# Novel in vivo techniques to visualize kidney anatomy and function

**János Peti-Peterdi**[1], **Kengo Kidokoro**[1,2], and **Anne Riquier-Brison**[1]

[1]Departments of Physiology and Biophysics, and Medicine, Zilkha Neurogenetic Institute, University of Southern California, Los Angeles, CA

[2]Department of Nephrology and Hypertension, Kawasaki Medical School, Kurashiki, Japan

## Abstract

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 to 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.

Corresponding author: J. Peti-Peterdi, M.D., Ph.D., Zilkha Neurogenetic Institute, ZNI335, University of Southern California, 1501 San Pablo Street Los Angeles, California 90033. petipete@usc.edu.

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 due to the use of pulsed infrared, low energy excitation lasers, and minimized phototoxicity also due to fluorescent excitation occurring only at the focal plane, etc. 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 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 non-invasive measurement of the magnitude and temporal oscillations in single nephron filtration rate,[6] changes in blood flow and 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] etc. 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 related other technologies.

## Regular oscillations in glomerular filtration and beyond

Time-lapse MPM imaging helped to visualize what most physiology textbooks avoid discussing, namely the dynamic temporal variations in individual nephron and cell functions. Non-steadiness, which is typical for most biological systems, is a particularly true feature of the kidney on the single nephron level, due to the regular oscillations in single nephron glomerular filtration rate (SNGFR). 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 SNGFR oscillations with a faster 0.12 Hz (cycle time about 10 seconds) and a slower (0.023 Hz, cycle time about 45 seconds) frequency.[27, 28] Due to the direct coupling of glomerular hemodynamics, the "parent" mechanism, to the filtered fluid flow, SNGFR 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] Supplement movie 1 shows one of the underlying mechanisms, the regular (once in every 5–10 s) myogenic constrictions of the afferent arteriole in the mouse kidney, while supplement movie 2 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 supplement movies 1–2). Mechanical forces are known to influence intracellular functions in most cell types including in renal epithelia, via mechanosensory organelles and calcium signaling.[30] While 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 CD.[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 (due to tissue depth penetration issues) to the use of Munich-Wistar rats, a special strain which 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] Due to the importance and tremendous clinical relevance of podocytes in the maintenance of normal GFB functions and in the development of glomerular diseases and albuminuria, MPM imaging studies applied a new visual approach to better understand the structure and dynamic function of podocytes in vivo in the intact kidney.[29, 32] Using iv infusions of the freely filtered fluid marker Lucifer yellow allowed investigators to negatively label podocytes and parietal epithelial cells (PEC) of the Bowman's capsule.[29] The application of this earlier labeling technique is shown in Figure 2a–c. Time-lapse MPM imaging of the intact healthy glomerulus found direct visual evidence for the slow migration

of a cell on the visceral layer of the Bowman's capsule (moving approx. 10 μm over 7 min), suggesting the possibility that some glomerular cells may be able to migrate in vivo.[29] Since increased cell motility (e.g. in podocytes) is involved in the development of glomerular pathologies,[33] further studies are needed to positively identify the migrating cell type(s) and the conditions under which they occur. In numerous follow-up studies by our laboratory the migration of a very few glomerular cells have been re-confirmed, however, migrating cells were observed extremely rarely (unpublished observation). The migrating cell may not be a podocyte, since time-lapse MPM imaging of zebrafish glomeruli found no evidence for podocyte motility or migration over a time period of up to 23 hours.[34]

MPM also helped the discovery of anatomical structures around the podocyte and to characterize their function, such as the sub-podocyte space (SPS), a labyrinthine fluid space between the underside of podocyte cell body and the foot processes.[32] The small connectors between the SPS and the Bowman's space, so-called exit pores, may have important functional implications in physiological (filter backwash, podocyte-to-endothelium crosstalk, in light of the above described oscillating filtered fluid flow around podocytes) as well as in pathological processes such as focal segmental glomerulosclerosis (FSGS). MPM studies visualized numerous pseudocysts in the PAN model of FSGS which developed under the podocytes (Fig. 2b–c), and showed delayed dynamics of filling and emptying of injected fluorescent tracers, similarly to those in the SPS of healthy glomeruli.[29] These results suggested that the podocyte pseudocysts that form as a result of PAN-induced FSGS may represent enlargements of the SPS.[29] The pathological relevance and role of these SPS-pseudocysts that ultimately become highly albumin containing is illustrated in Fig. 2c. The rupture of one of these enlarged pseudocysts was caught by MPM (Figure 2c), which results in the emptying of high amounts of albumin into the Bowman's space causing or contributing to albuminuria. The heterogeneous leaking of high molecular weight plasma markers into the Bowman's space that occurs only around damaged podocytes was demonstrated earlier[7, 29] including in time-lapse movies.[29] These findings suggested that the increased GFB permeability in FSGS is not generalized, but restricted to areas of podocyte damage.[29]

MPM imaging does have the necessary spatial and temporal resolution to study complex cell-to-cell interactions between different cell types of the GFB, and circulating blood cells and other cell and plasma factors in vivo. For example, MPM directly visualized the developing vascular dysfunction and increased glomerular albumin permeability due to the loss of the glomerular endothelial surface layer (glycocalyx).[19] Also, MPM is capable of imaging the immediate structural and functional alterations in response to single podocyte injury, including disruption of the glomerular basement membrane, the underlying endothelial cell function, and the formation of localized microthrombi in the affected glomerular capillaries.[29] Similar MPM approaches in the future are expected to uncover further mechanistic details of glomerular thrombotic microangiopathy which is critically important in various glomerular pathologies.[35] Direct in vivo visualization of the crosstalk between endothelial, mesangial cells and podocytes may help to better understand the cellular and molecular mechanisms of kidney disease, but also to gain insights for future repair strategies on "how to put the glomerulus back together".[36]

Also, time-lapse MPM imaging provided new details and important in vivo confirmation of podocyte shedding, replacement, and the role of PECs in the pathology of glomerulosclerosis. Direct visual evidence was shown in the PAN-model of FSGS for the rather rapid processes of podocyte detachment (possibly due to cell necrosis) and glomerular exit either downstream the tubular fluid or by breaking through the highly permeable PEC layer into the periglomerular interstitium.[29] Remarkably, MPM visualized a process what appeared to be replacement of a detached podocyte by a new cell.[29] Further confirmation and characterization of this potentially important process requires future studies.

