Chapter

Confocal Scanning Laser Microscopy in Medicine

Hasan Kiziltoprak, Dilara Ozkoyuncu, Kemal Tekin and Mustafa Koc

Abstract

Confocal Scanning Laser Microscopy (CSLM) offers high resolution morphological details and generates en-face images with excellent depth discrimination for visualizing different structures of the living human body non-invasively. There have been significant advances in technology since the CSLM was first defined. It has been used commonly, especially in ophthalmological area, in order to diagnose and give direction for the treatment of corneal pathologies. Ocular surface, corneal subbasal nerve plexus, filtering blebs of glaucoma surgery were also investigated widely by CSLM. With the improvements in CSLM technology over time, it is widely used in other fields than ophthalmology. The combined use of CSLM with the slit lamp biomicroscopy and optical coherence tomography will also lead to significant advances in the diagnosis and treatment of more diseases in the future.

Keywords: confocal scanning laser microscopy, laser imaging, medicine, ophthalmology, subbasal nerve plexus

1. Introduction

Confocal Scanning Laser Microscopy (CSLM) is a non-invasive imaging method for visualizing different structures of the living human body [1]. CSLM provides morphological details with high resolution and generates en-face images with excellent depth discrimination [1, 2]. CSLM is compatible with three-dimensional (3D) live imaging provided by sequential acquisition of tomograms along the depth direction [3]. There is a broad range of experimental and clinical applications on corneal analysis with CSLM. The imaging procedure may assess stromal changes in keratoconus patients [4], experimental full-thickness corneal 3D imaging [5], the quantification of morphological features of epithelial cell layers, and the subbasal nerve plexus [6–10] that has become very popular in recent years.

Since its commercialization in the late 1980's, CSLM has become one of the most applied fluorescence microscopy techniques for 3D-dimensional structural studies of biological cells and tissues [3]. Recent technological breakthroughs have led to the development of CSLM, and it has reached the current level of high resolution that can be used in many areas today. In recent years, there has been a vast increase in researchers using CSLM in many fields of medicine, especially in ophthalmology.

In this chapter, we have attempted to summarize the principles of CSLM and the application in ophthalmological and non-ophthalmological areas of medicine. Finally, it was discussed how it could give an essential direction to medical development in the future.

2. Principles of confocal scanning laser microscopy

Objects that share a conjugate focal plane are defined as with the term "confocal". In microscopic area, that means whereby the in-focus image plane can be seen from adjacent axial planes in case of coincidence between the focal plane of the objective lens and the detector. CSLM uses a diffraction-limited spot of light to illuminate the sample and an aperture in the collection light path at conjugate focus.

The first steps of CSLM were designed by Marvin Minsky in 1955 at his early education times and patented in 1957 [11]. However, there has been no significant improvement in CSLM technology over a long time as the required technologies which were either underdeveloped or non-existent at that time. Moreover, CSLM technology was new, and there was no pressing need for it by the scientific community. Therefore, the commercialization of CSLM occurred in the late 1980s. Petran *and colleagues* introduced the first tandem scanning confocal microscope in 1968 [12]. The Nipkow disk was used as the basis of a new sectioning microscope, and the field of view was achieved by simultaneously scanning multiple points on a stationary specimen using a rotating Nipkow disc. Even this allows for real-time imaging; it has the disadvantages of a very low light throughput and low image quality. In 1969, Svishchev produced the slit scanning confocal microscope based on an oscillating double-sided-mirror [13]. The Svishchev confocal microscope used two confocal adjustable slits. It was used to observe living neural tissue using an oscillating two-sided mirror for simultaneous scanning and de-scanning of the sample [13]. This design was subsequently further modified to enable real-time scanning. The slit scanning microscope was superior in tandem design in terms of shorter examining time and the requirement of low light intensity. The modern, and first commercially-successful CSLM was developed by Brad Amos and John White at the University of Cambridge. With this new technology, precise 3D visualization of ocular microstructures was achieved [14]. Modern digital image processing technology enables quantitative data to be stored noninvasively, rapidly, and with a low level of illumination.

