# A Microspectrophotometric Study of the Shielding Properties of Eyespot and Cell Body in *Chlamydomonas*

Klaus Schaller and Rainer Uhl
Abteilung Physikalische Biologie der Ludwig Maximilians Universität, D-80638 München, Germany

ABSTRACT The eyespot apparatus of the unicellular alga *Chlamydomonas* exhibits a clear directivity, i.e., it perceives light from different directions with different sensitivity. Using a newly constructed confocal microscope we have studied how absorption and reflection of eyespot and cell body shape this directivity. In agreement with previous results the eyespot was found to be highly reflectant, owing to its interference reflector design, but only for yellow light. Light of 490 nm, the maximum of absorption of the photoreceptor, was hardly reflected at all, even when the reflector was "tuned" to lower wavelengths by tilting it relative to the incoming light. The absorption of the carotenoids in the interference reflector also contributed little to the shielding properties of the cell, leaving the major contribution to the cell body. Thus most of the attenuation of light reaching the eyespot from the rear is due to chlorophyll and other pigments within the cell. In its peak around 490 nm the "contrast-ratio" reached a value of 8–10.

## INTRODUCTION

The ability of *Chlamydomonas* to sense the light direction and adjust its swimming direction accordingly requires some sort of directivity for its photoreceptor assembly. For most of this century this has been assumed to be due to absorption of light by both stigma and chloroplast (for a review see Nultsch and Häder, 1979). In 1980 Foster and Smyth came forward with the idea that the eyespot may act as an interference reflector, i.e., as a biological quarter-wave-stack, and subsequent confocal microscopy studies (Kreimer and Melkonian, 1990; Kreimer et al., 1992) and biochemical studies (Grung et al., 1994) have lent support to this notion. However, the observation that mutants lacking stigma clearly exhibit photoorientation (Morel-Laurens and Feinleib, 1983; Sineshchekov et al., 1989; Kreimer et al., 1992; Lawson and Satir, 1994) has already suggested that the interference reflector cannot be the only shielding factor.

We have recently been able to show (Schaller et al., 1997) that a stimulus consisting of continuous light acts with a different spectral effectivity than pulsed light, even though there is no evidence for the existence of more than one photoreceptor molecule (Uhl and Hegemann, 1990; Kröger and Hegemann, 1994). A plausible explanation would be that the shielding properties of the cell, i.e., the spectral dependence of all factors that prevent light from reaching photoreceptor molecules from the rear, shape the spectral characteristics of the response to continuous light. To examine this we have constructed a special confocal microscope which allowed us to hold a living cell in a drop of saline between two water immersion objectives and to study the spectral and angular characteristics of light reflection and transmission for eyespot and cell body.

*Received for publication 31 October 1996 and in final form 28 May 1997.*
Address reprint requests to Rainer Uhl, Abteilung Physikalische Biologie der Ludwig Maximilians Universität, Menzinger Strasse 67, D-80638 München, Germany. Tel.: +49-89-1786-1227; Fax: +49-89-1786-1185; E-mail: uhl@botanik.biologie.uni-muenchen.de.

0006-3495/97/09/1573/06 $2.00

## MATERIALS AND METHODS

*C. reinhardtii* strain 806 $mt^-$ cells were grown and differentiated into gametes as previously described (Uhl and Hegemann, 1990).

To characterize the reflective and absorptive properties of eyespot and cell body as a function of wavelength and angle of incidence, single cells were held in a drop of saline between two water immersion objectives (Achroplan water 63x, 0.90, Carl Zeiss, Jena, Germany), Fig. 1. The pipette used to hold the algae (Rüffer and Nultsch, 1985) had a smaller opening than that usually employed for electrophysiological studies (Harz and Hegemann, 1991) and was found to have no influence on the optical properties of the cell.

The long working distance (2 mm) of the water immersion objectives allows manipulation of the cells with the pipette holding them and coupling light into the drop from the side, i.e., at an angle of 90°. With the help of beamsplitters (dichroic or 50%/50%) in the infinity part of the optics or directly into the intermediate image plane, light could be coupled into the microscope through both objectives. The same positions could also be used as output ports, where the eye, photodiodes, video cameras, or slow-scan CCD-cameras served as detectors. The slow-scan frame transfer CCD-camera and the high-intensity, rapid scanning monochromatic light source (polychrome) have been described previously (Messler et al., 1996).

The numerous illumination and detection geometries are described in detail in the corresponding chapters.

