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Imaging retinal mosaics in the living eye

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Abstract

Adaptive optics imaging of cone photoreceptors has provided unique insight into the structure and function of the human visual system and has become an important tool for both basic scientists and clinicians. Recent advances in adaptive optics retinal imaging instrumentation and methodology have allowed us to expand beyond cone imaging. Multi-wavelength and fluorescence imaging methods with adaptive optics have allowed multiple retinal cell types to be imaged simultaneously. These new methods have recently revealed rod photoreceptors, retinal pigment epithelium (RPE) cells, and the smallest retinal blood vessels. Fluorescence imaging coupled with adaptive optics has been used to examine ganglion cells in living primates. Two-photon imaging combined with adaptive optics can evaluate photoreceptor function non-invasively in the living primate retina.
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Photoreceptors

Retinal imaging with adaptive optics allows for non-invasive imaging of single cells in the living eye. All eyes suffer from optical aberrations, mainly in the cornea and lens that limit the resolution of conventional ophthalmoscopes. Adaptive optics provides a means for dynamically correcting these aberrations in real time, allowing nearly diffraction-limited resolution. Adaptive optics is implemented with two key components: (1) a wavefront sensor that measures the aberrations of the eye and (2) a wavefront corrector (most often a deformable mirror) that compensates for the aberrations. Several methods have been developed to characterize the wavefront aberration of the eye, but the most convenient is the Shack–Hartmann wavefront sensor, which was first applied to the eye by Liang *et al*[1] and later implemented in the first closed-loop adaptive optics ophthalmoscope.[2] The transverse resolution of current adaptive optics systems is $<2\,\mu$m, (see ref. 3) theoretically allowing images of the smallest cells in the retina. The first cells to be imaged in the living eye using adaptive optics technology were the cone photoreceptors, and these continue to be the most studied of all cell types using this technology. Cones function as waveguides; this fundamental property makes them both excellent cells for collecting light and also ideal target cells for imaging. This feature of cones makes them relatively easy to image, with a portion of the light that is not absorbed by the photopigment being back reflected out of the eye through the cone waveguide.[4,5]

Adaptive optics imaging of cone photoreceptors has provided insight into the structure and function of the cone mosaic in both normal and diseased eyes. An early contribution made with adaptive optics imaging was the measurement of the distribution of different cone classes in the human retina.[6] A selective bleaching technique, combined with AO imaging, revealed that there is wide variability in the L- and M-cone ratio in persons with normal color vision.[7,6] Adaptive optics imaging of persons with mutations in the genes that encode the cone opsins have provided new insights about the cone mosaic.[8–10] Genetic techniques can determine specific mutations in the cone opsin, revealing the genotype of the affected individual. Adaptive optics imaging can then evaluate the retinal phenotype and examine variation among individuals with the same genotype. These genotype-phenotype correlations can provide powerful information about how mutations in the cone opsins affect retinal development and lead to visual

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dysfunction. Surprisingly, it has been revealed that many individuals with genetic perturbations in the photopigment molecule can have excellent vision despite significant cone loss (Figure 1).[8] This illustrates the potential for cellular imaging with adaptive optics in the early detection of retinal disease as it can reveal cell loss before clinically observable symptoms appear.

Although there was some success in imaging the smallest photoreceptors, the cones at the foveal center and the rods (which are comparable in size and morphology) using flood illuminated adaptive optics ophthalmoscopy,[11,12] these cells remained unresolved in adaptive optics scanning laser ophthalmoscopy (AOSLO) until only recently (Dubra *et al*, in preparation). Foveal cones are particularly interesting to study because along with the optical quality of the eye, it is these cells that underlie visual acuity.[13–15] Rods are of great importance as they are the most abundant of photoreceptors and are often the first cells disrupted in many types of retinal disease. Clearly, having an efficient means of evaluating the structure of these cell types is of critical importance. The newest generation AOSLO instrument at Rochester was designed to minimize astigmatism, a persistent aberration that is present in many other AOSLO designs.[16] This improved optical design, combined with the latest deformable mirror technology and new image registration methods, has provided the first images of the complete photoreceptor mosaic, including foveal cones (Figure 2a) and the complete peripheral photoreceptor mosaic, including rods (Figure 2b) (Dubra *et al*, in preparation).

