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Two-photon microscopy: Visualization of kidney dynamics

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The introduction of two-photon microscopy, along with the development of new fluorescent probes and innovative computer software, has advanced the study of intracellular and intercellular processes in the tissues of living organisms. Researchers can now determine the distribution, behavior, and interactions of labeled chemical probes and proteins in live kidney tissue in real time without fixation artifacts. Chemical probes, such as fluorescently labeled dextrans, have extended our understanding of dynamic events with subcellular resolution. To accomplish expression of specific proteins *in vivo*, cDNAs of fluorescently labeled proteins have been cloned into adenovirus vectors and infused by micropuncture to induce proximal tubule cell infection and protein expression. The localization and intensity of the expressed fluorescent proteins can be observed repeatedly at different time points allowing for enhanced quantitative analysis while limiting animal use. Optical sections of images acquired with the two-photon microscope can be 3-D reconstructed and quantified with Metamorph, Voxx, and Amira software programs.

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With the invention of the two-photon microscope, dynamic cellular processes in tissues of living organisms can be studied at increased depths with far less phototoxicity. Until now, it has been difficult to observe the distribution and behavior of specific proteins in tissues of living animals. Protein localization and functional studies have been primarily restricted to immunochemical analysis of fixed tissues collected at set time points, compromising the true nature of the proteins studied, owing to the fixatives used, and limiting the temporal resolution needed to capture dynamic events. In recent years, investigations of protein dynamics have been extended to cell culture. Although cell culture has indeed provided the advantage of visualization of fluorescently labeled protein dynamics through microinjection of fluorescently labeled proteins or transgene protein expression in cells, studies of immortalized cells in culture offer a restricted view of cellular protein dynamics in the living organism. With the introduction of three scientific advances: (1) two-photon microscopy;[1,2] (2) *in vivo* expression of fluorescently labeled protein probes;[3,4] and (3) sophisticated reconstruction computer software,[5] dynamic studies of cellular proteins in health and disease can now be pursued in live animals. Utilization of these exceptionally powerful scientific tools provides the opportunity for scientists to address important biological and clinical questions in whole animals. Increased understanding of cellular protein dynamics in the live animal opens the possibility for discovery of new diagnostic tools and treatments to fight disease processes. Therefore, this review will first focus on general applications of two-photon microscopy and then concentrate on near real-time studies addressing the dynamic nature of fluorescently labeled cytoskeletal proteins expressed in live animal kidney proximal tubule cells under physiological conditions and during ischemia and recovery.

TWO-PHOTON MICROSCOPY

The advent of the two-photon microscope now allows scientists to visualize fluorescent probes as deep as 150 μm in live animal kidneys and to gain a better understanding of the structural and functional changes observed in the kidneys in health and disease.[6–9] Until recently, studies of live animal

kidneys have been limited by the availability of a microscope that can image deep into tissues without causing significant phototoxicity. Microscopic analyses of protein localization and function have primarily been described with the aid of the wide-field epifluorescence or confocal microscopes. Although these studies provided fundamental information on specific cellular proteins, especially in cell culture and fixed thick tissue sections, these microscopes cannot be used to study labeled protein probes deep in live animal tissues. The information obtained on thick specimens using epifluorescence microscopes is notoriously hazy and unusable without the aid of deconvolution software. Although confocal optical image stacks can be acquired in live tissue, the data are compromised by severe photobleaching and tissue phototoxicity. The two-photon microscope can capture optical image stacks deep into biological tissue using minimally invasive techniques with limited photobleaching and tissue phototoxicity, as fluorescence occurs only within the plane of focus. Therefore, fluorescent molecules can be imaged repeatedly, enabling dynamic study of cellular events in the intact organs of live animals over variable time periods from seconds to months.[6–10]

The two-photon microscope accomplishes increased penetration into samples through simultaneous absorption of two low-energy photons.[1,2] Multiple fluorophores can be excited and imaged at the same time. High-resolution subcellular images can be obtained with less light scattering and reduced photodamage to surrounding biological tissues. Investigators have successfully used two-photon microscopy in the study of embryonic development,[11] tumor formation,[12] and structure and function studies of the brain,[13] kidney,[6] and skin.[14] These studies have provided researchers with a more realistic glimpse of the *in vivo* cell biology, biochemistry, and physiology of these tissues.

