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Fluorescence of Blowfly Metarhodopsin*

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Abstract. The visual pigment of blowfly peripheral photoreceptors displays a marked red fluorescence when the pigment is in the metarhodopsin state as demonstrated in vivo by simultaneous measurements of transmission and fluorescence. The rhodopsin state is non-fluorescent.

It is argued that fluorescence offers a unique means to study visual pigment properties in completely intact living animals and, furthermore, provides the opportunity for studying the photoreceptor optics of visual waveguides. As an example the action of the pupil mechanism of blowfly visual sense cells on the fluorescence signal is demonstrated.

Key words: Fluorescence – Fly – Photoreceptor – Metarhodopsin

Introduction

Autofluorescence of visual receptors has been observed in a wide variety of retinae (Liebman and Leigh 1969; Franceschini 1977) but its potential for studying visual pigments is clearly not yet generally recognized, as for instance in a recent handbook (Packer 1982, covering over 100 methodological papers) visual pigment fluorescence is not treated. The reason for this neglect may be that the fluorescence signal is weak (Guzzo and Pool 1968), and the high light intensities which are thus necessary for visual observation rapidly induce irreversible bleaching of visual pigment (see note p. 318).

Actually, however, this drawback only holds true for vertebrates since the visual pigments of invertebrates are photochromic substances, i.e., the molecules have two thermostable states, rhodopsin and metarhodopsin, which are photointerconvertible (rev. Goldsmith 1972; Hamdorf 1979; Stavenga and Schwemer 1983). This property makes fluorescence especially attractive for experimental investigations of invertebrate visual pigments. In fact, recognizing

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the possibilities Cronin and Goldsmith (1981, 1982a, b) have performed a series of detailed analyses of crayfish visual pigment in situ by using the distinct red fluorescence exhibited by the metarhodopsin state. The fluorescence of the pigment in the rhodopsin state appeared to be non-detectable.

Essentially the same situation occurs in flies: rhodopsins do not fluoresce and metarhodopsins fluoresce in the red (Stavenga and Franceschini 1981). This report describes experiments on blowfly photoreceptors illustrating the basic fluorescence properties.

Materials and Methods

Live blowflies *Calliphora erythrocephala*, wild type and mutant chalky from laboratory stocks reared on a vitamin-A-rich diet, were immobilised by placing the fly in a well-fitting plastic tube to which the head was glued.

The measurements were performed with a microspectrophotometer (MSP) using the deep pseudopupil phenomenon (e.g., Kirschfeld and Frachceschini 1969; Franceschini 1975; Franceschini and Kirschfeld 1976; Stavenga 1976, 1979). The MSP is a Leitz Orthoplan equipped with a Compact photometer head (photomultiplier Hamamatsu R928, multialkali cathode). Epi-illumination was applied via a half-silvered mirror and a Leitz NPL 10 objective. The illumination, alternating blue and red, caused changes in visual pigment composition, which were monitored by measuring the antidromic transmission and/or the induced fluorescence.

In the case of the transmission measurements a small piece of the back of the head capsule of the fly was removed to allow antidromic illumination via a flexible light guide. Identical interference filters (567 nm; Schott DAL) were put in the illumination beam and in front of the photomultiplier in order *i*) to

