# Long wavelength multiphoton excitation is advantageous for intravital kidney imaging

C.D. Schuh[1,5], D. Haenni[1,2,5], E. Craigie[1], U. Ziegler[2], B. Weber[3], O. Devuyst[4] and Andrew M. Hall[1]

*[1]Institute of Anatomy, University of Zurich, Zurich, Switzerland; [2]Center for Microscopy and Image Analysis, University of Zurich, Zurich, Switzerland; [3]Institute of Pharmacology and Toxicology, University of Zurich, Zurich, Switzerland; and [4]Institute of Physiology, University of Zurich, Zurich, Switzerland*

**Intravital multiphoton microscopy is a powerful tool to study kidney physiology in living animals. However, certain technical issues have curbed its usage to date, including limited depth of tissue penetration and high background emission of endogenous signals. Most previous studies have used the excitation range 700–1000 nm. Since newer longer wavelength excitation lasers may provide solutions to these problems we constructed a microscope coupled to a laser tunable up to 1300 nm and optimized for kidney imaging. This set-up offers substantial advantages for intravital studies, especially when coupled with newly available far-red probes. First, the background at longer wavelengths is markedly reduced, thus increasing the signal to background ratio. Second, the depth of tissue penetration is significantly increased, enabling detailed imaging of previously inaccessible structures, such as deeper glomeruli. Third, using a combination of two- and three-photon excitation, multiple different fluorescent probes can be imaged simultaneously in the same animal, with clear spectral separation. Application of these techniques helped visualize pathological aspects of tubular cell function in a well-established model of acute kidney injury (maleate toxicity). Thus, utilizing long wavelength excitation offers substantial advantages for intravital kidney imaging, which together enhance the capabilities of this powerful and increasingly used research technique.**

*Kidney International* (2016) **89,** 712–719; http://dx.doi.org/10.1038/ki.2015.323

KEYWORDS: acute kidney injury; intravital imaging; multiphoton microscopy



**Correspondence:** *Andrew M. Hall, Institute of Anatomy, University of Zurich, Winterthurerstrasse 190, Zurich 8057, Switzerland. E-mail: andrew.hall@uzh.ch*

[5]These authors contributed equally to this work.

**Received 26 June 2015; revised 14 August 2015; accepted 3 September 2015; published online 28 October 2015**

Multiphoton microscopy (MPM) is an imaging technique that has significant advantages over standard confocal microscopy for intravital studies, including increased depth of tissue penetration and decreased phototoxicity.[1] It offers a level of resolution that is currently unmatched by other intravital imaging techniques, and is thus a powerful tool to study cellular and organelle function in intact organs in living animals. MPM has been used in numerous previous studies to investigate kidney function and renal disease processes,[2,3] and has yielded fundamental insights into how the kidney works, some of which have challenged conventional paradigms.[4] However, significant technical limitations remain when using MPM for intravital kidney imaging. For example, the depth of tissue penetration is limited to the outer cortex and the vast majority of glomeruli cannot be routinely visualized in mice.[5,6] Moreover, signals emitted by fluorescent probes can be contaminated or masked by endogenous autofluorescence, which is relatively bright in the kidney. Therefore, there is a need to develop and utilize new technologies that offer some practical solutions to these problems, in order to realize the true potential of MPM for kidney research.

Most previous studies involving intravital MPM have used excitation wavelengths in the range of 700–1000 nm. A new generation of ultrafast tunable infrared lasers is now available, which provide a laser output that is stable up to about 1300 nm. Extended wavelength excitation light offers several theoretical advantages for intravital MPM.[7] Firstly, longer wavelength light is less scattered and absorbed in biological tissues, potentially increasing the depth of imaging.[8] Secondly, recently developed far-red fluorophores can be excited, which emit at wavelengths distinct from autofluorescence signals in the blue to red ranges.[9] Thirdly, important biological structures can be visualized using 'label-free' techniques, such as second and third harmonic generation signals.[10,11] Lastly, it has been suggested that phototoxicity in biological tissues is reduced at longer wavelengths.[11]

We have recently constructed an advanced microscope that is coupled to an extended wavelength excitation laser (tunable from 680 to 1300 nm), and is equipped with four distinct detector channels to simultaneously detect signals from blue to the far-red range (330–735 nm). In this study, we show how this set-up offers significant advantages for intravital kidney imaging in mice, and increases the amount of information that can be simultaneously acquired in temporal, spectral, and spatial dimensions from disease models.

## RESULTS

### Endogenous autofluorescence in the kidney is lower at longer wavelength excitation

When imaged, using MPM kidney tissue emits a high level of endogenous signals, comprising both autofluorescence and second harmonic generation, but these have not previously been systematically characterized. We assessed the tissue depth at which endogenous signals emitted by the kidney could be detected in our accessible spectral range, at various different excitation wavelengths from 700 to 1300 nm (Figure 1). As expected, we found a high emission in the blue/green/red ranges, which could be detected at depths down to 100 μm below the kidney capsule. However, the signal intensity decreased markedly in the blue/green/red ranges when the excitation wavelength was increased to greater than 1000 nm, although there was a slight increase in the blue signal at 1250 nm, most likely due to three-photon excitation and/or third harmonic generation. There was generally less endogenous signals detected in the far-red range at all excitation wavelengths.

