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In Vivo Confocal Microscopy of Corneal Nerves in Health and Disease

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Abstract

In vivo confocal microscopy (IVCM) is becoming an indispensable tool for studying corneal physiology and disease. Enabling the dissection of corneal architecture at a cellular level, this technique offers fast and noninvasive in vivo imaging of the cornea with images comparable to that of ex vivo histochemical techniques. Corneal nerves bear substantial relevance to clinicians and scientists alike, given their pivotal roles in regulation of corneal sensation, maintenance of epithelial integrity, and proliferation and promotion of wound healing. Thus, IVCM offers a unique method to study corneal nerve alterations in a myriad of conditions, such as ocular and systemic diseases and following corneal surgery, without altering the tissue microenvironment. Of particular interest has been the correlation of corneal subbasal nerves to their function, which has been studied in normal eyes, contact lens wearers, and patients with keratoconus, infectious keratitis, corneal dystrophies, and neurotrophic keratopathy. Longitudinal studies have applied IVCM to investigate the effects of corneal surgery on nerves, demonstrating their regenerative capacity. IVCM is increasingly important in the diagnosis and management of systemic conditions such as peripheral diabetic neuropathy and, more recently, in ocular diseases. In this review, we outline the principles and applications of IVCM in the study of corneal nerves in various ocular and systemic diseases.

Keywords

corneal dystrophies; corneal nerves; corneal sensitivity; corneal surgery; diabetes; keratoconus; in vivo confocal microscopy; pain; peripheral neuropathy

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I. INTRODUCTION

There is growing interest in noninvasive techniques to study live cellular physiology in health and disease, and the development and application of in vivo confocal microscopy (**IVCM**) has enabled ophthalmologists to add intravital corneal imaging to their armamentarium for the diagnosis and management of corneal disorders. With the ease of clinical set-up, high throughput, and a 800-fold magnification of live cellular architecture, IVCM holds promise of enhancing the quality of care provided to patients in an outpatient setting.

In this review, we illustrate the significant advances made to date by the use of this in vivo technique to promote a better understanding of corneal nerve morphology and host immune response. We discuss the utility of IVCM in assessing physiological nerve morphology and pathological alterations in a variety of corneal disorders ranging from localized infections to systemic diseases.

II. EMBRYOLOGY OF CORNEAL NERVES

Corneal nerves of the adult human eye have been studied extensively both ex vivo and in vivo.[1–4] An understanding of their origin and development helps to better address ocular pain and ocular surface health in conditions of altered physiology, such as after infections, trauma, and surgery. In 1957, Kitano demonstrated that innervation of the corneal epithelium first occurs at 5 months of gestation in humans,[5] whereas in chick embryos, corneal epithelial innervation is first seen at embryonic day (E) 11.[6] Neural crest cells differentiate from the lateral border of the neural plate, a process induced by bone morphogenic protein (**BMP**)-4 and BMP-7.[7] BMP signal transduction is mediated by cytoplasmic co-receptor SMAD proteins, which then regulate gene transcription in conjunction with co-Smad and SMAD4.[8,9] Differentiation of these cells leads to the development of cranial neural crest cells that migrate to specific pharyngeal arches based on their origin within the rhombomere. Guidance of neural crest cells to respective pharyngeal arches is controlled by the homeobox-b (Hoxb) gene complex, and OTX2 gene (*bicoid*-class homeobox gene).[10] The trigeminal ganglion is among the derivatives of these neural crest cells. Corneal innervation is largely sensory and derived from the ciliary nerves of the ophthalmic branch of the trigeminal ganglion.[11, 12]

During development, guidance of neuron axonal growth is provided by neurotrophins that attract axons into the cornea and promote their survival.[13–16] The human cornea expresses four major classes of neurotrophins: nerve growth factor (**NGF**), brain-derived neurotrophic factor (**BDNF**), and neurotrophins (**NT**)-3 and NT-4.[13] In addition, of recent interest have been factors that also serve to regulate corneal nerve growth and directionality, namely, semaphorins (Sema 3A, 3F, 7), slits (1–3), netrins (netrin 2) and ephrins (B2) along with their respective receptors, neuropilins (Nrp), roundabout (Robo), deleted in colorectal carcinoma (DCC), and eph receptors.[17,18]

Sema3A is a negative regulator of nerve growth and is involved in the initial stages of corneal innervation pertaining to formation of the pericorneal nerve ring (embryonic days 4–

8).[19, 20] Further studies have confirmed the role of Sema3A as a negative regulator of corneal innervation, preventing growth of nerves into the stroma, and in later stages of development, into the corneal epithelium.[6] Unlike the neuronal chemorepellent Sema3A,[21,22] Sema7 (**CDw108**) is unique in that it acts as a facilitator of axonal growth and tract formation in an integrin receptor-dependent manner, suggesting that integrin-driven semaphorin signalling may be one of the mechanisms in the development of the nervous system.[23] Sema7A is constitutively expressed in the mouse corneal epithelium and to a lesser degree in the stroma, at a concentration of 2.38 μM and 0.41 μM, respectively.[18] Jain and colleagues elegantly demonstrated increased Sema7A gene expression in corneas with nerve regeneration following lamellar surgery, which was also associated with an influx of CD45+, CD11b+ and CD3+ immune cells in vivo, indicating the neuro-immunomodulatory functions of Sema7A in corneal nerve regeneration.[18] It is now believed that Slit2 plays a dual role in the control of corneal innervation. In the chick embryo at E7, Slit2 serves as a negative regulator of neurite growth from the ophthalmic trigeminal ganglia (**OTG**), as seen by the reduced length and number of neurites from the OTG upon application of recombinant mouse Slit2, and enhanced neurite growth in OTG co-cultures with chicken-specific Slit2 antibody. Interestingly, by E9 Slit2 switches its role to induce branching of nerves in the corneal epithelium.[6]

BMPs also play a role in the development of the sympathetic nervous system via smad4-dependent and –independent pathways by regulating noradrenergic differentiation and proliferation, and survival of precursors of the sympathetic nervous system, respectively.[8] Murine,[24] primate,[25] and human studies suggest that these sympathetic nerves release neuropeptides that may be involved in corneal neurogenic inflammation and remodelling following corneal injury.[24,25]

III. CORNEAL SUBBASAL NERVE PLEXUS

A. Innervation of the Human Cornea

The cornea is the most densely innervated tissue in the human body, with approximately 7000 epithelial free nerve endings per square millimetre.[3,26] There have been elegant and meticulously thorough studies defining and characterizing the anatomy of human corneal innervation using light microscopy, electron microscopy and confocal microscopy.[1–4,26–28]

As the nerve bundles from the ophthalmic branch of the trigeminal ganglion approach the cornea in a radial pattern around the limbal circumference, they lose their perineurium and myelin sheath at approximately 1 mm after entering the corneoscleral limbus, traversing into the cornea encased only in Schwann cell sheaths at a mean depth of 293 ± 106 μm from the corneal surface.[4,26] Therefore, peripheral stromal nerves comprise both myelinated and unmyelinated nerve fascicles. The unmyelinated nerve fascicles in the central stroma contain axons enmeshed in an amorphous extracellular matrix.[26] These fascicles turn anteriorly towards the corneal surface at 90°, piercing the Bowman's layer, after which they extend unmyelinated nerve axons in the form of epithelial leashes at 90°, parallel to the corneal surface, between the Bowman's layer and basal epithelial layer, forming the subbasal nerve plexus that supplies the overlying corneal epithelium (Figure 1).[26] These nerve leashes

contain both beaded and unbeaded, straight nerve axons, where the beading represents foci of axon enlargements with collections of mitochondria.[26]

Nerves of the subbasal plexus run centripetally towards the corneal center in a clockwise direction and whorl towards the inferonasal paracentral area (Figure 2).[29] The centripetal migration of corneal nerves has been attributed to factors such as the "X-Y-Z" hypothesis,[30, 31] turnover of epithelial cells at the corneal center drawing in peripheral epithelial cells and nerves to the center,[32] migration pressure from the limbus,[33,34] and electromagnetic forces.[35] The inferonasal displacement is postulated to be a result of the shearing force imparted by the eyelid during blinking.[36] More recently, IVCM studies in patients with diabetic peripheral neuropathy have identified reduction in the length of nerves of the inferonasal whorl, comparable to the loss of central corneal subbasal nerve length.[37] While IVCM is sensitive in detecting corneal subbasal nerve length changes in diabetic neuropathy, neither central corneal subbasal nerve length nor subbasal whorl nerve length have proven to be strong diagnostic tests for diabetic peripheral neuropathy independently (area under the curve, AUC = 0.76–0.77; specificity = 0.5–0.6).[38] However, when taken together, there was moderate improvement in its specificity (0.71) but without an increase in its utility as a diagnostic test (AUC = 0.75).[37]

In addition to these sensory fibers, studies in mammals, including humans,[25,39] pigs,[25] cats,[40,41] rabbits,[40] rats,[40,42] and monkeys[40] suggest that the cornea also receives a sparse supply of autonomic sympathetic nerves fibers, which originate in the cell bodies of the superior cervical ganglion.[40,41] However, it is unclear whether the human cornea receives parasympathetic innervation as well.[26]

B. Types of Sensory Corneal Nerves

The cornea receives a large supply of functionally heterogeneous sensory nerves from the trigeminal nerve. These sensory nerves have both an anatomical and functional organization. Anatomically, subsets of these nerves run in parallel, while others are aligned perpendicular to the corneal surface.[26, 27] Functionally, they vary in their chemical composition, electrophysiological properties, and response to excitation stimuli. According to their myelination patterns and speed of impulse conductance, corneal nerves can be classified as:

- **a.** thin myelinated (A-delta type; fast conducting with average velocity of 6 ms^{-1})[43–46], and
- **b.** unmyelinated (C type; slow with average velocity of less than 2 ms^{-1}).[43–46]

Based on the stimuli that activate these nerve endings, they can be classified as:

- **a.** mechano-nociceptors (20% of all corneal sensory nerves; A-delta type, convey acute sharp pain in response to mechanical contact with the cornea),[43–46]
- **b.** polymodal nociceptors (70% of all corneal sensory nerves; majority C type; convey sharp and sustained pain in response to mechanical, heat, exogenous chemical and endogenous inflammatory irritants to the cornea),[44,47,48] and
- **c.** cold receptors (10% of all corneal sensory nerves; A-delta and C types; start firing in response to tear film evaporation, application of cold solutions or cold

air to the cornea and when corneal surface temperature decreases below 33°C).[45,49,50]

C. Function of Corneal Nerves

One of the functions of corneal nerves is to transduce thermal, mechanical, and chemical stimuli as perceptions of pain.[51,52] The high density of delicate sensory afferent endings interspersed within the corneal epithelium cover specific regions of both the stroma and corneal surface to form the receptive field of those nerve fibers, as first described in the cat by Belmonte and Giraldez in 1981.[44] The size of the receptive field varies with functional classification of the nerves. Polymodal nociceptors and mechano-nociceptors have large receptive fields, whereas cold receptors have smaller receptive fields in the cornea, which reach near pinpoint size in the perilimbal region. When a stimulus triggers nerve endings within these receptive fields, rapid depolarization and subsequent impulse conduction along the axon allow detection of even small magnitude stimuli.[48] Moreover, emerging evidence suggests that the idiosyncrasies of corneal nerve response to inflammation and trauma, both mechanical and chemical, may be attributed to specific genetic and molecular signatures of the primary sensory neurons within the trigeminal ganglion.[53]

Recently, there has been a growing interest in the understanding of the electrophysiological function and contribution of cold receptors in the maintenance and integrity of the ocular surface.[52,54] Cold receptors are now believed to be the key perpetrators of basal tearing as a function of their heightened sensitivity to small fluctuations in ambient temperature, mediated by the cationic channel Transient Receptor Potential Melastatin 8 (**TRPM8**).[54] Thus, corneal sensation is critical to protection of the ocular surface.

Investigations into the cellular and molecular mechanisms underpinning the pathogenesis of corneal epithelial disorders, such as in neurotrophic keratopathy, have elucidated the pivotal role of trigeminal nerves in the maintenance of corneal health and function. Corneal nerves not only protect the ocular surface through an elaborate mechanism of sensation and the blink reflex, but they also release various trophic factors, which regulate the modulation of epithelial integrity, proliferation and wound healing.[55–57] The human cornea has trophic factors, including NGF, BDNF, NT-3, NT-4, and glial cell line-derived neurotrophic factor (**GDNF**).[13] All of the above, except GDNF, belong to the neurotrophin gene family, whereas GDNF is a member of the transforming growth factor-beta family (**TGF-β**).[58, 59] Neurotrophins exist as homodimers, which upon binding with tyrosine kinase family of receptors (Trk A, B, C and E) induce phosphorylation and dimerization, leading to transduction of the signal cascade.[60]

You and colleagues demonstrated that NGF and GDNF promote epithelial colony formation and proliferation, whereas BDNF only enhances epithelial colony formation.[13] This is achieved by activation of the mitogen-activated protein kinase (**MAPK**) signalling pathway mediated by phosphorylation of epithelial extracellular signal-regulated kinase-1 (**ERK-1**) that in turn activates transcription factors.[13] Thus, NGF ensures integrity of the corneal epithelial surface, whose function, when compromised, e.g., as in cases of nerve damage, can lead to neurotrophic ulcers. Neurotrophic ulcers have been successfully treated with NGF in human case studies and clinical trials, thus reconfirming the rejuvenating and

protective effects of corneal nerves on the corneal epithelium.[61–63] Moreover, a recent non-human primate study found that following LASIK, corneas of LASIK-treated rhesus monkeys showed correlation of NGF protein and gene expression levels with corneal nerve density, demonstrating a possible role of NGF in early response (3 days post-operatively) toward nerve regeneration and eventual nerve recovery (1, 3 and 6 months post-operatively) following refractive surgery.[64] Currently, clinical trials are underway in Europe (REPARO study, ClinicalTrials.gov identifier: NCT01756456) and the United States (ClinicalTrials.gov identifier: NCT02227147) to assess the safety and efficacy of NGF in neurotrophic ulcers.

Immunochemistry has revealed the presence of various neurotransmitters, including substance P (**SP**), calcitonin gene-related peptide (CGRP), neuropeptide Y (**NY**), vasoactive intestinal peptide (**VIP**), galanin, methionine-enkephalin, catecholamines, and acetylcholine in the cornea.[65] SP released by corneal nerves has received increasing attention, given its effects on promoting corneal wound healing in synergistic conjunction with insulin-like growth factor-1 (**IGF-1**).[65] This has translated into extraction of short peptide sequences from SP (FGLM) and IGF-1 formulated into eye drops for the treatment of epithelial defects in neurotrophic keratopathy.[66,67] Thus, corneal nerves have an unequivocal role, both direct and indirect, in maintaining, protecting and promoting corneal epithelial integrity and, consequently, corneal transparency. It is hence of great importance to develop and apply methods by which we can better observe, analyse, and understand alterations in corneal nerve morphology and function.

