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New Approaches in Renal Microscopy: Volumetric Imaging and Super-resolution Microscopy

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Abstract

Purpose of review—Histologic and electron microscopic analysis of the kidney has provided tremendous insight into structures such as the glomerulus and nephron. Recent advances in imaging, such as deep volumetric approaches and super-resolution microscopy, have the capacity to dramatically enhance our current understanding of the structure and function of the kidney. Volumetric imaging can generate images millimeters below the surface of the intact kidney. Super-resolution microscopy breaks the diffraction barrier inherent in traditional light microscopy, enabling for the visualization of fine structures. Here, we describe new approaches to deep volumetric and super-resolution microscopy of the kidney.

Recent findings—Rapid advances in lasers, microscopic objectives, and tissue preparation have transformed our ability to deep volumetric image the kidney. Innovations in sample preparation have allowed for super-resolution imaging with electron microscopy correlation, providing unprecedented insight into the structures within the glomerulus.

Summary—Technological advances in imaging have revolutionized our capacity to image both large volumes of tissue and the finest structural details of a cell. These new advances have the potential to provide additional profound observations into the normal and pathologic functions of the kidney.

Keywords

multiphoton microscopy; CLARITY; super-resolution microscopy; STORM

Introduction

Much of what we know about the kidney is from careful morphometric studies performed by light microscopy. Work by anatomic microscopists of the 19th century helped to define the renal cortex and medulla, as well as the complex architecture of the renal nephron despite

the fact that light microscopy is actually poorly suited for visualizing the structures of the kidney.

Light microscopy requires sectioning kidney tissue into 3–5 μm slices, smaller than the thickness of a single cell (Figure 1). The nephron begins with the glomerulus which has a diameter of 100–200 μm , and then extends through a long tubular system that can be 1–3 cm in length. While the glomerulus and collecting duct were identified in the 17th century, it took another 200 years to discover the tubular segment connecting them, the loop of Henle. These studies required three-dimensional imagination of the nephron structure gleaned from thin serial sections.

Since the resolution of the light microscopy is around 200 nm, it would take the development of electron microscopy (EM) in the 1930's to reveal the fine architecture of the glomerular capillary wall. These studies showed us that the glomerular capillaries consist of three layers, the endothelial cell, the basement membrane and the epithelial cell (podocyte). Furthermore, these studies revealed the 60–80 nm diameter endothelial fenestrations, the porous, fibrillar basement membrane and the complex adhesive complexes between podocytes (about 40 nm) called the slit diaphragm. Podocytes themselves have a complex architecture with multiple extended cell processes that terminate with 100–150 nm in diameter foot processes. Up until recently, only EM could visualize these structures.

Advancements in microscopy are transforming approaches to viewing and studying the kidney. New lasers, microscopic objectives, and new methods of tissue preparation are allowing images that are several millimeters deep over areas that are centimeters in size. These approaches should be perfect for mapping and imaging of complete nephrons. At the other end of the scale, newly developed super-resolution imaging methods allows for imaging of fine structures not possible before using traditional light microscopic approaches. Progress in imaging technology is the start of a revolution in our understanding of kidney function in health and in disease. Here we will focus on these approaches that are allowing for imaging of large volumes of tissue as well as methods that break the diffraction barrier of the light microscope.

Volumetric imaging: viewing the kidney in three-dimensions

Imaging the nephron requires a depth that cannot be seen in the typical 3 μm section. Here we will review new methods that allow for more complete 3D imaging of the nephron.

Volumetric imaging using physical sectioning: serial sectioning and block-face imaging

The traditional approach to imaging a volume of tissue is to perform serial sectioning: the tissue is cut into many thin sections, imaged sequentially and assembled in 3D. Given an average glomerular diameter of 150 μm and average tissue section of 3 μm , imaging a complete glomerulus would require between 40–60 sections. This approach has been utilized with TEM [1,2], light microscopy [3], and in combination [4,5]. A related approach is block-face imaging where the tissue block is directly imaged followed by sectioning. This method was used by the Allen Brain Institute to image both human [6,7] and mouse brain [8]. In the kidney, scanning EM with serial sectioning [9] was used to image the whole

human glomerulus in three-dimensions [10] along with the basal surface of podocytes within rat glomeruli [11].

