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STRUCTURAL BASIS FOR WAVELENGTH DISCRIMINATION IN THE BANKED RETINA OF THE FIREFLY SQUID *WATASENIA SCINTILLANS*

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Summary

There is a greatly thickened region of retina in the ventral part of the eye of the firefly squid *Watasenia scintillans*, with an outer segment (OS) layer around 600 μm thick. The distal two-thirds of this OS layer is yellow and contains a visual pigment, based on 4-hydroxyretinal (A4), with an absorbance maximum at 470nm. The proximal third is pink and contains a visual pigment, based on dehydroretinal (A2), with an absorbance maximum at 500nm. Light and electron microscopic investigations demonstrate the presence of four types of photoreceptor cell. In the pink layer, three of the four types (α, β and γ) produce no microvilli and are columnar in structure. These cells form square microvillous rhabdoms only in the distal yellow layer. The fourth cell type (δ) produces microvilli in the pink layer only. These observations led us to propose that the A2-based visual pigment is contained in the pink-layer cells and that the A4-based visual pigment is contained in the three types of yellow-layer cells. The absorbance of fresh retina was determined by microspectrophotometry. The yellow OS layer, with an absorbance of 0.7 per 100 μm thickness at 470nm, is expected to act as a short-wavelength cut-off filter to the underlying pink OS cells, shifting their photosensitivity peak by an estimated 50nm to 550nm. Possible wavelength discrimination by this squid is discussed.

Introduction

The firefly squid *Watasenia scintillans* lives deep (below 250m in the daytime) in the open ocean around Japan. The mature squid has a mantle length of about 6cm. This species is known as the firefly squid because of its intense bioluminescence emitted from

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three large photophores at the tips of the fourth arms (Tsuji, 1985). It also has five relatively large photophores on the ventral side of each eye and numerous small photophores distributed mainly over the ventral surface of the body (Inamura *et al.* 1990), which provide a counter-illumination system to camouflage the silhouette of the body against downwelling light (Young, 1977; Herring, 1988).

The dorsal retina has a 200 μm thick outer segment (OS) layer, a 50 μm thick inner segment layer and contains a visual pigment with an absorbance maximum at 484nm, based on the chromophore retinal (A1). The ventral retina is thicker with, at most, a 600 μm thick OS layer and a 200 μm thick inner segment layer. The distal two-thirds of the thick OS layer is yellow and contains a visual pigment with an absorbance maximum at 470nm, based on 4-hydroxyretinal (A4). The proximal third of the OS layer is pink and contains a visual pigment with an absorbance maximum at 500nm, based on 3-dehydroretinal (A2) (Matsui *et al.* 1988*a*,*b*; Seidou *et al.* 1990).

This species is, therefore, an unusual cephalopod in having three visual pigments of markedly differing spectral sensitivity, two of which are located in the same (ventral) part of the retina. It has never been clear whether the two ventral pigments are contained in the microvilli of the OS of a single visual cell or whether each pigment is restricted to a different visual cell. We provide evidence that the latter is true, and therefore suggest that this squid has the potential for some form of wavelength discrimination.

Materials and methods

Large shoals of *Watasenia scintillans* come to Toyama Bay, in the Sea of Japan, for spawning in spring and are readily captured at night in shallow set nets. For experiments, live squid were placed in ice-cooled sea water in light-tight containers for transport to the laboratory. Under dim red light, they were killed by decapitation and the eyes were quickly excised. The lens was removed and the optic nerves were trimmed away before placing the eye into fixative A (2.5% glutaraldehyde and 0.25mol l^{-1} NaCl in 0.1mol l^{-1} cacodylate buffer, pH7.4). The row of five photophores on the ventral surface of the eye serves as a good marker for dissection of the ventral retina. After about 30min, the halved eye was sliced thinly along the dorso-ventral axis in the fixative under dim red light. After fixation for 1h at 4°C, the retinal slices were rinsed overnight in buffer (0.1mol l^{-1} cacodylate, pH7.4, containing 0.5mol l^{-1} NaCl) and separated into two groups: one for scanning electron microscopy (SEM) and the other for light microscopy (LM) and transmission electron microscopy (TEM).

The retinal slices for SEM observation were post-fixed for 2.5h in fixative B (1% osmium tetroxide in 0.1mol l^{-1} cacodylate buffer, pH7.4, containing 0.2mol l^{-1} NaCl) and then treated for 1h with dimethylsulphoxide. After rinsing in buffer, they were post-fixed again in fixative B for 2.5h. The tissues were then treated with 2% tannic acid and carefully fractured under a binocular microscope (Iino *et al.* 1987). The pieces obtained were dehydrated through an ethanol series, critical-point-dried and sputter-coated with gold. They were examined with a JEOL JSM-35C scanning electron microscope.

