Multiphoton Imaging Reveals Differences in Mitochondrial Function between Nephron Segments

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ABSTRACT
Mitochondrial dysfunction may play a role in the pathogenesis of several renal diseases. Although functional roles and metabolic demands differ among tubule segments, relatively little is known about the properties of mitochondria in different parts of the nephron. Clinically, the proximal tubule seems particularly vulnerable to mitochondrial toxicity. In this study, we used multiphoton imaging of live rat kidney slices to investigate differences in mitochondrial function along the nephron. The mitochondrial membrane potential was markedly higher in distal than proximal tubules. Inhibition of respiration rapidly collapsed the membrane potential in proximal tubules, but potential was better maintained in distal tubules. Inhibition of the $F_0F_1$-ATPase abolished this difference, suggesting that maintenance of potential via ATPase activity is more effective in distal than proximal tubules. Immunostaining revealed that the ratio of the expression of ATPase to IF1, an endogenous inhibitor of the mitochondrial ATPase, was lower in proximal tubules than in distal tubules. Production of reactive oxygen species was higher in proximal than distal cells, but inhibition of NADPH oxidase eliminated this difference. Glutathione levels were higher in proximal tubules. Overall, mitochondria in the proximal tubules were in a more oxidized state than those in the distal tubules. In summary, there are axial differences in mitochondrial function along the nephron, which may contribute to the pattern and pathophysiology of some forms of renal injury.


Mitochondria are intracellular organelles with a range of functions that go beyond the canonical function of ATP synthesis. They are involved in the regulation of intracellular pH and redox state, generation of reactive oxygen species (ROS), $[Ca^{2+}]_c$ signaling, and the regulation of cell death pathways.1 Mitochondrial dysfunction has been implicated in the pathogenesis of a range of renal diseases, including acute kidney injury after ischemia-reperfusion (IR) injury,2,3 the renal Fanconi syndrome,4 and some glomerulopathies.5 Thus, a better understanding of mitochondrial biology in the kidney may help us to develop a more rational approach to therapies.

The proximal tubule (PT) is especially vulnerable to mitochondrial dysfunction from insults such as genetic mitochondrial cytopathy6-8 or toxic xenobiotics (e.g., antiretroviral drugs5,9). The reasons for this are not properly understood; in vivo, the PT is thought to depend mainly on aerobic metabolism for its energy supply, with very little anaerobic capacity compared with distal tubule (DT) segments.2 This may explain why, after significant IR, PT cells tend to undergo necrosis, whereas apoptosis is more common in DT cells10; however, other aspects of mitochondrial function, or its regulation, that vary along the nephron could underlie these clinical observations. For example, the mitochondrial protein IF1 is thought to be an important regulator of ATPase activity during ischemia,11 but it is unknown whether its expression levels differ along the nephron.

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There is ongoing debate about the nature and site of injury in the kidney during IR,\textsuperscript{12} perhaps because studies have relied mainly on retrospective histopathologic examination\textsuperscript{13} rather than real-time imaging of the cellular processes that occur in response to ischemia. Multiphoton imaging uses a long wavelength excitation laser that permits significantly greater tissue penetration (with less phototoxicity) than conventional single-laser confocal fluorescence microscopy; therefore, it is an ideal technique for studying cellular physiology in intact tissue sections.\textsuperscript{14,15} We have used multiphoton imaging of freshly prepared rat kidney slices to investigate mitochondrial function in cells along the nephron, both at rest and in response to toxic stimuli, including chemical anoxia. To explore differences between the PT and DT (which for our study was mainly the thick ascending limb of loop of Henle [TAL]), we studied the medullary rays of the cortex and upper medulla, because the tubules in this region are arranged side by side, making direct comparisons easier within the same field of view. Dyes and reagents were loaded using an on-stage perfusion system, and changes in signal were imaged in real-time in response to reagents.

Using this method, we found significant differences in mitochondrial function (including mitochondrial membrane potential [$\Delta \psi_m$], ROS production and redox state) between the PT and DT. Furthermore, using immunostaining, we showed that the level of expression of the mitochondrial ATP synthase relative to IF1 (an endogenous inhibitor) is lower in the PT. These differences may be important in understanding the topographical patterns of injury observed in renal diseases or after injury, as in IR.

