博士論文

論文題目　Establishment of quantitative method for measuring intracellular oxygen tension in kidney

（腎細胞内酸素分圧の定量的測定方法の確立）

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Abbreviations

ATP	adenosine triphosphate
BOLD-MRI	blood oxygen level dependent magnetic resonance imaging
BTP	(acetylacetonatobis[2-(2'-benzothienyl)pyridinato-κN,κC3']-iridium(III))
CKD	chronic kidney disease
DO_2	oxygen delivery
ESAs	erythropoiesis stimulating agents
FL	fluorescence lifetime
HIF	hypoxia-inducible factor
HK-2	human kidney 2
HRE	hypoxia response elements
ICP-MS	inductively coupled plasma mass spectrometry
I/R	ischemia reperfusion
PHD	prolyl hydroxylase domain
PL	phosphorescence lifetime
PO_2	partial pressure of oxygen
PpIX	protoporphyrin IX
PTC	peritubular capillary
VHL	von Hippel-Lindau disease tumor suppressor
5-ALA	5-aminolevulinic acid

Abstract

Hypoxia plays crucial roles in pathogenesis and progression of many diseases including chronic kidney disease. Since the activity of hypoxia-inducible factor (HIF), a transcript factor which exerts as a master regulator of cellular response against hypoxia, is modulated in accordance to oxygen-dependent hydroxylation inside the cell, intracellular oxygen tension is a dominant factor in hypoxic conditions. Existing methods for detecting intracellular hypoxia *in vivo* such as pimonidazole protein adduct immunohistochemistry or detection of HIF activation are neither quantitative nor indicative of oxygen tension. Thus a method which can detect hypoxia in a quantitative way *in vivo* is wanted. Phosphorescence is a light and thought to be useful in detecting hypoxia since its intensity and lifetime depend on circumstantial oxygen tension. BTPDM1, a lipophilic cationic dye, distributes intracellularly, thus this probe is a candidate as an intracellular oxygen sensor *in vivo*. I affirmed this probe distributes in tubular cells after systemic administration. I determined phosphorescence lifetime measurement is more suitable than phosphorescence intensity measurement in case I attempted to detect hypoxia in kidney in living mice. Then I confirmed phosphorescence lifetime was indeed influenced by tissue oxygenation in three kidney hypoxia models and chronic kidney disease model. Finally, by using calibration line obtained in a cellular experiment, I determined intracellular oxygen tension in kidney. This novel method will greatly benefit research

about how hypoxia affects the pathogenesis and progression of diseases.

1. Introduction

1-1. Oxygen and hypoxia

Oxygen exerts as a powerful oxidant, and enough deliver of oxygen enables us to use efficient system of oxidative phosphorylation to obtain sufficient energy substrate, that is, ATP[1]. As well as acquiring ATP, fueling tricarboxylic acid cycle and subsequent activation of electron transfer system in mitochondria has been recently reported to be related with synthesizing certain metabolites, which is vital for cell proliferation[2,3]. Thus, cells should adjust to change in such changes if they are under reduced oxygen availability (hypoxia). Many transcriptional responses to hypoxia is mediated by hypoxia-inducible factors (HIF)[4]. HIF was originally found in searching some enhancers of the human *EPO* gene[5]. HIF consists of two subunits, α-subunit and β-subunit, and both belong to Helix-Loop-Helix per-arnt-sim family[6]. Before now, three isoforms of α-subunit were found, and the cellular concentration of all of these three subunits is dependent on oxygen concentration, whereas the β-subunit exists independently of oxygen concentration. The concentration of α-subunit is maintained strictly in a post-transcriptional manner. In the presence of oxygen, the α-subunit is hydroxylated at two proline residues by prolyl hydroxylases, and then von Hippel-Lindau disease tumor suppressor is recruited, followed by ubiquitination and proteasomal degradation[7,8]. Thus, the concentration of HIF-α is mainly regulated by hydroxylation at proline residues, which is dependent on

surrounding oxygen concentration, that is, intracellular oxygen concentration. Under hypoxic condition, the concentration of HIF-α increases, then HIF-α forms a heterodimer with HIF-β, resulting in transfer into nucleus and binding to the hypoxia response element of certain genes. By this regulation and function of HIF as oxygen-dependent transcriptional factor, HIF is called as a master regulator under hypoxia. What is important is that the concentration of HIF is maintained by oxygen concentration inside the cells, not outside.

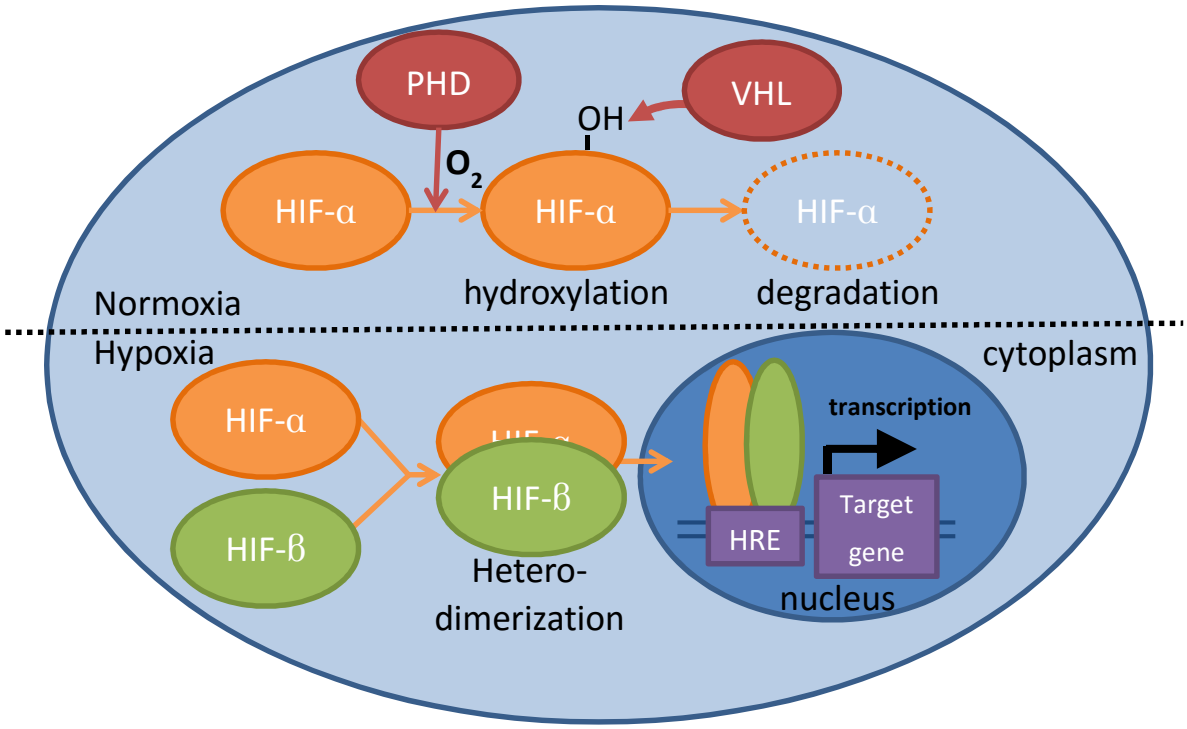

Figure S1. The regulation and function of HIF. HIF is a heterodimeric transcription factor. HIF-α is an oxygen sensitive unit while HIF-β is independent of oxygen. Under normoxia, HIF-α is hydroxilated by PHD with oxygen. Hydroxylated HIF-α is recognized by VHL, ubiquitinated and subsequently degraded. In hypoxic conditions, HIF-α is not degraded and forms a heterodimer with HIF-β. The heterodimer translocates into nucleus and bind to HRE. This bind activates the transcription of downstream genes. Abbreviations: HIF, hypoxia-inducible factor; HRE, hypoxia response elements; PHD, prolyl hydroxylase domain; VHL, von Hippel-Lindau disease tumor suppressor.

1-2. Hypoxia in CKD; CKD induces renal hypoxia.

Although kidneys receive approximately 20% of blood pumped out from heart in physiologic conditions, kidneys suffers from mild hypoxia by nature. This is multifactorial, and the most important factor is the oxygen shunt in renal artery and renal vein. The existence of this shunt is originally researched in the experiment using dog[9], and now it has been accepted that this shunt exists in murine[10] and human. In chronic kidney disease (CKD), the renal hypoxia is worsened[11,12]. This is also multifactorial. In CKD, tubulointerstitial fibrosis occurs, and it leads to loss of peritubular capillary (PTC)[13-15]. Loss of PTC means the distance becomes longer on average from arteriole to cells, which restrict oxygen deliver. Not only the density but also flow of PTC affects renal oxygenation. In CKD, reduction in PTC flow is seen and thought to be related to aggravation of renal hypoxia[16]. CKD frequently causes renal anemia, and anemia is a state of lower oxygen delivery, since oxygen delivery (DO_2) is determined by using the equation as below[17], where CO is cardiac output in liters per minute, [Hb] is hemoglobin concentration in grams per liter, SaO_2 is percentage of hemoglobin O2 saturation and PaO_2 is partial pressure of oxygen (pO_2) in arterial blood in millimeter of mercury.

$$DO_2 = CO \times (1.39 \times [Hb] \times SaO_2 + 0.003 \times PaO_2) \quad (1)$$

Since o.oo3 × PaO_2 is usually enough small to be ignored, DO_2 is approximately

proportional to [Hb] in the same respiratory state. In fact, it is proven that anemia itself cause renal hypoxia[18]. In addition to decrease of oxygen supply, increase of oxygen demand in renal tubular cells also exists in CKD[19].

