Ophthalmology

Imaging in DRY AMD

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Since the discovery of fluorescein angiography much progress has been made in the field of retinal imaging. For age-related macular degeneration in particular, the scientific and clinical communities are facing a revolution in diagnostic tools. Confocal scanning laser ophthalmoscopy and spectral domain optical coherence tomography have enabled the non-invasive visualization of the natural history of a disease. With the advent of adaptive optics it is now also possible to resolve the fine structure of the photoreceptor mosaic, giving new perspective to the understanding of future potential therapeutic strategies.

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Introduction

Age related macular degeneration (AMD) is a multifactorial disease involving the central region of the retina, and is associated with poor vision. It can be divided in two separate entities: wet and dry AMD, both of which may be diagnosed. To achieve this and understand retinal diseases in greater detail, imaging devices such as the confocal scanning laser ophthalmoscopy (cSLO) and optical coherence tomography (OCT) are now crucial. We are facing a revolution in retinal imaging currently, and early diagnosis of AMD has seen a considerable level of improvement. In the case of dry AMD, the combination of *en face* techniques with tomographic reconstructions have given the possibility to increase the diagnostic sensitivity, while these non-invasive techniques are a powerful tool to assess the efficacy of therapeutic strategies for cases of wet AMD. In the near future, the advent of adaptive optics (AO) and single-cell imaging technologies will give the opportunity to improve diagnostic and therapeutic approaches.
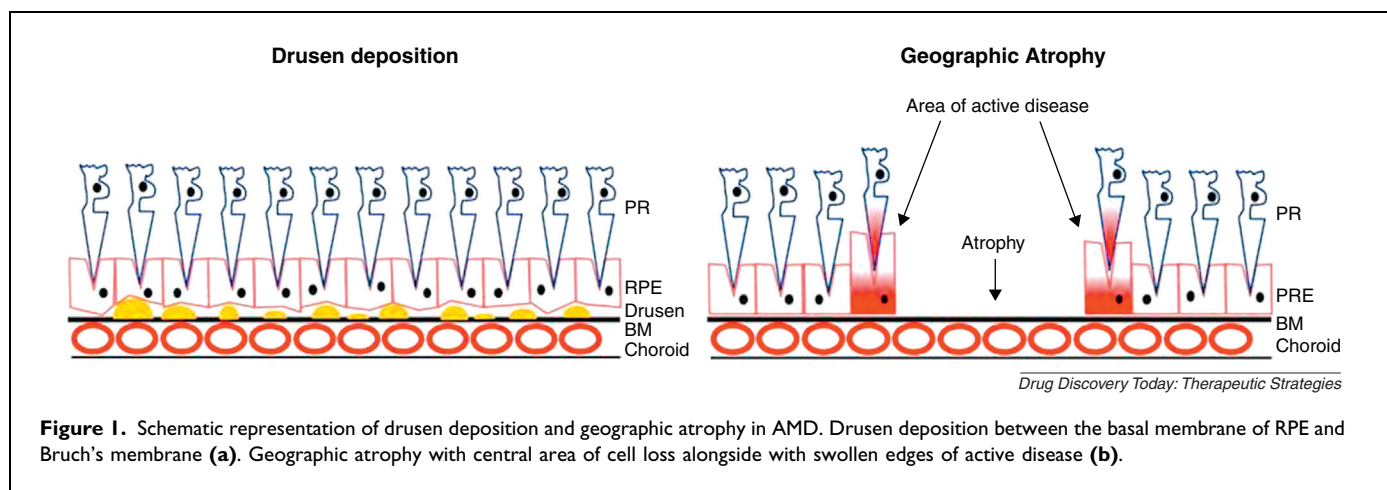
Age related macular degeneration

AMD is the leading cause of blindness in Western populations aged over 65 years [1]. This decline of vision will have increasingly important social issues as the population ages. Current studies estimate that approximately 1.75 million Americans are affected by a loss of vision from macular degeneration and that this number will grow to around three million in 2020 [2]. AMD has a prevalence of between 8.5% and 11% in the age group between 65 and 74, and 27% over 75 years.

