Cellular Characterization of Optical Coherence Tomography Outer Retinal Bands Using Specific Immunohistochemistry Markers and Clinical Implications

Nicolás Cuenca1,2,*, Isabel Ortuño-Lizarán1, Isabel Pinilla3,4

1 Department of Physiology, Genetics and Microbiology, Alicante University. San Vicente del Raspeig, Alicante, Spain.
2 Alicante Institute for Health and Biomedical Research (ISABIAL-FISABIO Foundation), Alicante, Spain.
3 Department of Ophthalmology, Lozano Blesa University Hospital, Zaragoza, Spain.
4 Aragon Health Science Institute. IIS Aragon, Spain.

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* Correspondence to: Nicolás Cuenca, Physiology, Genetics and Microbiology, University of Alicante, Alicante, San Vicente del Raspeig, E-03690 Alicante, Spain. Phone: +34-96-590-9916. E-mail: cuenca@ua.es


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ABSTRACT

Purpose: Optical coherence tomography (OCT) has been a technological breakthrough in the diagnosis, treatment and follow-up of many ocular diseases, especially retinal and neuro-ophthalmological pathologies. Until now, several controversies have arisen over the specific cell types that the bands observed in the OCT represent, especially over the four outer retinal bands. Design: The aim of this study was to correlate the four outer hyperreflective bands observed in the OCT with the histological structures using human retinal sections and immunocytochemistry at the fovea level, Subjects: Eyes from human donors. Methods: Vertical cryosections of human retinas were immunostained with antibodies specific for cones photoreceptors, bipolar cells, mithocondria, Müller and RPE cells, and visualized using confocal microscopy. Results and Main Outcome Measures: Triple immunolabeling allowed distinguishing between cells types and different cell compartments. Immunostaining with GNB3 and CRALBP antibodies showed all retinal layers at the foveola, especially the separation between the outer nuclear layer and the Henle fiber layer. CRALBP and cytochrome C immunolabeling revealed that the hyperreflective bands 1 and 2, observed in the OCT, correspond to the outer limiting membrane and the cone ellipsoids respectively, separated by the cone myoids. CRALBP, cytochrome C and GNB3 showed that the RPE interdigitations extend along the entire external segment of the cones, what discard them as the responsible structure that form the 3rd band. However, the identification of small fragments of cone outer segments within the RPE led us to characterize the 3rd band as the cone phagosomes located in the top of the RPE. Finally, we propose that the 4th band corresponds to the accumulation of mitochondria at the basal portion of the RPE as identified by cytochrome C immunoreactivity, and that the hyporeflective band between band 3 and 4 corresponds to the RPE nuclei and melanosomes zone. Conclusions: This work proposes a new interpretation of the outer retinal bands that leads to a more accurate interpretation of OCT images and could help to a better understanding of retinal diseases diagnosis and progression.
INTRODUCTION

The emergence of optical coherence tomography (OCT) has meant a technological breakthrough in the diagnosis, treatment and follow-up of many ocular diseases, especially retinal and neuro-ophthalmological pathologies. Prior to its clinical incorporation, the diagnosis of retinal pathologies was largely based on the observation of retinal fundus changes or alterations in retinal vascular angiography. OCT has allowed visualizing of the different retinal layers and their thickness in both macula and optic nerve locations. OCT can be used to study the loss or changes in photoreceptor compartments, edema, and cysts. It allows an evaluation of the vitreoretinal junction, the persistence of subretinal fluid after retinal detachment or macular hole surgery, or the presence of hyperreflective foci in vascular diseases. The low resolution of the original OCT technology did not allow us to distinguish retinal microscopical structures. The improvement of the technology with the arrival of spectral domain OCT (SD-OCT) or swept-source OCT (SS-OCT), with better resolution power, provides the opportunity to in vivo observe all the retinal layers and some cellular compartments in an image composed of several hypo and hyperreflective lines, including hyperreflective bands in the outer retina at the fovea level. The histological knowledge of the retina is crucial to understand images obtained from OCT examination. The analysis of the four outer hyperreflective bands at the fovea could inform us about the health and integrity of the cone compartments and their relationship with the retinal pigment epithelium (RPE). Healthy foveal cones are essential to provide good visual acuity and the state of their ellipsoids, their outer segments (OS) and their interaction with the RPE can be an indicator of how healthy cones are. Therefore, OCT images are useful to evaluate cell damage in retinal diseases and to establish a correct diagnosis and prognosis. Changes of the hyperreflective or hyporeflective lines thickness may have important clinical implications, as visual function was found to closely correlate with band integrity after surgical or medical treatment and the recovery of outer retinal OCT bands at the fovea has been correlated with the increase of visual acuity in different retinal pathologies. The rescue of the retinal layers after surgery or different medications may indicate the response to treatment and potential visual restoration, because it is a sign of how healthy the retinal cells are. There is an agreement on the interpretation of the main bands observed in the OCT, however, over time, the 4 hyperreflective lines in the outer retina in the fovea have generated various interpretations. These 4 bands represent different portions of cone photoreceptors and the retinal pigment epithelium (RPE) at the fovea.

