Adaptive optics optical coherence tomography at 120,000 depth scans/s for non-invasive cellular phenotyping of the living human retina

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Abstract

This paper presents a successful combination of ultra-high speed (120,000 depth scans/s), ultra-high resolution optical coherence tomography with adaptive optics and an achromatizing lens for compensation of monochromatic and longitudinal chromatic ocular aberrations, respectively, allowing for non-invasive volumetric imaging in normal and pathologic human retinas at cellular resolution. The capability of this imaging system is demonstrated here through preliminary studies by probing cellular intraretinal structures that have not been accessible so far with in vivo, non-invasive, label-free imaging techniques, including pigment epithelial cells, micro-vasculature of the choriocapillaris, single nerve fibre bundles and collagenous plates of the lamina cribrosa in the optic nerve head. In addition, the volumetric extent of cone loss in two colour-blinds could be quantified for the first time. This novel technique provides opportunities to enhance the understanding of retinal pathogenesis and early diagnosis of retinal diseases.

1. Introduction

Optical coherence tomography (OCT) has emerged as a leading technique in ophthalmic imaging due to its capability to non-invasively resolve tissue morphology with high sensitivity and high axial resolution [1–9]. Despite increases in axial resolution, monochromatic ocular aberrations [10–12] limited the transverse resolution for retinal imaging to ~20 μm, which is too large for visualization of cellular structures. Adaptive optics (AO) may be used to correct such aberrations, leading to an improvement in image contrast and lateral resolution, as demonstrated in works [13–31] that have improved our understanding of the normal and pathological retina.

However, these improvements are thwarted with broadband illumination [18,32] due to the longitudinal chromatic aberrations (LCA) of the human eye in the near-infrared [33]. Hence, an achromatizing lens [34] was designed [35,36] to compensate for this effect, and when combined with AO, leads to an appreciable increase in image contrast and both lateral and axial resolution [26,27,29–31].
It had previously been shown [22] that increasing the image acquisition speed to 75,000 depth scans/s can improve visualization of retinal features in AO-OCT with LCA correction. Considerable technological advances have pushed the imaging potential of OCT further with acquisition speeds reaching up to 106,382 depth scans/s and 312,500 depth scans/s [37] with ~2 μm and ~9 μm axial resolution (without AO for both cases), resulting in a significant reduction in motion artefacts. Despite such improvements, it has been shown [31] that saccades of up to ~360 °/s [38] can severely hinder image quality by disrupting volumetric images with motion artefacts in the form of discontinuities. This effect is most critical for AO-OCT since such artefacts can thwart any benefit from AO.

The purpose of this study is to further enhance the imaging potential of OCT with LCA compensation and an improved implementation of AO-OCT from that of a previous [27] system through the use of dynamic closed-loop AO in combination with a 6-fold increase in acquisition speed to 120,000 depth scans/s, demonstrating that only with ultra-fast acquisition speeds is it possible to benefit more fully from AO and LCA compensation for revealing cellular structures in the living human retina non-invasively. In a recent work [31] this system was used as part of a larger study to investigate how improved resolution (with AO and LCA compensation), speed (with 3D OCT at 800 nm) and penetration (with 3D OCT at 1050 nm) can provide complementary clinical information in selected subjects with gross retinal pathologies. In contrast, the focus of the current study is to demonstrate through preliminary data that with such improvements it is possible to visualize structural information at the cellular level in normal and pathologic retinas unattainable without AO-OCT with LCA compensation at ultra-high image acquisition speeds.

2. Methods

2.1 Optical set-up

The optical set-up is an improved implementation from that of a previous system [27] and shares similar features with LCA corrected AO-OCT set-ups used in other works [26,29,30]. The light source (FemtoLasers Produktions GmbH, Austria) is a Titanium:sapphire laser [39,16] with an ultra-broad spectrum of 140 nm full-width-at-half-maximum centred at 800 nm. This spectral bandwidth provides a theoretical axial resolution of 2.0 μm in tissue. The laser output is sent through 100 m of optical fibre to stretch the pulses, thereby reducing the peak power incident on the retina. An 80/20 fibre coupler divides the source light into two paths: the sample and reference arms (Fig. 1).

The sample arm contains the AO (Imagine Eyes, France) and achromatizing lens [35] for monochromatic and longitudinal chromatic aberration compensation, respectively. The AO closed-loop rate is ~1 Hz and consists of a 52-actuator deformable mirror (Mirao-52, Imagine Eyes, France) capable of providing ±35 μm of defocus correction and a Hartmann-Shack wavefront sensor (Haso-32, Imagine Eyes, France) utilizing a 32×32 sub-aperture array of microlenses with a pitch size of 110 μm. A 6 mm beam was used, allowing for a maximum theoretical transverse resolution of 2.7 μm at the central wavelength of 800 nm (assuming an effective focal length of 16.67 mm for the eye). The wavefront sensor and deformable mirror driver are interfaced to a computer and the AO command signals and associated processing is performed with the bundled software provided by Imagine Eyes (Casao, Imagine Eyes, France).

