Applications of Confocal Microscopy

An Essay Submitted
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ABSTRACT

Confocal microscopy is an important new imaging technique that offers tremendous promise for the study of in vivo system for corneal changes, general experimental cell biology and medicine.

Confocal microscopy provides the ability to section optically, living or in-vitro tissue non- invasively over time. Images are obtained from different depths within the thick specimen. Thus confocal microscopy has made it possible to view biological structure and function at the cellular level under physiological conditions than other methods.

Confocal microscopy allows non-invasive in vivo imaging of the ocular surface. Its unique physical properties enable microscopic examination of all layers of the cornea and have been used to investigate numerous corneal diseases: epithelial changes, numerous stromal degenerative or dystrophic diseases, endothelial pathologies, corneal deposits, infections, and traumatic lesions.

Key words:

Keratoplasty, The morphology of normal cornea, Corneal dystrophy, Infectious keratitis, Refractive surgery

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بسم الله الرحمن الرحيم

﴿وعلمك ما لم تكن تعلم وكان فضل الله عليك عظيماً﴾

صدق الله العظيم

سورة النساء آية (١١٣)

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LIST OF ABBREVIATION

1- AD : Autosomal dominant.
2- AR : Autosomal ressesive.
3- Conf. mic.: Confocal microscope.
4- CMTF : Confocal microscopy through focusing.
5- DLK : Diffuse lamellar keratitis.
6- ECM : Extracellular matrix.
7- LASIK: Laser in-situ keratomileusis.
8- LCM: laser confocal microscope.
9- PRK: photorefractive keratectomy .
10- SEIs: Subepithelial infiltrates.
11- TSCM :Tandem scanning confocal microscope.

INTRODUCTION

Background

The development of the microscope was one of the highly influencing advances in the history of science. For the first time, the realm of the truly small became accessible to observation and study. The limiting constraint of microscopy in determining the structural and functional interrelationships in living cells and tissues, however, is the need to section or otherwise process the specimens to be examined. Because the conventional microscope collects all of the light reflected back through the object imaged, out-of-focal-plane information above and below areas of interest creates noisy and unsatisfactory images in all but the thinnest specimens at high magnification. Furthermore, the need to section mechanically, fix, embed, or stain specimens introduces inherently uncontrollable processing artifacts and severely compromises or obviates the possibility of examining truly undisturbed physiologic processes in situ as they actually occur.[1]

Confocal microscopy is an important new imaging technique that overcomes some of these problems and offers tremendous promise for studies of in vivo systems for both ophthalmology and general experimental cell biology and medicine. As the emerging technology and applications rapidly continue to develop, the potential importance of this unique instrumentation is vast. Confocal microscopy provides the ability to section, optically, living or in vitro tissues noninvasively over time. Images are obtained from different depths within the thick specimen, thereby eliminating the need for processing and sectioning

procedures. Thus confocal microscopy has made it possible to view biologic structure and function at the cellular level under more physiologic conditions than heretofore possible. The most widespread current use of confocal microscopy has historically been the application to the in vitro or ex vivo study of cell biology with specific immunofluorescent molecular probes using coherent light. However, significant opportunities for in vivo, real-time applications using noncoherent light (white light) are beginning to be appreciated.

HISTORY OF CONFOCAL MICROSCOPY

The optical design of confocal microscopy is based on the principal of Lukosz,[2] which states that resolution may be improved at the expense of field of view. In 1955, Minsky[3] developed the first confocal microscope for studying neural networks in the living brain. The Minsky microscope condenser focused the light source within a small area of tissue, with concomitant focusing of the microscope objective lens on the same area. Because both condenser and objective lenses had the same focal point, the microscope was called confocal. Since the introduction of Minsky's original microscope, the optical theory of confocal microscopy has been more formally developed and improved. In modern confocal microscopy, a point (i.e., diffraction limited) light source is focused onto a small volume within the specimen, and a confocal point detector is used, to collect the resulting signal.

