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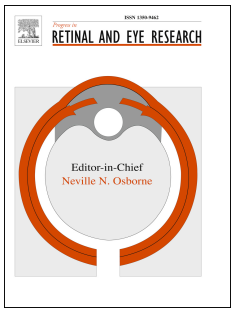

Corneal nerves in health and disease

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Abbreviations:

AChE	Acetylcholinesterase
BK	Bullous keratopathy
CGRP	Calcitonin gene-related peptide
EK	Endothelial keratoplasty
FECD	Fuchs' Corneal Endothelial Dystrophy
HSK	Herpes simplex keratitis
HZO	Herpes zoster ophthalmicus
IVCM	In vivo confocal microscopy
LASIK	Laser in situ keratomileusis
LEC	Limbal epithelial crypts
LNC	Limbal nerve corpuscles
NcP	Nociceptor pain
NGF	Nerve growth factor
NP	Neuropathic pain
NK	Neurotrophic keratopathy
PRK	Photorefractive keratectomy
SEM	Scanning electron microscope
SFSN	Small-fibre sensory neuropathy
SMILE	Small incision lenticule extraction
SP	Substance P
TEM	Transmission electron microscope
TRPM8	Transient receptor potential cation channel subfamily M member 8
TRPV1	Transient receptor potential cation channel subfamily V member 1

Abstract

The cornea is the most sensitive structure in the human body. Corneal nerves adapt to maintain transparency and contribute to corneal health by mediating tear secretion and protective reflexes and provide trophic support to epithelial and stromal cells. The nerves destined for the cornea travel from the trigeminal ganglion in a complex and coordinated manner to terminate between and within corneal epithelial cells with which they are intricately integrated in a relationship of mutual support involving neurotrophins and neuromediators. The nerve terminals/receptors carry sensory impulses generated by mechanical, pain, cold and chemical stimuli. Modern imaging modalities have revealed a range of structural abnormalities such as attrition of nerves in neurotrophic keratopathy and post-penetrating keratoplasty; hyper-regeneration in keratoconus; decrease of sub-basal plexus with increased stromal nerves in bullous keratopathy and changes such as thickening, tortuosity, coiling and looping in a host of conditions including post corneal surgery. Functionally, symptoms of hyperaesthesia, pain, hypoaesthesia and anaesthesia dominate. Morphology and function do not always correlate. Symptoms can dominate in the absence of any visible nerve pathology and vice-versa. Sensory and trophic functions too can be dissociated with pre-ganglionic lesions causing sensory loss despite preservation of the sub-basal nerve plexus and minimal neurotrophic keratopathy. Structural and/or functional nerve anomalies can be induced by corneal pathology and conversely, nerve pathology can drive inflammation and corneal pathology. Improvements in accuracy of assessing sensory function and imaging nerves in vivo will reveal more

information on the cause and effect relationship between corneal nerves and corneal diseases.

Key words

Corneal nerves
Trophic function
Sensory function
Limbal nerve corpuscles
Bullous keratopathy
Keratoplasty
Keratoconus
Dry eye
Corneal crosslinking
Diabetic keratopathy

1. Introduction

To serve its principal function of letting light in to the eye and providing two thirds of the eye's focusing power, the cornea must remain transparent (Meek and Knupp, 2015). Its anatomy and physiology are adapted towards this end. Intrinsic transparency of the cornea is related to the architecture of the stromal collagen, determined largely by the uniform diameter and spacing of the collagen fibres, the transparency of the keratocytes, which are the predominant cell population by far, the relative state of dehydration (78% water content) and the absence of blood vessels. The cornea also maintains a rich nerve supply with a high density of nerve fibres located within the epithelium, in the sub-basal plexus and anterior stroma, which do not interfere with the transmission of light (Al-Aqaba et al., 2010b; He et al., 2010; Marfurt et al., 2010). The major nerve bundles lose their myelin sheath soon after entering the cornea at the limbus and become transparent thereafter.

Being largely invisible on conventional examination techniques their clinical appearance in diseases of the cornea has not been widely remarked on. The advent of in vivo confocal microscopy (IVCM) has enabled en-face examination of corneal nerves in health and disease and bespoke software programmes have allowed quantification of various aspects of nerve morphology such as density, fibre length, width, tortuosity, beading, branching and reflectivity (Alzubaidi et al., 2016; Cruzat et al., 2017; Shaheen et al., 2014). Immunohistological and microscopy techniques of studying whole mount specimen of normal and diseased corneas have also contributed vastly to our knowledge of corneal nerve aberrations (Al-Aqaba et al., 2011a; Al-Aqaba et al.,

2011b; Al-Aqaba et al., 2010b; He et al., 2010; Marfurt et al., 2010). It is not surprising therefore that a whole range of corneal nerve pathology is now known, and descriptions and definitions are attributed to specific disease conditions. Whether the changes are a cause, or an effect of the disease is not always clear.

Corneal nerves serve sensory and trophic functions (Labetoulle et al., 2019). They release a large number of chemical substances (neuro-mediators) that preserve the health and integrity of the corneal cells, both epithelial cells and keratocytes. This 'nutritional function' is referred to as the 'trophic' function of the nerves. Sensory input is responsible for the normal and protective blink reflex and are the afferent components driving tear secretion from the tear glands. Trophic and sensory functions can be independently affected for example in lesions that affect the pathway between the trigeminal ganglion and the sensory cortex, the sensory function is compromised but the trophic function is maintained by the surviving pseudo-unipolar neurons in the ganglion. Distal lesions affect both sensory and trophic functions. When trophic function is compromised, the health of the cells is affected and neurotrophic keratopathy results. The epithelial cells and keratocytes in turn release molecules (growth factors and cytokines) that maintain the health and regeneration of the nerves. Nerve growth factor is an important example. There is thus a mutual cycle of support and dependence of the nerves and cells.

Assessing corneal nerve function by testing corneal sensitivity is an age-old practice with the non-standard use of a 'cotton wisp' as the mainstay. Touching the cornea with a finely drawn out wisp of cotton or soft tissue paper is satisfactory for a 'yes' 'no' response as assessed by the blink reflex or withdrawal

of the head by the patient. By this method, quantitative estimation of corneal sensitivity is very subjective to both the patient and the doctor. The Cochet-Bonnet aesthesiometer is a useful semi-quantitative tool to measure sensitivity (Murphy et al., 1998). The filament is subject to environmental factors such as temperature and humidity and it only assesses touch/pain sensations (Lum and Murphy, 2018). The Belmonte aesthesiometer is more reliable and can assess touch, cold and chemical stimuli but currently is largely viewed as a research tool (Golebiowski et al., 2011).

The cornea also has a significant autonomic, predominantly sympathetic, innervation. The limbal and perilimbal innervation is essentially vasomotor, causing the ciliary blush (circumcorneal injection) on corneal irritation (Labetoulle et al., 2019). This axonal reflex is part of the corneal defence system allowing extravasation of immune cells from the limbal vessels, which then infiltrate the cornea to combat the infectious/injurious agent. As the cornea itself is avascular, the role of sympathetic nerves within the cornea is not clear but alteration of sympathetic innervation can affect cell proliferation and alter the behaviour and course of infections and diseases (Labetoulle et al., 2019).

As our ability to study structure and function of corneal nerves in health and disease improves, understanding the pathophysiology of specific diseases will be enhanced and specific targeted therapies will develop. Undoubtedly it will be established that the causes and consequences of corneal nerve damage are integral to most if not all corneal pathology and addressing these will be important in managing corneal diseases and restoring corneal health.

2. Neuroanatomy of the cornea

2.1 Embryology and development of corneal innervation

The development of the human cornea starts with the formation of primitive epithelium and lens. The process involves invagination of surface ectoderm above the optic vesicles to form the crystalline lens. This primitive lens induces the formation of the overlying epithelium into the corneal epithelium at approximately one month of gestation. A week later, a bi-layered epithelium appears (O'Rahilly, 1983). The basal layer produces collagen fibrils and glycosaminoglycans that accumulate as the primary corneal stroma between the epithelium and the lens vesicle (Dodson and Hay, 1971; Hay and Revel, 1969). A wave of neural crest cells migrates between the lens vesicle and primary stroma to form the embryonic endothelium. A subsequent migration of neural crest cells, at around the seventh week of gestation, populate the primary stroma to form the primitive keratocytes that construct the definitive stroma (Cai et al., 1994; Hayashi et al., 1988). Descemet membrane is secreted by the endothelial cells and can be detected in utero as early 12 weeks of gestation (Eghrari et al., 2015).

Kitano has described the embryology of human corneal nerves and the following course of events has been observed (Kitano, 1957). In the one-month embryo the cornea has not taken the shape and no nervous tissue was found in the future orbit. At the two-month stage, the primary cornea was fashioned and at this point the ciliary nerves reached the edge of optic cup. Fine nerve branches began to appear in the slowly differentiating corneal stroma during the third month. A large number of nerves approach the anterior stroma just under Bowman’s layer by the

fourth month. These penetrated Bowman's layer and entered the epithelium at the fifth month stage. From the sixth to ninth month of gestation there was a progressive increase in the density of nerve fibres and their fine branches both in the stroma and epithelium.

2.2 Origin of corneal nerves

The human cornea is one of the most richly innervated structures in the body and is densely supplied by sensory and autonomic nerve fibres (Muller et al., 2003). It is 40 times more sensitive than dental pulp, 100 times more than the conjunctiva (Wells and Michelson, 2008), and over 400 times more than the skin (Bonini et al., 2003b).

2.2.1 Sensory nerves

The trigeminal nerve is the largest of all cranial nerves. It is the major sensory nerve of the head innervating the skin of the face, oral mucosa, nasal cavity, and paranasal sinuses. Its three sensory branches, the ophthalmic (V1), maxillary (V2), and mandibular (V3) nerves, emerge at the anterior convex aspect of the ganglion. The human trigeminal ganglion is canoe-shaped and has an average number of 27,400 pseudo-unipolar neurons [*neurons from which a single axon emerges and divides into two, one going to the central nervous system and the other to the peripheral end organ*] (LaGuardia et al., 2000). The corneal sensory fibres, which constitute most of the corneal nerve fibres, are mainly derived from cell bodies of the ophthalmic division of the trigeminal nerve. A relatively small number of neurons (about 1.5% of the total number of the neurons of the ganglion), which are located in the medial or neural crest component of the

trigeminal ganglion serve the cornea (Arvidson, 1977; Felipe et al., 1999; Keller et al., 1991; LaVail et al., 1993; Marfurt, 1981; Marfurt and Del Toro, 1987; Marfurt and Echtenkamp, 1988; Marfurt et al., 1989; Morgan et al., 1987b; Morgan et al., 1978; ten Tusscher et al., 1988). The sensory nerves exhibit a variety of afferent (e.g. pain and temperature sensation) and efferent (trophic and secretory) functions [*Unlike nerves, the terms afferent and efferent for blood vessels have the opposite connotation*]. Mechanical, thermal and chemical stimuli of the corneal nerves produce predominantly a sensation of pain in humans. (Lele and Weddell, 1959) In some cases the maxillary division of trigeminal nerve can provide innervation to part of the inferior cornea (Ruskell, 1974; Vonderahe, 1928). A single corneal sensory neuron can support an extremely large number of individual nerve endings. This number differs considerably among different mammalian corneas e.g. 200 in mouse (de Castro et al., 1998; Felipe et al., 1999) and 3000 in rabbit (Marfurt et al., 1989; Rozsa and Beuerman, 1982). Many retrograde nerve tracing studies have shown that 50-450 neurons innervate each cornea, the real number depending on species (Launay et al., 2015; LaVail et al., 1993; Marfurt et al., 1989; Morgan et al., 1978). These neurons are mainly small or medium size, averaging 20-23 μm in diameter in rodents and 31-33 μm in larger mammals (Keller et al., 1991; Marfurt et al., 1989; Nishimori et al., 1986; Sugimoto et al., 1988). Given that the human corneal surface area is around 123 square millimetres (Dua et al., 1994) this would equate to approximately 11,000 nerve endings per square millimetre (based on counts for mammalian (rabbit) nerve endings).

2.2.2 Autonomic nerves

All mammalian corneas receive sympathetic nerve fibres that are derived from the superior cervical ganglion. The density of the sympathetic innervation varies considerably among different species (Marfurt and Ellis, 1993). For example, a dense sympathetic innervation that constitutes up to 15% of the total corneal innervation has been demonstrated in rabbit and cat corneas (Ehinger, 1966b; Marfurt et al., 1989; Morgan et al., 1987a; Tervo and Palkama, 1978). However, a sparse corneal sympathetic innervation was found in humans and other primates (Ehinger, 1966b, 1971; Sugiura and Yamaga, 1968; Toivanen et al., 1987). They were predominantly located in the limbal stroma and in a close proximity to the blood vessels, except capillaries (Ehinger, 1966b). The function of the adrenergic input to the cornea, which is essentially avascular, is not yet known. Could the sympathetic nerves be an extension of the limbal vasomotor nerves that respond to corneal stimuli to cause dilation of the vessels, as part of a local axonal reflex arc usually mediated by the sensory nerves? Parasympathetic fibres, originate from the ciliary ganglion, have been shown to terminate in the peripheral part of rat and cat corneal stroma (Marfurt et al., 1998; Morgan et al., 1987a; Tervo et al., 1979). However, this kind of innervation has not been confirmed in humans.

2.3 Corneal nerve architecture

2.3.1 Limbal nerve plexus

The sensory nerves reach the eye mainly via the nasociliary branch of the ophthalmic division of the trigeminal nerve. This branch gives rise to 2-3 long ciliary nerves and a communicating branch or ramus taking sensory fibres to the

ciliary ganglion. Six short ciliary nerves from the ciliary ganglion and the long ciliary nerves course toward the posterior pole of the globe, penetrate it around the optic nerve and then pass anteriorly in the suprachoroidal space. Nerves in the suprachoroidal space are of two main groups: 1) the short uveal nerves form the suprachoroidal nerve plexus, which provide branches supplying the ciliary body. They appear as mixed fibres containing sympathetic, parasympathetic and sensory axons (May, 2004). 2) the long uveal nerves, accompanying the long posterior ciliary arteries, which provide innervation to the anterior segment. Seventy-five percent of these nerves are myelinated, while the remaining (25%) are unmyelinated. They contain both sympathetic and sensory nerve fibres.

Until recently, all the nerve fibres, innervating different structures within the globe, were thought to be postganglionic nerves. However, studies have shown neurons within the uveal tissue of human eyes, which are immunoreactive to nitric oxide synthase and vasoactive intestinal peptide (May et al., 2004; Tamm et al., 1995). Approximately 2000 of these neurons are present in the choroid and their postganglionic nerve fibres join the perivascular nerve plexus that supports the vasodilating effect on the choroidal vasculature (Flugel et al., 1994). A similar number of the neurons are located in the inner part of the ciliary muscle and are believed to be involved in fine regulation of accommodation (Tamm et al., 1995).

Branches from the long ciliary (uveal) nerves, together with nerve fibres from the sub-conjunctival plexus, form a complex peri-corneal (limbal) plexus, which is characterised by a dense ring-like network of nerve fibres that encircle the entire peripheral cornea (Fig. 1) (He et al., 2010). Most of the fibres in this plexus are believed to supply vasomotor innervation to the blood vessels in the

limbus, while others pass within the limbal stroma unrelated to blood vessels (Marfurt, 2000).

2.3.1.1 Limbal nerve corpuscles

Tortuous nerves fibres have been found to extend from the superficial limbal plexus and terminate in round or oval structures (Fig. 2), 20 to 100 μm in diameter, termed limbal nerve corpuscles (LNCs) (Al-Aqaba et al., 2018). These are generally confined to the peripheral 2 mm of the cornea and are randomly distributed along the limbal circumference in the subepithelial substantia propria. Their numbers are variable, ranging from 70 up to 300 per eye. They show an association with the palisades of Vogt and the limbal epithelial crypts (Fig. 3) (Dua et al., 2005).

On confocal microscopy they appear as bright, hyper-reflective ovoid structures singly or in clusters (Fig. 4) within the fibrous core of the palisades. On whole mount staining with the Acetylcholinesterase technique they show unevenly staining convoluted lamellae covering loculated spaces. On scanning electron microscopy (SEM) of macerated limbal tissue the LNC show a globular structure with a ruffled covering (Fig. 5). The location of the LNC and close association with the LEC and limbal palisades suggests that they form part of the stem cell niche microenvironment that maintains the stemness of the stem cells interacting with the blood vessels, stromal cells and local immune cells. The structure and function of the LNC is being studied and their exact role needs to be deciphered. They could also serve as special receptors for pressure and biomechanical stress at the limbus acting to restore limbal and corneal shape

following distortions induced by blinks, squeezing the eyelids and rubbing and globe; external pressures that the eye is constantly subjected to.

2.3.2 Corneal stromal nerves

A mixture of sensory and autonomic nerves passes through the limbus and enter the cornea at different levels. Nerves enter the cornea in the middle third of the stroma in a series of large, radially-oriented nerve bundles and run forward and anteriorly in a radial fashion toward the central area, giving rise to branches that innervate the anterior and mid-stroma (Fig. 6). About 33-90 fascicles, containing 900-1500 axons, enter the human cornea, while 20-40 fascicles enter the cornea of other mammals (Al-Aqaba et al., 2010b; Chan-Ling, 1989; He et al., 2010; Marfurt et al., 2010; Millodot, 1978; Zander and Weddell, 1951a). Additionally, other small nerve bundles enter the cornea more superficially in the episcleral and conjunctival planes, to provide innervation to the superficial stroma and epithelium of the peripheral cornea respectively (Chan-Ling, 1989; He et al., 2010; Zander and Weddell, 1951a). [*Nerve structure: An axon is the cytoplasmic extension of the neuron cell body. The cytoplasm is termed the axoplasm and the cell membrane covering the axoplasm is the axolemma. This is covered by myelin, which is white and relatively opaque. Myelin is surrounded by the Schwann cells that secrete the myelin. The Schwann cell layer is termed neurilemma. Delicate connective tissue (endoneurium, endoneurial sheath or Henle's sheath) containing connective tissue cells (endoneurial cells) surrounds the axon. Bundles of axons surrounded by a connective tissue sheath, the perineurium, are called a fascicle. Bundles of fascicles bound together with connective tissue, the epineurium constitutes a nerve (nerve bundle).]*

All corneal sensory nerves derive from finely myelinated (A-δ) and unmyelinated (C) axons determined by the size and presence of myelin sheaths in the axon (Felipe et al., 1999). [*Nerve fibres are of three types. A fibres, which can be the A alpha(α)[13-20 µm diameter and conduction velocity c/v of 80-120 metres per second m/s], which carry proprioception sensations from muscles; A beta(β) [6-12 µm diameter and c/v of 6-12 m/s], which transmit sensation of touch and A delta(δ) [1-5 µm diameter and c/v of 5-40 m/s], which transmit pain and temperature sensations. B fibres, which are preganglionic nerves of the autonomic system and visceral afferents. Nerve fibres of types A and B are myelinated and consequently have a faster conducting velocity. C fibres that carry sensations generated by pain, thermal and chemical stimuli. C fires are unmyelinated with a slower conduction velocity (0.5 to 2 m/s) and are comparatively thinner (0.2 to 1.5 µm)* (Tubbs et al., 2015).

In the human cornea, central stromal axons are unmyelinated and run in the anterior stroma as large bundles parallel to collagen bundles. Most of the axons in these bundles are about 0.5 µm in diameter. However, a few may be as large as 2.5 µm (Muller et al., 1996; Muller et al., 1997). On the other hand, more than 70% of the axons in rabbit corneas are unmyelinated (Beuerman, 1983). The rest are finely myelinated axons that lose their myelin sheath within 1 mm after penetrating the cornea (Lim and Ruskell, 1978; Rozsa and Beuerman, 1982; Zander and Weddell, 1951a). Interestingly, studies have shown that myelinated axons are present in the central cornea in some mammals (Rodger, 1950; Whitear, 1960).

As soon as they enter the corneal stroma, the nerve bundles lose their perineurium and continue centrally surrounded only by Schwann cells. The nerve bundles sub-divide and reconnect in a series of random bifurcations and trifurcations forming a multi-layered network that distributes nearly equally throughout all four corneal quadrants (Ishida et al., 1984). Each single sensory neuron can extend in to the cornea for up to three quarters of its diameter and supply as much as 20-50% of the corneal area (Zander and Weddell, 1951a, b). Several studies have shown that corneal stromal nerves have a tendency to concentrate in the anterior one third of stroma, where they form a dense sub-Bowman's plexus. However, the posterior stroma seems to lack innervation and only a few investigators have noticed a sparse innervation of corneal endothelium (Leon-Feliu et al., 1978; ten Tusscher et al., 1989; Wolter, 1957).

Are the stromal nerves merely passing through the stroma en route to the sub-basal plexus or are they supplying the stroma and keratocytes? Some nerves are seen to terminate in the stroma, in relation to a keratocyte but such endings are sparse compared to the density of nerves seen in relation to the epithelial cells. Like epithelial cells, keratocytes too depend on trophic support from the nerves implying neurotrophic factors released anteriorly can percolate through the stroma to support all keratocytes. The increased density of nerves in the anterior stroma corresponds with the increased density of keratocytes in the same location. This could be more than just a co-incidence and represent the gradient of trophic factor being more concentrated anteriorly.

The wide area covered by each neuron indicates that there is considerable overlap of innervation ensuring that no part of the cornea is deprived of nerve

supply following injury or insult to a single or few neurons or their axons. This probably explains why multiple limbal incisions of varying lengths performed during corneal or cataract surgery do not seriously affect corneal health.

Vascular and nervous systems share considerable similarities at the anatomical and cellular levels. They often run parallel to each other, exhibit a similar branching pattern (Ferrari et al., 2013). The developmental and growth of both networks require an intricate control, which is maintained by a variety of common molecules and trophic factors (Carmeliet et al., 2009; Tessier-Lavigne and Goodman, 1996). In healthy physiological state, the densely innervated cornea shows lack of vascular elements. However, conditions, that promote corneal vascularisation e.g. ocular surface inflammation, limbal stem cell deficiency, corneal graft rejection, infectious keratitis and chemical burns, often result in abnormalities of nerve structure and function (Epstein and Paton, 1968; Lim et al., 2009). Inflammation seems to be a common associated factor. A direct inhibitory cross-talk between the sensory nerves and angiogenesis has been confirmed in a relatively recent animal study, where inducing angiogenesis results in nerve loss and, conversely, denervation induces corneal vascularisation (Ferrari et al., 2013). This response appears to be mediated by reduced expression of corneal angiostatic molecules such as pigment epithelial-derived factor (PEDF) and epithelial vascular endothelial growth factor receptor 3 (VEGFR3).. However, there are contradictions as well. Herpes virus keratitis is the commonest cause of corneal vascularisation despite reduced corneal sensations (Faraj et al., 2016). Advanced keratoconus is associated with exuberance of stromal nerves yet rarely vascularised in the absence of acute hydrops (Al-Aqaba et al., 2011b). In

acanthamoeba keratitis nerve affection in the form of radial keratoneuritis is common yet it is an inflammatory condition least associated with vascularisation (Faraj et al., 2016). Stressed cells release chemokines that attract vascular endothelial cells suggesting that vascularisation is a multifactorial and complex process. There could also be an anatomical correlation between vessels and nerves. It has been shown that vessels in the cornea tend to follow tissue planes such as suture track and those created by incisions and penetrating and lamellar keratoplasty (Faraj et al., 2016). The micro channels through which nerves traverse the stroma may also provide channels for vessels to follow both in the presence or absence of the resident nerve.

Instillation of topical anaesthetic drops that predominantly affect the terminal sensory nerve endings, allows the surgeon to make full thickness corneal incisions near the limbus, transecting the thick limbal nerves without eliciting pain. The deeper stromal nerves may therefore be more trophic than sensory, and the superficial nerves the other way around.

Is pain response related to only the epithelium? With SMILE (small incision lenticule extraction, laser refractive surgery) and LASIK (laser in situ keratomileusis) procedures the pain is less when the anaesthesia wear off compared to photorefractive keratectomy (PRK) where the epithelium is removed and pain is severe when the anaesthetic wears off. This supports the notion that the sensory element of superficial nerve endings is much greater than in the stromal nerves/nerve endings. In bullous keratopathy (BK), episodic severe pain is attributed to rupture of epithelial bullae and exposure of nerve endings. Paradoxically, it has been shown that the sub-basal plexus in bullous keratopathy

is reduced or absent (see below Section 7.8). It is possible that the stromal nerves elicit pain response in an environment of chronic edema and inflammation. In 'radial keratoneuritis' of acanthamoeba infection the pain is severe. Here the stromal nerves are directly affected by the inflammation associated with surrounding edema.

A modest number of sympathetic nerves are mixed with corneal sensory nerves. The distribution of corneal sympathetic innervation has been studied using histochemical fluorescence techniques, immunohistochemistry and nerve tracing techniques (Ehinger, 1966a, b, c; Ehinger and Sjoberg, 1971; Klyce, 1986; Laties and Jacobowitz, 1964; Laties and Jacobowitz, 1966; Marfurt, 1988; Marfurt and Ellis, 1993; Tervo and Palkama, 1976). It has been found that sympathetic nerves are mainly distributed in the anterior one-third of the stroma and there is an inter-species difference in the overall density (Marfurt et al., 1989). Although it has been reported that there is insignificant corneal sympathetic innervation in adult primates including human cornea (Ehinger, 1966a, 1971; Ehinger and Sjoberg, 1971; Laties and Jacobowitz, 1966; Sugiura and Yamaga, 1968), ~~recent~~ other studies using antibodies against tyrosine hydroxylase reveal greater sympathetic innervation than has been previously identified (Marfurt and Ellis, 1993; Ueda et al., 1989).

There is ongoing evidence to support the physical and biochemical interaction between the nerves and the cellular constituents of the cornea. Stromal keratocytes are frequently found in the close vicinity of the nerves and occasionally enclose nerve bundles with their cytoplasmic processes (Fig. 7) (Muller et al., 2003). Metabolically active stromal fibroblasts have been recently

391 shown to promote neurite growth in a dose-dependent manner supporting the
392 notion that the density of stromal fibroblasts could influence nerve regeneration
393 (Yam et al., 2017). Conversely, neurite outgrowth following injury appears to be
394 delayed by scar-forming corneal myofibroblasts (Jeon et al., 2018). This effect is
395 believed to be mediated by transforming growth factor beta 1 (TGF-β1) secreted
396 by myofibroblasts.

397 Acetylcholinesterase (AChE) is present in both neuronal and non-neuronal
398 tissues, and is usually regarded as a membrane-bound enzyme (Taylor et al.,
399 1981; Wheeler et al., 1972). AChE activity has been demonstrated in the corneal
400 epithelium and keratocytes (Fig. 8). Evidence to suggest possible neuronal
401 function related role of intracellular AChE within the cornea include (1) the
402 association of acetylcholine and true AChE in cholinergic nerves; (2) loss of
403 corneal sensation when AChE synthesis is inhibited in the cornea; (3) lack of
404 acetylcholine and AChE content in denervated cornea; and, (4) localisation of true
405 cholinesterase in corneal epithelium by light and electron microscopy (Howard et
406 al., 1975).

