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The use of *in vivo* confocal microscopy in fungal keratitis – Progress and challenges

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ABSTRACT

Fungal keratitis (FK) is a serious and sight-threatening corneal infection with global reach. The need for prompt diagnosis is paramount, as a delay in initiation of treatment could lead to irreversible vision loss. Current "gold standard" diagnostic methods, namely corneal smear and culture, have limitations due to diagnostic insensitivity and their time-consuming nature. PCR is a newer, complementary method used in the diagnosis of fungal keratitis, whose results are also sample-dependent. *In vivo* confocal microscopy (IVCM) is a promising complementary diagnostic method of increasing importance as it allows non-invasive real-time direct visualization of potential fungal pathogens and manifesting infection directly in the patient's cornea. In numerous articles and case reports, FK diagnosis by IVCM has been evaluated, and different features, approaches, sensitivity/specificity, and limitations have been noted. Here, we provide an up-to-date, comprehensive review of the current literature and present the authors' combined recommendations for fungal identification in IVCM images, while also looking to the future of FK assessment by IVCM using artificial intelligence methods.

1. Introduction

Fungal keratitis (FK) is a serious, sight-threatening infection of the cornea, particularly prevalent in developing countries [1], but nevertheless with a global reach. The clinical presentation of patients with FK usually includes pain, photophobia, decreased vision, redness, and excessive tear secretion [2]. The classic findings are a whitish corneal infiltrate with feathery margins and satellite lesions [3], with eyelid edema, conjunctival hyperemia, chemosis, corneal epithelial defect, endothelial plaques [4] and an anterior chamber reaction with or without a hypopyon [2]. Typical examples of FK cases are shown in Fig. 1. Risk factors for FK include ocular trauma, contact lens wear, ocular surface disease, nasolacrimal duct obstruction, fungal skin infections, and long-term use of local or systemic steroids or antibiotics [1]. Agricultural workers are at a higher risk due to potential ocular trauma by soil and plant material [5]. More than 100 species of fungi are known to cause FK [6]. In tropical and subtropical regions, filamentous fungi (*Fusarium* and *Aspergillus* spp.) are most commonly seen [7],

Abbreviations: IVCM, In vivo confocal microscopy; AI, artificial intelligence.

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whereas yeast (*Candida* spp.) is more common in temperate climates [2]. In 2005, an outbreak of *Fusarium* keratitis in healthy eyes of contact lens wearers in many developed countries gained great interest [8,9]. The cause was traced to a contaminated contact lens disinfection solution (ReNu with MoistureLoc, Bausch & Lomb) [8].

The traditional diagnostic methods of FK are detection of fungal elements on corneal smears and isolation of fungus in culture [10]. The culture method, however, has drawbacks; for example, growth of fungi in culture takes several days to weeks, and may show a false negative result, especially in deep corneal infections where the sampled superficial cornea may not contain fungal elements [11]. Furthermore, corneal scrapes involve invasive tissue sampling. Delayed or false negative cultures can lead to a delay in the diagnosis of FK [1,6]. However, timely diagnosis and treatment is imperative as a prompt, targeted treatment decreases the risk of long-term damage to the eye. The high rate of vision loss following a fungal infection of the cornea, and limitations associated with traditional diagnostic methods, emphasize the need for a rapid and less invasive diagnostic option [1]. PCR is a complementary diagnostic method used to detect fungi [12], which provides a rapid diagnosis compared to the culture method [13–15].

In vivo confocal microscopy (IVCM) is a non-invasive imaging modality that provides *en face* images of layers through the thickness of the cornea. IVCM examination of the cornea renders a series of high-contrast and high-resolution images of living tissue, potentially allowing rapid identification and diagnosis of pathogenic infection. So far, IVCM has mainly been used as a diagnostic tool in the field of ophthalmology, but other established uses include examination of skin lesions [16] and the oral cavity [17].

The first confocal microscope introduced for clinical examination of the cornea was the tandem-scanning confocal microscope (TSCM) in 1990 [18]. The low light throughput of the TSCM gave way to more modern instruments used in clinical practice today, such as the white light slit-scanning confocal microscope (ConfoScan series, Nidek) and the laser-scanning confocal microscope (Heidelberg Retina Tomograph II or III with the Rostock Cornea Module (HRTII-III/RCM), Heidelberg Engineering). The Nidek ConfoScan microscope uses a slit-scanning design and allows for a non-contact exam, whereas the HRT-RCM is used with a sterile, disposable cap and an index-matching ophthalmic gel [19]. The gel makes gentle contact with the cornea, minimizing light scattering and reflections as well as providing additional stabilization of the eye. The HRT-RCM system uses a diode laser and has a lateral resolution of 1 μm. Generally, the laser-scanning technology, which scans the sample with a focused laser spot of a single wavelength, gives better confocality (axial resolution), higher contrast, and more uniform image illumination than the white light slit-scanning method [20].

In the present review, we examine the role of IVCM in the diagnosis of FK. We investigate the body of evidence reporting the use of IVCM in the identification, diagnosis, and management of FK, and discuss the advantages and limitations of the technique. We also discuss the evolution of the technique apparent in the literature, from considering IVCM solely as a method to confirm FK, to more recent studies applying advanced artificial intelligence techniques to analyze IVCM images to enable early and rapid FK diagnosis. We also present a classification scheme for suspected fungal elements in IVCM images, as well as recommendations for IVCM image-guided assessment of FK cases, based on agreement among several of the authors who are experienced IVCM operators and examiners.

2. Literature search

2.1. Literature search strategy

A literature search was conducted using the PubMed and Ovid EMBASE databases on September 7th, 2021, using the keyword groups 'fungal' AND 'keratitis' AND 'confocal microscopy OR IVCM'. The term 'fungal' included the words 'fungals' OR 'microbiology' OR 'fungal' or 'fungi'. The term 'keratitis' included the words 'keratic' OR 'keratitis' OR 'keratitides'. The term 'confocal microscopy' included the MeSH Terms 'microscopy, confocal' OR the words ('confocal' AND 'microscopy') OR 'confocal microscopy'. All published full-text articles in English were included in the initial search results, regardless of publication date. The relevance of the articles was first determined based on title and abstract. The initial search and screening were performed based on a consensus among three researchers (IB, NL, TPU).

2.2. Search results

The initial search yielded 251 results in PubMed and 268 results in Ovid EMBASE. These were further filtered by relevance, i.e., whether the article included FK and the use of IVCM. Most did not fulfill this criterion, with the majority of articles describing keratitis of other etiologies such as *Acanthamoeba*, viral or bacterial keratitis, FK after surgery, laser refractive surgery or crosslinking, or they did not describe the use of IVCM in the diagnostics. Through manual search of the reference list of the already included articles, additional relevant studies were identified and included. Excluding all non-relevant articles, the final list included 21 original articles and 29 case reports summarized in Table 1 and Table 2, respectively. Fig. 2 depicts the country of origin of the included studies and case reports.

Investigating if there were recent published reviews regarding FK and diagnosis by IVCM, we found two articles concerning the diagnosis of infectious keratitis by IVCM [21,22], both published in 2010 or earlier. Some recently published review articles do have a subsection of IVCM and fungal keratitis [23–27]. These reviews, however, were not specifically focused on FK diagnosis by IVCM. In this rapidly evolving field, 25 of 29 case reports and 15 of 21 original articles identified in our search were published after 2010, indicating a growing activity in the

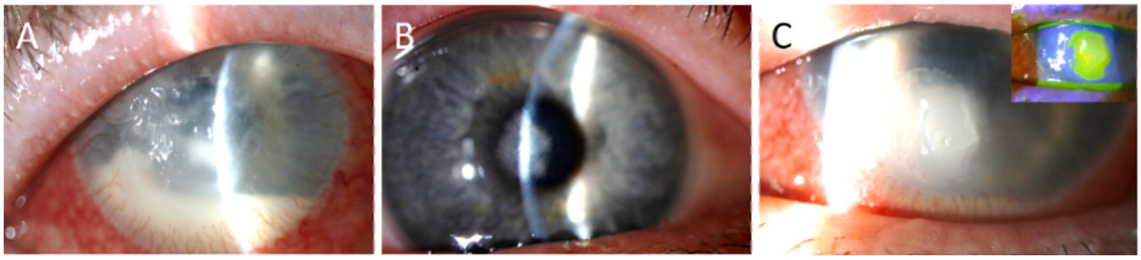

Fig. 1. Clinical examples of fungal keratitis in Sweden. (A) Combined *Fusarium* spp. and *Staphylococcus* spp. infection in a 78-year-old woman. Note the conjunctival hyperemia, hypopyon, peripheral corneal neovascularization, stromal infiltration, and irregular corneal surface. (B) *Fusarium* spp. infection in a 50-year-old woman due to ocular trauma from a direct strike by a pigeon. The infected region in this case has feathery margins and is confined to the central cornea. (C) A case of combined *Candida* spp. and *Staphylococcus* spp. infection due to fingernail trauma. Note the conjunctival hyperemia, hypopyon, peripheral corneal neovascularization, and thick, white protruding stromal infiltration with epithelial defect (fluorescein staining in inset). Images provided by Per Fagerholm, Linköping University, Sweden.

Table 1
Original articles reporting data from several patients as a clinical study.

