CONFOCAL MICROSCOPY OF THE HUMAN CORNEA

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Abstract Confocal microscope is an ophthalmic instrument that provides real-time and non-invasive serial imaging of cornea. With the principle of both projecting of light to the tissue and focusing lens have the same focal point, it has a high magnification of 40X to visualize many layers of cornea, in sequence, the epithelial layer, subepithelial nerve plexus (which has not demonstrated by any instrument before), stroma (the most thickness layer that comprised of keratocytes and matrix), and endothelium. Therefore, this instrument can apply for a clinical use in the studies of cornea. Chiang Mai Medical J 2007;46(2):83-91.

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The concept of confocal microscopy is obtained from a clinical need to visualize the living tissues noninvasively in a more dynamic way than that provided by conventional light microscopy.

In 1955, Marvin Minsky (1) developed the first clinical confocal microscope for studying neural networks in the living brain. His microscopic condenser focused on light source within an area of neural tissue, with concomitant focusing of the microscopic objective lens on exactly the same plane. According to both condenser and objective lens having the same focal point, the microscope was termed confocal.

The first practical application of ophthalmologic confocal optical theory was developed in 1974 by David Maurice, (2) who illustrated enhanced resolution and contrast in both endothelial and in situ keratocyte images that were obtained by narrowing a slit beam, thus reducing the volume of scattered light reaching the final image.

James Hill (3,4) obtained US Patent 5307203 on a confocal tandem scanning reflected light microscope in 1994, which is marketed as the Tandem Scanning Confocal Microscope for Ophthalmology.

The confoscan is a confocal microscope system that employs an oscillating slit aperture in an ophthalmic microscope configuration, especially suitable for cell layer analysis of the cornea. The confoscan4 comprises of a confoscan, non-contact endothelial microscope, and pachymeter.
Basic principles

On a regular microscopic examination of transparent fine structured tissue, especially cornea, this tissue is not visible. By using the confocal microscope setup, the fine structures can be seen in all layers. The light is projected onto the cornea and passes through one half of the front lens. Most of the light is concentrated inside the focal point. Only a small area inside the cornea is illuminated to minimize light scattering. A small illuminated slit is projected into the cornea, similar to the slit lamp. A small amount of reflected light passes through the other half of the front lens and a second slit, with the same size and optical setup as the illumination slit. Finally, this image is projected on PC monitor. According to this optical arrangement, all the unwanted light that comes from the unfocused layers is cut off by the second slit. A halogen lamp illuminates the first slit. A collector and condenser lens arrangement allows a homogenous illumination of the first slit. A tube lens between the front lens and the slit projects the illumination slit into the cornea. The reflected portion of light passes through the second half of the objective and is projected into the second slit by means of a second tube lens. The final corneal image is projected onto a highly sensitive camera with an achromatic lens (Fig. 1).

When the slit is in motion, it scans the cornea in order to see a larger area. The confocal microscope is able to demonstrate a separate layer of transparent organic structures and tissues of the cornea in high magnification. A standard 40X immersion lens delivers an image field of approximately 440x330 micron. Using other lenses, such as 20X, different magnification can be obtained.

Normal corneal morphology

In vivo confocal microscopy provides real-time and non-invasive serial imaging of corneal subsegments with resolution and imaging contrast. Generally, nearly all corneal layers are clearly visible through confocal microscopy. According to the variable thickness of the corneal structures, as soon as a single scan is done, a larger number of images are caught within the stroma, which generally represents 85% to 90% of the entire corneal thickness. If the scan is well executed and embraces the whole corneal thickness, it is possible to visualize, in sequence, the epithelium, subepithelial nerve plexus, stroma, and endothelial layer.

One important aspect that needs to be stressed is that even in eyes with significant corneal opacities resulting from corneal edema or scar, the endothelial pathology could be imaged with sufficient contrast.

The Bowman’s and Descemet’s membranes in normal cornea cannot be visualized directly because they are transparent to confocal microscopy; but it is possible to demonstrate abnormal features of these structures when they are affected by pathological processes like Bowman’s membrane dystrophy of congenital glaucoma. The best quality image of each corneal layer is supposed to be attained when the object is well-centered.

