



Research article

Electron microscopy study of the central retinal fovea in Pied flycatcher: evidence of a mechanism of light energy transmission through the retina

Lidia Zueva^{a,b,e}, Tatiana Golubeva^c, Elena Korneeva^d, Oscar Resto^a, Mikhail Inyushin^e, Igor Khmelinskii^f, Vladimir Makarov^{a,*}^a University of Puerto Rico, Rio Piedras Campus, PO Box 23343, San Juan, PR 00931-3343, USA^b Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, Thorez pr. 44, 194223, St-Petersburg, Russia^c Department of Vertebrate Zoology, Lomonosov Moscow State University, 119992, Moscow, Russia^d Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences, Butlerova str., 5a, 117485, Moscow, Russia^e Universidad Central del Caribe, Bayamón, PR 00960-6032, USA^f University of the Algarve, FCT, DQF and CEOT, 8005-139, Faro, Portugal

ARTICLE INFO

Keywords:

Biological sciences
Cell biology
Cytoskeleton
Developmental biology
TEM
Passeriformes
Inverted bird retina
Central fovea
Müller cell's intermediate filaments
Cone's intermediate filament bundles
Intermediate filament distribution

ABSTRACT

We present unique ultrastructural data on avian retinal cells. Presently and earlier (Zueva et al., 2016) we explored distribution of intermediate filaments (IFs) in retinal cells of the Pied flycatcher (*Ficedula hypoleuca*, Passeriformes, Aves) in the central foveolar zone. This retinal zone only contains single and double cone photoreceptors. Previously we found that continuous IFs span Müller cells (MC) lengthwise from the retinal inner limiting membrane (ILM) layer up to the outer limiting membrane (OLM) layer. Here we describe long cylindrical bundles of IFs (IFBs) inside the cone inner segments (CIS) adjoining the cone plasma membrane, with these IFBs following along the cone lengthwise, and surrounding the cone at equal spacing one from the other. Double cones form a combined unit, wherein they are separated by their respective plasma membranes. Double cones thus have a common external ring of IFBs, surrounding both cone components. In the layer of cilia, the IFBs that continue into the cone outer segment (COS) follow on to the cone apical tip along the direction of incident light, with single IFs separating from the IFB, touching, and sometimes passing in-between the light-sensitive lamellae of the COS. These new data support our previous hypothesis on the quantum mechanism of light energy propagation through the vertebrate retina (Zueva et al., 2016, 2019).

1. Introduction

The mechanism of light energy propagation in vertebrate retina has been discussed for more than 100 years. Both classical optics (Walls, 1942) and the theory describing Müller cells as optical light guides (Agte et al., 2011, 2018; Karl et al., 2018; Reichenbach and Bringmann, 2010, 2013; Reichenbach et al., 2012; Franze et al., 2007; Labin et al., 2014) have difficulties explaining low light scattering in the inverted retina of vertebrates. Recently quantum mechanism (QM) of light propagation in the retina was proposed (Makarov et al., 2017), which explained very effective light energy transmission to the photoreceptors, operating through intermediate filaments (IF). Zueva et al. (2016) reported a detailed morphological study of Müller cells (MCs) in the central fovea of the Pied flycatcher retina (*Ficedula hypoleuca*, Passeriformes, Aves). This area has a specialized structure, containing only red-sensitive single and

double cones, and no rods at all (Zueva et al., 2003; Coimbra et al., 2015). The authors (Zueva et al., 2016) found that intermediate filaments (IFs) traverse the entire length of the MCs. They suggested that IFs could be the structures responsible for the transmission of light energy by the MCs (Makarov et al., 2017; Zueva et al., 2016; Khmelinskii et al., 2015, 2017). These IFs appear in the MC endfeet that border the ocular vitreous body, and span the entire MC body, continuing up to its apical end and straight into the microvilli (Mv). However, the exact path of these IFs and the ultrastructure of the retinal fovea from the MC apical end to the membrane lamellae of the cone outer segment (COS) remained unknown.

Providing a comprehensive picture of light energy propagation in the retina, here we report a detailed ultrastructural analysis of the upper retinal layers in Pied flycatcher, in and above the layer of the outer limiting membrane (OLM), including the photoreceptor cells and

* Corresponding author.

E-mail address: vladimir.makarov@upr.edu (V. Makarov).

pigment cells, and explore the distribution of the IFs in these retinal layers.

The presently obtained morphological results combined with an earlier report (Zueva et al., 2016) give new insights into the ultrastructure of the retinal fovea, providing a complete picture of MC IF distribution, originating in the endfeet and extending to the photoreceptor cell COS by way of special structures we discuss. We believe that the earlier reported morphological data (Zueva et al., 2016) combined with the present results provide a new look at the mechanism of light energy transmission in an inverted retina.

2. Electron microscopy results

Based on our cytomorphologic studies of Pied flycatcher (*Ficedula hypoleuca*) retina, we show the schematics of a fragment of its outer layers in Figure 1. Presently our attention was focused on the

ultrastructure, with an objective of obtaining complete information on the structure of the foveal retina in Pied flycatcher eyes. The samples were taken from the foveal retina, where only single- and double-cone photoreceptors exist, with no rods present. These samples included the layers starting from the ILM and continuing up to the Bruch's membrane. The cone outer segments contain membranes with the photosensitive cone opsin chromophore and other molecules of the visual excitation cascade (Hunt et al., 2009; Wu et al., 2008; Dreher et al., 1992). This retinal layer also contains the Müller cell (MC) microvilli (Mv), and the pigment cells with their processes (PCp). The present study focuses on the distribution of the IFs within the cone cells (Figure 1, schematics of a single cone). We measured the parameters of these cellular structures and the distances between them, thus obtaining a functional description of this retinal layer. Therefore, we managed to follow the entire extension of the IFs in the retina, from the OLM to the cones and pigment cells.