All confocal microscopes share the same basic principle in their designs that enable optical sectioning of a relatively thick light scattering object. A directed light is crossed through an aperture and focused with the help of an objective lens onto a small area of the specimen. At each tissue location, light is reflected or backscattered and travels the same way back. It is separated from the incident beam by a beam splitter. The reflected light from that specimen was then directed onto a second aperture by a second objective lens. By this method, out-of-focus light is strongly reduced, improving image resolution and contrast considerably. The ability of this system to distinguish between light out of the focal plane yields images of higher lateral and axial resolution compared with light microscopy. As the illumination and detection paths are at the same focal plane, the term confocal is used [2, 3, 15].

The precision of CSLM is mainly based on the concept of the confocality of the investigated object with the light source and the detector plane. Such a system was limited because of its small field of view. By the time, a larger field of view obtained either by moving the specimen whereas the microscope remains stationary, or by moving the confocal system over a stationary specimen. Modern CSLM devices use the second technique. The microscope's temporal resolution determined by the speed at which a single image of the field is acquired. Poor temporal resolution is important as increased motion artifacts inevitable because of pulse, respiration, and eye movement when examining living human subjects [16].

3. Ophthalmological applications

Biological tissues usually slice with 2–5 μm thickness were cut, stained with various chemicals and examined by light transmission at high magnification as part of conventional microscopic evaluation. In ophthalmology, in vivo examination of semitransparent tissues is performed by slit lamp biomicroscopy thanks to the inventor Allvar Gullstrand [17]. With slit lamp biomicroscopy, optically cut planes are orientated sagittally and observed by a binocular microscope with the magnification up to 50-fold. However, single cell resolution is still impossible at this level of magnification. Nonetheless, a large number of corneal diseases could be diagnosed and followed up by slit lamp microscopy easily in many cases.

Since the corneal cells could not be evaluated by slit lamp biomicroscopy, CSLM has quite satisfactory use in this regard. CSLM provides the imaging of biological structures with up to a magnification of 800-fold that renders possible single cell evaluation (**Figure 1**). Secondly, the optical section is perpendicular to the slit lamp image as its direction is parallel to the corneal surface [2].

CSLM has been used in various ophthalmological conditions for corneal diagnostics. Corneal nerve degeneration and regeneration, assessment of corneal grafting and refractive surgery, contact lenses, diabetes mellitus, keratoconus, ocular surface disease, and normal anatomy are investigated by CSLM with a considerably high number of studies.