Two galvanometric mirrors (GSI Group, USA) raster scan the beam onto the retina, and a series of off-the-shelf lenses and spherical mirrors optically conjugate the iris of the eye with the above-mentioned elements. Since the same probing beam used for imaging was also used for wavefront sensing in a double-pass configuration, spherical mirrors were mostly used for optical conjugation to avoid back reflections from lenses that would disrupt the
wavefront measurements. Since the light returning from the eye is sampled after the deformable mirror, non-common path errors will result from the remaining optics in the imaging path (L<sub>1–3</sub> and AL) and wavefront sensing branch (L<sub>4–5</sub>). Thus, care was taken during alignment to avoid introducing spherical aberration and oblique astigmatism, for example, by selecting optics with sufficiently large clear apertures and by aligning lenses to the beam axis, respectively. In order to minimize aberrations induced by off-axis spherical mirrors, ≥300 mm focal lengths were chosen. The use of long relays with limited optical aperture sizes in turn imposed a restriction on the allowable scanning angle in the eye, which was approximately ±1º.

The reference arm contains mirrors and dispersive prisms (BK7 and SF11) to balance the path length and dispersion (due to L<sub>1</sub>, L<sub>2</sub> and AL) with that of the sample arm. A water cell was not included, as typically used for dispersion compensation of the eye, since this dispersion was compensated numerically [7,40]. The spectrometer (BaySpec Inc., USA) consisted of commercial compound lenses, a holographic transmission grating (600 lp/mm, BaySpec Inc., USA), and a high speed CMOS camera (Basler Vision Technologies, Germany). The camera was configured with a line rate of 120 k-A-lines/s (184 M voxels/s) utilizing 1536 pix. The corresponding axial sampling was 0.85 μm per pixel. The number (i.e. density) of scans along both transverse directions could be varied to achieve different acquisition volumes and hence the total acquisition time, as discussed below. This 6-fold increase in line rate speed from the system’s predecessor allowed to acquire a 512 × 768 pix (x x z) tomogram in 4.3 ms, or a volume of 256 × 256 × 768 pix (x x y x z) in 0.5 s, for example.

2.2 Experimental protocol

The experimental protocol was approved by the local safety and ethics committee. The entrance beam diameter was 6 mm and Tropicamide 0.5–1% was used to dilate the subject’s pupil and to paralyze accommodation. A bite-bar with headrest and fixation light helped stabilize the subject’s head and reduce eye movements. The incident light intensity (800 μW) was within the maximum permissible exposure set by ANSI [41]. Defocus was applied to the deformable mirror during closed-loop AO to change the focal plane depth, allowing to increase the signal throughput at layers of interest. The focussing control was applied subjectively by observing where approximately in the OCT image preview the layer of maximum back-scattered light was originating from. Retinal images were obtained from a small sample of normal and colour-blind subjects for this preliminary study. Data was acquired multiple times at several different retinal eccentricities for all subjects.

As mentioned in the Section 2.1, the density of the raster scanning pattern was varied to achieve different volume sizes acquired over different time scales. This allowed for compromise between over-sampling to reduce motion between successive raster scans and under-sampling to reduce overall acquisition time and thus overall motion. For the comparative study of normal vs. colour-blind subjects (3.2), for example, a 512 × 512 pix<sup>2</sup> scanning protocol was used with equal sampling densities of 394 pix<sup>2</sup> along the x and y direction. A 512 × 512 pix<sup>2</sup> scanning protocol was also used for imaging of the lamina cribrosa (3.5) but with unequal scanning densities along the x (197 pix<sup>2</sup>) and y (985 pix<sup>2</sup>) directions. Finally, to further reduce motion artefacts and speckle noise to improve signal-to-noise contrast, the retina was sometimes raster scanned along the fast-axis direction only (i.e. no slow-axis scanning) and the acquired tomograms were averaged. In this case, although a 3D volume was acquired, the same 2D (x x z) retinal section was scanned 512 times (aside from translations in retinal position due to eye motion). The speed of motion sufficient to decorrelate the speckle pattern with speckle diameter ξ can be approximated by the speed needed to shift the pattern by one speckle width (ξ) within the desired time frame τ. Thus assuming a speckle size of up to 4 μm and with τ = 4.3 ms, a speed of 0.31 mm/s

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would be sufficient to decorrelate the speckle field at each acquisition frame. In this case, even a modest eye motion velocity of 20 °/s or 5.8 mm/s (assuming 1° = 288 μm) would suffice. The various scanning patterns used in the presented results are summarized in Table 1.

2.3 Image processing

Spatial information was reconstructed from the spectral information by first applying spectral shaping, upsampling, resampling and dispersion correction followed by transformation to the spatial domain using zeropadding and the fast Fourier transform [7,40]. In cases where unequal scanning densities were used, the data was rescaled accordingly to obtain isotropic pixel dimensions. An adaptive wavelet shrinkage filter (A trous) [42,43] or a bandpass filter was used to remove excess speckle noise. Careful attention was paid towards the selection of the coefficients or parameters used during these filters to ensure that noise was removed without introducing gross artefacts. Although the exact parameters selected for bandpass filtering varied for the data presented in Section 3, structures smaller than 2–4 pix and greater than 500 pix were filtered. Since the speckle size is closely related to the diffraction-limited resolution of the imaging system, the expected speckle size is 2–3 μm, and hence, the chosen passband is reasonable. The coefficients chosen for a trous filtering were k1 = 3 and k2, k3= 1.3. For conciseness, details of the filter used will be denoted by ATF for “a trous filter” or BPFα–β for “bandpass filter”, where α and β represent the minimum and maximum limits in pixels of the passband. For example, with BPF2–500, structures smaller than 2 pix and greater than 500 pix are filtered out, while structures between 2 to 500 pix in size are passed. OCT images of both raw data and filtered data are presented in the Discussion to demonstrate that the cellular structures visualized are not a result of artefacts of the post-processing techniques utilized. Discontinuities (image tears) due to eye motion and bidirectional scanning were corrected using a modified pyramidal registration algorithm [44,45].

