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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