The first decade of using multiphoton microscopy for high-power kidney imaging

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Peti-Peterdi J, Burford JL, Hackl MJ. The first decade of using multiphoton microscopy for high-power kidney imaging. Am J Physiol Renal Physiol 302: F227–F233, 2012. First published October 26, 2011; doi:10.1152/ajprenal.00561.2011.—In this review, we highlight the major scientific breakthroughs in kidney research achieved using multiphoton microscopy (MPM) and summarize the milestones in the technological development of kidney MPM during the past 10 years. Since more and more renal laboratories invest in MPM worldwide, we discuss future directions and provide practical, useful tips and examples for the application of this still-emerging optical sectioning technology. Advantages of using MPM in various kidney preparations that range from freshly dissected individual glomeruli or the whole kidney in vitro to MPM of the intact mouse and rat kidney in vivo are reviewed. Potential combinations of MPM with micromanipulation techniques including microperfusion and micropuncture are also included. However, we emphasize the most advanced and complex, quantitative in vivo imaging applications as the ultimate use of MPM since the true mandate of this technology is to look inside intact organs in live animals and humans.

intravital imaging; juxtaglomerular apparatus; podocyte; two-photon microscopy

IT HAS BEEN MORE THAN 10 YEARS since two- or three-photon excitation, collectively called multiphoton microscopy (MPM), was first applied to the study of the living kidney tissue by the Peti-Peterdi-Bell (37, 38) and Dunn-Molitoris groups (6, 42) that pioneered several applications of this powerful new imaging technology. Over the past decade, MPM has provided stunning images and real-time movies of the structure and function of the intact kidney in unparalleled spatial and temporal resolution. Because of the ability of this noninvasive imaging approach to directly visualize dynamic intrarenal processes in vivo and in near real-time without causing tissue damage, MPM has revolutionized renal physiology research and served as the perfect complement to more traditional biological and histological techniques. MPM also opened the door to studying otherwise inaccessible cell types and complex tissue structures like the glomerular podocyte (30, 39) and the juxtaglomerular apparatus (JGA) (37) in their intact environment. Applications of MPM helped to change several paradigms in renal (patho)physiology. Imaging data have been incorporated into a number of textbooks on kidney function (3, 8, 41), and MPM videos (30, 37, 39, 40) have been used as visual aid and teaching material in graduate and medical student classrooms worldwide.

MPM offers a state-of-the-art imaging technique superior for deep optical sectioning of living tissues. The higher resolution and minimal phototoxicity of this method permit longer time periods of continuous tissue scanning with uses in near real-time imaging of intact organs in vivo. MPM has applications far beyond the generation of superior images: dynamic intracellular processes as well as more complex physiological functions on the organ level can be visualized over time in a noninvasive way and quantified. In fact, the quantitative imaging approaches (17, 61, 64, 65) have been the most critical developments in the field recently, which allowed the continuous monitoring of basic renal (patho)physiological parameters in the intact rat and mouse kidney.

Practical details and experimental protocols of using MPM in a wide range of renal and biological specimens from in vitro to in vivo can be found elsewhere (15). Also, details of the principles and advantages of MPM over other fluorescence optical or nonoptical imaging modalities have been reviewed before (5, 7, 27, 29, 32, 40, 49, 66). This review aims to highlight the most important scientific discoveries in kidney research during the past 10 years that were made possible by the use of MPM. Milestones of the technological development and the various experimental approaches of kidney MPM are also summarized. Figure 1 illustrates the timeline of the development of MPM technology in general, including the first MPM applications in the renal field.

