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In vivo confocal microscopy of the ocular surface: from bench to bedside

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Abstract

In vivo confocal microscopy (IVCM) is an emerging technology that provides minimally invasive, high resolution, steady-state assessment of the ocular surface at the cellular level. Several challenges still remain but, at present, IVCM may be considered a promising technique for clinical diagnosis and management. This mini-review summarizes some key findings in IVCM of the ocular surface, focusing on recent and promising attempts to move "from bench to bedside".

IVCM allows prompt diagnosis, disease course follow-up, and management of potentially blinding atypical forms of infectious processes, such as Acanthamoeba and fungal keratitis. This technology has improved our knowledge of corneal alterations and some of the processes that affect the visual outcome after lamellar keratoplasty and excimer keratorefractive surgery.In dry eye disease, IVCM has provided new information on the whole ocular surface morpho-functional unit. It has also improved understanding of pathophysiological mechanisms and helped in the assessment of prognosis and treatment.

IVCM is particularly useful in the study of corneal nerves, enabling description of the morphology, density, and disease- or surgically induced alterations of nerves, particularly the subbasal nerve plexus. In glaucoma, IVCM constitutes an important aid to evaluate filtering blebs, to better understand the conjunctival wound healing process, and to assess corneal changes induced by topical antiglaucoma medications and their preservatives.

IVCM has significantly enhanced our understanding of the ocular response to contact lens wear. It has provided new perspectives at a cellular level on a wide range of contact lens complications, revealing findings that were not previously possible to image in the living human eye.

The final section of this mini-review provides a focus on advances in confocal microscopy imaging. These include 2D wide-field mapping, 3D reconstruction of the cornea, and automated image analysis.

Key words: confocal microscopy, keratitis, dry eye, keratoplasty, glaucoma

In vivo confocal microscopy (IVCM) is a rapidly evolving imaging and diagnostic tool, which offers an exciting bridge between clinical and laboratory observations, enabling clinicians and scientists to gain insight into alterations of the ocular surface microstructure, both in health and disease.[1]

In contrast to conventional microcopy in which the image can be observed directly (all points in the specimen are imaged parallel), a CM optimizes illumination and detection for a single spot only.[2]

At present, the most widely used IVCM reported in the published literature are the Confoscan series (NIDEK, Gamagori, Japan) and the HRTII/Rostock Corneal Module (Heidelberg Engineering GmbH, Heidelberg, Germany).[1]

The firsts are white light slit scanning confocal microscopes. With this technology many points along the axis of the slit can be scanned in parallel, greatly reducing scanning times and the required intensity of the light source. However, these instruments are only truly confocal in the axis perpendicular to the slit height and provides lower transverse and axial resolution.[1]

The HRTII/Rostock Corneal Module is a laser scanning confocal microscope. This instrument uses a coherent high intensity light source and the laser beam is scanned over the back of the microscope objective by a set of galvanometer scanning mirrors.[1] Because of the high-depth resolution, optical sections of only a few micrometers can be imaged and precisely measured in combination with a high contrast.[2]

Studying the cornea, the Confoscan 4 (the last of the Confoscan series) produces high quality images throughout the depth of the cornea with accurate depth information.[3] In comparison, the HRT III/RCM produces very detailed images of the anterior cornea but is less accurate in determining depth or producing high quality images of the posterior cornea. The ability of the laser IVCM to examine the different components of the ocular surface has opened new lanes for studying the physiology and pathology of this complex morpho-functional unit.

This mini-review does not provide an exhaustive review of the literature but rather summarizes some key findings in IVCM of the ocular surface, focusing on recent and promising attempts to move “from bench to bedside”. The role of IVCM in the management of infectious keratitis, corneal transplantation, refractive surgery, dry eye disease, and imaging of corneal nerves, assessment of the ocular surface in glaucomatous patients and contact lens wearers are the main topics of this review. The final section provides a focus on advances in confocal microscopy imaging.

In Vivo Confocal Microscopy in Management of Infectious Keratitis

Infectious keratitis is a common condition that can lead to sight-threatening complications and blindness.[4] Early and accurate diagnosis, as well as subsequent initiation of appropriate therapy have been demonstrated to be the key in prevention of permanent vision loss.[4] Although clinically, patient history and slit-lamp examination are essential in raising suspicion for the underlying etiology, microbiology remains the gold standard. However, the high false-negative rate and delay of corneal cultures due to slow-growing organisms such as fungi and Acanthamoeba are important limitations to their diagnostic ability.[5] Given the importance of timing in diagnosis and initiation of therapy, there is an exciting emerging role for IVCM evolving not only for the diagnosis, but also potentially in the management of this disease.[6]

Acanthamoeba Keratitis

Acanthamoeba is a protozoan that is ubiquitously found in soil, water and air. While the infection rate was reported to be 1.2 per million adults and 0.2-1 per 10,000 contact lens wearers per year,[7] rates have recently risen to more than seven-fold in contact lens wearers.[8]Acanthamoeba keratitis is often misdiagnosed due to initially nonspecific presentation. The typical ring infiltrate and radial perineuritis may develop at later stages and raise suspicion, at which time the prognosis is guarded and cases require surgical intervention.[9] Therefore, there is great value in more rapid identification of the organism. Unfortunately, cultures can take weeks to become positive and sensitivity ranges from 0 to 68%.[9]Further, although polymerase chain reaction (PCR) diagnosis has a better sensitivity for more superficial cases,[10] it is costly, needs technical expertise and is not yet available in all laboratories. The pharmacotherapy for Acanthamoeba keratitis is expensive, prolonged, and toxic and toxicity may become difficult to distinguish from persistent infection. IVCM may thus be helpful in determination of therapeutic efficacy and persistence or absence of Acanthamoeba noninvasively, potentially improving patient outcomes and decrease the need for surgical intervention.[11]

Acanthamoeba presents as active trophozoites and/or quiescent cysts, with both forms being identifiable on IVCM, allowing for rapid, same-day diagnosis. Detection of Acanthamoeba by IVCM was first reported in 1992[12] and was recently supported by The American Academy of Ophthalmology as an adjunctive diagnostic modality.[13] Double-walled cysts appear as hyper-reflective, spherical, occasionally ovoid, structures, ranging 15-28 micrometers in diameter (Fig. 1 A, B). The double-wall may not always be apparent by IVCM, making it occasionally difficult to differentiate cysts from leukocyte or epithelial nuclei. Clustering (Fig. 1A) and rows (Fig. 1B) of cysts are typically suggestive of active proliferative disease. Trophozoites are 25-40 micrometers in

diameter,[7] and appear as hyper-reflective and ovoid structures on IVCM (Fig. 1 C, D) but are difficult to distinguish from leukocytes and keratocyte nuclei.

Several single-observer studies have demonstrated a sensitivity of 90.9-100%, and a specificity of 77.3-100% by IVCM.[14-16] Another study, studying grading by different observers with variable or no experience, demonstrated a sensitivity of up to 55.8% and specificity up to 84.2%,[17] underlining the importance of training and experience in evaluation of IVCM images.

Fungal Keratitis

The clinical presentation of fungal keratitis is nonspecific and indolent, resulting in delayed therapy and potential development into endophthalmitis and loss of the eye. Alternatively, the permanent scarring associated with the delay in treatment can lead to significant vision loss. The gold standard for diagnosis are corneal smear and cultures, both of which have a limited sensitivity and cultures can take several days to weeks to obtain growth.