The other group of retinal slices, for LM and TEM, were cut into pieces 0.5mm square in the rinsing buffer and promptly immersed in fixative A. After fixation for 2h at 4°C,

they were rinsed in buffer overnight and post-fixed in fixative B at 4°C for 4h. They were then block-stained in 5% uranyl acetate in 50% ethanol for 1h, rinsed and dehydrated through an ethanol series. Epoxypropane was then substituted for ethanol and the specimen was embedded in SUPER resin. Alternate series of ultra-thin and semi-thin (about 1 μm) sections were cut parallel to the retinal surface, from the rhabdom tips to the basement membrane of the retina. The ultra-thin sections were counterstained with lead citrate and observed with a JEOL 2000 FX transmission electron microscope at 120kV. The 1 μm thick sections were stained with Toluidine Blue and examined by LM.

For absorbance measurements, a fresh retina was unfolded onto filter paper and sliced perpendicular to its surface with a razor-blade guillotine (Matsui *et al.* 1988*a*). The thickness of the slice was measured with a micrometer and it was then dipped in physiological saline containing $0.1 \mathrm{mol\,l^{-1}}$ hydroxylamine. This promotes bleaching of squid visual pigment upon irradiation (Hubbard and St George, 1958). The difference spectrum of the OS layer of the retinal slice was measured with a microspectrophotometer (Unisoku, Osaka) before and after bleaching (i.e. until spectral changes discontinued). The wavelength of the irradiating light was longer than 560nm, using a VO-56 cut-off filter (Toshiba, Tokyo) with a 500W slide-projector lamp as the light source. The area of retinal slice measured was 10 μm in diameter.

Results

Fig. 1A is a scanning electron micrograph of the OS layer of the cleaved ventral retina. We expected to observe 'gaps' (discontinuities) such as those found in the banked OS layer of deep-sea fish retinae (Denton and Locket, 1989), but no such gaps were found. However, the outer two-thirds of the OS has a granular appearance which was not observed in the proximal third. Furthermore, these two regions appear to be separated by an abrupt transition zone (stars in Fig. 1A).

Banked outer segments were not observed in LM longitudinal sections of the ventral retina, which were indistinguishable from sections through the dorsal retina (results not shown), except by the obvious difference in length. The retinal surface was found to be covered with an amorphous outer limiting membrane. No rhabdomeric structures were found near the basement membrane, but there were many screening-pigment granules, which were not observed in other regions of the OS layer. The observed colour of the OS layer was therefore due to the visual pigment. This may be a specific feature of the deep-sea squid retina (cf. dark-adapted retina of *Loligo pealei*; Daw and Pearlman, 1974).

To investigate the arrangement of the OS cells of the ventral retina in more detail, a series of LM and TEM sections was taken, parallel to the retinal surface, providing cross sections of the OS cells. Fig. 1B,C shows LM and TEM cross sections at a depth of 10 μm from the retinal surface. Each rhabdom is constructed from four OS cells. The OS cytoplasm is contained in oval profiles 2–3 μm in diameter, designated as α′ type.

Fig. 1D,E shows cross sections 100 μm from the retinal surface. Each cushion-shaped rhabdom consists of microvilli from four OS cells. The cross-sectional area of cytoplasm and rhabdom for each OS cell is about twice that of the profiles in Fig. 1B,C and the shape of the cytoplasmic profiles is fusiform rather than oval. The OS cells shown in these