	First author, year	Focus of study	# eyes	Criteria to identify FK	Cmf FK	IVCM assessor	Key findings	IVCM sen/spe (%)	Study design
1	Hoffman et al., 2021 [41]	Comparing IVCM, PCR, and culture in diagnostics of MK.	15	N/A	IVCM, PCR, M	MO	IVCM was the most sensitive method to diagnose FK (and AK)	81.8%/100%	R
2	Tabatabaei et al., 2020 [11]	IVCM use in FK treatment, differentiation *Aspergillus* and *Fusarium* spp.	65	N/A	M	MO	IVCM is helpful to monitor FK treatment, but cannot differentiate *Aspergillus* and *Fusarium* spp.	N/A	P
3	Roth et al., 2020 [44]	Establishment of a simulator to practice IVCM.	9	N/A	N/A	Ophthalmologists, varying experience	A simulator is a good way to gain experience in IVCM assessment	N/A	N/A
4	Lv et al., 2020 [34]	Developing an intelligent system for IVCM diagnostics.	[a]	Fungal hyphae	M	ResNet	The system based on ResNet had good results in diagnosing FK.	91.86%/ 98.34%	R
5	Liu et al., 2020 [33]	CNN framework for automated diagnosis of FK.	[b]	N/A	N/A	CNN	CNN can improve performance in the diagnosis of FK.	N/A	N/A
6	Wang et al., 2019 [39]	Use of IVCM in IK	12	HR, septate, double-walled filaments, W = 3–8 μm.	C, M, T	MO, experienced	IVCM is as good as clinical assessment in diagnosing FK.	66.7%/100%	R
7	Chidambaram et al., 2019 [30]	FK clinical outcome indication by IVCM	143	HR, Br structures, W = 3–6 μm, L < 400 μm.	M, LM, IVCM	MO, experienced	IVCM can indicate clinical outcome in FK.	N/A	P
8	Anutarapongpan et al., 2018 [46]	Role of IVCM in *Pythium insidiosum* keratitis	21	HR, double-walled filaments, varying size.	M, PCR	Experienced corneal specialist	IVCM cannot differentiate *P. insidiosum* from FK.	N/A	R
9	Wu et al., 2018 [36]	Accuracy of AHD vs. corneal smear.	56	Fungal hyphae not specified.	M	IRBHD and ophthalmologist	AHD was superior to corneal smear in FK.	89.29%/ 95.65%	P
10	Chidambaram et al., 2018 [31]	IVCM cellular features of BK, FK, AK.	183	Fungal hyphae/ filaments, spore-like structures	M or LM	MO, experienced	Features observed by IVCM may be associated with different organisms.	N/A	P
11	Kheirkhah et al., 2017 [38]	The use of IVCM for detection of filamentous fungi.	21[c]	Based on observer's experience or literature.	T	MO, varying experience	IVCM is highly dependent on of the observer's experience.	42.9–71.4%/ 87.5–89.6%	R
12	Chidambaram et al., 2017 [28]	Using IVCM to distinguish *Fusarium* and *Aspergillus* spp.	98	Br hyphae	M	Single MO, unknown experience	IVCM cannot distinguish *Fusarium* and *Aspergillus* spp.	N/A	P
13	Chidambaram et al., 2016 [29]	Diagnostic accuracy of IVCM in MK.	176	Fungal filaments, W = 3–6 μm.	M, LM	MO, varying experience	IVCM is a valuable tool in detecting FK.	85.7%/81.4%	P
14	Nielsen et al., 2013 [42]	Introducing a grading system to interpretate IVCM in FK.	6	Li, HR, Br, bifurcating interlocking or isolated, well-defined linear structures	M, H	N/A	IVCM is superior to culture in FK. Forming three categories of IVCM characteristics of FK.	86%/N/A	N/A
15	Vaddavalli et al., 2011 [10]	IVCM use in diagnostics of MK.	93[d]	HR, Br, septate, double-walled filaments, W 3–8 μm	M	MO, unknown experience	IVCM is an accurate diagnostic method for AK and FK.	89.2%/92.7%	P
16	Takezawa et al., 2010 [45]	IVCM in the diagnostics and treatment of FK.	6	HR, Br, septate, interlocking white lines, W = 3–5 μm.	M	N/A	IVCM is helpful in diagnosis and evaluation of treatment in FK.	N/A	N/A
17	Hau et al., 2010 [40]	Accuracy of IVCM in MK.	12[e]	HR, irregular Br, Li objects.	M, H	MO, varying experience	IVCM diagnostic accuracy is dependent on observer experience.	27.9–55.8%/ 42.1–84.2%	R
18	Das et al., 2009 [32]	The role of IVCM in deep FK	6	HR, Br, septate, double-walled, filaments, W = 3–8 μm	M, H	N/A	IVCM is useful for diagnosing deep FK.	N/A	R
19	Shi et al., 2008 [35]	Evaluation of FK treatment response by IVCM	121	Br filaments	M, H	N/A	IVCM can guide anti-fungal therapy.	N/A	N/A
20	Kanavi et al., 2007 [37]	IVCM vs smear & culture.	16	HR, Br, hyphae-like lines, W = 4–8 μm	M	N/A	IVCM is useful in diagnosing FK and AK.	94%/78%	N/A
21	Brasnu et al., 2007 [43]	Benefit of IVCM in FK	5	HR, Br, lines, L = 200–300 μm, W = 3–5 μm	M	N/A	IVCM is a rapid technique of early FK diagnosis.	N/A	N/A

eyes = number of eyes included in study, AHD = Automatic hyphae detection, AK = acanthamoeba keratitis, BK = bacterial keratitis, Br = branching, C = clinical presentation, cfm = confirmatory methods, CL = contact lens, CNN = convolutional neural network, FK = fungal keratitis, HR = highly-reflective, HRTII-RCM IVCM = Heidelberg Tomograph II-Rostock Cornea Module, HRTIII-RCM IVCM = Heidelberg Tomograph III-Rostock Cornea Module, H = histopathologic examination/biopsy, IK = infectious keratitis, IRBHD = image recognition-based hyphae detection, IVCM = in vitro confocal microscopy, L = length, Li = linear, LM = light microscopy, LS-IVCM = laser scanning in vitro confocal microscopy, M = microbiologic examination (smear/culture), Mi = microscopy, MO = masked observer, MK = microbial

keratitis, N/A = not available, OSD = ocular surface disease, P = prospective, PCR = polymerase chain reaction, R = retrospective, sen = sensitivity, spe = specificity, T = response to specific treatment, TSM = tandem scanning microscope, W = width, μm = micrometers.

[a] Number of images included: 688 (number of patients not given). Control group = 1400 images negative for fungi.

[b] Number of images included: 994 (number of patients not given). Control group = 219 normal images.

[c] Number of controls (BK): 24.

[d] Number of FK/AK-negative controls: 45.

[e] Number of controls (BK): 19.

topic during the past decade and thus the need for an updated and focused review.

3. *In vivo* confocal microscopy methodology

The articles (Table 1) investigating the use of IVCM in FK were published in India (n = 6) [10,28–32], China (n = 4) [33–36], Iran (n = 2) [11,37], United States (n = 2) [38,39], United Kingdom (n = 2) [40,41], Denmark (n = 1) [42], France (n = 1) [43], Germany (n = 1) [44], Japan (n = 1) [45], and Thailand (n = 1) [46] (Fig. 3). The majority of these original articles (71.4%) reported the use of IVCM in a primary diagnostic capacity [10,11,28–30,32,35,37–41,43,45,46]. A group of articles (19.0%) aimed to improve the diagnostic accuracy of IVCM (including use of artificial intelligence) [33,34,36,44]. Other articles (9.5%) described specific IVCM features of FK [31] or introduced an image grading system [42].

Thirteen (66.7%) of the publications listed in Table 1 each included more than 10 patients [10,11,28–31,35–41,46]. Of the articles mentioning the number of patients examined, the largest study investigated the cellular features of fungal keratitis (FK), *Acanthamoeba* keratitis (AK), and bacterial keratitis (BK), and included 183 patients in total [31]. Assessing the original articles in Table 1, most studies included patients based on clinical findings, and IVCM appeared to play a small role in the inclusion of patients. "Clinically suspected" [10,11,28,36,37,43,45] or culture-verified FK [34,40,42,46] were found to be the criteria used most frequently to include patients. Regarding the criteria to exclude patients, descemetocele or excessive thinning of the cornea [10,11,28–31,37,46] and perforated ulcers [10,36,37,46] were the main reasons. Previous history of herpes virus keratitis [28–31] and visual acuity less than 20/200 in the unaffected eye [28–31] was described as exclusion criteria in 19.0% of the articles.

Of the included original articles in Table 1, 33.3% were retrospective studies [32,34,38–41,46]. These retrospective studies included patients who had undergone IVCM and were diagnosed with FK based on clinical presentation [39], response to therapy [38,39], verification by culture [32,34,39–41], biopsy [32,40], polymerase chain reaction (PCR) [41,46], or IVCM [41]. 33.3% of the articles in Table 1 were prospective [10,11,28–31,36], and 33.3% did not mention the study design [33,35,37,42–45]. Unknown study design is of concern as a retrospective design is known to be a source of bias. The studies of unknown design are mainly the early studies [35,37,43,45] evaluating the use of IVCM in FK, and the studies investigating the use of artificial intelligence [33], establishing a simulator for IVCM training [44], or introducing an image grading system to interpret the IVCM findings [42].

Table 2 summarizes the findings from the 29 case reports identified from the literature search. Although there is a low level of evidence available in case reports, they provide important information on less common species, and the interested reader is referred to the relevant references given in Table 2.

Several potential sources of bias in the original articles reviewed in Table 1 were identified. Regarding human observers/assessors, sources of potential bias included unknown experience of the IVCM assessor, not stating whether the assessor(s) were masked to the microbial result, not using multiple assessors and determining inter- and intra-assessor variabilities, and the lack of clearly defined positive and negative identification criteria for fungal hyphae in IVCM images. Another source of potential bias in many studies was the possibility of false-negatives or selection bias due to the use of positive fungal culture as a reference. On the other hand, the use of a reference standard as a comparison is important for generating diagnostic accuracy data for any emerging diagnostic imaging techniques. The use of a control group (non-fungus cases) would reduce the rate of false-negatives and should be a prerequisite in diagnostic accuracy study design. Of the investigated studies in Table 1, only 19.0% [10,38,40,44] included such a control group. A summary of the potential sources of bias in the articles analyzed in Table 1 is given in Table 3.

Of the articles listed in Table 1, 57.1% used the HRT3-RCM [10,11,28–31,33,34,36,38,39,42] and 23.8% used the HRT2-RCM [40,41,43–45] laser-scanning IVCM. The Nidek ConfoScan 4.0 [46], ConfoScan 3.0 [32,37], or ConfoScan 2.0 [35] slit-scanning system was used in 19.0% of the studies. The IVCM investigations were performed by "experienced investigators" [10,11,30,31,42,46], "confocal microscopists" [39,40], or "physicians"/"ophthalmologists"/"cornea experts" [29,34,36]. "Masked graders" [28] with varying experience [29,40] or "ophthalmologists"/"cornea specialists" with varying experience [36,38,46] reviewed the images. Four of the articles did not describe the IVCM-assessor [32,35,37,42]; and IVCM-investigator [32,35,37] was not described in three of the articles. A follow-up period was reported in four of the articles (19.0%). This period varied from 2 weeks [11] to 37 days [30], 7 weeks [45], and 2 months [35] after the initial IVCM examination and treatment initiation.

Considering that the multitude of studies use different terminology, we suggest that the persons performing the IVCM are called "IVCM-operator" and the persons assessing the images are called "IVCM-assessor", both may be masked or unmasked. Apart from the terminology, knowing the experience of both the investigator and the assessor is crucial, and we recommend future studies to specify this.

4. Use of IVCM in the diagnosis, prognosis, and follow-up of FK

4.1. Criteria for identification of filamentous fungal elements by IVCM

The first study describing the use of IVCM in the diagnosis of infectious keratitis was by Chew and colleagues in 1992 [47]. They used TSCM to examine live rabbit eyes infected with *Aspergillus* fungi. They observed characteristic branching hyphae, 3–5 μm in width and of variable length, surrounded by a zone of corneal edema. The team found that TSCM made it possible to examine infected corneas in real-time, noninvasively, and at high magnification even during the very early stages of the infection.

Recognition of morphologic features that are consistent with filamentous fungal hyphae in IVCM images is the key to the use of IVCM in assessment of possible cases of filamentous FK. Sixteen (76.1%) of the reviewed articles in Table 1 described morphologic criteria for identification of fungi using IVCM. The terms used to describe the morphology were 'filaments' [10,29,31,32,35,39,46], 'hyphae' [28], 'elements' [42], 'lines' [43,45], or 'linear' [40,42]. 'Structures' [30,37] were described as being 'highly'/'hyper-reflective' or 'high-contrast' [10,30,32,37,39,40,42,43,45,46], 'septate' [10,32,39,45], or 'branching' [10,28,30,32,35,37,39,40,42,43,45,46]. Filament diameter varied between 3 and 5 μm [43,45], 3–6 μm [29,30], 3–8 μm [10,32,39], 4–8 μm [37], and 1.5–7.5 μm [46]; and the length was up to 400 μm [30,46]. Takezawa and colleagues (2010) described fungal filament length as "hundreds of micrometers" [45].

FK caused by *Aspergillus* spp. is associated with a poorer clinical outcome compared to FK caused by *Fusarium* spp [28]. However, while

Table 2
Relevant case reports.