IVCM is a tool for identification of both filamentous fungi and yeast. Aspergillus hyphae are hyper-reflective 5-10 micrometers in diameter and have septate hyphae with dichotomous branches at a 45 degree angle (Fig. 2A).[6] In contrast, Fusarium typically branches at a 90 degree angle.[8] Paecilomyces have variable branching and demonstrate loops on IVCM as well (Fig. 2B).[18] The hyper-reflective elements seen on IVCM must be differentiated from subbasal corneal nerves, which have a more regular branching pattern, as well as from stromal nerves that are much larger in diameter (25-50 micrometers). Filamentous fungi are 200-400 micrometers long. In addition, yeast such as Candida Albicans, have round, budding bodies that may develop pseudohyphae, and are 10-40 micrometers in length and 5-10 micrometers in width.[8] Candida parapsilosis in contrast present as small hyper-reflective round 3-5 micrometers structures (Fig. 2C).[18]

Two recent studies[14, 16] in patients with microbial keratitis demonstrated that IVCM had a sensitivity from 89.2 to 94% and a specificity from 78 to 92.7% in patients with fungal keratitis. As with Acanthamoeba keratitis, IVCM can be applied not only for diagnosis of fungal keratitis, but for monitoring and guidance of treatment as well. In fungal keratitis, the depth of invasion is an important prognostic factor, and IVCM is currently the only method that allows determination of the depth of infection. This information helps the clinician to decide the timing for surgical intervention when the disease is progressing despite medical treatment.[6]

Conclusion

IVCM is a non-invasive tool that may allow for rapid diagnoses of potentially blinding atypical infections, such as Acanthamoeba and fungal keratitis, allowing prompt initiation of treatment.

IVCM may also provide the unique advantage of following the disease course and guiding treatment, as well as shed light on the pathogenesis in these potentially devastating diseases.[19, 20] Currently smaller organisms, including bacteria and viruses are not visible on examination, and the use of IVCM in these cases is not helpful. It is important to note that IVCM testing requires skilled operators, and interpretation requires an experienced and well-trained observer. Furthermore, in some cases, the evidence of its high sensitivity and specificity is still poor and therefore IVCM cannot at present replace microbiological testing.[6]

In Vivo Confocal Microscopy in Corneal Transplantation and Refractive Surgery

IVCM is a valuable imaging modality for examining corneal cells and ultrastructure after transplantation or other surgical procedures.

Confocal studies of the donor cornea after penetrating keratoplasty (PK) have revealed decreased innervation,[21, 23] increased backscatter,[23] and decreased cellularity.[22] With current interest in lamellar keratoplasty techniques, this review will concentrate more on recent findings after lamellar keratoplasty and other lamellar procedures.

Corneal Transplantation

Lamellar keratoplasty has resurged over the last decade for the treatment of anterior and posterior corneal disease.[24] While donor changes would be similar to those after PK, corneas after lamellar keratoplasty also contain residual host tissue and a new surgical lamellar interface. Currently, the most common lamellar keratoplasty procedure is endothelial keratoplasty (EK), specifically, Descemet stripping endothelial keratoplasty (DSEK), in which nearly all the host cornea is retained with the exception of host Descemet membrane and endothelium. IVCM has improved our knowledge of the residual changes in the host cornea after EK, and has enabled understanding of some of the processes that affect the visual outcome after EK, as discussed below.

Corneal Backscatter after Corneal Transplantation

Corneal backscatter refers to light scattered toward an observer from the cornea and is of interest after EK because backscatter from the surgical interface has been assumed to degrade postoperative visual acuity.[25] With appropriate calibration of the incident light source and the optical detection system, changes in corneal backscatter can be determined prospectively by using IVCM.[26] After DSEK for Fuchs dystrophy, significant corneal backscatter is noted from the surgical interface in the early period, but this decreases progressively over the first two years after surgery.[27] Contrary to popular belief, backscatter from this region has not been associated with changes in visual acuity or disability glare after EK.[27] In contrast, more backscatter can be measured from the subepithelial

and anterior stromal region of the cornea.[27, 28] High backscatter in this region is present preoperatively (in Fuchs dystrophy) and is caused by subtle basal epithelial edema, increased scatter from the anterior stromal extracellular matrix, and the presence of subepithelial cells (presumed fibroblasts), despite the cornea appearing clear by slit-lamp examination.[29] After DSEK, scatter from this anterior region declines over the first six months, mainly because of resolution of epithelial edema, but thereafter persists and remains elevated compared to normal through two years after surgery. Scatter from the subepithelial region has not been associated with visual acuity after DSEK, but has been associated with disability glare.[25, 27, 30]

Corneal Cellularity after Corneal Transplantation

Corneal nerves are not affected by EK except those traversing the site of the incision, whereas all the corneal nerves are severed during PK.[22, 31] Nevertheless, IVCM has revealed significant abnormalities of subbasal and stromal nerves before and after EK for Fuchs dystrophy. Stromal nerves are frequently tortuous and closely associated with keratocytes, suggesting interactions between these cell types.[31] In addition, an abnormal and depleted subbasal nerve plexus has been associated with decreased corneal sensitivity.[31, 32]

Abnormal subepithelial cells (presumed fibroblasts) are brightly reflective cells visible before and after DSEK for Fuchs dystrophy.[29] These cells contribute to corneal backscatter and possibly to increased anterior surface aberrations.[33] Abnormalities of keratocytes, which repair and maintain the cormealstroma, have been shown by IVCM. Keratocyte density is reduced in the most anterior cornea in Fuchs dystrophy, and remains reduced with no evidence of repopulation through three years after DSEK.[29, 34] The consequence of this loss of cells is unknown, but possibly results in impaired repair of the anterior stromal extracellular matrix after EK. Posterior host keratocytedensity has been found to decrease at two and three years after EK,[29] and might be the consequence of loss of proximity, and therefore disrupted communication, between these cells and the endothelial cells.

The donor corneal endothelium is easily examined by IVCM after EK, and it is an ideal imaging technique for examining these thickened and potentially hazy corneas when compared to non-contact specular microscopy.[35] Microfolds of the posterior donor cornea after EK can be visualized by confocal microscopy, but these folds have not been associated with postoperative visual acuity.[36]

Excimer Keratorefractive Surgery

Laser in-situ keratomileusis (LASIK) and photorefractive keratectomy (PRK) are the most common corneal surgical procedures. Both involve tissue removal by excimer photoablation, and LASIK

involves anterior corneal flap creation resulting in a lamellar interface.[37] Long-term changes in corneal backscatter and cellularity in both procedures have been studied by IVCM.

Corneal Backscatter after Excimer Keratorefractive Surgery

Aside from transient increases in corneal backscatter in the early period after LASIK and PRK, corneal backscatter is decreased over the longer-term, as measured by IVCM.[26, 38] The decrease can only be partly explained by decreased keratocyte density (see below).Other possible factors are also thought to contribute to decreased reflectivity in the LASIK flap (unpublished data). Of interest is that the anterior keratocyte depletion after LASIK and in Fuchs dystrophy is associated with opposite effects on anterior corneal reflectivity.

Corneal Cellularity after Excimer Keratorefractive Surgery

Keratocyte density decreases after LASIK in the stromal flap, and after PRK, because of removal of the highest density of anterior cells.[39] Loss of anterior keratocytes has not been associated with a detrimental visual outcome, with increased backscatter, or with unstable anterior surface aberrations.[26, 40] Corneal subbasal nerves become depleted after LASIK and PRK, with a slow recovery over several years, although decreased corneal sensitivity recovers within months.[41, 42] No long-term detrimental effects have been observed in the corneal endothelium by IVCM.[43, 44]

In Vivo Confocal Microscopy of Ocular Surface in Dry Eye

The International Dry Eye Workshop[45] (2007) and the International Workshop on Meibomian Gland Dysfunction[46] (2010) recently defined dry eye and meibomian gland dysfunction (MGD). Although these definitions provide clear descriptions of the disease components, in-depth investigation of the pathophysiological mechanisms are still required.[47]

IVCM is a minimally invasive and powerful tool which allows detection of changes in the ocular surface epithelium, immune and inflammatory cells, corneal nerves, keratocytes, and meibomian gland structures at cellular level.[44, 45]

IVCM studies show dry eye-related changes in the corneal epithelium,[49-52]including decreased superficial cell density and increased basal density. Changes in the stroma are also evident by IVCM including the presence of abnormal hyperreflective keratocytes.[50, 53, 54]Some authors referred to these as “activated” and interpreted them to be in a particular state of metabolic activation, induced by proinflammatory mediators.[50, 54, 55]

Confocal studies confirmed a key role of the sub-basal nerve plexus (SNP) in dry eye disease, reporting increased tortuosity of the nerves.[50, 51, 55]The nerves also show an increased density of bead-like formations[50, 51]that could reflect nerve damage or, alternatively, reflect increased

metabolic activity of nerve fibers in response to abnormal changes in the epithelium and to neuro-inflammation. The meaning of changes in subbasal nerve tortuosity and bead-like formations is currently subject to interpretation as there is conflicting data about those changes and how they correlate with sensitivity.[49, 50, 55-59] A more extensive discussion on this issue is included in the next section.

