Imaging outer segment renewal in living human cone photoreceptors

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Abstract

In vertebrate eyes, vision begins when the photoreceptor’s outer segment absorbs photons and generates a neural signal destined for the brain. The extreme optical and metabolic demands of this process of phototransduction necessitate continual renewal of the outer segment. Outer segment renewal has been long studied in post-mortem rods using autoradiography, but has been observed neither in living photoreceptors nor directly in cones. Using adaptive optics, which permits the resolution of cones, and temporally coherent illumination, which transforms the outer segment into a “biological interferometer,” we observed cone renewal in three subjects, manifesting as elongation of the cone outer segment, with rates ranging from 93 to 113 nm/hour (2.2 to 2.7 μm/day). In one subject we observed renewal occurring over 24 hours, with small but significant changes in renewal rate over the day. We determined that this novel method is sensitive to changes in outer segment length of 139 nm, more than 20 times better than the axial resolution of ultra-high resolution optical coherence tomography, the best existing method for depth imaging of the living retina.

1. Introduction

The outer segment (OS) of the vertebrate photoreceptor is composed of a stack of phospholipid membrane discs studded with opsins, the proteins that capture photons and initiate phototransduction—the origin of vision. Throughout the day, the photoreceptor adds new discs to the proximal end of its OS. At the distal end, dozens of discs are removed periodically by cells in the underlying retinal pigment epithelium (RPE), whose microvilli ensheathe the OS. These complementary processes—disc renewal and disc shedding—are thought to offset the extreme optical [1,2] and metabolic [3–5] demands placed on photoreceptors, and have been studied extensively in post-mortem tissue samples, using autoradiographic and histological techniques since their discovery in the late 1960s [6,7]. In rod photoreceptors, the rate of renewal is determined by radiolabeling the discs, sacrificing animals at fixed intervals, and determining the locations of radiolabel in the OS [6,8]. Autoradiography does not allow renewal to be observed in cones because the continuity of their disc membranes causes rapid diffusion of radiolabel throughout the OS [9]. However,
conce disc shedding has been quantified by dissecting the eye and measuring the size of disc-filled phagosomes in the RPE [10,11].

Our proposed method of *in vivo* detection of disc renewal depends on a simple optical model of light propagation through the cone OS [12]. It is known from studies using adaptive optics optical coherence tomography (AO-OCT) [13] and other non-invasive imaging modalities [14,15] that the bulk of the reflected light exiting an individual cone photoreceptor originates from the two ends of the OS, the connecting cilium (CC) and the posterior tip (PT). This is evident in the representative AO-OCT retinal cross-section shown in Fig. 1(b). The two lines of regularly spaced bright reflections occur at the CC and PT layers, with each bright reflection corresponding to an individual cone cell. This observation supports a two-surface model describing light propagation through the OS [Fig. 1(c)] and the subsequent reflectance of the cone. Based on this model, the cone reflectance $I$ can be predicted by the well known interference equation of two wavefronts: the sum of a DC component $I_0$ representing non-interfering reflections and a cosine component representing interference of the two surface reflections. Mathematically, this is expressed as

$$I = I_0 + 2 |\Psi_1 \parallel \Psi_2| \cos \left( \frac{2\pi}{\lambda} 2nL \right)$$

(1)

where $\Psi_1$ and $\Psi_2$ are the bright reflections from CC and PT, and $L$ and $n$ are the OS's length and refractive index, and $\lambda$ is the imaging wavelength. Throughout the paper we assume $n = 1.43$, following [16]. The optical delay between $\Psi_1$ and $\Psi_2$ is given by $2nL$, since the light reflecting from PT makes a round trip before it interferes with light reflecting from CC. An underlying assumption of the equation is that the temporal coherence length of illumination $L_c$, i.e., the distance over which light will interfere with itself, must be longer than $L$. We term this condition—where $\Psi_1$ and $\Psi_2$ interfere—“long coherence”. When $L_c$ is smaller than $L$, $\Psi_1$ and $\Psi_2$ do not interfere and the cosine term vanishes. We term this condition “short coherence”.

Importantly, Eq. (1) relates the cone reflectance $I$ to the length of the cone’s OS, $L$. Specifically, it predicts that linear changes in OS length lead to sinusoidal oscillations in reflectance and that random miniscule, $< \lambda/ (2n)$, differences among the OS length of different cones cause random variations in the initial phase of these oscillations.

In accord with the model presented in Eq. (1) and Fig. 1, we predict that cones, when imaged with a long coherence source, will exhibit sinusoidal reflectance oscillations caused by the elongation of the outer segment that accompanies daily renewal, and that the phase of these oscillations will vary randomly from cone to cone. We predict that these oscillations will not appear when the cones are imaged with a short coherence source.

2. Methods

To test the predictions above, we imaged the cone mosaics of three human subjects using a research-grade camera equipped with adaptive optics, and both long- and short-coherence, near infrared illumination sources. The Indiana University Committee for the Protection of Human Subjects approved all experimental protocols.

2.1 Adaptive optics retina camera

We designed a research-grade camera for measuring disc renewal, consisting of two main subsystems: an adaptive optics (AO) system for measuring and correcting ocular
aberrations, and an imaging system for collecting images of the subject’s retina. A schematic diagram of the system is shown in Fig. 2.