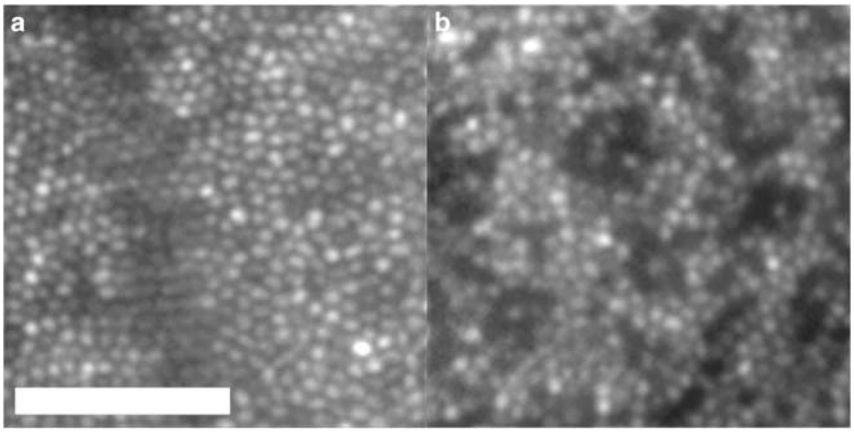

Figure 1 AO reveals cone loss in eye disease. (a) Dichromat with cone mosaic indistinguishable from a normal trichromat. (b) Dichromat with M pigment mutation showing dark regions where cones may be damaged or lost; despite the disruption in the cone mosaic this person has excellent spatial vision. Scale is identical for each panel; scale bar is 50 microns.

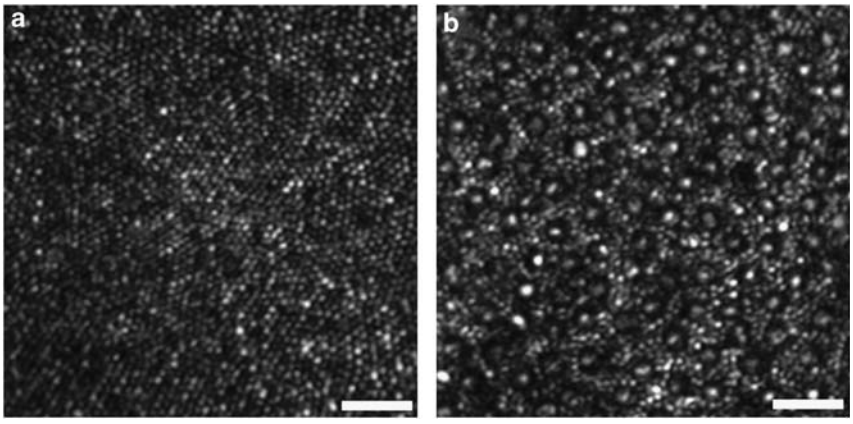

Figure 2 High-resolution images of the smallest photoreceptors obtained with the new Rochester AOSLO. (a) The complete foveal cone mosaic. (b) The complete peripheral photoreceptor mosaic showing both rods and cones, imaged at 10° temporal and 1° inferior. Scale bars are 20 microns.