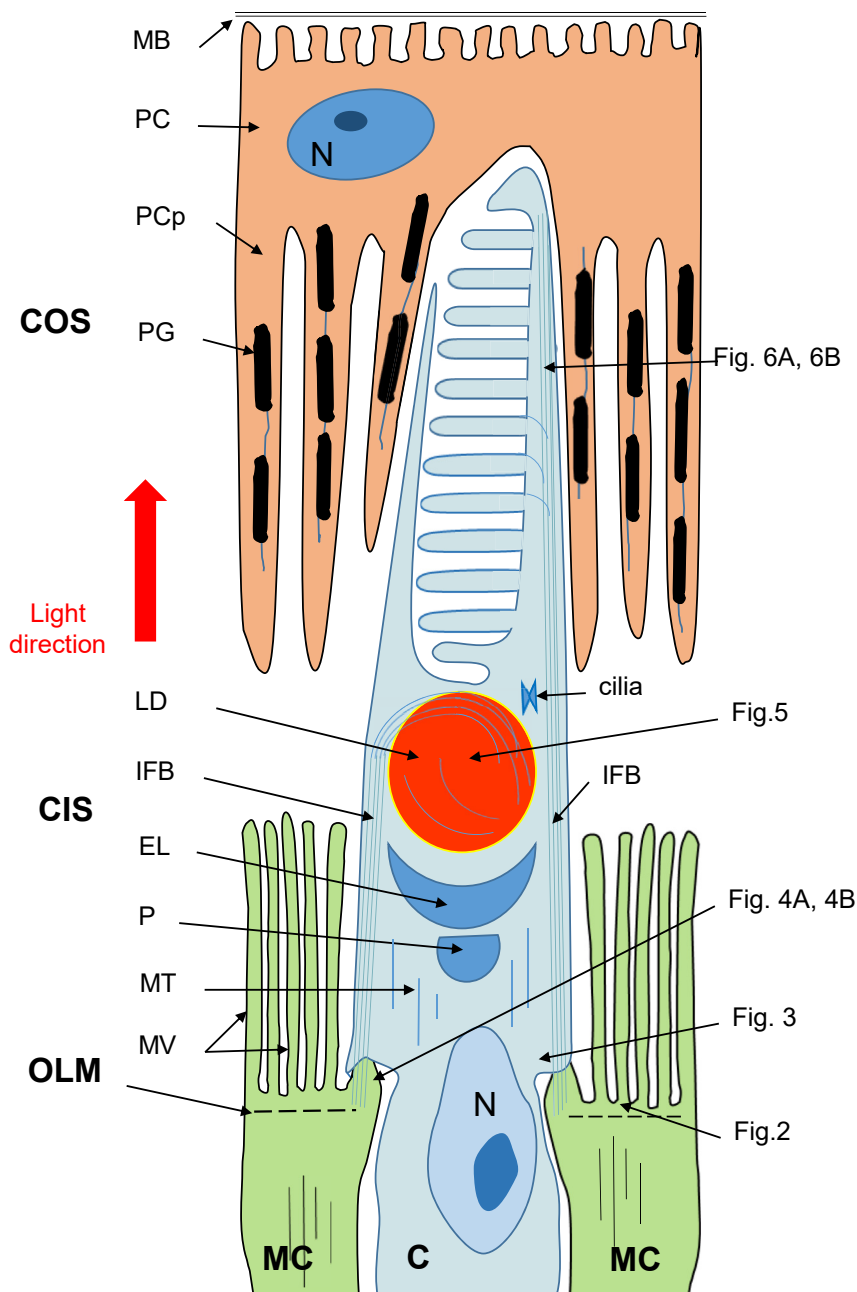


Figure 1. Schematics of the longitudinal section of the foveolar retina fragment in the Pied flycatcher (*Ficedula hypoleuca*) bird. The vertical axis of the diagram coincides with the light propagation direction. The light energy is passing through the whole thickness of the retina from the inner limiting membrane (ILM, not shown) to the outer limiting membrane (OLM) towards the pigment epithelium, as shown by the red arrow at left. The cone (C) inner segment (CIS) is surrounded in the OLM layer by microvilli (MV) of the Müller cells (MC). Some of nearby microvilli are jointly trapped by the cone ribs (see also Figures 2 and 4b), with the intermediate filaments (IFs) contained within the microvilli forming the bundles (IFBs.) Multiple microtubules (MT) are visible in the cone cytoplasm. The paraboloid (P) with its tubular network is located above, along with the ellipsoid (EL), built of densely packed mitochondria (M). A spherical lipid drop (LD) appears in the apical part of the inner segment. The inner segment transforms into outer segment (COS) above the lipid drop, with the cilium separating the two parts. Some of the IFBs are extended from CIS to COS. Individual IFs separate from these bundles (IFB), located to the right of the cilium and following into the cone outer segment (COS), these IFs touch membrane lamellae of the outer segment, or enter the space between lamellae by up to 30 nm. The cone outer segment (COS) with the light-sensitive membranes (lamellae) is surrounded by the pigment cells (PC), whose processes (PCp) are filled with the pigment granules (PG). Numbers of the electron micrographs and their location on the retina are indicated at the right side using long arrows. MB – Bruch's membrane, N – nucleus.

Individual IFs, each ca. 10 nm in diameter, group into bundles (IFBs) that first appear in the OLM layer (Herrmann et al., 2009). These long cylindrical bundles, each 180 ± 20 nm in diameter, appear in the base of cone inner segment (CIS) in the OLM layer. The IFBs join the plasma membrane of the CIS along its internal surface, and could be part of the cone cytoskeleton. We also observed separate IFs in the photoreceptor cytoplasm. According to their distribution in the cone cytoplasm, we suggest that some IFs may help organize the internal space of the CIS, concentrating endoplasmic reticulum (ER) into the paraboloid shape (P), clustering many mitochondria into an ellipsoid (EL) on the cone central axis, and forming the spherical basis for a lipid droplet (LD) in the apical part of the inner segment. According to classical optics, these organoids may be focusing the incident light onto the cone, even as they change their form and thus their optical focusing properties (Govardovskii et al., 1981).