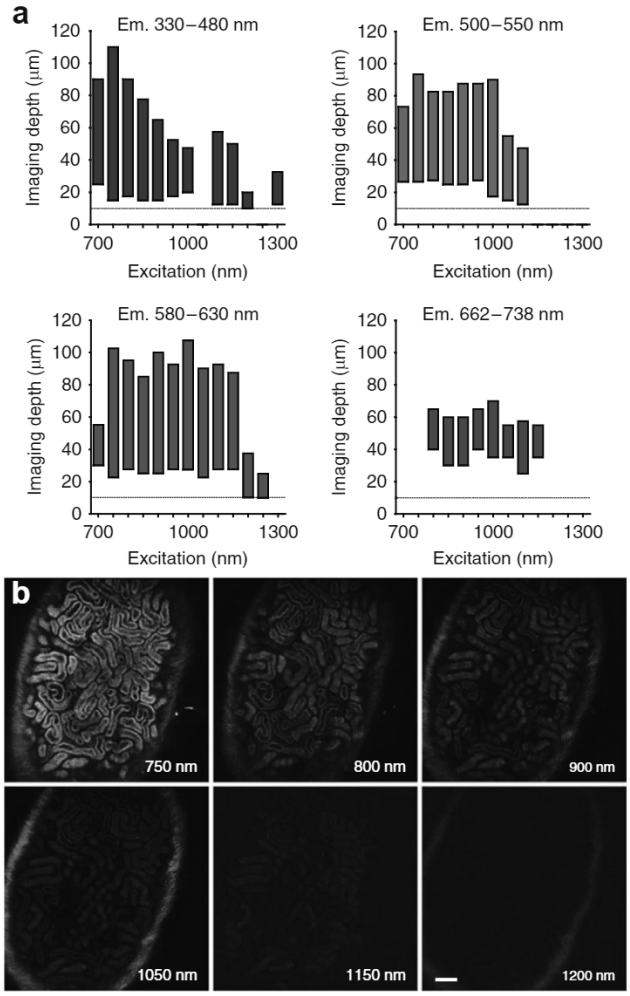


**Figure 1 | Endogenous autofluorescence in the kidney is lower at longer wavelength excitation. (a)** The depth at which endogenous signals could be structurally resolved in the kidney was assessed in the blue (330–480 nm), green (500–550 nm), red (580–630 nm), and far-red (662–738 nm) ranges, at excitation wavelength intervals of 50 nm between 700 and 1300 nm. The black lines indicate the renal capsule. Endogenous signals were considerably lower in all channels at longer wavelength excitation, and were also relatively low in the far-red channel across all excitation wavelengths. **(b)** Example images at different excitation wavelengths, showing the renal cortex and surrounding capsule. Collagen fibers in the capsule were visible with second harmonic generation. Scale bar = 100 μm.

### Signal-to-background ratio is higher in the far-red range

Selective imaging of fluorescent-labeled structures at high resolution *in vivo* requires a high signal-to-background ratio. A number of far-red fluorophores are now available, which can be excited with longer wavelength lasers. Given the notable decrease in background signal emission that we observed in the kidney when imaging at wavelengths greater than 1000 nm, we hypothesized that the signal-to-background ratio would be higher with far-red probes. To investigate this, we injected animals intravenously with albumin, labeled with either Alexa Fluor 488 (excited at 950 nm) or Alexa Fluor 647 (excited at 1150 nm). As expected, both probes were rapidly visualized in the renal vasculature, and there was also some evidence of apical uptake in proximal tubule (PT) cells (Figure 2). When corrected for laser power, the emitted intensity of both probes was similar. However, because the background signal was much lower at 1150 nm, this resulted in a much higher signal-to-background ratio. Thus, the usage of far-red probes and extended wavelength excitation allows a much cleaner signal to be obtained when imaging fluorescent-labeled molecules in the kidney *in vivo*.

### Depth of imaging is increased with longer wavelength excitation

Arguably the biggest limitation of MPM in the kidney to date has been the relatively poor depth of tissue penetration—in comparison to organs such as the brain—which has restricted imaging to the very superficial cortex. Importantly, the vast majority of glomeruli in mice lie too deep to be visualized,[4,6] and only very superficial glomeruli can routinely be studied.[12] Because of this, some researchers have resorted to innovative experimental measures to image deeper glomeruli, such as rendering kidneys hydronephrotic.[13] However, such approaches may induce fundamental changes to organ physiology and therefore confound the interpretation of data acquired. Limited depth of tissue penetration in the kidney is almost certainly due to scattering and absorption of both excitation and emission light, which is strongly wavelength dependent, so imaging at longer wavelengths should offer a distinct advantage. To perform a direct comparison, we simultaneously injected animals intravenously with identical concentration of a green–labeled albumin (Alexa 488 nm, excited at 950 nm) and a far red–labeled albumin (Alexa 647, excited at 1180 nm). We found that we were able to image considerably further into kidney tissue using the latter (Figure 3). Importantly, with the far-red albumin we could for the first time visualize deeper glomeruli, and could perform detailed imaging of glomerular structures, such as arterioles,

