Novel *in vivo* techniques to visualize kidney anatomy and function

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Intravital imaging using multiphoton microscopy (MPM) has become an increasingly popular and widely used experimental technique in kidney research over the past few years. MPM allows deep optical sectioning of the intact, living kidney tissue with submicron resolution, which is unparalleled among intravital imaging approaches. MPM has solved a long-standing critical technical barrier in renal research to study several complex and inaccessible cell types and anatomical structures *in vivo* in their native environment. Comprehensive and quantitative kidney structure and function MPM studies helped our better understanding of the cellular and molecular mechanisms of the healthy and diseased kidney. This review summarizes recent *in vivo* MPM studies with a focus on the glomerulus and the filtration barrier, although select, glomerulus-related renal vascular and tubular functions are also mentioned. The latest applications of serial MPM of the same glomerulus *in vivo*, in the intact kidney over several days, during the progression of glomerular disease are discussed. This visual approach, in combination with genetically encoded fluorescent markers of cell lineage, has helped track the fate and function (e.g., cell calcium changes) of single podocytes during the development of glomerular pathologies, and provided visual proof for the highly dynamic, rather than static, nature of the glomerular environment. Future intravital imaging applications have the promise to further push the limits of optical microscopy, and to advance our understanding of the mechanisms of kidney injury. Also, MPM will help to study new mechanisms of tissue repair and regeneration, a cutting-edge area of kidney research.

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For many decades, structure–function studies of the glomerulus and glomerular cells have been limited to the use of electron microscopy and classic histology techniques on fixed kidney tissues.[1] The first steps in the direction of studying glomerular cells live, for example, the motility and intracellular calcium ($[Ca^{2+}]_i$) changes of the critically important but anatomically complex podocyte, either in culture or in microdissected preparations *in situ*, were helped by the development of immortalized rodent/human podocyte cell lines[2] and confocal fluorescence imaging techniques.[3] Multiphoton microscopy (MPM, also called two-photon or three-photon excitation microscopy to distinguish it from conventional confocal fluorescence microscopy, which uses only one-photon excitation) became commercially available in 1996, which offered significant technical advantages for intravital imaging of intact organs. The most important MPM features include its deep tissue penetration capability owing to the use of pulsed infrared, low-energy excitation lasers, and minimized phototoxicity also owing to fluorescent excitation occurring only at the focal plane and so on. Altogether, MPM finally allowed investigators to perform deep optical sectioning of the intact, living kidney continuously, over longer time periods, without causing tissue injury. The detailed description of the technology, advantages, and early applications of MPM for kidney imaging studies can be found in previous reviews.[4–10] Figure 1 illustrates the timeline of the technical development of glomerulus and podocyte imaging, from the perspective of major milestones in fluorescence imaging in general. As shown in Figure 1, the first MPM applications using the freshly dissected and *in vitro* microperfused glomerulus[8,11,12] or the intact living kidney[6,13] were subsequently improved and shifted the focus to quantitative imaging. MPM studies were developed for the rapid measurement of the most basic, clinically relevant parameters of kidney function including glomerular filtration rate (GFR) using direct quantitative visualization of glomeruli and the volume of filtered plasma per unit time on the single-nephron level,[6] or indirectly by measuring plasma clearance kinetics of injected, fluorescent GFR markers.[14] The latter approach is currently under development for human clinical applications.[15] In addition, MPM techniques have been applied for the noninvasive measurement of the magnitude and temporal oscillations in single-nephron filtration rate,[6] changes in blood flow and

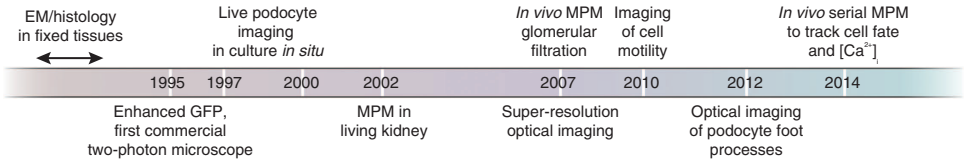

Figure 1 | Timeline of the technical advances in glomerular/podocyte imaging. After studying glomerular structure by electron microscopy (EM) and classic histology techniques on fixed kidney tissues, the first important milestone in 1997 was the functional (e.g., motility, calcium) imaging of the live podocyte in culture/*in situ* thanks to the development of immortalized mouse/human podocyte cell lines[2] and microdissection/confocal fluorescence imaging techniques.[3] In perspective, this was at the time when the enhanced green fluorescent protein (GFP) became available (in 1995) and when the first commercial two-photon microscope was built by Bio-Rad (in 1996). The first applications of multiphoton microscopy (MPM) of the intact living kidney in 2002[13] were subsequently improved to directly and quantitatively visualize glomerular filtration and permeability to macromolecules (in 2007)[6,14,18] and glomerular cell motility/migration in health and disease *in vivo* (in 2010).[29] Widely available genetic strategies allowed podocyte-specific expression of fluorescent reporters to study ultrastructural changes to foot processes after podocyte injury using conventional fluorescence (optical) microscopy.[42,50] Serial MPM imaging was established recently to track the function and fate, and also intracellular calcium changes, of podocytes over the course of various glomerular pathologies in the living intact kidney *in vivo*.[21,41] The application of modern imaging approaches, e.g., super-resolution nanoscopy established in 2007, in podocyte research is expected to further push the limits of podocyte imaging.

