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Intravital multiphoton microscopy as a tool for studying renal physiology and pathophysiology

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ABSTRACT

The kidney is a complex and dynamic organ with over 40 cell types, and tremendous structural and functional diversity. Intravital multi-photon microscopy, development of fluorescent probes and innovative software, have rapidly advanced the study of intracellular and intercellular processes within the kidney. Researchers can quantify the distribution, behavior, and dynamic interactions of up to four labeled chemical probes and proteins simultaneously and repeatedly in four dimensions (time), with subcellular resolution in near real time. Thus, multi-photon microscopy has greatly extended our ability to investigate cell biology intravitally, at cellular and subcellular resolutions. Therefore, the purpose of the chapter is to demonstrate how the use in intravital multi-photon microscopy has advanced the understanding of both the physiology and pathophysiology of the kidney.

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1. Introduction

Microscopy as a technique has evolved past established tasks such as histological analysis of pathology specimens to one capable of simultaneously studying various dynamic cellular and subcellular processes *in vivo*. This is due to continuing improvements in optics, lasers, computers, and hardware. In turn, this has led to increased usage by making these complex systems far more affordable and robust while simplifying their ease of use. Studies utilizing intravital multi-photon microscopy (MPM) have increased because it affords the researcher data that is by nature dynamic, emanating from attached, intact, whole organs still regulated by intrinsic factors such as hormones, chemokines, and metabolites. The capability of imaging includes up to 4 channels simultaneously (blue, green, red, and far-red emitting fluorophores and compounds) at depth (3D) at near real-time speeds (4D data) thanks to innovations such as extremely fast resonant scanners on newer microscope systems and computers capable of rapidly collecting and storing these data sets that easily exceed several gigabytes in size. For example a 12-bit system acquiring three channels generates an ~1.5 MB file per focal plane. Taking three 30 μm volumes for 8 glomeruli at both 60× and 20× magnification plus an initial 200-frame infusion movie will exceed 1 GB.

Our group has applied these techniques to studying renal physiology and pathophysiology within the intact, functioning kidney [1]. One limitation that varied from other organs previously studied, specifically the brain, is reduced optical penetration due to the increased cellular heterogeneity and blood flow of the kidney. Despite this limitation numerous studies have greatly advanced the understating of renal physiology and disease. Our study on fluorescent folate was the first to follow the transcytotic pathway of this essential vitamin after internalization by the proximal tubule. Previous studies relied on following the folate receptor due to the inability to fix folate in place for subsequent visualization via immunolocalization techniques [2]. Some of these discoveries have challenged long established dogma as disruptive technologies often do [3–8].

This chapter will review a number of techniques that focus on different aspects of renal morphology and physiology, and detail the methods used. The methods used to prepare the rat for imaging will not be discussed here having been carefully described in other publications [1,5,9]. First, an overview of the kidney structure, visualized by MPM, helps identify visual landmarks and establish differences between tubule types. Then studies that utilized numerous fluorescent probes to delineate various aspects of tubular function in health and disease will be reviewed. In particular, we will narrow our focus to the proximal tubule as the majority of studies have been on this segment of the nephron. This is the first part of the nephron to encounter the glomerular filtrate, and its functions to limit a wide range of important molecules and nutrients from being lost in the urine. Multi-photon microscopy quantitation of glomerular permeability, a key function of the

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kidney, will be described as well and key factors in assuring accurate measurement of this parameter.

Acute kidney injury (AKI) from renal ischemia and reperfusion (I/R) is an important area of study due to its clinical significance. Intravital MPM has helped elucidate a number of important findings relating to apoptotis and necrosis associated with ischemia/reperfusion. Alterations induced by this injury model that occur within the peritubular microvascular relating to red blood cell (RBC) flow, WBC rolling and adhesion, and vascular permeability can be studied using large molecular weight markers such as dextrans or albumin. One key finding made while utilizing this technique is the temporal and spatial heterogeneity in which intravenously infused fluorescent markers distribute throughout the microvasculature in pathologic conditions [10,11].

These examples serve to underscore the novel, complementary approaches intravital MPM uncaps to advance an investigator's research. However, they represent just a few examples of the true capabilities of this technique.