Based on a theoretical-bibliographic model, in 2011 Spaide and Curcio proposed an anatomical correlation of the four hyperreflective bands in the outer retina. They analyzed histological information from previously published papers to create a scale model drawing. According to their model, the 1st layer corresponded to the external limiting membrane (ELM), the 2nd one was aligned with the ellipsoids of photoreceptor inner segments (IS), the 3rd corresponded to the extensions of the retinal RPE cells surrounding the photoreceptors outer segment disc, and the 4th one matched the RPE. With the purpose of unifying the terminology, an international OCT panel of experts was appointed and a nomenclature for the basic anatomic landmarks seen in normal OCT was proposed. They reached a consensus after an open discussion and nowadays this nomenclature is the most internationally accepted. However, discrepancies between the naming of the hyperreflective bands of the outer retina persist. As an example, the term IS/OS junction for the 2nd band is still used in the bibliography. Spaide and Curcio indicated that this layer corresponds to the ellipsoids but Jonnal et al, in 2014, using adaptive optics OCT images, concluded that assignment of the 2nd outer retinal band to the IS ellipsoid was unjustified and proposed a return to the IS/OS terminology. This issue was further discussed in a letter to the editor. Other authors described the 3rd band as the Verhoef’s membrane instead of the RPE projections around OS, so confirming the lack of consensus about outer hyperreflective bands nomenclature. In order to clarify these interpretation discrepancies, several authors claimed that better histological studies are needed to elucidate the correlation between retinal anatomy and OCT images.

In conclusion, after revising the recent literature about the four OCT outer bands, some unresolved questions arise: 1) Does the 2nd band correspond to the cone ellipsoids or to the inner /outer segment boundary (IS/OS)? 2) Does the 3rd band represents the interdigitation zone of the apical processes of the RPE or does it correspond to the cone outer segment tips (COST)? 3) If the 3rd hyperreflective line corresponds to the COST, why are not the rest of the OS discs hyperreflective? 4) If the 3rd hyperreflective band corresponds to the COST and the 4th is the RPE, what is the anatomical correlation of the hyporeflective band found between the 3rd and 4th bands?

In order to answer these questions we investigated the correlation of the four hyperreflective bands in the outer retina with immunocytochemical techniques using specific markers for cells or cell organelles that could justify this light hyperreflection in OCT images. To our knowledge there is not a detailed description using both techniques in the human retina.
The aim of this study was to correlate the hyperreflective bands observed in the OCT at the fovea level with the retinal histological structures using immunocytochemistry.

Materials and methods

Human retina samples were obtained from anonymous donors. Informed consent was obtained from all subjects prior to participation in this study. All donations were performed in accordance with relevant guidelines and regulations. All procedures were approved by the Institutional Review Board/Ethics Committee from the University of Alicante and were conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Eyes were enucleated and immediately fixed in 4% paraformaldehyde for 2 hours at room temperature. After washing with 0.1 M sodium phosphate buffer (pH 7.4), they were cryoprotected by immersion in increasing concentrations of sucrose, until reaching the 30% sucrose. Eyeballs were dissected into eight portions according to the anatomical axes, and frozen. The central area containing the fovea and the optic nerve head was cut in a cryostat and transverse sections of 14µm thickness were obtained. Sections were carefully examined looking for the macula and those where the foveola was present were selected and immunostained.

Triple immunohistochemistry was performed using combinations of the following primary antibodies, which have been used in several previous studies and are widely characterized regarding cell type specificity: polyclonal rabbit anti-guanine nucleotide binding protein 3 (GNB3) at 1:50 dilution (Sigma-Aldrich Corp., St. Louis, MO, USA, #HPA005645)\textsuperscript{14}, monoclonal mouse anti-cone arrestin at 1:200 dilution (Dr MacLeish, Morehouse School of Medicine; Atlanta, GA, USA) monoclonal mouse anti-cytochrome C (CytC) clone 6H2.B4 diluted 1:1000 (Zymed Laboratories, San Francisco, CA, USA, #33-8200)\textsuperscript{17}, rabbit polyclonal anti-cellular retinaldehyde-binding protein (CRALBP) at a 1:50 dilution (Dr Saari, University of Washington, Seattle, WA)\textsuperscript{15,18}, and mouse monoclonal anti-CRALBP at a 1:100 dilution (Abcam, Cambridge, UK, #Ab15051)\textsuperscript{19,20}. Primary antibodies were incubated over night at room temperature at the stated dilutions in 0.1 M sodium phosphate buffer (pH 7.4), 0.5% Triton X-100. Thereafter, samples were washed and incubated for 1 hour at room temperature with Alexa Fluor 488 donkey anti-rabbit IgG, Alexa Fluor 555 donkey anti-rabbit IgG, Alexa Fluor 488 donkey anti-mouse IgG, Alexa Fluor 555 donkey anti-mouse IgG or Alexa Fluor 633 donkey anti-mouse IgG secondary antibodies from Molecular Probes (Eugene, OR, USA) at a 1:100 dilution, employed in the proper combinations depending on the primary antibodies used. TO-PRO®-3 iodide 642/661 (Molecular Probes) at a 1:1000 dilution was, in some cases, incubated together with secondary antibodies. Images were taken using a Leica TCS SP2 confocal laser-scanning microscope (Wetzlar, Germany).

OCT images were performed using Cirrus HD OCT (Cirrus; Carl Zeiss Meditec Inc, Dublin, CA). A single scan with high resolution in a healthy subject was obtained for comparison with the histological images. Cirrus software version was 5.0.