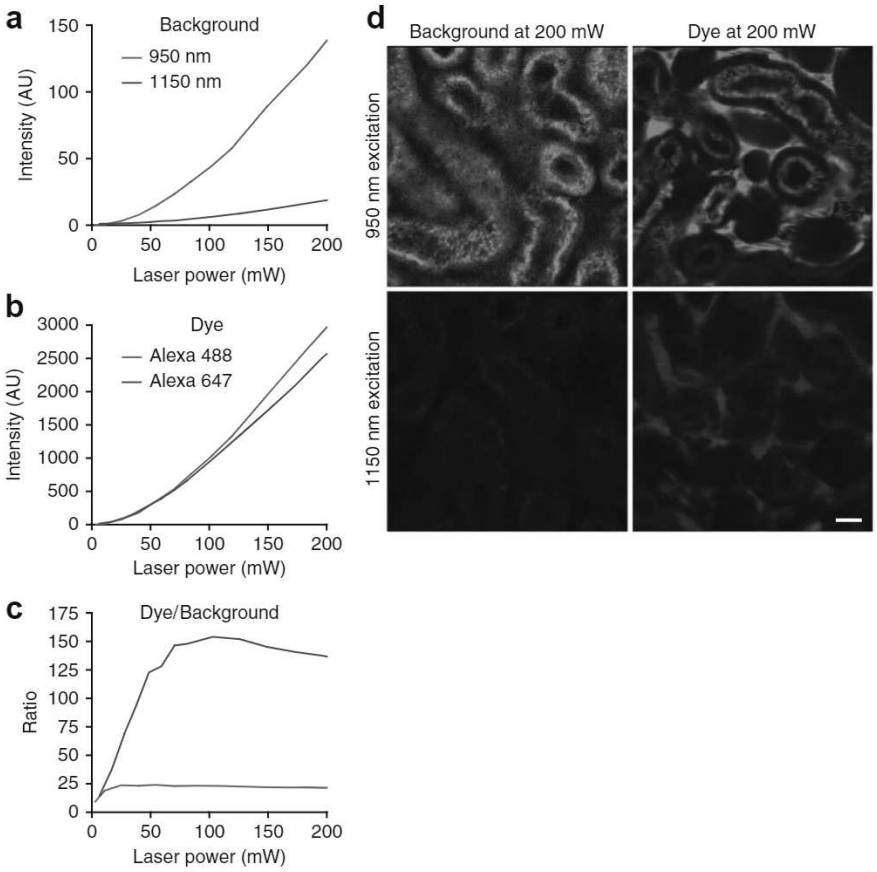


**Figure 2 | Signal-to-background ratio is higher in the far-red range. (a)** Background signal intensity increased in both green and far-red channels with increasing laser power; however, the magnitude of the increase was much greater in the former than in the latter. **(b)** Following simultaneous intravenous injection of Alexa 488–labeled albumin and Alexa 647–labeled albumin, the emitted signal from each probe was similar with increasing laser power. **(c)** Owing to the lower background signal, the dye/background ratio was much higher for Alexa 647 than for Alexa 488. **(d)** Representative images are depicted of background and dye signals at 200 mW laser power. Imaging was performed at a depth of 50 μm. Scale bar = 20 μm.

capillaries, and Bowman's space, along with the initial S1 portion of the PT (Figure 4a–d). Moreover, due to a combination of the stability of the tissue and the depth of imaging possible with this set-up, we were also able to acquire sequential stacks of images at 1-μm intervals in the *z*-plane, which were then amenable to de-convolution and three-dimensional (3D) rendering image analysis techniques, resulting in detailed reconstruction of the 3D architecture of the glomerulus (Figure 4e and Supplementary Movie S1 online). Furthermore, sufficient resolution was achieved to image dynamic processes within glomeruli in real time, such as the movement of individual blood cells through capillaries (Supplementary Movie S2 online). Thus, the combined usage of extended wavelength excitation and far-red probes provides an effective solution to what was previously a major technical obstacle in intravital kidney imaging.

### Extended wavelength excitation allows simultaneous acquisition of multiple readouts of cellular function in acute kidney injury

Our custom-designed MPM is equipped with four detectors that can acquire signals in the range of 330–735 nm, thus allowing simultaneous imaging of up to four different exogenous fluorophores with minimal signal overlap (Figure 5). To demonstrate the translational potential of this approach, we performed experiments to investigate how multiple readouts of tubular cell function are altered in a well-established model of acute kidney injury (AKI). First, in control animals, we determined that we could simultaneously image markers of fluid phase endocytosis (Cascade blue–labeled 10 kDa dextran), mitochondrial morphology (Rhodamine 123), lysosomal acidification (Lysotracker), and receptor-mediated endocytosis (Alexa 647–labeled albumin) in PTs *in vivo* (Figure 6). To induce AKI, we performed intraperitoneal injections of sodium maleate (600 mg/kg) 1 h prior to imaging, which is highly toxic to renal tubules in rodents.[14] Multiple striking abnormalities in PT structure and function were observed in mice post injection of maleate. PT cells appeared generally flattened, and there was no detectable uptake of dextran and albumin, implying complete inhibition of both fluid phase and receptor-mediated endocytosis. However, both dextran and albumin were clearly visualized in tubular lumens, consistent with maintenance of glomerular filtration. Mitochondria in PTs were noticeably shortened,

