Impact of motion-associated noise on intrinsic optical signal imaging in humans with optical coherence tomography

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Abstract: A growing body of evidence suggests that phototransduction can be studied in the human eye in vivo by imaging of fast intrinsic optical signals (IOS). There is consensus concerning the limiting influence of motion-associated imaging noise on the reproducibility of IOS-measurements, especially in those employing spectral-domain optical coherence tomography (SD-OCT). However, no study to date has conducted a comprehensive analysis of this noise in the context of IOS-imaging. In this study, we discuss biophysical correlates of IOS, and we address motion-associated imaging noise by providing correctional post-processing methods. In order to avoid cross-talk of adjacent IOS of opposite signal polarity, cellular resolution and stability of imaging to the level of individual cones is likely needed. The optical Stiles-Crawford effect can be a source of significant IOS-imaging noise if alignment with the peak of the Stiles-Crawford function cannot be maintained. Therefore, complete head stabilization by implementation of a bite-bar may be critical to maintain a constant pupil entry position of the OCT beam. Due to depth-dependent sensitivity fall-off, heartbeat and breathing associated axial movements can cause tissue reflectivity to vary by 29% over time, although known methods can be implemented to null these effects. Substantial variations in reflectivity can be caused by variable illumination due to changes in the beam pupil entry position and angle, which can be reduced by an adaptive algorithm based on slope-fitting of optical attenuation in the choriocapillary lamina.

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References and links


In my important eye diseases, such as age-related macular degeneration [1], retinitis pigmentosa [2] and Stargardt disease, pathologic changes of retinal photoreceptors are the hallmark of disease development. Although no treatments exist yet for retinitis pigmentosa and Stargardt disease, several research groups are currently developing gene augmentation therapy. A thorough evaluation of remaining retinal architecture and function is essential to select patients amenable for treatment. We therefore need imaging techniques that can determine miniscule changes in the structural and functional integrity of retinal photoreceptors, for patient selection but also to monitor the effects of therapy.

Current high-resolution imaging techniques, e.g. optical coherence tomography (OCT) and adaptive optics (AO) imaging, can already provide information on the structural tissue integrity. However, structural information alone may be insufficient to monitor functional decline [3]. It has been shown that intrinsic optical signal (IOS) imaging can help to detect neural activity (for instance, of the cortical surface of the mammalian brain [4, 5]). The principle of IOS-imaging is based on measuring the transient changes in light reflectance from tissue that are indicative of stimulus-induced alterations in neuronal activity. Reflectance changes in retinal tissue have been associated with slow (seconds to minutes) alterations in light-scattering and -absorption caused by hemodynamic activation [6], and
with fast (milliseconds to seconds) changes in the optical properties of photoreceptors [7–9]. Current in vivo measurements of phototransduction are either confined to the initial photon absorption kinetics (fundus densitometry [10]) or to the final membrane hyperpolarization (electroretinography, ERG [11, 12]). Imaging of fast IOS, however, provides a unique opportunity to investigate the intermediate processes in individual photoreceptors in vivo [13, 14]. At present, adaptive-optics ultrahigh-resolution OCT (AO-UHR-OCT) is the only in vivo technique capable of resolving cones in three dimensions close to the fovea [15].

In contrast to the numerous studies that demonstrated reproducible IOS in animals using ex vivo retina preparations or in vivo measurements under anesthesia, comparatively few studies were able to detect fast IOS in awake humans using various techniques [7–9, 16–21]. Several of these human studies showed low reproducibility due to a poor signal-to-noise ratio (SNR), caused by substantial confounding imaging noise relatively to the small IOS amplitudes. The robustness and reproducibility of the acquisition procedure are limited by its low tolerance for retinal motion and the presence of a narrow foveal avascular zone [8]. Eye motion adversely affects IOS-imaging in two important ways. First, the monitoring of the same tissue volume over time is complicated due to tissue displacements out of the field of view (FOV) and/or other motion artifacts (e.g. blurring and distortions). Second, the main outcome measure (reflectivity) can be significantly affected by specific eye- or head movements. The magnitude of motion-associated noise is of the same order as that of the expected signals [17]. Although several motion artifacts in SD-OCT-imaging were analyzed previously (e.g [22]), no study to date has determined how eye motion can induce noise in IOS-imaging by SD-OCT. This work aims to provide a comprehensive overview of known properties of the fast IOS and their relation to biophysical processes, and to analyze motion-associated IOS-imaging noise. Finally, we will suggest improvements to dedicated optical systems, as well image acquisition and post-processing, to facilitate studies on this promising technique in the living human eye.

2. Review of biochemical and biophysical correlates of the fast IOS

Using a variety of methods, fast IOS-imaging studies have found indications that several of the photoreceptor’s optical properties change upon visual stimulation. These changes may reflect different biochemical processes.

