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Novel fluorescence techniques to quantitate renal cell biology

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Abstract

Fluorescence microscopy techniques are powerful tools to study tissue dynamics, cellular function and biology both *in vivo* and *in vitro*. These tools allow for functional assessment and quantification along with qualitative analysis, thus providing a comprehensive understanding of various cellular processes under normal physiological and disease conditions. The main focus of this chapter is the recently developed method of serial intravital multiphoton microscopy that has helped shed light on the dynamic alterations of the spatial distribution and fate of single renal cells or cell populations and their migration patterns in the same tissue region over several days in response to various stimuli within the living kidney. This technique is very useful for studying *in vivo* the molecular and cellular mechanisms of tissue remodeling and repair after injury. In addition, complementary *in vitro* imaging tools are also described and discussed, like tissue clearing techniques and protein synthesis measurement in tissues *in situ* that provide an in depth assessment of changes at the cellular level. Thus, these novel fluorescence techniques can be effectively leveraged for different tissue types, experimental conditions as well as disease models to improve our understanding of renal cell biology.

1 Introduction

The presence of anatomically diverse cell types with wide-ranging functions and the relative inaccessibility of the kidney present a unique challenge in developing experimental approaches for studying renal cell biology, which requires sub-cellular resolution. Various microscopy techniques allow us to visually study, both qualitatively and quantitatively, different aspects of cellular biology—structural alterations, anatomical adaptations, cellular interactions, intracellular signaling cascades as well as cellular responses to environmental cues. Earlier approaches involved classical histology-based techniques, cell culture-based model systems and electron microscopy. Although these methods are effective in providing a static understanding of renal cell biology, it is imperative to employ advanced techniques that provide non-invasive or minimally invasive imaging of the intact living kidney tissue in order to provide a deeper understanding of the dynamic nature of cell biology (Peti-Peterdi, 2016). Multiphoton microscopy (MPM), a term which encompasses both two- and three-photon microscopy, is one such experimental approach. First used for biological applications in the early 1990s, MPM is a state-of-the-art fluorescence imaging tool that allows for deep

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optical non-invasive sectioning of biological tissue with a high degree of spatial and temporal resolution. MPM was established for kidney tissue imaging by the Peti-Peterdi-Bell and Dunn-Molitoris groups and over the last two decades, has been used to provide novel insights about renal tissue structure and function (Peti-Peterdi, Burford, & Hackl, 2012). In brief, MPM uses nonlinear pulsed lasers in the infrared spectrum for fluorescence excitation. The simultaneous absorption of two photons of same energy at the focal plane results in the excitation of a fluorophore equivalent to a single photon of double the energy. The use of long wavelength, low energy photons results in deeper tissue penetration with lower scattering and noise. Additionally, long wavelength photons are associated with less phototoxicity-related tissue damage, making it highly suitable for *in vivo* tissue imaging. In contrast, conventional confocal imaging techniques rely on the use of a single photon in the ultraviolet and visible spectrum, resulting in greater scattering, lesser optical tissue penetration with greater tissue damage. These unique features of MPM allow for the possibility of serial imaging of living tissue in a temporal fashion (Dunn & Young, 2006; Helmchen & Denk, 2005). In the recent years, monumental leaps in microscopy technology and engineering—ultrafast scanners, advanced optics, high sensitivity detectors, and longer wavelength lasers (Peti-Peterdi, 2016)—have resulted in even deeper optical tissue penetration (Schuh et al., 2016), third harmonic generation, and selective plane illumination (Buckley et al., 2017).

2 Overview of MPM

2.1 Glomerular imaging

The human kidney on average consists of 1 million nephrons that mediate the diverse renal functions. Present at the beginning of the nephron, the glomerulus acts as the renal sieve, regulating filtration of different molecules across the filtration barrier. Structurally, the glomerulus is composed of several different cell types including the endothelial cells of the glomerular capillaries and the podocytes lining the filtration barrier. Additionally, in the glomerular vascular pole region, there are two important cell types—the juxtaglomerular (JG) cells and the macula densa (MD) cells—which regulate among many other renal functions, renal and glomerular hemodynamics and the renin-angiotensin system (RAS). Thus, the glomerular region represents an important structural and functional unit that is responsible for regulating fundamental renal functions. The cellular and functional complexity as well as the inaccessibility of the glomerular unit has limited our understanding of its functions under normal physiological and pathophysiological conditions. In order to better understand kidney development, renal function and physiology, and the mechanisms responsible for pathophysiology and disease progression, it is necessary to visualize the interplay between different renal cells *in vivo*. The establishment of MPM and the rapid advances in the microscopy technology made it possible to study the glomerular cell populations in a living, intact kidney. With MPM, it is possible to observe different nephron segments—from the glomerulus to the tubular segments—and track the fate of the same single cells and region of the kidney over time or during the course of drug treatments. MPM thus provides a means to visualize temporal and spatial alterations in the kidney with sub-cellular resolution that would otherwise be impossible using conventional approaches.

