Novel light microscopy imaging techniques in nephrology

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Purpose of review

As more genomes are sequenced, the difficult task of characterizing the gene products of these genomes becomes the compelling mission of biological sciences. The melding of whole organ physiology with transgenic animal models, gene transfer methods and RNA silencing promises to form the next wave of scientific inquiry. A host of new microscopy imaging technologies enables researchers to directly visualize gene products, probe alterations in cell function in transgenic animals and map tissue organization. This review will describe these microscopy imaging techniques, their advantages, imaging properties and limitations.

Recent findings

New optical methods such as two-photon confocal microscopy, fluorescence resonance energy transfer, and total internal fluorescence reflectance microscopy are increasingly being applied to extend our understanding of whole organ and renal epithelial function. Two-photon confocal microscopy has been used to image directly into the kidney of living animals. Fluorescence resonance energy transfer has been used to directly visualize transcription factor complexes within the nucleus while total internal fluorescence reflectance microscopy has permitted direct observation of protein delivery to the plasma membrane.

Summary

The application of these optical techniques along with the ability to label virtually any protein with a fluorescent tag will enable researchers to study cellular processes and whole organ function *in vivo*. Light microscopy methods will allow an advance from semi-quantitative to quantitative approaches to problems of relevance to physiologists studying issues related to renal function.

Keywords

confocal microscope, fluorescence resonance energy transfer, green fluorescent protein, total internal reflectance fluorescence microscopy

Introduction

Recent technical advances have revolutionized light microscopic imaging. The advances have made possible observation of cellular processes that normally are beyond the resolution limit of traditional light microscopes. New optical arrangements allow investigators to acquire biophysical data, explore complex tissue organization and create three-dimensional reconstructions. These achievements are feasible due to improvements in computer speed, detector sensitivity, fluorescent probes and optical engineering. Application of these new technologies to problems specific to areas of interest to the nephrology community presents both opportunities and challenges. The challenges arise from the unique anisotropy of the kidney for light in the visible spectrum. Although many tissues have a homogeneous refractive index that simplifies image acquisition, fluorescently-labeled structures in the kidney are more difficult to image due to a heterogeneous network of intertwined or anastomosing tubules, vessels and nerves with intervening stroma.

In this review, we will discuss advances in light microscopy, which offer promising opportunities in areas of renal research. These advances include optimization of confocal microscope equipment, fluorescence resonance energy transfer (FRET), total internal fluorescence reflectance microscopy (TIFRM), fluorescence spectroscopy, new applications for novel forms of green fluorescent protein and data analysis methods. Using state-of-the-art imaging technologies, researchers can probe gene expression to bridge the gaps between whole organ structure and physiology.

Confocal microscopes

An exhaustive description of confocal microscopes cannot be contained in a review of this size, for that the reader is referred to several excellent papers [1,2]. A brief description of common confocal microscope designs is included here to provide the basis for later discussion. Most of the commercially available confocal microscopes are designed to acquire images from biological samples labeled with fluorescent probes. Some of these confocal microscopes can also function in a reflectance mode, which has been employed by some investigators to visualize cell-matrix interactions [3,4]. Laser scanning confocal microscopes follow the basic design proposed by Minsky in 1951 [5]. In the schematic diagram shown in Figure 1 one can see that the image of a pinhole is projected onto a sample with a projection lens at a focal distance ($f1$) from the sample. Emitted light, either

reflected or fluorescent, travels through a second projection lens at focal length $f2$. If the focal lengths of $f1$ and $f2$ are arranged to be the same in both the illumination and light collection pathways, a confocal optical arrangement is produced. Light coming from out-of-focus regions of the sample fall before or beyond the detector located behind the second pinhole and so there is removal of the out-of-focus information from the acquired image. In laser scanning confocal microscopes excitation light is provided by a laser aligned along the optical axis of the pinhole, projection lens and imaging pinhole. A dichroic mirror is placed in the optical pathway to discriminate between the excitation light and the emission light when fluorescent images are acquired. An advantage of this optical setup is that the objective lens functions both as an objective and a condenser, which optimizes two-point discrimination. This optical arrangement is excellent for imaging samples less than 20 μm thick [6]. Beyond that distance light scattering due to refractive index mismatch causes significant attenuation of image intensity and decreases image clarity [6]. Beyond 20 μm light scattering within the tissue by a mixture of objects with different refractive indexes degrades light transmission during both entry and exit. For some tissues this problem can