Retinal pigment epithelium

Although important scientific contributions continue to be obtained through imaging of the cone photoreceptor mosaic, investigators are increasingly setting their sights on other retinal structures. Imaging the retinal pigment epithelium (RPE) mosaic, for example, capitalizes on the autofluorescent properties of the lipofuscin intrinsic to these cells. As a byproduct of phagocytosis, all RPE cells accumulate lipofuscin[17–19] that fluoresces in the red when illuminated with blue or green light. Lipofuscin is composed of many different fluorophores with a broad excitation and emission profile.[17] Autofluorescence imaging has become an important clinical tool for evaluating the health of the RPE;[20] however, current clinical instruments lack the resolution to visualize individual RPE cells. Fluorescence adaptive optics scanning laser ophthalmoscopy (FAOSLO) overcomes the resolution limitations of current clinical instruments allowing individual RPE cells to be imaged in living macaque (Figure 3a) and human eyes.[21,22] The exclusion of lipofuscin from the nuclei of RPE cells and the fact that it is sequestered in granules in the cell cytoplasm generates contrast in images of single cells.[23,22]

FAOSLO uses two different sources that can be used for simultaneous high signal-to-noise ratio (SNR) reflectance imaging with low SNR fluorescence imaging. Reflectance imaging in the infrared can be used to image photoreceptors, whereas autofluorescence imaging with visible light excitation and emission can be used for RPE mosaic imaging.[23] It has been shown that high resolution RPE autofluorescence imaging in FAOSLO can reliably and repeatedly obtain images of the complete RPE cell mosaic.[22] In normal human retina, images were obtained at the same retinal location at different time points with 100% of the cells able to be identified in both images.[22] This technique was validated through comparison of *in vivo* FAOSLO images and *ex vivo* images at the same location.[22] The exquisite resolution capabilities afforded by adaptive optics has even allowed for individual lipfuscin granules or clusters of granules to be revealed in the cytoplasm of RPE cells *in vivo* (Figure 3b). Single cell resolution RPE imaging is an extremely valuable tool not only for evaluating the organization of the RPE mosaic in healthy eyes, but also for understanding both phototoxicity and disease pathogenesis and progression in diseases in which RPE cell dysfunction is implicated, such as age-related macular degeneration.

RPE imaging at microscopic resolution in macaque found that light levels that were thought to be safe based upon published guidelines[24] resulted in changes in the RPE mosaic.[25] These studies revealed that light levels that were below the ANSI maximum permissible exposure (MPE) caused disruptions in the RPE mosaic in macaque (Figure 4).[24,25] In addition, a reduction in the autofluorescence signal was observed at light levels well below the MPE.[26] This reduction in the autofluorescence signal either fully recovered or preceded RPE cell disruption. This light-induced change in the RPE could be hazardous, perhaps resulting from lipofuscin photooxidation and the creation of reactive oxygen species. Autofluorescence reduction may be the first visible change related to the RPE disruption observed at high light levels, potentially accelerating the accumulation of toxic products in the RPE. Alternatively, autofluorescence reduction may be benign, simply resulting from the photoisomerization of lipofuscin. On the basis of these investigations we are currently undertaking a rigorous study to examine the

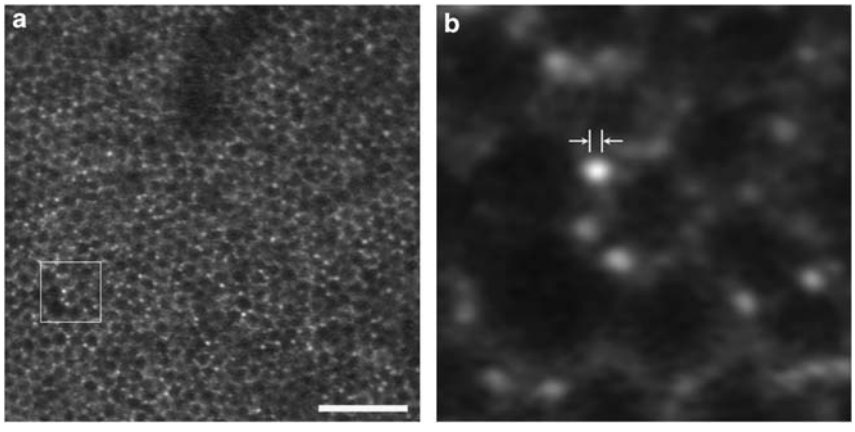

Figure 3 Retinal pigment epithelium and individual lipofuscin granules revealed in FAOSLO. (a) Individual RPE cells imaged using FAOSLO in macaque. Scale bar is 100 microns. (b) Outlined region from **a** showing individual lipofuscin granules; distance between arrowheads is 2 microns, on the order of the size expected for RPE granules.