Photons of the visible spectrum (400 – 780 nm) have a chance to be absorbed by the photoreceptor’s visual pigments and to initiate phototransduction by photoisomerization of rhodopsin. In the rod and cone outer segment (OS), the likelihood of photon absorption in basal disks is greater, relative to apical disks near the OS tips. This so-called self-screening process is less pronounced in the cone OS as a consequence of its tapered structure [23]. Key processes of the phototransduction cascade are schematized in Fig. 1(a). In the process of photoisomerization of the opsin molecule, the first relatively stable isomer (Metarhodopsin II, RMII) is formed about 1 ms after photon absorption. The speed of this formation is quite similar in rods and cones, regardless of their opsins’ wavelengths of maximum absorption [24–28]. RMII catalyzes activation of the G-protein transducin, which couples very rapidly with phosphodiesterase (PDE). This enzyme, in turn, catalyzes the hydrolysis of cyclic guanosine monophosphate (cGMP) to a very high rate for as long as it remains active (PDE*). The lifetime of PDE* depends on the inactivation of visual pigment and transducin, which in turn depend on the photoreceptor type and (in case of cones-) on light-intensity. The cytoplasmic concentration of cGMP is reduced very quickly, causing cyclic nucleotide gated (CNG) channel – bound cGMP to dissociate. In the dark, cGMP is responsible for holding open CNG channels to allow the influx of sodium, which maintains the OS membrane in a depolarized state, such that a ‘dark current’ flows through the cell. This dark current allows voltage-gated calcium influx channels to remain open. The resulting high intracellular calcium concentration causes constant release of the neurotransmitter glutamate in the dark. In the light then, cGMP hydrolysis causes closure of CNG channels leading to
hyperpolarization, which causes closure of voltage-sensitive calcium channels along the OS, resulting in cessation of the dark current and inhibition of glutamate release.

Up to now, two types of fast IOS have been described; high-frequency intensity reversals in human cones with a duration of 300-400 ms [7, 8], and changes in light backscatter from photoreceptors (of various species), that persist for 1-3 s [29, 30].

The first type of IOS was detected by Jonnal et al. (2007) [7]. Using a high-speed AO flood-illumination camera, they observed fast stimulus evoked-intensity fluctuations with random starting phase in individual cones. These results confirm that the OS can function as a ‘biological interferometer’ with sensitivity to sub-wavelength changes in cell length, refractive index, or scatter [7, 31]. The authors suggested that these intensity reversals are driven by interference of coherent light that is reflected off the posterior OS tips and the connecting cilium. Their detection required the source coherence length to exceed the optical path length between these structures [7].

Similar intensity fluctuations observed with shorter source coherence lengths [8, 9, 18, 21] indicate the presence of several scattering boundaries along the OS length [8]. Of interest, similar signals were detected in 80-100% of the assessed cones [8, 9], indicating the involvement of a common process among all types of cones. The fluctuation signals’ time of onset (2-7 ms) and duration (300-400 ms) closely matched those of single-flash, single-cone electrophysiological recordings [7, 33]. This could indicate changes in cone circulating current itself or associated processes, such as membrane polarization or changing concentrations of cGMP [8], although it is unknown whether this leads to changes in refractive index, scatter, or cell length through osmotic activity [7, 31]. The involvement of non-linear processes, such as changes in the concentration of activated opsin, transducin, or PDE*, has been ruled out, because the frequencies of these intensity reversals (which increased with stimulus strength) were variable [7, 8].

Neuronal osmotic swelling is well known to cause increased light transmission due to reduced light scattering [34]. However, the squid and amphibian photoreceptor diameters change only for about 100 - 200 ms upon stimulation [35, 36], which does not quite match the duration of the fluctuation-type IOS in humans [7, 8]. Unfortunately, we found no similar measurements in mammalian retinas. Further, single-cone ERG recordings in macaques show that photovoltages have largely dissipated by 200 ms [33], which indicates that the associated ion fluxes have reduced as well. Thus, the biomechanical and ERG results show a close temporal correspondence. The discrepancy in durations of IOS and osmotic changes in the outer segments suggests that other contemporary events contribute, such as altered light scattering.

