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Multiphoton Imaging Techniques in Acute Kidney Injury

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Abstract

Multiphoton microscopy allows investigators the opportunity to study dynamic events within the functioning kidney and during acute kidney injury. This enables investigators to follow complex multifactorial processes in the kidney with improved spatial and temporal resolution, and sensitivity. Furthermore, the ability to obtain volumetric data (3-D) makes quantitative 4-D (time) analysis possible. Finally, use of up to three fluorophores concurrently in multiphoton microscopy allows for three different or interactive processes to be observed simultaneously. Therefore, this approach compliments existing molecular, biochemical and pharmacologic techniques by allowing for direct visualization at the cellular and subcellular levels for molecules without the requirement for fixation. Its use in acute kidney injury is in its infancy but offers much promise for unraveling the complex interdependent processes known to contribute to cell injury and organ failure.

Developments in kidney multiphoton microscopy over the last decade have provided researchers new and tremendously in-depth insights into complex yet highly interdependent cellular and subcellular processes. A significant advance in these developments has been the emerging technology of studying cells within their natural living environment, rather than in isolated ex-vivo controlled settings. This gives the unique opportunity of analyzing cellular pathophysiology of acute kidney injury (AKI) and the effects of potential treatments in the context of an intact functioning organ. It also offers hope for additional diagnostic tools for diagnosis, stratification and prognostic purposes. The purpose of this review is to emphasize the use of multiphoton microscopy in understanding the pathophysiology and therapy of AKI.

Multiphoton Microscopy

The potential to image deeper, with far less phototoxicity, into biological tissue was accomplished by utilizing multiphoton microscopy where increased penetration (up to 150 μm in the kidney) occurs [1–3]. Multiphoton microscopy allows for utilization of multiple fluorescent probes simultaneously, enabling labeling of different physiological compartments. Distinguishing these fluorescent emissions from endogenous or autofluorescence is also easier and enhanced in multiphoton fluorescence microscopy, as compared to confocal microscopy, as the fluorescence excitation occurs only at the focal point of the excitation beam. Therefore, out-of-focus fluorescent excitation is eliminated [2]. The longer wavelength light used for multiphoton microscopy also penetrates deeper into the kidney.

Table 1 lists the possible types of data that can be acquired using multiphoton imaging of the kidneys. Migration of white blood cells (WBCs) out of the microvasculature has not been seen up to 24 h post injury.

Yu et al. [4] have developed a quantitative ratiometric approach using a generalized polarity (GP) concept that was implemented to analyze the multidimensional data obtained from multidextran infusion experiments, the concept being the comparison of relative intensities of two fluorescent dyes. Ratiometric imaging methods are relatively independent of the amount of fluorescent probes injected, the excitation power and the depth of field being imaged. These properties are particularly advantageous for the quantitative imaging of animals where there is variability in the quantity of dyes injected, the appropriate levels of laser power used, and imaging depth. In addition, using ratiometric techniques also minimizes spatial variations of the fluorescence signals across the field of view due to detector/sample non-uniformity. Finally, these investigators were able to quantify GFR based upon the disappearance (filtration) of a small molecular weight dextran, compared to a non-filtered fluoroscopy dextran, over time.

Intracellular Endocytosis, Trafficking, Transcytosis

Intracellular uptake, compartmentation and metabolism can be studied and quantified once the fluorescent probe has entered the cell. Using multiphoton microscopy it is now possible to observe and quantify endocytosis occurring across the apical membrane of the proximal tubule cells [1, 5]. Furthermore, it is possible to follow the intracellular accumulation and subcellular distribution over time in the same animal, and to undertake repeated observations in the same animal at varying intervals over days to weeks. Such experiments are particularly useful in understanding drug delivery for AKI states. We have utilized this approach to observe and quantify fluorescent siRNA

Table 1. Uses for multiphoton microscopy in the kidney

Glomerular
– Size/volume/cellularity
– Permeability/filtration
– Fibrosis/sclerosis
– Glomerular sieving coefficient determination
Microvasculature
– RBC flow rate
– Endothelial permeability
– WBC adherence/rolling
– Vascular diameter
– Clot formation
Cellular uptake
– Cell type-specific uptake
– Site – apical vs. basolateral membrane
– Mechanism – endocytosis vs. carrier/transporter-mediated
Cellular trafficking
– Intracellular organelle distribution
– Cytosol, nuclear localization
– Transcytosis
Cellular metabolism
– Fluorescence decay over time
Cell toxicity
– Cell injury with necrosis, apoptosis
– Surface membrane/blebbing
– Mitochondrial function
– Actin cytoskeletal dystruption, aggregation

uptake and metabolism differentiating endosomal and cytosolic concentrations [6].