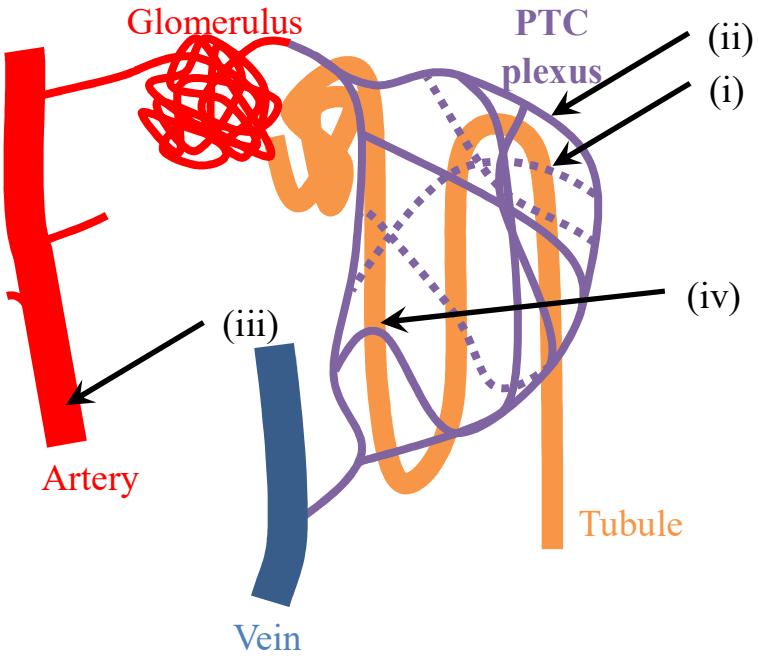

Figure S2. Factors involving aggravation of renal hypoxia in CKD. In CKD, many physiological or histological changes occur and are related to worsening renal hypoxia. Representative reasons of exacerbation of renal hypoxia are as follows; rarefaction of PTC (i), decrease of PTC flow (ii), insufficient oxygen delivery resulting from anemia (iii) and increased demand of oxygen in tubular cells (iv). Abbreviations: CKD, chronic kidney disease; PTC, peritubular capillary.

1-3. Hypoxia in CKD; does renal hypoxia deteriorate kidney fibrosis?

As mentioned above, CKD patients frequently suffer from renal anemia, and nowadays erythropoiesis stimulating agents (ESAs) are prescribed in order to raise their hemoglobin level and maintain their quality of life[20]. It is also indicated that higher hemoglobin level with ESA leads better renal prognosis[21]. Considering that renal fibrosis is a major characteristic of CKD[22], this result invokes the idea that renal fibrosis is ameliorated with improvement of renal hypoxia resulting from increase of oxygen supply. However, there is pleiotropic effect of erythropoietin other than erythropoiesis[23], hence this result of clinical study cannot absolutely support the idea that renal fibrosis is ameliorated by more oxygen supply. In another viewpoint, people in high altitude might be at high risk of CKD progression[24], although there is an objection[25]. It is still controversial that anemia or hypoxemia, which is thought to be closely related to renal hypoxia, itself affects CKD prevalence or prognosis in clinical researches.

In animal experiments, it is difficult to sustain anemia or hypoxemia for a long time, thus the effect of these inducer of hypoxia is not elucidated. Alternatively, effect of HIF-activation is eagerly examined in murine. Cobalt chloride is frequent used HIF activator, although it cannot be used to human. The administration of cobalt chloride protect kidney against various CKD models[26-29]. Although there is a report that HIF overexpression by

genetic *VHL* deletion deteriorates interstitial fibrosis[30], activation of HIF by not extreme extent is expected to protect kidney from fibrosis[31]. Recently some prolyl hydroxylase domain (PHD) inhibitors have been developed[32] and now under clinical trial, thus these drugs are anticipated to be powerful agents against CKD in human. However, the questions that when and how hypoxia initiates to influence the progression of renal fibrosis and when is the most appropriate moment to start hypoxia-oriented treatment are little known, partially because the methods for detection and evaluation of hypoxia in renal fibrosis are unsatisfactory even in animal experiments.

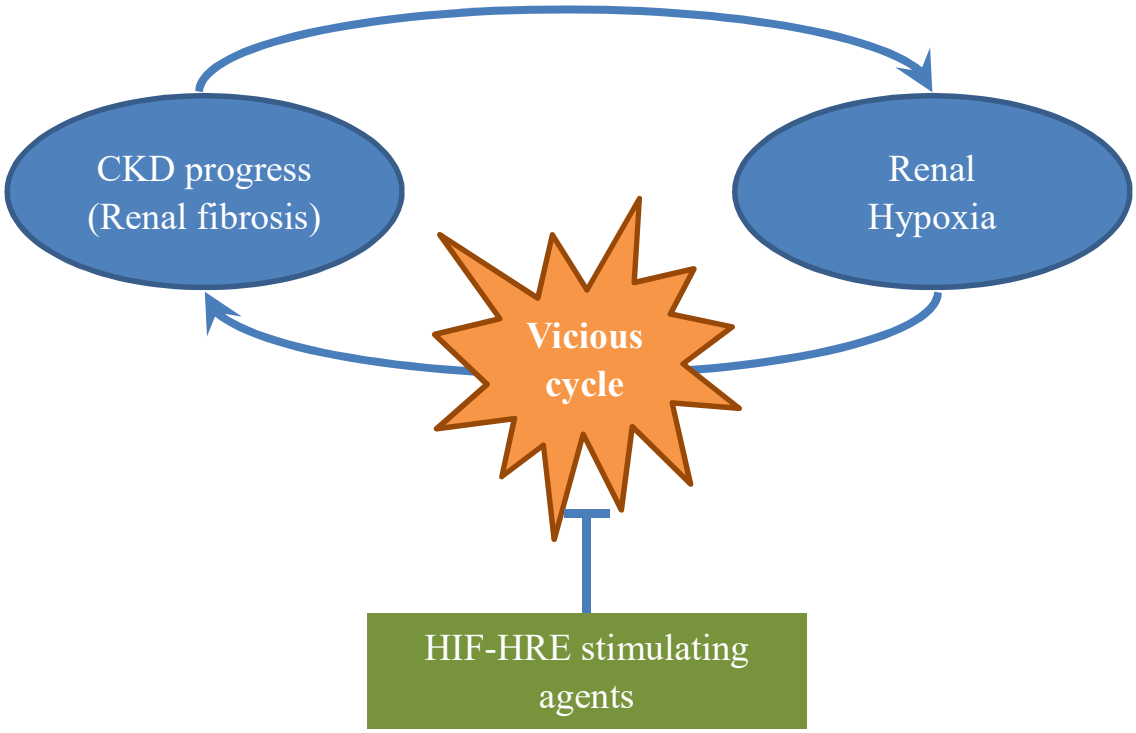

Figure S3. Vicious cycle between CKD progression and renal hypoxia. CKD progression aggravates renal hypoxia. In turn, renal hypoxia is supposed to deteriorate CKD progress. It is hoped that HIF-HRE axis stimulating agents improve renal fibrosis partly according to protecting renal tubular cells against hypoxia. Abbreviations: CKD, chronic kidney disease; HIF, hypoxia-inducible factor; HRE, hypoxia response element.

1-4. Existing methods for detecting hypoxia

There are many methods for detecting hypoxia in living animals. The methods can be divided into two groups; detector of intravascular hypoxia or detector of intracellular hypoxia. As I noted earlier, intracellular oxygen tension should modulate the concentration of HIF and subsequent the activity of HIF-HRE axis, hence intracellular oxygen tension should be a more important player compared with intravascular oxygen tension.

Needle electrode is a traditional method and it enables quantitative measurement of oxygen tension. In kidney research, it is frequently used for detect and quantitate hypoxia in renal cortex and medulla[10,33-35]. However, in addition to its high invasiveness, the biggest problem of the technique is that it cannot be understood of where oxygen concentration is measured. The fact that the electrode should be pulled a little after insertion to kidney parenchyma to obtain reliable value might implicate it reflects oxygen tension in blood in capillary. Blood oxygen level dependent magnetic resonance imaging (BOLD-MRI) is another often-used technique to detect renal hypoxia because of its minimum invasiveness and availability to human[36,37], although the change in BOLD-MRI is reported to fail detecting the progression of CKD[38]. The problems of BOLD-MRI are that what it measures is deoxyhemoglobin, which is influenced by the total concentration

of hemoglobin[39] and by some electrolyte/metabolite change such as pH and pCO2 (Bohr effect). As mentioned below, phosphorescence lifetime measurement is a promising technique for measuring oxygen concentration *in vitro* and *in vivo*. Phosphorescence lifetime measurement using PtP-C343, a phosphor which distribute only in the blood, is used for measuring oxygen concentration in microartery in bone marrow[40] and brain[41]. Oxyphor G2, another phosphor with intravascular distribution, is used for assessing renal hypoxia with monitoring the phosphorescence lifetime in renal vein[42], although the oxygen tension in renal vein is modified by oxygen shunt between renal artery and renal vein.

