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Visual pigment concentration and photoreceptor outer segment length in the human retina

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Abstract

Purpose: The Beer–Lambert law suggests that visual pigment optical density (OD) should be linearly related to the length of photoreceptor outer segments (POSs). Mammalian studies indicate that visual pigment concentration increases with POS length, but the nature of this relationship may vary due to factors such as visual pigment packing density or retinal eccentricity, and may not necessarily be linearly related. The purpose of this study was to establish the relationship between OD and POS length in humans.

Methods: Spectral domain optical coherence tomography (OCT) was used to image POS, and imaging retinal densitometry (IRD) was used to measure OD at corresponding locations in 19 healthy participants (age range 25–82 years). POS length and OD measurements were extracted from OCT and IRD images at 23 discrete locations spanning the central 9° of the retina. The averaged data from all participants were fitted with models based on the Beer–Lambert law to establish the relationship between OD and POS length.

Results: Visual pigment OD increased monotonically with POS length, but the relationship was non-linear, and a straight-line fit, based on a simple interpretation of the Beer–Lambert law, provided a poor description. A model allowing for different rod and cone visual pigment concentrations provided a superior fit. Specifically, the data were well described by a model where the molar concentration of visual pigment in cones and rods were 3.8×10^{-3} mol/L and 1.8×10^{-3}mol/L, respectively.

Conclusions: In accordance with the Beer–Lambert law, the results indicate that OD increases monotonically with POS length in humans, but the precise relationship is dependent on photoreceptor type. These results suggest that visual pigment concentration in rods is only about 48% of that found in cones. This may be due to the ubiquitous nature of artificial light that works to reduce the concentration of rhodopsin in rod photoreceptors.

KEYWORDS
densitometry, optical density, photoreceptor, physiology, retina, visual pigment

INTRODUCTION

The aim of this study was to determine the relationship between visual pigment optical density (OD) and photoreceptor outer segment (POS) length in humans using imaging retinal densitometry (IRD).[1] Mammalian studies have shown an increase in OD with increasing POS length,[2] but factors other than POS length play a role in determining the precise relationship. These include opsin packing density in the photoreceptor membranes,[3,4] photoreceptor packing density,[5] the molar extinction coefficient of rhodopsin and iodopsin[6] and retinal eccentricity.[7]

Several studies[2,4,5,8] have investigated the relationship between visual pigment concentration and POS length in mammals. Battelle and LaVail[4] studied the relationship between rod outer segment (ROS) length and rhodopsin

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content after the manipulation of light exposure history in albino rats, finding OD increased with ROS length, but the relationship was not linear. More specifically, the relationship between ROS length and OD was strongly dependent on light exposure history. Animals raised in the dark had 25% greater ROS lengths than animals raised in the light, but their rhodopsin levels were 50% higher. Penn and Williams[8] obtained a similar pattern of results with rods observed to be 78% longer in rats raised in low luminance conditions, coupled with a fourfold increase in rhodopsin concentration as determined using a spectrophotometer.

The breakdown of the direct relationship between POS length and OD suggested that visual pigment packing density on POS membranes can vary as a function of light exposure history, at least in rodents. This notion was confirmed by Organisciak and Noell[3] who found an increase in the concentration of opsin in ROS membranes in rats reared in the dark in comparison with rats raised in cyclic light conditions. A similar conclusion was made by Penn and Anderson,[5] who showed that three factors affected the dark-adapted rhodopsin content of POS. Specifically, for animals living in the light, ROS length was shortened, the number of rod photoreceptors was reduced and the packing density of rhodopsin in the ROS membrane was decreased.

To the authors' knowledge, there have been no previous attempts to measure human POS length and OD within the same participants. While a study has investigated the relationship between pigment density and cone packing density in the human fovea using interferometry and retinal densitometry, POS length was not measured.[7]

The purpose of this study was to establish the relationship between OD and POS length across the central retina in human participants. As a starting point, and in accordance with the Beer–Lambert Law,[9] we hypothesised that there would be a simple linear relationship between OD and POS length. Despite the declining cone-to-rod ratio with increasing eccentricity, a simple straight-line fit should describe the data providing the difference in the molar concentration and molar extinction coefficients between rods and cones is negligible. There is, however, one additional complication. Specifically, the outer segment layer of the retina contains the outer segment discs and an extracellular space known as the interphotoreceptor matrix (IPM), which is void of outer segment discs.[10] Given that visual pigment resides in POS, only that fraction of the retinal area occupied by the receptors will contribute to the measured OD. Nonetheless, provided the fraction of retinal area occupied by the IPM for rods and cones is similar, a linear relationship between OD and POS should still apply.