Figure 1. The confocal arrangement. (a: camera, b: light source, c: moving slits, d: front lens, e: cornea)
Confocal microscopy

perpendicularly on the central surface of the cornea, when the eye is steady and firm, and when the level of illumination is perfectly set, based on the level of intrinsic reflectivity of the different corneal structures.

These can vary among different individuals and within the same cornea, and this variation depends on the perpendicular or oblique position by which the scan is performed. Normally, the corneal endothelium is the most reflective and will appear as a bright structure, while other layers like the rear stroma present a lower level of intrinsic reflectivity. This means that if the intensity of light is set to give a good view of the endothelium, without excessive reflection, the stromal matrix could not have bright keratocytes and good contrast; thus the endothelial layer could appear excessively white due to huge scattering light, resulting in poor quality images. Therefore, it is important to pay attention to the light intensity setting, in order to obtain good quality images during a confocal microscopic examination. If the primary goal of the examination is to attain a good view of all corneal layers, it might be helpful to employ an intermediate level of light intensity, which can be varied to give the best result when focusing on a particular corneal structure.

There is physiological variability in morphology, size, and density of different corneal cellular populations (epithelium, stromal keratocytes, and endothelial cells) in healthy humans, but in normal eyes there are no statistically significant differences in cell densities or cell areas of any corneal layer between male and female patients, or between right and left eyes. Recent studies evidenced that only the endothelial cell density decreases with age, while the densities of the other corneal cell layers do not have a statistically significant correlation with age.\(^6\) Other studies, in contrast, showed that full-thickness keratocyte density is correlated with age; decreasing by 0.45% per year.\(^7\)

### Epithelium

The human corneal epithelium is composed of five to six cellular layers, in which three different kinds of cells can be recognized.

1. Flat, superficial cells (two to three layers)
2. Polygonal, intermediate cells (two to three layers)
3. Cylindrical, basal cells (one layer).

The last type, which lies on a basal membrane, is the only one capable of mitotic activity. The average thickness of the whole epithelium is approximately 50 micron. By means of confocal microscopy, it is possible to recognize and distinguish three different cellular types. In confocal bidimensional sections, superficial epitheliums appear polygonal in shape, generally with evident nuclei, well-defined cellular borders, and homogeneous density. Nuclei are usually brighter than the surrounding cytoplasm, and a perinuclear hyporeflective dark ring is typical (Fig. 2). Basal epithelial cells appear instead as smaller polygonal cells, thus presenting a higher density, without evident nuclei and with very well-defined and bright cellular edges. Intermediate cells present borderline characteristics.

Normal values of cell size and density are extremely variable in normal subjects. The area of superficial epithelial cells is 900±300 micron\(^2\) on average (range: 520 to 2,100); the mean superficial epithelial cell density is usually around 1,200±370 cells/mm\(^2\); the mean area of basal epithelial cells is generally 180±20 micron\(^2\) (range: 138 to 242); and the average basal epithelial cell density is 5,700±600 cells/mm\(^2\) (range: 4,135 to 7,267).\(^6\)
According to the higher reflectivity, in comparison with the underlying stroma, the epithelium usually presents a recognizable peak of reflectivity, thus it is possible to measure the entire epithelial thickness. The results of epithelial thickness measurement using in vivo confocal microscopy are generally reproducible and consistent with the ones obtained by microscopic anatomy, giving a normal central epithelial thickness of 48.6±5.1 micron.\(^7\)

**Subbasal and subepithelial nerve plexus**

It is impossible to visualize details of the acellular structures of the basal membrane of the epithelium and Bowman’s membrane. Until the advent of confocal microscopy in clinical practice, descriptions of the corneal innervation structure were possible. Confocal microscopy gives the opportunity to study the morphology of the corneal innervation in vivo.\(^8\)