Imaging the Microperfused Glomerulus, Kidney Tissue Ex Vivo

The very first two-photon imaging project of the Peti-Peterdi-Bell laboratories was performed in 1998 in collaboration with the Okada group in Okazaki, Japan and addressed the role and regulation of macula densa (MD) cell volume changes in the function of the juxtaglomerular apparatus (JGA) (38). This initial project (37, 38) and several follow-up studies (21,
30, 32, 44, 50) used the freshly dissected and in vitro microperfused JGA preparation with double-perfused thick ascending limb-MD and afferent arteriole (AA)-glomerulus since MPM allowed for the first time z-sectioning and three-dimensional reconstruction of the entire living and functioning glomerulus (diameter ~100 μm) (Fig. 2A). These studies identified several additional details of important JGA functions, including the control of renin release and tubuloglomerular feedback (TGF), and helped to change several paradigms in the field. The direct visualization of renin content, dynamics of exocytosis, and enzymatic activity using acidotropic fluorophores [for example, quinacrine and LysoTracker dyes (17, 33, 34, 57) and fluorogenic renin substrates (33, 40, 43)] provided important new information on the activation mechanisms of the intrarenal renin-angiotensin system (RAS) (16, 34, 40, 49, 58). These included the identification of the collecting duct as a new renin-producing anatomic site within the kidney under pathophysiological conditions. For example, MPM studies identified the collecting duct as the major source of (pro)renin in diabetes (18, 43). Other imaging studies using the in vitro microperfused JGA-glomerulus visualized the changes in MD cell volume during TGF (21, 37), the calcium wave of TGF (30), function of the subpodocyte space (47) (SPS) (Fig. 2C), and bulk fluid flow in the JGA which may facilitate the diffusion of renin into the renal interstitium (44). Calcium fluorescence studies directly demonstrated the tubular fluid flow-sensing capability of MD cells, indicating that not only salt but changes in tubular fluid flow may also directly trigger TGF responses (50). MPM identified tubular epithelial cells at the perimeter of the MD (perimacular cells) that exhibited spontaneous oscillations in cytosolic calcium concentration ([Ca²⁺]), enhanced by tubular flow and luminal sodium chloride (20). These cells and their oscillatory [Ca²⁺] patterns may play a role in JGA signaling in addition to the classic MD-mediated TGF (20).

Electrotonic vascular signal propagation in freshly dissected and microperfused pregglomerular blood vessels was studied recently with MPM (25) using ANNINE-6, a newly synthesized voltage-sensitive dye that was originally designed for ultrafast (1 ms) neural signal detection. ANNINE-6 is highly membrane specific in contrast to other commercially available voltage-sensitive fluorophores and responds to a membrane-depolarizing voltage with a fluorescence decrease (22). TGF activation depolarized afferent arteriolar smooth muscle cells, which spread to the cortical radial artery and other afferent arterioles (25). The combination of this experimental approach with mathematical modeling may further help our understand-

ing of the regulation of renal vascular function, one of the classic topics in renal physiology research.

In addition to the freshly dissected glomerulus and JGA, other ex vivo tissue approaches used MPM imaging for renal functional studies. Hall et al. (11) used MPM in live rat kidney slices to study mitochondrial (dys)function in different nephron segments and found that mitochondrial membrane potential was higher and more effectively maintained in distal vs. proximal tubules. The observed axial differences in mitochondrial function along the nephron may be important in the pathophysiological mechanisms of renal injury (11). More recently, the same group established MPM of the rat isolated, perfused kidney to avoid certain technical problems associated with loading of fluorescent dyes (10). The technique allowed the imaging of tubular functions including the production of reactive oxygen species (ROS), uptake of dextrans and proteins, and the effects of ischemia-reperfusion injury (10).