Flash stimulation, in the absence of guanosine triphosphate, can cause increased scatter from rod disk membranes due to a gain in mass of membranes when proteins are bound from solution (‘binding signals’) [14]. These proteins include transducin [14], rhodopsin kinase, and arrestin [37]. In the presence of guanosine triphosphate, transducin can dissociate (Fig. 1(a), step 2), which is measurable by a ‘dissociation signal’ opposite in polarity to the binding signal [14]. PDE-activity also increases light scatter, which initiates after at least 10 ms in rod disks. Kinetic analysis demonstrated that light scatter due to PDE-activity exceeds the dissociation signal’s reduction in light scatter [37]. Further, modeling indicates that PDE-activity in cones returns to baseline about 400 ms after photon absorption [38], which corresponds closely with the fluctuation-type of IOS [7]. Based on these results we hypothesize that light scattering boundaries can form locally in the OS, that can contribute to optical path length changes of the ‘biological interferometers’. This is schematized in Fig. 1(b). In summary, in addition to optical path length changes due to osmotic swelling, PDE-activity may be an important contributor to the interference phenomena reported earlier [7, 8], and merits additional investigation.
Fig. 1. (a) Simplified scheme of key processes involved in phototransduction (modified from Leskov et al., 2000) [32]. Upon absorption of visible photons by visual pigment, consisting of rhodopsin and an opsin chromophore (R) (step 1), rhodopsin undergoes conformational changes until Metarhodopsin II is formed (R_{MII}). Consequently, the G-protein transducin (G_t) is bound from solution and is enzymatically activated by R_{MII} by phosphorylation (step 2). Activated G_t (G_t*) then dissociates from R_{MII} (step 3). Next, two molecules of G_t* bind and activate the enzyme phosphodiesterase (PDE*) (step 4). PDE* catalyzes the hydrolysis of cyclic guanosine monophosphate (cGMP), which is responsible for holding open cyclic nucleotide gated (CNG) channels to allow for the influx of Na^+ and Ca^{2+}. As a consequence of cGMP-hydrolysis after visual stimulation, CNG-channel bound cGMP dissociates, leading to channel closure and membrane hyperpolarization (step 5). Consequently, voltage-sensitive calcium channels close, leading to cessation of the photoreceptor’s dark current and inhibition of the release of the neurotransmitter glutamate. (b) The hypothesized involvement of PDE-activity in the generation of the fluctuation-type of fast IOS described earlier [7]. In the dark (left image), light rays impinging on the photoreceptor outer segment (black lines) reflect principally off the inner-outer segment junction (IS/OS-junction) and the OS outer tips (blue lines), with the optical path length between these structures $\Lambda_1 = n L_1$. Upon stimulation (right image), osmotic swelling may cause changes in the optical path length $(\Lambda_2)$, as well as light scatter caused by localized PDE-activity $(\Lambda_3)$. This PDE-activity has a higher probability of occurring in basal disks close to the IS/OS-junction as a consequence of self-screening (see text for details).

The second type of fast IOS have been measured by depth-resolved OCT in different animals, where IOS of different polarities, i.e., increased or decreased reflectance, were measured from retinal photoreceptors [20, 29, 30, 39, 40]. Following on initial indications of the co-existence of IOS with opposite polarity in the same area [41], Wang et al. (2013) demonstrated significantly lowered IOS amplitudes in frog retina when the lateral resolution was worse than 2 µm. This signal loss is probably caused by the integration of opposite
signals in adjacent photoreceptors when groups of cells are imaged in one pixel due to low imaging resolution [42]. Of interest, the SNR remained equal regardless of resolution, due to the greater amplitude of positive IOS (increased reflectance). These findings could very well explain the generally poor reproducibility of previous studies in humans [16, 19, 20]. A sound recommendation for future studies is therefore to employ AO to resolve individual cones, and to avoid pixel crosstalk by using either point-scanning OCT systems or specialized en-face line-scanning OCT-systems [42].

Intriguingly, at the level of the OS, this type of fast IOS displays a random pattern (Fig. 2). Previous reports suggested that the positive IOS reflect cone activity. In human retina, standard resolution UHR-OCT revealed a fast (time to peak, about 100 ms) positive signal from the inner/outer segment (IS/OS)- junction of rods and cones [16, 17], whereas the outer segment layer showed a fast negative signal in human retina [16].The absence of the IS/OS junction response in light-adapted conditions and in the peripheral retina [16] suggests that the positive signal reflects cone-activity. Also, if scattering boundaries within the OS can explain the fluctuation-type signals that were measured with short-coherence length light sources, then they likely lead to increased backscatter in cone OS as well [8]. Further, this hints at the possibility that intracellular scattering boundaries are responsible for both types of fast IOS. High-resolution IOS-imaging studies that are able to separate rod and cone responses in humans are needed to clarify the cellular sources of positive and negative IOS.