Figure 1. Laser scanning confocal microscope

be significant even at imaging depths less than 20 μm. In an elegant attempt to solve this problem, researchers have developed adaptive optics for confocal microscopes [7]. A deformable membrane mirror was placed in the optical pathway. The mirror played two roles, first as a wavefront sensor-biasing element and secondly as an aberration correction element. Aberration correction was applied to both the illumination and collection paths of the confocal microscope. This system improved axial resolution of the microscope and enhanced image contrast [7]. The design is one that can easily be incorporated into commercial confocal instruments. However, this application was used to image a sample with a depth of less than 20 μm and is an unproven solution to the problem of imaging deep into tissue.

Multi-photon microscopes

Fluorescence occurs when a fluorophore absorbs light with specific quantum sufficient to bring electrons to a higher energy state. Return of electrons to their ground state is accompanied by photon emission. It is also possible for photons of lower energy to stimulate fluorescence if they are absorbed simultaneously. However, this is a low probability event and requires a high photon flux to occur. In microscopes, fluorescence at lower energy excitation only occurs at the point of highest photon density, which is the focal point of the objective. Because no fluorescence is stimulated outside this spot, a multiphoton microscope generates thin optical sections. This optical arrangement has the added benefit that unlike in confocal and conventional epifluorescence microscopes no photobleaching occurs in the out of focus planes, limiting phototoxicity and permitting the collection of thick three-dimensional volumes. Two-photon confocal microscopy was developed by Denk *et al.* [8]. Imaging within a single focal plane is accomplished by exciting fluorophores with a fast-pulsed femtosecond laser [8]. Rapid pulsed lasers supply photon densities in time and space necessary to stimulate fluorescence. The duty cycles employed in imaging deliver a time-averaged power comparable with a laser scanning confocal microscope. As mentioned above, because the excitation wavelengths lie in the near infrared range tissue penetration is greater. In our experience, 100 μm thick sections can be completely imaged using a two-photon microscope.

Despite these advantages, two-photon confocal microscopes do have some limitations. The resolving power of a microscope in the x–y plane is proportional to $\lambda/2$. Therefore, the longer imaging wavelength of the infrared light decreases the two-point discrimination of two-photon microscopy. This means that some intracellular constituents, generally less than 400 nm apart, are not well resolved in a two photon confocal system. Despite this limitation, two-photon microscopy is an
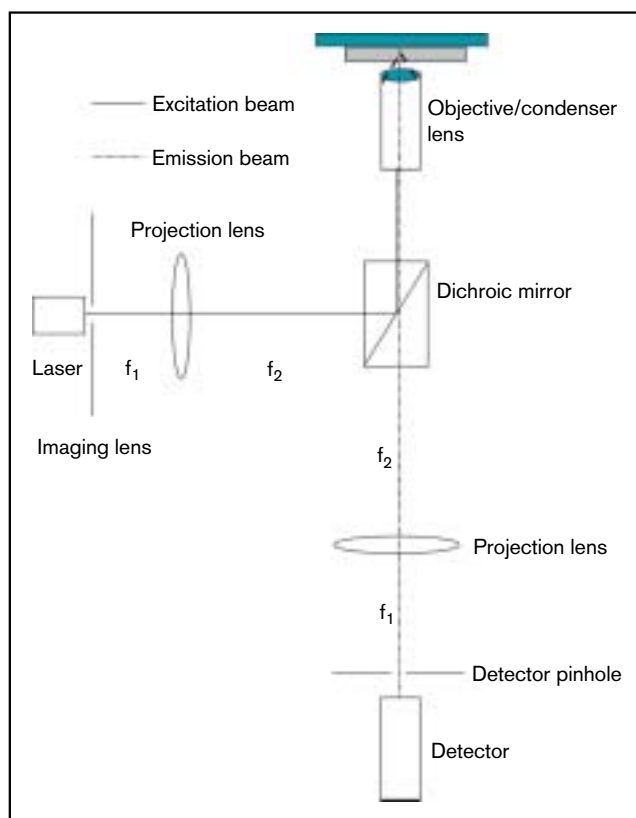
excellent way to observe large-scale tissue organization or to study biological processes *in vivo*.

