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CORNEA (T YAMAGUCHI, SECTION EDITOR)

In Vivo Confocal Microscopy Evaluation in Dry Eye and Related Diseases

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Abstract

Purpose of Review We reviewed recent findings on in vivo confocal microscopy (IVCM) of the ocular surface in dry eye and related diseases.

Recent Findings In dry eye disease, IVCM allows for corneal structure evaluation at the cellular level and is frequently used in diagnosis, disease course follow-up, and management. IVCM also enables a detailed examination of variations, such as abnormal hyperreflexia keratocytes and inflammatory cells, altered corneal superficial cell density, and basal cell density. In addition, several cellular alterations in ocular surface diseases have been detected using IVCM. Many studies have used IVCM to evaluate qualitative and quantitative changes in the corneal nerves associated with dry eye disease, enabling characterization of the morphology, density, and disease or surgically induced alterations of the subbasal nerve plexus.

Summary IVCM is a valuable and promising complementary method for clinical diagnosis and follow-up in dry eye and related diseases.

Keywords In vivo confocal microscopy · Dry eye diseases · Ocular surface · Corneal subbasal nerves

Introduction

Dry eye disease (DED) is one of the most frequently encountered diseases that affect hundreds of millions of people worldwide. Typical clinical presentation of severe DED is a limitation of daily activities, foreign body sensation, burning, itchiness, redness, pain, ocular fatigue, and visual disturbance. While the prevalence of DED in adults ranges from 10 to 20%, this rate is up to 33% in patients over 50 years old [1]. In the USA alone, approximately 7–10 million Americans require artificial tear preparations, with patients spending over $100 million/year [2].

The 2017 Dry Eye WorkShop (DEWS) report defined DED as a multifactorial disease of the ocular surface characterized by loss of the homeostasis of the tear film, accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles [3]. Despite the wide range of dry eye research described in the DEWS report, there are still many areas that require investigation at the pathophysiological level and in vivo confocal microscopy (IVCM) is a useful tool to help with these investigations.

In Vivo Confocal Scanning Laser Microscopy

IVCM is a noninvasive imaging method that allows for the study of corneal structures at the cellular level and is frequently used in the differential diagnosis and follow-up of healthy corneas as well as eye diseases. The Heidelberg Retina Tomograph (HRT) with the Rostock Cornea Module (Heidelberg Engineering, Heidelberg, Germany) is most frequently used for corneal surface layer examination. HRT uses a 670-nm red wavelength diode laser source, provides 400 × 400 μm real-time images of the cornea with a lateral resolution of 1 μm/pixel, and the total period of IVCM assessment is approximately 5–10 min per eye. Through IVCM, high-resolution images of epithelial cells, keratocytes, endothelial cells, corneal subbasal

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nerves, immune/inflammatory cells, and meibomian gland structures can be acquired, while also changes in these structures may also be monitored. With the increasing use of IVCM in clinics, many studies have begun to evaluate the structures of healthy and pathological corneas [4, 5••].

IVCM Evaluation of DED

IVCM enables the detailed examination of variations in the corneal layers associated with dry eyes and other ocular surface diseases, as well as the subbasal nerve plexus associated with corneal neuralgia [6].

In recent IVCM studies, many changes in meibomian glands in Sjögren syndrome [7], graft versus host disease [8], ocular demodicosis, superior limbic keratoconjunctivitis, contact lens use [9], and aging [10] have been reported. Studies using IVCM have shown changes in the corneal epithelium such as decreased superficial cell density and increased basal cell density in DED [11–13]. Abnormal hyperreflexia keratocytes in the stroma were also detected with IVCM [6, 14]. It was thought that these changes occurred as a result of pro-inflammatory mediators causing metabolic activation [6, 14].

Sjögren Syndrome

Sjögren syndrome (SS) is a chronic, systemic, autoimmune disease characterized by lymphocytic infiltration in all exocrine glands, especially in the lacrimal and salivary glands. The main symptoms of this disease, which is basically an autoimmune exocrinopathy, are mouth and eye dryness (sicca symptoms), but SS can also affect many organs and systems.