2.2 The development and renal applications of MPM technology

The functional assessment of the kidney traditionally involves quantitative measurements of important physiological parameters including renal blood flow, glomerular filtration rate, vascular permeability and tubular reabsorption, all of which have been conventionally measured at the whole-organ level. Changes in tissue architecture, cellular structure and anatomy have also been ascertained using classical histology and immunofluorescence techniques. In contrast to these traditional methods, MPM affords us the opportunity to study real-time changes in a living intact kidney in a nuanced and detailed fashion (Dunn et al., 2002; Molitoris & Sandoval, 2009; Peti-Peterdi, Kidokoro, & Riquier-Brisson, 2015, 2016). In the initial years of applying MPM for renal tissue imaging, an inherent complication was limited imaging depth, since the kidney parenchyma is not as optically clear as other organs like the brain. Additionally, attenuation of fluorescence signal due to increased light scattering and absorption in the heterogenous renal tissue, only allowed for imaging highly superficial structures within 100–150 μm of kidney surface. Thus, in the early 2000s, *in vivo* applications of MPM were limited to the Munich-Wistar-Froemter (MWF) strain of rats which are endowed with superficial glomeruli (Dunn et al., 2002; Peti-Peterdi et al., 2012; Schiessl, Bardehle, & Castrop, 2013). Albeit these limitations, the real-time imaging of entire living, intact glomeruli in mice has become possible with the advancement of MPM technology, as demonstrated by different research groups (Peti-Peterdi et al., 2012). The earliest glomerular MPM applications used freshly dissected, *in vitro* microperfused glomeruli and juxtaglomerular apparatus (JGA) preparations, in which optical sectioning and reconstruction of a functioning glomerulus, functional assessment of JGA control of renin release and tubuloglomerular feedback (TGF) was made possible using two-photon fluorescence microscopy (Peti-Peterdi, 2005; Peti-Peterdi, Fintha, Fuson, Tousson, & Chow, 2004; Peti-Peterdi, Morishima, Bell, & Okada, 2002; Rosivall, Mirzahosseini, Toma, Sipos, & Peti-Peterdi, 2006). Visual clues provided by MPM laid out a more detailed understanding of the regulation of the RAS. Additionally, MPM was instrumental in visualizing calcium waves during TGF and in perimacular cells in response to alterations in tubular flow and salt (Peti-Peterdi, 2006). Of special interest to our laboratory are the MD cells, and MPM has helped shed light on the regulatory role of MD volume changes in JGA function (Komlosi, Fintha, & Bell, 2006; Peti-Peterdi et al., 2002), calcium responses of MD cells due to tubular flow and salt sensing capability of MD cells (Sipos, Vargas, & Peti-Peterdi, 2010). With respect to *in vivo* imaging, the early MPM studies involved measurement of basic renal functions, mainly glomerular filtration and tubular reabsorption (Dunn et al., 2002; Dunn, Sandoval, & Molitoris, 2003; Molitoris & Sandoval, 2005). Further advancement in techniques allowed for quantitative assessment of single nephron glomerular filtration rates, glomerular hemodynamics, and vascular functions (Kang, Toma, Sipos, McCulloch, & Peti-Peterdi, 2006; Peti-Peterdi, 2009). While the initial MPM studies in the late 1990s were performed using the first commercial MPM system BioRad MRC1024MP equipped with a Tsunami laser (350-mW power @ 800 nm excitation wavelength) (Dunn et al., 2002; Peti-Peterdi et al., 2002), technical advances in faster scanners, more sensitive nondescanned external detectors, more powerful broad-band tunable excitation (680–1080 nm, >4-W @ 800 nm with the Ultra-II Chameleon laser) have become the standard imaging approach for MPM, and helped to improve image quality and resolution (Peti-Peterdi et al., 2012). Thus, within 15 years of the establishment of this

technique, tremendous advances were made in MPM technology and this allowed researchers to directly visualize different physiological parameters in several nephron segments *in vivo*, and to ascertain mechanisms of several renal diseases, including but not limited to, acute kidney injury (Basile et al., 2011; Gyarmati et al., 2018; Hall, Rhodes, Sandoval, Corridon, & Molitoris, 2013; Nakano & Nishiyama, 2016), glomerulosclerosis (Peti-Peterdi & Sipos, 2010), diabetic nephropathy (Kang et al., 2006; Toma et al., 2008), *etc.* These studies were not limited to the MWF strain of rats but were performed in several transgenic mouse models using the superior capabilities of later MPM models. Besides studying the JGA and its functions, MPM also made it possible to visualize podocytes, another inaccessible renal cell type present within the glomerulus. While there exist cultured podocyte cell lines, in order to accurately study the intricate structure and function of podocytes, it is necessary to observe them *in vivo* in their native environment and in the context of the other glomerular cell types. MPM enabled us to study the complex three-dimensional structure of the glomerular filtration barrier *in vivo* with functional assessment of albumin leakage in cases of pathophysiology (Peti-Peterdi & Sipos, 2010). Previous work also demonstrated visually the presence of a new anatomical structure, the “subpodocyte space” (SPS), using MPM (Salmon et al., 2007). Thus, MPM has shed light on novel structural and functional features of various renal cell types and more importantly, has allowed us to track dynamic changes in these features over a period of time. Currently, one of the most advanced MPM equipment is the Leica SP8 DIVE system featuring ultra-high sensitivity quad module four spectral (first in class) HyD SP GaAsP detectors, and compatible with extended infrared spectrum single-box lasers, such as the Discovery laser system (680–1300 nm tunable range). The most important advantage of using longer wavelength excitation lasers is increased tissue penetration and depth of imaging due to decreased scattering and absorption in the tissue. Using excitation wavelengths >1000 nm also allows second and third harmonic generation signals as well as far-red fluorophore excitation that can be easily distinguished from endogenous autofluorescence. These advantages of longer wavelength excitation make it possible to visualize deeper structures in the kidney tissue that were previously inaccessible. Schuh et al. used this extended wavelength excitation in a model of acute kidney injury (AKI) using a nephrotoxic agent. Using MPM, the authors reported simultaneous measurement of several physiological readouts—alterations in proximal tubule epithelia, inhibition of endocytosis and aberrations in intracellular organelles. Thus, the use of long-range wavelengths in MPM could help better understand different renal pathophysiologies and the mechanisms underlying the same (Schuh et al., 2016). As mentioned earlier, three-photon excitation microscopy has also improved thanks to using advanced optics and lasers with longer range excitation, namely > 1300 nm. Applying three-photon excitation, Ouzounov et al. demonstrated functional imaging of calcium activity in deep neurons beyond the imaging depth of two-photon microscopy in an intact mouse brain. Thus, high resolution spatial and temporal deep imaging of tissue is made possible using longer range excitation wavelengths (Ouzounov et al., 2017).