	First Author, year	Focus of study	# Pt	IVCM findings	Cmf FK	Key findings
1	Palioura et al., 2021 [61]	A case of deep FK diagnosed by endothelial biopsy	1	No identified fungal elements	EB	Deep FK diagnosed by deep corneal biopsy, by the aid of AS-OCT.
2	Roszkowska et al., 2021 [62]	Combined keratitis by *Acanthamoeba* and *Phialemonium curvatum*, a rare cause of ocular infection.	1	Fungal hyphae.	M, IVCM	First report of corneal coinfection by *P.curvatum* and *Acanthamoeba*, where prompt diagnosis by IVCM allowed early diagnosis and treatment.
3	Soifier et al., 2021 [63]	FK caused by *Purpureocillium lilacium*.	1	Hyperreflective septate branching hyphae	IVCM	Describing a case of FK caused by *P. lilacium*, a fungus not reliably responding to commonly used antifungals
4	Liu et al., 2021 [64]	FK caused by the rare pathogen *Myrothecium verrucaria*.	1	Massive interlocking white lines	M, IVCM, PCR	IVCM finding of the rare pathogen *M. verrucaria*, also presenting the therapy MICs.
5	Knutsson et al., 2021 [65]	Three cases presenting the effects of abrupt discontinuation of steroids in FK	3	N/A	M	Diagnosed FK should slowly taper corticosteroids, start antifungal therapy and maintain antibiotic therapy.
6	Raghavan et al., 2021 [66]	Combined Acanthamoeba and *Cladosporium* keratitis,	1	Multiple cysts	M	In keratitis cases with ring infiltrates and feathery edges one should suspect co-infection.
7	Massa et al., 2020 [67]	Describing the first reported case of *Phaeoacremonium parasiticum* keratitis	1	Filamentous infiltrate	ITSS	First report of *P. parasiticum* keratitis. IVCM was helpful in finding the right diagnosis.
8	Bayraktutar et al., 2020 [68]	A case of ophthalmia nodosa misdiagnosed as FK	1	Numerous HR, linear needle-shaped structures with small protrusions	N/A	IVCM findings was essential in making the right diagnosis.
9	Tabatabaei et al., 2018 [69]	Two rare cases of filamentous FK	2	HR, linear, branching, interlocking structures	M	*Pseudallescheria bodii* and *Colletotrichium coccodes* were discovered.
10	Rathi et al., 2018 [70]	Suspected FK not responding to maximal therapy, must raise the suspicion of *P. insidiosum.*	1	Branching hyphae	PCR, ITSS	*P. insidiosum* keratitis leading to fatal cavernous sinus thrombophlebitis.
11	Behaegel et al., 2018 [71]	A case of *Nocardia* keratitis, suspected only after poor response to protozoal and fungal treatment.	1	Long structures with possible fungal morphology	M	Nocardia keratitis should be a differential diagnosis in keratitis cases not responding to initial therapy.
12	Johansson et al., 2017 [72]	A keratitis case not responding to therapy		Branching filamentous structures	IVCM, B, M	IVCM gave the first evidence to support the clinical diagnosis of Nocardia keratitis.
13	Aggarwal et al., 2017 [52]	Post PKP keratitis caused by the rare fungi *Exophiala phaeomuriformis.*	1	Filamentous structures at a depth of 96–100 μm in the stroma	DNAS	*E. phaeomuriformis.*is a rare fungus to cause FK, and can be suspected when culture show black yeast.
14	Li et al., 2016 [73]	An FK-case by *Lasiodiplodia theobromae*, a rare cause of keratitis.	1	Fungal elements in superficial and mid-stroma.	M	Early IVCM allowed the initiation of anti-fungal therapy on day one.
15	He et al., 2016 [50]	*Pythium insidiosum* keratitis misdiagnosed as FK	1	High refraction filaments with irregular branching, 3–5 μm W, 200–400 μm L	PCR	*P. insidiosum* is a rare but destructive cause of keratitis, here misdiagnosed as FK
16	Lelievre et al., 2015 [49]	*Pythium insidiosum* keratitis initially misdiagnosed as FK.	1	Septate linear branching structures	ITSS	*P. insidiosum* is a rare but destructive cause of keratitis, surgical debridement being the most effective treatment
17	Mitani et al., 2014 [74]	A case of FK caused by the slow growing fungi *B. bassiana*	1	Interlocking, branching white lines	DNAS	IVCM showed filamentous fungi, confirmed by DNAS to be *B. bassiana.*
18	Hong et al., 2014 [75]	A case of non-typical *Pseudomonas aeruginosa* keratitis misdiagnosed as FK	1	HR, thin, branching interlocking structures, 5–8 μm W, 200–400 μm L	M, H	*P. aeruginosa* keratitis misdiagnosed as FK by findings on IVCM
19	Giovannini et al., 2014 [76]	FK caused by a rare fungus in human disease: *Rhodotorula mucilaginosa*	1	Round dumbbell shaped structures, extensive branching structures	M, IVCM, DNAS	*Rhodotorula* keratitis may be successfully treated with topical therapy is diagnosed early.
20	Arnoldner et al., 2014 [77]	Successful treatment of *Paecilomyces lilacinus* FK with posaconazole	1	Fungal hyphae	M, H	Posaconazole may be an effective treatment in refractory FK by *P. lilacinus*
21	Qiu et Yao 2013 [78]	Corneal coinfection by *Exserohilum mcginnisii* and *Candida parapsilosis*	1	Hyper-reflective, linear, highly branching structures	M	The first reported case of coinfection of filamentous fungi and yeast.
22	Bahadir et al., 2012 [79]	*Candida* infection after DALK	1	Hyperreflective deposits	M	*Candida* is an uncommon post-DALK infection but should be considered in cases where interface deposits are seen.
23	Martone et al., 2011 [80]	A case of *Alternaria alternata* keratitis	1	HR filamentous structures, narrow angle branching, 5–15 μm W, 100–500 μm L	M	Prompt diagnosis of FK by IVCM and AS-OCT is beneficial, also in the follow-up.
24	Labbé et al., 2011 [81]	Case of FK diagnosed by IVCM	1	HR, thin, Br, interlocking linear structures, 5–7 μm W, 200–400 μm L	M, IVCM	While culture was negative, IVCM could visualize the fungi in this case, indicating the usefulness of IVCM.
25	Tanhehco et al., 2011 [51]	A case of *P. insidiosum* keratitis acquired in Israel.	1	Highly reflective, elongated, branching structures	M, ITSS	*P. insidiosum* keratitis with the final action being enucleation.
26	Mauger et al., 2010 [82]	A case of AK and BK with development of FK on the contralateral eye	1	Fungal hyphae	IVCM	Bilateral keratitis cannot be assumed to be caused by the same organism.
27	Miller et al., 2008 [83]	An FK case tentatively linked to the contact lens solution Renu MoisureLoc	1	HR, branching, hyphae-like bodies, 5–10 μm in W.	IVCM	IVCM was used in the diagnostics, and to follow treatment.
28	Babu et al., 2007 [84]	A case of combined AK and FK	1	Linear fungal filaments, double-walled cysts and trophozoites.	M, IVCM	IVCM enabled early detection of etiology, also helpful in the follow-up in this mixed keratitis case.
29	Tu et al., 2007 [85]	Reporting clinical, IVCM and histologic features of *Beauveria bassiana* keratitis.	1	Extensive filamentary forms consistent with filamentous fungi	M	*B. bassiana* shows a slow growth on culture media, and IVCM may be helpful in identification of the fungi.

AK = acanthamoeba keratitis, AS = anterior segment, B = Biopsy, BK = bacterial keratitis, Br = branching, cmf = confirming, DALK = deep anterior lamellar keratoplasty, DNAS = DNA sequencing, EB = endothelial biopsy, FK = fungal keratitis, H = histopathology, HR = hyper-reflective, ITSS = internal transcribed spacer

sequencing, IVCM = *in vivo* confocal microscopy, L = length, M = microbiology (smear and culture), MICs = minimum inhibitory concentrations, N/A = not available, OCT = Optical Coherence Tomography, PCR = Polymerase Chain Reaction, PKP = penetrating keratoplasty, # Pt = number of patients in the report, W = width.

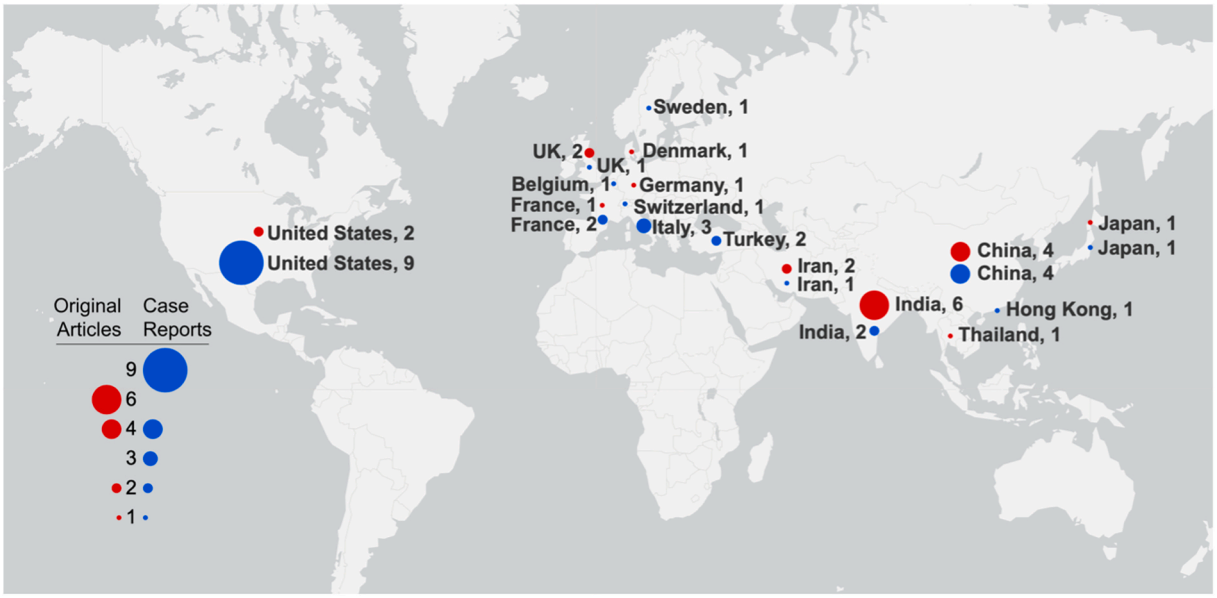

Fig. 2. Geographic representation of country of origin for the original articles (red) and case reports (blue) included in this review, reporting the use of IVCM in fungal keratitis assessment. Numbers indicate the number of articles of each type in the published literature, visually indicated by the size of the circle.

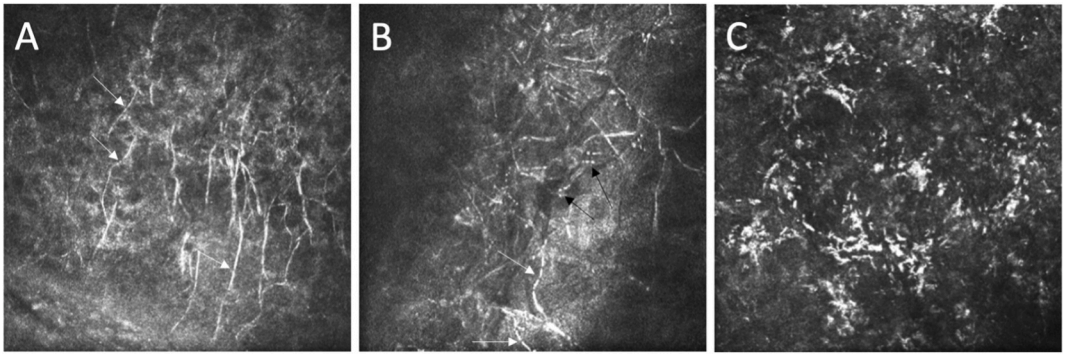

Fig. 3. Images of culture-positive cases of *Candida* spp. yeast infection. (A) Case of *Candida albicans* infection with linear structures of varying reflectivity and length up to 100 μm. Upon closer inspection of the structures, several have variations in intensity consistent with local constrictions or discontinuities (arrows) that are considered as a 'pseudohyphae' morphology, although this species can also produce 'true' hyphae [53]. Also note the linear and branching patterns. (B) A different case of *Candida albicans* which had shorter, reflective linear structures 10–40 μm in length representing pseudohyphae. Note the clear discontinuities (white arrows) along with pointlike features (black arrows) possibly representing buds (called blastoconidia). (C) A case of *Candida parapsilosis* where small hyperreflective inclusions <10 μm in length are present, along with small pointlike reflective features. All images 400 × 400 μm.