In SS, the most prominent eye finding is keratoconjunctivitis sicca (KCS), in other words, xerophthalmia or dry eye, which develops in the cornea and conjunctival epithelium, with symptoms such as burning, stinging, itching, and sensation of the presence of a foreign body. Photophobia, redness, mucous discharge in the morning, eyestrain, and blurred vision may also occur in the patients. Secondary infections, and rarely corneal perforations, can be seen and may lead to vision and even eye loss [15]. During diagnosis, the lack of tears is determined quantitatively with the most commonly used Schirmer test in practice. Ocular surface damage can be investigated with fluorescence in the cornea and with Rose Bengal and lissamine green vital staining in the conjunctiva.

During the examination of SS patients with the cornea, irregular corneal epithelium, decreased superficial corneal epithelial density, decreased subbasal cell count, increased tortuosity, bend-like formation in increased subbasal nerves, activated keratocytes, and decreased corneal thickness can be observed with IVCM [7, 16]. Furthermore, in patients with SS, subbasal nerve density was reported to be correlated with vital staining score and/or negative Schirmer test results [16]. In our previous study on patients with SS, we showed a significant increase in inflammatory cell density with IVCM, which is a sign of inflammation in the cornea and conjunctiva. These inflammatory cells were mostly polymorphonuclear cells, dendritic cells, and/or lymphocytes [17••].

Dendritic cells around conjunctiva and the limbus can be observed in many conditions, such as keratoconjunctivitis, ocular injury, use of contact lens, Sjögren syndrome DED (SSDE), non-Sjögren syndrome DED (NSSDE), atopic keratoconjunctivitis, and herpes keratosis. Another considerable finding was the notable reduction in the density of the superficial, intermediate, and deeper conjunctival epithelial cells, possibly due to the increase of the ocular surface inflammatory aspect and impairments in the overall turnover of epithelial cells. When we examined the positive Rose Bengal stained areas in the conjunctiva with IVCM, we clearly observed round, dark spots likely corresponding to micro cysts (Fig. 1). These epithelial micro cysts were significantly increased in patients with SS. These studies have shown that IVCM is a useful method for diagnosis and follow-up in SS, especially in the evaluation of inflammation and epithelial status.

Superior Limbic Keratoconjunctivitis

Although the cause of superior limbic keratoconjunctivitis (SLK) is not precisely known, it is thought to be caused by mechanical shear stress, which is caused by the friction between the upper eyelid and the superior bulbar conjunctiva. SLK was found to be associated with autoimmune thyroid disease [18]. SLK is a chronic and recurrent disease which causes ocular irritation and redness. This typically occurs in women between 20 and 70 years old and may act up between 1 and 10 years. This disease is usually bilateral; mild papillary reaction in the tarsal conjunctiva, thickening and injection in the upper bulbar conjunctiva, hypertrophy in the upper limbus, squamous metaplasia and above the limbus in the upper bulbar conjunctiva, and fluorescein and Rose Bengal staining on the superior area of the cornea is frequently observed [18–20].

As we have already mentioned, IVCM is a highly effective method for demonstrating corneal and conjunctival changes in patients with SSDE and non-SSDE disease. In a previous study, the mean individual epithelial cell area (MEICA), nucleocytoplasmic ratio (N/C), and inflammatory cell density in patients with SLK were investigated with IVCM and impression cytology (Fig. 2) [21]. Morphological changes such as cellular expansion, cell dropout, reduced cell cohesion, and shrunken nuclei were observed in both impression cytology and IVCM. Evaluation with IVCM showed that the inflammatory cell density was significantly higher in SLK patients

