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Techniques to study nephron function: microscopy and imaging

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Abstract Recent advances in optics, computer sciences, fluorophores, and molecular techniques allow investigators the opportunity to study dynamic events within the functioning kidney with subcellular resolution. Investigators can now use two-photon microscopy to follow several complex heterogenous processes in organs such as the kidney with high spacial and temporal resolution. Repeat determinations over time within the same animal are possible and minimize animal use and interanimal variability. Furthermore, the ability to obtain volumetric data (3D) makes quantitative 4D (time) analysis possible. Finally, use of multiple fluorophores concurrently allows for three different or interactive processes to be observed simultaneously. Therefore, this approach compliments existing molecular, biochemical, and pharmacologic techniques by advancing in vivo data analysis and interpretation to subcellular levels for molecules without the requirement for fixation. Its use in the kidney is in its infancy but offers much promise for unraveling the complex interdependent physiologic and pathophysiologic processes known to contribute to cell function and disease.

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Introduction

The kidney is an extremely complex heterogeneous organ consisting of vascular and epithelial components functioning in a highly coordinated fashion to mediate the regulation of a myriad of interdependent processes. Over the years, novel experimental approaches have been developed that allow for isolation, manipulation, and exploitation of the unique structure–function relationships that occur. Investigators have developed model systems allowing for isolation of specific variables of interest. This has led to mechanistic understanding of cellular processes and the identification of alterations of these processes under defined conditions that attempt to mimic either physiologic or disease states. However, these reductionary models often lack the organ-specific complexity and the dynamic nature of cell–cell interactions necessary for complete understanding of the processes under study. These limitations have minimized our ability to understand complex 4D processes and the therapeutic approaches to diseases resulting in difficulties translating preclinical data into therapeutic advances.

New imaging technologies, such as multiphoton microscopy, have equipped researchers with extremely powerful tools to uniquely address biological questions that can only be accomplished in whole organ studies [3, 7, 13, 18, 19]. In parallel with this, advances in fluorophores with increased quantum yields and ease of labeling [17], molecular and transgenic approaches, and new delivery techniques [1, 12] have allowed for the development of intravital studies that can follow and quantify events with enhanced spatial and temporal resolution. Furthermore,

exponential developments in computer sciences, specifically with applications to imaging, have removed many of the obstacles previously limiting the ability to utilize microscopy to study and quantify dynamic cellular processes. These imaging technologies enable the measurement of dynamic four-dimensional (3D plus time) structure and function in organs and cells [4, 5, 10], the measurement of chemical and biochemical composition of tissues, the expression of fluorescently labeled molecular agents including drugs and proteins, quantification of the rates of physiological processes such as microvascular perfusion rates, glomerular permeability, and the mechanism of cellular uptake and intracellular trafficking [1, 14, 20, 21, 24, 26].

Multiphoton microscopy offers the investigator a minimally invasive high resolution technique, with increased depth of penetration and markedly reduced phototoxicity, for visualization of cell–cell and intracellular events intravitally. The reduction in phototoxicity with multiphoton microscopy results as fluorescence excitation occurs only at the focal point thereby eliminating out of focus fluorescent excitation within the tissue as would occur with confocal microscopy. The genesis of these advances was covered in a previous article [13, 36] and in previous publications [7, 18, 19]. Second, improved detectors with increased sensitivity, enhanced software and faster hardware, and new computational algorithms for 3D analysis and quantification have allowed for more rapid, sensitive, and accurate data gathering, visualization, and interpretation [4]. Finally, the revolution in fluorophores capable of reporting on a growing number of cellular processes has markedly improved the capabilities available to the investigator [25, 31]. This is especially true for the biotech industry where small proteins and oligonucleotides can be labeled without affecting the pharmacokinetics or pharmacologic effects of the agent. In addition, fluorescent labeling of proteins, either by genetic or chemical means of attachment using a wide spectrum of colors, allows for simultaneous multicolored imaging of different cellular processes [8–11].