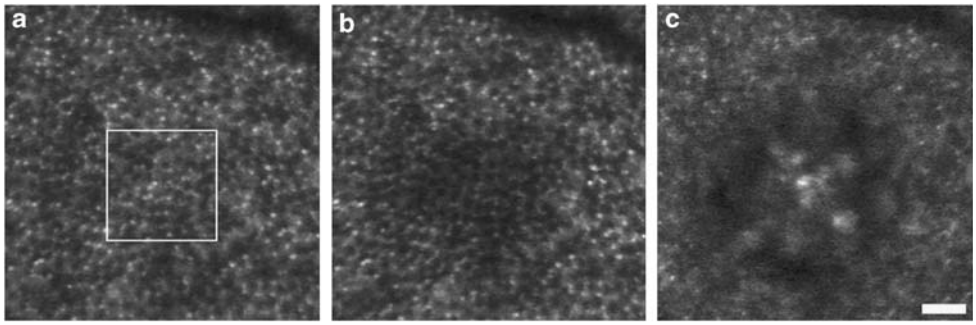

Figure 4 RPE changes caused by visible light exposure. Pre- (a), immediately post- (b), and 6-days post-exposure (c) images of the RPE cells in locations exposed by a uniform source to 150 μW of 568 nm light for 15 min over $\frac{1}{2}^{\circ}$. The white squares show the exposure locations. Scale bar is 50 microns.

phenomenon of autofluorescence reduction and RPE disruption. This work has already resulted in re-evaluation of the ANSI laser safety standards and may have direct clinical implications as eventual changes to the MPE have the potential to re-evaluate the light levels used in endoilluminators and other ophthalmic instruments.

Ganglion cells

As the photoreceptors are positioned near the outer surface of the eye, light must travel through several transparent layers of cells before reaching the photopigment molecules of the outer segment. The transparent nature of these inner cell types is a feature that is advantageous for vision but detrimental for imaging. Nerve fiber bundles are highly reflective and are readily imaged in the inner retina, and it is possible that some of the cell bodies within this layer, such as astrocytes or pericytes, may be revealed using reflectance imaging with AO (Figure 5). However, many of the most interesting cell types residing in the inner retina, such as ganglion cells have not been revealed directly. Contrast agents, often fluorescent molecules, are typically used in animal studies to reveal cell types that would otherwise be invisible. However, these techniques are often not suitable for work in humans as the methods to introduce these agents (such as viral vectors) are often too invasive. One of the more interesting targets for retinal imaging in the inner retina is the retinal ganglion cells (RGCs); these are the output cells of the retina, which carry signals directly to the brain.

There are numerous different types of ganglion cells,[27,28] the function of many of which have not yet been identified. Gray *et al* showed that individual RGC bodies, axons, and dendrites could be visualized using adaptive optics retinal imaging in living macaque eyes when a fluorescent dye was introduced into RGCs via retrograde transport after injection into the LGN (Figure 6).[21,3]

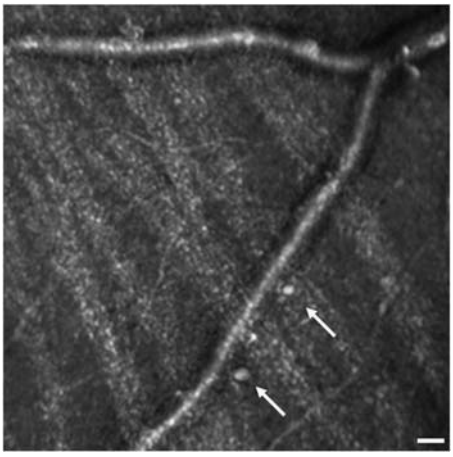

Figure 5 Cellular structures revealed in the nerve fiber layer. Nerve fiber bundles and cell bodies (indicated by the arrows). Scale bar is 20 microns.