RESULTS

Cone morphology at the fovea

Cone morphology was identified using antibodies against cone arrestin\textsuperscript{21}. In the foveal and parafoveal areas the entire cone could be identified: OS and IS, cell bodies, long axons forming the Henle Fiber Layer (HFL), and their axon terminals (pedicles) (Fig. 1A). Cones have several compartments that will reflect light or not depending on their content. In their apical portion, in close connection with the RPE, they have their outer segments (OS) formed by well-disposed parallel disc-shaped folds where phototransduction take place. Below the OS, and connected via an internal cilium, the inner segment (IS) is divided into two zones: the ellipsoid, fully occupied by large numbers of compacted mitochondria, and the myoid unit, where the organelles responsible for the synthesis of vesicles are located. Some vesicles are transported to the OS to form the discs and others, loaded with neurotransmitters, are transported to the axon terminal. The cell nucleus is located under the myoid, continuous with the axon and ending in the cone pedicle, where synaptic connections with horizontal and bipolar cells take place. As we approach the fovea cones adopt a stylized morphology, increase their density and OS, ellipsoids and myoids become thinner and elongated (Fig. 1A). Cones and bipolar cells were also identified using antibodies against guanine nucleotide-binding protein beta3 (GNB3). GNB3 is an isof orm of the β subunit of the heterotrimeric G protein second messenger complex that is commonly associated with G-protein-coupled transmembrane receptors. GNB3 is expressed...
in photoreceptors and in cone and rod ON-bipolar cells in different species\textsuperscript{14,22}.

In the human retina, GNB3 immunoreactivity is expressed in both cone and rod photoreceptors and in ON-bipolar cells. At the fovea, high expression of GNB3 is found in cone OS, and the entire morphology of cones from the OS to the pedicles can be identified. Their axons forming the HFL run long distances almost parallel to the surface of the retina to connect with ON-bipolar cells in the outer plexiform layer (OPL) (Fig. 1C). In the parafoveal zone only 2 or 3 cone rows can be observed (Fig 2C,D,E), but they are quickly reduced to one row in adjacent regions (Fig. 1C) where the outer nuclear layer (ONL) is very narrow and the HFL occupies around a quarter of the retinal thickness (Fig 2C,D,E). In the peripheral retina only one row of cone photoreceptors can be identified. The most relevant morphological difference between cones at the foveal level compared to peripheral cones is their long axons. The large number of cones accumulated in the foveal result in their axons elongating radially outwards to clear the foveal dimple and connecting with bipolar and horizontal cells in the perifoveal OPL. All these axons form an additional layer in the central retina, the HFL (Fig. 2D, 3C).

\textbf{Müller cells and RPE identification}

We used cellular retinaldehyde-binding protein (CRALBP) antibodies to immunostain Müller and retinal pigment epithelium (RPE) cells. CRALBP plays an important role in the visual cycle. Once 11-cis-retinal is photoisomerized at the OS level, it is converted to all-trans-retinal and further modified into all-trans-retinol. All-trans-retinol then diffuses into the RPE cells (by both rod and cone photoreceptors) or in retinal Müller glia (by cones only) to be converted back to 11-cis-retinol and further oxidize into 11-cis-retinol (both by CRALBP). Mutations in the CRALBP can lead to severe vision loss in patients affected by this degeneration\textsuperscript{23}.

Müller cells have their cell bodies located in the central part of inner nuclear layer (INL) and emit two extensions (Fig. 1B). One processes goes towards the outer retina, surrounds the cell bodies of other neurons and their synaptic contacts in the OPL and encloses photoreceptors to reach the junction area between the cone myoid, where it forms adherent bonds with the photoreceptor IS, the so-called external limiting membrane (ELM) (Fig. 1B arrowheads). The extensions that go towards the inner portion of the retina interdigitate the synaptic contacts of the inner plexiform layer (IPL), the ganglion cells and their axons. They join together at the level of the terminal endfeet secreting glycoproteins and forming the internal limiting membrane (ILM), which separates the retina from the vitreous. Müller cells and their columnar distribution also show morphological modifications at the fovea. Müller cell processes run long distances accompanying cone axons and together they form the HFL (Fig. 1B, 2C,E).

CRALBP antibodies also label the entire RPE cells forming a monolayer of polarized cells that extend microvilli apically that interdigitate with cone OS. These cells with a cuboid morphology are attached to a basement membrane called Bruch’s membrane (Fig. 1B).

\textbf{Identification of Mitochondria in the retina}

In order to identify the ellipsoid band in the human retina we used a specific antibody against cytochrome C (Fig. 1D, 2D). In the inner retina, mitochondria accumulations are located in the cytoplasm of ganglion cells, in the dendrites of bipolar, amacrine and ganglion cells at the IPL and in the cytoplasm of cells located in the inner nuclear layer (INL). In the outer retina some mitochondria are located running in the cone axon in the HFL, but the highest mitochondria immunoreactivity is found in the photoreceptor ellipsoids and in the basal portion of the RPE adjacent to Bruch membrane (Fig. 1D).

