Can Kidney Regeneration Be Visualized?

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Abstract

Background: Various cell types, including podocytes and parietal epithelial cells, play important roles in the development and progression of glomerular kidney diseases, albuminuria, and glomerulosclerosis. Besides their role in renal pathologies, glomerular cells have emerging new functions in endogenous repair mechanisms. A better understanding of the dynamics of the glomerular environment and cellular composition in an intact living kidney is critically important for the development of new regenerative therapeutic strategies for kidney diseases. However, progress in this field has been hampered by the lack of in vivo research tools. **Summary:** This review summarizes the current state-of-the-art in the application of the unique intravital imaging technology of multiphoton fluorescence microscopy for the dynamic visualization of glomerular structure and function over time in the intact, living kidney. Recently, this imaging approach in combination with transgenic mouse models allowed tracking of the fate of individual glomerular cells in vivo over several days and depicted the highly dynamic nature of the glomerular environment, particularly in disease conditions. **Key Messages:** The technology is ready and available for future intravital imaging studies investigating new glomerular regenerative approaches in animal models.

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The recent development and application of intravital fluorescence imaging approaches using multiphoton microscopy (MPM) have overcome a critical technical barrier in glomerular biology research. Until recently, most morphological and functional observations were based on cell culture models [1] and fixed tissue sections [2]. However, during the past few years, tremendous advances in the field of live imaging have helped to modernize kidney research. The ability of MPM to directly visualize the changes in the structure and function of the same glomerulus in an intact living kidney over time with unprecedented, subcellular detail is an important technological breakthrough. In this brief review, we first summarize the most exciting new developments in fluorescence imaging technology for glomerular studies and then highlight the key points of the new insights in the glomerular environment using MPM imaging, and the future directions in research and technology.

New Developments in Fluorescence Imaging Technology for the Study of the Glomerulus

MPM is a powerful minimally invasive imaging technique for deep optical sectioning of living tissues [3, 4]. The basic principles, applications, advantages, and limitations of this imaging technology for the study of living intact kidneys were recently described in detail [5]. During the last decade, improved applications of intravital

MPM have been developed and applied for quantitative imaging of basic functions in renal (patho)physiology in intact whole kidneys [6, 7], including measurement of the magnitude of and temporal oscillations in the single nephron filtration rate, changes in blood flow and tubular flow, vascular resistance and permeability, renin granule content, release, and tissue renin activity [3, 4, 7]. MPM imaging has also allowed the study of intracellular variables in cells in the intact living kidney, such as intracellular calcium levels [3, 8] and pH [5, 9].

Importantly, confocal fluorescence imaging of the cellular and subcellular elements of the intact glomerulus and the glomerular filtration barrier (GFB) has become possible not only in zebrafish [10] but also in the few surface glomeruli of most mouse strains [11]. In fact, the feasibility of routine performance of MPM imaging of glomeruli in the intact mouse kidney of the commonly used C57BL6 strain has been demonstrated in our previous publications [4, 5, 7], and it has also been confirmed by at least 3 independent laboratories [11–13]. The permeability of the GFB to various macromolecules, including leakage of the clinically relevant albumin from glomerular capillaries to Bowman's space, has been measured in healthy mouse kidneys and throughout the course of disease [14, 15]. Also, interactions between the glomerular endothelium (including its glycocalyx), the basement membrane, and podocytes have been visualized [4, 15]. In addition to the analysis of glomerular and GFB functions, nonspecific negative labeling techniques as shown in figure 1a have allowed the visualization of migrating single cells within intact glomeruli *in vivo* [4].

Another technical innovation and new milestone for glomerular imaging *in vivo* was the combination of widely available mouse genetic strategies with MPM imaging. The development of new transgenic mouse models in which the lineage of various glomerular cells has been fluorescently tagged has helped to properly identify and track individual cells within the glomerulus over time and to study the dynamics of cell motility as an addition to traditional genetic cell fate mapping [16]. For example, figure 1b–c demonstrates the use of Tomato-GFP and confetti [the combination of membrane-targeted CFP (blue), nuclear GFP (green), and cytosolic YFP (yellow) and RFP (red)] fluorescent reporters for labeling podocytes and cells of podocyte origin. Most recently, serial MPM imaging was performed on the same glomerulus over time in an intact Pod-GFP and Pod-confetti mouse kidney *in vivo* once every 24 h. This new approach was instrumental in depicting the dynamics and the dramatic changes in the morphology and cellular composition of