Quantitative analysis

Quantitative analysis requires careful attention to acquisition parameters. If the final goal is to determine a visual difference between groups, the acquisition parameters must be set to capture the desired organelle or structure even at the cost of either oversaturating (as long as the overall image quality is not compromised) or not detecting others. Determining quantitative differences in parameters such as accumulation of fluorescent agents requires far greater attention in maintaining standard acquisition parameters to allow for a fair and accurate comparison. If this is not feasible due to circumstances such as one experimental model internalizing far less of the fluorescent probe, it is crucial that the characteristics of the acquisition system be well characterized in order to make accurate mathematical compensations during processing and analysis. In quantitative studies relying mainly on determining accumulated quantities of probes, different methods of presenting those values are shown. Regardless of the method, background subtraction of noise from either the system or tissue is crucial in generating meaningful data. These studies include analysis of probe accumulation in either lysosomal or cytosolic pools by selectively thresholding the respectively intrinsically high or low intensity values [6, 19], and quantifying total integrated fluorescence for the two pools. This method can also be used to evaluate integrated fluorescence by the average intensity of the selected probes. Additionally, these average intensity values can be ratioed to determine accumulation of two different probes relative to each other.

Image processing

Image processing is an integral component of digital microscopy. Here, operations such as visualization of dim structures, assessing colocalization between multiple probes, measuring cellular volume changes, and quantifying fluorescence at the subcellular level are achieved [4, 5]. Universal Imaging's image processing software Metamorph (West Chester, PA, USA) has been utilized extensively in our studies for the operations listed above as well as preproduction of images and movies for publication. Then, composite images and figures are usually generated using Adobe Photoshop (Adobe, Mountain View, CA, USA). In addition, Voxx, developed at the Indiana Center for Biological Microscopy (Indianapolis, IN, USA), utilizes inexpensive high-end video cards on desktop PCs to render 3D volumes and allows rotation in free space at near real-time speeds [5]. This allows for analysis of intricate structures with subcellular resolution visualized as either solid object with shading to generate depth of field or intensity projections to determine co-localization. Finally, segmentation, surface rendering, and quantitative volume rendering can be accomplished with Amira (TGS, San Diego, CA, USA) [23]. The utility of such powerful software programs used in conjunction with multiphoton for optical sectioning and volume rendering in fixed tissues is illustrated by the work of Phillips et al. in studying the glomerulus and glomerular diseases [22]. The increased depth of penetration of longer wavelength light, compared to confocal imaging, allows for increasing the volume thickness from 40 to over 150 Mm. Segmentation analysis then allows one to isolate in on a particular cell for quantitative measurements. These approaches can be used under physiologic conditions in vivo or on fixed samples.

Applications

Types of applications

Table 1 lists the types of data that can be obtained using multiphoton microscopy of the kidney. Pharmacokinetic, mechanistic, structural, and functional observations are possible. Therefore, one can observe and correlate drug delivery, specific cellular uptake, cellular metabolism, and the functional effects of the substance being studied. Since tissue fixation is not necessary, these structural–functional studies can be undertaken with a large number of small as well as large molecules in a dynamic fashion. Furthermore, the same animal can be reevaluated over day to months.

Studying molecules that cannot undergo tissue fixation

In Fig. 1, we show the cell-specific uptake of rhodamine-labeled folic acid (R-FA) probe within the kidney. This agent does not bind to proteins, is freely filtered, and is rapidly taken up by proximal tubule cells across their apical membrane via endocytosis [24]. No other cell type within the kidney either bound or internalized R-FA, as shown by lack of fluorescence in endothelial cells (thick arrow) or in distal tubule cells. At 3 min, there was rapid binding to the apical membrane (arrowhead) of proximal tubule cells and this intense apical binding remained throughout the study. With increased time, there was enhanced cellular accumulation of R-FA, especially in lysosomal structures (thin arrows). Using total integrated fluorescence, it was possible to quantify the extent of cellular uptake and to even partition it into apical binding and cellular accumulation [24].