Fusarium spp. responds better to natamycin, which may account for the better outcome, it is found to be aggressive and might decimate an eye within weeks [48]. It may therefore be advantageous to differentiate these two fungal species. Brasnu et al. [43] reported hyphal branching angles to be 90° in *Fusarium* spp. and 45° in *Aspergillus fumigatus*. However, a follow-up study by Chidambaram et al. [28] found no significant difference in the branching angle of *Fusarium* and *Aspergillus* spp. In a more recent report from 2020, Tabatabaei and colleagues [11] also studied the use of IVCM to differentiate *Fusarium* spp. from *Aspergillus* spp., but similarly found no significant difference in their branching angles. They also found that the difference in mean hyphal diameter between treatment failure and success groups was under 1 μm, so the difference was not clinically significant as this is below the error of measurement in IVCM images.

Anutarapongpan and colleagues [46] further investigated the use of IVCM in distinguishing *Pythium insidiosum* keratitis from other true fungi. *P. insidiosum* is a fungus-like organism causing keratitis, with clinical features similar to those of true FK. *P. insidiosum* is often included in the list of potential infectious agents of FK. PCR is used to make the correct diagnosis, as it is difficult to distinguish this organism from true fungi on IVCM [49]. Confirming *P. insidiosum* by culture or PCR, the presence of hyper-reflective, double-walled filaments varying in size were the characteristics used to identify pathogenic hyphae on IVCM; however, the authors could not reliably distinguish *P. insidiosum* from *Aspergillus* or *Fusarium* filaments. Case reports describe *P. insidiosum* keratitis misdiagnosed as FK [50] or AK [51]. As *P. insidiosum* lacks ergostrol in its cytoplasmic membrane, the infection is recalcitrant to anti-fungal therapy, and the most effective treatment is surgical debridement of the infected tissue [51].

Table 3
Potential sources of bias in the articles listed in Table 1.

First author, year	Potential sources of bias
Hoffman et al., 2021 [41]	Retrospective study design, therefore only including keratitis cases with a positive result in either IVCM, culture or PCR. Small group of FK patients. Not all patients had all diagnostic tests done.
Tabatabaei et al., 2020 [11]	Unknown experience of IVCM assessor, and whether masked to the microbial result or not. No exclusion of patients with corneal perforation during treatment. Possible false-negative FK among the controls using culture as sole method to confirm FK.
Roth et al., 2020 [44]	Not real patients used in the simulator, cadaver corneas were placed in a holder. Unknown confirmatory method of FK.
Lv et al., 2020 [34]	Retrospective design. Identification of fungal strain and severity of the infection could not be achieved by the intelligent system. Unknown number of patients. Possible false-negative FK among the controls using culture as sole method to confirm FK.
Liu et al., 2020 [33]	Identification of fungal strain and severity of the infection could not be achieved by the intelligent system. Unknown number of patients. Unknown confirmatory method of FK.
Wang et al., 2019 [39]	Small group of FK patients. The IVCM examination was performed by different technicians each visit. Not all cases had confirmatory microbiological culture performed, only confirmed by response to treatment or clinical presentation.
Chidambaram et al., 2019 [30]	Only one IVCM assessor, not investigating the inter- and intra-observer agreement. Not confirming the inflammatory cell identity by immunohistochemistry.
Anutarapongpan et al., 2018 [46]	Descriptive study of *P. insidiosum*, no use of real FK as controls. Small group of included patients. Retrospective design.
Wu et al., 2018 [36]	Comparing the automatic results with only one IVCM assessor.
Chidambaram et al., 2018 [31]	Only including large ulcers. Large differences in the number of patients in the subgroups of infectious keratitis included.
Kheirkhah et al., 2017 [38]	Response to treatment was the criteria set to identify fungi. Knowing that some fungi respond to certain antibiotics there is a slight possibility for misdiagnosis. Retrospective design.
Chidambaram et al., 2017 [28]	Hyphal measurement only by a single assessor. Possible false-negative FK among the controls using culture as sole method to confirm FK.
Chidambaram et al., 2016 [29]	Focus on large ulcers/moderate to severe keratitis, many culture-positive FK. Excluding those whose images were not definite for FK would increase the diagnostic sensitivity.
Nielsen et al., 2013 [42]	Small group of included patients. Unknown number and experience of IVCM assessor, and whether masked to the microbial result or not.
Vaddavalli et al., 2011 [10]	A difference in the included number of patients in the subgroups of infectious keratitis. Fewer controls than the sample size required. Possible false-negative FK among the controls using culture as sole method to confirm FK.
Takezawa et al., 2010 [45]	Small group of included patients. Unknown IVCM assessor, whether it was more than one assessor, if masked to microbial result, or the level of experience. Possible false-negative FK among the controls using culture as sole method to confirm FK.
Hau et al., 2010 [40]	Retrospective design. Small group of included patients. Experienced confocal microscopists preselected images with clear pathogen for the assessors to view, possibly introducing selection bias. Preselected IVCM images, with the observers not investigating the infected corneas *in vivo*.
Das et al., 2009 [32]	Unknown experience of the IVCM assessor, and whether masked to the microbial result or not. Small group of included patients. Retrospective design.
Shi et al., 2008 [35]	Unknown experience of the IVCM assessor, and whether masked to the microbial result or not. Only including more superficial and smaller ulcers (<5 mm).
Kanavi et al., 2007 [37]	Unknown experience of the IVCM assessor, and whether masked to the microbial result or not. Small group of included FK patients. Possible false-negative FK among the controls using culture as sole method to confirm FK.
Brasnu et al., 2007 [43]	Unknown experience of the IVCM assessor, and whether masked to the microbial result or not. Small group of included patients. Possible false-negative FK among the controls using culture as sole method to confirm FK.

4.2. Yeast as a causative organism for FK

Corneal infection by yeast is more common in temperate climates, and in patients with ocular surface disease [2]. In IVCM images *Candida albicans* are found to have round budding bodies and may develop pseudohyphae [23]. Compared to *Candida parapsilosis* which is small hyper-reflective round 3–5 μm structures, *C. albicans* pseudohyphae are 10–40 μm in length, and 5–10 μm in width [23]. *Paecilomyces* hyphae present loops on IVCM, with variable branching [23]. Only a few of the reviewed studies investigated the use of IVCM in FK caused by yeast. Brasnu and colleagues [43] compared *C. albicans* infected donor corneas to an FK caused by the same organism in a patient's eye. IVCM showed *Candida* pseudofilaments to be high-contrast elongated particles measuring 10–40 μm in length and 5–10 μm in width. Wang et al. [39] found yeasts to be round, budding bodies possibly developing pseudohyphae (*C. albicans*) with the same length and width as above, or as small hyper-reflective round structures with a diameter of 3–5 μm (*C. parapsilosis*). As they did not perform a separate sensitivity and specificity analysis of yeast, but combined it with that of filamentous fungi, the results are further discussed in section 4.3. The first case report on FK caused by *Exophiala phaeomuriformis* was described by Aggarwal and colleagues [52] in 2017, finding filamentous structures in stroma on IVCM, highly suspicious for fungal elements. Light microscopy and 3D reconstruction revealed dimorphic fungi with branched hyphae of pigmented muriform cells with conidia. Establishing a simulator to train personnel in the diagnostics of FK by IVCM, Roth et al. [44] included images of *Candida albicans*, a topic more thoroughly described in section 5. Fig. 3 shows IVCM images of yeasts collected from our own archives. The specific detection of yeast elements, however, remains challenging, as these can easily be mistaken for classical fungal hyphae. Also, the same species, such as *C. parapsilosis*, can have varying appearance in different culture-confirmed cases [23].

4.3. Sensitivity and specificity of FK diagnosis by IVCM

The first clinical study of IVCM use in FK was in 2007, where Brasnu and colleagues [43] performed IVCM on FK patients with the diagnosis confirmed by culture (Table 1). They validated the IVCM method using FK-infected donor corneas. The same year, Kanavi et al. [37] was the first group to report sensitivity and specificity of detection of fungi by IVCM. Investigating fungal, bacterial, and *Acanthamoeba* keratitis cases ranging from mild to severe, the investigators included 16 patients of which were culture-positive for fungi. Examining these FK patients by IVCM, they reported a high sensitivity of 94%, and a specificity of 78% of IVCM findings leading to correct FK diagnosis. Das et al. [32] published a case series of six patients in 2009, concluding that IVCM is a good tool for diagnosis of patients with deep stromal lesions from FK infection. However, the experience of the IVCM assessor, or whether the assessor was masked to the microbiological result, was not stated in any of these early studies [32,37,43].

Using masked clinical and non-clinically trained assessors with a varying degree of experience in IVCM, Hau et al. [40] were the first to evaluate the diagnostic accuracy of IVCM as a stand-alone tool in 2010. They included patients with either BK, AK, FK, or *Microsporidia* keratitis, using BK as control images. Considering solely FK, the percentage of

correct diagnosis by IVCM was low (8.3–41.2%) in all assessors. Discovering an obvious difference in sensitivity between the experienced and inexperienced assessors, the authors found that a trained non-medical assessor with no clinical experience, had a higher sensitivity value than an untrained medical assessor. This was an important finding, raising the possibility of training non-medical personnel in performing and analyzing IVCM images. The authors concluded that the usefulness of IVCM as a stand-alone tool was limited as the diagnostic accuracy was highly dependent on the assessor's experience. Furthermore, the diagnostic accuracy of IVCM used alone without clinical assessment was determined to be too low to be a substitute for culture-based diagnosis. Another important consideration in this study is the fact that the IVCM images analyzed were preselected, meaning the assessors did not themselves investigate the infected corneas *in vivo* or decide which images were possibly indicative of pathology.

Following the introduction of masked assessors and the variable results demonstrated between experienced and non-experienced assessors by Hau et al. [40], the role of the masked assessor was further investigated in a large study published in 2011 by Vaddavalli et al. [10]. A total of 146 patients with clinically suspected microbial keratitis were included in the study, which investigated the use of IVCM in both FK and AK. Microbiological analysis revealed 93 cases of FK representing an almost 8-fold increase in the number of FK patients, relative to the study by Hau et al. [40]. Vaddavalli and colleagues included controls that either showed BK or no organism on smear and culture, and the sensitivity and specificity of IVCM were investigated in comparison with microbiology as the gold standard. The masked assessors were two cornea specialists with varying experience. On subgroup analysis, the authors reported a high sensitivity and specificity for IVCM detection of fungi, 89.2% and 92.7%, respectively. Although there was good inter- ($\kappa = 0.6$) and intra-assessor ($\kappa = 0.795$) agreement, the authors concluded that IVCM had limited use as a primary diagnostic modality due to its cost, limited availability, and the considerable amount of training required to both perform and analyze the IVCM images. Vaddavalli and colleagues stated that the subject, the instrument, and the assessor are three potential sources of error. Similar to Hau et al. [40], the authors found that the main source of error was the assessor with the results being highly dependent on assessor's experience. Vaddavalli and co-workers stated that their test was a one-time exam using the same confocal microscope by the same examiner, so they did not suspect other possible sources of error.