Volumetric imaging without physical sectioning: optical sectioning approaches

Serial sectioning methods are labor intensive and therefore not routinely used. The development of the confocal microscope was one of the first methods to use optical sectioning to image a volume [12]. Conventional fluorescence microscopy illuminates the entire field of tissue, resulting in fluorescent light from areas outside the focal plane that obscures detail [13]. Confocal microscopy utilizes a small pinhole to filter away out-of-focus light, allowing only the light emitted from the focal plane to be collected [14] (Figure 2). The depth of imaging depends on the penetration of the laser, which is in turn limited by the opacity of the tissue and laser power. While increased depth could theoretically be achieved with more powerful lasers, an important practical limitation is tissue damage inflicted from the power of the laser.

An important innovation was the development of multiphoton microscopy (MPM). Here, lasers deliver rapid pulses (~10 nanoseconds) of light of very short duration (< 1 picosecond) [15,16]. Excitation is achieved by the near-simultaneous absorption of two photons in a highly focused volume (~1 femtoliter) (Figure 2). Rastering in the X, Y and Z planes allows for a 3D image to be produced. While MP imaging is used for live imaging, MP imaging can also be used for volume imaging of fixed tissue. Imaging depth is dependent on the wavelength of light with longer wavelengths able to penetrate more deeply. While MP lasers were originally limited to 1,000 nm wavelength, newer lasers extend this range to 1,350 nm allowing for deeper penetration of tissue. Newer microscope objectives allow MPM to image over 1 mm in depth.

Light scattering significantly limits transparency of biologic tissue

What limits even deeper volumetric imaging is tissue opacity. When light hits the boundary between two heterogeneous media (differences in refractive index), it changes its velocity and scatters. The overall RI of biologic tissue is ~1.5 [17], and its opacity is driven by the heterogeneous composition of cells [18]. While lipids represent the major source of light scattering with a RI of 1.48 [19], numerous organelles within the cell including the nucleus (RI=1.39) and mitochondria (RI=1.39–1.47) [20,21] also contribute to light scattering. This limits the ability to image deep into tissues.

Approaches to reduce light scattering and optically clear tissue

For over 50 years, it has been known that imaging deep within tissues will require diminishing the heterogeneity of refractive indexes in the cell. Two major approaches have been used to solve this problem. The first involves incubating the tissue in a solvent that has a RI close to the tissue itself. For example, water has a RI of ~1.3, so replacing the water with solvent that has a RI of ~1.5 can decrease RI heterogeneity in the cell. The second method involves removal of lipids to help to homogenize the RI of the cell. In a recent advance, proteins and nucleic acids were fixed in tissue by crosslinking them together into a hydrogel followed by removal of lipids by electrophoresis. As an indication of success, homogenizing the RI converts an opaque specimen into a transparent one.

Index matching—Index matching reduces the amount of light scattering within tissues by eliminating RI differences in the tissue as well as in the solvent around it. The method involves incubating the tissue in a solvent that has a RI similar to the tissue.

Currently, both organic and water-based solvents are used for index matching. Organic approaches first require dehydration of the tissue, which increases the density of the sample and its RI (>1.5). The tissue is then incubated with an organic solution that removes some lipids but more importantly homogenizes the RI of the sample [22][•], [23–25]. Using water-based solvents is simpler to perform because it does not require a dehydration step but is slower and often incomplete [26–33]. A critical advantage however of using water-based solvents is their compatibility with most fluorescent probes and endogenous reporters.

Hydrogel embedding—An alternative strategy to render tissue transparent is to embed proteins and nucleic acids in a hydrogel, followed by removal of lipids. Karl Deisseroth's group pioneered this approach on the mouse brain, and termed this method CLARITY (clear, lipid-exchanged, acrylamide-hybridized rigid, imaging/immunostaining compatible, tissue hydrogel) [34].