Intracellular organelles such as mitochondria and lysosomes can be studied in acute injury states by specific labeling of these organelles and quantifying individual number and fluorescence potential of respective organelles. DNA fluorescent markers can help identify specific cell types based on their nuclear morphology (e.g. nuclei of podocytes are characteristically bean-shaped, while endothelial cells have characteristic flattened elongated morphology). It also permits evaluation of intranuclear uptake of other fluorescent compounds in disease and therapeutic states, and analysis of necrosis and apoptosis [7].

Glomerular Permeability

Glomerular permeability and filtration of different sized compounds across glomerular capillaries can be quantified and visualized using Munich-Wistar rats with surface glomeruli (100 μm in diameter). Areas of interest can be defined and isolated and fluorescence intensities measured to obtain quantitative date. By using the change in GP or ratiometric methods it is possible to quantify glomerular permeability by measuring relative change intensities of dyes in the bloodstream or within the Bowman's space [4]. Filtration and clearance of smaller size molecules is typically faster than larger size molecules. By using two different sized fluorescent dextrans, it is thus possible to measure glomerular filtration fraction by measuring the changes in concentration in the bloodstream over time of each dextran. In figure 1 we show glomerular filtration of both a truly filtered small MWT red fluorescent dextran and a large 500-kDa green fluorescent dextran in the same glomerulus following a 45-min renal pedicle clamp injury. Note the small dextran is seen throughout Bowman's space. The large dextran is also filtered in a portion of the glomerulus suggesting a worked increase in the glomerular sieving coefficient post ischemic injury, consistent with known changes in podocyte structure following ischemic injury [8]. A large clot fills the lower large capillary on the right.

Proximal Tubule Reabsorption

Since many AKI states affect the proximal tubules (PT), it is very important to be able to study the reabsorptive profile of the tubular epithelium and its recovery with or without therapeutic intervention. It is possible to evaluate the reabsorptive properties of the PT by (a) direct observation of the epithelial cells and lumen of the PT, (b) luminal GP changes in the PT, and (c) comparing the

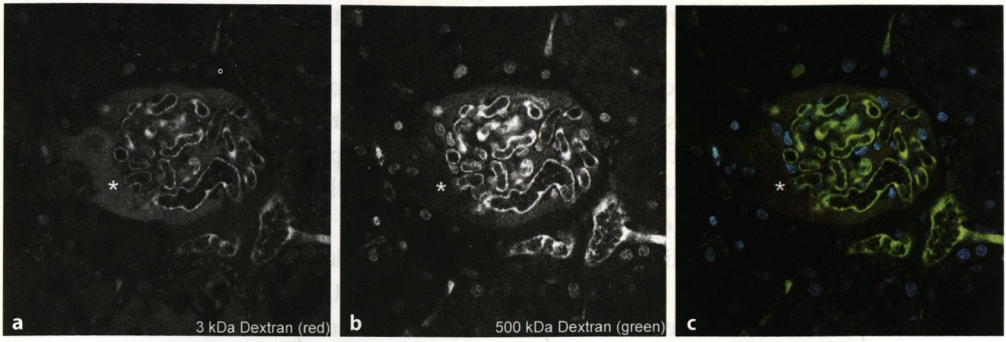

Fig. 1. Intravital multiphoton kidney imaging showing cortical tissue following ischemic injury. A rat following 45 min of renal pedicle clamping was administered intravenously a 500-kDa fluorescein-conjugated dextran (green) to label the vasculature, Hoechst 33342 (cyan) to label nuclei systemically, and a small filterable 3-kDa rhodamine-conjugated dextran (red). Circulating RBCs within the microvasculature appear as black streaks as the 500-kDa dextran (green) fills the plasma space but remains excluded from the RBCs. There is a noticeable flux of the 500-kDa dextran (green) into Bowman's space, except for the area on the left (*), implying injury to part of the glomerular capillaries resulting in an abnormally high glomerular sieving coefficient (bar = 20 μm). **a** 3-kDa dextran channel with uniform filtration into Bowman's space. **b** 500-kDa dextran channel. **c** Overlap of the two channels showing differential filtration of the large 500-kDa green dextran.

change in GP values from within PT lumen with those from within Bowman's capsule [1–4].