D. Alteration and Pathophysiology of Corneal Nerves

Human corneal nerves have been studied ex vivo by various groups using light and electron microscopy.[1,3,26,27,33,68] These techniques, however, may generate unreliable results, since human corneal nerves are known to degenerate within the first 14 hours of death.[3] The prevalent in vivo examination technique of the cornea is slit-lamp biomicroscopy, whose major limitation is its magnification factor (40x), precluding examination of the cellular and neural architecture of the cornea. This obstacle is now being circumvented with the invention of the IVCM, and more recently the laser IVCM, which offers a 800-fold magnification, allowing visualization of the corneal cellular architecture and corneal nerves, including the subbasal nerve plexus (Figure 3). Aside from advancing our understanding of the corneal cellular and nerve morphology, a great utility of this technique is that it provides the opportunity for quantitative assessment of corneal cellular and nerve properties in normal health, disease, and postoperative conditions.[69–72] IVCM has many advantages: it is rapid, noninvasive, precise, and quantitative assessment of corneal nerves has demonstrated low interobserver variability.[73]

IV. ASSESSMENT OF THE SUBBASAL CORNEAL NERVE PLEXUS

A. Morphological Assessment: Histology by In Vivo Confocal Microscopy

1. Principles of Confocal Microscopy—The principle of confocal microscopy was first described by Goldmann in 1940 and patented by Marvin Minsky in 1955,[74] after which it made its way to in vivo imaging of the living brain for studies of neural networks.[75,76] The principle underlying confocal microscopy is conjugate alignment of light rays focused on

the tissue by the condenser lens, with those reflected by the tissue and captured by the objective lens, hence the term “confocal.”

The first scanning confocal microscope was developed by Minsky in 1988.[74] Conventional light microscopes produce poor quality images due to increased reflections and scattered light from structures outside of the focal plane.[77] The confocal microscope actively eliminates the light from the planes outside the focal point, as those rays do not fall directly onto the detection aperture.[78] This feature of the confocal microscope is beneficial for corneal imaging in two important ways: first, lateral resolution (x,y) and axial (z) resolution are both increased; second, serial imaging of successive points with deeper focal planes allows three-dimensional reconstruction of corneal structures.[69,79]

The field of confocal microscopy has undergone development since the principle was first described by Minsky. In 1986, the tandem-scanning confocal microscope (**TSCM**) was applied to both ex vivo[80] and in vivo corneal imaging,[77, 81] followed by the slit-scanning confocal microscope (**SSCM**).[82] A more recent significant advance of this technique has been the use of a coherent light source to generate the laser-scanning confocal microscopes (**LSCM**).[83]

2. Types of In Vivo Confocal Microscopes

a. Tandem-Scanning Confocal Microscope: The first in vivo TSCM was created and described by Petran et al in 1968.[84] TSCM uses a spinning Nipkow disc with multiple conjugate sets of pinhole openings arranged in Archimedean spirals. This confocal design provides desirable axial and lateral resolution. With the small pinhole diameters and high numeric aperture of the objective lens, TSCM has a narrow depth of field and creates thin optical sections. However, due to numerous apertures, there is increased scattering of light with less than 1% light reaching the cornea. Hence, a very strong light source with a low light level camera is required for imaging. This design has now been discontinued due to new and improved technological advancements in the field.

b. Slit-Scanning Confocal Microscope: The Confoscan (Nidek Technologies) is an example of an SSCM. SSCM, first developed by Svischev in 1969–1971,[85,86] employs multiple vertical slit-like apertures for both illumination and observation of the tissue. This technique was first applied to in vivo corneal imaging by Masters and Thaer in 1994.[82] An advantage of this device is that increased light output and multiple points along the axis of the slit allow a greater area to be scanned simultaneously and in parallel, thus reducing the time taken to scan the tissue. In comparison to the TSCM, since the slit allows more light to reach the tissue, the required intensity of the light source is lower, and the images formed are sharper, brighter and present greater detail to the examiner. In addition, the SSCM yields clearer images of the corneal stroma[34] and allows for imaging of the corneal endothelial cell layer with improved quality, which could not be achieved with TSCM. The drawbacks of this design are that a) it has lower axial and transverse resolution than the TSCM and that the subbasal nerve plexus is not as clearly identified as it is with the LSCM (Figure 4), and b) it has a larger step size of 25 μm per image stack.

c. Laser-Scanning Confocal Microscope: The LSCM draws up on a coherent light source and its laser beam scans the back of the microscope objective using a set of scanning mirrors.[78] The Heidelberg Retinal Tomograph with the Rostock Cornea Module produced by Heidelberg Engineering, Germany (HRTII-III/RCM) is the only commercially available in vivo LSCM for imaging the cornea (Figure 3). The wavelength of the diode laser used in the HRTII-III/RCM is 670 nm and scans the imaging field in a raster pattern. With the combination of a high numerical aperture (0.9) and a 63x objective lens (exchangeable), this system offers a high-magnification imaging platform of up to 800-fold. Furthermore, what makes this system unique is its provision for sequence scans, which allows continuous, dynamic scanning at a user-specified depth. However, it is a contact-based applanation technique that may introduce some artefacts associated with flattening of the corneal surface if it is not operated properly.

This machine allows serial focal plane advancement and generates high contrast, very sharp and high quality images in comparison to SSCM (Figure 4). The subbasal nerve plexus can be viewed and imaged with excellent resolution by the LSCM, unaffected by corneal fluorescein staining.[87] A unique feature of the LSCM is its provision of sequence scans, which are dynamic scans at a user-determined depth of imaging. The utility of a 670 nm laser by LSCM also allows visualization of corneal immune and inflammatory cells in vivo.[88,89] The sequence scan mode allows the same region to be imaged continuously thereby allowing for a sensitive, accurate and rapid method of corneal nerve imaging in vivo.

3. Image Acquisition—When a patient is imaged, all the possible adaptations in the setting of the microscopes should be accounted for, in order to improve compliance, position, and comfort and decrease movement. For instance, focusing targets, dimmed lights in the room, a drop of topical anesthesia in each eye, and a drop of hydroxypropyl methylcellulose in each eye will ensure increased patient comfort. In the case of the laser IVCM, a drop of hydroxypropyl methylcellulose placed on the outside tip of the Tomo-Cap to improve optical coupling will further enhance image quality, decrease applanation of the cornea, and reduce compression artifacts.

For imaging the subbasal nerve plexus with the laser IVCM, a total of 6 to 8 volume and/or sequence scans are recommended from the center of each cornea in order to ensure availability of high-quality images. Three of these scans are recommended to be sequence scans with particular focus immediately beneath the basal epithelium, where a fine nerve plexus can be detected, typically at a depth of 50 to 80 μm.

4. Image Selection and Analysis—Image selection should be performed by an experienced observer. Considering that the typical field of view of a laser IVCM image is about 0.4 x 0.4 mm^2, or 0.16 mm^2, a single image represents only 0.15% of the total corneal area. Since local nerve fiber density can vary considerably across the cornea,[4,90] sufficiently reliable quantitative analysis of this parameter based on a single conventional image is unlikely.[91] Vargenas et al have shown that a minimum number of random central corneal images (not overlapping by more than 20%) is required to achieve an acceptable level of accuracy in the averaged measurement of corneal nerve fiber length and branch density.[92] Further, a recent study showed that the corneal subbasal nerve density is comparable

between a set of three representative standard IVCM images and wide-field mapped composite IVCM images.[93] Images should be selected from the layer immediately at or posterior to the basal epithelial layer and anterior to the Bowman's layer. The selected images should be the best focused and most complete images, with the whole image in the same layer, without motion, without folds, and with good contrast. Images should be de-identified and randomized prior to analysis to avoid observer bias.

As revised by Patel et al,[94] several software applications are available for quantifying IVCM images, each with specific advantages and disadvantages. There are also multiple ways of defining morphological parameters, and there is currently limited consensus regarding "gold standard" definitions of parameters such as subbasal nerve length or density, main nerve trunks or nerve branches, making comparisons between different studies difficult. Standardization of IVCM image analysis through centralized reading centers will be crucial in the future.[94] Commonly used software in IVCM studies include: Image J (National Institutes of Health, Bethesda, MD), a free public domain open source software; Adobe Photoshop (Adobe Systems Inc, San Jose, CA); AnalySIS (Soft Imaging System GmBH, Münster, Germany); and AMIRA (Visage Imaging GmbH, Berlin, Germany).[94]

Our group currently performs the nerve analysis by manually tracing all visible nerves using the semi-automated tracing program NeuronJ,[95] a plug-in for ImageJ (http://www.imagescience.org/meijering/software/neuronj/). Nerve density is assessed by measuring the total length of the nerve fibers in micrometers per mm^2. Main nerve trunks are defined as the total number of main nerve trunks in one image after analyzing the images anterior and posterior to the analyzed image to confirm that these do not branch from other nerves. Nerve branches are defined as the total number of nerve branches in one image. The number of total nerves measured is defined as the number of all nerves, including main nerve trunks and branches in one image.

Research groups have developed automated algorithms for analyzing the subbasal nerve plexus by IVCM,[96–100] but, to date, there is no commercially available software for automated analysis of IVCM images. Scarpa et al[101] first devised a method for automatically tracing corneal nerves of confocal microscopy images, showing that automatic nerve length estimations were very well correlated to manual quantification. Kallinikos et al[102] were the first to describe an objective, semi-automated technique for quantifying subbasal nerve tortuosity. Scarpa et al[103] then modified their algorithm for the automatic recognition of corneal nerve structures and grading of corneal nerve tortuosity.

B. Functional Assessment: Corneal Sensitivity by Esthesiometry

1. Principles of Corneal Esthesiometry—Measurement of corneal sensation is a method for assessing corneal nerve function.[104] The abundant sensory nerve supply to the cornea makes it 300–600 times more sensitive than skin.[105] Efforts to measure corneal sensation date back to 1894, when von Frey used horse hair to test corneal sensation.[106]

Corneal esthesiometry uses mechanical stimuli to stimulate mechano-nociceptors by applying pressures of varying forces to the corneal surface. The mechanically induced stimulation allows corneal sensitivity to be quantitatively assessed. However, corneal

sensation is not limited to that induced by mechanical stimuli; specialized corneal nerves can be stimulated by chemical, cold, and thermal stimuli.[49,107]

Corneal sensation is mediated by Aδ and C type nerve fibers that originate from the trigeminal ganglion. Based on the type of nociceptors expressed on the nerve terminals (mechanical, thermal, polymodal), these nerve fibers depolarize and fire electrical impulses in response to mechanical, thermal, and chemical stimuli applied to the corneal surface.[48,108] Currently, the most popular and extensively used esthesiometers are the Cochet-Bonnet contact esthesiometer, which tests corneal sensitivity to mechanical stimuli, and the Belmonte esthesiometer and its modified version the CRCERT-Belmonte esthesiometer, which can provide mechanical, chemical, and thermal stimuli.

2. Types of Esthesiometers

a. Cochet-Bonnet (Contact Esthesiometer): In 1960, Cochet and Bonnet developed a commercialized device for measuring corneal sensation,[109] based on Boberg-Ans' prototype introduced in 1955,[110] which used various lengths of a nylon filament of to exert increasing amounts of pressure on the corneal surface.[110,111] The nylon filament, typically of 0.12 mm diameter, is gently applied with an applicator onto the corneal surface at the desired position and then withdrawn. The initial stimulus is delivered with the longest length of the filament at 6 cm, which applies the lowest amount of pressure onto the corneal surface. With incremental shortening of the filament, the force imparted increases. The unit of measurement is in centimeters and is directly proportional to corneal sensation; the longer the length of the filament that elicits sensation, the more sensitive the cornea. The patient is asked to respond if the stimulus is felt. The mechanical stimulus is applied successively until a positive response is generated with stepwise shortening of the filament by 5 mm to 10 mm with each application. Afterwards, the filament length is increased by 5 mm until no response is elicited. It is speculated that given the mechanical nature of the stimulus delivered, the Aδ fibers are stimulated and largely responsible for corneal sensation. These fibers lie posterior to the corneal surface within the wing cell layer,[27] which may explain the device's suboptimal performance in accurately measuring corneal sensitivity at low-intensity stimuli.[112] Our group established that corneal sensation, an index of corneal nerve function, and corneal subbasal nerve density have an initial nonlinear relationship in eyes with higher nerve density, but a linear relationship in patients with lower nerve density,[70,113,114] which may also contribute to this phenomenon.

The Cochet-Bonnet esthesiometer is now widely used in ophthalmology for both clinical and research purposes.[115–118] One of the prime reasons for the popularity of this device is its portability and ease of use. However, the Cochet-Bonnet esthesiometer is less than ideal for its intended purpose due to its design, limited stimulus intensity range, user-dependency, variation in stimulus delivered, restrictive stimulation of only mechanoreceptors, and lack of reproducibly measuring corneal sensation at low thresholds of stimuli.[112,119]

b. Belmonte Esthesiometer: Because of the shortcomings of the Cochet-Bonnet esthesiometer, efforts were made to develop noncontact corneal esthesiometers. To address the issue of limited lower-intensity stimuli testing, the noncontact corneal esthesiometer

(**NCCA**) offered a viable alternative.[120] The Belmonte esthesiometer and its modified version, the CRCERT-Belmonte esthesiometer, are among the newer devices that are built upon the principle of the noncontact pneumatic esthesiometry; a jet of air stimulates corneal nerves by compressing the surface.[121] The threshold to stimulus is measured in flowrate (ml/min). The change in force exerted by the CRCERT-Belmonte esthesiometer is consistent with increasing stimulus, unlike the Cochet-Bonnet esthesiometer; the force exerted by the jet of air is quadratically related to the flow rate.[119] The design and mechanism of the CRCERT-Belmonte esthesiometer ensure repeatability of stimulus and thus more reliable measurements of corneal sensitivity.[119, 122] However, it is recognized that the force delivered by the jet of air decreases laterally from the central core of the air jet stream, which would impart unequal pressure on the corneal area being tested.[119] Furthermore, as recognized by Belmonte et al, when results from this esthesiometer are compared to others, it should be noted that stimulation of a greater corneal area reduces the mechanical corneal threshold because a greater number of receptive fields are recruited and larger number of neurons are activated.[119,122] The CRCERT-Belmonte esthesiometer also measures the chemical response in corneal polymodal receptors that respond to changes in pH by gas jets containing carbon dioxide (CO_2).[122] The application of CO_2 to the corneal surface forms carbonic acid, which leads to a pH drop, generating a chemical gradient.