Over the last two decades since the commercialization of MPM, tremendous advances have been made in this technology. Since the initial studies that were limited to *ex vivo* tissue

preparations to the current *in vivo* imaging depths, MPM remains the mainstay for dynamic, real-time intravital imaging for structural and functional analyses of biological tissues.

3 Advantages of serial MPM

Taking advantage of the latest advances in MPM technology with essentially zero cytotoxicity, improvements in animal surgery and maintenance with minimally or non-invasive intravital imaging approaches, our laboratory has developed and applied a new modality named “serial MPM.” Serial MPM involves *in vivo* MPM imaging of the same area (*e.g.*, glomeruli) of the intact living kidney in the same animal over the course of longer time periods, *e.g.*, several days and weeks. Serial MPM of the intact living kidney tissue makes it possible to visualize several slow dynamic processes including cell fate tracking and migration. The combination of transgenic mouse models with multicolor fluorescent reporters such as Confetti, and intravital MPM allows researchers to tag a single cell (unique cell ID based on color combinations) and follow the progression of that specific cell or a renal cell type (*e.g.*, renin lineage cells, podocytes, endothelial cells, interstitial cells, *etc.*) in the same exact region over the course of a disease. In fact, serial MPM is the only current research technology that enables the study of the dynamics and patterns of single cell movements in the intact living kidney during several days of tissue remodeling. Along with cell-fate tracking, serial MPM can also be used for functional phenotyping—vascular permeability, albumin leakage across the glomerular filtration barrier, alterations in renal blood flow and glomerular filtration rate. Since serial MPM involves visualizing the same area of the kidney tissue over time, this technique overcomes the issue of renal tissue heterogeneity. A combination of structural and functional measurements *in vivo* can thus help better understand the dynamics of renal pathophysiology at a single cell level that cannot be ascertained using whole organ measurements and histology techniques (Peti-Peterdi, 2016; Peti-Peterdi et al., 2016).

4 Advanced microscopes for deep tissue serial MPM imaging

Our laboratory currently makes use of the Leica SP8 DIVE system (Leica Micro-systems, Wetzlar, Germany), a state-of-the-art MPM setup, for intravital kidney tissue imaging. The SP8 DIVE microscope is equipped with a single-box extended infrared range excitation Discovery laser system (Coherent, Palo Alto, USA) with 680–1300 nm tunable excitation as well as a fixed wavelength 1040 nm laser line. This advanced microscope is capable of increased optical penetration in highly scattering biological tissues such as the kidney. Using ultra-high speed resonant scanning (8 kHz), ultra-high sensitivity quad module spectral HyD SP GaAsP detectors along with a fully-motorized scanning stage, it is possible to perform highly efficient serial MPM with a high degree of optical resolution in a non-invasive manner. The first-in-class 4Tune spectral detector of the SP8 DIVE system enables the simultaneous detection of up to four different fluorescence channels with 1 nm spectral precision, thus making it possible to track the fate of cells in the Confetti mouse model efficiently and with extreme precision. The SP8 DIVE system provides the user the maximum possible optical tissue penetration with very low phototoxicity and enhanced fluorescence signal capture. Additionally, using the fully motorized imaging stage, single tile scan images of large surface areas of kidney cortex (approx. 3–4 mm in diameter) can be

generated that serve as a “map” for registering and re-identifying the same glomeruli for subsequent serial MPM sessions.

For *in vivo* imaging, it is imperative that the animal is maintained in an optimal physiological state with respect to maintenance of body fluids, blood pressure, and body temperature. Our imaging system is equipped with the CODA tail-cuff blood pressure monitoring system that can non-invasively measure the systolic, diastolic and mean blood pressure, heart rate, tail blood volume as well as blood flow, and uses far infrared warming pads (Kent Scientific Corporation, Torrington, USA) to efficiently maintain body temperature while imaging is in progress. A combination of this sophisticated MPM setup and transgenic fluorescent reporter mouse models thus makes it possible to study several aspects of renal physiology in a living intact mouse kidney.

5 Examples of serial MPM

Using transgenic mouse models in which specific renal cell types—podocytes, renin lineage cells, endothelial cells, interstitial cells, immune cells—are fluorescently tagged, various cell-fate tracking and migration studies can be performed that help provide visual clues regarding endogenous remodeling and the dynamic glomerular environment.