The COS, as a derivative of the cilium, begins at its base from the centriolar apparatus. This is the place where the light-sensitive membrane is forming constantly, folding into lamellae of the growing COS (Eckmiller, 1987, 2004). The IFBs follow from the CIS and along the COS to the cone tip, with single IFs separating from the IFBs, and touching and sometimes passing in-between the light-sensitive membrane lamellae of the COS.

The cone outer segments are surrounded by pigment cells (PC), whose processes (PCp) are filled by pigment granules (PG), able to respond to changes in light intensity with movements along the processes. These processes of the pigment cells (PCp) extend in the inverse direction to that of the Müller cell microvilli, and towards the incident light.

The structure of MCs is quite well known by now, including the existence of the IFs in their cytoplasm (Reichenbach and Bringmann, 2010; Zueva et al., 2016; Wu et al., 2008). These IFs extend along the entire MC, from its endfoot adjacent to the inner lamina of the retina, to the apical ends of the microvilli above the OLM (Figure 9 from Zueva et al., 2016).

Initially these filaments were perceived as a purely mechanical skeleton, supporting the shape of the cell (Ishikawa et al., 1968; Yuan et al., 2009; Liem, 2013), with disturbances in their mechanical function causing certain diseases (Fuchs and Weber, 1994). Recently we used the quantum confinement theory (Khmelnikii et al., 2015, 2017) to justify that some of these IFs may also transfer light energy in the form of excitons, notwithstanding their very small diameter, typically only about 10 nm.

Note that according to the data obtained by light and electron microscopy, the microvilli of the MCs do not reach the layer of the outer segments with the light-sensitive membranes containing opsin molecules (Terakita, 2005; Hill and McGraw, 2006; Hunt et al., 2009), terminating at the distances far exceeding the 24 nm of the typical distance between the two adjacent membranes (Wolken, 1995). Therefore, we assume that light energy absorbed by the IFs is transported in the form of excitons to the OLM by the IFBs that we found in the CIS cytoplasm.

These long bundles with the 100–200 nm diameter are not enveloped by a common membrane and therefore can't be classified as membrane organoids; they should rather be classified as cytoskeletal polymers. The IFBs first appear in the cone inner segment in the OLM layer. They extend along the cone inner segment and then inside the cone outer segment along its outer plasma membrane, barely touching it but providing anchorage, distributed around and extending along the inner and the entire outer segment of the cone (Figures 2 and 3).

Such IFBs were not detected below the OLM either in the body or the synaptic terminals of the cones, although single IFs are found in this part of the retina inside all of the Müller cells, along with other cytoplasmic organoids and skeletal microstructures – microtubules and microfilaments (Agte et al., 2011, 2018; Reichenbach and Bringmann, 2010, 2013; Reichenbach et al., 2012; Franze et al., 2007; Labin et al., 2014). These single IFs are distributed homogeneously within the cytoplasm and directed along the cell, following the curves of the cellular shape (Makarov et al., 2017; Zueva et al., 2016).

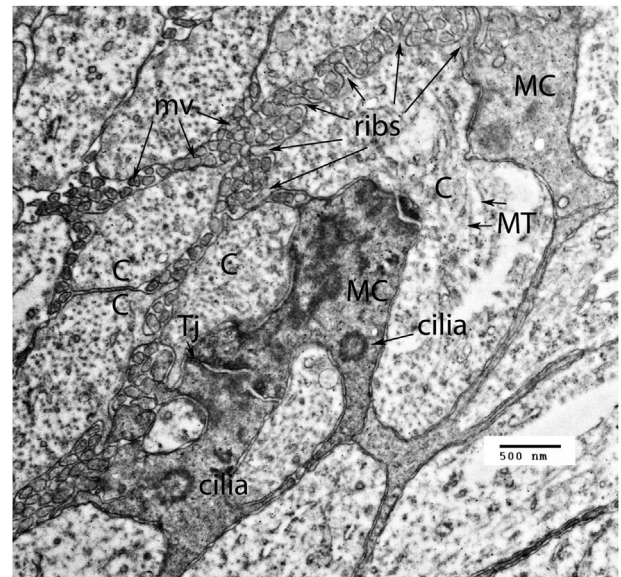


Figure 2. Transverse section of Pied flycatcher retina in the outer limiting membrane (OLM) layer. OLM is the zone of intensive cellular contacts, appearing here as darker osmiophilic apical surfaces of the Müller cells (MC) forming a continuous layer, united to each other and to cones by the binding complexes of the cell junctions and held in place by the tight junctions (Tj). There is a cilium in each of the MC bodies in this layer. Due to the oblique cut, we see both the MC bodies and the crosscut microvilli (Mv). Cones form ribs that separate several microvilli in this layer – transverse cuts of ribs and microvilli are in the upper part of the figure. We never observed any microvilli of Müller cells (MC) in the space between the membranes of the Principal cone and the Accessory cone of the double cones (C/C). C – cone, C/C – double cone, MC – Müller cell, Mv – Müller cell microvilli, MT – microtubules, Tj – tight junction.

Note that all of the figures refer implicitly to the schematics of Figure 1; therefore, the locations and descriptions of the organoids in all of the other figures also refer to Figure 1.

The OLM is usually easily noticeable, because its contrast is higher than that of the surroundings in the light-microscopy preparations, the OLM being denser due to the presence of numerous microfilaments (MFs) and IFs. Note also that the OLM is also the most osmiophilic part of glial MCs in the electron micrographs. The Müller cells envelop tightly the photoreceptor cells, and they separate the intercellular space of the outer retinal layer from that of its inner layer (Wei et al., 2006) due to the massive presence of the IFs, MFs, and structural proteins organized into junctions, including tight junctions (Tj), both between the adjacent MCs and between MCs and photoreceptors. The cytoplasm of the cones appears lighter than that of MCs on the electron micrographs (Figure 2), where Mt, IFs, Golgi apparatus (G), ribosomes, coated vesicles and other intercellular structures are evident in the sample cross-sections studied. The cone membrane ribs also appear in this thin layer; these cone ribs that protrude outside are clearly visible in this thin layer; the ribs stabilize the cell positions in a square-mosaic pattern, limiting their rotational and translational motion (Figures 2 and 3, ribs). Looking at both the MC and the cone, we note that the more osmiophilic (dark) MCs send their microvilli (Mv) into the intercellular space above the OLM. Every MC also carries a cilium in its cytoplasm in the OLM layer.