FLUORESCENT REAGENTS

The development of nontoxic fluorescently labeled probes amenable to visualization in live animals has further enhanced the ability to visualize cellular processes *in vivo*. For instance: (1) nuclei of intact live kidney cells have been stained with Hoechst 33 342 dye, or propidium iodide to study apoptosis;[6,15] (2) the proximal tubule brush border and endocytic vesicles have been labeled with Texas Red-folate in a study of folate uptake;[16] (3) intracellular pH has been determined with pH indicator probes;[17,18] (4) single nephron glomerular filtration rate has been measured using fluorescein isothiocyanate-inulin or Lucifer yellow;[19] and (5) glomerular permeability, proximal tubule endocytosis, and blood flow rates have been studied with labeled albumin and dextrans.[6–8,19–23] All of these fluorescent probes are markers that provide vital information on kidney structure and function in health and disease. To understand intracellular molecular mechanisms of pathophysiology and disease processes in live animal tissues and organs, continued research to identify new methods to observe dynamic cellular components is crucial.

Until recently, visualization of fluorescently labeled proteins in live animal kidney proximal tubule cells has not been possible. The development of adenoviral vectors containing cDNA sequences that encode for labeled cytoskeletal proteins[24] and the utilization of micropuncture to infuse the adenoviral vectors into the proximal tubule lumen or Bowman's space has provided a feasible technique to induce expression of fluorescently labeled cytoskeletal proteins in live animal kidney proximal tubule cells.[4] The combination of a classical method (kidney micropuncture) with newer molecular biology techniques has facilitated *in vivo* investigations of cytoskeletal protein structure and function. Extending this approach to the numerous membrane and cytosolic proteins found in kidney cells will provide novel insights and will increase our understanding of cellular dynamics under normal and pathophysiological conditions.

OPTICAL STACK RECONSTRUCTION

Although the development of two-photon microscopes and incorporation of fluorescent probes in live animal tissue cells are fundamental to the study of cellular dynamics, the availability of computer hardware and software capable of reconstructing massive optical section stacks is essential for analysis, quantitation, and presentation of the collected data. Optical sections are typically obtained at an interval of 1 μm over a vertical distance of 60–80 μm. In our laboratory, we use (1) Metamorph software (Molecular Devices, Sunnyvale, CA, USA) to measure fluorescence intensities of cellular structures and for 2-D and 3-D image processing, (2) Voxx[5] and Amira (Template Graphics, San Diego, CA, USA) 3-D reconstruction software to build optical stacks for surround views of the collected images, and (3) Amira software for segmentation and measurements of fluorescent volumes. These optical stack reconstruction software packages have been successfully used for fixed tissues, but live animal image collections still present challenges owing to plane shifts that can result from very slight respiratory movements of the animal. For 4-D reconstructions (3-D over time), this problem is amplified further, making data comparisons between time points challenging. To gain an understanding of dynamic changes, however, the time factor is critical and explorations of methods to quiet the animal, or tissue being studied, or development of high-speed two-photon systems are central to forwarding this research. Also, collection of large optical stacks over time can lead to fluorescent probe bleaching at the focal points, requiring care be taken in study design and analysis.