In one of the first reports of serial intravital imaging, we tracked the fate of two renal epithelial cells—podocytes and parietal epithelial cells (PECs)—in two disease models—unilateral ureteral ligation (UUO) and Adriamycin nephropathy. In the Pod-GFP model, serial MPM demonstrated that the GFP-labeled podocytes form contacts with the surrounding PECs and eventually migrate into the parietal Bowman’s capsule after UUO. Additionally, in a Pod-Confetti mouse model, the fate of individual podocytes was tracked over time. In this mouse model as well, UUO induced the bridging of podocytes from the visceral to the parietal layer that remained unaffected by alterations in hemodynamics. Thus, serial MPM of the intact kidney is an effective tool to overcome the inaccessibility barrier in podocyte research and also has demonstrated the dynamic nature of glomerular epithelial cells, at least in disease conditions (Burford et al., 2014; Hackl et al., 2013). Other studies have used continuous intravital MPM to study podocyte motility in zebrafish (Endlich et al., 2014) and mouse models (Brahler et al., 2016). Intravital MPM has been an effective research tool also to visualize the dynamic interplay between circulating and resident immune cells and the local renal tissue microenvironment, and to improve our understanding of immune-mediated pathologies (Brahler et al., 2018; Devi et al., 2013; Gyarmati et al., 2018; Hato, Winfree, & Dagher, 2018).

An important cell type near the vascular pole of the glomerulus are the cells of the renin lineage (CoRL). In the Ren1d-Confetti model, serial MPM revealed that multiclonal CoRL migrate into the intraglomerular space in the FSGS model of podocyte depletion. Furthermore, the migrating CoRL present within the glomerulus express several podocyte proteins while the small subset that migrate toward the Bowman’s capsule express PEC markers. Serial MPM helped to track the fate of every CoRL (on the basis of their fluorescent tag) as well as confirm classic functional markers of podocyte depletion pathology—albumin leakage and increased uptake of albumin by the proximal tubule

segment. Directly visualizing and tracking the fate of genetically labeled CoRL *in vivo* provided evidence supporting the migration of CoRL into the glomerulus as well as the Bowman's capsule (Kaverina et al., 2017).

Serial MPM imaging techniques using the abdominal imaging window (AIW) technique (Schiessl et al., 2018) have provided visual evidence of renal repair and regeneration processes, including regeneration of the renal tubular epithelium. In the inducible Pax8-Confetti mouse model, lineage tracing experiments using serial MPM have demonstrated the presence of large monochromatic cell clusters around the site of laser-induced injury in the proximal tubule epithelium 7 days after injury. Furthermore, MPM visualized PDGFR β ⁺-interstitial cells migrating toward the injury site that helped restore tubular function. Thus, under conditions of tubular injury, cell fate tracking and migration experiments with serial MPM can describe the dynamic nature of endogenous regeneration by specific renal cell types (Schiessl et al., 2018).

The above-mentioned studies provide a snapshot of the diverse applications of serial MPM and the visual insight provided by this powerful technique. MPM is not only effective in describing changes in tissue architecture, it can also be successfully applied for quantitative functional measurements at the level of a single nephron. Serial MPM is the only current technology that can visualize and investigate dynamic cellular remodeling and repair processes that occur after injury with repeated imaging of the same area of the kidney tissue over time, thus providing a unique perspective of endogenous cellular processes that operate in different tissue types.

5.1 In vivo fluorescence imaging with serial MPM

Serial MPM is a technique that allows investigators to visualize and track the same area of the living intact kidney in a temporal fashion, over several days and weeks. Thus, using this technique, it is possible to determine the fate of specific fluorescently tagged cell types in response to injury, drug treatment, or to follow the progression of disease conditions.

5.1.1 Protocol—The method involves the surgical implantation of an abdominal window over the exposed kidney tissue and this serves as the imaging area for serial MPM. The following protocol has been adapted from Ritsma et al. (2013) specifically for the kidney tissue and has been effectively used by several research groups for intravital imaging of the renal tissue (Ritsma et al., 2013; Schiessl et al., 2018).

5.1.2 Materials—C57BL6 background (fluorescent reporter) mice

Customized titanium ring

12 mm coverslips

Cyanoacrylate-based glass glue

PLL-g-PEG solution

Eye ointment

Isoflurane

Plasma dye

Surgical tools and equipment

5.1.3 Method

A. Preparation of AIW for implantation:

1. To prepare the AIW, carefully glue a 12 mm coverslip on the customized titanium ring using cyanoacrylate-based glass glue and forceps. Ensure that the entire surface of the ring is covered with glass-glue before placing the coverslip. Apply gentle pressure for about 1 min using a cotton swab to firmly attach the coverslip to the ring (Fig. 1A).
2. Allow the glass-glue to air-dry for 1 h. If required, remove any excess glass-glue using acetone.
3. Ensure that the AIW is water-tight by placing it on a dry paper towel and filling it with saline. If the coverslip is appropriately glued onto the ring, no saline should leak onto the paper towel.
4. Before surgically implanting the AIW, sterilize the window by incubating it in 70% ethanol for approximately 30 min. AIWs can be sterilized and stored in a sterile tube for 1 week.
5. An optional step in the AIW preparation is incubating the AIW in a 1 ng/mL solution of PLL-g-PEG for coating purposes. Add 150 μ L of PLL-g-PEG to the interior of the AIW and incubate it for 1 h under sterile conditions. Remove the solution after 1 h and wash the AIW with sterile PBS.