The imaging advantages of two-photon microscopy would not be realized without sample preparation techniques that permit labeling of thick tissue sections. Phillips and co-workers [9] labeled embryonic and new-born mouse kidneys with antibodies, fluorescent-conjugated phalloidin or lectins. Large-scale analysis of branching morphogenesis could be performed with the images volumes obtained from these samples. Additionally the technical details of sample labeling for greater than 100 μm thick tissues were described in this paper [9]. A similar approach was taken to analyze innervation patterns within epidermis [10]. Two-photon microscopy of whole mounts of the murine developing genital-urinary tract revealed trigone development in mice expressing green fluorescent protein in the collecting duct, developing ureter and bladder [11••]. Ureter positioning in the bladder was shown to be dependent upon the vitamin D and Ret signaling pathways [11••].

Deep tissue penetration permits direct visualization into living tissue [12••,13–15]. Intravital staining of tissue compartments using a variety of fluorescent probes allowed investigators to directly image glomerular filtration, endocytic uptake of fluorescent dextrans, and regional blood flow [12••]. By exteriorizing the kidney and injecting a variety of vital fluorescent stains, the author obtained simultaneous image information on receptor mediated endocytosis, apoptosis, blood flow, leukocyte trafficking and fluid phase endocytosis [12••]. Significantly these investigators showed that several parameters could be analyzed simultaneously and this method has the potential to link gene expression to whole organ physiology.

Petráň tandem scanning confocal microscopes

Petráň tandem scanning confocal microscopes have an optical plate with pinholes cut in an Archimedes spiral pattern (Fig. 2). The disk is placed at a spot equidistant from the light source and detector. When the plate is rotated at high speed the movement of pinholes scans light across the sample. The major problem with this optical arrangement is the low light levels that eventually reach the detector [16,17]. To circumvent this limitation and increase scientific utility, scanning disc confocal microscopes have been outfitted with improved charged-coupled device cameras and lasers. The Perkin-Elmer Life Sciences (Perkin Elmer Life Sciences, Boston, MA) confocal microscope employs a Yokogawa spinning disk system. This scan head has two disks that are arranged to limit the amount of illumination light that reaches the detector. This confocal microscope suffers from the disadvantage that only two high numerical aperture lenses can be used to image in the confocal mode. Only the 60X and 100X objectives can be used in this system and because they are high numerical aperture objectives, the working distance is low. Despite this limitation, this microscope allows for extended imaging of cells and near video rate data acquisition. The low-light levels ameliorate phototoxicity during imaging and it is possible to observe entire three-dimensional data sets at near video rate. A useful comparison of scanning disc confocal microscopes and multifocal multiphoton microscopes has been published [18•].

Live cell imaging has been used extensively in the past 2 years. The development of green fluorescent protein as a vital stain for virtually any cellular protein has greatly aided the utility of live cell imaging, which has been particularly useful for studies of membrane traffic [19]. The dynamics of Golgi coat assembly were studied in living cells. This study showed that Arf1 (ADP-ribosylation factor) catalyzes delivery of COP1 (Coat protein-1) to Golgi membranes and coatomers remain on Golgi membranes even after Arf1 has hydrolyzed GTP [19]. Assembly of protein complexes in cells is also easily studied with the scanning disc confocal microscopes. For example, modulation of an inward rectifying potassium channel, Kir2.1, by A kinase-anchoring protein 79 during cell signaling was studied in HEK-293 cells [20]. A kinase-anchoring protein anchors kinases near phosphorylation sites on the membrane channel [20].

