Multiphoton imaging of renal tissues in vitro

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Peti-Peterdi, János. Multiphoton imaging of renal tissues in vitro. Am J Physiol Renal Physiol 288: F1079–F1083, 2005; doi:10.1152/ajprenal.00385.2004.—The highly inhomogeneous and light-scattering structure of living renal tissue makes the application of conventional imaging techniques more difficult compared with other parenchymal organs. On the other hand, key physiological processes of the kidney, such as regulation of glomerular filtration, hemodynamics, concentration, and dilution, involve complex interactions between multiple cell types and otherwise inaccessible structures that necessitate visual approaches. An ideal solution is multiphoton excitation fluorescence microscopy, a state-of-the-art imaging technique superior for deep optical sectioning of living tissue samples. Here, we review the basics and advantages of multiphoton microscopy and provide examples for its application in renal physiology using dissected cortical and medullary tissues in vitro. In combination with microperfusion techniques, the major functions of the juxtaglomerular apparatus, tubuloglomerular feedback and renin release, can be studied with high spatial and temporal resolution. Salt-dependent changes in macula densa cell volume, vasoconstriction of the afferent arteriole, and activity of an intraglomerular precapillary sphinter composed of renin granular cells are visualized in real time. Release and tissue activity of renin can be studied on the individual granule level. Imaging of the living inner medulla shows how interstitial cells interconnect cells of the vasa recta, loop of Henle, and collecting duct. In summary, multiphoton microscopy is an exciting new optical sectioning technique that has great potential for numerous future developments and is ideal for applications that require deep optical sectioning of living tissue samples.

multiphoton excitation; fluorescence microscopy; juxtaglomerular apparatus; tubuloglomerular feedback; renin release; inner medulla

Multiphoton microscopy is one of the newest innovations in fluorescence microscopy, in which fluorescence excitation is strictly confined to the optical section by the process of two- or more photon absorption. To encompass the possibilities of two-photon and three-photon fluorescence, we use the term multiphoton microscopy. To date, the world’s most advanced microscope, called the two-photon 4Pi-confocal laser-scanning microscope (4), is capable of revealing the structure of genetic material within a cell in three dimensions. The application of multiphoton excitation in biological specimens originated from W. Denk, J. Strickler, and W. W. Webb at Cornell University in 1990 (2). The first two-photon systems became commercially available around 1995 and were applied to living renal tissue shortly thereafter (3, 8, 11–15). Two-photon microscopy involves simultaneous absorption of two photons of one-half energy (double wavelength), and this happens only at the focal plane. The sample is illuminated with light of a wavelength approximately twice that of the absorption peak of the particular fluorophore being used. In terms of the energy necessary for excitation of the fluorophore, the absorption of two photons of a long wavelength is equivalent to the absorption of one photon of short wavelength, and so it can lead to fluorescence excitation. For example, the popular nuclear counterstain 4′,6-diamidino-2-phenylindole, which has an absorption peak at ~360 nm, can be excited at 720 nm using two-photon microscopy. The two-photon effect requires sufficiently high photon density at the focal point, which is achieved by use of a laser source emitting very short pulses (in the range of femtoseconds, $10^{-15}$ s) and with a pulse repetition rate of 50–100 MHz. The full advantages of multiphoton microscopy (particularly 3-photon excitation) are best achieved by using recently available femtosecond as opposed to earlier picosecond lasers. This is demonstrated by its suitability for three-photon excitation of UV fluorescent probes such as the nuclear stains 4′,6-diamidino-2-phenylindole and Hoechst 33342. We have found that quinacrine, the probe used to stain individual renin granules, which has an absorption peak at ~295 nm, can be excited at 880 nm using three-photon fluorescence (triple wavelength). The fully automated, widely tunable femtosecond lasers (such as the Mai-Tai from Spectra-Physics (Mountain View, CA) and the Chameleon from Coherent) are very versatile; the Mai-Tai allowed us to image UV dyes (by 3-photon excitation) simultaneously with visible probes (by 2-photon excitation) for multicolor applications.