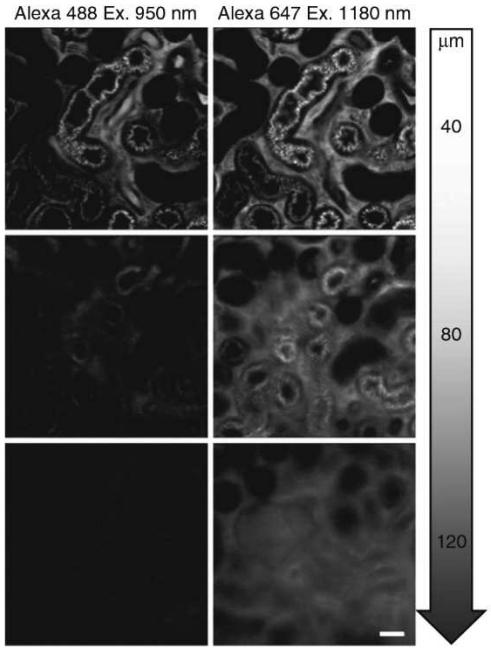


**Figure 3 | Depth of imaging in kidney tissue is increased with longer wavelength excitation and far-red dyes.** To directly compare relative depth of tissue penetration at shorter and longer wavelengths, Alexa 488–labeled albumin and Alexa 647–labeled albumin were co-injected into the same mouse and were excited at 950 and 1180 nm, respectively. Serial images were performed in the *z*-plane at 1 μm intervals from the surface into the kidney to investigate how the signal intensity changed in each channel with increasing tissue depth. Representative images are displayed showing that the depth of imaging possible was greater with Alexa 647. Scale bar = 30 μm.

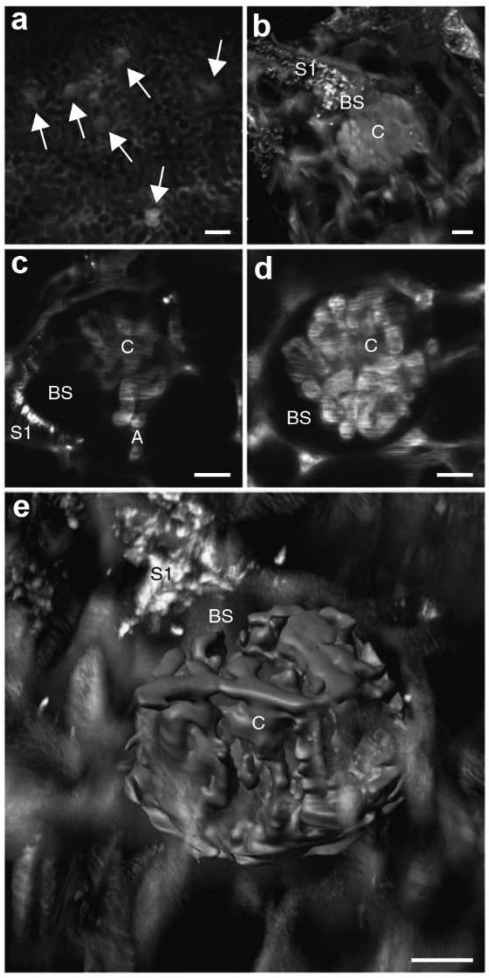


**Figure 4 | Intravital imaging of glomeruli in the mouse.** Increased depth of penetration with far-red probes and long wavelength excitation enabled detailed imaging of glomeruli in mice. **(a)** Multiple glomeruli (arrows) were visualized with Alexa 647–labeled albumin excited at 1180 nm. **(b)** Three-dimensional (3D) *z*-stack of a single glomerulus, showing capillaries (C), Bowman's space (BS), and the early portion of the proximal tubule (S1). (**c** and **d**) Selected single plane images of the same glomerulus at a superficial (90 μm) **(c)** and deeper level (125 μm) **(d)** showing these structures in more detail, and also an arteriole (A) entering the glomerulus. **(e)** Three-dimensional architecture of the glomerular capillaries reconstructed from the *z*-stack using both image deconvolution and 3D rendering processes. Scale bar = 100 μm in **(a)**, 20 μm in all other images.

signifying damage to these organelles, upon which PT cells are dependent for ATP generation to drive solute transport. Moreover, acidified lysosomes were no longer visible in PTs. Finally, no abnormalities were observed in the vasculature, consistent with the notion that maleate toxicity is specifically targeted at kidney tubules.

In summary, these experiments show that intravital imaging with MPM and long wavelength excitation allows multiple important physiological readouts to be acquired simultaneously in animal models of AKI, which could provide important new insights into underlying pathogenic mechanisms.

## DISCUSSION

Intravital imaging with MPM is a powerful tool to study physiology and pathophysiology at a cellular level in intact functioning organs in living animals. However, certain technical and biological issues have constrained its application in the kidney to date, including limited depth of tissue penetration and a high level of background signals. Newly available longer wavelength excitation lasers and far-red probes offer potential solutions to some of these problems. Using a custom-built microscope coupled to a laser fully tunable from 700 to 1300 nm, we have shown that the achievable depth of imaging is significantly increased at longer wavelengths, allowing for the first time visualization of deeper glomeruli in mice. Moreover, we have found that endogenous signals are significantly lower at excitation wavelengths greater than 1000 nm, resulting in a much greater signal-to-background ratio when working with far-red probes, in comparison with standard green fluorophores. We therefore believe that these technical advancements will significantly improve the quality of imaging achievable in future studies of the kidney.