Key points

- Visual pigment optical density increases monotonically with photoreceptor outer segment length in humans, but the relationship is non-linear.
- This complex relationship is explained by differences in the spatial distribution and concentration of visual pigment in rod and cone photoreceptors.
- We estimate the molar concentration of visual pigment in rods (1.8×10^{-3} mol/L) is approximately 48% that of cones (3.8×10^{-3} mol/L).

METHODS

Study design and sample

Nineteen healthy participants between 25 and 82 years of age were included in this cross-sectional study. To be eligible, participants were required to have visual acuity (VA) better than 0.20 logMAR in the test eye, a normal retinal appearance, no history of medication likely to affect retinal function and no significant media opacity (LOCS III[11] grade ≤ 2). Individuals with a known allergy to tropicamide or phenylephrine, narrow iridocorneal angles (Van Herrick[12] grade 0–1), neurological disorders affecting the understanding of the test, fixation instability, known colour vision defect (which can affect the OD measured[13]) and refractive error outside the range of ±8D were excluded. Ethical approval was obtained from the Cardiff University School of Optometry and Vision Sciences, Research Ethics Audit Committee and the study adhered to the tenets of the Declaration of Helsinki.

POS length and OD assessment

This study used IRD[1] to measure OD topographically across the central retina and OCT to determine POS length. When considering POS length, the first step was to identify appropriate boundaries in OCT images. In accordance with the Spaide and Curcio[14] review of the anatomical correlates of the bands seen in OCT, we have assumed that the distance between the anterior border of band 4 (the RPE) to the posterior border of band 2 (the ellipsoid zone) defines POS length (see Figure 1). The fovea was located by visually identifying the horizontal B-scan with the lowest point of the foveal depression. Having identified the B-scan that best captured the anatomy of the fovea, this image, together with the two adjacent B-scans, were averaged to improve the visibility of the separate layers of the retina before the measurement of POS length.

To extract POS length data to correspond spatially with IRD data, which have a lower image resolution, an overlay was applied to the averaged OCT foveal B-scan to identify an 11-pixel wide area between measurement points. Twenty-three measurements were obtained at 11-pixel intervals, at the fovea and then 11 points extending both nasally and temporally, covering a total retinal area of 9°. This area was selected because the OCT image resolution at greater retinal eccentricities was too poor to distinguish

reliably the separate layers which would have led to inaccurate estimates of POS length.

The measured pixel values (e.g., OS length) were then converted to μm by multiplying by 1.96, the axial conversion factor for pixels to μm for the OCT system. A limitation of using OCT scans to obtain POS length measurements is that separate photoreceptor types (i.e., rods and cones) cannot be differentiated.

A detailed description of the IRD, workflow and associated calculations is provided elsewhere.[1] In brief, the device measures retinal reflectance at nine wavelengths (from 475 to 725 nm), before and after exposure to a 'white' light source that bleaches >95% of rod and cone visual pigment. Reflectance measurements are corrected for instrument noise and back scatter before calculating the density difference at each wavelength. Rod- and cone-specific OD data are determined by fitting the known spectral absorbance profile for each receptor type to the wavelength-dependent density difference data. The estimate of cone OD is based on the combined spectral absorbance profile of long and medium wavelength-sensitive cones and is unique for each participant. The IRD device determines this absorbance profile at the fovea by fitting the known spectral absorbance profiles of long and medium wavelength-sensitive cones to the density difference observed at this location. Our cone OD measurements assume that this combined cone spectral absorbance profile is consistent across the macula and ignores any contribution from short-wavelength-sensitive cones, which account for only about 0.7% of all photoreceptors in the macular region.[15] Among other metrics, the device outputs topographical heat maps describing rod and cone OD across the macular region. In this analysis, because POS length measurements could not differentiate receptor type (a limitation of using OCT), the rod and cone OD data were combined to produce heat maps describing total OD (see Figure 2). These circular plots (45 × 45 pixels) provided OD data out to 10° from the fovea. The pixel with the highest cone OD value was taken as the location of the fovea.

