



Intravital imaging in the kidney

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Purpose of review

The review aims to provide a brief summary and evaluation of the current state of research that uses multiphoton fluorescence microscopy for intravital kidney imaging.

Recent findings

Direct visualization of the glomerular filter, proximal and distal tubule segments, and the renal vasculature in the living, intact kidney in zebrafish, mouse, and rat models with high temporal and spatial resolution provided new insights into the function of the normal and diseased kidney. New technical developments in fluorescence excitation and detection, in combination with transgenic animal models for cell function and fate mapping, and serial imaging of the same glomerulus in the same animal over several days further advanced the field of nephrology research, and the understanding of disease mechanisms.

Summary

Intravital multiphoton imaging has solved many critical technical barriers in kidney research and allowed the dynamic portrayal of the structure and function of various renal cell types *in vivo*. It has become a widely used research technique, with significant past achievements, and tremendous potential for future development and applications for the study and better understanding of kidney diseases.

Keywords

calcium, cell fate, glomerular filtration barrier, multiphoton microscopy, podocyte

INTRODUCTION

The last few years have seen the continuation of the trend of intravital imaging of the kidney using multiphoton microscopy (MPM) becoming more and more appreciated and used by a growing number of nephrology research laboratories worldwide. Owing to its ability to directly visualize dynamic changes in tissue morphology and function with subcellular resolution, and over time, intravital MPM imaging remained a preferred experimental technique for investigators to study renal physiology and disease. Over the past 15 years, several nephrology laboratories have applied this high power imaging technique and developed various modalities of MPM to quantitatively visualize renal glomerular, tubular, and vascular structures and their functions. These include single nephron glomerular filtration rate, blood flow, tubular flow, the concentrating and diluting mechanism, renin granular content, release, and tissue renin activity, cell and mitochondrial metabolism, cell migration and fate, intracellular processes, and parameters such as endocytosis, pH, calcium, and many others that can be directly and quantitatively visualized in the intact living kidney with high temporal, spatial resolution in noninvasive optical tissue sections

[1,2,3[■],4–7,8[■],9–11]. These are significant and unique capabilities of this technology, and, currently, no other experimental methods provide this amazing morphological and functional detail of the mouse and rat kidney *in vivo*. The basic principles, advantages, and examples of using multiphoton excitation fluorescence microscopy have been reviewed elsewhere [3[■],5–7,8[■],10,12,13]. Here, we present a brief summary of the most recent intravital MPM imaging studies of the kidney, emphasizing the most important aspects and future directions of technical developments and research applications, and discussing the scientific breakthroughs that were made possible by the use of intravital MPM.

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KEY POINTS

- Intravital imaging using MPM has become a widely used experimental technique in nephrology research.
- Owing to its capabilities and versatility, MPM remains the first choice high power imaging method for studying the intact living kidney.
- Direct visual clues on changes in cell and tissue morphology and function observed by MPM imaging *in vivo* have provided important insights into kidney disease mechanisms.

Multiphoton microscopy imaging of the glomerulus

The main focus of recent *in-vivo* glomerular imaging studies has been the glomerular filtration barrier (GFB), and the podocyte in particular. This is well justified by their importance in the development of glomerular and renal diseases, delicate three-dimensional structure, and inaccessibility by other *in-vivo* research techniques. One important technical advance has been the application of MPM for imaging glomeruli in the intact living mouse kidney [9,14], which overcame the technical limitation and necessity to study the Munich-Wistar-Fröter rat model that features superficial glomeruli [2,4]. Although the structure and function of the rat glomerulus resemble the human condition, which is advantageous for translational studies, the availability of an arsenal of mouse genetic tools has made the mouse MPM model better suited for mechanistic studies. The accessibility of multiple mouse glomeruli for MPM imaging studies is demonstrated in Fig. 1 a, and has also been confirmed by several laboratories [15–17,18[•],19,20]. Furthermore, significant improvements in imaging technology, mainly in fluorescence detection (availability of high sensitivity gallium arsenide phosphide and hybrid detectors) now allow routine MPM imaging of glomeruli in adult male mice, and not only in adolescent female mice as before. The application of long wavelength multiphoton excitation (up to 1300 nm) can further improve depth penetration and the routine *in-vivo* MPM imaging of deep rather than superficial glomeruli [21^{••}].