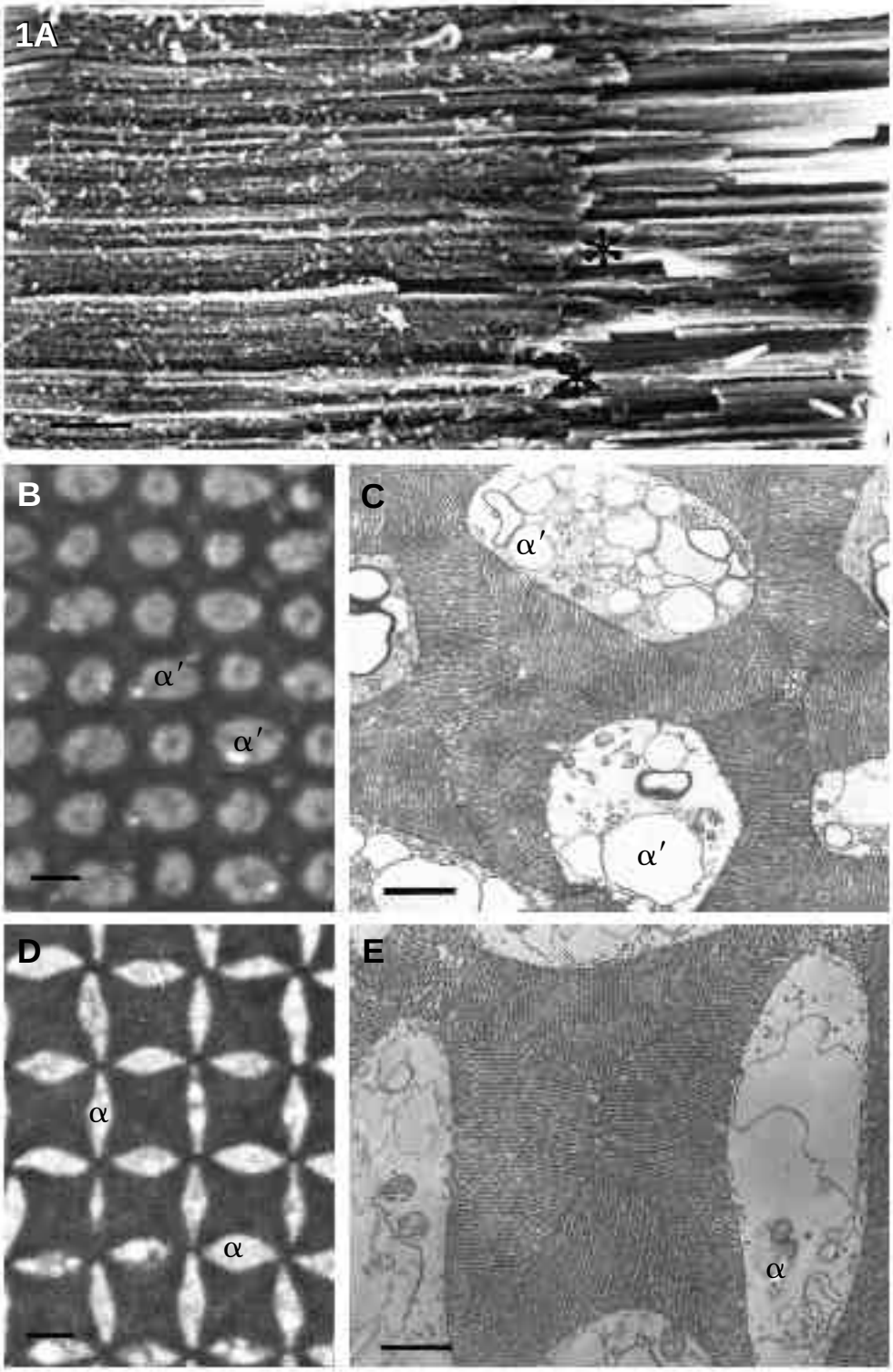

Fig. 1. For legend see p. 6

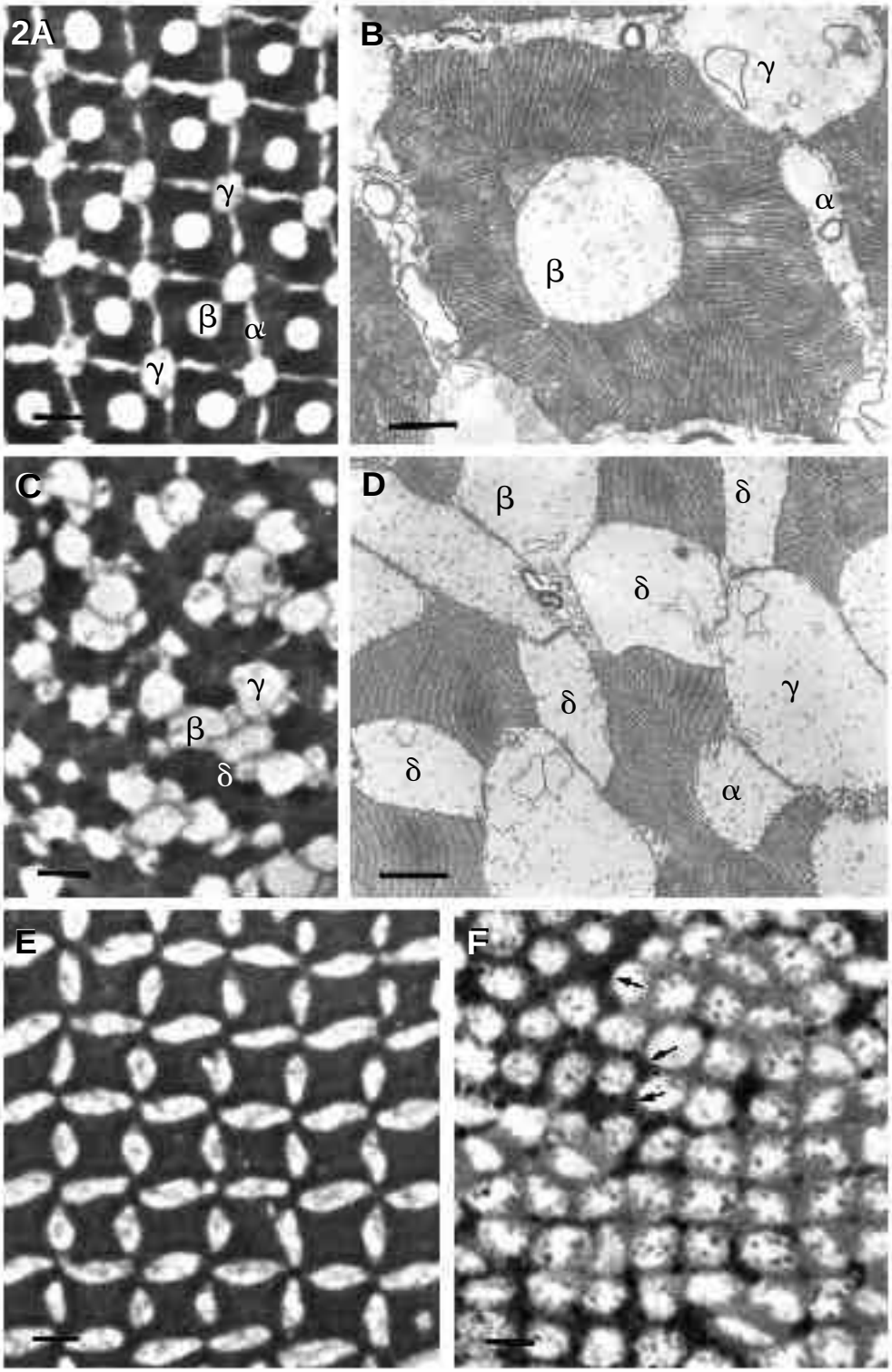

Fig. 2. For legend see p. 6

Fig. 1. (A) Scanning electron micrograph of the thick outer segment (OS) layer of the ventral retina of *Watasenia scintillans*, fractured longitudinally (vitreal surface to the left of the figure). Note the presence of many vesicles in the distal OS layer and the filamentous appearance of the proximal OS layer. The stars mark the transition zone between the proximal and distal OS cells. Scale bar, 100 μm. (B) Light micrograph of a cross section 10 μm from the surface of the ventral retina of *W. scintillans*. Rhabdoms are densely stained by Toluidine Blue. Scale bar 10 μm. (C) Transmission electron micrograph of a cross section at the same depth as in B. The α′-type OS cells have oval cytoplasmic profiles. Scale bar, 1 μm. (D) Light micrograph of a cross section at 100 μm depth from the surface of the ventral retina, showing the regular rhabdom. In this region, rhabdoms and cytoplasmic profiles are arranged in a highly regular pattern. Scale bar, 10 μm. (E) Transmission electron micrograph of a cross section corresponding to that in D. Each photoreceptor cell (α) has two rhabdomeres on each side, oriented in the same direction. Each rhabdom is composed of four rhabdomeres, with microvilli orthogonal to those of adjacent rhabdomeres. Note that the rhabdom is about twice the size of those in C. Scale bar, 1 μm.