Taking into account the various possible manifestations, classification of AMD can be divided into two types: non-exudative or dry AMD, characterized by drusen, retinal hyperpigmentation and retinal pigmented epithelium (RPE) atrophy, with or without geographical pattern (Fig. 1); and exudative or wet AMD, which involves choroidal neovascularization, RPE detachment and disciform scar formation. The advent of anti-VEGF therapy has revolutionized the treatment of wet AMD [3]. In the early stages of dry AMD, specific symptoms may not be present and good visual acuity may be still preserved, so many efforts are aimed at the early detection of the disease [4–6]. Despite approximately 10% of patients exhibiting the wet form, the vast majority of legal blindness from AMD – almost 90% – is through dry AMD [7].

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Figure 1. Schematic representation of drusen deposition and geographic atrophy in AMD. Drusen deposition between the basal membrane of RPE and Bruch's membrane **(a)**. Geographic atrophy with central area of cell loss alongside with swollen edges of active disease **(b)**.

The most common symptoms of AMD are metamorphopsia (central vision distortion) and the reduction of visual acuity which can have various degrees of severity, and a progression rate which depends on the type and the area of the injury. Overall vision loss is more gradual in dry AMD compared with wet AMD, in which vision loss is commonly accompanied by sudden central vision disturbance. Drusen, meaning lump, can be considered as the first detectable clinical manifestation of dry AMD. These yellowish growths, formed by an abnormal deposition of lipid, are situated beneath the retinal pigmented epithelium (RPE) particularly in the innermost part of the basal membrane between the RPE and inner collagenous layer [8] (Fig. 1a). Drusen can however, be found anywhere at the back of the eye, though most frequently in the posterior pole. Drusen are usually distributed symmetrically in both eyes, close to the macular region and confluent, with a wide range in size from tiny point deposits to 250 μm in diameter. There are different types of drusen: hard drusen which are small, yellow, rounded deposits, of less than 50 μm diameter and usually associated with alteration of the pigment epithelium; and soft drusen which are larger (Ø >125 μm), with pale edges and a raised surface, and a tendency to coalesce and form serous pigment epithelial detachment (PED). The confluence of drusen may be associated with an increased risk of progression to the wet form of AMD.

Fundus photography and fluorescein angiography
Imaging has a leading role in the diagnosis of dry AMD. The first techniques applied historically were fundus photography [9] and since the 1960s, fundus fluorescein angiography (FFA) [10,11]. Both remain useful in the diagnosis of AMD despite their limitations [12].

Drusen are seen as small yellow areas in a colour photograph [13]. Using FFA, hard drusen show as small hyperfluorescent dots visible during the early phase due to a window effect caused by thinning, atrophy or depigmentation of the RPE. Soft drusen are hypofluorescent during the early phase

of FFA, becoming brighter in the late phase because of pooling of fluorescein between the basal membrane of RPE and the Bruch's membrane [14]. Fundus photography and FFA have also been used for the identification and follow-up of geographic atrophy which appears as an area of hypo pigmentation on the fundus photograph and as a hyper fluorescent region (a window effect) on the FFA due to the loss of RPE [15].

The limitations of fundus photography centre on its low resolution and an inability to enhance the retinal signal. To acquire a good retinal image it is also necessary to employ a powerful light which is uncomfortable for patients. FFA carries the risk of potential serious side effects, including anaphylaxis.

Confocal scanning laser ophthalmoscopy
During the past two decades, the advent of the confocal scanning laser ophthalmoscopy (cSLO) has increased the ability to diagnose dry AMD significantly [16], offering the possibility of stereometric analysis of intraocular structures [17]. The basic principle of cSLO, invented in the 1980s [18], is to illuminate the retina with a dot point light source that can be swept over the retinal surface. The returned light passes through a pinhole in a plane conjugated to the point of interest. Only light from this focal plane can be detected, therefore enhancing image quality greatly, through tomographic imaging [19].