**RESULTS**

Viability and Orientation

We used tissue for up to 6 h after removal of the kidney. Various structures could be imaged by slicing at different levels, and we confirmed cell viability by uptake and retention of the dye calcein-AM (Figure 1, A through C). We identified structures by their characteristic morphology, location, and green autofluorescence emission pattern at 800-nm excitation (Figure 1D). Our identification of tubules was confirmed in preliminary experiments by using fixed slices stained with specific antibodies (Figure 1, E and F). PT cells were labeled using an antibody to aquaporin 1,\textsuperscript{16} whereas DT cells (TAL) were labeled with an antibody to Tamm-Horsfall protein.\textsuperscript{17}

**Mitochondrial Membrane Potential**

$\Delta \psi_m$ lies at the heart of mitochondrial function, determining rates of ATP synthesis, $\text{Ca}^{2+}$ accumulation and protein im-

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**Figure 1.** (A through C) Live rat kidney slice loaded with calcein and excited at 800 nm to demonstrate viability and showing glomerulus (A), cortical medullary ray (B), and medulla (C). (D) In addition to differing morphology, PTs and DTs can be further distinguished by the green autofluorescence emission pattern when excited at 800 nm. Antibody labeling in fixed slices of kidney was used in initial experiments to confirm the identity of nephron sections. (E) Anti–Tamm-Horsfall protein (red) staining in DTs demonstrated the clear difference in morphology from PTs (green autofluorescence) in the slice preparation. (F) Anti–aquaporin 1 (green) staining was used to confirm the identity of PTs. Arrowhead, PT; arrow, DT. Bar = 20 $\mu$m.
port. We used the cationic lipophilic indicator tetramethyl rhodamine methyl ester (TMRM) to explore the distribution of $\Delta \psi_m$ in the tubules. The dye clearly concentrated in mitochondria, as expected, showing the characteristic distribution described in the literature. The signal intensity at any given pixel is a function of dye concentration, which in turn is a function of potential. The mitochondrial signal was isolated by applying threshold settings to remove the background cytoplasmic signal. The mean signal intensity in the remaining pixels within mitochondria is therefore independent of mitochondrial density in any given tubule.

The signal was clearly heterogeneous across the slice and was considerably higher in cells of the DT, giving a mean fluorescence intensity per $\times 40$ objective field of $1038 \pm 80$ arbitrary units (AU), compared with a mean in convoluted PT of $566 \pm 23$ AU ($P < 0.001$), which in turn was significantly higher than straight PT, for which the mean was $385 \pm 3$ AU ($P < 0.001$; data are from three fields per slice, randomly selected using the bright-field setting, from a total of three slices; Figure 2, B, C, and E). These observations suggest that mitochondria in the DT are more polarized compared with the PT. A similar pattern of signal was also observed with rhodamine 123 (Figure 2D), another cationic dye that functions in the same way as TMRM, showing that this difference was not specific to TMRM.

It was shown previously that PT cells in isolated tubules are vulnerable to hypoxia, after which $\Delta \psi_m$ can be partially maintained by reversal of the $F_1F_0$-ATP synthase. Another possibility is that the reduced potential reflects an increase in ATP turnover in PT compared with DT, in which case oligomycin may increase the potential in the PT more than in the DT; however, oligomycin (an inhibitor of ATPase and ATP syn-

![Figure 2](image-url)
thase activity) addition (5 μg/ml) had no significant effect on potential after 30 min and did not alter the differences in Δψₘ between PT and DT (Figure 2E).

Both Rhodamine 123 and TMRM are substrates for the multidrug resistance transporter (MDR),²⁰ so we were concerned that the dye was being actively exported from the PT cells via the MDR. Verapamil, an inhibitor of MDR, enhanced loading of TMRM in both the PT and DT, with the fluorescence intensity rising in the PT from a mean value per ×40 objective field of 357 ± 11 AU before verapamil to 522 ± 65 AU after verapamil (P < 0.03) and in the DT from a mean of 613 ± 46 to 1054 ± 165 AU after verapamil (P < 0.03; in each case, three randomly selected fields were imaged per slice, from a total of three slices); however, verapamil did not affect the clear signal differences between the PT and DT (Figure 3A). Cimetidine, an inhibitor of renal organic transporters,²¹ did not have any significant effect on TMRM loading (Figure 3B).