B. Surgical implantation of AIW:

1. Prepare the surgery station by sterilizing all the surgical tools using 70% ethanol and a hot glass bead dry sterilizer or an autoclave. Clean the entire surgery workstation using a disinfectant or 70% ethanol solution.
2. Anesthetize the mouse using an induction chamber flooded with 2% isoflurane.
3. Once sufficiently anesthetized, carefully shave the left flank of the mouse using electrical shaver or hair removal cream.
4. Ensure that the surgery platform is heated before placing the mouse on its side on the platform. Induce the mouse using 1.5% isoflurane *via* a nose cone to maintain anesthesia.
5. Carefully apply an eye ointment to lubricate the eyes of the anesthetized mouse.

6. Clean the shaved surface of the mouse three times with an antiseptic solution and sterile saline alternately using sterile cotton swabs.
 7. Before the surgery, treat the mouse with a subcutaneous injection of 1 mg/kg body weight of Buprenorphine-SR in the neck fold of the mouse for proper analgesia.
 8. Cover the mouse with a surgical drape and using sterile surgical scissors make a 1 cm flank incision to exteriorize the left kidney.
 9. Make a purse-string suture to attach the skin and muscle layers using a non-absorbable 6-0 Prolene surgical suture along the edges of the incision.
 10. To implant the window, first coat the inner surface of the titanium ring attached to the coverslip with a thin layer of cyanoacrylate glass-glue using a 10 μ L micropipette.
 11. Using a pair of forceps, gently place the window over the exposed kidney surface and hold it in place for about 5 min to ensure proper implantation of the window.
 12. Following the window implantation, place the abdominal wall and muscle in the groove of the titanium ring (Fig. 1B) and then carefully pull on the ends of the purse-string suture to fasten the window in place.
 13. Place a double-knot at end of the purse-string suture to tightly secure the window in place and then hide the knotted end of the suture in the titanium ring to ensure that the mouse does not loosen the suture knot (Fig. 1C).
 14. Allow the mouse to recover on a heating pad for about 30 min before placing it back in its housing cage for postoperative monitoring.
- C. Serial MPM imaging of the mouse kidney:
1. After the surgical implantation of the AIW, prepare the microscope stage by placing the microscope stage insert. The microscope stage insert should have a hole that is similar in size to the AIW and should be fitted with a coverslip for imaging.
 2. Ensure that the warming blanket and anesthesia nose cone are set up and ready to use.
 3. If required, inject the mouse with a plasma dye (Alexa594-BSA, Alexa680-BSA or similar) retroorbitally and then move the mouse onto the microscope stage.
 4. Place the mouse in such a way as to align the AIW with the coverslip of the microscope insert and ensure that the mouse is asleep using 1.5% isoflurane supplied *via* the nose cone. For serial MPM, it is important to

align the mouse and AIW in the same orientation for each imaging session.

5. Carefully cover the mouse with the warming blanket and if required, use the non-invasive tail-cuff blood pressure monitoring system (Kent Scientific Corporation, Torrington, USA) to monitor blood pressure during imaging.
6. Using the eyepiece, adjust the *z*-plane of the objective in order to locate the kidney tissue. The green autofluorescence of the kidney tissue is often useful to establish the appropriate *z*-plane.
7. Depending on the genetic reporters expressed in the transgenic mouse model, establish the optimal microscope settings for imaging.
8. For serial imaging, it is necessary to establish a “map” of the kidney tissue so as to image the same area of the tissue over time. In order to locate the same area of the kidney over time, several methods can be used. Morphological clues like the organization of superficial glomeruli, unique patterns of tubules and branching of large blood vessels can help to find the same area in subsequent imaging sessions. In case of genetic reporter mouse strains, re-identifying the imaging area is simplified by the distribution of different colored cells based on their fluorescent tag. Finally, using the motorized microscope stage, one can generate a tile scan image of the entire available imaging area and this serves as an efficient “map” for registering and re-identifying the same glomeruli in subsequent serial imaging sessions (Fig. 1D).
9. After the imaging session, allow the animal to recover on a heating pad before placing it back in its cage before the next imaging session.

Using the Leica SP8 DIVE system, it is possible to visualize fluorescence signals in four different spectral regions simultaneously. Taking advantage of this capability of the imaging system, we have successfully demonstrated the principle (Fig. 2) and feasibility of performing serial MPM-based single cell migration and fate tracking in several transgenic mouse models including Pod-Confetti and Ren1d-Confetti (in which podocytes or CoRL respectively express cytosolic RFP or YFP, membranous CFP or nuclear GFP (Fig. 3A–F)) (Hackl et al., 2013; Kaverina et al., 2017). As demonstrated in Fig. 3, serial intravital MPM imaging can identify single glomerular epithelial cells based on their unique Confetti color (either blue/green/yellow/red), and changes in the relative position of the same cells can be tracked in consecutive imaging sessions. Top-to-bottom optical sectioning (*z*-stack) of the same glomeruli as shown in Fig. 3A and D are available in Supplementary Videos 1 and 2 in the online version at <https://doi.org/10.1016/bs.mcb.2019.04.013>, respectively. The initial applications of this technology provided visual clues on the dynamic glomerular environment, including podocyte-to-parietal epithelial cell (Hackl et al., 2013) and CoRL-to-podocyte (Kaverina et al., 2017) transitions.