This technique results in a reduction of the amount of out-of-focus signal from above and below the focal plane, which contributes to the detected image and produces a marked increase in both lateral (x,y)

and axial (z) resolution.[4]

The use of a point source/detector in the confocal optical design affects field of view for the enhanced resolution; therefore a full field of view must be built up by scanning. The first scanning confocal microscope, developed by Petran et al,[5] used a modified Nipkow disk containing thousands of optically conjugate pinholes arranged in archimedian spirals (Fig.1*).* Light from a broad band source passes through the pinholes on one side of the disk and is focused into the specimen. Detector pinholes on the opposite side of the disk prevent light from outside the optical volume, as determined by the objective lens and pinhole diameter, from reaching a camera or eyepiece. Rotation of the disk results in even scanning of the tissue in real-time. Because illumination and detection of light through conjugate pinholes occurs in tandem, this microscope was named the tandem scanning confocal microscope (TSCM).

Most confocal systems in use today use a focused laser beam as the point source and are thus called laser confocal microscopes (LCM***).***[7] In scanned beam LCM, rotating or vibrating mirrors are used to scan a stationary specimen. These systems have been used for a variety of biologic applications in recent years.[8]

The most common use has been the localization of fluorescently labeled structures in biologic tissue or cell culture. The optical sectioning ability of confocal microscopy provides images with improved clarity as compared to conventional epifluorescent microscopy, especially within thick tissue specimens. Furthermore, confocal microscopy can be used to characterize the three-

dimensional organization of labeled structures by acquiring a series of thin optical sections through a sample and reconstructing and projecting the images on a computer. [9]

CURRENT IN VIVO IMAGING SYSTEMS

Although the superiority of confocal imaging over conventional light microscopy for many biologic applications ex vivo and in vitro is well recognized, one area that has not received significant attention is the application of confocal imaging techniques to the study of cells within intact organ systems. In vivo confocal imaging is challenging because movement of the cells or tissue due to heartbeat, pulse, or respiration can cause blurring of the detected images; real-time imaging therefore is required. Unfortunately, most beam scanning LCMs require nearly one second to acquire a single 512 x 512 image. However, several vendors have recently developed real-time laser confocal microscopes that allow video-rate image acquisition in both reflected and fluorescent modes. Despite their real-time capabilities, these systems have not yet been applied in vivo. One limitation of these systems is that the high energy laser light source can potentially damage living tissue because of light toxicity.[1]

Most of the in vivo imaging to date has been accomplished with the TSCM design. The TSCM is well suited for in vivo applications because it generates images in real-time, and uses a broad band light source that causes less tissue damage than laser sources. Petran and Hadravsky obtained the first images of cells from uncut and unstained tissue blocks, including the brain, retina, and other

organs. [10] These initial observations were not repeated until 1985 when Boyde demonstrated the dramatic optical sectioning abilities of the TSCM by imaging osteocytes in intact bone without demineralization, grinding, or other destructive processing techniques.[11] In 1986 Lemp et a1[12] were the first to apply confocal imaging techniques to the study of the cornea ex vivo. This work led to the design of a TSCM with a horizontally oriented objective, which was more suited to use in ophthalmology[13] (Fig. 2).

One of the principal applications of the TSCM has been the imaging of the cornea in vivo. High resolution ophthalmic images have been obtained previously using a specular microscope, which scans the cornea with a narrow slit beam, thus reducing the volume of scattered light contributing to the final image.[14] These microscopes are in widespread use today in most eye banks and clinics. The major difference between specular microscopy and confocal microscopy is that the specular microscope uses a slit aperture, whereas the confocal microscope uses a diffraction limited spot. As shown by Sheppard and Cogswell,[15] confocal imaging provides a higher signal/noise ratio with concomitantly improved resolution in the *z-axis* compared to the slit design. Even with a very narrow slit, where there is a marked improvement in imaging, resolution can be maximized only by reducing the slit aperture to its theoretical limit (i.e., a pinhole aperture). The clinical specular microscope is used primarily to provide images of the posterior (endothelial) surface of the cornea, which provides a bright specular reflection.[16] With some difficulty, it also can be used to image the anterior (epithelial) corneal surface.[16]