Responses to Chemical Anoxia
By using an on-stage perfusion system, the effects of chemical anoxia (a model of in vivo ischemia) on mitochondrial function in renal tubules were also imaged in real time. Chemical anoxia was induced using a buffer (gassed with nitrogen rather than oxygen) containing 1 mM sodium cyanide (CN⁻). Δψₘ in PT mitochondria fell rapidly in response to inhibition of respiration (Figure 4A); however, in cells of the DT, Δψₘ fell very slowly and was then maintained at a plateau for long periods. Even after 1 h of chemical anoxia, DT mitochondria still maintained a significant potential, as shown by a further response to p-trifluromethoxy carbonyl cyanide phenyl hydrzone (FCCP) (Figure 4B). (An experimental example demonstrating these differences is shown in Supplemental Movie 1.)

Immunostaining for IF1
During anoxia, reversal of proton flow through the ATP synthase (ATPase activity) allows maintenance of Δψₘ at the expense of ATP. The mitochondrial protein IF1 has been shown to inhibit this ATPase activity.¹¹ Immunostaining for both IF1 and its binding partner, the β subunit (ATP5b) of the ATPase, in fixed 3-μm slices of rat kidney showed that whereas levels of IF1 seemed to be similar in both the PT and DT, there was a striking difference in levels of ATP5b, which were higher in the DT (Figure 5, A and B); therefore, the ATP5b/IF1 ratio was higher in the DT (mean 1.15 ± 0.05; n = 18 tubules) than in the PT (mean 0.67 ± 0.03; n = 15 tubules; P < 0.001; Figure 5, C and D). This finding indicates that the amount of IF1 in the mitochondria of the PT, relative to the amount of its target molecule, is higher in the PT than DT, suggesting a potential mechanism for more rapid depolarization of PT mitochondria after inhibition of the RC.

ROS Production
Mitochondria are a major source of intracellular ROS, which may play an important role in renal pathology. We measured rates of ROS generation in tubules using dihydroethidium (HEt), which becomes fluorescent on oxidization by superoxide (O₂⁻).²² Hence, in cells producing ROS, HEt fluorescence signal increases with time, and the rate of increase is proportional to the rate of ROS production. Because the ROS-dependent oxidized product of HEt binds to DNA, most of the signal change observed occurs in the nucleus; therefore, we measured the mean rate of fluorescence intensity change in the nuclei of

Figure 3. (A and B) Loading of TMRM was enhanced by 10 μM verapamil in both PTs and DTs (A) but not by cimetidine (B). Data are means ± SE signal per ×40 objective field from three medullary ray regions (randomly selected using the bright-field setting) per slice from a total of three (verapamil) or four (cimetidine) slices per experiment (*P < 0.03).
renal tubular cells. Thus, the data acquired reflect the total rate of ROS production per cell and are not normalized to intracellular mitochondrial mass (see the Discussion section). Under resting conditions, the basal rate of HEt oxidation was higher in the PT cells (mean fluorescence intensity increase per hour 70.9 ± 3.8%; n = 35 cells from three separate slices) and DT cells (mean increase per hour 51.3 ± 3.6%; n = 31 cells from three separate slices; P = 0.14); however, in response to rotenone, there was again a significantly greater increase in the rate of ROS production in the PT cells (mean 7.03 ± 0.68-fold increase) than in the DT cells (mean 2.69 ± 0.74-fold increase; P < 0.001; Figure 6A).

Within mitochondria, complexes I and III of the RC are thought to be the main sources of ROS. Exposure to 10 μM rotenone (an inhibitor of complex I of the RC) increased the rate of ROS generation in both tubule segments (Figure 6, A and C). The ratio of the rate of change of HEt signal after rotenone compared with the rate before rotenone was significantly greater in PT cells (mean 5.01 ± 0.64-fold increase) than DT cells (mean 1.62 ± 0.45-fold increase; P < 0.001), consistent with a much bigger response to rotenone in the PT than in the DT. This observation could be explained by differences in either the amount or the resting redox state of complex I in the two tubule segments (see the next section).