Figure 3 shows the retinal structure within an oblique transversal cross-section made 2–4 μm above the outer limiting membrane (OLM). Due to the angle of the cut, we see both the square mosaic pattern of the double cones in the retinal plane and the structural changes developing along the length of the cones. The overall form of the cone in this section resembles a gearwheel, with microvilli (Mv) forming mechanical contacts with the cone ribs in the layer containing myoids of the main and accessory cones in the basal part of the inner segments, above the OLM.

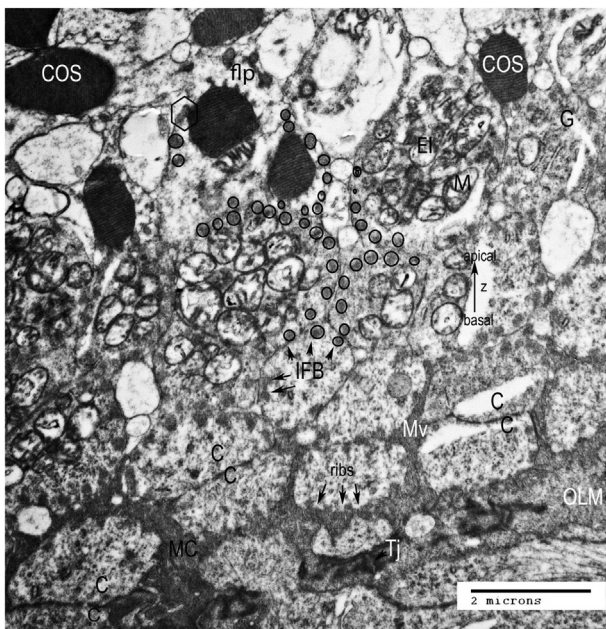


Figure 3. Oblique section of the Pied flycatcher retina 2–4 μm above the OLM with the cone inner and outer segments. The long arrow (light, see Figure 1) shows the direction from the basal part of the cone inner segments (CIS) towards the apical part of the cone outer segments (COS). The basal part of the CIS above the outer limiting membrane (OLM) has a characteristic ribbed zone. These ribs protrude into the inter-photoreceptor matrix, filled by the Müller cell (MC) microvilli. Individual microvilli appear between the double cone (CC) ribs closer to the OLM. Grey rounded bundles of intermediate filaments (IFB, arrows) have no surrounding membrane. The bundles (IFBs) are indicated by circles on the electron microphotograph in the layer of mitochondria-filled ellipsoids and in the layer of the outer segments, to facilitate visual identification within the matrix of the cytoplasm. Note the regular distribution of the IFBs. The hexagon outlines the bridge joining the IFB with the membranes of the COS. On the other side of the same outer segment we see a row of fingerlike cytoplasmic processes (flp), supporting the COS. G – Golgi apparatus, OLM – outer limiting membrane, C/C – double cone, Mv – Müller cell microvilli, M – mitochondria, El – ellipsoid, Tj – tight junction.

The gear teeth may be seen on the transverse section of the cone as elongated outgrowths, with the spaces between them filled by the MC microvilli. The ribs above the OLM in the cone are marked with arrows. The CIS is slightly wider in the ellipsoid layer, where its membrane is smooth, with no ribs present. In this layer, we see grey rounded structures inside the cones, which are cross-cut bundles of IFs, with these bundles (IFBs) regularly spaced along the plasma membrane at an approximately equal distance from each other on the side of the cytoplasm. We marked these formations by surrounding black circles in the middle of the electron micrograph. No such bundles (IFBs) appeared in between the principal and the accessory cones, as they were concentrated along the plasma membranes facing the interphotoreceptor matrix, delimiting the double cone in its entirety.

As a rule, individual IFs are tightly packed into a pseudo-crystalline structure of a bundle, as was demonstrated for other cells (Walko et al., 2013), because of their unique cross-bridges (side arms) and ability to align in parallel arrays. It was shown also that some IFs are well-suited to support the specific shapes of neurons (Yuan et al., 2009; Goldman, 1990; Goldman et al., 2012), and expand axons for optimal electric pulse conduction (Perkins et al., 2008).

We see the same IFBs are continuing inside the COS cytoplasm visible in the upper part of the oblique section (Figure 3), where we have crosscut the outer segments of the double cones. Some of the IFBs pass close to invaginations of the COS membranes containing chromophores of the photosensitive molecular system (receptor membranes), touching these membranes by way of the cross-bridges (one of the cross-bridges is

marked by a hexagon). Finger-like processes surround the same cone outer segment on its opposite side.

Only the cone photoreceptors are present in the investigated zone of the retina, and there is an asymmetry of the bundle distribution around the cone outer segment. Indeed, bundles are only existent on the side of the outer segment where the cone cytoplasm is present. The cone outer segment is open into the intercellular space on its opposite side, with only the thin finger-like cytoplasmic processes (that have no bundles) supporting it there.

Comparing the transverse (Figure 3) and the longitudinal (Figure 4a) sections, we see that we are dealing with a cylindrical bundle composed of IFs. Such bundles have no surrounding membrane, with about ten IFBs for each of the two elements of the double cone. Similar multiple IFBs are also present in single cones (figures not shown).

Interestingly, both single and double cones carry similar bundles of IFs in the central part of the retina of the great tit (*Parus major*) and of the reptile agama (*Agama caucasica*) (L. Zueva, personal communication). Therefore, at least some of the reptiles and many birds have IFBs in their cones.