2. Morphology of the unlabeled renal cortex

Most modern turnkey confocal and multi-photon microscopes are equipped with an epi-fluorescent mercury arc lamp that allows for wide-field visualization and orientation of fluorescent samples prior to image acquisition. The standard filters typically included are a blue pass (for Hoechst and DAPI), a green pass (Fluorescein) and a red pass (Rhodamine and Texas Red), which will allow only those specific corresponding wavelengths through to the eyepiece. For intravital MPM it is highly advantageous to replace a filter (if necessary) with a dual pass red/green. The predominant feature of the kidney surface is the lysosomal autofluoresence localized within the proximal tubules facilitating their identification (Fig. 1A and B). Autofluorescence has a broad red/green emission which gives it a combined yellow-orange color when viewed through a dual pass filter. The dual pass filter is useful when discriminating a true fluorescent signal (a dye or a fluorescent protein) from the red or green portion of the endogenous autofluorescence present in that particular band pass. Our laboratory sets red and green detector gain settings when acquiring images via confocal or multi-photon excitation to match the yellow-orange autofluorescent signal seen through the eyepiece. This normalizes the appearance of the images from experiment to experiment. Excitation power of the laser should be kept relatively low to prevent photo-toxicity. We typically keep laser power between 12–15% when using an excitation emission of 800 nm. Power can be increased as longer, red-shifted excitation wavelengths are used because of a decrease in power output.

Other tubule types such as collecting ducts and distal tubules are indistinguishable from each other, lack any visible landmark and appear as large empty patches similar in size to proximal tubules. The Munich Wistar rat strains (Simonsen's and Frömter) have in addition visualizable surface glomeruli; the Frömter strain having roughly two to three times more than the Simonsen's strain. Surface glomeruli lack any fluorescence and are discerned by the large circular void among proximal tubules (Fig. 1A & B). Focusing through proximal tubules reveals the tubular lumen. A rough approximation of hydration status can be gauged by noting the lumen diameter. The lumenal space from a hypovolemic hypoperfused rat will be collapsed and the autofluorescent lysosomes will appear to touch from side to side. Surrounding the tubules is a network of peritubular capillaries and associated interstitial space. The use of a large molecular weight fluorescent dextran or protein will demarcate the vessel from the interstitial space (Fig. 1C & D).

3. Selecting a range of intensities for quantitation via thresholding

Data obtained from intravital MPM is information intensive and quantifying a specific parameter can present challenges. Selecting regions of interest to quantify fluorescence intensity or area can be extremely labor intensive and time consuming especially if the areas are discontinuous. For example, drawing individual regions around endosomes in a field containing 60 cells, with 100 endosomes per cell, and analyzing 10 fields per condition (control and experimental) would make analysis prohibitively time consuming. Fortunately, many modern image processing and analysis software packages allow for quantitation of a selected range of intensities within an image while excluding the rest. This process would allow the aforementioned example to be analyzed in a few minutes by selecting the upper and lower range encompassing the endosomal associated fluorescence. After selection, parameters such as average fluorescence intensity, total thresholded area, total number of fluorescent spots, and average spot area (in pixels) are easily attained. In this chapter we will illustrate two examples of this technique. The first will be in the section detailing the method for quantifying the uptake of compounds within the proximal tubule via endocytosis. The second involves the determination of albumin plasma intensity within the renal vasculature where circulating RBCs blur the margins of the plasma. When conducting this analysis the user must be aware of the mode the software is in when reporting values. See Fig. 2.

Drawing a region of interest around an area can be done in a stringent manner to trace a well-defined margin and isolate the area based on those margins. This is typically done when the entire region needs to be analyzed for area and/or intensity and there are structures in close proximity that have similar intensities. When drawing a region make sure the software is presenting values associated with that particular region only and not for the entire image.

When employing thresholding to select a range of intensities within a region, the margins of the region do not have to be drawn precisely if the intensity values between the area of interest and surrounding area vary markedly. When analyzing data in this manner, make sure the software is presenting values for that specific region and only for the intensities within the threshold. It is easy to overlook these subtle details and make errors in data collection.

4. Proximal tubule endocytosis and transcytosis

Materials: Small molecular weight dextrans 3 kDa (Invitrogen, Carlsbad, CA) 4 kDa (TdB Consultancy Uppsala, Sweden) 2.5–4.0 mg, Fluorescent albumin 2.5–4.0 mg.

One of the main functions of proximal tubules is the retrieval of fluid, electrolytes and macromolecules, that are filtered by the glomerulus, to prevent loss via urinary excretion. Once internalized across the apical membrane via receptor mediated or fluid phase endocytosis, endocytic trafficking within proximal tubule cells sorts material into two main pathways. These include sorting to the lysosomes for degradation and sorting to various other non-degradative compartments or locales within or across the cell. The inability of the proximal tubule to reclaim some of the filtered nutrients has been implicated in certain disease states and injury models. Intravital MPM has helped expand the investigative focus, beyond glomerular dysfunction, to elucidate the role tubular injury plays in proteinuric and albuminuric diseases that were previously thought to be associated solely with damage to the filtration barrier. In this section we will outline methods to study both pathways [8,9]. In quantifying uptake, it is important not to saturate the intensity of the endosomal pool (particularly lysosomes) as this will underestimate the accumulation of the material therein [12]. Care-