Cross-sections were averaged to further reduce speckle noise and to highlight prominent features. Image averaging can detrimentally blur out details of the very structures of interest due to, for example, inaccuracies in image registration, or due to OCT signals originating from neighbouring structures. In turn, this can thwart any beneficial impact of AO on image resolution. For this reason, the number of sections averaged depended on the size of the structures of interest and are specified in Section 3. For example, the axial sampling of 0.85 μm/pix allowed for averaging of 15 axial cross sections in order to increase the visualization of the RPE cell mosaic without blurring out details of the RPE cell spacing. Similarly, a raster scanning density of 394 pix/° (Table 1), or 0.73 μm/pix, allowed for averaging of ~5 transverse cross sections to improve the appearance of cones without introducing significant blurring due to overlapping of signals from cones in more distant layers.

2.4 Cell counting and estimation of cell-to-cell spacing

Cells were counted by searching for maxima in ImageJ [46]. This was found to provide an accurate cell count, judged qualitatively by overlaying spots representing the locations of maxima over the image. To improve signal-to-noise contrast, cell counts were always performed on images obtained from averaging of multiple sections. The number of images included in the average was limited so as to not average out the details of interest. Due to the presence of vessel shadowing or residual motion artefacts in some parts of the processed data, smaller areas were always selected from the entire (averaged) image area and the cell count was performed only within this smaller area. Since the cells in question were larger than the speckles (< 4 pix across), the (averaged) image used for cell count analysis was bandpass filtered. For the case of RPE cell counts, since foveal retinal pigment epithelium (RPE) cells are ~10 pix in diameter, 15 en face slices were averaged then filtered with
BPF2–500. These additional steps of averaging and filtering helped to improve confidence in cell counts.

In cases where the algorithm produced inaccurate results based on the above-mentioned qualitative check, the cells were counted manually. For the cell counts used in Section 3.2, 300 × 300 pix² areas were considered for the automated cell count, while in Section 3.4, a 360 × 500 pix² area was considered for the manual count. All reported cell counts were obtained by averaging five estimates. In the case of automated counting, the ‘noise tolerance’ parameter in the Find Maxima tool of ImageJ was adjusted over a range of values to account for the somewhat arbitrary choice in that parameter. But only cell counts that produced good qualitative matches between the data and the perceived cell centres were considered in the average. This was determined by overlaying spots corresponding to cell centres over the data.

The cell-to-cell spacing of RPE cells was inferred from one of two ways: From the radius of Yellott’s ring [47] in the power spectrum of the (averaged and bandpass filtered) cell mosaic, and based on the expected cell-to-cell spacing for a triangularly packed mosaic with a given cell density. To reduce the effect of speckle noise for the former method, the cell mosaic (obtained by averaging of 15 en face slices) was filtered using BPF2–500. For the former, the radius of Yellott’s ring was measured 20 times at roughly equiradial positions and averaged. For this calculation as well as others that required a conversion from microns to degrees, it was assumed that 1° of arc corresponds to 288 μm at the retina.

In order to infer the cell spacing s from the cell density D, it was assumed that the RPE cell packing is triangularly arranged. In this case, the cell spacing is given by [48]:

\[ s = \left( \frac{2}{\sqrt{3} D} \right)^{\frac{1}{2}}. \]

Finally, Voronoi domain analysis [49] was used to investigate the packing arrangement of cells as described elsewhere [50]. This also provided an additional independent means of estimating the mean cell-to-cell spacing. For this, a script written in Matlab was used to perform the analysis based on the manually determined coordinates of the cell centres. The analysis was performed on all five sets of coordinates to obtain an average.

Results

3.1 In vivo phenotyping in red/green colour-blinds in 3D

En face slices at 2.9° and 5.8° temporal in a normal and a red/green colour-blind subject with cone degeneration due to a Cys203Arg genetic mutation are shown in Fig. 2(a)–(h). Individual images were obtained after averaging of several en face slices and additionally applying bandpass filtering to reduce speckle noise (see figure captions for details). The presence of speckle noise has interrupted some of the underlying structure of the photoreceptor mosaic – particularly for the colour-blind data. The degree to which this has occurred is difficult to assess due to the extent of cone degeneration for that subject.

Photoreceptor densities were estimated at two different depths: the inner/outer segment (I/OS) junction and at the distal-most part of the outer segment (OS tips). The latter is where the outer segment interfaces with the RPE cell and is located approximately 28 μm and 48 μm below the I/OS junction in the normal and colour-blind subject, respectively, at 5.8° temporal. The photoreceptor densities in mm² are indicated at the top-right corner of the panels in Fig. 2(a)–(h) with standard deviations <5%, and they revealed a considerable
reduction in cell counts for the colour-blind case. For comparison, normal photoreceptor densities at 2.9° and 5.8° have been reported to be approximately 24,000 cells/mm² and 16,000 cells/mm², respectively [52]. This 42–58% reduction in cell density at 2.9–5.8° is consistent with a similar finding of 51% by Carroll et al. [53] using adaptive optics flood-illumination ophthalmoscopy in the same colour-blind subjects and different normals.