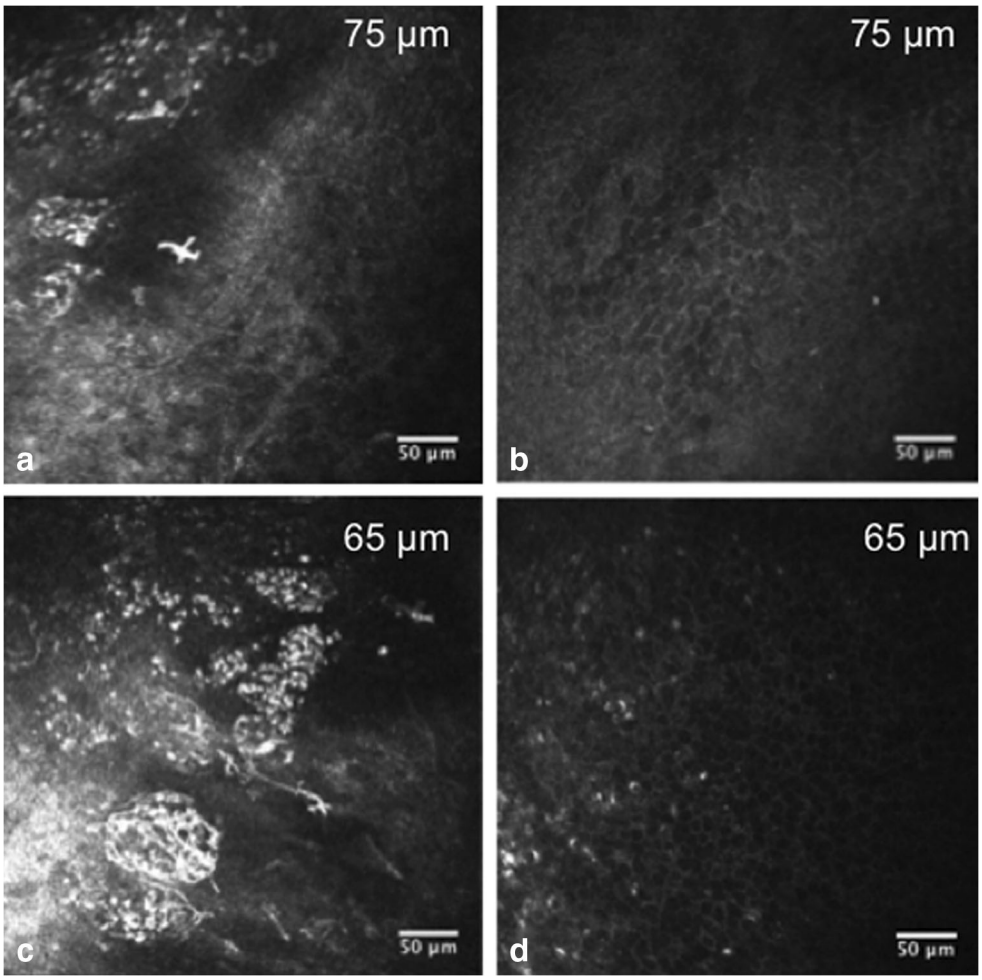

Fig. 1 **a**, c Widespread inflammation including dendritic cells, polymorphonuclear cells, and/or lymphocytes can be observed in the conjunctival epithelium in a 52-year-old woman with SS. **b**, **d** After 1-month dry eye treatment, significant improvement of conjunctival inflammatory infiltrates at two conjunctival epithelial depths (65 and 75 m) was observed [48]. Reprinted with permission from Wakamatsu TH, Sato EA, Matsumoto Y, Ibrahim OM, Dogru M, Kaido M, Ishida R, Tsubota K. Conjunctival in vivo confocal scanning laser microscopy in patients with Sjögren syndrome. Invest Ophthalmol Vis Sci. 2010 Jan;51(1):144–50

than in the control group. Based on IVCM, MEICA values were significantly higher and N/C ratio was significantly lower in SLK patients compared to the control group. Confocal microscopy in patients with SLK revealed that superficial epithelial cells were enlarged and had pyknotic nuclei. Additionally, round, white bodies, presumably goblet cells, were observed in normal control eyes. Such cells were not found in SLK patients [21].