The kidney emits a high level of endogenous signals when imaged using MPM, and we have performed the first systematic study of these spanning all the visible spectrum from 330 to 735 nm, at excitation wavelengths from 700 to

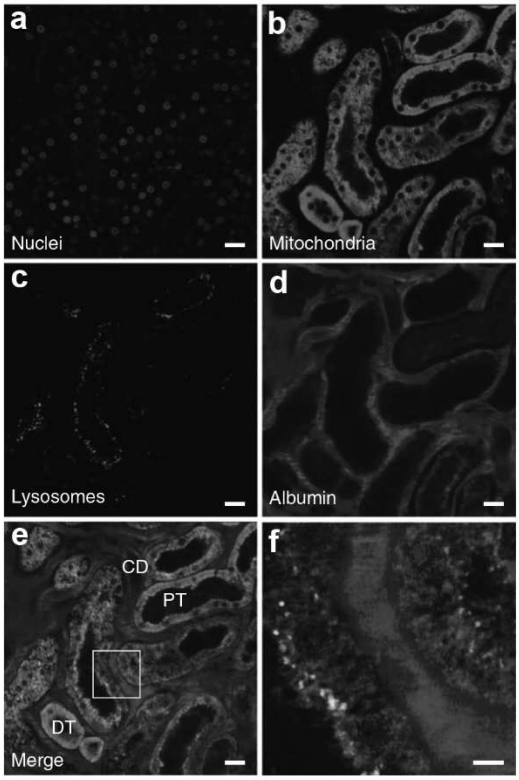


**Figure 5 | Simultaneous imaging of 4 different fluorescent probes in the kidney.** By using long wavelength excitation and a custom-designed detector system, it was possible to simultaneously image up to 4 different probes in the kidney. (**a** and **d**) Hoechst was used to label nuclei **(a),** rhodamine 123 was used to label mitochondria **(b),** Lysotracker was used to label acidified lysosomes **(c),** and Alexa 647–labeled albumin was used as a vascular marker and also as a substrate for receptor-mediated endocytosis **(d)**. All 4 probes were excited simultaneously at a single wavelength of 1120 nm. (**e** and **f**) Overlay images showing different tubule types, including proximal tubules (PTs), distal tubules (DTs), and collecting ducts (CDs) **(e),** and detailed subcellular structure of the PT **(f)**. Scale bars = 20 μm in **(a–e)** and 5 μm in **(f)**.

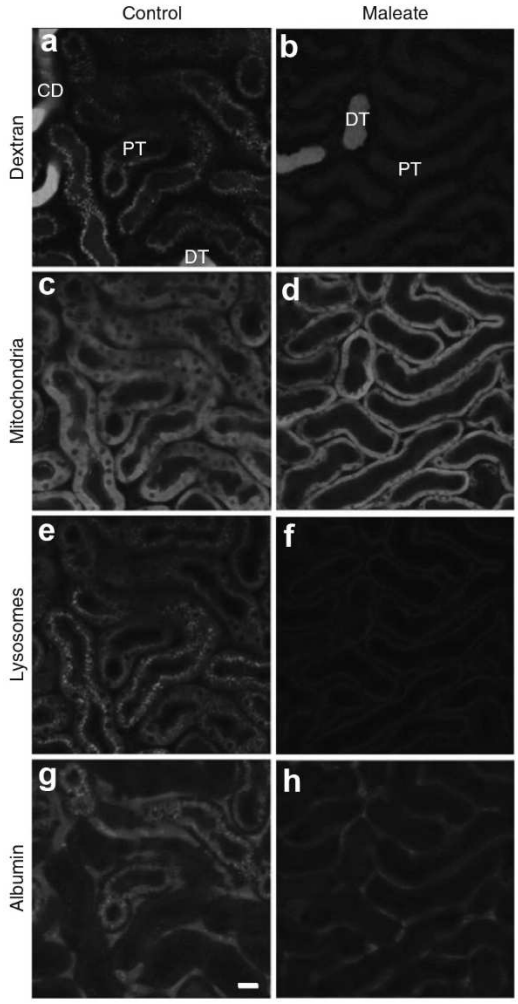


**Figure 6 | Intravital imaging of tubular cell function in maleate-induced acute kidney injury.** (**a, c, e,** and **g**) Representative images from a control mouse showing uptake of a 10 kDa Cascade Blue–labeled dextran in proximal tubules (PTs), a marker of fluid phase endocytosis **(a),** mitochondrial energization and morphology (rhodamine 123) **(c),** lysosomal acidification (Lysotracker) **(e),** and albumin uptake, a marker of receptor-mediated endocytosis in the PT **(g)**. Some filtered dextran was also visible in the lumens of distal tubules (DTs) and collecting ducts (CDs). (**b, d, f**, and **h**) Corresponding images from a mouse injected with sodium maleate 1 h prior to imaging. Uptake of dextran into PTs was markedly reduced, but dextran was still visible in DT lumens, implying preservation of glomerular filtration **(b)**. Mitochondria were shortened, but still appeared energized **(d)**. Acidified lysosomes were no longer visible in PTs **(f)**. Albumin was still visible in the vasculature, but no uptake was apparent in PTs **(h)**. Scale bars = 30 μm.

