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Intravital multiphoton microscopy of dynamic renal processes

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Molitoris, Bruce A., and Ruben M. Sandoval. Intravital multiphoton microscopy of dynamic renal processes. *Am J Physiol Renal Physiol* 288: F1084–F1089, 2005; doi:10.1152/ajprenal.00473.2004.—Recent advances in microscopy and optics, computer sciences, and the available fluorophores used to label molecules of interest have empowered investigators to utilize intravital two-photon microscopy to study the dynamic events within the functioning kidney. This emerging technique enables investigators to follow functional and structural alterations with subcellular resolution within the same field of view over seconds to weeks. This approach invigorates the validity of data and facilitates analysis and interpretation as trends are more readily determined when one is more closely monitoring indicative physiological parameters. Therefore, in this review we emphasize how specific approaches will enable studies into glomerular permeability, proximal tubule endocytosis, and microvascular function within the kidney. We attempt to show how visual data can be quantified, thus allowing enhanced understanding of the process under study. Finally, emphasis is given to the possible future opportunities of this technology and its present limitations.

glomerular filtration; endocytosis; proximal tubule cells

NEW IMAGING TECHNOLOGIES, such as multiphoton microscopy, have equipped researchers with extremely powerful tools to uniquely address biologically important questions that can only be accomplished in whole organ studies (1, 11, 16). In parallel with this, advances in fluorophores with increased quantum yields utilized to tag individual molecules (7), transgenic approaches, and new delivery techniques (5) have allowed for the development of intravital studies that can follow and quantify events with subcellular resolution. Furthermore, exponential developments in computer sciences, specifically with applications to imaging, have removed many of the obstacles previously limiting the ability to utilize microscopy to study and quantify dynamic cellular processes (12, 14). In particular, developments in hardware, software, bandwidth, and data storage now provide the life scientist with systems that possess the necessary speed to effectively attack data intensive processes utilized in digital image acquisition and analysis. These imaging technologies enable the dynamic four-dimensional (4D) analysis (3D plus time) of structure in organs and tissues (4), the measurement of chemical and biochemical composition of tissues, the expression of fluorescently labeled functional proteins, and quantification of the rates of physiological processes such as microvascular perfusion rates, glomerular permeability, or endocytic uptake rates. Therefore, it is now possible to utilize these minimally invasive technologies across a spectrum of organs to facilitate the translation of new knowledge into the intact animal. Researchers equipped with these unique and ever-improving tools can utilize optical microscopy and digital imaging to study events within cells, cell-cell interactions and integrative organ physiology, biochemistry, and molecular and cellular biology. This will greatly enhance the understanding of physiological and disease processes and will hasten and improve the reliability and interpretation of preclinical data.

The kidney is an extremely complex heterogeneous organ consisting of vascular and epithelial components functioning in a highly coordinated fashion that allows for the regulation of a myriad of interdependent processes. Over the years, talented and creative individuals have developed novel and imaginative experimental approaches that allow for isolating, understanding, and integrating the unique structure-function relationships that occur. Investigators have developed model systems to enable enhanced manipulation and isolation of specific variables of interest. This has led to a mechanistic understanding of cellular processes and the identification of alterations of these processes under defined conditions that attempt to mimic either physiological or disease states (Fig. 1). However, these models are not without their shortcomings as recently delineated for acute renal failure (8). Although absolutely essential, these models often lack the organ-specific complexity and the dynamic nature of cellular processes and cell-cell interactions necessary for an adequate understanding of a disease process. Models also severely limit our ability to test therapeutic approaches to disease states, and this has led to difficulties in translating preclinical data into therapeutic advances.