Table 1 Investigational uses for multiphoton microscopy

Glomerular
Size/volume
Permeability/filtration
Fibrosis/sclerosis
Microvasculature
Blood flow rate
Endothelial cell injury and loss
Endothelial permeability
WBC adherence/rolling
Vasoconstriction
Afferent efferent dynamics
Cellular uptake
Cell type specific uptake
Site—apical vs. basolateral membrane
Mechanism—endocytosis vs. carrier/transporter mediated
Transcytosis
Intracellular trafficking
Intracellular organelle distribution
Cytosol localization
Cellular metabolism
Fluorescence decay over time
Cell toxicity
Cell injury in necrosis, apoptosis
Surface membrane/blebbing
Mitochondrial function
Cell volume

Since these studies are independent of tissue fixation, one can observe compounds that do not form cross linkages during chemical fixation. There is also less concern about possible tissue fixation artifacts that are known to occur especially in the kidney where the tissue osmolality of specific anatomic regions varies widely. Additional studies with fluorescein isothiocyanate (FITC)–folic acid revealed rapid loss of intracellular fluorescence following cellular uptake. These data did not indicate that FA was rapidly catabolized but rather that FA was taken up into acidic compartments resulting in FITC quenching and loss of its fluorescent signal. In fact, using the R-FA probe, we documented transcytosis as a mechanism for reclamation of filtered FA. These data emphasize that understanding probe characteristics is essential in order to appropriately interpret the data obtained. Therefore, it is necessary to thoroughly characterize the probe prior to these labor intensive in vivo studies. We have previously emphasized the importance and utility of probe characterization in cell culture studies [18]. Competitive binding, uptake, and functional characterization in simple model systems improves efficiency and minimizes costs prior to in vivo animal studies requiring larger amounts of probe. Furthermore, this extensive characterization maximizes data interpretation and limits study replication in vivo. Another example is a unique fluorescent probe recently used by Horbelt et al. [14] to quantify organic cation transport. They identified a cationic styryl dye (ASP+) as a fluorescent substrate of the renal organic cation transport system. This probe also undergoes a spectral shift between aqueous and lipid environments. This allowed the investigators to observe the substrate entering into basolateral membranes from the peritubular capillaries due to a shift in its emission spectra to shorter wave lengths (blue-shift). In the cytosol, the fluorescent emission shifted back, and then again in the apical membrane, the blue shift was seen.

Using multiple fluorophores

A major advantage of multiphoton microscopy is the simultaneous use and detection of multiple fluorescent probes. Giepmans et al. and Hadjantonakis et al., respectively, have recently reviewed the growing assortment of fluorescent probes and techniques for studying protein location and function [11, 12]. However, many of these same approaches and reagents can be utilized to label nonprotein molecules with fluorophores that allow for