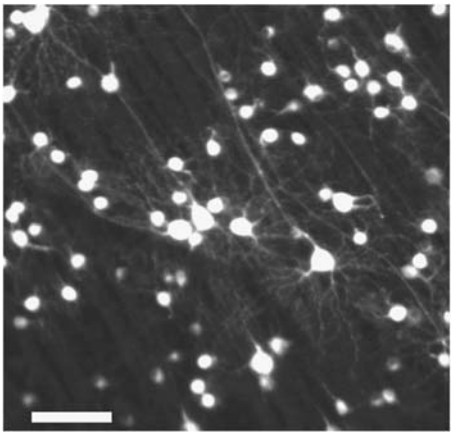

Figure 6 Rhodamine-labelled RGC cell bodies, axons, and dendrites imaged in FAOSLO. Scale bar is 100 microns.

Ganglion cell bodies, axons, and dendrites labeled with eGFP have also been visualized in living rat eyes using FAOSLO.[29] These techniques are too invasive for use in humans, but many of the studies that are particularly

relevant for understanding RGC function are perhaps best applied in animal models. The two most commonly studied types of RGCs are midget and parasol ganglion cells, and it would be quite useful to distinguish between these two cell types *in vivo*. Midget and parasol RGCs are anatomically very distinct and therefore, if these cells could be imaged they could be classified based upon their anatomical appearance alone. The ON and OFF sub-classes of these cells could further be identified by the stratification of the different cell classes in different layers of the retina.[30] Ganglion cell structure is interesting, but it is RGC function, and particularly RGC responses to visual stimuli that will provide insight into the role of the different classes of RGCs. Viral vectors can be tailored to target retinal ganglion cells with fluorescent markers, such as GFP.[31] These vectors can further be modified to introduce fluorescent indicators of cellular function (such as the calcium indicator, G-CaMP [32]). Using sophisticated stimulus delivery techniques in AOSLO, retinal ganglion cell activity could eventually be monitored optically *in vivo* as different stimuli are delivered to the retina. This method may eventually accelerate our understanding of the role that different classes of ganglion cells have in vision.

Retinal vasculature

High-resolution imaging using adaptive optics can also be used to visualize the fine structure of blood vessels that reside within the tissues of the inner retina. Fluorescein angiography, combined with adaptive optics imaging in FAOSLO has been used to obtain images of the smallest capillaries near the foveal avascular zone (Figure 7a) and of the radial peripapillary capillaries (Figure 7b) in macaque.[23,33] Recently, parafoveal capillaries were visualized with advanced video processing techniques in AOSLO.[34] These motion contrast methods use the change in reflectance caused by blood flowing through the vessels (that is, the variance in the image) to map vessel paths. Tam *et al*[34] have shown that this technique can reveal the smallest vessels in a way that is superior to conventional fluorescein angiography and with vessel resolution that is comparable to that seen using FAOSLO.[21,33] The greatest advantage of this method over fluorescein angiography is that this technique can be done non-invasively and repeatedly, in both normal and diseased eyes.[34]

Multi-photon Imaging

Extrinsic fluorophores are extremely versatile in that they can be selectively introduced into specific cell types and can be tailored to visualize either structure or function (or both). However, these methods are often inappropriate for use in humans; the exception may be fluoroscein angiography, which can be used in humans when clinically indicated, but utility of which is probably limited in AOSLO, given the advancements in extracting vasculature topography using motion contrast, as discussed above. Fortuitously, there exist multiple intrinsic fluorophores within the different cell types in the retina that can be used for retinal imaging. These include NAD(P)H, FAD, and retinol.[35] Some fluorophores, such as the components of lipofuscin, including A2-PE, can be readily imaged using conventional linear (that is, single photon) imaging methods.[21,22,25] However, many intrinsic fluorophores have emission profiles that require single-photon excitation using wavelengths of light that are shorter