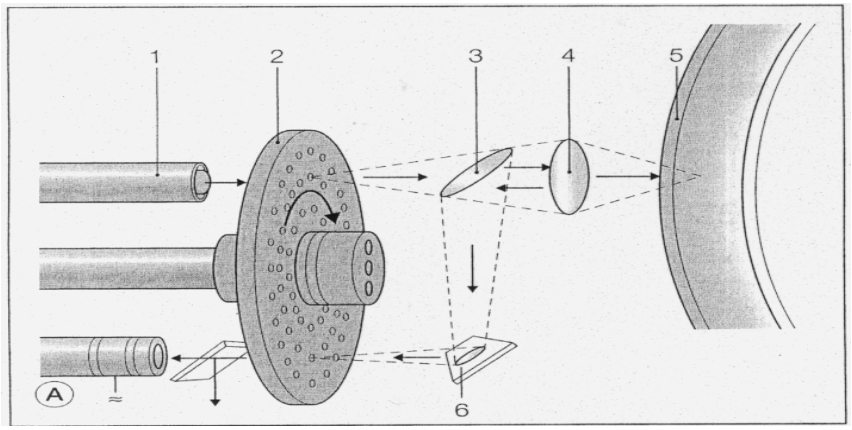

Fig. 1. An illustration of the optical pathway used in tandem scanning confocal microscopy (TSCM). Light from a broad band source (1) passes through the pinhole one side of a Nipkow disk (2) and a beam splitter (3), and is focused by an objective lens) (4) into the specimen (5). The reflected or emitted signal is then reflected by the beam splitter (3) and front surface mirror (6) to the conjugate pinholes on the opposite' side of the disk, which prevent light from outside the optical volume from reaching camera or eyepiece. Rotation of the disk results in even scanning of the tissue in real-time.[6]

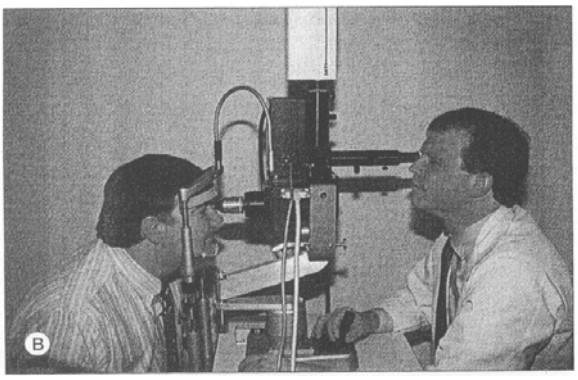

Fig. 2. TSCM adapted for use in ophthalmology.[1]

Confocal microscopy, on the other hand, provides for noninvasive optical sectioning and observation of the corneal epithelium, epithelial basal lamina, stromal keratocytes, and nerves, as well as the corneal endothelium. Furthermore, because of its improved optical sectioning, the TSCM can better detect images through a cornea that is partially opaque as a result of edema or scarring. This characteristic of the TSCM makes it ideally suited for dynamic studies of corneal pathology.

The primary disadvantage of confocal microscopy is that it is more difficult to operate and has more sophisticated image processing requirements than specular microscopy.

Recently, Koester et al [17] have redesigned their bifurcated optical path objective lens to improve the optical sectioning ability of the specular microscope, allowing images to be obtained at good spatial resolution for all cellular layers of the cornea. The greatest advantage of this system is low cost; a standard clinical microscope is generally already available in most ophthalmology clinics and can be updated relatively inexpensively with added optical accessories. For many user applications, both clinical and biologic, this technique may offer the least expensive way to use this new technology.[18] This system, however, uses a slit detector instead of a point detector and the split objective path, which divides in half the effective numerical aperture. Thus it is unclear at this time whether it can provide as high a lateral and axial optical resolution as TSCM imaging using the same numerical aperture objective. One potential advantage of using the slit design is that flash photography can be used for image detection, thus eliminating the need for a complex image acquisition system.