Chidambaram et al. [29] investigated the accuracy of IVCM in diagnosing moderate to severe microbial keratitis (AK and FK) using five masked experienced confocal assessors in a study published in 2016. Using a positive culture result or presence of fungal hyphae on light microscopy as the reference method for diagnosis of FK, the team reported a pooled sensitivity of 85.7% and a pooled specificity 81.4% for all assessors. One of the masked assessors who also performed the IVCM and examined the ulcer with the slit-lamp had the highest overall sensitivity (89.8%). The team further found that cases with earlier presentation and shorter symptom duration (4 days or less) had the highest sensitivity (95%) but the lowest specificity (53%), whereas cases with symptom duration over 10 days had a lower sensitivity (72%) and a higher specificity (91%). Where IVCM assessors were able to detect a pathogen (either FK or AK) on IVCM images and the confirmatory method (culture or light microscopy) was negative, cases were defined as true infections due to the deep localization making superficial corneal scraping less likely to gain access to the pathogen. This illustrates the usefulness of IVCM in the correct diagnosis of deep corneal infections. In their study, Chidambaram and colleagues included FK patients only with moderate to severe infection, and this may at least partly explain their much higher sensitivity rate compared to that reported by Hau et al. [40] six years earlier. In addition, Chidambaram et al. included a larger number of FK patients. The finding of improved sensitivity in detection by an assessor who was also the IVCM investigator and viewed the ulcer by the slit-lamp suggests that the IVCM examination should ideally be guided by a slit-lamp examination and be conducted in close consultation with the clinical examiner/specialist where possible.

It has been recognized that a potential source of bias in FK diagnosis by IVCM could be attributed to the possibility of false negatives among controls, using smear and culture as the 'gold standard' method to confirm FK [10]. In other words, the low sensitivity of fungal culture is a source of bias, as the culture false negatives are not included in the studies. Kheirkhah and associates [38] eliminated possible false negative controls by defining positive response to anti-fungal therapy as confirmation of FK. Like Hau et al. [40], assessors in the study by Kheirkhah and colleagues were masked and had a varying degree of IVCM experience [38]. BK images were used as controls, but no specific criteria for identification of fungi were set and the observers judged the IVCM images based on personal experience or images from the literature. The results showed that the sensitivity was highly dependent on the experience of the assessor, ranging from 42.9% for the inexperienced assessor, to 71.4% for the experienced assessor. The inter-assessor agreement was found to be good ($\kappa = 0.77$) for the experienced assessor, and moderate ($\kappa = 0.51$) for the inexperienced assessor. These findings once again illustrate the importance of previous experience in viewing and interpreting IVCM images, and that a lack of experience cannot be overcome simply by studying the scientific literature. At the very least, assessing many raw, non-preselected images from IVCM exams taken in different subjects would be desirable. Finding that the average sensitivity was higher for patients with positive fungal culture (82%) than those with negative cultures (60%), it was shown that the inclusion of only the culture-positive patients results in an overestimation of IVCM sensitivity for detection of FK. In contrast to Chidambaram et al. [29], Kheirkhah and colleagues [38] found a higher positive rate of fungal elements in those with a longer duration of the disease, probably due to replication and spreading of the organism. The apparently conflicting findings reported by Chidambaram and associates may be explained by their inclusion of only culture- or light microscopy-positive deep fungal infections, where the deep infections may have been accompanied by significant inflammation and edema, thus complicating the evaluation of IVCM images.

Wang and colleagues [39] investigated the use of IVCM in fungal-, bacterial-, viral-, and *Acanthamoeba* keratitis, using clinical presentation, available microbial analysis, and response to treatment to determine the final diagnosis. A total of 49 eyes were included, with 10 of them being diagnosed as FK and two diagnosed as combined FK and AK. On subgroup analysis, the sensitivity of IVCM detection of FK was 66.7%, with a specificity found to be 100%. Pooled (for all types of keratitis) intra-assessor agreement was excellent ($\kappa = 0.94$), with a good inter-assessor agreement ($\kappa = 0.68$). Using both the clinical presentation and response to treatment to determine the final diagnosis, the results revealed seven non-culture proven FK cases. Clinical assessment missed four of the culture-negative FK cases, and interestingly three of these did not have any fungal elements identified by IVCM. The small sample size of FK patients in that study was a limitation; however, the authors could conclude that neither clinical assessment nor IVCM should be used alone, but that IVCM is nevertheless a powerful tool in FK diagnosis.

A retrospective study recently published by Hoffman et al. (2021) compared the use of culture, IVCM and PCR in routine hospital use for BK, AK, and FK diagnosis [41]. In the FK subgroup, IVCM had superior sensitivity in detecting 14 cases, with the authors concluding that IVCM was the most accurate tool for diagnosing both AK and FK compared to culture and PCR.

We reviewed the articles in Table 1 to determine if there was any relation between the sensitivity of FK diagnosis and any of the defined criteria for identification of fungi using IVCM, for example if the IVCM sensitivity was increased in studies defining more "open" criteria, e.g., larger width of fungal elements, but no such association was found. Several articles found IVCM to be a good supportive diagnostic method for FK but concluded that its use as a stand-alone tool was limited [10,11,29,32,37,38,40,42,46]. These studies concluded that IVCM should

not be used as a substitute for the gold standard methods (i.e., smear and culture). However, bias was potentially introduced in some studies by the omission of FK cases deemed falsely negative by the culture method, and sensitivity was reported to be highly dependent on the experience of the assessor. To address this latter limitation, more recent studies have shifted focus to the development of artificial intelligence systems to help overcome sources of human bias in the clinical setting (see Section 5). Addressing the limitation of reduced sensitivity and specificity in even the 'gold standard' swab-culture approaches - which are highly dependent upon the inclusion of viable intact microbes in the swabbed region - may prove to be more difficult. Therefore, a reassessment of what can be considered a 'gold standard' in FK diagnosis is warranted.

4.4. Morphologic features of filamentous FK on IVCM

In contrast to the two earlier studies [10,40], in 2013 Nielsen et al. [42] reported that use of IVCM was superior to the culture method in diagnosing filamentous FK, with a sensitivity of 86%. A positive diagnosis of FK was based on culture or histopathology taken as the gold standard. Introducing an image grading system for interpretation of IVCM images in FK, the authors addressed the issue of sensitivity being highly dependent on the assessor's experience in evaluating IVCM mages. With support from prior published studies (that included published IVCM images) and based on their own experience, Nielsen et al. defined three categories of results: 1) clearly positive for fungi, 2) inconclusive, and 3) negative for fungi. They recommended that Category 1 should be designated as pathognomonic for filamentous FK, and that anti-fungal therapy should be initiated at once, without waiting for

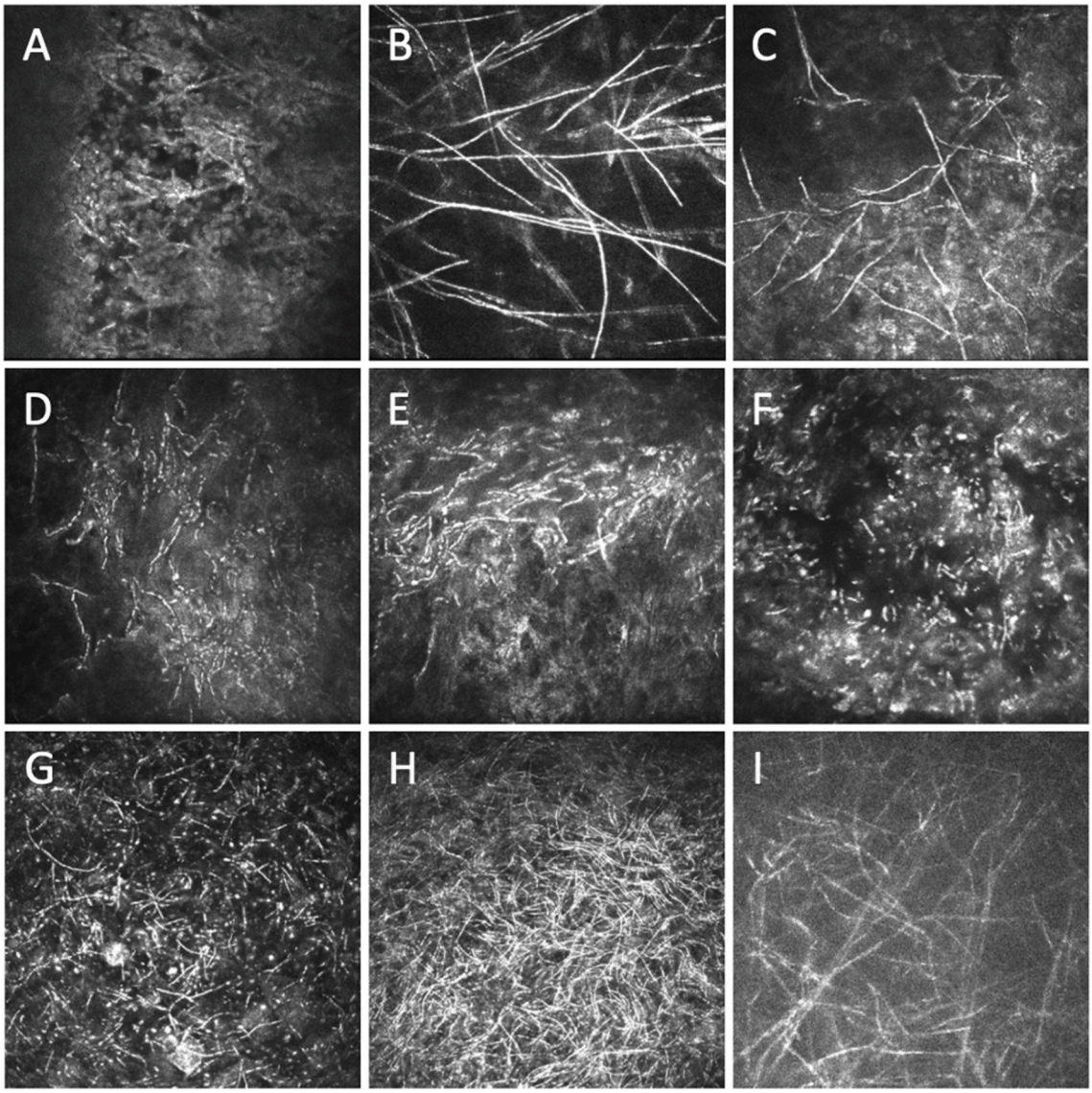

Fig. 4. Examples of positive detection of fungal hyphae by *in vivo* confocal microscopy. (A) A case of *Aspergillus* spp. keratitis, epithelial depth. (B, C) Two separate cases of *Fusarium* spp. keratitis illustrating varying length, reflectivity, and width of hyphae. (B) is in the stroma, whereas (C) is in the epithelium. (D–F) Three images obtained from a single corneal examination in a case with a culture-negative keratitis which resolved following a course of treatment with voriconazole antifungal therapy. Note the differing appearance of fungal hyphae or pseudohyphae in different layers. (D) Reflective linear and curvilinear branching hyphae in the mid-stroma, with variable reflectivity along the hyphae length. (E) Thicker, highly reflective hyphae in an anterior stromal region. (F) In the most superficial epithelial layer, short, reflective linear formations are visible. (G–I) Three images obtained from a single corneal examination with subsequent culture-positive fungal findings (failure to detect species), with images taken at differing depths in the cornea. The eye was eviscerated shortly thereafter. (G) Superficial epithelial hyphae with short, linear and curved segments at a depth of 14 μm from the corneal surface. (H) Dense hyphal infiltration in the anterior stroma at a depth of 79 μm. (I) Longer, thicker hyphae in the mid-stroma at a depth of 150 μm. All images 400 × 400 μm.

the result of cultures.

