Optical coherence tomography reveals light-dependent retinal responses in Alzheimer’s disease

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ABSTRACT

Spectral-domain optical coherence tomography (SD-OCT) is an accessible clinical tool for measuring structural changes to the retina, and increasingly as a biomarker for brain-predominant neurodegenerative diseases like Alzheimer’s. Information about retinal function can also be extracted from OCT images, but is under-studied, with literature examples often employing challenging protocols or requiring specialized hardware. The first goal of this study was to verify that functional retinal imaging was feasible with a commercially-available SD-OCT device and a clinically practical protocol. Inspired by methods from other functional imaging modalities, we acquired images while repeatedly cycling lights on and off, and spatially normalized retinas to facilitate intra- and inter-individual analyses. In eight healthy young adults, light-dependent increases in reflectivity were easily demonstrated at photoreceptor inner and outer segments, changing by ~7% in bright light and ~3% in dim light. Bright light elicited a subtle (~2%) but consistent light-dependent increase in reflectivity through much of the rest of the retina, including the avascular outer nuclear layer (ONL). We speculated that some of these changes are influenced by glial function – as through water management – a topic of high interest in neurodegenerative diseases that may involve the glymphatic system. Functional abnormalities in patients with antibodies against aquaporin-4 (n = 3) supported this interpretation. We next compared patients with early-onset Alzheimer’s disease (n = 14) to age-matched controls (n = 14), revealing that patients had a relatively exaggerated light-induced change in ONL reflectivity (p < 0.05). Because these measurements can be obtained within 30 min, regular use in research and limited clinical settings is feasible.

1. Background

There are subtle stimulation-induced changes in how neural tissues reflect and absorb light. These changes, often described as “intrinsic optical signals” (IOS), alter the tissue’s appearance in modalities like optical coherence tomography (OCT). In the retina, some of these changes are very fast – arising and returning to baseline within a few seconds – and can be observed in excised preparations lacking the retinal pigment epithelium (RPE) (Bizheva et al., 2006). Different, slower, IOSs have been observed in the photoreceptor layers of the retina (Yao and Wang, 2015). These build over several seconds and then wane over seconds-to-minutes. Unnoticed or absent in early studies of the excised retina (Bizheva et al., 2006), these changes were first reported at photoreceptors of the intact rat eye (Srinivasan et al., 2006), and later demonstrated in macaques (Suzuki et al., 2013) and humans (Tumlinson et al., 2009; Srinivasan et al., 2009). Our initial focus was on this slow light-dependent increase in reflectivity in avascular layers of the outer retina.

Being distinct from fast IOSs, non-vascular, and potentially dependent on the RPE, the precise origin of these slower IOSs (sIOSs) remained uncertain. Parallel to those observations on retinal reflectivity, a light-dependent increase in the distance between the photoreceptor ellipsoid zone and the RPE was documented in 2013 (Abramoff et al., 2013). In normal subjects, this volumetric change builds over a couple of minutes, then reverses after 10 min, before eventually normalizing (Abramoff et al., 2013; Lu et al., 2017). In patients with Best disease, who have abnormal chloride movement across the RPE, the initial light-dependent volume increase is exaggerated and prolonged. The demonstration of a similar light-dependent volumetric increase in mice (Li et al., 2016) was pursued by Zhang et al. (2017), who analyzed both reflectivity and volume changes in the same animals. They found a striking similarity in the time-courses and the specific graded responses of reflectivity and
volumetric changes to different light intensities, arguing that they share the same underlying physiology. Under a common hypothesis (Lu et al., 2017; Li et al., 2016), those structural and reflectivity changes result from light-dependent changes in hydration of the outer retina: Magnetic resonance imaging (MRI) of the mouse and rat retina demonstrates light-dependent increases in water diffusion and water content (Berkowitz et al., 2017, 2018; Bissig and Berkowitz, 2012), in agreement with older invasive studies of cat (Li et al., 1994), chick (Gavodovski et al., 1994), and frog (Huang and Karwoski, 1992) retinas that revealed a light-dependent increase in extracellular fluid volume. When the MRI-measured change in water diffusion is abolished by preventing phototransduction (Berkowitz et al., 2016), the volumetric OCT change is also abolished (Zhang et al., 2017). Recently, Berkowitz et al. (2018) showed that mouse strain differences in retinal mitochondrial activity—the necessary precursor to the metabolic water and waste production thought to drive this volumetric change—predicted strain differences in light-dependent retinal hydration, water diffusion, and OCT thickness changes. Importantly, there is at least one alternative hypothesis for the biology underlying sIOSs at the photoreceptors (Zhang et al., 2017), and the smaller-magnitude sIOSs reported over non-photoreceptor regions of the retina (Suzuki et al., 2013) are under-studied.

Regardless of the underlying biology of the sIOSs in avascular layers of the retina, a non-invasive marker of neuronal activity should have been enticing for exploratory studies of neurodegenerative disease, since functional changes generally precede structural changes. However, despite its discovery over a decade ago, we are aware of only a few studies of this phenomenon in healthy humans (Tumlinson et al., 2009; Cohen et al., 2011). The protocol used in prior experiments might discourage clinically-oriented exploration of this functional biomarker. This was one motivation for the current work, where we test the hypothesis that light-dependent reflectivity changes in avascular layers of the retina are detectable with commercially-available hardware and a practical stimulation protocol.

The second part of this study explores Alzheimer’s disease. In Alzheimer’s, the forty-two amino acid amyloid beta peptide (Aβ1–42) accumulates in the interstitial space, oligomerizes, forms plaques, and causes neuronal dysfunction and injury (Jarrett et al., 1993; Palop and Mucke, 2010; Iwatsubo et al., 1996; Minati et al., 2009). High tissue levels of Aβ1–42 are mirrored by low levels of Aβ1–42 in cerebrospinal fluid (CSF) (Strozyk et al., 2003), suggesting that there is impaired clearance from the interstitial fluid to the CSF. Akin to the retina, brain interstitial fluid volume is modified by neuronal activity, which seems to drive the sleep-dependent changes in Aβ clearance in mice (Xie et al., 2013). This waste clearance pathway is facilitated by astrocytic aquaporin-4 (AQ4): Animals lacking this channel have impaired clearance of Aβ (Iliff et al., 2012), and the sleep-dependent clearance of Aβ observed in humans (Shokri-Kojori et al., 2018) is modulated by AQ4 polymorphisms (Mazucchelli et al., 2018). This glymphatic (glia-dependent lymphatic-like) system is implicated in Alzheimer’s disease: Patients with Alzheimer’s have increased but mis-localized AQ4 channels (Zeppenfeld et al., 2017), and AQ4 polymorphisms predict their rate of cognitive decline (Burfeind et al., 2017). The retina generates the same Aβ isoforms as the brain, and the retinas of Alzheimer’s patients inappropriately accumulate Aβ1–42, although plaque formation is sparse (Alexandrov et al., 2011; Hoh Kim et al., 2010; Koronyo et al., 2017; Tsai et al., 2014). We therefore anticipate that the dysregulation of interstitial water and waste observed in the Alzheimer’s brain is also present in the retina. Insofar as sIOSs report on activity-dependent changes in interstitial fluid volume, we hypothesized that this functional OCT outcome would be abnormal in Alzheimer’s disease.

In the retina, only glia express AQ4 (Schev et al., 2014). The Müller glia predominate, and participate in water and waste management for most retinal layers (Bringmann et al., 2006). Since some retinal layers are glia-free, we realized that Alzheimer’s-related abnormalities may be retinal layer specific: Inferring from Best disease (Abramoff et al., 2013) a toxic effect of Aβ1–42 on the RPE (Liu et al., 2015) might exaggerate responses in the glia-free layers occupied by photoreceptor inner and outer segments. For the remaining glia-rich layers, we realized that Aβ4 may be both overexpressed and mis-localized in Alzheimer’s disease (Zeppenfeld et al., 2017). We were therefore unable to predict whether sIOSs would be exaggerated or blunted (or unchanged), and sought a separate clinical population to clarify expectations: We recruited patients with neuromyelitis optica and circulating auto-antibodies to AQ4. These patients are expected to have Müller glial injury and AQ4 downregulation (Felix et al., 2016) underlying electroretinogram abnormalities consistent with selective Müller glia dysfunction (You et al., 2019).

2. Methods

The study was approved by the Oregon Health and Science University (OHSU) IRB. Informed consent was obtained from all subjects prior to the study.

2.1. Experiment 1, light-dependent changes in healthy young adults

Participants: We imaged the right eye of eight healthy young adults (mean ± sd age 32.8 ± 5.1 yr, range 25–42 yr) without a history of ophthalmologic complaints (aside from corrected refractive error). These participants included three of the authors.