The versatility of the cSLO allows for the use of lasers of different wavelengths and specific filters; for example, red-free light (532–540 nm) which is particularly helpful in highlighting vessels, drusen, hard exudates and epiretinal membranes; red light (660–680 nm) which is suitable for imaging the RPE and choroid; infrared reflectance or IR or NIR (790–820 nm) which is useful in detecting the presence of cataracts; and blue auto-fluorescence or AF or FAF (Fundal Auto Fluorescence) (488 nm) (Fig. 2b) and near infrared auto-fluorescence (NIA) (Fig. 2c) which are particularly useful for the detection of geographic atrophy [20]. The limitations of the cSLO depend mostly on ocular media opacities and pupil diameter. In presence of cataract for example, FAF shows poor
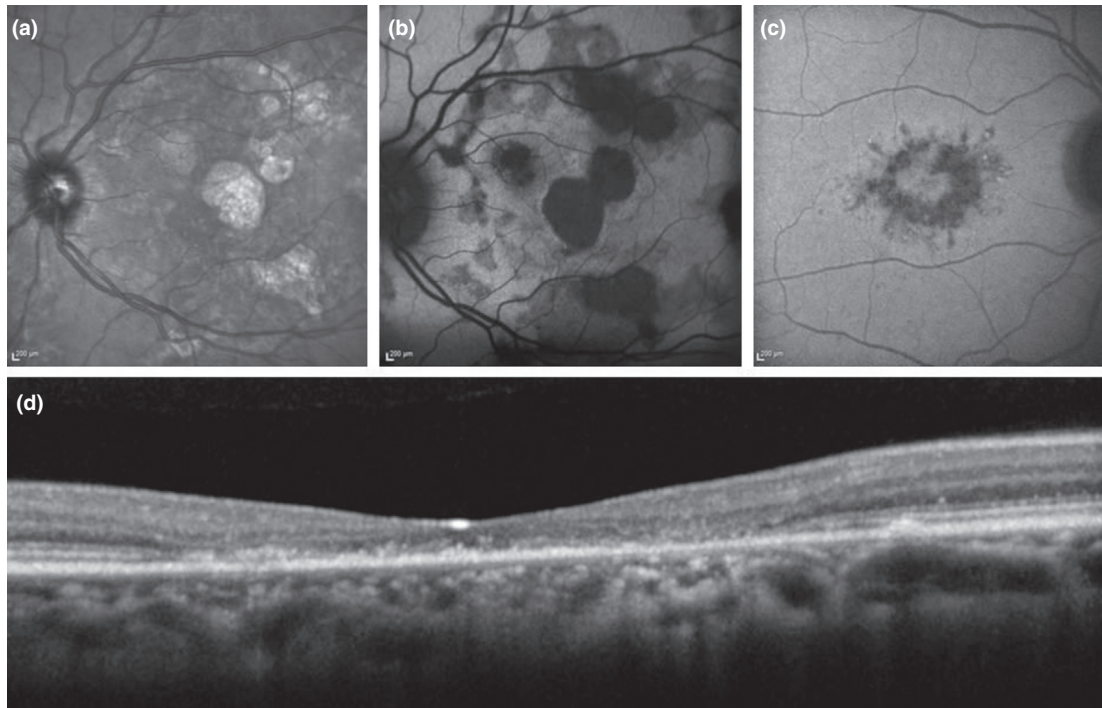
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Figure 2. Imaging modalities for Geographic Atrophy. Using cSLO (Spectralis OCT) it is possible to appreciate the NIR hyperfluorescence of GA **(a)** with a corresponding hypoflurescent window effect at the FAF **(b)** in the same patient. The dark NIA area of GA **(c)** corresponds with loss of RPE at the OCT **(d)**.

results because the blue light is absorbed by the crystalline lens.

Using the Spectralis HRA + OCT (Heidelberg Engineering, Heidelberg, Germany) large drusen appear having an either increased or decreased NIR, FAF and NIA with evidence of RPE elevation on the OCT. Small drusen have an enhanced NIR, FAF and NIA with an OCT focal thickening of the RPE. Drusenoids show increased NIR, decreased NIA and FAF and are usually circumscribed in the subretinal space at the OCT. PED exhibits patches of hypo/hyper florescence at NIR, NIA and FAF with multiple elevation of the RPE at the OCT [42].

Auto-fluorescence in the retina is derived from both lipofuscin (LF) and melanin [21,22]. These molecules accumulate in the cytoplasm of RPE cells during the aging process. LF is a polymer of high molecular weight, consisting essentially of residues of lipids, proteins and carbohydrates. LF cannot be degraded by lysosomes or eliminated by exocytosis [23]. LF is excited by 488 nm light, making blue AF particularly useful in detecting variations in the metabolism of RPE cells. Melanin can be excited by light of 790 nm, with emission occurring at 810 nm [22], which means that melanin can be used as an indirect marker for the presence of RPE. Since insults to the RPE generate modification to the auto-fluorescence, these variations can be used as an indirect measure of the state of health and activity of the RPE and thus provide a better understanding of the pathophysiology of dry AMD in which the RPE plays a fundamental role [24–26] (Table 1).