The initial studies with high power MPM imaging of the GFB used highly fluorescent molecular tracers of different sizes to label the filtrate, measure GFB permeability, and to visualize the podocytes in negative [2,9,19,22–26,27[•]]. This approach also helped to shed light on new microanatomical details of the GFB, the presence and function of the subpodocyte space [24], its role in puromycin-

induced focal segmental glomerulosclerosis [7,9], and angiotensin II-induced albumin vesicle transcytosis in podocytes [28^{••}]. In addition, unidentified migrating cells were seen in both in the parietal [29^{••}] and visceral layers of the Bowman's capsule [9], which may play a role in the cellular remodeling of the GFB.

Positive identification of cells of the glomerulus and the GFB using genetically expressed fluorescent lineage tags (genetic cell fate mapping) in combination with intravital MPM imaging was another major technical advance [30^{••},31^{••}]. This approach was able to unequivocally identify and visualize parietal epithelial cells and podocytes expressing green fluorescent protein (GFP) [31^{••},32[•]]. Further, the unique identification and tracking of the fate of single podocytes was made possible by the expression of multicolor fluorescent reporters, for example the Confetti construct of cyan, green, yellow, and red fluorescent protein (CFP/GFP/YFP/RFP) as shown in Fig. 1b [31^{••}]. Serial MPM imaging of the exact same glomerulus in the same kidney over several days shed light on the highly dynamic glomerular environment, the robust cellular remodeling of the glomerulus by migrating parietal epithelial cells and podocytes in disease conditions, including adriamycin nephropathy (which resembles human focal segmental glomerulosclerosis) and obstructive nephropathy. Other MPM studies used the genetically encoded calcium indicator GCaMP3 (a fusion protein containing the calmodulin-binding domain from the myosin light chain kinase also called M13 peptide, the circularly permuted green fluorescent protein, and the calmodulin) expressed in podocytes, and established the role of purinergic calcium signaling via purinergic receptor type Y2 receptors in primary and secondary (propagating) podocyte injury, cell clustering, and migration (Fig. 1c) [30^{••}]. Future studies with this serial MPM approach to study the cellular remodeling of the same glomerulus over several days will be essential to identify the renal progenitor cell types and their mechanisms involved in glomerular repair and regeneration.

Multiphoton microscopy imaging of renal tubules

Owing to the easily accessible S1–2 segments of the proximal tubule, which are close to the kidney surface, several research groups have used MPM imaging to visualize dynamic cellular processes *in vivo* in proximal tubule cells. Recent studies focused on proximal tubule albumin uptake, processing, transcytosis of filtered albumin, and elements of a reclamation pathway that minimizes urinary loss