IVCM demonstrated that the density of epithelial dendritic cells, which are interpreted as antigen presenting cells, and other inflammatory cells increases in the central and peripheral cornea in patients with dry eye.[20, 60] Dynamic in vivo assessment of the central corneal inflammatory cell density may not only be of help in evaluating dry eye severity, but can likely guide clinical treatment and aid in the evaluation of the efficacy of anti-inflammatory drugs in clinical trials.[47, 61]

IVCM has recently been used to examine the meibomian glands (MGs). With this application, it provides a new tool to assess morphologic changes and allows the quantitative study of MG acinar unit diameter and density, orifice diameter, and periglandular inflammatory cells density.[62-65] The method also allows semi-quantitative assessment of meibum secretion reflectivity and the inhomogeneity of the glandular interstices and acinar wall.[64, 65] Confocal data showed the potential to diagnose simple MGD (Fig. 3) with high sensitivity and specificity and to assess MGD response to treatment.[63-66] Finally, confocal examination provided new information about particular patterns of MG changes associated with Sjogren syndrome,[64] graft-versus-host disease,[67] contact lens wear,[65] and aging.[68]

Recent IVCM reports studied the conjunctiva in dry eye patients, focusing on confocal correlations with impression cytology, inflammatory glands, and goblet cell evaluation. In Sjogren syndrome, cellular changes resembling squamous metaplasia were present, and the mean cell area and nucleus-cytoplasm ratio in impression cytology showed a significant correlation with the corresponding confocal microscopy parameters (Figure 4).[69] IVCM was effective in the study of inflammatory conjunctival cell density and response to treatment in Sjogren patients,[70, 71] while data are conflicting about the evaluation of goblet cells (repeatability, inter-observer agreement, images interpretation and chances to discriminate these cells.).[70, 72] Finally, IVCM was successfully applied to lacrimal gland examination in some Sjogren patients, revealing inflammatory cell infiltration and perilobular fibrosis.[73]

In conclusion, IVCM is providing new opportunities to better understand the complex pathophysiological mechanisms of dry eye. Recent confocal studies have shown several potential clinical applications of this technology in the assessment of dry eye disease grading and prognosis,

in the improvement of differential diagnosis, in the optimization of a tailored approach to treatment, and in the evaluation of the responses to treatments.[74]

In Vivo Confocal Microscopy of Corneal Nerves

The cornea is the most densely innervated tissue in the body with a density of nerve endings 200-300 times greater than the skin.[75] The majority of nerves in the cornea have a sensory function. Sensory nerve endings termed "nociceptors" function in maintaining homeostasis, in wound healing and in sensing the environment to regulate tear secretion and distribution.

IVCM allows the description of the morphology, density, and disease- or surgically-induced alterations of corneal nerves, particularly the subbasal nerve plexus.[76, 77]It is particularly useful for imaging corneal nerves near the corneal apex because the central cornea is more easily applanated to obtain high quality tangential images. Although IVCM is capable of providing excellent images, it lacks sufficient resolution to image nerve terminals in the epithelium and very small diameter nerves in the subbasal plexus. Furthermore, the Heidelberg Engineering HRT laser scanning confocal microscope that has been used in many of the published studies is only capable if imaging a 400x400 μm area, making it difficult to locate and serially reimage the same region of the cornea.

IVCM imaging of nerves in Ocular Surface and Corneal Diseases

IVCM has been used to image corneal nerves in a variety of corneal and ocular surface diseases/conditions, including non-Sjögren syndrome (SS) or SS-associated aqueous tear deficiency,[49, 50, 56, 78] following LASIK surgery[41, 79-84] (including post-LASIK dysesthesia) and neurotrophic epitheliopathy before and after treatment with autologous serum.[85]

Benıtez del Castillo and associates reported decreased density of nerves in the subbasal plexus in both non-SS and SS aqueous tear deficiency (ATD) compared to normal subjects < 60 years of age.[78] Furthermore, both ATD groups had a greater number of beaded nerves in the subbasal plexus than control subjects less than and greater than age 60. Similar findings were also observed by Villani and colleagues who reported a decreased number and increased tortuosity of subbasal nerves compared to the control group.[50]Tuominen and colleagues observed nerve sprouting in the SNP in 40% of eyes with SS.[49] The authors hypothesized that this finding may represent sensory nerve regeneration. Nerve sprouting was noted in neonatal rat corneas after treatment with capsaicin which induces release of neuropeptides from sensory nerve endings,[86] and overexpression of nerve growth factor (NGF) was found to induce hypertrophy of the peripheral nervous system.[87]

In another study by Tuisku and associates, corneal subbasal nerve density was similar in patients with SS and control subjects; however, thickening and cone-like structures were seen in nerves and this was accompanied by hyperalgesia to an air jet esthesiometer and increased density of dendritic cells (DC) surrounding corneal nerves in the SS corneas.[56] The density of DCs correlated with the severity of irritation symptoms.

Post LASIK Hypoesthesia or Dysesthesia

Following LASIK, patients may rarely develop neurotrophic epitheliopathy or chronic pain, termed "keratoneuralgia".[88-90] This may be due to abnormal regeneration or increased sodium ion channels that lower the threshold to excitation, resulting in greater sensitivity to normal environmental stimuli (e.g. air drafts, temperature change).[90]IVCM can confirm loss of corneal innervation or the presence of abnormal nerve regeneration. It may identify patients who would benefit from protective and/or regenerative therapy. Confocal microscopy of the cornea after LASIK has shown decreased corneal nerve density in the early postoperative period that increases with time, albeit at a slower rate than return of corneal sensitivity.[41]The subbasal nerve fiber density is barely detectable for up to six months post LASIK,[79] and one year after LASIK, the number of subbasal and stromal nerves in the corneal flap are less than half the pre-operative density.[80] Immediately after LASIK, most patients develop a relatively hypoesthetic cornea. Studies have found that corneal sensitivity progressively improves and approaches the preoperative or "normal" range by 6-12 months when measured with Cochet-Bonnet esthesiometry[91, 92] and by up to 2 years when measured by gas esthesiometry.[93]

Nerve morphology has been found to correlate with corneal sensitivity after LASIK. One study found that low sensitivity in specific corneal areas was associated not only with the absence of nerves in confocal images, but also with short nerves lacking connections between nerve bundles.[81] Another study found a significant association in LASIK patients between long nerve fiber bundles and greater sensitivity by noncontact esthesiometry.[82] Finally, Stachs et al. concluded that "normal" sensation returns to eyes by 6 months post-LASIK, despite a markedly abnormal SNP with abnormally curved bundles or with thin nerves without the connections between nerve bundles as seen in healthy, non-surgical eyes.[83]

Tuisku et al. found that 2-5 years after LASIK for high myopia, most patients experienced ocular surface discomfort consistent with dry eye syndrome, but in the absence of clinical signs of ocular surface disease and with normal sensitivity measured with noncontact esthesiometry.[84]IVCM of these corneas showed abnormal nerve morphology with excessive nerve sprouting. Interestingly, patients with diabetes mellitus and severe peripheral neuropathy have also been found to have

tortuous subbasal nerves which may represent nerve regeneration.[81] Many of the patients with post-LASIK dysesthesia, evaluated at the Ocular Surface Center of the Cullen Eye Institute (Houston, TX, USA), have an abnormal subbasal plexus with small, branched nerve bundles that lack interconnections.

Neurotrophic Keratopathy

IVCM was found to be useful in monitoring corneas for evidence of nerve regeneration in patients with neurotrophic keratopathy who were treated with autologous serum drops.[85] In a study performed at the Ocular Surface Center of the Cullen Eye Institute (Houston, TX, USA), 11 eyes of 6 patients with neurotrophic keratopathy from a variety of conditions, including diabetes mellitus, herpes zoster ophthalmicus and trigeminal nerve ablation were evaluated. Four images of the SNP in the central cornea were randomly selected for analysis of the corneal nerves. Autologous plasma drops were instilled 6 to 8 times per day. Autologous serum therapy resulted in improved best-corrected visual acuity, decreased mean corneal fluorescein staining scores and significantly increased corneal sensitivity measured by the Cochet-Bonnet and modified Belmonte esthesiometers. The mean number, length, width and density of subbasal nerves increased significantly after serum treatment.