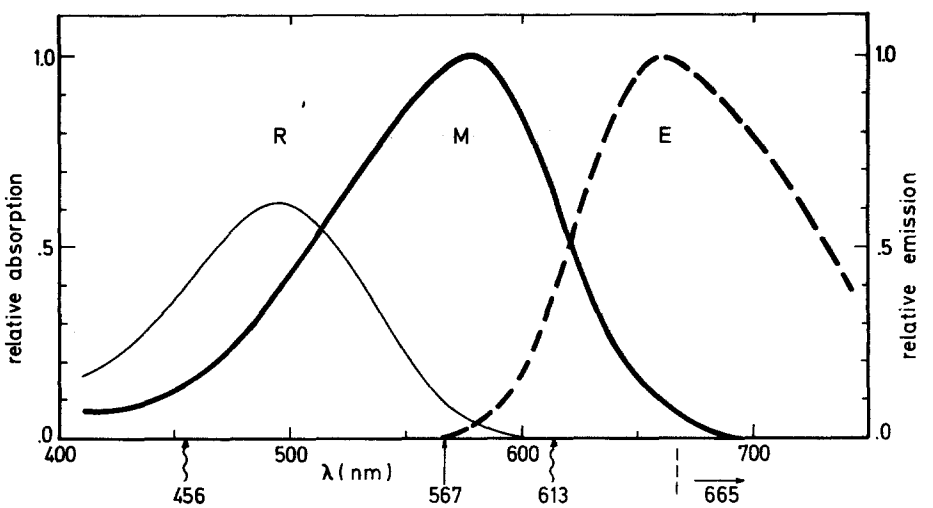

Fig. 1. Absorption spectra of blowfly rhodopsin R and metarhodopsin M, and emission spectrum of metarhodopsin E, normalised to the metarhodopsin absorption peak

measure near the peak of the difference spectrum resulting from conversion of rhodopsin to metarhodopsin (see Fig. 1), and *ii*) to block the blue and red epi-illumination light reflected from the microscope optics.

The fluorescence was measured by placing a RG 665 (Schott) or a Wratten 70 (Kodak) far-red transmitting filter in front of the photomultiplier. The relevant spectra of blowfly visual pigment are given in Fig. 1. Rhodopsin R495 is photointerconvertible with metarhodopsin M580. The wavelengths of the epi-illumination (adapting light) used for converting visual pigment in Fig. 2 are indicated in Fig. 1 by wavy arrows. The induced emission was measured above 665 nm in Fig. 2; indicated in Fig. 1 by the horizontal arrow. The approximate emission spectrum of metarhodopsin is given by the dashed curve (from Stavenga, in preparation).

Results

Monitoring Visual Pigment Conversions by Fluorescence Measurements

The classical method for measuring photochemical changes in the visual pigment composition in photoreceptors relies on the different absorption characteristics of the various visual pigment states. Transmission measurements thus have yielded the absorption spectra of blowfly rhodopsin and metarhodopsin given in Fig. 1 (see Hamdorf 1979; Stavenga 1976).

Figures 2a and 2b show measurements of the transmission at 567 nm of the eye of a blowfly mutant chalky. The experiment of Fig. 2a demonstrates that

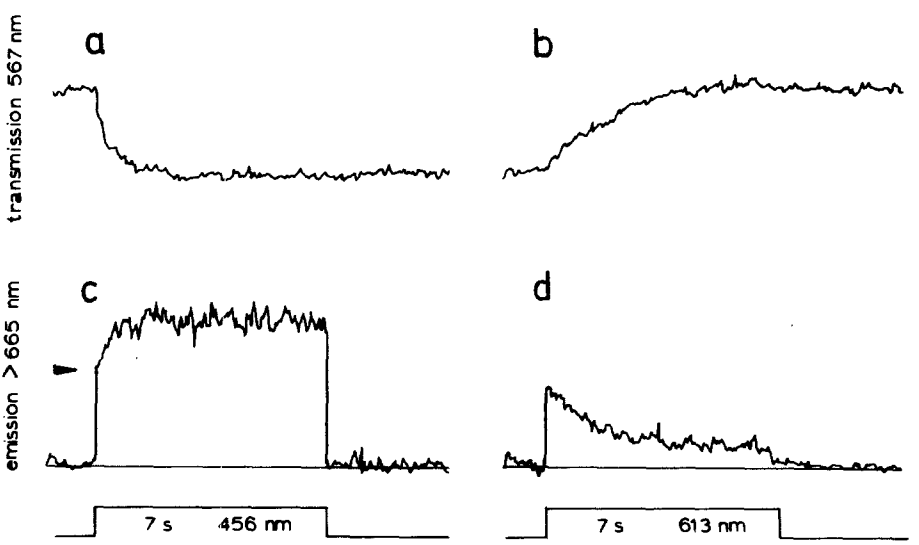

Fig. 2. Simultaneous transmission and fluorescence measurements performed on a blowfly mutant chalky in vivo. The baselines of traces **a** and **c** and of **b** and **d** are identical. For further explanation see text