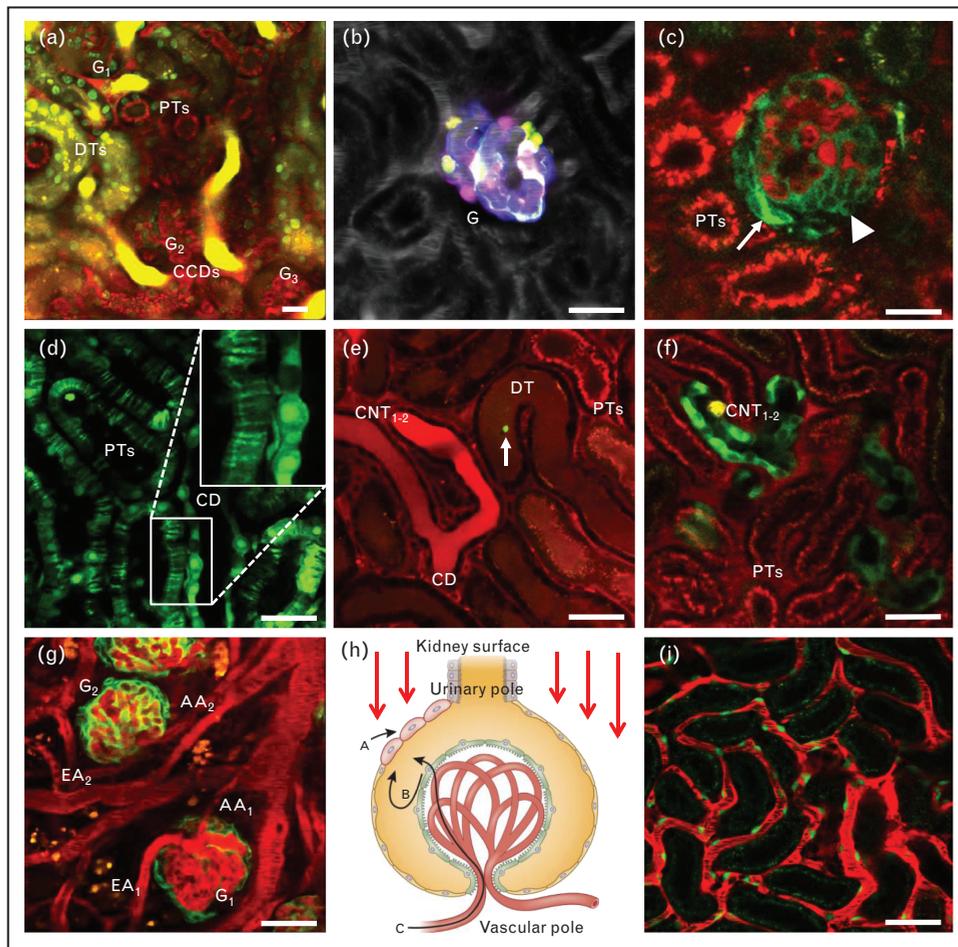


FIGURE 1. Intravital multiphoton microscopy (MPM) images of glomeruli (a–c), tubules (d–f), and vasculature (g–i) in the intact C57BL/6 mouse kidney. In all animals and images, plasma was labeled by Alexa594-albumin (red) given in i.v. bolus. (a), Deep optical section of three adjacent glomeruli (G_{1-3}), round-shaped PTs, elongated DTs with intensely nucleic acid stain (Hoechst33342)-labeled cell nuclei (green), and cortical collecting ducts (CCDs) containing highly concentrated Lucifer Yellow, a freely filtered dye injected i.v. in bolus. (b), Multicolor labeling of podocytes in the transgenic Podocin-Confetti mouse model, either by genetically encoded membrane-targeted cyan fluorescent protein (CFP), nuclear green fluorescent protein (GFP), cytosolic yellow fluorescent protein (YFP) or cytosolic red fluorescent protein (RFP). Plasma label is shown in greyscale. (c), Spontaneous glomerulosclerosis in a Podocin-GCaMP3 mouse kidney, with high calcium (intense green) in a subset of podocytes that cluster (arrowhead) or invade the parietal Bowman's capsule (arrow). Albumin uptake is visible in proximal tubule segments (PTs, red). (d), 2',7'-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-loaded PTs and a CD featuring a striated pattern in PTs, and diffuse homogenous labeling of whole cells in the CD (magnified area shown in inset) in Sprague-Dawley rat kidney. (e), Podocyte injury-induced leakage of plasma albumin (red) into the renal tubular system in a Podocin-GFP mouse. High albumin uptake in PT segments, and the transit of high amounts of albumin are visible downstream the nephron in DT, and connecting tubules (CNT_{1-2}) merging into CDs. A detached podocyte (green, arrow) is seen flowing downstream in the DT lumen. (f), MPM imaging of cytosolic calcium changes in tubular epithelial cells *in vivo* in kidney-specific cadherin-GCaMP3 mice. The genetically encoded calcium indicator GCaMP3 (green) is seen highly expressed in two adjacent connecting tubules (CNT_{1-2}) and in some cells of PTs. (g), Studying renal cortical vascular responses in Podocin-GFP mice after unilateral ureter obstruction. Several glomeruli and their adjacent afferent (AA) and efferent arterioles (EA) are clearly visible. (h), Illustration of the model of glomerular imaging and cell fate tracking by the combination of genetic lineage tracing and serial MPM. Red arrows indicate the direction of fluorescence excitation light coming from the microscope objective at the kidney surface. Visual clues on the migration pattern and dynamics of the same genetically labeled single stem/progenitor cells and their progenies [e.g., unique single cell identity by RFP (red) expression] can help to determine if a new cell (and its identical color progeny) that appeared on the parietal layer of Bowman's capsule originated from the renal interstitium (a), or the visceral podocyte layer (b), or from a circulating cell population (c). Modified with permission from [8]. (i), Peritubular capillaries in a receptor tyrosine kinase (Tie2)-GFP mouse kidney. Capillary endothelial cells are labeled green. Bars = 20 μm . CT, collecting duct; DT, distal tubules; PT, proximal tubules.