These studies indicate that IVCM is a useful technology to image the SNP in normal and diseased corneas. Additional studies are necessary to standardize examination methods and confirm the findings of these preliminary studies.

Peripheral Neuropathy

A promising future clinical application of IVCM is the assessment and quantification of peripheral neuropathies, related to diabetes mellitus or to other conditions known to cause small nerve fibre damage, including hereditary sensory and autonomic neuropathy,[94] autoimmune neuropathy,[95] and chemotherapy-associated neuropathy.[96]

Diabetic neuropathy, specifically, is a common and significant clinical problem, as highlighted by The American Diabetes Association, which reports that 8.3% of US population is affected by diabetes and that 60-70% of people with diabetes have mild to severe forms of nervous system damage.[97] The accurate detection, characterization and quantification of this condition are important in order to define at-risk patients, anticipate deterioration, monitor progression, assess new therapies, and potentially reduce the incidence of foot ulcerations and amputations.[98]

Several confocal studies reported corneal nerves changes in diabetic patients, including reduced nerves density and branching and increased tortuosity.[81, 99-101] The most recent researches showed

early detection,[101, 102] severity stratification,[100, 103] and moderate to high sensitivity and specificity of IVCM for diagnosis of diabetic neuropathy[99, 104], suggesting the prospect to use in vivo confocal features of corneal nerves as biomarkers of diabetic neuropathy.[105, 106] Larger longitudinal studies and a larger normative database, together with ongoing IVCM technical progress, promise to allow the optimization and diffusion of the clinical use of this approach.

In Vivo Confocal Microscopy for Assessing the Ocular Surface in Glaucoma

In glaucoma, IVCM constitutes an interesting aid to evaluate filtering blebs and to better understand the conjunctival wound healing process. IVCM has also been used to assess corneal changes induced by topical antiglaucoma medications and their preservatives.

The long-term success of glaucoma filtering surgery is mainly dependent on the development of a functioning filtering bleb. The formation and the maintenance of this functioning bleb, with regard to wound healing and conjunctival scarring, are therefore of primary importance. Because in some cases the appearance of the bleb is not correlated to intraocular pressure (IOP) control and because the reason of failure is often unclear, some authors have looked for new *in vivo* evaluation techniques such as IVCM. This imaging technique allows the visualization of epithelial microcysts, sub-epithelial connective tissue, blood vessels, and inflammatory cells within the conjunctival bleb tissues.[107-110] Functioning blebs show a normal conjunctival epithelium with numerous microcysts and a loose hyporeflective sub-epithelial tissue with a high number of optically clear spaces. In contrast, non-functioning blebs, show no or very few microcysts with hyper-reflective subepithelial tissue, dense connective tissue and numerous blood vessels.[107, 108] In functioning blebs with mitomycin C, numerous clear spaces corresponding to large confluent microcysts and a loosely arranged connective tissue with numerous large clear spaces may also be observed.[107, 108, 110] The infiltration of bleb tissues by inflammatory cells can be also observed and monitored during the post-operative period.[109] Clinicians, with images at a cellular level, would be able to better predict the outcome of these blebs and eventually provide specific treatments in order to enhance the success rates of their surgical procedures.[107]

Moreover, the chronic use of topical intraocular pressure (IOP)-lowering drugs and their preservatives is known to cause significant changes in the ocular surface. Several studies have evaluated the subbasal corneal nerves with IVCM in patients treated for glaucoma or ocular hypertension. IVCM analysis showed ocular surface alterations in glaucomatous patients treated chronically with Benzalkonium chloride (BAK)-containing eye drops. These changes included a reduction of the density of superficial epithelial cells, activation of stromal keratocytes, decrease in subbasal nerves and increase in subbasal nerve tortuosity.[111] Baratz *et al.* showed a decrease in the

number and density of subbasal corneal nerves in the medication group of the Ocular Hypertension Treatment Study (OHTS).[112] Martone *et al.* observed similar nerve modifications but in association with an increased number of nerve beadings and tortuosity.[111] These authors also demonstrated a direct relationship between nerve tortuosity and corneal sensation in glaucoma patients. Labbe et al. recently evaluated the relationship between the *in vivo* confocal microscopic morphology of subbasal corneal nerves and corneal sensitivity in patients with dry eye and patients treated for glaucoma and ocular hypertension.[113] The density and number of subbasal corneal nerves were significantly decreased in dry eye and glaucoma patients compared to controls. In the dry eye group, corneal sensitivity correlated with the density and the number of nerves. However, in the glaucoma group, corneal sensitivity correlated only with the tortuosity of subbasal nerves. Although patients with dry eyes and those medically treated for glaucoma experience similar ocular surface symptoms and signs, the different correlations found between corneal sensation and nerve morphology in these two groups suggest that nerve alterations and/or dysfunction could also be different. Despite the decreased number of subbasal nerves observed in those patients, an additional anesthetic effect of IOP-lowering medications and preservatives could explain these results.[113]

In relation to drug induced toxic effects, IVCM has also been widely used by researchers to explore the ocular surface changes induced by topical medications and their preservatives. Clinical observations of toxic keratopathy are important findings in glaucoma patients. Additionally IVCM may be used indifferent animal models of ocular toxicity, in order to compare several formulations of topical antiglaucoma eyedrops and preservatives.[114, 115] Liang et al. also investigated with IVCM the effects of antiglaucoma prostaglandin analogs with or without BAK preservative on the conjunctiva-associated lymphoid tissue (CALT).[116] The CALT reaction after instillation of BAK-containing eye drops was characterized by inflammatory cell infiltration in the dome and intrafollicular layers and by cell circulation inside the lymph vessels confirming the concentration-dependent toxic effects of BAK. IVCM analyses of the ocular surface in animal models as well as in humans could thus become pertinent tools in the future for evaluating and understanding immunotoxicologic challenges on the ocular surface and would provide useful criteria for investigating newly developed eye drops.

Confocal Microscopy in Contact Lens Wear

IVCM has significantly enhanced our understanding of the ocular response to contact lens wear in two ways. First, it has provided new perspectives, at a cellular level, on a wide range of contact lens complications that have already been well documented in the literature, such as stromal edema and changes in endothelial cell morphology. Second, it has revealed phenomena that were not

previously possible to image in the living human eye, such as the appearance of stromal microdots and changes in keratocyte cell density. At present, IVCM is a precious complementary tool in contact lens wearers' evaluation and management.[53] This technology, in addition to its previously discussed essential role in contact lens related infectious keratitis, allows early detection of ocular surface changes, finer identification of the pathogenic mechanisms, and improved tailored clinical approach. As for several other areas, clinical utility of IVCM in contact lens wearers is suggested by a strong background and supported by preliminary clinical experiences, but a satisfactory level of evidence is still an unmet need. The following is a brief review of contact lens associated ocular changes observed using IVCM in relation to the various substructures of the anterior eye.

Tear Film

Mucin balls form in the tear layer in patients wearing silicone hydrogel lenses. They have been demonstrated using IVCM to vary in size from 40 µm to 80 µm,[117] and to display a highly reflective core with a more poorly reflective, apparently translucent, outer layer.[118] These inclusions are spherical and can apparently penetrate the full thickness of the epithelium,[117,119] leading to activation of keratocytes in the underlying anterior stroma.[117]Such observations indicate a potential for mucin balls to render the cornea to be more susceptible to infection.