blue adaptation light (456 nm) converts a substantial fraction of the rhodopsin molecules into the metarhodopsin state, whereas Fig. 2b testifies that subsequent red light (613 nm) reconverts the metarhodopsin molecules back, so that a photo-equilibrium is established with a high rhodopsin (and low metarhodopsin) fraction; this was actually the initial situation in Fig. 2a. The experimental sequence performed in a live blowfly mutant chalky can be repeated numerous times with perfect reproducibility, so that transmission changes and emission changes induced by the converting flashes can be measured alternately. The changes in far-red emission occurring during the blue and red illuminations are given in Fig. 2c and 2d respectively. Evidently, the emission increases with an increase in metarhodopsin and decreases when metarhodopsin is converted back to rhodopsin. Both the initial emission signal in Fig. 2c and the remaining final emission in Fig. 2d are due to autofluorescence originating from substances other than visual pigment as was determined by *i*) measuring from eye areas other than the deep pseudopupil, and *ii*) measuring from the deep pseudopupil in vitamin-A deprived flies. In both cases no changes in transmission or fluorescence were detected.

The conclusion that blowfly metarhodopsin exhibits a red emission is corroborated by the experiment of Fig. 3. The wavelenghts of the alternatively delivered illuminations were now 477 nm and 606 nm respectively. The fluorescence measurements were performed at two intensities differing by 1 log unit in order to compare amount and kinetics of fluorescence as a function of

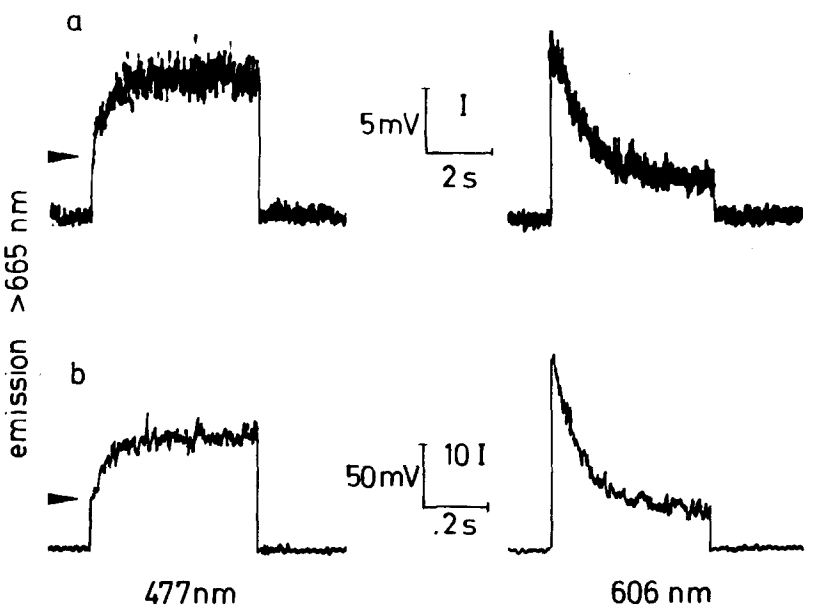

Fig. 3. Emission above 665 nm measured from the eye of a blowfly mutant chalky during photoconversion induced by blue (477 nm) and red (606 nm) light of intensities differing by approximately 1 log unit. The magnitudes and time courses of the signals differ accordingly, but identical curves, except for the improved signal-to-noise ratio at the higher intensity, are obtained by adjusting the recorder settings

intensity. Therefore the recorder settings were adjusted so that in the high-intensity experiment (Fig. 3b) the sensitivity was reduced by 1 log unit and the speed increased by 1 log unit. Both sets of curves in Fig. 3a und 3b appear identical in size and shape except for the improved signal-to-noise ratio attained at the high intensities. These results show that emission intensity is proportional to excitation light intensity, and that the conversion speed of the fluorescing pigment is proportional to light intensity. This demonstrates that the usual photochemical description of blowfly visual pigment (e.g., Hamdorf and Schwemer 1975) is applicable to the fluorescence kinetics.