Triple immunostaining using the combination of three antibodies, GNB3, Cytochrome C and CRALBP, allowed us to identify different retinal layers, including the relationship between cones and RPE. Figures 2A and 2B show an histological picture from the fovea to optic nerve (Fig. 2A) and an OCT image (Fig. 2B) at the same magnification to compare the retinal layers. At high magnification the main retinal layers at the foveola can be especially well correlated with the OCT profile (Fig. 2C,D,E,F). When comparing a vertical section of human retina and OCT image (Fig. 2E vs 2F), the ganglion cell layer, IPL, OPL are easily identified. In the immunostained retina, the difference between the long axon forming the HFL and the two or three cone nuclei in the parafoveal portion of the retina is easy to identify (Fig. 2E). However in the OCT images this difference is not clear (Fig. 2F) and both the HFL and nuclei are part of the same hyperreflective band. When the OCT is taken in a oblique direction the HFL can be observed as a hyperreflective band\textsuperscript{24,25}. The HFL thickness includes the cone axons and the Müller cell processes (Fig. 2C,D,E).
The first hyperreflective band at the outer retina:

CRALBP immunolabels the whole RPE cell including its apical microvilli (Fig. 2C, arrowheads points the microvilli). GNB3 antibody shows the complete cone morphology. GNB3 antibodies also immunostain cone midget bipolar cells, which connect with the pedicles in the OPL and extend their axon terminal into the IPL (Fig. 2D,E).

Using this double staining with CRALBP and GNB3 we could also identify the endfeet of Müller cells forming a continuous layer at the junction with the vitreous (Fig. 2C arrows). In the opposite site, at the outer retina, the end of the Müller cell processes end at the boundary between cone cell bodies and their myoids (Fig. 2C,F, Fig 3A,B). The apical processes of these cells form the external limiting membrane (ELM) (Fig. 2C). Comparing the immunolabeled picture (Fig 2E) and the OCT image (Fig 2F) we can see that the 1st OCT hyperreflective band is located at the same level as the ELM.

High magnification of the ELM (Fig. 3A) shows outer Müller cells processes ending at the ELM surrounding cone photoreceptors nuclei and extending as far as to their myoids (Fig. 3B arrowheads). In all vertebrate retinas, adherent junctions and desmosomes have been identified at the ELM. Indeed, the apical processes of Müller cells are attached to each other and to the photoreceptor IS by continuous heterotypic adherens junctions that collectively form the ELM\(^{26,27}\). Figure 3D shows the ELM structure in the fovea where thin Müller cell processes pass between thin cone myoids forming the ELM (Fig. 3D). This ELM corresponds with the 1st hyperreflective band observed in the OCT images: it is a thin hyperreflectant layer with the lowest intensity of the four outer bands (compare Fig. 2E vs 2F, and Fig. 4D, vs 4E).

Second hyperreflective band

To identify the 2nd band seen in OCT and to verify that this line corresponds to the ellipsoids, we used cytochrome C antibodies that label all the mitochondria in the retina (the ellipsoids are full of these organelles) and GNB3 antibodies to see cone morphology (Fig. 3A,B, 4A,B,C). The two compartments that form the cone IS could be distinguished because the ellipsoids are immunostained with cytochrome C (stained in blue) and the myoids located between the ELM and the ellipsoids are not (Fig. 3A,B, 4A,C). Comparing the immunostaining with the OCT lines, it seems clear that the 2nd hyperreflective band is generated by the ellipsoids and probably due to the high number of mitochondria that they contain. The myoid would then be represented by the hyperreflective band between the ELM (1st hyperreflective band) and the cone ellipsoids (2nd hyperreflective band) (Fig. 4D,E).

The third hyperreflective band

To clarify which is the histological correspondence of the 3rd and 4th bands observed in the OCT we used antibodies to identify the relationship between cones and the RPE cells. Triple immunostaining using a combination of antibodies against GNB3, cytochrome C and CRALBP allowed us to study this in the fovea. Figure 5A,D,E show the fovea, where it can be seen how thin cone OS are wrapped by the RPE microvilli that take care of the OS sheading, forming the so-called interdigitation zone. This interdigitation zone covers all the length of the cone OS: it originates in the RPE and goes from the COST to the ellipsoids (Fig. 4A,B,C, Fig. 5A,D,E). Figure 5B and 5C show high magnification images where it is clear that RPE interdigitations cover the whole length of the cone OS.

If the RPE interdigitation covers the whole OS to the ellipsoids, then it can’t be responsible of the 3rd hyperreflective line as it has been interpreted to date: in these images (Fig. 5) we can see that RPE microvilli do not form a contact cylinder around the OS tips as previously suggested\(^4\), but it goes all along the OS to the IS. As microvilli occupy all the space between ellipsoids (2nd band) and RPE, they can’t be responsible for the 3rd hyperreflective band. So, what does this 3rd OCT hyperreflective band represent? To verify if the 3rd line corresponds to the COST we performed double immunostaining with antibodies against cone arrestin and CRALBP. At the foveal level we found a continuous line of OS inside the RPE (Fig. 6A-D). This line is formed by arrestin immunoreactive circular structures, separated and with no continuity with the OS (Fig. 6B,C).

High magnification using cone arrestin, cytochrome C and CRALBP confirmed that these arrestin-immunoreactive structures were located inside the RPE (Fig. 6E) and some of them near the nucleus (Fig. 6F). Figures 6G and 6H show double immunolabeling of the RPE cell with arrestin and CRALBP antibodies showing the empty space inside the RPE when we take out the green channel that corresponds to the
structures expressing cone arrestin. The orthogonal projection also demonstrates that arrestin immunoreactive structures are located inside the RPE (Fig. 6I): there is no colocalization between CRALBP and arrestin and arrestin structures are surrounded by CRALBP RPE in all axes and directions. Because they are totally encompassed by the RPE (Fig. 6G,H,I) we suggest that arrestin staining corresponds to phagosomes that are engulfed by the RPE during the OS shedding process. This findings suggest that the 3rd hyperreflective band corresponds to the phagosomes zone and not the COST or the RPE interdigitation as previously thought.