Pimonidazole staining is a technique for detecting hypoxia in tissue section[43]. It is frequently used for detection of hypoxia in kidney section[44-46]. This technique is based on that 2-nitroimidazoles bind to thiol when they keep reductive state without oxygen[47]. We should keep it in mind that this response might be influenced by redox state in the cell and that this method is not indicative of oxygen concentration. Detection of HIF-HRE pathway is another frequently used technique for detecting hypoxia in kidney[45], although HIF-HRE pathway does not depend absolutely on oxygen concentration[48]. In addition, this technique is also qualitative one.

Protoporphyrin IX (PpIX) is an intrinsic protein which is precursor of heme protein.

Since PpIX emit fluorescence and its lifetime depends on oxygen concentration, local application of 5-aminolevulinic acid (5-ALA, precursor of PpIX) on the skin and measure fluorescence lifetime is a promising method for measure intracellular oxygen tension quantitatively in skin[49,50]. 5-ALA is clinically used for intraoperative tumor marker because PpIX is accumulated to tumor after 5-ALA administration. When we want to measure fluorescence lifetime of PpIX other than tumor, we should employ local application but not systemic administration. Maybe by this property, fluorescence lifetime measurement using 5-ALA has not been used for detecting hypoxia in abdominal or retroperitoneal organs.

In summary, there are no established methods for detecting intracellular hypoxia. The fact that BOLD-MRI fails to detect hypoxia in clinical trial might mean what is important is intracellular hypoxia, thus quantitative method for measuring intracellular oxygen tension *in vivo* is desired.

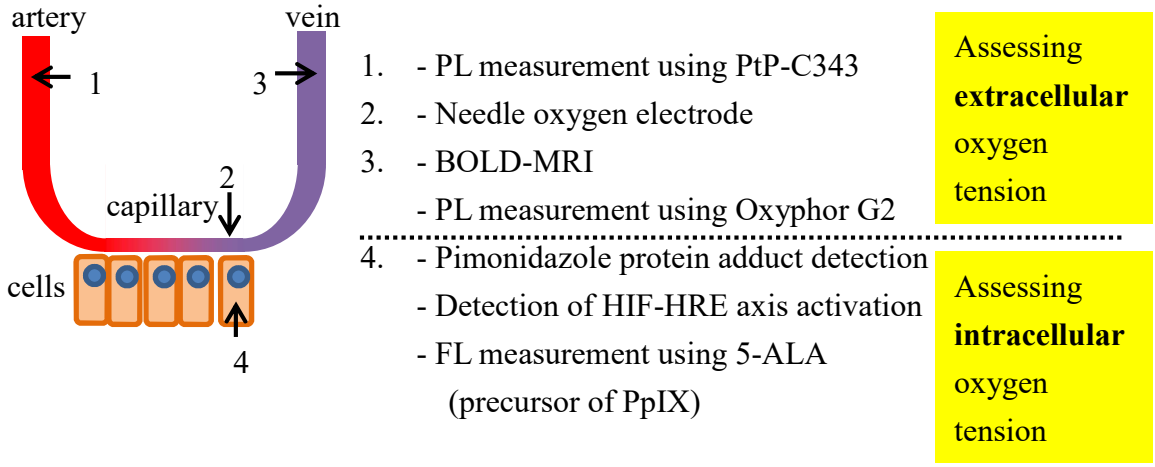

Figure S4. Overview of hypoxia detecting techniques in murine. Hypoxia assessing techniques are written and classified according to measuring site of the techniques. Abbreviations : BOLD-MRI, blood oxygen level dependent magnetic resonance imaging; FL, fluorescence lifetime; HIF, hypoxia-inducible factors; HRE, hypoxia responsive elements; PL, phosphorescence lifetime; PpIX, protoporphyrin IX; 5-ALA, 5-aminolevurinic acid hydrochloride.

1-5. The characteristics of phosphorescence

Here I report the availability of BTPDM1, a phosphorescence dye, to estimate intracellular oxygen tension *in vivo*. Phosphorescence is a kind of light which is emitted from a molecule in triplet excited state. Generally, the molecules in singlet excited state can lose its energy by emitting fluorescence more easily than molecules in triplet excited state by emitting phosphorescence, thus the lifetime of phosphorescence is much longer than that of fluorescence. The lifetimes are usually microseconds in phosphorescence and nanoseconds in fluorescence.

When a molecule is excited to triplet excited state, the triple state returns to ground state by light emission (phosphorescence) or energy transition to other molecules, mainly oxygen in biological samples[51]. With the existence of oxygen, the triplet excited molecules rapidly lose its energy by energy transfer to oxygen, thus phosphorescence lifetime becomes shorter. Provided that the energy of triplet excited state loses by phosphorescence emission or collision with oxygen, Stern-Volmer equation holds[52],

$$[O_2] = \frac{1}{k_q}\left(\frac{1}{\tau} - \frac{1}{\tau_0}\right) \qquad (2)$$

where k_q is the rate constant (varies depending on solvent of phosphorescence dye), τ is the phosphorescence lifetime at an oxygen concentration $[O_2]$ and τ_0 is the phosphorescence lifetime in the absence of oxygen. According to this equation, if one can

determine k_q and τ_0, τ can be converted to oxygen concentration. For this aspect, phosphorescence lifetime measurement is superior to measuring phosphorescent intensity albeit it needs particular experimental systems.

Many of the phosphorescence probes are heavy metal complexes such as Pt(II), Pd(II), Ir(III) and Rb(II)[53], and frequently-used phosphorescence dyes are nanoparticles which contains these heavy metals in the center of the molecule[54,55]. These molecules contains numerous polyethylene glycol (PEG) group to improve solubility in water and decrease interference by serum albumin, hence these phosphorescence dyes are fairly good when used as sensors of intravascular oxygen concentration.

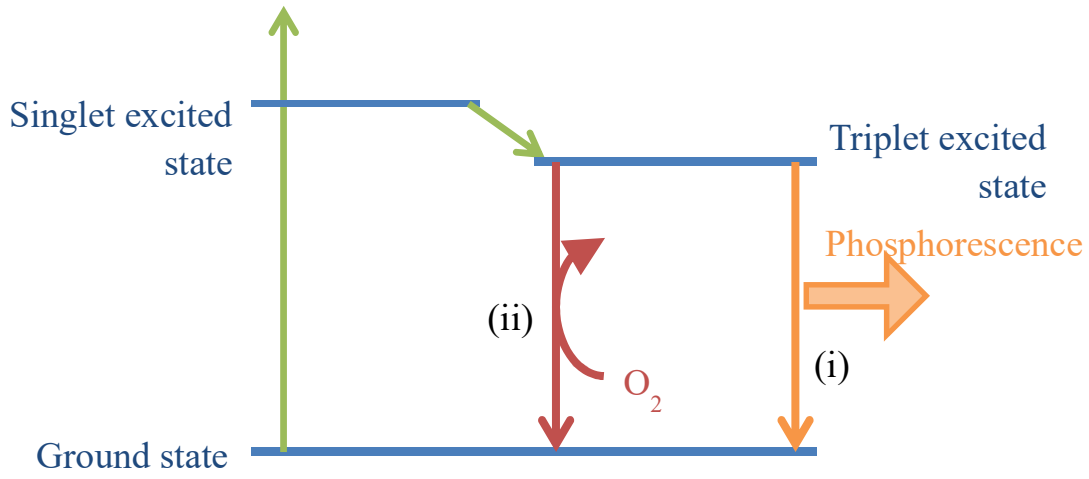

Figure S5. Energy state of phosphorescent molecule and involvement of oxygen. Like many fluorescence probes, phosphorescence probes can excited by photon of a certain wavelength. Phosphorescence probes have unique characteristics that they can move to triplet excited state by losing some energy from singlet excited state. In triplet excited state, the molecules can lose their energy not only by emitting phosphorescence (i) but also being hit by oxygen (ii). Thus, the lifetime of triplet excited state depends on circumstantial concentration of oxygen. This is why phosphorescence lifetime is dependent on surrounding oxygen.

1-6. The characteristics of BTPDM1 and aim of this research

Here I use BTPDM1, a lipophilic cationic phosphorescence probe, to detect and assess hypoxia in the kidney. BTPDM1 is developed from a red-emitting Ir complex, BTP (acetylacetonatobis[2-(2'-benzothienyl)pyridinato-κN, κC3']-iridium(III)) and has dimethylamino group. BTPDM1 is reported to distribute inside cultured cells when administered into media, and also reported to distribute in tissue and disappear from blood when administered to murine[56]. By using this phosphorescence probe, it is hoped that I can detect and assess quantitatively intracellular hypoxia in kidney, which is thought to be much important factor in progression of CKD than hypoxia in blood. This phosphorescence probe is expected to be more sensitive to mild hypoxia which occurs in kidney, especially in pathological conditions, than present intracellular hypoxia-detecting methods such as pimonidazole staining or other compounds which is hypoxia-sensitive with their nitroimidazole skeleton.