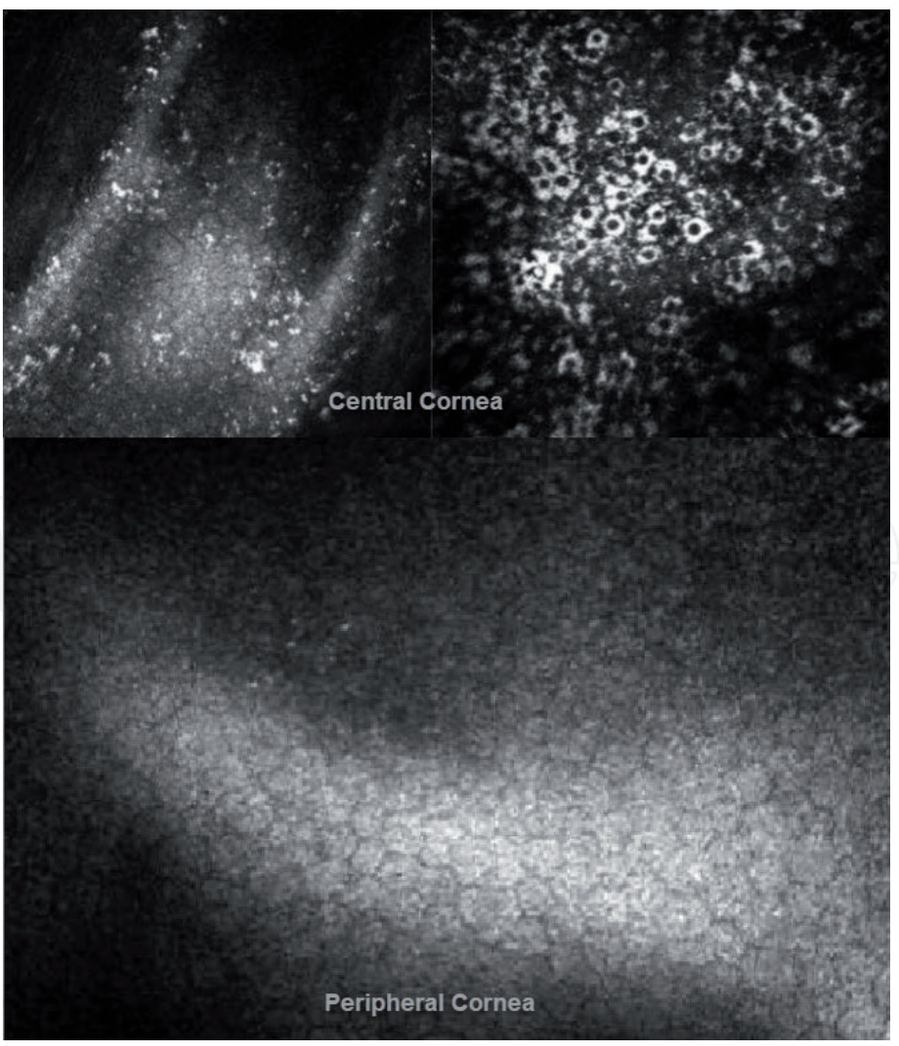

Figure 1.
Normal endothelial cells. Endothelial pigment appeared as hyper-reflective spots in some frames of the central cornea, while peripheral endothelium appeared normal. (courtesy by Mustafa Kosker).

3.1 Corneal fungal infections

Fungal keratitis can be a significant problem in especially developing countries as its slow course and treatment resistance. The clinical findings are nonspecific, and there are difficulties in diagnosis due to its delayed growth even in specific cultures. Despite their infrequent nature, in industrialized countries proper management of fungal keratitis due to prolonged diagnostic procedures ends up with devastating results [18–21]. Moreover, after initiation of antimicrobial therapy, it is still difficult to assess therapeutic response of some ulcers based upon clinical appearances by slit microscopy alone.

CSLM has been reported to be useful in diagnosis and follow up of patients in fungal keratitis [18–21]. CSLM has provided instant diagnosis without long lasting preparations of sample cultures. Also, CSLM demonstrates activated keratocytes and directly proven fungi in the corneal ulcer in differential diagnosis of non-fungal keratitis [18–21]. Although it is a rapid and noninvasive method of diagnosis of routine as well as deep-seated corneal infiltrates, its use as a primary diagnostic modality may not be possible due to its cost and limited accessibility.

3.2 Keratoconus

Keratoconus is an ectatic corneal disorder characterized by progressive thinning of cornea, which leads to an apical corneal protrusion, irregular astigmatism, superficial scar formation, and progressive decreased vision [22]. The diagnosis of keratoconus is now easy with the development of corneal topography systems. However, CSLM is another approach for the diagnosis and follow up of keratoconus. Quantitative and qualitative structural alterations were seen in all corneal layers in eyes with keratoconus, and the alterations were more prominent as the severity of disease increased [22–25].

In the keratoconus, main pathologic changes evaluated by CSLM included elongated, exfoliating superficial epithelial cells; brightly reflective material deposition within the basal epithelial cells; prominent, thickened subbasal nerves; structural changes in subbasal nerve fibers; pronounced reflectivity and irregular arrangement of stromal keratocytes; structurally abnormal anterior stromal keratocyte nuclei; folds in the anterior, mid, and posterior stroma; folds in Descemet's membrane; pleomorphism and enlargement of endothelial cells; and endothelial guttata [22]. Moreover, keratocyte density is significantly lower in subjects with keratoconus and correlated with disease severity [26]. CSLM's noninvasive nature allows the opportunity to study early microstructural changes in the keratoconic cornea and to understand its pathophysiology (**Figure 2**).