**Quantitative Imaging of Kidney Functions In Vivo**

The adage “a picture is worth a thousand words” was the title of a recent American Society of Nephrology imaging
symposium, and it may sound particularly appropriate for the field of kidney MPM. In fact, multicolor images and videos of the living intact kidney provided enormous amounts of new information on renal function in the past decade. However, in recent years the state of the art in the field shifted to the saying “data are worth a thousand pictures,” which emphasizes the importance of quantitative imaging. Accordingly, the initial MPM observations of various cell and kidney functions, including glomerular filtration and tubular processing of substances (6, 7, 29, 54), and cell apoptosis and endocytosis (6), were later followed by quantitative, more complex approaches. These included the quantitative imaging (17) of the single-nephron glomerular filtration rate (SNGFR), red cell velocity in glomerular and peritubular capillaries for blood flow estimations, oscillations in SNGFR and tubular fluid flow, changes in renal concentration-dilution, and the highly debated measurement of the glomerular sieving coefficient of albumin (3, 31, 39, 45, 46, 52, 53). The Molitoris group established the ratiometric measurement of glomerular filtration function using various GFR and plasma markers (61, 64, 65). Their work ultimately resulted in the development of a novel, clinically useful diagnostic approach for the rapid quantification of GFR, for example, in acute kidney injury (60).

Improvements in technical, surgical, and imaging approaches in the past several years resulted in further developments in MPM. In contrast to other organs with optically clear parenchyma (e.g., the brain) in which deep (>1 mm) tissue imaging is possible, MPM of the renal cortex is limited to the initial 150–200 μm from the surface of the kidney. This is due to the high level of light scattering and absorption by the renal tissue, which are the result of its specific anatomic and functional features, such as the dense capillary network, high blood flow and local hematocrit, convoluted tubules, fibrous renal capsule, etc. Therefore, glomerular and JGA imaging (Fig. 2, B and D) has been mainly performed in Munich-Wistar rat strains which feature surface glomeruli (6, 17). Recently, MPM imaging of close-to-surface glomeruli in intact C57BL6 mouse kidneys has also become possible, and this technical advance allowed the visualization of podocytes and parietal cells of Bowman’s capsule in vivo in great detail (39). The combination of MPM with mouse genetics creates endless possibilities for future research.

**Imaging Cellular Parameters and Organelle Function**

MPM allows us to look inside kidney cells and visualize dynamic intracellular processes in vivo in their intact environment. The function of organelles such as endocytotic vesicles (6, 10, 29), lysosomes (33), nuclei (6, 15, 32), secretory granules (18, 33, 40, 43, 57, 58), mitochondria (11), and cell membranes (21, 25, 32, 38, 51) can be directly observed and quantified. The fate of locally delivered adenovirus-cDNA constructs (55) or the mainly proximal tubular uptake of systemically injected siRNA (28, 40) can be followed by time-lapse MPM imaging.

The acidotropic dyes (for example, quinacrine, LysoTracker dyes) that are weak bases and can penetrate through cell membranes and accumulate in acidic organelles have been used to visualize renin-containing secretory granules in both the JGA and collecting duct (16–18, 32, 33, 40, 43, 51, 57, 58). Although these dyes label all acidic organelles (lysosomes and nuclei, too) and therefore are not specific for renin vesicles, the unusual large size (1–2 μm) and low pH of these vesicles make them extremely brightly fluorescent. With proper imaging settings (low detector offset), however, the faintly labeled other organelles can be excluded. In addition to renin vesicle cargo, the release and tissue activity of renin have been successfully visualized using fluorescent renin substrates (33, 40).

The acetoxyethyl ester (AM)-based intracellular delivery of fluorophores has been extremely popular and made it easy to study various cell functions in vitro. However, their use in vivo is limited due to the interference by extracellular esterases which cleave the AM into the free dye form before uptake by the target cell (14). Unlike systemic injection into the plasma, the local tissue delivery of high concentrations of AM dyes around the cells of interest can actually achieve great results. For example, injection of the calcium-sensitive dye Rhod 2-AM under the renal capsule can result in sufficient dye uptake into proximal and distal tubule segments, which allows visualization of [Ca<sup>2+</sup>], (40). Another way to avoid plasma degradation of AM dyes and achieve even more specific local delivery is the combination of MPM with classic renal micropuncture techniques (55). Figure 3 demonstrates this possibility for the study of [Ca<sup>2+</sup>], or cell pH in nephron segments in vivo.