1300 nm. As expected, we found a high level of emission in the blue, green, and red channels throughout the excitation range used in previous studies of the kidney (700–1000 nm). These endogenous signals consist of both autofluorescence and second harmonic generation and can provide some useful structural and functional information, pertaining to structures such as mitochondria, lysosomes, the apical membrane brush border of PTs, and collagen fibers.[15,16] However, they can also potentially contaminate and mask signals of interest emitted by exogenous probes. By increasing the excitation wavelength to >1000 nm, we found that the intensity of autofluorescence decreased markedly in all channels, while in the far-red channel it remained relatively low across all excitation wavelengths. We observed the emergence of some blue signals at excitation >1000 nm, which most likely represent three-photon excitation and third harmonic generation.[17]

We do not know at present the reason(s) why endogenous signals are so much lower in intensity at longer wavelength excitation. Presumably, this reflects the fact that naturally occurring fluorophores in the kidney are more optimally excited at shorter wavelengths. Alternatively, it could be explained in part by a decrease in available laser power at longer wavelengths; however, the power in this range was still easily sufficient to excite far-red probes, and the net result was a greatly increased signal-to-background ratio. In practical terms, this means that when working with far-red probes cleaner signals can be acquired in the kidney (relative to shorter wavelength fluorophores), which can be more

confidently ascribed to the fluorophore, rather than any other endogenous sources. This has significant potential advantages for future imaging studies. For example, detailed studies of solute uptake and trafficking in the PT endosomal–lysosomal system using labeled proteins could be enhanced by avoiding the very bright autofluorescence signals typically emitted by lysosomes in the green/red ranges. Moreover, it has been suggested that longer wavelength light is better tolerated by biological tissues, increasing the capabilities to perform repetitive dynamic imaging without inducing photo-damage.[11]

Perhaps the biggest limitation to date of intravital kidney imaging with MPM has been the relatively poor depth of tissue penetration, which has constrained studies to the very outer layer of the cortex. Crucially, the vast majority of glomeruli in mice are situated beyond the limit at which structures can be clearly resolved,[4,6] and studies to date have been limited to very superficial glomeruli,[12] which might not be representative of the greater population. Rendering kidneys hydronephrotic enhances visualization of glomeruli *in vivo*,[13] but while this is an innovate solution, there is obviously a risk that such procedures may introduce confounding factors into experiments. Superficial glomeruli can be visualized in certain species of rat, but these animals have a preponderance to develop complications such as proteinuria and hypertension.[4,18] Moreover, most existing transgenic and disease models relevant to the kidney are murine. Thus, there is a pressing need to increase the depth of tissue penetration possible in the kidney. The biggest factor that limits this is almost certainly light scattering and absorption, which is strongly wavelength dependent.[1] We have found that increasing the excitation wavelength to >1000 nm significantly increases the depth to which exogenous probes can be imaged in the kidney. Importantly, by using a far red–labeled albumin, we were able to visualize deeper glomeruli in mice for the first time, which represents a significant technical breakthrough in the field. Moreover, due to the combined advantages of a customized set-up (including tissue stability and increased imaging depth), we were able to reconstruct the 3D architecture of glomeruli and related structures with a high level of detail. By performing these studies in living animals rather than fixed specimens we could avoid the possibility of structural artifacts caused by tissue fixation techniques.

Although we have now shown that depth of imaging in the kidney is significantly increased with longer wavelength excitation, it still remains considerably less than that has been reported in some other organs, such as the brain.[1] The depth of imaging that can be achieved in tissues is dependent on a number of factors in addition to excitation wavelength, including detector sensitivity, laser power, and the quantum yield and emission wavelength of the fluorescent probe. In our study we used highly sensitive gallium-arsenide-phosphide detectors, which could have been partly responsible for the increased imaging depth achieved; however, when we performed side-by-side comparisons between green and far-red probes using the same detectors, we found that long wavelength light still provided a significant advantage. The reason(s) why the kidney is a relatively optically opaque organ are unclear, but we speculate that they could relate to the potential light scattering effects of dynamic blood flow in superficial renal vessels. Adaptive optics approaches might permit deeper imaging in the future,[19] but successful implementation of such techniques in highly dynamic systems such as functioning organs will be a considerable technical challenge.

The major translational strength of intravital imaging with MPM lies in the ability to study dynamic cellular events in disease processes in ways that are not otherwise possible.[16] Other imaging techniques, such as single-photon emission computed tomography, can also provide useful information about tubular function in rodents,[20] but do not offer the same level of cellular and subcellular resolution as MPM. We have shown that by using longer wavelength excitation and a highly sensitive custom-designed detector system, up to four different fluorophores can be imaged simultaneously, and thus multiple functional readouts can be acquired concurrently from kidney cells. This is made possible by the fact that many fluorophores have relatively broad two-photon excitation spectra.[10] Theoretically, it is possible to image multiple signals with any excitation wavelength,[10] and this has been demonstrated previously in the kidney;[12] however, by extending excitation to the far-red range, we were able to ensure that the four channels were spectrally well separated (reducing the amount of cross-talk between the channels), and could also perform three-photon excitation of blue probes.