Advanced MPM techniques have been used recently to directly and quantitatively visualize the key barrier function of intact glomeruli of Munich-Wistar rats and C57Bl6/J mice in vivo by estimating the permeability of the GFB to fluorophore-conjugated macromolecules including high molecular weight dextran, albumin and angiotensinogen.[16, 19, 29, 37–39] This has been a very important but challenging task due to the complex experimental approach it requires (including proper animal maintenance, preparation of the fluorescent tracer, controls and calibrations of the imaging technique, etc.) and limitations in the MPM imaging technology caused by the highly light scattering and absorbing renal tissue structure and environment.[29] It should be emphasized that, unfortunate for renal research, the kidney is one of the most optically challenging organs when performing intravital microscopy techniques. In comparison to other tissues, for example the optically clear parenchyma of the brain where in vivo MPM imaging is possible >1 mm under the tissue surface,[40] useful MPM imaging is limited to the initial <200 μm depth in the highly light scattering kidney tissue. As a result of the technical limitations, but also due to differently applied MPM imaging approaches, several investigator groups measured different absolute values for the albumin permeability of the GFB. Combining all results from the various MPM studies, the glomerular sieving coefficient (GSC) of albumin was found in a wide range (0.0004–0.0341) which led to different, debated interpretations of the extent of GFB albumin permeability in the healthy and diseased kidney.[16, 18, 19, 29, 37–39] As a potential solution for the technical issues associated with the determination of absolute GSC values, a recent study measured relative increases in GFB albumin permeability in the same glomerulus over time (compared to baseline) during the development of podocyte/glomerular injury.[21]

## Intravital imaging of mouse and zebrafish glomeruli using transgenics

More recently, intravital imaging of positively labeled renal cells (cell lineage) has been developed by cell-specific expression of fluorescent proteins in new transgenic animal models as shown for mouse podocytes in Figure 3a–d. Not surprisingly, this was established mainly in mouse[21, 41–44] and zebrafish models,[34, 45, 46] the species with the most developed and reproducible transgenic technologies. However, it should be noted that the plasmid or viral vector-mediated delivery of transgenes (including those of fluorescent reporter proteins) to certain target cells in the rat kidney is becoming more and more popular and shows great promise not only for future gene therapy, but intravital imaging approaches as well.[47] The high expression of intensely fluorescent reporters in podocytes (highly intense cell signal) helped investigators to perform MPM imaging of mouse glomeruli in vivo, which lie deeper under the kidney surface compared to those in the Munich-Wistar rat. In fact, the feasibility of routinely performing MPM imaging of the few surface glomeruli in

the intact mouse kidney of commonly used strains has been confirmed by several groups independently.[16, 21, 41–43, 48, 49]

In contrast to the above described negative cell labeling approach (Figures 2a–c), the development of new transgenic mouse models in which the lineage of various glomerular cells has been fluorescently tagged helped to properly, and positively identify individual cells within the glomerulus. For example, Figures 3a–d demonstrate the use of the Tomato-GFP, and the Confetti (the combination of membrane-targeted CFP (blue), nuclear GFP (green), and cytosolic YFP (yellow) and RFP (red)) fluorescent reporters for labeling, and MPM imaging of podocytes and cells of podocyte origin in the intact living mouse kidney as described recently.[41] Monochromatic labeling of the podocyte layer made it possible to visualize the development of podocyte clusters (Figure 3a) and parietal podocytes, visual clues of ongoing glomerular tissue remodeling in vivo after unilateral ureteral obstruction (UUO),[21, 41] Adriamycin nephropathy,[41] and cytotoxic IgG-induced podocyte injury, a model of FSGS.[21] In contrast, the use of the multicolor Confetti reporter allows the identification of podocytes on the individual cell level (Figure 3b), which is necessary if investigators aim to track the fate of single cells.[41] Also, multicolor labeling of directly adjacent podocytes was used recently to follow alterations in podocyte morphology over time (down to foot process resolution) after podocyte injury using conventional fluorescence (optical) microscopy in both fixed kidneys and ex vivo.[42, 50] This is an important technical advance which will make it much easier to perform ultrastructural analysis of podocyte morphology, in contrast to the gold-standard, but cumbersome technique of electron microscopy. Also, it may allow the future, time-lapse visualization of the dynamics and mechanisms of podocyte foot process effacement in vivo.[51] Supplement movie 3 shows optical z-sectioning of a glomerulus (starting at the kidney surface and cutting deeper and deeper into the glomerulus) in the normal, healthy kidney of a multicolor Podocin-Confetti mouse.[41] In this mouse model, each podocyte is labeled in one of four colors, either by genetically encoded membrane-targeted CFP (cyan), nuclear GFP (green), cytosolic YFP (yellow) or cytosolic RFP (red).