The fourth hyperreflective band

If the 3rd band corresponds to the phagosomes located in the apical portion of the RPE, what structures inside the RPE are responsible for the 4th hyperreflective band and the hyporeflective band between both of them? A high cytochrome C immunoreactive intensity was found in the basal portion of the RPE (Fig. 1D, 4B, 5A, 6E,F, blue), generated by the accumulation of mitochondria in the basal RPE. In the same way that mitochondria in the ellipsoid provoke its hyperreflectivity, we think that mitochondria in the basal portion of the RPE could explain the 4th line of hyperreflectivity (Fig. 6E,F,G). In these images, thanks to the melanosome green autofluorescence we could identify melanosomes accumulated between apical phagosomes and the basal mitochondria (Fig. 6E,F,G). The hyperreflective band between the 3rd and 4th hyperreflective bands could be the RPE space between phagosomes and mitochondria, mainly composed of the nucleus and the melanosomes. We propose that the melanosome zone forms the hyporeflective line between the 3rd and the 4th lines.

DISCUSSION

OCT has become one of the most important tools in Ophthalmology, providing clinically relevant images with a clear histological correlation. It has also been an important tool in the investigation of therapies in animal models of neurodegenerative retinal diseases. After the introduction of the SD-OCT, several studies have looked for the relationship between visual outcomes, after different pathologies and treatments, and the status of the outer hyperreflective bands in the human retina. These outer retina OCT bands have been debated in numerous papers with conflicting interpretations. It would be good to have a consensus in the terminology to facilitate communication in the Ophthalmology field. To our knowledge this is the first immunohistochemical study that correlates the OCT bands with the cell micro-structure in the fovea. We propose herein a new nomenclature according to our histological observations using confocal microscopy. Figure 7 illustrates our proposal of the structures and organelles that are responsible for the visualization of the four hyperreflective bands in the external retina.

First hyperreflective band: The ELM comprises clusters of junctional complexes and microvilli between the Müller cells and the photoreceptors. We histologically verify that this OCT hyperreflective line corresponds to the Müller cell contacts and their microvilli surrounding the photoreceptors. The percentage of contribution of the junctional complexes or the microvilli to the hyporeflection of this band is unknown. Alterations in both cell types can generate changes at the ELM, which are critical for photoreceptor maturation and polarization. For example, retinal inherited diseases originated by Crb1 or CRALBP mutation will change the ELM configuration and genetic and chemical dysfunction of the ELM will generate rosette formations.

Changes at this level have relevant clinical implications: ELM is a barrier formed by proteins from both adherent and tight junctions that can be disrupted in pathological conditions. During vascular diseases, like diabetic retinopathy, Müller cells are not only swollen and reactive but also they lose their occluding junctions and have an important contribution to diabetic macular edema. ELM has been found to be the best predictor for final visual acuity after resolved diabetic macular edema, followed by basal visual acuity and HbA1c level. Besides, ELM damage has also been correlated to the damage at the ellipsoid layer and changes at the ELM have been described in age-related macular degeneration. In eyes with geographic atrophy a preserved internal retinal layer can be seen, but they present alterations in the external bands where the ELM seems to be disintegrated in the junctional zone and absent in areas of atrophy. Also, ELM and photoreceptor disruptions have been related to the visual acuity recovery after macula off retinal detachment.

Second hyperreflective band: Years ago this band was described as the IS/OS junction and this term is still used in some papers, but it is conceptually impossible based on histological knowledge: the IS/OS junction is only a boundary or passage from one to another cone compartment, not an anatomical structure,
and cannot generate hyperreflective changes. Our results corroborate the finding that this layer is generated by the IS ellipsoids (the ISe layer) in agreement with Spaide and Curcio\textsuperscript{6}. Optical scattering is a property of a heterogeneous medium like the retina that occurs due to the different refractive indices within the tissue. These index variations can be caused by subcellular structures or bundles of structures, like the retina nerve fibers\textsuperscript{46}, and they will be represented as hyperreflective or hyporeflective lines at the OCT image. Wilson \textit{et al} have studied the contributions of different cell organelles to light scattering and using different models they determined that both mitochondria and lysosomes are the most important structures scattering light inside the cell\textsuperscript{37,38}. Our suggestion that ellipsoids, a region full of mitochondria, are responsible of the 2\textsuperscript{nd} hyperreflective band matches their light scattering findings.

Clinical implications of the ISe band: The status of the 2\textsuperscript{nd} band has been the most extensively studied and correlated with visual acuity. Before knowing the exact histological interpretation, and using it as a marker of the integrity of the photoreceptor layer, the loss or attenuation of this band has been related to decreased visual acuity in patients with advanced Retinitis Pigmentosa\textsuperscript{39,40} and in patients with diminished cone function\textsuperscript{41}. Also it has been related to poor visual recovery in resolved central serous chorioretinopathy\textsuperscript{42,43} and employed as a functional prognosis indicator following macular edema resolution in a branch retina vein occlusion\textsuperscript{44}, or after anti-VEGF treatment together with the ELM status\textsuperscript{45}. All authors suggested that the incomplete visualization of this band reflected a deterioration or disorganization of photoreceptor cells. The fact that the 2\textsuperscript{nd} band is generated by the ellipsoids could have other important clinical implications, as it also reflects the state of mitochondria and their membranous structure and have a clear implication for adequate photoreceptor function. Wilson \textit{et al} demonstrated that changes in mitochondria morphology could modify the amount of light scattering\textsuperscript{38} and then modify the appearance of the OCT band. Mitochondria generate the energy for the cell and their integrity is clearly related to cell function and health. Besides, mitochondria can divide generating new organelles, and the recovery of this layer after injury indicates that the photoreceptor health and function is restored demanding energy production. An example could be the recovery of visual acuity months after macula off retinal detachment surgery correlated with the progressive better visualization of this ellipsoids band\textsuperscript{7}.