In an attempt to isolate the mitochondrial contribution to ROS production in the tubules, we repeated the experiments in the presence of 500 μM apocynin (an inhibitor of NADPH oxidase, another major source of intracellular O$_2$). As expected, the HEt fluorescence signal was greatly reduced in the presence of apocynin, and the microscope settings had to be adjusted to bring the signal back within the working range. In the presence of apocynin, there was no longer any significant difference in the rate of ROS production between PT cells (mean fluorescence intensity increase per hour 42.2 ± 3.8%; n = 35 cells from three separate slices) and DT cells (mean increase per hour 51.3 ± 3.6%; n = 31 cells from three separate slices; P = 0.14); however, in response to rotenone, there was again a significantly greater increase in the rate of ROS production in the PT cells (mean 7.03 ± 0.68-fold increase) than in the DT cells (mean 2.69 ± 0.74-fold increase; P < 0.001; Figure 6B).

Steady-state ROS production depends on a balance of ROS metabolism and catabolism. Glutathione (GSH) is a major intracellular antioxidant able to reduce ROS. Monochlorobimane (MCB) forms a fluorescence adduct with GSH in a reaction catalyzed by glutathione-s-transferase (GST), allowing measurement of GSH cellular content. In an attempt to isolate the mitochondrial contribution to ROS production in the tubules, we repeated the experiments in the presence of 500 μM apocynin (an inhibitor of NADPH oxidase, another major source of intracellular O$_2$). As expected, the HEt fluorescence signal was greatly reduced in the presence of apocynin, and the microscope settings had to be adjusted to bring the signal back within the working range. In the presence of apocynin, there was no longer any significant difference in the rate of ROS production between PT cells (mean fluorescence intensity increase per hour 42.2 ± 3.8%; n = 35 cells from three separate slices) and DT cells (mean increase per hour 51.3 ± 3.6%; n = 31 cells from three separate slices; P = 0.14); however, in response to rotenone, there was again a significantly greater increase in the rate of ROS production in the PT cells (mean 7.03 ± 0.68-fold increase) than in the DT cells (mean 2.69 ± 0.74-fold increase; P < 0.001; Figure 6B).

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Figure 4. (A) PTs loaded with TMRM showed rapid depolarization of Δψm after chemical anoxia. Bar = 20 μm. (B) In the DT, the decrease was slower and Δψm was not completely depolarized after 60 min of anoxia; however, in the presence of oligomycin (5 μg/ml), Δψm depolarized rapidly in distal tubular cells when exposed to anoxia. Data are means ± SE signal per tubule from a total of 15 PTs, 15 DTs without oligomycin, and 29 DTs with oligomycin from three separate slices for each experiment. The data were normalized from 1 (value at t = 0, taken as resting Δψm) to 0 (minimum value after FCCP, taken as 0 mV).

Figure 5. Immunostaining for IF1 (red, A) and the ATP5b subunit of the mitochondrial ATPase (green, B). The ratio of the ATP synthase image with respect to the IF1 image (color coded as indicated between values of 0 and 2) (C) reveals that the ratio of ATP5b to IF1 is significantly higher in distal than proximal tubules (D). Results displayed are mean ratio per tubule, from a total of 15 proximal and 18 distal tubules (**P < 0.001). Arrowhead, PT; arrow, DT. Bar = 20 μm.
steady-state fluorescence signal was reached by 50 min, and the signal was significantly greater in the PT (mean fluorescence intensity increase per tubule after 60 min 48.8 ± 2.5%; n = 40 tubules from four separate slices) than in the DT (mean increase per tubule 17.0 ± 3.6%; n = 26 tubules from four separate slices; P < 0.001; Figure 6, D and E). These findings are consistent with a higher level of GSH in the PT than in the DT.

**Autofluorescence and Mitochondrial Redox State**

Changes in $\Delta \psi_m$ and ROS production may reflect differences in oxygen consumption that might in turn reflect changes in the redox state of NADH and FADH$_2$ (e.g., see reference$^{25}$), which are the substrates for complexes I and II of the RC, respectively. At 720 nm of excitation, the autofluorescence signal emitted between 435 and 485 nm (blue) arose predominantly from mitochondrial NADH, whereas at 458 nm of excitation, the signal emitted between 500 and 550 nm (green) arose partly from FAD$^{2+}$. These interpretations were supported by the typical pattern of the signal, consisting of striations of signal at the basal pole of the cell, matching the known distribution of mitochondria in the tubule and the signal seen with TMRM; further confirmation was provided by the characteristic changes in signal observed in response to either RC inhibition or uncoupling (Figure 7, A through F).