Fluorescence resonance energy transfer

FRET relies on the ability of one fluorophore to absorb light at its excitation wavelength and emit light at a wavelength that lies within the excitation spectrum of a second fluorophore. If the second fluorophore is oriented in the correct direction, and close enough to the emitting fluorescent molecule, then the second fluorophore will emit fluorescent light at its characteristic emission wavelength. Both fluorescent molecules must be within 60–80 nm [21]. This requirement for FRET extends the ability of the microscopist to observe interactions that would normally be below the resolution limit of a light microscope. Using mixtures of cyan fluorescent protein and yellow fluorescent protein to perform FRET analysis, investigators have extended the utility of this method. However, the increasing popularity of this experimental approach must be cautiously scrutinized. The experimental system must be carefully characterized before the results of FRET analysis can be interpreted. Cyan fluorescent protein has a relatively low quantum yield as compared with yellow fluorescent protein. In addition yellow fluorescent protein has a large excitation spectrum, which partially overlaps with cyan fluorescent protein (even though the efficiency of excitation is low at these wavelengths). Under these

Figure 2. Diagram of a spinning disk confocal microscope
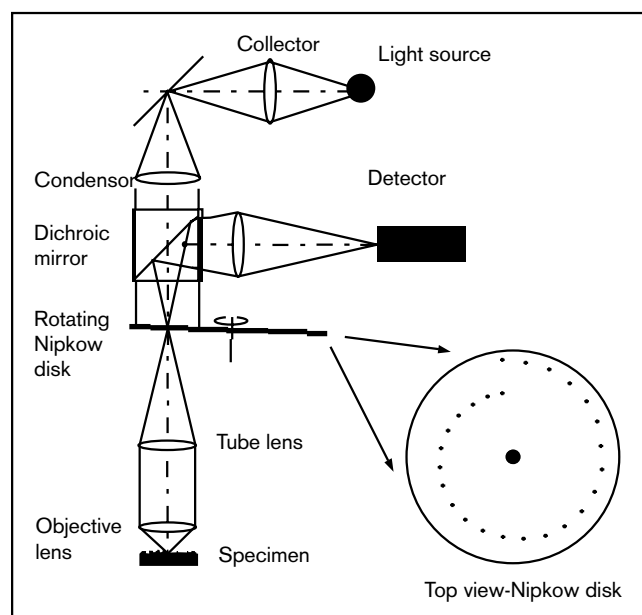
A disk with pinholes placed in a spiral configuration (inset) is located in an optical pathway midway between specimen and detector. Spinning the optical disk results in scanning of the illumination light across the sample. This type of confocal arrangement usually results in low light level illumination. The Perkin-Elmer Life Sciences confocal microscope has two spinning disks in the scan head. Cooled charge coupled devices (CCDs) are employed as light detectors and high powered lasers provide sufficient light input for live cell imaging.

conditions it is easy to conclude that a fluorescent signal in the yellow fluorescent protein channel is due to FRET when the fluorescence may be due to direct stimulation. However, if the experimental conditions are correctly characterized significant protein interactions can be ascertained [21].

Particularly elegant applications of FRET have been used to demonstrate a direct interaction between the pituitary specific transcription factor, Pit-1, and c-Ets-1 transcription factor within the nucleus of transfected cells [22]. A similar approach was used to map alpha helices containing the consensus sequence (LXXLL) in activating ligands with the hydrophobic cleft domain of the estrogen receptor [23]. Interestingly the specificity of FRET, were confirmed by demonstrating that FRET did not occur when anti-estrogen ligands, tamoxifen or ICI 182,780, were incubated with the cells. Conversely FRET was enhanced when estrogen receptor ligands, estradiol, diethylstilbestrol, ethyl indenestrol A, and 6,4′-dihydroxyflavone, were incubated with the cells [23].

Total internal reflection fluorescence microscopy

TIRFM, also known as evanescent wave microscopy, is a powerful surface imaging technique that can achieve

high image resolution in the axial direction. When an incident light reaches the interface between two optical media with different indices of refraction (η), depending upon the incident angle $\theta 1$ a portion of the light will be transmitted and travel in the second medium in a different angle $\theta 2$ (Fig. 3). The relationship between the two angles has the following form:

$$Sin\theta 1 / Sin\theta 2 = \eta 2 / \eta 1$$

Total internal reflection is a condition when the incident light reaches a critical angle θc and light does not travel into the second medium, or put another way, $\theta 2 = 90°$.
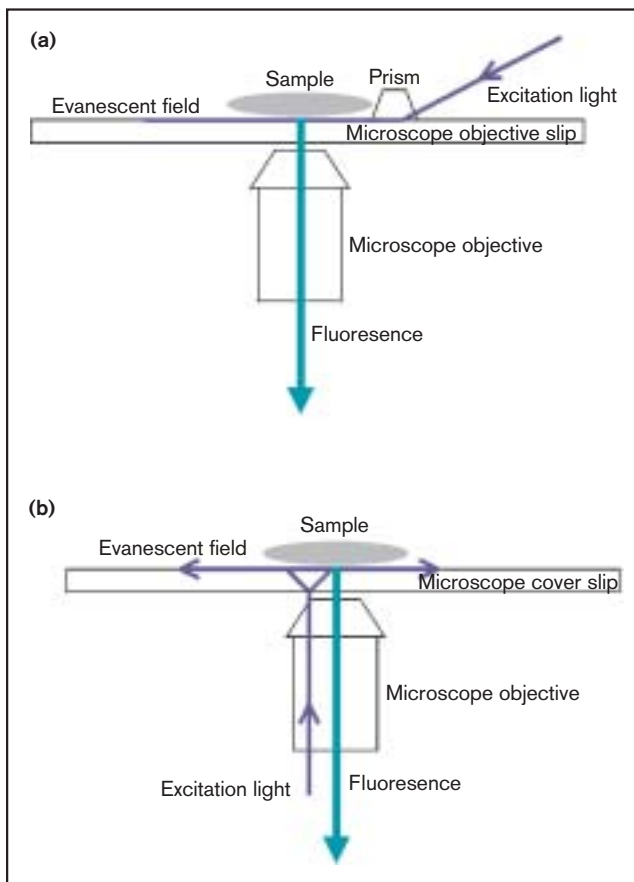
Under this condition, the electrical field (evanescent field) of the optical wave is perpendicular to the surface of the two interfacing optical mediums. The strength of the evanescent field decays exponentially in the direction away from the interface.

There are two major approaches to generate evanescent waves for microscopy imaging: (1) using a coupling prism; and (2) using a special microscope objective with high numerical apertures. The basic idea of a prism coupler-based TIRFM is illustrated in Figure 3(a). One may also fulfill the condition to generate total internal reflection at the cover slip surface through a high numerical aperture objective as illustrated in Figure 3(b).

The reason that TIRFM can achieve high axial resolution is, typically, that the effective distance at which the strength of the evanescent field can still produce enough fluorescence for imaging is less than 100 nm. By comparison, this resolution is at least a factor of five better than a typical one-photon confocal system.

TIRFM is typically used to monitor events at the plasma membrane. It is particularly useful to monitor membrane fusion events [24,25,26••,27••]. Time-lapse TIRFM was used to follow exocytosis of post-Golgi transport carriers during development of epithelial polarity in Madin-Darby canine kidney (MDCK) cells [28••]. TIRFM data was correlated with three dimensional reconstructions of confocal data to show that apical and basolateral membrane protein transport vesicles were randomly distributed in the cytoplasm of non-polarized MDCK cells. In polarized cells, transport vesicles containing cargo destined for assembly in the lateral membrane were predominantly distributed along the lateral membrane below the tight junction [28••]. Conversely apical transport vesicles were distributed in the apical region of the cytosol. Microtubule depolymerization induced a redistribution of syntaxin 3 which mediated delivery of apical transport vesicles to the lateral membrane domain [28••].

Figure 3. Total internal reflection microscopy

(a)

Sample
Prism
Evanescent field
Excitation light
Microscope objective slip

Microscope objective

Fluoresence

(b)

Sample
Evanescent field
Microscope cover slip

Microscope objective

Excitation light
Fluoresence

(a) Using a coupling prism. The incident light is shown in blue. Through the prism, at the critical angle, the incident light generates total internal reflection at the surface of a microscopy cover slip. Sample fluorescence, shown in green, caused by evanescent field excitation is collected through a microscope objective. (b) Total internal reflection microscopy optical pathway using a microscope objective with high numerical aperture.