![Fig. 2. Fast IOS-pattern in frog retina observed with line-scanning en face OCT after the application of a circular stimulus. The IOS in this image have similar amplitudes across the image in a random arrangement. Adapted from [42] with permission.](image)

The biophysical underpinnings of both types of fast IOS require closer examination. The suggestion that the different polarities of the second type of fast IOS (Fig. 2) resemble binding and dissociation signals of transducin [14, 43] is unlikely, since the dissociation signal is undetectable in the presence of PDE-activity that directly follows it [37]. It is possible to selectively examine the contribution of PDE-activity in IOS, by measuring IOS in functionally intact rod-dominated retinas in the presence of PDE-inhibitors such as 3-isobutyl-1-methyl-xanthine and papaverine. The important question on the previously hypothesized involvement of cell swelling in the generation of fast IOS [7] can be examined, by measuring fast IOS in the presence of CNG-channels blockers such as pseudechetroxin for rods [44], which selectively inhibits ion fluxes and associated cell swelling. These experiments will aid in elucidating the biophysical nature of fast IOS.

### 3. Analysis of motion-associated noise

This section gives an overview of technical and biological sources of dominant noise in in vivo IOS-imaging in humans by SD-OCT. This noise can be addressed by modifications of the imaging and stimulation concept or by image post-processing to increase the SNR. In Sections 3.1 and 3.2, the topic-related literature is reviewed. In Sections 3.3 and 3.4, our analytical work is presented.

#### 3.1 Motion artifacts, image distortions, and recording discontinuity

Involuntary eye motion during fixation is a major cause of artifacts in OCT imaging [45], even in highly-cooperate and healthy subjects. When the transversal velocity of the sample is high relative to the OCT-beam size and camera integration time, the transverse resolution
and SNR deteriorate [46, 47]. Although high imaging speeds can reduce these effects [47], it does not eliminate them, as they affect even multi-MHz scanning systems [48].

Eye motion can occur as a slow shift in gaze (drift), high-frequency motions (tremor), or large, nearly instantaneous shifts in fixation (saccades) [49]. Microsaccades cause the most severe motion artifacts: they occur roughly every second in healthy subjects with amplitudes of up to 30 arcmin. Eye motion can produce blurring and discontinuities of structures in B-scans, and saccades appear as ripples in OCT volumes. More complicated motion artifacts include distortions and warping in features of interest [50]. This hinders resolving and monitoring the same photoreceptors over time, which is necessary to avoid crosstalk of fast IOS [42]. That capability is offered by a combination of high-speed AO-UHR-OCT (125 kHz A-scan rate) and dedicated registration/dewarping algorithms [51]. An alternative solution is online stabilization of retinal imaging, which has been achieved by real-time tracking and compensation for retinal motion with either hardware based systems [52] or trackers based on simultaneous scanning-laser ophthalmoscope (SLO)-imaging [53]. Although the latter can stabilize imaging up to 3.2 μm root-mean-square residual error, which is close to the level of individual parafoveal cones, this tracker has not yet been combined with AO-UHR-OCT. Therefore, at present, the application of appropriate registration methods remains necessary. One possibility is the manual tracking of landmark cones over time in en face projections, followed by lateral registration of image strips demarcated by those cones [51]. Automation of this method is possible by making use of the high statistical power provided by numerous strips from images of the same location [54], which is relatively insensitive to changes in cone reflectivity and therefore advantageous for IOS-imaging. With respect to B-scan time series, cross-correlation analysis has frequently been used to select out B-scans that were acquired at a different location than the reference location [40].

Finally, the capacity to monitor the same tissue over time is limited when the OCT’s field-of-view (FOV) is small relative to the amplitude of displacement. Cellular resolution OCT-imaging requires sufficient oversampling to maintain feature contrast and high SNR. Therefore, the detector speed typically limits the FOV. It is also limited by the size of the isoplanatic patch, which depends on the accuracy of wavefront aberration correction [55]. Therefore, a useful FOV of AO-OCT typically spans no more than 60 arcmin retinal eccentricity. Microsaccadic displacements of the retina relative to such a small FOV can be substantial because those movements commonly spread to 24 arcmin [56], i.e. nearly half of the FOV. Several techniques can increase the stability of fixation and reduce the amplitude of microsaccades, such as using a combination of a bulls eye and a crosshair as a fixation target [57], and by using a bite bar, instead of a chin rest [58, 59]. Also, large, bright stimuli used for IOS-induction in the fovea can significantly reduce fixational stability.

Many of the potential technical difficulties posed by these eye movements can be avoided by imaging of IOS at larger foveal eccentricities. In eyes endowed with good optics, individual cones can be seen at 0.5° retinal eccentricity even without adaptive optics [60], which also holds for SD-OCT ([61]). This comes with the benefit of larger FOV’s with less pronounced influences of eye movements. For this promising imaging technique to gain clinical relevance, though, robust imaging of IOS at the macula lutea is required. We anticipate that this will occur with 1) future improvements of wavefront aberration correction to enable enlargement of the isoplanatic patch, with 2) the development of faster cameras to allow these systems to enlarge the FOV, and 3) by improved stabilization of AO-OCT-imaging by either hardware-based retinal tracking or automated registration methods.