The fovea was used as a common reference point to align the IRD and OCT images. IRD measurements were

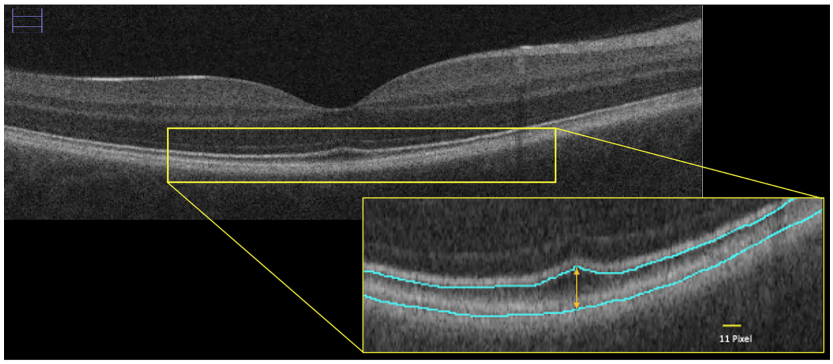

FIGURE 1 Part of an optical coherence tomography (OCT) image for participant PQM027 highlighting the photoreceptor outer segment region of interest. The double-headed yellow arrow locates the foveal centre, identified as the highest peak in the inner segment/outer segment junction layer. The horizontal bar at the bottom right indicates an 11-pixel scale bar which was the spacing used between measurements (0.4° of visual angle). The blue lines indicate the layers used to measure the photoreceptor outer segment length.

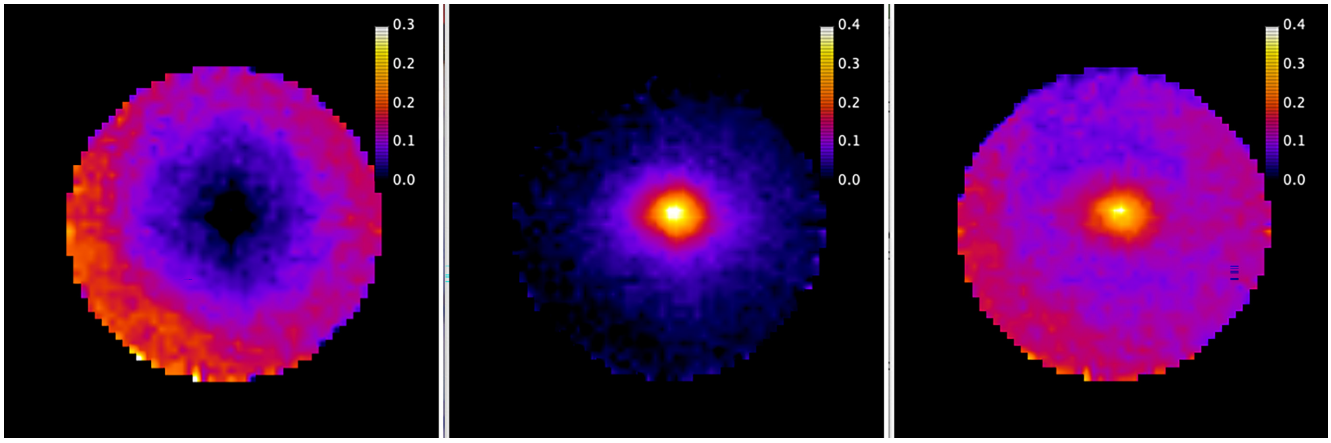

FIGURE 2 Optical density plots for rod data (left), cone data (centre) and combined rod and cone data (right) for participant PQM027. The units of optical density can be seen in the scale bars displayed at the top left of each plot, where higher optical density is indicated by lighter colours and lower optical density by darker colours.

taken at locations corresponding to the POS length measurements, that is, 11 measurements either side of the foveal measurement.

