Intravital phosphorescence lifetime imaging of the renal cortex accurately measures renal hypoxia

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Renal tubulointerstitial hypoxia is recognized as a final common pathway of chronic kidney disease and is considered a promising drug target. However, hypoxia in the tubules is not well examined because of limited detection methods. Here, we devised a method to visualize renal tubular oxygen tension with spatial resolution at a cellular level using the cell-penetrating phosphorescent probe, BTPDM1 (an iridium-based cationic lipophilic *dye),* and confocal phosphorescence lifetime imaging microscopy to precisely assess renal hypoxia. Imaging with BTPDM1 revealed an oxygen gradient between S1 and S2 segments in mouse kidney. We also demonstrated that our microscopy system can detect subtle changes of hypoxemia and reoxygenation, and the acquired phosphorescence lifetime can be converted to partial pressure of oxygen. This new method allows, for the first time, visualization of intravital oxygen gradients at the renal surface with high spatial resolution. Thus, the confocal phosphorescence lifetime imaging microscopy platform, combined with BTPDM1, will promote an accurate understanding of tissue hypoxia, including renal hypoxia.

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Renal tubulointerstitial hypoxia is regarded as the worsening factor of chronic kidney disease (CKD) progression.[1] The response of the kidneys against hypoxia is mainly via the tubules. The tubules are composed of several subsets that differ in terms of their energy sources.[2] Among these, the proximal tubules obtain adenosine triphosphate from aerobic energy metabolism, which is implicated to be the reason for the susceptibility of the proximal tubules to hypoxia. Therefore, it has been postulated that an oxygen gradient may exist between the tubule subgroups, which, if established, could account for the subgroups' differing levels of susceptibility to hypoxia.

Phosphorescence originates from the long-lived excited triplet state, and thus its lifetime depends significantly on oxygen tension.[3,4] We have recently developed a method to assess intracellular oxygen tension in mouse renal tubules using a cell-penetrating phosphorescent probe (BTPDM1, Supplementary Figure S1) based on Ir(III) complex and a bifurcated fiber system.[5] One limitation of this technique was that it provided only the average oxygen tension of numerous proximal tubules in the circle with the diameter of approximately 3 mm. Phosphorescence lifetime imaging microscopy (PLIM) is a promising tool for detecting an oxygen gradient with high spatial resolution and has been applied to the bone marrow, cerebral vessels,[6,7] and tissue fragments as well as cultured cells.[8,9] However, there have been no reports regarding its application to organs in living animals, probably due to the lack of suitable probes.

Therefore, we attempted to take intravital PLIM images following systemic BTPDM1 administration in mice. Fifteen minutes after BTPDM1 administration, sufficient phosphorescence signals were detected at the renal surface with single exponential decay curve fitting (Figure 1a and b and Supplementary Figures S2 and S3). The PLIM image shows that BTPDM1 distributes intracellularly, mainly in the apical side of the tubules, and not in the peritubular capillary or urinary spaces, which is consistent with previous reports.[5,10,11] The tubules seem to be divided into 2 subgroups: the tubules of 1 group consist of thick epithelial cells and display shorter phosphorescence lifetimes and the tubules in the other group consist of thin epithelial cells and display longer lifetimes (Figure 1c and Supplementary Figure S4). PLIM measurements in the liver surface were also attempted,