3.3 Subbasal nerve plexus

There has been an increased interest in using CSLM, that non-invasive technique as an objective diagnostic tool for peripheral neuropathies due to the capability to acquire high-resolution in vivo images of the densely innervated human cornea [27–29]. Also, the evaluation of the subbasal nerve plexus of the cornea has led to a significant rise in CSLM use to help clinicians diagnose various diseases (**Figure 3**). Morphological alterations of the corneal subbasal nerve plexus may correlate with the progression of neuropathic diseases and even predict future-incident neuropathy.

Corneal nerves are affected in cases with limbal stem cell deficiency, infection, corneal surgery, keratoconus, diabetes mellitus, lysosomal storage diseases, and keratitis [2]. Moreover, the evaluation of systemic diseases could also be possible by

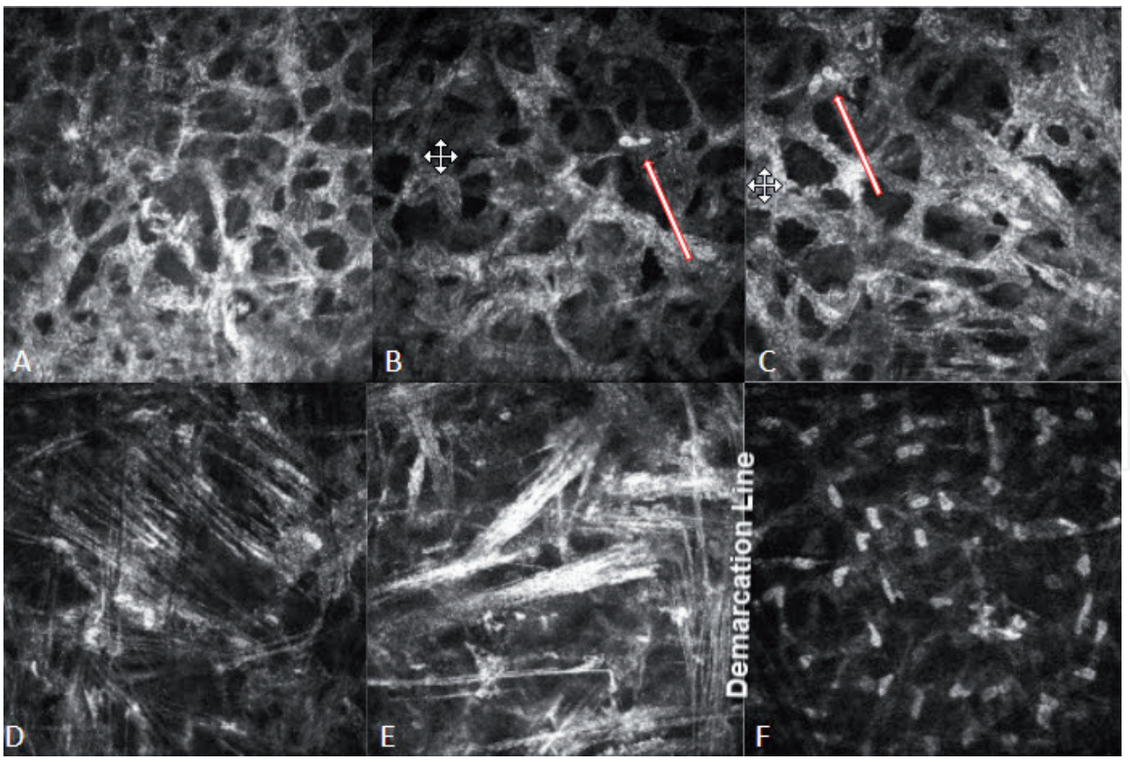

Figure 2.
Keratoconus patient that underwent corneal cross-linking treatment. Hyperreflective cytoplasm, extracellular spaces, and anterior stromal edema give a honeycomb appearance and can be observed until the 3rd month. Although almost all of the keratocytes undergo apoptosis, sporadic keratocytes are observed (arrows). Demarcation line in confocal microscopy: The long, thin, hyperreflective, needle-like structures in the middle stroma and the transition area from the wide hyperreflective stromal bands to normal keratocytes appear as the demarcation line. These hyperreflective bands can be seen in the first six months. While these changes occur in anterior stromas, there is no significant change in the keratocyte density and endothelium count behind the demarcation line. (courtesy by Mustafa Kosker).

observing corneal subbasal nerve plexus. In particular, the use of CSLM in diabetic patients, who are at risk of small fiber neuropathy leading to limb amputation, may be helpful in the early detection of small fiber neuropathy, and some preventions can be taken to slow down or eliminate the incident in both industrialized and developing countries [27].