To demonstrate the translational potential of this approach, we used a well-established model of AKI (maleate toxicity). We found that maleate induced near complete inhibition of PT solute uptake via both fluid phase and receptor-mediated endocytosis. While it has been well documented previously that maleate causes renal Fanconi syndrome in rodents,[21] the magnitude of this effect was striking. Moreover, we also observed rapid shortening of mitochondria and a loss of lysosomal acidification in the PT. Although PT cells were markedly flattened post-maleate, we did not observe extensive cell shedding, which typically occurs during ischemic AKI, implying that maleate mainly produces functional changes in PT cells. Overall, our observations on the effects of maleate on PT cell ultrastructure were in agreement with findings from older studies using electron microscopy, which reported abnormalities in the appearance of mitochondria and the endosomal–lysosomal system.[22]

As we only acquired images at a single time point, we cannot ascertain from these experiments the temporal sequence of events caused by maleate. Previous studies have suggested that maleate toxicity is primarily targeted to mitochondria,[14] and that PT cells are almost entirely dependent on aerobic respiration for ATP generation;[23] thus, it is plausible that changes in solute uptake and lysosomal acidification could have occurred secondary to a decrease in intracellular ATP levels, leading to a decrease in endosomal/lysosomal

V-ATPase activity, but this will need to be investigated in future studies. Interestingly, although maleate induced changes in mitochondrial structure, it did not appear to cause these organelles to depolarize. This is in stark contrast to our findings in previous studies of ischemia–reperfusion induced AKI, where we found that mitochondria in the PT depolarize rapidly in response to cessation of blood flow.[15] Thus, there may be differences in how mitochondria respond to different insults *in vivo*, but this also requires further investigation. In the meantime, it seems apparent that intravital imaging with MPM offers the potential to further elucidate the cellular mechanisms underlying different types of AKI, and fill a vital gap in translational kidney research.

In summary, we have shown that utilizing a long wavelength excitation laser offers substantial practical advantages for intravital kidney imaging, which together significantly enhance the capabilities of this powerful and increasingly used research technique.

## MATERIALS AND METHODS

### Dyes and reagents

All dyes used in this study were purchased from Molecular Probes (Eugene, OR, USA) and all dyes were bolus injected intravenously at a maximum injection volume of 200 µl. The amount of injected dye were as follows: Hoechst 33342 4 mg/kg bodyweight (bw), Dextran Cascade Blue 10,000 MW 12.5 mg/kg bw, Rhodamine 123 1–1.5 mg/kg bw, Bovine Serum Albumin Alexa Fluor 488 conjugate 25 mg/kg bw, Lysotracker Red DND99 16–40 µg/kg, and Bovine Serum Albumin Alexa Fluor 647 7.5–25 mg/kg bw. Sodium maleate was purchased from Sigma-Aldrich (St Louis, MO, USA).

### Intravital imaging

All experiments were performed on 8- to 12-week-old male C57Bl/6JRj mice (supplied by Janvier, Le Genest, France), and were performed in accordance with the regulations of The Zurich Cantonal Veterinary Office. Animals were anesthetized with 2.5% isoflurane (Attane, Provet AG, Switzerland) in 100 ml/min oxygen, and the left kidney was externalized for imaging using previously established protocols.[2] The internal jugular vein was cannulated to allow intravenous injections of dyes and reagents. Animals were placed on a custom-built temperature-controlled stage, specially designed to dampen transmission of movements to the kidney (e.g., from breathing), and thus reduce movement artifacts while performing dynamic imaging of cellular events. Body temperature was monitored throughout experiments.

All imaging was performed on a custom-built multiphoton microscope operating in an inverted mode.[24] A broadband tunable laser (InSight DS Dual, Spectraphysics, Santa Clara, CA, USA) was used as an excitation source. The excitation laser power was modulated using an electrooptical modulator (350-80, Conoptics, Danbury, CT, USA). A × 25 water immersion objective with a numerical aperture of 1.05 and a working distance of 2 mm was used for imaging (Olympus, Tokyo, Japan). For separating the excitation from the emission light, a 665-nm single-edge dichroic beamsplitter (AHF Analysentechnik AG, Tübingen, Germany) was used for most experiments. This beamsplitter was chosen to be able to use the full tunable range of the laser system (680–1300 nm). By allowing short wavelength excitation, this filter ultimately limited the far-red detection at around 675 nm. To circumvent this limitation and therefore to expand the range of observable fluorescence further into the far-red region we also used an alternative dichroic with a reflection up to 735 nm (AHF Analysentechnik AG). The emitted fluorescence light was collected in a non-descanned epifluorescence detection mode. The detection unit was outfitted with four highly sensitive gallium-arsenide-phosphide photomultiplier tubes (Hamamatsu, Japan). The collected fluorescence light was split by three dichroic beam splitters: BS 488 (F38–488), BS 560 (F38–559), and BS 650 (AHF Analysentechnik AG). Following the dichroic beam splitters, the fluorescence light was further filtered by bandpass filters: 405/150, 525/50, 605/50, 700/75 (AHF Analysentechnik AG). To exclude excitation light on the detectors, 680/SP or 770/SP (AHF Analysentechnik AG), shortpass filters were placed in front of the bandpass filters and detectors. Image acquisition was controlled using the ScanImage 3.8 software package.[25] Further functionality such as controlling the detector gain and the movement of the custom-built microscope body were implemented in LabVIEW 12.0 (National Instruments, Austin, TX, USA). Images were converted to hyper-stacks and saved using FIJI image analysis software,[26] and further processing was performed using Imaris version 7.6.4 (Bitplane AG, Zurich, Switzerland).