The resting NADH and FAD$^{2+}$ fluorescence signals were calibrated in relation to the full available dynamic ranges, which were defined by inducing a maximally reduced state with an RC inhibitor (CN$^-$) and a maximally oxidized state with an RC uncoupler (FCCP). This allowed us to “calibrate” the resting signal as a redox index. We generated single z-stack images at (1) time 0, (2) after 10 min of perfusion with 1 mM CN$^-$ and after a 10-min washout period, (3) after 10 min of perfusion with 1 $\mu$M FCCP.

*Figure 6.* (A) Fluorescence signal increased over time in the nuclei of cells in kidney slices loaded with HEt, as a result of the production of ROS, and the basal rate of ROS production was higher in the PTs than in the DTs. After 20 min, 10 $\mu$M rotenone was added to the perfusate. This caused a relatively greater increase in the rate of ROS production in the PTs than in the DTs. Data are means ± SE fluorescence signal per nuclei (expressed as a percentage of the starting signal) from a total of 54 PT and 26 DT cells from four separate slices. (B) Experiments were repeated in the presence of 500 $\mu$M apocynin, an inhibitor of NADPH oxidase. As expected, the rates of ROS production were lower, so different microscope settings were used. Under these conditions, the basal rate of ROS production was found to be similar in both tubule types, but, again, a relatively greater rise in the rate of ROS production was observed in PTs in response to rotenone; mean values given are from a total of 37 PT and 31 DT cells from three separate slices. (C) A representative image is depicted of the HEt signal 10 min after rotenone (in the absence of apocynin). (D) Levels of the antioxidant GSH were higher in the PTs than in the DTs, as measured using the fluorescence indicator monochlorobimane. Steady state was reached after 50 min. Data are means ± SE signal per tubule from a total of 40 PTs and 26 DTs from four separate slices. (E) A representative image after 50 min is depicted. Arrowhead, PT; arrow, DT. Bar = 20 $\mu$m.
Using this method, we found the resting mitochondrial NADH pool to be in a more oxidized state in PT (mean value \( [f_0 - f_{\text{FCCP}}]/[f_{\text{CN}} - f_{\text{FCCP}}] \) 0.57 ± 0.03; \( n = 38 \) tubules from five separate slices) than in DT (mean value \( [f_0 - f_{\text{FCCP}}]/[f_{\text{CN}} - f_{\text{FCCP}}] \) 0.76 ± 0.02; \( n = 29 \) tubules from five separate slices; \( P < 0.001 \)). Furthermore, this was mirrored by the finding of a more reduced FAD\(^{2+}\) pool in the DT (mean value \( [f_0 - f_{\text{CN}}]/[f_{\text{FCCP}} - f_{\text{CN}}] \) 0.39 ± 0.02; \( n = 42 \) tubules from four separate slices) than in PT (mean value \( [f_0 - f_{\text{CN}}]/[f_{\text{FCCP}} - f_{\text{CN}}] \) 0.61 ± 0.03; \( n = 43 \) tubules from four separate slices; \( P < 0.001 \); Figure 7, G and H). Overall, these data suggest that mitochondria in the PT are in a more oxidized state at rest, which might explain why a relatively greater response in ROS production was observed in this nephron segment after inhibition (and reduction) of complex I by rotenone.

**DISCUSSION**

Multiphoton imaging of the kidney *in vivo* or *in vitro* is a powerful and relatively new approach to understanding basic renal physiology.\(^{14,15}\) Imaging of live, intact kidney slices is a useful technique to access all major nephron segments within the
cortex and medulla (which is not possible in vivo), as well as to investigate mitochondrial function in what is a highly aerobic and heterogeneous tissue. Using this approach, we identified differences in $\Delta \psi_m$ among tissue slices of cortex and medulla. These differences in $\Delta \psi_m$ are likely to reflect differences in mitochondrial function, as established by others.23,24 Using monochlorobimane (MCB), we found that levels of MCB fluorescence were significantly higher in the PT than in the DT, which is consistent with our findings in the PT. A possible explanation is that the results shown in Figure 4 are due to higher mitochondrial ATP production in DT. ROS are thought to have wide-ranging physiologic and pathophysiologic effects in the kidney, including in ischemic acute kidney injury.30 HET is predominantly oxidized by O$_2^-$, and regional differences in the origins of O$_2^-$ production (e.g., mitochondrial versus NADPH oxidase) in the kidney have been reported using tissue homogenates.31 We observed an increased basal rate of ROS production per cell in PT compared with DT within the same region of kidney, although the rates were similar when corrected for the contribution of NADPH oxidase. Given the higher resting $\Delta \psi_m$, it might be expected that mitochondrial ROS production would be lower in the PT; however, it is important to stress that we measured total cellular ROS production, and this was not adjusted for intracellular mitochondrial mass. Although the density of mitochondria in DT is generally a little higher than in PT, the ratio of mitochondrial volume to nuclear volume is much greater in the PT because the cells are larger; therefore, it is likely that basal ROS production per mitochondrion is lower in the PT.