Fig. 2. (A,B) Cross sections of the ventral retina 300 μm from the surface. (A) Light micrograph. Scale bar, 10 μm. (B) Transmission electron micrograph. The β OS cytoplasmic profile at the centre of the rhabdom bears two sets of microvilli, which are extended at 45° to those of the α-type OS cells. A γ OS cell profile appears in the corner of the rhabdom of the α OS cell. Scale bar, 1 μm. (C,D) Cross sections of the ventral retina 450 μm from the surface. (C) Light micrograph. Scale bar, 10 μm. (D) Transmission electron micrograph. The fourth cell type, γ can also be seen, with two rhabdomeres, one either side of the cytoplasmic profile. The α, β and γ OS cells have no microvilli in this section. The bundles of α, β and γ cytoplasmic profiles are arranged irregularly. Scale bar, 1 μm. (E) Light micrograph of a cross section at 70 μm from the surface of the dorsal retina. Rhabdoms and cytoplasmic profiles are arranged in a highly regular pattern. This arrangement of OS cells is very similar to that of the ventral retina (Fig. 1D). Scale bar, 10 μm. (F) Light micrograph of a cross section of the OS layer near the basement membrane of the dorsal retina. The OS cells have oval cytoplasmic profiles, which are arranged regularly. No rhabdomeric structures are found and there are many screening-pigment granules (arrows), which are not observed in other regions of the OS layer. Scale bar, 10 μm.

