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Quantifying Dynamic Kidney Processes Utilizing Multi-Photon Microscopy

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Abstract

Multi-photon microscopy and advances in optics, computer sciences, and the available labeling fluorophores now allow investigators to study the dynamic events within the functioning kidney with subcellular resolution. This emerging technology, with improved spatial and temporal resolution and sensitivity, enables investigators to follow complex heterogenous processes in organs such as the kidney. Repeated determinations within the same animal are possible minimizing their use and inter-animal variability. Furthermore, the ability to obtain volumetric data (3D) makes quantitative 4D (time) analysis possible. Finally, use of up to three fluorophores concurrently allows three different or interactive processes to be observed simultaneously. Therefore, this approach compliments existing molecular, biochemical, and pharmacologic techniques by advancing data analysis and interpretation to subcellular levels for molecules without the requirement for fixation.

New imaging technologies, such as multi-photon microscopy, have equipped researchers with extremely powerful tools to uniquely address biologically important questions that can only be accomplished in whole organ studies [1–3]. In parallel with this, advances in fluorophores with increased quantum yields and ease of labeling [4], molecular and transgenic approaches, and new delivery techniques [5] have enabled 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 4-dimensional (3D plus time) structure and function in organs and tissues [6],

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, 2, 7].

The kidney is an extremely complex heterogeneous organ consisting of vascular and epithelial components functioning in a highly coordinated fashion that enables the regulation of a myriad of interdependent processes. Over the years talented and creative individuals have developed novel experimental approaches that enable the isolation, understanding and integrating the unique structure–function relationships that occur. Investigators have developed model systems to enable enhanced manipulation and isolation of specific variables of interest. This has lead to a mechanistic understanding of cellular processes and the identification of alterations in these processes under defined conditions that attempt to mimic either physiologic or disease states. However these models often lack the organ-specific complexity, the dynamic nature of cellular processes and cell–cell interactions necessary for adequate understanding of the process under study. For example, proximal tubule cells in cell culture undergo dedifferentiation resulting in reduced metabolic rates and alterations in many cellular processes. This has limited our ability to test therapeutic approaches and has resulted in difficulties translating preclinical data into therapeutic advances.

Multi-photon 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 multi-photon 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 [3] and in our previous publications [1, 2, 8]. Second, improved detectors with increased sensitivity, enhanced software and faster hardware, and new computational algorithms for 3D analysis and quantification have enabled more rapid, sensitive and accurate data gathering, visualization and interpretation [9]. Finally, the revolution in fluorophores capable of reporting on a growing number of cellular processes has markedly improved the capabilities available to the investigator. 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, makes simultaneous multi-colored imaging of different cellular processes possible [1–7].

Table 1. Investigational uses for multi-photon microscopy

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

Applications

Table 1 lists the types of data that can be obtained using multi-photon microscopy of the kidney. Both dynamic structural and functional observations are possible. Therefore, one can observe and correlate cell–cell interactions, structural changes, glomerular filtration, permeability, reabsorption, cellular metabolism, microvascular flow and the functional effects of a substance being administered. Since tissue fixation is not necessary these structural-functional studies can be undertaken with a large number of small as well as large molecules that cannot be fixed in tissues. In figure 1a we show the use of multiple fluorescent molecules to follow several processes simultaneously. Glomerular capillary blood flow is easily seen and can be quantified in the capillary network of Munich Wistar rat surface glomeruli. At the same time filtration of a small molecular weight (MW) Texas Red dextran is seen in Bowman's space. We have used this approach to quantify glomerular permeability, show that