and catabolism of albumin, therefore prolonging its serum half-life [33]. Under different albumin loading conditions, investigators identified a complex molecular regulatory system that responded rapidly to different physiologic conditions to minimize alterations in serum albumin levels [34[■]]. Figure 1e demonstrates the use of MPM for tracking the renal tubular processing of filtered albumin.

In other studies, reduced proximal tubule tubular flow because of cell swelling and luminal obstruction was found in a model of septic acute kidney injury, which helped the better understanding of the mechanism of oliguria in the early phase of endotoxemia [35[■]]. Megalin-mediated endocytic uptake of filtered lipotoxic substances in a high-fat diet was shown to cause proximal tubule cell injury and retrograde glomerular dysfunction [36[■]]. In addition to imaging proximal tubule endocytosis, MPM has been very useful in studying alterations in mitochondrial structure and function, and the relevant aerobic and anaerobic metabolic pathways of both proximal and distal tubular epithelial cells in acute kidney injury [3[■],37]. These MPM imaging studies of many intracellular organelles were instrumental in uncovering several new proximal tubule mechanisms and their roles in a variety of kidney diseases.

New intravital MPM imaging approaches have been established to investigate cytosolic parameters of proximal tubule cells, including pH [38[■]] and calcium [39[■]]. MPM imaging of proximal tubule segments in the rat kidney loaded with the pH sensitive dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxy-fluorescein (BCECF) visualized the development of a high pH microdomain near the bottom of the brush border in response to an acute rise of blood pressure [38[■]], which may be a new important mechanism in pressure natriuresis (inhibition of proximal tubule sodium reabsorption). BCECF loading of proximal tubules also reflected on major differences in tubular epithelial cell microanatomy between different tubule segments. Figure 1d demonstrates the typical striated pattern of proximal tubules on thin confocal optical sections when loaded with a pH dye, which was due to the uneven loading (and pH) of adjacent proximal tubule cells interdigitating via their many lateral cell processes. In contrast, collecting duct cells with continuous cytoplasm space show diffuse homogenous labeling (Fig. 1d). These anatomical differences need to be considered when analyzing intracellular vesicle movements in proximal tubule cells.

MPM imaging of cytosolic calcium changes in tubular epithelial cells *in vivo* has been established using transgenic rats [39[■]] or mice expressing the genetically encoded calcium indicator GCaMP

proteins. Basal levels, and ligand and drug-induced alterations in cell calcium levels in proximal tubule epithelial cells were measured successfully in a rat model, which opens new possibilities for future physiologic and pharmacologic investigations [39[■]]. Figure 1f demonstrates the use of a Cre-lox-based mouse model for intravital calcium imaging of tubular epithelia, using kidney-specific cadherin-GCaMP3 mice [kidney-specific cadherin (Ksp)-DNA recombinase enzyme (Cre)/GCaMP3-fl mice, unpublished]. These GCaMP-based transgenic approaches have several advantages over earlier calcium imaging techniques that used classic fluorophores [40].

