# Long wavelength multiphoton excitation is advantageous for intravital kidney imaging

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**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.**

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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

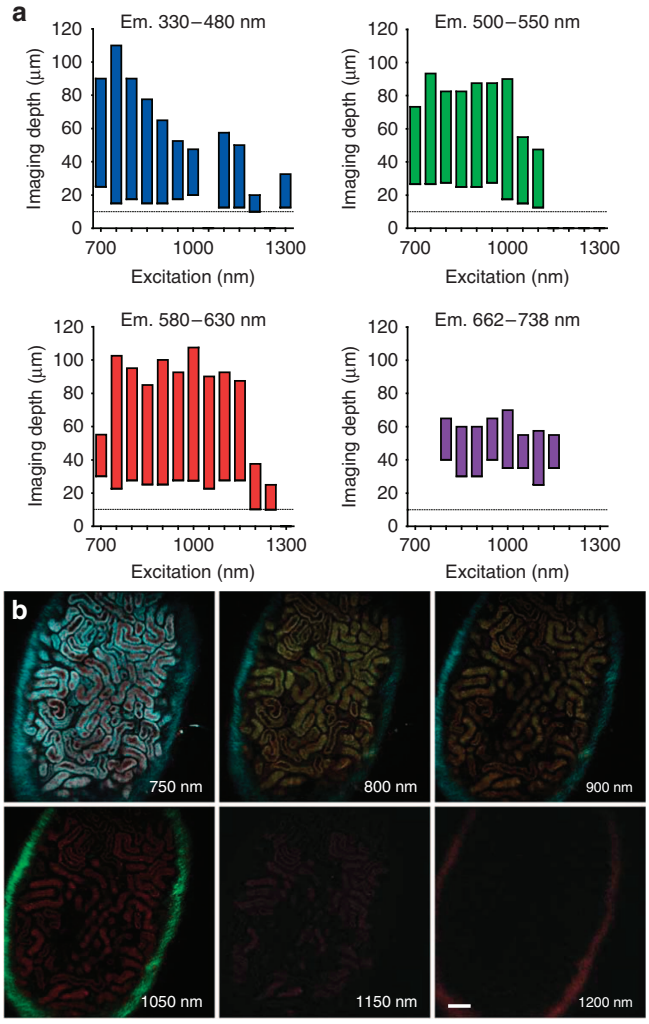


**Figure 1 | Endogenous autofluorescence in the kidney is lower at longer wavelength excitation.** (**a**) The depth at which endogenous signals could be structurally resolved in the kidney was assessed in the blue (330–480 nm), green (500–550 nm), red (580–630 nm), and far-red (662–738 nm) ranges, at excitation wavelength intervals of 50 nm between 700 and 1300 nm. The dotted 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.

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.

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

Selective imaging of fluorescent-labelled 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, labelled 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-labelled 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-labelled albumin (Alexa 488 nm,