The AO system is described in detail in previous publications [12,17,18]. In short, it consisted of a near-infrared light source for projecting a narrow beacon of light into the eye, a Shack-Hartmann wavefront sensor (SHWS) for measuring ocular aberrations, and a deformable mirror (DM) for correcting the ocular aberrations. We minimized refractive error of the subject’s eye using a Badal configuration, realized by axially translating the subject’s head and the lens in front of the eye using the bite bar and XYZ translation stage. The AO system ran in closed loop at 15 Hz, correcting subjects’ ocular aberrations to 0.10, 0.12, and 0.15 m root mean square (RMS) residual error (subjects 1, 2, and 3, respectively).

The imaging arm of the system consisted principally of two light sources for illuminating the subject’s retina and a high-speed, high-sensitivity electronic camera. The “long coherence” source consisted of two near-infrared laser diodes (combined output: $\lambda = 810$ nm; $\Delta \lambda = 3$ nm; $L_c = 68 \mu$m in OS, combined output; EM4, Inc.) with a maximum power of 3.0 W each, combined using a custom multimode fiber combiner coupled to 200 m of multimode fiber that scrambled the light, reducing its spatial coherence. The “short coherence” source consisted of a near-infrared SLD ($\lambda = 842$ nm; $\Delta \lambda = 22$ nm; $L_c = 10 \mu$m in OS; Superlum, Inc.) with a maximum power of 150 mW. The SLD was coupled to 25 m of multimode fiber. We connected the multimode fibers, one at a time, to a 2 m, 600 $\mu$m core multimode patch fiber. The tip of this patch fiber was magnified and imaged onto the retina for illuminating a 1.7 deg (510 $\mu$m) region. The powers of the sources were 2.0 mW and 1.8 mW, respectively, at the cornea. Both incident powers were more than a factor of ten below the ANSI limit for the 1.2 s exposures used in these experiments.

Coherence lengths of the light sources, in the outer segment, were computed using $L_c = 2(\ln 2)\lambda^2 / (n \pi \Delta \lambda)$, where $\lambda$ and $\Delta \lambda$ were provided by the source manufacturer. The illuminated retinal patch was imaged through the system onto a back-illuminated CCD (Sarnoff, Inc., CAM1M100-SFT), which operated in trigger mode and acquired images at a rate of 192 Hz.

The desired retinal location was positioned in the beam path by instructing the subject to gaze at specified points on a calibrated fixation target. The target was placed upstream of the eye and at a plane conjugate to the retina.

### 2.2 Image acquisition

Dental impression bite bars were fabricated for each subject. The bite bars were attached to an XYZ translation stage that allowed accurate alignment of the subject’s eye to the optical axis of the retina camera. Subjects were given hourly drops of Tropicamide 1% for dilatation and cycloplegia, over the course of the imaging sessions. For periods of 5 or 24 hours, images were collected and time stamped at fifteen minute (±1.5 minutes) intervals. For each 5 or 24 hour experiment, the subject fixated at the same location (1.8 deg from the foveal center—superior-temporal, superior-nasal, and superior-temporal, for subjects 1, 2, and 3, respectively). This retinal eccentricity was chosen as a compromise between the foveal center, where the cones are numerous but near the resolution limit of the system, and the parafovea, where cones are easily resolvable, but cone density is reduced tenfold and cones are often obscured by vasculature. The AO system was monitored until residual wavefront error fell below a selected threshold. At that time, the subject was asked to temporarily refrain from blinking. A pulse/delay generator (Berkeley Nucleonics Corporation) simultaneously triggered a mechanical shutter (Vincent, Assoc.; Uniblitz) in the illumination channel to open for 1.2 s, and a waveform generator (Agilent, Inc.) to send a train of 200 square 5 ms TTL pulses (192 Hz) to the external trigger input of the imaging CCD. This resulted in the acquisition of 200 retinal images in 1.04 s.
2.3 Experiment protocol

In three subjects, we illuminated a 1.7 degree patch of retina, located 1.8 degrees from the foveal center, with the long coherence laser diode and collected images every 15 minutes over five hour intervals in the afternoon or evening. To determine if oscillations are present throughout the day, and to explore variations in oscillation frequency over the course of the day, we imaged one subject every 15 minutes over 24 hours (noon – noon). To test the effect of source coherence we switched to the short coherence SLD of similar wavelength, and imaged one subject every 15 minutes over five hours. Table 1 summarizes the six trials.

2.4 Image processing and analysis

All software for image processing and analysis was developed in MATLAB® (Mathworks, Inc.) and Python/NumPy/SciPy. To correct for unwanted etaloning (an artifactual effect caused by the interference of reflections inside the thin silicon substrate of the Sarnoff CCD), images were flat-fielded. To correct for image-wide variations in retinal reflectance, such as those due to variations in source power, pupil size, or AO correction, each pixel's intensity was divided by the image's mean pixel intensity.

The 200 images, acquired within 1.04 s at each fifteen minute interval, were registered to correct translational eye motion artifacts, then averaged to improve the signal-to-noise ratio (SNR). The average images (one from each 15 minute interval) were next registered to correct translational and torsional eye motion artifacts. All temporal analysis of cone reflectance was performed using this registered set of average images. To identify cone locations a pseudo-incoherent composite image was generated by averaging the registered set. The composite image benefited from averaging over temporal oscillations in cone reflectance, such that the instantaneous variance among cone brightnesses, largely due to coherent effects, was greatly reduced. An automated cone location algorithm was used on the composite image to locate center coordinates of cones. These coordinates were determined to have total error rates less than 3%, including false positives, misses, and position errors, which were found and corrected manually by visual inspection. Flat-fielding and registration procedures are described in detail in a previous publication [12].