An MPM imaging study of mouse glomeruli in vivo found low glomerular permeability to albumin and angiotensinogen, and suggested that, depending on the type of kidney disease and severity of GFB damage, the majority of urinary angiotensinogen originates from the tubules rather than glomerular filtration.[16] Another intravital imaging model was established for the simultaneous assessment of glomerular filtration and barrier function in live zebrafish, and found glomerular leakage of 500 kDa dextran into the tubules only after disrupting the GFB.[52]

In addition to imaging whole cells, intravital imaging approaches have been developed for the time-lapse visualization of cell organelles in the intact living kidney. For example, tissue and cell type-specific GFP labeling of primary cilia created new opportunities for using live imaging of cilia function and to better characterize the many genetic models of ciliopathies.[53] Also, MPM imaging visualized nanotubes between visceral (podocytes) and parietal (PEC) layers of the Bowman's capsule which may function in cell migration or cell-to-cell communication.[41]

## Intravital cell $Ca^{2+}$ imaging

Intravital MPM imaging is also able to look inside single cells in the intact living kidney to quantitatively visualize intracellular variables and cell signaling. While the use and localized tissue delivery of classic acetoxymethyl ester-based fluorophores to visualize cell $[Ca^{2+}]$[8, 22] and pH[23] in vivo is feasible, this approach has numerous technical issues.[7, 22, 54] Again, the development of transgenics tools, genetically encoded fluorescent protein indicators provided a breakthrough for in vivo imaging of cell calcium,[55, 56] chloride,[57] pH,[58] voltage,[59] and other cell variables.[60]

The role of cell $[Ca^{2+}]$ signaling in (patho)physiological processes has been established in multiple organs including in the kidney and podocytes, but the technology to study podocyte $[Ca^{2+}]_i$ in vivo was sorely lacking. Recently, our laboratory has developed and applied minimally invasive in vivo MPM imaging techniques to study podocyte $[Ca^{2+}]_i$ in the intact kidney of Pod/Cre-GCaMP3/fl mice which express the genetically encoded calcium indicator GCaMP3 selectively in podocytes.[21] Examples of this new model are shown in Figure 3c–d. This approach allowed the detailed investigation of the role of podocyte $[Ca^{2+}]_i$ and the related cellular and molecular mechanisms in glomerular disease. In fact, this was the first time that changes in $[Ca^{2+}]_i$ were followed in any organ or disease state in vivo. Steady and low baseline, and small angiotensin II-induced changes in podocyte $[Ca^{2+}]_i$ were detected, suggesting highly active $Ca^{2+}$ extrusion and intracellular $Ca^{2+}$ buffering mechanism in these cells.[21] The application of podocyte injury models, including UUO and FSGS, revealed new features and molecular mechanisms including cell-to-cell propagation of a podocyte $[Ca^{2+}]_i$ wave, and the key role of P2Y2 receptor-mediated purinergic $[Ca^{2+}]_i$ signaling in podocyte pathology.[21] The development of the heterogeneous, focal segmental pattern of highly elevated podocyte calcium in response to podocyte injury and FSGS is shown in Figure 3d. Importantly, the in vivo MPM imaging of podocyte $[Ca^{2+}]_i$ can be performed together with the quantitative imaging of glomerular functional parameters (e.g. glomerular tuft contraction, albumin leakage) for the complex visual analysis of renal and glomerular pathology.[21] This new in vivo visual approach is a great complement to recent in vitro imaging studies of the freshly harvested living glomeruli which characterized the role of other molecular players in podocyte $[Ca^{2+}]_i$ signaling, including purinergic mechanisms[61] and TRPC5/6.[62, 63]

## Serial MPM imaging of the same glomerulus over days

Recently, our laboratory has developed serial MPM, which we believe is an important technical advance to better understand cellular dynamics in the living kidney tissue. The technique allows for the visualization of the exact same kidney region (e.g. glomerulus) over days, during the course of disease in the intact living kidney.[41] No other current technology is capable of accomplishing this. Advantages include (i) overcoming glomerular heterogeneity issues, (ii) establishing the dynamics and pattern of individual cells' motility and migration over time, and (iii) combination with functional measurements (e.g. glomerular albumin leakage).

The development and first applications of serial MPM made it possible to track the fate of single cells in the intact kidney in vivo using novel fluorescent lineage tagged mouse models, and to study the dynamics of cell motility. This new approach was instrumental in depicting the dynamics and the dramatic changes in the morphology and cellular composition of the injured glomerulus.[41] The bridging, and significant migration of visceral podocyte cell clusters to the parietal Bowman's capsule, proximal tubule and beyond, and the appearance of new visceral podocytes within 24 and 48 hours supported the highly dynamic rather than static nature of the glomerular environment and cellular composition in glomerular disease.[41] Figure 4 illustrates the added benefit of the combination of classic genetic lineage tracing techniques and serial MPM. By tracking the same single, genetically labeled cell using serial MPM, the origin and migration pattern of cells can be established. This visual information can provide new, important clues regarding the pathomechanism of glomerular and tubulointerstitial diseases, but also it can be useful to understand intrinsic mechanisms and dynamics of renal cell/tissue turnover and repair. Future applications of serial MPM imaging for the dynamic tracking of individually labeled renal stem and progenitor cells may provide important new knowledge that could be used for the development of novel strategies of nephron and kidney regeneration.[51]

## Use of the multiphoton laser as a micromanipulator

In addition to the use of the powerful multiphoton laser for fluorescence excitation, it also has less-known, non-traditional uses as a micromanipulator. Similarly to the wide use of femtosecond pulsed lasers in the industry for materials microprocessing and nanofabrication,[64] they can be used for the extremely precise micro or nano-manipulation of the living tissues as well, without the need for additional instrumentation around the experimental setup. Accordingly, the focusing of the multiphoton laser beam on small regions of interest has been used for the selective ablation of single podocytes,[29] or causing extremely localized injuries to submicron regions of a single endothelial cell[29] or podocytes,[21] or for more robust disruption of the entire GFB that causes transient, single nephron hematuria.[7] The generation of laser-induced marks in the renal cortical interstitium has been instrumental in identifying and locating individual glomeruli in repeat imaging sessions when performing serial MPM imaging of the same glomerulus.[41]