Environment and Stimulation: Data collection started in the morning (n = 3) or afternoon (n = 5). After consenting the participant under typical indoor clinical lighting, ambient lighting was minimized to 0.1 lx, with doors closed, room lights off, and a blackout curtain separating the OCT operator from the subject. Along with fixation targets, a residential white light emitting diode (LED) bulb was mounted at eye level on a flat white foam poster board, which was positioned 42 cm away from the participant. We chose an LED for light stimulation because it emits essentially no light in the near-infrared spectrum used for OCT imaging (Behar-Cohen et al., 2011). The “bright” condition used a 2,600 lx stimulus (1, 600 lm, generated by a 100 W-equivalent bulb), whereas the “dim” condition used a 140 lx stimulus (135 lm, from an 11 W-equivalent bulb). By comparison, a much more luminous LED light source viewed at a shorter distance (7,000 lm at 20 cm) for several minutes is regarded as “low risk”, typical daytime illuminance is > 10,000 lx, and retinal injury emerges in primates only after exposing a pharmacologically dilated eye to > 5900 lx for 12 h (Behar-Cohen et al., 2011; Sykes et al., 1981). The layout of fixation targets is shown in Fig. 1, and is meant to preferentially illuminate the span of retina between the fovea and the optic disc during light stimulation. Fixation on a separate target during image acquisition centers the same section of retina on the OCT beam. This portion of the retina is easy to locate based on retinal surface landmarks in infrared fundus images acquired during OCT imaging, and is populated by both rods and cones (Wells-Gray et al., 2016).

The protocol began with acquisition of a test image while the stimulus light was on, and after fixation just left of the stimulus light for 5 s. This duration of continuous light exposure is used in macular photostress recovery testing (Hammond et al., 2013), and is of middling duration compared to prior studies of the avascular sIOSs in the human retina: Whereas single brief (< 12 ms) flashes of light were used in one recent study (Lu et al., 2017), other studies use ~ 0.4 s (Tumlinson et al., 2009), 1.5 s (Srinivasan et al., 2009), or several minutes (Abramoff et al., 2013; Messner et al., 2019) of continuous light exposure. In macaques (Suzuki et al., 2013), both flash and continuous (> 16 s) light stimulation are effective, though the longer duration appeared to yield more robust responses in the near-foveal region, which is our focus. Although flickering
After the test OCT image was acquired, the stimulus light was turned off and the participant was asked to keep his or her eyes closed for the next 120 s. After this period of darkness, the participant fixated just left of the OCT beam, and the first (lights-)OFF image was acquired. Next, the stimulus light was turned on, and after briefly acclimating to this lighting change (for 15 s; see Fig. 1) the participant centered the stimulus light on span of retina that was just imaged. After 5 s of light stimulation, the participant again fixated just left of the OCT beam, and the operator collected the first ON image. Immediately thereafter, the stimulus light was turned off, and the participant again closed his or her eyes for 2 min, thereby starting the next OFF/ON cycle. In the “bright” condition, fifteen OFF/ON cycles (thirty OCT acquisitions) were collected per participant.

The “dim” condition (which preceded “bright” for half of the participants) used five OFF/ON cycles (ten OCT acquisitions).

**Acquisition Parameters:** High-resolution (3.89 μm, axially) images were acquired on a Heidelberg Engineering (Franklin, MA) SPECTRALIS OCT machine, which uses a broadband superluminescent diode (centered at 870 nm). A second infrared diode (815 nm) is used to acquire simultaneous fundus photographs. The SPECTRALIS Slice Planning Tool was used to select a single slice of the retina per subject, which was automatically re-selected for each acquisition. That single slice spanned 30° eccentricity, including and angled through the fovea and the center of the optic disc, generating the cross-sectional view seen in Fig. 2. Using the high speed (“HS”) mode on this device, we collected images at 8.8 frames per second. Automatic Real Time (ART) averaging was set to the maximum (100).

**Image Processing:** Retinas were cropped from raw reflectivity maps in ImageJ (v.1.47) (Schneider et al., 2012), resampled from the native image resolution to isometric pixels (3.89 μm × 3.89 μm) and the approximate position of the following structures was manually marked: the border between the vitreous and the retina, the border between the retinal nerve fiber layer (RNFL) and the ganglion cell layer (GCL), the border of the optic disc, generating the cross-sectional view seen in Fig. 2. Using the high speed (“HS”) mode on this device, we collected images at 8.8 frames per second. Automatic Real Time (ART) averaging was set to the maximum (100).
Fig. 2. Illustrative example of light-dependent changes in retinal reflectivity. Left: Average of images collected with bright lights ON and OFF for a young adult in Experiment 1. The difference image (ON minus OFF; lower left) shows a clear and contiguous band of tissue where reflectivity is greater with lights ON than lights OFF. Red rectangles mark the location of narrow strips of data used on the right of this figure. Note that these images are generated using the SPECTRALIS Scan Planning Tool, and – unlike all other analyses in this manuscript – utilize the Heidelberg Eye Explorer’s default 8-bit TIFF images. The scale for this difference image is therefore based on 8-bit grayscale values, which bear a complicated relationship to raw reflectivities. While this is convenient for illustrative purposes, since each acquisition is well-aligned at the time of export, all of our subsequent analyses were based on raw data extracted from “.sdb” files. Middle: an illustration of the different cell and tissue types in each retinal layer, which is aligned with the enlarged strips to the figure right. In pink, intersecting blood vessels depict vascular networks that are connected by small vertical anastomoses (Savastano et al., 2015; Campbell et al., 2017). All layers external to (below) the outer plexiform layer are avascular, until the choriocapillaris external to the RPE (Campbell et al., 2017). In gray, a Müller glial cell spans from the retina/vitreous border to the OLM, and RPE cells are shown just external to the photoreceptors. Neurons are drawn in white, depicting photoreceptors (spanning OPL to INL), bipolar cells (spanning IPL to OPL), and ganglion cells (dendrites in the IPL and axons continuing in the RNFL towards the optic nerve). To limit figure complexity, some cell types (e.g., horizontal cells and amacrine cells) are omitted, and Müller glia are depicted vertically, instead of gently following the contour of neuronal structures in the HFL. Right: Standard OCT layer labels are placed alongside strips of data from the ON and OFF conditions (shown side-by-side to contrast the photoreceptor layers) and from the difference image to the far right of the figure. RNFL = retinal nerve fiber layer; GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer, HFL = Henle fiber layer, OLM = outer nuclear layer, OLM = outer limiting membrane, ELIP = ellipsoid zone, INT = interdigitation layer, RPE = retinal pigment epithelium. Layers occupied by neuronal nuclei tend to be less-reflective than adjacent layers. The clearest light-induced increase in reflectivity occurs at the ellipsoid zone and interdigitation layer.

The in-plane beam angle can significantly impact reflectivity (Lujan et al., 2011). Because the length of each OFF/ON cycle exceeded 2 min, we realized that the alignment of OCT beam and the retina would drift over time as participants shift and settle in place. The angle was therefore re-measured in every image, and used as a covariate in all analyses of “angle-adjusted” reflectivities.

Of note, the image acquisition strategy used in this Experiment does not encode the beam angle perpendicular to the image plane (for these horizontally-oriented strips of retina, this beam angle in the vertical direction). This choice balanced reports that vertical beam angle has only modest influence on retinal reflectivities in this portion of the retina (Lujan et al., 2011; Wartak et al., 2017), with software limitations in the SPECTRALIS system. An alternative acquisition strategy was used in Experiment 2, allowing us to quantify the influence of the vertical “out-of-plane” beam angle. Because that acquisition strategy makes it harder for the operator to optimize image position and quality, it was not pursued in Experiments 3 and 4.

As seen in Fig. 3, retinas were linearized based on the contours of the RPE. In these flattened retinas, the position of aforementioned layers/structures was automatically refined based on local signal features. Subsequent steps were applied to the section of the retina that was preferentially illuminated when the stimulus light was ON: Measuring along the RPE, this spanned 500 μm to 2750 μm away from the center of the fovea, towards the optic nerve head. Since layer thickness varies between participants, and within each participant as a function of distance from the fovea, images were spatially normalized to a common template. At each distance from the fovea, we sampled signal at a specific number of points spaced evenly between the aforementioned anatomical borders: (a) 8 points between the vitreous/RNFL and the RNFL/GCL borders, (b) 22 points between the RNFL/GCL border and the IPL/INL border, (c) 16 points between the IPL/INL border and the OPL/HFL border, (d) 16 points between the OPL/HFL border and the OLM, and (e) 18 points between the OLM and the RPE. Where a layer is thick (e.g., RNFL near the optic disc), those points are spaced farther apart than where the layer is thin (e.g., RNFL near the fovea). By dynamically oversampling based on layer thickness, a universal map of signal intensities as a function of location is generated. Finally, this map is averaged to create a profile of reflectivity as a function of %Depth into the retina for each image (with the retina-vitreous border at 0%Depth, and the RPE at 100%Depth, and intervals of 1.25%Depth). For completeness, three points are evenly sampled between 4 μm and 12 μm interior to retina (into the vitreous) and six points between 4 μm and 24 μm exterior to the RPE (into the choriocapillaris) and placed at 1.25% intervals on this %Depth scale.