sections were designated α. Many vesicles are found within the cytoplasm of the OS cells at this level (Fig. 1C,E). The tubular structures with round profiles found at the centre of each rhabdom probably include processes of the slender supporting cells (Yamamoto *et al.* 1965). The α′- and α-types of OS cell are the same, although they showed different cross-sectional patterns seen at 10 and 100 μm depth. At around 10 μm depth, the α′- and α- type rhabdom patterns are mixed in an oblique section.

Two other types of OS cells are present in sections taken 300 μm from the retinal surface, as shown in Fig. 2A,B. One type (designated β) occupies the centre of the rhabdom, and the other (γ) the corner. At this depth, the α-type cytoplasmic profiles are reduced to thin bands about 0.5 μm across. The β-type begins to appear at around 100 μm from the retinal surface; the γ-type somewhat deeper, at 150 μm. Both have circular cytoplasmic profiles about 3 μm in diameter and extend their microvilli at 45° to those of the α-type. The β and γ cell microvilli are only about one-third of the length of the α-type microvilli and, in their most distal regions, these cells produce no microvilli at all.

Below about 400 μm, the cytoplasmic profiles of all types of OS cell (α, β and γ) contain microtubules, lack the vesicles observed in more distal sections and do not extend

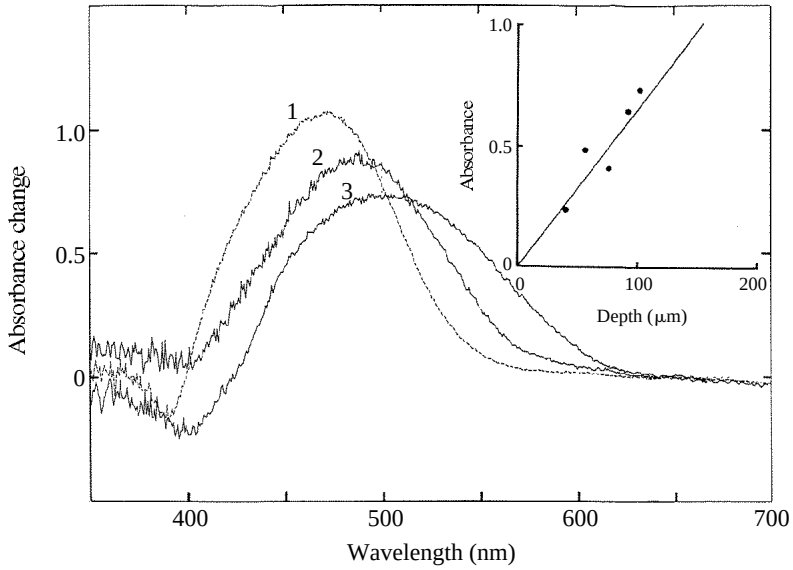

Fig. 3. Difference spectra of the OS layer of the retina of the firefly squid, before and after bleaching of visual pigment. Curve 1, distal yellow OS layer of the ventral retina at 200 μm from the surface. Curve 2, centre of the OS layer of the dorsal retina. Curve 3, proximal pinkish OS layer of the ventral retina 100 μm above the proximal layer of screening-pigment granules. The inset shows the relationship between depth and maximal absorbance of slices of the yellow OS layer.

microvilli. A fourth type of OS cell (δ) also appeared in such sections, as illustrated in Fig. 2C,D. The δ-type cytoplasmic profiles are ellipsoidal in cross section, with a rhabdomere extending from the flattened sides. The δ-type OS cells appear among bundles of rhabdomless proximal regions of α, β and γ OS cells. The δ-type OS cells form smaller rhabdoms, each consisting of only two rhabdomeres, in which the microvilli are perpendicular to one another.