Additionally, serial MPM is a powerful tool for functional assessment of intracellular calcium changes over time. Intracellular calcium acts as a secondary messenger of several

signaling pathways that regulate various cell functions including proliferation and motility. Elevations in levels of intracellular calcium have been measured by using serial MPM, associated with several pathological alterations in podocytes, suggesting the vital role of Ca^{2+} in podocyte dynamics (Burford et al., 2014). Using a highly calcium-sensitive genetically encoded reporter, namely GCaMP5, here we demonstrate the use of this technology for detecting podocyte calcium elevations in response to hypertensive and podocyte-toxic injury (Fig. 4). Damage to podocytes (*e.g.*, in response to Adriamycin treatment, Fig. 4) can cause an elevation in podocyte intracellular calcium, and these changes can be quantitatively assessed *in vivo* in the same glomerulus over time using serial MPM. Thus, serial MPM is an invaluable tool for qualitative assessment of renal cell biology, and can also be combined with quantitative measurements (hemodynamics, glomerular filtration rate, intracellular parameters such as calcium) at a single cell and nephron level *in vivo*.

5.2 In vitro fluorescence imaging techniques

While *in vivo* MPM imaging techniques are powerful tools to interrogate cell fate and migration patterns, complementary *in vitro* fluorescence imaging methods allow researchers to characterize molecular mechanisms and their downstream effects, alterations in cellular anatomy, cell-cell interactions and cellular responses to environmental signals. These include traditional cell-culture based experiments and immunofluorescence techniques, many of which have been extensively used to study specific cellular mechanisms (cell migration and proliferation, calcium responses, cytosolic volume changes, *etc.*) (Kang, Toma, Sipos, & Peti-Peterdi, 2008). Tissue clearing techniques have made it possible to accurately quantify changes in specific cell number in 3D (Puelles et al., 2016) while fluorescence techniques can also be applied to determine changes in rates of protein synthesis at the whole organ and animal level (Liu, Xu, Stoleru, & Salic, 2012). Thus, fluorescence microscopy can be used for quantitative readouts of cellular biology at a cellular as well as tissue level.

5.2.1 OPP assay to measure protein synthesis—A cellular process that is fundamental to all cell types is regulated gene expression *via* gene transcription culminating in protein synthesis. Rates of translation and protein synthesis are an important metric to ascertain cellular biology and quantification of these parameters is also useful in determining cellular biology and physiological function. The development of a novel fluorescence method-O-propargyl puromycin (OPP)-based quantification of protein synthesis has now made it possible to visualize and quantify changes in rates of protein synthesis in the kidney *in situ* at a cellular and tissue level (Liu et al., 2012).

Developed in 2011 by Liu et al., OPP is an alkyne analogue of the antibiotic puromycin that is incorporated into the nascent polypeptide strand being synthesized by the ribosomes. As described by the authors, OPP offers several advantages over other methods to measure translation and rate of protein synthesis. Previous methods relied on the incorporation of modified analogues of amino acids, but these methods are limited to cell-culture systems and cannot be used in whole animals. Using fluorescently tagged copper azides to visualize OPP incorporation in the nascent polypeptide chain, this method makes it possible to accurately

image and quantify nascent proteins in cells as well as tissues (Liu et al., 2012). Taking advantage of this fluorescence method to visualize translation in tissues, our laboratory recently adapted this technique to measure rate of protein synthesis in various renal cell types in the kidney tissue *in situ* under different physiological conditions.

5.2.1.1 Protocol: The following protocol describes an approach to measure overall rates of protein synthesis in the kidney tissue of mice using OPP incorporation and fluorescence microscopy. This protocol can also be used to study translation rates and patterns in other tissues or in cell-culture based systems. The final steps of development involve the use of the Click-iT Plus OPP Alexa Fluor 488/594 Protein Synthesis Assay Kit (ThermoFisher Scientific, Waltham, USA) in which the Alexa Fluor 488 or Alexa Fluor 594-tagged copper azides (click chemistry) respectively, help to visualize the OPP incorporation in the nascent polypeptides strands.

5.2.1.2 Materials: C57BL6 mice

O-propargyl puromycin

DMSO

PBS

Formalin

Ketamine/Xylazine solution

Click-iT Plus OPP Alexa Fluor 488/594 Protein Synthesis Assay Kit

27 gauge Insulin syringes

27 gauge needles

1cc syringes

VectaShield mounting medium

Glass slides

Coverslips

Surgical equipment, peristaltic pump

5.2.1.3 Method

1. Prepare a 20 mM solution of OPP (Mol. Wt. = 495.53 g/mol) by dissolving OPP in DMSO and then vortexing it. Make sure to aliquot the OPP solution in dark tubes protected from light at 4°C.
2. Carefully restrain a mouse and using an Insulin syringe, inject 30 µL of the OPP solution intraperitoneally.

