective measurement *in vivo*.

C. pO_2 changes in tumors during oxygen inhalation

Figure 5 shows the changes in pO_2 at a fixed point in the tumor (a) as well as the pO_2 profile across the tumor tissue before and after oxygen inhalation (b). Oxygen inhalation immediately increased tumor pO_2 from approximately 20 mmHg to 60 mmHg. This pO_2 level was maintained for approximately 20 minutes. However, the pO_2 of the oxygenated tissue began to decrease gradually thereafter, and finally it returned to the initial hypoxic state despite continuous oxygen inhalation.

We also determined the pO_2 profile of the tumor tissue before and after oxygen inhalation (Figure 5(b)). The profile indicated that the center of the tumor, not the normal tissue, was hypoxic (10 mmHg or below vs. 40 mmHg) before oxygenation (red line). After oxygenation, pO_2 in the normal tissue remained at 80 mmHg but in the tumor tissue it decreased to a hypoxic level, indicating that repeat hypoxic phenomenon occurred solely in the tumor tissue. These results could mimic the repeat hypoxic phenomenon identified in clinical therapy.

IV. Discussion

We designed a microscopic system for mapping pO_2 using a laser-assisted phosphorescence technique. Our system enables measurement of temporal changes in pO_2 as well as 2D mapping of pO_2.

In tumors, the angiogenic vasculature results in heterogeneous pO_2, resulting in hypoxic conditions in the center of the tumor. In a previous report, apoptosis was demonstrated to be inhibited by a genetic defect in the tumor that ensured survival of tumor tissue under hypoxic conditions [6]. Application of pO_2 imaging can be a powerful tool for revealing the mechanisms behind tumor oxygen metabolism. In our murine model, continuous tumor oxygenation could not be achieved despite continuous oxygen inhalation. Clinical experience has shown a similar phenomenon that reduces the effectiveness of radiation therapy, but the mechanism remains unknown. Hypoxic conditions in tumors switch the ATP production from oxidative phosphorylation to glycolysis; this process differs in aerobic and anaerobic respiration [7]. We hypothesize that the repeat hypoxic 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, ATP, glucose assay and metabolome analysis have been used to analyze oxygen metabolism, but there are several unknown mechanisms of oxygen metabolism in tumor tissues. Using our stage-controlled pO_2 mapping system, which may possibly help to elucidate the oxygen metabolism mechanism in tumor, we were able to observe a repeat hypoxic phenomenon in a murine model. However, because continuous inhalation of oxygen worsens blood flow in microcirculation [8], hemodynamics during oxygenation also should be analyzed.

Quantitative analysis of tumor oxygenation could help reveal the mechanisms of oxygen metabolism in tumor tis-

sues. In future, oxygenation of tumor tissues may possibly be controlled and thus enhance radiation therapy. Moreover, because angiogenesis and metastasis are believed to be influenced by oxygen conditions, new strategies may be proposed for anti-angiogenesis and anti-metastasis therapy in future.

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