Laser scanning confocal microscopy seems to be an efficient noninvasive tool in the evaluation of phenotypic alterations of the conjunctival epithelium in SLK and may serve as an alternative for impression cytology. N/C ratio and inflammatory cell density appear to be two new promising parameters of in vivo confocal microscopy in the assessment of ocular surface disease in SLK.

Examination of Corneal Nerves with IVCM

The 2017 DEWS report extensively discussed the role of neuronal involvement and neurosensory abnormalities in DED [3]. The cornea is the most densely innervated tissue in the body and it is supplied primarily by the ophthalmic terminal branch of the trigeminal nerve [22]. CSN fibers have a substantial function in corneal homeostasis and have a primary role in the maintenance of ocular surface sensation and epithelial integrity via regulation of epithelial cell proliferation and wound healing [23].

Many studies have used IVCM to evaluate DED-dependent qualitative and quantitative changes in the corneal nerves [24, 25]. These studies usually were focusing on the density of the corneal subbasal nerve. Although a decrease in corneal nerve density was detected in the majority of studies [10, 26–29], there are studies reporting an increase in nerve density in patients with SS [30]. However, Hoşal et al. [31] and Tuominen et al. [11] compared DED patients with control groups in terms of corneal subbasal nerve density and did not detect any changes. The results of these studies are thought to depend on the severity and the different stages of DED, the difference in neural regeneration/degeneration patterns, the level of inflammation, and the change in paralgesia and allodynia as a result of repetitive effects on the corneal nerves.