The ribs of the inner segments of the three cones are well visible under high magnification in the longitudinal section of the retina (see Figure 4a). The ribs of the left and right cones on this Figure 4a were sectioned along the rib axis; as we know, the ribs are located above the OLM, tightly strapping the cones. The cone in the centre was sectioned so that the rib is overhanging the OLM, being surrounded by the apical part of the MC that has its microvilli (Mv) on both sides of the rib. The cone cytoplasm contains microtubules (Mt), inclusions delimited by a double membrane, and also ribosomes and polysomes. There is an IFB in the left cone, oriented along the cell, starting from the OLM (shown by the arrowhead) and going along the plasma membrane. Comparing the shape, size and osmiophilicity of these bundles with the crosscut of the same structures lying along the plasma membranes of the double cones in Figure 3, we conclude that all of these IFBs have similar cylindrically-shaped architecture. Notably, TEM images of Figures. 3, 4a, b and 5 also reveal the presence of microtubules (Mt) in the cones in the same layer of the retina. These images illustrate clear differences between the IFs and the Mt as regards their structure and diameter.

Figure 4b shows a transverse section of the Pied flycatcher retina, detailing the mechanism of the IF bundle formation in the cone inner segment directly above the OLM. On this figure the ribs of the principal member of the double cone in the OLM layer are surrounding several microvilli of the MC that are clustered nearby. These ribs are elongating and extending themselves to separate and envelop the IFs. Thus, the bundle of filaments forms out of several microvilli of the MC due to the two cone ribs that join one another, forming a closed tunnel. We see that the membranes that surround the bundle of microvilli disappear promptly within the cone (two long arrows). About 2 μm above this layer, the membrane around IFBs has disappeared completely. Thus, the IFBs are formed in the retinal CIS, as we also illustrate in Figure 1.

The interaction between the IFs and Mt is apparent in Figure 5, where both are clearly visible. Note that the IFB forms inside the cone cytoplasm in the OLM layer (Figure 4a,b).

The IF bundles adjacent to the plasma membrane extend along the CIS, passing the layers of myoids, ellipsoids and lipid droplets, with the myoid cytoplasm interspaced by numerous microtubules. These myoids also contain a well-expressed rough endoplasmic reticulum (RER), framed by ribosomes. (Note once more that all figures refer to the scheme of Figure 1, therefore the locations of the organoids and the directions are always denoted in the same way as on that scheme.) The ellipsoid located above the myoid is composed of mitochondria, tightly tied together by several IFs. The ellipsoid pushes the IF bundles out of the central part of the cone, pressing them tightly to the plasma membrane.

A round lipid droplet is located over the ellipsoid in the main element of double cones and also in single cones. Lipid droplets have no external membrane and may look colorless or colored – red, orange or yellow – in fresh non-fixed total retinal preparations (there are only red-colored

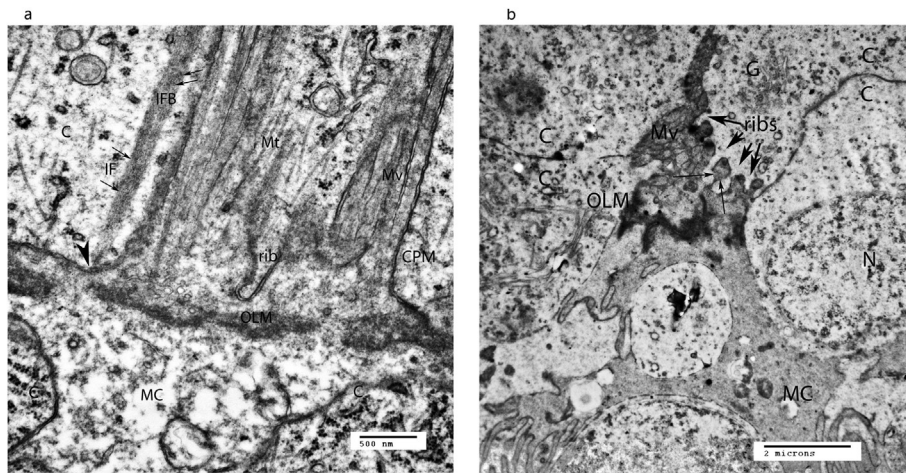


Figure 4. Longitudinal (a) and Transversal (b) sections of the Pied flycatcher retina in the OLM layer. a). The section shows one Müller cell and three cone ribs overhanging the OLM. The two lateral ribs were cut lengthwise, while the central rib was cut crosswise. Note at the left a bundle of intermediate filaments (IFB) (arrowhead) starting directly at the cone plasma membrane and extending parallel to it. One can see separate filaments (IFs) within the bundle (IFB) under high magnification in the longitudinal section. Note a cone that has a visible rib in the centre, with its specific plasma structures, microtubules (Mt), ribosomes and polysomes. b). Double cone (C/C) on the transversal section could be identified as two tightly connected cells with the principal member looking like a crescent surrounding the round section of the accessory member. In the zone of the outer limiting membrane (OLM) the ribs of the principal member of the double cone surround several Müller cell microvilli (Mv) located nearby. The development of this bundle formation may be followed: a pair of neighboring ribs elongate and extend, separating Müller cell Mv. Next, some of these Mv appear inside the cone without their surrounding membranes. Note that part of the membrane of one of the microvilli has dissolved (thin arrows). Thus, the filaments of several microvilli of the Müller cell enter the cone cytoplasm and form the bundle (IFB) via a closed tunnel formed by two cone ribs that trap microvilli with the filaments and join together, keeping the filaments inside. The membrane that surrounded the bundle (IFB) is absent inside the cone. N - nucleus, G - Golgi apparatus.