The potential of this imaging device for phenotyping is also demonstrated in Fig. 2(i)–(m), which presents cross-sectional images obtained by integrating 5 (Fig. 2(i), (m)) and 200 (Fig. 2(j), (l)) tomograms from a normal (Fig. 2(i)–(j)) and a colour-blind subject (Fig. 2(l)–(m)) at 2.9° and 5.8° temporal. The integrated portions help to emphasize the transversal density of the reflectors, revealing a significant reduction in back-scattered light from the OS in the colour-blind retina, as indicated by lower cell counts relative to the normal. Media 1 and Media 2 present a 424 × 240 μm² stack of cross-sectional images filtered with ATF from a normal and colour-blind subject at 5.8° temporal.

Referring to Fig. 2(k), the thickness of the IS was defined by the distance between the centres of the first and second bright bands, corresponding to the ELM/IS and IS/OS junction, respectively. The thickness of the OS was defined as the distance between the centres of the second and third bright bands, the latter of which likely corresponds to the average depth at which the OS interface with the RPE (i.e. the “OS tips”). And the thickness of the RPE was defined by the distance between the centre of the third bright band and the most distal part of the fourth bright band. Comparisons in the thickness of the IS, OS and RPE layers between the normal and colour-blind subject at 5.8° temporal indicate that these layers are 25%, 71% and 15% greater in the colour-blind retina. A similar trend was observed at 2.9° eccentricity and in a second colour-blind individual. This may be caused by the absence of cones or disrupted waveguiding of present but non-functional cones [49,51] but further studies are needed to verify this.

Finally, the 2D power spectrum of the cone mosaic presented in Fig. 2(c) is shown in Fig. 2(n). Based on the radius of Yellott’s ring from averaging of 20 approximately equiradial measurements, the cell spacing was 8.0±0.6 μm (36.2±2.5 cycles/°). The limits of the passband correspond to 0.6 cycles/° and 96 cycles/° for the filter utilized (BPF3-500).

3.2 3D Visualization of foveal nerve fibre layer and ganglion cells

Figure 3 presents visualization of foveal stratification centred at 1° eccentricity. A depth dependent colour-coded image is shown in Fig. 3(a), composed of a greyscale image of nerve fibre bundles and a red pseudo-coloured image of vessels ~52 μm below. Nerve fibre bundles are highly reflective and thus show up as striations. Due to the steep foveal slope at this eccentricity, 22 "en face" sections were integrated in order to include nerve fibre bundles across the entire ~2 × 2° patch while also reducing noise, and speckle noise was further reduced using BPF2–500. Similarly, due to the multiple layers of vessels, 60 "en face" sections were integrated for the composite vascularization image.

An average of 30 slow-scan-axis tomograms filtered with ATF within the blue region in Fig. 3(a) is shown in Fig. 3(b), tracing a microvessel and its intersections in the inner retina. Averaging of 3 slow-axis-scan tomograms filtered with ATF within the yellow region in Fig. 3(a) revealed a fine structure extending from the ganglion cell layer (GCL) to the interface of the inner plexiform layer (IPL) and inner nuclear layer (INL). Given by the location and rarity of the structure, and that it appears to bifurcate at the GCL/IPL and IPL/INL interfaces, it likely corresponds to a microvessel.

As described in Section 2.2, the retina was also raster scanned along the fast-axis direction only and 30 tomograms were filtered with ATF and averaged (Fig. 3(d)) to further reduce...
motion artefacts and speckle noise. Even at this magnification, dark circular shaped structures (denoted by red arrows in Fig. 3(e)) likely corresponding to ganglion cell bodies emerge beneath the GCL. The size of the structures purported to be ganglion cells in Fig. d–e, which are approximately 10–15 μm in diameter, are considerably larger than the speckles (2–3 μm) and are consistent with the expected size of ganglion cells [54].

3.3 Three-dimensional visualization of retinal pigment epithelial (RPE) cells and choriocapillaris

The 3D volume rendering in Fig. 4(a) shows a slightly off centre retinal patch size of 1.3 × 1.8° from a 28 year old normal male caucasian, extending from the external limiting membrane (ELM) to the choroid. Speckle noise was suppressed by filtering with ATF and by averaging of 5 en face slices. After passing the ELM, IS, and OS, the light is weakly backscattered at the central level of the RPE soma (Fig. 4(b), (d)–(e)). After averaging of 10 en face sections filtered with ATF, its irregular appearance mimics structures found in histology [55] (Fig. 4(f)).

At the deeper RPE soma level (Fig. 4(g)–(h)), corresponding to the layer containing RPE nuclei, a dominantly structured arrangement of dark round bodies are visible. To improve signal-to-noise contrast, 15 en face slices were averaged then filtered with BPF4–500. Also shown for comparison are light micrographs of 18 year old (Fig. 4(i)) and 42 year old (Fig. 4(j)) male caucasian RPE cell mosaics from Gao et al. [56]. Some irregularity in the cell mosaic (Fig. 4(h)) is present due to some residual speckle noise and motion artefacts, but Voronoi domain analysis confirmed a dominantly triangular cell arrangement based on the average number of sides per Voronoi cell (5.98±0.81). Based on that same analysis, the average nearest-neighbour-distance (NND) was 9.5±1.6 μm.