We quantified the oscillations in terms of their contrast, frequency, variation in frequency, and phase. To start, we determined the locations of all cones and assessed cone reflectance as the mean pixel value over a circular region of the image whose radius was chosen based on the observed spacing between cones in the image, which agreed closely with spacing values reported in the literature [19].

Next, to analyze all the cones in each trial, we computed mean power spectra. We computed the power spectrum of each cone reflectance series by subtracting the mean reflectance from the series (resulting in a zero-mean reflectance series), padding with zeros to generate a vector of 1024 points, computing the Fourier Transform of the vector, and then squaring the resulting magnitude. The power spectra from all cones were averaged to generate the mean power spectrum.

We fit a four-parameter cosine expression, \( F = A + B \cos(2\pi C + D) \), analytically, to the cone reflectance series. We computed the fit \( F \) by setting \( A \) to the mean cone reflectance, \( B \) to \( \sqrt{2\sigma_r} \) (where \( \sigma_r \) was the standard deviation of the measured reflectance series), \( C \) to the peak frequency in the power spectrum, and \( D \) to the phase component of the Fourier transform at the peak temporal frequency. As the fitting was performed analytically, no optimization or error minimization procedures were necessary. The cosine fits were used to estimate the system's sensitivity to changes in OS length, as follows. The cosine fit of each reflectance series was subtracted from the measured reflectance to give a residual error \( \varepsilon \),

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with standard deviation $\sigma_e$. The amplitude of the measured oscillation was estimated using
\[ \sqrt{2\sigma_e} \] as above. Thus the minimal detectable reflectance change is
\[ R_{\delta} = \sigma_e / \left( \sqrt{2} \sigma_e \right). \]
Considering that the full amplitude of oscillation occurs when the outer segment's length
changes by $\lambda/2$, and the round trip effects of OS length and refractive index, the smallest
detectable length change is estimated by
\[ R_{0\delta} \lambda / (4n). \]

In order to measure changes in oscillation rate over the course of the day, we computed
variations in frequency over the 24-hour data set. This was realized by multiplying the 24-
hour reflectance series of each cone by Gaussian windows ($\sigma = 1$ hr) centered about each
hour from 2pm (two hours after the experiment began) to 10 am (two hours before the
experiment's end). The power spectrum of each windowed reflectance series was computed,
and the peak frequency located. We averaged the peak frequencies of all cones, at each time,
and computed the standard error of the mean. These values resulted in the data points and
error bars plotted in Fig. 6. No additional statistical tests were employed.

3. Results

3.1 Cone reflectance oscillates with a period of 2.5 to 3 hours

Images acquired during the five hour experiments showed clear oscillations in reflectivity of
individual cones. A representative example is given in Fig. 3, showing cone images at 0.75
hr intervals for one subject. A corresponding time-lapsed video of all the images of the cone
mosaic over five hours is given in Fig. 4 (Media 1). Similar oscillations in reflectivity were
observed in all trials on all subjects when we used the long coherence source. The period of
oscillations ranged from 2.5 to 3 hours.

Oscillations in reflectance are clearly visible in Fig. 5(a), which shows temporal changes in
reflectance of eight sample cones from trial 4. The 2.5 to 3 hour sinusoidal periodicity is
evident, as are differences in phase. Shown at the bottom Fig. 5(a) is the average reflectance
of all 1626 cones from the trial (diamonds). No oscillations are visible in this average. The
washout of oscillations in the average confirms that the oscillation phase is randomly
distributed, supporting the interferometric model shown in Fig. 1(c) and Eq. (1).

3.2 Cone reflectance temporal power spectrum peaks between 0.3 and 0.4 cyc/hr

The power spectra of the eight sample cones are shown in Fig. 5(b). The power spectra of
most cones peaked at a frequency between 0.3 cyc/hr and 0.4 cyc/hr in all long coherence
trials. Because the power spectrum is phase insensitive, we were able to average the spectra
of all cones for a given trial and determine the overall peak frequency. The average power
spectrum for all cones for trial 4 is shown in Fig. 5(b) (dark line), revealing a frequency peak
at 0.37 cyc/hr. Similar peak frequencies were found in all trials using the long coherence
source; these are shown in Table 1. In short, the thousands of cones that compose a given
retinal patch were found to share common frequencies of oscillation. In striking contrast,
when the short coherence source was used, no peak was evident in either the spectra of
individual cones or their average. A long temporal coherence length is clearly a prerequisite
for oscillations and further supports our interferometric model of the OS.

3.3 Oscillations present over 24 hours, with significant differences in frequency with time
of day

In the 24-hour trial, oscillations occurred throughout the day and a distinct peak was present
in the average power spectrum at 0.40 cyc/hr. Figure 6 shows daily variations in frequency,
with the lowest frequency at 6pm and the highest at 3am. Though measured on just one
subject, the observed small (17%) changes in frequency were significant, indicating the
general capacity of our method to measure such diurnal changes and the potential to test for
daily variations in disc renewal and shedding.