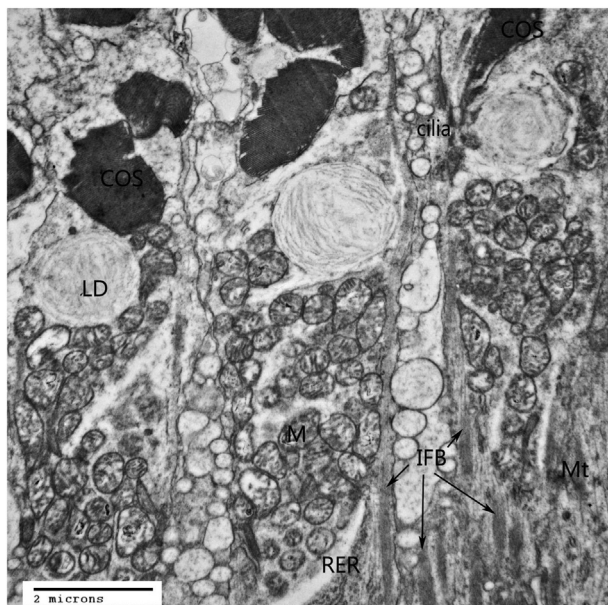


Figure 5. Longitudinal section of Pied flycatcher retina: the apical part of the cone inner segment in the ellipsoid layer. There are three cones (right, middle, left) present. The myoid of the right cone is filled by a large number of microtubules (Mt) and intermediate filament bundles (IFB) that are extending parallel to the cone plasma membrane. Round lipid drops (LD) appear in the apical part of the inner segments (CIS) of each of the three cones above the respective ellipsoids (El) constituted by tightly packed mitochondria (M); there is no membrane delimiting the lipid drop from the cytoplasm. Prolonged fixation exposed the filamentous matter filling the drop matrix. The cilium in the base of the cone outer segment (COS) is also connected to the filaments of the lipid drop.

droplets in the avian foveal areas). Their color is due to carotenoids dissolved in lipids. Our prolonged fixation made visible the filamentous material that was holding together the droplet inner structure, as carotenoids and lipids were washed out during fixation and dehydration processing. Thus, our data suggest that the droplet was formed around filaments wound into a loose round ball. We suggest that these filaments coming from the cytoplasm both to the lipid droplet and (later, in COS, Figure 6a) to the lower membrane lamellae of the outer segment are the same filaments (IF) of the bundle passing nearby and lending some IFs to this organoid. A cilium (another essential component of the cone) is visible in the upper part of the image inside the right cone, where the COS begins. It has been proposed that all of these cell organelles have such optical parameters like specific coefficients of refraction and spherical, ellipsoidal, and paraboloidal forms that allow them to focus light on the COS (Govardovskii et al., 1981; Nakata et al., 1991).

Figure 6a shows the interaction of the IFs, detaching from the IFB, with the light-sensitive membrane of the photoreceptor cell.

A bundle of filaments (IFB) located within the cell extends along the COS at the left (Figure 6a). The distance from the bundle to the membranes of the outer segment does not exceed 80 nm. Individual intermediate filaments, detached from the bundle, are present between the membrane folds (lamellae). Small strands of filaments directly touch the membranes of the lamellae of the outer segment, sometimes penetrating into the spacing between the photoreceptor membranes by up to 30 nm (1.5% of the COS diameter, Figure 6a).

Dark pigment granules are visible in the COS layer (Figure 6b), being situated in the pigment cell processes (PCp). These are mobile melanin inclusions that may absorb the excess EMF energy that reaches the layer of pigment cells, without being absorbed by the photoreceptor cells. The pigment granules migrate upwards along the processes in low light, filling the body of the pigment cell and vacating its processes. At higher luminosity these granules move downwards, almost to the base of the outer segment, absorbing excess light and thus improving image contrast and resolution (Kolb, 1995).