Experiments undertaken to detect emission changes proportional to changes in rhodopsin concentration, specifically in the green, i.e., outside the metarhodopsin emission range, have failed. Consequently, rhodopsin fluorescence can be neglected and the important conclusion to be drawn is that metarhodospin can be studied separately from rhodopsin through fluorescence.

Pupil Effects Interfering with Fluorescence Measurements in Wild Type Flies

In the experiments described above the white eyed mutant chalky was used. This mutant lacks the pupil mechanism of wild type flies which consists of migratory pigment granules inside the photoreceptor cells (Kirschfeld and Franceschini 1969). Intense illumination drives the granules towards the rhabdomeres resulting in attenuation of light reaching the visual pigment. The bright light is necessary for fluorescence measurements but also activates the pupil mechanism. Contamination of the fluorescence measurements by the pupil accordingly seems inevitable in wild type flies.

The experiment of Fig. 4, performed on a wild type blowfly, demonstrates the pupil action. Preceding the trace in Fig. 4a a blue (488 nm) adapting light was given followed by a 1-min dark period. At the start of Fig. 4a the visual pigment molecules were thus predominantly in the metarhodopsin state. Therefore the red (613 nm) illumination initially induced a high emission, but the decrease of the signal shows that the illumination rapidly converted the metarhodopsin molecules into the rhodopsin state (compare Figs. 2, 3). The subsequent blue (488 nm; Fig. 4b) illumination initially induced a low emission due to metarhodopsin absence, but the transient emission increase demonstrates that metarhodopsin was produced. Whereas in the corresponding experiments on the mutant chalky the signal reached a stable level when a photoequilibrium was established (e.g., Fig. 2c), a second phase emerged in Fig. 4b, due to the pupil mechanism of the wild type fly. The activated pupil diminished the effective intensity of the exciting light, and thus the emission from the photoreceptors was reduced.

The action of the pupil is seen even more clearly in Fig. 4c. Between Figs. 4b and 4c an interval of 1-min darkness was given. During such a time negligible changes occur in the visual pigment (Stavenga et al. 1973) and hence a high metarhodopsin content was maintained. However, the pupil relaxes during

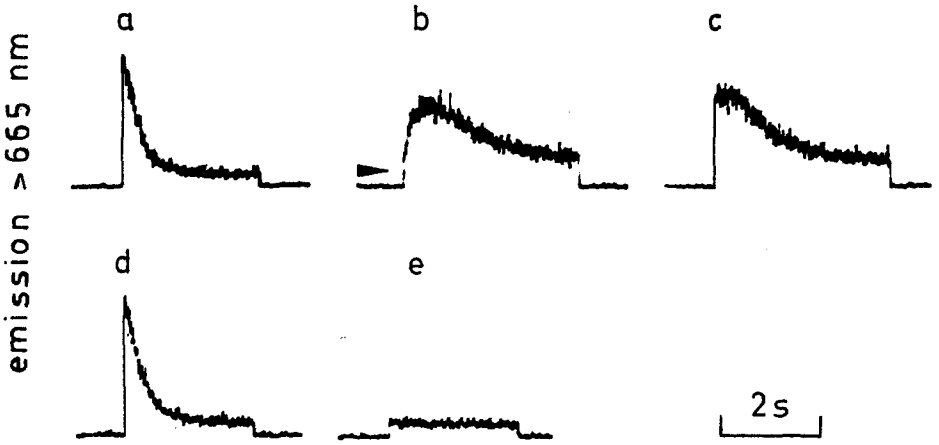

Fig. 4. Emission above 665 nm induced by red (613 nm; **a, d, e**) and blue (488 nm; **b, c**) light; wild type blowfly. The (constant intensity) illumination activates the pupil, which subsequently relaxes during the 1-min dark time given between the illuminations. Photoconversions occur transiently in **a, b,** and **d.** The pupil influence is distinctly noticeable with blue illumination and negligible with red illumination, since the pupil absorbs highly in the blue and little in the red (Stavenga et al. 1973)