Thus the aims of this research are as follows; to establish a method which detects intracellular hypoxia even in mild hypoxia in kidney and to obtain intracellular oxygen concentration in kidney in living murine.

2. Materials and Methods

Cell experiment

HK-2 cells were incubated in a mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture (DMEM/F12) containing 10% Fetal Bovine Serum (FBS) and 100U/ml penicillin and 100μg/ml streptomycin. They are incubated at 37˚C in humidified 5%-CO_2 enriched atmosphere unless specific oxygen concentration was written.

BTPDM1 was synthesized in the laboratory of Professor Seiji Tobita as previously described[56]. For phosphorescence imaging or phosphorescence lifetime measurement, the media was changed to DMEM/F12, no phenol red without FBS. BTPDM1 was added as final concentration to be 500nM, and washed by Hank's Balanced Salt Solution (HBSS) 30 minutes later. After that, phosphorescence intensity or lifetime was measured in FBS- and BTPDM1-free DMEM/F12, no phenol red. To determine cellular distribution of BTPDM1, LysoTracker DND-99 (L-7528, Life technologies, Carlsbad, CA) was added after BTPDM1 administration. To investigate cellular distribution of BTPDM1 and LysoTracker Red, epifluorescence microscope (BZ-710, Keyence, Osaka, Japan) was used. The wavelength of excitation filter, dichroic mirror and emission filter used to detect BTPDM1 were 500/20nm, 570nm and 600nm, respectively.

Animal Experiment

All animal experiments were conducted in accordance with the guidelines for the care and use of laboratory animals approved by the University of Tokyo Graduate School of Medicine and Gunma University, and approved by both universities (M-P13-040 for University of Tokyo, 13-031 for Gumma University).

C57BL/6J male mice at 8 weeks of age were used in this study. General anesthesia was done with intraperitoneal injection of pentobarbital (40 mg/kg as initial dose; if necessary, 8mg/kg as additional dosage). In all of the experiments of phosphorescence lifetime measurement and ischemia-reperfusion injury, mice are always on heater mat after opening abdomen. For phosphorescence intensity measurement using Maestro In-Vivo imaging system (ParkinElmer Inc.) equipped with 465nm band-pass filter for excitation and 580nm long-pass filter for emission or phosphorescence lifetime measurement, medial incision was placed and abdomen was opened. Then gut was moved to expose kidneys, followed by irradiation of excitation laser. For acute ischemia/reperfusion, left renal artery and renal vein were ligated by a suture, and the suture was cut 20-30 minutes later. When making chronic ischemia/reperfusion model, ischemic time was fixed to be 30 minutes.

For measuring phosphorescence lifetime in various oxygen concentration of inhaling

atmosphere, mice were at first placed inhaling room air, and then change the inhaling atmosphere to mixture of air and N_2 using a digital gas mixing system GM-8000 (TOKAI-HIT Co., Ltd. Shizuoka, Japan). Phosphorescence lifetime was measured 5 minutes after O_2 concentration was changed. Anemic model was made by phlebotomy. 300μl of blood was discarded by tail cutting in two consecutive days, and phosphorescence lifetime or hematocrit was measured in the next day. Hematocrit was measured using heparinized capillary tubes for hematocrit (Terumo Co. Ltd., Tokyo, Japan) and centrifugation for 5 minutes by 12,000 rotations per minute.

Checking the distribution of BTPDM1 in kidney

To macroscopic investigation, a stereoscopic microscope (Leica M165 FC, Leica Microsystems, Heerbrugg, Switzerland) was used for examining the distribution of BTPDM1. 30 minutes after the injection of 250nmol of BTPDM1, kidney was excised, halved and placed on the microscope. The signals from BTPDM1 were checked with fluorescence filters (Excitation; 470/20, Emission; 510LP).

To determine the distribution precisely, epifluorescence microscope (BZ-710, Keyence, Osaka, Japan) was used. After euthanasia, kidney was cut and embedded with O.C.T. Compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), then frozen on liquid nitrogen.

Kidney sections of 4μm thickness were made, and observed with the microscope. The filters used to detect the signals from BTPDM1 were the same to what used in cellular experiments.

Phosphorescence lifetime measurement

For incubating cells, phosphorescence lifetime was measured using an inverted microscope (IX71, Olympus, Tokyo, Japan) with a stage top incubator (INUB-ONICS-F1-H2, GM-8000, Tokai Hit CO., Ltd, Shizuoka, Japan). This stage top incubator can maintain the temperature and oxygen concentration when combined with a digital gas mixing system. Excitation light is produced by a laser diode (iBeam smart-S 488-S: 488nm; pulse width, 20ns; repetition rate, 40kHz, TOPTICA Photonics AG, Gräfelfing, Germany), and phosphorescence was detected by a time-correlated single photon counting (TCSPC) system (Quantaurus-Tau C11367, Hamamatsu Photonics K.K., Shizuoka, Japan). The same system was used to measure phosphorescence lifetime *in vivo* except that irradiation of excitation laser was through a seven-way branched fiber and detection of phosphorescence was made by the same fiber. During the measurement, the mouse and the fiber were under black-out curtain in order to reduce background signals.

All of the decay curves obtained from either incubated cells or living animals could not

be fitted as single exponential curves, thus they were fitted as biexponential. As for the calculation for averaged lifetime (<τ>), amplitude-averaged lifetime was adopted, that is expressed in the equation below, where A1 and A2 are the preexponential factors of each component and τ1 and τ2 are the lifetimes of each.

$$< \tau >= \frac{A_1\tau_1+A_2\tau_2}{A_1+A_2} \quad (3)$$

All of the phosphorescence lifetime measurements were done quarterly and the average was defined as the lifetime in the condition. I considered unexpectedly long lifetimes as outliers, because those lifetimes were resulted from the wrong irradiation area which includes other organs such as guts.

Immunohistochemistry

Histological analysis was evaluated by optical microscope of formalin-fixed, paraffin embedded sections of 3μm thickness with periodic acid-Schiff staining (PAS staining) and Trichrome-Masson staining. For immunohistochemistry, pimonidazole (Hypoxyprobe, HP3-100, Hypoxyprobe, Inc., Burlington, MA) was injected intraperitoneally 120 minutes before euthanasia. Kidneys were fixed by formalin or methyl Carnoy's solution, and embedded in paraffin. Kidney sections of 3μm thickness were incubated in Hypoxyprobe-Omni antibody (HP3-100, Hypoxyprobe, Inc.,

Burlington, MA) or CD-31 antibody (DIA310, Dianova, Hamburg, Germany) overnight at 4˚C. Then they were soaked in biotinylated goat anti-rabbit IgG antibody (BA-1000, Vector Laboratories, Inc., Burlingame, CA) followed by HRP-conjugated avidin D (A-2004, Vector Laboratories, Burlingame, CA). For color development, diaminobenzidine (045-22833, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used.

3. Results

3-1. The distribution of BTPDM1 in kidney

I first examined the distribution of BTPDM1 in kidney in macroscopic approach. After systemic administration of BTPDM1, kidney was excised and halved, then observed in stereoscopic fluorescence microscope. It was revealed that cortex is stained with orange, which indicates BTPDM1 distribution and that there are many black circular areas in the cortex, indicating that this probe distributes in the tubules and not in the glomeruli (Figure 1A). For further examination, I checked the distribution in frozen section. Since BTPDM1 is highly lipophilic and its mechanism of intracellular distribution may be only by diffusion, fixing the frozen section by paraformaldehyde, methanol or acetone should be avoided, otherwise BTPDM1 flows out to fixing agents. Thus I observed frozen section without fixation. By this method, it was revealed that BTPDM1 distributed inside tubules but not in the glomeruli and vessels (Figure 1B). This result was consistent with the result of inductively coupled plasma mass spectrometry (ICP-MS) in previous paper in the point that BTPDM1 did not persist in the blood[56].

In tubular cells, BTPDM1 seemed to distribute in cytosol, however determining intracellular distribution in this method is not reliable because the distribution might change after death.

By these findings, it was proven that phosphorescence intensity and phosphorescence

lifetime in kidney after BTPDM1 administration is affected by intracellular oxygen tension in the tubular cells. Since tubulointerstitial hypoxia is considered as major concern in CKD progression, this probe was favorable to assess renal hypoxia.