The subbasal nerve plexus, which runs between the basal cellular layer and the most anterior part of the Bowman’s membrane, is clearly visible in healthy cornea. Corneal nerves derive from the long ciliary nerves that come from the ophthalmic branch of the trigeminal nerve. At the level of the sclerocorneal limbus, nerve fibers that derive from the long ciliary nerves are arranged into a circular plexus from which other fibers originate. These fibers run radially with several anastomosis within the rear stroma, forming the deep corneal plexus. Thin vertical fibers originate from this plexus and then form the subepithelial nerve plexus. By perforating the Bowman’s membrane at the level of the basal epithelium, the fibers form the subbasal nerve plexus. These fibers run superficially, providing innervation to the basal epithelial cell layer and terminating within the superficial epithelial layers.\(^9\) Oliveira-Soto et al proposed classifying the microscopic nerve anatomy of the cornea, as visible by confocal microscopy, due to a subdivision of the corneal layer into different sublayers.\(^9\) The subbasal epithelial nerve plexus has a close anatomical relationship with the basal epithelial cell layers and the underlying basal lamina. The subepithelial nerve plexus communicates with most of the anterior stroma below the Bowman’s membrane. The stromal nerve fibers, instead, are located in the anterior-mid and mid stroma. The plexus is generally visualized by confocal

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**Figure 2.** Superficial epithelial cells. Nuclei are bright with perinuclear hyporeflective dark ring.
Confocal microscopy, and thin bright and reflective nerve fibers, disposed into a vertical or oblique parallel orientation with several bifurcations, are visible. The thickness of single subbasal nerve fibers usually ranges from 2 to 4 microns, while the subepithelial fibers are on average 3 to 7 microns in width. Recently, several parameters of normal human corneal nerve, such as normal density, width, tortuosity, reflectivity, orientation, and branching patterns, have been investigated by using confocal microscopy.\(^{(9)}\) Normally, it is possible to observe long, parallel-running interconnected bundles.\(^{(10)}\) The nerves are often bright and well-contrasted against an opaque dark background. The subbasal epithelial nerve plexus may appear well-defined, with homogenous reflectivity distinct from the background, or it may have a blurred appearance (Fig. 3). Nerve reflectivity may not be homogenous within the width of the nerve fibers.\(^{(9)}\)

**Stroma**

Corneal stroma represents, on average, 80% to 90% of the whole corneal volume. It is possible to recognize three different components within its structure:

1. **Cellular**
2. **Acellular**
3. **Neurosensory.**

The cellular component is composed of keratocytes which represent approximately 5% of the entire volume. The acellular part (90% to 95% of the stroma) includes regular collagen lamellar structures (collagen type I mostly, but type II and V are also present) and interstitial substance. The last part is represented by stromal nerve plexus together with isolated stromal nerve fibers. The typical confocal microscopic image of the stroma shows several bright, irregularly oval-shaped bodies, which represent the keratocyte’s nuclei, spread within a nearly transparent (dark grey or black) acellular matrix (Fig. 4). This usually means that in the absence of stromal pathologic conditions, direct visualization of components such as the extracellular matrix, interstitial substance, and collagen lamellae is impossible because of the intrinsic transparency of these structures to light. In confocal microscopy, the corneal stroma can be divided into four different sublayers: anterior stroma (right below...
Bowman’s layer), anterior-mid stroma, mid stroma, and posterior (or rear) stroma. The mean density of keratocytes is higher in the anterior stroma (generally between 600 and 1,600 cell/mm², mean value 1,058±217 cell/mm²), whereas it decreases while moving toward the rear stroma, where it usually ranges from 500 to 1,100 cell/mm² with an average value of 771±135 cell/mm². Keratocyte density is much higher in the anterior of 10% of the stroma. By means of confocal laser-scanning fluorescence microscopy, recent studies showed that, whereas cell density progressively decreases from the anterior (100%) to the posterior (53.7%) stroma, volume density tends to be at its highest in the posterior stroma, and its lowest in the central stroma. The varying density (decreasing with depth) of keratocyte nuclei is evident, as well as the different morphology of these cells in the anterior and posterior stroma. In the anterior stroma, keratocyte nuclei present as a rounded bean-like morphology, while they assume an oval shape in the rear stroma. Often, the keratocytes present with a different brightness, and some of them appear particularly reflective. The difference in brightness (reflectivity) between keratocytes is thought to depend on their metabolic activation (and, of course, on the direction of the incident light ray); and this is the reason why they are called activated keratocytes. They are present in healthy corneas (the meaning and role of these highly reflective keratocytes in normal corneas has not yet been clarified). However, they represent a typical feature of inflammatory or scarring processes such as wound healing after excimer laser refractive surgery.