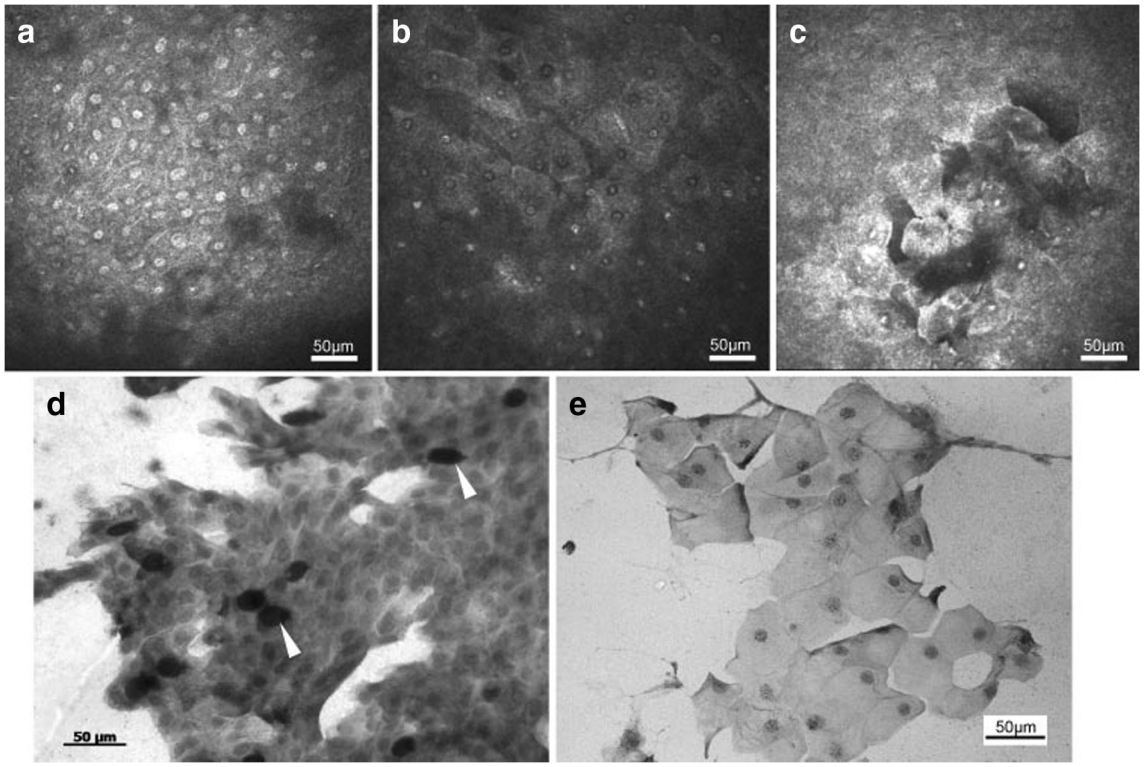

Fig. 2 Comparison of conjunctival epithelial cell differentiation between SLK patients and the control group. **a**–**c** Representative conjunctival epithelial cell images in confocal microscopy. **a**, **d** Eyes of a healthy individual. **b**, **c**, **e** Representative IVCM and impression cytology images of eyes in SLK patients. **b**, **e** Remarkable expansion of cell size with pyknotic nuclei was demonstrated in patients with SLK. **c** In some areas, sloughing of the superficial conjunctival epithelium was observed. White arrowheads indicate the presumable goblet cells with glycogen overload [21]. Reprinted with permission from Kojima T, Matsumoto Y, Ibrahim OM, Sato EA, Dogru M, Tsubota K. In vivo evaluation of superior limbic keratoconjunctivitis using laser scanning confocal microscopy and conjunctival impression cytology. Invest Ophthalmol Vis Sci. 2010 Aug;51(8):3986–92

Corneal sensitivity is very important for maintaining a healthy ocular surface. Corneal epithelium becomes more susceptible to external factors, when there is a decrease in blink reflex and tears due to any reason. As a result of excessive evaporation and cooling, tear osmolarity increases. Local inflammation and peripheral nerve damage occur as a result of stress in the ocular mucosal epithelium depending on a decrease in the amount of tears and an increase in osmolarity [32]. Local inflammation and nerve damage can cause short- and long-term genetic and molecular changes in primary sensorial neurons [33]. Sensorial nerve terminals are located in a densely and superficially between the epithelial cells on the corneal surface. Therefore, corneal superficial nerves are vulnerable to environmental factors (air pollution, low humidity), trauma (cataract and refractive surgery), and ocular surface diseases (pterygium, conjunctivochalasis, keratoconus) [14, 34, 35]. However, changes in the density and structure of subbasal nerves and epithelial nerve terminals depend on changes in tear secretion, according to studies in animal models [36, 37]. Similarly, studies on patients with tear deficiency due to various etiological reasons have shown changes in the number, tortuosity, branching pattern, and reflectivity of subbasal nerve fibers [7, 38, 39].

Other morphological parameters associated with corneal subbasal nerve density with IVCM were tortuosity, reflectivity, and beading pattern [7, 28, 30, 38, 40, 41]. These studies have shown an increase in these parameters and this increase is thought to be caused by neural regeneration after damage in the subbasal corneal nerves. In patients with dry eyes, an increase in the density of other immune cells along with DCs was determined in studies with IVCM [24, 25, 28, 42]. In these patients, clinical symptoms were parallel with the increase in DC density [43]. Therefore, IVCM can be considered as an important tool that can help diagnosis and treatment of DED. In our recent study, we evaluated the changes in tortuosity, reflective, and DC cell density in mice with dry eyes exposed to scopolamine for 1 month and revealed significant changes in these parameters. We thought that these changes occurred due to regeneration as a result of dry eye stress in the cells as a consequence of scopolamine application [44•].

Meibomian Gland Disease

Recently, IVCM has also been frequently used in the examination of meibomian gland dysfunction (MGD). Meibomian gland disorders are common in daily ophthalmology practice [45]. MGD is a term commonly used for obstructive meibomian gland disease. A decrease in meibomian gland lipid secretion, increase in tear evaporation, and decrease in tear stability can lead to deterioration in the lubrication of the ocular surface and damage to the corneal epithelium. In the etiology of DED, MGD is a significant factor [46].

IVCM was useful in characterizing phenotypic alterations in MGD such as subepithelial fibrosis and obstruction of meibomian gland (MG) orifices [47]. Additionally, our group previously reported that IVCM was efficient in describing phenotypic alterations in MGD by devising new diagnostic parameters such as acinar unit density and acinar unit diameter reflecting histopathological changes such as glandular atrophy and acinar/ductal dilatation [48].