An example of IVCM image features indicative of FK from our image archive is given in Fig. 4. Clearly the fungal hyphae can vary in appearance, based on the relative size, distribution, density, form (branching or curving), and reflectivity relative to the background. Notably, images from the same examination can have a different appearance, where the size, form, reflectivity, density, and distribution have different appearance in the same cornea, as seen in images D – F in Fig. 4. Images obtained at different depths in the same cornea can also differ in their morphologic appearance (Fig. 4 G – I).

Unfortunately, in routine clinical practice, not all cases exhibit clear, unambiguous features, as acknowledged by Nielsen et al. [42]. Cases may instead present with ambiguous, inconclusive findings (Category 2) based on IVCM images that are difficult to interpret and do not match the 'classical' fungal morphologies. We present some of these type of images from our archive in Fig. 5. As suggested by Nielsen et al. [42] some of the linear features could masquerade as hyphae or alternatively they may in fact be hyphae, but not appearing in the 'typical' morphology as in Fig. 4. Background inflammation, stromal edema, scarring, or enhanced extracellular matrix reflectivity may either alter the appearance of genuine fungal hyphae, or these factors may produce features that can be mistaken for fungal hyphae (for example, elongated dendrites of dendritiform cells). In such cases, culture findings, the clinical course of the infection and treatment, and repeat IVCM imaging would be valuable in determining the likelihood of true FK, but unfortunately such reports of inconclusive IVCM cases with clinical follow-up are absent in the scientific literature. Longitudinal IVCM examinations can not only be used to confirm the efficacy of anti-fungal treatment but can also be used to evaluate the failure of medical treatment or incorrect initial diagnosis by persistence of detected microbial structures (see Section 4.6).

Other morphologic features including subbasal nerve alterations and epithelial dendritiform cell (DC) changes during the course of FK can be observed by IVCM. In their prospective study from 2011, Cruzat et al. [54] found a significant decrease of the subbasal corneal nerve density in both FK, AK, and BK, as well as an increase in epithelial DC density, compared to healthy controls. During the recovery phase, the total number of nerves increased significantly, but despite this the nerve density remained significantly lower than in a healthy control group [55]. Investigating the contralateral eye in patients with microbial keratitis [56], a subclinical involvement in the contralateral, clinically non-affected eye was found, with diminished corneal subbasal nerves and an increase in DC density with no significant difference between FK, AK, or BK. Five morphological types of inflammatory cells forming infiltration in infectious keratitis have described by Smedowski et al. [57], who found FK to be associated with the most dense infiltration of inflammatory cells (compared with AK, BK, and viral keratitis). Together with viral keratitis, FK was associated with lowest density of subbasal nerves, with FK showing thinning of the nerves with decreased number of nerve fibers and stimulation of DC not covering the nerve fibers. Combined analysis of inflammatory cells, changes in the corneal epithelium, and the morphology of different species may increase the specificity of keratitis diagnostics. Another study published in 2018 investigated coinfection by AK in other forms of infectious keratitis, especially in FK [58], finding AK coexistence to be more prevalent than previously suspected.

4.5. A new scheme for FK diagnosis by IVCM

Here, we propose a scheme generated by expert IVCM operators and assessors to aid in identifying and distinguishing features in IVCM images, based on recent studies and our combined experience with hundreds of cases of FK. A summary of the new scheme is given in Table 4. When a patient with keratitis is referred to a specialist, a standard

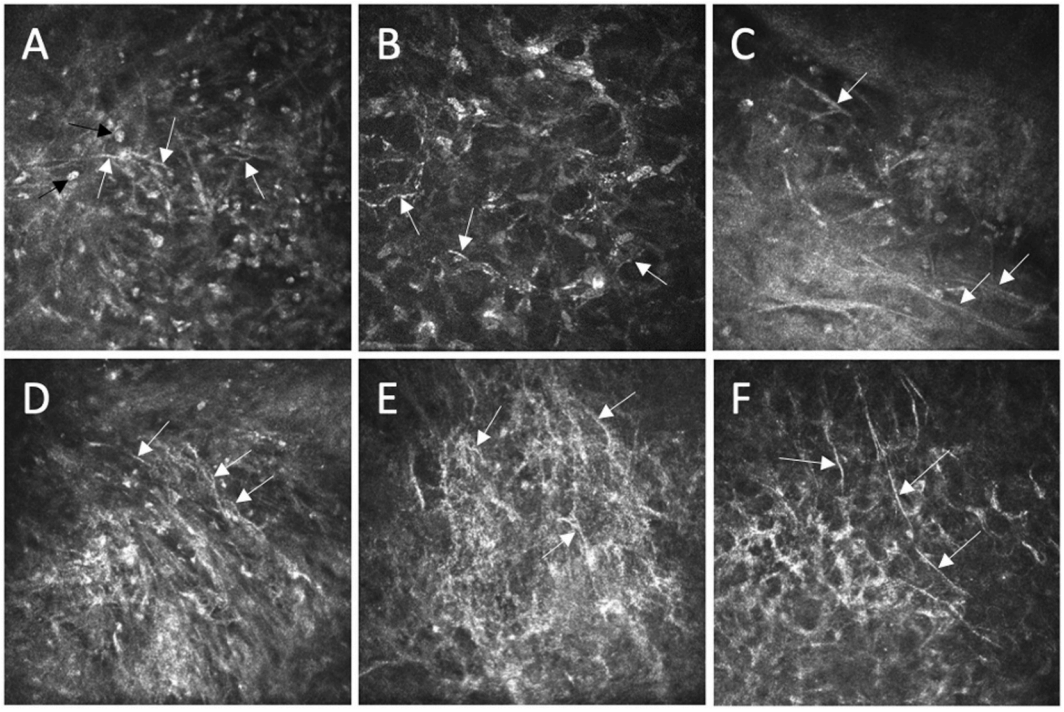

Fig. 5. Examples of inconclusive findings of fungal hyphae by IVCM. (A) Thin, threadlike structures (white arrows) and presumed leukocytes (black arrows) visible. The case was culture-negative for fungus but responded to antifungal treatment with natamycin. (B) Reflective curvilinear structures (arrows). The case was culture-negative for fungus and positive for *Streptococcus pneumoniae*. (C) Linear formations with low, diffuse reflectivity (arrows). The case was culture-negative for fungus and bacteria, and the structures could represent stromal collagen bundles. (D) Thin strand-like structures (arrows). The case was culture-negative for fungus but positive for *Pseudomonas aeruginosa*. The strand-like structures can be dendrites of dendritiform cells, (E) Thin, reflective curved structures (arrows). The case was culture-negative for fungus but positive for *Moraxella nonliquefaciens*. The structures could be dendrites of dendritiform cells. (F) Reflective linear and branching structures (arrows). The case was culture-negative for fungus and bacteria but responded well to natamycin antifungal treatment. All images 400 × 400 μm.

Table 4
Expert advice – summary of IVCM assessment categories for suspicion of fungal keratitis.

Category 1: Consistent with FK, showing highly reflective, branching/bifurcating, well-defined, interlocking structures, measuring 3–10 μm in diameter, not seen in isolation (Fig. 4). Immediate initiation of anti-fungal therapy is recommended.
Category 2: Possible FK, showing features resembling fungal elements, i.e., isolated or several linear/curvilinear structures, of varying reflectivity, lacking the well-defined branching and smooth features in Category 1 (Fig. 5). Follow-up examination is warranted, and if a Category 1 image is obtained, the recommendation above is followed.
Category 3: Unlikely FK, showing normally occurring structures or inflammatory response that does not raise suspicion of fungal elements. In this category the experience of the assessor is crucial, as e.g., linear or curved reflective branching structures could confuse the untrained assessor (Fig. 6). In Category 3 cases, we recommend repeating the IVCM examination only in cases refractive to treatment where diagnosis by other methods remains elusive.
IVCM scan should include the central and peripheral parts of the infiltrate, at various depths.
The number of IVCM images acquired should be at least 1000 images (ten 100-image scans).
The roles should be clearly defined:
- Confocal microscope operator = person performing the confocal scan
- Confocal assessor = person assessing the images
The role (physician, ophthalmologist, optometrist etc.) and the experience level should be specified.
Specify when the assessment of the images took place (real-time, retrospective), and if the assessment was based on the full-examination, or a selection of the saved images.
- The number of images reviewed should be reported.
The experience of the confocal microscope operator and assessor should be reported.

operating procedure for IVCM should be followed. At minimum the IVCM scan should include the central and peripheral parts of the infiltrate and surrounding corneal region at various depths. We suggest repeating the IVCM examination throughout the course of the infection from referral until resolution, on a daily to weekly basis depending on the disease severity and frequency of clinical visits. In particular, the IVCM examination should be performed at various corneal depths (if fungal elements are not immediately visible) and at different corneal locations, particularly at the border of the infiltrate. The number of distinct images acquired, at least at the initial visit, is recommended to be at least 1000 individual images, especially if no clear fungal features are immediately evident. This recommendation is also relevant for subsequent visits, to form a basis for assessing whether a case is likely or unlikely to have fungal infection. This is also dependent on the ability of the patient to tolerate the examination. Further examinations during the course of treatment and follow-up may require adjustment of the recommended number of images, based on the patient's disease course.

The following classification categories are defined:

- Category 1 – Consistent with FK (see for example Fig. 4 B, C, H, I). This category is defined by highly reflective, irregular branching or bifurcating, well-defined, interlocking structures [42], usually not seen in isolation and may appear double-walled [39] with a diameter of 3–10 μm and the length raging from short filaments of only a few micrometers long to several hundred micrometers. For non-filamentous fungi, the definition may be broadened to include pseudohyphae structures with discontinuities or constrictions, or alternatively reflective point-like structures sometimes appearing diffusely reflective and completely covering a large area [23]. In Category 1, we recommend immediate initiation of anti-fungal therapy (based on filamentous or yeast subtype), while in parallel taking a sample for smear and culture. Although the culture results take time to obtain, they may indicate specific species of fungi that can guide further anti-fungal treatment.
- Category 2 – Possible FK (see for example Fig. 5C, D, E). Features resembling fungal elements are flagged as 'suspicious', however the appearance differs slightly from Category 1, so as to cause doubt of the origin (for example just one or a few randomly distributed features are present, or features resemble dendrites). The specific features could be isolated or could comprise several linear or curvilinear structures of varying reflectivity, lacking the well-defined branching and smooth features described in Category 1, and may only be visible in one or a few IVCM images. Here, there is ambiguity in fungal features or image interpretation, or fungal elements could be sparsely distributed and may be difficult to detect by IVCM. In addition to Nielsen et al. [42], who recommended waiting for the culture result in this category, we suggest repeating the IVCM examination as described above. Attention should be paid to scanning at different depths and in locations at the border of the affected area of the cornea, according to the local standard operating procedure. If during any of the follow-up examinations a Category 1 image is obtained, then the Category 1 recommendation should be followed, even if the smear/culture results are unavailable or are negative.
- Category 3, Unlikely FK (see for example Fig. 6). Normally occurring structures or an inflammatory response may be present but do not raise suspicion for fungal elements. These features could be bright curvilinear structures with multiple branches and of variable thickness and/or reflectivity, which may only be present at a single depth or in a single image. Here, experience and training in identification of corneal features in IVCM images is important to exclude non-fungal structures as being suspicious or indicative of FK. In Category 3, there is no doubt to the trained assessor that the IVCM images obtained are not indicative of FK. Corneal subbasal or stromal nerves are an example that could confuse the untrained assessor. They are seen as long structures, sometimes with periodic or bead-like reflectivity that can be branching and are frequently interspersed with dendritiform cells, often with a background of basal epithelial cells. The depth location of nerves in the subbasal plexus is invariably near the basal epithelial layer or epithelial-stromal junction. Active inflammation with highly reflective dendritiform cells bearing long dendrites is another feature that could be mistaken for fungal hyphae, as well as straight collagen bundles without branching, which are common and often confusing for inexperienced observers. For this category, no action is to be taken based on IVCM findings. It is important to consider, however, the possibility of a false negative IVCM examination. Category 3 images may require repeated IVCM examination on different occasions, but typically only in cases refractive to treatment, where clinical diagnosis by other methods remains elusive. If not originally targeted to the proper corneal location and/or depth, a repeated IVCM examination could potentially yield Category 1 images as long as the clinical condition remains unresolved.