The cross-sectional pattern in Fig. 1D,E is similar in appearance to sections through the dorsal regions of this retina. Fig. 2E,F shows LM cross sections at 70 μm depth from the retinal surface and near the basement membrane of the dorsal retina. The rhabdom pattern in Fig. 2E is found throughout the OS layer of the dorsal retina from the retinal surface to about 10 μm above the section shown in Fig. 2F. The OS cells in this region are about 200 μm long, and the rhabdom arrangement appears to be almost uniform throughout the layer. The dense pigment granules are found only near the basement membrane (Fig. 2F), so the rest of the OS layer is highly transparent.

Microspectrophotometry (MSP) of the OS layer of the ventral retina yields difference spectra for the yellow OS layer (curve 1), for the pink layer (curve 3) and for a comparison with the OS layer of the dorsal retina (curve 2) such as those in Fig. 3. The inset shows the relationship between thickness and maximal absorbance of slices of the yellow OS layer. The maximal absorbances per 100 μm thickness were at 470 nm (0.7) for the yellow layer, 500 nm (0.49) for the pink layer and 484 nm (0.7) for the dorsal OS layer.

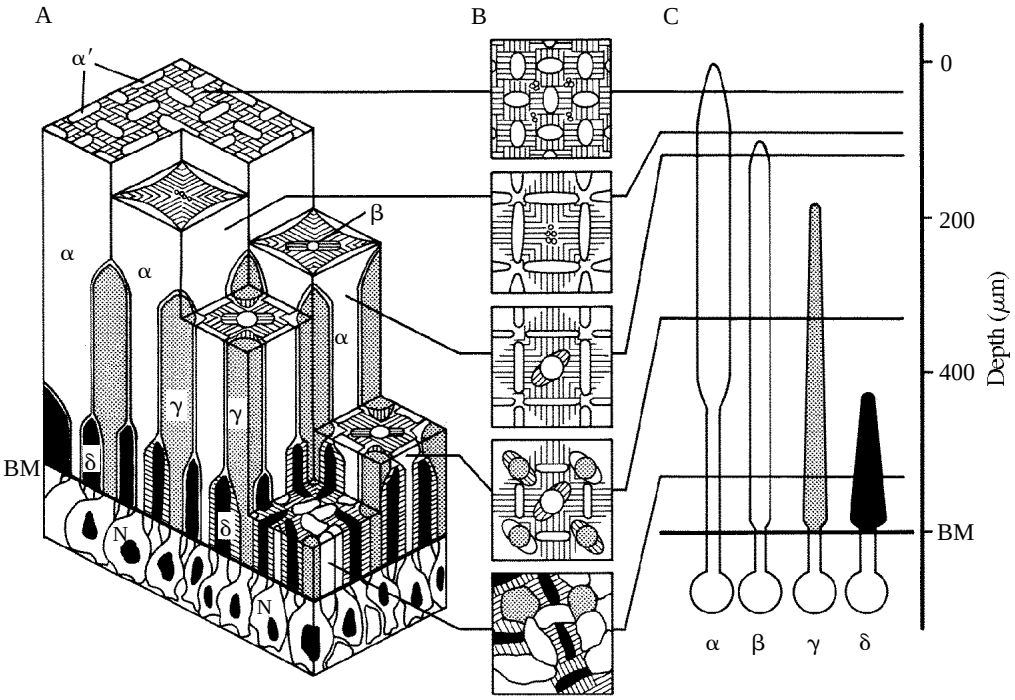

Fig. 4. Diagram to illustrate the arrangement of various outer segments in the ventral retina of the firefly squid *Watasenia scintillans*. (A) Block diagram of the retinal elements. (B) Cross sections at the indicated levels. (C) Representation of the relative positions of α, β, γ and δ photoreceptor OS cells. The longest (α) OS has a slender head (α′) at the distal end, where smaller-sized rhabdoms appear in cross sections. Parallel lines indicate the orientations of microvilli in the rhabdom. The right-hand scale indicates the depth from the surface of the ventral retina. BM, basement membrane; N, nucleus.

Discussion

Previous studies by our group have shown that the A2 (500nm) and A4 (470nm) visual pigments of *W. scintillans* are restricted to the proximal and distal OS layers of the ventral retina, respectively (Seidou *et al.* 1990). If individual visual pigments are localized in different photoreceptor cells, the eye may have the capacity for wavelength (colour) discrimination.

Our observations on the remarkably thick OS layer of the firefly squid ventral retina are summarized in Fig. 4. The α-, β- and γ-type OS cells produce their microvilli only in the distal two-thirds of the OS layer, and the δ-type OS cells lay down microvilli only in the proximal third. This strongly suggests that the A2 and A4 pigments are segregated into the outer segments of different visual cells. Therefore, *W. scintillans* may indeed be able to perform some form of wavelength discrimination.