In these new experiments, Munich-Wistar-Fromter rats were anesthetized and surgically prepared for renal micropuncture and tubular microperfusion delivery of the calcium- or pH-sensitive fluorophores Rhod 2-AM or SNARF-1-AM (10 μM each), respectively. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Southern California. Intravital MPM was performed using a Leica TCS SP5 multiphoton confocal fluorescence imaging system powered by a Chameleon Ultra-II MP laser at 860 nm (Coherent) and a DMI 6000 inverted microscope’s external nondes canned detectors with a TRITC filter (Leica Microsystems) and previously published techniques (17, 36, 55). Briefly, anesthetized Munich-Wistar-Fromter rats (120 mg/bdy wt kg, Inactin ip) were placed on a homeothermic operating table (Vestavia Scientific), and whole body temperature was kept at ~37°C. A ~22-mm dorsal incision on the left
subcostal flank and two horizontal midline incisions were made for placement of a kidney cup. The left kidney was gently exteriorized, and the renal pedicle and kidney were cleanly dissected of debris and fat. A 22-mm kidney cup (Vestavia Scientific) was placed within the retracted peritoneal cavity opening, and the left kidney was placed within the kidney cup and continuously bathed with warm 0.9% saline. Glass capillary tubes (ID 0.084 × 0.064 in., Drummond Scientific) were pulled with a PP-830 pipette puller (Narishige), the tip ground to an OD of ~2 μm, filled with the fluorophores (Invitrogen), and put on a micromanipulator (Leitz). Tubule segments were selected using a Stemi 2000 stereomicroscope (Zeiss, ×250 magnification), micropunctured with the glass micropipette, and microperfused with the fluorescein dyes at a rate of 5–10 nl/min for 15–20 min. Figure 3 demonstrates that the combination of MPM and micropuncture delivery of fluorophores can be used for direct visual monitoring of intracellular parameters and signaling pathways in different nephron segments in vivo. To avoid potential artifacts that may result from technical problems (such as fluorophore loading, bleaching, secretion, cell volume changes, etc.), ratiometric imaging is recommended similar to the use of a fluo 4 and fura red dye pair in cells of the glomerulus and JGA (30).

For the detection of freshly released chemical mediators (e.g., ATP, PGE2) in the local tissue environment in situ that are hardly detectable using other conventional methods, the use of an imaging-based biosensor approach can be a good alternative. Biosensor cells loaded with calcium fluorophores and overexpressing a calcium-coupled receptor of the ligand which needs to be detected can amplify the endogenous signal of the mediator. After calibration, the ligand’s concentration in the medium can be quantified (2, 35, 51, 56).

**Imaging Renal Pathology**

MPM imaging in different animal models helped to further our understanding of the mechanisms of several kidney diseases including acute kidney injury (1, 28, 61), ischemia-reperfusion (10, 13, 28), diabetic kidney disease (17, 18, 48, 58), glomerulosclerosis (39), renal vascular defects (62), kidney transplantation (13), and urinary bacterial infection (26). The pathogenic roles of the phenotypic transition of peritubular capillary endothelium combined with an impaired regenerative capacity in ischemia-reperfusion were recently visualized with MPM using a YFP reporter transgenic mouse model (1). Also, MPM provided direct visual evidence for the protective effect of temporary inhibition of the proapoptotic p53 signaling in both ischemic- and cisplatin-induced acute kidney injury (13, 28). Administration of small interfering RNA for p53 resulted in the conservation of renal function and amelioration of structural and functional damage to proximal tubular epithelial cells (13, 28). Cell apoptosis, necrosis, increased vascular permeability, leukocyte adhesion, and rouleaux-forming red blood cells under these conditions have been demonstrated earlier (6, 9, 29). Treatment with activated protein C improved LPS-induced acute renal failure, endothelial dysfunction, and vascular inflammation (9). Glomerular and peritubular vascular defects and increased capillary permeability were observed in mice deficient of Crim1, a cell-surface transmembrane protein that binds a variety of growth factors, including VEGF (62).