Missing and Excluded Data: No participants had gross signs of retinal pathology on infrared fundus images, nor on OCT images. Images were individually inspected to verify proper acquisition, adequate signal-to-noise, and the absence of imaging artifacts. In total, 16 of 320 images were excluded. Each participant had at least twelve “bright” images in both ON and OFF conditions, and at least four “dim” images per condition.

Reflectivity Normalization: In each profile of reflectivity as a function of %Depth into the retina, reflectivities were ratio-normalized to the RPE (i.e., the reflectivity at each specific %Depth was divided by the reflectivity at 100%Depth, and then multiplied by 100). After log-transformation, this sets the RPE’s reflectivity to 4.6 (i.e., In(100)) in all profiles, as depicted in Fig. 4. This planned approach matches that of two prior human studies (Tumlinson et al., 2009; Srinivasan et al., 2009). Preliminary analyses of pre-normalization “bright” data offered further reassurance: While there was no evidence that lights ON versus OFF affected RPE reflectivity (p = 0.28), much of the neural retina already showed the light-dependent changes (p < 0.05) catalogued below. Section 2.5 details additional post-hoc assessment of reflectivity normalization.

First Pass Analyses: We compared normalized reflectivities when the stimulus light was ON versus OFF. Due to inter-individual differences
shared by each condition – possibly due to melanin content (Wilk et al., 2017) – the groupwise estimate of the \%change between lights ON versus OFF is our primary focus. We test for a graded light-dependent response from the retina by testing whether that \%change is larger with the OFF is our primary focus. We test for a graded light-dependent response with \%Depth in

**Reflectivity and In-Plane Angle:** We used apparent retinal angle as a covariate while comparing ON and OFF normalized reflectivities. Based on prior work (Lujan et al., 2011), and qualitative observations of our own data, we suspected that it would sometimes be best to use the raw calculated angle, and other times to use the absolute value of the angle. We tested this at each \%Depth in “bright” data using a statistical model with OFF vs ON and |angle| as main effects, and compared this to a larger model that also included the sign of the angle. For a \%Depth where the |angle| is significant but the sign of the angle is not, microstructures are oriented either parallel or perpendicular to the retinal surface, and only the magnitude of the in-plane angle between the retina and the OCT beam is important. Where both the sign and magnitude of the angle matters – as expected for the HFL (Lujan et al., 2011) – microstructures are oriented oblique to the retinal surface.

**General Statistical Approach:** Using the geepack library for R, serial repeated measures from each OFF/ON cycle are entered into generalized estimating equation (GEE) models with an autoregressive correlational structure. Since models are created at most \%Depths – excluding the vitreous, the \%Depth used for normalization (RPE at 100\%Depth) and

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**Fig. 3. Workflow for raw OCT images.** Using ImageJ, 16-bit raw data are imported from “.sdb” files generated by the SPECTRALIS system. These images show the retina before any alignment/registration steps, allowing the apparent retinal angle to be calculated (see Fig. 5), and values of each pixel are directly proportional to log-transformed reflectivities. One such image is shown in A. The center of the fovea is manually marked (near-vertical white line), and the retina is linearized using the gently curving RPE as a landmark (for illustrative purposes, the white line is placed exterior to the RPE). Only the span of retina between the fovea and the optic nerve head is analyzed. The result of the linearization step, B, spans from 0 to 2750 μm from the fovea’s center. Since each image collected from a participant is processed the same way, this step aligns images, regardless of apparent retinal angle. However, combining data near versus far from the fovea would be challenging, as the thickness of each layer changes. Inter-individual comparisons are similarly complicated by person-to-person differences in layer thicknesses. The solution is to resample each “column” of pixels in B, proportional to the size of the span between specific anatomical landmarks, which are labeled at the far right of this figure. For instance, eight samples are collected between the vitreous/RNFL border and the RNFL/GCL border, selected at equidistant intervals proportional to the thickness of the RNFL. (To make a legible figure, these are illustrated by four white hash marks, which are farthest apart where the RNFL is thickest.) Between 500 and 2750 μm from the fovea’s center, these data are mapped onto a standardized template image that approximates the relative position of anatomical landmarks midway between the fovea and the optic nerve, according to how deep they are into the retina. As shown in C, the vitreous/RNFL border is placed at 0 \%Depth, and data from the RNFL occupies 0 to 10 \%Depth. The hash marks in the RNFL are now equidistant to one-another. The GCL and IPL occupy 10 to 37.5\%Depth. The INL and OPL occupy 37.5 to 57.5\%Depth. The avascular layers with glia – the HFL and ONL – occupy 57.5 to 77.5\%Depth. The avascular layers lacking glia – photoreceptor inner and outer segments, and midway through the RPE – occupy 77.5 to 100\%Depth. Inspired by analyses used in MRI (Bissig and Berkowitz, 2012), this approach maps OCT data onto a uniform template. From here, data can be averaged to make a single reflectivity-versus-\%Depth profile for each image. Those profiles can be averaged within a single subject, or compared across subjects as in Fig. 4.
These first-pass analyses clearly illustrate the main findings of Experiment 1, although they do not account for variance introduced by the in-plane angle of the infrared OCT beam, as illustrated in Fig. 5. **Top:** Group-average reflectivities from the “bright” light condition (upper chart) and “dim” light condition (lower chart), after ratio-normalization to the RPE (set to 100) and log-transformation. The vertical expanse of each mark shows the mean ± standard error, while marks for OFF data (black) are thicker, so as to be visible behind marks for ON data (white). In this view, much of the variance in reflectivities is explained by person-to-person differences that persist whether lights are on or off, obscuring small but reliable light-dependent changes that are visible when calculated for each participant. **Middle:** Illustration of cell types within each retinal layer, as in Fig. 2. Here, the illustration is aligned with %Depth and layer markers for the plots. **Bottom:** The %change in reflectivity when the stimulus light is ON versus OFF (i.e., 100% [on – off]/off). Means ± standard errors (dot and vertical lines) are connected to better-visualize the band of data generated from “bright” (red) and “dim” (blue) conditions. Significant effects of light are found through most of the retina (red * indicates p < 0.05 for the OFF versus ON comparison in bright light); light decreases reflectivity interior to (in this figure, to the left of) the OLM, and increases reflectivity over the photoreceptor inner and outer segments. Analyzing only “dim” data, significant effects of light were noted in the ONL, ellipsoid zone, and just exterior to the RPE (blue *; p < 0.05). Light-dependent changes were significantly larger in “bright” than “dim” conditions in the ellipsoid zone (black *; p < 0.05). There, the constellation of findings demonstrates a graded light-dependent increase in reflectivity. At every %Depth with significant findings, the GEE model (“bright” versus “dim”, lights OFF versus ON, and when it fell below p < 0.05) the interaction term, fell below an FDR threshold of q = 0.05. To simplify significance markings, we did not mark the only area (82.5–83.75%Depth) where there was a main effect of light intensity (bright > dim reflectivities): The effect was driven by the significant “bright”>”dim” difference when the light was ON, that was unopposed by a trend (p > 0.05) in the same direction when the light was OFF.

Fig. 4. Light-dependent changes in retinal reflectivity in young adults. These first-pass analyses clearly illustrate the main findings of Experiment 1, although they do not account for variance introduced by the in-plane angle of the infrared OCT beam, as illustrated in Fig. 5. **Top:** Group-average reflectivities from the “bright” light condition (upper chart) and “dim” light condition (lower chart), after ratio-normalization to the RPE (set to 100) and log-transformation. The vertical expanse of each mark shows the mean ± standard error, while marks for OFF data (black) are thicker, so as to be visible behind marks for ON data (white). In this view, much of the variance in reflectivities is explained by person-to-person differences that persist whether lights are on or off, obscuring small but reliable light-dependent changes that are visible when calculated for each participant. **Middle:** Illustration of cell types within each retinal layer, as in Fig. 2. Here, the illustration is aligned with %Depth and layer markers for the plots. **Bottom:** The %change in reflectivity when the stimulus light is ON versus OFF (i.e., 100% [on – off]/off). Means ± standard errors (dot and vertical lines) are connected to better-visualize the band of data generated from “bright” (red) and “dim” (blue) conditions. Significant effects of light are found through most of the retina (red * indicates p < 0.05 for the OFF versus ON comparison in bright light); light decreases reflectivity interior to (in this figure, to the left of) the OLM, and increases reflectivity over the photoreceptor inner and outer segments. Analyzing only “dim” data, significant effects of light were noted in the ONL, ellipsoid zone, and just exterior to the RPE (blue *; p < 0.05). Light-dependent changes were significantly larger in “bright” than “dim” conditions in the ellipsoid zone (black *; p < 0.05). There, the constellation of findings demonstrates a graded light-dependent increase in reflectivity. At every %Depth with significant findings, the GEE model (“bright” versus “dim”, lights OFF versus ON, and when it fell below p < 0.05) the interaction term, fell below an FDR threshold of q = 0.05. To simplify significance markings, we did not mark the only area (82.5–83.75%Depth) where there was a main effect of light intensity (bright > dim reflectivities): The effect was driven by the significant “bright”>”dim” difference when the light was ON, that was unopposed by a trend (p > 0.05) in the same direction when the light was OFF.