Estimates for the cross-sectional area occupied by outer segments

To determine the fraction of the outer segment layer that was occupied by photoreceptor discs, rather than IPM, we evaluated images from the literature using ImageJ. For foveal cones, we used the human flat mount epifluorescence imagery produced by Hollyfield et al.,[16] who stained for IPM and determined that 37% of the area was occupied by cones, with the remaining 63% being IPM. This estimate was corroborated by an analysis of the adaptive optics imagery of foveal cone outer segments by Zhang et al.,[17] who indicated that cone discs account for about 36.7% of the area at this retinal depth. Data on IPM surrounding parafoveal human rods are more limited, and so to calculate the area occupied by rod discs, we assumed the diameter of a ROS to be 2 μm and the spacing between adjacent rods to be 2.65 μm about 4° from the fovea.[18] Thus, for rods in the parafovea, approximately 45% of the available area is occupied by rod discs. To test our original hypothesis, that there would be a simple linear relationship between POS length and OD, at the outset we chose to ignore this difference and assumed that the photoreceptors on average covered approximately 41% of the retinal area.

Study procedures

After obtaining informed consent, all participants underwent a detailed medical history to establish ocular history, medication, general health and recent light exposure history. Autorefraction, best-corrected distance vision (using the participants' spectacles), axial length, intraocular pressure and Van Herrick estimates of the anterior angle were also obtained. Prior to imaging, pupils were dilated using one drop of 1% tropicamide and one drop of 2.5% phenylephrine. Participants were dark adapted for a period of 30 min before measuring visual pigment OD using IRD. Subsequently, macular OCT scans for a corresponding area (out to 10° from the fovea), at a resolution of 512 × 128 pixels, were obtained using a Cirrus Carl Zeiss Meditec AG 07740 SD-OCT (zeiss.com).

Analysis

The OD and POS length measurements for all 19 participants were averaged at each retinal location, and plots of OD as a function of POS length were established. In accordance with a simple interpretation of the Beer–Lambert law,[9] we expected to summarise the data with the straight-line equation:

$$\mathrm{OD}_e = ECL_eA, \tag{1}$$

where OD_e is the optical density at each eccentricity, E is the molar extinction coefficient, C is the molar concentration, L_e is the POS length at each eccentricity and A is the fractional area occupied by outer segment discs. The molar extinction coefficient of visual pigments was taken to be 40,600 cm^2/mol,[19] and the average fractional area of the measured location occupied by outer segments was assumed to be 41%. While this was our starting point, from the outset we appreciated that this simple description may not be optimal. More specifically, Equation 1 assumes that differences in the molar concentration, or molar extinction coefficients, of the visual pigments in rod and cone photoreceptors are negligible. It also fails to respect the subtle differences in the fractional area occupied by photoreceptor outer segments.

Hence, an alternative model, which respected the potential differences between rods and cones, was developed. The alternative model utilised information about the topographical distribution of rods and cones to work out the likely contribution of each receptor type at varying distances from the fovea. For example, we know that only cones are found at the fovea and at an eccentricity of 1.5°, the proportion of rods and cones is nearly equal. More specifically, we established the fraction of rods and cones at different distances from the fovea by modelling data from Curcio et al.[20] with the Van Genuchten–Gupta function[21]:

$$\mathrm{FC}_e = \frac{1}{1 + \left(\frac{E}{C_{50}}\right)^p} \tag{2}$$

where FC_e is the fraction of cones at a given eccentricity, E is the eccentricity from the fovea in degrees, C_{50} is an empirical constant and p determines the steepness of the curve. Hence, the fraction of rods (FR_e) is described by Equation (3).

$$\mathrm{FR}_e = 1 - \mathrm{FC}_e, \tag{3}$$

Having established the fraction of cones and rods as a function of eccentricity, and with the aim of quantifying the molar concentration of different pigments, the revised model became:

$$\mathrm{OD}_e = \left(\mathrm{FC}_e\, E_c\, C_c\, L_eA_c\right) + \left(\mathrm{FR}_e\, E_rC_rL_eA_r\right), \tag{4}$$

where FC_e is the fraction of cones at each eccentricity, FR_e is the fraction of rods at each eccentricity, E_c is a constant describing the molar extinction coefficient of cones, E_r is a constant describing the molar extinction coefficient of rods, C_c is the molar concentration of visual pigment in cones, C_r is the molar concentration of visual pigment in rods, A_c is the area occupied by cone outer segments and A_r is the area occupied by ROSs. Having differentiated between rods and cones, the molar extinction coefficients were revised to respect the subtle differences that have been reported.[9] Specifically, E_c and E_r were assumed to

be 47,200 cm^2/mol and 40,500 cm^2/mol,[6] respectively. The area occupied by cone and ROSs was taken to be 37% and 45%, respectively. The alternative model, despite its apparent complexity, includes just two free parameters, namely C_c and C_r.