As described in 2.4, a manual count of RPE cells was obtained by estimating the number of dark circle-like bodies in the RPE cell mosaic (Fig. 4(g)), yielding a cell density of 7017±187 cells/mm². Since the mosaic was trianularly packed, the expected cell spacing (s) was 12.8±1.1 μm (Eq. (1)). The cell spacing was also inferred from the radius of Yellott’s ring in the power spectrum of the RPE cell mosaic, obtained from the average of 15 power spectra from 15 unfiltered en face sections piercing through the RPE cell layer (Fig. 3(c)). Since the average power spectrum is itself quite speckled, filtering with BPF4-500 was used to help emphasize Yellott’s ring. The radius of Yellott’s ring was estimated 20 times at approximately equiradial orientations and averaged to yield a cell spacing of 10.7±0.3 μm (27.0±0.8 cycles/°). This analysis was repeated on the non-filtered power spectrum (Fig. 6) resulting in a similar cell spacing of 11.0±0.5 μm (26.2±1.2 cycles/°). For Fig. 4(c), the limits of the passband correspond to 0.6 cycles/° and 72 cycles/° for the filter utilized (BPF4-500).

An additional metric for the packing arrangement was the ratio of the cell spacing based on Voronoi analysis (i.e. NND) to the expected cell spacing based on the cell count (i.e. s): NND/s = 0.81, providing further indication that the RPE cell arrangement conformed to a trianularly packed mosaic.

Distal to the RPE is a layer containing mesh-like structures (Fig. 4(k)) consistent with that of the choriocapillaris, although the presence of speckle has interrupted the otherwise continuous connections of microvessels, shown here after averaging of 10 en face sections filtered with ATF. Upon inverting the OCT image (Fig. 4(l)), the now dark interrupted network resembles features evident from histology (Fig. 4(m)) of human choriocapillaris [57].
3.4 Revealing the fine 3D structure of the lamina cribrosa

Nerve fibres form bundles on the inner retina and congregate at the optic nerve head (Fig. 5(a)–(d); Media 3). To counteract mechanical forces from the vitreous, the lamina cribrosa (LC) – a collagenous extension of the sclera, forms a 3D meshwork, which can be seen in Fig. 5e as bright structures in histology [58] (Fig. 5(e), left), and in OCT (Fig. 5(e) right; Media 4) as bright horizontal lines or dots depending on their orientation (average of 5 cross sections filtered with ATF). Before the nerve fibres form the optic nerve they are redistributed from larger thick bundles – seen as dark regions due to the axial illumination in the en face slices (filtered with ATF; Fig. 5(b)–(d)), to fine fascicles which can be followed and reconstructed in 3D (filtered with ATF; Fig. 5(g)). Media 3 and Media 4 present a 418 × 101 μm² and 418 × 1234 μm² stack of en face and cross-sectional images (filtered with ATF) of the LC. In Fig. 5(a), the coordinates of the pore boundaries were manually selected along one transverse direction for each en face slice within the volumetric stack. A spline algorithm was used to join the points and to smooth the connections to create the white contours that trace the paths taken by the pores.

4. Discussion and conclusions

Regions of high mitochondrial density in the ellipsoids give rise to strong intensity signals in OCT. Inner segments of rods and particularly those of cones, i.e. the ellipsoids, contain a high concentration of mitochondria [59,60] which are known to play a role in waveguiding due to their high refractive index. Similarly, the presence of melanosomes in RPE cells [60] and the tapering of cone outer segments might contribute to an increase in back-scatter in more distal regions.

The difficulty in discerning structure (or signal-carrying speckle) from noise (including signal-degrading speckle) is a very challenging issue for OCT imaging. Several factors may contribute to the wide variation in image quality observed within and across subjects. One possible factor may be the degree of correction of LCA. It is known [26] that improper subject alignment can result in deviations from optimal correction, and overall subject compliance is one common contributing factor to misalignments. Further work is needed to better understand the factors responsible for the variability in image quality within and across subjects.

Image averaging was used to improve visibility of retinal structures, but averaging alone cannot remove most of the speckle noise present. To better combat this noise, bandpass and a trous filters were utilized. As described in section 2.3, carefully chosen parameters were used to avoid the introduction of artefacts that could confuse artificial structure for real structure. To demonstrate the effect of these two filters, some sample data before and after some postprocessing steps is presented in Fig. 6. A 100 × 100 μm² patch of RPE mosaic obtained by averaging 15 en face slices is shown in Fig. 6(a). The 15 en face sections were minimally processed – only image registration was applied to compensate for image motion, followed by rotation of the data by <4° along both orthogonal directions to adjust the tilt of the retina. The power spectrum of Fig. 6(a) is shown in Fig. 6(b), while in Fig. 6(c), the power spectrums from each of the 15 en face slices was averaged. Since the power spectrums also appear speckled, the power spectrum in Fig. 6(c) was filtered with BPF4-500 resulting in Fig. 6(d). A similar sequence of results are shown in Fig. 6(e)–(h) and in Fig. 6(i)–(l) where the 15 en face sections were filtered using BPF4-500 or ATF, respectively. Although a detailed analysis of the effects of the bandpass and a trous filters is beyond the scope of this work, Fig. 6(e)–(l) demonstrates that the use of these filters has not added spurious artefacts to the RPE mosaics (Fig. 6(e), (i)), nor is there any significant change in the radius of Yellott’s rings in the power spectrums (Fig. 6(f)–(g), (j)–(k)), even after
applying bandpass filtering to the power spectrums themselves (Fig. 6(h), (l)). Rather, the ability to discern Yellott’s ring is improved with the use of the filters.