3.4 Oscillations likely due to elongation of cone outer segment

The model expressed in Eq. (1) suggests that a sinusoidal oscillation in cone reflectance
requires a linear change in $(2\pi / \lambda)2nL$. While small changes may occur in the refractive
index $n$ of the cone OS, they cannot plausibly explain the multiple cycles of reflectivity
change we observed in the 24-hour experiment, which would require at least an 8% change
in the refractive index. For reference, an 8% increase or decrease, from an assumed baseline
of 1.43, would result in refractive indices of 1.54 or 1.31, approximately those of pure lipid
[20] and water, respectively. Linear shifts in the wavelength of the source $\lambda$ could also cause
reflectance oscillations, but the source spectrum was carefully monitored and was stable
within 1 nm over at least 5 hours. Thus we conclude that the cause of the oscillations in cone
reflectance is a linear change in outer segment length $L$. The model is indifferent to the sign
of $L$—either contraction or elongation of the OS would cause sinusoidal reflectance
changes. Given that elongation of the OS—due to disc renewal—over periods of hours is a
well-established phenomenon [6] and that comparable contraction has not been reported, we
conclude that the oscillations are due to the renewal of OS discs over the course of the day.
Abrupt contraction of the OS can occur, as for example from disc shedding, but there is
substantial evidence that this process occurs quickly, certainly much faster than the
measurement period (hours) of our experiment. According to our interferometric model of
the outer segment, disc shedding events would appear as abrupt shifts in the phase of the
sinusoidal reflectance oscillation. Our data were not fully analyzed for these features, and in
light of the wealth of evidence that lighting conditions play a crucial role in the timing of
shedding events (see, for example [8,21–27], ), the present experiment was not designed to
elicit an observable burst of disc shedding.

Based on this conclusion, it follows from Eq. (1) that the rate of elongation of the cone OS
(velocity of the outer segment PT relative to CC), can be expressed as

$$v_{PT} = \frac{f\lambda}{2n}$$

(2)

where $f$ is the frequency of the reflectance oscillation measured in our experiments. Note
that $f$ can also be thought of as the Doppler frequency generated by $v_{PT}$.

Using our measurements of $f$, the corresponding PT velocities were found to range from 93
nm/hr to 113 nm/hr, giving a range of daily disc renewal of 2.2 $\mu$m to 2.7 $\mu$m, daily
variations in renewal rate notwithstanding. At the retinal locations imaged, the outer
segments were measured with optical coherence tomography (Spectralis, Heidelberg
Engineering) to have average lengths of 29 $\mu$m, 27 $\mu$m and 29 $\mu$m, in subjects 1, 2 and 3,
respectively. The daily renewal measurements correspond to 8.2% to 8.7% of the outer
segment length, suggesting that the outer segment is completely replaced every 11 to 12
days.

Using $R_0\lambda / (4n)$ to estimate the smallest change in length detectable by our system, we
found that the system was capable of detecting length changes in the cone OS of 139 nm,
more than sufficient for detection of the daily renewal amounts of 2.2 to 2.7 $\mu$m.
4. Discussion

We found that over the course of the day, the reflectance of nearly all cones, under long coherence illumination, oscillated sinusoidally. Moreover, we observed that the phase of these oscillations varied randomly from cone to cone. These observations were predicted by the interferometric model of cone reflectance presented in Fig. 1(c) and Eq. (1), and thereby lend confirmation to the model. The model appears to describe accurately the reflectance of most cones under long coherence illumination. The model further predicts that no such oscillations in reflectance should occur when the cones are illuminated with short coherence light. The lack of oscillations in our short coherence trial (Trial 6) lends additional support to the model. Indeed, other investigators conducted an experiment in which the cone mosaic was imaged every hour over a 24-hour period using an incoherent visible illumination source [15]. While that study revealed changes in cone reflectance, both rapid changes occurring over matters of minutes and slow changes occurring over matters of hours, it did not show the highly prevalent, sinusoidal reflectance oscillations we observed using the long coherence source. Neither did that study claim to observe disc renewal or its optical correlates. While the experimental protocols—regular imaging over periods of hours—were similar between that experiment and our own, the differences in illumination sources—theirs incoherent and visible, ours coherent and near-infrared—make comparisons between the findings difficult. Based on current evidence, we believe the mechanisms underlying the main changes in cone reflectance observed in the two experiments to be disjoint. In terms of Eq. (1), changes in the cone's brightness due to temporally incoherent illumination are described by $I_o$, whereas temporally coherent effects are described by $2 | \Psi_1 \parallel \Psi_2 | \cos(2\pi / \lambda)2nL$. We determined that the main changes we observed in cone reflectance, viz. sinusoidal oscillations, were an effect of temporally coherent illumination. We did not observe the temporally incoherent effects observed by Pallikaris et al. There are many potential reasons for this: the incoherent cone effects they observed may require the visible stimulation of cones present in their experiment but not ours; alternatively, these effects were present but undetectable because they were either masked by the high amplitude coherent effects or the relatively low cone SNR imposed by near-infrared illumination [28].

Using the outer segment as a biological interferometer, we were able to track minute changes in its length, significantly smaller than the wavelength of the illumination source. We found that the rate of renewal can be determined within five hours and that renewal can be continually tracked over 24 hours while monitoring small but significant changes in its rate over the course of the day.