In quantitative terms, it is difficult to compare the Cochet-Bonnet esthesiometer to the Belmonte esthesiometer, since the units of measurement of each instrument cannot be equated adequately. Caution must be exercised when interpreting and comparing results from each of the esthesiometers discussed above, given the fundamental differences in design of instrument, delivery method, type of stimulus, and the types of corneal neuro-receptors activated.[112,119–122]

V. Corneal Nerves in Normal Subjects with and without Contact Lens Wear

A. Normal Human Cornea

In the past, the study of normal human corneal nerves has been restricted, as it depended on ex vivo analysis after death due to deterioration and degeneration of corneal nerves starting immediately after death and occuring within 13.5 hours.[3,26] However, with IVCM, the corneal nerves can now be studied in vivo.[90]

Determination of normal values for the corneal subbasal nerve density and morphology of the living human cornea is important to establish a baseline for use in early detection and follow-up of corneal and systemic disorders that affect corneal nerves. Some limitations of IVCM should be noted. Variations in the method of quantification can make it difficult to compare the results of different studies.[71] First, the definition of nerve density has been inconsistent. Most studies have defined subbasal nerve density as the total length of nerves visible within a defined area (mm/mm^2 or $\mu m/mm^2$), but some have included only nerve branches longer than 50 μm in their measurements, and the nerve definition criteria may vary.[123] Second, subbasal nerve densities vary depending on the type of in vivo confocal microscope used. Studies using LSCM have reported densities of 19.1 ± 4.5 mm/mm^2,[124] and as high as 25.9 ± 6.7 mm/mm^2,[73,125] whereas studies using TSCM and SSCM have reported densities of 5.87 to 15.18 mm/mm^2,[71,126] and many of these studies do not state the

image selection criteria. Our group reported subbasal corneal nerve density in a normative database of 20.1 mm/mm^2 (18.8–21.4) in the central cornea, which is significantly higher in comparison to peripheral areas (10.5 mm/mm^2 [8.8–12.2]), and we showed that all peripheral areas demonstrated similar distribution of the subbasal nerve plexus.[127] Measurements of subbasal nerve diameter range from 0.52 to 4.68 mm, and those for subbasal nerve beading frequency vary from 90 to 198 beads/mm in healthy subjects.[71] Quantitative analysis of stromal nerves remains controversial and difficult, given the changing orientation, with stromal nerve density ranging from 0.31 to 3.61 mm/mm^2 and diameters ranging from 5.5 mm to 11.4 mm in the normal cornea.[71]

The method of quantification and the area scanned must also to be taken into consideration.[90,124] Manual analysis of subbasal corneal nerve parameters is time-consuming and is subject to human bias and variations. One reason for the variations of the assessed parameters might be the relatively small size of the subbasal nerve plexus area evaluated.[91] However, a recent study has shown that a certain minimum number of random central corneal images (not overlapping by more than 20%) is required to achieve an acceptable level of accuracy in the averaged measurement of corneal nerve fiber length and branch density.[92] Further, although IVCM has been criticized for having poor topographic reproducibility, our group has demonstrated that there are no significant differences in the mean subbasal nerve density between the average values of three representative standard IVCM images and wide-field mapped composite images.[93]

Current methods are subjective, particularly in the quantification of nerve tortuosity, beading, and nerve branches. For instance, some research groups quantify nerve branches by the branching points, while other groups quantify according to the branch length. Development of automated methods of analysis and quantification that do not require any user intervention,[101] and more sophisticated techniques for subbasal nerve image acquisition and visualization,[90, 128–131] are currently under way. Scarpa et al developed a fully automated algorithm for analyzing subbasal nerve length.[101]

A rigorous study by Parissi et al attempted to overcome all of the problems discussed above. It analyzed the subbasal nerve density in a large cohort of healthy subjects with laser IVCM, using a standardized method, comparing the manual and the automated algorithm.[124] For image analysis, representative images must be selected by experienced specialists in the field in order to achieve a consistent and more objective analysis. For this purpose, reading centers are required to analyze large sets of images for multicenter studies.[94] IVCM is limited by poor topographic reproducibility and the difficulty of ensuring the exact same locations tested. Finally, lack of automated analysis and interpretation of IVCM images prohibits its wider clinical utility.

Interestingly, some groups have studied the dynamics of the subbasal corneal nerve plexus, showing that the nerves migrate in a centripetal fashion, converging on a whorl (vortex pattern) in the lower nasal quadrant of the paracentral cornea (Figure 2).[29,34,90] Several studies have quantitatively analyzed the subbasal nerve plexus by IVCM and its relationship with corneal sensitivity in normal human corneas.[28,55,90,126,132,133] Studies performed in normal subjects demonstrated that corneal sensation decreases with age (Figure

5).[127,132–135] However, the data regarding the correlation of aging to reduction in subbasal nerve density are variable, most likely due to differences in imaging techniques. Laser IVCM has shown decreased subbasal nerve fiber density with age, while slit- and tandem-scanning IVCM suggest that subbasal nerve density is maintained in an age-independent manner. [28,71,123,124,136–138] Further, a study performed by Niederer et al showed a corresponding linear decline in subbasal nerve density of 0.9% per year, while Parissi et al showed a decline of 0.25% to 0.30% per year.[124,138]

Corneal nerve tortuosity has been of interest to clinicans and scientists for many years. A recent study that analyzed corneas of healthy subjects in vivo identified tortuous stromal nerves in 38% of the sampled cohort. This finding was found to be independent of aging,[137] Patel et al found increased nerve tortuosity with age.[71] Tortuosity is becoming extensively used as a feature to describe subbasal nerves in healthy and pathological corneas.[139] Lagali et al performed an interesting study, focusing on the perception of experts and definitions of tortuosity, and concluded that reproducibility in tortuosity analysis can be subject to sampling bias regardless of the definition used and that further efforts are required to develop standardized quantification strategies.[139]

Our group has recently demonstrated a novel system for estimating and interpreting automated tortuosity.[140] In this method, a tortuosity plane on a two-dimensional continuous scale, onto which each image is mapped, is used for interpretation. This automated system stratifies images by four tortuosity levels (discrete scale) matching or exceeding the accuracy of experienced observers. Moreover, it allows assessment of micro- and macro-tortuosity on a continuous and more sensitive scale.[140]

It is important to point out that the study of healthy normal subjects requires a detailed slit-lamp and ocular surface examination of the normal subjects to rule out any abnormality, such as asymptomatic dry eye ,that could be missed and may influence the subbasal nerve density or induce morphological changes.

B. Contact Lens Wear

A few studies have investigated the effect of contact lens wear on the subbasal nerve plexus.[108,141–144] With tandem-scanning IVCM, Patel et al showed in a mixed group of CL wearers that there was no change in the subbasal corneal nerve density, although corneal sensitivity was decreased as compared to control subjects.[142] Similarly, Oliveira-Soto et al, using slit-scanning IVCM, and Dogru et al, using a laser IVCM, showed that contact lens wear does not appear to affect corneal nerve density, distribution, or morphology. Oliveira-Soto et al reported some qualitative differences, such as slight blurring of nerves and less contrast with the background.[143, 145] Mocan et al demonstrated by slit-scanning IVCM that patients with keratoconus with or without contact lens wear had no difference in subbasal nerve density.[146]

However, some more recent studies suggest concomitant decrease in both corneal sensitivity and corneal subbasal nerve density with use of both silicone hydrogel[147] and orthokeratology contact lenses.[145,148, 149] Liu et al demonstrated by slit-scanning IVCM that the subbasal nerve plexus density of contact lens wearers with and without dry eye was

significantly reduced.[147] Lum et al showed by laser IVCM in orthokeratology lens wearers a decrease in central subbasal nerve density.[148] Patel et al demonstrated by laser IVCM that keratoconic contact lens wearers had a lower subbasal nerve density than non-contact lens wearing controls.[126]

Corneal sensitivity alterations have been shown to vary between different types of contact lenses, with rigid gas permeable lenses associated with a lower corneal sensitivity than polymethyl methacrylate (**PMMA**) lenses.[141,142] Overall, long-term contact lens wear, including PMMA, rigid gas permeable, orthokeratology, and conventional hydrogel lenses, has been associated with a considerable reduction in corneal sensitivity, while silicone hydrogel and disposable hydrogel lens materials have not shown changes in corneal sensitivity in short- and long-term wear.[108,117,141,147,150–152] Cessation of contact lens wear is related to a recovery of corneal sensitivity.[144] Short-term contact lens wear does not induce morphological changes in the corneal subbasal plexus, although it has been shown to induce tear film instability, which in turn may have long-term implications on the ocular surface health.[145]

The subcommittee of neurobiology of the TFOS International Workshop on contact lens discomfort suggested that the mechanisms of corneal sensitivity change as a result of contact lens wear were perhaps related to neurobiological mechanisms.[108] The contact lens-induced stimuli to the ocular surface are complex and multifactorial, including components of osmolarity, solution effects, desiccation, thermal effects, inflammation, friction, and mechanical stimulation.[108] Studies also suggest that it could be due to altered levels of oxygen available, altering corneal metabolism, due to a mechanical etiology or a sensory adaptation of peripheral neuroreceptors.[72,105,108,117,141]

VI. Correlation of Corneal Nerve Alterations to Corneal Sensation in Corneal Diseases

A. Keratoconus

The pathophysiology of keratoconus has not been completely elucidated, although it appears that both environmental and genetically predisposing factors are associated with this disease.[153] In the last decade, IVCM has been increasingly utilized to evaluate the corneal changes in keratoconus. Several studies have shown a decreased subbasal nerve density in corneas with keratoconus and demonstrated more tortuous nerves in keratoconic corneas as compared to controls, with abnormal architecture in the region of the cone (Figure 6).[126,146,154–156] The diminishment of nerve density has been significantly correlated with loss of corneal sensation in keratoconic patients, which is more pronounced in patients wearing contact lenses.[126,156] Even in patients with asymmetrical keratoconus, a decrease in corneal sensitivity has been found, both in clinical and subclinical keratoconus, compared to normal eyes. A positive correlation between nerve density and the severity of the disease has been reported,[155] as well as a significant correlation between decreased central corneal sensation and severity of keratoconus.[157,158]

Corneal subbasal nerve alterations may be involved in the pathogenesis and progression of the disease.[126,159] Several studies have reported decreased epithelial cell density, with a concurrent increase in the epithelial cell area, which may be led by the corneal nerve changes.[126,146,155,160] Brookes et al have shown that the destructive process in keratoconus involves the nerves, or their associated Schwann cells, which express proteolytic enzymes (cathepsin B and G) more extensively in keratoconus compared to normal corneas.[159] Al-Aqaba et al confirmed the previous IVCM findings by studying corneal nerves in advanced keratoconus by immunohistochemistry. They showed that subbasal nerves presented loss of radial orientation and increased tortuosity at the cone apex.[161] The histological evidence of the involvement of corneal nerves in the pathology of keratoconus suggests that corneal nerves may play a role in the pathophysiological features and progression of the disease. Hence, the noninvasive assessment of keratoconic patients by IVCM could be useful in evaluating the corneas of these patients for forme fruste keratoconus, disease severity, progression, and possibly the development of neurotrophic ulcers in the cone.

Corneal collagen cross-linking (**CXL**) is the first treatment available to mechanically strengthen the cornea and thus slow the progression of keratoconus, combining the use of riboflavin and ultraviolet light type A (**UVA**).[162] The originally described corneal CXL method involves the removal of the epithelium ("epi-off") prior to UVA crosslinking irradiation treatment to facilitate riboflavin penetration into the stroma.[162,163] Investigations have revealed significant alterations in the architecture and histology of the anterior 300 μm of the cornea. IVCM studies have revealed disappearance of subbasal and anterior stromal nerves immediately after CXL, due to destruction secondary to the mechanical scraping of the epithelium and CXL treatment.[164–166] On the other hand, while the transepithelial ("epi-on") CXL approach produces less nerve damage, the overall effect of crosslinking is reduced, i.e., there is less collagen reorganization and limited cytotoxic keratocyte loss.[164] Regeneration of nerves has been observed within 6 months to 1 year after CXL, while corneal sensitivity seems to recover faster, returning to normal levels between 3 months to a year.[162,164–171] In a recent study comparing the recovery of corneal sensitivity following epi-off and epi-on approaches, both caused hypoesthesia, but corneal sensitivity was significantly reduced for up to 3 months after epi-off CXL and gradually returned to normal levels, while the recovery time was shorter (1 month) for eyes treated using epi-on CXL.[172]

B. Dry Eye Disease

The International Dry Eye WorkShop[173] defined dry eye disease (**DED**) as a disorder of the lacrimal functional unit (**LFU**), an integrated system comprising the lacrimal glands, ocular surface (cornea, conjunctiva, and meibomian glands) and lids, and the sensory and motor nerves that connect them. The LFU controls the key components of the tear film in a regulated fashion with the aim of preserving the integrity of the ocular surface. A vital portion of the LFU is the role played by sensory impulses arising from the ocular surface in the maintenance of resting tear flow. Disease or damage to any component of the LFU (the afferent sensory nerves, the efferent autonomic and motor nerves, and the tear-secreting glands) can destabilize the tear film and lead to ocular surface disease that expresses itself as DED.[173] There are several published IVCM studies on corneal nerves of dry eye patients,

which try to elucidate the alterations in corneal innervation and the clinical significance (Table 1).[174, 175]

In dry eye patients some studies have demonstrated that hyposecretion of tears may lead to a decline in corneal sensitivity, [176–178] while other studies have shown increased corneal sensation.[179, 180] Similarly, studies present conflicting results regarding the effect of DED on subbasal nerve density by IVCM. Most studies have observed a significantly reduced subbasal nerve density in dry eye patients (both Sjögren syndrome and non-Sjögren syndrome) compared to controls, correlating to corneal sensation in these patients.[178, 180–185] However, a few studies noted no difference in subbasal nerve density but revealed abnormal nerve morphology (presence of nerve sprouts, abnormal tortuosity, increased bead-like formation, and thinning of nerve fiber bundles).[177,186] In contrast, Zhang et al showed an increased nerve number and nerve density in patients with DED. In comparison to normal subjects, they observed abnormal morphologic changes in the subbasal nerves of dry eye patients with Sjögren syndrome, suggesting an underlying attempt of corneal nerves to regenerate, presumably subsequent to the nerve degeneration in dry eye patients.[187]

Injured nerves are known to develop hypersensitivity (hyperalgesia) or become the source of spontaneous discharge (allodynia), explaining the hyperalgesia of some patients with DED and the discrepancies in various IVCM studies noted earlier. Regenerative activity is manifested by sprouting from endbulbs and the formation of microneuromas,[188] seen as abrupt swelling of injured nerve endings and neurite sprouting.[180,186] Aggarwal et al reported that the treatment of patients with corneal neuropathy with autologous serum eyedrops showed restoration of nerve topography through nerve regeneration, correlating with improvement in symptoms of photoallodynia. This supports the notion that corneal nerve damage results in alterations in afferent trigeminal pathways to result in photoallodynia.[189] Given the significant overlap of corneal neuropathic disease withDED, additional IVCM studies in more homogenous populations are required.