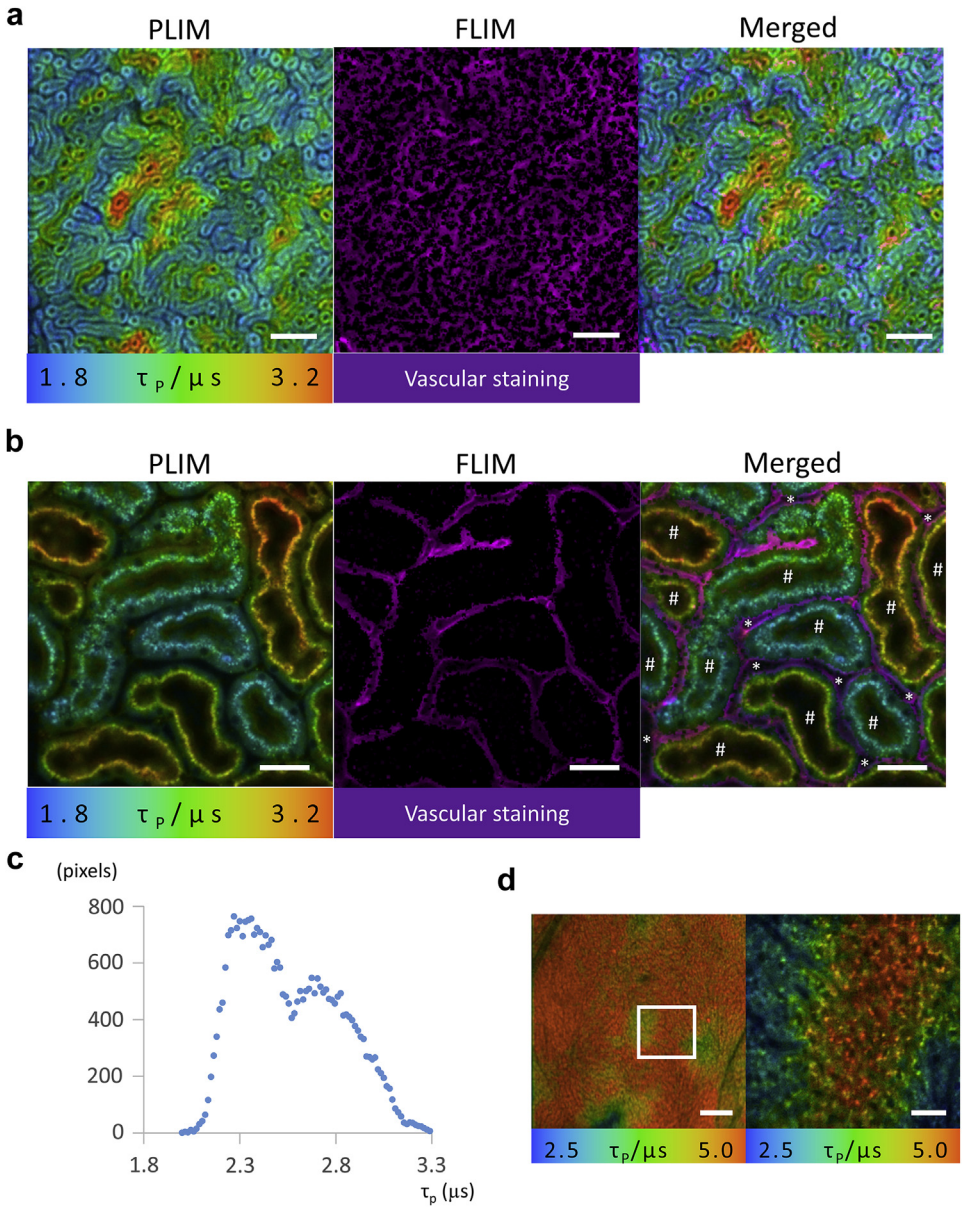

Figure 1 | Renal and hepatic phosphorescence lifetime imaging microscopy (PLIM) images. Renal pseudocolored PLIM images obtained with (**a**) ×10 objective lens and (**b**) ×40 objective lens are shown in the left panel. Color scales are shown under the image. Signals from fluorescein isothiocyanate (FITC)-conjugated *Lycopersicon esculentum* lectin, which binds to vascular endothelium, are shown in the center panels as vascular counterstaining. Signals of FITC are defined as the pixel whose fluorescence lifetime is over 2.5 ns. Merged images are shown in the right panels. FLIM, fluorescence lifetime imaging microscopy. (**b**) In a high-magnification view, phosphorescence signals are seen only inside the tubular cells and absent in the (*) peritubular capillary and (#) urinary space. Bar = 200 μm in (**a**) and 50 μm in (**b**). (**c**) Distribution histogram of phosphorescence lifetime for the PLIM image in (**b**). Bimodal peaks exist, which support that tubules are divided into 2 subgroups according to their phosphorescence lifetime. (**d**) Hepatic PLIM image. The PLIM image in the hepatic surface with ×10 objective lens (left image) and ×40 objective lens (right image). The field of the image in the right panel corresponds to the box in the left panel. The same gradient was seen in 2 more mice. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

and a phosphorescence lifetime gradient was also detectable (Figure 1d). This result was compatible with previous reports that visualized the existence of oxygen tension gradient in the hepatic lobule.[12,13]

We found that photographing at the depth of 10 μm from the renal surface is most suitable for our PLIM system (Supplementary Figure S5A). Considering that almost all tubules within 10 μm depth in the frozen section of mouse kidneys are proximal tubules (Supplementary Figure S5B), the tubules observed in our PLIM system are assumed to be S1 and S2 segments of the proximal tubules.[14] To identify the subgroups of tubules, the urinary excretion dye fluorescein isothiocyanate (FITC)-conjugated 4 kDa dextran was administered i.p. in living mice. At first, the FITC signals were located only inside the tubules with shorter phosphorescence lifetimes, and later the fluorescence signals of FITC appeared also inside the tubules with longer lifetimes (Figure 2a–c and Supplementary Figure S6). This result suggested that the S1