Three major areas of development have now been combined to provide investigators studying the kidney with unique approaches to understanding biological processes in a renal-specific fashion at cellular and subcellular levels. First, multiphoton microscopy offers the investigator a minimally invasive, high-resolution technique with increased depth of penetration and markedly reduced phototoxicity for visualization of cell-cell and intracellular events intravitally. The reduction in phototoxicity results as fluorescence excitation occurs only at the focal point, thereby eliminating out-of-focus fluorescent excitation within the tissue as would occur with confocal microscopy. The genesis of these advances was covered

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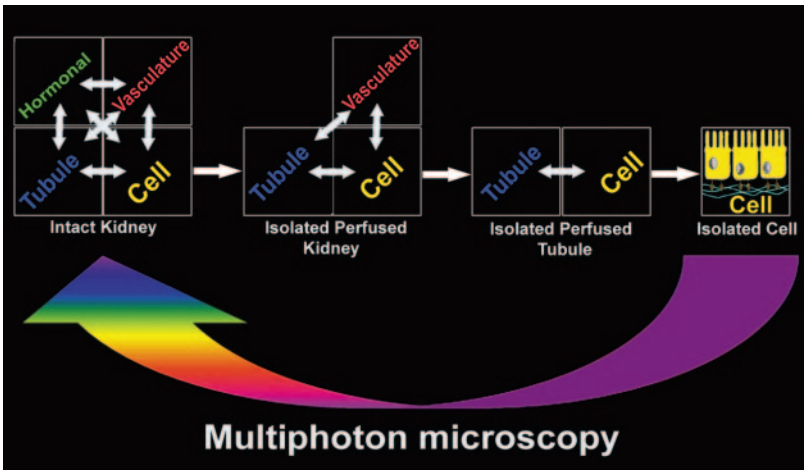

Fig. 1. Reversing reductionism. Understanding renal physiological and pathological processes has benefited tremendously from the development and use of models that allow for a reductionist approach. Multiphoton microscopy now offers the ability to study processes with up to subcellular resolution intravitally, with minimal phototoxicity, using multiple fluorescent probes at one time in 3 and 4 dimensions. This, combined with previously developed approaches, such as micropuncture, will allow for delivery of specific probes to areas of interest within the functioning kidney.

in the previous article (16) and in our previous publication (3). Second, improved detectors with increased sensitivity, enhanced software and faster hardware, and new computational algorithms for 3D analysis and quantification have allowed for more rapid, sensitive, and accurate data gathering, visualization, and interpretation (2). Finally, the revolution in fluorophores capable of reporting on a growing number of cellular processes has markedly improved the capabilities available to the investigator. Fluorescent labeling of proteins, either by genetic or chemical means of attachment using a wide spectrum of colors, allows for simultaneous multicolored imaging of different cellular processes. The utility of such powerful combinations is seemingly endless but not without challenges. Therefore, the remainder of this review will be used to highlight some of the areas of progress and remaining challenges relating to kidney imaging using intravital two-photon techniques.

METHODS

Optimal success in the development and use of intravital two-photon techniques and subsequent interpretation of the data require an integrated investigative team of biologists/physicians, microscopists, engineers, and computer scientists. Additional experience and expertise in surgery and anesthesia are also beneficial for conducting live animal studies. Therefore, these studies are best undertaken in a specialized imaging facility or institute, where daily attention is directed toward assisting investigators, maintaining equipment in maximal working condition, and developing new techniques, software, and their applications. We have been afforded a unique opportunity to develop such a facility with funds provided by Indiana University, INGEN, and the National Institutes of Health through a George M. O'Brien Center of Excellence Award. This latter grant offers kidney and urological related studies requiring intravital multiphoton microscopy to be accomplished at our center without cost to the principal investigator for technical assistance or microscopic beam time. We presently have three commercial multiphoton instruments that allow for a wide spectrum of studies. We also have an integrated team of scientists and engineers dedicated to ensuring optimal instrument performance and software development, allowing for maximal data acquisition and analysis. Access to such a facility allows the individual investigator opportunities to discuss, understand, and "personalize" the approaches necessary for success. For example, cell culture studies requiring 3D dynamic data (4D) are probably best accomplished using a spinning disc confocal microscope and not a two-photon microscope. The spinning disc microscope has a faster image-capture rate and can easily image through one layer of cultured cells in the *z*-axis. However, use of multiphoton microscopy offers numerous advantages for intravital organ-specific studies, such as a greater depth of penetration. Both types of microscopy offer similar resolution, limited phototoxicity, and the ability for repeated observations of the same site over hours, days, or weeks. These advantages will be demonstrated in the specific examples described later.