**Third hyperreflective band:** The phagosome zone (PhaZ).

We suggest that the 3\textsuperscript{rd} hyperreflective line correlates with the PhaZ. The PhaZ is visible in OCT as a more subtle hyper-reflective line between the ellipsoid zone and the RPE mitochondria. In fact, the internal structure of a phagosome, composed of membranes of disassembling discs is similar to the internal crista of mitochondria which could explain its hyperreflectivity\textsuperscript{46,47}. The process of recycling the OS of the cones by phagocytosis is essential for normal vision. Inside these phagosomes the OS discs adopt a non-parallel distribution, consisting of irregular membranes, similar to the crista inside mitochondria. This especial arrangement, similar to mitochondria, could cause the light reflection of these structures. We showed that the RPE interdigitations cover the whole length of the cone OS, then they can’t be responsible of the 3\textsuperscript{rd} hyperreflective line as it has been interpreted to date. We discard that this 3\textsuperscript{rd} band could be the Verhoeff’s membrane because it is not a membrane itself: it is originated by the complex unions between RPE cells that cannot induce enough light reflection to form the band, unlike the ELM to which microvilli would contribute much of its reflectivity. Regarding the hypothesis of the COST as the structure responsible of the 3\textsuperscript{rd} band, COST and phagosomes are mainly constituted from the same structures: folded discs membranes. In the COST these membranes are homogeneously parallel distributed limiting the refractive ability of this region, while in the phagosomes the OS have lost their organized membrane configuration and are now composed by irregular folded membranes, with higher ability to reflect light. So, we believe that the phagosomes band is a more accurate interpretation of these 3\textsuperscript{rd} hyperreflective band than the COST.

Clinical implications: RPE shedding activity is a sign of a healthy photoreceptor-RPE unit. If the RPE is not able to phagocytose the photoreceptor discs for their renewal, the outer retina won’t work properly. Some authors found that the distance between photoreceptor IS/OS junction and RPE layer, known as PROS, is a better sign of visual function than the central foveal thickness in patients affected by diabetic macular edema\textsuperscript{48}. The recovery of line 3 after a retinal detachment or other pathologies is important in relation to the visual recovery, because it would indicate the reestablishment of the relationship between the outer segments of the photoreceptors and the pigment epithelium, when the shedding of the RPE is recovered. The lack of an adequate phagocytosis function of the outer segments can lead to photoreceptor degeneration, as occurs with the MERTK mutation. The loss of phagosome activity, as described in MERTK, in the βA3/βA1-crystallin gene, or in the CLN3 mutation among others, leads to the photoreceptors death or malfunction\textsuperscript{49-51}. Disappearance of this 3\textsuperscript{rd} line has also been described after retinal detachment. This fact support our theory, because if the RPE loss their connections with the photoreceptor cells, photoreceptor discs can’t be phagocytized and the phagosome band won’t appear in the OCT images. The lack of reconnection between
RPE and photoreceptors can be one of the causes of vision loss reported after anatomical success in retinal detachment surgery. In experimental retinal detachment it has been demonstrated that the recovery of a normal photoreceptor-RPE relation is followed by an increase in phagosome number and recovery of their size and apical processes. Lack of kinesin-1 light chain (KLC1), which contributes to the phagosome movement inside the RPE, impairs phagosome localization and degradation, leading to an AMD-like model. Impaired phagosome function or number has been related to retinal degeneration in other animal models of different pathologies, like vitiligo, Niemann-Pick, rd mouse or Nuc 1 rat (mutation in the βA3/A1-crystallin gene). Kominami and collaborators reported the correlation between the restoration of the interdigitation zone with the improvement of the macula EGR after fovea-OFF rhegmatogenous retinal reattachment. We suggest that improvement of visual function is due to the recovery of cone outer segments shedding by the RPE and the development of phagosomes. The presence of new phagosomes would indicate that the relationship between the photoreceptors and the pigment epithelium has been reestablished. Because the visual function improve, we can infer that important functions as formation of new discs, reisomerization of all-trans retinal into 11-cis-retinal (what is crucial for the visual cycle), secretion of essential neurotrophic factors and transport of nutrients, ions, and water have been recovered. The gradual recovery of the 2nd (IScZ) and 3rd band (PhaZ), that respectively indicate that the energy metabolism of cones and the shedding by the RPE have been recovered, are two factors that must be evaluated in the interpretation of the OCT images to perform an accurate diagnosis and good prognosis of retinal pathologies.