While it has not previously been possible to measure OS renewal rate in living animals, let alone humans, numerous ex vivo studies have been conducted to study these rates in mammalian rods. Daily rod renewal rates have been reported to be 1.8 to 2.2 μm /day in mice [6,29,30], 1.8 to 2 μm /day in dogs [31,32], and 2.6 to 2.8 μm /day in rhesus monkeys [33]. In mammalian cones, electron microscopy has been employed to measure the size of phagosomes in the RPE cells underlying the cone OS, found to be 1.6 μm /day in the squirrel [11], 1.15 um/day in the domestic cat [34], and 1 to 3.23 μm /day in the Rhesus monkey [35,36].

The rates of renewal we report here lie squarely within the range of renewal rates reported in the literature and agree with the estimates of daily disc shedding in cones, suggesting that we have developed an accurate method for measuring disc renewal in the living human eye. This is the first observation of renewal in a living eye and the first direct observation of renewal in cones. While the measurement period is currently long (5 hours), improvements are possible to significantly shorten it, which will enhance the technique's practicality for measuring larger numbers of subjects.
Our technique could be used to measure normal rates of outer segment renewal in a population study, and given such normative data, be extended to investigate the relationship between renewal and disease. Defective phagocytosis of outer segments has been shown to play a role in retinitis pigmentosa (RP) [37]. Disruptions of phagocytosis and associated signaling between RPE and photoreceptor has been shown to play a role in age-related macular degeneration (AMD) [38,39]. Measuring rates of renewal in AMD and RP patients’ retinas could help determine the role of disc renewal in these diseases, and may be useful in early detection and monitoring of progression in these leading causes of blindness. Moreover, the method could be employed to investigate the cyclic properties of renewal and shedding in human cones and the governing roles of circadian and light cues, none of which has yet been characterized. Recent years have witnessed considerable interest in optical measurements of photoreceptor function; see, for example [12,40–47]. While the dependence of electroretinogram (ERG) signals on daily renewal has been measured [48], it would be interesting to use the current system to explore the relationship between renewal and stimulus-evoked optical changes, at the level of single photoreceptors.

The best existing method for depth imaging of the living retina is ultrahigh-resolution optical coherence tomography (UHR-OCT), whose axial resolution has been reported to be 3 m in retinal tissue [49,50]. Combined with AO, UHR-OCT can measure outer segment lengths of individual cones [50–52]. However, the axial resolution of UHR-OCT is likely insufficient to detect daily OS renewal, let alone which occurs over matters of hours or daily variations in rate. Spectral-domain phase microscopy has demonstrated sub-nanometer resolution in in vitro applications [53], but has not been demonstrated in vivo, likely because of the substantial phase noise introduced by eye motion even when the subject’s head is well stabilized. Our instrument may be thought of as a phase-sensitive common-path interferometer that uses the two opposing sides of the structure of interest (OS) as sample and reference surfaces. As such, the instrument is sensitive only to those phase changes that occur between the two surfaces. While we have targeted disc renewal, our method can be readily applied to other physiological processes that exhibit minute length or refractive index changes in the OS, as for example the processes of disc shedding and phototransduction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References and links

1. Noell WK, Walker VS, Kang BOKS, Berman S. Retinal damage by light in rats. Invest. Ophthalmol. Vis. Sci. 1966; 5:450–473.
2. Organisciak DT, Winkler BS. Retinal light damage: practical and theoretical considerations. Prog. Retin. Eye Res. 1994; 13(1):1–29.
3. Daemen FJM. Vertebrate rod outer segment membranes. Biochim. Biophys. Acta. 1973; 300(3):255–288. [PubMed: 4587475]
4. Futterman, S. Metabolism and photochemistry in the retina. In: Moses, RA., editor. Physiology of the Eye. 6th ed.. C. V. Mosby Co.; St. Louis: 1975. p. 406-419.

5. Anderson B Jr. Ocular effects of changes in oxygen and carbon dioxide tension. Trans. Am. Ophthalmol. Soc. 1968; 66:423–474. [PubMed: 5720847]

6. Young RW. The renewal of photoreceptor cell outer segments. J. Cell Biol. 1967; 33(1):61–72. [PubMed: 6033942]

7. Young RW, Bok D. Participation of the retinal pigment epithelium in the rod outer segment renewal process. J. Cell Biol. 1969; 42(2):392–403. [PubMed: 5792328]

8. LaVail MM. Rod outer segment disk shedding in rat retina: relationship to cyclic lighting. Science. 1976; 194(4269):1071–1074. [PubMed: 982063]

9. Bok D. Retinal photoreceptor-pigment epithelium interactions. Friedenwald lecture. Invest. Ophthalmol. Vis. Sci. 1985; 26(12):1659–1694. [PubMed: 2933359]

10. Steinberg RH, Wood I, Steinberg RH. Phagocytosis by pigment epithelium of human retinal cones. Nature. 1974; 252(5481):305–307. [PubMed: 4431450]

11. Anderson DH, Fisher SK. Disc shedding in rodlike and conelike photoreceptors of tree squirrels. Science. 1975; 187(4180):953–955. [PubMed: 1145180]

12. Jonnal RS, Rha J, Zhang Y, Cense B, Gao W, Miller DT. In vivo functional imaging of human cone photoreceptors. Opt. Express. 2007; 15(24):16141–16160.