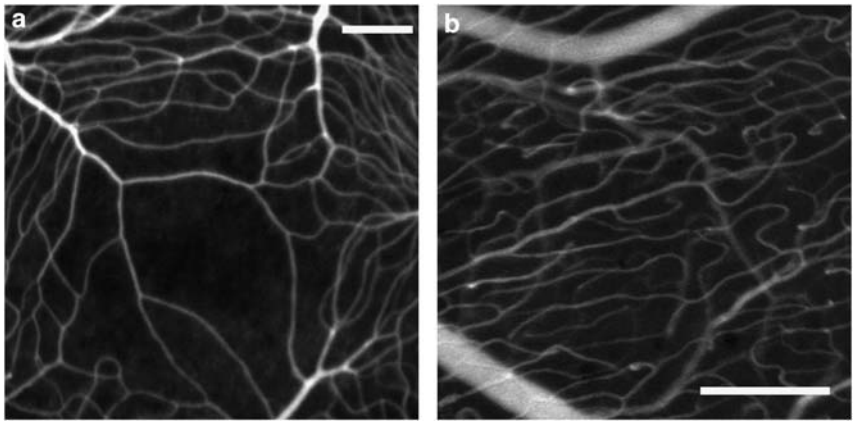

Figure 7 Fluoroscein angiography reveals the smallest perfused capillaries in FAOSLO. (a) Blood vessels around the foveal avascular zone; scale bar is 150 microns. Reproduced from ref. 21 with permission from OSA. (b) Radial peripapillary capillaries; scale bar is 200 microns.

than the ocular transmission window. The eye readily passes wavelengths greater than 400 nm and less than 900 nm, but there is a sharp cutoff for frequencies lower than 400 nm, and a lesser reduction in transmittance for wavelengths greater than 900 nm.[36] The low frequency cutoff limits the UV exposure to the retina and thus prevents retinal damage from short wavelength light; however, this also limits the spectral window for single-photon fluorescence. The solution to this is two-photon excitation.

In two-photon fluorescence excitation a wavelength of light is used for excitation that is double the wavelength that would be used for conventional single-photon excitation.[37] This allows for UV excitation *in vivo* with near infrared light. Unfortunately, the process is much less efficient than single-photon excitation. Thus, two-photon fluorescence excitation requires higher light levels and results in lower emission than single-photon fluorescence. An advantage, however, is that two photon excitation only occurs very close to the location in the tissue where the excitation beam is focused, obviating the need for a confocal aperture, and thus allowing all of the emitted light passing back through the dilated pupil to be collected. Recently, the Rochester FAOSLO was configured for two-photon imaging and obtained the first functional measurements of cone photoreceptors in living primate eyes (Figure 8).[38] This technique could potentially be used to image cone function in the living human eye, providing a non-invasive method for measuring photoreceptor health and monitoring photoreceptor dysfunction in disease. However, progress must be made towards making two-photon imaging in the living eye more efficient as the work in macaque used light levels that are $\sim$4.5 times greater than the MPE, preventing this method from being implemented in humans in its current form. Current work that is underway will improve the efficiency of multi-photon imaging to explore the feasibility of implementing this method in the living human eye.

Future directions

The potential advantages that adaptive optics can provide for imaging the retina are rapidly expanding as adaptive optics continues to be coupled with existing imaging modalities. Adaptive optics has been combined with OCT to exquisitely reveal the axial structure of the retina, revealing many features that are not visible in current clinical instruments and showing, in particular, excellent details of the inner and outer segments of cone photoreceptors in the living eye.[39] Drawing on techniques developed in microscopy, phase imaging methods have the potential to reveal invisible cells without requiring extrinsic contrast agents.[40] New adaptive optics imaging system designs have made the acquisition of data from large areas of the retina faster and easier.[41] The most important advances may well be yet to come, as advanced retinal imaging methods with adaptive optics become more clinically relevant.