CLARITY achieves tissue transparency through three steps: 1) hydrogel embedding, 2) lipid removal and 3) index matching [34,35] (Figure 3). During hydrogel embedding, proteins and nucleic acids are crosslinked to the gel using a solution of acrylamide and formaldehyde. Lipids are then removed after incubation in SDS solution using electrophoresis. The cleared tissue is then placed in an index-matched solution for imaging. The method is time consuming as both the lipid removal step and the immunostaining step can take days to weeks, but this approach has been used successfully by several groups to image mouse organs including kidneys [36,37].

Several improvements to the original CLARITY protocol have been published. These approaches passively remove lipids without the need of electrophoresis [35]^{••}, [38]^{••}. Additionally, by increasing the pore size of the hydrogel vis-à-vis decreased crosslinker density, they can dramatically reduce the amount of time required to immunolabel tissue [38]^{••}.

Advances in objective lens technology: a critical innovation for imaging cleared tissue

Prior to advances in tissue clearing, most objective lenses emphasized high numeric aperture (NA) as long working distances (WD) were unnecessary. Following the recent innovations in tissue clearing, advances in objective lenses allow for significant increases in WD. Until a few years ago, the best objectives for deep imaging had a WD of 2 mm. Now, most of the microscope manufacturers sell objectives with longer WD, up to 8 mm. Thus, the major limitation to deep imaging today is the penetration of the tissue to antibodies for staining.

Successful volume imaging can allow structures like the complete glomerulus or complete nephron to be visualized. This could be important in conditions where there is a focal lesion or where the infiltration of inflammatory cells is moderate or focal. Finally, changes in the morphology of the complete nephron due to disease processes are a relatively unexplored area that could be important in disease pathogenesis.

Super-resolution microscopy in the kidney

The advent of electron microscopy revealed key structures in the glomerulus critical for effective blood filtration including the endothelial fenestrations, slit diaphragms and the fine processes of the podocyte. Light microscopy cannot resolve any structure smaller than 200 nm in diameter. This is important as podocyte foot processes, slit diaphragms, and the GBM are all smaller than the diffraction limit. New methods called super-resolution imaging now allow this barrier to be broken using fluorescent microscopes. In recognition of this breakthrough, the Nobel Prize in Chemistry in 2014 was awarded for this achievement.

Breaking the diffraction barrier

In the 19th century, Abbe discovered a mathematical formula that could explain the theoretical resolution limit of light microscopy. This formula showed that the resolution limit correlated with the wavelength of light and the NA of the lens, which incorporates both the refractive index of the media as well as the size of the cone of light that can enter the objective. For practical purposes, Abbe's law states the resolution is about ½ of the wavelength of illuminated light. Since the wavelength of visible light is between 400–800 nm, the theoretical limit of light microscopy is about 200–300 nm. Abbe predicted that smaller wavelengths of light such as that used for EM (0.008 nm) would allow for higher resolution. However, EM requires high power illumination, very thin tissue sectioning (100 nm), specialized fixation methods and instrumentation. These methods are unwieldy and often damage the tissue limiting their widespread use.

Recently, new methods allow light microscopes to image below the diffraction limit. The three major methods, STED, STORM and PALM are all variants of fluorescence microscopy and are based on single molecule detection. STED and STORM require specific fluorescent antibody reagents while PALM uses green fluorescent protein. Each method has limitations that will determine what the best applications are.

STED—Stimulated emission depletion microscopy (STED) uses two lasers and specialized optics to illuminate a spot between 30–80 nm [39,40]. Essentially, a donut shaped excitation ring functions to bleach the area outside of the spot, leaving the central spot to fluoresce by itself. Like a confocal microscope, methodically moving the spot around allows the image to be generated. This method is limited by the need for a specialized microscope and dyes for illumination. Using fluorescent proteins expressed in cells, the method is optimal for live cell imaging. Its utility for imaging of tissue is still unknown.