## RESULTS AND DISCUSSION

### The eyespot as interference reflector

When viewing *Chlamydomonas* with white, transmitted light, the eyespot appears as an orange-red spot of ~1 μm diameter (Buder, 1917; Mast, 1927). In reflection, i.e., when the eyespot is viewed from the side of the light source, a bright, yellow/green reflex appears (Mast, 1927; Foster and Smyth, 1980; Kreimer and Melkonian, 1990; Kreimer et al., 1992). By placing a 50%/50% beamsplitter in the illumination path of the microscope it was possible to shine light onto the eyespot apparatus and simultaneously examine the reflected light, either by eye or, quantitatively, with the help

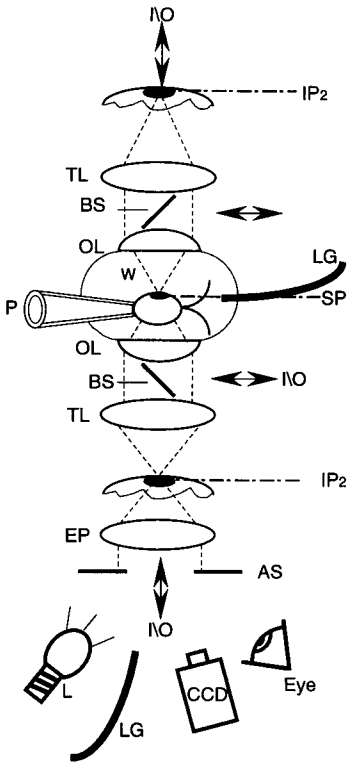


FIGURE 1 Optical set-up of the confocal microscope used for the microspectrophotometric and microspectroreflectometric measurements. The following abbreviations were used: I/O, optical input/output; IP, image plane; TL, tube lens; BS, beam splitter; OL, objective lens; W, drop of water; P, holding pipette; LG, light guide; SP, specimen plane; EP, eye piece; AS, aperture stop; L, lamp.

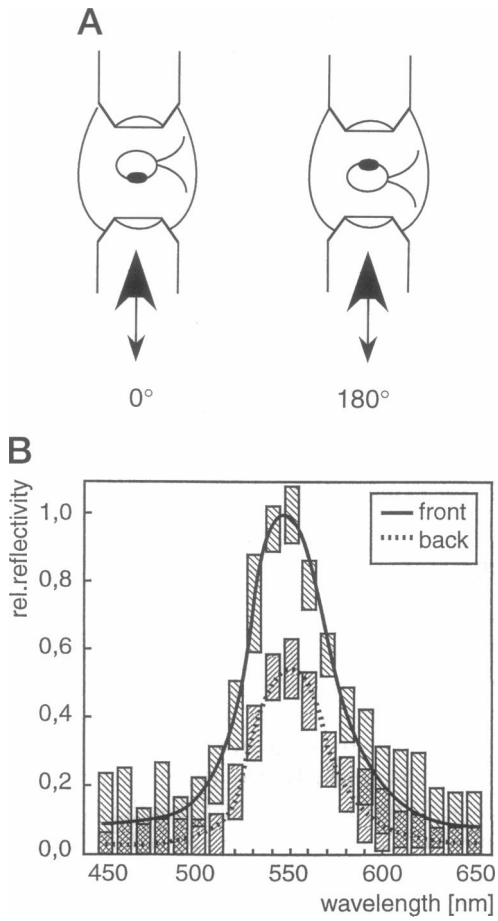


FIGURE 2 (*a*) Optical set-up for the confocal measurement of the stigma-reflectivity at 0° and 180°. The back-focalplane of the objective was fully filled, i.e., the cone of light corresponded to the numerical aperture of the water immersion objective of 0.9. (*b*) Wavelength dependence of the relative eyespot reflectivity, measured for light reaching the stigma directly from outside the cell or indirectly after passage through the cell body. The relative amplitudes of the two cases can be compared directly to each other.

of a slow-scan CCD-camera. Given its tiny dimensions, a clearly visible eyespot apparatus required the use of the full aperture of the objective, i.e., its numerical aperture of 0.9. With bare eyes the reflex "shone" like a star on an otherwise black sky.