## Future directions of technology development

The future use of the constantly advancing laser, optics, and imaging technology and intravital approaches are expected to further push the limits (including tissue depth limits) of in vivo fluorescence microscopy. There are commercially available newer multiphoton microscope systems with fully integrated OPO (optical parametric oscillator) lasers which extend the usual, tunable fluorescence excitation range of 680–1080 nm to 1300 nm. Longer wavelength excitation light can penetrate deeper into the living tissue due to less absorption and scattering, and the extended OPO range now allows for label-free live tissue imaging with third-harmonic generation.[65] The use of high sensitivity gallium arsenide phosphide (GaAsP) detectors, high-speed resonance scanning, dual scanning, improved microscope objectives specifically designed for MPM imaging are also available for high quality and advanced MPM imaging applications. Other exciting new developments in MPM and other

intravital imaging modalities include fiberoptic delivery of photons[66], minimally invasive intravital endomicroscopy of the kidney,[67] super-resolution analysis (nanoscopy) of the molecular building blocks of the GFB using stochastic optical reconstruction microscopy (STORM),[68] and new optical tissue clearing techniques (CLARITY) for high-resolution 3D analysis of intact entire organs.[69]

In summary, in vivo MPM imaging of the intact living kidney has been an innovative, pioneering experimental technique in kidney research for more than a decade. MPM has now become a common, routinely used intravital microscopy technique available in most renal research centers worldwide, and it allows investigators to perform comprehensive, quantitative visualization of the structure and function of the kidney in vivo with amazing detail and over time. The many different uses and modalities of MPM will continue to help the renal research field and the better understanding of normal kidney function and the mechanisms of kidney diseases.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

## References

1. Mundel P, Kriz W. Structure and function of podocytes: an update. Anat Embryol (Berl). 1995; 192:385–397. [PubMed: 8546330]

2. Mundel P, Reiser J, Kriz W. Induction of differentiation in cultured rat and human podocytes. J Am Soc Nephrol. 1997; 8:697–705. [PubMed: 9176839]

3. Nitschke R, Henger A, Ricken S, et al. Angiotensin II increases the intracellular calcium activity in podocytes of the intact glomerulus. Kidney Int. 2000; 57:41–49. [PubMed: 10620186]

4. Dunn KW, Young PA. Principles of multiphoton microscopy. Nephron Exp Nephrol. 2006; 103:e33–40. [PubMed: 16543762]

5. Hall AM, Molitoris BA. Dynamic multiphoton microscopy: focusing light on acute kidney injury. Physiology (Bethesda). 2014; 29:334–342. [PubMed: 25180263]

6. Kang JJ, Toma I, Sipos A, et al. Quantitative imaging of basic functions in renal (patho)physiology. Am J Physiol Renal Physiol. 2006; 291:F495–502. [PubMed: 16609147]

7. Peti-Peterdi J, Burford JL, Hackl MJ. The first decade of using multiphoton microscopy for high-power kidney imaging. Am J Physiol Renal Physiol. 2012; 302:F227–233. [PubMed: 22031850]

8. Peti-Peterdi J, Toma I, Sipos A, et al. Multiphoton imaging of renal regulatory mechanisms. Physiology (Bethesda). 2009; 24:88–96. [PubMed: 19364911]

9. Sipos A, Toma I, Kang JJ, et al. Advances in renal (patho)physiology using multiphoton microscopy. Kidney Int. 2007; 72:1188–1191. [PubMed: 17667980]

10. Molitoris BA, Sandoval RM. Intravital multiphoton microscopy of dynamic renal processes. Am J Physiol Renal Physiol. 2005; 288:F1084–1089. [PubMed: 15883167]

11. Peti-Peterdi J. Multiphoton imaging of renal tissues in vitro. Am J Physiol Renal Physiol. 2005; 288:F1079–1083. [PubMed: 15883166]

12. Peti-Peterdi J, Morishima S, Bell PD, et al. Two-photon excitation fluorescence imaging of the living juxtaglomerular apparatus. Am J Physiol Renal Physiol. 2002; 283:F197–201. [PubMed: 12060602]

13. Dunn KW, Sandoval RM, Kelly KJ, et al. Functional studies of the kidney of living animals using multicolor two-photon microscopy. Am J Physiol Cell Physiol. 2002; 283:C905–916. [PubMed: 12176747]

14. Yu W, Sandoval RM, Molitoris BA. Rapid determination of renal filtration function using an optical ratiometric imaging approach. Am J Physiol Renal Physiol. 2007; 292:F1873–1880. [PubMed: 17311910]

15. Wang E, Meier DJ, Sandoval RM, et al. A portable fiberoptic ratiometric fluorescence analyzer provides rapid point-of-care determination of glomerular filtration rate in large animals. Kidney Int. 2012; 81:112–117. [PubMed: 21881552]

16. Nakano D, Kobori H, Burford JL, et al. Multiphoton imaging of the glomerular permeability of angiotensinogen. J Am Soc Nephrol. 2012; 23:1847–1856. [PubMed: 22997258]

17. Rosivall L, Mirzahosseini S, Toma I, et al. Fluid flow in the juxtaglomerular interstitium visualized in vivo. Am J Physiol Renal Physiol. 2006; 291:F1241–1247. [PubMed: 16868308]

18. Russo LM, Sandoval RM, McKee M, et al. The normal kidney filters nephrotic levels of albumin retrieved by proximal tubule cells: retrieval is disrupted in nephrotic states. Kidney Int. 2007; 71:504–513. [PubMed: 17228368]

19. Salmon AH, Ferguson JK, Burford JL, et al. Loss of the endothelial glycocalyx links albuminuria and vascular dysfunction. J Am Soc Nephrol. 2012; 23:1339–1350. [PubMed: 22797190]