Models were fitted to the data on a least squares basis using the Solver function in Microsoft Excel (microsoft.com). To accommodate the use of non-linear models, we chose to assess the goodness of fit by calculating the standard error of the estimate (SEE) rather than the R^2 statistic, which is only valid for linear regression. Specifically, the SEE was taken to be:

$$SEE = \sqrt{\Sigma(OD_{data} - OD_{fit})^2 / n}, \quad (5)$$

where OD_{data} is the measured OD, OD_{fit} is the OD predicted by the model and n is the number of paired data points. Helpfully, the SSE statistic describes the average error between the data and the model's prediction, with smaller values indicating a better fit.

RESULTS

Optical density and POS length data obtained at each of the locations studied are described in Figure 3. Optical density increased monotonically with increasing POS length, but the relationship was non-linear (see Figure 4a). The optical density of the longest POS, which measured approximately 45 μm and was located at the fovea, was approximately 0.3. Shorter POS were found outside the foveal region and typically had an OD of about 0.15. Fitting Equation 1 to the data produced an estimate for the molar concentration of visual pigment of 2.9×10^{-3} mol/L, and the goodness-of-fit evaluation (SEE) indicated that the average discrepancy between the data and model fit was 0.0387, that is, a typical

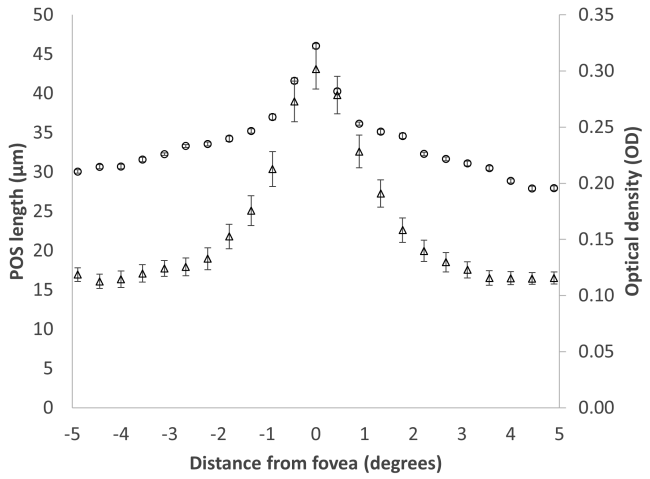

FIGURE 3 Optical density and photoreceptor outer segment length as a function of distance from the fovea. Open circles describe the average photoreceptor outer segment length and triangles, the optical density. The vertical error bars describe the magnitude of the standard error.

error of about 25%. However, a visual inspection of the fit (see Figure 4b) clearly highlights the deficiency of our original hypothesis and the limitations of Equation 1.

In contrast, the alternative model (Equation 4) that respected differences in rod and cone topography allowed for potentially different molar concentrations and differences in IPM area, producing a much more satisfying description of the data (see Figure 4b). The fit is not smooth because the model is based not only on POS length but also on the proportion of rods and cones at different eccentricities.[20] The SEE for the alternative model was 0.0104, indicating a typical error of about 7%. The fitting procedure returned molar concentration values for cones and rods of 3.8×10^{-3} mol/L and 1.8×10^{-3} mol/L, respectively. This alternative model is consistent with the established boundaries of rod and cone photoreceptors determined using OCT by Spaide and Curcio,[14] that is, the regression goes to zero.