All animal work was reviewed and approved by the Institutional Animal Care and Utilization Committee.

When intravital studies using two-photon techniques are performed, adequate anesthesia is essential as any kidney movement limits the ability to obtain useable data, particularly 3D and 4D data. We have found that using an inverted microscope with an exteriorized left kidney (longer renal pedicle facilitates use) limits kidney movement (3). We have recently started to use halothane anesthesia as it provides for rapid induction, rapid cessation, and greater control over the depth of anesthesia. This is particularly important when one deals with diseased animals that are highly sensitive to anesthesia. Maintenance of core body temperature and volume status is also essential for all studies involving the kidney, as renal blood flow is markedly decreased when they are not maintained.

Only water-immersion objectives can be used, and this limits the effective magnification to approximately ×60, with a high numerical aperture of 1.2 or greater preferred for improved resolution of subcellular structures. One can use at least three different colored fluorescent probes at once with simultaneous excitation, although one has to be careful and monitor for bleed-through of fluorescence emissions from one channel into another. The spectrum of emitted fluorescence is split into three channels centered around 605, 525, and 455 nm, which are collected in separate photomultiplier tubes. We typically use excitation wavelengths in the 800- to 850-nm range for two-photon studies, with higher wavelengths providing less autofluorescence from pigments within proximal tubule cells (3, 10). Laser power can be varied over a broad range and standards should be run each day to monitor output. This is especially important when quantitative studies are to be undertaken and compared with data obtained on other days. Another limitation minimizing the depth and quantitation of imaging is the loss of fluorescence due to light scatter as one images more deeply into any organ. This scatter is organ specific, with the kidney having a higher level of scatter compared with other organs such as the brain. Under normal physiological conditions, we have been able to image up to 200 μm into the kidney of a rat or mouse. This works well for 3D visualization and quantification of superficial glomeruli in Munich-Wistar rats but does not allow for visualization of the corticomedullary regions even in mice. Furthermore, correction of fluorescence intensity for depth of field is not yet available to allow for quantitative studies comparing different depths within the kidney.

Image processing is an integral component of digital microscopy. Here, operations such as visualizing dim structures, assessing colocalization between multiple probes, measuring cellular volume changes, and quantifying fluorescence at the subcellular level are achieved. Universal Imaging's image processing software Metamorph (West Chester, PA) has been utilized extensively in our studies for the operations listed above as well as preproduction of images and movies for publication. Then, composite images and figures are usually generated using Adobe Photoshop (Adobe, Mountain View, CA). In addition, Voxx, developed at the Indiana Center for Biological Microscopy (Indianapolis, IN), utilizes inexpensive high-end video cards on desktop PCs to render 3D volumes and allows rotation in free space at near real-time speeds (2). This facilitates analysis of intricate structures with subcellular resolution. Data sets are visualized as either solid objects with shading to generate depth of field or intensity projections to determine colocalization. Finally, segmentation, surface rendering, and quantitative volume rendering can be accomplished with Amira (TGS, San Diego, CA) (9).