3. After 1 h, inject a ketamine/xylazine solution (80 mg/kg body weight ketamine and 10 mg/kg body weight xylazine) intraperitoneally using a 27 gauge needle and 1cc syringe in order to anesthetize the animal.
4. Ensure that the animal is fully anesthetized by checking for a toe pinch-response and then carefully open the abdominal cavity by making a 5–6 cm incision using a clean surgical platform.
5. Make a small incision in the diaphragm using curved, blunt scissors and then completely open the diaphragm to expose the pleural cavity.
6. Carefully expose the heart tissue and then insert the needle of the peristaltic pump tubing into the left ventricle of the heart. Make sure that the tubing is free of bubbles and is completely filled with ice-cold PBS.
7. Make a small incision in the descending abdominal aorta using sharp scissors and then open the outlet port of the peristaltic pump to perfuse the animal slowly with ice-cold PBS.
8. Once the animal is completely perfused (as observed by the clearing of the liver and the kidneys), close the outlet port of the peristaltic pump.
9. Switch the PBS solution to ice-cold formalin solution and perfuse the animal for about 2 min.
10. Carefully harvest the kidney tissue and any other organs as required.
11. Incubate the organs in formalin for 2 h at room temperature or at 4°C overnight.
12. Switch the organs to a 70% ethanol solution and prepare paraffin-embedded tissue blocks by following several steps of ethanol dehydration.
13. Cut 8–10 μ m thick tissue sections on glass slides and de-paraffinize them using xylene and a series of increasing concentrations of ethanol.
14. Following de-paraffinization, follow the protocol described in the Click-iT Plus Alexa Fluor 488/594 Protein Synthesis Assay kit to develop the tissue.
15. Mount the slides using VectaShield mounting medium and a coverslip.
16. Using a fluorescence confocal microscope, image the slides at 488 nm (519 nm emission) or 594 nm excitation wavelength (615 nm emission) as required.
17. In order to quantify the rate of translation, place an ROI and measure the fluorescence intensity in the red and green channel. Using a ratiometric approach compare the mean fluorescence intensity to the background/autofluorescence intensity.

For example, we compared rates of protein synthesis in C57BL6 mice treated with a regular diet or a salt deficient diet combined with Enalapril (an ACE inhibitor). As seen in Fig. 5A, under control conditions, rates of protein synthesis are quite low except in collecting duct cells and in some podocytes. However, after treatment for 14 days, there is a significant increase in the overall rate of protein synthesis as visualized by an increase in red fluorescence intensity (Fig. 5B and C). For quantification, all tissue sections were imaged

using the same imaging settings and the red fluorescence intensity in each ROI was normalized to the green autofluorescence in the same ROI using the Leica LAS X relative fluorescence intensity calculator. Thus, the OPP method allows us to visualize patterns of protein synthesis in single cells at a whole animal level and quantify changes in protein synthesis rates in response to alterations in dietary conditions or any other stimulant.

5.3 Cell-specific marker based quantification of cell number

A major limitation in cell-specific marker-based quantification of cell density on tissue samples is the limited tissue depth of paraffin or frozen tissue sections. In order to accurately quantify changes in cell number, it is necessary to analyze cell numbers in 3D. Earlier stereologic methods to quantify changes in cell number involved serial sectioning of tissue slices followed by quantification of cell number in each slice. The advent of tissue clearing techniques combined with immunofluorescence has now made it possible to accurately quantify changes in specific cell type numbers in response to injury or drug treatment (Puelles et al., 2016). Several tissue clearing techniques have been developed over the recent years—solvent-based methods, aqueous-based clearing, *etc.*—and these can be optimized according to the nature of the tissue, compatibility with immunostaining, *etc.* (Richardson & Lichtman, 2015). For example shown here, we have used established markers for podocytes like WT1 and p57 (Kaverina et al., 2017) and a newly identified nuclear marker for endothelial cells, Meis2 for endothelial cells (Karaikos et al., 2018) in conjunction with the widely used BABB clearing technique to perform 3D quantification of cell number in approximately 500 μm thick kidney slices of different transgenic mouse strains. Several software applications can be used to quantify cell numbers in an unbiased fashion including Leica LAS X and IMARIS. In case of IMARIS, after visualizing the entire glomerular volume in 3D (Fig. 6A), draw a ROI over the entire volume (Fig. 6B) and use the “Spots” feature to automatically threshold and count the exact cell number (Fig. 6C).

Another important parameter used to determine the viability of a cell is its ability to replicate, *i.e.*, cell proliferation. There are several markers for cell proliferation including cell cycle markers like PCNA and Ki67, DNA synthesis markers like BrdU and EdU as well as cell metabolism assays like MTT assay. Using markers for DNA synthesis like EdU or BrdU is one of the most accurate methods to visualize proliferation at a cell or tissue level. These are thymidine analogues that are incorporated in the DNA of a dividing cell instead of thymidine and this can then be visualized using an antibody or fluorescently-tagged copper azides (click chemistry). The advantage of using EdU over BrdU is that EdU can be developed using click chemistry and does not require DNA denaturation, thus making it amenable for *in vivo* use (Salic & Mitchison, 2008). Using EdU and click chemistry-based methods, we determined cumulative changes in renal cell proliferation over 5 days in C57BL6 mice either on a regular diet (Fig. 6D) or a salt deficient diet combined with Enalapril (an ACE inhibitor) for 14 days (Fig. 6E). For quantification, at least 10 fields per animal were imaged and quantified (Fig. 6F). Mice treated with the diet had a significantly increased rate of cell turnover as compared to mice on control diet. The advantage of using EdU over PCNA or Ki67 is that the latter provide a static picture of cell proliferation while the former presents a picture regarding the cumulative and dynamic nature of proliferation and remodeling. Additionally, we have also used the click chemistry-based EdU method

along with the BABB clearing technique to quantify the number of proliferating cells in 3D in 500 μm thick formalin-fixed kidney tissue slices. Thus, tissue clearing techniques combined with immunofluorescence labeling represent the most accurate method to quantify specific cell numbers for comparative analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIG. 1.

AIW implantation for serial intravital MPM. (A, B) Representative images of AIW used for serial MPM highlighting the customized titanium ring with the fitted coverslip (A) and the groove (B). (C) Representative image of a C57BL6 mouse after surgical implantation of AIW on the left kidney. (D) Tile scan image map of the entire available kidney surface of a Pod-Confetti mouse as visualized *via* the AIW. Tile scan image consisting of 15×15 individual full xy frames (using a $40\times$ water immersion objective) was generated by the tile scan function of Leica LAS X software and by using a motorized stage. A two-channel preview (one GFP/YFP and one RFP) of the four confetti channels is shown. Region of interests (ROIs) are highlighted by circles and are annotated for easy identification of different glomeruli.