Assuming rods and foveal cones have a POS diameter of ~2 μm[22] and ~1 μm,[23] respectively, we estimate the number of visual pigment molecules in foveal cones to be ~8.2×10,[7] and for rods ~5° from the fovea ~9.7×10.[7]

DISCUSSION

This study provides the first description of the relationship between visual pigment OD and POS length in humans. The main conclusion is that a physiologically plausible model based on the Beer–Lambert law, which accommodates differences in the area occupied by the IPM and the molar concentration of rod and cone visual pigments, readily explains the data and produces plausible estimates for the number of visual pigment molecules present in rods and cones. Our values for the molar concentration of visual pigment in human rods and cones are similar to those from other species, which are typically around 3×10^{-3}mol/L.[24] Intriguingly, the model suggests that the molar concentration of rod visual pigment is only about 48% of that observed in cones.

There are several possible explanations for the observation that the concentration of rod visual pigment is less than that of cones. The first is that the near-continuous exposure of humans to light works to reduce the concentration of rhodopsin in rods. This explanation is consistent with a body of work that shows that animals raised in the light have a significantly lower concentration of rhodopsin in their rod photoreceptors than animals raised in the dark.[3–5,8,25] This literature is largely based on studies of rodents; however, there is also some evidence showing that environmental light levels play a role in regulating human scotopic sensitivity.[26] More specifically, humans working in relatively light-adapted conditions have reduced scotopic sensitivity, as determined by the electroretinogram. This may be attributed to rods reducing their length and/or decreasing their rhodopsin concentration in more light-adapted conditions.

An alternative explanation is that the relative reduction in rhodopsin concentration is an artefact due to incomplete dark adaptation at the start of the experiment or the Stiles–Crawford effect, which works to funnel light into cone photoreceptors. Dealing with each of these in turn, incomplete dark adaptation seems an unlikely explanation because our estimate of OD is based on the ratio of dark-adapted to light-adapted retinal reflectance where the dark-adapted measurement is taken before bleaching and after 30 min of dark adaptation. Given that rod visual pigment regeneration has been shown to be complete 30 min after a full bleach, the notion that rod visual pigment regeneration was incomplete before our dark-adapted measurement seems to be unlikely.[27] The possibility that the Stiles–Crawford effect could have exaggerated our estimate of cone OD also seems unlikely because the densitometer had been designed to minimise this effect by ensuring that light entering the eye does so towards the periphery of the pupil.[28] One additional explanation is that our estimates regarding the area of the retina occupied by the IPM were erroneous. We could not measure this directly using OCT and so had to rely on an assessment of images in the literature for cones, as well as data on POS spacing and size for rods.[16–18] The cone data for IPM appear robust and consistent across studies. However, for rods, we based our estimate of the IPM area on human adaptive optics scanning laser ophthalmoscopy data on rod POS separation and the assumption that parafoveal rods have a diameter of 2 µm.[18] Parity between the molar concentration of visual pigment in cone and rod POS can only be achieved by assuming that the diameter of rods is 1.4 µm or less, which is an improbably small value.[29] Hence, it seems unlikely that our finding that the concentration of visual pigment in cones is greater than in rods is an experimental artefact. Our continuous exposure to light during waking hours remains a compelling explanation.

Participants in this investigation covered a large age range. Previous studies have shown that over the life span, the OD of cone pigments declines with age,[30] rhodopsin OD slightly increases[31] and there are no significant changes in POS length.[32] These age-related changes in visual pigment OD will likely contribute to some variability in our data as exemplified by the vertical error bars in Figure 4. However, it was unlikely to distort the shape of the underlying relationship.

One limitation of this study is that OCT B-scans were unable to distinguish between rod and cone photoreceptor types. Therefore, our description of the relationship between OD and POS length (Equation 4) relied on existing data on human photoreceptor topography[20] to tease apart their characteristics. Thus, the longest POSs were found at the fovea and were assumed to be exclusively cones, out to approximately 0.7°, and the shorter POSs were found approximately 5° from the fovea where rods were assumed to outnumber cones, approximately 10:1.[20] The histological data are well established, and we have shown that they correspond to estimates of rod and cone populations determined via densitometry.[1] Therefore, we are confident that our understanding of the fraction of cones and rods as a function of eccentricity is robust. What is more challenging, however, is the estimate of POS length, particularly for cones in the parafovea where they are known to be shorter than rods.[33] To understand how this might impact our conclusions, we considered an extreme case where cone POS length reduced from 100% of the measured length out to 0.7°, to just 50% of the measured POS length at an eccentricity of 5°. In this instance, our estimates for the molar concentration of visual pigment in cones and rods become