2.2. Experiment 2, other experimental parameters in healthy young adults

**Participants:** We scanned the right eye of four healthy young (32.2 ± 5.6 yr) adults, including three who were scanned in Experiment 1.

**Environment and Stimulation:** The colorful fixation targets from Experiment 1 subtended <2.7° visual angle (19 mm diameter). While target features conceivably could influence the central-most fovea during fixation, they should not influence the slightly more eccentric region of retina analyzed throughout the present work. It was nevertheless desirable to test for an effect, as alternative targets may be preferable in future studies. Three blocks of five bright light OFF/ON cycles (totaling thirty OCT acquisitions) were collected per participant. For one block, we replaced each fixation target from Experiment 1 with a small (2 mm diameter) dot of glow-in-the-dark Elmer’s glue. The overall environment and bright light stimulation parameters were unchanged.

**Acquisition Parameters:** Here, we used an imaging mode that allowed acquisition of two large (spanning 30° eccentricity) slices of the retina in perpendicular image planes centered on the fovea. Because software limitations prevent rotation of slice orientations in this mode, we could not follow the practice in other experiments of selecting a slice that intersects both the fovea and optic disc. In its place, the horizontal slice centered on the fovea offered a fair approximation. The vertical slice was only of interest because of its ability to report on the OCT beam angle out-of-plane from the horizontal slice. In an attempt to maximize the range of out-of-plane angles, one block of five OFF/ON cycles were collected after manually tilting the OCT beam/camera apparatus >10°. However, the angle at which the beam meets the retina is also influenced by entry point of the beam through the pupil, which is optimized by experienced operators (by monitoring image quality, and translating the apparatus up or down as needed). In retrospect, it was therefore unsurprising that this manipulation had no impact on the vertical angle of the OCT beam with
the retina ($p > 0.4$; interquartile range of the apparent retinal angle was $1.26^\circ$ to $2.32^\circ$ in the tilted setup, versus $2.79^\circ$ to $1.15^\circ$ in the usual setup), and these two five-cycle blocks were therefore combined. All other parameters were identical to Experiment 1.

Image Processing and Missing and Excluded Data: Raw images of the vertically-oriented slices were used to measure the apparent angle of the central 500 $\mu$m of the foveal RPE. This value reports on the vertical angle of the OCT beam, which is out-of-plane for our horizontal slice of the retina. All other analyses used the horizontal slice of the retina, processing it just as we did for fovea-to-disc images in Experiment 1. In total, 9 of 60 images were excluded (five OFF and four ON images). This higher percentage of exclusions (15%, compared to 5% in Experiment 1) is explained by a second software limitation of this imaging mode: Only the vertically-oriented slice is presented in real time for the operator to optimize image quality.

Reflectivity Normalization, First Pass Analyses, and the General Statistical Approach were unchanged from the first experiment, except that both in-plane and out-of-plane angle were available as covariates.

2.3. Experiment 3, light-dependent changes in patients with neouromyelitis optica

Participants: Anti-AQ4 antibody titers guided patient recruitment: Disease severity is generally correlated with titer (Takahashi et al., 2007), and so those with marginal lab results were not considered. Since the antibodies themselves cause a direct, selective, and at least partially reversible Müller glial injury (Felix et al., 2016), it would therefore be difficult to interpret responses in a patient who has become seronegative, as sometimes occurs after treatment with rituximab (Valentino et al., 2016). We therefore only considered those for whom there was recent verification of seropositivity. As it is uncommon to follow titers longitudinally as part of routine clinical care, this limits the pool of participants for this already uncommon neuroinmunological disease. Finally, because we wanted to avoid the complicated immunopathology of an acute flare of this disease, only those at their neurological baseline and receiving care in our outpatient clinic were considered.

We scanned the right eye of all three patients (age: $30.6 \pm 9.4$ yr) that met our search criteria: One man in his mid-20s had been diagnosed over ten years prior, and had been persistently seropositive despite years of treatment with rituximab. There had been no recent changes in his therapy, and seropositivity (1:1000 titer) was last documented 57 d before OCT. He retained only a slight pupillary response and no conscious light perception in the scanned eye. One woman in her mid-20s had carried her diagnosis for eight years, and had been persistently seropositive despite years of treatment with rituximab. Seropositivity (1:10000 titer) was documented on the same day as OCT, when she had subjective color desaturation, full visual fields, and 20/40 Snellen acuity. The third participant, a woman in her early-40s, was diagnosed after two lifetime clinical events (vision loss in the left eye, and later loss in the right eye) occurring within the last two years, from which she had full subjective recovery and intact visual fields with 20/20 Snellen acuity on the day of her OCT scan. Her scans occurred four months after documented seropositivity (>1:10,000 titer), at which time she was treatment naive. The only intervening treatment had been intravenous corticosteroids with a brief oral taper. Because that treatment has an incomplete and predictable effect of reducing anti-AQ4 antibody titers (Takahashi et al., 2007), we calculate that titers were in excess of 1:1000 on the day of the scan.

Environment and Stimulation: Five bright light OFF/ON cycles (ten OCT acquisitions) were collected per participant. All other parameters were unchanged from Experiment 1.

Acquisition Parameters, Image Processing, Reflectivity Normalization, the General Statistical Approach, the assessment of Retinal Thicknesses, and handling of Missing and Excluded Data were essentially unchanged from Experiment 1. Only one image was excluded, an ON image from the third patient. The first patient had extensive degeneration of the GCL, and data assigned to the GCL area by the spatial normalization program were therefore censored.

Analyses: We tested whether light-dependent changes in reflectivities differed between patients seropositive for anti-AQ4 antibodies versus the similarly-aged control participants in Experiment 1. This comparison (i.e., OFF/ON × group interaction) was performed both with and without using in-plane angle as a covariate. At each %Depth, the decision on whether to use the apparent in-plane angle versus its absolute value was carried over from Experiment 1. Individual patient data (with intra-individual means and standard errors) were also examined in detail.

2.4. Experiment 4, light-dependent changes in Alzheimer’s patients

Participants: We enrolled sixteen patient-plus-healthy-control dyads from the OHSU Aging and Alzheimer’s clinic. We focused on patients with early-onset Alzheimer’s dementia: Compared to late-onset patients, this relatively younger cohort will have less coincidental ocular and systemic disease burden that might influence results. Their study partner, usually a similarly-aged spouse, is likely to be free of prodromal Alzheimer’s disease, but shares the patient’s lifestyle and socioeconomic status. Patient demographics, clinical information, and exclusions are detailed in Table 1. Two study partners were excluded, one had Parkinson’s disease, and dyskinesias limited participation. The other had a self-reported history of right eye retinal detachment affecting his central vision. Groups were well-matched for age and sex in the final analyses, which used right eye images from 14 patients (57% women; age 60.5 ± 5.1 yr) and 14 controls (50% women; age 60.4 ± 7.0 yr).

Environment and Stimulation: Five bright light OFF/ON cycles (ten OCT acquisitions) were collected per participant. The choice to use five cycles was based on the fairly strong results in Experiment 1, and to facilitate participant recruitment and comfort: Because both a patient and study partner were scanned sequentially, sometimes after a planned clinic visit, we anticipated that spending more than 30 min per person acquiring images would have been onerous. All other parameters were unchanged from Experiment 1.

Acquisition Parameters, Image Processing, Reflectivity Normalization, the General Statistical Approach, and assessment of Retinal Thicknesses were unchanged from Experiment 1. For statistical purposes, each patient is presumed to be unrelated to their study partner.

Missing and Excluded Data: The Alzheimer’s patient in the first patient/partner dyad had a sub-RPE deposit (presumably drusen) near the fovea, but the rest of the retinal strip had a benign appearance, visual fields were full, and Snellen acuity was 20/30-2. Reflectivities were calculated using the span from 1000 to 2750 $\mu$m from the fovea, bypassing the deposit. Although the Alzheimer’s patient in the 11th dyad had an abnormally thin RPE directly at the fovea (within 150 $\mu$m of its center; outside of our area of interest), all data were retained due to the otherwise benign appearance of the retina, normal visual fields, and 20/30-2 Snellen acuity. Again, all images were reviewed for quality, and only five were excluded (each in a different participant), leaving 275 images.

Analyses: We tested whether light-dependent changes in reflectivities differed between patients with Alzheimer’s disease versus age-matched controls. Analyses were run both with and without in-plane angle as a covariate. At each %Depth, the decision on whether to use the apparent in-plane angle versus its absolute value was carried over from Experiment 1.