The variability of results in regard to the correlation of corneal sensitivity and subbasal nerve density may be attributed to different stages and severity of DED, or to the level of inflammation in patients enrolled in these studies. However, the studies agree that subbasal corneal nerve tortuosity is significantly increased.[178,181,185,187,190] Increased number of beadlike formations have been noted in patients with DED, and are interpreted as metabolically active transmitter-containing nerve fibers, which attempt to improve the abnormal epithelial trophism.[186,191] Alternatively, the beadlike formations are thought to represent nerve damage due to inflammatory processes.[181] Previous studies have demonstrated that immune changes and inflammation play an important role in the pathogenesis of DED.[192] A recent study by our group has demonstrated differential alterations in both dendritic cell density and morphology in subtypes of DED.[193] These changes, which reflect the degree of immune activation and inflammation in DED, may be involved in the subbasal nerve damage and changes observed. For instance, Villani et al showed that patients with DED, including patients with primary Sjögren syndrome, non-Sjögren syndrome dry eye, and meibomian gland disease, have decreased subbasal nerve fibers and higher beading.[185] They also observed increased tortuosity in both primary

Sjögren syndrome and meibomian gland disease, as well as increased density of dendritiform cells.[185] Interestingly, in a separate study, Villani et al demonstrated that both the clinical symptoms (Ocular Surface Disease Index score) and the dendritiform cell density significantly decreased after steroid treatment and correlated to the baseline dendritiform cell density, particularly in patients that responded to treatment.[194]

Clinical correlation of the nerve damage to slit-lamp biomicroscopy findings has been shown in several studies. Benitez et al demonstrated that the number of subbasal nerves and the level of corneal sensation correlated with Schirmer test results.[178] Further, Zhang et al have shown that corneal rose Bengal staining is inversely related to beading of nerves.[187] More recently, Labbe et al showed a significant correlation between the severity of dry eye and the subbasal nerve density and corneal sensitivity.[184] For a summary of these findings, see Table 1. Interestingly, a recent study by our group showed that the response of patients with DED to the treatment may be dependent on the individual patient's subbasal nerve density. Those with near-normal subbasal nerve density showed a better response to DED therapy.[195]

In summary, the results of IVCM studies in patients with DED strongly suggest a role of corneal nerve function, density, and morphology in the pathogenesis of this disease. The discrepancy between signs and symptoms, as well as the increase in patient symptoms in the face of corneal sensation loss, could be explained by injury of corneal nerve endings due to inflammatory processes, followed by altered excitability in regenerated nerves, as well as due to neuropathic symptoms in these patients.[189,196,197] On the other hand, hyposecretion of tears in dry eye may lead to pathologic alterations in corneal nerves and a decline in corneal sensitivity, which subsequently perpetuate the dry eye state.

C. Neurotrophic Keratopathy and Infectious Keratitis

Several studies have demonstrated the role of corneal nerves in patients with neurotrophic keratopathy, including patients with herpes simplex keratitis (**HSK**)[132,198, 199] and herpes zoster ophthalmicus (**HZO**; Figure 7).[133,200] Patel et al reported a case showing the scarcity of the subbasal corneal nerves in the affected eye of a patient with HZO.[200] Martone et al[199] described subbasal nerve changes in patients with bilateral HSK, and Rosenberg et al[198] compared pathologic corneal changes in HSK eyes and the contralateral eyes of 16 patients and found no significant difference in the subbasal nerve plexus between the two eyes. Further, Hamrah et al demonstrated a significant decrease in total number and density of subbasal nerve fibers, in both HZO and HSK eyes, strongly correlating with the decrease in corneal sensation. Interestingly, the contralateral unaffected eyes also presented with a loss of subbasal nerve plexus as compared with normal subjects.[132,133] Moreover, profound HZO- and HSK-induced changes were observed in the superficial epithelium, which showed increase in cell size, decrease in cell density, and squamous metaplasia in both HSK and HZO, strongly correlating with decreased corneal sensation and nerve density.[133, 201] Interestingly, in these studies the abnormal corneal sensation is only noted when the nerve density is approximately ≈1000 μm/frame or lower (by Confoscan), explaining why the sensation in some eyes is perceived as normal by patients, despite significant decrease in nerve density and number.[132,133]

Similarly, a profound diminishment of the subbasal corneal nerve plexus was observed in patients with fungal and *Acanthamoeba* keratitis.[202] More recent prospective studies demonstrated that the decrease in subbasal corneal nerve density is associated with increased density and morphological changes of central epithelial dendritic cells in patients with infectious keratitis, including bacterial, fungal and *Acanthamoeba* keratitis, suggesting a potential direct interaction between the immune and nervous system in the cornea (Figure 7).[88,203] Kobayashi et al studied patients with cytomegalovirus corneal endotheliitis by IVCM and also found reduced subbasal nerves.[204] Recently, our group showed that patients with infectious keratitis who sustain profound loss of corneal nerves during the acute phase of infection experienced corneal nerve regeneration of subbasal nerves during the first 6 months after the resolution of infection. Regeneration rate between the acute phase and the cessation of the antimicrobial treatment was of 0.61 mm/mm^2 per month, whereas between the cessation of treatment and the recovery phase, the regeneration rate almost tripled, reaching 1.60 mm/mm^2 per month.[205]

Recent reports have shown increase in corneal sensation in a variety of patients with neurotrophic keratopathy.[206–208] Using IVCM, Rao et al showed corneal nerve regeneration and increase in corneal sensitivity in neurotrophic keratopathy of different ethiologies following autologous plasma therapy.[208] NGF eye drops are also of great interest and potential efficacy. Bonini et al, in an uncontrolled study, found that treatment with murine NGF healed the neurotrophic keratopathy in 45 corneas of 47 patients, with return of corneal sensation and/or healing of all ulcers.[206]

D. Corneal Dystrophies

Current methods for diagnosis of corneal dystrophies involve slit-lamp characteristics, genetic analysis, and invasive biopsy. However, IVCM analysis of corneal dystrophies is helpful in evaluating the morphological characteristics of corneal dystrophies, degenerations, or developmental abnormalities of the cornea at the histological level, where there is limited availability of corneal tissue for examination and when the final diagnosis is difficult to obtain with conventional methods.[209] IVCM shows nerve abnormalities in several corneal dystrophies and may be useful for diagnosis and determination of progression, as well as for understanding the pathophysiology of disease (Table 2).

1. Epithelial and Subepithelial Corneal Dystrophy—IVCM of corneas in patients with recurrent erosions or epithelial basement membrane dystrophy has shown decreased subbasal nerve density, with short or abnormally shaped nerve fiber bundles (Figure 8).[210] In Meesmann corneal dystrophy, a case series study demonstrated fragmented appearance of the subbasal nerve plexus beside the finding of hyporeflective areas in the basal epithelial layer, corresponding to the multiple epithelial cystic lesions seen by slit-lamp bio-microscopy.[211]

Gelatinous drop-like dystrophy (**GDLD**) is a rare autosomal recessive disease characterized by the deposition of amyloid material in the subepithelial space of the cornea. Jing et al investigated two brothers with GDLD by IVCM, showing an overall mild disorganization of the epithelial architecture and reduced subbasal nerves.[212]

A study performed in a family with Dystrophia Helsinglandica, an autosomal dominant corneal disease characterized by recurrent corneal erosion episodes and progressive subepithelial fibrosis, showed an alteration in the subbasal nerve morphology and decreased corneal sensitivity.[213]

2. Stromal Dystrophies—In Schnyder crystalline corneal dystrophy, Vesaluoma et al reported that the normal corneal architecture becomes disturbed by large extracellular crystalline deposits and accumulation of highly reflective extracellular matrix, resulting in central opacity and disruption of the subbasal nerve plexus. Furthermore, they showed that neural regeneration after keratectomy appears delayed in these cases.[214] Similarly, Ciancaglini et al confirmed that the corneal nerves in these patients present with an irregular and tortuous appearance.[215] IVCM images of granular corneal dystrophy show reflective breadcrumb deposits between epithelial and Bowman's layer, as well as in the anterior stroma.[216–218] Traversi et al reported thin subbasal nerve fibers in between these deposits.[217] Similarly, lattice corneal dystrophy has been described as having decreased long nerve fiber bundles in the subbasal nerve plexus, which should not be mistaken for reflective linear branching filaments in the stroma, characteristic of this dystrophy, in these patients.[219, 220]

In patients with pre-Descemet's dystrophy, Lanza et al described prominent subbasal nerves,[221] while studies performed in Fleck corneal dystrophy by IVCM demonstrated that decreased corneal sensitivity found in some of these patients is associated with reduced subbasal nerve density and branches.[222]

3. Endothelial Dystrophies—Several studies in eyes with Fuchs' endothelial corneal dystrophy (**FECD**) have shown decreased subbasal nerve plexus and morphological alterations (Figure 9). In a series of 11 patients, 8 of 17 eyes showed absence of subbasal nerve plexus as well as pathological changes in all the other corneal layers by slit-scanning IVCM.[223] Ahuja et al, in a study including 69 eyes with FECD, demonstrated decreased corneal sensitivity that is likely to be related to loss of subbasal nerves and abnormal nerve morphology, which persist after endothelial keratoplasty.[113,114,224,225]

Alomar et al performed a study correlating the histological and IVCM changes in chronic corneal edema and FECD patients. They described subepithelial fibroblasts and reduced subbasal corneal nerves both in the edema and FECD patients.[226] Likewise, Al-Aqaba et al studied patients with bullous keratopathy by IVCM and histology, confirming that the density, branching pattern, and diameter of subbasal nerves were significantly lower compared with normal corneas. These alterations were unrelated to any specific etiology of bullous keratopathy.[227]

Schrems-Hoesl et al demonstrated that in patients with early stage FECD, subbasal corneal nerves are diminished, which suggests alterations in corneal innervation and a potential role of corneal nerves in the pathophysiology of this disease.[114] Similarly, Bucher at al analyzed the corneal nerve alterations in different stages of FECD, and found that increasing severity of FECD is concurrent with marked attenuation of the density, as well as mild diminishment of the function, of the subbasal corneal nerve plexus in late stage of the disease.[225] Aggarwal et al showed that different stages of FECD and pseudophakic bullous keratopathy

had profound diminishment of the subbasal nerve plexus and is correlated to decreased sensation.[113] These studies suggest that corneal nerves may be involved somehow in the pathogenesis of FECD, but additional studies are necessary to elucidate whether nerve alterations are caused by nonspecific corneal edema or decreased endothelial cell density, or whether the nerves are potentially leading to loss of endothelial cells.

Although Salzmann nodular degeneration of the cornea is not considered a dystrophy, Roszkowska et al observed that these patients also have decreased subbasal nerve plexus, in addition to more typical changes observed in the epithelium, basement membrane, and Bowman's layer.[228] Likewise, analysis by IVCM of cases with Terrien's marginal corneal degeneration have found a decrease in the subbasal nerve density and branching.[229]

E. Other Ocular Diseases

IVCM provides detailed images of the corneal layers in diverse ophthalmic pathologies, promoting a better understanding of the underlying pathophysiology, aiding in the diagnosis of disease, assessing therapeutic response, and demonstrating unexpected alterations of corneal nerves.

Chronic, severe allergic conjunctivitis, such as vernal keratoconjunctivitis (**VKC**) and atopic keratoconjunctivitis (**AKC**), can affect both children and adults. Both AKC and VKC are associated with profound changes in the corneal subbasal plexus and stromal nerves.[230,231] Both conditions are associated with decreased subbasal nerve density and increased stromal nerve tortuosity, whereas eyes with VKC also have tortuous subbasal nerves and thicker stromal nerves.[230,231]

Glaucoma is a chronic condition associated with keratopathy. Patients with glaucoma have reduced subbasal nerve density, with tortuous nerves independent of the effects of treatment, while reduction in corneal nerve reflectivity remains debatable.[232,233] Further, the chronic use of antiglaucoma medications with preservatives causes significant changes in the ocular surface.[185] Several studies have shown decreased subbasal nerves and increase in nerve tortuosity and beading by IVCM in patients treated for glaucoma or ocular hypertension.[183,233,234] Some of these studies also demonstrated a correlation between nerve tortuosity and corneal sensation in glaucoma patients.[183,233] Rossi et al confirmed the lessened side effects in corneal nerves of preservative-free antiglaucoma drops after 1 year of treatment, showing that previously treated patients had an improvement in number of corneal nerves and tortuosity and that naïve patients did not show significant changes with the preservative-free medication.[235] Villani et al[236] showed that in stable primary open-angle glaucoma patients without a history of DED, there are subclinical ocular surface changes due to antiglaucoma medications. Interestingly, they observed increased subbasal nerve length and tortuosity, as well as dendritic cell density, compared to controls. Active ingredients, preservatives, number of concomitant drugs, and number of eye drops instilled per day are all elements that can induce ocular surface changes.[236]

Of significant interest and potential impact has been the recent work on the utility of IVCM in the diagnosis of corneal and conjunctival intraepithelial neoplasia (**CIN**). Alomar and colleagues demonstrated a high correlation of cellular findings between standard invasive

histological techniques and noninvasive corneal IVCM in the diagnosis of CIN.[237] In addition, they observed that the corneal subbasal nerves were absent in regions of the corneal epithelium affected by CIN, which they interpreted as a limitation of the imaging technique, wherein the high reflectivity of the CIN cells is close to the high reflectivity of the nerves, rendering them difficult to detect. As the lesions resolve and CIN cells are replaced by normal cells, the subbasal nerves "reappear," probably related to the increased contrast.[237]

Vera et al reported corneal epithelial abnormalities and absence of the subbasal nerve plexus in patients with chronic Stevens-Johnson syndrome, toxic epidermal necrolysis, and limbal stem cell deficiency (**LSCD**).[238] IVCM can be useful in monitoring early-to-late-stage degenerative changes in LSCD. Lagali et al also showed that progression of LSCD in aniridia correlates with corneal nerve deficit.[239] Among Swedish families, Eden et al observed that in approximately 19% of cases with early congenital aniridic keratopathy there was increased subbasal nerve density.[240] A combination of morphological changes in the corneal epithelium and a significant reduction in both basal epithelial cell density and subbasal nerve density might be the early signs of LSCD.[241]

Wang et al showed morphologic alterations of the subbasal nerve plexus in patients with pterygium.[242] Furthermore, in diseases affecting the anterior chamber, alterations in the corneal nerves have been found. In patients with Cogan syndrome, a reduction of the subbasal corneal nerve plexus has been shown. These are associated with thin and poorly reflective nerves, as well as with interruptions and lack of the typical branching patterns.[243] Furthermore, a study in patients with pseudoexfoliation syndrome showed a decrease in the subbasal nerve plexus density that was significantly correlated to reduction in corneal sensitivity.[244]

F. Corneal Pain

Corneal discomfort and pain has recently generated great interest because of their high incidence in post-refractive surgery patients[245–247] and patiennts who suffer from DED.[248–250] In addition to its adverse effect on quality of life and physical function, central sensitization of pain leads to altered physiology of organ systems.[251] A spectrum of corneal stimuli that damage corneal nerves, such as DED, contact lens use, infections, epithelial erosions, and corneal surgery, may lead to centralized pain by providing continuous peripheral nociceptive stimuli.[252, 253] Belmonte et al have done extensive research in the neural basis of corneal sensation, demonstrating that when the cornea is stimulated, the various functional types of sensory nerve fibers evoke conscious sensations of different quality, including ocular dryness, discomfort, and pain.[254] Nerve damage leads to an altered expression of membrane Na^+ channels at the injured and regenerating nerve fiber terminals of microneuromas, giving rise to aberrant spontaneous and stimulus-evoked nerve impulse firing, which forms the basis of ectopic firing.[48,254] Microneuromas can form because of mechanical trauma to corneal nerves, e.g., refractive surgery or in systemic disease as diabetes and HSV,[188] making nociceptor-mediated corneal pain a prevalent condition that requires attention.