FIG. 2.

Schematic illustration of single cell migration and fate tracking using various applications of serial MPM. Genetic identification and fate tracking of different single renal cells or cell populations including podocytes and renin-lineage cells is made possible using unique multi-color fluorescent tags (*e.g.*, using Confetti construct consisting of membrane-targeted CFP (blue), nuclear-targeted GFP (green), cytosolic YFP (yellow) or RFP (red)) expressed by the cells in lineage-specific transgenic mouse models. Intravital imaging of the same area and tissue volume of the glomerulus over time allows the tracking of alterations in cell distribution and migration patterns (illustrated by arrows), and the dynamics of tissue turnover in response to injury or stress. Serial MPM-based cell fate tracking can also be complemented by functional assessment of hemodynamics, filtration rates and albuminuria. Serial MPM is a powerful tool to visualize and quantitate alterations in the renal tissue at a single cell and nephron level, thus overcoming limitations of tissue heterogeneity.

FIG. 3.

Representative serial MPM images of single cell fate tracking using fluorescent reporter mouse models. (A) Representative single projection image of multiple optical sections (z-stack, Supplementary Video 1 in the online version at <https://doi.org/10.1016/bs.mcb.2019.04.013>) of a Pod-Confetti mouse glomerulus. Single podocytes can be identified based on their unique Confetti color (either blue/green/yellow/red). (B,C) Representative images of the same glomeruli (G1–G3) of a Pod-Confetti mouse visualized by serial MPM after AIW implantation (B) and 6 days later (C). The same blue (CFP+), green (GFP+), yellow (YFP+), and red (RFP+) podocytes can be re-identified in consecutive imaging sessions (arrows). (D) Representative single projection image of multiple optical sections (z-stack, Supplementary Video 2 in the online version at <https://doi.org/10.1016/bs.mcb.2019.04.013>) of a Ren1d-Confetti mouse glomerulus. (E,F) Representative images of the same glomeruli (G1–G2) of a Ren1d-Confetti mouse visualized by serial MPM after AIW implantation (E) and 5 days

later (F). Changes in the relative position of the same blue (CFP+), yellow (YFP+), and red (RFP+) parietal epithelial cells can be tracked in consecutive imaging sessions (arrows). Plasma was labeled with iv injected Alexa680-BSA converted to grayscale in the images. Bars are 25 μm .

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FIG. 4.

Demonstration of cell calcium measurements with serial MPM in podocytes *in vivo* and their changes in response to injury. (A, B) Representative MPM images of the same *xy* plane in the same kidney of a Pod-GCaMP5/Tomato mouse receiving L-NAME (1 g/L in drinking water *ad libitum*) at baseline (A) and 14 days after single Adriamycin (ADR 12 mg/kg body weight iv) injection (B). Arrows highlight podocytes in which GCaMP5 fluorescence intensity (green/yellow) increased between time points compared to the steady levels of Tomato (red), reflecting elevations in cell calcium. (C) Scatter plot summary of changes in podocyte intracellular calcium at baseline and 14 days after ADR injection. Each data point represents a ratio of the GCaMP5 (G5) to Tomato (T) intensity of a single podocyte. Data represents mean $\pm$ SEM. L-NAME group: $n = 3$; L-NAME + ADR group: $n = 4$, >10 podocytes/animal, unpaired *t*-test, ns: not significant, **** $P < 0.0001$.

FIG. 5.

Fluorescence-based quantification of overall rate of single cell protein synthesis in the kidney *in situ*. (A, B) Representative fluorescence images of kidney tissue of C57BL6 mice treated with control diet (A) and salt deficient (NS) diet with angiotensin converting enzyme inhibitor (ACEi) (B) after OPP injection (20 mM OPP ip for 1 h). Patterns of protein synthesis are visualized in red. ROIs highlighted in circles demonstrate collecting duct segments. (C) Scatter plot summary of OPP relative fluorescence intensity as a measure of protein synthesis. Relative fluorescence intensity is calculated as a ratio of red (594/615 nm excitation/emission) to green (488/519 nm excitation/emission) fluorescence intensity in each ROI using Leica LAS X software. Data represents mean $\pm$ SEM ($n = 3$ each, 10 fields/animal, 5 ROIs/field, unpaired t -test, $*P = 0.0113$).

FIG. 6. Cell-specific marker based quantification using fluorescence techniques. (A–C) Stepwise depiction of cell-specific marker based quantification using IMARIS software (WT1 for podocytes). After rendering the multiple optical scans (z-stacks) in 3D in IMARIS (A), a 3D ROI was defined (B). “Spots” tool was used to obtain a count of the total number of WT1⁺ cells within the ROI. Each white spot represents a single nucleus (C). (D, E) Representative fluorescence images of kidney tissue of C57BL6 mice treated with control diet (D) and NS +ACEi diet (E) after five consecutive EdU injections (100 μ L of 1 mg/mL ip every day). EdU⁺ nuclei appear in red. (F) Scatter plot summary of total number of EdU⁺ cells/field. Data represents mean $\pm$ SEM ($n = 4$, 10 fields/animal, unpaired t -test, ** $P = 0.0024$).