High-resolution adaptive optics retinal imaging holds significant promise for the study of human disease. The ability to simultaneously image multiple retinal mosaics has the potential to determine the temporal sequence of retinal disease at a microscopic scale. The clinical relevance of adaptive optics imaging is poised to increase dramatically as improvements in system design and operation, such as the advances made by Dubra *et al* make the technology more clinically accessible. Currently, imaging methods using adaptive optics are being applied to study several diseases including macular telangiectasia (Figure 9), cone-rod dystrophy, retinitis pigmentosa, and age-related macular degeneration. The clinical utility of adaptive optics

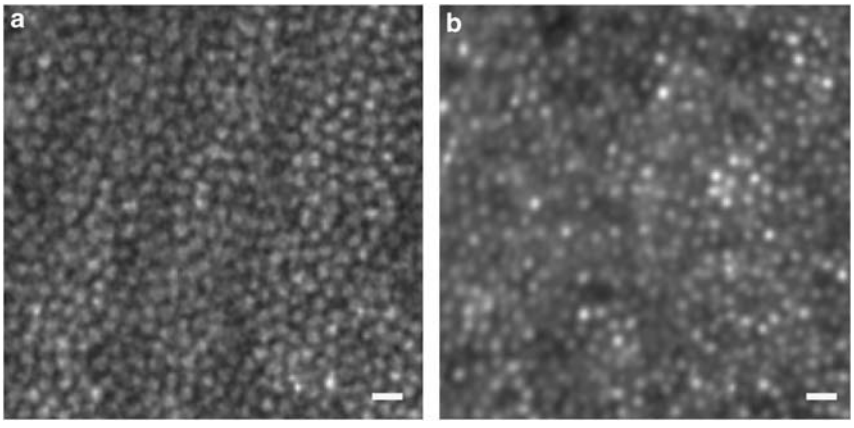

Figure 8 Functional measurements of photoreceptors using two-photon imaging with adaptive optics. (a) Two-photon image of cone inner segments. (b) Cone IR reflectance image of the same retinal location. Scale bar is 10 microns.

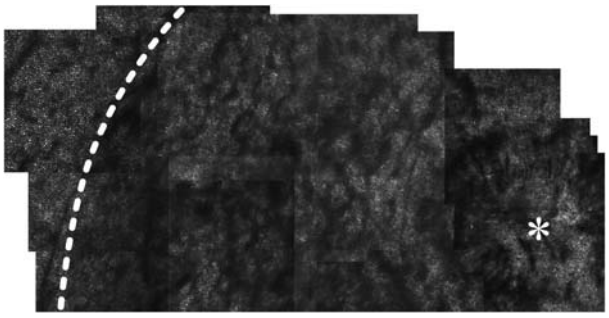

Figure 9 Adaptive optics IR reflectance image of macular telangiectasia. Dotted white line indicates a demarcation between an area of healthy appearing cones (to the left) and disrupted cones (to the right). A radial pattern of cystic changes is present surrounding the foveal center (asterisk). Scale bar is ~300 microns.

imaging to: (1) reveal retinal changes in disease that cannot be observed through any other method and (2) monitor the efficacy of clinical interventions, is perhaps best illustrated through the recent work of Duncan and colleagues at UCSF and Berkeley.[41] They monitored the cone mosaic using AOSLO in three retinitis pigmentosa patients undergoing an experimental treatment with sustained-release of ciliary neurotrophic factor over 24 months and demonstrated that significant differences in cone spacing could be measured as the disease progressed differentially in the treated eye and in the fellow control eye.[42] The broadening range of cell classes that can be monitored in disease using advanced retinal imaging techniques with adaptive optics further enhances the potential for this technology to provide unique and unprecedented insight into disease pathogenesis and progression and for monitoring novel treatment strategies.

Conflict of interest

David Williams and Alfredo Dubra are inventors on patents held by the University of Rochester in the field of ophthalmoscopy and high-resolution retinal imaging with adaptive optics.

Acknowledgements

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