The above analysis has demonstrated that the filters used for speckle noise reduction have not introduced gross artefacts in the resulting RPE cell mosaics or in their power spectrums. To further confirm that the structures visualized were attributed to RPE cell packing and not due to speckle noise or artefacts of the speckle noise filtering process, the en face slice central to the sections piercing through the RPE cell nuclei was cross-correlated to other layers of comparable signal intensity above and below it. A depth dependent plot of the peak cross-correlations is presented in Fig. 7. Since the same filtering was applied to all depth scans considered in this analysis, these results demonstrate that the observed structural details were attributed to real structure and not speckle noise or artefacts due to noise filtering.

Two possible categories of mutations can lead to dichromatic colour vision (colour-blindness) – one which results in photoreceptor loss, while in the other, the substitution of an absent cone class with another [49,51]. Although it has been possible to show that these two different genotypes, which have classically been associated with the same dichromat class, can represent different cellular phenotypes, little information was known of the extent of the degeneration distal to the ellipsoids due to a limited depth range. Image quality varied between subjects and within repeated acquisitions at the same or different eccentricities for each subject (Fig. 2).

For the case of photoreceptor imaging in normals and colour-blinds, a significant reduction in signal-producing elements (photoreceptors, in this case) may help to explain why some presentations better contrasted structure from noise than others. Despite this, averaging of several (200) cross-sections (Fig. 2(c), (d)) suggested that the majority of signals evident in en face (Fig. 2(a)) and transverse tomograms (Fig. 2(b), (e)) were attributed to real structure. Furthermore, this demonstrated that a significant reduction in back-scattered signal was obtained from the photoreceptor layer in two colour-blind individuals, supporting the former model of dichromatic colour vision. Although other imaging techniques [49,51] can detect such cone loss, it is only possible to confirm that there has been a complete degeneration of the cones with the high-resolution depth profiling capability of OCT.

A general thickening of the retina was observed in the colour-blind retina relative to that of the normal retina. The thickness of the IS, OS and RPE layers were defined by the centre of the bright bands associated with the ELM and IS/OS junction, and the bottom of the bright band associated with the RPE (Fig. 2(k)). At 5.8° eccentricity (Fig. 2(b)–(e)), the thickness of the IS, OS and RPE layers were 25%, 71% and 15% greater in the colour-blind relative to the normal retina. This observed thickening of retinal layers in the colour-blind retina possibly resulted in part from the phagocytosis of non-functional cones but further studies at multiple retinal locations and with larger sample sizes are needed to determine how these differences relate to normal variations with the human population.

The capability of selectively focussing the probing beam onto the nerve fibre layer improved visualization of the nerve fibre bundles and underlying retinal structures. This was previously demonstrated by Zawadzki et al. [25] using AO-OCT. A means to track changes in the structure of retinal ganglion cells would offer a useful diagnostic tool for early detection of glaucoma. But due to their dense packing and low-scattering, it is difficult to distinguish ganglion cells without invasive contrast-enhancing agents. Cordeiro et al. [61] used a prototype Zeiss confocal SLO to image ganglion cells in rats and anesthetized macaque monkeys in vivo after intravitreal injection of Annexin 5-bound fluorophore. Gray
et al. [62] also imaged ganglion cells in the macaque monkey in vivo using SLO and AO-SLO with Rhodamine or Alexa fluorescent dyes.

To improve signal-to-noise, 1D scanning patterns were employed. This technique has a distinct advantage from averaging of tomograms obtained with 2D scanning patterns since for the former, the averaging occurs over the same section of sample (except for translations due to eye motion), thus enhancing the visibility of structures pierced by the sectioning plane. This is in contrast to averaging across a series of adjacent planes, which will blur out details if the average is performed over a layer thickness that exceeds the dimensions of the structures of interest. If eye motions are sufficiently high, this will lead to some spatial averaging of speckle noise (after applying image registration to compensate for any residual motion) while also averaging out detector noise. Although larger sample sizes are needed to verify this result, circular bodies approximately 10–15 μm in size have been observed near the fovea in a normal individual. Averaging across 30 repeated tomograms acquired with only fast-axis scanning significantly reduced speckle noise and improved image contrast. Since no further processing was performed, the structures, with sizes consistent with that of ganglion cells [54], cannot be attributed to post-processing artefacts.

In vivo visualization of RPE cells may help to identify and detect early indicators of diseases associated with their function and to track progression. The RPE nuclei are surrounded by highly scattering organelles (Fig. 4(i)–(j)) including melanosomes (contained within microvilli that cover phagocytosed OS fragments), mitochondria and lysosomes (Fig. 4(b)). In OCT, these dense, highly scattering structures give rise to bright intensity signals that surround a structured arrangement of dark circular bodies that correspond to the cell nuclei. While it had previously only been possible to image RPE cells in vivo using autofluorescence imaging [61,62], or in a patient with cone loss [63], RPE cell structure has been probed using OCT for the first time in a subject with a normal and complete photoreceptor mosaic without autofluorescence. The RPE cell spacing was estimated from a manual cell count and this was in agreement with the spacing inferred from the power spectrum of the RPE cell mosaic. And the observed cell density and spacing was consistent with estimates in primates [61,62] and humans [62,63] using other imaging systems as well as histology in humans [54,55]. Moreover, the combination of an ultra-high imaging speed and ultra-high resolution allowed for selectivity in depth sectioning to reveal not only the RPE cell mosaic, but also layers containing structures consistent with the RPE cell soma (Fig. 4(d)–(e)) and choriocapillaris (Fig. 4(k)–(l)), demonstrating the potential of this imaging modality when compared to other technologies with limited depth resolution.