Functional changes in the early phase of diabetes have been visualized in the rat kidney using MPM (48) or conventional confocal fluorescence microscopy (24). Glomerular hyperfiltration, altered glomerular hemodynamics (increased ratio of efferent to efferent arteriole diameter), glomerular hypertrophy, and increased glomerular permeability to macromolecules have been confirmed (24, 48). The beneficial effects of RAS blockade were also demonstrated (24, 48). Other morphological and functional changes in the glomerulus (for example, in glomerulosclerosis) can be visualized using MPM (see below).

**Imaging Podocytes and the Glomerular Filtration Barrier**

Similar to cells of the JGA, the podocyte is another good example of inaccessible renal cell types that have been studied only in cultured cell systems in vitro and in fixed static tissue samples. However, their relevance in vivo is not fully clear. For many years, our understanding of important glomerular disease processes and their mechanisms, for example, albuminuria and glomerulosclerosis, has been limited due to the technical difficulty in studying the glomerular filtration barrier (GFB) in its native environment. Thanks to MPM, it is now possible to study the dynamic three-dimensional structure of the glomerulus and the cellular complexity of the GFB in vivo.

![Fig. 4. MPM imaging of glomerular pathology in vivo. In a puromycin aminonucleoside (PAN)-induced model of focal segmental glomerulosclerosis (FSGS) in Munich-Wistar-Fromter rats, the intravascular space (plasma) marker Alexa 594-albumin (red) was given in a bolus, and Lucifer yellow was infused continuously into the carotid artery to label the primary filtrate in Bowman’s space (yellowish green). Numerous large cysts in dark, unlabeled podocytes (asterisk) are visible after PAN treatment (A). The nuclear stain Hoechst 33342 was given (iv) to help the identification of various cells (B). Examination of the linear profile of Alexa 594-labeled albumin fluorescence intensity in Bowman’s space around glomerular capillaries (indicated by white line in B) suggests that the increased albumin permeability of the glomerular filtration barrier in this model is restricted to focal areas (C). Scale bar = 20 μm.](http://ajprenal.physiology.org/)

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Glomerular endothelial and mesangial cells and podocytes in the intact living rat and mouse kidney can be visualized in great detail (39).

Time-lapse MPM imaging recently provided new details and important in vivo confirmation of the dynamics of podocyte movement and migration (39). Using the puromycin amino-nucleoside (PAN)-induced model of focal segmental glomerulosclerosis (FSGS), cystic podocyte damage (Fig. 4A), podocyte detachment (shedding), and replacement were observed (39). Advanced MPM determinations of the glomerular sieving coefficient of albumin found very low levels (31, 39, 52, 53) in contrast to earlier work (45, 46). GFB permeability to macromolecules was found to be restricted only to areas of podocyte damage (Fig. 4, B and C), consistent with the generally accepted role of podocytes and the glomerular origin of albuminuria (39).

Time-lapse MPM imaging of both the in vitro microperfused glomerulus (Fig. 2C) and the Munich-Wistar-Fromter rat kidney in vivo confirmed the function of the newly discovered SPS, a lymphine fluid space between the underside of the podocyte cell body and the foot processes (47). These studies demonstrated that the SPS constitutes a highly significant restriction to flow, consistent with the view that the SPS is a new layer of the GFB (47). The small connectors between the SPS and Bowman’s space, so-called exit pores, may have important functional implications in physiological (filter backwash, podocyte-to-endothelium cross talk) as well as pathological processes (glomerulosclerosis) and will be further studied in future work.