13. Zhang Y, Cense B, Rha J, Jonnal RS, Gao W, Zawadzki RJ, Werner JS, Jones S, Olivier S, Miller DT. High-speed volumetric imaging of cone photoreceptors with adaptive optics spectral-domain optical coherence tomography. Opt. Express. 2006; 14(10):4380–4394. [PubMed: 19096730]

14. Gao W, Cense B, Zhang Y, Jonnal RS, Miller DT, Miller DT. Measuring retinal contributions to the optical Stiles-Crawford effect with optical coherence tomography. Opt. Express. 2008; 16(9): 6486–6501. [PubMed: 18516251]

15. Pallikaris A, Williams DR, Hofer H. The reflectance of single cones in the living human eye. Invest. Ophthalmol. Vis. Sci. 2003; 44(10):4580–4592. [PubMed: 14507907]

16. Snyder W, Pask C. Stiles-crawford effect: explanation and consequences. Vision Res. 1973; 13(6):1115–1137. [PubMed: 4713922]

17. Zhang Y, Rha J, Jonnal RS, Miller DT. Adaptive optics parallel spectral domain optical coherence tomography for imaging the living retina. Opt. Express. 2005; 13(12):4792–4811. [PubMed: 19495398]

18. Rha J, Jonnal RS, Thorn KE, Qu J, Zhang Y, Miller DT. Adaptive optics flood-illumination camera for high speed retinal imaging. Opt. Express. 2006; 14(10):4552–4569. [PubMed: 19516608]

19. Curcio CA, Sloan KR, Kalina RE, Hendrickson AE. Human photoreceptor topography. J. Comp. Neurol. 1990; 292(4):497–523. [PubMed: 2324310]

20. Ross KFA, Chou JTY. The physical nature of the lipid globules in the living neurones of Helix aspersa as indicated by measurements of refractive index. J. Cell Sci. 1957; 3:341.

21. LaVail MM. Circadian nature of rod outer segment disc shedding in the rat. Invest. Ophthalmol. Vis. Sci. 1980; 19(4):407–411. [PubMed: 7358492]

22. Bobu C, Hicks D. Regulation of retinal photoreceptor phagocytosis in a diurnal mammal by circadian clocks and ambient lighting. Invest. Ophthalmol. Vis. Sci. 2009; 50(7):3495–3502. [PubMed: 19234351]

23. Goldman AI, Teirstein PS, O’Brien PJ. The role of ambient lighting in circadian disc shedding in the rod outer segment of the rat retina. Invest. Ophthalmol. Vis. Sci. 1980; 19(11):1257–1267. [PubMed: 7429762]

24. Grace MS, Chiba A, Menaker M. Circadian control of photoreceptor outer segment membrane turnover in mice genetically incapable of melatonin synthesis. Vis. Neurosci. 1999; 16(5):909–918. [PubMed: 10580726]

25. Nandrot, F.; Finnemann, SC. Altered rhythm of photoreceptor outer segment phagocytosis in b5 integrin knockout mice. In: Hollyfield, JG.; Anderson, RH.; LaVail, MM., editors. Advances in Experimental Medicine and Biology: Retinal Degenerative Diseases. Springer; New York: 2006. p. 119-123.

*Opt Express. Author manuscript; available in PMC 2011 June 13.*
26. Tosini G, Fukuhara C. The mammalian retina as a clock. Cell Tissue Res. 2002; 309(1):119–126. [PubMed: 12111542]

27. Young RW. The daily rhythm of shedding and degradation of rod and cone outer segment membranes in the chick retina. Invest. Ophthalmol. Vis. Sci. 1978; 17(2):105–116. [PubMed: 624604]

28. Choi SS, Doble N, Lin J, Christou J, Williams DR. Effect of wavelength on in vivo images of the human cone mosaic. J. Opt. Soc. Am. A. 2005; 22(12):2598–2605.

29. LaVail MM. Kinetics of rod outer segment renewal in the developing mouse retina. J. Cell Biol. 1973; 58(3):650–661. [PubMed: 4747920]

30. LaVail MM. Photoreceptor characteristics in congenic strains of RCS rats. Invest. Ophthalmol. Vis. Sci. 1981; 20(5):671–675. [PubMed: 7216680]

31. Buyukmihci N, Aguirre GD. Rod disc turnover in the dog. Invest. Ophthalmol. Vis. Sci. 1976; 15:579–584.

32. Aguirre GD, Andrews L. Nomarski evaluation of rod outer segment renewal in a hereditary retinal degeneration. Comparison with autoradiographic evaluation. Invest. Ophthalmol. Vis. Sci. 1987; 28(7):1049–1058. [PubMed: 3596987]

33. Young RW. The renewal of rod and cone outer segments in the rhesus monkey. J. Cell Biol. 1971; 49(2):303–318. [PubMed: 19866760]

34. Fisher SK, Pfeffer BA, Anderson DH. Both rod and cone disc shedding are related to light onset in the cat. Invest. Ophthalmol. Vis. Sci. 1983; 24(7):844–856. [PubMed: 6683265]

35. Anderson DH, Fisher SK, Erickson PA, Tabor GA. Rod and cone disc shedding in the rhesus monkey retina: a quantitative study. Exp. Eye Res. 1980; 30(5):559–574. [PubMed: 7409012]

36. Guérin CJ, Lewis GP, Fisher SK, Anderson DH. Recovery of photoreceptor outer segment length and analysis of membrane assembly rates in regenerating primate photoreceptor outer segments. Invest. Ophthalmol. Vis. Sci. 1993; 34(1):175–183. [PubMed: 8425823]