407 *2.3.3 Bulb-like structures at the junction of the stromal and sub-basal nerves*

408 The junction of the stromal nerves and the sub-basal nerves at the sub-
409 basal plane demonstrate interesting anatomical features. As they approach the
410 anterior stroma, the unmyelinated nerves subdivide in a characteristic branching
411 pattern. After passing through Bowman's layer they terminate in bulb-like
412 structures from which a leash of sub-basal nerves arises (Fig. 9). The perforation
413 sites are predominantly located in the mid-peripheral cornea. Relatively fewer

perforation sites are found in the central cornea. The average number of perforation points is 185 per cornea (Al-Aqaba et al., 2010b). The bulb-like structures do not show any nuclei and probably represent the termination of the endoneurium which appears to be folded upon itself. However, the demonstration of neurons in the choroid should prompt another look at these structures in greater detail. Interestingly, the epithelial cells surrounding these structures differ from the remaining epithelium in that they often show a denser nuclear staining with AChE (Fig. 10). While the unmyelinated nerves maintain their Schwann cell sheath throughout the stroma, the sub-basal nerves project for several millimetres within the sub-basal and intraepithelial planes without Schwann cell support. Corneal epithelial cells function as surrogate Schwann cells for the sub-basal and intraepithelial nerves during homeostasis and in response to injury (Stepp et al., 2017). The morphological similarity between the sub-basal nerves and intraepithelial nerve plexus and the dendritic lesion of herpes simplex keratitis suggests the latter is determined by the former, which would not be surprising given the affinity of the virus for the nerves. Pseudodendrites are seen in acanthamoeba keratitis and with healing corneal abrasions. The mechanisms of these lesions are different. In the former they represent the migration path taken by acanthamoeba as they infect and ingest the epithelial cells and in the latter they represent the meeting sites of healing epithelial sheets. Though sub-basal nerve changes have been described with acanthamoeba infection (Kurbanyan et al., 2012) the nerves are unlikely to determine the pseudodendritic pattern.

The appearance of the bulbous end of the stromal nerves has been confirmed using a variety of techniques including AChE staining, Nanozoomer

scanning, (Fig. 10) (Al-Aqaba et al., 2010b), SEM, TEM (Fig. 11) and IVCM (Fig. 12). The morphological features revealed differ with the stain used. AChE staining demonstrated the blub-like terminations of the nerves as they emerge anterior to the Bowman's layer but these are not shown by staining with anti-Beta III tubulin immunohistology. This is most probably related to the intracytoplasmic location of this neuron-specific major cytoskeleton protein, which would show the axons but not the surrounding sheath (He et al., 2010; Marfurt et al., 2010).

2.3.4 Sub-basal nerve plexus

The corneal epithelium receives sensory nerve fibres either from the sub-Bowman's nerves via the sub-basal plexus or in the peripheral cornea, directly from the conjunctival nerves (Marfurt, 2000). The majority of these nerve fibres are of C type (Richter et al., 1997). The sub-basal nerve plexus is situated between the basal epithelial cells and Bowman's layer (Fig. 13). Morphologically, each nerve consists of non-beaded straight nerve fibres in the centre and beaded fibres located in the periphery of the nerve (Fig. 13). Several electron microscopy studies have shown that these beads represent axonal efferent and sensory terminals with accumulations of mitochondria and glycogen (Matsuda, 1968; Muller et al., 1997; Ueda et al., 1989).

These nerves have a highly characteristic appearance, when viewed by SEM and IVCM, as sharply defined linear structures of homogenous reflectivity with Y shaped bifurcating branches and H shaped thinner connecting nerve fibre bundles (Fig. 13 & Fig. 14) (Oliveira-Soto and Efron, 2001). At their point of origin, the sub-basal nerves project downward (superior to inferior) for fibres

461 arising from perforation sites in the superior and central cornea and upward
462 (inferior to superior) for those originating in the inferior cornea. The nerves fibres
463 then converge towards the infero-central cornea to form a characteristic clockwise
464 whorl pattern (Al-Aqaba et al., 2010b; Patel and McGhee, 2005). The whorl is
465 located at the junction of the upper two thirds and lower one third of the cornea
466 and has been demonstrated by IVCM and AChE staining (Fig. 15) (Al-Aqaba et
467 al., 2010b; Patel and McGhee, 2005). In vortex keratopathy and hurricane keratitis
468 the epithelial lesions replicate the pattern of the underlying nerves. The epithelial
469 whorls, like the underlying whorl of the sub-basal plexus are almost always
470 'clockwise' in disposition and are believed to be a response to the electromagnetic
471 fields of the eye (Dua and Gomes, 2000; Dua et al., 1996; Dua et al., 1993).

472 The response of the migrating corneal epithelial cells to ocular
473 electromagnetic fields generated by the dipole of the eye along its antero-posterior
474 axis has been suggested as the likely driving force for the whorl appearance (Dua
475 et al., 1993). A difference in electric potential of nearly 6 mV exists between the
476 cornea and the back of the eye, with the cornea positive to the posterior segment
477 (Berson, 1981). The resultant flow of electricity would generate electro-magnetic
478 forces that are disposed in a clockwise direction. The directional movement of
479 epithelial cells is known to be influenced by electric (galvanotaxis) and magnetic
480 (magnetotaxis) fields. During epithelial cell turnover e.g. wound healing
481 following corneal abrasion, the radial centripetal slide of corneal epithelial cells
482 from the limbal region together with the tendency of the electric field to arrange
483 them in concentric circles would eventually result in the formation of clockwise
484 whorls (Dua et al., 1993). As the sub-basal nerves give rise to intra-epithelial

nerve branches which are perpendicular to the sub-basal plane, during the centripetal movement of the corneal epithelium both the intra-epithelial nerves and epithelial cells must advance in the same direction and velocity. This would also explain the whorl-like configuration of the sub-basal nerves (Patel and McGhee, 2005).

Furthermore, by virtue of the vertically oriented intraepithelial nerves and nerve endings there is an intricate association of the epithelial sheet with the nerves. This would necessitate the two tissues to have a complementary configuration. Whether the former determines the latter or vice versa is unknown. In epithelial defects, the epithelial sheet comes off with the corresponding intraepithelial nerves. Both epithelium and nerves must regenerate. Whether this occurs concurrently, which is more likely, or sequentially, with the nerves following the epithelial healing, is also unknown.

2.3.5 Corneal nerve endings

Nerve fibres arising from the sub-basal plexus innervate the corneal epithelium through all its layers. The intra-epithelial fibres closer to the basal epithelium run in a more or less horizontal direction and those that terminate more superficially are vertically oriented and terminate in three different ways: Simple terminals, that do not branch after leaving the sub-basal plexus and present a bulbar swelling at their termination within or below the superficial squamous cells. Ramifying terminals, where the single intraepithelial nerve fibre arising from the sub-basal plexus divides into 3 - 4 branches, which run horizontally for a hundred microns or less and end with a bulbous swelling like the simple

terminals. Complex terminals, from nerve fibres that have multiple branches starting in the wing cells and terminating with multiple larger bulbous endings within the wing or squamous cells of the epithelium. The nerve endings are not fixed structures as they change and regenerate in accordance with epithelial turnover (Ivanusic et al., 2013; Marfurt et al., 2010; Rozsa and Beuerman, 1982). It is often believed that corneal free nerve terminals of C- and Aδ-neurons could only be differentiated through their function. However, immunofluorescent study on pig corneas suggest that these nerve terminals can also be distinguished morphologically (Alamri et al., 2018). TRPM8 immuno-labelled cold thermoreceptors have more complex endings than TRPV1 immuno-labelled polymodal nociceptors. (Alamri et al., 2018).

2.3.6 Corneal neural receptors

Corneal receptors can be categorized into polymodal nociceptors (receptors that respond to more than one noxious stimulus), cold thermoreceptors and selective mechano-nociceptors, based on their electrophysiological and functional properties (Belmonte et al., 2017).

2.3.6.1 Polymodal nociceptors

Polymodal nociceptors are most abundant type of corneal receptors. They normally fire in response to a wide range of noxious external stimuli including mechanical energy, heat, and chemical irritants. They can also be activated in response to endogenous chemical mediators released by inflammation and trauma (MacIver and Tanelian, 1993). Polymodal nociceptors have been shown to be excited by temperatures over 37°C and exhibit sensitisation to repeated heating

(Gallar et al., 1993). They can also respond to a mechanical stimulus of slightly lower threshold than pure mechano-nociceptors. A prolonged mechanical indentation of the cornea can produce a sustained discharge of polymodal nociceptors (Belmonte et al., 1997). It has been found that acidic solutions of pH 5.0–6.5 or gas jets containing CO_2 also excite corneal polymodal nociceptors, supporting the hypothesis that excitation of nociceptors by low pH is the cause of pain following injury (Chen et al., 1995).

2.3.6.2 Mechano-nociceptors

They represent 20–30% of the corneal nerve terminals. They respond only to strong mechanical stimuli that can potentially result in damage to corneal epithelial cells (Belmonte et al., 2017). In contrast to polymodal nociceptors, brief or persistent indentations of the corneal surface produce a single or a few impulses from the mechano-nociceptors. This phasic sensory response helps to signal the presence of a stimulus and to produce the immediate, sharp sensation of pain induced by touching or scratching the corneal surface (Belmonte et al., 2017).

2.3.6.3 Cold thermoreceptors

Cold thermoreceptors account for 10-15% of the total number of corneal sensory neurons (Belmonte et al., 2017). They are continuously firing when the temperature of the ocular surface is at its normal range (34-35°C). Moderate cooling or heating can alter the electrical activity of these receptors (Brock et al., 2006; Tanelian and Beuerman, 1984). In addition, recent evidence has shown that abnormal activity of corneal cold thermoreceptors provides a plausible

554 explanation for the unpleasant sensations in dry eye disease (Kovacs et al., 2016).
555 Increased tear osmolarity, as a result of ocular surface dryness, enhances cold
556 thermoreceoptors activity and eventually results in compensatory changes in
557 lacrimation and blink rate (Kovacs et al., 2016; Parra et al., 2014).

558

559 **3. Neurochemistry of the cornea (Neurotrophins, Neurotransmitters and**
560 **Neuropeptides)**

561 Like all tissues in the body corneal innervation is associated with
562 neurotrophins, neuropeptides and neurotransmitters. The neurotrophins are growth
563 factors, produced by neurons and by the tissues served by the nerves that play
564 important roles in the development, maintenance, survival, regeneration and
565 integration of neuronal function. They activate the p75 and Trk tyrosine kinase
566 receptors, which through several signalling pathways bring about the diverse
567 aspects of neural function (Huang and Reichardt, 2001; Mastropasqua et al., 2017;
568 Muller et al., 2003). Nerve growth factor is the prototype neurotrophin, which has
569 gained prominence with regard to the cornea as a recombinant NGF is now
570 available as eye drops for clinical use (Dua et al., 2018). Clinical trials have
571 shown that it is effective in inducing healing of corneal epithelial defects in stage
572 2 (moderate) and stage 3 (severe) neurotrophic keratopathy (Bonini et al., 2018b;
573 Dua et al., 2018). Brain derived neurotrophic factor (BDNF), Glial-derived
574 neurotrophic factor (GDNF), NT3, NT4 are other neurotrophins that have been
575 demonstrated in the cornea (You et al., 2000). Besides supporting neurons in both
576 the central and peripheral nervous systems, neurotrophins also modulate functions

577 in the immune and reproductive systems. They are also implicated in
578 neurodegenerative diseases, neoplasia, inflammation and pain.

579 Neurotransmitters and neuropeptides are polypeptide derivatives that serve to
580 transmit the nerve impulse from one neuron to another via the synaptic junction.
581 Examples of neurotransmitters are acetylcholine, dopamine, serotonin and
582 histamine. These are small molecular weight molecules that are synthesised at the
583 synaptic junction in relatively high concentrations, bring about their effect over a
584 short period of time and are rapidly metabolised. They induce acute short-term
585 response and act on specific receptors. Neuropeptides also have neurotransmitter
586 function. They have a slow release, bring about a prolonged effect and act on
587 multiple receptors. Unlike neurotransmitters, their action extends beyond the
588 immediate vicinity of their site of release. Examples of neuropeptides are
589 Substance P (SP) and calcitonin gene-related peptide (CGRP), which are the most
590 abundant neuropeptide in the mouse cornea (He and Bazan, 2016). In humans, a
591 relationship between CGRP in tears and corneal nerve density and sensitivity has
592 been demonstrated (Golebiowski et al., 2017). Oxytocin, vasopressin, insulin and
593 glucagon are other examples of neuropeptides. SP has been shown to modulate
594 corneal vascularisation in humans and murine models (Barbariga et al., 2018).

595 The list of neurochemicals associated with corneal innervation is large and
596 growing (Shaheen et al., 2014). A wide range of neuropeptides (SP and
597 neurokinin A), neurotransmitters (Ach, adrenaline, nor-adrenaline and dopamine)
598 and their receptors are expressed by human keratocytes both in vitro and in vivo
599 (Sloniecka et al., 2015). CGRP and vasointestinal peptide but not SP were found
600 in significantly higher concentrations in keratoconus corneal extracts compared to

normal and scarred (leucoma) corneas suggesting that altered corneal innervation and consequent trophic effects can contribute to corneal pathology (Sacchetti, 2014). Involvement of neuropeptides and neurotransmitters in cell proliferation, migration and vascularisation show that they participate in wound healing responses as well.

Evidence suggests that these chemical mediators of nerve responses have biological effects that extend beyond the transmission of a nerve impulse, affecting corneal health and disease. Given the extensive ramification of nerves in corneal stroma and their association with keratocytes and epithelial cells, it is not surprising that they contribute to physiological homeostasis of the cornea and are involved in pathogenesis of disease, response to injury and repair, regeneration and recovery therefrom. For many years, the reduction in corneal sensitivity was the only objective (and subjective) measure of nerve health. It was the surrogate measure of all nerve functions. It is now known that trophic functions, mediated by the chemical mediators, can be dissociated from sensory functions and in vivo confocal microscopy has enabled direct visualisation of nerve morphology revealing a range of alterations in corneal pathology. While all the above sounds logical and is supported by reasonable evidence, the question that remains unaddressed is how these effects translate to the posterior cornea, which is almost completely devoid of nerves? Lack of innervation would imply lack of sensitivity yet the need for trophic support would be no less. Neurotrophins and neuropeptides produced in neurons and released at nerve terminals anteriorly could diffuse posteriorly and support the posterior cornea. Equally, non-neuronal

cells (keratocytes) could produce the chemical mediators in the absence of nerves or respond via their receptors, to chemical released in the anterior stroma.

4. The role of corneal nerves

4.1 Sensory and reflex function

The cornea is considered as the most densely innervated and most sensitive structure in the human body. A ‘blink’ is a rapid closure and opening of the eyelid involving the orbicularis and levator palpebrae muscles. Spontaneous blinking, occurring about 15-20 times a minute, is centrally driven in the pre-motor brain stem. It occurs without any external stimuli and is involuntary. Reflex blinking is a protective response to external stimuli that can be tactile, optical or auditory. It occurs faster than a normal blink and is also ‘involuntary’. Corneal nerves carry afferent impulses generated by touch, temperature (cold), pain and chemical stimuli (McKay et al., 2018). Light touch to the cornea causes rapid bilateral blink response by contraction of the orbicularis muscle. This a protective reflex mediated between the ophthalmic division of the trigeminal nerve and the facial nerve(s). Afferent fibres from the cornea synapse in the spinal trigeminal nucleus or the main sensory nucleus and neurons project directly to the facial nuclei on both sides. Efferent fibres from the facial nuclei innervate the orbicularis oculi muscle. Affection of the trigeminal nerve, trigeminal nucleus or brainstem (cerebello-pontine angle or posterior fossa tumours, multiple sclerosis or brain stem strokes), slow down the reflex response (Prasad and Galetta, 2007). A general belief is that afferent pathway lesions affect the blink reflex bilaterally

while efferent (VII nerve) lesions affect the blink reflex unilaterally. However, it is absent on one side in 8% of normal individuals (McGee, 2017).

The blink reflex is considered to have two components, one that can be elicited by touching the cornea (corneal reflex) and the other by stimulating the supraorbital region (Blink reflex, R2 Component.). Afferent nerves for the reflex have been identified in the supraorbital nerve. Both components are regarded as cutaneous reflexes but could have different neural connections as the latency and duration of the corneal reflex have been shown to be longer than the R2 component (Berardelli et al., 1985).

The sensory nerves of the ocular surface also provide afferent impulses for the reflex tear arc. Lacrimation reflexes involve three nerves, the 5^{th}, 7^{th} and parasympathetic, and three ganglia, the geniculate (VII), spheno-palatine and the Gasserian (V). The efferent pathway is through the parasympathetic fibres from the superior salivary nucleus in the pons. The role of the sympathetic nervous system in lacrimation is ill understood. Lacrimation too is a protective reaction to wash out or dilute noxious agents and irritants. Tears bring with them a whole range of antimicrobial molecules that prevent adhesion of organisms, destroy organisms and avert infection.

4.2 Axonal reflex and neurogenic inflammation

A classic reflex arc involves a sensory receptor at the point of contact of the nerve terminal with an exciting stimulus (the starting point), an effector terminal at the destination (the end point) and an integration centre, usually a synapse, connecting the two (orthodromic transmission) (Yaprak, 2008). Unlike

the classic reflex, the axonal reflex does not pass via a synapse. The stimulus propagates an impulse that travels centrally along the nerve to the point of bifurcation from where it reverses direction and is transmitted down the nerve branch to an end organ (antidromic transmission) such as a blood vessels or gland (Langley, 1923). Early evidence to support this phenomenon came from experiments on the conjunctiva; which showed that the axon reflex was abolished by instillation of topical anaesthesia and remained intact after trigeminal nerve transection (Bruce, 1913).

The triple response of Lewis involves the appearance of a *red* line within a few seconds of injury to skin with a sharp object representing immediate capillary dilatation in response to release of histamine; followed in a few minutes with surrounding redness or flare due to neurogenic arteriolar dilatation, which is followed by a white *wheal* resulting from exudation of plasma from the capillaries (Mtui, 2015). The axonal reflex is responsible for the wheal and flare responses. The pathophysiology involves a sequence of events starting with the stimulation of polymodal nociceptors by a stimulus, which generates impulses that are transmitted to both the central nervous system orthodromically and to the neighbouring skin or conjunctiva (limbus) antidromically. Antidromic stimulation induces a release of neuropeptides, mainly CGRP and SP, which act directly on vascular endothelial and smooth muscle (Chiu et al., 2012; McCormack et al., 1989). CGRP induces vasodilation by binding to receptors on the arteriolar walls producing the flare response and SP binds to receptors on mast cells inducing release of histamine (Columbo et al., 1996), which increases capillary permeability causing the wheal response. When the stimulus is sustained the

694 neuropeptides can mediate neurogenic inflammation through attraction and
695 subsequent activation of the innate (mast cells, dendritic cells) and adaptive
696 immune cells (T cells) (Ansel et al., 1993; Mikami et al., 2011).

697 Neurogenic inflammation is a term used to describe inflammation mediated by
698 neuropeptides and neuromediators released from afferent fibres of sensory nerves
699 and also from autonomic, mainly sympathetic postganglionic nerves. The key
700 substances are tachykinins, CGRP and proinflammatory cytokines especially IL6
701 (van der Kleij et al., 2009). These mediators act directly on the adjacent blood
702 vessels to promote vasodilation and extravasation of inflammatory cells. Such a
703 mechanism has been shown to drive joint, colon, lung and bladder inflammation.
704 Interestingly, neurogenic inflammation can be countered by neurogenic
705 cholinergic anti-inflammatory response mediated via parasympathetic efferents
706 for example of the vagus nerve. Acetylcholine can interact with nicotinic
707 receptors on macrophages and monocytes to inhibit proinflammatory cytokine
708 release (Basbaum, 2009; Labetoulle et al., 2019).

709 A strong body evidence point to the fact that ocular immune privilege is
710 under neural control (Streilein et al., 2000). Several neuropeptides e.g. vasoactive
711 intestinal peptides (VIP) and CGRP, that are circulating in the aqueous humour,
712 have been demonstrated to inhibit the functions of T lymphocytes and
713 macrophages (Taylor, 2007). Transection of the corneal nerves have been shown
714 to disable corneal CD11c+ T regulatory cells (Tregs) by converting them into
715 contrasuppressor (CS) cells and also to suppress the production of
716 immunomodulatory factors in the anterior chamber (Neelam et al., 2018; Streilein
717 et al., 1996). CD11c+ Tregs cells normally inhibit CD8+ Tregs cells, which are

induced by introducing antigens into the anterior chamber. Nerve regeneration is associated with improved level of these factors.

4.3 Trophic function

In addition to their well-known sensory and reflex functions, corneal nerves also have important trophic effects on the cornea and play a significant role in the maintenance of a healthy ocular surface through the stimulation of corneal wound healing after corneal injuries. Damage or dysfunction of the corneal sensory innervation produces a degenerative condition known as neurotrophic keratopathy (NK) (Duke-Elder, 1965) characterised by, in mild or stage 1 NK, decreased epithelial thickness, varying degrees of epithelial degradation, decreased epithelial cell mitosis, and impaired wound healing after corneal injuries (Beuerman and Schimmelpfennig, 1980; Bonini et al., 2003b; Lim, 1976; Sigelman and Friedenwald, 1954; Sugiura and Matsuda, 1967). This leads on to moderate or stage 2 NK where there is a frank non-healing epithelial defect extending to stromal involvement (severe or stage 3 NK) with melting and eventual perforation of the cornea. A large number of conditions, both ocular and systemic, can be associated with NK. Peripheral or corneal causes, wherein the pathology is largely confined to the cornea such as herpetic viral infection and corneal surgery; corneal sensations are impaired but conjunctival sensations and those of the surrounding skin are preserved. With ‘central’ damage to the trigeminal nerve for example with head trauma, intracranial surgery, aneurysms or tumours (Davis and Dohlman, 2001) sensitivity of the cornea, conjunctiva and facial skin is impaired. Certain types of lens and retinal surgery, and some ophthalmic laser treatments (e.g. panretinal photocoagulation and cycloablation)

742 can also cause NK by damaging the ciliary nerve fibres as they pass anteriorly
743 within the globe between the sclera and the choroid (Johnson, 1998; Menchini et
744 al., 1990; Weigt et al., 2002).

745 Laser refractive surgery has focused intense attention on corneal
746 innervation and consequence of damage to corneal nerves that is inevitable with
747 any type of laser refractive surgery both by the incisions made to prepare a
748 corneal flap mechanically with a blade or with femtosecond laser; and by the
749 excimer laser applied to achieve the refractive change. It has been reported that
750 procedures like radial keratotomy, photorefractive keratectomy, and LASIK
751 produce localised injury of thick stromal nerves and the sub-basal plexus resulting
752 in transient mild to severe epithelial changes with neurotrophic and/or dry eye
753 features (Tervo and Moilanen, 2003; Wilson, 2001; Wilson and Ambrosio, 2001).

754 Several studies have been conducted to investigate the exact mechanism
755 by which the corneal nerve fibres maintain a healthy cornea and promote wound
756 healing after corneal injuries. The results of these studies suggest that there is a
757 trophic relationship between corneal nerves and epithelial cells and each one
758 supports the other. Corneal nerves can stimulate epithelial cell growth,
759 proliferation, differentiation and type IV collagen formation through the release of
760 neurotrophins and neuropeptides (Baker et al., 1993). Conversely, corneal
761 epithelial cells can affect nerve survival and growth through the release of certain
762 factors (e.g. NGF and GDNF) (Emoto and Beuerman, 1987).

763 The clinical presentation of nerve damage, both symptoms and signs, can
764 be diverse and result from consequences of sensory damage affecting tear

765 secretion with features of dry eye disease; allodynia and hyperaesthesia due to
766 nerve inflammation, aberrant firing and aberrant or hyper regeneration. Corneal
767 sensations may correspondingly be decreased or increased though there is no
768 clinical means yet of measuring hyperaesthesia. In some instances this might
769 represent different ends of the spectrum of the same condition, starting with
770 increased sensitivity and ending in complete anaesthesia. Affection of trophic
771 function can lead to a cycle of epithelial and nerve damage with frank NK
772 progressing through the three stages. The clinical sign of superficial punctate
773 keratitis (SPK), which are essentially tiny epithelial defects, is generally more
774 centrally located in post-laser corneal refractive surgery compared to classical dry
775 eye disease where SPK affect the inferior third of the cornea. The former is more
776 likely to be a direct manifestation of the nerve damage in the corresponding area
777 with symptoms and signs of dry eye disease being a secondary occurrence (Dua et
778 al., 2018).

779 Interestingly, several experimental and clinical studies have shown that
780 there is a bidirectional control of corneal epithelium proliferation: sensory
781 neurotransmitters enhance epithelial cell mitosis, while sympathetic mediators,
782 epinephrine and norepinephrine, decrease epithelial cell mitosis (Bonini et al.,
783 2003a; Cavanagh and Colley, 1989). This is supported by the finding in animal
784 studies that cervical sympathetic denervation reduces corneal epithelial changes
785 induced by lesions of sensory nerves (Fujita et al., 1987).

786

787 **5. Manifestations of corneal nerve dysfunction**

5.1 Definitions

The International Association for the Study of Pain (IASP) defined pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." (Loeser and Treede, 2008). Pain syndromes can be classified into two main categories: neuropathic pain (NP) and nociceptive pain (NcP). NP is induced by a disease or lesion of the peripheral or central somatosensory nervous system. Typical examples of peripheral NP are post-herpetic neuralgia, diabetic neuropathy, chemotherapy induced neuropathy and trigeminal neuralgia. NP syndromes of central origin include multiple sclerosis pain, post-stroke pain and following spinal cord injury. Syndromes such as atypical chronic fatigue, irritable bowel, facial pain, interstitial cystitis and fibromyalgia, are types of neuropathic pain where the primary underlying pathophysiology is not fully understood.

Patients with NP can present with positive and/or negative sensory symptoms. Positive symptoms are usually due to excessive neuronal activity, for examples allodynia *[sub threshold or a normally non-painful stimulus provokes pain]*, hyperalgesia *[Increased response from a stimulus that normally provokes pain]* and paraesthesia *[Non-painful ongoing sensation]*. Negative symptoms are the result of deficit of nerve function and may include hypoesthesia *[Decreased sensitivity to stimulation]*, anaesthesia *[absent sensitivity to stimulation]*, hypoalgesia *[Diminished pain in response to a normally painful stimulus]* and analgesia *[Absence of pain in response to a normally painful stimulus]*. Patients with NP often experience a combination of these paradoxical symptoms.

NcP is defined as a pain that results from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors. It occurs in the setting of tissue inflammation, acute trauma or surgery.

5.1.1 Corneal pain representation in the somatosensory cortex

The human cornea has a high density of nociceptors that transduce noxious stimuli. Painful stimulation of the human cornea induces neural activity in the contralateral primary somatosensory cortex homunculus (S1); a region consistent with the location of the eye representation at the mid-cortical region (Moulton et al., 2012). It is believed that it is through these nociceptive fibres that the somatotopic representation of the human cornea is conveyed. Localisation of the site of pain in the cornea is not as refined as elsewhere. It is difficult for any patient to tell which part of the cornea is pain originates form, i.e. whether is it central, superior, inferior, nasal or temporal. This is likely related to the extensive overlap of innervated areas from single neurons.

