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# *In vivo* multiphoton imaging of mitochondrial structure and function during acute kidney injury

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**Mitochondrial dysfunction has been implicated in the pathogenesis of acute kidney injury due to ischemia and toxic drugs. Methods for imaging mitochondrial function in cells using confocal microscopy are well established; more recently, it was shown that these techniques can be utilized in *ex vivo* kidney tissue using multiphoton microscopy. We extended this approach *in vivo* and found that kidney mitochondrial structure and function can be imaged in anesthetized rodents using multiphoton excitation of endogenous and exogenous fluorophores. Mitochondrial nicotinamide adenine dinucleotide increased markedly in rat kidneys in response to ischemia. Following intravenous injection, the mitochondrial membrane potential–dependent dye TMRM was taken up by proximal tubules; in response to ischemia, the membrane potential dissipated rapidly and mitochondria became shortened and fragmented in proximal tubules. In contrast, the mitochondrial membrane potential and structure were better maintained in distal tubules. Changes in mitochondrial structure, nicotinamide adenine dinucleotide, and membrane potential were found in the proximal, but not distal, tubules after gentamicin exposure. These changes were sporadic, highly variable among animals, and were preceded by changes in non-mitochondrial structures. Thus, real-time changes in mitochondrial structure and function can be imaged in rodent kidneys *in vivo* using multiphoton excitation of endogenous and exogenous fluorophores in response to ischemia–reperfusion injury or drug toxicity.**

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Mitochondria have a variety of important intracellular functions, including ATP production (via oxidative phosphorylation), synthesis of reactive oxygen species (ROS), modulation of $Ca^{2+}$ signals, and regulation of cell death pathways (for review see Duchen *et al.*[1]). Mitochondrial dysfunction has been implicated in the pathogenesis of a range of renal diseases, including ischemic-[2–7] and drug-induced[8,9] acute kidney injury (AKI). The proximal tubule (PT) is densely packed with mitochondria and is frequently the major site of damage in AKI. To understand more about the underlying mechanisms and develop novel protective strategies, new technological approaches are required that will enable the study of mitochondrial function in the kidney *in vivo* in animal models of AKI.

Previous studies on mitochondria in the kidney have relied mainly on morphological analysis with electron microscopy, on measures of tissue oxygen consumption, or on respiratory chain function tests conducted on isolated organelles (which can become damaged during the isolation process).[10] Methods for studying a variety of mitochondrial functions *in situ* in intact cells using fluorescence microscopy are well established;[11] however, PT-derived immortalized cell lines can differ greatly in their metabolism from native PT,[12] and the usage of primary tissue is therefore preferable. Confocal microscopy of freshly isolated PTs has been used to gain important insights into the effects of hypoxia on mitochondrial function.[6,13] Multiphoton microscopy allows greater tissue penetration, with less phototoxicity, compared with conventional confocal imaging; it thus provides the potential to transfer imaging approaches established in isolated cells and tubules to intact sections of tissue. It has been demonstrated recently that a range of aspects of mitochondrial function can be imaged *ex vivo* in live slices of rat kidney[14] and intact isolated perfused organs[15] using multiphoton microscopy. Imaging intact tissue with a preserved architecture enables the direct comparison of signals between different cell types, and an initial study using this novel approach has suggested that mitochondrial function varies along the nephron, both at rest and in response to ischemia.

Multiphoton microscopy of the rodent kidney *in vivo* is now an established and powerful technique in renal research (e.g. see Molitoris *et al.*[16] and Sipos *et al.*[17]), and in the