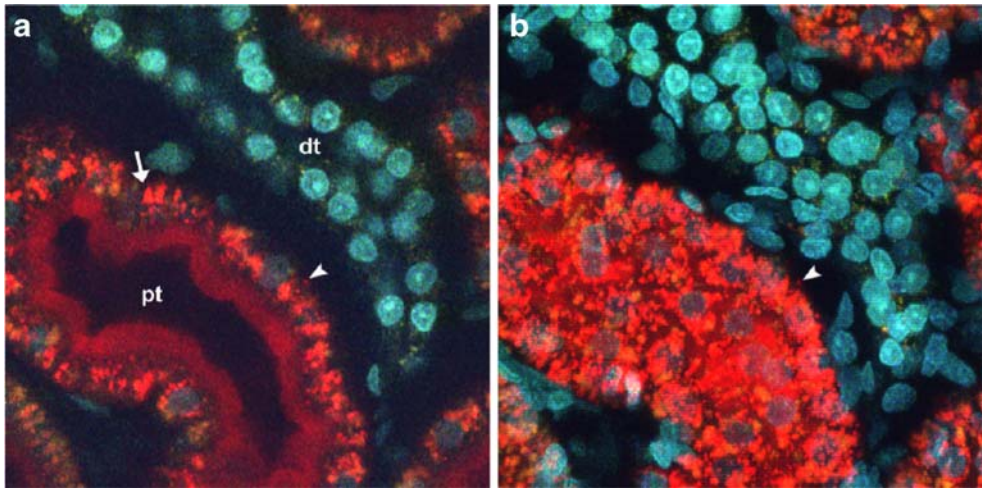

Fig. 1 Binding, internalization, and transcytosis of Texas Red folic (TR-F) acid. **a** Binding of TR-F to the apical membranes of proximal tubule cells (*pt*) and no binding or internalization in distal tubule cells (*dt*) or endothelial cells 60 min post-IV injection. Receptor-mediated endocytosis of TR-F and trafficking to the endosomal/lysosomal pool is evident. Small discrete punctate vesicles are seen (*arrowhead*) while endocytosis and trafficking to the lysosomes (*arrow*) has shifted the baseline orange autofluorescence spectra to red as more of the TR-F is sequestered in this organelle. **b** A 15-μm 3D volume reconstruction of the same area and again the small vesicle undergoing transcytosis can be visualized

observations using multiphoton microscopy in living animals at the subcellular level [32]. We have made use of this approach to label nonfixable molecules like RNAi, antisense oligonucleotides, and folic acid to observe cellular uptake and processing. We have also used this approach to evaluate and quantify glomerular permeability to dextrans of different size and charge [33–35], vascular permeability [26, 27], and label multiple intracellular compartments like mitochondria with molecular reporters like rhodamine 123.

The use of fluorescent ratioing to study events within the kidney has been recently advanced by Yu et al. [33–35]. These studies have demonstrated specific ways to evaluate glomerular permeability, glomerular sieving coefficients, and tubular reabsorption of different compounds as affected by size and charge selectivity using a Generalized Polarity concept. The use of this ratiometric concept has several advantages including minimizing errors secondary to the effect of intensity attenuation by tissue depth, fluctuations in excitation intensity, and detector sensitivity on the overall quantitative process. As variations in fluorescence intensity can be a major problem in quantification, the use of ratiometric techniques for quantitative analysis is of great importance in in vivo fluorescence microscopy. It can also be used to directly compare similar compounds directly if each can be labeled with a different fluorophore.

Intracellular distribution of molecules

Once the fluorescent compound is within a cell, it then becomes possible to quantify its intracellular distribution and metabolism. Previously, we have quantified the intracellular distribution of a fluorescently labeled siRNA to total, lysosomal, and cytosolic compartments [19]. Furthermore, it is possible to follow the intracellular accumulation and subcellular distribution over time in the same animal and to undertake repeated observations in that animal at varying intervals over days to weeks. Finally, analysis of volumetric data, obtained by collecting images along the *z* axis, with quantitative software such as VOXX [5] or Amira [29] can yield additional information regarding cellular uptake and intracellular distribution [29]. These studies can be particularly helpful in the pharmacokinetic understanding of drug delivery and metabolism at the individual cell level. However, one must remember that the fluorescence half-life and biologic half-life of the molecule may vary and that specific studies are required to relate these to important parameters. From previous experience, we can also indicate that utilizing cell culture data on fluorescent probe t1/2 and biologic t1/2 may not be valid in vivo. This may relate to any number of differences between cultured cells and cells in organs in vivo.

Specific intracellular organelles can be studied utilizing fluorescent dyes that have been developed to selectively label these organelles. For example, rhodamine 123 can be utilized to label the mitochondria of tubular epithelial cells. As the fluorescence of this compound is directly related to the potential difference across the mitochondrial membrane, one can then develop quantitative assays for mitochondrial function for both acute and chronic studies. It is also possible to selectively label the mitochondria of endothelial cells and circulating white blood cells (WBC), utilizing

rhodamine B hexyl ester which stays within the microvascular compartment. Again, quantitative assays can be developed to look at the individual number and fluorescence potential of mitochondria. This is an example of where developing an internal standard for ratiometric imaging would be advantageous for quantitative analysis.

It is also possible to use the DNA fluorescent marker Hoechst to specifically evaluate intranuclear uptake of other fluorescent compounds and to identify specific cell types based upon their nuclear morphology. Identifying apoptosis in vivo, utilizing standard nuclear condensation criteria, can also be done following an acute injection of Hoechst 33342 [15, 16]. Finally, different cellular nuclei have different morphologies allowing one to identify podocyte, distal tubule, proximal tubule, and endothelial nuclei.