APPLICATIONS

Table 1 lists a few of the many renal processes that can be studied utilizing two-photon microscopy. Figure 1 shows examples of the ways that multiphoton microscopy has been utilized to study dynamic cellular processes in the functioning kidney. In Figure 1a, we used 3 kDa Cascade Blue-labeled dextran to evaluate filtration in a surface glomerulus of a Munich–Wistar rat. This small molecular weight conjugate is

Table 1 | Applications for two-photon microscopy within the kidney

Glomerular structure and function
Filtration rate
Size/volume of glomerulus
Permeability characteristics
Fibrosis/sclerosis
Cell function
Endocytosis – quantitative analysis
Intracellular trafficking and subcellular localization
Transcytosis
Exocytosis
Sites of tubular reabsorption and secretion
Signal transduction
Ion concentrations
Metabolic state
Microvasculature
Blood flow rate
Endothelial permeability
WBC adherence/rolling
Vasoconstriction
Rouleaux formation
Renin release
Cell toxicity
Cell injury including necrosis or apoptosis
Surface membrane blebbing or internalization
Mitochondrial function

WBC, white blood cell.

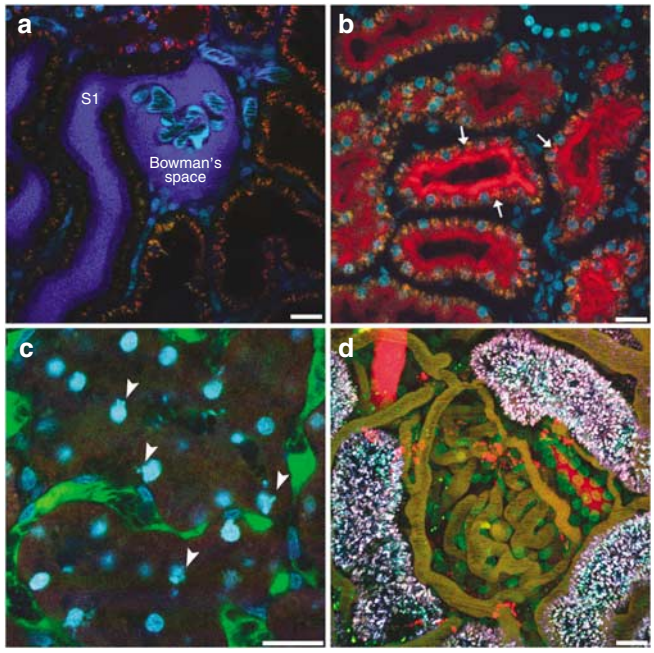

Figure 1 | Demonstration of glomerular filtration, endocytosis, and apoptosis in the anesthetized rat using two-photon microscopy, fluorescent probes, and 3-D reconstruction computer software. In (**a**), kidney filtration of a 3 kDa Cascade Blue-labelled dextran is shown. The dextran has been filtered by the glomerulus into Bowman's space and is present in the S1 segment of the proximal tubule. In (**b**), Texas Red-folate has bound to the apical brush border membrane of proximal tubule cells, was endocytosed, and appears in lysosomes in the cell's cytoplasm. In (**c**), fragmented nuclei (arrows), a hallmark of apoptosis, are shown in a rat proximal tubule cell in a sepsis model. In (**d**), a 3-D reconstruction of a surface glomerulus in a Munich–Wistar rat is shown. Intravenous infusion of small molecular weight dextrans conjugated to red and blue fluorophores was followed by later delivery of 500 kDa fluoresceinated dextran and 40 kDa Texas Red-dextran. The glomerulus in the center is surrounded by proximal tubule cells displaying white to multicolored lysosomes containing the small-sized dextrans. High concentrations of Texas Red-dextran can be seen in the distal tubule lumen, but dextrans are not internalized into the distal tubule cells. The capillary loops of the glomerulus and adjacent microvasculature appear reddish-orange, a mixture of the 500 kDa fluoresceinated dextran and the smaller, slowly filtered 40 kDa Texas Red-dextran. The nuclei appear teal in color. Bar = 20 μm.