APPLICATIONS

Glomerular filtration and the permeability of differently sized compounds across glomerular capillaries can be visualized and quantified using Munich-Wistar rats with surface glomeruli. Because a rat glomerulus is ~100 μm in diameter, and two-photon microscopy can visualize up to 200 μm into the kidney, it is possible to record events within the majority of the glomerulus. Figure 2*A* shows one photomicrograph from a series of images, collected at the rate of ~1 image/s, demonstrating filtration of a 3-kDa Texas red-labeled neutral dextran (red) across glomerular capillaries, into Bowman's space, and flow through the S1 proximal tubule segment. The vasculature contains a large nonfilterable 500-kDa fluorescein-labeled dextran (green). The dark objects within the vasculature are cells, primarily red blood cells, that displace the green fluorescent dye and therefore appear as dark objects. Although it is instructive to visualize the glomerular filtration rate, especially from a teaching point of view, several unique opportunities are now available to the scientist interested in glomerular function in the normal and diseased state. For example, it is now possible to use dextrans of different sizes with fluorophores of different colors with a known net charge (neutral, cationic, or anionic) or proteins of different molecular weights to observe and quantify glomerular permeability (15). Detection of permeability defects at the glomerular capillary surface also eliminates the confounding effects of proximal tubule protein reabsorption (6). This may be particularly important in quantifying proteinuria in early stages of a disease process or in assessing therapeutic responses. Finally, it is possible to observe effects over hours, days, weeks, or months within the same glomerulus of a Munich-Wistar rat. This has obvious advantages in increasing sensitivity and specificity and eliminating the need for euthanizing a large number of animals at each time point within a study. A movie constructed from consecutive images (URL http://filtration-movie1) shows the dynamics of this process. The ability to simultaneously excite and acquire three different fluorescent probes using infrared light is an important aspect of two-photon microscopy.

Figure 2, *B* and *C*, taken at ~10 s and 2 min after initiation of cascade blue dextran infusion, respectively, shows filtration of rhodamine-labeled albumin (red) and 3-kDa cascade blue anionic dextran (blue) across glomerular capillaries. The small cascade blue dextran is easily seen crossing the glomerular capillaries and filling Bowman's space, whereas the rhodamine-labeled albumin has limited permeability and therefore is not visible within Bowman's space. However, rhodamine albumin is easily visualized in proximal tubule cells after endocytic uptake across the apical membrane as variably sized intercellular vesicles, shown in red. These data imply that proximal tubule cell uptake and concentration of filtered material can be used as a more sensitive indication of glomerular filtration, especially in Fig. 2*B* nuclei labeled with Hoechst (cyan) and, as previously reported (3), distal tubule nuclei stain more intensely with Hoechst. Also, as previously reported by Tanner et al. (13), water removal during transit along the nephron results in a higher concentration of cascade blue dextran in distal tubules with a concomitant increase in fluo-