**Fourth hyperreflective band:** the RPE mitochondria zone (RPEmit).

We have already talked about the main role of mitochondria as energy generators for the cell. They will change their morphology and function under stress conditions and are able to divide and generate new organelles after recovery. As Wilson and coworkers found, mitochondria are the main light scattering organelle from cells. Mitochondria are the main structure scattering the light at the ellipsoids and after our study, we suggest that they are also the main contributor to the forth hyperreflective band at the outer retina. Therefore we suggest that the 4th band is generated by the basal mitochondria of the RPE. We cannot rule out the contribution of Bruch’s membrane to the hyperreflectivity of the fourth band. Bruch’s membrane is a thin lamina composed by five layers of extracellular matrix located between the choroid and the pigment epithelium. It is formed by the two basal laminas of the RPE and the choriocapillaris, two collagenous layers and an elastic layer in the center, being a layer of acellular structures with small thickness of 2-4 μm. As it thickness is below the resolution power of the OCT, it is difficult to assess its contribution to the hyperreflectivity of the 4th band. The new nomenclature of the 3rd and 4th bands here proposed clarifies the question raised about what is the nature of the hyperreflective band between them: we suggest that this band is formed by the accumulation of melanosomes in the inner most portion of the RPE.

Several authors claim that the 4th hyperreflective band corresponds to the RPE cell but when comparing OCT with immunohistochemistry images (2E vs 2F; 4D vs 4E) it can been seen that this band occupies only a portion of the total RPE cells thickness. Nevertheless, if we consider both the 3rd and the 4th bands, their combined thickness in the OCT is similar to the RPE thickness in the histological section. In addition the staining of the mitochondria (with high hyperreflectivity) in the lower portion of the RPE cells, seen in figure 6, makes clear the assumption that the 4th band cannot be the whole RPE cell but only a portion of it. Furthermore the 3rd and 4th lines are spatially separated parts of the RPE cell: phagosomes zone (PhaZ) and mitochondria zone (RPEmitZ) respectively. The space between both regions is occupied by other RPE structures including the nuclei and melanosomes, and represent the hyporeflective band between the 3rd and 4th hyperreflective. Besides, the presence of 4 hyperreflective bands occurs in albinos patients’s OCTs similar to that of pigmented individuals. This is evidence that none of these 4 layers is produced by the reflection of melanin. This fact is consistent with our suggestion that the melanosome zone contributes to the hyporeflective band rather than to the hyperreflective band.

Clinical implications of this 4th hyperreflective band are similar to the function already mentioned in the ellipsoid mitochondria: normal mitochondria function is necessary for the normal RPE physiology. These organelles are responsible for ATP storage and energy generation. Any disease affecting to RPE would show changes in both 3rd and 4th lines of the outer retina. The disruption of this band indicates RPE cells damage with loss of their mitochondria and consequent death of the RPE, loss of photoreceptors and visual dysfunction.

**Proposed nomenclature for the OCT retinal bands.**
After analyzing our findings we proposed a new nomenclature for the four hyperreflective bands at the outer retina seen in OCT images: the 1st and innermost one will be the External Limiting Membrane (ELM), the 2nd hyperreflective correspond to the cone ellipsoid zone (ISeZ), the 3rd one will correspond to the phagosome zone (PhaZ) and the last one will be the RPE mitochondria zone (RPEmitZ) in the basal portion of the RPE. Between these hyperreflective bands three hyperreflective bands can be recognized: the inner one located between the ELM and ISeZ correspond to the cone myoids, the 2nd hyperreflective band is formed by the cone outer segments and the RPE interdigitation and the thin 3rd hyperreflective band between the PhaZ and RPEmitZ represent the melanosomes of the RPE (Fig.7).

Figure 8 summarizes the OCT interpretation here proposed and shows the correlation between all retinal bands observed in an OCT picture and a human section immunostained with specific antibodies to identify all retinal layers. From the most internal to the external retina we can see the following layers: Nerve fiber layer, Ganglion cell layer, Inner plexiform layer, Inner nuclear layer, Outer plexiform layer, Henle fiber layer, Outer nuclear layer, External Limiting membrane, Cone myoid, Cone ellipsoids, Cone outer segments, Phagosome zone, Melanosome zone of the RPE, Mitochondria zone of the RPE, Choriocapillaris and Choroid /sclera junction.

In conclusion, studying different retinal markers and comparing the results with OCT images we demonstrate that the four hyperreflective bands seen at the outer retina in OCT images are: the external limiting membrane, the inner segment ellipsoid zone, the phagosome zone at the RPE and finally, the basal RPE mitochondria zone. This new nomenclature provides a better interpretation of the outer retinal OCT bands and will help to obtain accurate information about photoreceptor health and the relationship between photoreceptors and pigment epithelium, being relevant to evaluate the improvement of visual function.

FIGURE LEGENDS
Figure 1: A) Vertical section of human retina showing cone morphology at the parafoveal zone with long axons identified by antibodies against cone arrestin. Only one row of cones can be observed. B) Human retina immunostained with antibodies against CRALBP showing Müller cells with their cell bodies located in the INL the inner processes crossing the IPL and their endfeet making contacts at the inner surface of the retina. Their outer processes form the external limiting membrane (arrowheads). CRALBP antibodies also immunostain retinal pigment epithelium and their interdigitation around the photoreceptor outer segments. C) GNB3 immunoreactivity is found in cones and ON bipolar cells. Long cone axons form the Henle fiber layer at the foveal region. D) Vertical section of human retina using antibodies anti cytochrome C labeling mitochondria located in different cell types. Accumulation of mitochondria in the ellipsoids (arrows) and basal portion of the RPE can be identified (arrowheads). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; HFL, Henle fiber layer; ONL, outer nuclear layer; IS, inner segment; OS, outer segment; RPE, retinal pigment epithelium. Scale bar 40µm