2.5. Post-hoc assessment of life-span changes

Control data from Experiments 1 and 4 were combined, and select analyses re-run with age as a covariate. We first tested for the expected slow thinning of the retina with age (uncorrected threshold p-value of 0.05, given prior positive findings in other studies) (Neuville et al., 2009) and compared these aging trends in Alzheimer’s patients. Upon co-plotting the light-dependent and in-plane beam angle-dependent
calculations based on a per condition per participant. A best-
on average (AON) and the ellipsoid zone (sampled at 86.25\%Depth). We RPE-normalized bright light Experiment 1 data for the ONL (sampled at another 100 simulations excluded all but four deviations from these simulations were stored and later averaged. Another 100 simulations excluded all but five OFF and ON images per participant, and another 100 simulations excluded all but five OFF and ON images per participant, and so on, until twelve images were permitted per condition per participant. A best-fit line of these data described how increasing the number of OFF/ON cycles could reduce variance. Power calculations based on a t-test were used to translate these data into changes in reflectivity in younger (Experiment 1) and older (Experiment 4) controls, we noted a few layers where the groups appeared to diverge. We therefore tested for the influence of age on those reflectivity changes, and clustered findings (i.e., multiple contiguous \%Depths wherein p < 0.05) from this unplanned analysis are briefly discussed.

### 2.6. Post-hoc assessment of alternative normalization strategies

Combining Experiments 1 and 4, we collected bright light OFF vs ON data for n = 36 participants without a sight-threatening condition. With this large dataset, we evaluated the general pattern of light-dependent changes in retinal reflectivity under different normalization schemes: (1) no normalization of reflectivity, (2) normalization to the inner plexiform layer as in one prior study (Lujan et al., 2011), (3) normalization to the RPE (Tumlinson et al., 2009; Srinivasan et al., 2009) as detailed above and favored throughout, and (4) use of the average reflectivity across the retina, analogous to the “total retinal reflectance” used in an alternative analysis of one prior human study (Srinivasan et al., 2009). Data were adjusted for in-plane beam angle.

### 2.7. Post-hoc consideration of acquisition strategies

The present data can offer guidance for future studies on how many OFF/ON cycles to use and how many participants to enroll. We reviewed RPE-normalized bright light Experiment 1 data for the ONL (sampled at 72.5\%Depth) and the ellipsoid zone (sampled at 86.25\%Depth). We performed a sensitivity analysis, and randomly excluded all but three OFF images and all but three ON images per participant, and re-ran GEE analyses of light-dependent reflectivity changes with in-plane beam angle as a covariate. That was repeated 100 times, and mean and standard deviations from these simulations were stored and later averaged. Another 100 simulations excluded all but four OFF and ON images per participant, and another 100 simulations excluded all but five OFF and ON images per participant, and so on, until twelve images were permitted per condition per participant. A best-fit line of these data described how increasing the number of OFF/ON cycles could reduce variance. Power calculations based on a t-test were used to translate these data into participant sample size estimates.

Since five OFF/ON cycles were used in Experiments 3 and 4, sample size estimates for replication studies presumed the same number of cycles.

Finally, while our approach was to include OCT beam angle as a covariate, and then to calculate what reflectivity would be if the beam were perfectly perpendicular to the retina, we realized that some may prefer to simply exclude any images collected with the beam tilted beyond a certain threshold. This is included as a sensitivity analysis, with a threshold of [1°], and aggregates RPE-normalized data from Experiments 1 and 4.

### 3. Results

#### 3.1. Experiment 1

**First Pass Analyses:** As shown in Fig. 4, virtually the entire retina shows significant light-dependent changes. Patterns cluster by anatomical layer:

- In the area occupied by Müller glia – from the RNFL to the OLM – reflectivities are lower with the stimulus light ON than with it OFF. Although the strength of this main effect varies somewhat between anatomical layers (e.g., until we corrected for beam angle, variance remained high in the HFL) median p-values extracted from each layer consistently show p < 0.003. This effect is driven largely by “bright” data: When “dim” data are analyzed in isolation, only the ONL has OFF/ON comparisons where p < 0.05. Indeed, portions of the IPL, INL, and OPL show significant OFF/ON × “bright”/“dim” interactions.

- In the area between OLM and the RPE, which is avascular, glia-free, and is occupied only by photoreceptor inner and outer segments, reflectivities are higher with the stimulus light ON than with it OFF (at several \%Depths, p < 0.00001). Again, this effect is driven largely by “bright” data, although when “dim” data are analyzed in isolation, the OFF/ON comparison falls below p < 0.05 at the ellipsoid zone. At the same place, there is an OFF/ON × “bright”/“dim” interaction. Taken together, these findings demonstrate a graded response to light.

- Exterior to the RPE – in the area occupied by the choriocapillaris – reflectivity is slightly higher with the stimulus light ON than with it OFF.
and not impacted by light intensity.

Reflectivity and In-Plane Beam Angle: Using “bright” data, we found that reflectivity in several retinal layers was influenced by the in-plane angle of the OCT beam, which is encoded in images by the apparent angle of the retina. In most cases, |angle| provided an excellent description of this effect. Consistent with expectations (Fig. 5) the exception was data at %Depths of 55–67.5 inclusive, corresponding to the HLF: There, the sign of the angle significantly improved statistical models. For subsequent comparisons of reflectivities, we included either the angle of the retina (at %Depths of 55–67.5) or |angle| (all other % Depths) as a covariate. For concise presentation, angle-adjusted data from this experiment are visualized alongside those from Experiment 3 (Fig. 7), which shows the different retinal layers impacted by the in-plane angle of the OCT beam. Light has a clear impact on angle-adjusted reflectivities, with statistical conclusions essentially unchanged from the first-pass analyses of “bright” data (compare Figs. 4 and 7). Upon adding angle as covariate in “bright”/“dim” comparisons, the ellipsoid zone OFF/ON × “bright”/“dim” interaction was attenuated (p = 0.051) but most of the interactions noted in layers occupied by the Müller glia remained significant.

Probing the data for OFF/ON × in-plane angle interactions, these were noted in the RNFL (p < 0.05 from 6.25 to 8.75% depth) where reflectivities became insensitive (p = 0.39) to |angle| with lights were ON, but remained angle-dependent when lights were OFF. They were also noted in more external layers of the HFL (65–67.5%Depth), where reflectivities remained sensitive to angle in both conditions, but angle had slightly greater influence on reflectivity while the lights were ON than OFF.

Retinal Thicknesses: We tested GEE models with main effects of lights ON versus OFF and “bright” versus “dim” light stimuli. No interactions were significant, and these were dropped from the models. Whole retinal thickness was unaffected by experimental conditions (p > 0.05; all condition-specific mean ± sem values were either 332 ± 4 μm or 333 ± 4 μm). The distance between the OLM and the RPE was similarly unaffected (p > 0.05; all condition-specific values were 71 ± 1 μm). RNFL thicknesses showed a statistical change (p = 0.00002), but the magnitude change fell far below available image resolution of 3.89 μm (for the bright condition, thickness with lights OFF was 35.4 ± 1.2 μm, increasing by 0.4 ± 0.2 μm with lights ON; for the dim condition, this increase was by 0.7 ± 0.2 μm, from the OFF value of 34.5 ± 1.1 μm).

3.2. Experiment 2

Fixation Target: In “first pass” models that ignore beam angle, and in
models including either in-plane or out-of-plane beam angle, we found no effect of the type of fixation target on light-dependent changes within the retina (at all %Depths, \( p > 0.07 \) for the \( \text{OFF/ON} \times \text{target interaction} \)). Target type was therefore ignored, and data combined for the subsequent analyses.

First Pass Analyses: Findings in these young adults shown “bright” light are similar to Experiment 1: In the area occupied by Müller glia – spanning both vascular and avascular layers – reflectivities are lower with the stimulus light ON than with it OFF. The opposite pattern is found in the avascular and glia-free layers between the OLM and the RPE (Fig. 6).

Reflectivity and In-Plane Beam Angle: Like in Experiment 1, the sign of the beam angle improved model fits through the HFL and immediately-adjacent tissue (here, 47.50 to 68.75%Depth). The sign of the angle also improved fits in a few scattered depths near the OLM and the interdigitation layer. For simplicity, we followed the convention from Experiment 1 of using the |angle| at all %Depths outside of the HFL (55–67.5%Depths). We again found reflectivity in several retinal layers was influenced by the in-plane angle of the OCT beam (Fig. 6), especially in layers where there are densely-packed and well-aligned microstructures; the RNFL, the HLF, the ellipsoid zone, and the interdigitation layer.

Of uncertain significance, some inner retinal layers (GCL through INL) and the OML appeared slightly more reflective when the in-plane beam angle was slightly tilted.

Reflectivity and Out-of-Plane Beam Angle: The sign of the beam angle did not improve model fits at the pre-selected statistical threshold. We therefore used the |angle| at all %Depths. For most layers, the cylindrical microstructures thought to mediate the influence of beam angle on retinal reflectivity course within the image plane (e.g., photoreceptor axons in HFL; see Fig. 5), leading us to predict minimal effect of out-of-plane beam angle. Consistent with expectations, effects of out-of-plane beam angle were sparse and low-magnitude (Fig. 6).