Vascular imaging

The afferent arteriole and the juxtaglomerular apparatus (JGA) are key anatomical structures that regulate renal hemodynamics and the rennin-angiotensin system. They are therefore important targets in a variety of nephrology studies. However, MPM imaging of the far side of the glomerulus, the vascular pole, and glomerular arterioles (Fig. 1g) has been technically challenging, because it requires the deepest optical sectioning through the highly light scattering glomerular capillaries (Fig. 1h). However, *in-vivo* imaging of the terminal, renin-positive part of the afferent arteriole has been successful in a few nephrons in both the rat and mouse kidney. Several MPM imaging studies helped to directly visualize and better understand the regulation of renin granular content in the JGA and collecting ducts in various conditions, including diabetes [4,10,14,41] and rennin-angiotensin system inhibitors [41,42], and the role of bulk ultrafiltration of plasma and fluid flow in the JGA [43]. Recent MPM studies of glomerular capillaries found increased blood flow, but constant shear stress in rats after nephrectomy because of increased capillary diameter and alterations in capillary blood rheology [44].

Perhaps the most widely used and characterized animal model in studying renal cortical blood flow, glomerular microcirculation, and vascular functions, is the unilateral ureteral obstruction model and the consequently developing hydronephrotic kidney, as established by Buhle *et al.* [45]. This model has been used extensively for the past 25 years to study the renal vascular effects of a number of antihypertensive drugs on the afferent and efferent arteriole. The combination of this classic experimental model with intravital MPM imaging provides a new dimension for future renal hemodynamics studies, including single cell and subcellular resolution of morphological, intracellular calcium, membrane voltage changes of vascular smooth

muscle cells [46], and the propagation of vascular signals along glomerular arterioles and nephron-to-nephron coupling [8^{***}]. Figure 1g demonstrates that glomerular hemodynamic parameters, vascular functions, including acute changes in glomerular, and afferent and efferent arteriole diameter can be directly visualized over time in this model. Commercially available mouse genetic tools (e.g., Cre-lox mouse models) will allow cell-type specific expression of fluorescent reporters (e.g., calcium-sensitive GCaMP proteins) in various vascular cell types, including smooth muscle, endothelial, and mesangial cells and podocytes [30^{***}], and their direct visualization with high temporal and spatial resolution using MPM imaging (Fig. 1g–i). Future studies using these visual experimental approaches will undoubtedly help to a better understanding of renal vascular (patho)physiology, and will be a tremendous aid in renal drug discovery, target validation, and testing. By tracking the same single, genetically labeled cell (by a unique color identifier) using serial MPM over several days, the origin, fate, and migration pattern of cells can be established (Fig. 1h).

Another hot topic in nephrology research, and an area where MPM imaging can be very useful in further development, is the role of vascular endothelium in kidney diseases. In the past, intravital imaging studies provided visual clues on the role of endothelial fenestrations of the terminal afferent arteriole (nanopores) [43], and the glomerular capillary endothelial surface layer (glycocalyx) in vascular dysfunction, and albumin leakage [23]. Also, the density and function of peritubular capillaries, which are easily accessible on the kidney surface for intravital MPM imaging, are critically important in the context of kidney disease. The role of peritubular capillary rarefaction, which causes kidney ischemia and accelerated nephron injury and interstitial fibrosis, is well established in the progression of chronic kidney disease. Rouleaux formation by red blood cells, leukocyte sticking and rolling in peritubular capillaries, and the leakiness of these blood vessels, have been visualized by intravital imaging studies in several experimental models of acute renal injury [1,47] which helped to evolve our understanding of the mechanisms of tubular epithelium-vascular endothelium crosstalk, inflammatory cell interactions, and the development of effective therapeutic strategies. Other MPM studies visualized renal erythropoietin producing cells that tightly associate with the capillary endothelium, and found that erythropoietin synthesis can be restored after renal injury by activating the hypoxia signaling pathway [48^{*}]. Figure 1i illustrates the high detail imaging of peritubular capillaries,

endothelial cells, and various blood cells that can be achieved by using intravital MPM.

CONCLUSION

Intravital MPM imaging of the rat and mouse kidney has become a routine nephrology research technique available in most basic biomedical research facilities and is now being used worldwide. It has provided tremendous contributions to our better understanding of the structure and functions of the normal and diseased kidney, and the many pathological mechanisms of kidney disease. Constant improvements in laser, optics, and microscopy technology, the development of new MPM imaging modalities, in combination with genetic animal models, will further advance its use for nephrology research and, perhaps, for clinical diagnostics.

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Conflicts of interest

There are no conflicts of interest.

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