IVCM can be used to measure morphological changes in the meibomian glands, the diameters and densities of the MG acinar unit, diameter of the MG orifices, and the density of periglandular inflammatory cells [7, 9, 48, 49]. IVCM also enables semi-quantitative evaluation of meibum secretion reflectivity and the heterogeneity of the glandular interstices and acinar wall [7, 9]. In patients with MG disease, a decrease in acinar cell density, an increase in acinar unit diameters, and an increase of reflectance in meibomian secretions were observed [7, 9, 49]. However, in patients with SS, small acinar units, increased inflammatory cell density, and decreased homogeneity in periglandular interstitium were demonstrated [7].

IVCM parameters showed acceptable sensitivity and specificity, and the cutoff parameters certainly helped clinicians in MGD diagnosis [49]. Similarly, another IVCM parameter described by our group in a recent study is periglandular inflammatory cell density, which suggested the potential of this novel technology in differentiating inflammatory obstructive MGD from non-inflammatory subtypes as well as the potential for evaluating the outcome of different treatment protocols [7].

Graft Versus Host Disease

Graft versus host disease (GVHD) is the most important complication of allogeneic hematopoietic stem cell transplantation, as a result of donor cell recognition by the host immune system as foreign antigens and subsequent attack. GVHD is a condition that affects many organs and systems, is characterized by high morbidity, and can lead to a wide variety of clinical outcomes [50, 51]. The eye is mostly affected by GVHD with an incidence between 60 and 90% [52, 53]. GVHD can cause various ocular conditions such as corneal epitheliopathy, MGD, ocular surface inflammation, conjunctival cicatricial disease, lacrimal gland dysfunction, hyperemia, uveitis, scleritis, and retinal microvasculopathy in the eye [54]. Additionally, the most common pathology in GVHD is DED, which has been reported to occur in 40–70% of GVHD patients [52, 53, 55].

In patients receiving hematopoietic stem cell therapy (HSCT) by our group, GVHD and non-GVHD patients were compared using IVCM, and significant differences were observed [56]. Subbasal corneal nerve density was significantly lower in patients with GVHD compared to individuals with normal eyes. These findings were consistent with Kheirkhah et al. [57]. This change in subbasal corneal nerve cell density was thought to be related not only to GVHD-related DED, but also to the immune reaction that occurred. In the same study, the tortuosity and branching patterns of the subbasal nerves were evaluated and a significant increase was observed in both. It is thought that this increase is caused by a regeneration process initiated by subcutaneous nerves by nerve growth factors and cytokines released during inflammation due to ocular surface damage [56]. Another change in the ocular surface of patients with GVHD is the density changes in dendritic cells, which played an important role in the primary immune response. These cells primarily play a role in the initiation of immune responses as antigen presenting cells. As a result, in the same study, the density of DCs in both the central cornea and limbal epithelium was found to be significantly high in patients with DED due to GVHD. In addition, significant morphological changes were observed in distinct subbasal nerves in patients with dry eye associated with GVHD receiving HSCT [56]. In light of all these findings, IVCM is considered as a potentially helpful technique in the evaluation of ocular surface changes in the eyes especially during inflammation in GVHD.

Ocular Demodicosis

Demodex folliculorum (DF) and *Demodex brevis* (DB) are mites that are parasites only in humans and settle in human hair follicles and pilosebaceous units [58]. It is widely believed that there may be a potential risk for skin diseases [59, 60]. DF and DB can be found in humans, especially in the face, nose, eyelashes, ears, and genital area [59]. Although they can remain in the intact skin, in the hair follicles, and in the meibomian glands without any pathogenic effects, they can be pathogenic in certain cases that skin cleaning is not performed well and the immune system is suppressed, causing inflammatory dermatitis, contributing to the development of keratosis and epithelioma, cuasing acne and acne rosacea [60–64]. Occasionally, they can cause strong skin reactions and prominent pigmentation [60]. Moreover, a large number