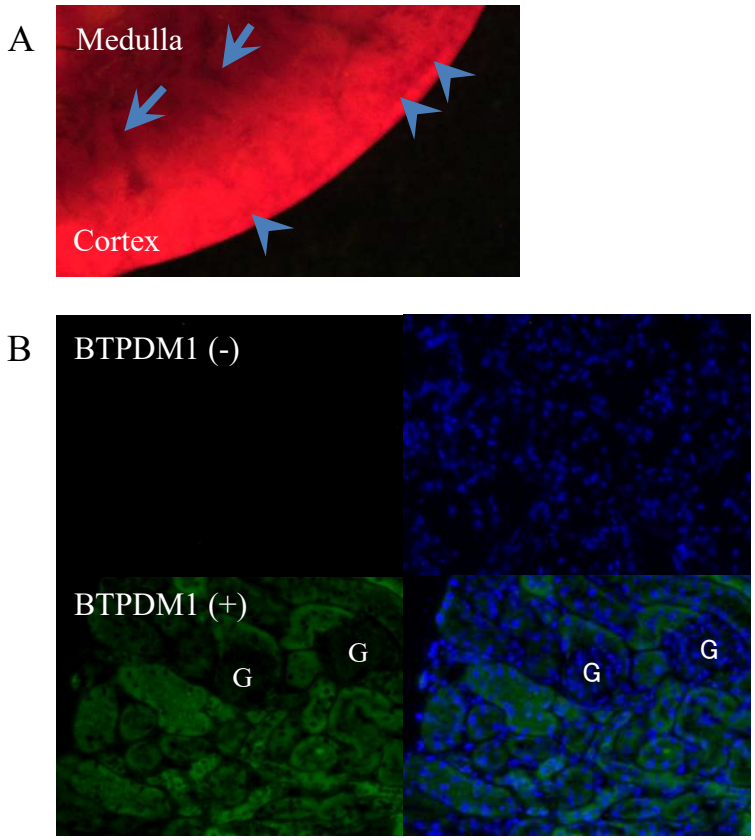

Figure 1 The distribution of BTPDM1 in kidney. The image obtained by stereoscopic microscope is shown (A), and it indicates its distribution in renal tubules and not in glomeruli and urine, since dark columns (arrow) were seen in cortico-medullal border zones and small dark circular areas (arrowhead) were in cortex. The images by epifluorescence microscope (B) showed BTPDM1 distribution in kidney in finer way. Upper panels were the images of mice kidney without BTPDM1 and lower panels were those with BTPDM1. In each panel, left panel indicates only signals from BTPDM1 (green) and in right panel BTPDM1 signals were merged with nuclear staining (blue). The character "G" denotes glomerular area. Exposure time; 1/2s (green) and 1/3.5s (blue). Original magnifications, ×400.

3-2. An attempt to detect hypoxia by phosphorescence intensity measurement

To investigate whether I can detect renal hypoxia by phosphorescence intensity measurement, I checked phosphorescence intensity in acute ischemia using Maestro. This time I adopted acute ischemia by clipping renal pedicles after BTPDM1 administration, since it can be a key test. Contrary to expectation, I could not detect the increase of phosphorescence signals, even color of kidney changed from red to black after clipping, which indicated tissue ischemia (Figure 2). I carefully checked the images, and find that there was one area which indicates low phosphorescence signals in these three images (indicated in blue arrow in Figure 2). There was blood clot in the surface of this area in the kidney. From this finding, it is realized that the phosphorescence can be absorbed by deoxyhemoglobin. It is well known that phosphorescence (or fluorescence) intensity can be affected by many factors such as optical scattering and probe concentration [57,58], and this time phosphorescence intensity was thought to be reduced by increased reabsorption by deoxyhemoglobin. By ischemia or other hypoxic state, the ratio of oxy- and deoxyhemoglobin should change, thus we concluded this phosphorescence intensity measurement is not suitable for detecting hypoxia *in vivo*, especially in the organ which contains large quantity of blood.

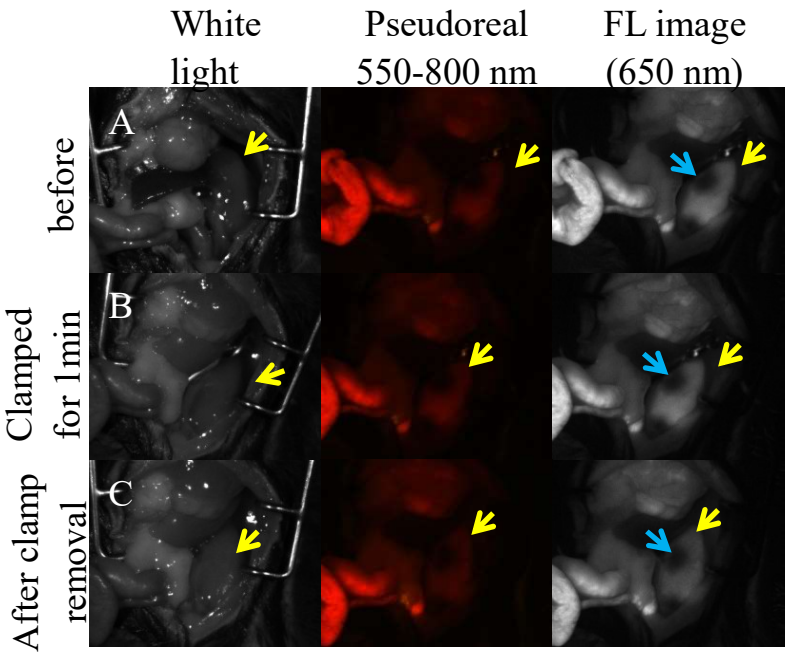

Figure 2. Phosphorescence intensity imaging in acute ischemia in kidney. Fluorescence/phosphorescence images of before clipping (A), 1 minute after clipping (B) and after removal of clip (C) were shown. Images in left were white light images, those in middle were pseudocolored images according to fluorescence spectrum and those in right were intensity at the wavelength of 650nm. Yellow allows indicate right kidney and blue allows in FL image indicates dark area on the kidney. Abbreviation; FL, fluorescence.

3-3. Phosphorescence lifetime measurement in kidney *in vivo* using BTPDM1.

After it had been revealed that phosphorescence intensity measurement is not suitable for hypoxia detection in kidney, I attempted phosphorescence lifetime measurement in kidney using BTPDM1, since phosphorescence lifetime is not influenced by probe concentration and the degree of reabsorption of phosphorescence which particularly occurs by hemoglobin in kidney. This time I measured phosphorescence lifetime by time-correlated single photon counting (TCSPC) system that illustrates a decay curve of phosphorescence. The decay curves were approximated as biexponential curves, and each lifetimes and preexponential factors were calculated, then averaged lifetimes were determined by amplitude based calculation (see Method for detail). In normal mouse, phosphorescence lifetime was well detected after administration of BTPDM1 (Figure 3A, 3B). Given that BTPDM1 distributes inside tubular cells, this phosphorescence lifetime reflect intracellular oxygen tension.

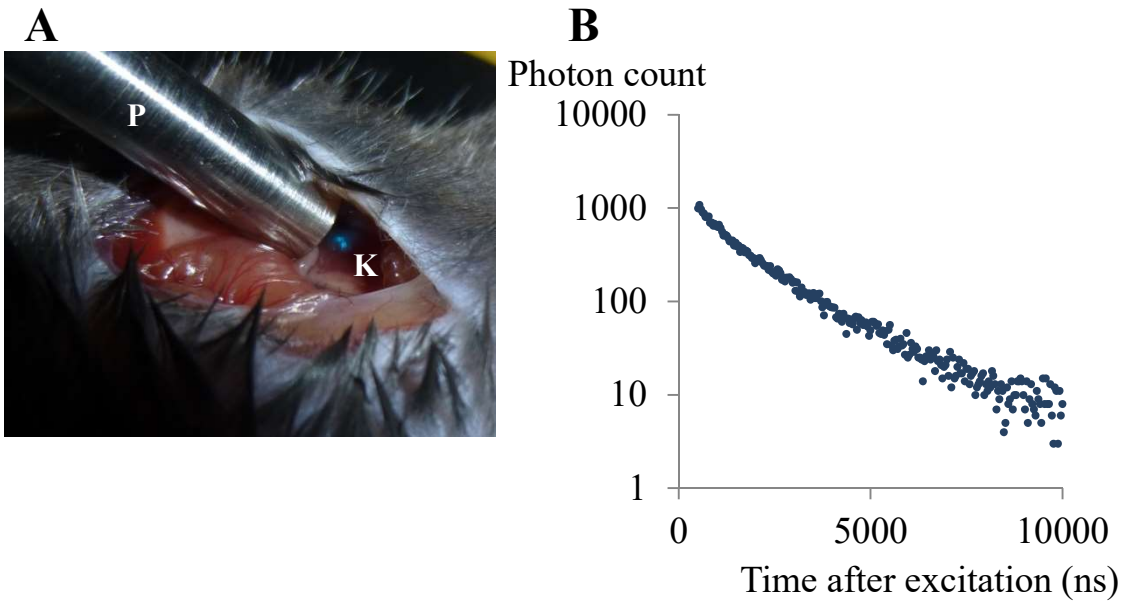

Figure 3. Representative experimental image and phosphorescence decay curve in kidney. (A) An experimental image of phosphorescence lifetime measurement. Laser probe (denoted as P) was inserted near the kidney (denoted as K) and excitation light was spotted on the surface of the kidney. The diameter of the irradiated laser seemed to be around 3μm. (B) A decay curve obtained in phosphorescence lifetime measurement in kidney. Vertical bar means photon count expressed in log-scale and horizontal bar means time after excitation. This decay curve seems not to be approximated as single-component exponential curve. Indeed, this curve and all of the decay curves obtained in cellular experiments and animal experiments need to be approximated as double-component exponential curves.