20. Toma I, Kang JJ, Sipos A, et al. Succinate receptor GPR91 provides a direct link between high glucose levels and renin release in murine and rabbit kidney. J Clin Invest. 2008; 118:2526–2534. [PubMed: 18535668]

21. Burford JL, Villanueva K, Lam L, et al. Intravital imaging of podocyte calcium in glomerular injury and disease. J Clin Invest. 2014; 124:2050–2058. [PubMed: 24713653]

22. Svenningsen P, Burford JL, Peti-Peterdi J. ATP releasing connexin 30 hemichannels mediate flow-induced calcium signaling in the collecting duct. Front Physiol. 2013; 4:292. [PubMed: 24137132]

23. Brasen JC, Burford JL, McDonough AA, et al. Local pH domains regulate NHE3 mediated Na+ reabsorption in the renal proximal tubule. Am J Physiol Renal Physiol. 2014 ajprenal 00174 02014.

24. Sandoval RM, Kennedy MD, Low PS, et al. Uptake and trafficking of fluorescent conjugates of folic acid in intact kidney determined using intravital two-photon microscopy. Am J Physiol Cell Physiol. 2004; 287:C517–526. [PubMed: 15102609]

25. Hall AM, Crawford C, Unwin RJ, et al. Multiphoton imaging of the functioning kidney. J Am Soc Nephrol. 2011; 22:1297–1304. [PubMed: 21719788]

26. Hall AM, Rhodes GJ, Sandoval RM, et al. In vivo multiphoton imaging of mitochondrial structure and function during acute kidney injury. Kidney Int. 2013; 83:72–83. [PubMed: 22992467]

27. Holstein-Rathlou NH, Leyssac PP. TGF-mediated oscillations in the proximal intratubular pressure: differences between spontaneously hypertensive rats and Wistar-Kyoto rats. Acta Physiol Scand. 1986; 126:333–339. [PubMed: 3962682]

28. Marsh DJ, Sosnovtseva OV, Chon KH, et al. Nonlinear interactions in renal blood flow regulation. Am J Physiol Regul Integr Comp Physiol. 2005; 288:R1143–1159. [PubMed: 15677526]

29. Peti-Peterdi J, Sipos A. A high-powered view of the filtration barrier. J Am Soc Nephrol. 2010; 21:1835–1841. [PubMed: 20576805]

30. Praetorius HA, Frokiaer J, Leipziger J. Transepithelial pressure pulses induce nucleotide release in polarized MDCK cells. Am J Physiol Renal Physiol. 2005; 288:F133–141. [PubMed: 15367389]

31. Toma I, Kang JJ, Peti-Peterdi J. Imaging renin content and release in the living kidney. Nephron Physiol. 2006; 103:71–74.

32. Salmon AH, Toma I, Sipos A, et al. Evidence for restriction of fluid and solute movement across the glomerular capillary wall by the subpodocyte space. Am J Physiol Renal Physiol. 2007; 293:F1777–1786. [PubMed: 17804486]

33. Mundel P, Reiser J. Proteinuria: an enzymatic disease of the podocyte? Kidney Int. 2010; 77:571–580. [PubMed: 19924101]

34. Endlich N, Simon O, Gopferich A, et al. Two-photon microscopy reveals stationary podocytes in living zebrafish larvae. J Am Soc Nephrol. 2014; 25:681–686. [PubMed: 24309184]

35. Keir L, Coward RJ. Advances in our understanding of the pathogenesis of glomerular thrombotic microangiopathy. Pediatr Nephrol. 2011; 26:523–533. [PubMed: 20949284]

36. Schlondorff D. Putting the glomerulus back together: per aspera ad astra ("a rough road leads to the stars"). Kidney Int. 2014; 85:991–998. [PubMed: 24786868]

37. Sandoval RM, Wagner MC, Patel M, et al. Multiple factors influence glomerular albumin permeability in rats. J Am Soc Nephrol. 2012; 23:447–457. [PubMed: 22223875]

38. Tanner GA. Glomerular sieving coefficient of serum albumin in the rat: a two-photon microscopy study. Am J Physiol Renal Physiol. 2009; 296:F1258–1265. [PubMed: 19211688]

39. Schiessl IM, Castrop H. Angiotensin II AT2 receptor activation attenuates AT1 receptor-induced increases in the glomerular filtration of albumin: a multiphoton microscopy study. Am J Physiol Renal Physiol. 2013; 305:F1189–1200. [PubMed: 23946289]

40. Helmchen F, Denk W. Deep tissue two-photon microscopy. Nat Methods. 2005; 2:932–940. [PubMed: 16299478]

41. Hackl MJ, Burford JL, Villanueva K, et al. Tracking the fate of glomerular epithelial cells in vivo using serial multiphoton imaging in new mouse models with fluorescent lineage tags. Nat Med. 2013; 19:1661–1666. [PubMed: 24270544]

42. Hohne M, Ising C, Hagmann H, et al. Light microscopic visualization of podocyte ultrastructure demonstrates oscillating glomerular contractions. Am J Pathol. 2013; 182:332–338. [PubMed: 23246153]

43. Khoury CC, Khayat MF, Yeo TK, et al. Visualizing the mouse podocyte with multiphoton microscopy. Biochem Biophys Res Commun. 2012; 427:525–530. [PubMed: 23022193]

44. Kistler AD, Caicedo A, Abdulreda MH, et al. In vivo imaging of kidney glomeruli transplanted into the anterior chamber of the mouse eye. Sci Rep. 2014; 4:3872. [PubMed: 24464028]

45. Vasilyev A, Liu Y, Mudumana S, et al. Collective cell migration drives morphogenesis of the kidney nephron. PLoS Biol. 2009; 7:e9. [PubMed: 19127979]