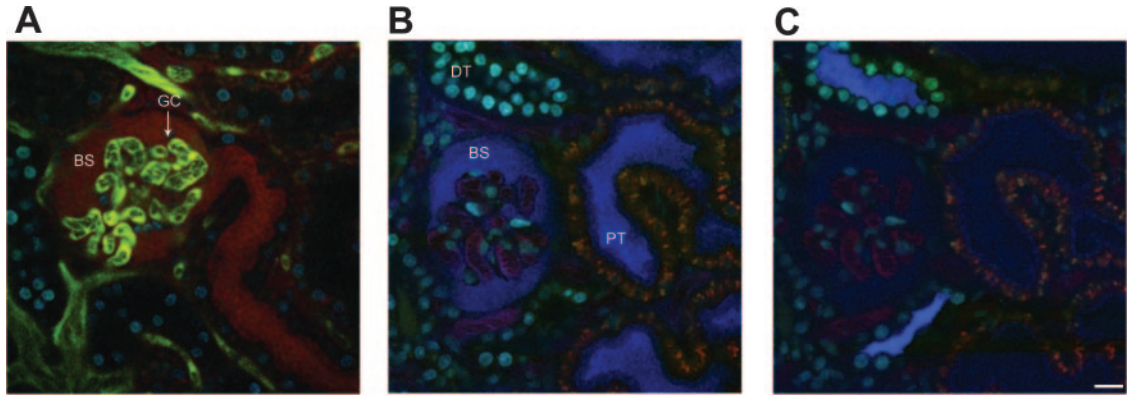

Fig. 2. Glomerular filtration of small-molecular-mass dextrans into Bowman's space (BS) and into the leading S1 proximal tubule segment in Munich-Wistar rats. In all 3 panels, the nuclei of all cells were labeled with Hoechst 33342 (cyan). In *A*, a large-molecular-mass (500 kDa) fluorescein dextran (green) and a 3-kDa Texas red dextran (red) were given simultaneously via a femoral venous access line. The vasculature is outlined with the 500-kDa dextran and red blood cells appear as dark shadows in both glomerular capillary loops (GC) and surrounding blood vessels. The 3-kDa dextran is rapidly filtered into BS and travels down the lumen of the early S1 segment of the proximal tubule. In *B*, cascade blue dextran can be seen filtering into BS and the lumen of proximal tubules (PT) ~10 s after initiation of infusion. DT, distal tubule. In *C*, the DT at the *top left* and lower *middle*, previously empty in *B*, became filled with cascade blue dextran ~2 min after initiation of infusion. As water is reabsorbed along the tubule segment, the fluorescent intensity of the dextran increases as it reaches the distal tubule lumen. Bar = 20 μm. Videos for image sequences in this figure are available for broadband download at http://ajprenal.physiology.org/cgi/content/full/00473.2004/DC1. Note that in all studies, dextrans were infused in a bolus and required ~5 s for delivery.

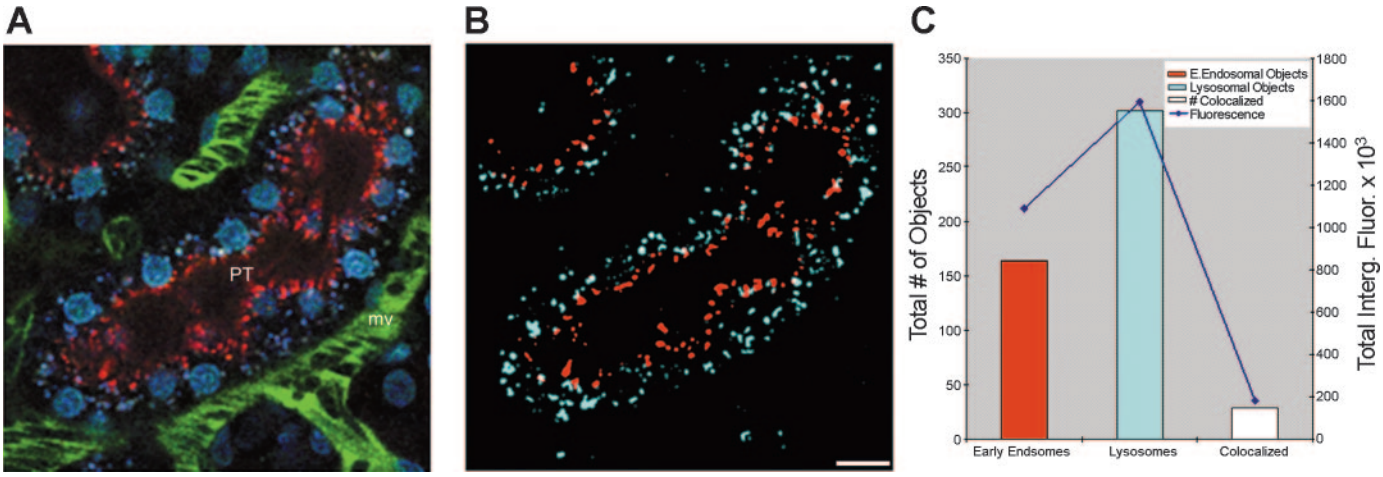

Fig. 3. Quantification of lysosomes and early endosomes in Sprague-Dawley rats using 3-kDa dextrans given 24 h apart. In *A*, microvasculature (MV) labeled with a 500-kDa fluorescein dextran (green) with red blood cells appearing within as dark shadows. The nuclei of all cells were labeled with Hoechst 33342 (cyan). A 3-kDa cascade blue dextran (blue) was given via a tail vein injection 24 h before imaging. During the intravital imaging session, a 3-kDa Texas red dextran (red) was given along with the 500-kDa fluorescein dextran. Approximately 15 min after infusion, small red vesicles containing the Texas red dextran were seen localized just below the apical membrane in early endosomes within PT cells. The cascade blue dextran given 24 h prior is shown localized near the basal membrane in lysosomes. In *B*, the 2 dextrans demarcating the early endosomes and lysosomes are shown after background subtraction in preparation for quantification. These 8-bit masks clearly show almost complete segregation of the early endosomes (red) and lysosomes (cyan), with some colocalization of the 2 endocytic pools appearing in white. In *C*, values for total integrated fluorescence (Integr. Fluor.) within each compartment and an average total integrated fluorescence for the amount colocalized are delineated by the dark blue line. The number of objects for each compartment is show in the histogram with the respective colors. All values were generated using Metamorph. Bar = 20 μm.