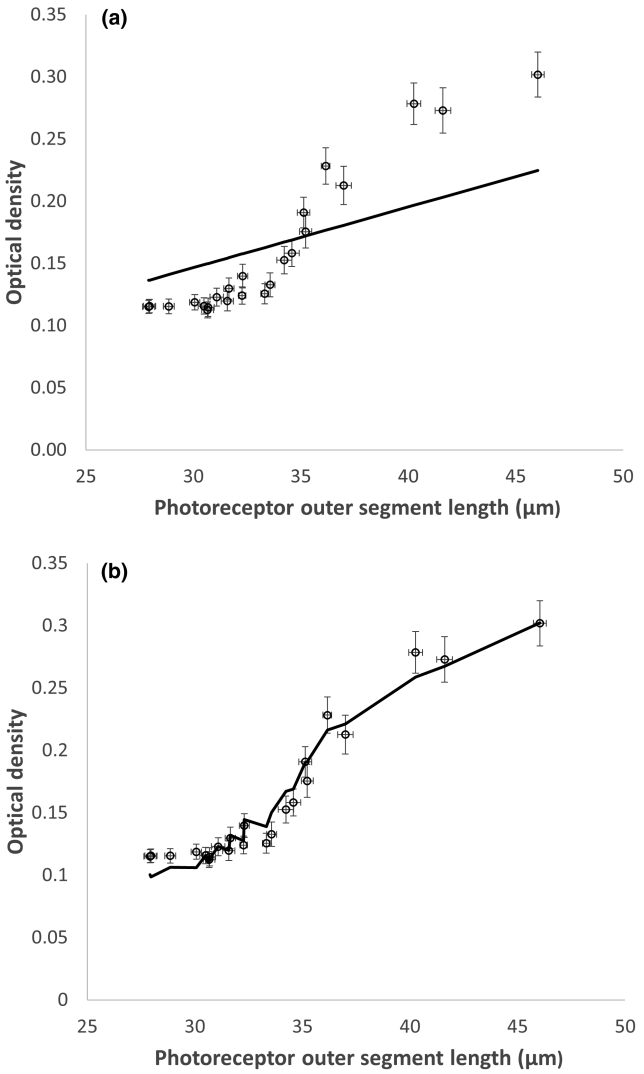

FIGURE 4 Plot of visual pigment optical density as a function of photoreceptor outer segment length in humans. As can be seen, visual pigment optical density increases monotonically with increasing photoreceptor outer segment length. Panel A exemplifies the limitations of Equation 1 producing a standard error of the estimate (SEE) of 0.0387. Panel B shows the revised model based on the known distribution of rods and cones across the central retina and allowing for different molar concentrations between receptor types. The revised model provides a good description of the data producing an SEE of 0.0116.

3.7×10^{-3} mol/L and 2.0×10^{-3} mol/L, respectively. This relatively modest difference indicates that our initial conclusion, namely that visual pigment concentration is lower in rods than cones, is robust despite the possibility that cone POS in the parafovea may be shorter than the OCT based POS length measurement might suggest.

In conclusion, we have shown that a physiologically plausible model based on the Beer–Lambert law provides a good explanation for the relationship between POS length and visual pigment optical density in humans. The apparently complex relationship is accounted for by a difference in the molar concentration of visual pigment between receptor types; specifically, by cones having a higher concentration of visual pigment than rods. We suggest the most likely explanation for this difference is that the human rods have adapted to the near-ubiquitous use of artificial light by reducing the concentration of rhodopsin in their outer segments.

AUTHOR CONTRIBUTIONS

Krishna Pattni: Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); project administration (supporting); validation (equal); visualization (equal); writing – original draft (lead); writing – review and editing (equal). **Ashley Wood:** Conceptualization (equal); funding acquisition (lead); investigation (equal); methodology (supporting); project administration (lead); supervision (equal); writing – review and editing (equal). **Nicola Cassels:** Data curation (lead); investigation (equal); methodology (supporting); project administration (supporting); writing – review and editing (supporting). **Tom Margrain:** Conceptualization (equal); formal analysis (lead); funding acquisition (supporting); investigation (equal); methodology (lead); project administration (equal); resources (lead); software (lead); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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