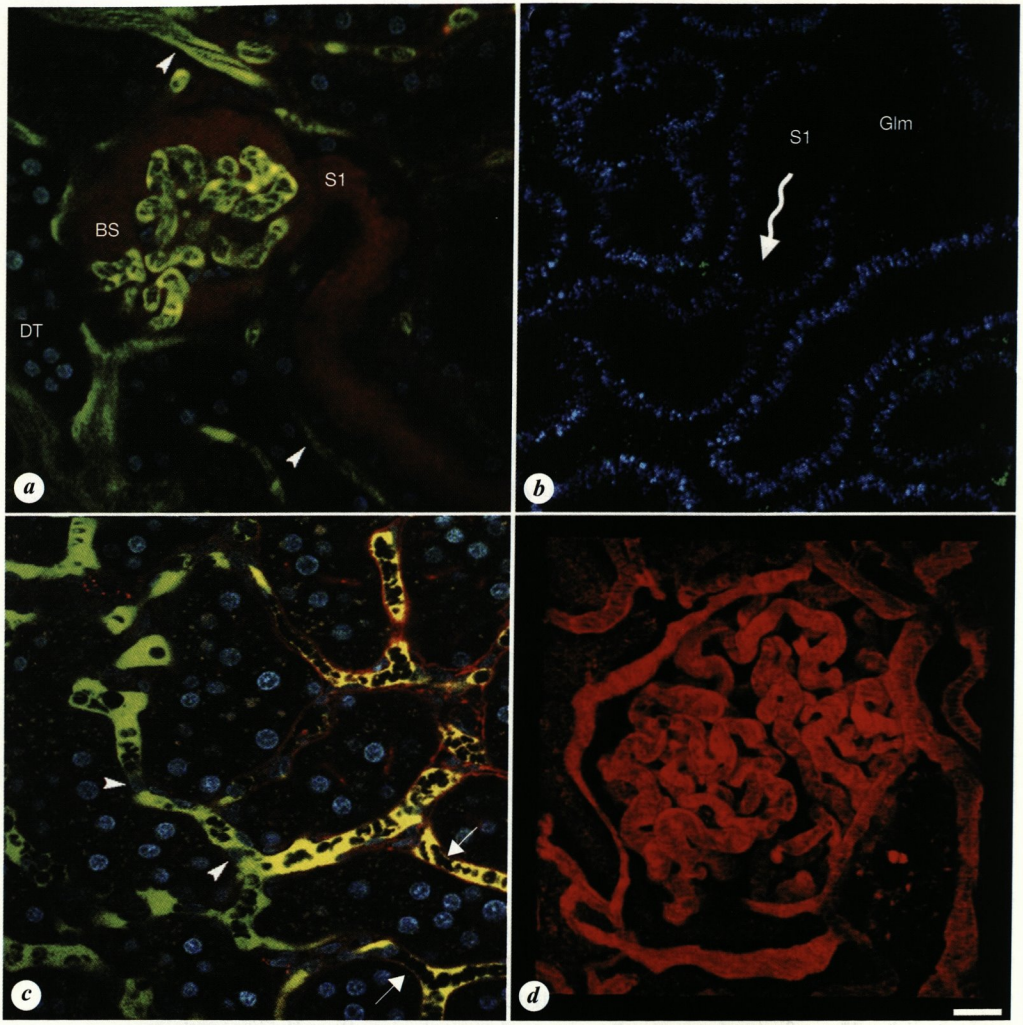

Fig. 1. Physiologic and morphologic parameters in the superficial rat kidney identified by intravital 2-photon microscopy. ***a*** Glomerular filtration and movement along a nephron segment. A large, non-filtering 500,000 MW fluorescein dextran (green) is retained within the capillary loops in the glomerulus (center) and the microvasculature (arrowheads). Within both of these structures circulating red blood cells (RBCs) appear as black oblong streaks because of exclusion of the large MW dextran. A small, freely filtered 3,000 MW Texas Red dextran (red) is seen filtering into Bowman's Space (BS) around the glomerulus and down into the S1 segment. A variation in nuclear morphology between cells types, labeled with Hoechst 33342 (cyan), can be seen between proximal tubule cells (S1), distal tubule cells (DT), and podocytes around the glomerulus (center). ***b*** Endocytic uptake by proximal tubule cells. A small, freely filtered 10,000 MW Cascade Blue dextran (blue) was given 24 h prior to imaging. The bulk of

molecular charge is not a determinant of filtration and the dissociation of protein-bound molecules prior to filtration [2, 10]. In figure1b we show the cell-specific uptake of a Cascade Blue-labeled dextran probe within the kidney. This agent is not bound to proteins, is freely filtered and rapidly taken up by proximal tubule cells across their apical membrane via endocytosis. No other cell type within the kidney either bound or internalized the dextran as shown by the lack of fluorescence in endothelial cells or in distal tubule cells. Within seconds of intravenous injection there was filtration and rapid binding to the apical membrane of proximal tubule cells. With increased time there was enhanced cellular accumulation, especially in lysosomal structures. Using total integrated fluorescence it is possible to quantify the extent of cellular uptake and to even partition it into apical binding and cellular accumulation [1, 2, 7]. Additional studies with folic acid (FA)-FITC 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 [11]. In fact, using the R-FA probe we documented transcytosis as a mechanism for reclamation of the reabsorbed FA [11]. Finally, recent data indicate that serum albumin undergoes glomerular filtration at a much greater level than previously believed [12]. Thereafter, it is rapidly reabsorbed by PTC and a fraction undergoes transcytosis, a process of reclaiming without intracellular catabolism.