46. Zhou W, Hildebrandt F. Inducible podocyte injury and proteinuria in transgenic zebrafish. J Am Soc Nephrol. 2012; 23:1039–1047. [PubMed: 22440901]

47. Corridon PR, Rhodes GJ, Leonard EC, et al. A method to facilitate and monitor expression of exogenous genes in the rat kidney using plasmid and viral vectors. Am J Physiol Renal Physiol. 2013; 304:F1217–1229. [PubMed: 23467422]

48. Devi S, Li A, Westhorpe CL, et al. Multiphoton imaging reveals a new leukocyte recruitment paradigm in the glomerulus. Nat Med. 2013; 19:107–112. [PubMed: 23242472]

49. Schiessl IM, Bardehle S, Castrop H. Superficial nephrons in BALB/c and C57BL/6 mice facilitate in vivo multiphoton microscopy of the kidney. PLoS One. 2013; 8:e52499. [PubMed: 23349687]

50. Grgic I, Brooks CR, Hofmeister AF, et al. Imaging of podocyte foot processes by fluorescence microscopy. J Am Soc Nephrol. 2012; 23:785–791. [PubMed: 22362911]

51. Peti-Peterdi J, Burford JL, Hackl MJ. Can kidney regeneration be visualized? Nephron Exp Nephrol. 2014; 126:86. [PubMed: 24854647]

52. Kotb AM, Muller T, Xie J, et al. Simultaneous Assessment of Glomerular Filtration and Barrier Function in Live Zebrafish. Am J Physiol Renal Physiol. 2014 ajprenal 0002902014.

53. O'Connor AK, Malarkey EB, Berbari NF, et al. An inducible CiliaGFP mouse model for in vivo visualization and analysis of cilia in live tissue. Cilia. 2013; 2:8. [PubMed: 23819925]

54. Peppiatt-Wildman CM, Crawford C, Hall AM. Fluorescence imaging of intracellular calcium signals in intact kidney tissue. Nephron Exp Nephrol. 2012; 121:e49–58. [PubMed: 23147410]

55. Gee JM, Smith NA, Fernandez FR, et al. Imaging activity in neurons and glia with a Polr2a-based and cre-dependent GCaMP5G-IRES-tdTomato reporter mouse. Neuron. 2014; 83:1058–1072. [PubMed: 25155958]

56. Dana H, Chen TW, Hu A, et al. Thy1-GCaMP6 transgenic mice for neuronal population imaging in vivo. PLoS One. 2014; 9:e108697. [PubMed: 25250714]

57. Arosio D, Ratto GM. Twenty years of fluorescence imaging of intracellular chloride. Front Cell Neurosci. 2014; 8:258. [PubMed: 25221475]

58. Bencina M. Illumination of the spatial order of intracellular pH by genetically encoded pH-sensitive sensors. Sensors (Basel). 2013; 13:16736–16758. [PubMed: 24316570]

59. Han Z, Jin L, Chen F, et al. Mechanistic Studies of the Genetically Encoded Fluorescent Protein Voltage Probe ArcLight. PLoS One. 2014; 9:e113873. [PubMed: 25419571]

60. Ai HW. Fluorescent-protein-based probes: general principles and practices. Anal Bioanal Chem. 2014

61. Peti-Peterdi J. Calcium wave of tubuloglomerular feedback. Am J Physiol Renal Physiol. 2006; 291:F473–480. [PubMed: 16495210]

62. Schaldecker T, Kim S, Tarabanis C, et al. Inhibition of the TRPC5 ion channel protects the kidney filter. J Clin Invest. 2013; 123:5298–5309. [PubMed: 24231357]

63. Ilatovskaya DV, Palygin O, Chubinskiy-Nadezhdin V, et al. Angiotensin II has acute effects on TRPC6 channels in podocytes of freshly isolated glomeruli. Kidney Int. 2014; 86:506–514. [PubMed: 24646854]

64. Kang, S.; Vora, K.; Shukla, S., et al. Femtosecond Laser Nanofabrication of Metal Structures Through Multiphoton Photoreduction. In: Di Bartolo, B.; Collins, J., editors. Nano-Optics for Enhancing Light-Matter Interactions on a Molecular Scale. Springer; Netherlands: 2013. p. 411-412.

65. Witte S, Negrean A, Lodder JC, et al. Label-free live brain imaging and targeted patching with third-harmonic generation microscopy. Proc Natl Acad Sci U S A. 2011; 108:5970–5975. [PubMed: 21444784]

66. Croix CS, Zipfel WR, Watkins SC. Potential solutions for confocal imaging of living animals. Biotechniques. 2007; 43:14–19. [PubMed: 17933097]

67. Xi J, Chen Y, Zhang Y, et al. Integrated multimodal endomicroscopy platform for simultaneous en face optical coherence and two-photon fluorescence imaging. Opt Lett. 2012; 37:362–364. [PubMed: 22297353]

68. Suleiman H, Zhang L, Roth R, et al. Nanoscale protein architecture of the kidney glomerular basement membrane. Elife. 2013; 2:e01149. [PubMed: 24137544]

69. Tomer R, Ye L, Hsueh B, et al. Advanced CLARITY for rapid and high-resolution imaging of intact tissues. Nat Protoc. 2014; 9:1682–1697. [PubMed: 24945384]
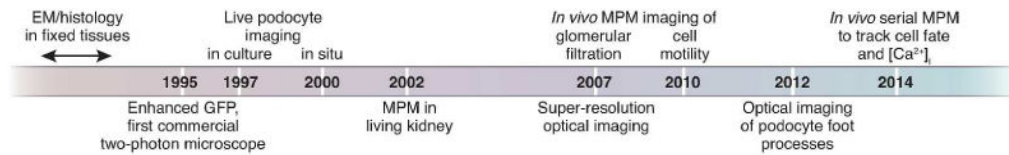
**Figure 1. Timeline of the technical advances in glomerular/podocyte imaging**