3-4. Phosphorescence lifetime measurement in the models of renal hypoxia.

In order to prove that phosphorescence lifetime after BTPDM1 administration was dependent on intracellular oxygen tension even *in vivo*, I next succeeded to measuring phosphorescence lifetime in kidney in three models in which kidney was clearly hypoxic. The first model was acute ischemia and reperfusion. The baseline phosphorescence lifetime was around 1.5μs and it actually elongated to 3.1μs followed by shortening again to 1.5μs after reperfusion (Figure 4A). This result indicated that phosphorescence lifetime can detect renal hypoxia which phosphorescence intensity imaging could not detect. It was also proven that phosphorescence lifetime is reversible depending on oxygen tension according to the shortening of phosphorescence lifetime after reperfusion. The second model was acute hypoxemia induced by decreased oxygen concentration in inhaling atmosphere. In this experiment, phosphorescence lifetime elongated from 1.3μs in normoxic condition to 1.6μs and 1.9μs under 15% and 10% oxygen concentration, respectively. In addition to demonstration that phosphorescence lifetime was dependent on oxygen concentration, this result elucidated that this technique was so sensitive that I could detect even mild hypoxia induced by inhalation of atmosphere containing only 15% oxygen. Prompt change of phosphorescence lifetime was a significant characteristic of this method, since phosphorescence lifetime changed just after change of oxygen

concentration, and it came to plateau within only 5 minutes. Another finding was that phosphorescence lifetime was not affected by depth of anesthesia because the phosphorescence lifetime in control group in which mice always inhaled air was not changed over 1 hour. The third model was anemia. The anemia was induced by phlebotomy from tail in two consecutive days. The hematocrit measured in the next day was 35.4±4.0% in anemic group whereas it was 51.4±6.1% in the control group. Phosphorescence lifetime was also increased in anemic group (Figure 4C). When compared to an earlier report which measured renal oxygen concentration by needle oxygen electrode[59], this result could indicate that this technique was more sensitive to hypoxia in anemic model, because in the report oxygen electrode indicated only a few change of oxygen concentration in kidney in 35% of hematocrit.

From the results described above, phosphorescence lifetime in kidney using BTPDM1 was really dependent on tissue oxygen concentration. In addition to oxygen dependency, rapid change and reversibility were proven.

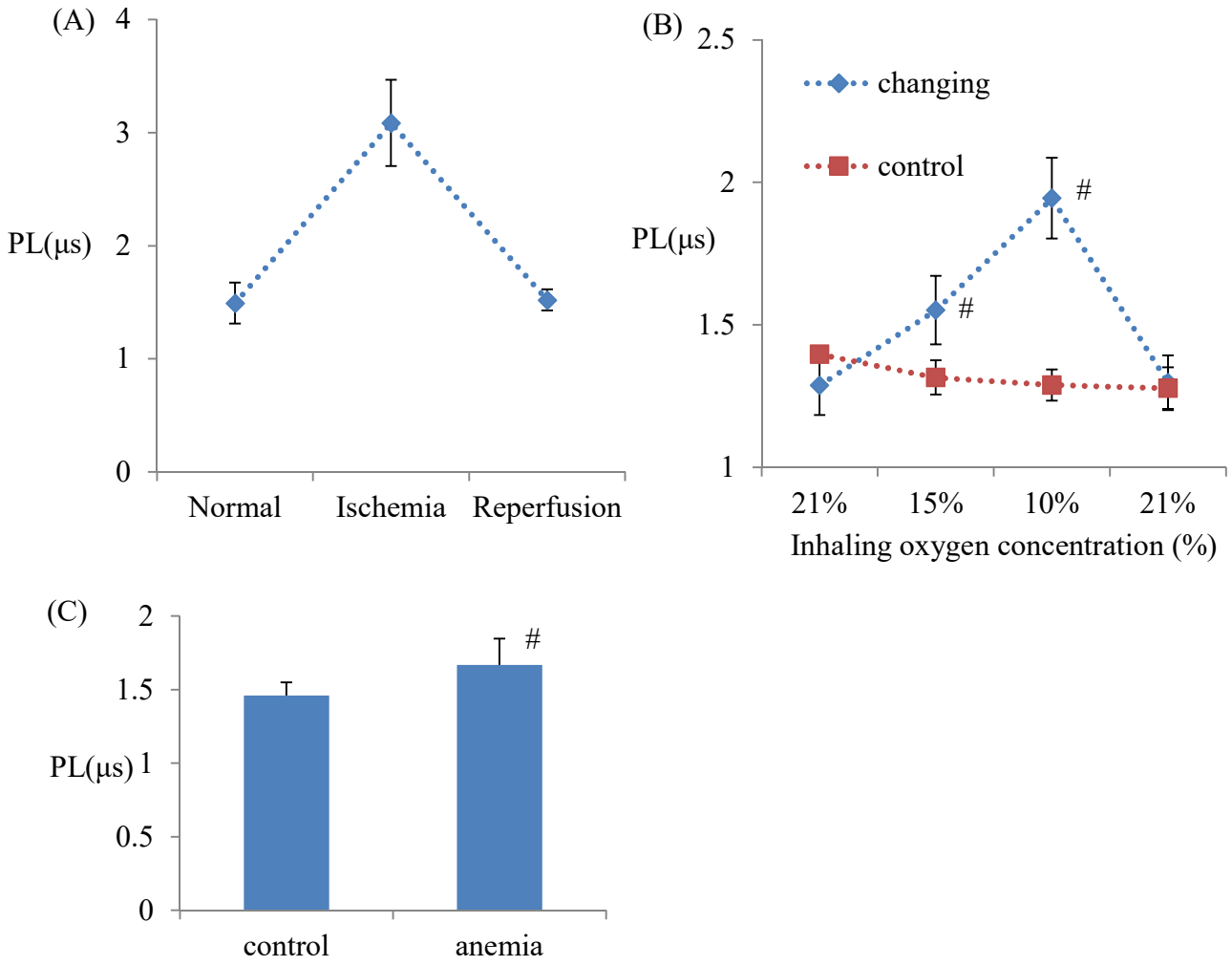

Figure 4. Phosphorescence lifetime in renal hypoxia models. (A) The transit of phosphorescence lifetime in acute renal ischemia/reperfusion. Phosphorescence lifetime significantly increased in ischemia and recovered after reperfusion. N = 3. Error bar; S.D. (B) The change of phosphorescence lifetime in hypoxemia. Phosphorescence lifetime elongated in 15% of oxygen, and more in 10% of oxygen, followed by returning under normoxic condition. N = 4 (changing group) and 3 (control group). Error bar: S.D. #; $P < 0.05$ compared with control group by two-tailed unpaired t-test. (C) The difference of phosphorescence lifetime in anemia and control group. Phosphorescence lifetime is longer in anemia group. N = 6 (anemia group) and 5 (control group). Error bar: S.D. #; $P < 0.05$ compared with control group by two-tailed unpaired t-test. PL, phosphorescence lifetime.

3-5. Detection of renal hypoxia in renal fibrotic model

The results so far indicated phosphorescence lifetime measurement in kidney using BTPDM1 enabled detection of hypoxia with good sensitivity, thus I examined whether this technique could detect renal hypoxia in renal fibrotic model or not. This time, unilateral ischemia-reperfusion model and examination in the day seven days after disease induction was employed. In this time point, the fibrosis in the affected kidney was shown to be mild (Figure 5A). Before measuring phosphorescence lifetime, the distribution of BTPDM1 after systemic administration should be checked, since phosphorescence lifetime could be affected by difference of probe distribution. The distribution was again inside tubular cells in this model, although complicated by increase of the signals in BTPDM1 (-) kidney, which could be interpreted as increase of autofluorescence (Figure 5B). Then I measured phosphorescence lifetime in kidney, and it significantly elongated (Figure 5C). Provided that the distribution of BTPDM1 was same, this elongation of phosphorescence lifetime indeed indicated decreased intracellular oxygen tension in tubular cells in this disease model. Examining the renal hypoxia by other techniques is important to conclude this elongation of phosphorescence lifetime really reflect change of renal oxygenation. I performed immunohistochemistry of pimonidazole protein adduct and CD-31 (also known as PECAM-1). There were more

stained tubules in I/R injured kidney by pimonidazole immunohistochemistry and CD-31 staining elucidated that many peritubular capillaries were distorted and disrupted (Figure 5D, E). These findings strongly supported that I/R injured kidney was more hypoxic than contralateral kidney and were collateral evidences that phosphorescence lifetime measurement could indeed detect hypoxia in I/R injured kidneys.