3.2 Optical Stiles-Crawford effect

In 1933, Stiles and Crawford [62] discovered that a beam of light, when displaced from the pupil center, is perceived less bright (reduced luminous efficiency), in the absence of changes in focus, illumination, or pupil diameter. The general consensus is that this effect, apparent
only in photopic conditions, originates from the orientation of the cone optical apertures. They point roughly towards the same location [63] - usually the center of the pupil, although marked differences between individuals exist [64]. Its optical counterpart, the optical Stiles-Crawford effect (OSCE) [65, 66], measures the directional dependence of fundus reflections to determine cone directionality. Gao et al. [67] used OCT (source \( \lambda_{\text{mean}} \), 842 nm) to measure cone directionality. The principal sources of retinal reflections were identified in the OS tips and the IS/OS- junction, and reflections along the OS length may also contribute [68]. In the infrared, the magnitude of the OSCE in the dark-adapted- and in the bleached state is comparable [69, 70]. Although the OSCE is quite faint in the infrared, normalized reflectance data in OCT indicate that cone reflectance changes substantially upon displacement of the OCT-beam entry position from the pupil center, as a consequence of reduced waveguiding of the imaging light through the cones [67]. These alterations may mask or mimic IOS-related dynamic optical changes and therefore contribute to motion-associated IOS-imaging noise. The magnitude of the OSCE is fairly constant from 0.5° to at least 3° retinal eccentricity, which is likely related to the more cone-like shape of the parafoveal cones [71]. However, and this is important, SLO measurements at \( \lambda = 514 \) nm indicate that the directionality of cones across the central retina (0.5 - 3° retinal eccentricity) is not symmetrical, with directionality varying between 0.05 and 0.3 [66]. This suggests that, even with information on the beam pupil entry position, it is not possible to normalize for the OSCE in the stimulated region, based on the reflectivity change of a reference region at equal retinal eccentricity.

To minimize this impact, maintaining alignment with the peak of the OSCE function is important. Due to the peakedness of the OSCE in the perifoveal area, bite-bars may be critical to abolish optical misalignments caused by head motions. Since the OSCE significantly diminishes beyond 3° retinal eccentricity [66], SD-OCT imaging of single cones in these locations, which is possible even without adaptive-optics [61], is anticipated to benefit IOS-imaging in humans due to a reduced influence of this noise factor.

### 3.3 Sensitivity fall-off

Typically, IOS-imaging studies of fixed samples determine the presence of a dynamic optical change by pixel-wise subtraction of the intensity at time \( t \) by the mean intensity at baseline, and by normalizing to that intensity as [41]:

\[
\frac{\Delta I_{x,z,t}}{I_{x,z,\text{baseline}}} = \frac{I_{x,z,t} - I_{x,z,\text{baseline}}}{I_{x,z,\text{baseline}}}. \quad (1)
\]

Equation (1) calibrates for the intensity of background light (\( I_{\text{baseline}} \)) of each sampled tissue volume, thus allowing for the extraction of dynamic optical changes (\( \Delta I / I \)). However, its assumption that the optical conditions governing the background intensity remain invariant over time is questionable in the case of sample motility. Characteristic for Fourier-domain OCT is the depth dependent sensitivity fall-off by which image quality rapidly degrades in regions corresponding to deeper locations of the sample. Its causes have been discussed in detail by Nassif et al. [72]. When the head is relatively unfettered (i.e., when using a chin- and headrest), axial displacements of the sample over time caused by heartbeat [22], breathing [22], and bulk head motion will cause it to be sampled at different sensitivities as a consequence of depth-dependent sensitivity fall-off, thus changing the background light intensity. Fall off, \( S(z) \), can be modeled with a Gaussian (Fig. 3 (a)):

\[
S(z) = \exp \left( -\frac{z^2}{\sigma^2} \right), \quad (2)
\]

where \( z \) represents the distance from zero optical path length delay, i.e. the position in the eye at which the optical path length differences of sample and reference arms are matched to
within one coherence length. The width $\sigma$ is the axial distance at which the sensitivity has dropped by 3 dB. Sensitivity fall-off can be measured with a mirror sample and by axial displacement of either the sample or reference arm. Signal decay with depth can be corrected by first averaging many A-scans and subtracting the result from each A-scan to remove fixed pattern noise, after which the power magnitude of each A-line can be calculated by squaring the complex depth profile. Each A-scan is then divided by $S(z)$ [73].