PALM and STORM—Photoactivated Localization Microscopy (PALM) [41] and Stochastic Optical Reconstruction Microscopy [42,43] are both based on a similar principle in which single molecules are imaged and mathematics is used to calculate the position of the molecule in the sample. In both methods, excitation of only a small number of fluorescence molecules occurs. This increases the probability that a fluorescent signal detected is from a single molecule and not from two or more closely localized molecules. In the case of PALM, the fluorescent molecule is bleached returning it to the ground state. In the case of STORM, the ability of fluors like Cy3 and Cy5 to blink is used. Since the diffraction limited spot (>200 nm) has a Gaussian distribution, careful mapping of the

position and calculation for the center of the spot allows the fluorescent molecule to be placed in the image with high precision, typically around 20–25 nm. This process is repeated thousands of times and the computer creates the final image by overlaying all of the results together.

Imaging kidney cells and tissue

While super-resolution imaging techniques have been extensively applied to cells in culture, their use on tissue sections is infrequent. Recently, Dani et al. used STORM to image the neurological synapses in brain sections [44]. They used 10–12 μm thick brain slices mounted on glass slides in order to map different molecules to the presynaptic and postsynaptic terminals.

Attempts to use this protocol to image the kidney were unsuccessful, requiring a new methodology of sample preparation. In contrast to the neuronal study, examination of the glomerular structures necessitated a method of preservation also suitable for EM imaging. Tissue preservation using Tokuyasu's method of sucrose protection was found to be suitable for both super-resolution and EM microscopy. Semi-thin sections between 75–200 nm were also required to allow for robust antibody staining and allowing EM correlation imaging.

STORM was first used to study the organization of the GBM using three different antibodies simultaneously [45]. This showed that the GBM is organized into layers with two layers of proteoglycans on the outside surface (Figure 4) and collagen and laminin localized to the inside. The resolution of STORM could also reveal the orientation of molecules in the GBM. Lastly, using genetic models, the source of molecules in the two sides of the GBM appeared to be distinct, with the podocyte side contributing to the surface of the GBM on that side and presumably, endothelial cells contributing to molecules on the other side [45–47].

Super-resolution/electron microscopy correlation

Since super-resolution approaches aim to replace EM, a key component of super-resolution microscopy imaging is EM correlation. This is especially true when using super-resolution imaging techniques on structures such as glomerular podocytes. Many different approaches have been reported using transmission electron microscopy (TEM) [41,48], scanning EM [49,50], platinum replica EM contrast [45,51] and cryo-EM [52]. For kidney tissues, deep-etch freeze-fracture (DEFF) was used successfully. For DEFF, a platinum replica of the surface of the tissue slice is made and imaged by TEM [45–47]. It provides superb resolution, but is technically challenging and more expensive than other EM techniques. Recently, focused ion beam-scanning electron microscopy (FIB-SEM) has been used to image podocytes and the glycocalyx with excellent resolution [10,11,53]. FIB-SEM is capable of providing global high-resolution three-dimensional EM images of the area of interest. Technically, FIB-SEM is less challenging and it is much cheaper than the DEFF. If combined with super-resolution microscopic technique, we anticipate that FIB-SEM could serve as a good EM method for super-resolution/EM correlation studies.

Conclusion

We are already appreciating the kidney at a more detailed level with new advances in imaging. The rate of innovation in imaging continues to accelerate, and newer technologies will enable further probing of homeostatic and pathogenic processes within the kidney.

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Conflicts of interest

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Key Points

1. Volumetric imaging is possible due to advances in laser technology, deeper working distances of objective lens, and tissue clearing approaches such as CLARITY.
2. Volumetric imaging of the kidney can help image whole 3D structures of nephrons or glomeruli that currently have to be inferred using serial thin sections.
3. Super-resolution microscopy enables the visualization of very fine structures that cannot be discerned through traditional light microscopic techniques.
4. Super-resolution microscopy has revealed the complicated architecture of the glomerular basement membrane, including the orientation of molecules within it.