The optical slicing of CSLM is parallel to the surface of the cornea. Therefore, it provides an ideal condition to display and to quantify structures of the subbasal nerve plexus which is located between Bowman membrane and the basal lamina of the corneal epithelial cells. It has been proposed that the imaging of the subbasal nerve plexus will be possible to find new treatment strategies and more effective prevention of serious disease.

3.4 Keratoplasty and refractive surgery

Keratoplasty is still a common method in the treatment of corneal pathologies. It has been possible because of the increase in knowledge about corneal anatomy, improvement in instruments, and advancements in technology. Today, development of modern technologies, especially in microscopy, has reached a very good position in terms of success in keratoplasty. With the widespread use of CSLM, it was possible to image a graft's microstructure as well as calculation of endothelial cell density. CSLM detected some changes such as declining of subepithelial plexus nerves, keratocytes, and endothelial cells in the central clear graft following keratoplasty [30–32]. The graft is in a stress condition which affects the normal physiological function of keratocytes and leading to the graft failure [30–32]. Activated immune

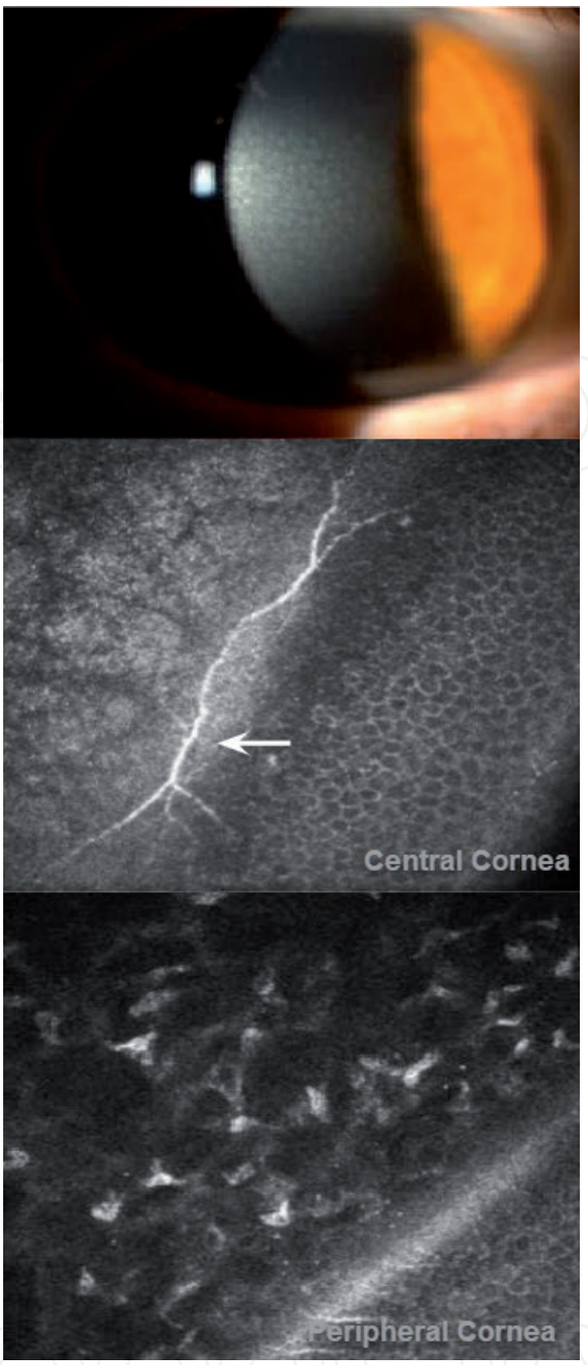

Figure 3.
Central mosaic dystrophy in a case with Megalocornea: Central cornea: The epithelium appeared normal morphologically. (white arrow): The subepithelial nerve fibers seemed to be thickened and appeared more prominent. In the stroma, starting just below Bowman's membrane, polygonal, moderately reflective areas of opacification separated by diagonal hyporeflective striations were observed. Peripheral cornea: The epithelium, bowman membrane, and anterior stroma appeared normal morphologically. (courtesy by Mustafa Kosker).

cells could also be detected in some of the clear grafts, which clearly showed that the subclinical stress of immune reaction took part in the chronic injury of the clear graft failure without any rejection episode. Therefore, morphologic alterations of corneal grafts after keratoplasty detected by CSLM enables us to be aware of corneal graft rejection and to intervene early in a possible rejection.