We illustrate the influence of fall-off on IOS-imaging with a timelapse SD-OCT measurement (without stimulation). Based on a measurement of fall-off of our system, we predict an intensity change of 2% by axial movements of only 10 $\mu$m. This system is a standard resolution (~21 $\mu$m lateral, axial ~6 $\mu$m, assuming a tissue refractive index of 1.38), high speed (70 kHz, 137 B-scans/s) SD-OCT operating at $\lambda_{\text{mean}} = 840$ nm. It is equipped with transversal motion compensation by a synchronized line- scanning ophthalmoscope (LSO)-based active retinal tracker, which is detailed further in [74]. Mis-tracked B-scans that showed a sub-threshold cross-correlation with a scan through the foveal pit were selected out. Figure 3(b) shows a representative average of 900 B-scans after flattening to the IS/OS-junction. We segmented this junction with OCT-SEG, a standalone MatLab OCT-segmentation application [75] and coupled it with additional automated scripts written in MatLab (R2011b, The MathWorks, Inc., Natick, MA) to correct for segmentation errors and for axial registration. For each B-scan, the retinal depth was calculated as the mean depth of the IS/OS-junction. Figure 3(c) shows a scatter plot of mean intensity in a region of interest (red box in Fig. 3(b)) versus retinal depth, before and after fall-off compensation. The data sets were fitted with models of the expected intensity decay, and of a linear (representing no correlation with depth), respectively. The correlation (Pearson’s R: $-0.54$, $P < 10^{-4}$) indicates that 29% of the signal variance is accounted for by axial motion. The correlation disappeared after compensation (R: $-0.008$, $P = 0.79$). In Fig. 3(d), intensity and retinal depth are plotted versus time, in both the corrected and uncorrected case, which shows that depth-dependent intensity fluctuations are substantially reduced.

Fig. 3. Effect of sensitivity fall-off on time-resolved SD-OCT imaging. (a) Measured (blue dots) sensitivity fall-off versus distance from zero optical path length delay and a Gaussian-fit (line) by using Eq. (2). (b) Mean image (n = 900) of motion-tracked B-scans after axial flattening to the IS/OS-junction. Red box: region of interest (ROI) of the IS/OS-junction, used to plot intensity data in (c) and (d). (c) Mean intensity versus retinal depth. Data is plotted before correction of sensitivity fall-off (red) and fitted with a model of the intensity decay with depth, which was calculated from sensitivity measurements shown in (a). The corrected intensity (blue) was fitted with the expected linear curve (dotted line). (d) Comparison of original (red line) and corrected (blue line) mean intensity of the ROI over time, plotted together with retinal depth (grey line), showing reduced variation with retinal depth after fall-off compensation. However, substantial variability in reflectivity remains. See text for details.
It should be noted that heartbeat causes axial shifts of $81 \pm 3.5 \, \mu m$ [22], in our case corresponding to about 17% intensity change. As compared to the frequently reported IOS-amplitudes of a few percent, and even compared to the largest reported IOS amplitudes (40% in individual OS in frog retina [42]), fall-off related noise can be substantial. Our results agree with previous suggestions [19] that compensation for fall-off may benefit in vivo IOS-measurements. It should be emphasized that conditions for which the spectrometer is sensitive, such as alignment, temperature and air flow, should be stabilized to avoid significant variations in fall-off. A hardware solution to the problem is offered by swept-source OCT, which inherently suffers less from fall-off by eliminating the sensitivity of the spectrometer to higher fringe modulation [76]. However, these systems operate at longer wavelengths (1050 nm), which comes with the disadvantage of a lower axial resolution.

3.4 Illumination artifacts

Variations in the eye’s light path and optical alignment can contribute to large-scale alterations in light transmission. These variations include head drifts that lead to optical axis misalignments, accommodation changes, media turbidity, floaters, and tear film quality, which colloquially contribute to illumination artifacts. A severe case of this variability can be observed when comparing the intensity of epiretinal reflections visible in Fig. 4(a) and 4(b) and the corresponding intensity of the outer retinal layers in two mean images corresponding to two different time periods of a B-scan time series. As explained in section 3.2, to allow for the extraction of IOS by calculating $\Delta I / I$ by means of Eq. (1), the differences in background intensity ($I$) caused by variable illumination must be reduced. Srinivasan et al. (2009) proposed a normalization method, by dividing the linear amplitude reflectance of a layer of interest to that of an inert (irresponsive to stimulation) reference layer. This was helpful, but not required to detect IOS by SD-OCT [16] This type of normalization rests on the assumption that reflectivity changes in both layers show a highly linear correspondence. To verify this, we analyzed (fall-off corrected) scans without stimulation according to [16] (Fig. 4). As shown in Fig. 4(c), there was a lack of linear correspondence in reflectance changes in the OS and RPE-layers, leading to inadequate compensation of illumination-related intensity fluctuations by normalization to the RPE-reflectance, shown in Fig. 4(d), indicating that this normalization method does not sufficiently normalize for illumination artifacts. This is probably due to differences in the intrinsic scattering properties of the two layers, such as angle-dependent scatter or tissue-specific attenuation coefficients.
Fig. 4. Influence of variable illumination on OCT reflectivity. Epiretinal specular reflections are prominent in mean intensity projections of scans at $t_{\text{end}}$ (a), but less so at $t_{\text{early}}$ (b), together with increased reflectivity in deeper layers. (c) The fractional reflectivity changes of a layer of interest, such as the OS (blue dots) and a reference layer, the OS/RPE-complex (red dots) do not show a highly linear correspondence. The axial positions of these layers (inset in the top right of (c)) were identical for each B-scan. We tested if normalization to the OS/RPE complex band is sufficient to suppress noise caused by variable illumination, as proposed previously [16]. The median value of the linear amplitude reflectance of these bands was determined for each A-scan (excluding vessel shadows). A random set of these values was averaged per B-scan, and the fractional reflectance relative to the first B-scan was determined. (d) The linear amplitude reflectance of the OS-band (blue dots) still shows the change in reflectance after normalization (green dots), although a partial compensation effect is evident.