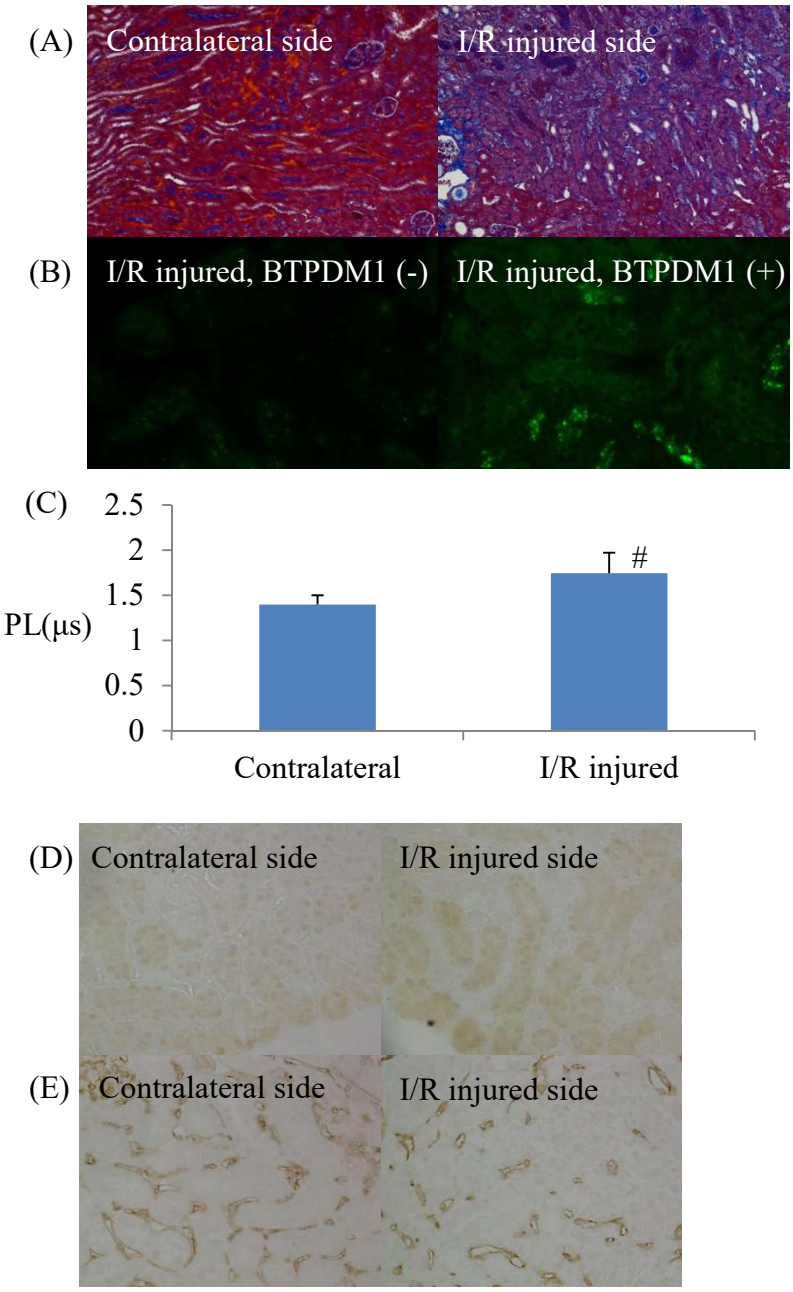

Figure 5. Detection of hypoxia in unilateral I/R model. (A) Trichrome-Masson staining of kidney. Cortical images of I/R injured kidney (right) and contralateral kidney (left) are shown. Original magnification, ×100. (B) The distribution of BTPDM1 in unilateral I/R injured model. Signals from BTPDM1 were pseudocolored as green. Exposure time; 3s. Original magnifications, ×400. (C) Phosphorescence lifetime of I/R injured kidney and contralateral kidney. PL, phosphorescence lifetime. N = 5. Error bars; S.D. #; $P<0.05$ by paired two-tailed t-test. (D, E) Pimonidazole protein adduct immunohistochemistry (D) and immunohistochemistry of CD-31 (E). I/R injured kidneys (right panels) and contralateral kidneys (left panels) are shown. Original magnifications, ×400.

3-6. Characteristics of BTPDM1 in cultured proximal tubular cells and conversion from phosphorescence lifetime to oxygen tension.

To achieve the conversion of phosphorescence lifetime to oxygen tension, calibration curve between phosphorescence lifetime and oxygen tension must be drawn. How to make it was a problem, since *ex vivo* experiments cannot make sense mainly on the grounds that the distribution of BTPDM1 should change in dead cells and that oxygen cannot diffuse into excised kidneys. Thus I examined the phosphorescence characteristics in immortalized cultured proximal tubular cells, HK-2 (human kidney 2) cells. BTPDM1 distributed inside HK-2 cells by only addition to medium without carrier, and the distribution seemed to be in lysosomes in view of the merged image with LysoTracker (Figure 6A). Then, it was found that phosphorescence lifetime elongated in hypoxic conditions in oxygen concentration dependent manner (Figure 6B). I expected that the inverses of phosphorescence lifetime could be approximated linearly given that Stern-Volmer equation (equation 2) holds even in intracellular conditions and indeed it could (Figure 6C). The preparation of cell specific calibration curve is proven to be important since the calculated k_q and τ_0 were different from those in SCC-7 cells[56]. Using this calibration line, I determined intracellular oxygen tension in tubules in the animal experiments above. Table 1 shows the value of oxygen tension in each situation.

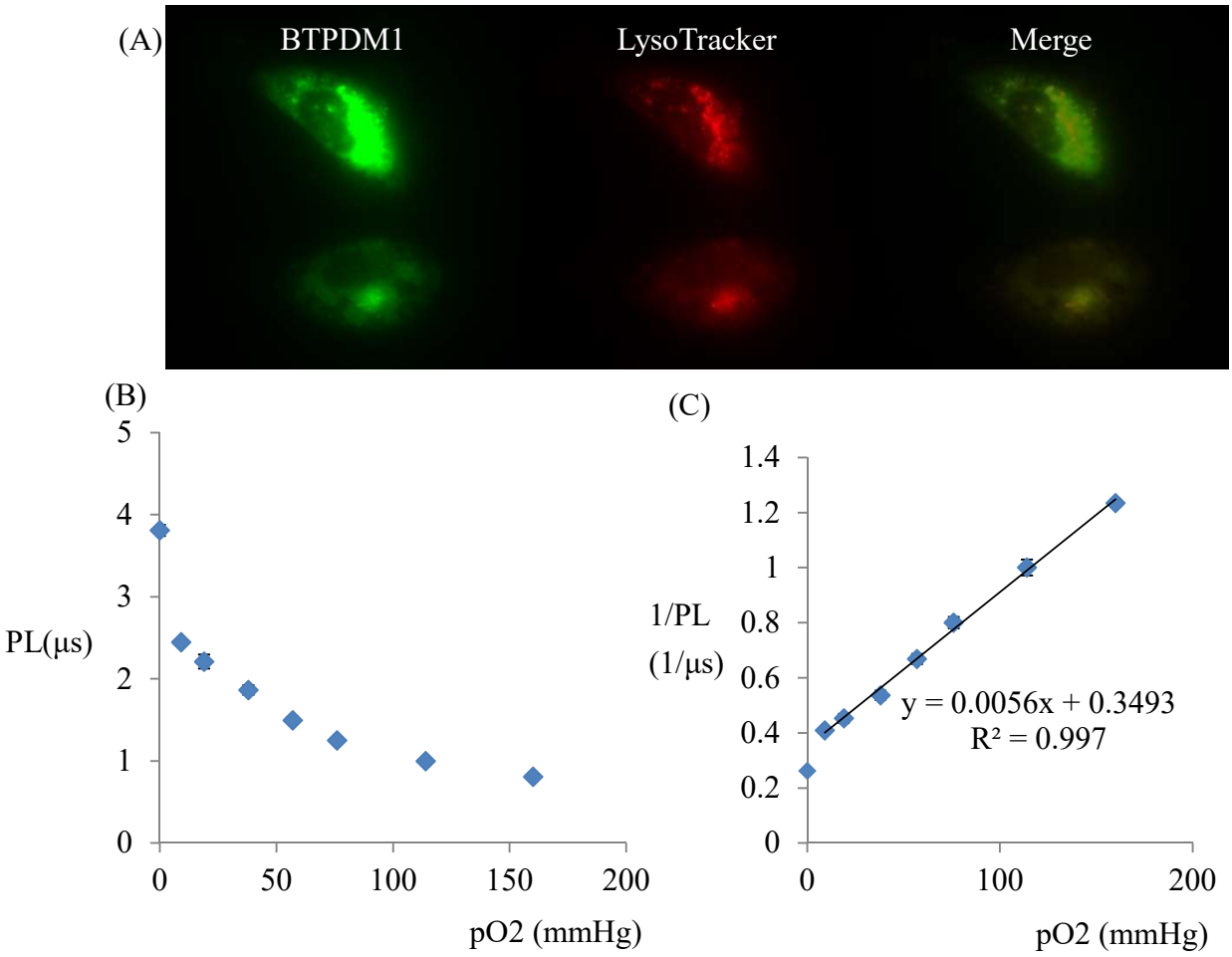

Figure 6. Characteristics of BTPDM1 in HK-2 cells. (A) The distribution of BTPDM1 in HK-2 cells. The signals of BTPDM1 (left) and LysoTracker (center) are merged in the right panel. Exposure time; 1/2s for both BTPDM1 and LysoTracker. Original magnifications, ×400. (B) Phosphorescence lifetime in various oxygen concentrations. Phosphorescence lifetime decreased in accordance to increase of oxygen concentration. (C) The linear approximation between the inverse of phosphorescence lifetime and oxygen concentration. The approximation formula and the coefficient of determination were written in the graph. Abbreviations: PL, phosphorescence lifetime; pO_2, partial pressure of oxygen.