5.2 Corneal pain in the absence of ocular surface disease "Pain without stain"

Dry eye disease is a complex, multifactorial, and heterogeneous diagnosis that covers a wide variety of clinical presentations (Galor et al., 2018). Symptoms includes ocular sensations of dryness, discomfort, pain, and ocular surface abnormalities such as reduced tear production and increased tear evaporation (Smith et al., 2007). Dry eye symptoms are common complaints in the general population. They are more frequently encountered in females than in males (Moss et al., 2000), and with advancing age (Schaumberg et al., 2003). In the US, it was found that 14.4% of people between the ages of 48 and 91 years reported dry eye

symptoms (Schaumberg et al., 2003). Most cases of dry eye disease have associated clinical signs on slit-lamp examination that resolve following appropriate therapy. However, there remain a subset of patients that demonstrate gross disparity between the scale of symptoms and clinical signs on examination (Hamrah et al., 2017). Such patients are troubled with profound ocular discomfort and pain despite having a clinically unremarkable examination. Due to the chronicity of the pain and its subsequent effect on the quality of life, these patients end up being labelled as having “chronic pain syndrome”. Anti-neuropathic and antidepressant medications are commonly prescribed to help with the ocular symptoms. These factors add to the complexities and challenges around the management of these cases.

In our experience such patients with longstanding symptoms of ocular pain, discomfort and burning without obvious clinical signs on examination, who were referred with a diagnosis of central neuropathic ocular pain show evidence of subclinical corneal pathology on IVCM [Ross A, Al Aqaba A, Said D and Dua HS, unpublished observations] (Fig. 16). The changes include keratocyte activation with swelling and enlargement of syncytial cell bodies of the keratocytes, giving rise to a honeycomb-like pattern in the anterior stroma (Fig. 16 A to C). Dendritic-like cells are seen closely associated with stromal nerves (Fig. 16 D-F), which show segments of hyper-reflective outgrowths and hypo-reflective darker bands along their course. These findings signify a form of subclinical inflammatory corneal neuropathy of unknown aetiology. Hamrah et al. have recently reported significant nerve alterations in patients with severe neuropathic corneal pain. The changes include reduction in the total length and number of

858 nerves, increased reflectivity and tortuosity of sub-basal nerves and the presence
859 of sub-basal microneuromas (Aggarwal et al., 2019; Hamrah et al., 2017).
860 Interestingly, treatment with autologous serum tears induces nerve regeneration,
861 which correlates with improvement in patient symptoms (Aggarwal et al., 2019).

862 Despite, the ocular pain symptoms, corneal sensation was paradoxically
863 reduced in some of the patients. These findings, together with the prompt
864 alleviation of the ocular symptoms upon instillation of topical anaesthetic, suggest
865 that the cause of the pain symptoms is likely to be peripheral i.e. corneal, in
866 origin. The precise mechanism and the pathophysiology of these microstructural
867 alterations are still unknown. Hormonal and metabolic influences may play a role
868 in the development of the corneal nerve pathology noted (Auro et al., 2014).
869 Another possible explanation is central sensitisation of pain pathways as a result
870 of prolonged periods of unexplained damage to the peripheral somatosensory
871 system (Galor et al., 2018). Furthermore, corneal inflammation has been shown to
872 induce activation of a specific neuronal pathway within sensory trigeminal
873 complex, which might play a priming role in the central sensitisation of ocular
874 related brainstem circuits and the development of neuropathic corneal pain
875 (Launay et al., 2016). The use of topical anti-inflammatory medications e.g.
876 NSAIDs and ciclosporin result only in partial resolution of the subclinical
877 inflammation and ocular pain (unpublished data). When pain becomes ‘central’ in
878 origin, topical anaesthesia does not alleviate the patient’s symptoms.

879 *5.3 Anaesthetic cornea with intact corneal nerves (pre-ganglionic damage)*

Corneal anaesthesia or hypoesthesia in the presence of intact corneal nerves (sub-basal plexus) can be observed in patients with damage to the pre-ganglionic nerve fibres of the ophthalmic division (V1) of the trigeminal nerve or its CNS nucleus (Dhillon et al., 2014; Dhillon et al., 2016). This phenomenon can be seen following surgical interventions for trigeminal neuralgia and cerebellopontine angle tumours or post-brainstem stroke. Corneal sensation is impaired as the afferent conduction to the cortex is disrupted. However, the surviving neurons within the trigeminal ganglion possibly continue to produce neurotrophic factors, which may sustain and promote the outgrowth of distal axons and also help to maintain the integrity of healthy ocular surface. Therefore, clinically significant symptoms or signs of neurotrophic keratitis are less likely to develop in preganglionic V1 lesions (Dhillon et al., 2016). This is in contrast to the postganglionic nerve damage that occurs following laser surgery or corneal transplantation, in which the distal axons degenerate with subsequent loss of both the sensory and trophic nerve functions. This suggests that the sensory and trophic functions of V1 are independent and can be dissociated by injury or disease (Dhillon et al., 2016). Anaesthesia dolorosa is a rare “positive” pain phenomenon, which can also be observed after traumatic or surgical injury to trigeminal nerve. It is characterised by a persistent “deafferentation” central neuropathic pain with numbness along the territory of the respected nerve damage. (Elahi and Ho, 2014). In relation to the cornea this can also manifest as pain in an eye with no corneal sensation but an intact sub-basal plexus (Dhillon et al., 2014).

5.4 Anaesthetic/hypoesthetic cornea with absent nerves and abnormal ocular surface “neurotrophic keratopathy, stain without pain”

Pain is regarded as a manifestation of active disease and resolution of pain is an indicator of improvement of the underlying condition. This is true from the patient's perspective where pain is indicative of active disease. However, clinically and pathologically this can be a sinister occurrence indicating further nerve damage and risk of corneal melting. Neurotrophic keratopathy (NK) has been defined as a disease related to alterations in corneal nerves leading to impairment in sensory and trophic function with consequent breakdown of the corneal epithelium, affecting health and integrity of the tear film, epithelium and stroma (Dua et al., 2018). Three stages or grades have been described (section 4.3), which can progress from one to the other or, if the pathology or insult is severe may manifest directly in the moderate or severe grade (stage 2 or 3).

From the evidence in the literature and experience of patients and clinicians alike, it would be reasonable to conclude that corneal nerve disease is a spectrum representing hyperaesthesia or paraesthesia at one end and complete anaesthesia with loss of sensory and trophic functions at the other. Symptomatically it can progress along this path, arrest at any one point for a prolonged period or indefinitely or manifest at any point in the path at first insult and progress thereafter. The precise pathophysiological changes that correspond to these clinical manifestations and subjective symptoms need to be better understood.

6. Visualisation of corneal nerves

6.1 In vitro/ex vivo techniques

927 Most of the knowledge of corneal nerve morphology, distribution and
928 ultrastructure in the last century was the result of light and electron microscopic
929 studies of corneas processed with different types of stains (Jones and Marfurt,
930 1991; Schimmelpfennig and Beuerman, 1982; Zander and Weddell, 1951a).
931 Corneal axons appear morphologically homogeneous when they are demonstrated
932 using routine histological methods or electron microscopy techniques. However,
933 immunocytochemical staining shows the presence of different neuropeptides
934 within the cytoplasm and peripheral axonal fibres of corneal neurons (Belmonte et
935 al., 2004). For example, about 58% of corneal neurons are immunoreactive to
936 Calcitonin gene-related peptide; 20% of them also contain Substance P (Felipe et
937 al., 1999; Muller et al., 2003; Stone et al., 1986; Tervo et al., 1981).

938 There are several staining techniques for the demonstration of corneal nerve
939 distribution (Table 1). Some of these stains are used to demonstrate corneal
940 sensory nerves while others are used specifically to stain corneal autonomic
941 nerves.

942 *6.1.1 Whole mount Acetylcholinesterase staining technique*

943 This is an enzyme histochemical staining technique which has been proven
944 to be excellent for the histological demonstration of cholinesterase containing
945 corneal nerves (Ishida et al., 1984). The cholinesterase enzymes are found along
946 the corneal nerve axons and believed to be responsible for the maintenance of the
947 ionic gradient along the axons during propagation of the nerve impulse
948 (Morishige et al., 2009). Whole mount preparations have allowed excellent in
949 vitro three-dimensional visualisation of the distribution and spatial arrangement of

the nerve bundles and characterisation of the structure of certain individual nerves within large trunks (Robertson and Winkelmann, 1970).

In general, this technique is relatively simple and yields reproducible results. Its reaction product is constant and stable making it ideal for reliable quantitative studies (Ishida et al., 1984). Basically, there are two types of reactions; specific and non-specific cholinesterase reactions. The latter can demonstrate Acetylcholinesterase as well as Butyrilcholinesterase (Karnovsky and Roots, 1964) and so allow adequate visualisation of individual axons and their terminations, as well as large nerve fibre bundles.

Specific inhibitors are used with the former reaction and can differentially demonstrate any of the above enzymes depending on the type of inhibitor used (Robertson and Winkelmann, 1970). The non-specific reaction is preferred in quantitative studies for two reasons. Firstly, it can demonstrate as much of the total nerve population as possible and therefore, non-specificity is an advantageous feature. Secondly, it allows for a comparison with other methods used for quantification of corneal nerves such as gold chloride impregnation and methylene blue which are also non-specific (Ishida et al., 1984). Several studies have been conducted to investigate the corneal nerves in different mammalian corneas qualitatively (Chiou et al., 1999; Linna et al., 1998; Morishige et al., 2009; Robertson and Winkelmann, 1970; Tervo, 1976) and quantitatively (Hernandez-Quintela et al., 1998; Ishida et al., 1984).

Using this modified technique several novel findings of corneal innervation in health and disease have been discovered and presented (Al-Aqaba

et al., 2011a; Al-Aqaba et al., 2012a; Al-Aqaba et al., 2018; Al-Aqaba et al., 2011b; Al-Aqaba et al., 2010b; Al-Aqaba et al., 2012b; Tervo and Palkama, 1978; Tervo et al., 1983). The other popular technique has been the neuronal class III beta-tubulin technique (βIII-tubulin). The latter technique has largely been used to demonstrate normal innervation patterns in animal corneas and some in normal human corneas (Chucair-Elliott et al., 2015; He et al., 2010; Kubilus and Linsenmayer, 2010; Marfurt et al., 2010). The most extensive studies on human corneal pathologies have used the AChE technique. Distinct features demonstrated by the AChE technique were not visible by the βIII-tubulin. For example, the bulb like termination of sub-Bowmans nerves at the perforation sites have been shown using AChE technique (Al-Aqaba et al., 2010b) and confirmed by IVCM (Al-Aqaba et al., 2010a) but were not seen with βIII-tubulin. Similarly, two recent studies on the architecture of corneal nerves using βIII-tubulin failed to show the limbal nerve corpuscles (He et al., 2010; Marfurt et al., 2010). This is most probably related to the intracytoplasmic location of this neuron-specific major cytoskeleton protein, which would be stained in the axons but not in the surrounding sheath. We, however, were able to demonstrate these large structures both en face and on cross section (light and transmission electron microscopy) using AChE method (Al-Aqaba et al., 2018) and confirmed by IVCM (Al-Aqaba et al. Clinical and In Vivo Confocal Microscopic features of Neuropathic Corneal Pain, Revision under review, BJO 2019). Features that are visible by both techniques, AChE and βIII-tubulin, show strong correlation. A similar correlation between the en-face imagining confocal microscopy technique and AChE findings in corneal diseases such as keratoconus, bullous keratopathy, post penetrating

keratoplasty and after CXL is also demonstrated (Al-Aqaba et al., 2011a; Al-Aqaba et al., 2012a; Al-Aqaba et al., 2011b; Al-Aqaba et al., 2012b).

AChE is intrinsic to blood vessels and nerves, thus where both are present the picture can be confusing. Differentiation between these two structures stained by AChE technique has been reported (Al-Aqaba et al., 2012b). Red blood cell membranes also stain with AChE hence it is possible to visual red blood cells in the lumen of blood vessels. Blood vessels lack the linear, longitudinal striations that are seen in nerves only. Cross section histological examination of 'nerves' do not show a lumen whereas that of vessels show a lumen. This approach has been used to corroborate the observed difference between nerves and vessels during optimisation of the staining techniques in vascularised corneas. Additionally, NanoZoomer scanning of the corneal whole mounts allows back tracing of all tortuous structures. Only those structures where the origin could be traced to major nerve trunks, with longitudinal striations were labelled as nerves. Aberrant nerves in the cornea seen in disease states often end in closed loops (Al-Aqaba et al., 2011a; Al-Aqaba et al., 2011b; Wolter, 1964, 1966), which is not a feature of blood vessels. Correlation of the AChE structures with clinically photographed nerves and blood vessels in particular, also allows differentiation of nerves and vessels in specimen removed during corneal transplantation and processed for AChE staining (Al-Aqaba et al., 2011a; Al-Aqaba et al., 2011b; Al-Aqaba et al., 2010b). In these studies all corneas were also examined clinically with the slit lamp prior to surgery.

6.1.2 Methylene blue staining technique

In a review of several histochemical techniques used for staining neural tissue, Weddell and Zander demonstrated sufficient details of corneal nerves most precisely with methylene blue (Weddell and Zander, 1950). This method is difficult to perform and requires high skill and attention to the technical procedure. In addition, non-neuronal corneal tissue like keratocytes may be stained with this dye and can be a source of confusion. Unlike cholinesterase, methylene blue can be used for in vivo staining (Weddell and Zander, 1950).

6.1.3 Sliver staining technique

This technique is less favourable because it requires thin tissue sections for staining and final nerve ramifications, such as those present in the epithelium, are difficult demonstrate. This is because epithelial cells usually stain deeply with silver. However, stromal nerves are fairly well demonstrated by this method (Weddell and Zander, 1950).

6.1.4 Gold chloride staining technique

These demonstrate the nerve pattern nicely in whole mounts but this technique has been superseded by the cholinesterase technique because of greater detail obtained with the latter method (Robertson and Winkelmann, 1970).

6.2 In vivo techniques: In vivo confocal microscopy (IVCM)

Although the human cornea is well known to be extremely sensitive, relatively few aspects of corneal nerve parameters, i.e. spatial arrangement and nerve density, have been illustrated by classical histological techniques. This is mainly because corneal nerves start to degenerate very quickly after death (Muller et al., 1997). In addition, these techniques require the specimen to be physically

cut and stained so considerable information about their spatial arrangement may be lost during specimen preparation (Guthoff et al., 2005). IVCM offers a great opportunity to study corneal nerves and other corneal structures in vivo. The basic principle of IVCM is to decrease light scattering from tissues outside the focal plane enabling optical sectioning of thin layers of the cornea resulting in producing two- or three-dimensional images. Intraepithelial nerves cannot be visualised with current devices but the sub-basal plexus is clearly demonstrated. As it allows enface imaging and montaging of images from over a large surface area, the complete picture is manifest. Indicators such as length, density, tortuosity, beading, reflectivity and branching patterns can be measured with dedicated software to give quantitative or semi-quantitative values. Not all confocal microscopes allow the same sub-basal nerve plexus quality studies. This is related to resolution, the ability to scan the periphery of the cornea and the same site repeatedly. The Rostock cornea module which we have used in all our studies has a side camera that allows localisation of the objective lens on the same site of the cornea (Petroll and Robertson, 2015).

7. Nerve affection in corneal pathology

VCM has become the gold standard technique for studying the morphology and morphometry of the human corneal nerves in health and disease (Cruzat et al., 2017; Patel and McGhee, 2009; Shaheen et al., 2014). Several factors can affect the accuracy of the morphometric measurements of corneal nerves. The type of confocal microscope, the quantification method and the area scanned must all be

taken into consideration (Cruzat et al., 2017; Labbe et al., 2012). Three types of in vivo confocal microscopes have been developed since the principle was first described by Minsky in 1955 (Petropoulos et al., 2019). These are tandem scanning, slit scanning and laser scanning confocal microscopes, the latter being more accurate and sensitive method for evaluating corneal nerves in vivo and allowing the detection of corneal immune and inflammatory cells as well (Qazi et al., 2015).

In normal subjects, the average sub-basal nerve density is 20 mm/mm^2 (18.8–21.4) in the central cornea and 10.5 mm/mm^2 (8.8–12.2) in the periphery (Cruzat et al., 2017). The diameter of sub-basal nerves has been reported to range from 0.52μm to 4.68 μm. Sub-basal nerve beading, which represents accumulation of mitochondira, can vary from 90 to 198 beads/mm^2 (Patel and McGhee, 2009).

Quantitative IVCM examination of the corneal stromal nerves remains controversial and challenging. This is probably due to the fact that stromal nerves cross the cornea obliquely, relative to the en face plane of confocal images. Image through the cross-centre of the nerve is, therefore, not always feasible, especially when saccadic eye movements are present (Patel and McGhee, 2009). The density of stromal nerves can range from 0.31 to 3.61 mm/ mm^2 and diameters from 5.5 mm to 11.4 mm in the healthy subjects (Cruzat et al., 2017).

7.1 Post-surgical conditions

7.1.1 Laser in situ keratomileusis (LASIK) and small incision lenticule extraction (SMILE)

In LASIK, nerve damage results from the transection of the nerves during creation of the flap with a blade or femtosecond laser pulses, and the subsequent ablation of stromal tissue and the nerves contained therein. In SMILE the extracted stromal lenticule brings with it a considerable number of anterior-mid stromal nerves (Mehta J, Al Aqaba M, Jung C, Nubile M and Dua HS, unpublished observations) (Fig. 17 and 18). Denoyer et al. have compared SMILE to LASIK for post-refractive ocular surface health. They reported a higher incidence of mild to moderate dry eyes in LASIK group up to 6 months after surgery (Denoyer et al., 2015). Furthermore, corneal sub-basal nerve parameters, including nerve density, number of long fibres, and branching as assessed by IVCM were significantly higher in the SMILE group compared with the LASIK group 1 and 6 months post-operatively. They concluded that LASIK has more profound impact on corneal innervation and subsequently ocular surface health particularly in early post- operative period when compared with SMILE. These findings have to be reconciled with the fact that a substantial number of stromal nerves are excised with the SMILE lenticule, as indicated above. This can be explained on the basis that the entire sub-basal plexus is an interconnected network with several larger nerve trunks carrying impulses via the stromal nerves to the limbal afferents and beyond. With SMILE several stromal nerves are cut but some remain and can carry information from the entire sub-basal plexus, retaining functional integrity. In LASIK, all the nerves along the entire circumference (except the hinge) of the flap are transected thus affecting sensitivity of the flap.

Experimental animal studies have shown a significant loss of epithelial, sub-basal, and superficial stromal nerves in the LASIK flap excluding the hinge area at 3 days after surgery. However, initial regenerative nerve fibres are also identified at that time. At five months, a near normal nerve architecture of the epithelial, sub-basal, and anterior stromal innervation is achieved. (Linna et al., 1998). One to two years after LASIK, the corneal regions with better sub-basal nerve morphology show better corneal sensitivity. These regions are near the flap hinge or the central cornea. According to Donnenfeld et al., transection of nasal and temporal oriented long ciliary nerve bundles from the corneal nerve plexus following creation of a superior-hinge flap leads to more marked loss of corneal sensation and more pronounced dry eye signs and symptoms than that with a nasal-hinge flap (Donnenfeld et al., 2003). The same feature has been reported with a narrow nasal-hinge flap compared with a wider hinge flap in which more nerves are left intact (Donnenfeld et al., 2004). However, in ~~recent~~ human IVCM studies on sub-basal nerve regeneration after LASIK, long sub-basal nerves are still identifiable in the central cornea 3 days after LASIK (Linna et al., 2000). Thereafter, they showed a great reduction in number, or were entirely absent by seven days (Avunduk et al., 2004; Calvillo et al., 2004; Lee et al., 2002; Linna et al., 2000; Mitooka et al., 2002; Pisella et al., 2001). With continued nerve regeneration, there is a gradual increase in the number of visible sub-basal nerves, in the central cornea by the first six months (Calvillo et al., 2004; Darwish et al., 2007c; Mitooka et al., 2002). However, two studies have shown that the morphometric features of this plexus had not returned to preoperative levels 6 months after surgery (Darwish et al., 2007a; Darwish et al., 2007c). On the other

hand, corneal sensitivity was decreased after LASIK but returned to normal levels 3 months after surgery. Therefore, it was concluded that there was no direct correlation between the nerve fibre length and density of sub-basal plexus and central corneal sensitivity (Darwish et al., 2007a; Darwish et al., 2007c). Other studies have revealed a significant correlation between central corneal sensitivity and sub-basal nerve morphology (Linna et al., 2000) and density (Lee et al., 2006; Perez-Gomez and Efron, 2003). This discrepancy might be due to difference in the ways by which the correlation was established. In studies where no correlation was found, the investigators have correlated the postoperative state of corneal sensitivity and sub-basal nerve morphology with their preoperative state, while in those studies that documented a strong correlation, the authors have compared the degree of corneal sensation with the level of sub-basal nerve regeneration postoperatively only. It is worth noting that in one long term follow-up study, there has been incomplete regeneration of the sub-basal nerves for up to 5 years following LASIK (Erie et al., 2005).

It is likely that in some individuals the nerves regenerate to preoperative levels and in others they do not. Despite the reduced density postoperatively corneal sensations have largely been reported to return to preoperative levels after an initial drop. Moreover, the physical recovery of nerves appears to lag behind the return of sensations. This suggests that there is redundancy in the density of nerves the cornea is endowed with or that our methods of testing corneal sensitivity (Cochet Bonnet aesthesiometer) is relatively crude and not sensitive enough to distinguish between finer degrees of loss of sensitivity. Transected nerve segments in the flap and stroma tend to be visible on IVCM for a variable

number of hours before they disappear, functional loss preceding morphological loss of the nerves. Depending on the timing of the examination, these segments may be counted as 'normal', compounding the nerve density measurements.

The contention that superior-hinge flaps are worse than nasal-hinge flaps is based on the premise that main bundles of nerves originating from the long posterior ciliary nerves that traverse in the nasal and temporal meridians are both transected in superior-hinge flaps and the nasal nerves are preserved in nasal flaps. This is not entirely accurate as the depth at which the main bundles enter the corneal periphery is mid to two-thirds of the corneal thickness, which is well posterior to the thickness of the flap either by mechanical keratomes or femtosecond laser. Hence it is unlikely that the major trunks are transected during making of the flap. Histological studies on human corneas that have undergone LASIK have not been described (to the best of our knowledge). We had the opportunity to study one eye 'a few years' post-LASIK by the AChE whole mount staining technique. Disrupted nerves with irregular thickening and aberrant regeneration in the form of thick closed loops were noted, primarily within the margins of the LASIK flap (Fig. 19) (Dua HS, Dhillon V unpublished observations).

7.1.2 Laser epithelial keratomileusis (LASEK)

There is a significant reduction in the sub-basal nerve diameter and density following LASEK and these do not recover to preoperative states even 6 months after surgery (Darwish et al., 2007a; Darwish et al., 2007c; Lee et al., 2006). Although tortuosity showed a significant decrease after LASEK, the sub-basal

nerve tortuosity measured by tortuosity coefficient returns to its normal preoperative level by 3 months (Darwish et al., 2007a). When comparing the regeneration of sub-basal nerve following LASIK and LASEK, different studies have reported different results. Whilst, Lee et al noted slower sub-basal nerve regeneration following LASIK in comparison with LASEK (Lee et al., 2006), Darwish et al found no differences in the recovery of sub-basal nerves following these procedures (Darwish et al., 2007c).

7.1.3 Photorefractive keratectomy (PRK)

Several studies using confocal microscopy have been conducted to investigate the corneal nerve regeneration and morphology over time following PRK. The immediate postoperative finding is the evident stromal nerve damage fifteen minutes after PRK (Heinz et al., 1996). At 1-2 months, the first sub-basal nerve bundles can be seen (Linna and Tervo, 1997) together with sprouting nerve fibres in the anterior stroma at the edge of the wound, directed toward the centre of cornea (Heinz et al., 1996; Kauffmann et al., 1996). At 5-8 months following PRK, regeneration of stromal nerves and sub-basal nerve plexus seems to be completed. However, abnormal branching and accessory thin nerve fibres were present (Heinz et al., 1996; Kauffmann et al., 1996).

Some investigators have noted that the sub-basal nerve morphology returned to its preoperative state at 1 year (Moller-Pedersen et al., 2000), while others have reported a bizarre pattern compared to controls 2 years following PRK (Bohnke et al., 1998). Regarding sub-basal nerve density after PRK, a 59% reduction in the density at 1 year has been reported but it returned to its pre-

operative density 2 years following PRK (Erie et al., 2005). Moreover, no significant difference in the number and density of central sub-basal nerve fibres were found when compared to controls at 5 years following PRK (Moilanen et al., 2003). In this study, 71% of patients showed a normal branching pattern.

In LASEK and PRK there is the added element of epithelial healing in addition to anterior stromal healing. Cytokines and growth factors related to epithelial healing are known to influence keratocyte 'activation' and inducing haze with an exaggerated healing response in the stroma (Lim et al., 2003). What effect these molecules have on nerve regeneration is unknown. Irregular nerve sprouting and persistence of abnormal thin nerves could be related to the epithelial wound healing response. As far as the causes of nerve damage in these procedures are concerned, it seems likely that the microkeratome cut causes most of the nerve damage in corneas after LASIK. However, in PRK all epithelial nerves are removed with the removal of the epithelium by whatever means, sub-basal nerves photoablated, and a certain degree of stromal nerve injuries is induced by photoablation as well (Tervo et al., 2002).

7.1.4 Corneal crosslinking

Corneal crosslinking (CXL) for keratoconus has various techniques with different initial effects on sub-basal and stromal nerves. The immediate effect of corneal crosslinking was studied in an ex-vivo model where corneoscleral buttons were subjected to epithelium-on or -off corneal crosslinking. There was complete absence of the sub-basal nerves in the epithelium-off group while they were intact in the epithelium-on group. Stromal nerves although present, showed localised

swelling, axonal discontinuity, and interruption of axonal membrane in the area that was treated with epi-off CXL (Fig. 20) (Al-Aqaba et al., 2012a).

Other studies have shown that crosslinking whether epithelium-off or -on leads to initial reduction of anterior stromal keratocytes and nerve densities. Regeneration starts at 7 days post-operatively by nerve sprouting from neighbouring non-injured nerves. At 3 months, there is excessive stromal nerves regeneration in the anterior stroma which reaches preoperative levels at 6 months (Xia et al., 2011). Sub-basal nerves density was found to be markedly reduced in the first 6 months following epithelium-off CXL but complete regeneration was seen by IVCM after 12 months with return of corneal sensation to baseline (Mazzotta et al., 2015) while trans-epithelial crosslinking with iontophoresis (I-CXL) was associated with less damage to the sub-basal nerves and return to normal density within 6 months (Bouheraoua et al., 2014).

The ex vivo study on human corneas would suggest that almost all the damage that occurs to nerves during CXL is related to the removal of the epithelium. However, IVCM based in vivo studies suggest damage to the anterior stromal nerves as well, which implies that this may take a relatively longer time to manifest and is likely to be associated with the host inflammatory response that will be absent in the ex vivo model.

7.1.5 Corneal transplantation

Penetrating keratoplasty (PK) is a major surgical procedure wherein all corneal nerve bundles entering the cornea are severed. This often performed in the background of a corneal pathology itself would have caused alterations in both

function and structure of the nerves. The knowledge of corneal nerve regeneration and repair in this context is therefore important. In a 12 month follow up study after penetrating keratoplasty, no clinically observable sub-basal nerves were identified over. (Darwish et al., 2007b). In a smaller longitudinal study of a series of cases series, presumed nerve structures have been observed at the level of Bowman's layer at one year post operatively (Hollingsworth et al., 2006). In both studies, stromal nerves were observed in the central cornea from 6 months after surgery. The longest longitudinal study was performed by Richter et al (Richter et al., 1996), documenting re-innervation of the central cornea with stromal nerves at 7 months and sub-basal nerves at 2 years post operatively. The long-term effects of penetrating keratoplasty on sub-basal corneal nerves have also been investigated in larger cross sectional studies. Findings are sub-basal nerves appear tortuous and disoriented and nerve density is reduced, even four decades after surgery (Niederer et al., 2007; Patel et al., 2007).