Non-invasive imaging of the lamina cribrosa is of interest since it has been suggested that larger pores and thinning of the connective tissue support might implicate nerve fibre damage due to glaucoma [64]. In vivo imaging of the lamina cribrosa was performed by Vilupuru et al. [65] using AO-SLO in anesthetized normal and glaucomatous Rhesus monkeys, and in humans by Srinivasan et al. [66] and Potsaid et al. [37] using OCT, and by Zawadzki et al. [29] using AO-OCT. AO-SLO presents a limited depth of field and without AO, uncompensated ocular aberrations severely impair lateral resolution in OCT. For the first two cases, the axial resolution was restricted (~100 μm and 8 μm, respectively), due to spectral bandwidth limitations. In Zawadzki et al. [29], the axial resolution was limited to 6 μm due to uncompensated LCA, and to avoid instability in the AO correction (the AO was applied and fixed to a different retinal location before the subject’s eccentricity was adjusted for imaging of the optic nerve head). We have demonstrated that with a high axial resolution offered by an ultra-broadband light source and LCA compensation, and a high transverse resolution offered by stable AO correction is it possible to visualize collagen fibre bundles in the human lamina cribrosa in vivo (Fig. 5).
The main limitations inherent in the imaging technique demonstrated here include residual motion artefacts due to eye movements, accuracy of image registration and signal-to-noise, including the deleterious effects of speckle noise. The impact of the first two limitations may be reduced by a further increase in the acquisition speed, but not without posing a further compromise on the third limitation. The signal-to-noise primarily depends on how efficiently the optical system collects and passes the light back-scattered off the retina to the spectrometer (via an optical fibre coupling, in this case), which is primarily affected by optical aberrations. But even a signal free of aberrations will contain OCT signals composed of both signal-carrying and signal-degrading speckle. A great challenge for OCT imaging is in discerning one speckle type from the other.

Although the light incident on the eye is strictly limited to prevent retinal damage [67], there is possible room for improved image resolution through a better AO correction. Studies [68] have observed dynamics in ocular aberrations as high as 30 Hz, and thus, enhancements in image resolution could be had by increasing the AO closed-loop speed. Nevertheless, when compared to the system’s predecessor [27], the implementation of AO in this work was considerably improved since the closed-loop AO correction was performed in real-time, and thus, the geometric aberrations were compensated dynamically throughout the entire duration of image acquisition. Although the subjects’ heads were constrained and accommodation was paralyzed in that earlier work, it would be expected that any microfluctuations in accommodation, tear film changes, or line-of-sight alterations that may have occurred within not only the time elapsed for image acquisition but also between the “freezing” of the AO loop and the commencing of data acquisition would have resulted in aberration changes left uncompensated. When compared to the current system, this effect would have been exacerbated by the fact that the previous acquisition speed was 6X slower.

This paper demonstrates the successful combination of ultra-high resolution OCT with adaptive optics and longitudinal chromatic aberration compensation and an ultra-high speed image acquisition system to provide detailed morphological structure at the cellular level in normal and pathologic retinas. A high axial resolution offered by an ultra-broadband light source and correction of geometrical and longitudinal chromatic aberrations had been shown [26,27,29,30] to provide a means of probing cellular structure non-invasively in the living human retina. But only with an ultra-fast image acquisition speed (120,000 depth scans/s) is it possible to overcome deleterious eye motion artefacts to expose weakly-scattering cellular structures in the in vivo human retina such as ganglion cells and RPE cells. Finally volumetric phenotyping of colour-blinds at the cellular level was accomplished, illustrating the potential of this imaging modality to provide detailed insight of the living retinal micromorphology, which should help to contribute to a better understanding of pathogenesis as well as to improve early diagnosis and therapy monitoring of a variety of retinal diseases. Although it has been shown through preliminary data that the system performance has been markedly improved from its predecessor [27], further studies are needed to better demonstrate the capabilities of this imaging modality.

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Fig. 1. Optical set-up. AL: achromatizing lens, BS: beam-splitter, DG: diffraction grating, DM: deformable mirror, L: lens, PM: plane mirror, PP: polarization control paddles, RR: retro-reflector, SM: spherical mirror, WFS: wavefront sensor.
Fig. 2.
Cellular phenotyping in a normal and red/green colour-blind subject at 2.9° and 5.8° temporal. 

- **a–h:** En face images at the I/OS and OS tips after averaging of 15–22 slices filtered with BPF2–500 (a–b, e–f) or BPF3–500 (c–d, g–h). Photoreceptor densities per mm² are indicated at the top-right corner of each panel.

- **i–m:** Comparison of photoreceptor distributions in a normal (i–j) and colour-blind (l–m) subject at 5.8° temporal. Tomograms obtained by averaging 5 cross-sections filtered with ATF are shown in i and m, while in j and l, portions of integrations obtained from 200 fast-scan-axis tomograms filtered with AFT are shown to emphasize transversal cell densities (stack of 424 × 240 μm² cross-sectional images filtered with ATF from a normal and colour-blind subject at 5.8° temporal, presented in Media 1 and Media 2).

- **k:** Layer thicknesses for the IS, OS and RPE are depicted in k. 