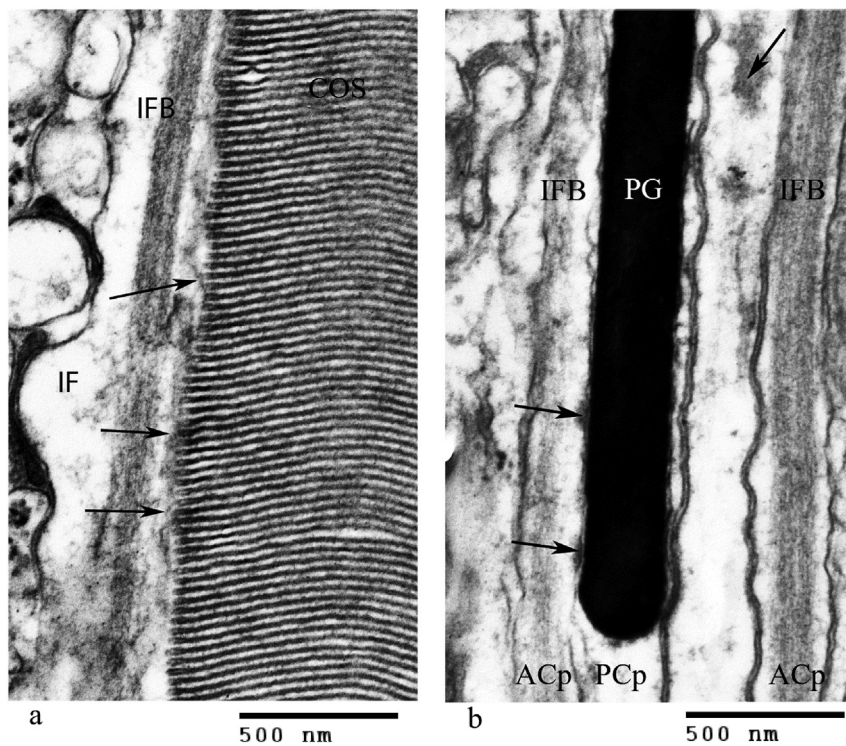


Figure 6. Longitudinal section in the layer of interspaced cone outer segments (COS) and pigment cell processes (PCp). a). Cone outer segment (COS), composed of lipid membrane lamellae with the molecules of red-light-sensitive cone opsin. A bundle of filaments (IFB) located inside the cone cell extends along the outer segment. The distance between the bundle (IFB) and COS membranes does not exceed 80 nm. The inter-membrane gap between the COS membrane lamellae (receptor membranes) is equal to 24 nm. Individual filaments that separate from the bundle (arrows) follow to these gaps. These filaments directly touch the membranes of the outer segment, either individually or as small tufts, sometimes penetrating into the gaps by up to 30 nm. b). A pigment cell process (PCp) with pigment granule (PG) inside, on the electron micrograph is situated near the cone apical processes (ACp), with osmiophilic hemidesmosome compactions visible on their plasma membranes (arrow). It was yet impossible to see the other half of the desmosome inside the pigment cell, due to the high density of melanin. Usually both halves of the desmosome are built of skeletal microfibrils ~10 nm thick, positioned at right angles to the membrane. The hemidesmosome in the left process was cut along the membrane, and we see a gap below it, separating the pigment granule from the ACp that has an intermediate filament coming to it. The hemidesmosomes in the right process were cut along the surface. We believe that these inter-membrane contacts may dump the unused energy arriving along the bundles onto the pigment granules, as otherwise this energy would be reemitted back into the eye. Note that the entire section of the process (ACp) in the far right is filled by the intermediate filament bundle (IFB).

Pigment granules have desmosome-like plackets; similar desmosome plackets in keratinocytes are the attachment points of the granules to the filaments that move these granules (Van Den Bossche et al., 2006). We see a dark pigment granule in the pigment cell process on the right side in Figure 6b. To the left of it, inside another process, we see an intermediate filament bundle and structures resembling hemi-desmosomes, adjacent to the pigment granule, being separated from it by two plasma membranes. The section cuts along the surface of one of these plackets (arrow) within the process at right. In the previous study we had suggested that these structures may transfer excess excitons onto the pigment granules. As the cones are surrounded by the pigment cell processes (PCp), excess energy from the cone IF bundles apparently may be transferred onto pigment granules, accelerating the photosensor recovery.

The experimental data presented here show that MCs microvilli conduct the IFs through both cellular membranes and into the cone, by means of the special morphological structures built of cone ribs, and then IFs join each other inside the cone cell forming IF bundles. These bundles prolong throughout the entire CIS close to the plasma membrane, and next within the COS, where some of the IFs get separated from the bundle. Some of these individual IFs interact with the lipid droplet, some with the membrane folds (lamellae), touching their surface and sometimes getting deeper into the space between the membranes with the photosensitive chromophores (cone opsins). Thus, the detailed retinal structure based on the morphological data reported earlier (Zueva et al., 2016) and those obtained here leads to the conclusion that individual IFs, which may transport light energy through the retina, may also transfer energy directly to cone opsins. Note that presently we did not study either the morphology of IF – rod assemblies, or the mechanism of efficient light energy transport in an inverted retina outside of the foveal zone. Both of these issues will be addressed in future studies.

3. Discussion

We examined the ultrastructure of the central fovea of the bifoveal retina in one-month Pied flycatcher nestlings (*Ficedula hypoleuca*). It was shown earlier (Zueva et al., 2003) that this region specialized for sharp

vision contains only single and double cone photoreceptors, and no rods, as usually happens in other diurnal Passerines. Recently it has been reported that the rod-free foveolar region is also easily observable in several other Australian species of Passeriformes (Coimbra et al., 2015). Therefore, the present discussion refers to cones only.

As it was shown earlier (Walls, 1942; Zueva et al., 2014; Bringmann, 2019), the central fovea in the Pied flycatcher has a much sharper and deeper conical shape than that in other vertebrates. Such shape of the foveolar retina enhances visual resolution by reducing the effective area of the zone directing light energy to an individual cone photoreceptor (Zueva et al., 2014). The cones in the central foveolar zone are longer and thinner than those in other retinal zones. The foveae in general are specialized for increased visual acuity. In birds with lateral eyes like Pied flycatcher the central fovea provides for long-focus and short-focus lateral vision, while the temporal fovea – for binocular vision. The presently explored retinal region contains cones only, with no rods present. We found that the IFs traverse all of the retinal layers (Figures 1, 2, 3, 4, 5 and 6), starting from the ILM (Zueva et al., 2016) and interacting directly with the cone membrane lamellae. Taking into account these morphological results, we proposed that some specialized IFs have an additional function as conduits of light energy. Thus we have a very well organized system, where the IFs function as natural nanofibers, which absorb light, passing into their excited electronic states, and transfer these excitations (excitons) to the photoreceptor chromophores (energy acceptors).

Intermediate filaments (IFs) are the primary components of the cytoskeleton, and they may have different additional functions. We observed that IFs passing the OLM are forming IF bundles (IFBs) in the OLM layer (Figures 2, 3, 4, 5, and 6), the latter were of special interest for us in this study. Each of the bundles formed was about 180–200 nm in diameter (see Figure 6a, b). These bundles started to be visible in the electron microscopy apically to the OLM (Figure 4a, b), allowing a 3D-reconstruction. OLM consists of different junction proteins and maintains the integrity of the photoreceptor layer (Wei et al., 2006).

Above the OLM layer, cones form the ribs that are closely overhanging OLM and Müller cells, with the latter protruding their microvilli

through the OLM. We had found ribs both in single and double cones (Figure 4a, b), with 6 and 16 ribs on each cone unit, respectively. The ribs in this layer surround several nearby microvilli of the respective Müller cell (MC). Then the ribs elongate and extend themselves to separate and envelop the microvilli (Figures 2 and 4a, b). Two nearby ribs join together, surrounding a group of microvilli and forming a tunnel. Further on, the microvilli start losing their respective membranes (Figure 4b), with intermediate filaments appearing inside the cone inner segment (CIS). Thus, bundles of intermediate filaments are formed inside the CIS, just on the inside of its plasma membrane (Figures 3 and 4b).