Among different indications for penetrating keratoplasty, patients with keratoconus showed greater regeneration of the sub-basal nerve plexus (Niederer et al., 2007). This might be due to the fact that corneal nerves in the peripheral host rim are relatively healthy allowing relatively rapid regeneration after PK. Considerable changes, in the form of thickening and looping and coiling, have been noted in corneal nerves in keratoconus. These changes are largely restricted to the ectatic area of the cornea while the periphery remains essentially normal. (Patel and McGhee, 2006) The regenerating nerves have to traverse the scar tissue between donor and host tissue to reach the centre of the donor cornea (Escapini, 1955; Rexed, 1951). The scar tissue may have a barrier effect slowing the

progression of the regenerating nerves or may provide a plane of less resistance directing nerves to grow along the graft host junction as is classically seen with any invading vessels after PK (Fig. 21) (Al-Aqaba et al., 2012b). Where the underlying disease has resulted in attrition and attenuation of peripheral nerves it is to be expected that the regenerating response would be delayed, aberrant and incomplete.

After PK, sub-basal nerves are seen to approach the central cornea travelling directly from the host sub-basal plexus to the donor cornea and also from regenerated stromal nerves, passing anteriorly through the Bowman's layer as they normally do (Fig. 21). Varied patterns of stromal nerve regeneration are seen with in the same transplanted corneal button, these may be straight and angular resembling normal nerves, coiled and tortuous resembling aberrant regeneration and some area may be devoid of nerves (Fig. 22). Central corneal sensitivity is reduced in grafted eyes and returns to near normal levels after 12 months. There is no direct association between the sub-basal nerve regeneration and recovery of central corneal sensitivity. This could be due to the limitation of IVCM in detecting fine regenerating nerve fibres that contribute to the restoration of the corneal sensitivity postoperatively. (Darwish et al., 2007b) Other alternative explanations could be the redundancy of corneal nerve density or the inadequate sensitivity of the Cochet-Bonnet aesthesiometer as discussed in the context of laser refractive surgery in section 7.1.1.

Although tear film function has been shown to recover significantly faster in DALK patients compared to PK patients, there was no significant difference in corneal sensitivity between the two groups at 12 months postoperatively.(Lin et

al., 2014). The earliest appearance of the sub-basal nerve was documented at about 6 months after surgery in both groups. In addition, there was no statistical difference in the number and density of the sub-basal nerves, even 5 years following DALK and PK (Zhang et al., 2013). This is expected as the transection of tissue and nerves contained therein is identical for DALK and PK barring the retention of pre-Descemets layer (Dua's layer, DL) of 15-20 microns, which like the deep stroma does not contain any nerves (Dua et al., 2015).

Descemet's membrane endothelial keratoplasty (DMEK) have been shown to result in short term reduction of the sub-basal nerve density and corneal sensitivity which return to their preoperative values between 4 to 20 months (Bucher et al., 2014). The authors' suggest that surgical trauma including corneal incisions and descemetorhexis are the most likely reason for the transient nerve alteration. Neurotrophic factors released by the normal corneal endothelial cells of the graft may contribute to nerve regeneration. Ahuja et al found a similar trend in patients following Descemet-stripping endothelial keratoplasty (DESK). The sensitivity diminishes 1 month postoperatively and returned to preoperative values by 24 months. However, the sub-basal nerve regeneration appears retarded with abnormal branching pattern (Ahuja et al., 2012). Endothelial keratoplasty procedures, DSEK, pre-Descemets endothelial keratoplasty (PDEK) and DMEK, are the main stay interventions for endothelial failure (Singh et al., 2018). These procedures involve small incisions at the limbus as for cataract surgery. Though some nerve transection can and does occur at the incision sites the damage in never as much as in DALK and PK. In DSEK a finite amount (100-150 microns) of stroma is also transplanted; in PDEK, DL and Descemets membrane (DM) is

transplanted and in DMEK only the DM is transplanted. Hence, it is almost certain that no donor nerves are transplanted as in DALK and PK. This could have a bearing on the nerve changes seen in the immediate and late postoperative periods. While evaluating nerve changes following corneal transplantation it is important to consider that much of these will relate to the underlying pathology and not just the type of procedure performed.7.1.6 Cataract surgery

Following extracapsular cataract surgery, a significant reduction in the sensitivity is confined to a wedge-shaped area extending from the scleral wound and encompassing the central cornea (John et al., 1988). This is in contrast to a small area of reduced sensation around the incision site in phacoemulsification. When compared to the superior clear corneal incision, temporal incision seems to induce a larger decrease in corneal sensitivity, albeit not significant (Kim et al., 2009). Corneal sensation returns to its preoperative levels by 3 months, despite suboptimal regeneration of sub-basal nerve fibres as confirmed by confocal microscopy (Kim et al., 2009). There is notion that the temporal long ciliary nerve is damaged with temporal incisions (see also section on LASIK 7.1.1). If this were the case, innervation in a significant area would be affected. It has been shown that the long ciliary nerves arborize far posterior form the limbus and the branches enter the limbus equally all around (Al-Aqaba et al., 2010b; He et al., 2010; Marfurt et al., 2010). The risk of damaging the main trunk of the long ciliary nerve in limbal incisions is low or none at all.

7.2 Diabetic keratopathy

Diabetic keratopathy is characterised by abnormal innervation of the cornea that results in decreased sensitivity and impaired epithelial wound healing (Bikbova et al., 2018). It represents a form of neurotrophic keratopathy where patients are at a higher risk of ocular complications such as surface irregularities, corneal erosions, corneal infections, and stromal opacification. In neurotrophic keratopathy the balanced mutual dependence of epithelial cells and nerves is disrupted leading in damage to both structures (Dua et al., 2018).

Sub-basal nerve plexus is frequently affected in diabetic patients with neuropathy and the changes depend on the severity of neuropathy. It has been found that the number of fibres, number of beadings, and branching pattern of fibres in the sub-basal plexus decreases in cases of mild to severe neuropathy (Hossain et al., 2005; Malik et al., 2003; Midena et al., 2006) but corneal mechanical sensitivity decreases only in cases with severe neuropathy (Rosenberg et al., 2000). The epithelium of diabetic patients with advanced neuropathy is much thinner than that of diabetic patients without neuropathy, (Rosenberg et al., 2000) which could influence the subjective response to sensitivity testing. Moreover, it has been shown that there is a direct correlation between corneal sensitivity and the number of nerve fibres in the sub-basal plexus and between corneal diabetic neuropathy with peripheral diabetic neuropathy. Quantitative analysis has also shown greater tortuosity in patients with greater severity of peripheral diabetic neuropathy (Chang et al., 2006; Kallinikos et al., 2004). The visibility of corneal nerves by IVCM enables their assessment and serves as a useful clinical marker of peripheral diabetic neuropathy (Boulton, 2007; Pritchard et al., 2015). Proliferative diabetic retinopathy is also significantly associated with

lower sub-basal nerve densities (Mocan et al., 2006). Surprisingly, diabetic patients with normal corneal and vibration sensation show significant changes in the sub-basal nerve parameters compared to healthy controls (Messmer et al., 2010). Therefore, IVCM is capable of detecting diabetic nerve fibre damage earlier than corneal aesthesiometery and perception of vibration in the lower limb (Messmer et al., 2010). This reinforces the notion of redundancy of corneal innervation wherein for a measurable loss of sensitivity as assessed by the Cochet-Bonnet aesthesiometer, there has to be a significant loss of visible nerves in the sub-basal plexus. Interestingly, IVCM can detect abnormalities of sub-basal nerve fibres in the inferior whorl complex in diabetic patients even before the development of clinical peripheral diabetic neuropathy (Petropoulos et al., 2015). In addition to corneal nerve alteration, diabetic cornea demonstrates decreased basal epithelial cell density, reduced anterior stromal keratocyte counts, and endothelial cell density (Bitirgen et al., 2014). These cellular changes can only been seen in diabetic patients with retinopathy, while nerve alterations seem to precede the development of diabetic retinopathy. It is worth noting that increasing duration of type 2 diabetes has been linked to the progressive degeneration of sub-basal nerve plexus (Lagali et al., 2017).

Other cellular changes haven been observed in diabetic corneas. Qu et al showed an increase in the densities of Langerhans cells in patients with type 2 diabetes mellitus who have corneal punctate epitheliopathy. These changes were related to the reduction in basal epithelial cell density leading to a delayed corneal wound healing (Qu et al., 2018).

A previous study has provided evidence that panretinal photocoagulation for diabetic retinopathy is associated with further reduction in sub-basal nerve density and this damage seems to be due to the effect of argon laser on the long ciliary nerves as they traverse the suprachoroidal space and could be directly hit by the laser pulses or be involved in the induced scarring in the choroid (De Cilla et al., 2009).

7.3 Herpetic corneal disease

Herpes simplex infection of the ocular surface is the leading cause of neurotrophic keratopathy and infectious corneal blindness in the developed world (Farooq and Shukla, 2012; Looker and Garnett, 2005). Corneal nerves are at the heart of the pathophysiological mechanism and the phenotypical manifestation of the disease. During primary infection, corneal nerves serve as a conduit for the herpes virus to track into the trigeminal ganglion via retrograde axonal transport, where a latent HSV-1 infection is established. Following reactivation, viral particles travel down sensory nerve axons to the ocular surface where they replicate and induce cytopathic effect.

In patients with herpes simplex keratitis (HSK), IVCM has shown a significant reduction in the density, number and branching of the sub-basal nerve plexus (Hamrah et al., 2010). The chronicity of the infection episodes has been associated with greater nerve damage. In acute phase of the disease, loss of corneal sensation correlates strongly with profound reduction of the sub-basal nerve density. However, anatomical nerve regeneration is often associated with poor functional recovery, even 3 years following the last infection episode

(Chucair-Elliott et al., 2015; Moein et al., 2018). The contralateral, clinically unaffected eyes also show a diminishment of sub-basal nerve plexus as compared with normal controls, suggesting bilateral nerve alteration in a clinically unilateral disease (Hamrah et al., 2010). Furthermore, Tear function has been shown to be impaired in the unaffected eyes of patients with unilateral recurrent herpetic keratitis, even when the disease is apparently quiescent (M'Garrech et al., 2013). This finding suggests that recurrent disease induces a reduction in the afferent pathways of the tear reflex from the affected eye, leading to tear abnormality in the unaffected eye.

Following resolution of HSK, from a month to 12 months later, a majority of patients have a normal looking sub-basal plexus though a few patients can show either complete absence of sub-basal nerve plexus or reduced number of nerve fibre bundles (Rosenberg et al., 2002).

In an experimental animal model, sympathetic hyper-innervation of the infected cornea is causatively linked to the pathological changes observed in HSK including thinning and scarring (Yun et al., 2016). Surprisingly, sympathetic nerves have also been shown to prevent corneal reinnervation by sensory nerves and promote severe and persistent stromal inflammation. Surgical sympathectomy is shown to halt or reverse the process of corneal neovascularisation, scarring and thinning and is often associated with restoration of sensory innervation (Yun et al., 2016). Following penetrating keratoplasty and deep anterior lamellar keratoplasty, corneal nerves are cut along the entire circumference. Aberrant and limited nerve regeneration in corneal grafts has been observed even 14 years following surgery, where a large number of nerves failed to regenerate and extend

beyond the area of graft host junction (Al-Aqaba et al., 2012b). This blocks the free access of viral particles to the donor cornea. Extensive virus replication and shedding have been detected at the graft host junction without clinical signs of disease (Nicholls et al., 1996). This regeneration pattern possibly explains the altered clinical appearance of recurrent HSK in corneal grafts where lesions can start only in the vicinity of trephination line, often geographic, and lack of typical dendrites and terminal bulbs (Remeijer et al., 1997; Rezende et al., 2004). Late recurrences are located more centrally inside the donor button corresponding with further progression of the regenerating nerves.

In herpes zoster virus keratitis (HZVK), the reduction of sub-basal nerve density is strongly linked to the profound changes in corneal epithelium e.g. increase cell size, decrease epithelial cell density and squamous metaplasia (Hamrah et al., 2015). The recovery of corneal sensation and innervation is possible, though many years after the last infection episode (Cruzat et al., 2016). The dendritic pattern of HZVK is similar but not identical to HSVK. The dendrites are described as ‘elevated’ rather than ‘ulcerated’ but are likely to be related to the epithelial nerves as (Piebenga and Laibson, 1973) both herpes zoster and simplex viruses have an affinity for nervous tissue.

7.4 Dry eyes

Aspects of dry eye are covered in sections 5.2 and 5.4. The Tear Film Ocular Surface society Dry eye workshop-2 (TFOS DEWS II) report defines dry eye disease thus “Dry eye is a multifactorial disease of the ocular surface characterised by a loss of homeostasis of the tear film, and accompanied by ocular

symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles" (Craig et al., 2017). Inflammation and neurosensory abnormalities play a role in the pathophysiology of DED and are both accentuated by DED being the principle factors contributing to pain, soreness and other symptoms. Hyperosmolarity leads to inflammation and nerve damage, which when perpetuated over time lead to altered gene expression of ion channel and receptor proteins, sensitisation of nociceptors, altered excitability and abnormal firing of impulses (Craig et al., 2017).

It is well known that Sjogren's syndrome is associated with increased prevalence of peripheral and cranial neuropathy (Meijering et al., 2004). In addition, a relation has been suggested between corneal innervation and aqueous tear production. Therefore, different studies using IVCM have been conducted to investigate the sub-basal nerve plexus in patients with aqueous tear deficiency. With regard to the effect of dry eye on sub-basal nerve density, results are inconsistent. While some studies report a significantly reduced sub-basal nerve density in both Sjogren's and non-Sjogren's syndrome dry eyes compared to normal (Benitez del Castillo et al., 2004; Shin, 2002), others have observed no significant differences in the density (Hosal et al., 2005; Meijering et al., 2004; Zhang et al., 2005). One study even reported an increased number of nerves per frame in patients with Sjogren`s syndrome compared to controls (Zhang et al., 2005). Another finding reported is an increased sub-basal nerve tortuosity in Sjogren`s syndrome (Benitez del Castillo et al., 2004; Shin, 2002; Zhang et al., 2005), which was hypothesised to be due to the release of nerve growth factors in

response to the inflammatory process (Shin, 2002). The conflicting reports of increased or decreased nerve density and other parameters in dry eye and their return to normal in response to treatment (Cagini et al., 2018) could be reconciled by considering that dry eye disease is a spectrum starting with increased nerves and/or increased sensitivity (hyperaesthesia) in the early stages followed by reduction in nerves and hypoaesthesia as the disease progresses (Iaccheri et al., 2017; Stepp et al., 2018).

Active DED affects not only the nerves but epithelial cells and keratocytes as well. Laccheri B (2017) noted increase in cell density of the intermediate corneal epithelial cells, activation of keratocytes and increased density, tortuosity and reflectivity of sub-basal nerves; all of which returned to normal levels on treatment with cyclosporine drops indicating that altered behaviour to sensory stimuli and trophic effects, both play a role in DED but can be reversed in the early stages of disease but the same may not be possible later in the course when permanent structural changes and nerve loss occur (Belmonte et al., 2017).

7.5 Keratoconus

Early IVCM studies regarding alteration in the corneal nerve morphology in patients with keratoconus have been limited to qualitative analysis, with observations such as " sub-basal nerve fibres running in and out of the plane of the field in the central cornea" (Tervo et al., 1982). Quantitative studies have shown that patients with keratoconus exhibit a significant decrease in the sub-basal nerve density (Patel et al., 2008; Patel and McGhee, 2006; Simo Mannion et al., 2005; Tervo, 1977). However, a considerable reduction in corneal sensation

has been revealed only in patients with keratoconus who wore contact lenses routinely (Patel et al., 2008). Stromal nerve bundles are thicker than normal, especially within the area of the cone explaining why prominent corneal nerves are often seen using slit lamp biomicroscopy. (Mannion et al., 2007). Some authors have linked these nerve changes to progression of keratoconus (Brookes et al., 2003). IVCM and immunohistological studies on keratoconus buttons removed during corneal transplant surgery has revealed abnormal architecture with a tortuous network of nerve fibre bundles at the apex of the cone; many of these bundles formed closed loops. The sub-basal blub like structures too are thickened in keratoconus (Al-Aqaba et al., 2011b; Patel and McGhee, 2006). At the topographic base of the cone, nerve fibre bundles appeared to follow the contour of the base, with many of the bundles running concentrically in this region. On histology, stromal nerves within the conical region showed a series of changes of varying severity and were classified into 3 grades based on the extent and severity of the morphologic alterations. In grade 3, there is an excessive overgrowth of tortuous nerves forming a very complex network within the central cornea (Fig. 23). In grade 1, the changes are in the form of mild looping and coiling of the central stromal nerves. These findings provide an evidence of the involvement of corneal nerves in keratoconus and suggests further that they may play a role in the pathophysiology of the disease progression (Al-Aqaba et al., 2011b).

7.6 Contact lens wear

Although associated with reduced corneal sensation (Patel et al., 2002), long term contact lens wear does not appear to affect the morphology,

distribution or number of corneal nerves (Oliveira-Soto and Efron, 2003; Patel et al., 2002). Non-structural functional nerve changes have been attributed to the decreased corneal sensitivity in these subjects. This may be a sensory adaptation of the nerves to the constant presence of the contact lens much like the unawareness of the clothes we wear on a daily basis (Graczyk et al., 2018).

7.7 Fuchs' Endothelial Corneal Dystrophy (FECD) and Bullous keratopathy (BK)

Corneal nerve changes in conditions associated with chronic corneal edema have been studied in pseudophakic bullous keratopathy, Fuchs' endothelial corneal dystrophy (FECD) and failed corneal grafts. Though all conditions have persistent corneal edema they fundamentally differ in relation to transection of corneal nerves in those that have had penetrating keratoplasty compared to those that have not. Some may have had an endothelial keratoplasty procedure where the nerve trauma is expected to be different to those who have undergone penetrating keratoplasty. Ahuja et al. studied sensitivity and nerve morphology in patients with FECD and reported low sensitivity and density prior to endothelial keratoplasty (EK), a further drop after the operation and slow recovery to pre-operative levels (Ahuja et al., 2012). As the patients underwent EK it is presumed that the indication was endothelial cell dysfunction and corneal edema, implying an association of the reported changes to edema. Data on corneal thickness and endothelial cell counts is not provided. They noted that stromal nerves were frequently tortuous and formed loops with the abnormalities persisting up to 36 months post EK. Kobayashi et al (Kobayashi et al., 2008) noted that patients who underwent Descemets stripping automated keratoplasty surgery had a change in

the morphology from honey comb pattern, consistent with edema, to normal not activated keratocytes.

Al Aqaba et al. (2011) studied corneal nerve changes in BK with in vivo confocal microscopy and AChE staining of buttons removed for BK eyes during PK (Al-Aqaba et al., 2011a). They reported that the nerve density was lower, the branching pattern less apparent and the sub-basal nerves were thinner in BK compared to normal. Other striking anomalies such as localised thickenings or excrescences and abnormal twisting, coiling, and looping of the mid-stromal nerves were also noted. The localised thickenings along the course of the nerve were interesting as they represented either the stub of a degenerated nerve or the starting point of aberrant nerve re-generation. It is not just the nerves that show structural changes in BK (Fig. 24 and 25). Subepithelial fibroblasts with scarring, altered keratocyte cell bodies and absent or decreased sub-basal nerves were seen both histologically and on IVCM in patients with BK of long standing, secondary to FECD and cataract surgery (Alomar et al., 2011). With the popularity of EK these findings raise an interesting question. Are we leaving behind a normal cornea following EK compared to PK? How long do the changes described above persist and do they every return to normal is unknown? From clinical experience it appears that vision improves despite reduced sensitivity and residual changes in nerves and resident cells, raising once again the question of structure-function correlation, redundancy and what constitutes normal as far as vision is concerned?

BK is often encountered as a unilateral disease, with patients presenting to ophthalmic emergency care due to an acute onset of pain in the background of blurred or misty vision. It was thought that the ocular pain in BK is caused by

defects in the corneal epithelium exposing the corneal nerve endings as the fluid filled epithelial bullae rupture or due to nerve stretching and irritation by the epithelial and subepithelial bullae. However, Al-Aqaba et al (Al-Aqaba et al., 2011a) have demonstrated that sub-Bowman's nerves rather than sub-basal nerves may be contributing to the symptoms of pain. This explanation was based on their finding of reduced sub-basal nerves (diameter and branching) indicating attrition of sub-basal plexus in chronic corneal edema which does not reconcile with the increased pain experienced by these patients. Aberrant morphology and hyper-regeneration of the sub-Bowman's and mid-peripheral stromal nerves may contribute to the symptom of pain in BK.

7.8 Neurotrophic keratopathy (NK)

Aspects of NK are covered in sections 4.2 and 4.3.3. The hallmark of NK is affection of corneal nerves, which manifests as reduced or absent corneal sensations associated with epitheliopathy, epithelial defect and ultimately stromal ulceration (Dua et al., 2018; Mertsch et al., 2018). Absence of corneal sensations with preservation of trophic functions carriers a good prognosis compared to conditions where the nerve damage is such that both trophic and sensory functions are affected. (Dhillon et al., 2016) Although stromal involvement is traditionally regarded as stage 3 (severe grade) of NK, recent evidence with OCT examination has shown that stromal involvement occurs early in the course of the disease and is present throughout (Mastropasqua et al., 2018). Understanding of pathogenesis of NK stimulated the development of recombinant nerve growth factor (rhNGF) eye drops, for the treatment of stage 2 and 3 NK. Replacement of deficient nerve

growth factor promotes the healing of epithelium and nerve regeneration (Bonini et al., 2018a; Bonini et al., 2018b; Dua et al., 2018).

Corneal neurotisation is a surgical approach that restores corneal sensation by reinnervating the stromal and sub-basal layers of the cornea. The first report of IVCM in patients with NK and corneal neurotisation was published by Fung et al (Fung et al., 2018; Malhotra et al., 2019), where it was shown to restore sensory and trophic functions of corneal nerves, effectively halting the progression of NK and preventing further visual loss. Another case report by Ting et al (Ting et al., 2018) pointed out that the findings on IVCM and histopathological examination suggest that partial regeneration/maintenance of corneal nerves occurring after corneal neurotisation surgery is likely attributed to the paracrine neurotrophic support, instead of direct sprouting, from the perilimbal transplanted nerve fascicles.

7.9 Glaucoma

IVCM has been used to evaluate microstructural changes in different types of glaucoma as well as to assess corneal changes induced by topical antiglaucoma medications and their preservatives and study conjunctival wound healing process specially in filtering blebs. Gatzioufas et al. studied the corneal morphology in primary congenital glaucoma, and found that the anterior and posterior stromal keratocyte density was significantly reduced, compared with normal controls but there were no observed differences in the keratocyte morphology(Gatzioufas et al., 2013). In pseudoexfoliation syndrome and glaucoma, studies have shown marked reduced density of sub-basal nerves and keratocytes in the anterior and

posterior stroma as well as endothelial cells with increased endothelial cell polymegathism and pleomorphism (Kocabeyoglu et al., 2016; Yuksel et al., 2016).

Several studies have shown reduced density and increased tortuosity and reflectivity of sub-basal nerves in patients on antiglaucoma drops (Baghdasaryan et al., 2018), thus highlighting the importance of IVCM as a potential tool to assess the level of toxicity of topical antiglaucoma drugs specially those containing preservatives and to identify those patients who would best benefit from laser trabeculoplasty or minimally invasive surgery to control the disease without detrimental effect on the ocular surface and corneal innervation (Labbe et al., 2012; Zhang et al., 2019). Patients on preservative containing antiglaucoma medications have a greater reduction of sub-basal nerve density and corneal hypoesthesia, when compared to preservative free drugs (Martone et al., 2009). Although hypoesthesia is often associated with improvement in patient symptoms despite presence of signs, this is not always the case as nerve pathology can be associated with allodynia and hyperalgesia in the presence of hypoesthesia. Another study conducted in France compared patients on antiglaucoma medications and dry eyes to normal subjects and found that corneal sensation was significantly decreased in the former two groups. In the dry-eye group, corneal sensitivity correlated with the density and the number of nerves, whereas in the glaucoma group, corneal sensitivity correlated only with the tortuosity of sub-basal nerves. This highlights the importance of understanding the pathophysiology of the disease process to understand the correlation between corneal sensation and sub-basal nerve morphology. Cyclo-destructive procedures to control advanced

glaucoma such as cyclo-photocoagulation did not seem to affect the morphology or density of the sub-basal nerves (Raivio et al., 2002).

7.10 Corneal dystrophies

Corneal dystrophies are a heterogenous group of genetic, often progressive, bilateral non-inflammatory corneal diseases, which are characterised by deposition of abnormal material (Klintworth, 2009). Out of 25 types of corneal dystrophies, two are often associated with significant alterations of nerve structure and/or function. These are lattice corneal dystrophy (LCD) and epithelial basement membrane dystrophy (EBMD). Keratoconus and FECD are discussed in sections 7.5 and 7.7 respectively.

Lattice dystrophy typically starts as superficial fine nodular opacities in the central cornea, which appear to have stellate-shaped extensions when examined with slit lamp at high magnification (Wolter and Hendrson, 1962). Coalescence of the opacities form radially oriented linear deposits extend from the centre to the periphery, sparing the limbus. The latter appearance closely resembles the branching pattern of corneal nerves as demonstrated by several histological staining methods (Al-Aqaba et al., 2010b; Marfurt et al., 2010). A previous study has revealed that hyalinisation of the corneal nerves is responsible for the clinical appearance of linear opacities (Wolter and Hendrson, 1962). Furthermore, the clinical observation of the direct continuity of corneal nerves with the linear opacities at the periphery provides further evidence of the involvement of nerves. In addition, many patients with lattice dystrophy suffer from corneal hypoesthesia or anaesthesia (Meretoja, 1972), which probably

accounts for the development of recurrent corneal erosions and poor wound healing in these cases (Martin and Safran, 1988). Therefore, degeneration and hyalinisation of the corneal nerves is often considered the primary pathology of lattice dystrophy.

In familial amyloidosis, slowly progressive involvement of cranial nerves including the trigeminal, facial, glossopharyngeal, accessory, and hypoglossal nerves is clinically evident in patients with asymptomatic corneal lattice dystrophy (Boysen et al., 1979). Impaired corneal sensitivity in those patients is correlated with the reduction in density of sub-basal nerve plexus and the number of long fibre bundles on IVCM (Rosenberg et al., 2001).

EBMD is a common anterior corneal dystrophy, with a prevalence of at least 2% in the population (Waring et al., 1978). The majority of EBMD cases are asymptomatic and only 10% of patients present with recurrent corneal erosions. IVCM shows a reduction of up to 50% in the sub-basal nerve density when compared to the healthy population (Germundsson and Lagali, 2014). The poor adhesion of the basal epithelium to the underlying basement membrane or the hemidesmosome complexes to the Bowman's zone via anchoring filaments, with subsequent erosions result in disruption and abnormal regeneration of the sub-basal nerves (Germundsson and Lagali, 2014). Stromal nerves remain intact in this condition (He and Bazan, 2013). Interestingly, the improved sub-basal nerve density following phototherapeutic keratectomy has been attributed to regeneration from the peripheral sub-basal nerves.