Refractive surgical procedures are being used frequently in the light of the increasing incidence of myopia and technological developments in refractive surgical devices. It is possible to assess the wound healing response in the living human cornea that may help in unraveling the mechanisms of corneal haze and refractive regression observed following refractive surgery. Studies are carried out in the field

of CSLM in order to increase the success rate of this surgery, to detect and manage possible complications at early period [33, 34].

3.5 Contact lens

Contact lenses are used today for many different purposes. The effects of contact lenses on the eyes were evaluated with CSLM. Contact lens biocompatibility, its effects on cornea, limbal stem cells or conjunctiva, early diagnosis of devastating infections such as acanthamoeba keratitis are investigated by CSLM [35–38].

Acanthamoeba keratitis is a serious, sight-threatening corneal infection that can cause significant corneal damage and vision loss. Its incidence is on the rise because of the increasing usage of contact lenses. The diagnose of acanthamoeba keratitis is essential as its devastating nature, and CSLM can be used as an adjunct modality to the clinical data for diagnosing acanthamoeba keratitis [38].

3.6 Ocular surface diseases

CSLM has been widely used to visualize the morphology of the cornea and conjunctiva and detect changes of the ocular surface in pathological conditions such as infectious, metabolic, and trauma. The micromorphology of the corneal epithelium and stroma can be changed by infections, metabolic diseases, and genetic disorders. The progression of diseases can be observed and monitorized via CSLM [39, 40]. Chemical burns, which may result in irreversible damage to the ocular surface, constitute a large part of ocular trauma. CSLM can provide images of the goblet cells on the corneal surface which is a hallmark of limbal stem cell deficiency. The application of CSLM on chemical burns also allows for evaluation of the limbal structures and ocular surface changes after reconstructive ocular surgery [39].

Dry eye disease is another area of research for CSLM. CSLM is an effective non-invasive tool for evaluation of phenotypic alterations of the conjunctival epithelium. The use of CSLM is also crucial in the diagnosis of meibomian gland disfunction [41, 42]. It demonstrated the importance of meibomian glands for the healthy ocular surface and was also used for the effective treatment modalities of dry eye disease.

3.7 Glaucoma surgery

The formation of a filtering bleb, which attains by postoperative wound healing process, is a key factor in surgical procedures for glaucoma. Clinical and histological evaluation of these blebs has been investigated by CSLM to visualize functioning or nonfunctioning blebs at the cellular level [43, 44]. The CSLM images of filtering blebs have good consistency with the findings from previous studies. The implantation of CSLM in glaucoma surgery will be enlightened the histological processes responsible for filtration or failure.

4. Non-ophthalmological applications

CSLM is mainly improved for ocular and ocular adnexal surface structures. However, it can be suited for analyzing any surface of the human body in case of convenient for the device to reach at. On the other hand, the application of CSLM in non-transparent tissue is limited due to light-tissue interaction including reflection and refraction, absorption, and scattering of photons. In human tissues, water molecules and macromolecules such as proteins and chromophores are the main

factors that affect penetration depths of the device. Therefore, CSLM images with cellular resolution can only be obtained at depths up to 300 μm [2].

CSLM has been used to evaluate the oral and pharyngeal mucosal membranes and showed promising results in dentistry applications [45–47]. Studies describing the cellular morphology and pathological alterations of the oral cavity, cervix, and esophagus also showed promising results [48, 49]. Cell morphology, tissue architecture of the epithelium, and a number of pathological skin conditions were investigated [50–52]. The amelanotic epithelial tissue of the gastrointestinal tract, lip and tongue, and the oropharynx demonstrated with CSLM [52]. CSLM identified intraepidermal blisters and acantholytic cells in pemphigus vulgaris [53]. Sinonasal inverted papilloma could also be detected noninvasively by CSLM [54]. The combination of CSLM with endoscopy is helpful in detection of schistosomiasis [55].