Figure 1. Comparison of tissue section depth, size of structures in the kidney, and depth of various imaging approaches

Imaging using traditional sectioning approaches are unable to easily capture a whole glomerulus: ~30 histological slides are necessary to completely image a glomerulus. Vibratome sectioning requires MPM to satisfactory image a section this thick, but entire glomeruli can be obtained unaltered. Tissue clearing approaches push the limit of current objective lens technology, with the capacity to image up to 8 mm in depth. EM=electron microscopy, MPM=multiphoton microscopy.

Figure 2. Differences between wide-field, confocal, and multiphoton microscopy approaches

A) Wide-field excites a column of the tissue section, which causes emission fluorescence from both inside and outside the focal plane. Out-of-focus fluorescence contributes to detail obscurity, limiting the depth of tissue that can be imaged. B) In confocal microscopy, a pinhole placed just proximally to the detector blocks all out-of-focus light, allowing for in-focus fluorescence to be collected from the sample and dramatically improves visual detail. C) Multiphoton microscopy delivers light in short, rapid pulses from the near-infrared range, which penetrates tissue more deeply. Excitation occurs within a femtoliter volume due to the near simultaneous absorption of two photons. This reduces tissue photobleaching compared to wide-field and confocal approaches while maintaining high level of detail.

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“Tshikawa-Ankerhold HC, Ankerhold R, Drummen GP: **Advanced fluorescence microscopy techniques--FRAP, FLIP, FLAP, FRET and FLIM.** *Molecules* 2012, **17**:4047–4132.”(citation13 within text).

Figure 3. Application of CLARITY on the kidney

A) Schematic of CLARITY. Tissue is first incubated in a solution of acrylamide, bis-acrylamide, and formaldehyde. This solution formed an adduct which covalently attached to proteins and nucleic acids, leaving lipids untouched. The hydrogel is completed by acrylamide polymerization, leaving a gel by which proteins and nucleic acids are stabilized. Lipids are removed in an 8% SDS solution either passively or through an electrophoretic tissue chamber (ETC). Cleared tissue can be immunolabeled afterwards. Once the tissue is washed, clarified tissue is placed in index matched polar solution (FocusClear) for imaging. This method can require a few weeks to render organs

transparent, but is compatible with numerous fluorescent markers. B) Mouse kidney before and after CLARITY.

EDITORS: Note Figure 3A is going to need permission for reproduction. Original citation: “Tomer R, Ye L, Hsueh B, Deisseroth K: **Advanced CLARITY for rapid and high-resolution imaging of intact tissues.** *Nat Protoc*2014, **9**:1682–1697.” (citation 36 within text).

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Figure 4. Difference in resolution between standard immunofluorescence and super-resolution imaging of the mouse glomerular basement membrane

The principle on STORM imaging is outlined in (A). The labeled molecule of interest is located specifically at the center of the red circle, but its excitation generates a broad area of fluorescence (depicted by the whole red circle). In STORM, a location selected at random (designated by the hatched marks on the right) is excited, and the fluorescence measured. If fluorescence is detected (step 1), algorithms identify the location of the peak fluorescence (step 2), and the process is repeated (step 3). The final image shows areas of peak fluorescence only, as shown by the yellow dot located in the center of the red circle where

the molecule of interest is located. Conventional fluorescence (B) and STORM (C) images of a kidney glomerular capillary loop labeled with an antibody to agrin showing two agrin leaflets resolved by STORM imaging.

EDITORS: Note Figures 4B and 4C are going to need permission for reproduction. Original citation: “Suleiman H, Zhang L, Roth R, Heuser JE, Miner JH, Shaw AS, Dani A, McNeill

H: **Nanoscale protein architecture of the kidney glomerular basement membrane.**

*eLife*2013, **2**. <http://dx.doi.org/10.7554/eLife.01149>” (citation 45 within text).

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