darkness, so that initially in Fig. 4c the blue light flux was unimpeded. Therefore an initial high emission was induced, which was reduced due to the action of the pupil. After Fig. 4c another 1-min darkness was given. A subsequent red illumination (Fig. 4d) yielded a process identical to that of Fig. 4a. Figure 4e taken after another 1-min darkness shows that the remnant red induced fluorescence was not affected by the pupil and therefore originated from eye tissue outside the rhabdomeres.

Figure 4 demonstrates two points. First, metarhodopsin can be reliably investigated in wild type animals when the action of the pupil is accounted for, and second, the existence of visual pigment fluorescence facilitates investigations of the pupil mechanism. For instance, the curve of Fig. 4c shows that pupil closure occurs within a few seconds (room temperature), a finding already known from antidromic transmission experiments (e.g., Stavenga 1979). Indeed simultaneous transmission and fluorescence measurements, as that of Fig. 2, performed in wild type blowfly (unpublished), yield very similar pupillary effects on both signals.

Discussion

Fluorescence of Fly Metarhodopsin

The discovery of fluorescing fly photoreceptors (Franceschini 1977; Stark et al. 1977) has opened a new research field in invertebrate vision. The bright red fluorescence observable after irradiation with bright UV or blue light (Franceschini et al. 1981a), however, is not the fluorescence of metarhodopsin M demonstrated in the present paper, but originates from a different visual

pigment state. We presume that this state, coined metarhodopsin M′ (Franceschini et al. 1981a; Franceschini 1983), is a photoproduct of one of the short-lived intermediates in the photochemical cycle, probably of lumirhodopsin (see Kruizinga et al. 1983), as M′ can build up only at extreme light intensities. From a physiological viewpoint M′ may be considered a completely unimportant and artificially produced state, but its importance in retinal studies is unquestionable, as Franceschini et al. (1981a, b) have succesfully utilised the bright fluorescence as a distinct marker in their classification studies of fly photoreceptor cell types. Both metarhodopsin M and M′ are found in fly peripheral photoreceptors in general, as e.g., the blowfly *Calliphora erythrocephala,* the housefly *Musca domestica* (Stavenga and Franceschini 1981) and the fruitfly *Drosophila melanogaster.* Actually, simultaneous transmission and fluorescence measurements on fruitfly eyes failed to yield a fluorescence increase accompanying the transmission decrease indicating metarhodopsin formation (Stark et al. 1979). Also no change in fluorescence signal was observed in the blowfly mutant chalky. The reason for this failure was the use of an inadequate filter in front of the photomultiplier which passed a very broad wavelength range, namely above 570 nm, instead of > 665 nm as used in the experiments presented here. With an orange filter the metarhodopsin signal appears to be swamped by the background. By using a far-red transmitting filter, which favours the metarhodopsin signal over that of the background, the fluorescence of fruitfly metarhodopsin can indeed be clearly demonstrated (unpublished observations performed together with W. S. Stark).

The basic differences between the metarhodopsins M and M′ have as yet to be clarified, but it seems that the excitation and emission spectra are remarkably similar; the maxima are located at 570–580 nm and ≈ 660 nm respectively; only M′ fluoresces (> 3 times) more strongly than M, whereas the photosensitivity of M′ is much smaller (Franceschini et al. 1981a; Stavenga, Franceschini and Kirschfeld, submitted). Furthermore, the fluorescence lifetimes of both M and M′ determined by yellow laser flashes is < 20 ns (in vivo; Kruizinga, Kamman and Stavenga, in preparation).