We found that small strands of IFs separate from the bundles on their way along the CIS, reaching the paraboloid, the ellipsoid and the lipid droplet (Figure 5). Passing along the entire CIS, some IFBs are crossing to the COS in the layer of cilia. Later, IFBs liberate some IFs that touch the lamellae, and even go into the ca. 30 nm wide gaps between the two neighboring lamellae, Figure 6a.

We did not study the biochemical origin of the intermediate filaments involved. But we may suggest that different classes of IFs exist within the retina (Herrmann et al., 2007, 2009; Traub, 2012; Chernyatina et al., 2012). Some of these IFs may only have mechanical functions, while some others may be participating in energy transmission as we described earlier (Zueva et al., 2014, 2016, 2019).

Thus, we have found that microvilli formed on the apical extremity of Müller cells are penetrating into cones, as was shown above. This resembles the mechanism described for the podosome formation by cells that penetrate some cellular barriers, including immune cells crossing tissue barriers, or cells moving in bone remodeling (Seano and Primo, 2015), or cancer cells forming filament-rich protrusions of the plasma membrane that are associated with degradation of the extracellular matrix in cancer invasiveness and metastasis (Rizzolo, 2007; Rahner et al., 2004; Schoumacher et al., 2010). Now it is possible to suggest that MC microvilli similarly transfer IFs into cones, thus the invadopodia (microvilli) allow IF-sized filaments to traverse the OLM at right angle to it, in the direction of light propagation.

In our previous study of the MC endfeet structure (Zueva et al., 2014, 2016), we have suggested that each MC endfoot collects light as a conical lens and focuses it onto the IF extremities in the vertex of the same endfoot. Thus, taking into account that the smallest diameter of the MC is about 500 nm and the distance between neighboring IFs in this area is about 50 nm (Zueva et al., 2016), we estimated the number of IFs passing along a MC equal to about 82. Taking into account the IF cross-section and the MC diameter at its narrowest part, we estimated the fraction of the MC cross-section occupied by IFs of about 3.3%. However, since light propagates along MC IFs, the IF absorption cross-section for light would be much larger than its geometrical cross-section, providing for complete absorption of incident light in the ILM layer. We therefore believe that the quantum mechanism described in our previous publication (Zueva et al., 2019) supports high-contrast vision of vertebrate eyes, as almost all of the light energy received by IFs of a certain MC will be transferred exclusively to the photoreceptor cell coupled to this MC. These results and the earlier reported morphological data on the IF distribution in MCs (Zueva et al., 2016) produce a complete picture of IF distribution in the central foveal zone of the Pied flycatcher retina.

4. Experimental methods

Histological tissues were collected in the Moscow region (Russia) in the years 2013 and 2015. The experimental protocol complied with the general principles of bioethics, namely to the EC Convention 2010 (the Directive 2010/63/EU). Wild Pied flycatcher nestlings (*Ficedula hypoleuca*, Passeriformes, Aves) were taken from artificial nests in which parents died as a result of predator activity and raised in laboratory conditions. At the age of 27–30 days after hatching, the birds were decapitated; eyeballs were incised and placed in the fixative: 2.5% glutaraldehyde, 4% paraformaldehyde in 90 mM sodium cacodylate buffer with 0.02 mM CaCl₂, pH 7.4, and stored for about 2–6 months at

+4 °C. In stationary laboratory conditions the right and left eyeballs were washed separately in 90 mM sodium cacodylate buffer, oriented relative to the position of the pecten, sectioned into nasal, central, and temporal parts, photographed, and each of the parts individually marked. The central fovea was cut out of the retina in a piece containing both the optical nerve entrance and the foveal central pit, for better orientation. The pieces of tissue were postfixed in 1% osmium tetroxide (OsO₄) with 1.5% K₄[Fe(CN)₆] for 30 min in the same buffer, washed, incubated in 1% OsO₄ for 30 min, washed in distilled water, and then incubated in a 2% aqueous solution of uranyl acetate [UO₂(CH₃COO)₂·2H₂O] for 1h and washed. After dehydration through a graded series of acetones, the oriented pieces of retina were embedded in Embed 812 EMS epoxy resin (all of the chemicals were ordered from Electron Microscopy Sciences, Hatfield, PA, USA). Sections 60 nm thick were prepared using an LKB Ultratome (LKB-Produkter, Bromma, Sweden), mounted on Formvar/carbon copper blends or clear grids (EMS) and examined using JEM 100B and JEM 1011 electron microscopes (JEOL Ltd., Tokyo, Japan).

5. Conclusions

Using electron microscopy, we described morphologically the intracellular structures assembled of intermediate filaments (IFs) in the central foveal retinal cells of the Pied flycatcher bird. Namely, the intermediate filaments in cones are grouped into bundles (IFB), which are the continuation of the IFs running along the Müller cells. We followed the entire route of the intermediate filaments from the Müller cell endfoot and into the respective cone. Individual IFs detach from bundles within the cones, touching different organoids and the photosensitive membrane lamellae. We suggest that the presently described morphological structures based on IFs, jointly with the previously described IFs running from the MC endfeet to the microvilli at the opposite MC extremity (Zueva et al., 2016), are the morphological basis for the quantum mechanism of light energy transfer from the optical system of the eye to the photoreceptor cells. This mechanism explains high-contrast vision of avian eyes in Passeriformes.

Declarations

Author contribution statement

L. Zueva, T. Golubeva and E. Korneeva: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

O. Resto: Conceived and designed the experiments; Performed the experiments.

M. Inyushin, I. Khmelinski and V. Makarov: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

L. Zueva was supported by the Russian Science Foundation (16-14-10159). E. Korneeva was supported by the Russian Science Foundation (17-06-00404). V. Makarov was supported by PR NASA EPSCoR (80NSSC19M0049) and Puerto Rico Space Grant Consortium (NNX15AII1H). M. Inyushin was supported by the National Institutes of Health (SC2GM111149).

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

T.G. is grateful to G.N. Davidovich and A.G. Bogdanov (Electron microscopy laboratory, Faculty of Biology, Lomonosov Moscow State University) for technical assistance.

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