rapidly filtered and is seen in Bowman's space and in the S1 segment of the proximal tubule. Additional studies utilizing a ratiometric technique, developed by Yu *et al.*[23] has allowed for determining a rate constant for inulin clearance from the blood[25] as well as quantification of glomerular permeability characteristics. Recently, Russo *et al.*[21] determined the glomerular sieving coefficient of fluorescently labeled albumin in Munich–Wistar rats and provided evidence that glomerular filtration of albumin is ~50 times greater than previously thought; tubular reabsorption via endocytosis and transcytosis prevents its excretion and loss. Thus, it is possible to visualize and quantify many aspects of glomerular filtration and tubular reabsorption of proteins in the same nephron in a dynamic manner. This includes single nephron filtration rates.[19]

In Figure 1b, we demonstrate the binding of a Texas Red-folate conjugate, following filtration, to the apical membrane of proximal tubular cells and subsequent endocytosis. The endocytosed folate is concentrated in lysosomes, however, a fraction of the endocytosed material undergoes transcytosis.[16] Note the specific localization within proximal tubular cells, but not endothelial cells or distal tubule cells (upper right hand corner). In Figure 1c, fragmented nuclei, a hallmark of apoptosis, are shown by the use of Hoechst 33 342 labeling. This approach has allowed investigators to quantify the effect of different pathophysiologic processes on apoptosis within the kidney.[15]

Three-dimensional reconstruction of structures within the kidney is also possible and allows for segmentation and quantitative analysis of 3-D processes. In Figure 1d, multiple fluorescent dextrans have been utilized to label different structures, including a glomerulus and tubules of a Munich–Wistar rat. Approximately, 1½ h before imaging, freely filterable 3 kDa dextrans conjugated to Texas Red and Cascade Blue (red and blue, respectively) were infused sequentially. These dyes, now localized within lysosomes of proximal tubule cells, appear cyan to lavender to white as these colors colocalize within these autofluorescent structures. Also during this time, a large, non-filterable, 500 kDa fluoresceinated dextran (green) was given to label the microvasculature and capillary loops of the glomerulus. Approximately ½ h before imaging, a bolus of slowly filtering 40 kDa Texas Red-dextran was given intravenously. This dye is visible in the glomerular capillary loops, peritubular

capillaries, and distal tubule lumens, giving these structures a yellow-orange appearance. Distal tubule lumens contain high concentrations of the dextran, because of tubular water reabsorption, and are seen adjacent to the glomerulus. Hoechst 33 342 dye was used to label all the nuclei within the kidney (teal in appearance). Note the staining of the podocyte nuclei around the outside of the glomerular capillaries and the round distal tubule cell nuclei. The robust lysosomal staining within proximal tubule cells obscures the nuclei in this portion of the nephron.

Molecular protein labeling and dynamics

We have explored the behavior of cytoskeletal proteins in rat proximal tubule cells under physiological and non-physiological conditions using two-photon microscopy, newly designed fluorescently labeled protein reagents, and computer software. To understand the *in vivo* interactions of globular (G-actin) and filamentous actin (F-actin) with the two actin-binding proteins, actin-depolymerizing factor/cofilin (an actin depolymerizing and severing protein) and tropomyosin (an F-actin stabilizing protein), we expressed these proteins in the live animal kidney. The cDNAs for these proteins, with either an amino or carboxy terminal fluorescent tag, were cloned into an adenovirus vector system for infection and protein expression in the kidney proximal tubule cells. Initial studies demonstrated that bypassing the glomerulus was crucial to achieving adenoviral infection and protein expression in the proximal tubule cells. Utilizing micropuncture techniques in Munich–Wistar rats with surface glomeruli, we were able to choose our point of infusion.[4] The adenovirus was infused directly into the proximal tubule lumen and also into the urinary space of Bowman's capsule to achieve maximal proximal tubule cell adenovirus infection. We also successfully infused adenovirus into the vascular welling point to selectively infect endothelial cells. This micropuncture approach to gene transfer greatly reduced the number of viral particles injected and the resulting inflammation compared with the renal artery route. Adenovirus infection resulted in optimal fluorescent protein expression within 48 h and persistence of protein expression for longer than 2 weeks. In addition to fluorescent protein expression in the proximal tubule and endothelial cells, occasional fluorescent protein expression was detected in glomerular and distal tubule cells. Using this approach, we have successfully expressed green fluorescence protein (GFP)-actin and the actin-binding proteins, XAC(wt)-GFP and tropomyosin-EYFP, in proximal tubule cells. These labeled proteins localized to the cellular regions predicted by earlier immunolocalization studies. XAC(wt)-GFP localized to the cytoplasm in the proximal tubule cells and tropomyosin-EYFP had a terminal web localization. GFP-actin localized diffusely in the cytoplasm, and higher concentrations were found adjacent to the basolateral membrane and in apical brush border microvilli (Figure 2a). Localization of these fluorescently labeled proteins to previously identified cellular sites indicates the proteins function normally in this regard.