5. Future developments

Presently, CSLM has been a potential source of many researches and has received a high level of scientific and clinical attention in ophthalmology. Since CSLM can show high resolution images of various cellular structures within the living cornea non-invasively, it is mainly used for diagnostic purposes. However, ongoing research on this area is under development in order to improve their diagnostic potential and the usability of this technology.

5.1 Multiphoton microscopy

Corneal cell differentiation can be evaluated under various conditions by CSLM. However, the detailed information will not be satisfactory. Multiphoton microscopy, which uses a non-linear interaction mechanism, can be more useful for evaluation of cellular morphology [2]. According to multiphoton absorption, the background signal is strongly suppressed and leads to an increased penetration depth for this technique [56]. Multiphoton microscopy can be a superior alternative to confocal microscopy due to its deeper tissue penetration, efficient light detection, and reduced photobleaching. Multiphoton microscopy is reported as a promising technique for non-invasive detection of diabetic neuropathy [56, 57]. Information derived from this technology may help to develop new drugs for the treatment of diabetic neuropathy.

5.2 Slit lamp microscopy on a cellular level using CSLM

Slit lamp microscopy is a revolution for ophthalmology. Despite whole anterior segment structures can be evaluated clinically, information on cellular level cannot be attained. Recently, an in vivo method for 3D volumetric reconstruction of the cornea on a cellular level with volume sizes up to around 250 × 300 × 400 μm^3 has been reported. [58]. A piezo actuator is implanted to the microscope objective for image acquisition. Moreover, the automated, closed-loop control of the focal plane enables fast and precise focus positioning. Additionally, a novel contact cap with a concave surface has been presented that reduced eye movements by up to 87%. Therefore, the cuboid volume of the generated 3D reconstruction significantly increased. The possibility to generate oblique sections using isotropic volume stacks opened the window to slit lamp microscopy on a cellular level. The diagnosis can be made at cellular level during examination, and the treatment of diseases can be planned more effectively with the widespread useage of this technology,

5.3 Optical coherence tomography guided CSLM

CSLM is valuable for studying corneal morphology at cellular level non-invasively. However, certain drawbacks such as small field of view limit its usability. The exact CSLM image location and orientation inside the cornea are difficult to locate. Therefore, a combination with optical coherence tomography (OCT) was adapted to the conventional CSLM in order to overcome this limitation.

The combination of both technologies renders it possible to track image position and orientation in real-time [2, 59]. Real-time evaluation of CSLM image plane position and its orientation within the cornea through the OCT section provides an enhanced location-based diagnosis. It is now possible to specify the angle between the corneal surface and the image. Further studies will be necessary for optimizing the system design and OCT scan patterns. In the future, the combination of these technologies will be used widely for diagnostic purposes and will give direction to the treatment.

6. Conclusion

CSLM allows ocular structures and ocular surfaces to be assessed at cellular level. 2D tessellation or 3D reconstruction of the ophthalmic as well as non-ophthalmic tissue evaluation is possible. This technology is still promising, and close and direct collaboration between clinical science and basic science as well as industry partners can help it to reach its potential. CSLM's combination with several other technologies will also affect our understanding of diseases, diagnosis, and treatment options in the near future.

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Conflict of interest

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Author details

Hasan Kiziltoprak[1*], Dilara Ozkoyuncu[2], Kemal Tekin[3] and Mustafa Koc[4]

1 FEBO, FICO, Bingol Maternity and Child Diseases Hospital, Ophthalmology Department, Bingol, Turkey

2 FEBO, Bilecik Training and Research Hospital, Ophthalmology Department, Bilecik, Turkey

3 Ophthalmology Department, Hatay Mustafa Kemal University, Hatay, Turkey

4 Kayseri Mayagoz Hospital, Kayseri, Turkey

*Address all correspondence to: hsnkzltprk21@gmail.com

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