rescence, as seen in Fig. 2*C*. Therefore, comparing dye concentrations in early proximal tubules to distal tubules allows one to measure the concentrating ability of the kidney at different sites within the nephron (13). A movie of this image sequence can be seen at http://filtration-movie2.

Observation and quantification of endocytosis occurring across the apical membrane domain of proximal tubule cells are now possible intravitally using two-photon microscopy. We have previously shown that utilizing a fluorescent derivative of folic acid, the apical uptake and transcytosis of folate could be visualized in proximal tubular cells with this method. In Fig. 3*A*, we have utilized the uptake of two differentially labeled dextrans given at different time points to differentiate the intracellular locations of recently and previously endocytosed material. Specifically, the uptake of 3-kDa cascade blue dextran (blue) given 24 h before the study and a 3-kDa Texas red-labeled dextran (red) given 15 min before image acquisition. Hoechst was given to label the nuclei (cyan), and a 500-kDa fluorescein-labeled dextran (green) was also given intravascularly to label the microvasculature. A close-up of a proximal tubule reveals rapid uptake of the rhodamine dextran, appearing within the apical domain as red vesicles, with the previously endocytosed uptake of the cascade blue dextran visualized at the basal region within proximal tubular cells.

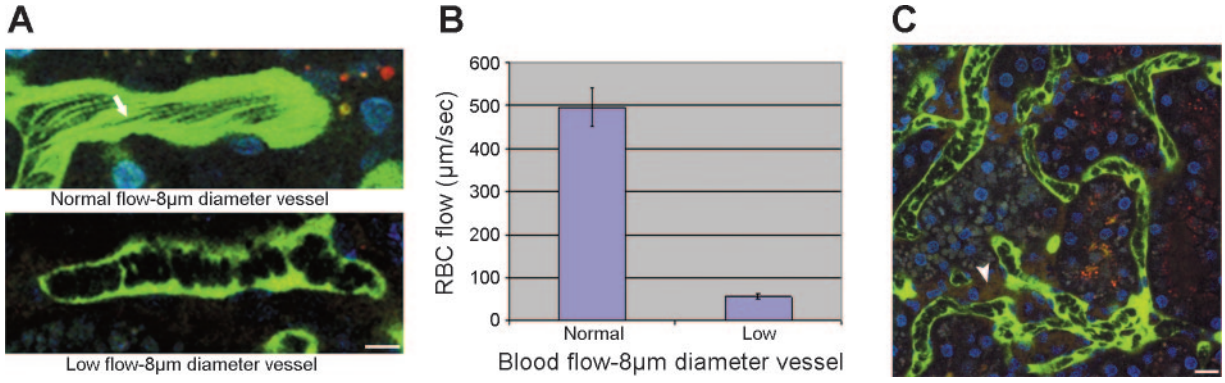

Fig. 4. Renal blood flow measured in Sprague-Dawley rats using a variant of the linescan method on preexisting images. In all panels, rats were given Hoechst 33342 to label all the nuclei (cyan), a 500-kDa fluorescein dextran was given to label the vasculature (green) and show red blood cell (RBC) flow, and a 3-kDa Texas red dextran (red) was used to discern rates of endocytosis in proximal tubule cells. In *A*, the *top* vessel shows a more normal flow rate, as delineated by the shallow angles of the RBC traversing the vessel (arrow). The flow rate for the *bottom* vessel is much slower, as can be discerned by the steeper angles of the RBC. This visual phenomenon is a function of the time an RBC dwells in the same space as the 512 horizontal lines that comprise the static image that are collected at 2-ms intervals. In *B*, a histogram of the average RBC flow rate for the images in *A* is shown. Values are means ± SE expressed as μm/s. In *C*, an area demonstrating injury after a suprarenal parietal aortic clamp with microvascular damage is shown. In this photomicrograph, leakage and accumulation of both small- and large-molecular-mass dextrans into the interstitial space (arrowhead) are seen. Note the lack of uptake of the 3-kDa Texas red dextran by the PT cells, indicating reduced endocytic activity in these cells. Also, one can clearly see that RBC morphology closely matches that of the *bottom* vessel in *A*, indicating reduced blood flow. Bars = 10 (*A*) and 20 μm (*C*).

