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
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 pathological 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 ([Ca^{2+}]i), enhanced by tubular flow and luminal sodium chloride (20). These cells and their oscillatory [Ca^{2+}]i 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 esti-
mations, oscillations in SNGFR and tubular fluid flow, changes
in renal concentration-dilution, and the highly debated mea-
surement of the glomerular sieving coefficient of albumin (3,
31, 39, 45, 46, 52, 53). The Molitoris group established the
ratimetric 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 ap-
proaches in the past several years resulted in further develop-
ments 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 func-
tional 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 combina-
tion 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 environ-
ment. The function of organelles such as endocytic 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 acridocyanin 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 [Ca2+] (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 mi-
cropuncture techniques (55). Figure 3 demonstrates this possi-
ability for the study of [Ca2+] 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
nondescanned detectors with a TRITC filter (Leica Microsys-
tems) and previously published techniques (17, 36, 55).
Briefly, anesthetized Munich-Wistar-Fromter rats (120 mg/
body wt kg, Inactin ip) were placed on a homeothermic
operating table (Vestavia Scientific), and whole body tem-
perature was kept at ~37°C. A ~22-mm dorsal incision on the left

Fig. 3, MPM of cytosolic parameters in the kidney in vivo. Micropuncture
delivery of Rhod 2-AM (A) or SNARF-1-AM (B) directly into the tubular fluid
in different nephron segments in Munich-Wistar-Fromter rats allowed direct
visualization of intracellular calcium (A) or pH (B; both are red), respectively.
Plasma was labeled green using 500-kDa dextran-FITC. A: 2 adjacent
nephron’s connecting tubule segments (CNT) merge into a common cortical
collecting duct (CCD). B: intense SNARF-1 fluorescence is observed in select
bulging epithelial cells of the CCD (arrows, most likely type A intercalated
cells). PT, proximal tubule. Scale bar = 20 μm.
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 Steini 2000 stereomicroscope (Zeiss, ×250 magnification), micropunctured with the glass micropipette, and microperfused with the fluorescent 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 afferent 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.
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 labyrinthine 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-mW 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. This
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, phototivated 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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