It is known that many deep-sea fishes have a multi-bank retina (Denton and Locket, 1989), the individual OS cells of which are clearly observed even at the LM level. However, in *W. scintillans*, it has proved more difficult to demonstrate any discontinuity

of the OS layer. This may be because the individual OS cells are tightly bound to their neighbours not only in the rhabdom region but over the whole cell surface, explaining why the cleaved OS layer of the retina (Fig. 1A) revealed intracellular surfaces rather than structures such as microvilli. In the distal part of the OS layer, cytoplasmic profiles contain many vesicles which might be involved in the processing of microvillous membrane components, including visual pigments, since these were conspicuous only in the distal OS layer (Fig. 1B–E), where all the OS cells bear microvilli.

The OS cells of the dorsal retina of *W. scintillans* are comparable in length to those of other cephalopods living successfully in photic regions of the deep sea. For example, in our observation, *Enoploteuthis chunii*, called the pseudo-firefly squid, lives a little deeper (below 300m in the daytime) in the Sea of Japan, but the retina does not have specialized dorsal and ventral parts, having uniform OS cells of about 200 μm in length. Also, the intensity of downwelling light is strongest in the '12 o'clock', vertical direction in the deep sea (Kampa, 1970; Munz and McFarland, 1977). This suggests that the long rhabdom in the ventral retina of *W. scintillans* is not merely to compensate for the dimness of the environmental light, because the ventral retina is the region receiving the strongest incident light.

Denton and Locket (1989) proposed that the multi-bank retinae of deep-sea fishes make it theoretically possible for them to discriminate colour even with a single visual pigment. This is because the overlying OS cells would act as a filter and shift the photosensitivity curves of the underlying OS cells. They also mentioned that the use of more than one visual pigment in the banked retina would be even more effective. This seems to be the case in *W. scintillans*. If the visual pigment in the underlying OS cells had an absorbance maximum at a wavelength longer than that of the overlying 'filter' pigment, the shift of the photosensitivity spectral maximum towards longer wavelengths would be pronounced, with little loss of photosensitivity.

We tried to determine the absorbance spectra of visual pigments in the fresh retina, as shown in Fig. 3. The difference spectrum would be very near to the true absorbance spectrum, but the spectra we obtained were disturbed at shorter wavelengths, presumably as a result of the scattering effect and the dense absorbance of the tissue slices. Judging from the absorbance spectra before bleaching of slices of various thickness, and from the spectrum of the A1 pigment extracted from the dorsal retina (Matsui *et al.* 1988*a*), the spectra are reliable in the wavelength range longer than 420nm. The absorbances of 0.7 at 470nm for the yellow A4 pigment layer and 0.49 at 500nm for the pink A2 pigment layer were obtained from measurements taken perpendicular to the long axis of the OS layer. These values are not expected to differ markedly from measurements taken along the technically difficult vertical axis, because this squid retina has dense pigment granules only near the basement membrane and the OS layer is fairly transparent. The yellow OS layer was 400 μm thick, so the total absorbance of this layer would reach 2.8. Fig. 5B shows the absorbance spectrum of the A4 pigment with an absorbance of 2.8; this figure was prepared from data on the absorbance of the A4 pigment solution (Kito *et al.* 1992*a*). The transmittance spectrum of this pigment is also shown in the figure, and the filter would shift the photosensitivity maximum of the underlying A2 pigment OS cells towards 550nm, as shown in Fig. 5C. The pronounced shift of the sensitivity peak is