For the purpose of IOS-imaging, it is possible to estimate the background illumination by modeling its attenuation as it propagates through an inert medium with relatively stable scattering. Calibration for changes in background illumination over time allows for an approximation of the tissue’s inherent scattering potential. Scattering declines exponentially with depth, and can be modeled as (modified from [77]):

$$I(z) = A_0 \exp(-2\mu z) + A_1,$$

where $I(z)$ is the reflectivity after light has traveled through a distance $z$ in a medium with attenuation coefficient $\mu$, and with $A_0$ and $A_1$ accounting for the illumination intensity of the incident light beam and the noise floor of the OCT image, respectively. The factor 2 is due to round trip attenuation. This single-scattering model is suited for modeling the exponential decline in the human retina [73]. By slope-fitting Eq. (3) to the decline of reflectivity with depth, with $\mu$, $A_0$ and $A_1$ as running parameters, changes in background illumination can be estimated. Compensation for the axial confocal point spread function [78] was not taken into account in the model, since our estimated ‘apparent’ Rayleigh length of 860 $\mu$m in tissue would affect the estimate of $\mu$ by only 0.29 mm$^{-1}$, which is small in comparison to typical $\mu_{\text{choroid}}$ values of 27 mm$^{-1}$ [79]. Since this approach is sensitive to speckle noise, A-scan averaging helps to lower speckle noise. For IOS-imaging, spatial averaging is preferable over temporal averaging. To estimate $\mu$ with sufficient confidence, reflectivity data from a minimum axial distance $d = \frac{1}{2} / \mu$, of the same tissue type, must be used [73].

The capillary lamina directly below the RPE/Bruch membrane, indicated in Fig. 5(a), appears to be a suitable reference layer for several reasons. First, it provides a sufficient...
fitting range \( d \) of 18.2 μm. Second, choriocapillary blood flow (0.3 – 3.6 mm/s) is slow relative to the choroidal blood flow, virtually steady, and this layer appears to be relatively inert for the fast IOS duration, as suggested by a recent fMRI study in rats showing slow choroidal blood volume changes upon single-flash stimulation (time to onset, \( \sim 1.5 \) s, peak, \( \sim 27 \) s). Flicker stimulation induced no changes [80]. Third, changes in light scattering in this layer caused by changes in \( \mu \) are expected to be small, because capillary pulsation is very weak (to diminish the danger of rupturing the thin capillary walls) and due to the uniformity in blood flow.

Speckle noise is a major source of noise in OCT images, and it is usually suppressed by spatial or temporal averaging. In the former, neighboring pixels in one image are spatially averaged (e.g., mean, median, or moving average). In the latter, the averaging occurs in time, for instance by averaging of B-scans. We suppressed speckle noise by A-scan averaging. Since changes in illumination typically occur slowly relative to the B-scan rate, the largest differences most likely arise from speckle noise. Figure 5(b) shows a representative averaged and modified A-scan with a curve fit.

Finally, the effect of background illumination on the pixel intensity is normalized:

\[
I_{\text{normalized}}(x,z,t) = \left( \frac{I_{x,z,t} - \overline{A}_t}{\overline{A}_0} \right) + \overline{A}_0, \tag{4}
\]