37. Vollrath D, Gal A, Li Y, Thompson DA, Weir J, Orth U, Jacobson SG, Apfelstedt-Sylla E. Mutations in MERTK, the human orthologue of the RCS rat retinal dystrophy gene, cause retinitis pigmentosa. Nat. Genet. 2000; 26(3):270–271. [PubMed: 11062461]

38. Kindzelskii AL, Elner VM, Elner SG, Yang D, Hughes BA, Petty HR. Toll-like receptor 4 (TLR4) of retinal pigment epithelial cells participates in transmembrane signaling in response to photoreceptor outer segments. J. Gen. Physiol. 2004; 124(2):139–149. [PubMed: 15277575]

39. Zareparsi S, Buraczynska M, Branham KEH, Shah S, Eng D, Li M, Pawar H, Yashar BM, Moroi SE, Lichter PR, Petty HR, Richards JE, Abecasis GR, Elner VM, Swaroop A. Toll-like receptor 4 variant D299G is associated with susceptibility to age-related macular degeneration. Hum. Mol. Genet. 2005; 14(11):1449–1455. [PubMed: 15829498]

40. Grinvald A, Lieke E, Frostig RD, Gilbert CD, Wiesel TN. Functional architecture of cortex revealed by optical imaging of intrinsic signals. Nature. 1986; 324(6095):361–364. [PubMed: 3785405]

41. DeLint PJ, Berendschot TT, van de Kraats J, van Norren D. Slow optical changes in human photoreceptors induced by light. Invest. Ophthalmol. Vis. Sci. 2000; 41(1):282–289. [PubMed: 10634632]

42. Tsunoda K, Oguchi Y, Hanazono G, Tanifuji M. Mapping cone- and rod-induced retinal responsiveness in macaque retina by optical imaging. Invest. Ophthalmol. Vis. Sci. 2004; 45(10):3820–3826. [PubMed: 15452094]

43. Bizheva K, Pfug R, Hermann B, Povazay B, Sattmann H, Qiu P, Anger E, Reitsamer H, Popov S, Taylor JR, Unterhuber A, Afnelt P, Drexler W. Optophysiology: depth-resolved probing of retinal physiology with functional ultrahigh-resolution optical coherence tomography. Proc. Natl. Acad. Sci. U.S.A. 2006; 103(13):5066–5071. [PubMed: 16551749]

44. Srinivasan VJ, Wojtkowski M, Fujimoto JG, Duker JS. In vivo measurement of retinal physiology with high-speed ultrahigh-resolution optical coherence tomography. Opt. Lett. 2006; 31(15):2308–2310. [PubMed: 16832468]

45. Yao XC, George JS. Near-infrared imaging of fast intrinsic optical responses in visible light-activated amphibian retina. J. Biomed. Opt. 2006; 11(6):064030. [PubMed: 17212553]
46. Grieve K, Roorda A. Intrinsic signals from human cone photoreceptors. Invest. Ophthalmo.
Vis. Sci. 2008; 49(2):713–719. [PubMed: 18235019]
47. Abrámoff MD, Kwon YH, Ts'o D, Soliz P, Zimmerman B, Pokorny J, Kardon R. Visual stimu-
lus-induced changes in human near-infrared fundus reflectance. Invest. Ophthalmo.
Vis. Sci. 2006; 47(2):715–721. [PubMed: 16431972]
48. Birch DG, Berson EL, Sandberg MA. Diurnal rhythm in the human rod ERG. Invest. Ophthalmo.
Vis. Sci. 1984; 25(2):236–238. [PubMed: 6538188]
49. Cense B, Nassif N, Chen T, Pierce M, Yun SH, Park B, Bouma B, Tearney G, de Boer J. 
Ultrahigh-resolution high-speed retinal imaging using spectral-domain optical coherence 
tomography. Opt. Express. 2004; 12(11):2435–2447. [PubMed: 14975080]
50. Cense B, Koperda E, Brown JM, Kocaoglu OP, Gao W, Jonnal RS, Miller DT. Volumetric retinal 
imaging with ultrahigh-resolution spectral-domain optical coherence tomography and adaptive 
optics using two broadband light sources. Opt. Express. 2009; 17(5):4095–4111. [PubMed: 
19259249]
51. Zawadzki RJ, Cense B, Zhang Y, Choi SS, Miller DT, Werner JS. Ultrahigh-resolution optical 
coherence tomography with monochromatic and chromatic aberration correction. Opt. Express. 
2008; 16(11):8126–8143. [PubMed: 18545525]
52. Fernández EJ, Hermann B, Považay B, Unterhuber A, Sattmann H, Hofer B, Ahnelt PK, Drexler 
W. Ultrahigh resolution optical coherence tomography and pan correction for cellular imaging of 
the living human retina. Opt. Express. 2008; 16(15):11083–11094. [PubMed: 18648422]
53. Choma MA, Ellerbee AK, Yang C, Creazzo TL, Izatt JA. Spectral-domain phase microscopy. Opt. 
Lett. 2005; 30(10):1162–1164. [PubMed: 15945141]
Fig. 1.
The reflective structure of the retina. (a) A diagram depicting the major layers of the neural retina, consisting of the inner limiting membrane (ILM), nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), external limiting membrane (ELM), the inner segments (IS) and outer segments (OS) (which make up the photoreceptor layer), the connecting cilia (CC) and posterior tip (PT) layers (which bound the outer segment), the retinal pigment epithelium (RPE), and the choroid (CH). (b) An AO-OCT B-scan (log intensity) from Subject 1, showing a cross-section of the full retinal thickness, aligned with the layers depicted in (a), and an enlarged view (linear intensity) of the cone outer segments. While OCT images are typically shown in log intensity, the linear intensity view of the outer segments demonstrates vividly that the bulk of the cone reflection originates at the CC and PT layers: the bright, patterned reflections at the CC and PT layers are the most visible structures in the linear intensity image; their peak intensity is more than two orders of magnitude greater than the average intensity of all other layers in the image. Each distinct reflection in the pattern represents a single cone cell. (c) A model of light propagation through the OS. Two bright reflections ($\Psi_1$ and $\Psi_2$) originate from the CC and PT layers, creating a biological interferometer in the retina that is sensitive to small ($\ll \lambda$) changes in the outer segment length $L$ whenever the temporal coherence length of the illumination source $L_c$ is longer than $L$. 