Corneal Epithelium

Epithelial cell size has been observed with the IVCM to increase in response to all forms of lens wear, with the greatest effect seen in rigid lens wearers. Lenses of higher oxygen transmissibility (Dk/t) have been shown to interfere least with the normal process of epithelial desquamation.[120] The degree of epithelial disturbance in response to various concentrations of preservatives used in contact lens disinfecting solutions depends on the duration of lens wear.[121] IVCM studies have confirmed that extended wear of high Dk/t rigid lenses induces more epithelial thinning than hydrogel lenses, which in turn induces more thinning than silicone hydrogel lenses.[122] Basal cell morphology is unaffected by short[123] or long[124] term wear of high Dk/t soft lenses, but visible alterations to basal cells (less regular in appearance) is associated with long term wear of low Dk/t soft lenses.[124]

Corneal Nerves

The current consensus is that contact lens wear does not alter corneal nerves;[125,126] however, a qualitative difference in appearance, such as slight blurring of nerves and less contrast with the background, have been noted among contact lens wearers, which have been attributed to an artifact due to lens-induced edema.[127]

Corneal Dendritic Cells

Among contact lens wearers, a higher density of dendritic cells, which are presumed to be Langerhans' cells, occurs in the layer of the SNP in both the central and peripheral cornea. This suggests that contact lens wear can alter the immune status of the cornea.[128]Increased numbers of dendritic cells have also been observed in association with various combinations of soft contact lenses and lens care solutions.[129]

Corneal Stroma

Dark lines and folds are observed with the IVCM in the edematous cornea in response to contact lens wear.[130]The lack of keratocyte clarity at high levels of edema probably corresponds with the corneal haze observed with the slit-lamp biomicroscope.[130] Apparent keratocyte loss following overnight lens wear[131] is most likely an artifact due to uni-dimensional volumetric stromal expansion, causing keratocytes to be more 'spread out within the tissue. Consequently, when viewed by IVCM, fewer keratocytes are observed within the fixed depth of focus (this phenomenon can be explained by explained by the binomial expansion theory[130]). As well, a degradation of image quality at higher levels of edema renders keratocytes more difficult to detect.[130] These artifacts need to be taken into account, or properly controlled for, in studies of keratocyte loss during contact lens wear.

Only a few of the studies that have investigated keratocyte loss associated with contact lens wear have attempted to account for these artifacts. Nevertheless, most of these studies have concluded that contact lens wear results in keratocyte apoptosis, although there is some disagreement as to the magnitude of this effect..[125, 130-137] Mechanical stimulation of the corneal surface, due to the physical presence of a contact lens, and the consequent release of inflammatory mediators, is likely to be the main cause of the observed reduction in the number of keratocytes.[133, 138] Keratocyte loss may play a role in contact lens-induced stromal thinning in view of the role of keratocytes in maintaining the structural integrity of the cornea.

Highly reflective stromal 'microdot deposits' can be observed with the IVCM throughout the entire stroma of lens wearers and non-lens wearers (Figure 7);[139-142] however, microdots appear in greater numbers in lens wearers and in patients with some disorders such as chloroquine induced keratopathy,[143] exfoliation syndrome [144] and mucopolysaccharidoses.[145] These findings' interpretation is still unclear in many cases and we may hypothesize that, in different conditions, similar images are due to different mechanisms. Contact lens related microdot deposits may represent granules of lipofuscin-like material within the corneal stroma of long-term contact lens wearers, formed as a result of chronic oxygen deprivation and chronic microtrauma to the cornea.

Corneal Endothelium

Endothelial blebs are an acute response to lens wear which take on a different appearance with the IVCM compared with the slit lamp biomicroscope due to the different optical configuration of these two instruments. With the IVCM, endothelial blebs have a bright center and dark annular surround, set against a bright background field of the remainder of the 'non-blebbing' endothelium.[142] Optical models have been constructed to explain these differences.[146] Chronic morphological changes to the endothelium in response to lens wear observed with the IVCM,[147] such as increased polymegethism and increased light scatter, confirm previous observations made using specular microscopy.

Limbus

Long-term soft contact lens wear can induce many changes in corneal limbus, such as microcystic formations in epithelial cells, microdot deposits in corneal stroma, increased Langerhans cell density and decreased of keratocyte density.[148] There are more rolling leukocytes in limbal vessels in patients wearing low Dk/t lenses (versus non-lens-wearers),[149] highlighting the potential of this measure as an indication of sub-clinical inflammation.

Contact Lens Associated Keratitis

Because bacteria involved in contact lens related infections are beyond the resolution of the IVCM, this instrument cannot be used to diagnose contact lens-associated bacterial keratitis. However, the IVCM may be used to view damage to the various corneal layers caused by bacterial infection, such as needle-like deposits in crystalline keratitis,[150] and to indirectly assess the infectious process by monitoring the activity of Langerhans' cells.[151]

Contact lens wear is a significant risk factor for *Acanthamoeba*and fungal keratitis. IVCM role in these severe affections is discussed in a previous section of this review.

Investigation of the chronic morbidity of corneal infiltrative events associated with contact lens wear by IVCM revealed no significant impact upon basal epithelial cell density, anterior or posterior keratocyte density, or endothelial cell density, polymegethism or pleomorphism.[152] However, markedly reduced pan-corneal cell counts and increased endothelial polymegethism were observed in the affected eye of a patient who had suffered from a severe corneal infiltrative event.[152]

Conjunctiva

Contact lens wear can induce changes in the bulbar conjunctiva as observed by IVCM. For instance, epithelial thinning, accelerated formation and enlargement of microcysts (Fig. 8), and increased

epithelial cell density occur and can be documented by IVCM.[153] A preliminary study found no impact of soft contact lens wear on bulbar conjunctival goblet or Langerhans cell density. Examination of MGs of contact lens wearers showed decreased basal epithelial cell density, lower acinar unit diameters, higher glandular orifice diameters, greater secretion reflectivity and greater inhomogeneity of the periglandular interstices.[65] These morphologic changes are interpreted as signs of MGs dropout, duct obstruction, and glandular inflammation.[154]

Advances in In Vivo Confocal Microscopy Imaging

Current developments of IVCM include (1) 2 dimensional (D) wide field mapping, (2) 3D reconstruction of the cornea, and (3) automated image analysis with the resulting quantification of cornea parameters, including the SNP.

2D Wide Field Mapping

One of the crucial limitations of the confocal microscopy is the small field of imaging. Images generated by confocal microscopes are relatively small (HRTII/RCM 400x400 µm; Confoscan 4/Nidek 460x345 µm)[155] and contain only a very small portion of the SNP. The analysis of the depicted nerve fibers may result in misleading findings after quantification. Therefore, several studygroups are working on solutions with off- and on-line mapping procedures for corneal structures.

Real-time mapping of the corneal emphasizing structures of the SNP was presented by Zhivov et al.[156] Source data (384x384 pixels, 400x400 µm) were used to create large-scale maps of the scanned area by selecting the Automatic Real Time (ART) composite mode. The algorithm is based on an affine transformation. The maximal ART composite image size using source images is 3072x3072 pixels (3.2x3.2 mm) and corresponds to 8x8 non-overlapping images. The acquisition time for a single composite image was up to 3 min. Given that the total CLSM time for a single composite image is less than 3 min with no post-processing, the advantages of the online system are apparent.

Another wide-field mapping technique was shown by Edwards et al.[157] The method uses the same source data (384x384 pixels, 400x400 µm, max. number of 100 images, duration 20 sec) and images could be mapped with a post-processing time of about 10 min.

3D Reconstruction

The idea for 3D imaging of the living cornea was originally suggested by Petroll *et al.* in 1993 when the first micrographs of rabbit cornea were published.[158] A further image processing algorithm based on phase correlation was used by Allgeier et al.[159] to analyze and reduce motion

distortions in volume scan image sequences (30 images, volume depth 60 µm, constant interslice distance 2µm, duration 4 sec). 3D tracing of the SNP was performed in order to reconstruct images containing only the SNP layer in humans.

Petrollet al. used the real time "streaming mode" of the HRT/RCM[160]. A device modification allowed z-scans through the whole thickness of the rabbit cornea with a 2 µm interslice distance. The external software (ImageJ, http://rsbweb.nih.gov/ij/; Imaris, Bitplane Inc.) allowed the determination of the epithelial and total corneal thickness as well as keratocyte density data.

Imaging and Quantification of the SNP

Presently, an automatic analysis of the SNP structures is the promising standard in SNP image analysis. Zhivov et al.[161] performed the evaluation of SNP on the basis of the best artifact-free single image using in-house developed software tool. The analysis was based on morphological (length, diameter, density) and topological (continuity and connectivity) parameters derived from segmented nerve fibers. It also used the skeletonized medial lines that were transformed into networks of undirected graphs consisting of nerve paths and various types of connecting nodes. A more recent approach (Winter, unpublished) employs different local adaptive image filters for image contrast homogenization during image pre-processing and an adapted Gabor filter model for final image segmentation.