- **n:** 2D power spectrum of the cone mosaic shown in c. ELM: external limiting membrane, IS: inner segments, OS: outer segments, RPE: retinal pigment epithelium, CC: choriocapillaris, BM: Bruch’s membrane. Scale bars: 50 μm.
Fig. 3.
Visualization of foveal stratification centred at 1°. a: Depth-dependent colour-coded composition of a grayscale image of retinal nerve fibre bundles (integration of 22 en face sections filtered with BPF2–500) and a red pseudo-coloured image of blood vessels (integration of 60 en face sections and filtered with BPF2–500). The white cross denotes the foveal centre and black graduations denote intervals of 1°. b: Average of 30 slow-scan-axis tomograms filtered with AFT within the blue region in a, piercing through a microvessel. Locations of intersecting vessels are denoted by blue arrows. NFL: Nerve fibre layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer. c: Average of 3 slow-axis-scan tomograms filtered with ATF within the yellow region in a, possibly showing a microvessel extending from the GCL (top yellow arrow) to the IPL, where it appears to branch out near the IPL/INL boundary (bottom yellow arrow). d: Average of 30 fast-axis-scan tomograms filtered with ATF and without slow-axis scanning centred at ~1° temporal exposing a zone of dark circular shaped structures likely corresponding to ganglion cell bodies, highlighted in e (magnification of d) by red arrows. Scale bars: 100 μm for a, b, d; 20 μm for c, e.
Fig. 4. Visualization of foveal retinal pigment epithelium (RPE) cells and choriocapillaris.  

- **a**: 3D rendered OCT volume of a 28 year old normal male caucasian retina at the fovea after filtering with ATF.  
- **b**: Schematic of RPE cell. OS: outer segments, MV: microvilli, SO: inner portion of RPE soma, NU: nuclear (basal) portion of RPE soma, BM: Bruch’s membrane, CC: choriocapillaris. Also indicated are the levels corresponding to panels c–d, f–g and j–k.  
- **c**: 2D power spectrum of the RPE cell mosaic obtained by averaging of 15 en face slices followed by filtering of the resulting power spectrum with BPF4–500.  
- **d**: Sectioned image at the level of the RPE soma (average of 15 en face sections filtered with ATF). At this depth, signal-producing elements are mainly melanin granules inferior to the inner portion of the RPE cell, also magnified in e.  
- **f**: Histological section from a normal human fovea (selected portion of Fig. 1(a) from Harman et al. [55]) for comparison.  
- **g**: Basal RPE exposing structure of the RPE cell mosaic at the level of the cell nuclei (average of 14 en face sections the filtered with BPF4–500).  
- **h**: Enlarged portion of g with yellow circles enclosing 7 hexagonally arranged clusters of RPE cells.  
- **i–j**: Light micrographs of tangentially sectioned tissue showing an en face view of human RPE cells from an 18 and 42 year old caucasian male, respectively (Fig. 3(i)–(j) reproduced from Gao et al. [56]).  
- **k**: Sectioned image at the level of the choriocapillaris (averaging of 5 en face sections filtered with ATF). The emerging, structure possibly corresponds to microvessels.  
- **l**: Inverted and enlarged selected portion of k.  
- **m**: Histology of a human choriocapillaris in alkaline phosphatase preparation (selected portion of Fig. 1(a) from McLeod et al. [57]).  

Scale bars: 100 μm for a, d, g, k; 20 μm for e–f, h–j, l–m, 10μm for b. White cross-hairs in d, g and k denote the foveal centre.
Fig. 5.
Pores and collagenous fibres of the lamina cribrosa (LC). 

- **a**: 3D rendered volume of the LC (filtered with ATF).
- **b-d**: Single en face sections (filtered with ATF) denoted by yellow rectangles in **a** (Media 3 presents a $418 \times 101 \, \mu m^2$ stack).
- **e**: Lateral section of the LC (left) as seen by histology of a human LC (selected portion of Fig. 1 from Kotecha et al. [58]) for comparison with OCT (right), obtained after averaging 5 fast-scan-axis tomograms filtered with ATF (Media 4 presents a $418 \times 1234 \, \mu m^2$ stack). Collagen fibre bundles oriented orthogonal to the probing beam appear bright.
Fig. 6. Visualization of RPE cell structure with and without filtering for speckle noise reduction. 

a: A 100×100 μm² patch of RPE mosaic obtained by averaging 15 (unfiltered) en face slices.
b: Power spectrum of a. c: Obtained by averaging power spectrums from each of 15 (unfiltered) en face slices. 
d: Result of filtering the power spectrum in c with BPF4-500. e: A 100×100 μm² patch of RPE mosaic obtained by averaging 15 en face slices filtered with BPF4-500. f: Power spectrum of e. g: Obtained by averaging power spectrums from each of 15 en face slices filtered with BPF4-500. h: Result of filtering the power spectrum in g with BPF4-500. i: A 100×100 μm² patch of RPE mosaic obtained by averaging 15 en face slices filtered with ATF. j: Power spectrum of i. k: Obtained by averaging power spectrums from each of 15 en face slices filtered with ATF. l: Result of filtering of the power spectrum in k with BPF4-500.
Fig. 7.
Depth-dependent peak cross-correlation between the central most *en face* section piercing through the RPE cell nuclei and sections at other depths.
Table 1

Summary of pixel number (N) and raster scanning densities (η) along the x and y direction for the data presented in Section 3.

| Figure number | N_x [pix] | N_y [pix] | η_x [pix/°] | η_y [pix/°] |
|---------------|-----------|-----------|-------------|-------------|
| 2             | 512       | 512       | 394         | 492         |
| 3, 4a–c, 5    | 512       | 512       | 394         | 394         |
| 4d–f          | 512       | -         | 394         | -           |
| 6             | 512       | 512       | 197         | 985         |