Future Directions

During the past decade, the progression of MPM technology has been enormous. This is well exemplified by the quick comparison of the commercial systems that our laboratory used in 1998–2002 (37), a Bio-Rad MRC1024MP using a Tsunami laser with maximum 350-nmW power output and high 800-nm excitation wavelength, to the currently used Leica TCS SP5 (Leica Microsystems, Heidelberg, Germany), which uses a >4-W Ultra-II Chameleon laser (Coherent) with tunable excitation between 680 and 1,080 nm. Even newer versions of this system are available with a fully integrated optical parametric oscillator, which extends the application range of MPM (excitation at 1,300 nm and beyond). Continued development of MPM technology, new infrared dyes (long wavelength penetrates deeper due to less absorption and scattering), ultra-sensitive hybrid detectors, and microscope objectives designed specifically for MPM may soon allow imaging deeper in the living, intact renal cortex and perhaps in the renal medulla. In addition to its research use in animals, MPM continues to be applied in newer and newer areas of human diagnostics, such as for the evaluation of skin diseases (19) and in endoscopy (63). Thus MPM offers unmatched versatility for biological and medical imaging studies of the living organism.

Future MPM imaging studies will take advantage of the available transgenic mouse models, for example, ones with cell-specific expression of various fluorescent proteins. Figure 5 demonstrates one of the possibilities for tracking the function and fate of specific cell types within the living kidney. The expression of enhanced green fluorescent protein driven by the Tie-2 promoter in mice labels the endothelial cell lineage (the mice are available from the Jackson Laboratory but were a generous gift from Dr. Young Kwon Hong at the University of Southern California). Endothelial dysfunction and proliferation within the kidney can be studied and directly visualized using MPM in various pathological states as reported recently (1). The model is readily applicable to study other renal cell types.

The powerful MP lasers that are normally used for fluorescence excitation of a probe within the sample can also have unconventional uses. Figure 6 demonstrates one such possibility: the use of the MP laser as a micromanipulator. By briefly focusing the multiphoton excitation laser beam on small anatomic structures, for example, on the wall of a glomerular capillary, the local tissue damage (nanosurgery) can cause disruption of the capillary wall and consequently single-nephron hematuria. This may help the study of the effects of blood factors on nephron function, or the streaming red blood cells can help to estimate tubular fluid flow rate, etc. The use of this tool on the luminal surface of the glomerular endothelium can artificially form a capillary thrombus that may partially and temporarily occlude a glomerular capillary loop.

Fig. 5. MPM imaging of cell lineage in vivo in transgenic mouse models. In Tie-2 GFP mice, which express the enhanced GFP in the vascular endothelium, the peritubular capillaries in the renal cortex are visualized in green. The intravascular space (plasma) was labeled with Alexa 594-albumin (red). Scale bar = 20 μm.

Fig. 6. Example for the use of the MP laser as a micromanipulator tool. In a Munich-Wistar-Fromter rat, the intravascular space (plasma) was labeled with Alexa 594-albumin (red). After a control image (A) was taken, a high-power laser beam was focused on one small part of a glomerular capillary (labeled X), which caused disruption of the capillary wall and single-nephron hematuria (B). The plasma and the unlabeled red blood cells are seen entering Bowman’s space and PT segments. The distal convoluted tubule (DCT) was labeled green by a previous Lucifer yellow bolus injection into the carotid artery.
approach may aid the more accurate determination of the local fluorescence intensity of a plasma marker, as was recently demonstrated for the measurement of the glomerular sieving coefficient of macromolecules (39).

We are in the age of superresolution fluorescence microscopy, also called confocal nanoscopy or nanoimaging. Imaging subcellular components at or beyond the diffraction limit (<250 nm in size) became possible by using stimulated emission depletion, photoactivated localization microscopy, or stochastic optical reconstruction microscopy (59). These and other emerging superresolution modalities (23) continue to push the limits of optical imaging technologies, and their use in kidney research will undoubtedly result in new discoveries in the future.

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Author contributions: J.-P.-P. provided the conception and design of research; J.-P.-P., J.L.B., and M.J.H. performed experiments; J.-P.-P., J.L.B., and M.J.H. analyzed data; J.-P.-P. interpreted results of experiments; J.-P.-P. prepared figures; J.-P.-P. drafted manuscript; J.-P.-P., J.L.B., and M.J.H. edited and revised manuscript; J.-P.-P., J.L.B., and M.J.H. approved final version of manuscript.

REFERENCES