Developments in green fluorescent protein utility

Green fluorescent protein and the newer mutant forms, cyan fluorescent protein and yellow fluorescent protein, have greatly extended the ability of researchers to study protein activity and subcellular localization of virtually any protein. New forms of green fluorescent protein can now be used as in-vivo probes for pH and calcium [29–32]. These novel forms of green fluorescent protein open up the possibility of in-situ analysis of calcium signaling and pH regulation particularly in transgenic animals.

Volume reconstruction

The major advantage of confocal microscopy is the ability to collect three-dimensional volumes. Data sets collected by confocal microscopes translate into stacks of image intensity values arranged in 512×512 arrays. Reconstruction of three-dimensional data sets to form volume renderings is computationally intensive and tended to require mainframe computers. A new image-processing program that utilizes an open source program to access the Nvidia Gforce 3 (*n*Vidia, Santa Clara, CA) video card has been released as shareware. This low cost alternative to commercial volumetric rendering [33••] allows investigators to perform real time volumetric rendering using a standard personal computer and a low-cost video board. An example of a volume rendered image is shown in Figure 4.

Future directions

New fluorescent probes are under development, which may replace green fluorescent protein [34•,35•]. Improvements in far red fluorescent proteins from sea corals may result in more reliable FRET analysis *in vivo* [34•]. Newer approaches to fluorescent labeling using quantum dots have the advantage that less photobleaching occurs

Figure 4. Three-dimensional rotations showing membranous glomerulopathy

Optical Z-stacks were collected from a human biopsy specimen using two-photon fluorescence microscopy. Voxx rendering permits exploration of the thickened glomerular basement membranes labeled with *lens culinaris* lectin (brown). The capillary loops are carpeted with subepithelial deposits of immunoglobulin G (blue). The volume measures 205 μm (x-axis) x 205 μm (y-axis) x 50 μm (z-axis).
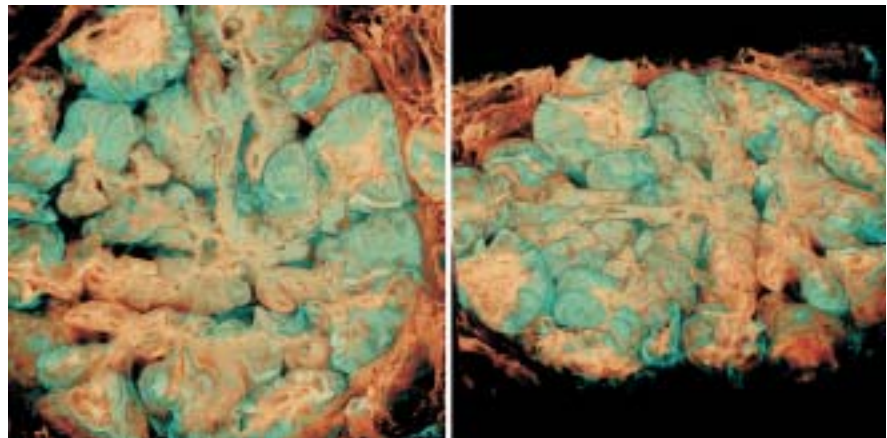
with these fluorophores [36**]. Nanocrystal fluorescent probes could be used *in vivo* and due to their small size have been used to specifically label DNA sequences [36**].

Traditional applications of fluorescence microscopy use only a small amount of the informational content of a fluorescent signal. Fluorescence spectroscopy can be used to measure local effects on fluorescent time constants and to give a direct reading on local pH or ionic strength conditions. Additionally the polarization of the emitted light can also provide information about local conditions. While fluorescence spectroscopy has been used to characterize biochemical reactions in a cuvette, it may be possible to use this powerful method for analysis of living specimens. Increasingly these methods will be brought to bear on research problems of interest to the nephrology community.

Conclusion

New optical arrangements offer unique opportunities to address questions relevant to clinical nephrology. These technologies' power lies in the ability of light microscopy to convert every cell into a biochemical crucible, directly assessable to observation. Creative use of green fluorescent protein chimeric constructs will enable researchers to observe how signaling pathways are integrated and where in the cells signaling pathways are organized. It is clear that every protein in the cell has specific activities but these activities have a spatial context that is important for modulating activities. Light microscopy methods offer unique opportunities to address these questions.

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