Fluorescence of Invertebrate Metarhodopsins

The findings on blowfly metarhodopsin are closely related to those obtained by Cronin and Goldsmith (1981, 1982a) for crayfish metarhodopsin which also shows a distinct red fluorescence. It seems quite likely that invertebrate metarhodopsins generally fluoresce in the red, as red emission can be observed from a wide variety of insect photoreceptors (Franceschini et al. 1981a). In accordance with this view we have found that in the specialised dorsal eye of the male mayfly *Cloeon dipterum* (e.g., Wolburg-Buchholz 1977) a distinct red fluorescence can be elicited by UV-illumination. Preliminary measurements indicate that the emission originates from a blue absorbing metarhodopsin ($\lambda_{max} \approx 480$ nm) which was formed by photoconversion from a UV rhodopsin ($\lambda_{max} \approx 365$ nm), confirming electrophysiological data on a related mayfly (Horridge and MacLean 1978).

Fluorescence as a Tool for Photoreceptor Studies

The main advantage of the fluorescence measurements described is that the method is non-invasive; it allows photoreceptor studies on completely intact, living animals over a long period of time. The experiment of Fig. 4 on the pupil mechanism existing inside the photoreceptor cells provides an example of how visual pigment fluorescence can be utilised. Similar experiments performed in hymenopterans confirmed the distinctly slower time course of the pupil in that insect order. Whereas the half time of pupil closure in flies is 1–3 s (Fig. 4; e.g., Franceschini and Kirschfeld 1976; Stavenga 1979), it takes in hymenopterans 10–20 s (Stavenga and Kuiper 1977). The cricket *Gryllus bimaculatus* has an even slower pupil: half time 1–2 min (unpublished; for anatomical evidence for the existence of the pupil see Burghause 1979). It is especially in animals like crickets where transmission measurements are virtually impossible that fluorescence measurements yield data which otherwise could not have been obtained.

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Note added in proof: A brief review on rhodopsin emission has been presented recently by Lewis and Perrault.

Lewis A, Perrault GJ (1982) Emission spectroscopy of rhodopsin and bacteriorhodopsin. In: Packer L (ed) Biomembranes. Academic Press, New York (Methods in enzymology, vol 82, Part H II, pp 217–229)

References

Burghause FMHR (1979) Die strukturelle Spezialisierung des dorsalen Augenteils der Grillen (*Orthoptera, Grylloidea*). Zool Jahrb Abt Allg Zool Physiol Tiere 83: 502–525

Cronin TW, Goldsmith TH (1981) Fluorescence of crayfish metarhodopsin studied in single rhabdoms. Biophys J 35: 653–664

Cronin TW, Goldsmith TH (1982a) Photosensitivity spectrum of crayfish rhodopsin measured using fluorescence of metarhodopsin. J Gen Physiol 79: 313–332

Cronin TW, Goldsmith TH (1982b) Quantum efficiency and photosensitivity of the rhodopsin ⇄ metarhodopsin conversion in crayfish photoreceptors. Photochem Photobiol 36: 447–454

Franceschini N (1975) Sampling of the visual environment by the compound eye of the fly: Fundamentals and applications. In: Snyder AW, Menzel R (eds) Photoreceptor optics. Springer, Berlin Heidelberg New York, pp 98–125

Franceschini N (1977) In vivo fluorescence of the rhabdomeres in an insect eye. Proc Int Union Physiol Sci XIII, 237. XXVIIth Int Congr Paris

Franceschini N (1983; in press) In vivo microspectrofluorimetry of visual pigments. In: Cosens DJ, Vincent-Prue (eds) The Biology of Photoreceptors, Symposia of the Society for Experimental Biology. Cambridge University Press, London

Franceschini N, Kirschfeld K (1976) Le contrôle automatique du flux lumineux dans l'oeil composé des Diptères. Propriétés spectrales, statiques et dynamiques du mécanisme. Biol Cybern 21: 181–203