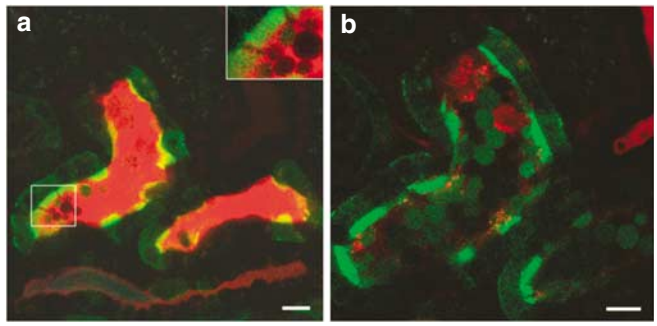

Figure 2 | Apical vesicles or blebs form during ischemia. In (**a**), proximal tubule cells infected with adenovirus express high concentrations of GFP-actin in the apical brush border. Texas Red-dextran (red, 40 kDa) signal fills the proximal tubule lumen, highlighting the GFP-actin (green) vesicles or blebs. Two microvilli (inset) formed bulbous tips. In (**b**), numerous blebs are shown in the proximal tubule lumen; only some express detectable levels of GFP-actin. Also, the red dextran can be seen within blebs, indicating cellular endocytosis before ischemia and subsequent blebbing. Bar = 10 μm.

These studies are being used to further our understanding of actin dynamics and protein interactions under physiologic and pathophysiologic conditions in intact proximal tubule cells.

Renal ischemia studies

Using these newly developed techniques, we pursued localization of labeled GFP-actin, XAC(wt)-GFP, and tropomyosin-EYFP in live animal kidney proximal tubule cells in real-time studies of actin dynamics during ischemia-induced acute renal injury and recovery. To study renal ischemia in the live rat, we anesthetized the rat 2 days after micropuncture and infusion of adenovirus into the rat's left kidney. The treated kidney was exposed by a flank incision and placed beneath the rat on the microscope stage.[6] To minimize the difficulties encountered from repositioning the rat on the stage, we utilized a suprarenal-aortic clamp approach. This approach obviated the need to remove the rat from the microscope stage in the middle of the experiment and increased the probability of obtaining correlating image stacks. A limitation of this approach, however, is that the clamp induces ischemia not only of the kidneys but also the lower half of the body, including the intestines. Using the suprarenal-aortic clamp model, localization of GFP-actin (Figure 2), XAC(wt)-GFP, and tropomyosin-EYFP was pursued during temporary renal ischemia and recovery.