Reflectivity when Accounting for Both Beam Angles: Statistical models using both angles as covariates showed non-zero influence of out-of-plane beam angle at the HFL and over the photoreceptor inner and outer segments, but the magnitude of the effect remained quite small (e.g., at the HFL, signal changes by less than 0.4% per 1° of out-of-plane tilt) (Fig. 6).

Overall, statistical conclusions regarding the effect of light on reflectivity were unchanged by accounting for beam angle.

3.3. Experiment 3

First Pass Analyses: Data from each subject are shown alongside the mean control data from Experiment 1 (Fig. 7). Trends visible in the group average data corrected for in-plane angle are also visible in each individual: All show a grossly normal light-dependent increase in reflectivity in the glia-free layers occupied by photoreceptors (between the OLM and RPE), but an abnormal and quite large light-dependent increase in reflectivity in the most vitread portions of the RNFL. Combined AQ4 Ab + data indeed showed an abnormal light-dependent increase in RNFL reflectivity (e.g., \( p = 0.0003 \) at 1.25%Depth), but a normal-appearing light-dependent decrease in the ONL (e.g., \( p < 0.0001 \) at 71.25% Depth) and light-dependent increase at the ellipsoid zone (e.g., \( p < 0.0001 \) at 86.25%Depth). The interaction (\( \text{OFF/ON} \times \text{group} \)) was significant at the RNFL (e.g., \( p < 0.0001 \) at 1.25%Depth) and at the OLM (e.g., \( p = 0.0059 \) at 76.25%Depth).

Reflectivity and In-Plane Beam Angle: Including in-plane beam angle into statistical models did not change the overall pattern of light-dependent changes documented in the first pass analyses of AQ4 Ab + patients (e.g., \( p < 0.0001 \) at 1.25%Depth, \( p = 0.0002 \) at 71.25%Depth, and \( p < 0.0001 \) at 86.25%Depth) which is shown in Fig. 7. Again, the light-dependent change in these patients differed significantly from the reflectivity change observed in similarly-aged controls from Experiment 1, with the interaction (\( \text{OFF/ON} \times \text{group} \)) significant at RNFL (e.g., \( p < 0.0001 \) at 1.25%Depth) and at the OLM (e.g., \( p = 0.0030 \) at 76.25% Depth).

For much of the retina, in-plane beam angle had a similar influence on reflectivity as seen in controls (e.g., \( p < 0.0001 \) through most of the HFL and the exterior-most portions of the interdigitation layer) with the notable exceptions of the area at and around the OLM (e.g., \( p < 0.0001 \) at 76.25%Depth) and what seems to be an exaggerated sensitivity to beam angle at the RNFL (Fig. 7). Due to low recruitment and the risk of model overfitting, we opted against post-hoc group comparisons of the angle-dependence of reflectivity.

3.4. Experiment 4

Reflectivity and In-Plane Beam Angle: When analyzing Alzheimer’s and control participants aged >45 yr, we again found that reflectivity in several retinal layers was influenced by the in-plane angle of the OCT beam. These findings are detailed in Fig. 8, which also shows the impact of light on angle-adjusted reflectivities for each group. Examining the main effect of the stimulus light being OFF versus ON, these data generally reproduced the finding that light reduces reflectivity in the much of the span occupied by Müller glia, but increases reflectivity between the OLM and the RPE.

Critically, in the ONL (68.75–75%Depth) and directly adjacent to the OLM (78.75%Depth) light decreased reflectivity significantly more so in patients with Alzheimer’s than in controls (\( p < 0.05 \) for \( \text{OFF/ON} \times \text{group} \) interaction). This finding persisted when we included in-plane beam angle in statistical models, and is well-captured by the angle-adjusted data in Fig. 8.

Retinal Thicknesses: Whole-retinal thickness, RNFL thickness, and the distance from the OLM to the RPE were unaffected by group (\( p > 0.2 \)) and there were no significant group \( \times \) lighting interactions (\( p > 0.05 \)). Only for whole retinal thickness was there a statistical effect of light (\( p = 0.018 \)), which was driven by a magnitude change in the Alzheimer’s group that fell far below available image resolution of 3.89 \( \mu \text{m} \) (thickness with lights OFF was 324.2 \( \pm \) 4.7 \( \mu \text{m} \), which decreased when the stimulus light was turned ON, changing by \( -0.7 \pm 0.3 \mu \text{m} \), \( p = 0.006 \)). In age-matched controls, no such pattern emerged (OFF 318.5 \( \pm \) 3.7 \( \mu \text{m} \), change by \( -0.6 \pm 0.2 \mu \text{m} \)).

3.5. Post-hoc assessment of life-span changes

Combining control participants from Experiments 1 and 4, we found age-related declines in whole retinal thickness (per decade change of \(-4.0 \pm 2.0 \mu \text{m} \); \( p = 0.047 \)) and in the distance between the OLM and the RPE (per decade change of \(-1.3 \pm 0.3 \mu \text{m} \); \( p = 0.0002 \)), but RNFL thickness remained stable (\( p = 0.7 \)). When Alzheimer’s patients were compared to these lifespan trends, no significant group \( \times \) age interactions emerged.

In some retinal layers, the light-dependent changes in reflectivity appeared greater in younger (Experiment 1) versus older (Experiment 4) control patients, as plotted in Fig. 8. Statistical testing generally supported this visual impression (\( p < 0.05 \)) at the outer nuclear layer (67.5–78.75%Depth). In other retinal layers, the impact of the in-plane OCT beam angle seemed stronger in in older versus younger control patients’ retinas. Statistical testing generally supported this visual impression (\( p < 0.05 \)) at the Henle fiber layer (62.5–67.5%Depth).

3.6. Post-hoc assessment of alternative normalization strategies

The \( p \)-values and light-provoked %change in reflectivity are summarized with representative points from several layers in Table 2. Regardless of normalization strategy, the overall pattern observed in Experiments 1 and 4 remains: There is a light-dependent decrease in reflectivity in multiple retinal layers occupied by Müller glia, but a light-dependent increase in reflectivity in glia-free layers (represented in Table 2 by data from the ellipsoid zone). In the absence of a normalization step, there was no light-dependent change in the RPE.
Fig. 6. Light-dependent changes in retinal reflectivity persist after accounting for the in-plane and out-of-plane angles of the OCT beam, which influence reflectivity in a layer-specific fashion. These “bright” data are from Experiment 2. For each y-axis, “%change” refers to the %change in RPE-normalized reflectivity. Top: Illustration of cell types within each retinal layer, aligned with %Depth and layer markers for the plots. A: Means ± standard errors (dot and vertical lines) of the %change in reflectivity when the stimulus light is ON versus OFF (i.e., 100 * [(on – off)/off]). In this “first pass” analysis before accounting for beam angle, patterns are broadly similar to those in Fig. 4, in that light decreases reflectivity interior to (in this figure, to the left of) the OLM, and increases reflectivity over the photoreceptor inner and outer segments (*; p < 0.05). B: The data presented in panel A are re-analyzed after including out-of-plane beam angle as a covariate. When beam angle is included as a covariate, the %change values for the ON/OFF comparison are estimates for when the beam is perfectly perpendicular to the retina within the stated plane (i.e., apparent angle of retina = 0°). This step has little effect on the reflectivity changes measured for the ON versus OFF comparison. Just below that plot, the retinal layer-specific %change (means ± standard errors) in reflectivity per ±1° change in out-of-plane beam angle is plotted. Small-magnitude but significant (*p < 0.05) effects of out-of-plane beam angle appear in the RNFL and interdigitation layer (“INT”). C: The data presented in panel A are re-analyzed after including in-plane beam angle as a covariate. Statistical conclusions are essentially unchanged in the ON versus OFF comparison, but variance is visibly improved in some layers, like the HFL and INT, compared to panel A. Also, compared to the “first pass” analysis, magnitudes of the %change in reflectivity are closer to those seen in Experiment 1 (red line in Figs. 4 and 7). Just below that plot, the retinal layer-specific % change (means ± standard errors) in reflectivity per ±1° change in in-plane beam angle is shown. In most layers, the absolute value of the in-plane beam angle was adequate predictor, driving the use of |angle| as a predictor outside of the yellow band. For instance, just as seen in Experiment 1 (Fig. 7), in-plane tilt of the OCT beam by ±1° in either direction leads to a ~4% drop in reflectivity in the interdigitation layer. Within the yellow band, the sign of the angle is included. By the sign convention in Fig. 5, a +1° change in angle places the OCT beam more parallel to microstructures in the HFL, causing a ~2.5% drop in reflectivity. Equally, within the yellow band the result could be read as a −1° change in angle causing a ~2.5% increase in reflectivity, as expected when the OCT beam is more perpendicular to the microstructures in the HFL. In this experiment, the GCL and IPL seem to become a bit more reflective as the beam is tilted away from a perpendicular alignment. This effect is of uncertain significance, as it was not documented in the larger Experiment 1 (Fig. 7; red), nor in healthy controls in Experiment 4 (Fig. 8; orange). D: The data presented in panel A are re-analyzed after including both in-plane and out-of-plane beam angle as covariates. Patterns here are essentially the same as seen in panels B and C, except for out-of-plane beam angle: It no longer seems to have
3.7. Post-hoc consideration of acquisition strategies

For bright data from Experiment 1, increasing the number of OFF/ON cycles predictably reduces variance. At the ellipsoid zone, angle-adjusted reflectivity increases by ~5.4% when the retina is exposed to bright light (compared to darkness; red data to the right of Fig. 7) regardless of the number of cycles included in the analyses. The standard deviation (SD) for that light-dependent %change was approximated by the equation \( SD = 3.3 + 8.0/#cycles \). Power calculations (for \( 1 - \beta > 0.80 \), given \( \alpha = 0.05 \)) with these figures argue that a replication study of light-dependent changes at the ellipsoid zone should recruit at least \( n = 12 \) participants if only three OFF/ON cycles are used, but will be adequately powered with \( n = 9 \) participants if five cycles are used. Further increases in the number of cycles improve power, but with diminishing returns: We estimate that \( n = 6 \) participants are adequate when anywhere from nine to seventeen cycles are used.