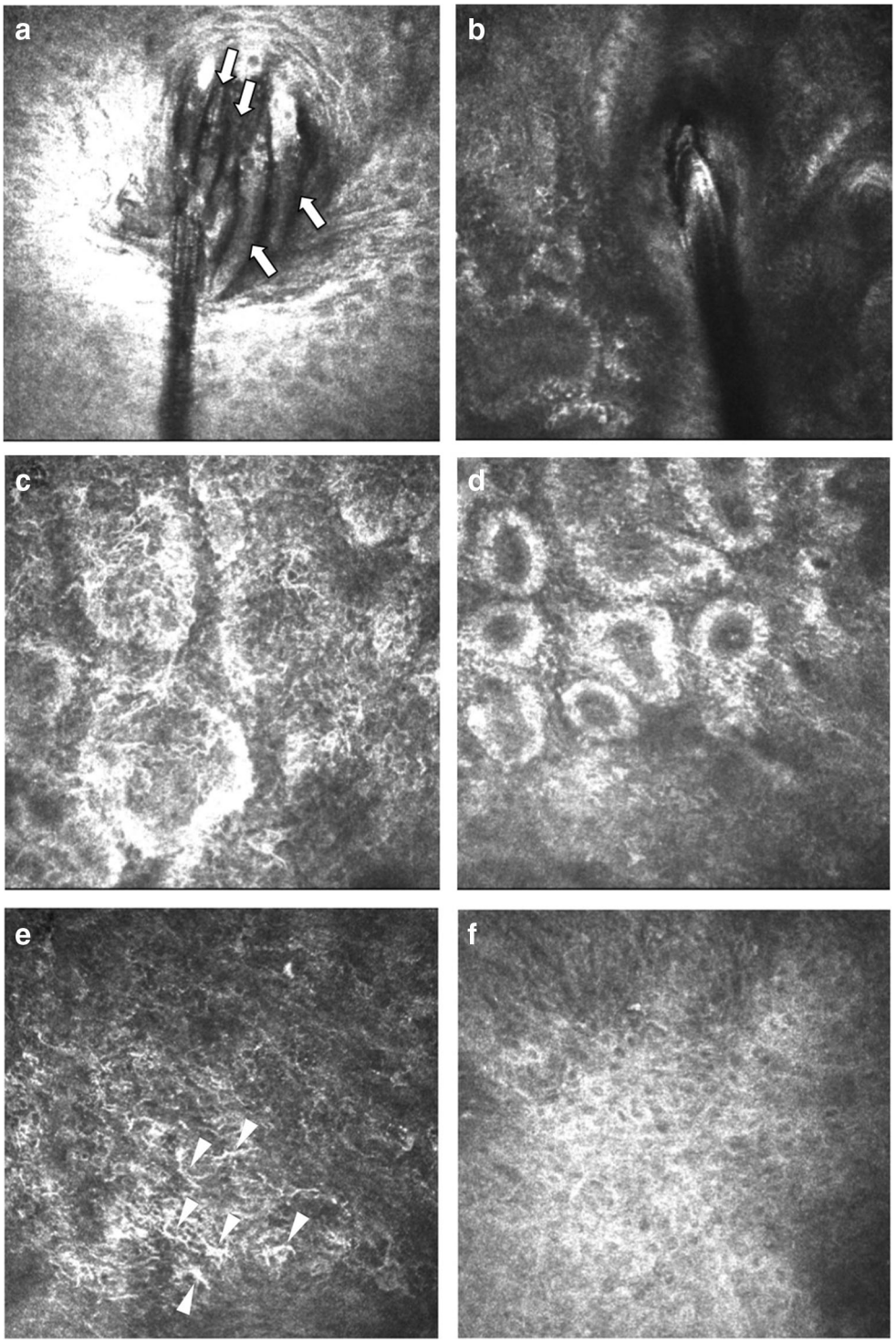

Fig. 3 **a** Heavy demodicosis infestation of eyelash follicles in a 72-year-old female patient shown by in vivo confocal microscopy. Note the presence of several *Demodex* colonies. **b** Representative IVCM images of eyelash bulbs in a healthy female control individual; note the absence of mites. **c**–**f** IVCM images of meibomian gland acinar units and palpebral conjunctiva before and after tea tree oil treatment of the patient in panel **a**. **c** Heterogeneous reflectivity of the gland lumen and dilatation of meibomian gland acinar units can be seen. **e** Prominent inflammatory infiltrates can be observed in the palpebral conjunctiva near the eyelid margin. **d**, **f** After treatment with tea tree oil, a significant improvement was observed in acinar dilatation and conjunctival cell infiltration [66]. Reprinted with permission from Kojima T, Ishida R, Sato EA, Kawakita T, Ibrahim OM, Matsumoto Y, Kaido M, Dogru M, Tsubota K. In vivo evaluation of ocular demodicosis using laser scanning confocal microscopy. Invest Ophthalmol Vis Sci. 2011 Feb 1;52(1):565–9

of mites may be located in the eyelid follicles and may cause keratosis, hyperplasia, and melanocyte aggregation [65].

Demodex mites can cause pathogenic conditions such as papulopustular rosacea, pityriasis folliculorum, rosacea-like demodicosis, demodicosis gravis (granulomatous rosacea-like demodicosis), and blepharitis when they over-proliferate or penetrate the dermis [66]. Previous studies have shown that the number of DFs in eyelashes was higher in blepharitis patients than in the control group, and it was determined that pruritus occurred parallelly with the ovulation period of *Demodex* in itchy blepharitis cases [62, 67–70].

In our previous study, we evaluated the feasibility and efficacy of IVCM in diagnosis and follow-up in patients with blepharitis-related cylindrical dandruff. According to

our results, IVCM was a highly effective method for the evaluation of meibomian glands/conjunctival diseases, acinar dilatation, periglandular inflammation, and conjunctival inflammation as well as the demonstration of embedded mites in the bulb. In the evaluation of patients with demodicosis with IVCM, dilution in meibomian gland acinar units and periglandular inflammatory infiltrates with DCs were observed (Fig. 