This new approach in fluorescence microscopy has a number of important advantages, particularly when living cells are being studied. Because fluorescence is confined to the focal region, 3-dimensional imaging is possible solely by excitation; there is no need for a confocal aperture (pinhole). In addition, with two-photon fluorescence imaging there is no out-of-focus
fluorescence, and no bleaching of the fluorophore will occur in the bulk of the sample. Although bleaching can be higher in the focal plane (i.e., due to the high photon density), bleaching is restricted to the focal region. All these features of multiphoton microscopy result in high image resolution and reduced phototoxicity of living specimens. Also, longer time periods of continuous tissue scanning are possible, which provide for real-time imaging. Near-infrared and infrared light are used for excitation, and the longer wavelengths allow for deeper penetration into tissues with much less scattering, while avoiding the deleterious effects of conventional ultraviolet or visible illumination on living specimens.

In summary, multiphoton excitation imaging is an exciting, new optical sectioning imaging technique for fluorescence microscopy. This technique offers considerable advantages over conventional confocal imaging for applications that require the maximum information from images of deep optical sections in living tissue. Here, we provide examples for its application in renal physiology using dissected cortical and medullary tissues in vitro, whereas the accompanying paper reviews in vivo applications (6a).

MULTIPHOTON EXPERIMENTAL MODEL

Our multiphoton laboratory consists of a Leica TCS SP2 MP confocal microscope system (Leica Microsystems, Heidelberg, Germany) equipped with a high-rate K scanner, acoustooptical beam splitter, and acoustooptical tunable filter. A Leica DMIRE2 inverted microscope is powered by a wide-band, fully automated, infrared (710–920 nm) combined photodiode pump laser and mode-locked titanium:sapphire laser (Mai-Tai, Spectra-Physics) for multiphoton excitation, and/or by red (HeNe, 633 nm/10 mW); orange (HeNe, 594 nm/2 mW); green (HeNe, 543 nm/1.2 mW); and blue (Ar, 458 nm/5 mW; 476 nm/5 mW; 488 nm/20 mW; 514 nm/20 mW) lasers for conventional, one-photon excitation confocal microscopy. Images were collected using three internal, two external (NDD1–2, nondescanned detectors), or a transmitted light detector photomultiplier in a time (xyz) or z-series (xyc), depending on the purpose of study, with the Leica LCS imaging software. The purpose, excitation, and loading parameters of fluorescent probes used are summarized in Table 1.

Afferent arterioles with attached glomerulus preparations containing the macula densa segment were dissected from New Zealand White rabbit (0.5–1.0 kg) kidneys, and the juxtaglomerular apparatus (JGA) was microperfused as described before (9–14). Briefly, dissection and bath media were prepared from DMEM (DME mixture F-12; Sigma) with the addition of 1.2 g/l NaHCO3. Before use, this solution was aerated with 95% O2-5% CO2 for 45 min, and pH was adjusted to 7.4. When used as a dissection medium, 3% fetal bovine serum (Hyclone) was added. The arteriolar perfusate was a modified Krebs-Ringer-HCO3 buffer containing (in mM) 115 NaCl, 5 KCl, 25 NaHCO3, 1.6 NaH2PO4, 0.4 Na2HPO4, 1 MgSO4, 1.5 CaCl2, and 5 d-glucose. Perfusion pressure was maintained at ~35 mmHg throughout the experiment. The tubule segment containing the macula densa was subsequently cannulated and perfused with a modified Ringer solution containing 10 mM NaCl (control) or 60 mM NaCl to initiate tubuloglomerular feedback (TGF). The bath was continuously aerated with 95% O2-5% CO2 and exchanged at a rate of 1 ml/min. Temperature was kept at 4°C during dissection; after cannulation it was maintained at 37°C. All animal protocols have been approved by the Institutional Animal Care and Use Committee at the University of Southern California.

Table 1. Summary of fluorescent probes used

<table>
<thead>
<tr>
<th>Probe</th>
<th>Labeled Structure</th>
<th>One-Photon/Multiphoton Excitation, nm</th>
<th>Loading</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMA-DPH</td>
<td>Cell membrane</td>
<td>375/755</td>
<td>1 μM perfusate</td>
</tr>
<tr>
<td>R18</td>
<td>Cell membrane</td>
<td>543/800</td>
<td>2 μM perfusate bath</td>
</tr>
<tr>
<td>Hoechst 33342</td>
<td>Nucleus</td>
<td>380/760</td>
<td>2 μM perfusate bath</td>
</tr>
<tr>
<td>Quinacrine</td>
<td>Acidic (renin) granules</td>
<td>290/880</td>
<td>5 μM perfusate bath</td>
</tr>
<tr>
<td>LysoTracker Red</td>
<td>Acidic (renin) granules</td>
<td>598</td>
<td>5 μM perfusate bath</td>
</tr>
<tr>
<td>Renin substrate (EDANS)</td>
<td>Renin activity</td>
<td>360/720</td>
<td>2 μM bath</td>
</tr>
<tr>
<td>Fluo 4</td>
<td>Calcium</td>
<td>488/850</td>
<td>10 μM perfusate bath</td>
</tr>
<tr>
<td>Fura red</td>
<td>Calcium</td>
<td>488/850</td>
<td>10 μM perfusate bath</td>
</tr>
<tr>
<td>Nile red</td>
<td>Neutral lipids</td>
<td>543/800</td>
<td>2 μM bath</td>
</tr>
</tbody>
</table>

TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluensulphonate; EDANS, 5-(2-aminoethylamino) naphthalene-1-sulfonic acid. All fluorophores were purchased from Molecular Probes (Eugene, OR) except quinacrine (Sigma, St. Louis, MO) and renin substrate (DABCYL-y-Abu-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-EDANS, AnaSpec, San Jose, CA).

Fig. 1. Multicolor labeling of the in vitro microperfused juxtaglomerular apparatus with attached glomerulus. Cell membranes of tubular epithelium [cortical thick ascending limb (cTAL) containing the macula densa], vascular endothelium of afferent arteriole (AA), and glomerulus (G) are labeled with R18 (red), renin granules with quinacrine (green), and cell nuclei with Hoechst 33342 (blue).
Inner medullary tissue containing the papillary region was dissected using similar methods. A similar dissection medium was used as above, modified to contain an additional 300 mM NaCl (final osmolality ~900 mosmol/kgH2O). Small medullary rays were loaded with fluorophores for 1 h, transferred to a thermoregulated chamber heated to 37°C, and superfused with a modified Krebs-Ringer solution containing an additional 300 mM NaCl (900 mosmol/kgH2O) and continuously gassed with 95% O2-5% CO2.

IMAGING THE JGA

The JGA is an important regulatory site of renal salt and water conservation and blood pressure maintenance, and it well represents the complexity of regulatory mechanisms that exist in the renal cortex. The structure of the JGA has multiple cell types that communicate with each other and exhibit a complex array of functions to regulate the process of filtrate formation and renal hemodynamics (TGF) and the activity of the renin-angiotensin system (renin release) (16). With the help of multiphoton microscopy, we are now able to look inside the filtrating glomerulus and JGA and perform three-dimensional imaging and therefore superior to 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene R18 is highly fluorescent and photostable with minimal bleach-contrast (DIC) and fluorescence imaging with high magnification. Simultaneous transmitted light-DIC images. C: fluorescent, produce large Ca2+ elevations in tubular NaCl concentration that happen during TGF activation has been recently reconfirmed using the cyto-solic calcium method (5). Labeling cell membranes with the rhodamine derivative R18 (Fig. 1) provides for simultaneous, multicolor labeling of morphology, renin granules, and cell nuclei without overlapping fluorescence. In our experience, R18 is highly fluorescent and photostable with minimal bleaching and therefore superior to 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate, the membrane probe we used before (11–13).

Multiphoton imaging in combination with microperfusion techniques (12) provided direct evidence for a “sphincter-like” response of the terminal, intraglomerular part of the AA during TGF activation, as suggested earlier by other investigators (7). Simultaneous transmitted light-differential interference-contrast (DIC) and fluorescence imaging with high magnification (Fig. 2) demonstrates that the sphincter, an almost complete closure of the AA during TGF, is indeed located in the renin-secreting segment of the AA. Also, further details of JGA function can be examined using calcium imaging. We found that newly available calcium probes such as fluo 4 and fura red offer several advantages over UV light-excitable indicators such as fura 2 and indo-1. Fluo 4 and fura red are highly fluorescent, produce large Ca2+-dependent changes in fluores-

![Image](http://ajprenal.physiology.org/)

Fig. 2. Constriction of the terminal AA, an intraglomerular precapillary sphincter, in response to elevations in distal tubular salt content. A: control (NaCl concentration at the macula densa is 10 mM). B: NaCl concentration is increased to 60 mM, resulting in an almost complete closure of AA. Transmitted light-DIC images. C: fluorescence image of same preparation (as shown in B). Vascular endothelium and tubular epithelium are labeled with R18 (red), renin granules with quinacrine (green), cell nuclei with Hoechst 33342 (blue). Note that renin-positive granular cells constitute the sphincter. MD, macula densa. Bar = 10 μm.