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.
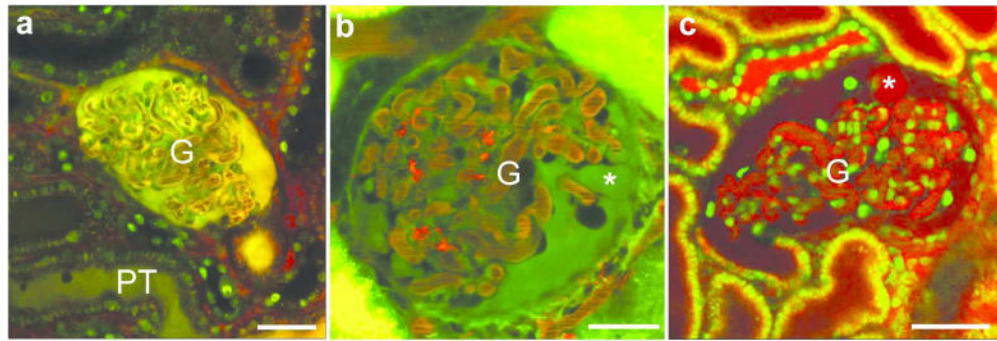
**Figure 2. Live imaging of podocytes and the glomerular filter in the intact rat kidney in healthy and disease conditions using MPM**

(a) MPM images of glomeruli (G) in vivo in intact control (a) or PAN-treated (b–c) Munich-Wistar-Fromter rat kidneys. Lucifer Yellow, a freely filtered dye injected iv in bolus labeled the Bowman's space and early proximal tubule (PT) identifying podocytes and parietal cells (which do not take up the dye) based on negative labeling (a–b). Cell nuclei were labeled using Hoechst33342 (green). The intravascular space (plasma) was labeled red by 70 kDa dextranrhodamine B (a–b) or Alexa594-albumin (c) given in iv bolus. In contrast to normal glomerular structure (a), numerous pseudocysts develop in podocytes (asterisk) after PAN treatment (b–c). (c) A pseudocyst is seen rupturing (asterisk) that results in the emptying of the high albumin containing cyst fluid into the Bowman's space (red) and downstream the nephron. Albumin uptake is visible in proximal tubule segments, but massive amounts of albumin make it to the collecting duct (intensely red labeled fluid-containing tubule at the top of the glomerulus) causing albuminuria. Hypertrophied podocytes (large dark cells) and numerous mesangial macrophages (intensely red colored cells due to plasma dye endocytosis) are visible after PAN-treatment (b). Dark objects in blood vessels are streaming red blood cells. Bars= 20 μm.
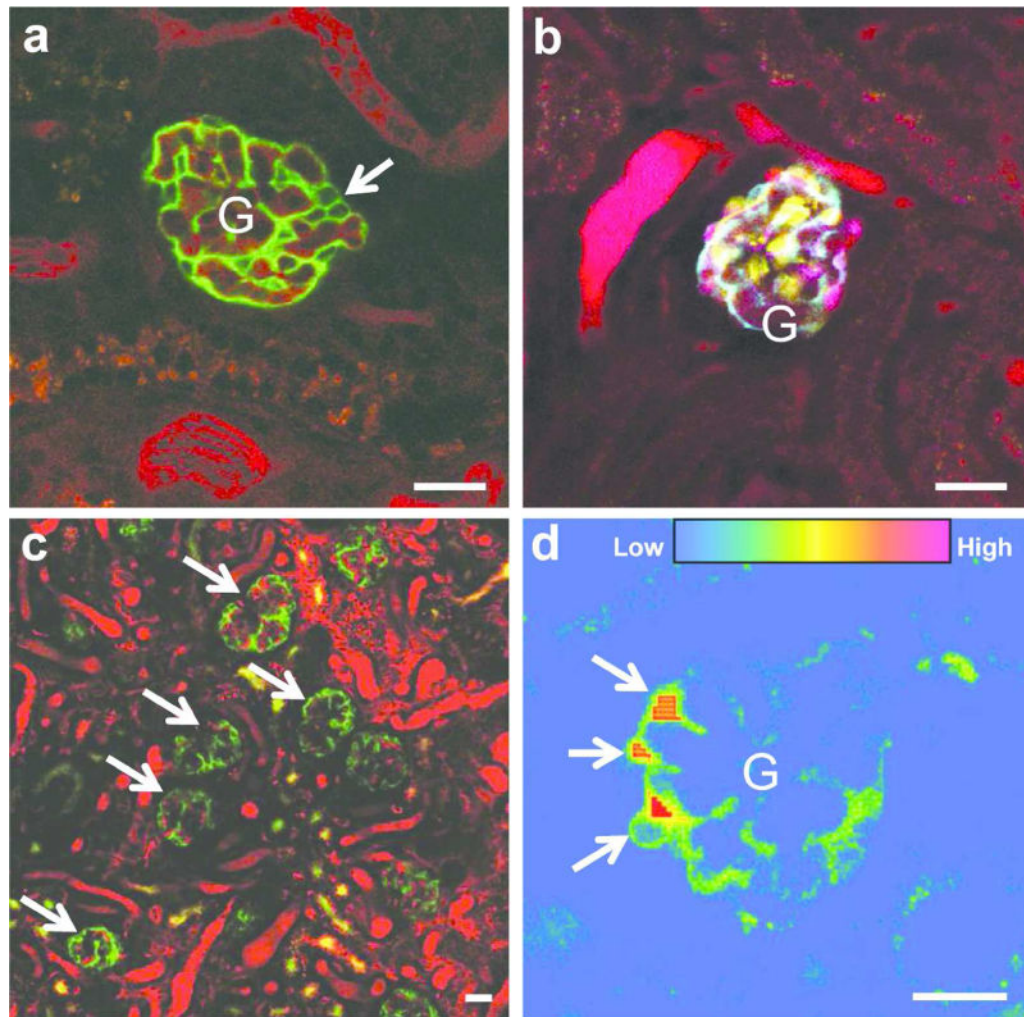
**Figure 3. Intravital imaging of genetically labeled podocytes in the intact mouse kidney using MPM**