In addition to peripheral sensitization of pain, chronic stimulation of central pain pathways can have detrimental effects on the management and treatment of corneal pain. Once the pain is centralized, treatment becomes complicated, as methods to reverse centralization of pain are currently not optimal.[255, 256] IVCM allows detection of changes in the subbasal nerve plexus that can be monitored for disease severity and response to treatment (Figure 10).[189] Monitoring corneal subbasal nerves with IVCM has shown that these nerves regenerate with treatment such as in autologous serum-treated corneas of patients with dry eye and photoallodynia.[189] Hence, IVCM has further utility in monitoring the corneal neurogenerative response to treatment.

An emerging and highly promising role of IVCM involves the clinically challenging differentiation between ocular discomfort and/or light sensitivity associated with DED and that occurring with corneal neuralgia and/or photoallodynia in corneal neuropathy, as these conditions may have similar clinical presentation or even overlap.[188,249,256,257] In patients with ocular pain in whom severity may be incongruent with clinical signs on slit-lamp examination,[258–260] corneal IVCM may contribute to a diagnosis of corneal neuropathy among these patients. Recent studies by our group have revealed quantifiable and significant changes in corneal subbasal nerve metrics[197, 261] and morphology in patients with corneal neuropathy.[189] Compared to healthy, asymptomatic controls, patients with corneal neuropathy demonstrate the presence of neuromas, increased reflectivity, a greater frequency of nerve beading, and typically a more profound loss of subbasal nerves.[189] In the current absence of other clinical tests, the presentation of severe ocular pain or photoallodynia, with minor-to-absent clinical signs on slit-lamp examination, as well as morphological and densitometric nerve changes on IVCM, should alert the physician to consider a diagnosis of corneal neuropathy or corneal neuralgia. These conditions have a treatment trajectory remarkably different than DED.

In addition to diagnosing corneal neuropathic pain using IVCM, our group successfully used IVCM-guided treatment of corneal neuropathic pain with autologous serum tears,[196] demonstrating improved subbasal nerve metrics,[189,196] morphology, and pain scores.[196] Furthermore, we were able to quantify the impact of reducing pain scores on facilitating and restoring quality of life in patients with corneal pain.[262]

VII. Corneal Nerve Alterations after Corneal Surgery

The application of IVCM for the correlation of nerve morphology and function goes beyond corneal infections or pathology; IVCM allows us to visualize, quantitate and monitor progressive changes in nerve and cellular immune responses before and after corneal surgical procedures, whether the underlying indication is therapeutic or refractive.

The corneal subbasal nerve plexus is a dynamic structure.[29,34] These nerves travel centripetally, towards the inferocentral whorl complex, with nerve branch point migration velocities as high as 26 μm/week near the periphery of the cornea in normal subjects, causing significant changes in corneal nerve architecture that can be observed over a period as short as 6 weeks.[29] It is of interest that a centripetal pattern of corneal epithelial cell migration has been observed both in normal adult mice[263–265] and in certain human corneal

pathological states, such as corneal verticillata and toxic keratopathies.[35, 266] This allows IVCM to be useful in clinical practice to monitor post-operative nerve damage and recovery.

Corneal re-innervation after a procedure may be affected by several factors, including the time elapsed after surgery, the patient's age, the preoperative diagnosis, other local or systemic comorbidities, the level of inflammation, and the surgical procedure.[267]

B. Therapeutic Corneal Surgery

1. Penetrating Keratoplasty—Corneal transplantation is the most common and successful form of transplantation in humans. The past decade has seen a shift from full-thickness penetrating keratoplasty (**PKP**) to tailored anterior and posterior lamellar graft procedures that replace only the diseased portion of the cornea, leaving any healthy cornea untouched [268,269] and the advent of cutting-edge research into corneal tissue engineering and artificial substitutes.[270] However, PKP still accounts for >90% of grafts in the UK and Australia, and 47% in the US.[271]

Indications for corneal transplantation differ among countries. Keratoconus is the most common indication in several developed countries, such as the USA and Australia, and infectious diseases and corneal scarring are the most common indications in developing countries.[272,273] Bullous keratopathy, or corneal edema, is a leading indication for PKP and corneal transplantation in general.[271] With advancement in the field of corneal surgery, IVCM provides a sensitive in vivo tool to provide cellular monitoring of the graft for best visual and surgical outcomes.[274,275]

PKP involves transection of the subbasal plexus and stromal nerves, in both the donor and recipient corneas, leading to abnormalities of the subbasal nerve plexus that include decreased density, increased beading, sprouting, tortuosity and, subsequently, compromised ocular surface health (Figure 11).[276] The nerves regenerate over time and resume subbasal nerve morphology, especially in the graft periphery. There are conflicting opinions on corneal sensation, which may return within 1 year or remain abnormal, indicating that recovery of morphology is not an indicator of recovery in function.[277–279] Thus, it is hypothesized that collateral organization of the subbasal plexus is a critical determinant of corneal sensation in lieu of subbasal nerve anatomy.

Cross-sectional studies have shown reduced subbasal nerve density, decreased nerve branching, and increased nerve tortuosity up to 40 years after surgery.[125,275,278,280] In a longitudinal study, Ruben and Colebrook followed 48 patients post-PKP for a period of up to 10 years.[281] Their findings are consistent with later work in the field, where they established that even after 3 years post-PKP, recovery of corneal sensitivity was incomplete. Later, Richter et al studied 46 grafts, which were followed for a period of 3 years.[267] Although their patients had a gain in subbasal nerve fibers, their findings echoed those of Ruben and Colebrook, in that none of the patients achieved normal corneal sensitivity. However, recovery in nerve function was better in the periphery, as compared to the center of the graft. Stromal nerves appeared 7 months after the procedure, whereas the subbasal plexus of the central cornea resumed morphology at 24 months postoperatively. At the 24-month follow-up, one-third of the grafts had normal corneal sensitivity at the graft center,

and 25% of the graft periphery remained anesthetic.[267] Using slit-scanning IVCM, Darwish et al found that corneal sensation in their patients improved over the 12-month period to near normal levels, although no central subbasal nerves were detected at 12 months. This suggests that slit-scanning IVCM may not be able to detect some of the finer regenerating subbasal nerve fibers.[279] Alternatively, given the limitation of the slit-scanning IVCM to visualize peripheral nerves, these patients may have had peripheral nerves that could not be detected. Slit-scanning IVCM has inferior image contrast and quality at a mid-stromal level to that of laser-scanning IVCM, leading to lower detection of the subbasal plexus. Thus, the lack of subbasal nerve detection may reflect mechanical inability of the instrument rather than an accurate anatomical state. Interestingly, in an animal (pig) study comparing innervation of corneal constructs and allografts to controls, Lagali et al observed that significantly fewer nerves were detected in the operated animals than in controls at 12 months. However, by 6 months, both constructs and allograft-implanted corneas responded to touch.[282]

In the case of lamellar surgery, such as deep anterior lamellar keratectomy (**DALK**), there is a complete trephination of the subbasal nerve plexus. A comparative study by Ceccuzi et al showed that the recovery of corneal sensitivity in the graft following DALK is similar to PKP, with good corneal sensitivity achieved by 2 years after surgery.[283] In contrast to PK and DALK, in endothelial keratoplasty (**EK**), corneal nerves are not affected, except those on the site of the incision, and hypoesthesia does not occur.[136,224,280,284] However, Bucher et al showed that Descemet's membrane endothelial keratoplasty (**DMEK**) diminishes the density and the function of subbasal corneal nerves early after transplantation, but a complete recovery up to preoperative values occurs within 4–10 months.[285]

2. Phototherapeutic Keratectomy—Phototherapeutic keratectomy (**PTK**) is indicated in patients with conditions such as recurrent corneal erosions, map-dot-fingerprint dystrophy, and some stromal dystrophies, where only partial ablation of the cornea is required, limited to the corneal epithelium and anterior stroma. Germundsson et al showed that subbasal nerve density in epithelial basement membrane dystrophy is reduced by 45% and recovers only to the reduced level in the long term after PTK treatment.[286] Lagali et al showed by IVCM that with complete removal of the Bowman's layer (15 μm ablation), there is a significant reduction in subbasal nerve density at 4 months post-operatively, with recovery at 8-months. However, with the integrity of the Bowman's layer partially maintained (7μm ablation), corneal nerve density was not significantly altered compared to pre-operative levels , thus increasing chances of corneal transparency and quick wound healing post-PTK,. [287]

B. Corneal Refractive Surgery

Corneal nerve regeneration and recovery of corneal sensitivity after refractive surgery are pivotal considerations, given their importance in wound healing and the possible development of severe dry eye, among other complications of neurotrophic corneas. The damage to the subbasal nerve plexus is assumed to depend partly on where and at which depth the nerves are severed. In photorefractive keratectomy (**PRK**), the corneal epithelium is removed and discarded and the outermost layer below the epithelium is treated with a

laser, with the epithelium regenerating after surgery. In laser-assisted sub-epithelial keratectomy (**LASEK**), the epithelium is not removed; an alcoholic solution is used to dislodge the epithelium as a sheet, exposing the anterior stroma for laser treatment, after which it is placed back into position. These procedures are distinct from laser-assisted in-situ keratomileusis (**LASIK**), where a permanent flap of 80 to 180 micrometers in thickness is created in deeper layers of the cornea, and repositioned after laser is applied to the stroma.

Various studies have examined corneal sensitivity and subbasal nerve plexus reductions in patients after the different refractive corneal procedures. The extent of corneal hypoesthesia after corneal refractive surgery depends on the ablation depth.[288–290] Consequently, not only the refractive procedure but also other factors, e.g., different preoperative refractive errors, can affect the recovery of central corneal sensation and the subbasal corneal nerve plexus.

As discussed below, various factors should be considered in selecting the refractive procedure, particularly under circumstances where the surgeon can expect an already reduced subbasal nerve plexus, i.e., contact lens users with dry eye disease, who are likely to develop severe DED after the refractive procedure.

1. Photorefractive Keratectomy—The recovery of corneal sensitivity in the central cornea after PRK has been reported to start at 4–6 weeks after surgery and appears to be completed to the pre-operative value 1–12 months after surgery.[291–293] Studies have shown that subbasal corneal nerves regenerate between 1–8 months after PRK, with faint subbasal nerves found in the central corneal with abnormal branching.[90,292,294] Erie et al found that at 12 months after PRK the subbasal nerve density was decreased 60%, with return to normal nerve density at 24 and 36 months after PRK (Figure 12).[295,296] After 5 years, 71% of post-PRK corneas showed a branching pattern of regenerated subbasal nerves closely resembling that observed in normal control corneas, even though some surgical corneas still showed a reduced nerve fiber bundle density without reaching the complete neural recovery.[295–298]

Haze formation is a common side effect of PRK. In a 5-year prospective study, Gambato and colleagues used IVCM to evaluate the safety of adjuvant mitomycin C in retarding the development of haze post-PRK.[299] They established that the corneal epithelium and subbasal nerve plexus remain largely unaffected even 5 years after mitomycin C-assisted PRK, thereby indicating its corneal safety.[299]

2. Laser-Assisted In Situ Keratomileusis—Evidence of corneal nerve plexus damage has been noted a few hours after LASIK, with degeneration of nerve structures, characterized by thinning or even complete absence of subbasal nerve fibers in the flap area.[277,300] One week after LASIK, the nerve plexus is not detectable, and 1 month after the procedure, very thin nerve fibers are visualized.[277] However, the numbers of subbasal and stromal nerve fiber bundles are decreased by 90% compared to the preoperative values.[277,301] The central subbasal corneal nerves start to regenerate about 2 weeks after LASIK,[302] although Slowik et al did not observe subbasal nerves in the central cornea during the first four months after LASIK.[303] After LASIK, re-innervation occurs with corneal nerves being detected in the central cornea by 6 months (Figure 13).[304,305] However, the nerve density after LASIK remains less than half of the pre-operative values even at 12

months.[277,301,302] Decreased subbasal nerve density remains even at 2 and 3 years after LASIK.[304] Stachs et also showed that at 2 years after LASIK, the whorl-shaped configuration of the subbasal nerve plexus was not visualized and that nerves were abnormally curved, thin, and non-branching.[277] Quantification of subbasal nerve density demonstrated a reduction by 51%, 35%, and 34% at 1, 2, and 3 years, respectively.[295] At 5 years following LASIK surgery, nerve regeneration appeared to be completed.[295]

A significant decrease in corneal sensation has been verified 1 week postoperatively.[306,307] The recovery of corneal sensation after LASIK to the preoperative value has been estimated to occur in 3–16 months, with an average of approximately 6 months.[136,290,293,300,308–313] Stachs et al showed that corneal sensation plateaus at 12 months postoperatively, as it reaches 90% to 100% of esthesiometry values measured in normal corneas, although at earlier time points corneal sensation is markedly lower.[277]