Within the stroma, particularly in the anterior-mid, and mid-stroma, it is possible to visualize nerve fibers belonging to the deep corneal plexus (Fig. 5). Usually, nerve fibers are absent in the posterior stroma. They appear to be three- to five-fold thicker than the thin nerve of the subepithelial and subbasal plexus, and they generally run linearly alone as single fibers, even though the “Y-shaped” bifurcation of the nerve is often visible. The fiber’s thickness usually ranges from 4 to 14 microns, and it is easy to perform reproducible measurements using confocal microscopy.

Figure 4. Cornea stroma. Oval-shaped nuclei appear as bright bodies in the keratocytes.
By using the 20X objective lens, a different magnification of the corneal structures can be obtained. This frontd lens provides a lower magnification, but a greater field of each frame. Thus, a wider space of corneal tissue can be visualized within each image. It is possible to recognize small bright objects, which represent the stromal keratocytes, and long, linear nerve fiber at the anterior-mid stroma layer. Although this magnification provides a lower contrast of the cellular and extracellular structures, its main advantages are represented by the wide field of view and the possibility of studying nerve fibers along a greater length. It is often possible to visualize two parallel stromal nerve fibers within the same frame, which is rarely done with the standard 40X magnification.

**Endothelium**

The corneal endothelium is the largest posterior layer of the cornea and is formed by a single layer of cells that derive from the neural crest. Descemet’s membrane and the endothelium are not innervated in humans.\(^{(13)}\)

Endothelial cell density of up to 7,500 cell/mm\(^2\) is at its highest at birth, and despite declining rapidly during the first years of life, it reaches average values of 2,700 cell/mm\(^2\) in individual adults.\(^{(14,15)}\) The normal value in healthy adult humans ranges from 1,600 to 3,200 cell/mm\(^2\). When cell density decreases below the level of 300 to 500 cell/mm\(^2\), due to pathological conditions, corneal decompensation is likely to occur.\(^{(16)}\) In childhood, endothelial cells present in a uniform hexagonal shape and homogeneous size, with a density that is generally higher in comparison to the one in older subjects.\(^{(15)}\) During life, a percentage of endothelial cells may assume different polygonal shapes as the cell density decreases.\(^{(16)}\) Polymegathism is an irregularity in the normally regular mosaic pattern. Endothelial cells present in different sizes and a significant part of them lose hexagonal morphology. This feature can also be qualified as the coefficient of variation (CV) of cell size and, despite being sometimes present in normal eyes, it is more often associated with several corneal pathological processes.

By using confocal microscopy, corneal endothelium is easily identifiable. It appears as a bright, unicellular layer composed by

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**Figure 5.** A thick stromal nerve fiber in the central stroma.
hexagonal or polygonal flat cells, disposed in a regular arrangement (Fig. 6). Cell nuclei are not recognizable, and the cellular body is generally homogeneously bright and white. The edges of the endothelial cells are visible as thin, regular, nonreflective lines that follow the cellular borders. The higher the cell density, the smaller the size of the cells that are visualized, using the same lens-objective. The use of a 20X objective lens provides lower magnification power, but a greater field of view.

It is possible to perform a manual or automatic cell count within a desired region of interest, thus obtaining values of mean cell density and mean cell area; it is also possible, with particular software, to calculate the CV of cell size. Recent results, attained by using in vivo confocal microscopy, showed that in healthy adult corneas, the average endothelial cell area is $344\pm51$ micron$^2$ (range: 273 to 553), and cell density $3,055\pm386$ cell/mm$^2$ (range: 1,809 to 3,668). In the same study, the endothelial cell density was found to have a negative, statistically significant correlation with age.

The important role of confocal microscopy in the evaluation of normal and pathological microscopic corneal anatomy is evident, not only because it provides detailed imaging of all corneal layers in healthy and diseased cornea, but also because it allows a prospective and reiterative examination in time, thus permitting the study of evolution and change of the clinical microscopic features that may occur during the follow-up period, without any invasiveness.

**References**

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