Table 1. Intracellular pO2 extrapolated from PL

Situation	PL (μs, mean±S.D.)	pO2 (mmHg)
Inhaling room air	1.29±0.10	70 (61 - 80)
Inhaling 15% O2	1.52±0.09	52 (46 - 58)
Inhaling 10% O2	1.94±0.14	30 (24 - 36)
Anemia	1.67±0.18	43 (34 - 54)
I/R injured model (I/R injured side)	1.75±0.23	38 (28 - 52)
I/R injured model (Contralateral side)	1.40±0.10	60 (53 - 69)

The values of PL are from the experiment of Figure 4B (inhaling room air, 15% O_2 and 10% O_2), Figure 4C (anemia) and Figure 5C (I/R injured model). Values of pO2 are written as A (B - C), where A is the pO2 corresponding to mean PL and B and C are the pO_2 which corresponds to (mean + S.D.) and (mean - S.D.), respectively. Abbreviations: I/R, ischemia-reperfusion; PL, phosphorescence lifetime; pO2, partial pressure of oxygen.

4. Discussion

I achieved phosphorescence lifetime measurement in kidney with BTPDM1, a cationic lipophilic phosphorescence probe. According to its intracellular distribution in tubular cells and rapid disappearance from blood, it exerts as an intracellular oxygen tension indicator *in vivo*. Indeed, this probe could detect renal hypoxia in acute ischemia, acute hypoxemia, anemia and CKD model. I also suggested a method to determine intracellular oxygen tension by extrapolating the calibration line obtained in the experiment of cultured cells to phosphorescence lifetime of living murine. This technique is superior to pimonidazole staining, a traditional hypoxia detecting method, because this is a quantitative evaluation, this enables detect of hypoxia in living murine and this is not affected by redox state in the tissue. There is also significant difference between this technique and needle oxygen electrode, a gold standard way to determine oxygen tension in tissue, in terms of the difference of the site in which the technique measures oxygen concentration, that is, my technique measures intracellular oxygen tension and needle oxygen electrode measures oxygen tension in the blood.

There are some reports which use phosphorescence probe to determine oxygen tension in cultured cells[60-62]. My probe and technique are different from these reports mainly in two points. The first point is how to determine intracellular oxygen tension with phosphorescence probe. Intracellular oxygen concentration is frequently determined by

phosphorescence intensity, after standardized by concentration in according to the signals from another fluorophore in the same molecule (often described as “ratiometric probe”). This method can almost diminish the weakness of phosphorescence intensity measurement that the intensity depends not only on oxygen tension but on probe concentration, however, this method works only in the experiment of inorganic environment or cultured cells. In the experiment of living animals, there is not a little autofluorescence, thus additional devices are needed to apply this technique to living murine. I overcame this wrinkle with measuring phosphorescence lifetime, which is not influenced by autofluorescence. The other point is that BTPDM1 was proven to diffuse into cells and, more importantly, rapidly flow out from blood stream. For measuring phosphorescence lifetime in this technique, it is needed to irradiate excitation laser on the surface of kidney and the irradiated area is not so small (around 3mm) that the area can include not only tubules but also glomeruli and vessels. Thus, if a phosphorescence probe lingers in blood, the obtained phosphorescence lifetime cannot be the indicator of intracellular oxygen. In this viewpoint, the proof of probe distribution out of vessel in Figure 1 and previous report by ICP-MS is quite important.

Some previous reports determined oxygen tension in murine by phosphorescence lifetime measurement[40,42,63]. The phosphorescence probes used in these reports were

hydrophilic dyes[54,55,64], and thus these probes are considered to distribute only in blood. As for water soluble phosphorescence dyes, only measuring phosphorescence lifetime of the probe solved in water with bovine serum albumin under various oxygen concentration is needed to make calibration curves between phosphorescence lifetimes and oxygen concentrations, since the circumstance around phosphorescence probes *in vivo* is enough mimicked[40,42,65]. On reflection, BTPDM1 is highly lipophilic and intracellular oxygen tension is expected to be measured, thus how to make the calibration curve is a major concern, since any single material cannot imitate intracellular lipid bilayer membrane which BTPDM1 is thought to distribute in because lipid bilayer membrane in cells contains numerous kinds of protein. My solution is to use cultured cells as simulant material of tubules *in vivo*. There should be a criticism that cultured cells cannot have perfectly same intracellular environment to living organ.

In this report, I focused on intracellular oxygen tension, but there should be some criticism that it is not definitive whether intracellular oxygen tension is different from extracellular oxygen tension. It is reported that decreased access to normoxic media can lead to intracellular hypoxia in cultured cells[56,66]. In these reports, placing coverslip on cultured cells leads to intracellular hypoxia, and the intracellular oxygen tension is decreased in a distance dependent manner from the edge of the coverslip, insisting that

when far from oxygen suppling media, intracellular oxygen tension is decreased, even if the oxygen tension in oxygen suppling media is not hypoxic. In CKD model, I showed rarefaction of PTC, and this situation can be imitated by experiments in cultured cells with coversplips.

To certify the value of oxygen tension *in vivo* with this technique, the obtained oxygen tension should be compared to previous reports with other techniques. However, there are no other established methods which can determine intracellular oxygen tension *in vivo.* This time the values of pO_2 obtained in this experiment are compared to the values in the experiment of needle oxygen electrode[10,29,33,34,59], even if they are not strictly able to be compared with my results because of the difference of measurement site.

Table 2 shows the values in each experimental method. Looking at this table comprehensively, although the value of pO2 in normal state by this technique is slightly larger than that by needle oxygen electrode, the values of pO2 might be convincing. This might support the method to extrapolate the calibration curve made in cultured cell experiment to tissue pO2 *in vivo*.

Table 2. The comparison of pO_2 in each technique.

Situation	pO2 by PL measurement with BTPDM1 (mmHg)	pO2 by needle oxygen electrode (mHg)
Normal state	51 - 70	45-50
Inhaling 15% O2	46 - 58	No report
Inhaling 10% O2	24 - 36	35
Anemia	34 - 54	Around 45

The values of pO_2 by PL measurement with BTPDM1 are converted from the PL in the experiment of Figure 4A, 4B and 4C for "normal state", Figure 4B for "inhaling 15% O2" and "inhaling 10% O2" and Figure 4C for "anemia". The values of pO_2 by needle oxygen electrode are from Ref. 10, 28 and 32 for "normal state", Ref. 33 for "inhaling 10% O_2" and Ref. 58 for "anemia". Abbreviations: PL, phosphorescence lifetime; pO2, partial pressure of oxygen.

The limitation of this report is that I assumed intracellular oxygen tension in tubules *in vivo* by only one method. It requires future research with other techniques to determine whether this technique can indicate exact pO_2 or not. Another limitation is that the phosphorescence from kidney is emitted from tubules, however there are many kinds of tubular cells. Determining accurate distribution in which tubular cells this probe distributes *in vivo* is needed to examine the role of hypoxia in CKD in greater depth. In terms of probe distribution, I cannot determine which organelle this probe distributes *in vivo*. To determine intracellular probe distribution is required when intracellular, interorganelle oxygen gradient matters. Moreover, in this technique I cannot assess oxygen tension in medulla, while medullary oxygen tension is also important in several renal diseases[67].

Allowing that there are some limitations in this reports described above, this technique provides a new look at oxygen biology and research on kidney. Assumption of intracellular oxygen tension by this technique should help to elucidate when and how hypoxia initiates to affect CKD progression by clarify when hypoxia in kidney emerges. This technique can be applied to determination of intracellular oxygen tension in other organs, since difference of probe concentration in each organ does not matter in phosphorescence lifetime measurement.

Application to other organs is future prospects of this technique. Hypoxia and HIF-activation is reported to influence on pathological conditions in such as heart[68], and liver[69]. In heart, HIF-α is reported to have protective role in ischemic preconditioning. On the other hand, HIF-α has a fibrogenic role in liver fibrosis caused by bile duct ligation. These two reports emphasize the importance of HIF. As mentioned above, oxygen tension is the main regulator of HIF-HRE activity, but it is also the case that HIF is also regulated by other factors such as inflammation[48]. Thus, this technique should be of help to research whether hypoxia itself has protective role in heart and fibrogenic role in liver. Deterioration of pancreatic islet function is reported to be related to hypoxia[70]. As for pancreas, the mechanism of emergence and progression of hypoxia is not well investigated, thus my technique may be helpful to investigate whether treatments against hypoxia in pancreas are promising or not.

Hypoxia or HIF-activation is reported to influence on pathological conditions of some organs other than kidney such as heart liver and pancreas, thus this technique can also be of help to elucidate the effect of hypoxia in these organs.

In summary, I achieved determination of intracellular oxygen tension in kidney *in vivo*. This technique should help to figure out the association of intracellular oxygen tension and CKD progression.

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