Dabbah et al.[162, 163] presented an analysis and classification system for detecting nerve fibers in confocal images. It was based on a multi-scale adaptive dual-model detection algorithm that exploited the curvilinear structure of the nerve fibers and adapted itself to the local image information. Holmes et al.[164]proposed a segmentation and skeletonization algorithm for the detection of nerve fibers based on ridge map calculation. During the process nerve fibers are identified, gaps are reconnected and finally quantified.

In summary, automated analysis of corneal nerve fibers employed and generated many sophisticated methods and strategies in the field of medical image processing. Currently, a number of different approaches in relation to the principal problem of small field of imaging of the SNP is in progress which hopefully will provide promising solutions. Imaging of large areas of the SNP will soon enable a considerably more accurate and thus more reliable quantification of nerve fiber networks in the context of research and clinical diagnosis (Figure 9).

Closing Remarks

The application of IVCM to the ocular surface opened a new era in ocular science.

The slit lamp biomicroscopy has been the mainstay of ophthalmic examination of the anterior eye for more than half a century, and this is unlikely to change. With its full colour, wide-field view, low-to-medium magnification, binocular objective, highly versatile illumination system and relatively low cost, the slit lamp will probably remain the instrument of first choice for examining any ocular disease.[53]

At present, IVCM provides new opportunities to view ocular surface structures at a cellular level, through a quick, minimally-invasive (maybe non-invasive at all, in the next future), and steady-state respectful examination.

The impact of IVCM on cornea and ocular surface research has been immediate and rapidly growing, with 907 papers published from 1985 (when Lemp MA first reported confocal examination of the cornea[165]) to July 2013, 455 of them published in the last 5 years (data by PubMed; research performed with the key-words "in vivo confocal microscopy cornea [OR] in vivo confocal microscopy ocular surface"). This quantitative progression has been associated to a qualitative evolution, moving from corneal apex to the whole ocular surface, from qualitative assessments to attempts to standardize and, in some cases, automatize quantitative evaluations, from small case series or pilot studies to well-constructed clinical trials.

Several challenges still remain. One of the main limitations of IVCM is the small field of view. In vivo confocal microscopes currently used in ophthalmology can image only a very small area. For instance, the HRTII/RCM (Heidelberg Engineering GmbH, Heidelberg, Germany) images a 400×400 μm field (0.160 mm2) and the Confoscan 4 ([NIDEK, Gamagori, Japan] images a 460×345 μm field (0.159 mm2). As a consequence of small fields of view and limitations of applicability in eyes with small palpebral apertures, the reproducible investigation and quantification of the same areas over time is virtually impossible. Additional concerns exist about the standardization of image acquisition, interpretation, and quantification.[1]

At present, these difficulties, together with the high cost of this technology, are factors preventing widespread deployment and use of IVCM in clinical practice. However, despite the open challenges, IVCM is ready to be used as a precious complementary in vivo technique for clinical diagnosis and management and its clinical applications seem to be extremely promising and still largely to be explored.

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The authors report no conflicts of interest.

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Figure Legends

Figure 1. Laser IVCM appearance of Acanthamoeba cysts demonstrating clustering of cysts (A), Acanthamoeba cysts demonstrating linear alignment of cysts (B), and Acanthamoeba trophozoites C, D).

Figure 2. Laser ICVM appearance of Fusarium Solani hyphae (A), Paecilomyces Lilacinus hyphae (B), and Candida Parapsilosis (C).

Figure 3. Representative confocal microscopy of meibomian glands scans of normal control subject (A), meibomian gland dysfunction (MGD) patients (B), and Sjogren Syndrome (SS) patient (C). Please note the decreased density and the enlargement of acinar unit and the increased secretion reflectivity in MGD patient (B). SS patient shows small acinar units, with increased density of inflammatory cells and increased inhomogeneity of the periglandular interstice (C).

Figure 4. Representative impression cytology imprint and confocal microscopy scans of bulbar conjunctiva in normal control subject (A, C) and Sjogren syndrome patient (B, D). The squamous metaplasia was observed both in impression cytology and confocal microscopy examination.

Figure 5. IVCM appearance of microcysts of aqueous humor in the conjunctival epithelium over a filtering bleb after trabeculectomy

Figure 6. Corneal epithelial changes in glaucoma patient, showing anisocytosis and inflammatory cells.

Figure 7. Extensive formation of microdots in the corneal stroma of a rigid lens wearer.

Figure 8. IVCM appearance of microcysts in the bulbar conjunctiva of an asymptomatic soft contact lens wearer.

Figure 9. Real-time mapping of SNP structures (A) and results of automatic quantification of SNP structures using in house developed software (B).

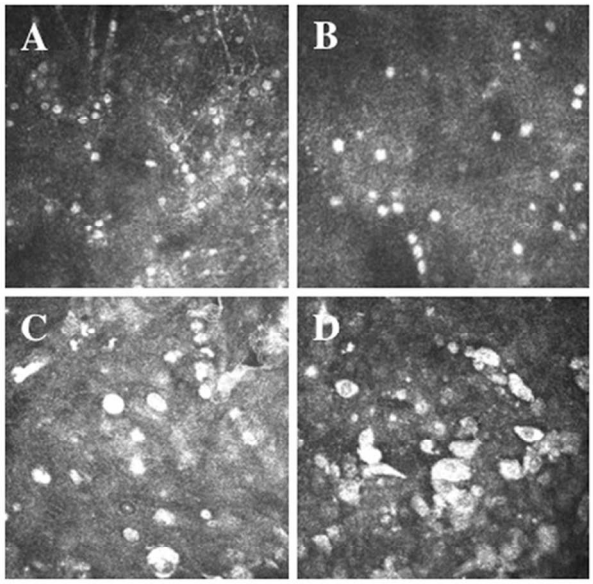

Figure 1. Laser IVCM appearance of Acanthamoeba cysts demonstrating clustering of cysts (A), Acanthamoeba cysts demonstrating linear alignment of cysts (B), and Acanthamoeba trophozoites C, D).
169x127mm (150 x 150 DPI)

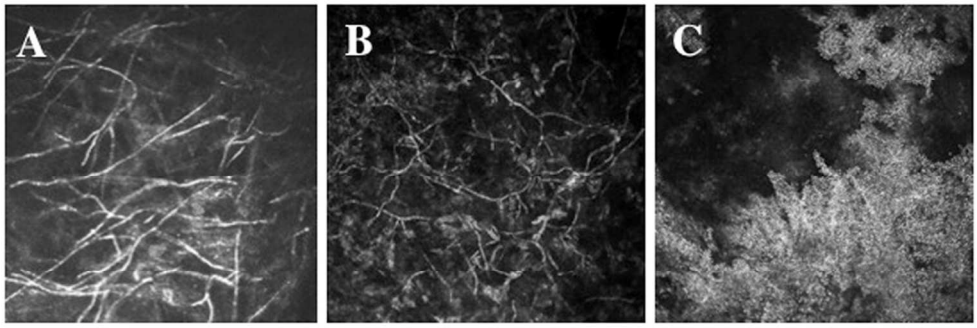

Figure 2. Laser ICVM appearance of Fusarium Solani hyphae (A), Paecilomyces Lilacinus hyphae (B), and Candida Parapsilosis (C).
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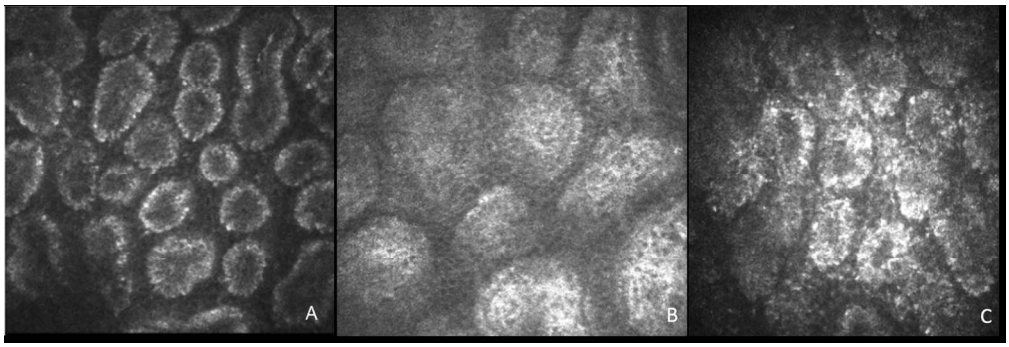