Given the above scheme, a re-evaluation is warranted as to what can be considered as 'positive FK'. Although microbiological confirmation is considered most robust (i.e., culture, smear, or PCR), there exists a large proportion of cases with negative microbiological results. Given this reality, we support in cases of negative microbiological confirmation a diagnosis of 'clinical FK' to represent at minimum, a positive response to antifungal treatment and a positive FK finding on IVCM.

Recognizing also that the utility of IVCM is dependent on the experience of those performing the examination and those assessing images, we further recommend that future studies clearly identify the roles of confocal microscope operator, the person(s) selecting images for analysis, the person(s) assessing the images, and the treating physician. The role and experience level of each of these persons should be specified, also where overlapping roles exist. We think it is also important to specify whether the assessment of IVCM images was performed in real-time or afterwards, and whether the assessment was based on the full examination, the saved images only, or a selection of the saved images. The approximate number of images reviewed should be reported. We believe that there is value in observing the IVCM examination real-time, and in the time shortly thereafter when images are reviewed without preselection. As the real-time IVCM examination is often conducted in

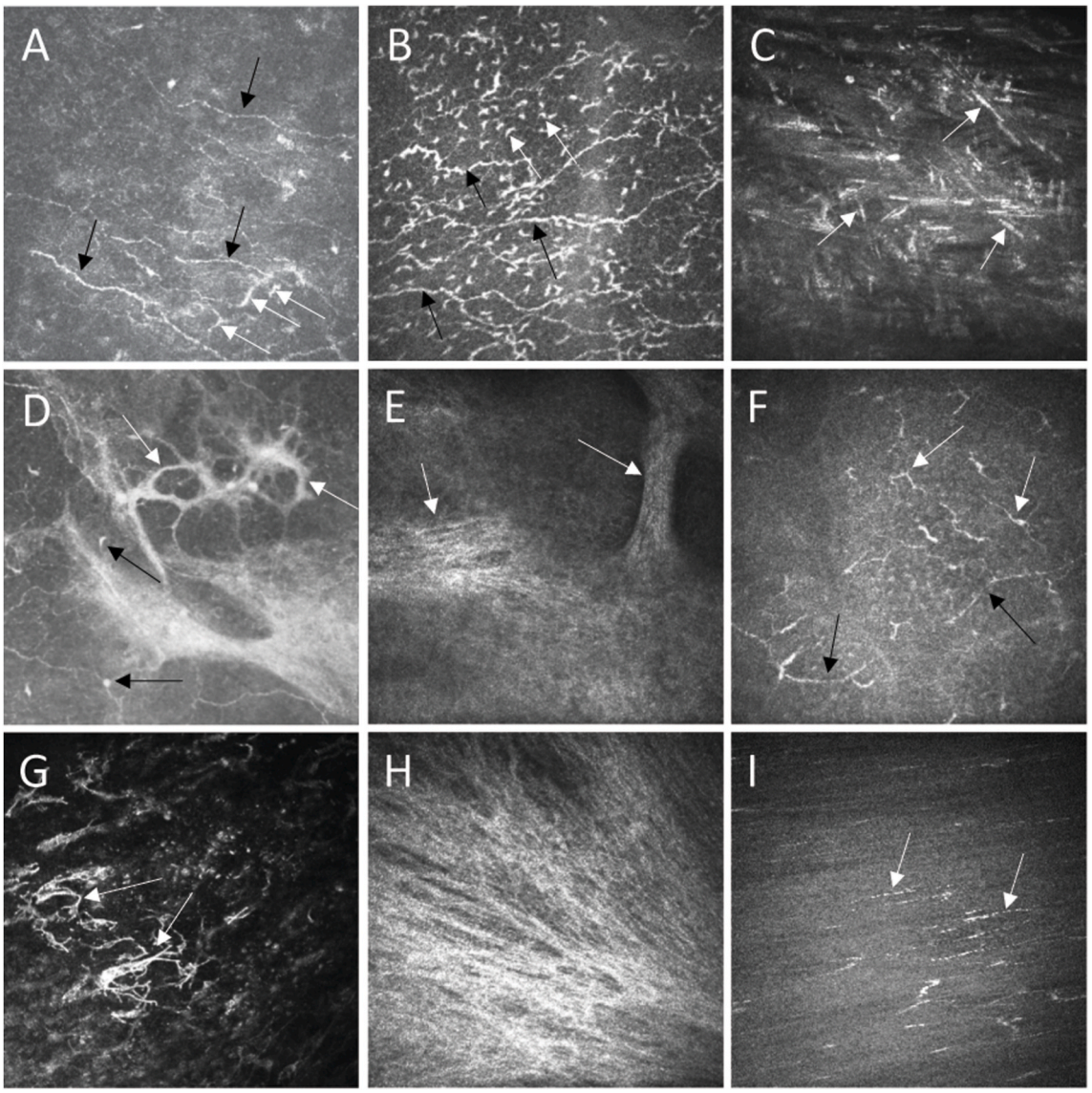

Fig. 6. Structures in corneas without fungal keratitis, that could masquerade as fungal structures. (A) Subbasal nerve plexus with subbasal nerves (black arrows) and dendritic cells (white arrows). (B) Subbasal nerve plexus with sometimes tortuous subbasal nerves (black arrows) and dense distribution of dendritic cell bodies (white arrows). (C) Anterior stromal keratocyte cell death indicated by short, reflective, non-branching linear structures (white arrows). (D) Subepithelial fibrosis, with nerve segments, fibrotic regions (white arrows) and dendritic cell bodies (black arrows) visible. (E) Anterior stromal fibrosis with linear structures visible within fibrotic regions (arrows). (F) Subbasal nerve segments (black arrows) and mature dendritic cells (white arrows). (G) Dendritiform or Langerhans' cells (white arrows) in active inflammation. (H) Fibrous tissue layer of the conjunctiva exhibiting densely packed linear reflective structures. (I) Linear structures (arrows) observed in an IVCM image taken during excessive eye motion (motion artifact).

conjunction with other clinical tests and examinations, the unfiltered IVCM examination may be valuable as a complementary test, along with other clinical evidence, in establishing an initial diagnosis.

Regarding experience of the confocal microscope operator and person performing the assessment of images, we feel that this depends on the number of cases examined and confirmed, rather than a specific period of time. Different centers will have varying incidence of infections, and experience with rare infections may sometimes take several years to accrue. Moreover, experience in image interpretation would additionally require a good understanding of corneal anatomy and physiology, as well as the underlying clinical conditions (such as microbial keratitis, including their treatment) and close contact with patients and/or close collaboration with medical professionals. Training in medical imaging and interpretation and image-based diagnosis is desirable, but in the absence of specific training or certification for IVCM, it is difficult to develop criteria for when an assessor is 'experienced' in image interpretation. Nonetheless, we agree that proficiency in operating the confocal microscope could be gained after three months of performing several examinations per day. The microscope operator, however, should also be familiar with images published in the literature, have familiarity with corneal anatomy and basic interpretation of images, and ideally should examine a broad mix of cases affecting different regions of the cornea. It is also important here to emphasize that regardless of who performs the microscope examination and interpretation of images, the final decision on diagnosis and treatment is to be made by the treating/prescribing clinician who is legally permitted to do so and is responsible for the treatment and follow-up of the patient in the country where the patient is treated.

4.6. *The role of IVCM in follow-up and management of FK*

Besides initial detection and diagnosis of FK, IVCM can have a role in

the continued clinical assessment and management of FK, including assessing the response to anti-fungal therapy. Based on slit lamp examination alone, it can be difficult to determine the therapeutic effect and when the anti-fungal therapy should be tapered. The reported follow-up period after FK diagnosis varied in the reviewed studies from two weeks to two months. Shi et al. [35] were the first to report follow-up of patients using IVCM during treatment and indicated that IVCM was a valuable tool in the follow up of FK. The authors followed patients for two months following initiation of treatment, noting a decrease in hyphal density and breaking up of hyphae into smaller segments in patients responding to treatment. Conversely, hyphal density increased in those not responding to treatment. Further into the recovery period, IVCM enabled continued assessment of corneal transparency, resolution of edema, regeneration of epithelium, and reduction or eradication of the hyphae [35].

In 2010, Takezawa et al. [45] also reported the use of IVCM to monitor the effect of therapy in three cases of FK during anti-fungal treatment. One week after initiation of treatment, they observed a decrease in hyphal density on IVCM in all three cases, whereas slit-lamp examination showed very little change. In one of the reported cases, IVCM revealed a delay in regeneration of the epithelium and a high load of hyphae after three weeks, indicating the need for surgical debridement. Based on these findings, Takezawa and colleagues concluded that IVCM was a helpful tool in evaluation of FK treatment.

Chidambaram et al. [30] in 2019 reported the use of IVCM to examine patients at baseline, and at days 7, 14, and 21 after receiving treatment for large ulcers; the authors found several features in IVCM images could predict the outcome in FK. The authors thoroughly described morphological features of the cellular changes in the cornea during FK, correlating these to the clinical outcome. The authors found that features indicating poor outcome were the presence of stellate inter-connected cells with absent nuclei, inflammatory cell infiltrate forming a honeycomb distribution at the final visit, and detection of fungal hyphae at the final visit. The appearance of dendritiform cells in the basal epithelial layer at the final visit, when not present at the first visit, was also found to be associated with deterioration. Furthermore, they found that the presence of dendritiform cells and, surprisingly, normal keratocyte morphology at the final visit was associated with corneal perforation. A limitation of the study, however, was that the authors did not definitively confirm the cell type associated with each morphological feature by immunohistochemistry. Focusing on large corneal ulcers in that study raises the need for further investigation of ulcers at earlier stages to determine if the same morphological abnormalities are present in early FK. The authors further reported that a major drawback of IVCM was the difficulty in reimaging exactly the same location in follow-up visits, indicating that the confocal operator's experience and/or patient compliance during examination can also play a role in the clinical utility of IVCM. In terms of grading of images, Chidambaram and colleagues stated in a previous study that there was variability in inter-grader agreement based on experience of the grader [29], but in their 2019 study [30] they did not assess the inter- and intra-observer agreement. This indicates the need for further research in assessing the diagnostic accuracy of graders in detecting different morphologies. A correlation between the number of filaments detected on IVCM images and the response to treatment was found by Olivier and colleagues in 2021 [59] in their retrospective study on FK in France, where the presence of numerous filaments on IVCM was associated with poor clinical outcomes. The outcome was also found to be correlated with the patient age and presence of a deep infiltrate at presentation.

Clinical examples from our own image archive are shown in Fig. 7. Fig. 7A indicates initial suspicion of fungal hyphae by IVCM, whereas Fig. 7B shows an image from the same patient two weeks after initiating anti-fungal treatment, with the features still present and culture-negative results, indicating the need for possible alternative diagnosis and treatment. Fig. 7C shows the IVCM image of a different patient at an initial visit, exhibiting suspected fungal hyphae. Following three weeks of anti-fungal therapy IVCM was again performed, revealing the resolution of the fungal infection in the same corneal region while culture results were positive for fungus and bacteria (Fig. 7D).