(a) High-power image of a single glomerulus (G) in a Podocin-GFP mouse kidney[41] after UUO shows restriction of GFP fluorescence to podocytes which surround the glomerular capillaries. Plasma is labeled red with Alexa594-Albumin. Dark objects in capillaries are streaming red blood cells. Multi-cell podocyte clusters develop after UUO (arrow). (b) In the normal healthy (non-UUO) kidney of a multi-color Podocin-Confetti mouse,[41] each podocyte is labeled in one of four colors, either by genetically encoded membrane-targeted CFP (cyan), nuclear GFP (green), cytosolic YFP (yellow) or cytosolic RFP (red). Highly red fluorescent tubular fluid-containing collecting ducts are visible around the top half of the glomerulus. (c) Low-power image of glomeruli (arrows) from a Podocin-GCaMP3 mouse kidney[21] in which the genetically encoded calcium indicator GCaMP3 was expressed only in glomerular podocytes, and its fluorescence is shown in green. Plasma marker (red) illuminates the renal vasculature, and the renal tubular fluid after its glomerular filtration. (d) Pseudocolor (gradient) map of GCaMP3 fluorescence intensity in a glomerulus from a Podocin-GCaMP3 mouse kidney after cytotoxic IgG treatment (a model of podocyte injury

and FSGS). The heterogeneous, focal segmental pattern of highly elevated podocyte calcium is shown in three adjacent podocytes (arrows). Bars= 20 μm.
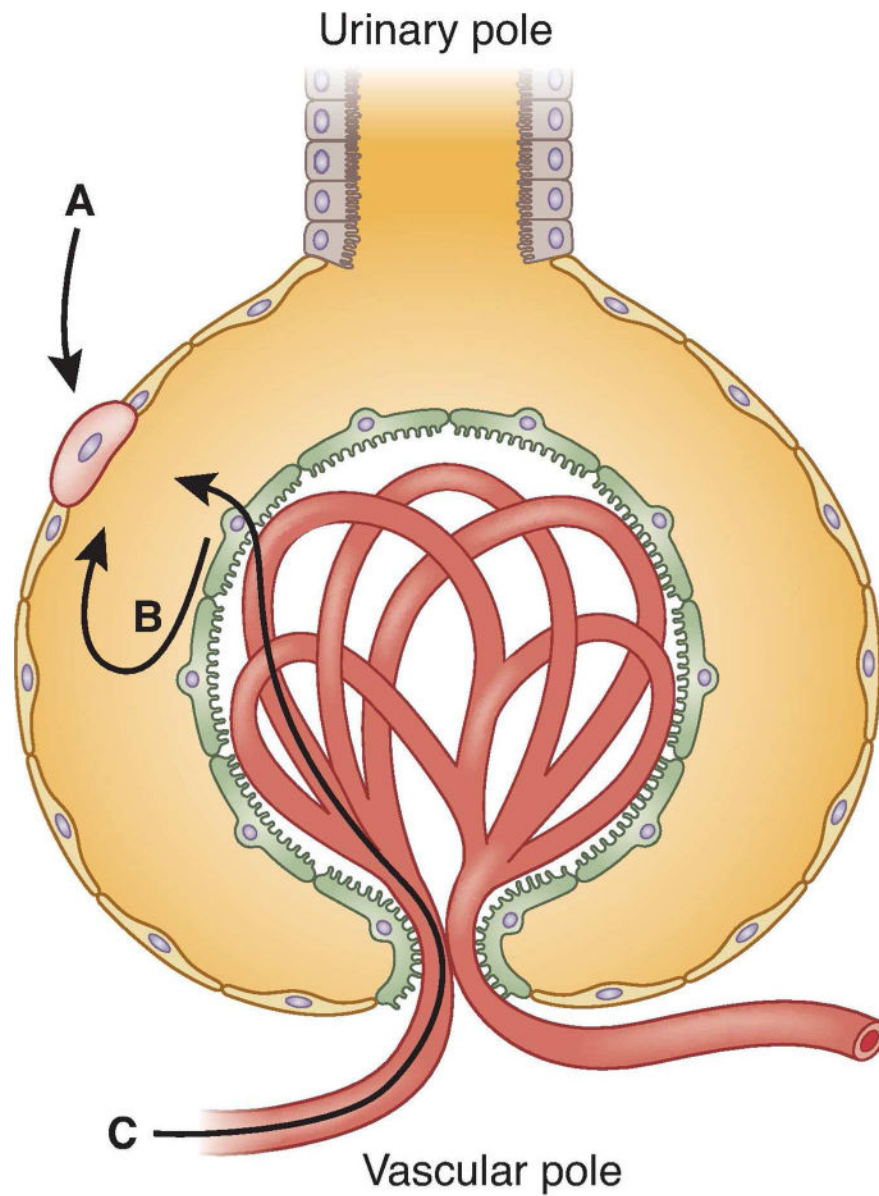
**Figure 4. Illustration of the principle of individual cell migration and fate tracking using the combination of genetic lineage tracing and serial MPM imaging**

By tracking the same single, genetically labeled cell (by a unique color identifier) using serial MPM, the origin and migration pattern of cells can be established. For example, serial MPM can determine if a newly appeared cell 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).