7.11 Limbal stem cell deficiency (LSCD)

Limbal stem cells play a pivotal role in the maintenance of normal homeostasis of the corneal epithelium and wound healing (Deng et al., 2019; Dua et al., 2000). LSCD is defined as "an ocular surface disease caused by a decrease in the population and/or function of corneal epithelial stem/ progenitor cells; this decrease leads to the inability to sustain the normal homeostasis of the corneal epithelium" (Deng et al., 2019).

On IVCM, LSCD is associated with a magnitude of changes in the corneal microstructure. These include a decrease in basal epithelial cell density and epithelial thickness, the presence of metaplastic cells with hyper-reflective nuclei and the presence of goblet cells within the conjunctivalised corneal epithelium (Chan et al., 2015a; Chan et al., 2015b; Deng et al., 2012; Miri et al., 2012).

Alterations in sub-basal nerve plexus have been the primary focus of nerve research in LSCD. Several studies confirmed the reduction in the sub-basal nerve density and increased nerve tortuosity in stage-dependent manner (Chuephanich et al., 2017; Miri et al., 2012). In one study, sub-basal nerves were detected in 22% of patients with total LSCD and in 100% of patients with partial LSCD (Miri et al., 2012). The density of sub-basal nerves was 4.26 ± 0.66 mm/mm^2 in cases of total LSCD and 9.70 ± 6.32 mm/mm^2 in cases of partial LSCD. Both figures fall significantly below the normal sub-basal nerve density previously published using laser scanning IVCM.

In normal corneas, sub-basal nerves and their associated intraepithelial nerve terminals run for several millimetres within the corneal epithelium without Schwann cell support. Emerging evidence suggest that corneal epithelial cells

function as surrogate Schwann cells during normal homeostasis and wound healing (Stepp et al., 2017). Therefore, loss of corneal epithelial phenotype could possibly explain the nerve dropout in the sub-basal plane in LSCD. In addition, the abnormal corneal and limbal epithelial cells in LSCD are unable to produce glial cell-derived nerve growth factors and nerve growth factors, which are necessary molecules to maintain neurite growth and regeneration (Qi et al., 2007).

There is another aspect to visualisation of sub-basal nerves through multiple epithelial cell layers by IVCM. In conjunctival intraepithelial neoplasia, the hyper-reflective nature of the epithelium and the thickness was considered to 'mask' the sub-basal nerves, which 're-appeared' as the CIN responded to treatment with mitomycin C. The central segmented nature of the re-appearing nerves corresponding to return of normality of the epithelium showed that a masking effect and visibility are factors to be considered (Alomar REF).

7.12 Small-fibre sensory neuropathy (SFSN)

Small-fibre sensory neuropathy (SFSN) refers to a spectrum of peripheral neuropathic conditions of diverse aetiologies that are characterised by damage to small-calibre sensory and/or autonomic nerve fibres (Hoitsma et al., 2004; Tavee and Zhou, 2009). It exclusively involves finely myelinated and unmyelinated fibres (Holland et al., 1998). Patients often suffer from burning, pain and autonomic deficits but with normal strength, proprioception, and reflexes (Gorson and Ropper, 1995). Symptoms are usually distributed in a stocking/glove pattern, suggesting a length-dependent neuropathy (Bucher et al., 2015). Alternatively, SFSN may manifest as an asymmetrical patchy pattern of sensory symptoms

primarily affecting the arms or even in the face, implicating a non–length-dependent ganglionopathy (Gemignani, 2012; Khan and Zhou, 2012).

SFSN can be caused by hereditary or acquired conditions (Holland et al., 1998). Hereditary causes include autosomal recessive hereditary sensory neuropathy, familial amyloidosis, and Fabry's disease. Acquired conditions include diabetes mellitus, systemic amyloidosis, and human immunodeficiency virus infection, and exposure to certain neurotoxic medications. However, no underlying cause can be identified in up to 47% of cases (Devigili et al., 2008), therefore these cases are categorised as “idiopathic.” Currently, the assessment of intraepidermal nerve fibre density through skin biopsy is the gold standard tool to establish the diagnosis (Lauria et al., 2010). Due to its invasive nature, skin biopsy has a limited application for longitudinal studies. More importantly, intraepidermal nerve fibre density does not often correlate with the disease severity (Bucher et al., 2015). It therefore becomes apparent that non-invasive measures are required to objectively evaluate the morphology of small sensory nerve fibres.

In a prospective single centre study, Bucher et al studied 14 patients with histologically confirmed SFSN. Corneal nerve parameters and dendritic cell density were assessed with IVCM and compared with age-matched healthy controls and correlated with symptoms, disease course, and histopathological findings (Bucher et al., 2015). They reported a reduction in nerve fibre density and total number of nerves with increased nerve tortuosity. However, these changes were not correlated with intraepidermal nerve fibre density or clinical symptoms. Similarly, Tavakoli et al evaluated corneal neuropathy in Fabry’s

disease and found that IVCM and non-contact corneal aesthesiometry provide novel means to detect early nerve fibre damage and dysfunction in patients with Fabry disease (Tavakoli et al., 2009).

IVCM evidence of corneal small fibre neuropathy has also been reported in Wilson disease (Sturniolo et al., 2015), fibromyalgia (Ramirez et al., 2015), sarcoidosis (Oudejans et al., 2017) and Parkinson's disease (Podgorny et al., 2016).

8. Future directions

Although numerous studies have contributed to our knowledge and a lot is known about corneal nerves in health and disease the one conclusion that can be drawn without any controversy is that a lot more needs to be learned, both in health and in disease. Part of this challenge is to dispense with some old conceptions and reconcile others with new discoveries and emerging knowledge. The long ciliary nerves, travelling anteriorly along the nasal and temporal meridians were believed to divide into multiple branches at the 3 and 9 O'clock positions to innervate the cornea leaving a watershed zone at the 12 and 6 O'clock positions. This fed the debate between preference for the nasal hinge (where the nasal fibres are not transected by the keratome blade) over the superior hinge (where both the 3 and 9 O'clock meridian are transected) in LASIK. As anatomical clarification emerged that the nerves were equally distributed along the entire circumference and the main trunks lay below the depth at which most keratome blades made their pass it became apparent that the location of the flap

was not paramount but thinner flaps were better. Inadequate knowledge drive hypotheses that become accepted as the 'norm' until proved one way or the other.

The terminal bulb-like structures located in the sub-basal plane from which the nerve bundles of the sub-basal plexus arise are there to be seen but their structure and function if any, needs to be deciphered. Their visualisation with some stains but not with others indicates that they relate to the nerve sheath and not the axoplasm or axolemma but that is where the tract runs cold. Ultrastructural studies supplemented with immunohistochemical studies should reveal information on structure, function and possible role in the afferent pathway of the nerves. Specific involvement of these structures in pathology should cast light on the basis of some clinical signs such as the multiple and isolated lesions of nummular keratitis and lesions of Thygeson's keratitis. Some clinical features of conditions such as lattice dystrophy and dendritic ulcers of herpetic eye disease relate to major trunks or fine terminal branches of corneal nerves. In the latter example the exact mechanism by which virus particles exit the nerve to infect the epithelium along the final length of the terminal branches is unclear. A better understanding of this association will provide insight in to nerve function and nerve pathology.

The intimate relationship between epithelial nerves and epithelial cells implies that with any abrasion, whatever the cause, the lost epithelium will take with it a network of nerve fibres, ripping them off their attachments to the sub-basal plexus. Though it is known that the terminal fine bundles and axons are always in a state of flux, how they respond to loss of large patches of cells is unclear. The whorl pattern of the entire corneal epithelial sheet and a

corresponding whorl pattern of the underlying sub-basal nerve plexus reinforces the close association of the nerves and epithelial cell. Regeneration of lost tissue to recreate the same association must require complex directional signalling in which the epithelial cells could direct the nerves or the nerves the cells. Research to monitor and understand the regeneration and re-association of nerves and cells is warranted to understand better the association and to expose clues related to nerve regeneration.

The LNC are recently discovered nerve related structures that are located at the limbus, have a distinct morphology and are intimately related to the limbal epithelial crypts. Here again the close association of nerves and epithelium is inescapable. Though a little is known of the structure of the LNC, nothing is known of their function. They resemble pressure sensors in the skin opening an exciting possibility of the existence of pressure sensors along the limbus, which may provide feedback on changes in corneal shape and curvature in response to external pressure applied by the lid blinks, lid squeezing, eye movement and eye rubbing. Any relationship to intraocular pressure would be pure speculation and blue-sky but as a notion, it cannot be brushed aside. Research to address these questions will gather pace and answers found to reveal the true role of these corpuscles.

The lack of correlation demonstrated in many studies, between corneal epithelial nerve density and corneal sensitivity needs an explanation. This discrepancy is noted both when nerves are lost as part of a disease process and during regeneration after attrition related to surgery, especially laser refractive surgery. The explanations put forth are either a redundancy between nerve supply

that exists and what is needed for the cornea to respond normally to environmental stimuli or the limitation of the instruments we have at our disposal, to assess corneal sensitivity. Exploration in both directions will yield scientific dividends, a better understanding of the catchment area of major nerve bundles and the overlap between the sensory fields on the one hand and the invention of better devices to measure and quantify not just normal and subnormal sensitivity but also hypersensitivity, which in some patients is a cause of significant morbidity and suicidal thoughts. Studies with functional magnetic resonance imaging together with sub-threshold stimuli are being contemplated to understand this hitherto inadequately explored area in the clinical setting.

Linked to the above is the correlation between function and structure. Many abnormalities are seen in corneal disease some by IVCM, some by whole mount histology and some by both techniques. How the structural changes relate to function is ill understood. Advances in measuring sensitivity will help to understand association of sensory function with structure for example why in BK, with absent sub-basal plexus there is intense pain? Is this related to gross stromal nerve anomalies seen in this condition? Conversely, in keratoconus, gross thickening of stromal nerves and sub-basal bulb-like structures are seen with almost no sensory symptoms. However, such changes could be a reflection of altered trophic function beyond that what is noted in NK. The predominant stromal nerve changes in keratoconus are seen in the area of ectasia. Can ectasia be a manifestation of altered trophic influences on the keratocytes? Trophic effects on corneal epithelium in NK are countered by treatment with recombinant NGF with effects that last well beyond the duration of administration of NGF

drops. Clinical trials on a number of different conditions are contemplated or required to ascertain effects of altered trophism and reversal thereof, on the disease condition being treated. Studies such as these should provide some answers to the question whether corneal nerve pathology drives the disease or altered nerve morphology is a consequence of the disease? Terms such as 'activated' or 'resting' nerves and keratocytes are attributed to these structures based on IVCM appearances, implying a structure-function relationship. These are based on some available evidence but at best are hypotheses that need to be proved or disproved. Besides keratocytes, there are other cells in the stroma notably dendritic cells that are also 'activated' and migrate to the corneal stroma in response to inflammatory stimuli. The role of these cells in modulating corneal nerve responses is being studied and should help to build our understanding of inflammation and induced symptoms originating from the cornea.

Most IVCM kits only capture a very small area of the cornea and image 'stitching' software is used to build a 'bigger picture' unlike whole mount staining of corneal buttons that provide a complete picture of the nerve pathology. As the latter can only be done in-vitro it can only provide a snapshot of the pathology at time the cornea was removed for grafting, which is usually in a more advanced stage of the disease process. Advances in technology related to hardware and software of IVCM will allow longitudinal examination over wider surface areas both during the course of the disease process and its response to treatment. It is more than likely that the studies indicated will progress and accrue data at pace such that answers to some of the questions posed are probably just around the corner.

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12. Legends for Figures

Fig. 1. Acetycholinestrase stained corneoscleral rim from a normal donor showing a dense network of fine and thick nerve bundles forming the limbal nerve plexus. They are derived from the long ciliary (uveal) nerves. Bar = 500 microns (Nanozoomer digital pathology microphotograph)

Fig. 2. En face sequential light micrograph of whole mount Acetylcholinestrase stained cornea extending into limbal stroma showing a group of limbal nerve corpuscles (LNC, brown stained dots) and their nerve branches originating from the superficial limbal plexus anterior to the palisades. Bar = 250μm (Nanozoomer digital pathology microphotograph, 5 micron sections from A to F). Inset in 'A' illustrates a cross section of a subepithelial LNC, which is solid and shows a neuronal extension attached to it.

Fig. 3. En face sequential light micrograph of whole mount Acetylcholinestrase stained cornea extending into limbal stroma showing limbal nerve corpuscles (LNC, arrows) that reside within the palisades of Vogt (P), which are all connected to fine nerve fibres (arrowheads) arising from the superficial limbal plexus anterior to the palisades. Bar = 100μm (Nanozoomer digital pathology microphotogra, 5 micron sections from A to F). Inset in 'A' illustrates a cross section of a subepithelial LNC, which is solid and shows a neuronal extension attached to it.

Fig. 4. In vivo confocal microscopy images of limbal nerve corpuscles (LNCs). LNCs appear as hyper-reflective ovoid or elongated structures (arrows). Bar =

100μm. Frame depth; A=45 μm, B=40 μm. Bar = 100μm. (Images were taken with Heidelberg Retina Tomograph II Rostock Corneal Module [RCM]; Heidelberg Engineering GmbH, Heidelberg, Germany).

Fig. 5. Scanning electron micrographs of a limbal nerve corpuscle (LNC) and its single terminal nerve branch using tissue maceration technique. LNC appears as an oval structure with a ruffled outer surface. Bar; A = 100μm, B = 20μm.

Fig. 6. Cross section photomicrographs of Acetylcholinestrase stained corneoscleral disc. A. A large (brown stained) stromal nerve is seen in the mid-stroma of the peripheral cornea. B. Shows the image of a large stromal nerve within the anterior stroma (arrow) bifurcating into two nerves (white and black arrowheads) C. The same branches as in 'B' as they approach the surface of the peripheral cornea and conjunctiva. The photomicrographs were obtained from a corneoscleral disc used for endothelial keratoplasty. It is standard practice to remove the epithelium to increase the depth of cut to obtain thinner donor lenticules for Descemets stripping endothelial keratoplasty, hence the epithelium is not seen. Sparse endothelial cells were seen in some sections, the loss being related to processing through the two steps. Bar = 200 microns (light microscopy with haematoxylin and eosin counter stain).

Fig. 7. Low (A) and high (B) magnification transmission electron micrographs of an Acetycholinesterase (AChE) stained normal human cornea showing a stromal keratocyte (K) in close contact (arrow) with a vertically-oriented stromal nerve (N). In this experiment, the tissue was first formalin fixed and taken through the steps of AChE staining, then fixed in EM fixative and processed for TEM. The

black dots are AChE stained particles. Although these particles can be seen scattered around the area of interest, they are clearly concentrated on either side of the nerve (not in the nerve itself) illustrating that the sheath is stained and nerve itself is not, with the AChE technique.. The pattern is regarded as strongly positive. Bar; A=20μm, B= 2μm.

Fig. 8. Nanozoomer digital pathology photomicrographs of Acetylcholinestrase stained normal human cornea. The nuclei of the epithelial cells are clearly demonstrated in enface sections from the central and peripheral cornea, respectively (A & B). At a higher magnification, the epithelial cell nucleoli can also be seen (B). Enface sections of the anterior stroma show the heterogeneous morphology of keratocyte nuclei (C & D). A stromal nerve (arrow) is seen in section (C). Bar; A=50 μm, B=25μm, C=200μm, D=100μm.

Fig. 9. Nanozoomer digital pathology photomicrograph of whole human corneal mount stained by the Acetylcholinesterase technique. A. Two bulb-like structures are seen (arrowheads) at the point where the sub-Bowman's nerve emerges in the sub-basal plane. B. A sub-Bowman's nerve is seen to emerge anterior to Bowman's layer and bifurcate with one branch terminating in two bulb-like structures and the other in four blub-like structures (arrowheads). In both A and B, fine axon bundles are seen to arise from the blub-like structures, divide and reconnect to form the sub-basal plexus. Bars = 50 μm. (reproduced with permission from the BMJ publishing group Ltd., author's own publication Al Aqaba et al. 2010).

Fig. 10. Sequential enface nanozoomer digital pathology photomicrographs of Acetylcholinestrase stained cornea (A-F at 5 micron intervals) showing the bulb-like termination of a stromal nerve at the perforation site anterior to Bowman's layer, from which a single sub-basal nerve bundle arises (arrowhead in section F). The epithelial cells in the close proximity to the bulb-like structure show dense nuclear staining with Acetylcholinestrase (arrows in C, D and E). Bar =10 μm.

Fig. 11. Electron microscopy images of the bulb like structures in normal human cornea. A, a scanning electron microscopy (SEM) image of a bulb-like structure (arrow) at the perforation site, from which a single sub-basal nerve arises. B, a SEM image of a bulb-like structure, (arrow) lying in the sub-basal plane anterior to a perforation site, from which two sub-basal nerves are seen to emerge. The basal surface of the basal cells of the epithelium are seen beneath the bulb-like structure as the specimen of the epithelial sheet has been flipped around to scan the basal surface. C & D, a transmission electron microscopy (TEM) image of a bulb-like structure at two different magnifications. A single stromal nerve bundle (arrowhead) is seen to run obliquely and anteriorly to perforate Bowman's layer and end in a bulb like structure (arrow). E-G, TEM images of a single bulb like structure at different magnifications. E&F, A single stromal nerve bundle (arrowhead) is seen to run anteriorly to perforate Bowman's layer (P) and end in a bulb like structure (arrow) in the sub-basal plane (Epi = basal epithelial cells,, K = keratocyte). G, the bulb-like structure appears to be made of convolutions of endoneurium surrounding bundles of neurofilaments (NF). Bar; A= 10 μm, B= 10 μm, C= 10 μm, D=2 μm, E=10 μm, F=2 μm, G=1 μm. The letters and numbers in A and B relate to power and exposure during SEM imaging. C to G are sections of

Acetylcholinestrase (AChE) stained whole mounts of corneal samples. The black dots are deposits of copper thiocholine crytals (CT in image G), which is an end product of the AChE staining method, and are electron dense.

Fig. 12. In vivo confocal microscopy (IVCM) images of normal human corneas showing the termination of sub-basal nerves in bright hyper-reflective bulb-like structures at the basal epithelial plane (arrows). Bar 100µ. Frame depth; A=40µ, B=44µ, C=58µ, D=46µ, E=40µ, F=46µ. These structureswere first demonstrated in whole mount Acetylcholinesterase stained corneas but were missed on IVCM. IVCM confirmation was then possible on cadaver whole eyes and finally in living human eyes. (Images were taken with Heidelberg Retina Tomograph II Rostock Corneal Module [RCM]; Heidelberg Engineering GmbH, Heidelberg, Germany).

Fig. 13. Scanning electron micrographs of the sub-basal nerve plexus. A. Nerve fibres are seen running parallel on the Bowman's layer. B. A beaded nerve fibre (arrow) approximately 2µ diameter bifurcating from a 5µ non-beaded straight nerve fibre (arrowhead) is seen as it ascends from the Bowman's layer anteriorly towards the basal epithelial layers. C. Beaded appearance of a single fibre is seen (arrow). D. Larger and thinner fibres (arrows) bifurcating and interconnecting with adjacent fibres are seen posterior to the basal surfaces of the basal epithelial cells. The specimen of the epithelial sheet has been flipped around to scan the basal surface. Bar; A=1µ, B=10µ, C=1µ, D=1µ. The letters and numbers at the bottom of the images relate to power and exposure during SEM imaging.

Fig. 14. In vivo confocal microscopy of the sub-basal nerve plexus in normal human subjects. The sub-basal nerves form a series of complex anastomosis with

characteristic Y shaped bifurcations and H shaped interconnections. Bar = 100 µ. Frame depth; A=49 µ, B=52µ. (Images were taken with Heidelberg Retina Tomograph II Rostock Corneal Module [RCM]; Heidelberg Engineering GmbH, Heidelberg, Germany).

Fig. 15. Nanozoomer digital pathology photomicrograph of whole human corneal mount stained by the Acetylcholinesterase technique showing a clockwise whorl pattern of the sub-basal nerves in the infero-central cornea. Perforation sites and bulb-like structures in the sub-basal plane in the inferior (white arrowheads) and central (black arrowhead) cornea are seen. . A dark brown line (arrow) represents an artefactual corneal fold secondary to flattening of the cornea during processing. Bar = 0.5 mm. (reproduced with permission from the BMJ publishing group Ltd., author's own publication Al Aqaba et al. 2010).

Fig. 16. In vivo confocal microscopy images of a patient with "pain without stain" A-C. Show activated anterior stromal keratocytes, giving rise to honeycomb-like pattern. D & E. Anterior stromal nerves show patchy areas of hyper-reflectivity (arrow). F. Dendritic cells appear as hyper-reflective bodies with an amorphous stroma. Bar = 100µm. Frame depth; A=82 µm, B=59 µm, C=46 µm, D= 85 µm, E=119 µm, F= 43 µm. (Images were taken with Heidelberg Retina Tomograph II Rostock Corneal Module [RCM]; Heidelberg Engineering GmbH, Heidelberg, Germany)

Fig. 17. Nanozoomer digital pathology photomicrographs of Acetylcholinestrase stained rabbit cornea showing corneal nerve damage induced by 'small incision lenticule extraction' femtosecond Laser surgery. A. The nerves have three zones

of progressive changes indicated by rectangles B, C and D. B. Normal peripheral zone (inset B) where the nerve maintains the normal structural features including nerve fibre striation. C. Abnormal mid-peripheral zone (inset C), where the nerve shows moderate damage including disruption of the Schwann cell tube and reduction of nerve fibre thickness. D. A central zone (inset D) where the nerve undergoes severe disintegration with extrusion of its contents. Bar: A = 200 µm; B and C = 50 µm and D = 100 µm. (unpublished observations Mehta J, Al Aqaba A, Jung C, Nubile M and Dua HS).

Fig. 18. Image of a lenticule extracted by 'small incision lenticule extraction' femtosecond Laser surgery in a rabbit cornea stained with Acetycholinestrase. A significant number of medium and large stromal nerve bundles are removed by this procedure. The long-term consequences of this is unknown. Bar =1mm (unpublished observations Mehta J, Al Aqaba A, Jung C, Nubile M and Dua HS).

Fig. 19. Nanozoomer digital pathology photomicrographs of whole-mount human cornea that had undergone Laser in situ keratomileusis with mechanical keratome for correction of myopia 'a few years' ago. A-C. The peripheral corneal stromal nerves show disruption (A), looping (B) and irregular thickening (C). D-F. Two adjacent stromal nerves show aberrant regeneration (D), which is magnified to demonstrate thickening, formation of loops and coiling, just central to the flap edge (E, F). (*) in figure A marks the area in figure B, (†) in figure A marks the area in figure C. (*) in figure D marks the area in figure E, (†) in figure D marks the area in figure F

Fig. 20. Nanozoomer digital pathology photomicrographs of whole-mount Acetylcholinesterase stained control and collagen cross-linked corneas. A, Normal-appearing central stromal nerves in the control cornea. B and C, Anterior stromal nerves terminate in bulb-like structures (arrows) from which no sub-basal nerves are seen to arise. Thickened stumps of the origin of sub-basal nerves are seen in 'C'. D, Central stromal nerves (arrowheads) show localised swelling and loss of nerve continuity (arrows). E and F, localised nerve thickenings (arrows) are seen probably representing disruption and discontinuity of nerves. G, These changes are more numerous in some areas (arrows). H, A prominent localised swelling (arrow) of a midperipheral stromal nerve within the treatment zone with loss of nerve continuity. I, Similar changes were not observed in corneas treated by transepithelial corneal crosslinking (CXL). A = control cornea; B-F = standard CXL (1/2 hour between CXL and fixation); G = standard CXL (1 hour between CXL and fixation); H = standard CXL (2 hours between CXL and fixation; I = transepithelial CXL. Bar: A-F = 100 μm, G = 25 μm, H-I = 100 μm. (reproduced with permission from Elsevier Inc., author's own publication Al Aqaba et al. 2012)

Fig. 21. Nanozoomer digital pathology photomicrograph of a whole-mount Acetylcholinesterase-stained corneal graft button from an eye that underwent a regraft. A. radially oriented sub-basal nerves (arrowheads) from the peripheral cornea of the host extend across the graft-host junction (GHJ) into the peripheral part of the graft (arrows). B. Radially oriented sub-basal nerves (arrowheads) from the peripheral cornea of the host extend across the graft-host junction in to the peripheral part of the graft (arrows). C. Two thin and tortuous sub-basal

nerves are seen running in the central zone of the graft. D. Stromal nerves from the host tissue stop at the peripheral edge of the GHJ. Some of them divide at the GHJ into branches (arrowheads) that run along the GHJ. A buried nylon suture related to the previous graft surgery is seen (asterisk). E. One stromal nerve has crossed the GHJ into the graft and extends for about 600 μm toward the graft centre (black arrow). Two thick stromal nerve trunks (black arrowheads) appear to extend for several millimetres along the GHJ, accompanied by a large blood vessel (blue arrowhead). A complex vascular arcade (blue arrows) in the graft is also seen. F. This image represents a higher magnification of the region outlined by a rectangle in (E). It shows a large nerve trunk (black arrowhead) and a blood vessel (blue arrowhead) running together at the edge of the GHJ but within the host tissue. Bars: A = 200 μm, B = 100 μm , C = 100 μm, D = 500 μm, E = 500 μm, F = 100 μm. (reproduced with permission from Elsevier Inc., author's own publication Al Aqaba et al. 2012).

Fig. 22. Nanozoomer digital pathology photomicrograph of whole-mount Acetylcholinesterase-stained corneal graft button from an eye that underwent a regraft. Excessive regeneration of two types of nerves is seen. One type representing an attempt at normal regeneration with large straight nerve trunks, which demonstrate abrupt angulations along their course (arrows), and the other type, which demonstrates a highly tortuous and complex network of interconnecting nerves (arrowheads). In the centre of the button is an area that is relatively sparsely innervated. Scale bar = 2 mm. (reproduced with permission from Elsevier Inc., author's own publication Al Aqaba et al. 2012).

Fig. 23. Nanozoomer digital pathology photomicrograph showing a whole-mount Acetylcholinestrase stained advanced keratoconus cornea. A. Exuberant overgrowth of the stromal nerves (grade 3) is seen to form a complex network of small and large nerve bundles at the central cornea corresponding to the area of the conical ectasia. The peripheral nerves demonstrate a near normal morphology. Bar = 2 mm. B. An overtly thickened central stroma nerve is seen with sprouting of short, tortuous aberrant nerves (arrows), (reproduced with permission from Elsevier Inc., author's own publication Al Aqaba et al. 2011)

Fig. 24. In vivo confocal micrographs of the corneal nerves. A. Normal appearance of the sub-basal nerve plexus seen in a healthy control. Bulb-like termination of sub-basal nerves is shown in the inset. B. Sub-basal nerve plexus appearance in bullous keratopathy. There is a reduction in the density and thickness of the nerves. C. Tortuous sub-basal nerves in bullous keratopathy. D-F. Coiling, looping and twisting of tortuous stromal nerves, some surrounding dark lacunae, are seen at different depths within the stroma. Frame level; A= 59μm, B=32, C=30 μm, D = 189 μm, E, 380 μm, F =331 μm. Bar =100 μm. (reproduced with permission from Elsevier Inc., author's own publication Al Aqaba et al. 2011a).