Running those analyses for the ONL, where bright light elicits a ~2.1% decrease in angle-adjusted reflectivity (Fig. 7), we found that the standard deviation for this effect was approximated by the equation SD = 3.3 + 8.0/#cycles. Thus, a replication study of light-dependent changes at the ONL would require 21 participants if only three cycles were used, and \( n = 17 \) if five cycles are used – similar to recruitment for each group in Experiment 4 – and we estimate that \( n = 10 \) participants are adequate when twelve to nineteen cycles are used.

Power calculations were applied for the largest unique findings in Experiments 3 and 4, providing a lower bound for recruitment goals of any replication study. For Experiment 3, the ~15% light-dependent increase in reflectivity at the RNFL (sampled at 1.25%Depth) may need only four participants to replicate, given its standard deviation of 5% when we acquired five cycles per participant. For Experiment 4, the light-dependent decrease in reflectivity at the ONL is exaggerated in Alzheimer’s disease. At most points sampled from the ONL, those control participants >45 yo had minimal response to having the lights ON versus OFF, whereas Alzheimer’s patients showed about a 1.5% decrease in reflectivity when the stimulus light was turned on. Standard deviations for each group’s light-dependent change were ~1.5%. Using the same five OFF/ON cycles as this experiment, a replication study with 17 participants per group should be appropriately powered. On one hand, this figure can be reduced if more OFF/ON cycles are collected per participant. On the other hand, if multiple %Depths of the retina are analyzed, one should target a lower alpha, which would permit appropriate statistical thresholding for multiple comparisons.

We found that it was problematic to restrict analyses to only those images with an in-plane beam [angle] < 1°. As might be expected from the above analyses, power was reduced when we analyzed fewer images per participant. Moreover, requiring at least one OFF and one ON image from each participant lead to exclusion of 44% (16/36) of participants across Experiments 1 and 4. Although some light-dependent changes appeared blunted in this sensitivity analysis, especially in the interdigitation layer, we nevertheless still found significant (\( q < 0.05 \)) light-dependent decreases in reflectivity in the OPL and ONL, and a light-dependent increase in ellipsoid zone reflectivity.

4. Discussion

Across four experiments that used a clinically-viable protocol and a commercially-available OCT machine, we reproduced previous findings (Tumlinson et al., 2009; Srinivasan et al., 2009) of a light-dependent increase in retinal reflectivity at the photoreceptor inner and outer segments. Including prior studies of light-induced volumetric changes – which may share the same biological underpinnings as the reflectivity changes (Zhang et al., 2017), but fall below the image resolution available in this study – prior human data on these avascular sIOSS is limited to few dozen young adults (Tumlinson et al., 2009; Srinivasan et al., 2009; Abramoff et al., 2013; Lu et al., 2017; Messner et al., 2019). The present work roughly doubles the literature experience with this phenomenon, newly verifies its presence in older adults, and newly demonstrates that the human retina’s response is graded according to light intensity.

In contrast to the light-dependent reflectivity increase over the photoreceptor inner and outer segments, much of the rest of the retina demonstrated a light-dependent decrease in reflectivity. This is not an artifact of normalization to the RPE, since it largely persists with alternative normalization strategies (Table 2). The light-dependent decrease in reflectivity spans ganglion cells, bipolar cells, and much of the photoreceptors (except for the inner and outer segments). Since all have very different responses to continuous light, it seems unlikely that an activity-dependent change in neuronal reflectivity is the primary source of the ~2% light-induced drop in reflectivity. Instead, we suspect any influence of neuronal reflectivity is limited to those smaller perturbations that appear to respect neuronal layers, like the consistent finding that the light-dependent drop in reflectivity is about twice as large in the OPL than in the IPL (Figs. 4 and 6-8, and Table 2). It is even harder to attribute this change to the retinal vasculature, as has been previously suggested (Suzuki et al., 2013): In all experiments, at least a portion of the avascular ONL shows a similar-magnitude light-dependent decrease in reflectivity. Results at the avascular but glia-occupied HFL and OLM were more variable, but also showed a robust decrease in reflectivity in Experiment 1, which persisted when averaged together with data from Experiment 4. The only cell type common to all of these layers is the Müller glia, which may have unique optical properties (Franze et al., 2007). This led us to the intriguing possibility that some reflectivity changes directly report on Müller glial function.

We tested whether Müller glial cell dysfunction altered light-dependent reflectivity changes by imaging patients with neuromyelitis optica and circulating anti-AQ4 antibodies. These patients did not show the light-dependent decrease in the RNFL and the OLM reflectivity seen in age-matched controls. Indeed, in most vitread portions of the “RNFL” – close to the fovea, a non-negligible portion of the span between the vitreous and the RNFL/GCL border is occupied by the Müller end-feet, which form the inner limiting membrane – a dramatic light-dependent increase in reflectivity was seen in all three patients (Fig. 7). The light-dependent change was also abnormal at the outer limiting membrane. Overall, then, our findings suggest that immune-mediated disruption of Müller cell water management causes abnormal light-dependent reflectivity changes at the basal and apical borders of the Müller glia. This...
illustration of cell types within each retinal layer, group mean (Fig. 4), in that light decreases reflectivity; red). Of the healthy controls, the calculated effect of light when the stimulus light is ON versus OFF (i.e., 100 \% \text{change} (means \pm \text{standard deviations}) of refraction per 1° change of in-plane beam angle, versus the \sim 2\% loss in controls). However, due to concerns about model over-fitting in the setting of low patient recruitment, we did not perform among-group comparisons of the angle-dependence of retinal reflectivities.

Light-dependent changes in retinal reflectivity are abnormal in those seropositive for anti-AQ4 antibodies. Healthy control data (red) are from the “bright” condition in Experiment 1. As in Fig. 4, a finding is only marked as significant if the GEE model at that depth fell below an FDR threshold of \( q = 0.05 \). \textit{Top:} Illustration of cell types within each retinal layer, aligned with \%Depth for the plots. \textit{Left:} For each anti-AQ4 Ab positive patient, we plot the intra-individual means \pm standard errors (green dots and vertical lines) of the \%change in reflectivity when the stimulus light is ON versus OFF (\textit{i.e.}, 100 \% \text{change} \sim \text{on} – \text{off} ). Patient sex and Snellen acuity are listed at the top of each plot. For context, we co-plot the mean of age-matched controls from Experiment 1 (red line; carried over from Fig. 4) in these “first pass” calculations, which do not account for beam angle. Note that the y-axis scale is compressed relative to Fig. 4 to visualize unexpectedly large light-dependent increase in RNFL reflectivity in patients with anti-AQ4 antibodies. Visible thinning of the man’s RNFL – a not uncommon finding in this disease, due to accumulated injury to retinal ganglion axons after they enter the optic nerve (Sotirchos et al., 2013) – likely lead to over-representation of the inner limiting membrane in the spatially-normalized span from 0 to \%Depth. Because he had essentially no GCL, that span of his spatially-normalized profile is censored. Even with no light perception, he generated a normal-appearing light-dependent increase in reflectivity over the inner and outer segments, arguing that his photoreceptor-RPE complex remains healthy. \textit{Right:} Group mean \pm standard error effects of light and in-plane beam angle are plotted for anti-AQ4 Ab positive patients (green) and healthy controls from Experiment 1 (“bright” light condition; red). Of the healthy controls, the calculated effect of light when the beam is perpendicular to the retina is very similar to the pattern seen in Experiments 2 and 4, the RNFL, HFL, and photoreceptor inner and outer segments are quite sensitive to in-plane beam angle. However, including beam angle into statistical models tends not to alter the statistical findings for the effect of light. In anti-AQ4 Ab positive patients, we also find that the RNFL, HFL, and photoreceptor inner and outer segments are sensitive to in-plane beam angle (green \*; \( p < 0.05 \)). This group also shows an unexpected effect of in-plane beam angle at the OLM, and the effect at the RNFL appears exaggerated (\sim 6\% loss in reflectivity per 1° change of in-plane beam, versus the \sim 2\% loss in controls). However, due to concerns about model over-fitting in the setting of low patient recruitment, we did not perform among-group comparisons of the angle-dependence of retinal reflectivities.