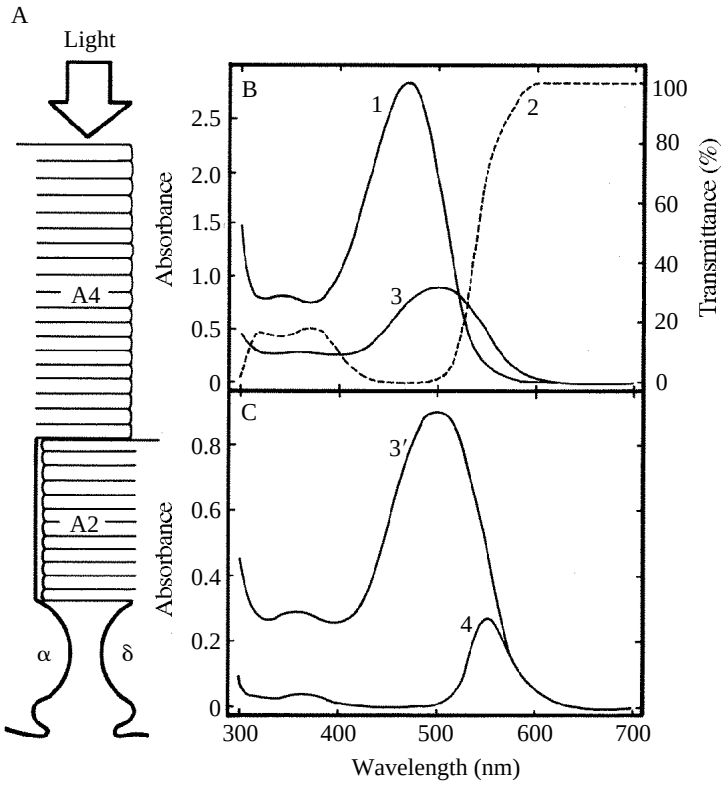

Fig. 5. The filter effect of the overlying OS layer with A4 pigment on the underlying visual cell with the A2 pigment. (A) Diagram showing that the α-type OS cell with A4 pigment overlies the δ-type OS cell with A2 pigment. (B) Curve 1, absorbance spectrum of the A4 pigment with a maximal absorbance of 2.8 at 470nm. Curve 2, transmittance spectrum of the A4 pigment deduced from curve 1. Curve 3, absorbance spectrum of the underlying A2 pigment with a maximal absorbance of 0.98 at 500nm, constructed on the basis of the vertebrate A2 pigment (Dartnall and Lythgoe, 1965). (C) Curve 3′, same as curve 3, differing in vertical scales. Curve 4, the resultant photosensitivity of the A2 pigment cell, derived from curves 2 and 3′.

attained at the expense of a considerable loss of sensitivity, but the underlying OS containing A2 pigment still seems to have some effective sensitivity in the region of longer wavelengths.

The separation of the maximal wavelength of photosensitivity between visual cells in the A4 and A2 pigment OS layers should be more than sufficient to provide for some form of wavelength discrimination by the squid. In particular, the red-shifted photosensitivity is remarkably close to the bioluminescent green light produced by these squids, which emit not only a 470nm blue light but also a 540nm green light from the different classes of ventral photophores (Kito *et al.* 1988, 1992*b*).

Furthermore, it is also clear that, although the distal yellow OS layer is composed of three OS types using a single visual pigment with an absorbance maximum at 470nm, the

medium-sized β and γ OS cells, which are about 250 μm long, are overlain by the longest α-type OS layer, which is at least 100 μm thick. The β and γ photosensitivities would thus also be modified to some extent by the yellow filter of 0.7 absorbance, as calculated on the basis of the spectra shown in the Fig. 5. This suggests that the receptor system of *W. scintillans* is more complex than for a simple two-banked retina.

It should be noticed that in the animal kingdom, insects and crustaceans have rhabdomeric photoreceptors and that many of them have fused rhabdoms consisting of rhabdomeres of several retinula cells, differing in their spectral sensitivities (Arikawa *et al.* 1987). These fused rhabdoms would be essentially similar in structure and function to the banked OS cells of the ventral retina of *W. scintillans.*

Another point of interest is the rhabdomere orientation in the firefly squid retina. As shown in Fig. 4, the rhabdomeres of the α-type OS cells are perpendicular both to each other and to those of the β- and γ-type OS cells. Furthermore, the rhabdomeres of the deeper δ-type OS cells are paired and perpendicular to each other. The relative orientation of the δ rhabdomeres to other types of rhabdomeres is uncertain, because their transition zone was difficult to locate. The highly regular orientation of the rhabdomeres of cephalopod photoreceptors, which appear to be very similar in both the sublittoral octopus (Yamamoto *et al.* 1965) and the firefly squid, reflects the high degree of polarization-sensitivity of the cephalopod eye (Moody and Parriss, 1960; Tasaki and Karita, 1966; Snyder, 1973). In the sky and in clear water, light has a significant component of plane-polarized light resulting from Rayleigh scattering. Near the surface of the sea, animals with polarization-sensitivity should therefore be able to utilize this property. However, deeper in the sea, the large amount of suspended matter would tend to produce random scattering, reducing the amount of polarization. It is, therefore, very interesting to investigate how the firefly squid is able to make use of its inherent polarization-sensitivity. So far, we know little about the life of this deep-sea animal and can only marvel at the fact that, in addition to polarization-sensitivity, the firefly squid has developed three visual pigments in a banked retina as part of its strategy of adaptation to the deep-sea environment.

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