Fig. 25. Correlation of confocal microscopy findings (Left column) with those observed on histology of whole mounts (Right column) in corneas with bullous keratopathy. A and B. A mid-stromal nerve characterised by localised nerve excrescences or thickenings (arrowheads) suggestive of early sprouting or representing stubs of degenerated nerves (arrows). C. A relatively thick stromal nerve with ill-defined margins at its bifurcation seen on confocal microscopy at

the level of 126 μm. D. The confocal image in ‘C’ corresponds with extensive axonal sprouting seen at a stromal nerve bifurcation on histology. E and D. Confocal microscopy and histology images show looping and coiling of tortuous aberrant stromal corneal nerves. Bar = 100 μm. (reproduced with permission from Elsevier Inc., author’s own publication Al Aqaba et al. 2011a).

Table 1. A list of techniques used for in vitro demonstration of corneal nerves.

Sensory nerves

1-Conventional light microscopy.

- Gold Chloride technique.
- Methylene blue staining technique.
- Sliver staining technique.
- Toluidine blue.

2-Light and electron microscopy after peptidergic and classic neurotransmitter staining.

- Whole mount cholinesterase technique.
- Calcitonin gene-related peptide detection.
- Substance P detection.
- Calcein acetoxymethyl ester
- Mixed staining technique.

3-Immunohistochemical staining.

- Anti-Class III β Tubulin (neuronal specific).

4-Retrograde nerve tracing technique.

- Horseradish peroxidise-wheat germ agglutinin (HRP-WGA) technique.

Autonomic nerves

1-Light and electron microscopy in ultrastructural immunohistochemical preparation.

- Antibodies against tyrosine hydroxylase.
- Sodium-potassium-glyoxylic acid-induced fluorescence technique.
- Formaldehyde-induced fluorescence technique.

2-Retrograde nerve tracing techniques.

- Horseradish peroxidise-wheat germ agglutinin (HRP-WGA) technique.

Highlights

- Sub-basal bulb-like structures and Limbal nerve corpuscles are two relatively recent discoveries where the structure has yet to be attributed a function.

- Dissociation of trophic and sensory functions, depending on the pre-ganglionic or post-ganglionic location of the lesion; and a discordance between objective signs and subjective symptoms in the clinical entities of 'pain without stain' and 'stain without pain' reflect the possible evolution of nerve pathology from hyperaesthesia to anaesthesia.

- In-vivo confocal microscopy and whole mount staining of normal and diseased corneas/corneal buttons has revealed that corneal nerves are not passive bystanders incorneal disease. It is likely that structural and/or functional nerve changes occur in all forms of corneal pathology.

- A wide range of pathology ranging from hyper-regeneration, aberrant regeneration, thickening, tortuosity, looping and coiling to thinning, disruption and complete absence, has been demonstrated in a number of conditions such as bullous keratopathy, keratoconus, dry eye disease, diabetic keratopahty, limbal stem cell deficiency, post-keratoplasty, refractive surgery and corneal infections.

- Inflammatory responses of resident epithelial cells, keratocytes and dendritic cells areintegral to corneal nerve responses.

Author Statement

All authors contributed to the content of the manuscript as detailed below:

Al Aqaba M: Contributed to writing of the paper, montaging images and referencing.

Dhillon V: Contributed to the section on scanning electron microscopy of the sub-basal nerves and bulb-like structures.

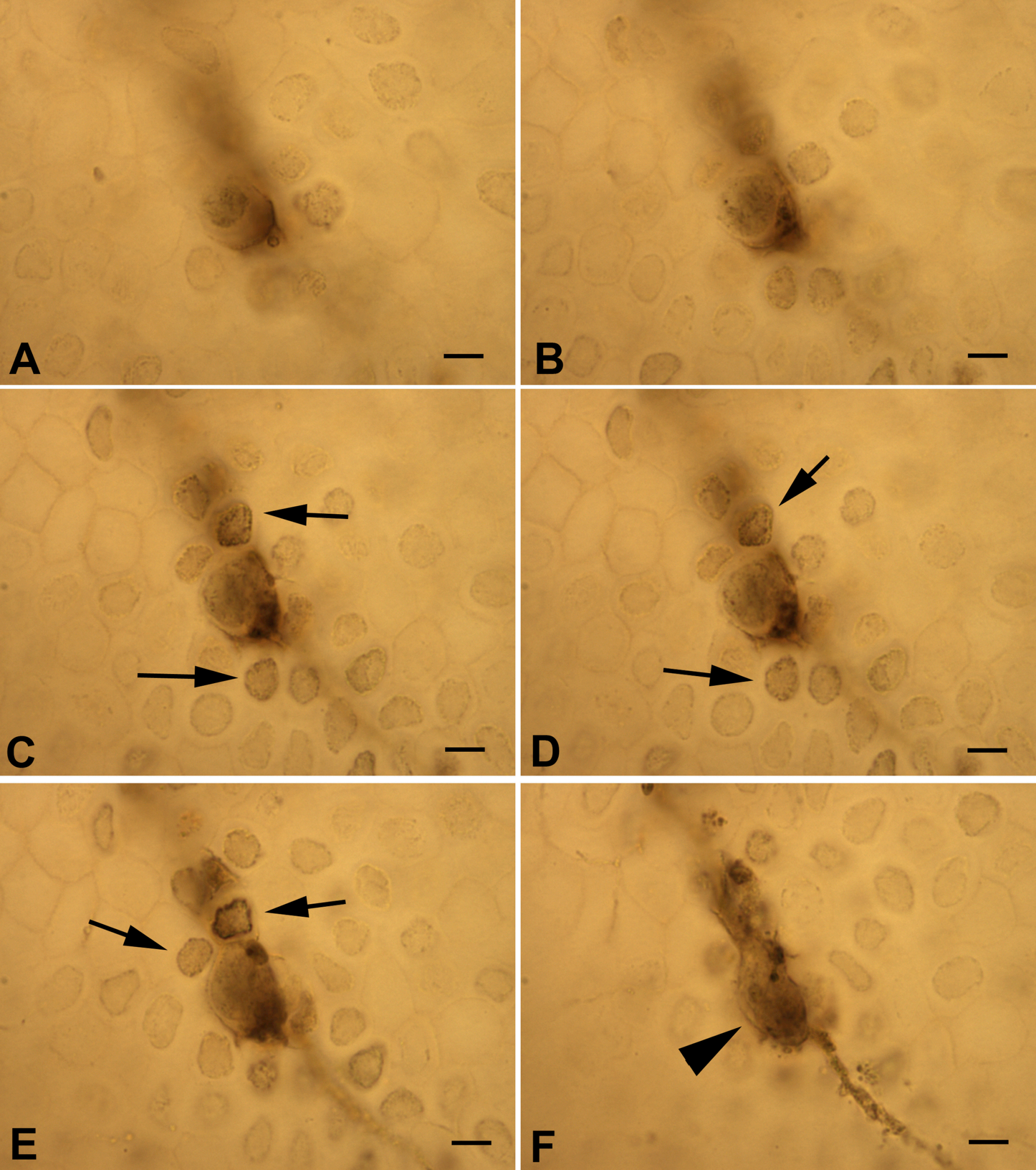
Mohammed I: Contributed to the section on Limbal nerve corpuscles.

Said DG: Contributed to writing of the paper, reviewing the paper and montaging of images and referencing.

Dua HS: Contributed to the concept, outline, writing, reviewing and editing, critical discussion related to sections of the paper and montages of some images and part referencing.

All authors agree to the content of the paper.