Development of apical membrane blebs

As a result of these *in vivo* fluorescent protein studies, we have gained new insights into the cellular changes that occur during ischemia and recovery. We observed apical membrane breakdown and noted formation of apical vesicles/blebs during renal ischemia (Figure 2a). These apical blebs form when the bundled actin filaments degenerate and the overlying apical membrane swells, forming a bulbous tip that is pinched off into the proximal tubule lumen. Previous

immunofluorescence studies of fixed ischemic rat kidney tissues demonstrated that actin and the actin-binding proteins, actin-depolymerizing factor/cofilin and tropomyosin, were present in these blebs. In live animal studies, we visualized formation of a GFP-actin-containing bleb with the aid of intravenously injected Texas Red-dextran 40 kDa to outline the lumen of the tubule. The 40 kDa dextran was slowly filtered by the glomerulus and filled the proximal tubule lumen, outlining the bleb as it was forming. Also, in similar experiments, the flow of blebs down the proximal tubule lumen immediately after release of the suprarenal-aortic clamp was demonstrated, suggesting again that apical blebs are formed and released during ischemia (Figure 2b). Blebs that expressed GFP-actin show green fluorescence, whereas those that appear black lack this protein construct.

Rouleaux formation

On occasion, unexpected findings are seen during the use of two-photon microscopy. Therefore, to study disease processes, the investigator has to be open-minded and observant. We have detected the widespread occurrence of rouleaux (rolls of red cells like a pile of coins) within the kidney cortex microcirculation in several different acute renal injury models, including ischemia, sepsis with cecal ligation and puncture, and either lipopolysaccharide or radiocontrast administration. Rouleaux formation in the kidney microvasculature is shown after ischemic injury in Figure 3a. In Figure 3b, we show rouleaux in a superficial kidney cortex venule. Rouleaux formation has been observed following ischemic injury to the cremaster muscle[26] and in the renal medulla following ischemic injury. The roles that rouleaux play in local cortical microvascular flow disturbances and distant organ injury remain to be determined. We have noted them within renal biopsies read out as ischemic injury (unpublished observations) and others have reported findings in the lungs consistent with either local formation of rouleaux or trapping of preformed red blood cell aggregates arising from an injured organ.[27]

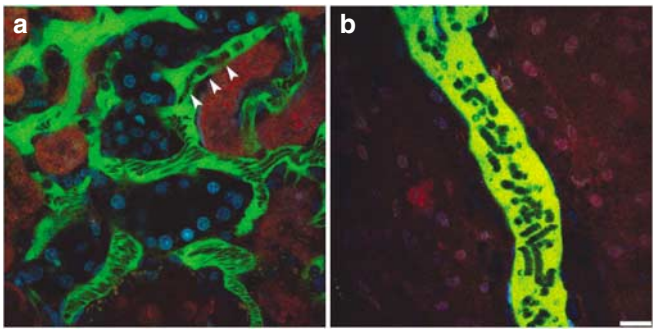

Figure 3 | Ischemia induces formation of red blood cell rouleaux or 'stack of coins' in the peritubular capillaries. In (**a**), these abnormal red blood cell formations can block blood flow in the kidney microvasculature as demonstrated by arrows. (**b**) Rouleaux are more readily observed in a superficial cortical venule following ischemia. Bar = 20 μm.

FUTURE OPPORTUNITIES AND CONCLUSION

Intracellular and intercellular dynamics can now be studied in the kidneys of live animals, during health and disease, using the two-photon microscope, fluorescent protein probes, and novel 3-D rendering software. No longer is kidney research on dynamic cellular processes restricted by the study of fixed tissues or cultured cells, by the availability of antibody probes, and by limited time points. Opportunities exist to increase the depth of penetration so as to allow visualization of the outer stripe of the kidney medulla. This is particularly important in ischemia research given the importance of lack of reperfusion to this region.[28] It should also be possible to view and study events in the inner medulla, as the renal papilla can be exposed in young rats and desert-living rodents. Increasing data acquisition rates will enhance the ability to follow rapid cellular processes with the necessary temporal resolution. Finally, the development of additional molecular and transgenic approaches will allow hypothesis testing presently not possible. Future developments within the field of multiphoton microscopy will continue to open new avenues of investigation resulting in more efficient and effective translation of basic science findings into preclinical observations and therapeutic advances.

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