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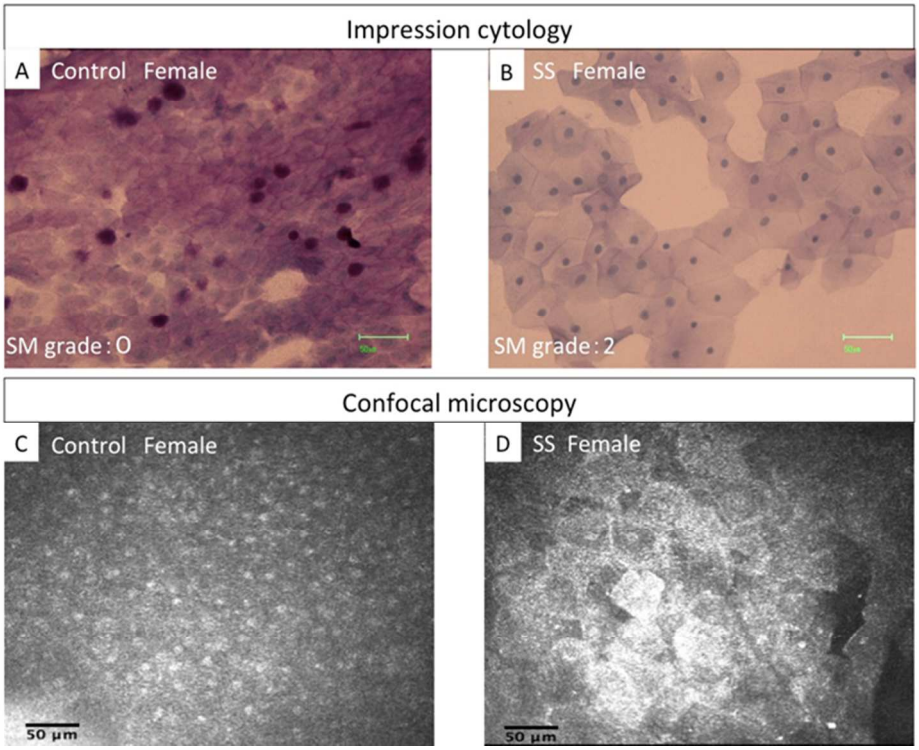


Figure 4. Representative impression cytology imprint and confocal microscopy scans of bulbar conjunctiva in normal control subject (A, C) and Sjogren syndrome patient (B, D). The squamous metaplasia was observed both in impression cytology and confocal microscopy examination.
149x112mm (150 x 150 DPI)

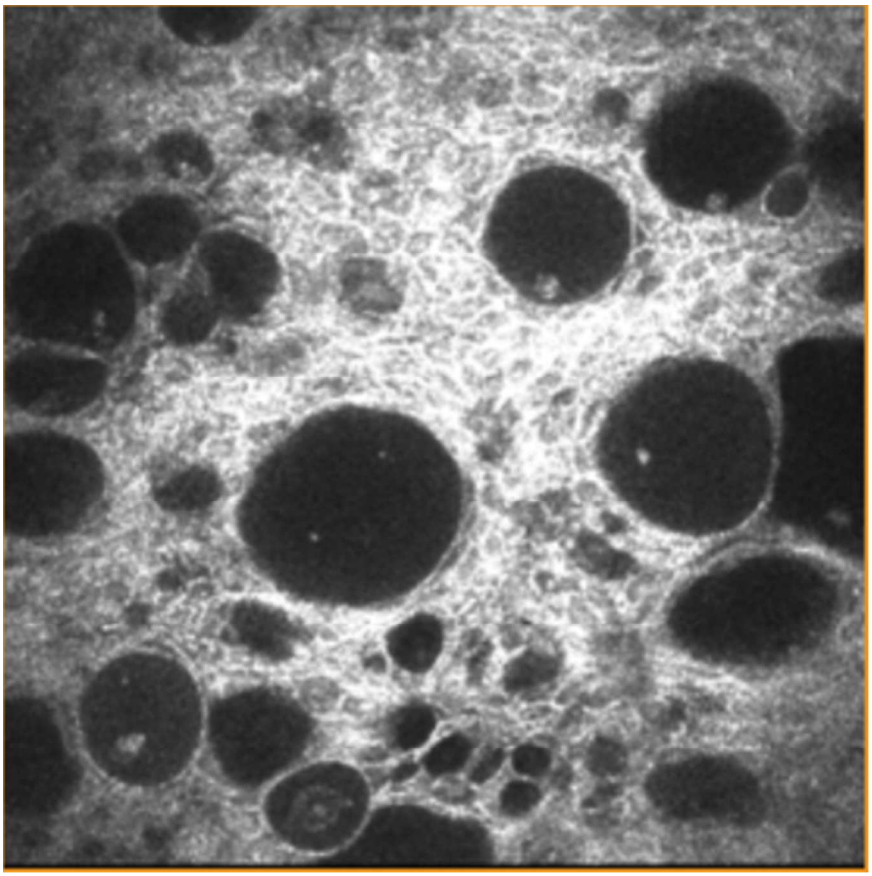


Figure 5. IVCM appearance of microcysts of aqueous humor in the conjunctival epithelium over a filtering bleb after trabeculectomy
73x73mm (150 x 150 DPI)