the injured glomerulus [16]. Also, podocyte-specific expression of fluorescent reporters allowed the study of the ultrastructural changes in primary, secondary, and foot processes after podocyte injury using conventional fluorescence (optical) microscopy both in fixed kidneys and *ex vivo* [17, 18]. This approach is exemplified in figure 1d–f. This is a significant technical advance which will ease podocyte research since fluorescence microscopy is generally available in contrast to the cumbersome electron microscopy, which used to be the standard technique for podocyte foot process imaging.

Intravital Imaging of Glomerular Remodeling: What Did We Learn So Far?

Time-lapse MPM imaging has provided new details and important *in vivo* confirmation of podocyte shedding and replacement and the role of parietal epithelial cells (PECs) in the pathology of glomerulosclerosis [4]. Direct visual evidence was shown in the puromycin model of focal segmental glomerulosclerosis for the rather rapid processes of podocyte detachment (possibly due to cell necrosis) and exit either downstream from the tubular fluid or by breaking through the highly permeable PEC layer into the periglomerular interstitium [4]. Remarkably, MPM visualized the replacement of a detached podocyte by a new, negatively labeled cell which appeared to originate from the parietal Bowman's capsule [4]. These findings suggested the presence of dynamic regenerative mechanisms within the injured glomerulus, consistent with the recent identification of a subset of highly proliferative PECs at the urinary pole of Bowman's capsule within the rodent and human kidney functioning as renal progenitors [19–25]. These progenitor cells were proposed to proliferate, differentiate, and generate new podocytes by progressively migrating from the urinary pole to the vascular stalk [20, 21, 23]. Facilitation of podocyte regeneration and therefore promotion of functional repair of the injured GFB is a promising new therapeutic strategy; however, several questions and mechanistic details remain to be clarified and understood in this process. Using a negative labeling technique due to dye exclusion, MPM imaging was able to visualize a few cells localized at the glomerulo-tubular junction which appeared to be loosely attached to the PEC layer (fig. 1a). However, the exact identity and function of these cells are unclear and require further study.

Most recently, tracking the cell fate using serial MPM imaging of the same glomerulus over time *in vivo* in novel transgenic mouse models provided new visual clues on cell

Fig. 1. Intravital MPM imaging of the structure and function of the glomerulus and the GFB in an intact living kidney. **a** A dye exclusion technique negatively identified podocytes around glomerular capillaries (arrowheads) in the Munich-Wistar-Fromter rat kidney, while Bowman's space (BS) was filled with systemically injected and filtered Lucifer Yellow (yellow). In addition to podocytes, several other dark cells can be seen attached to the parietal layer of Bowman's capsule at the tubulo-glomerular junction (arrows). PT = Proximal tubule. Plasma was labeled with Alexa594-albumin (red). **b** Podocyte-specific expression of GFP (green) in Pod-GFP mice positively identified visceral epithelial cells around the glomerular capillaries. However, after UUO, podocytes migrated from the visceral to the parietal layer of Bowman's capsule (arrowheads), leading to the appearance of GFP-positive cells on both layers. The glomerular permeability of systemically injected markers of varied molecular weights through either the visceral or the parietal layer of Bowman's capsule could be quantitatively vi-

sualized. Shown here is the accumulation of the i.v. injected, freely filterable Lucifer Yellow (yellow) into Bowman's space. **c** Multi-color labeling of podocytes in Pod-confetti mice. Individual podocytes are labeled in 1 of 4 different colors due to CFP (blue), GFP (green), YFP (yellow), or RFP (red) expression. The plasma dye is shown in gray scale. **d-f** Optical imaging of the ultrastructural details of podocyte primary, secondary, and foot processes. Podocyte-specific expression of GFP in Pod-GFP mice shows podocytes in green in great detail (**d**). **e** Magnification of the inset in **d** illustrating that optical sectioning horizontally through the surface of the capillary wall is able to spatially resolve the cell processes of podocytes. The dark objects in the glomerular capillaries (Cap) are streaming red blood cells. Plasma was labeled with Alexa594-albumin (red). **f** The interdigitating foot processes of 3 adjacent podocytes are shown in Pod-confetti mice, with each cell labeled by a different fluorescent protein (CFP/YFP/RFP). Scale bars = 20 μ m (**a-d**) and 5 μ m (**e, f**).