Table 1. Dry AMD characteristics: large drusen, small drusen, druseniod, pigmented epithelium detachment and geographic atrophy appearance using the Spectralis HRA + OCT

	Drusen				Geographic atrophy
	Large	Small	Drusenoid	PED	
Near infrared reflective	Increased/reduced	Increased	Increased	Patchy hyper/hypo	Bright
Near infrared autofluorescence	Normal/reduced	Increased	Reduced	Patchy hyper/hypo	Dark with bright edges
Fundus autofluorescence	Increased and well defined	Increased	Reduced	Patchy hyper/hypo	Dark with bright edges
OCT	Elevation of RPE	Local thickening of RPE	Subretinal localization	Multiple elevation of RPE	Thinning/loss of RPE with thicker edges

It has been shown recently that the hyperfluorescent edges of GA are areas of active accumulation of LF (Figs 1b and 2b,c) [22,27]. Longitudinal studies have demonstrated a direct correlation between the hyperfluorescence of GA margins and the rate of progression of the disease which is about two mm^2 per year [28–30]. It has been also shown that smaller atrophic areas progress at a slower rate than larger GAs. However it was noted that patients with irregular, convoluted areas of GA tend to grow faster in those areas with a growth rates of 1.1–2.8 mm^2/year [31]. These findings would be important to consider when designing therapeutic clinical trials; thus, a fast progressing patient can provide the researcher with more information in a shorter period of time. It may be that FAF can provide an accurate rate of progression for AMD. As recently highlighted, the rate of progression in eye diseases may be a better endpoint in early (pilot) proof-of-concept trials lasting less than a year, compared to current recognized biomarkers. [32]. Image analysis software (e.g. 'Region Finder'; Heidelberg Engineering) is also able to follow-up automatically the progression of the disease allowing the possibility of reproducible measurements [33]. The assessment of GA via cSLO is currently a primary outcome in several clinical trials including: NCT00393692, defining phenotypic variations in atrophic AMD and identifying predictive factors for disease progression based on FAF; NCT01229215, defining the growth rate of GA lesion areas from baseline; and NCT00429936 to determine the efficacy of 'fenretinide' in the treatment of GA'.

Optical coherence tomography

Another important tool for the diagnosis and follow-up of dry AMD is OCT, which is especially useful when associated with FAF [34] (Fig. 2c,d). OCT, as FAF, allows retinal imaging without dye injection and enables the visualization and quantification of cross-sectional anatomical details; as quantifiable measurements are not possible with traditional retinal imaging methods [35], this makes OCT useful in diagnosing and managing a wide variety of retinal conditions [36].

The operation of OCT is based on the principle of low-coherence interferometry. In this technique, the distances and sizes of different structures in the eye are determined by measurement of the time taken for light, backscattered from the different structures that compose the back of the eye, to return to the detector. This is analogous to A-scan ultrasound, in which the axial length of the eye is measured using sound rather than light [37].

Spectral domain optical coherence tomography (SD-OCT) technology uses low-coherence interferometry to detect light echoes, depending on a spectrometer and high-speed camera. It is based on the Fourier transformation, which describes the decomposition of a periodic function into a sum of simple sinusoidal-based oscillating functions [38]. The SD-OCT instrument is able to access the depth information – optical A-scan – of an object without mechanical scanning parts. Increased amounts of data allow image registration and 3-dimensional (3D) reconstruction, which aids in improved visit-to-visit measurement reproducibility [39]. Furthermore, several of the SD-OCT devices incorporate built-in software that permit correlation of OCT images with photographic, angiographic and auto-fluorescent investigations [20]. For instance, using OCT, the GA appears as an area of thinning or absence of the RPE with increased NIR and decreased NIA and FAF. The edges of the GA have increased NIA and FAF, with a thickening of the RPE at OCT [34] [42].

Current on-going clinical trials include: NCT00734487 to identify whether changes in AMD over time as seen with SD-OCT imaging, can be used to predict vision loss and the advancement of AMD in people at moderate to high risk for progression, and NCT01272076 to compare Cirrus HD-OCT automated measurements of the illumination area under the RPE to expert manual measurements of areas of hypofluorescence typical of GA in FAF images.