where \( \overline{A}_t \) and \( \overline{A}_0 \) are the time-averaged values of the illumination intensity \( A_t \) and the OCT noise floor \( A_0 \) at time \( t \), respectively. In Fig. 5(c), the mean intensity of the combined IS/OS-junction, outer segment tips, and RPE/Bruch membrane band at either sides of the foveal pit are shown. After correction by Eq. (4), the large-scale intensity changes have been reduced, although the corrected curves in Fig. 5(c) exhibit a temporal increase in reflectivity of equal magnitude, probably due normalization for reduced reflectivity of the choriocapillaris. In Fig. 5(d), both normalization techniques are directly compared, where more high-frequency noise is apparent after RPE/OS layer normalization. The coefficient of variation after normalization by the proposed technique was 6.88% versus 9.94% after RPE/OS layer normalization, which indicates the benefit of the proposed noise-suppression technique. Previous studies on slow IOS have shown that visual stimuli can induce a slow reduction of fundus reflections that are indicative of hemodynamic activation [81]. However, because slow IOS typically onset several seconds after visual stimulation, which is well beyond the signal duration of reported fast IOS, their effect on this correction procedure is of limited significance. Inconsistent sampling of the same tissue volume may be an additional factor, since a rotation or shift of the eye, compensated by the tracker, will change the angle of incidence of the beam onto the retina. Both factors may have affected the estimation of illumination parameters. A solution is to employ volumetric imaging with orthogonal scan patterns to verify consistent recording of the same tissue volume, and alternatively, a bite-bar can be used to abolish bulk displacements of the eye.
Fig. 5. Compensation for variation in background illumination in timelapse OCT-recordings.
(a) Mean image of axially flattened B-scans (n = 400). 125 A-scans per choriocapillary bed, indicated in yellow, were averaged and used to estimate the illumination parameters (see text). Vessel shadows were blotted out (b) Example curve fit of reflectivity with depth using Eq. (3). Shown in blue is the intensity with depth after noise suppression by A-scan averaging. The fitted curve is shown in green. (c) Effect of compensation. The mean intensity of a region-of-interest, comprising the IS/OS-junction, OS/RPE and RPE bands of the same A-scans used for averaging in (a), was used as an indication of general reflectivity changes over time (light blue: left side, orange: right side). After normalization using Eq. (4) of the same regions of interest on the left side (blue) and the right side (red), the large-scale intensity variations caused by misalignments of the optical axes of OCT and the eye have been reduced. (d) Direct comparison of normalization for reflectivity in the RPE/OS layer (blue line) and of normalization by the proposed technique (red line).

4. Conclusions
In this study, we analyzed the literature for significant findings in IOS-imaging, and we analyzed methods to compensate for noise derived from different sources. This led us to propose a hypothesis of the biophysical underpinnings of the fluctuation-type and reflectivity change-type of fast IOS. The hypothesis incorporates light scattering changes due to the potential involvement of protein binding and PDE-activity, which have thus far not been considered. This involvement could be the cause of stimulus-evoked scattering boundaries within the outer segments of cones [8, 9, 18, 21]. Further, this involvement could explain the existence of reflections within the outer segment on OCT and the random pattern in which fast IOS occur (Fig. 2) [42], although a differential polarity of fast IOS among rods and cones could serve as an alternative explanation. Important unresolved questions include 1)
whether the fluctuation and non-fluctuation types of fast IOS reflect (some of) the same processes; 2) whether PDE-activity and osmotic swelling are principally responsible for the generation of both types; and 3) whether IOS generated by rods and cones are distinct in signal polarity.

We analyzed important sources of noise in SD-OCT IOS-imaging. While illumination artifacts and the optical Stiles-Crawford effect have received only minor attention earlier [16], sensitivity fall-off and 1/f noise were in the focus [17, 19, 20]. Our data suggest that compensation for sensitivity fall-off suppresses noisiness in in vivo IOS-imaging by SD-OCT. We found that normalization by the RPE-reflectivity inadequately compensates for illumination artifacts. An alternative approach, based on slope-fitting of optical attenuation though the choriocapillaris, was found more adequate. By effective suppression of these illumination artifacts, it facilitates comparisons of reflectivity between stimulated and unstimulated areas.

Several lines of evidence suggest that the combination of lateral resolution and stability of retinal imaging are critical factors, which have been limited and so have prevented the acquisition of robust and reproducible IOS-measurements in awake humans by OCT. It is anticipated that with future improvements in hardware-based retinal trackers for high-speed AO-UHR-OCT setups [52], implementation of trackers providing higher imaging stability [53], and/or the application of appropriate registration methods [51, 54], these issues can be resolved. Our analysis of the various causes of imaging noise indicates that significant fluctuations in photoreceptor reflectivity may be caused by sensitivity fall-off or variability in illumination even in highly cooperative and healthy subjects. Results from previous studies [66] suggest that cone reflectivity can be substantially affected by the OSCE. The application of bite-bars addresses these three issues, and is likely to adequately suppress this noise. For the clinical implementation of IOS-imaging, however, the complexity and costs of these setups must be significantly reduced. Also, customized bite-bars are impractical to use in daily clinical practice. The currently proposed algorithms can serve as a basis for dedicated correctional post-processing algorithms that are most likely needed in the absence of bite-bars. Alternative means to calibrate for the OSCE other than bite-bars, however, remain to be explored.

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