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Fig. 2.
Schematic diagram of the adaptive optics (AO) retina camera. Abbreviations used: superluminescent diode (SLD), laser diode (LD), multimode fiber (MMF), mirror (M), dichroic beam splitter (DBS), pellicle beam splitter (PBS), and charge-coupled device (CCD). The AO system consists of the 788 nm SLD, the Shack-Hartmann wavefront sensor, and the deformable mirror. The imaging system consists of the 810 nm LD, the 842 nm SLD, and the science CCD.
Fig. 3.
Visible oscillations in cone reflectance over hours. (a) Cone mosaic image acquired from subject 1 with the AO retina camera. The image, an average of 21 images acquired over five hours, is 324×264 μm and each bright spot is a single cone cell. (b-f) Enlarged images of a sample region (location indicated by white box in (a)), acquired at 0h, 0.75h, 1.5h, 2.25h, and 3h. Most of the cones can be observed to go through approximately one full cycle of reflectance change. For example, cone 1 is bright at times 0h and 3h, but dark at time 1.5h, while cone 5 is dark at times 0h and 3h, but bright at time 1.5h.
Fig. 4.
Video showing the visible oscillations in cone reflectance over five hours (Media 1). The clock in the upper right shows the time each image in the video was acquired. In order to emphasize oscillations at the peak frequency while reducing the visible effects of noise near the sampling frequency, a Gaussian temporal lowpass filter ($\sigma = 12$ min) was used. Most cones appear to go through nearly two full cycles of reflectance oscillation. The amplitude and phase of oscillation appear to vary randomly among cones.
Fig. 5. Cone reflectances and their power spectra (plots offset vertically for ease of viewing). (a) Reflectance as a function of time of eight sample cones taken from trial 4. Superimposed on each plot is a cosine fit (gray line). The black bar in the upper left shows 1/10th of the average DC component, $I_0$, of cone reflectance. The oscillation of reflectance in all cones had a visible period of 2.5 – 3 hours, while the amplitudes and phases appeared to vary randomly. At the bottom is a plot of the average reflectance of all cones (diamonds), nearly flat (contrast 0.18%), which is predicted by the model shown in Fig. 1(c) and Eq. (1). (b) Power spectra of mean-subtracted cone reflectance traces shown in a, and the average spectrum of all 1626 cones (dark line). Most cones in this trial had a visible peak in the power spectrum around 0.37 cyc/hr, and this peak is visible in the average power spectrum as well. Similar peaks were seen in power spectra of individual cones, and the average power spectrum, in all trials in which the long coherence source was used (these frequencies are summarized in Table 1). When the short coherence source was used, neither the power spectra of individual cones nor the average power spectrum showed comparable peaks.
Fig. 6.
Plot of the average frequency of oscillation over 24 hour period. Each data point was generated by computing the power spectrum of every cone reflectance series over a four hour window, locating the peak in each power spectrum, and averaging those peak frequencies together. The data point at 2pm, for instance, is the average frequency between the hours of noon and 4pm. Standard deviation (σ) of peak frequencies was computed. Error bars show one standard error of the mean in the distribution of frequencies, (σ/√N, with N = 806). Oscillations in reflectance were present throughout the 24 hour experiment.
### Table 1

Summary of experimental trials and average renewal velocities measured (see text for details).

| Trial | Subj. | Start Time | End Time | Coherence of light source | # of cones | Avg contrast (%) | Peak freq (cyc/hr) | Re-newal velocity (nm/hr) | Re-newal velocity ($\mu$m/day) |
|-------|-------|------------|----------|---------------------------|------------|------------------|---------------------|----------------------------|-------------------------------|
| 1     | 1     | Noon       | 5pm      | Long                      | 1,253      | 6.5              | 0.35                | 99                         | 2.4                           |
| 2     | 2     | Noon       | 5pm      | Long                      | 742        | 4.7              | 0.32                | 93                         | 2.2                           |
| 3     | 3     | Noon       | 5pm      | Long                      | 1,232      | 4.5              | 0.37                | 105                        | 2.5                           |
| 4     | 1     | 5pm        | 10pm     | Long                      | 1,626      | 4.2              | 0.36                | 102                        | 2.4                           |
| 5     | 1     | Noon       | Noon + 1 day | Long                  | 806        | 7.7              | 0.40                | 113                        | 2.7                           |
| 6     | 1     | Noon       | 5pm      | Short                     | 937        | 1.6              | N/A                 | N/A                        | N/A                           |