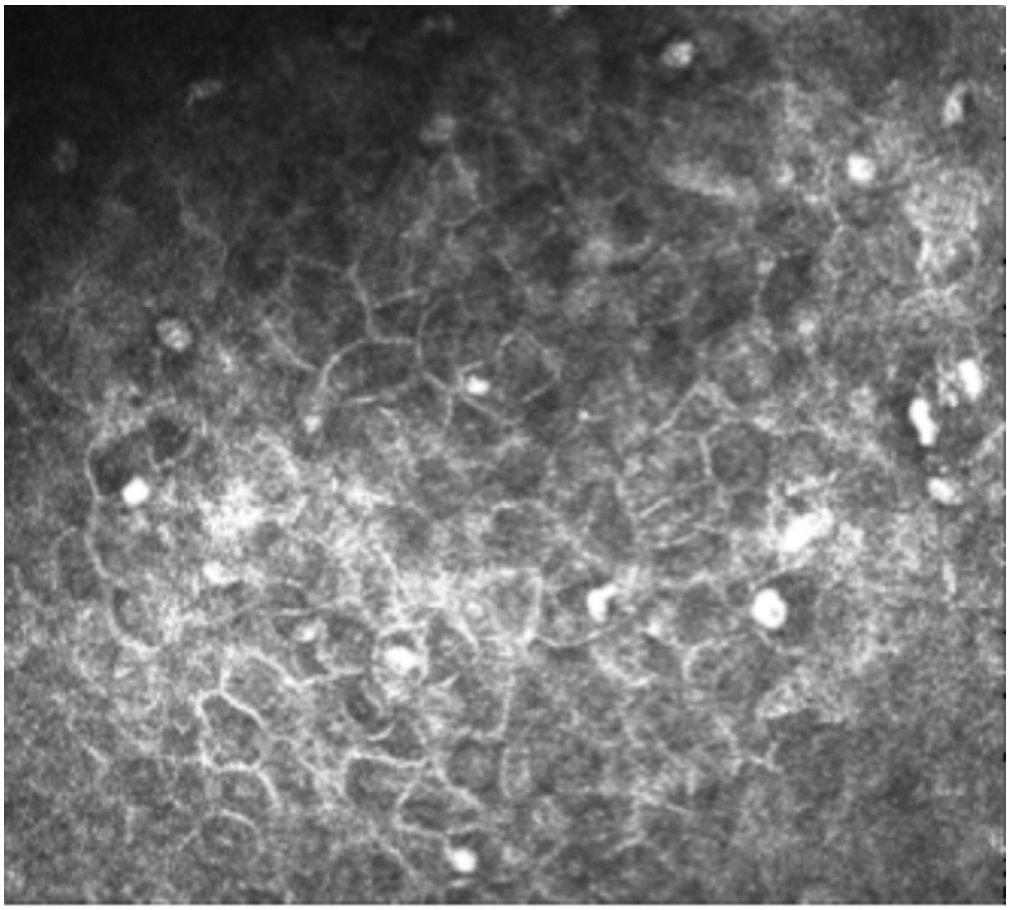
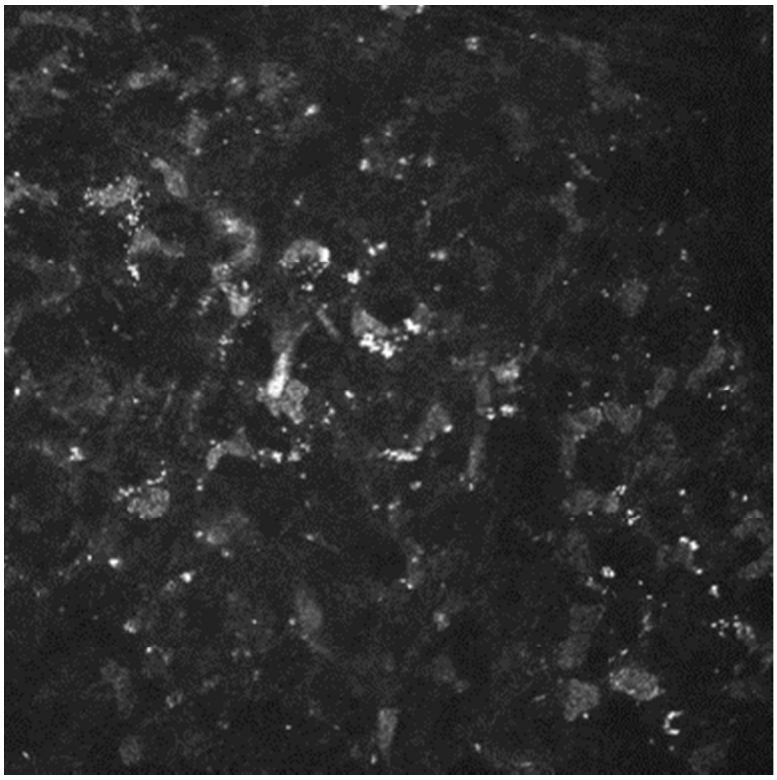


Figure 6. Corneal epithelial changes in glaucoma patient, showing anisocytosis and inflammatory cells.
130x117mm (150 x 150 DPI)



Extensive formation of microdots in the corneal stroma of a rigid lens wearer.
101x101mm (96 x 96 DPI)

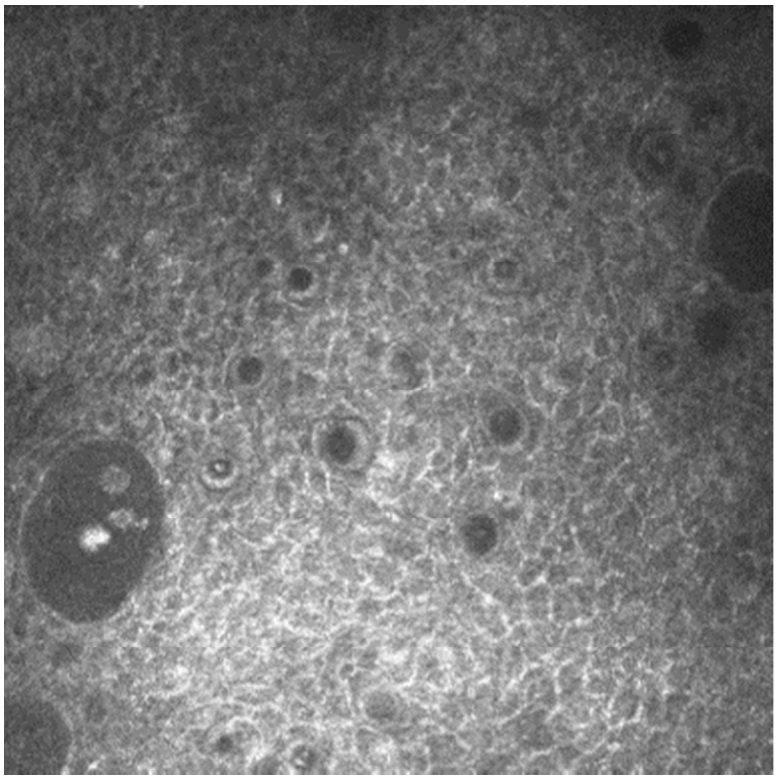


Figure 8. IVCM appearance of microcysts in the bulbar conjunctiva of an asymptomatic soft contact lens wearer.
101x101mm (96 x 96 DPI)

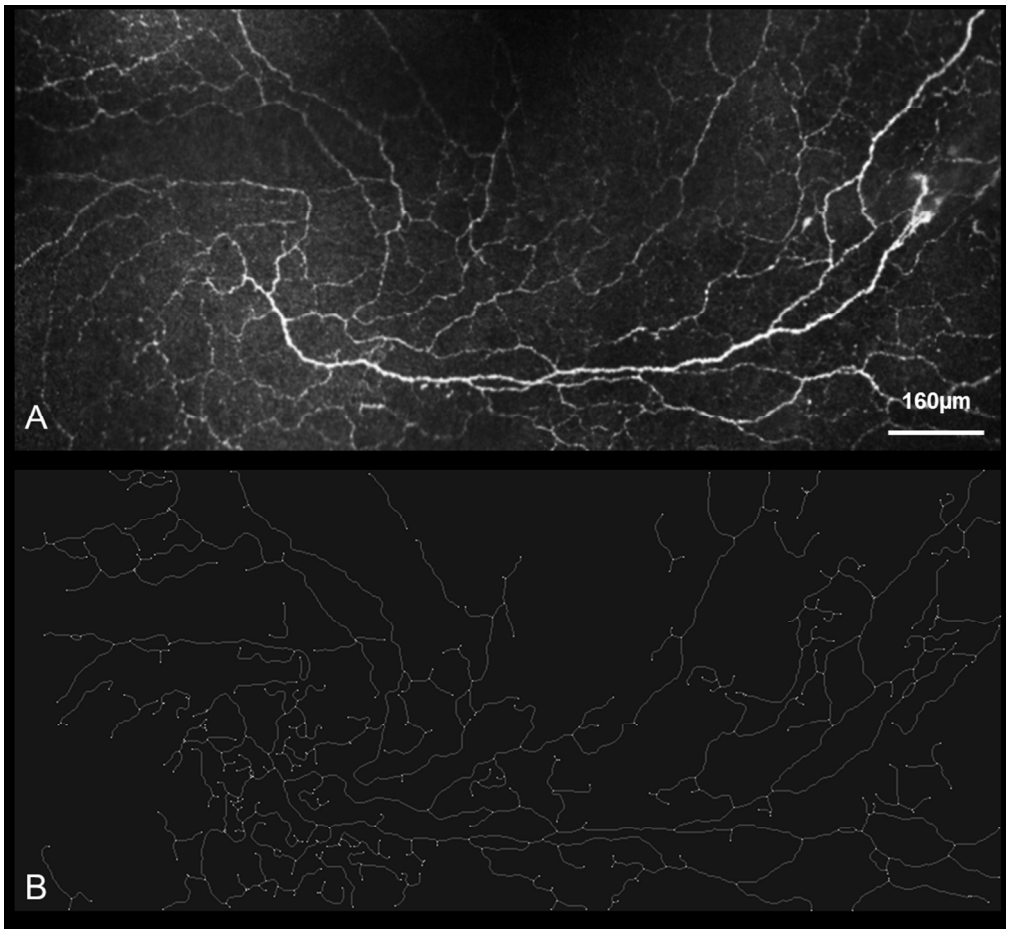


Figure 9. Real-time mapping of SNP structures (A) and results of automatic quantification of SNP structures using in house developed software (B).
187x172mm (150 x 150 DPI)