3). Similarly, prominent inflammatory infiltrates were found in the palpebral conjunctiva adjacent to the eyelids. In addition, IVCM has been shown to be a very useful technology in the follow-up of changes in the eyelids, meibomian, and conjunctival diseases following tea tree oil treatment [66]. As a consequence, laser scanning confocal microscopy is an efficient noninvasive tool in the diagnosis and follow-up of ocular demodicosis infestation.

Conclusions

Recently, the number of studies involving IVCM has increased dramatically [71••]. In the light of all these studies, IVCM is thought to be an extremely promising method, easy to implement, cost-effective, and minimally invasive providing rapid outcomes for the evaluation of ocular surface structures in dry eye and related diseases at the cellular level. Although it is a very practical method, there are some difficulties in evaluation with IVCM. IVCM can display a small area of the entire cornea. In order to see the entire cornea, it is necessary to acquire many shots from different regions and these areas may sometimes overlap. In addition, it is very difficult to reevaluate the same area which has been previously imaged. In addition, stable contact with the patient's eye sometimes can be difficult, even if local anesthesia is used. Additional concerns include the standardization of image acquisition, interpretation, and quantification [72]. Despite these difficulties, IVCM is a valuable and promising complementary method in clinical diagnosis and follow-up in DED.

Compliance with Ethical Standards

Conflict of Interest Simsek C, Karalezli A, and Dogru M each declare no potential conflicts of interest.

Kojima T has received personal fees from Santen pharmaceutical, Otsuka pharmaceutical, Alcon, Eye Lens, Carl Zeiss Meditec, and Echo electricity.

Human and Animal Rights and Informed Consent All reported studies/experiments with human or animal subjects performed by the authors have been previously published and complied with all applicable ethical standards (including the Helsinki declaration and its amendments, institutional/national research committee standards, and international/national/institutional guidelines).

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- • Of importance
- •• Of major importance

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