the dextran can be seen localized within lysosomes (large punctate structures) at the basal portion of proximal tubule cells. The leading S1 proximal tubule segment with the open connection to Bowman's space is seen here adjacent to an unlabeled glomerulus (Glm). The arrow indicates the direction of flow. ***c*** Microvascular injury following exposure to endotoxin. With the same dyes used in (***a***), alterations in microvascular dynamics, primarily RBC flow are seen. The oblong streaks seen in (***a***) become more defined as RBCs due to the reduced flow and the presence of obstruction causing Rouleaux formations (arrows), and white cells adhering to the microvascular walls (arrowheads). The heterogeneity of this alteration is seen by the color profile of the dextrans in the blood. On the left half of the image, the blood, although slow, has circulated sufficiently that the small 3,000 MW dextran (red) has been cleared and only the large non-filtering 500,000 MW dextran (green) remains in the plasma. On the right half of the image, stagnation of flow in those vessels has prevented that pool of blood from reaching a glomerulus to filter out the 3,000 MW dextran (red). As a result, the color profile is yellow, a combination of the large (green) and small (red) dextrans occupying the same space in the plasma. Also visible is the vasoconstriction of the vessels to some degree, and small MW dextran leaking into the interstitial space around the microvasculature. ***d*** A 3D rendering of a surface glomerulus. Using VOXX software (developed at the Indiana Center for Biological Microscopy), focal planes taken at 1-μm intervals were rendered to produce a solid appearing composite. The complex inter-weaving of the capillary loops is readily seen along with the surrounding microvasculature. Bar = 20 μm.

Figure 1c shows the renal cortical microvasculature following endotoxin injection. Again, quantitation of erythrocyte flow, permeability alterations, and white blood cell (WBC) rolling, adherence and infiltration are possible. WBC within the microvasculature can be identified using Hoechst 33342 as a nuclear marker. This is also true for all nucleated cells within the tissue. Since the staining intensity is different for PTC and distal tubule nuclei we can use this to identify tubular segments. Rouleau formation can be noted. This is a common occurrence in several types of acute kidney injury including ischemia, sepsis, lipopolysaccharide and radiocontrast (unpublished observations, B.A.M.).

Figure 1d shows a 3D volume reconstruction of multiple z-axis sections of a cortical section containing surface glomeruli. This enables quantitative analysis of structural changes over time as multiple volumes can be collected in a time series. Finally, using powerful software programs like Amira one can segment out and quantify individual cells or areas of interest within cells [13, 14].

Numerous other approaches to understanding protein function and gene regulation have been developed. This is a rapidly growing field that will continue to make use of molecular and transgenic advances to selectively label individual proteins. These approaches will enable the study of specific proteins, compartments and processes in a dynamic fashion.

The use of fluorescent rationing to study events within the kidney was recently advanced by Yu et al. [10]. Their studies have outlined 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 or fluctuations in excitation intensity or detector sensitivity on the overall quantitative process. As variations in fluorescence intensity can be a major problem in quantification, the use of ratiometric techniques is of great importance.

Once the fluorescent compound is within a cell, it then becomes possible to quantify its intracellular distribution and metabolism. Furthermore, it is quite 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. Furthermore, analysis of volumetric data, obtained by collecting images along the z axis, with quantitative software such as VOXX [9] or Amira [13] can yield additional information regarding cellular uptake and intracellular distribution. 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 may vary and that specific studies are required to relate these to important parameters.

Specific intracellular organelles can be studied utilizing fluorescent dyes that have been developed to selectively label these organelles [1]. For example rhodamine-123 is 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 WBCs 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 area where development of an internal standard for ratiometric imaging would be advantageous.

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]. As is shown in figure 1, different cellular nuclei have different morphologies allowing one to identify podocyte nuclei, distal tubule nuclei, proximal tubule nuclei and endothelial nuclei.

Challenges and Future Opportunities

Many challenges remain to maximize the ability to study and quantify cell–cell and subcellular processes at the cellular level within the kidney using multi-photon microscopy. Two major areas include image acquisition rates and the 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 of study, but this often sacrifices other important data. The depth of imaging possible, although 4–5 times greater than confocal imaging, remains limited to less than 200 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 lenses specifically designed for multi-photon microscopes will allow enhanced 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. Finally, cost remains an obstacle for the individual PI, with core imaging facilities and expertise generally required [1].

In summary, recent developments in intravital multi-photon 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. This approach will lead to enhanced understanding of renal physiology and the pathophysiology of disease processes and their therapy. This will result in more efficient and effective translation of preclinical data into therapeutic advances.

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