tubular flow,[6,8] tubular concentration and dilution,[6] vascular resistance and glomerular permeability to macromolecules,[6,8,16–19] renin granule content, release, and tissue renin activity,[6,8,17,20] and so on. MPM imaging also allowed investigators to study intracellular variables and processes within cells of the intact living kidney, such as intracellular $[Ca^{2+}]$ [8,9,21,22] and pH levels,[7,9,23] endocytosis,[10,18,24] and mitochondrial functions.[5,25,26]

In the next sections, we highlight some of the most exciting recent developments and findings in glomerular research that used MPM imaging, and the future directions in intravital imaging of the kidney and other related technologies.

REGULAR OSCILLATIONS IN GLOMERULAR FILTRATION AND BEYOND

Time-lapse MPM imaging helped visualize what most physiology textbooks avoid discussing, namely the dynamic temporal variations in individual nephron and cell functions. Nonsteadiness, which is typical for most biological systems, is a particularly true feature of the kidney on the single-nephron level, owing to the regular oscillations in single-nephron GFR. The two classic physiological regulatory mechanisms of GFR and renal blood flow autoregulation, the myogenic tone of the afferent arteriole, and the kidney-specific tubuloglomerular feedback maintain these regular nephron GFR oscillations with a faster (0.12 Hz, cycle time about 10 s) and a slower (0.023 Hz, cycle time about 45 s) frequency.[27,28] Owing to the direct coupling of glomerular hemodynamics, the 'parent' mechanism, to the filtered fluid flow, nephron GFR oscillations are in turn transmitted to the Bowman's capsule and tubular flow rates throughout the nephron and collecting ducts. As a consequence, no single blood vessel, tubule segment, or nephron epithelial cell is steady in the kidney (otherwise, it must be dead). Time-lapse MPM imaging focusing on single glomeruli and nephrons was able to visualize these regular oscillations in vascular hemodynamics, tubular flow, and diameters.[6,8,22,29] Supplementary Movie S1 online shows one of the underlying mechanisms, the regular (once in every 5–10 s) myogenic constrictions of the afferent arteriole in the mouse kidney, whereas Supplementary Movie S2 online shows the downstream effect in a rat kidney, the oscillations in tubular fluid flow rates, which are visible as passive dilatations and collapse of tubular diameters.

What the regularly oscillating hemodynamics and tubular flow mean is that podocytes, the glomerular filter (including the slit diaphragm), and all tubular epithelial cells are constantly exposed to regularly fluctuating high mechanical forces such as stretch and shear stress (visible in Supplementary Movies S1 and S2 online). Mechanical forces are known to influence intracellular functions in most cell types including in renal epithelia, via mechanosensory organelles and calcium signaling.[30] Although healthy podocytes appear to maintain steady and low $[Ca^{2+}]_i$ even in hemodynamically oscillating glomeruli,[21] epithelial cells of the proximal and distal tubules and collecting ducts generate regularly oscillating $[Ca^{2+}]_i$ elevations simultaneously with the endogenous tubular flow stimuli.[8,22] Recent research suggested that the physiological oscillations in tubular fluid flow may function as an important endogenous diuretic mechanism that involves ATP release, purinergic $[Ca^{2+}]_i$ signaling, and causes inhibition of tubular salt and water reabsorption in the collecting ducts.[22] Therefore, the regular oscillations in glomerular filtration and tubular flow may be important in the control of body fluid and electrolyte balance and blood pressure.[22]

MPM IMAGING OF THE RAT KIDNEY

Initially, MPM imaging of glomeruli *in vivo* has been limited (owing to tissue depth penetration issues) to the use of Munich–Wistar rats, a special strain that features superficial glomeruli under the renal capsule.[6,10,13] Earlier *in vivo* MPM studies visualized the glomerular filtration of injected, various molecular size fluorescent tracers,[6,10,13] the glomerular arterioles, and their renin content,[6,8,31] the bulk fluid flow in the juxtaglomerular apparatus,[17] and later the structure and function of the glomerular filtration barrier (GFB).[29,32]