Declaration of interests have been detailed in the title and affiliation page.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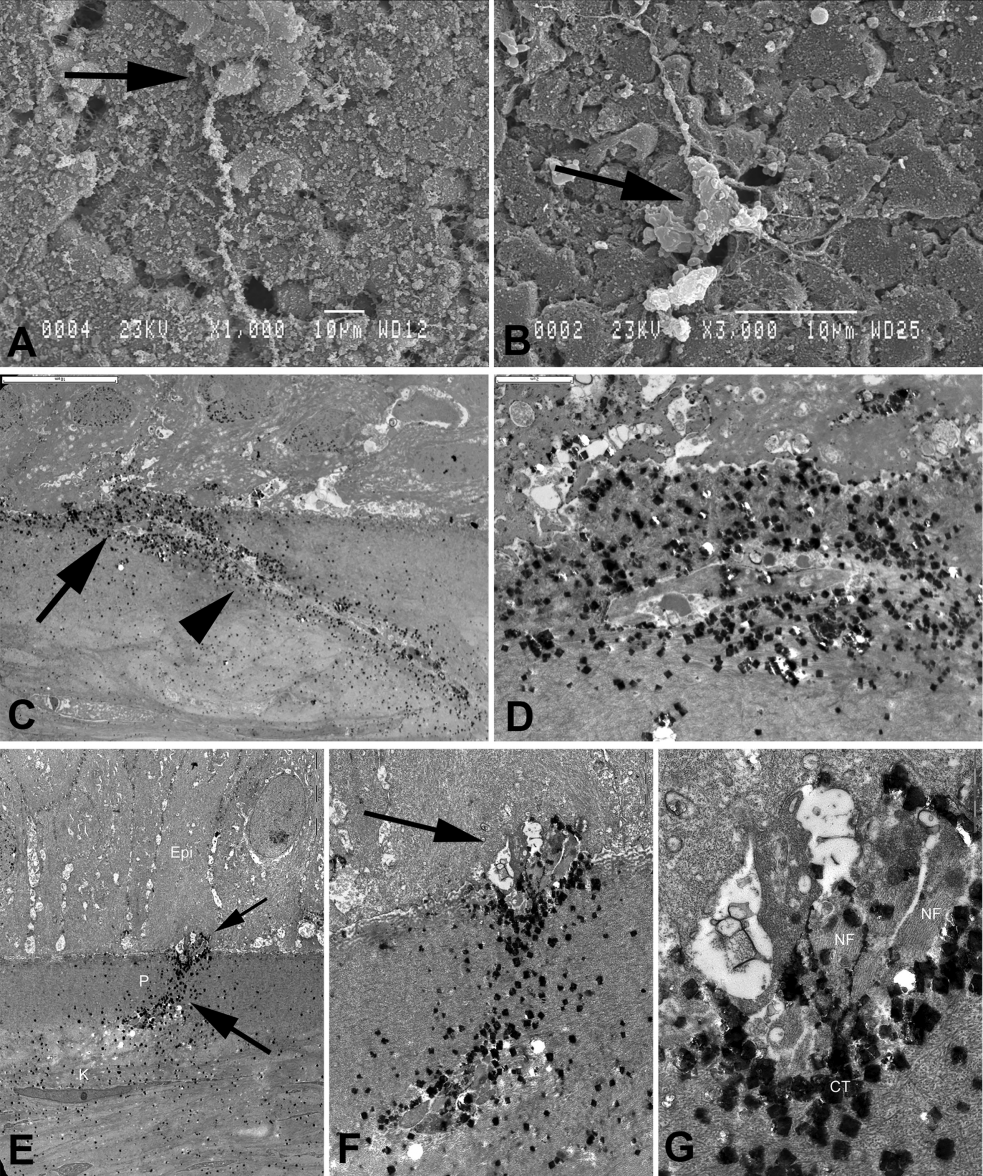
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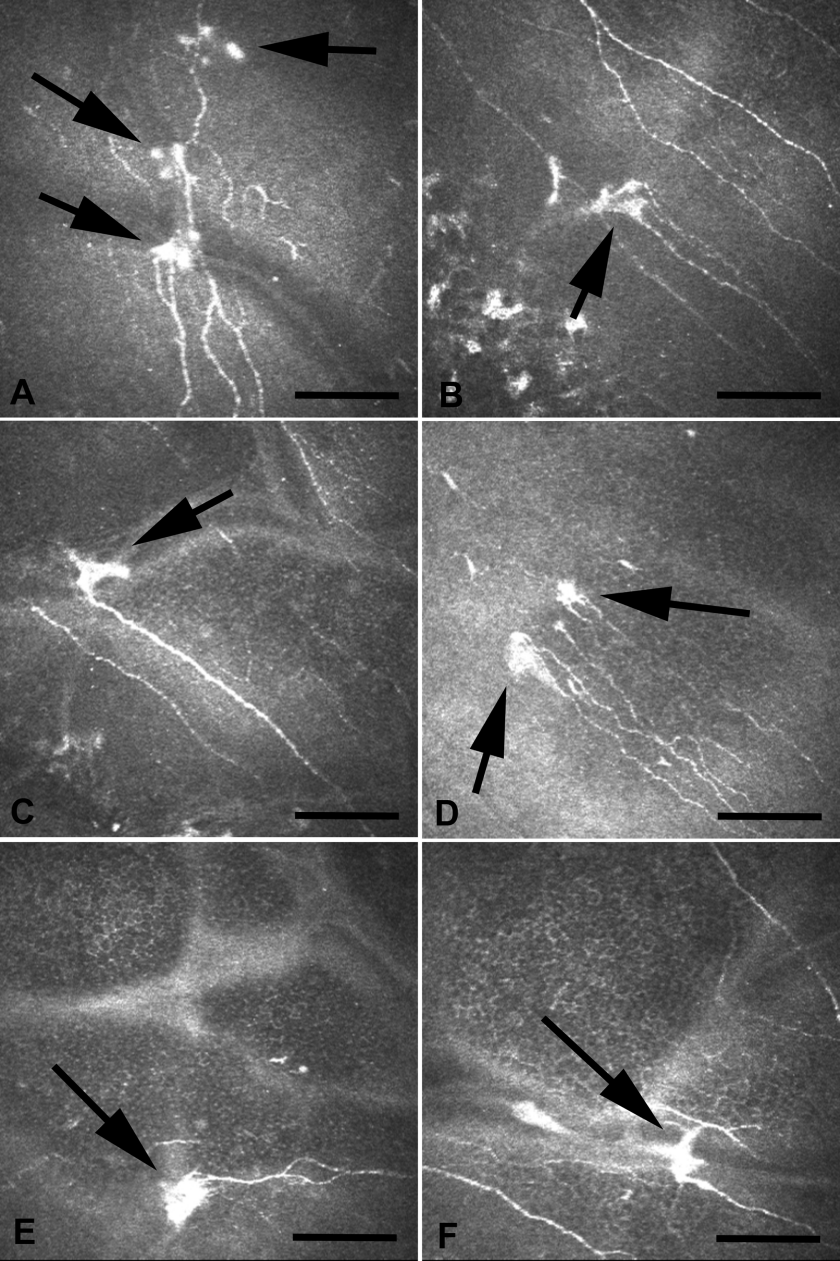
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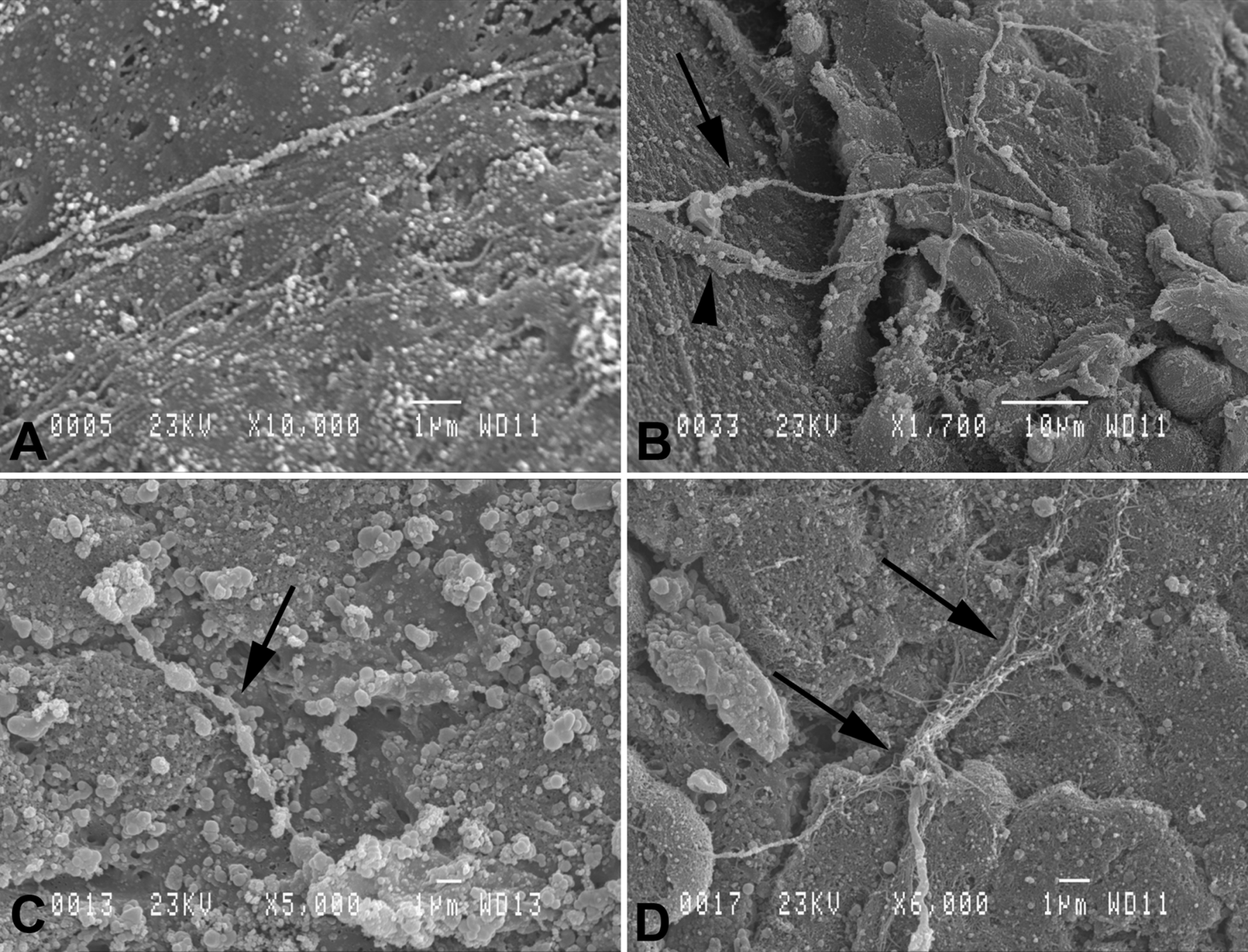
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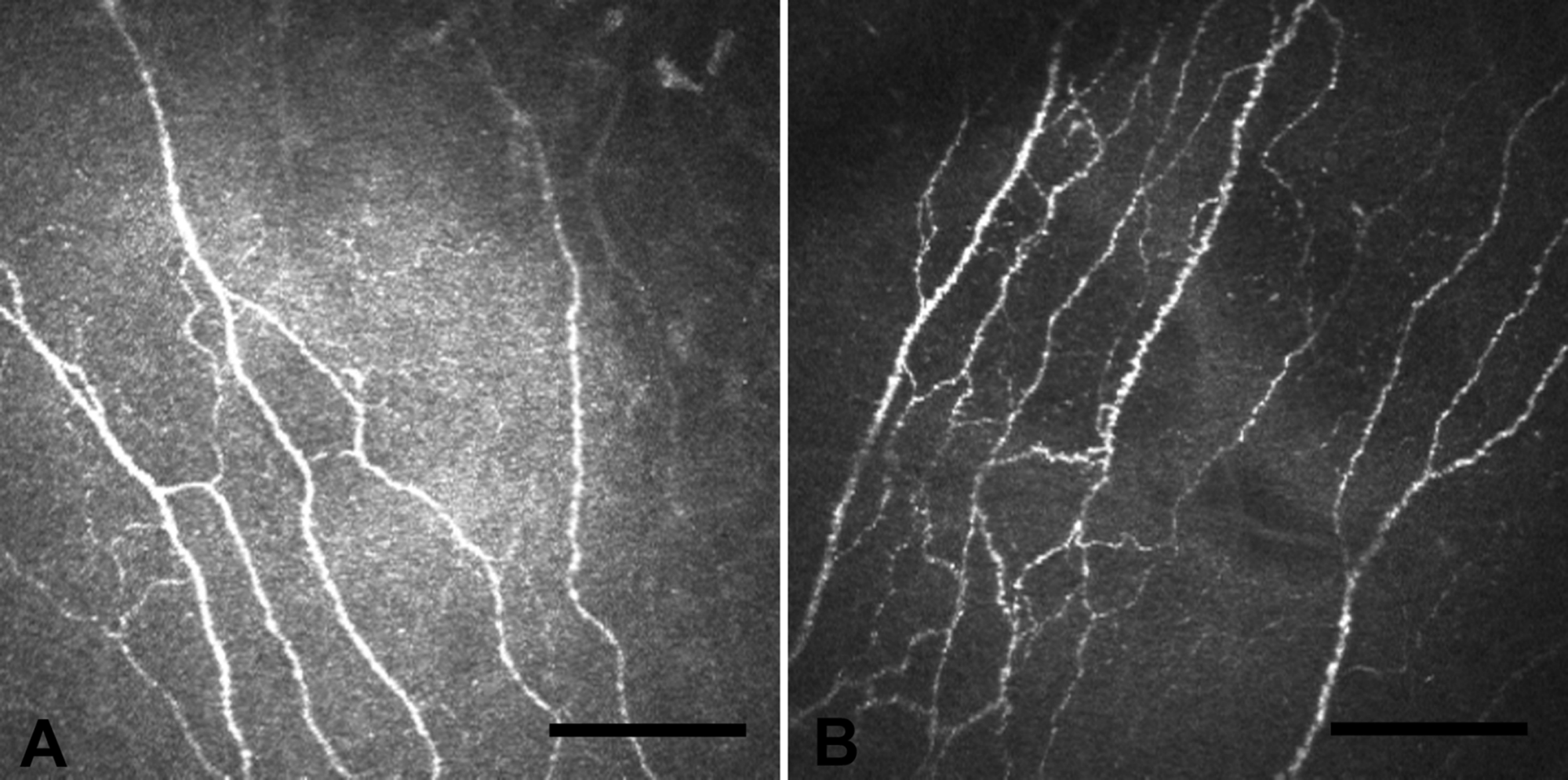
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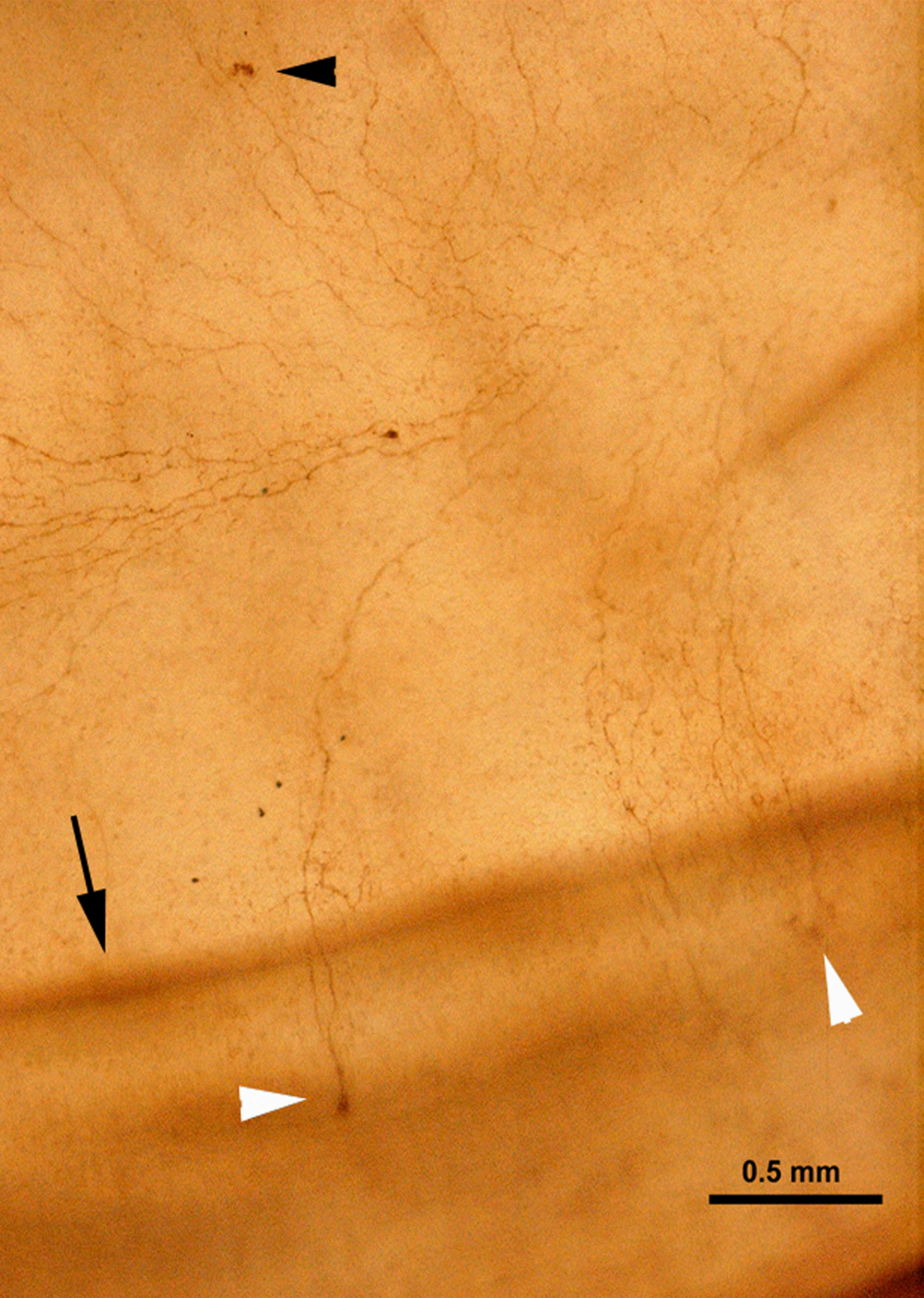
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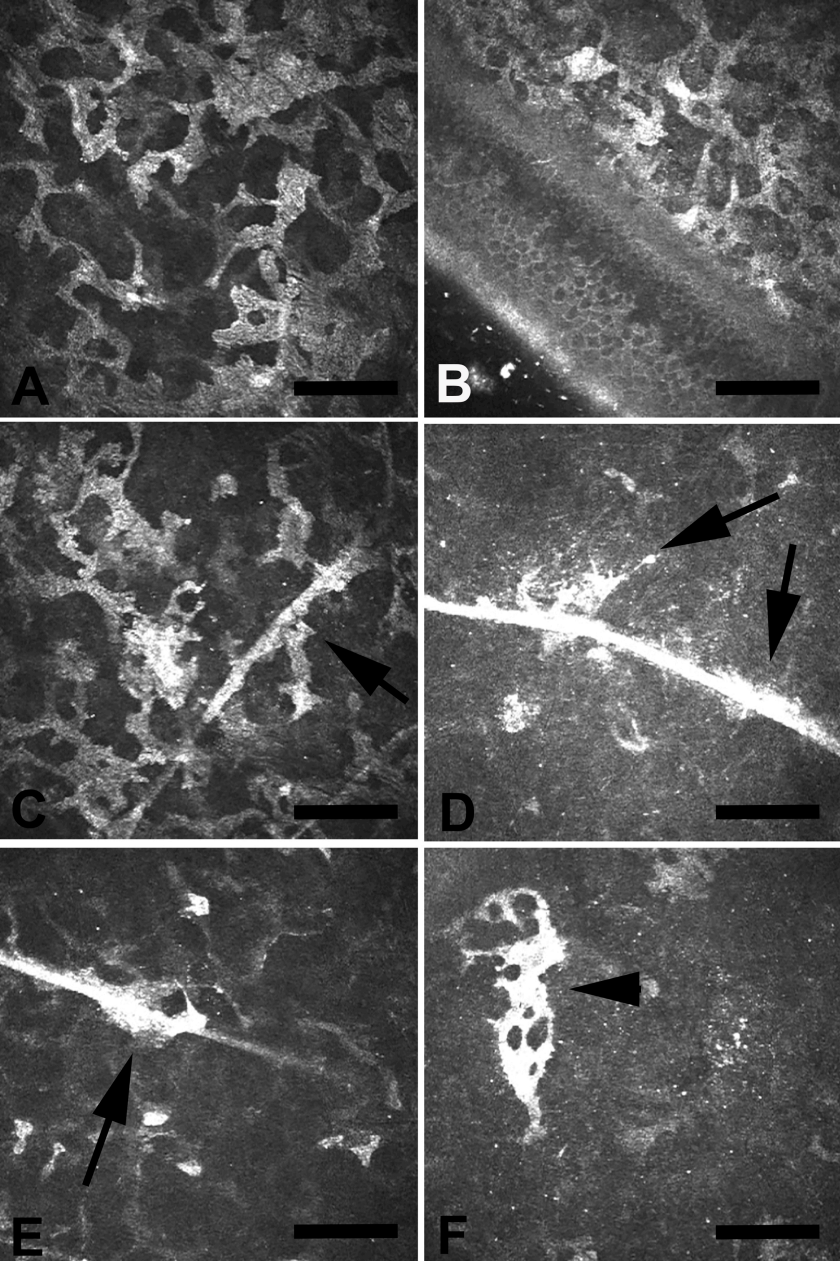
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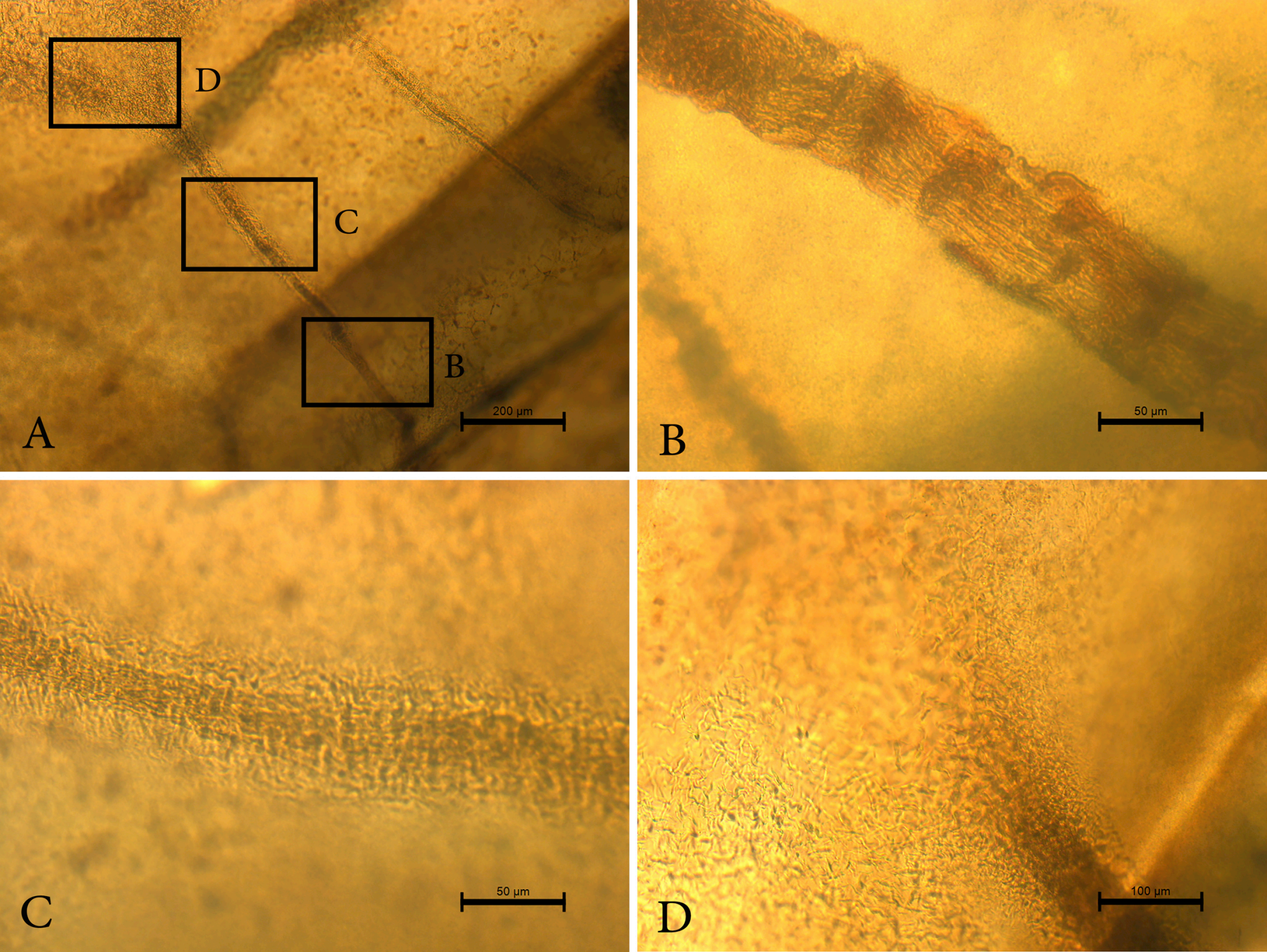
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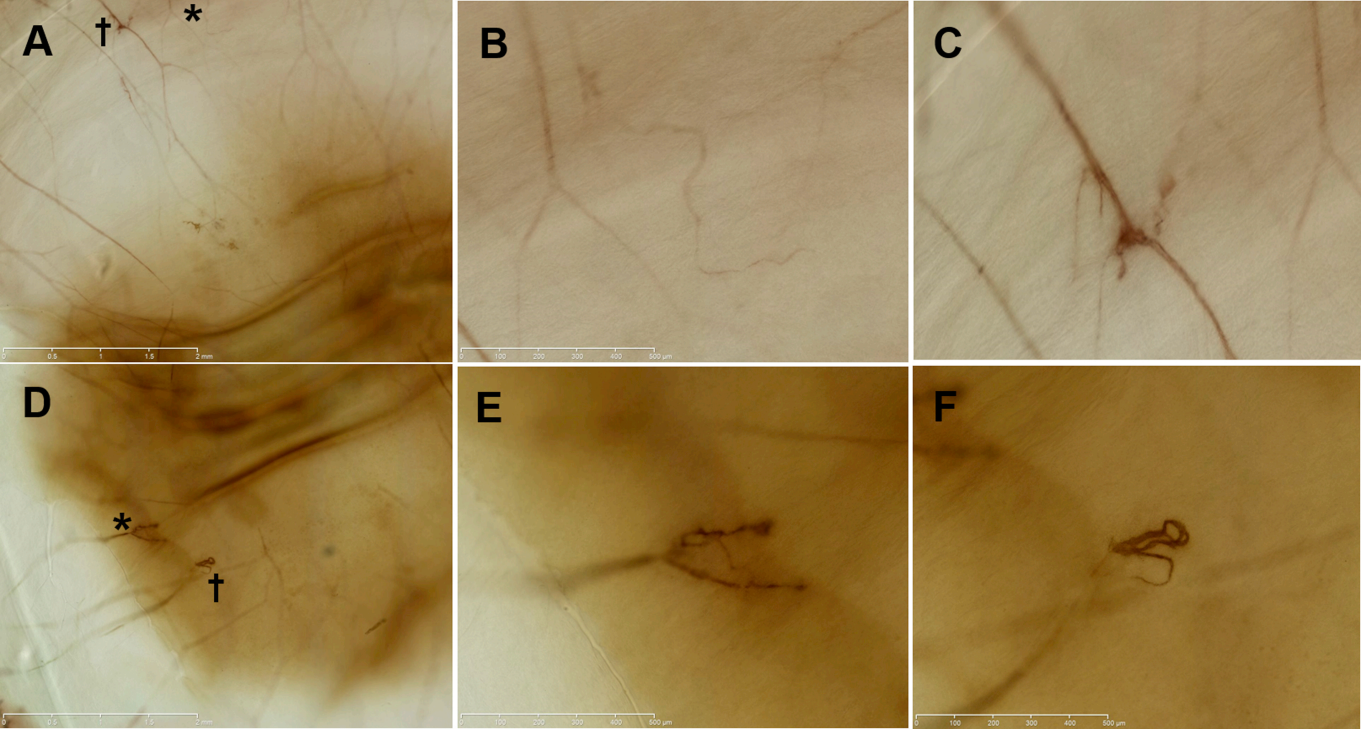
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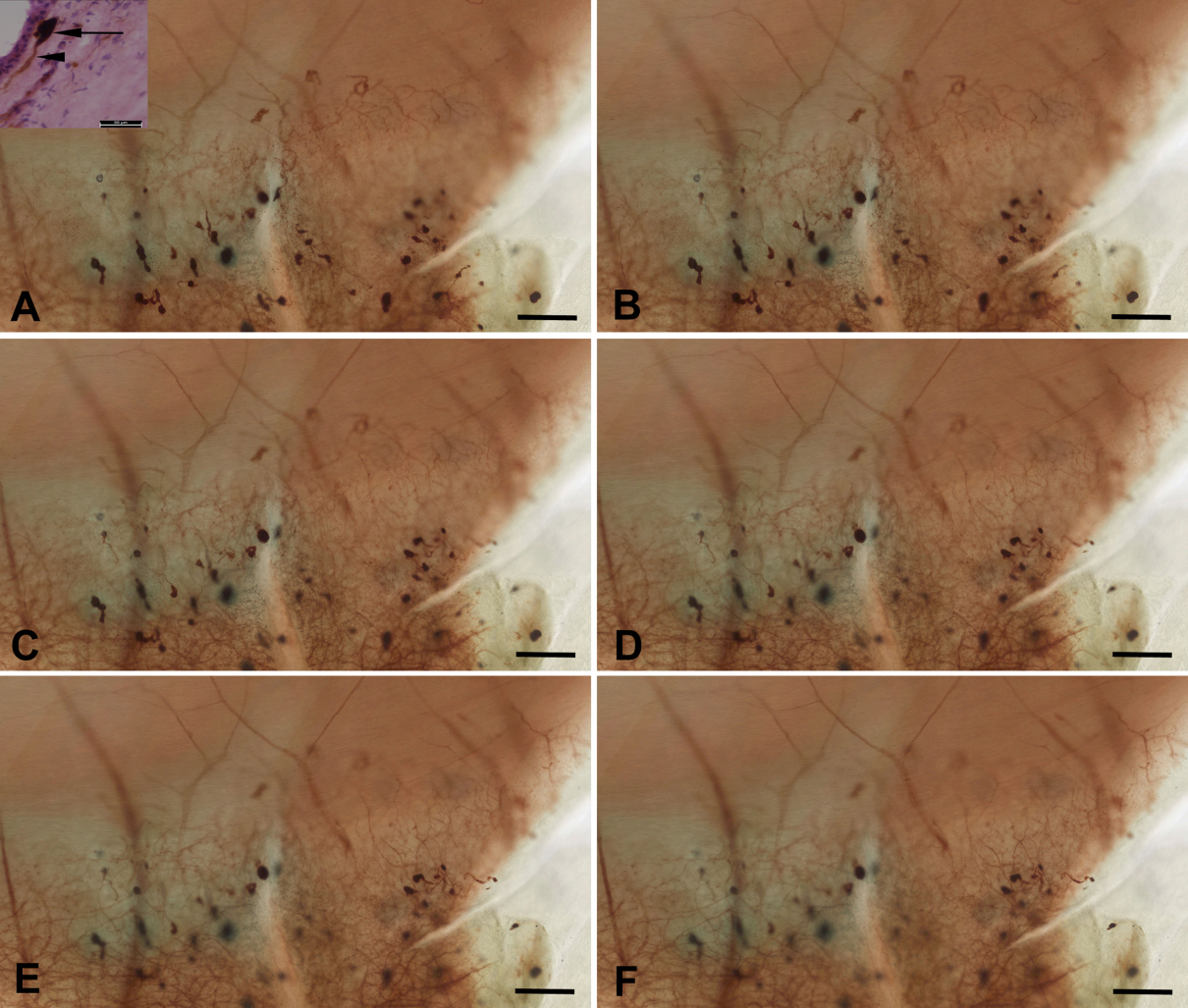
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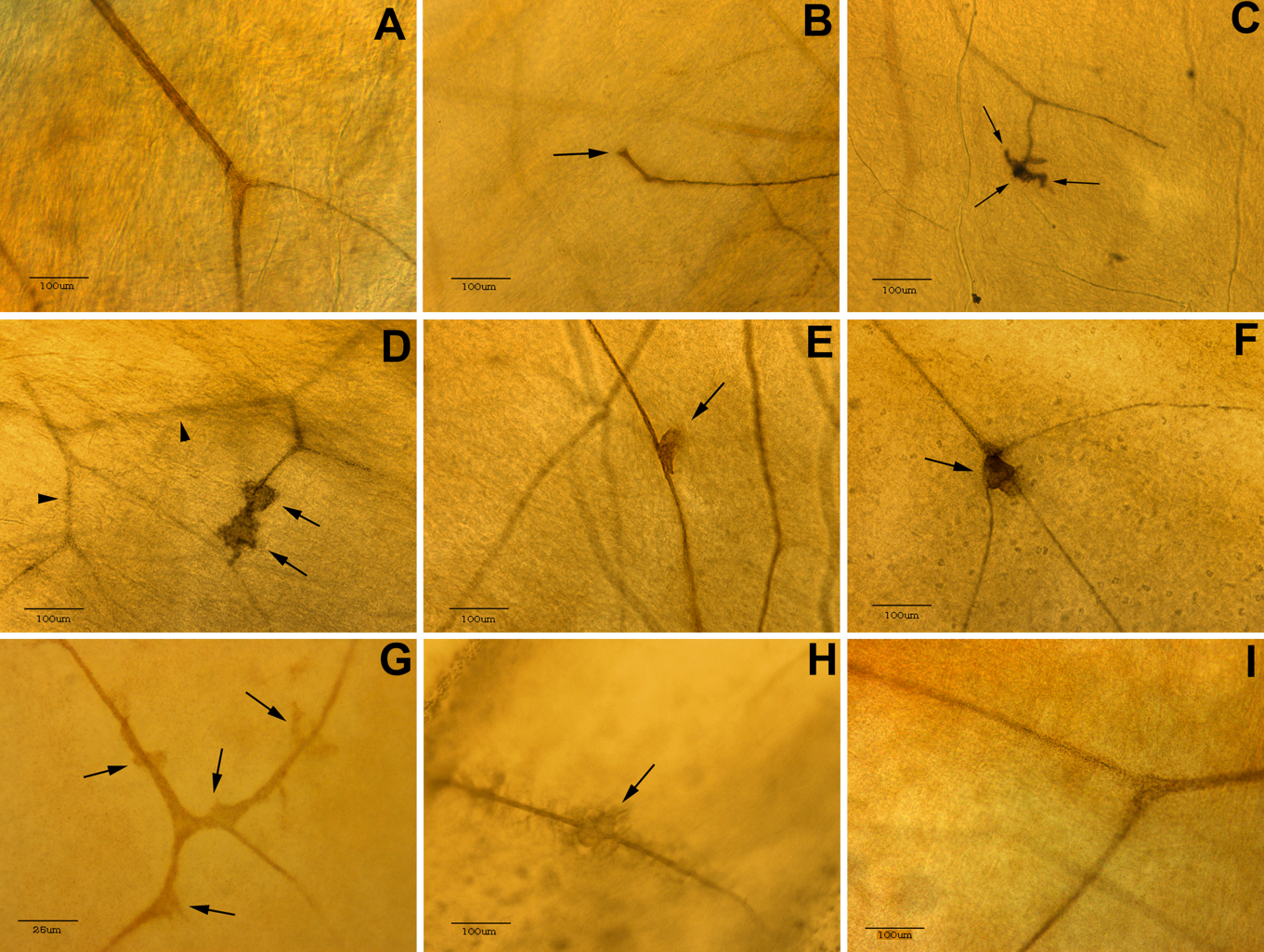
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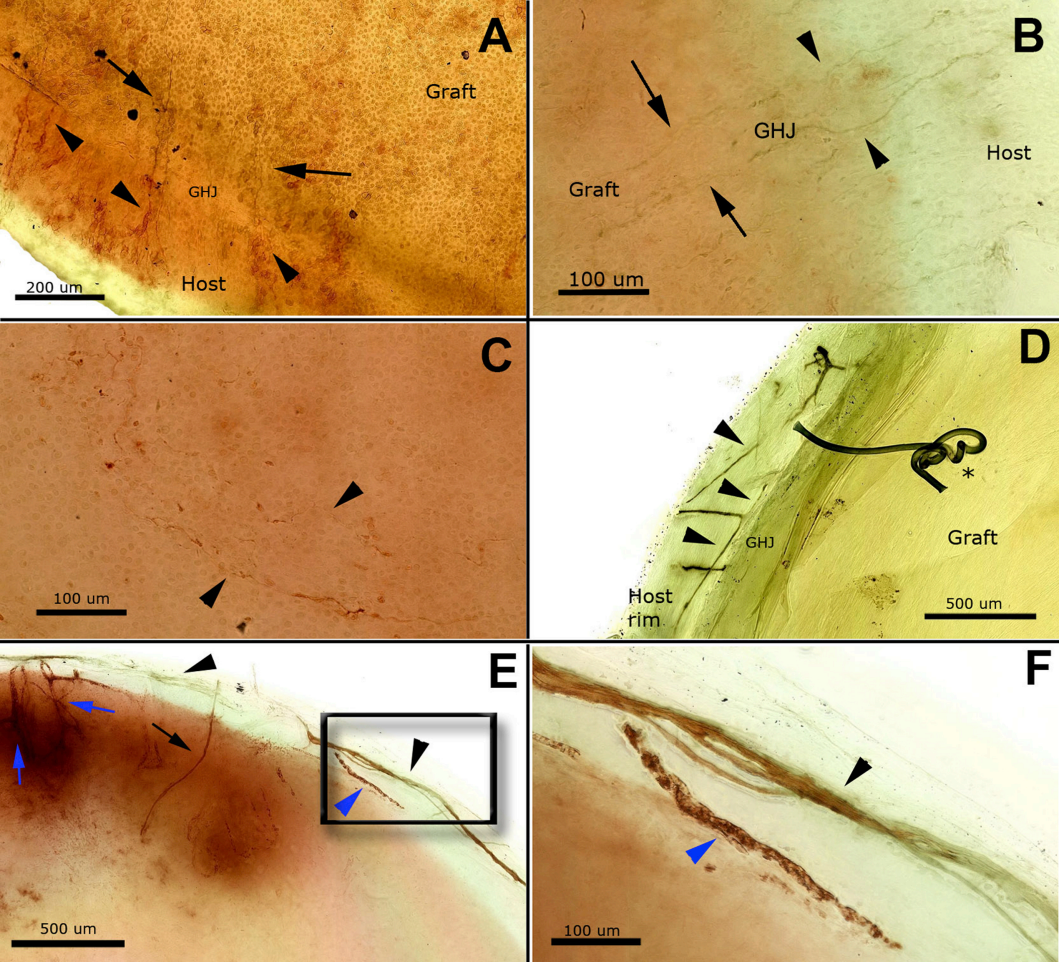