It is important to remember how OCT has changed the approach to AMD. This highly reproducible technique is limited only by any media opacity in the examined eye, and patient cooperation. For wet AMD, OCT is frequently preferred to FFA in assessing the efficacy of therapeutic strategies due to its non-invasiveness. OCT is also used as a screening tool to exclude the presence of wet AMD [40,41]. Combining SD-OCT with FAF or NIA images, enables the correlation of the topographic distribution of GA with cross sectional alterations in real-time [42,43]. Future innovations in OCT include the incorporation of a wide range of scanning wavelengths including the infrared band, which should provide increased penetration of the probe light through pigment and retinal haemorrhage, potentially allowing the imaging of choroidal neovascular membranes beneath thick intra-retinal or sub-retinal blood [44]. Broad-bandwidth light sources can be used to create cross-sectional OCT images with improved axial resolutions as fine as 3 µm, termed ultrahigh resolution OCT (UHR-OCT). UHR-OCT allows better visualization of the intra-retinal layers, particularly at the level of the photoreceptors.

Adaptive optics

Adaptive optics (AO) is a technique that was developed in the field of astronomy to reduce the effect of atmospheric turbulence on images obtained using a telescopic lens [45]. Subsequent development of AO led to its application in other fields, and it was recently applied to ophthalmology. The resolution achieved through AO has revolutionized retinal imaging through reduction of optical aberrations. This allowed visualization of the human foveal photoreceptor mosaic for the first time [48]. AO opens up a wide and interesting range of diagnostic and therapeutic possibilities

for diseases of the retina, increasing typical lateral resolution of current retinal cSLO devices (200 μm) to 2 μm, which is the average distance in the fovea between two photoreceptors [46,47]. In comparison, OCT lateral resolution currently is between 7 μm and 20 μm, although its axial resolution of 2–3 μm is sufficient for the determination of most retinal structures [44].

AO-OCT is rapidly developing and different variants have been reported, finally achieving a nearly isotropic resolution of 2–3 μm in axial and lateral directions, allowing resolution of cone photoreceptors in the human retina as close as 2° to the fovea [48]. The possibility of better resolution achievement using AO, will allow for more detailed studies of the RPE and the choroid, with the possibility of more specific answers to the pathogenesis and natural history of neovascular membranes, and retinal vascular changes in systemic diseases. The optical dynamic correction of ocular aberrations with AO systems has shown a marked improvement in the quality of retinal imaging when compared to current imaging techniques (FAG, ICG, OCT, ultrasound) [49].

AO may not only be able to provide significant knowledge of anatomy and physiology of the living human retina, but above all, be introduced into clinical practice as a powerful tool for early diagnosis and treatment of many retinal diseases, specifically those of the macula region. In fact AO imaging has provided accurate measures of RPE cell density of ~5500 cells/mm^2 [50]. So a growth rate of 1.1–2.8 mm^2/year would be about 6000–16 000 RPE cell deaths per year.

AO cSLO can currently image a small region of the retina only, and thus patient cooperation is crucial. For healthy subjects, the acquisition of montages of approximately 13° × 10° takes 30 min on average [51] and thus is highly labour intensive for the patient and imager. The amount of post-processing needed is also very time consuming [52]. One other limitation for AO is the need for a near perfect optics. Patients with cloudy corneas or cataracts cannot be imaged using AO methods. AO-cSLO could however be extremely useful in assessing the efficacy of potential therapeutic strategies after selection of an adequate population with a fast rate of GA enlargement by regular cSLO examination; AO could lead to a better focus on those potential responders to therapy. In patients with dry AMD macular rods degenerate faster than cones, thus the possibility to visualize affected rods in the early stage of the disease could lead to better focused clinical trials.