Figure 2: A) Panoramic view of human retina section from the foveal area to optic nerve, immunostained with antibodies against GNB3 (green), Cytochrome C (blue) and CRALBP (red). B) Spectral domain-OCT profile through the fovea to optic nerve for comparison with the Figure. 2A. C,D,E: High magnification of the cross section of the foveal zone immunostains with antibodies against GNB3 (green), Cytochrome C (blue) and CRALBP (red) showing the retinal layer at the fovea. The Henle fiber layer is formed by the axons of cones and long processes of Müller cells. F) OCT profile detail at the same area to compare the location of the four hyperreflective retinal bands with the immunohistochemical findings. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; HFL, Henle fiber layer; ONL, outer nuclear layer; IS, inner segment; OS, outer segment; RPE, retinal pigment epithelium. Scale bar in A and B, 200 µm. C,D,E,F, 50 µm

Figure 3: A,B): Human vertical section immunostained with antibodies against GNB3, CRALPB and Cytochrome C. A) The external compartments of cones myoids, ellipsoids and outer segments are easy of identify. B) High magnification of the external limiting membrane making junctions around cell bodies of cones and their microvilli (arrowheads). C) Müller cell processes forming the external limiting membrane (arrowheads) at the foveola. D) Magnification of inset in C. Scale bar 25 µm. ELM, external limiting membrane; OS outer segment.

Figure 4: A,B,C) Triple immunostaining with GNB3, CRALBP and Cyt C antibodies showing the
accumulation of mitochondria in the cone ellipsoids and basal portion of the RPE. D,E) OCT profile with the four hyperreflective bands compared with the immunostained section at D at the same magnification. The 1st band corresponds to the external limiting membrane; the 2nd one to the cone ellipsoids; the 3rd band to the RPE apical zone, where the phagosomes are located (PhaZ) and the 4th one to the accumulation of mitochondria at the basal portion of the RPE (RPEmitZ). Scale in A,B,C,D is 20 µm and in E, 100 µm. ELM, external limiting membrane; RPE, Retinal pigment epithelium.

Figure 5: A) Human foveola showing the RPE interdigitation identified by anti-CRALBP antibodies surrounding the cone outer segments and reaching the ellipsoids. B,C) Detail of RPE interdigitations in the pigment epithelium covering the entire length of the outer segment of cones and rods (arrowheads) D,E) High magnification at the foveola (inset of A) showing the cone outer segments surrounded by the RPE interdigitations. Scale bar is 20 µm in A,D,E and 10 µm in B and C. ELM, external limiting membrane, OS, outer segment, PhaZ, phagosomes zone, RPEmitZ, basal mitochondria.

Figure 6: A,B,C) Vertical immunosection of the human retina stained using cone arrestin (red channel), CRALBP (green channel) and TOPRO (blue channel). Fragments of cone OS inside the RPE are the phagosomes (arrows). B,C) Magnification of inset in A. D: Vertical immunosection of the human retina stained using cone arrestin (green channel), CRALBP (red channel) and TOPRO (blue channel). Arrowheads show the continuity of the band formed by phagosomes within the RPE. E) High magnification image of the RPE using antibodies against GNB3 (green), Cytochrome C (blue) and CRALBP (red) showing the band where the phagosomes are located (PhaZ), the middle zone of the RPE occupied by the melanosomes (MelanosomesZ), identified by their autofluorescence, and the mitochondria zone located in the basal zone of the RPE (RPEmitZ, arrows). F) Detail of two RPE cells showing phagosomes (green) inside the RPE, melanosomes (small granules, arrowheads) and mitochondria accumulation in the basal portion of the RPE (arrows). G,H) Two cone outer segments phagocytized by the RPE and leaving an empty space when the green channel is removed (compare G vs H). I) Orthogonal projection of the RPE with phagocytized cone outer segments located at the apical area, indicating that they are totally surrounded by the RPE cell. Scale bar represents 20 µm in A, D, E; 10 µm in B, C, F and 5 µm in G, H, I.

Figure 7: Cartoon representing the different hyperreflective and hyporeflective bands at the outer retina. The 1st band corresponds to the external limiting membrane (ELM); the 2nd one to the cone ellipsoids (ISeZ); the 3rd bands to the phagosomes at RPE apical area (PhaZ), and the 4th one to the RPE mitochondria (RPEmitZ).

Figure 8: Comparative nomenclature of OCT bands correspondence of the OCT image (A) to retinal immunohistochemistry (B).

References

tomography findings and visual outcome after primary rhegmatogenous retinal detachment repair. Retina 2012;32:43–53.


Figure 1

(A) GCL
IPL
INL
OPL
HFL
ONL
IS
OS
RPE

(A) Arrestin

(B) GCL
IPL
INL
OPL
HFL
ONL
ELM
IS
OS
RPE

(B) CRALBP

(C) GCL
IPL
INL
OPL
HFL
ONL
IS
OS
RPE

(C) GNB3

(D) GCL
IPL
INL
OPL
HFL
ONL
IS
OS
RPE

(D) Cyt C

[Arrows and arrowheads indicating specific cellular structures]
Figure 4
Figure 7

- External limiting membrane
- Myoid zone
- Ellipsoid zone
- OS
  + Interdigitations
- Phagosome zone
- Melanosome zone
- RPE Mitochondria zone

Light reflection

1. ELM
2. ISeZ
3. PhaZ
4. RPEmitZ