Background subtraction of Fig. 3*A* reveals the individual red and blue intracellular vesicles, with white areas corresponding to an overlap between the two probes given 24 h apart. Quantitative analysis using Metamorph software yields total integrated fluorescence, shown as a line in Fig. 3*C* for early endosomes, lysosomes, or those colocalized, respectively. The number of objects, shown as a histogram, allows the investigator to compare uptake of the two probes and the overlap between early endosomes and lysosomes. Although not shown, this could be done at different time points, allowing one to quantify uptake and movement into colocalized vesicles.

Two-photon microscopy can also be utilized to quantify microvascular flow rates within tissues. As indicated previously, infusion of a nonfilterable intravenous fluorescent dye results in intravascular cells appearing as dark objects. As is shown in Fig. 4*A*, under conditions of normal flow as the laser scans across a field of moving red blood cells, the cells appear as thin elongated objects, with the slope correlating to flow rate; the more shallow the slope, the faster the flow rate. Previous investigators have used line-scanning techniques (16) to accurately quantify flow based on this characteristic of the laser-scanning pattern inherent in many confocal microscope systems. In vessels with very low flow rates, red blood cells appear more normal as concave discs (Fig. 4*A*), having much steeper slopes. Quantification of flow rates for these two vessels is shown as a graph in Fig. 4*B*. For these studies, it is important to quantify red cell flow patterns in vessels of similar diameter, as increasing diameter results in increasing flow under physiological conditions. This technique, a derivative of the linescan method, allows for the quantification of flow rates from previously acquired images. Additionally, trigonometry can be used to correct for vessels not running perfectly horizontally, although we have found with vessels at angles of 30° or less, the correction factor is marginal (data not shown).

Endothelial cell dysfunction within the microvasculature can also be observed and quantified using the infusion of variously sized, differently colored dextrans or proteins. Subsequent movement out of the microvasculature and accumulation within the interstitial compartment are readily observed during injury or disease. As previously published (13), the movement of small and large dyes can be quantified with a ratio of the two dyes being used as an indicator of the relative permeability defect across the endothelium. This allows for further quantification of the relationship between the endothelial permeability defect and alterations in flow rates. These studies are not possible in fixed tissues as the interstitial dyes are rapidly washed away during the fixation process. Finally, using intravital techniques, one can correlate the blood flow in a given area with corresponding, adjacent permeability defects.

Many challenges remain to maximize one's ability to study and quantify dynamic renal processes using multiphoton microscopy. These areas include image-acquisition rates. The studies presented in this review were recorded at approximately 1 frame/s, and videos generated from these data are shown in 10:1 time compression (http://ajprenal.physiology.org/cgi/content/full/00473.2004/DC1). One can increase the acquisition rate by limiting the microscopic field of study, but this often sacrifices other important data. The depth of imaging possible, although four to five times greater than confocal imaging, remains limited to <200 μm for the kidney. Thus we are unable to visualize the corticomedullary area from the surface of the kidney. Perhaps, new external detectors and lens specifically designed for multiphoton microscopes will allow enhanced depth of penetration. Phototoxicity does remain a potential problem at the focal plane, and this must always be considered during study design. Quantifying the recorded results is also a major area under development. Continuing improvement in software and hardware has the ultimate goal of automation for data collection, processing, and analysis to eliminate any bias and improve efficiency. Finally, cost remains an obstacle for the individual principle investigator, with Core Imaging Facilities generally being required.