A strong correlation has been observed between corneal sensation and subbasal nerve morphology and density after LASIK. Corneal sensitivity improves as the subbasal nerves regenerate, although it seems that function recovers faster, approaching normal levels of corneal sensitivity in about 6 months.[290,302,308] Interestingly, corneal wound healing and nerve regeneration showed no difference between flaps created with femtosecond laser compared to mechanical microkeratome.[314–316] However, flaps created using One Use-Plus Sub-Bowman's keratomileusis (OUP-SBK) had faster nerve regeneration compared to those created with a femtosecond laser or an M2 90 microkeratome.[315] Several studies have shown the effect of the hinge position on the recovery of corneal nerves and sensitivity. Nerve density diminishment varies in different regions within the LASIK flap, being spared on the side of the hinge, and influencing nerve regrowth and corneal sensitivity recovery.[307,312] Linna et al showed that corneal nerves from the central and temporal region regrow 1–2 weeks after surgery, while nerves at the nasal region appear sooner, approximately on the third day after LASIK.[302] The recovery of sensitivity appears faster with nasal hinge flaps for up to 3 months after LASIK.[300]

The role of corneal innervation in patients suffering dry eye after LASIK was addressed in a review by Chao et al.[300] They suggested that the alteration of corneal nerves after LASIK is the most likely cause of the subjective symptoms of LASIK-induced dry eye, even though corneal sensitivity and the clinical indicators of dry eye return to apparently normal values within a year due to the partial recovery of the corneal nerve plexus. They hypothesize that dry eye symptoms following LASIK may result from abnormal sensation due to LASIK-induced corneal neuropathy.[300]

3. Laser-Assisted Subepithelial Keratectomy (LASEK)—Few IVCM studies have compared the LASEK procedure with PRK and LASIK in terms of the corneal subbasal nerve plexus and sensitivity. Corneal sensation after LASEK has been reported to be recovered to the pre-operative value between one[317] to 3 months after surgery,[318] although subbasal nerve density was still at half of pre-operative values 6 months following surgery.[318,319] However, Darwish et al found no difference in nerve recovery between LASIK and LASEK.[317,319] In a study comparing LASIK and LASEK, Lee et al found that at 6 months after surgery in the LASIK group, corneal sensitivity was still reduced from

preoperative levels, while in the LASEK group, there was no difference between baseline and 6-month postoperative values, and that there was a greater decrease in the nerve plexus in the LASIK group compared with the LASEK group at this timepoint. The recovery of corneal sensation in both groups correlated strongly to the regeneration of corneal nerves, although the tear film break-up time, Schirmer values, and epithelial thickness did not correlate with corneal nerve regeneration.[305] According to several studies reporting corneal nerve regeneration and the recovery of corneal sensation in LASEK and PRK separately, little difference was found between these procedures.

The comparison of nerve recovery after the different refractive procedures adds further evidence to the effect of ablation depth on postsurgical healing. More rapid recovery of central subbasal nerve density is observed in PRK eyes (2 years to return to preoperative levels) compared with LASIK eyes (approximately 5 years).[300]

VII. Systemic Diseases

A. Autoimmune Diseases

Autoimmune diseases negatively affect corneal nerve parameters, including density, tortuosity, beading, and function.[136] In patients with Grave's orbitopathy, corneal sensitivity correlated inversely with proptosis,[320] decreased subbasal nerve fibers, increased tortuosity, and beading.[320,321] Likewise, in patients with rheumatoid arthritis (**RA**) with and without secondary Sjögren syndrome, IVCM of corneal nerves showed increased beadlike formations that correlated to the systemic disease activity. In these patients, the number of corneal nerves correlated to the corneal sensitivity, Schirmer test, and fluorescein corneal staining.[322] However, in patients on chloroquine therapy for RA, increased branching of subbasal nerves has been shown; the density correlates with chloroquine therapy in a dose-dependent manner.[323] It is unclear if corneal nerve damage occurs as a primary component of these autoimmune diseases, or whether it is secondary to other orbital pathophysiology.[136]

B. Peripheral Neuropathy

IVCM is useful for early detection and assessment of the progression of systemic diseases with peripheral neuropathy, such as diabetes or polyneuropathic conditions. Zhao et al used IVCM to assess various types of polyneuropathies, characterized by clinical neurological and ophthalmic examinations, as well as by electroneuromyography, demonstrating significant alterations and reduction of the corneal subbasal nerve plexus.[324] Lalive et al used IVCM to follow a patient with peripheral neuropathy after treatment, revealing improvement marked by decreased thickness and reduced tortuosity of the stromal nerves, which correlated to the results of clinical and electrophysiologic assessments.[325] Gemignani et al described decreased corneal nerve density with IVCM in a small series of patients with non-length-dependent small fiber neuropathy related to Crohn's disease, impaired glucose tolerance, and Sjögren syndrome.[326] Similarly, Tavakoli et al demonstrated decreased corneal innervation in patients with idiopathic small fiber neuropathy and showed that these patients have significant intra-epidermal nerve fiber loss and an increased prevalence of impaired glucose tolerance.[327] Moreover, in chemotherapy-induced peripheral neuropathies,

it has been reported that IVCM demonstrated significant anomalies in morphology and number of the corneal subbasal nerve plexus.[326,328]

Use of IVCM has also been reported in patients with hereditary sensory and autonomic neuropathy (**HSAN**) type IV and type, both autosomal recessive disorders that are characterized by the loss of pain sensation. HSAN-IV involves a combination of hereditary sensory neuropathy and anhidrosis, and is also called *congenital insensitivity to pain with anhidrosis* (**CIPA**). HSAN-V is a phenotypically similar disorder to HSAN-IV but is characterized by congenital sensory neuropathy that mainly affects pain perception without leading to anhidrosis and is associated with the selective loss of small myelinated fibers.[329] Mimura et al have shown the correlation of clinical nerve dysfunction and corneal nerve reduction by IVCM. Superficial keratopathy accompanied by impairment of corneal sensation and tear film instability correlated with findings by IVCM in these patients, which included large keratinized cells in the superficial corneal epithelium and complete loss of the central subbasal nerve plexus.[329]

The usefulness of IVCM has also been shown in leprosy, a granulomatous infectious disease of the peripheral nerves and mucosa of the upper respiratory tract caused by *Mycobacterium leprae* and *Mycobacterium lepromatosis.*[330] Leprosy has a high incidence of ocular complications, including corneal lesions, lagophthalmos, iridocyclitis, and cataract. IVCM has shown changes in stromal nerve density, irregularities in epithelial nerves, and corneal nerve thickening, tortuosity, and beading, accompanied by hypoesthesia.

C. Diabetes Mellitus

Examination of corneal nerves by IVCM in patients with diabetic neuropathy has illustrated the application of the technique in systemic diseases. Diabetic peripheral neuropathy, or diabetic polyneuropathy (**DPN**), is defined as “the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes.”[332] The neuropathic disorder includes manifestations in the somatic and/or autonomic parts of the peripheral nervous system, and a minimum of two abnormalities from symptoms, signs, nerve conduction abnormalities, quantitative sensory tests, or quantitative autonomic tests is recommended.[333–335]

The diabetic neuropathies are heterogeneous, affecting different parts of the nervous system that result in diverse clinical manifestations. They may be focal or diffuse, but most common among the neuropathies are chronic sensoriomotor distal symmetric polyneuropathy and the autonomic neuropathies. DPN occurs in both type 1 and type 2 diabetes and is more common with increasing age and duration of diabetes. In a large population survey, Harris et al reported that 30% of type 1 diabetic patients and 36% of male and 40% of female type 2 diabetic patients experienced neuropathic symptoms.[336] In the Epidemiology of Diabetes Intervention and Complications (**EDIC**) study,[337] an epidemiologic follow-up of the Diabetes Control and Complications Trial (**DCCT**) in diabetic type 1 patients, showed that neuropathy, as assessed by symptoms, abnormal deep tendon reflexes, autonomic function, or vibration perception (measured with a bioesthesiometer) developed over 7.5 years in 24.6% of the patients, with independent risk factors of age, duration, HbA1c, triglyceride and body mass index.

The focus in relation to the consequences of nerve damage in diabetes has been the loss of sensation in the feet, predisposing to the development of diabetic foot ulcers and lower extremity amputation.[333] However, the cornea is 300–600 times more sensitive than the skin.[105] In diabetic patients, corneal sensitivity is reduced,[338] due to a loss of corneal nerve fibers,[198] which leads to diabetic keratopathy and a susceptibility to injury, with recurrent erosions and ulcers.[335,339] One or more of the following are used to assess sensory function: pinprick, temperature, and vibration perception (using a 128-Hz tuning fork), or pressure sensation (using a10-g monofilament pressure sensation at the distal halluces).[340] Combinations of more than one test have >87% sensitivity in detecting DPN.[333,335] Only biopsy of the sural nerve[341] and skin biopsy[342,343] currently permit a direct examination of nerve fiber damage. The use of punch skin biopsies demonstrating a decrease in intraepidermal nerve fiber density has also been shown to be useful in identifying patients with small fibre neuropathy.[341,342,344]

Increasing literature on the use of IVCM to quantify diabetic neuropathy has demonstrated a reduction in corneal subbasal nerve fiber density and an increase in nerve fiber tortuosity in diabetes, correlated with the stage or severity of peripheral neuropathy.[102,198,345–350] Changes in corneal nerve fibers have been associated with a reduction in corneal sensation in patients with type 1 diabetes.[198]

Recently, a correlation between the loss of corneal nerve fibers and the severity of diabetic retinopathy has also been demonstrated.[346] In addition, IVCM allows detection of early peripheral neuropathy,[350] as decreased nerve density has been shown to precede impairment of corneal sensitivity.[351] Recovery of the corneal subbasal nerve plexus has also been demonstrated with IVCM 6 months following simultaneous pancreas and kidney transplantation in diabetic patients, presumably as a result of improved systemic glycemic control.[351] Significant correlation between corneal and dermal nerve degeneration in diabetic peripheral neuropathy have strengthened the evidence that IVCM is a valuable tool in the diagnosis and assessment of diabetic neuropathy.[344]

In summary, to date, several groups have employed IVCM of the corneal subbasal nerves in diabetic patients to identify patients with minimal neuropathy, quantify the severity of the neuropathy, and follow progression or assess the therapeutic response in diabetic neuropathy.[352] IVCM has shown moderate-to-high specificity for diagnosing diabetic neuropathy,[348] and the utility of using corneal nerves as a biomarker of diabetic neuropathy.[175,348,353]

D. Other Systemic Diseases

Multiple endocrine neoplasia (**MEN**) 2A and B are associated with prominent corneal nerves, which are thickened due to a putative genetic alteration and axon and Schwann cell abundance, respectively.[136] In MEN2B, IVCM demonstrated an increased density of subbasal nerves.[354]

Patients with progressive supranuclear palsy and patients with Parkinson's disease frequently manifest signs of dry eye, yet remain asymptomatic. Reddy et al showed that these patients have lower blink rates and decreased corneal sensitivity compared to controls.

However, they did not find a reduction of the corneal subbasal nerve plexus density by slit-scanning IVCM.[355]

Fabry disease is an inherited metabolic disorder characterized by progressive lysosomal accumulation of lipids in a variety of cell types, including neural cells. Small, unmyelinated nerve fibers are particularly affected in this disease. While small fiber peripheral neuropathy often clinically manifests at a young age, patients with Fabry disease often remain undiagnosed until severe complications involving the kidney, heart, peripheral nerves and/or brain have arisen. Tavakoli et al demonstrated that IVCM and non-contact esthesiometry were useful tools to detect early nerve fiber damage and dysfunction compared to established tests of neuropathy.[356]

In Darier-White disease, known as Darier disease or keratosis follicularis spinulosa decalvans, a rare dominantly inherited skin disorder, corneal IVCM has shown subbasal nerve plexus abnormalities before ocular symptoms or evident corneal abnormalities have developed.[357] Ocular involvement in Darier-White disease includes eyelid and corneal abnormalities, such as punctate epithelial opacities, peripheral intraepithelial opacities, faint lines of central epithelial irregularity, and prominent corneal nerves, whose observation is limited to slit-lamp examination.[358,359] Lagali et al observed by IVCM perpendicular penetration of thick, beaded, subbasal nerve fiber bundles into the epithelium, suggesting that in the absence of an intact basement membrane (providing both a physical and biochemical barrier between epithelium and stroma), thicker subbasal nerve fiber bundles may proceed unimpeded into the more superficial wing cell layers before branching into thinner nerve strands.[357]

VIII. Conclusion

The factors and mechanisms regulating nerve morphology and response to the tissue microenvironment are complex. With subclinical changes that can be appreciated only at a cellular level, IVCM provides a window into live histology, a tool that holds immense potential to better evaluate and understand disease processes for improved therapeutic outcomes. With current functional assessment of corneal nerves being limited to esthesiometry, when taken together with IVCM, a robust and quantitative assessment of the tissue state can be made with direct viewing of changes in response at a cellular level. This translates into in vivo examination and monitoring of corneal nerve morphological parameters such as nerve inflammation, density, branching patterns, and local host immune response. Thus, IVCM allows for direct correlation between clinical findings, patient feedback, and corneal innervation. Changes in corneal innervation are not limited to corneal disease; the corneal subbasal plexus is a window to potential early diagnosis or late complications of neurological, immunological and infectious systemic conditions that would otherwise require invasive testing. Therapeutic medical or surgical interventions also lead to alteration of corneal nerve homeostasis making pre- and post-therapeutic and surgical management using IVCM an attractive adjunct to clinical practice or clinical trials.

Extensive studies have been performed to understand and determine the relationship between corneal innervation and sensation, both in physiologic and disease states. Some of

the widely studied conditions include but are not limited to dry eye syndrome, keratoconus, and diabetes. Efforts to investigate correlation between clinical function of the nerves using corneal esthesiometry with morphology of corneal innervation have yielded conflicting results. The differences in the findings of various groups can be attributed to the variation in the type of in vivo confocal microscopes used. Each generation of these microscopes has differences in axial resolution, image contrast, image quality and depth of field, creating a large variation in their respective abilities to capture and image the corneal subbasal plexus. This requires the development of a standardized method of analysis following validation of the method. In order for data from different centers to be comparable with meaningful interpretations, we propose the use of a consistent method of measuring corneal sensitivity and of measuring the subbasal nerve plexus at experienced reading centers in a masked fashion. It is important that the methodology is quantitative and reproducible.

In summary, IVCM empowers the clinician to make accurate conclusions about corneal nerves and immune response to disease and injury by allowing direct in vivo viewing and quantitation of critical nerve and inflammatory parameters, which can be correlated with nerve function and clinical ocular surface findings. Corneal IVCM introduces objectivity and standardization to clinical research, practice and clinical trials, an essential component in making meaningful comparisons and drawing accurate inferences from the results across research centers.