We observed a striking difference in response to inhibition of complex I of the RC by rotenone, the resulting increase in the rate of ROS production being significantly greater in the PT than in the DT. Using an established calibration method,25 we also showed that mitochondria in the PT are more oxidized at rest. Inhibition of complex I of the RC by rotenone is more likely to produce a greater change in the rate of ROS production in mitochondria that are in a more oxidized state than in a more reduced state.33

At present, we can only speculate on the reasons for a difference in $\Delta \psi_m$ between the PT and the DT. $\Delta \psi_m$ can be affected by a number of factors in intact cells, such as substrate supply, proton leak across the mitochondrial inner membrane, and the ATP/ADP ratio.34 In the PT, the more oxidized state and lower $\Delta \psi_m$ could be explained by a greater workload and more ATP hydrolysis to support the large amount of solute transport that occurs in this nephron segment; therefore, we were surprised that differences in $\Delta \psi_m$ were not corrected by inhibition of the mitochondrial ATP synthase with oligomycin, especially because we knew that oligomycin was working in our slice preparation from its profound effect on the DT in response to anoxia. However, it is unclear to what extent solute transport does occur in kidney slices in vitro. Indeed, if little transport is occurring under these conditions, then the ATP/ADP ratio could be high, which may inhibit RC function via a feedback mechanism35; oligomycin would have relatively little effect on $\Delta \psi_m$ in this setting.

Using monochlorobimane (MCB), we found that levels of the antioxidant GSH were significantly higher in the PT than in the DT, which is consistent with previously published biochemical data.36 MCB is metabolized by GST, and subtypes of this enzyme may vary along the nephron,37 which could affect the rate of increase in fluorescence observed; however, we were careful to make measurements only after a steady state was reached. In conclusion, multiphoton imaging of fresh intact kidney slices demonstrates significant differences in mitochondrial function between proximal and distal nephron segments. These differences may underlie the apparent clinical vulnerability of the PT to mitochondrial toxicity. Our findings will need to be extended in future studies and their potential significance for mechanisms of renal injury explored in more detail.
CONCISE METHODS

Materials
Calcein-AM, TMRM, Rhodamine 123, and HEt were obtained from Molecular Probes, Invitrogen (Paisley, UK). Antibodies to aquaporin 1 and Tamm-Horsfall protein were a gift from Dr. Clare Turner (University College London, London, UK). An antibody to IF1 was a gift from Prof. Sir John Walker (Cambridge University, Cambridge, UK); an antibody to ATP5b was obtained from Abcam (Cambridge, UK). All other reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK).

Preparing Renal Tissue Slices
All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986. Male adult Sprague-Dawley rats (weighing 250 to 400 g) were anesthetized using intraperitoneal pentobarbitone (50 to 100 mg/kg). After adequate anesthesia, the left kidney was removed and immediately placed in ice-cold HEPES-buffered solution containing (in mM) 118 NaCl, 10 NaHCO3, 4.7 KCl, 1.44 MgSO4, 1.2 KH2PO4, 1.8 CaCl2, 10 HEPES, 5 glucose, and 5 pyruvate. One pole of the kidney was sectioned and mounted on a stage. Slices were cut at 200 μm in oxygenated ice-cold HEPES-buffered solution using a Microm 650V tissue slicer. They were then incubated in a chamber filled with HEPES-buffered solution supplemented with 5 mM sodium butyrate and gassed with carbogen (95% O2/5% CO2) at 37°C until used.