Suspecting that some of the sIOSs in Experiment 1 were indeed related to water and waste management by Müller glia or the RPE (depending on the layer in question), and finding support for this in the RNFL and near the OLM in Experiment 3, we tested whether sIOSs are abnormal in Alzheimer’s disease. For those sIOSs confined to the photoreceptor inner and outer segments, the effect of light was grossly similar in Alzheimer’s patients and age-matched controls. Our data therefore imply that the photoreceptor-RPE unit is relatively unaffected by early-onset Alzheimer’s disease. In contrast, Alzheimer’s affected the sIOSs observed at the OLM, which is an avascular portion of the retina occupied exclusively by photoreceptor cells bodies and Müller glia. There was also overlap in the location of Alzheimer’s-related abnormalities and anti-AQ4-related abnormalities directly adjacent to the OLM, at \sim 78.75\% Depth. Minimally, these findings represent a novel functional OCT biomarker of Alzheimer’s disease, and complement existing literature linking Alzheimer’s and aquaporin-4 (Zeppenfeld et al., 2017; Burfeind et al., 2017). Further study is needed to determine its spatial and temporal relationship with other OCT biomarkers of Alzheimer’s (Alber et al., 2020), like changes in retinal vascular beds (Yoon et al., 2019), direct visualization of plaques (Koronyo et al., 2017), and thickness changes (Chan et al., 2018). We suspect our novel biomarker will precede thickness changes, since we did not detect retinal thickness differences between patients with early-onset Alzheimer’s and age-matched controls in this study.

In cross-sectional analyses of lifespan changes among control patients, we noted modest age-adjusted thinning of the retina (Neuville et al., 2009). Comparisons of the effects of OCT beam angle and bright light on reflectivity suggested additional effects of age in the avascular portions of the retina interior to the outer limiting membrane, which are occupied by Müller glia alongside photoreceptor cell bodies and axons. Insofar as some of the sIOSs here are caused by Müller glia, perhaps these attenuate with age due to declines in the inward rectifying potassium currents (Bringmann et al., 2003) that drive water flux through AQ4. The fine alignment of photoreceptor axons is thought to explain the influence of beam angle. It could variously be altered by subtle age-adjusted neuron loss and stretching of the retina (Harman et al., 2000) or the hydration status of adjacent cellular compartments (Müller glia and the interstitial space). Minimally, these findings emphasize the importance of age-matching clinical groups.

Suspecting that aging and Alzheimer’s-related changes near the OLM are indeed related to the integrity of the aquaporin system, we generate the following synthesis: When the system is impaired by immune-mediated attack, this blunts light-dependent changes in reflectivity (Experiment 3). Diminished use of the same system, as expected in normal aging (Bringmann et al., 2003), also blunts light-dependent changes in reflectivity (Fig. 8). Since Alzheimer’s patients have an exaggerated light-dependent response for their age – mimicking young adult responses – perhaps we are witnessing the effects of pathologically increased AQ4 expression, which has been documented in the brain (Zeppenfeld et al., 2017).
Future MRI and OCT experiments may clarify the origin of the sIOs, and to what degree they are related to retinal water content and compartmentalization. MRI-detectable manipulation of aquaporin expression is possible in animal models (Badault et al., 2011). Some of the retina’s light-dependent changes in interstitial fluid volume and water diffusion are modulated by hyperoxia or acetazolamide (Berkowitz et al., 2016; Gao et al., 1996). Human OCT measurements could be performed under similar conditions to see whether they impact reflectivity in avascular portions of the retina.

Updated image analysis techniques complimented the present work: First, we employed a spatial normalization step (Fig. 3) that allowed us to average across a broad segment of the central retina, and easily combine different individuals’ data for statistical testing. This approach builds on the relatively small but growing set of spatial normalization approaches employed in retinal OCT (Chen et al., 2014), and may have helped to reveal the −2% light-dependent decrease in reflectivity overlaying the Müller glia. Second, we included in-plane OCT beam angle in our analyses of retinal reflectivities. Although the operator generally optimizes beam angle during regular OCT image acquisition, small (~1°) deviations from perpendicular are difficult to avoid. Statistically accounting for this nuisance variable removed significant variance from OFF/ON comparisons and strengthened results (e.g., interdigitation layer error bars in Fig. 6A versus 6C). Consistent with prior studies (Lujan et al., 2011; Wartak et al., 2017) the influence of in-plane beam angle was strongest in retinal layers with well-aligned and tightly-packed microstructures – the RNFL, HFL, and the photoreceptor inner and outer segments (Fig. 5) – and there was relatively little influence of out-of-plane OCT beam angle (Fig. 6). Because of the radial symmetry of the eye, we expect this pattern to be influenced by retinal eccentricity, and those studying the retina just superior to the optic nerve may favor a vertical image plane (Jack et al., 2018). To our knowledge, ours is the first study in which the mostly-independent effects of light stimulation and beam angle on retinal reflectivity were analyzed together. Post hoc testing implied that bright light exposure could transiently modulate the effect of OCT beam angle on RNFL and HFL reflectivity, but this was only noted in Experiment 1. Future study of this phenomenon may employ deliberate and extreme manipulation of beam angle, or dedicated multi-directional techniques (Wartak et al., 2017). Such studies will benefit from acquiring more OFF/ON cycles than used in the present work: At extreme beam tilts – like the those explored in mice (Melepap et al., 2019) – we suspect that non-linearities will emerge in the relationship between beam angle and reflectivity, and these may be challenging to characterize.

One potential weakness of the present study is the low patient recruitment in Experiment 3, and the ad hoc estimation of recruitment
goals for Experiment 4. At the planning stages of these experiments, formal power calculations were not possible due to the paucity of pre-existing human data on the avascular light-dependent changes in retinal reflectivity. It is extremely reassuring that the core result of Experiment 1—that bright light stimulation increases reflectivity over the photoreceptor inner and outer segments, but decreases reflectivity in multiple layers occupied by Müller glia—was replicated in Experiments 2 and 4. In anticipation of future studies, we used a sensitivity analysis of Experiment 1 data to gauge the appropriate number of OFF/ON cycles for a replication study. Where the ONL is the focus of a planned statistical comparison, and five cycles are used, seventeen participants may be enough for a well-powered study. We are reassured that recruitment for Experiment 4 was not far from this target, and that note prior studies (Suzuki et al., 2013; Tuminson et al., 2009; Srinivasan et al., 2009) likely did not have adequate power to detect light-dependent changes at the ONL. It will be important to replicate Experiment 4 findings, and we estimate that similar recruitment goals (n = 17 per group) are appropriate. Greater effects at the ellipsoid zone imply that fewer subjects are needed for a well-powered study of light-dependent changes there (n = 9 may be adequate with five OFF/ON cycles). Even fewer participants may be needed to replicate our finding of unusual light-dependent changes in the RNFL of AQA-Ab positive patients, which is fortunate due to the uncommon nature of this condition, and others that may selectively affect glial health. For many clinical populations, the relatively short time needed to acquire five OFF/ON cycles may strike a fair balance between comfort and statistical power. Further reductions in sample size can be achieved by collecting more OFF/ON cycles, but the added acquisition time may not be worthwhile beyond twelve cycles. For highly engaged participants in a research setting, we suggest collecting roughly fifteen OFF/ON cycles for most research questions: Because artifacts or poor signal-to-noise will inevitably lead to the exclusion of a few images, a small excess ensures that the remaining images will provide adequate power. Second, while the operator typically tries to minimize beam angle, there may be value in deliberately collecting a few cycles with a small but perceptible in-plane beam tilt in each direction (e.g., nasal and temporal for a horizontally-oriented image plane): This step may make it easier to estimate and mitigate the effects of beam tilt, since some layers (e.g., HFL) are better characterized by the raw in-plane angle, and others need only the absolute value of the angle.

5. Conclusions

These experiments used a relatively brief and easy-to-implement protocol to replicate and expand upon previous functional OCT findings in healthy adults, uncovering a potential new biomarker for Alzheimer’s disease. Due to their spatial distribution, and due to the impact of anti-AQ4 antibodies, we conclude that at least some light-dependent changes in retinal reflectivity report on glial cell function. This represents a novel and non-invasive approach to study glial function, which may be important in the pathobiology of several other neurodegenerative diseases.

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CRediT authorship contribution statement

David Bissig: Conceptualization, Methodology, Formal analysis, Investigation, Data curation. Clarice G. Zhou: Investigation. Vy Le: Investigation. Jacqueline T. Bernard: Conceptualization, Resources, Supervision.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuroimage.2020.117022.

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