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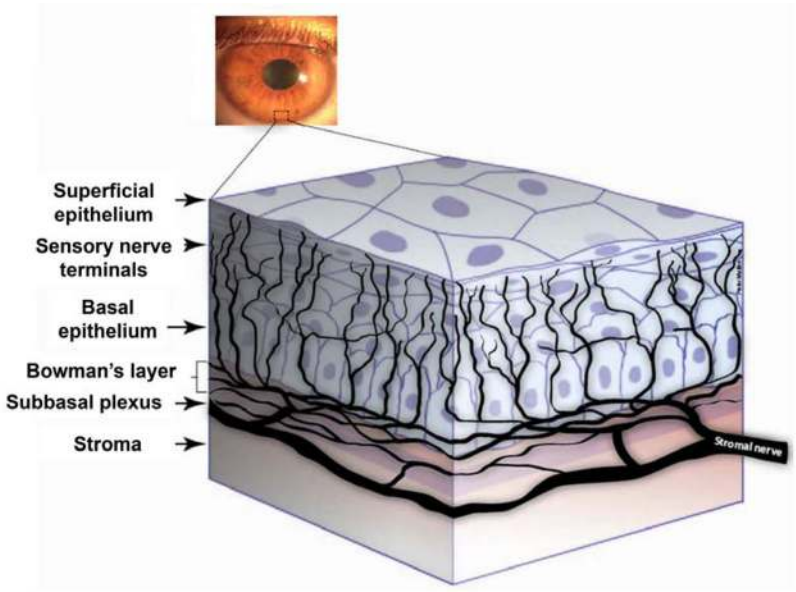

Figure 1.
Diagrammatic representation of human corneal nerves.

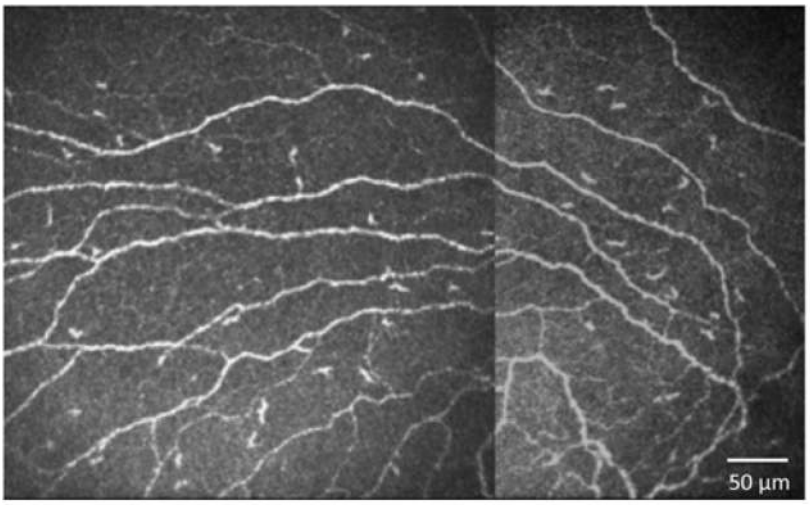

Figure 2.

Corneal apical whorl-like pattern of the human corneal subbasal nerve plexus seen with laser in vivo confocal microscopy. A whorl of subbasal nerves is seen at the corneal apex towards the inferonasal paracentral area.

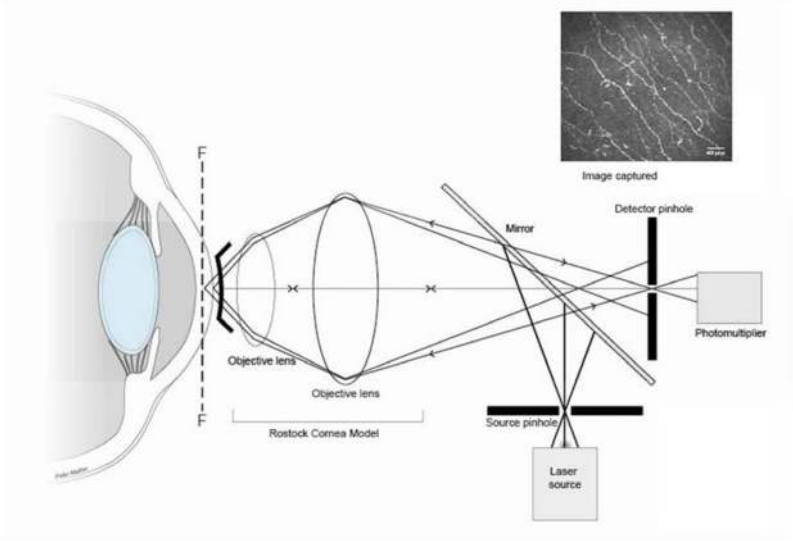

Figure 3.

Schematic diagram of the principle of laser-scanning corneal in vivo confocal microscopy (IVCM). Images acquired by the laser-scanning in vivo confocal microscope (Heidelberg Retinal Tomograph 3/Rostock Cornea Module, Heidelberg Engineering) provide an 800-fold magnification of the corneal tissue and subbasal nerve architecture as represented by the confocal micrograph at the top right-hand corner.

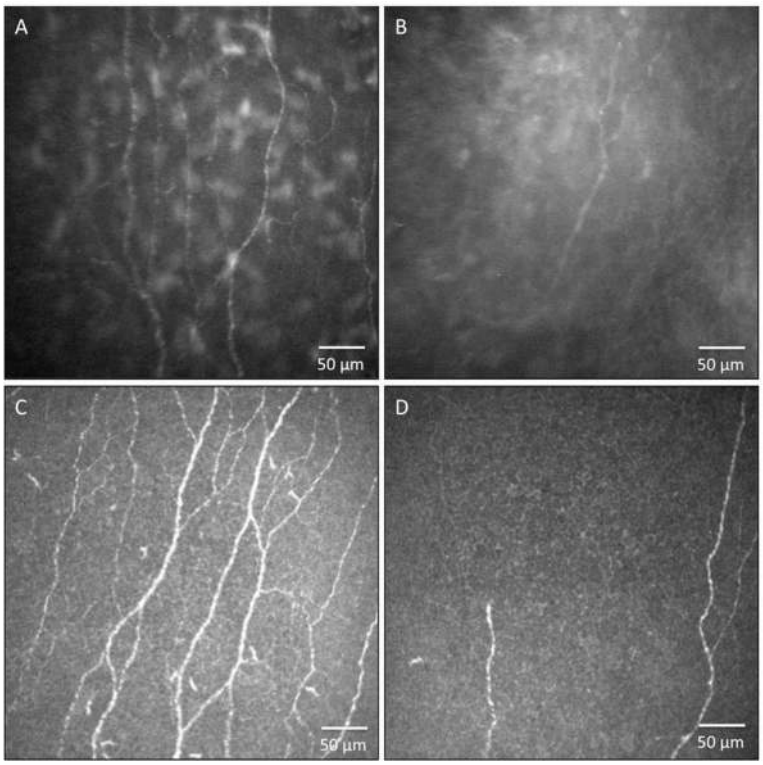

Figure 4.

In vivo confocal microscopy of corneal subbasal nerves and immune cells in health and disease. (A) Normal subbasal nerve plexus (SSCM; Confoscan4, Nidek Technology); (B) Subbasal nerve plexus in herpes simplex keratitis (HSK) (SSCM; Confoscan4, Nidek Technology). Note the decrease in total nerve count, length and branching associated with severe loss of corneal sensation; (C) Normal subbasal nerve plexus and immune cells (LSCM; HRTIII/RCM, Heidelberg Engineering) in same patient as panel A.; (D) Subbasal nerve plexus and immune cells in HSK associated with severe loss of corneal sensation and decrease in total corneal nerve count, length and branching (LSCM; HRTIII/RCM, Heidelberg Engineering) in same patient as panel B.

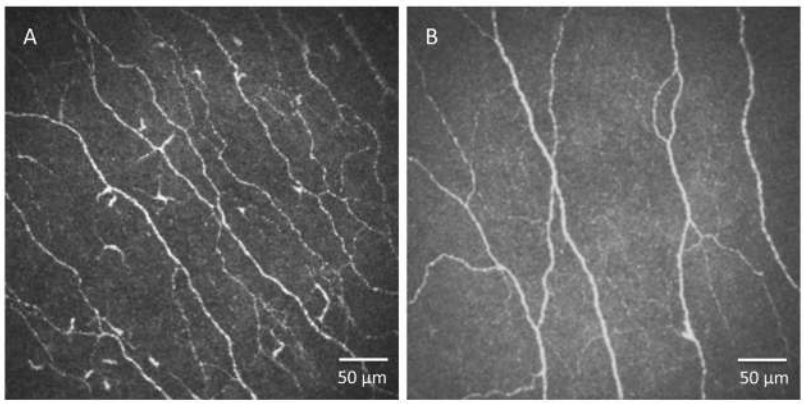

Figure 5.

Normal age-related changes in the corneal subbasal nerve plexus. (A) 25 year old patient, (B) 60 year old patient, by laser scanning in vivo confocal microscopy (HRTIII/RCM).

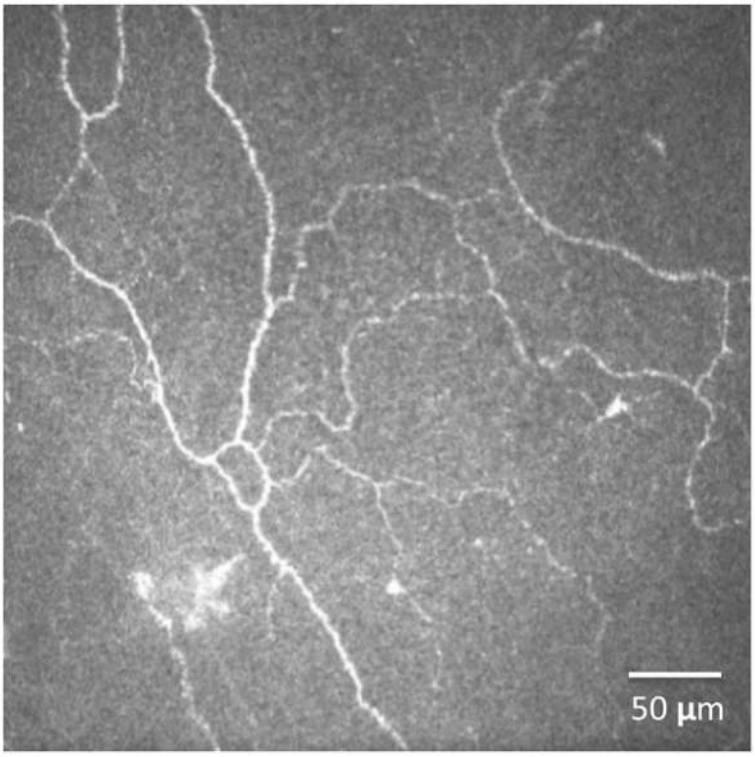

Figure 6.
Keratoconus. Laser scanning in vivo confocal microscopy (HRTIII/RCM) demonstrates a decrease in nerve density and increased tortuosity in keratoconus patients.

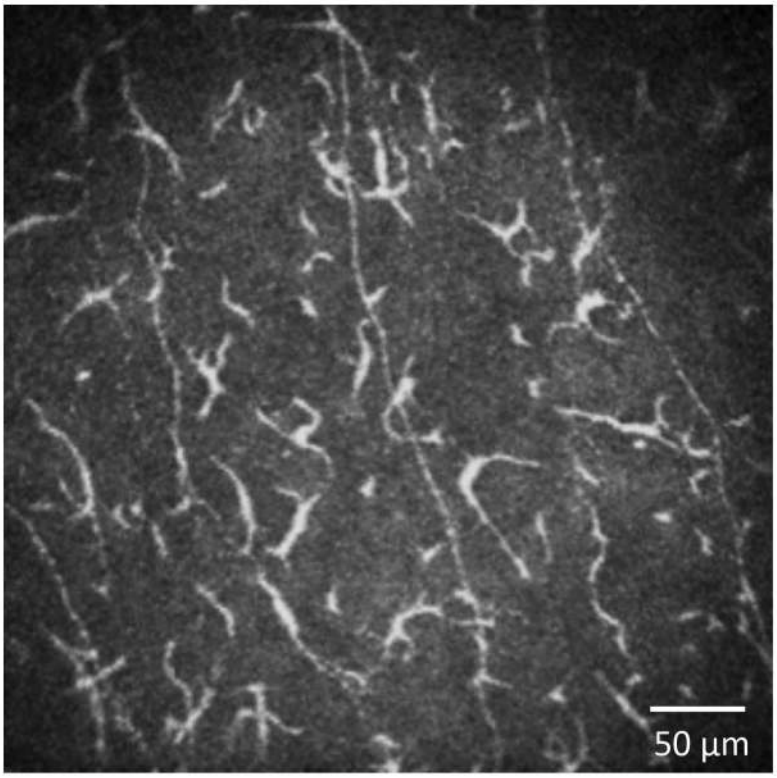

Figure 7.
Infectious keratitis. Laser scanning in vivo confocal microscopy (HRTIII/RCM) demonstrates a decrease in nerve density and increase in dendritic cells.