In summary, recent developments in intravital multiphoton studies within the kidney now allow investigators to utilize unique techniques and fluorescent probes, previously developed for cell culture studies, to visualize the functioning kidney and characterize cellular and subcellular events in a dynamic fashion. Incorporating intravital multiphoton microscopy with other established methodologies will offer investigators a novel, multifaceted approach to understanding physiological and disease processes with a greater ability to translate preclinical data into therapeutic advances.

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REFERENCES

1. **Bacallao RL, Yu W, Dunn KW, and Phillips CL.** Novel light microscopy imaging techniques in nephrology. *Curr Opin Nephrol Hypertens* 12: 455–461, 2003.
2. **Clendenon JL, Phillips CL, Sandoval RM, Fang S, and Dunn KW.** Voxx: a PC-based, near real-time volume rendering system for biological microscopy. *Am J Physiol Cell Physiol* 282: C213–C218, 2002.
3. **Dunn KW, Sandoval RM, Kelly KJ, Dagher PC, Tanner GA, Atkinson SJ, Bacallao RL, and Molitoris BA.** Functional studies of the kidney of living animals using multicolor two-photon microscopy. *Am J Physiol Cell Physiol* 283: C905–C916, 2002.
4. **Gerlich D and Ellenberg J.** 4D imaging to assay complex dynamics in live specimens. *Nat Cell Biol Suppl*: S14–S19, 2003.
5. **Lippincott-Schwartz J, Altan-Bonnet N, and Patterson GH.** Photobleaching and photoactivation: following protein dynamics in living cells. *Nat Cell Biol Suppl*: S7–S14, 2003.
6. **McCarthy E, Sandoval R, Reddy S, Dunn K, and Molitoris B.** Two-photon microscopy and albumin permeability (P_{alb}) assay confirm that increased glomerular protein permeability precedes proteinuria in puromycin aminonucleoside (PAN) nephrosis (Abstract). *J Am Soc Nephrol* 15: 490A, 2004.
7. **Miyawaki A, Sawano A, and Kogure T.** Lighting up cells: labelling proteins with fluorophores. *Nat Cell Biol Suppl*: S1–S7, 2003.
8. **Molitoris B, Weinberg J, Venkatachalam M, Zager R, Nath K, and Goligorsky M.** Acute renal failure. II. Experimental models of acute renal failure: imperfect but indispensable. *Am J Physiol Renal Physiol* 278: F1–F12, 2000.
9. **Phillips CL, Miller KJ, Filson AJ, Nurnberger J, Clendenon JL, Cook GW, Dunn KW, Overbeek PA, Gattone VH II, and Bacallao RL.** Renal cysts of inv/inv mice resemble early infantile nephronophthisis. *J Am Soc Nephrol* 15: 1744–1755, 2004.
10. **Sandoval RM, Kennedy MD, Low PS, and Molitoris BA.** Uptake and trafficking of fluorescent conjugates of folic acid in intact kidney determined using intravital two-photon microscopy. *Am J Physiol Cell Physiol* 287: C517–C526, 2004.

11. **Stephens DJ and Allan VJ.** Light microscopy techniques for live cell imaging. *Science* 300: 82–86, 2003.
12. **Swedlow JR, Goldberg I, Brauner E, and Sorger PK.** Informatics and quantitative analysis in biological imaging. *Science* 300: 100–102, 2003.
13. **Tanner GA, Sandoval RM, and Dunn KW.** Two-photon in vivo microscopy of sulfonefluorescein secretion in normal and cystic rat kidneys. *Am J Physiol Renal Physiol* 286: F152–F160, 2004.
14. **Weijer CJ.** Visualizing signals moving in cells. *Science* 300: 96–100, 2003.
15. **Yu W, Sandoval R, and Molitoris B.** A new concept of measuring glomerular permeability and molecular filtration rate in vivo with a fluorescence microscope (Abstract). *J Am Soc Nephrol* 15: 211A, 2004.
16. **Zipfel WR, Williams RM, and Webb WW.** Nonlinear magic: multiphoton microscopy in the biosciences. *Nat Biotechnol* 21: 1369–1377, 2003.