Functional observations

Glomerular permeability, RBC flow rates, and vascular permeability

Table 1 lists many of the in vivo functional parameters that can be quantified using multiphoton techniques. We have previously reviewed this application of multiphoton microscopy [7, 18]. This is a rapidly growing area providing investigators with approaches to evaluate physiologic and disease processes. For example, it is now possible to quantify microvascular parameters including red blood cells flow rate, permeability, vascular tone, and leukocyte adhesion prior to and at variable intervals after administration of compounds of interest [2, 18, 26–28]. One technique of particular importance and interest within nephrology is the ability to quantify glomerular permeability of specific compounds. We have previously used this approach to determine the effect of molecular size and charge on the glomerular permeability of fluorescently labeled dextrans [33, 35]. Use of the generalized polarity concept allowed us to quantify glomerular sieving coefficients. It also allowed us to dispel the previously held concept regarding negative charge minimizing filtration [33, 35]. We were able to document no effect in glomerular filtration of a neutral and anionic 40-kD dextran. However, the anionic dextran was far more quantitatively reabsorbed by proximal tubule cells leading to reduced urinary concentrations. This was not true for neutral dextrans. Therefore, if one only quantifies what is in the urine, one cannot differentiate the effects of filtration, tubular reabsorption, or secretion.

Another example of using multiphoton microscopy to study the dynamic aspects of kidney function relates to a series of elegant studies by Peti-Peterdi [20, 21]. In these studies, renin release was visualized and quantified, as was tissue renin activity.

In summary, the use of multiphoton techniques to develop functional assays allows direct observation of the effects of administered agents and their effects. This will greatly enhance the effectiveness and efficiency of preclinical studies. The ability to restudy the same animal repeatedly over hours, days, and months will also help to reduce interanimal variability and the number of animals needed for the study.

Challenges and future opportunities

Many challenges remain to maximize the ability to study and quantify the pharmacokinetic aspects of large and small molecules at the cellular level within the kidney using multiphoton microscopy. Two major areas include image acquisition rates and correction for depth of penetration within the kidney. The studies presented in this review were recorded at approximately one frame per second. One can increase the acquisition rate by limiting the area under study, but this often sacrifices important data. The depth of imaging possible, although four to five times greater than confocal imaging, remains limited to less than 150 μm for the kidney. Thus, we are unable to visualize the cortical–medullary area from the surface of the kidney. Perhaps new external detectors, access to longer wave length light sources, and objectives specifically designed for multiphoton microscopes will allow enhanced depth of penetration [9]. Microscope objectives optimized for near-infrared light transmission, collection of both scattered and unscattered fluorescent light, and techniques that allow for focusing of the laser pulse to a narrow point within the tissue are needed to improve efficiency and depth of penetration. Phototoxicity does remain a potential problem at the focal point of excitation and this must always be considered during study design. Quantifying the recorded results is also a major area under development. Continuing improvement in software and hardware has a goal of automation of data collection, segmentation, and analysis. However, correction of fluorescence intensity for depth has not been delineated. Also, different fluorophores have different rates of intensity loss with depth. Therefore, great care must be taken to make sure comparisons between animals, treatments, and investigators are controlled. We have further delineated these considerations elsewhere [8, 18]. Finally, cost remains an obstacle for the individual principle investigator, with core imaging facilities generally required.

Numerous advances in multiphoton microscopy have occurred and applications are growing at a very fast rate. The ability to utilize viral gene transfer and molecular techniques will rapidly enable investigators to ask new far reaching questions [12, 29, 30]. The ability to utilize different present day imaging technologies in a synergistic

fashion with existing techniques is a future frontier with vast opportunities for discovery and development.

Conclusion

In summary, recent developments in intravital multiphoton studies within the kidney now allow investigators to utilize unique techniques and fluorescent probes to visualize the functioning kidney and characterize cellular and subcellular events in a dynamic fashion. Advancements in many areas of microscopy, probe development, and software and hardware development have occurred. However, these changes represent the beginning of a new era in biology as it relates to the intact organism. This approach will lead to enhanced understanding of physiologic and disease processes and their therapy with pharmacologic agents. It will result into more efficient and effective translation of preclinical data into therapeutic advances.

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