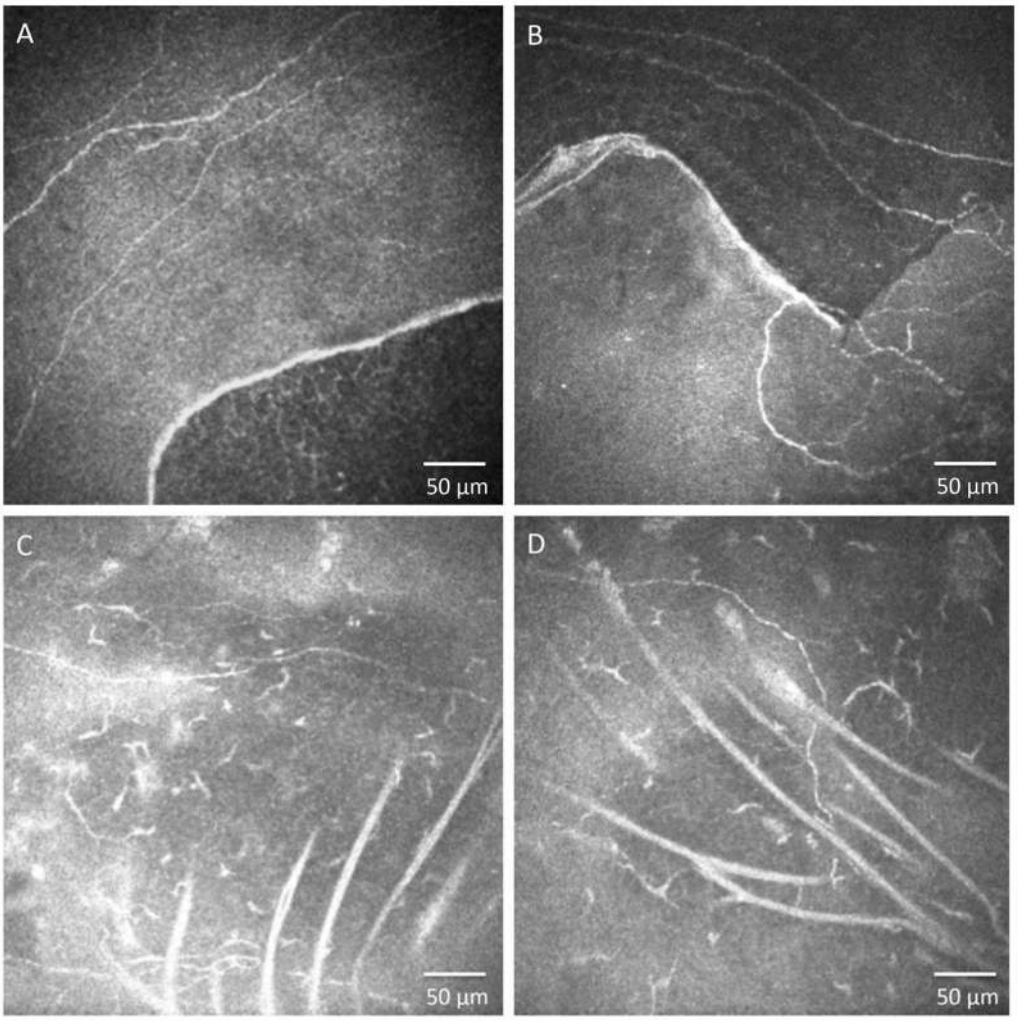

Figure 8.
Map Dot Fingerprint Dystrophy. Subbasal nerve plexus and basal epithelial membrane alterations by laser in vivo confocal microscopy (HRTIII/RCM).

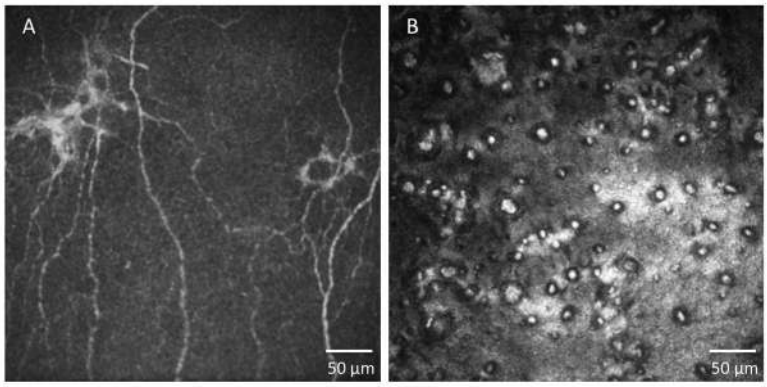

Figure 9.

Fuchs' endothelial corneal dystrophy. (A) Corneal subbasal nerve plexus alterations and (B) guttae observed in the endothelium by laser in vivo confocal microscopy (HRTIII/RCM).

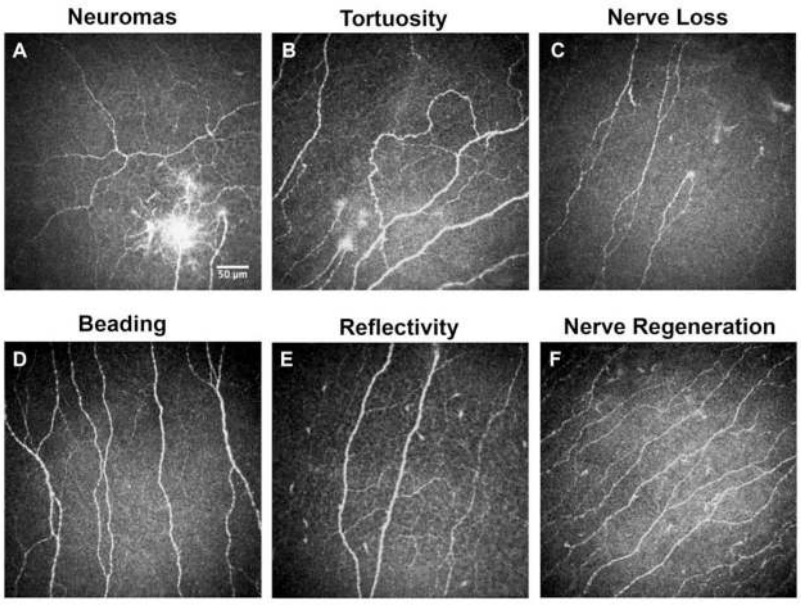

Figure 10.

Morphology of central corneal sub-basal nerves in corneal allodynia using in vivo confocal microscopy (IVCM). Central corneal IVCM of patients with corneal allodynia revealed presence of multiple neuromas (A), increased nerve tortuosity (B), stark decrease in sub-basal nerve density (C), nerve beading (D), and increased reflectivity (E). After treatment of corneal allodynia with 20% autologous serum tears, in addition to self-reported symptomatic improvement, IVCM revealed nerve regeneration, and reduced tortuosity, beading, and reflectivity (F).

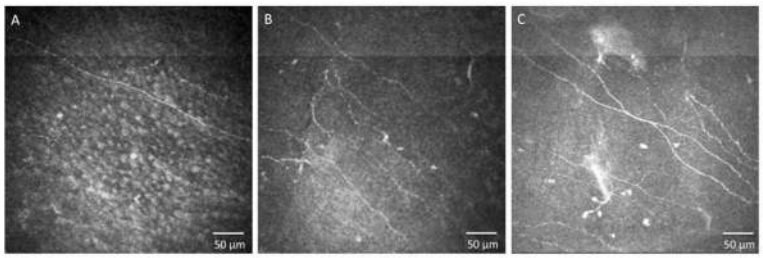

Figure 11.

Corneal graft re-innervation following penetrating keratoplasty. (A) Incomplete re-innervation of the corneal graft center 1 month after transplantation, a thin, sub-basal nerve is observed in the graft center. (B) Incomplete corneal graft re-innervation of the center 12 months after transplantation. (C) Partially re-innervated corneal graft at 18 months. Images taken by laser in vivo confocal microscopy (HRTIII/RCM).

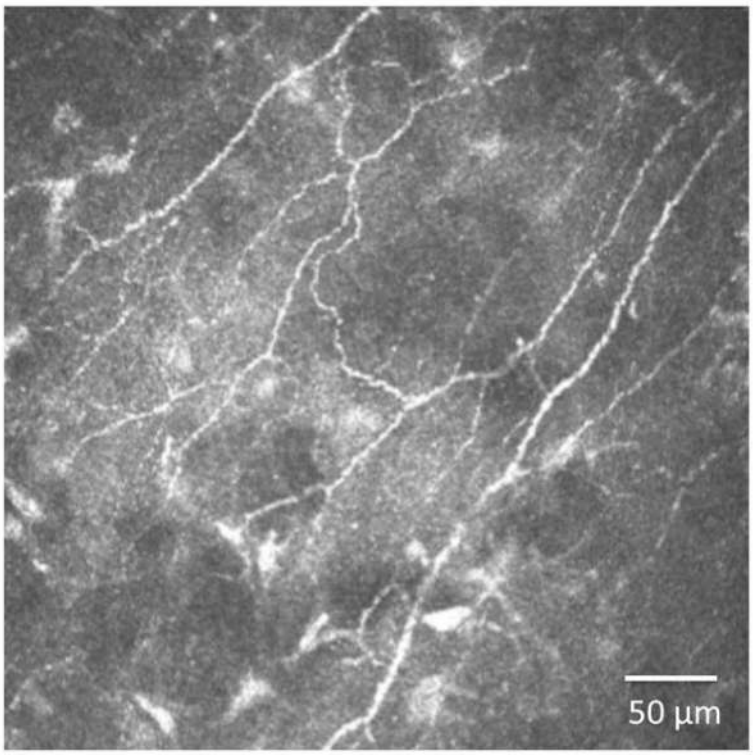

Figure 12.
Subbasal corneal innervation after photorefractive keratectomy (PRK). Subbasal corneal nerve plexus regenerates to normal nerve density between 24–36 months after surgery. Images taken by laser in vivo confocal microscopy (HRTIII/RCM).

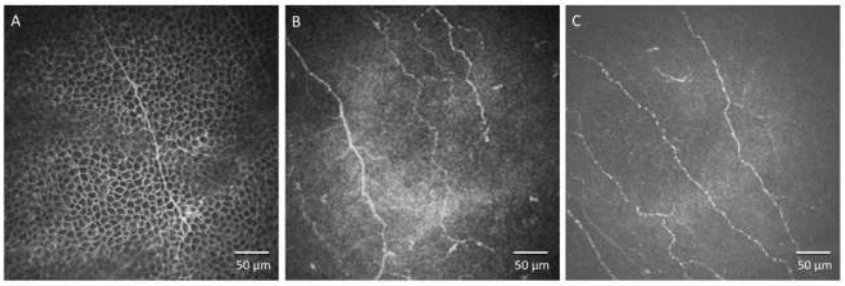

Figure 13.

Sub-basal corneal re-innervation after laser-assisted in situ keratomileusis (LASIK). Regenerating nerve fibers in the central cornea, (A) two months, (B) six months and (C) eight months after LASIK. Images taken by laser in vivo confocal microscopy (HRTIII/RCM).

Table 1

Subbasal corneal nerve plexus in dry eye disease

Study/Type of IVCM	Dry eye disease, patients studied	Subbasal nerve density	Morphology	Corneal Sensitivity	Association with clinical findings
Tuominen et al. (2003) TSCM	PSS (n=10) Normal (n=10)	No difference	Increased tortuosity, nerve sprouting and decreased nerve diameter in PSS	--	--
Benitez del Castillo (2004) SSCM	PSS (n=11) NSS (n=10) Normal (n=21)	Decreased nerve number	Increased beadlike formations	--	Schirmer test associated to number of nerves
Zhang et al. (2005) SSCM	PSS (n=8) NSS (n=30) Normal (n=30)	Increased nerve number	Increased tortuosity, beading and branching	--	Corneal fluorescein and rose Bengal staining associated to nerve density
HoŞal et al. (2005) SSCM	PSS (n=20) NSS (n=12) Normal (n=19)	No difference	Increased nerve diameter in PSS (non significant)	Decreased (Cochet-Bonnet)	--
Villani et al. (2007) SSCM	PSS (n=15) SS (n=20) Normal (n=20)	Decreased nerve number	Increased tortuosity and beadlike formations	--	--
Benitez del Castillo et al. (2007) SSCM	PSS (n=10) NSS (n=11) Normal (n=20)	Decreased nerve density	Increased tortuosity and beading	Decreased (non-contact esthesiometer)	Sensation associated to nerve density
Erdelyi et al. (2007) LSCM	NSS (n=26) Normal (n=10)	Decreased nerve density	Increased tortuosity, bead-like formations	--	Corneal fluorescein staining associated to epithelial thickness
Tuisku et al. (2008) SSCM	PSS (n=20) Normal (n=10)	No difference	Nerve sprouting, thickened stromal nerves and cone-like structures	Increased (modified Belmonte non-contact esthesiometer)	
Zhang et al. (2011) LSCM	MDE (n=20) MSDE (n=20) Normal (n=20)	Not measured	Increased tortuosity	--	--
Labbe et al. (2012) LSCM	SS (n=3) NSS (n=9) Glaucoma (n=14) Normal (n=10)	Decreased nerve density	No difference	Decreased (Cochet-Bonnet)	Sensation associated to nerve density
Villani et al. (2013) LSCM	PSS (n=15) NSS (n=15) MGD (n=15) Normal (n=15)	Decreased nerve fibre	Increased tortuosity and beading	--	--
Labbe et al. (2013) LSCM	NSS (n=43) Normal (n=14)	Decreased nerve density	Increased tortuosity, beading and width	Decreased (Cochet-Bonnet)	Oxford score (dry eye severity) associated to nerve density

IVCM: In vivo confocal microscopy; TSCM: tandem scanning confocal microscopy; SSCM: slit scanning confocal microscopy; LSCM: Laser scanning confocal microscopy; PSS: Primary Sjögren's syndrome; SS: Sjögren's syndrome; NSS: Non-Sjögren's syndrome; MDED: Mild dry eye disease; MSDED: Moderate to severe dry eye disease. DED: Dry eye disease.

Table 2

Subbasal corneal nerve plexus in corneal dystrophies

Epithelial and subepithelial dystrophies	Subbasal nerve plexus findings	Other findings	Study	Number of patients	Type of IVCM
Epithelial basement membrane dystrophy	Abnormal		Rosenberg et al. 2000	N=8	Tandem scanning
Meesmann corneal dystrophy	Fragmented appearance		Patel et al.	N=3	Slit scanning
Gelatinous drop-like dystrophy	Decreased number		Jing et al.	N=2	Laser scanning
Dystrophia Helsinglandica	Alteration in nerve morphology	Decreased sensitivity	Neira et al.	N=9	Laser scanning
Stromal dystrophies	**Subbasal nerve findings**		**Study**	**Number of patients**	**Type of IVCM**
Schnyder's crystalline corneal dystrophy	Irregular and tortuous Disrupted		Ciancaglini et al. Vesaluoma et al.	N=1 N=4	Slit scanning Tandem scanning
Granular corneal dystrophy	Thin nerve fibres		Traversi et al.	N=2	Laser scanning
Lattice corneal dystrophy	Decreased long nerve fibre bundles		Rosenberg et al. 2001	N=20	Tandem scanning
Fleck's dystrophy	Reduced nerves with hyperreflective inclusions	Decreased sensitivity	Frueh et al.	N=3	Slit scanning
Pre-Descemet's corneal dystrophy	Prominent subbasal nerves		Lanza et al.	N=1	Slit scanning
Endothelial dystrophies	**Subbasal nerve findings**		**Study**	**Number of patients**	**Type of IVCM**
Fuchs' endothelial (FECD)	Absent nerve plexus		Mustonen	N=11	Slit scanning
	Decreased, abnormal morphology	Decreased sensitivity	Ahuja et al.	N=69	Slit scanning
	Reduced nerves		Alomar et al.	N=11	Laser scanning
	Reduced nerves in early FECD		Schrems-Hoesl et al.	N=30	Slit scanning
	Decreased nerve plexus	Decreased sensitivity	Aggarwal et al.	N=33	Laser scanning
	Decreased density concurrent to increased severity of FECD	Decreased sensitivity	Bucher et al.	N=30	Laser scanning

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