Red Blood Cell Flow Rates

Multiphoton microscopy can also be utilized to study and quantify red blood cell (RBC) flow rates in the microvasculature. It is possible to image and differentiate the motion of RBCs in the renal cortex microvasculature. RBCs exclude the non-filterable fluorescent dye used to label the circulating plasma and consequently appear as dark, non-fluorescent objects on the images. The acquisition of repetitive scans along the central axis of a capillary (line-scan method) gives the flow of RBCs, which leaves dark striped bands in the images where the slope of the bands is inversely proportional to the velocity; the more shallow the slope, the faster the flow rate [9]. Using standardized vessel length, diameter and angles, it is possible to calculate the RBC flow rates in different states, e.g. ischemia, sepsis, and response to specific treatments [10–14]. We have recently quantified the effect of soluble thrombomodulin on endothelial protection from ischemic injury and the resulting improvement in RBC velocity and reduced WBC adherence [15].

Inflammation and Leukocytes

Inflammation is being increasingly recognized as an important and central process in the initiation, maintenance and progression of AKI and chronic kidney injury. Hence understanding the dynamic roles and functions of different leukocytes and their interactions with soluble and endothelial cell factors is key to develop and test preventive and therapeutic strategies. Multiphoton microscopy provides the opportunity to observe these crucial processes in vivo. The most effective method to image leukocytes in the kidney involves direct fluorescent labeling of specific WBC lineages. Fluorescent agents such as rhodamine 6G, acridine red or orange are preferentially concentrated in WBCs and allow intravascular detection. The DNA dye (Hoechst) used to label all nuclei, also permits detection of WBCs and differentiation of RBCs in the vascular space. Various markers are available for B lymphocytes, CD8+ T lymphoblasts, macrophages, naive T cells, etc. [12]

Vascular Pathology

AKI resulting from ischemia is associated with microvascular permeability defects and this is eventually linked with microvascular dropout [14]. Various other pathological conditions affect the vascular space, especially the glomerular vasculature. Apart from studying flow rates as mentioned above, using mixtures of different sized fluorescent-labeled dextrans, we can now examine the effect of injury on the microvasculature by observing and measuring the extravasation of these dextrans into the interstitium as elegantly demonstrated by Sutton et al. [16]. Furthermore, using multiple fluorescent labeling there is also the opportunity to study the effect of injury on the intricate and dynamic interaction of the endothelium with the matrix proteins such as metalloproteinases [17]. It also allows for correlation and further quantification of the relationship between the endothelial permeability and alterations in blood flow rates.

The regulation of glomerular hemodynamics is dependent on the interplay between the microvasculature, tubule and the juxtaglomerular apparatus. Peti-Peterdi and colleagues [18] have now extended their studies into actually being able to quantify the renin content of the juxtaglomerular apparatus in vivo in anesthetized Munich-Wistar rats using quinacrine tagging of the renin-secreting granules. They have also been able to measure the diameters of the afferent and efferent arterioles as well as glomerular volume.

Fluorescence microscopy application also extends its application to gene transfer into specific targets, e.g. tubular or endothelial cells. Green fluorescent protein linked with the protein of interest, e.g. actin, that is cloned into an adenovirus vector, can be delivered into the urinary Bowman's space, proximal tubule lumen or superficial efferent arteriole by micropuncture techniques. The

expression of these fluorescent-labeled proteins is an invaluable tool in studying dynamic changes in the cell in various disease models [19, 20].

Conclusion

In summary, recent advances in fluorescent imaging techniques, especially in intravital multiphoton microscopy, have now enabled investigators to employ and develop unique, tailor-made techniques to visualize the functioning kidney and analyze, in a dynamic fashion, the cellular and subcellular processes that occur in various AKI states and the response to therapy. Application of some of these techniques to patients is close at hand and will add to the diagnostic repertoire of the clinician.

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