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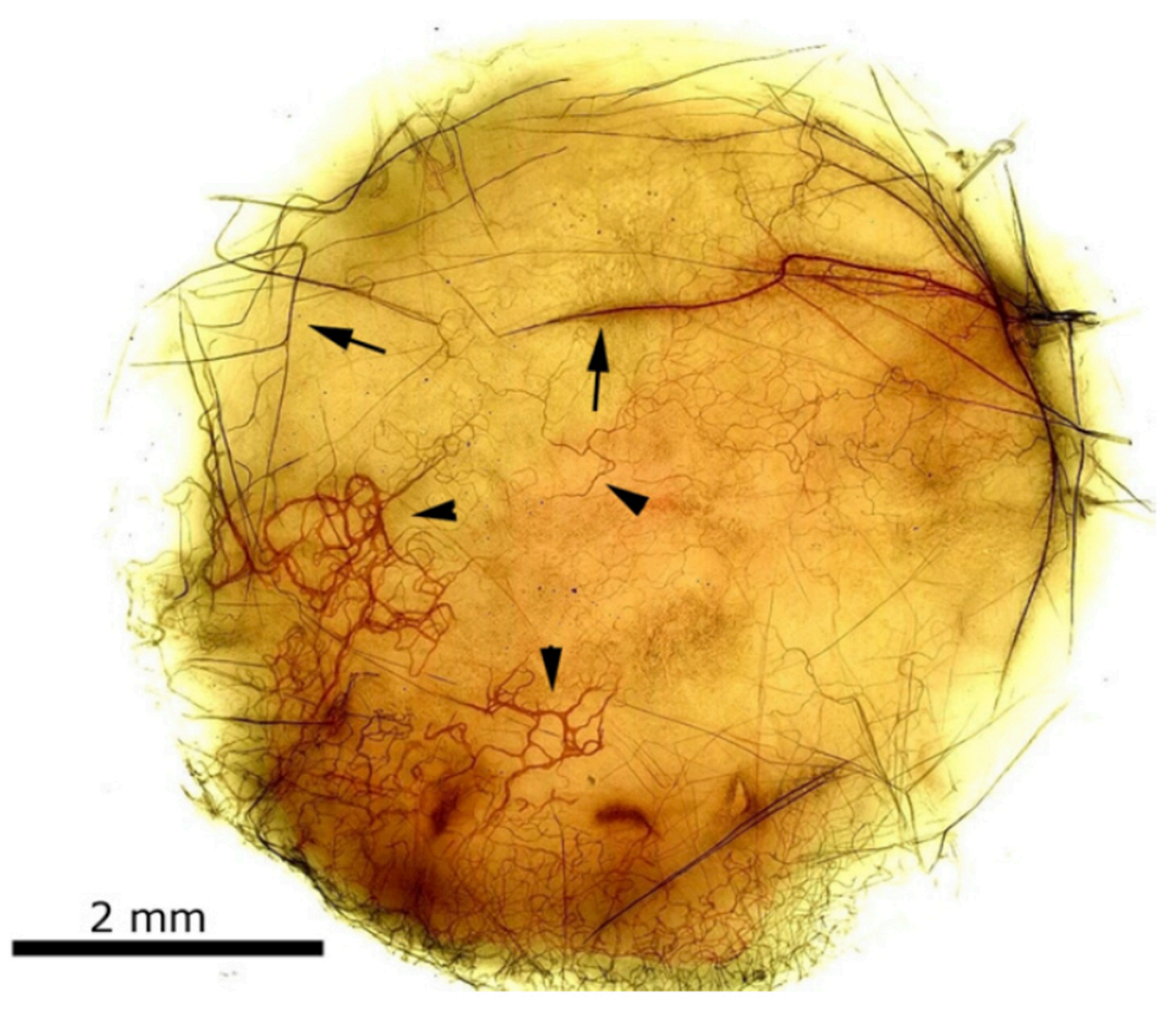
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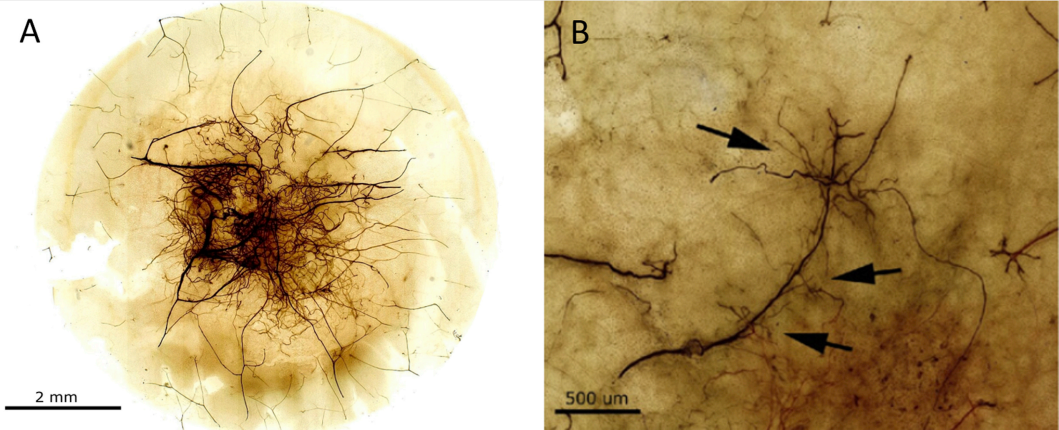
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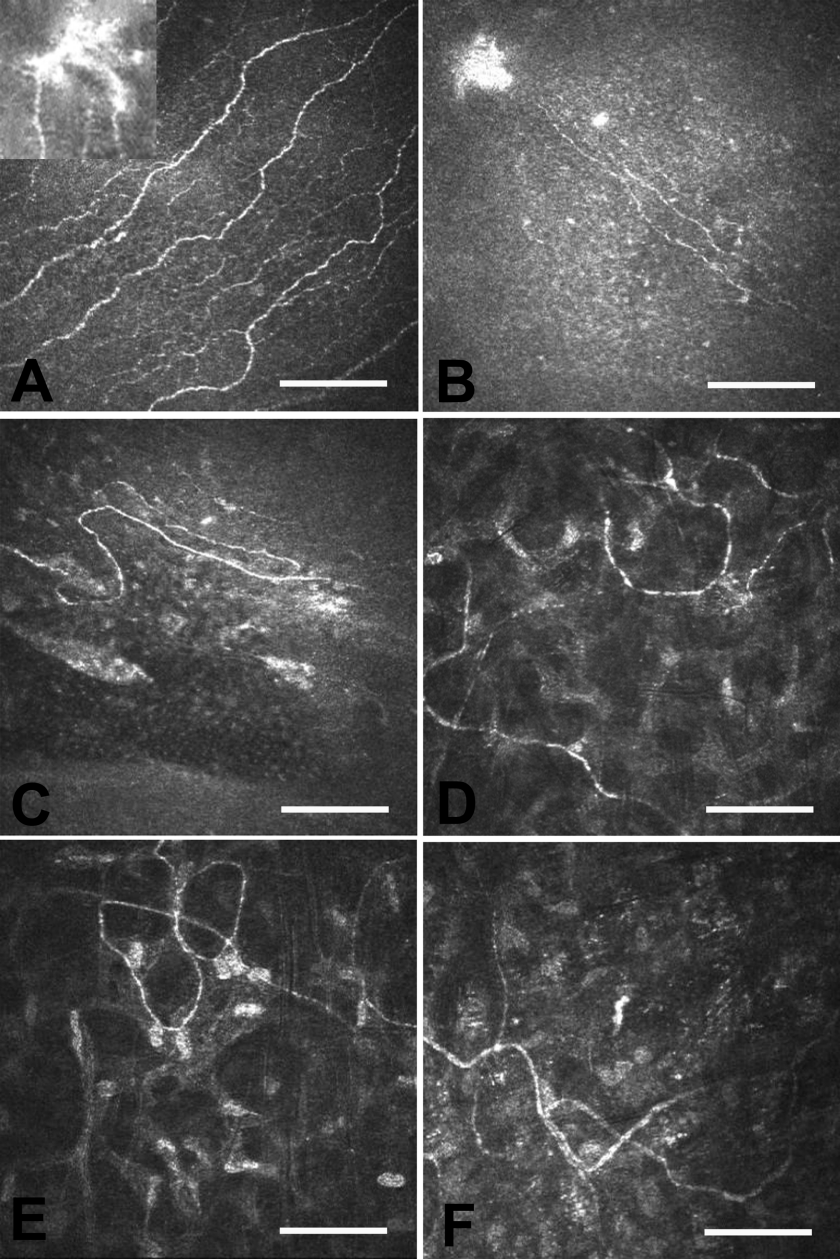
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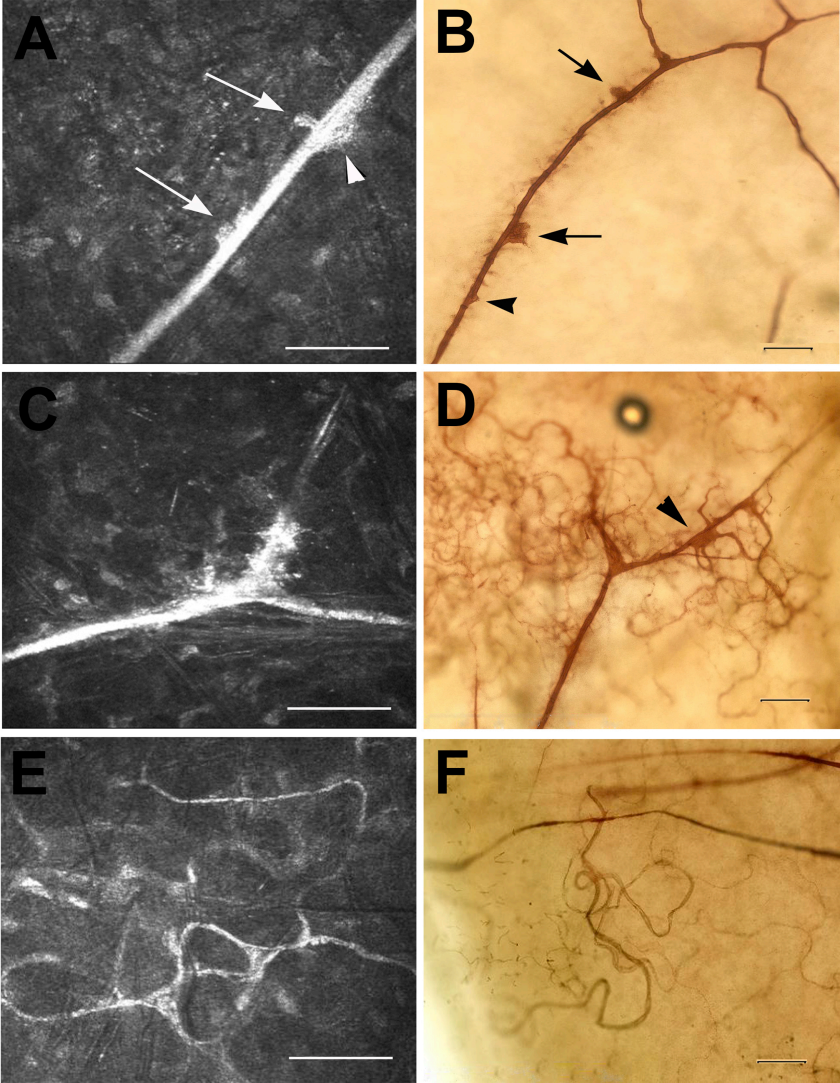
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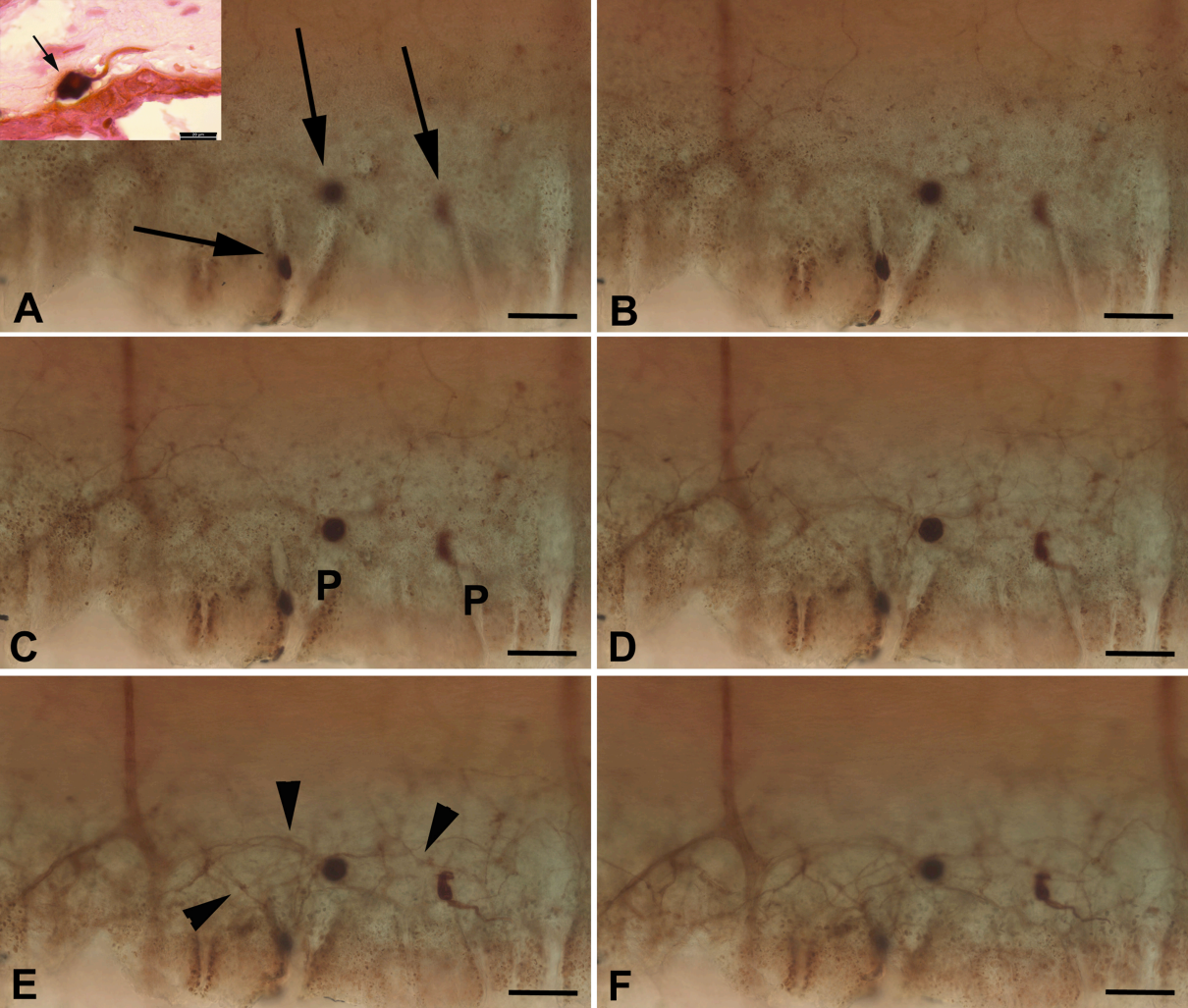
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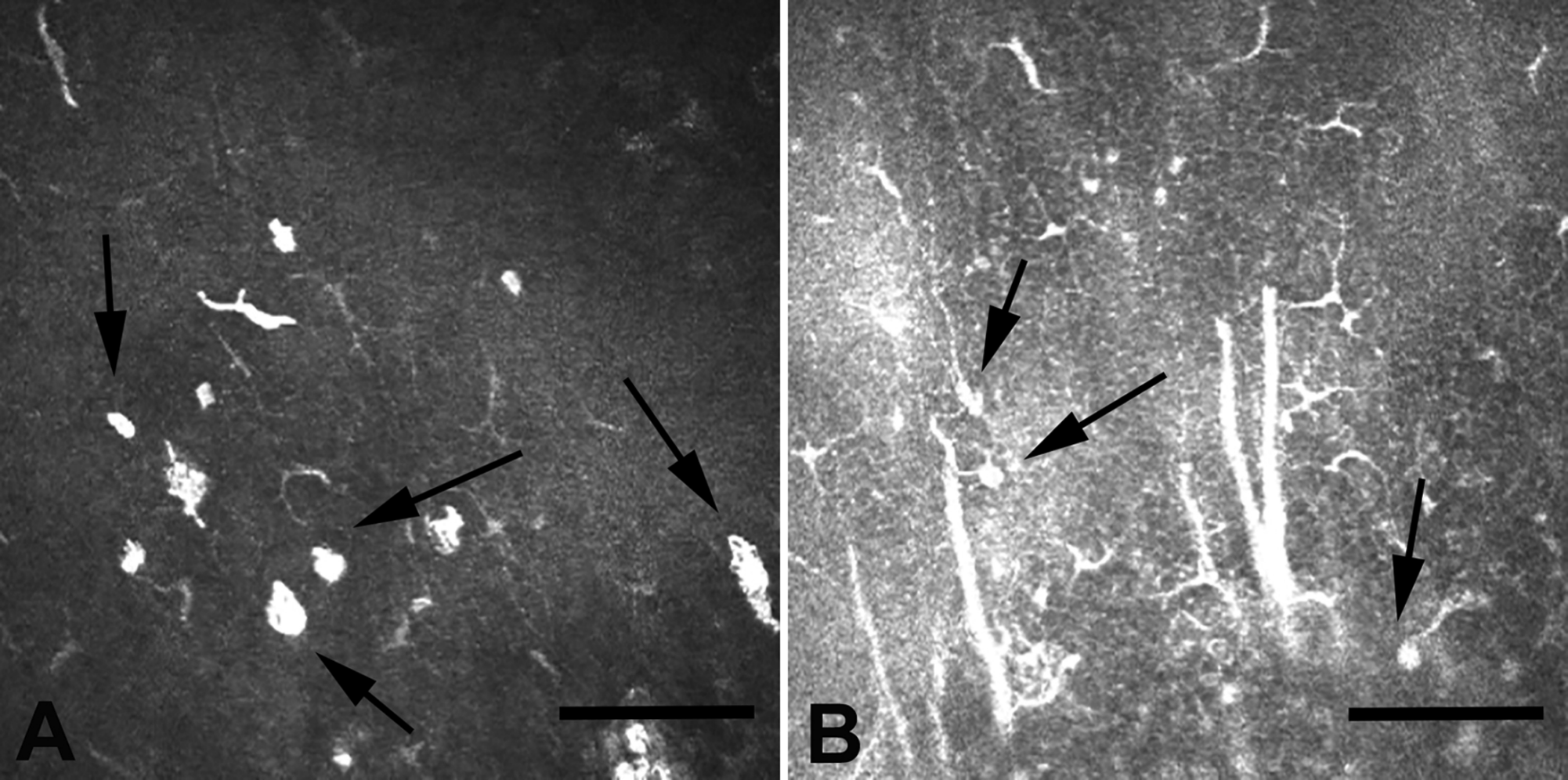
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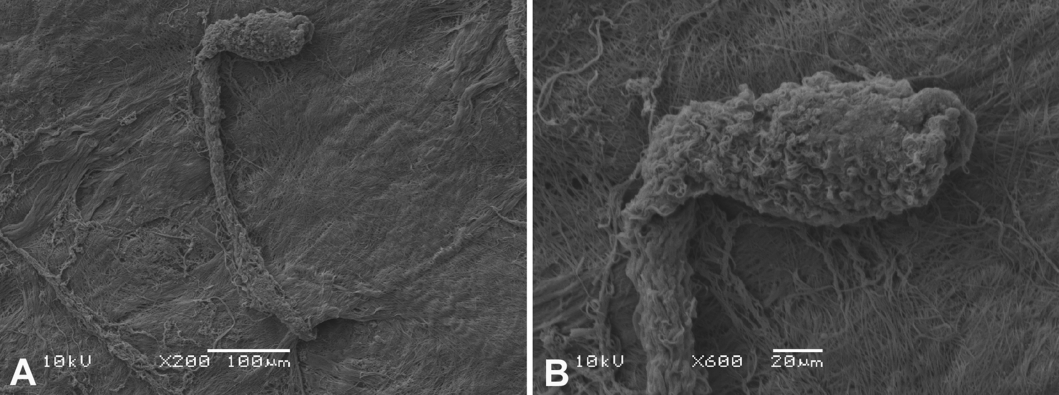
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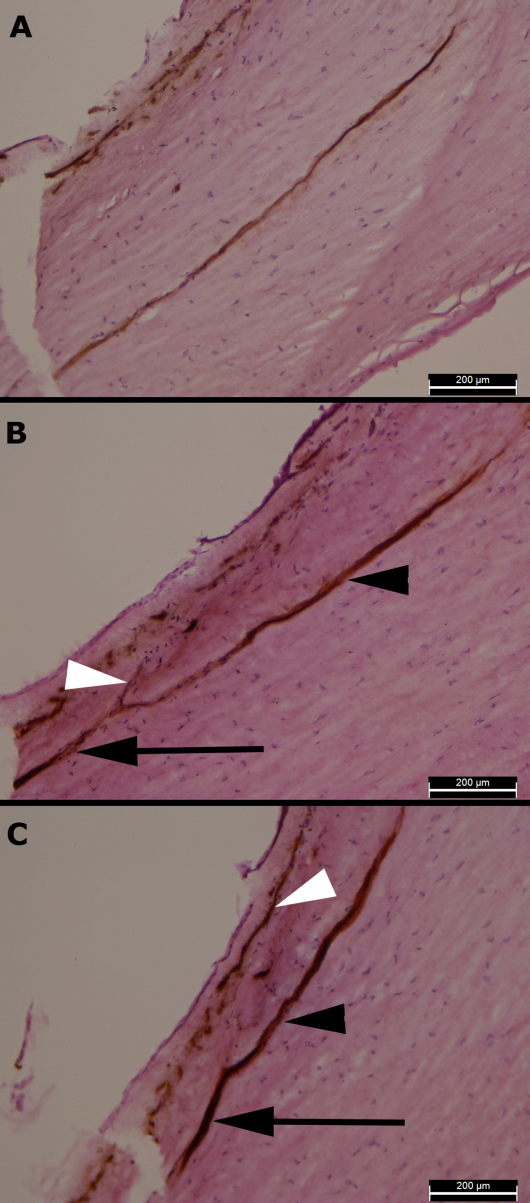
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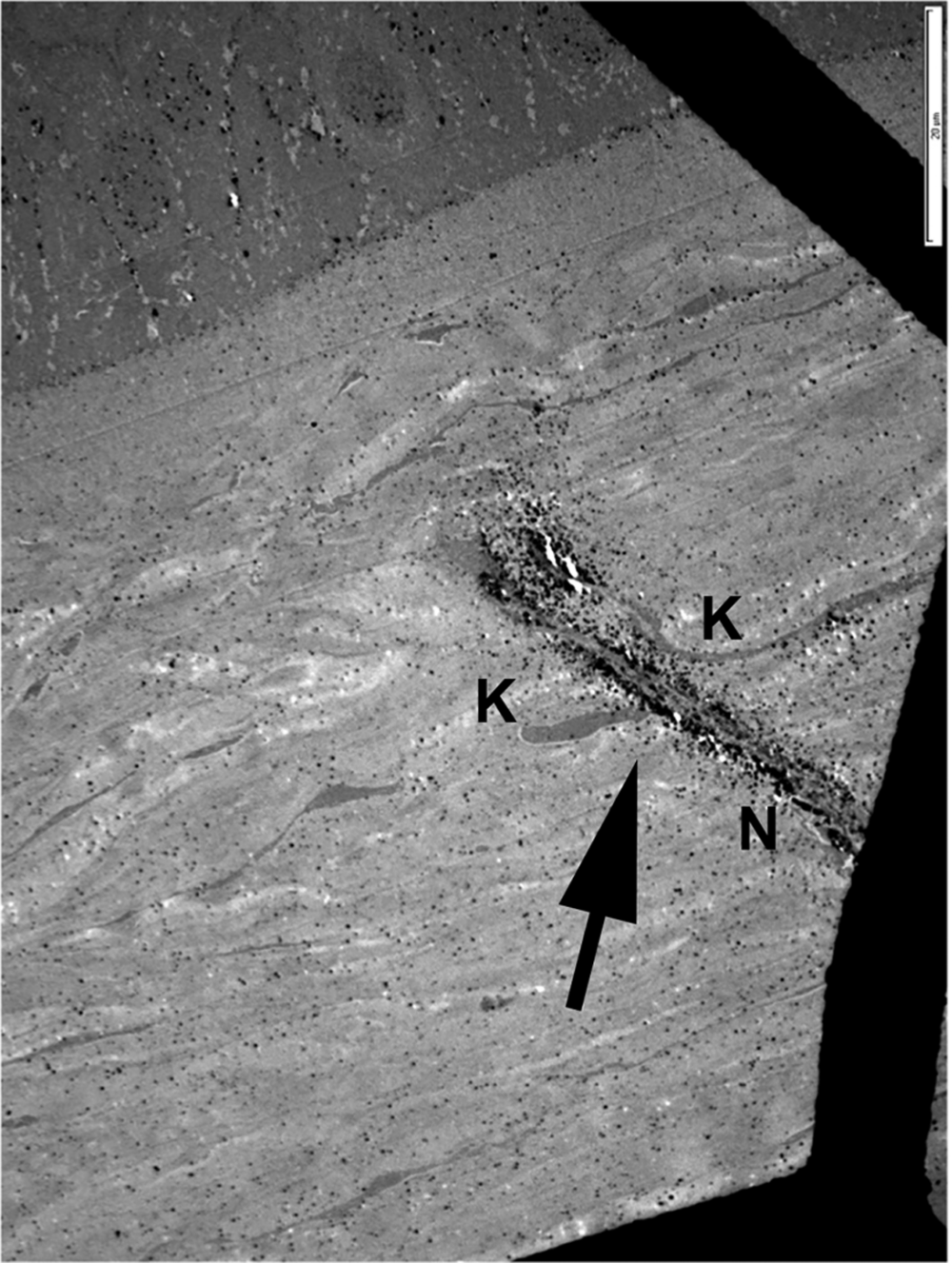
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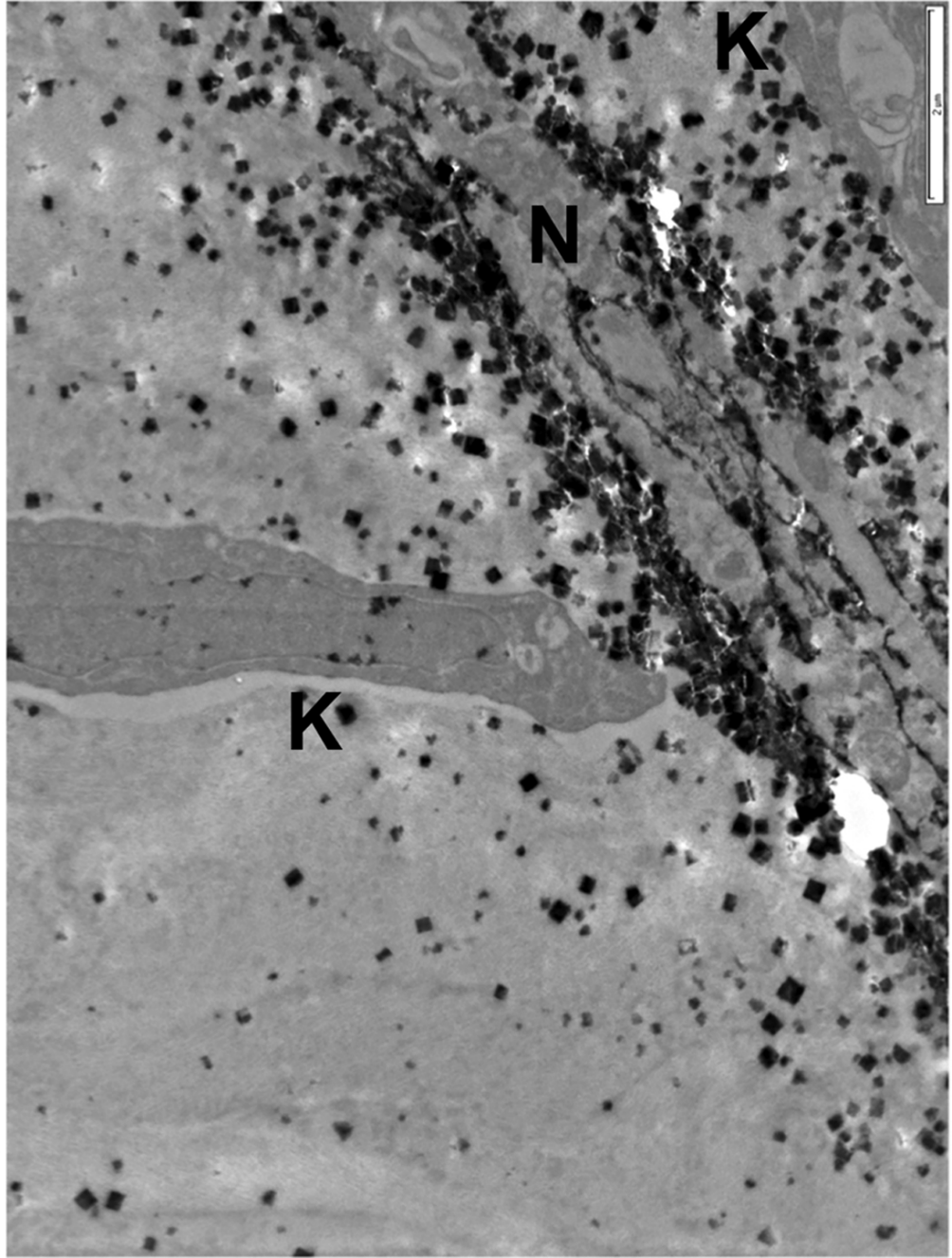
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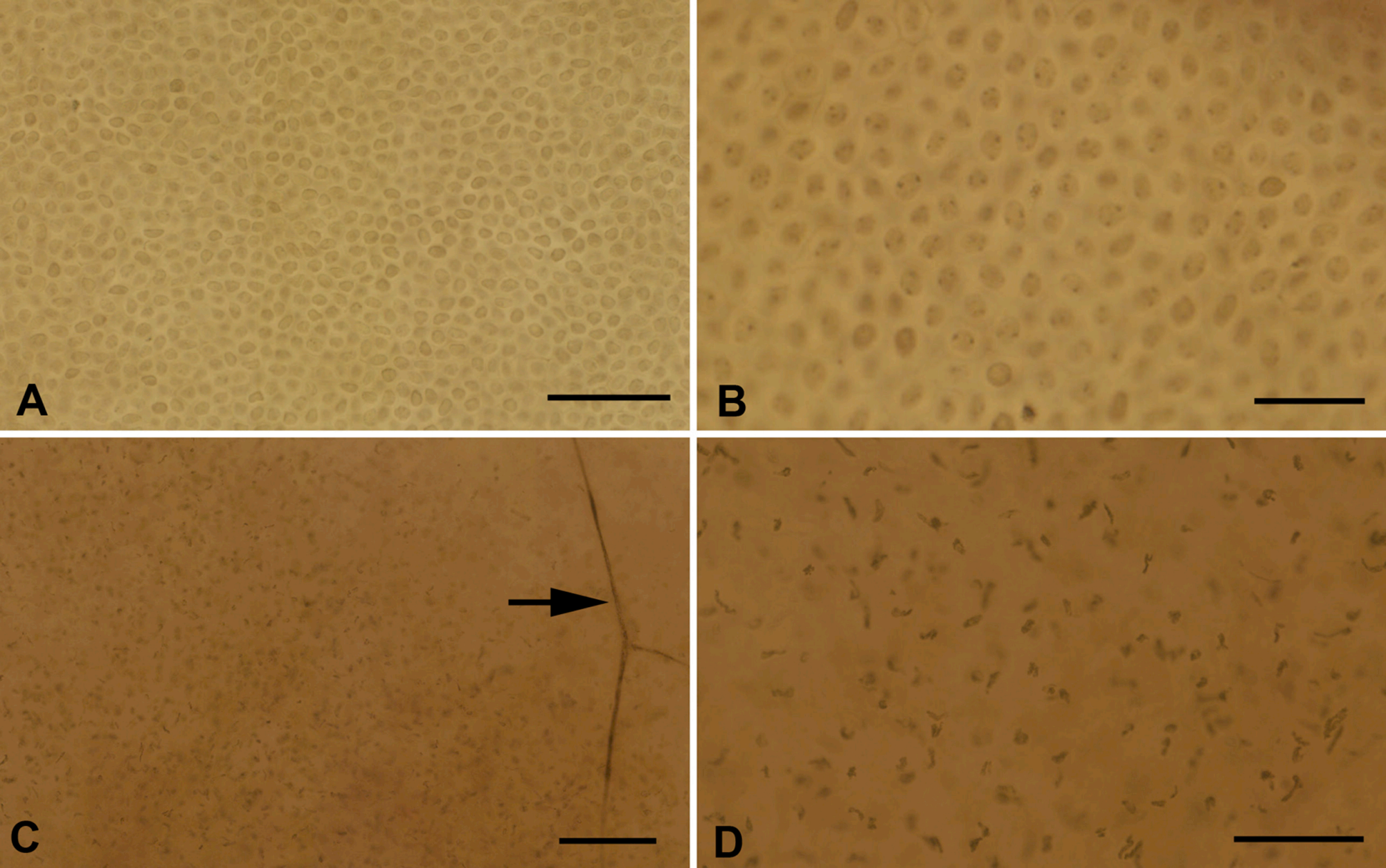
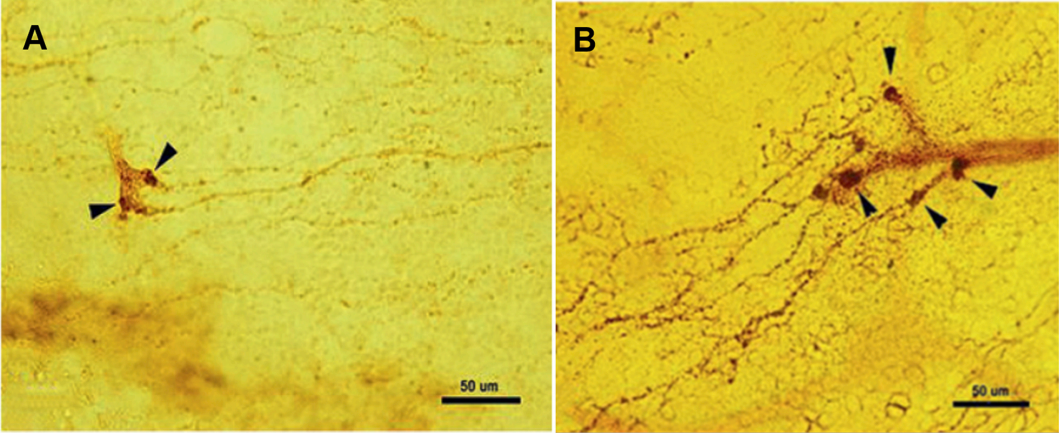

A
B
C
D


A
50 um
B
50 um