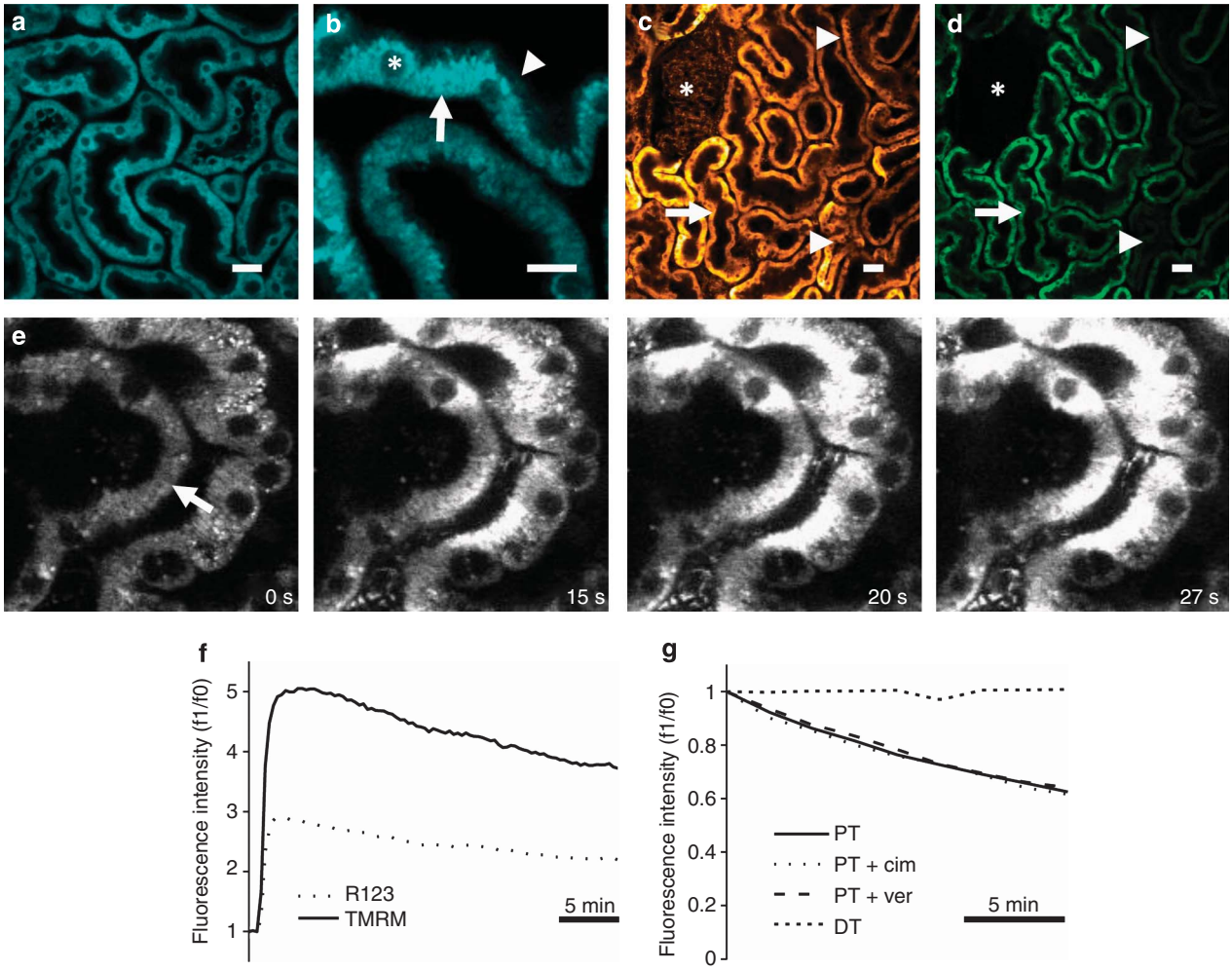


**Figure 1 | *In vivo* imaging of mitochondrial nicotinamide adenine dinucleotide (NADH) and membrane potential in the kidney.** (**a**, **b**) Mitochondrial NADH was visible at 720 nm excitation in both mouse (**a**) and rat (**b**) kidneys and showed a characteristic basolateral mitochondrial distribution in tubular cells (arrow), with very little fluorescence signal observed in non-mitochondrial structures including the apical brush border (arrowhead) or cell nuclei (asterisk). (**c**–**g**) The mitochondrial membrane potential ($\Delta\Psi_m$)-dependent dyes tetramethyl rhodamine methyl ester (TMRM) and rhodamine 123 loaded into rodent kidney tubules and localized to the mitochondria; TMRM loaded well into rat proximal tubules (PTs—arrow), distal tubules (DTs—arrowheads), and glomeruli (asterisk) following intravenous injection (**c**), whereas rhodamine 123 loaded well into PTs (arrow), but not DTs (arrowheads) or glomeruli (asterisk) (**d**). Uptake of TMRM into tubules occurred initially from the basolateral side (arrow) (**e**); images depicted were acquired shortly after an intravenous injection of the dye into a mouse. Representative traces are depicted showing the rapid increase and subsequent slow decrease in fluorescence that occurred in rat PTs following intravenous injection of TMRM and rhodamine 123 (**f**). Representative traces are depicted showing that the decline of TMRM fluorescence in rat PTs was not prevented by prior intravenous injection of either cimetidine or verapamil (**g**); no decline in TMRM fluorescence was observed in DTs. Bars = 20 μm in (**a**, **b**) and 40 μm in (**c**, **d**).

present study we have demonstrated that it can be applied to image mitochondrial function in the kidneys of anesthetized rodents, both at rest and in models of ischemia- and gentamicin-induced AKI, by using appropriate endogenous and exogenous fluorophores.

## RESULTS

### *In vivo* imaging of mitochondrial nicotinamide adenine dinucleotide (NADH) and membrane potential

The PT in the rodent kidney emits a large amount of autofluorescence *in vivo*; we found that the blue autofluorescence signal in mice and rats was dominated by mitochondrial NADH (Figure 1a and b), which was optimally excited at 720–760 nm. NADH is the substrate for respiratory chain complex I, but the oxidized form of the molecule ($NAD^+$) is not fluorescent;[18] hence, the emitted fluorescence signal reflects the proportion of the total NAD pool that is in a reduced state at any given time. The origin of the NADH signal was confirmed by colocalization with tetramethyl rhodamine methyl ester (TMRM) and also by a marked increase in intensity in response to ischemia (see below—'Real-time *in vivo* imaging of mitochondrial structure and function during ischemia-reperfusion'). Nicotinamide adenine dinucleotide phosphate (NADPH) has similar spectral properties to NADH; however, we observed relatively little blue autofluorescence signal outside the