Dye Loading and Microscope Settings
Kidney slices were placed in an open bath chamber (Harvard Apparatus, Edenbridge, Kent, UK) and secured with a slice anchor. An on-stage perfusion system was used to load dyes and reagents in the slice. For induction of anoxia (and to model in vivo ischemia), the perfusate was gassed with nitrogen instead of carbogen, the pH was reduced to 6.9, and metabolic substrates were replaced with choline chloride. Because of difficulties in producing true anoxia in an open bath chamber, 1 mM CN− was added to the perfusate to produce chemical anoxia.

Slices were imaged using a Zeiss LSM 510 NLO axiovert microscope coupled to a tunable Coherent Chameleon laser, and light emitted from the specimen was detected using external (nondescanned) detectors. Experiments were carried out at room temperature (21 to 23°C) because we found that excessive movement of the tissue occurred when perfusing at higher temperatures, which made it extremely difficult to follow accurately changes in signal over longer periods of time. For single measurements of resting Δψm (in Figures 2 and 3), we selected regions of a slice randomly using the bright-field setting, then recorded the fluorescence signal at the plane of maximal overall intensity. To compensate for the effects of movement and changes in focal plane in timed series experiments, we imaged and collected serial z-stacks of tissue and then reconstructed these as two-dimensional images of the average signal emitted from the entire z-stack at each time point. Regions of interest were drawn around individual tubules to obtain the mean fluorescence intensity per tubule. As stated in the Results section, for experiments involving HEt, regions of interest were drawn around the nuclei, where the bulk of the signal change was observed. All values given are the mean signal, per image pixel, within the relevant region of interest.

For all probes used, we established the optimal excitation wavelength by generating two-photon excitation spectra. Calcein-AM was used at a concentration of 2 μM (with 0.002% pluronic) and was excited at 800 nm. TMRM was used at a concentration of 50 nM; 10 μM verapamil was used to inhibit dye export from the cells by the MDR. TMRM was optimally excited at 860 nm. Rhodamine 123 was used at a concentration of 100 nM and excited at 800 nm. HEt was loaded at a concentration of 5 μM, and MCB was used at a concentration of 50 μM; both were excited at 720 nm. Mitochondrial NADH fluorescence was excited at 720 nm; FAD2+ was excited at 458 nm (single-photon excitation, because we found that the signal was masked by other sources of green autofluorescence when imaging with two-photon excitation). Emitted light was collected with the following filters: Band pass 435 to 485 nm (NADH and MCB), long pass 515 nm (calcein), band pass 500 to 550 nm (FAD2+), and band pass 575 to 640 nm (TMRM, Rhodamine 123, and HEt). Image processing, including three-dimensional reconstruction, was performed using Zeiss LSM (Carl Zeiss Ltd., Welwyn Garden City, Hertfordshire, UK) and Image J (http://rsbweb.nih.gov/ij/) software.

Immunofluorescence
Standard antibody labeling was used to identify tubules. Fresh 200-μm slices of rat kidney were fixed overnight in 4% paraformaldehyde. A mouse antibody to aquaporin 1 (1:200) and a sheep antibody to Tamm-Horsfall protein (1:500) were used to identify PTs and the TAL, respectively. For IF1 immunostaining, rat kidney was processed with paraformaldehyde fixation and paraffin embedding. Three-micrometer sections were cut and rehydrated. After antigen retrieval (pH 9.0 Tris/EDTA buffer [10 mM Tris Base, 1 mM EDTA, and 0.05% Tween] in a microwave oven for 25 min at 250 W), standard fluorescence immunohistochemistry was carried out using rabbit anti-bovine IF1 (1:500) and mouse monoclonal anti-human ATP5b (1:500). Cy3-conjugated donkey anti-rabbit (1:250) and Cy2-conjugated donkey anti-mouse (1:25) were used as secondary antibodies. The specificity of the antibody for IF1 in immunofluorescence was demonstrated previously.29 The specificity of both the IF1 and ATP5b antibodies was further checked in this study using Western analysis of mouse kidney (see Supplemental Figure 1). Negative controls, performed by omission of primary antibody, showed little or no signal.

Statistical Analysis
The results are calculated as means ± SEM. The significance of differences between groups was examined using the two-sample t test.

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29. Supplemental information for this article is available online at http://www.jasn.org/.