Detection of apoptosing retinal cells (DARC)
In 2004, a technique to visualize a retinal ganglion cell (RGC) undergoing apoptosis directly was developed, detection of apoptosing retinal cells (DARC) [53]. This technology uses fluorescence-labelled Annexin A5 to bind apoptosing retinal cells, detected by cSLO. More recently, different phases of cell death in the retina have been demonstrated using the same
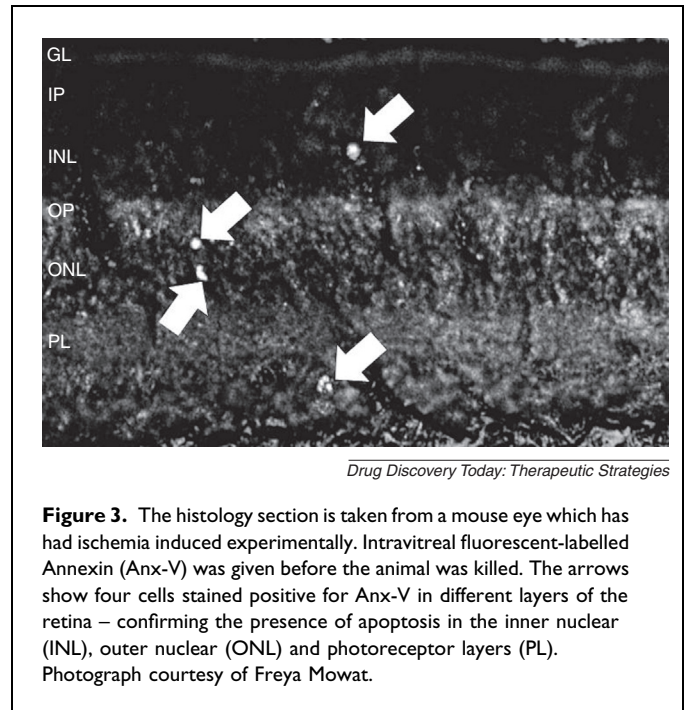

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Figure 3. The histology section is taken from a mouse eye which has had ischemia induced experimentally. Intravitreal fluorescent-labelled Annexin (Anx-V) was given before the animal was killed. The arrows show four cells stained positive for Anx-V in different layers of the retina – confirming the presence of apoptosis in the inner nuclear (INL), outer nuclear (ONL) and photoreceptor layers (PL). Photograph courtesy of Freya Mowat.

technique with several fluorescent probes [49]. To date, DARC has been used to image RGC apoptosis *in vivo* alone, and indeed, this is the basis of the phase I clinical trial due to start in 2013.

The greatest advantages of DARC are its *in vivo*, real time, high resolution and non-invasive nature, suggesting that this technique, due to the ability to visualize a single apoptosing cell, could become a future diagnostic tool which could enhance and anticipate diagnosis of several ocular diseases including AMD.

Recently, in a mouse model of retinal ischemia, it was shown that intravitreally injected fluorescent-labelled Annexin A5 was able to bind to the surface of apoptosing cells in different layers of the retina, as demonstrated histologically. Apoptosis was identified in the inner nuclear (INL), outer nuclear (ONL) and photoreceptor layers (PL) (Fig. 3). This has not yet been visualized *in vivo* due to the optical limitation of conventional cSLO; however, looking into the eye at cellular level, in combination with the introduction and application of AO-cSLO, may enable the DARC technology to be used in the evaluation of patients with dry AMD, providing a new biomarker for the clinic.

Conclusions
Retinal imaging has undergone a revolution in the last two decades. OCT and cSLO have given clinicians the opportunity to understand retinal diseases in greater detail. cSLO offers the opportunity to visualize changes such as FAF, macular pigment and with AO photoreceptor patterns. OCT provides information on structural changes (akin to an *in vivo* histological section of the retina). Evidence of the impact of these

technologies can be seen in the considerable level of improvement in the diagnosis of GA with FAF, NIA and OCT. By combining *en-face* techniques with the tomographic reconstruction of OCT, it is possible not only to improve diagnostic sensitivity of the disease but, with the use of dedicated software, to have a robust follow-up tool. These techniques are extremely useful in the case of wet AMD where there are established therapeutic strategies [54] and both are non-invasive techniques that are easy to use and patient-friendly. We strongly suggest that, as both technologies are complementary their use should be combined in clinical trials.

Unfortunately, current imaging techniques only monitor the natural history of dry AMD. Commercially available cSLO and SD-OCT, due to their non-invasive nature, high-test speed and high sensitivity and specificity in detecting pathological alterations, could be a useful tool for population screening and clinical trial patient selection. There are some limitations in the use of these techniques, of which patient cooperation is one of the most important. For cSLO the presence of cataract is a major limiting factor when FAF is performed; for AO, image size and the time needed to acquire and process the images are the biggest limiting factors. For the OCT, the eye tracking methods, high-speed acquisition and long scanning wavelengths have greatly improved patient-dependent factors, although media opacities remain a problem. DARC and other potential technologies could help in the identification of early disease and response to treatment, before the onset of irreversible vision loss. We hope that in future, a better understanding of the disease process in dry AMD and the development of new sophisticated imaging devices will enable the development of new therapeutic strategies through monitoring with AO-cSLO, DARC and AO-OCT.

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