For the assessment of depth of imaging of endogenous signals, serial *z*-stacks were recorded with intervals of 10 µm. The outer border of the renal capsule was taken as the starting point, and the qualitative criterion for maximum depth was the point at which structures within tubules could no longer be clearly resolved when the laser power was increased to 100%. The results presented are mean values from three animals.

## DISCLOSURE

All the authors declared no competing interests.

## ACKNOWLEDGMENTS

AMH is supported by The Swiss National Centre for Competence in Research (NCCR) Kidney Control of Homeostasis. OD is supported by a Swiss National Science Foundation project grant (310030 146490). The authors also acknowledge support from The Clinical Research Priority Program 'Molecular Imaging Network Zurich' and The Zurich Centre for Integrative Human Physiology.

## SUPPLEMENTARY MATERIAL

**Movie S1.** Three-dimensional imaging of the mouse glomerulus.
**Movie S2.** Dynamic imaging of the mouse glomerulus.
Supplementary material is linked to the online version of the paper at www.kidney-international.org.

## REFERENCES

1. Helmchen F, Denk W. Deep tissue two-photon microscopy. *Nat Methods*. 2005;2:932–940.
2. Ashworth SL, Sandoval RM, Tanner GA, et al. Two-photon microscopy: visualization of kidney dynamics. *Kidney Int*. 2007;72:416–421.
3. Peti-Peterdi J, Burford JL, Hackl MJ. The first decade of using multiphoton microscopy for high-power kidney imaging. *AJP Ren Physiol*. 2012;302:F227–F233.
4. Dickson LE, Wagner MC, Sandoval RM, et al. The proximal tubule and albuminuria: really! *J Am Soc Nephrol*. 2014;25:443–453.
5. Molitoris BA, Sandoval RM. Techniques to study nephron function: microscopy and imaging. *Pflugers Arch Eur J Physiol*. 2009;458:203–209.
6. 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.
7. Andresen V, Alexander S, Heupel W-M, et al. Infrared multiphoton microscopy: subcellular-resolved deep tissue imaging. *Curr Opin Biotechnol*. 2009;20:54–62.

8. Smith AM, Mancini MC, Nie S. Bioimaging: second window for in vivo imaging. *Nat Nanotechnol*. 2009;4:710–711.
9. Peti-Peterdi J, Kidokoro K, Riquier-Brison A. Novel in vivo techniques to visualize kidney anatomy and function. *Kidney Int*. 2015;88:44–51.
10. Zipfel WR, Williams RM, Webb WW. Nonlinear magic: multiphoton microscopy in the biosciences. *Nat Biotechnol*. 2003;21:1369–1377.
11. Friedl P, Wolf K, von Andrian UH, et al. Biological second and third harmonic generation microscopy. *Curr Protoc Cell Biol*. 2007; Chapter 4: Unit 4.15.
12. 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.
13. Devi S, Li A, Westhorpe CLV, et al. Multiphoton imaging reveals a new leukocyte recruitment paradigm in the glomerulus. *Nat Med*. 2013;19: 107–112.
14. Zager RA, Johnson ACM, Naito M, et al. Maleate nephrotoxicity: mechanisms of injury and correlates with ischemic/hypoxic tubular cell death. *Am J Physiol Renal Physiol*. 2008;294:F187–F197.
15. Hall AM, Rhodes GJ, Sandoval RM, et al. In vivo multiphoton imaging of mitochondrial structure and function during acute kidney injury. *Kidney Int*. 2012;83:72–83.
16. Hall AM, Molitoris BA. Dynamic multiphoton microscopy: focusing light on acute kidney injury. *Physiology (Bethesda)*. 2014;29:334–342.
17. Débarre D, Supatto W, Pena A-M, et al. Imaging lipid bodies in cells and tissues using third-harmonic generation microscopy. *Nat Methods*. 2006;3:47–53.
18. Remuzzi A, Puntorieri S, Alfano M, et al. Pathophysiologic implications of proteinuria in a rat model of progressive glomerular injury. *Lab Invest*. 1992;67:572–579.
19. Rueckel M, Mack-Bucher JA, Denk W. Adaptive wavefront correction in two-photon microscopy using coherence-gated wavefront sensing. *Proc Natl Acad Sci USA*. 2006;103:17137–17142.
20. Jouret F, Walrand S, Parreira KS, et al. Single photon emission-computed tomography (SPECT) for functional investigation of the proximal tubule in conscious mice. *Am J Physiol Renal Physiol*. 2010;298: F454–F460.
21. Nissim I, Weinberg JM. Glycine attenuates Fanconi syndrome induced by maleate or ifosfamide in rats. *Kidney Int*. 1996;49:684–695.
22. Christensen EI, Maunsbach AB. Proteinuria induced by sodium maleate in rats: effects on ultrastructure and protein handling in renal proximal tubule. *Kidney Int*. 1980;17:771–787.
23. Bagnasco S, Good D, Balaban R, et al. Lactate production in isolated segments of the rat nephron. *Am J Physiol*. 1985;248:F522–F526.
24. Mayrhofer JM, Haiss F, Hänni D, et al. Design and performance of an ultra-flexible two-photon microscope for in vivo research. *Biomed Opt Express*. 2015;6:4228–4237.
25. Pologruto TA, Sabatini BL, Svoboda K. ScanImage: flexible software for operating laser scanning microscopes. *Biomed Eng Online*. 2003; 2:13.
26. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. 2012;9:676–682.