Franceschini N, Kirschfeld K, Minke B (1981a) Fluorescence of photoreceptor cells observed in vivo. Science 213: 1264–1267

Franceschini N, Hardie R, Ribi W, Kirschfeld K (1981b) Sexual dimorphism in a photoreceptor. Nature (London) 291: 241–244

Goldsmith TH (1972) The natural history of invertebrate visual pigments. In: Dartnall HJA (ed) Photochemistry of vision. Springer, Berlin Heidelberg New York (Handbook of sensory physiology, vol VII/1, pp 685–719)

Guzzo AV, Pool GL (1968) Visual pigment fluorescence. Science 159: 312–314

Hamdorf K (1979) The physiology of invertebrate visual pigments. In: Autrum H (ed) Invertebrate photoreceptors. Springer, Berlin Heidelberg New York (Handbook of sensory physiology, vol VII/6A, pp 145–224)

Hamdorf K, Schwemer J (1975) Photoregeneration and the adaptation process in insect photoreceptors. In: Snyder AW, Menzel R (eds) Photoreceptor optics. Springer, Berlin Heidelberg New York, pp 263–289

Horridge GA, McLean M (1978) The dorsal eye of the mayfly *Atalophlebia (Ephemeroptera)*. Proc R Soc London Ser B 200: 137–150

Kirschfeld K, Franceschini N (1969) Ein Mechanismus zur Steuerung des Lichtflusses in den Rhabdomeren des Komplexauges von *Musca*. Kybernetik 6: 13–22

Kruizinga B, Kamman R, Stavenga DG (1983) Laser induced visual pigment conversions in fly photoreceptors measured in vivo. Biophys Struct Mech 9: 301–309

Liebman PA, Leigh RA (1969) Autofluorescence of visual receptors. Nature (London) 221: 1249–1251

Packer L (ed) (1982) Visual pigments and purple membranes. In: Biomembranes. Academic Press, New York (Methods in enzymology, vol 81, Part H 1)

Stark WS, Ivanyshyn AM, Greenberg RM (1977) Sensitivity and photopigments of R1–6, a two-peaked photoreceptor, in *Drosophila, Calliphora* and *Musca*. J Comp Physiol 121: 289–305

Stark WS, Stavenga DG, Kruizinga B (1979) Fly photoreceptor fluorescence is related to UV sensitivity. Nature (London) 280: 581–583

Stavenga DG (1976) Fly visual pigments. Difference in visual pigments of blowfly and dronefly peripheral retinula cells. J Comp Physiol 111: 137–152

Stavenga DG (1979) Pseudopupils of compound eyes. In: Autrum H (ed) Invertebrate photoreceptors. Springer, Berlin Heidelberg New York (Handbook of sensory of physiology, vol VII/6A, pp 357–439)

Stavenga DG, Franceschini N (1981) Fly visual pigment states, rhodopsin R490, metarhodopsins M and M′, studied by transmission and fluorescence microspectrophotometry in vivo. Invest Ophthalmol Vis Sci (Suppl) 20: 111

Stavenga DG, Kuiper JW (1977) Insect pupil mechanisms, I. On the pigment migration in the retinula cells of *Hymenoptera* (suborder *Apocrita*). J Comp Physiol 113: 55–72

Stavenga DG, Schwemer J (1983; in press) Visual pigments of invertebrates. In: Ali MA (ed) Photoreception and vision in invertebrates. Plenum Press, New York

Stavenga DG, Zantema A, Kuiper JW (1973) Rhodopsin processes and the function of the pupil mechanism in flies. In: Langer H (ed) Biochemistry and physiology of visual pigments. Springer, Berlin Heidelberg New York, pp 175–180

Wolburg-Buchholz K (1977) The superposition eye of *Cloeon dipterum:* The organization of the *lamina ganglionaris*. Cell Tissue Res 177: 9–28

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