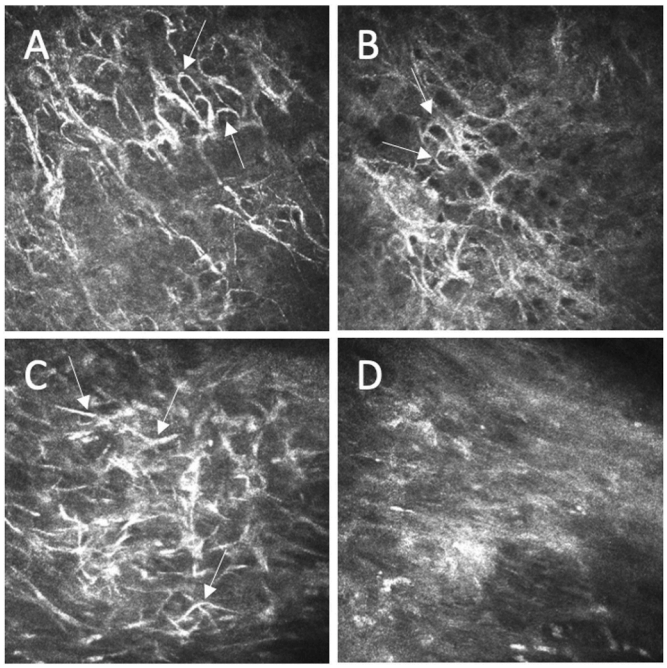

Fig. 7. Examples of clinical follow-up of suspected fungal keratitis cases using IVCM. (A, B) Images from approximately the same corneal region in a 51-year-old man. (A) Initial detection of U-shaped suspected fungal elements (arrows) in the patient led to initiation of natamycin treatment. (B) After 2 weeks of anti-fungal treatment, IVCM examination confirmed the continued presence of the structures (arrows). At that time, culture results were first available, and were negative for fungus but positive for *Staphylococcus aureus* and *Group G Streptococcus,* suggesting a change in diagnosis and treatment was indicated. (C, D) Images obtained on different occasions, representing approximately the same corneal region in a 40-year-old man. (C) At first presentation, fungal-like elements were detected (arrows). (D) After 3 weeks of natamycin treatment, no fungal hyphae could be found and instead a diffuse reflectivity was present in the infected stromal region. Culture results were positive for fungus, but identification of the genus and species was unsuccessful. The sample was additionally positive for *Cutibacterium acnes*. All images 400 × 400 μm.

The conclusion drawn from these studies and from our own experience is that IVCM can be an effective tool in confirming or refuting an FK diagnosis and guiding anti-fungal therapy or signaling that a change in therapy is required. IVCM allows for real-time evaluation of inflammation and the continued presence of fungal hyphae, so that therapy can be altered according to the findings on a case-by-case basis. One challenge is that it may be difficult to re-image the same location in the cornea at follow-up for accurate comparisons over time. Use of an IVCM standard operating procedure (consisting of a detailed scanning protocol) could partially compensate for this limitation. An experienced confocal microscope operator can also document localization to a particular region, for instance referencing to a certain part of an ulcer/lesion whose borders can be clearly identified during the IVCM scan. In addition, a comprehensive sweep of the entire lesion and border regions can be performed at the relevant depth during each follow-up examination, thereby providing the broadest possible image set to ensure that a particular region or feature is not overlooked. Nevertheless, the limitation of human subjectivity in identification and relative grading of fungal hyphae density, degree of inflammation and other relevant features remains. This motivates the need for possible automation of assessing morphologic features by IVCM.

5. The application of artificial intelligence approaches to IVCM

Substantial differences exist in the literature regarding the sensitivity and specificity of IVCM for FK diagnosis, and this depends partly on the experience of the examiner in recognizing features and interpreting IVCM images. This human limitation has led to the recent proposal of using artificial intelligence (AI)-based techniques for image classification. The first article to describe an intelligent system for the diagnosis of FK was Wu et al. [36] in 2018. Wu and colleagues developed an automated image recognition system to identify fungal hyphae on IVCM images and evaluated its accuracy in comparison to corneal smear and culture. Criteria for positive identification from IVCM images included "fungal hyphae" with no categories or further description as to how these structures were defined. A total of 56 FK cases were diagnosed by fungal culture. Among these, 38 FK cases were detected by corneal smear, whereas IVCM images assessed by an experienced ophthalmologist detected 53 of the 56 culture-verified FK cases. The intelligent system based on image recognition detected 50 cases of FK, thus identifying fungi in a much greater proportion of cases than corneal smear, with the sensitivity and specificity found to be high, 89.29% and 95.65% respectively. Importantly, no statistically significant difference was found between the automatic hyphae detection system and manual detection by the experienced ophthalmologist. The developed intelligent system could therefore be considered especially useful in cases of insufficient experience in IVCM image assessment (i.e., outside of specialized centers). An important limitation to note, however, is that only culture-positive cases were considered in that study, and thereby the matching of IVCM with positive culture results. The presence of deeper fungal infiltration and culture-negative cases were explicitly excluded.

Further addressing the limitation that IVCM as a diagnostic tool is highly dependent on experience of the image assessor, Roth et al. [44] developed a training simulator that allowed examiners to practice analyzing IVCM images of FK and AK. The assessors completed a questionnaire before and after the training, and the results showed a significant (self-reported) overall improvement in assessment skill level following training with the simulator. In contrast to real patient examinations, a simulator is an effective means of gaining experience as the assessor is not limited in time, can interrupt or restart an assessment session, and can make mistakes in a safe environment.

Lv et al. [34] developed an AI system to automatically diagnose FK based on a deep learning algorithm. Deep Residual Learning for Image Recognition (ResNet) was used to build an intelligent system for the automatic diagnosis of FK. The authors addressed the problem of time-consuming manual assessment of IVCM images by developing a robust diagnostic system relieving clinicians from examining images individually. The system developed by Lv et al. was limited, however, to identifying fungal hyphae (which were not clearly defined in the study) and could not identify FK severity or determine different species of fungi. Further optimization of the intelligent system will improve its accuracy, but the authors concluded that culture of corneal smears remains the gold standard. Similarly, Liu and associates [33] developed a deep learning network for analyzing IVCM image data. They used a convolutional neural network (CNN), which is a class of deep neural networks commonly used in analysis of medical images. It consists of multiple hidden layers and overcomes the shortcomings of traditional machine learning where image features and parameters are extracted manually. The team investigated confirmation of infection using their network and found the diagnostic accuracy to be almost perfect, at 99.95%. Similar to the limitations described by Lv and colleagues [34], identification of different fungal species and determination of the severity of the infection could not be achieved. A web-based medical image management and analysis system was designed by Hou et al. [60] in 2021, to help in managing and classifying medical images. With a gradual increase of users, the system would become more and more exact in automatically diagnosing FK from IVCM images, as it is a self-learning system implemented with deep learning algorithms. Table 5 summarizes the results from articles on artificial intelligence.

Suggestions for future research include establishing a large volume of IVCM images representing a multitude of confirmed fungal genus and species, which can provide training data. Another suggestion is to further explore deep learning networks, as these have been shown to have strong self-learning ability in many different fields. Still, given the variability in algorithms, the expanding volume of raw image data used as input to human or machine-based systems, and the ultimate need for humans to identify relevant parameters for algorithm development (how to teach the machine to identify hyphae), the examination and assessment of fungal elements from IVCM images based on morphologic characteristics will still be required. Artificial intelligence systems are not yet harmonized, widespread, or based on broad and validated datasets from multiple centers; thus, the need remains for identification of fungal features in real-time, in a rapid manner and in a clinical setting. It is imperative then, that morphologic IVCM criteria continue to be refined and communicated, to improve the probability of correct diagnosis and assessment of FK by humans or intelligent systems in the future.

6. Conclusions

The diagnosis of FK in a clinical setting is known to be difficult and time consuming, with the current standard diagnostic tests being corneal smear and culture. This traditional approach, however, is dependent upon the location of infection being superficial and the sample containing the causative agent in a form that allows downstream detection. A large gap in the diagnosis and assessment of FK thus remains. IVCM, a technique gaining more widespread use in recent years, represents a non-invasive and rapid technique enabling possible early detection and diagnosis of FK, that has been shown to be useful in the majority of the articles and case reports reviewed here. Overall, the bulk of the published articles we analyzed have reported a yes/no answer for the diagnosis of FK by IVCM, but here we emphasize the need for a third category reflecting the uncertainty in IVCM findings in a proportion of cases. We additionally provide international expert advice for issues relating to IVCM examination and classification of FK. More studies are needed to compare IVCM to reference methods as well as compare agreement in categorization across observers. Furthermore, effort should be directed towards standardizing and unifying the conditions for expert examination, assessment, classification, and terminology. In the future, more published cases with IVCM images of specific fungal species may facilitate identification of features specific to different species. Comparing confocal microscopy of culture plates *in vitro* and IVCM images *in vivo* may be a method to determine the morphological features of different species, but to date such information is lacking in the published literature. Limitations of IVCM are its expense, the requirement to perform high-quality imaging, and having experienced assessors available. These factors result in IVCM not being available in all but the largest or most specialized centers. Another limitation in the use of IVCM could stem from subjects experiencing pain during the examination. The use of local anesthetic eye drops before the procedure limits this issue, and experienced IVCM operators rarely encounter this issue in the context of FK. However, inability to perform an IVCM examination may arise more often with inexperienced operators. The rare cases of patients not tolerating the IVCM examination, however, could be a source of bias as these patients are excluded from studies. Also, not widely mentioned or accounted for in the reviewed studies, is the experience of the IVCM operator performing the imaging. Even where image assessors are well-trained, the IVCM image dataset itself is limited by the quality of the operator's imaging. As such, considerable effort should be expended in training the operator to perform a comprehensive corneal imaging including different regions of the cornea and different depths according to a standard operating procedure, to provide ample, high-quality and high-contrast images free of artifacts. Performing

Table 5
Summary of studies using artificial intelligence for detecting FK from IVCM images.

First author, year	Neural network	Training set	Confirming FK	Sensitivity	Specificity	AUC
Roth et al., 2020 [44]	[a]	9 eyes	N/A	N/A	N/A	N/A
Lv et al., 2020 [34]	ResNet	688 images	M	91.86%	98.34%	0.9875
Liu et al., 2020 [33]	CNN	994 images	N/A	N/A	N/A	N/A
Wu et al., 2018 [36]	[a]	56 eyes	M	89.29%	95.65%	0.946

AUC = area under the receiver operating characteristic curve, CNN = convolutional neural network, FK = fungal keratitis, M = microbiologic examination (smear/culture), N/A = not available, ResNet = deep residual learning for image recognition.
[a] No neural network specified.

several sessions of imaging at different times during the disease course and treatment is also advised, as important information can be gained that could impact treatment and prognosis.

IVCM is not yet able to replace fungal culture as a standard clinical diagnostic method; however, clinics actively using IVCM with experienced operators and assessors have a distinct advantage over clinics without IVCM, in terms of diagnosing and/or managing FK. A growing number of cases with culture-negative results and unresolved pathology are being referred for IVCM. The new classification scheme and related recommendations we present here can guide both the microscope operator and the ophthalmologist in FK management. Still, a set of generally accepted criteria for positive identification of various types and species of fungi is lacking and large uncertainties still exist in the assessment of images due to the presence of confounding features. In the past few years, research exploring the use of AI in IVCM image assessment has emerged in an attempt to standardize the process. As these systems become more common, however, it is even more critical to ensure the quality of the clinical exam, to remove sources of bias in process steps conducted by humans, to further develop and refine the criteria for positive and negative FK detection, and to understand the reasons and caveats underlying the successful results of AI systems in classifying FK.

Declaration of competing interest

The authors declares that they have no competing interests.

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