migration, remodeling, and the highly dynamic glomerular environment. In podocin-GFP mice, podocytes formed sporadic multicellular clusters after glomerular injury, caused by either unilateral ureteral ligation or Adriamycin administration, and migrated into the parietal Bowman's capsule [16] (fig. 1b). Podocytes migrated to the PEC layer either via continuous transition at the vascular pole or by forming bridges across Bowman's space close to the urinary pole or anywhere around the glomerular tuft (summarized in fig. 2). Tracking of single cells in podocin-confetti mice featuring cell-specific expression of CFP, GFP,

YFP, or RFP (fig. 1c) revealed the simultaneous migration of multiple podocytes [16]. Importantly, podocyte-to-PEC migration appeared to be a continuously ongoing phenomenon between 2 and 12 weeks after injury, which progressively increased the coverage of the parietal Bowman's capsule by podocytes [16]. Although this is an established pathological process in the model of unilateral ureteral obstruction (UUO) [26], the robust cell migration and likely the proliferation of a yet-to-be-identified precursor cell type may provide new clues for future regenerative approaches. Also, MPM imaging visualized nanotubes be-

tween the visceral (podocytes) and parietal (PEC) layers of Bowman's capsule which may function in cell migration or cell-to-cell communication [16].

Perhaps the most important MPM imaging finding, an *in vivo* visual clue suggesting podocyte regeneration, was the first-time visualization of *de novo* podocyte formation. Using serial MPM imaging of podocin-confetti mice in the UUO model of renal fibrosis, we were able to observe the appearance of a new podocyte within 24 h of the previous imaging session [16]. Altogether, these findings further challenged the previous static view of podocytes and demonstrated that at least some podocytes have a highly dynamic, motile, and migratory phenotype.

What Are the Future Needs and Research Directions?

Although the technology is in place to directly visualize the process of glomerular regeneration in the living kidney, and we have gained substantial new insights into this process, there are more questions than answers. How can intravital imaging help in the future to better understand the molecular and cellular mechanisms of glomerular regeneration? Positive identification and fluorescent tagging (lineage tracing) of various types of systemic and/or renal progenitor cell populations combined with serial MPM imaging will help to determine the origin of glomerular podocytes and other cell types and the dynamics of cell replacement. Future work may visualize podocyte replacement by migrating PECs as proposed and shown recently [20, 27] or by other cells of extraglomerular origin, e.g. the renin cell as suggested by the Shankland group [28]. In addition to tracking cells, MPM imaging can quantitatively visualize the dynamics of not only morphological but also functional regeneration of the same glomerulus and GFB over time. For example, the permeability of the GFB to various molecular tracers can be evaluated simultaneously by following the process of glomerular remodeling (fig. 1b). Also, the use of new transgenic mouse models with multicolor podocyte labeling [17] allows the optical resolution of podocyte foot processes (fig. 1d–f) for further studies of GFB ultrastructure changes during glomerular regeneration.

The use of future, ever-developing imaging techniques and approaches (e.g. long-wavelength infrared lasers, extremely sensitive detectors, and super-resolution nanoscopy) are expected to further push the limits of intravital, functional podocyte imaging. We anticipate that serial MPM of novel podocyte mouse models with fluorescent lineage tags as a novel, unique, state-of-the-art imaging

Fig. 2. Schematic illustration of the migration of podocytes and PECs in an intact kidney as observed with intravital MPM imaging. In addition to PEC-to-podocyte transition and cell replacement as established in the literature, MPM imaging allowed observation of the clustering and migration of podocytes (green) to the parietal layer of Bowman's capsule (red), which was most robust after glomerular injury. Podocytes migrated to the PEC layer either via continuous transition at the vascular pole or by forming bridges across Bowman's space close to the urinary pole or anywhere around the glomerular tuft (arrows). Nanotubes exist between the visceral (podocytes) and parietal (PEC) layers of Bowman's capsule (green lines) which may function in cell migration or cell-to-cell communication.

approach will be used in the near future to directly and quantitatively visualize novel mechanisms of glomerular regeneration *in vivo*.

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