![Image](http://ajprenal.physiology.org/)

Fig. 3. Ratiometric calcium imaging of the juxtaglomerular apparatus and attached glomerulus using fluo 4 and fura red. Compared with control (A), addition of 100 μM ATP to the bath (B) caused significant elevations in cytosolic calcium and consequent vasoconstriction of both the AA and efferent arteriole (EA) and spreading of calcium signals toward intraglomerular elements, including podocytes.
Fig. 4. Renin granular cells in the AA stained with quinacrine before (A) and after (B) the addition of 100 μM isoproterenol. A number of individual renin granules localized in the granular cell cytoplasm are clearly visible and disappear in response to the β mimetic. Bar = 10 μm. C: time course of renin release as measured by reductions in quinacrine (quin.) fluorescence. Basal rate of release (control) and effects of low salt (from 60 to 10 mM) at the macula densa [low Cl concentration ([Cl]) MD] and reductions in AA perfusion pressure (stop-flow AA) are shown.

Fig. 5. Visualization of LysoTracker Red (red, rare) fluorescence. Data collection: 1 image/10 s. Borrowed from Ref. 14.

Using a fluorescence resonance energy transfer-based, 5-(2-aminoethylamino)naphthalene-1-sulfonic acid-conjugated renin substrate in the bath, an increase in 5-(2-aminoethylamino)naphthalene-1-sulfonic acid fluorescence (renin activity) was observed around granular cells in response to isoproterenol (14). This novel fluorogenic renin substrate has great potential to directly visualize the activity of the intrarenal renin-angiotensin system. It can be applied together with a marker of renin granules (Fig. 5), and both the release and tissue activity of renin can be visualized simultaneously.

IMAGING THE INNER MEDULLA

Due to its anatomic position, visualization of the renal medulla requires invasive experimental approaches. We dissected and superfused in vitro papillary tubular rays and visualized the highly complex structure of the inner medulla on the individual cell level with multiphoton microscopy (13). Here, we show (Fig. 6) various tubular and vascular segments and how they are interconnected by a dense population of renal medullary interstitial cells. Fluorescence labeling of acidic or neutral organelles indicates a mixed population of intracellular granules; acidic granules were observed in most collecting duct cells and cells of the vasa recta. Neutral lipid inclusions were visualized in interstitial cells and in the collecting duct. Most neutral lipid granules stained with Nile red (red) appeared yellow (Fig. 6A), indicating colocalization with quinacrine (green). These images exemplify how the morphology and function of the renal medulla can be visualized. Measurement of vasa recta vascular diameter and the effects of vasoactive substances, calcium imaging, movement, and possible release of acidic or lipid granules are a few examples of what can be done using multiphoton microscopy in this preparation.

In summary, multiphoton excitation fluorescence microscopy is an excellent imaging technique for studying complex mechanisms of renal physiology in living tissue both in vivo and noninvasively.
and in vitro. When one is imaging the JGA, multiphoton microscopy compared with conventional confocal techniques (13) provides superior image resolution from deeper optical sections and an unmatched versatility. The same single excitation wavelength can be used to excite several fluorescent probes simultaneously, either by two-, or three-photon excitation, providing for multicolor applications. One of the most important questions for multiphoton users is how deep we can go. As always, the key is a good sample with intense labeling. In terms of hardware requirements, in our experience femtosecond lasers, external nondescanned detectors where prisms absorb much less emitted fluorescence, and water-based immersion objectives with proper correction for coverglass and ionic solutions are a must. We had no problem to z-scan through an entire glomerulus, with a diameter of ~100 μm. However, much deeper sectioning is expected with in vivo applications and as new techniques in fluorescence imaging emerge. The recent development and use of highly fluorescent, semiconductor nanocrystal particles called quantum dots (1), as an alternative to organic and conventional fluorescent probes, have great potential to selectively label single cells or molecules of interest.

**GRANTS**

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**REFERENCES**


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Fig. 6. Visualization of various tubular and vascular structures in the inner medulla. Fluorescence labeling of cell nuclei with Hoechst 33342 (blue), acidic organelles with quinacrine (green), and neutral lipids with Nile red (red), A: interconnection of thick ascending limbs (TAL), descending thin limbs (DTL), and vasa recta (VR) by renal medullary interstitial cells (RMIC). B: transmitted light-differential interference-contrast image of a tip of Henle’s loop. Left, descending thin limb; right, thick ascending limb. C: tip of the papilla and transition of collecting duct (left) into the epithelium of renal pelvis (right).