Given the pixel size of the CCD-camera of 23 $\mu$m and the magnification of the water immersion objective of 63×, the light reflected by a 1-$\mu$m spot should have been distributed over 3 × 3 pixels. This was experimentally verified. For quantitative purposes, however, only the central 2 × 2 pixels were used. A silvered mirror, which had a reflectivity of >98% over the spectral range in question, served as reference in the specimen plane. Fig. 2 *a* shows the two optical measuring configurations and Fig. 2 *b* the wavelength-dependence of the relative reflectivity of an eyespot which was illuminated directly or through the cell body. Note that in both measurements the angle of illumination and reflection was 0 ± 42.5°. The reflectivity peaked around 545–550 nm and declined steeply toward the blue and the red. This is in contrast to theoretical results of Foster et al. (1980), but in agreement with unpublished experimental results from the same laboratory (Foster, personal communication). It is interesting to note that reflexes from the eyespot could not only be seen when the light struck the eyespot from the outside of the cell, but also when it passed the cell body before it hit the interference reflector (see also Kreimer and Melkonian, 1990). Assuming that the reflective properties were similar for light coming from outside and from within the cell, i.e., for an extracellular respectively intracellular saline-eyespot interface, the data in Fig. 2 *b* suggest that light of 550 nm is attenuated only by a factor of two when passing the cell body twice, giving a first indication that the "contrast-ratio" is very low for yellow light, but increases toward the blue. This is further elaborated in the next paragraph.

An interference reflector should change its wavelength of maximal reflectivity when tilted with respect to the incoming light (see Hecht, 1987). To measure light reflected at angles substantially different from 0° required a completely different optical set-up, in which confocality could no longer be maintained (Fig. 3 *a*). Relatively parallel, monochromatic light was coupled into the drop of saline with the help of a quartz fiber, and the reflected light was determined using a CCD-camera as before. It should be kept in mind,

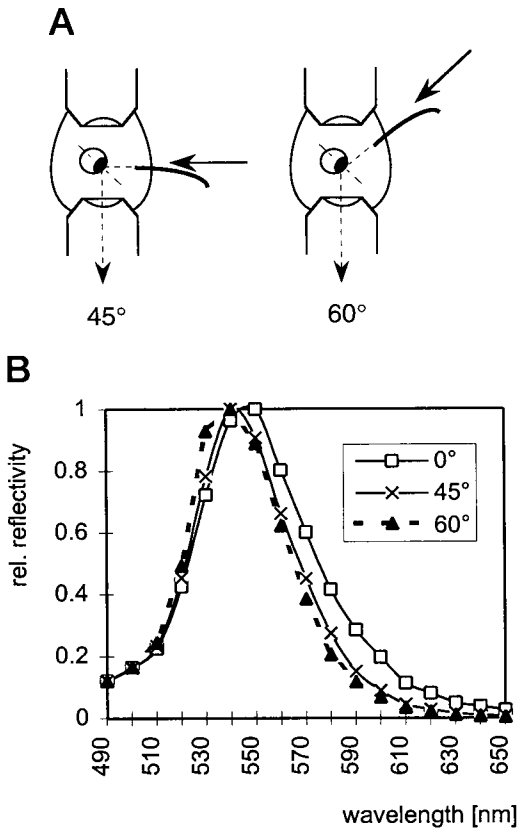


FIGURE 3 (*a*) Optical set-up for the measurement of eyespot-reflectivity using parallel light and an angle of incidence of 45° and 60°. The measuring beam was parallel to within ±7.5°. (*b*) Wavelength dependence of the relative eyespot reflectivity at two different angles of incidence, namely 45° and 60°, compared to the 0° reflexion from Fig. 2. Only fitted curves are shown in order not to obscure the results. The scatter of data is as in Fig. 2.

however, that the dimensions of the interference reflector were so close to a diffraction-limited spot that light was not only reflected, but also diffracted. When the eyespot was tilted, its projection became still smaller and diffraction became even more pronounced. The reflection effectivity could therefore not been determined in absolute terms; instead, the spectra had to be normalized to the maximal achieved value. Only when comparing direct reflexion and reflexion through the cell body a common reflectivity scale could be established. The results in Fig. 3 *b* show the expected blue-shift, which increased with increasing angle, but it was not as pronounced as one would have expected it from a quarter wave stack. While the reasons for this only small blue-shift are unclear, it can be stated with confidence that the reflectivity properties of the eyespot cannot provide much shielding against blue light reaching the rear end of the eyespot.

## The absorption properties of eyespot and cell body

When viewing *Chlamydomonas* with white, transmitted light, the eyespot appears as an orange-red spot in front of a green background. We have seen in the previous paragraph that light reaching the eyespot from outside the cell has a chance to pass the photoreceptor layer twice, while light reaching it from the reverse side is attenuated on its way through cell body and eyespot. We have used two different experimental approaches to determine quantitatively the degree of attenuation, and we have determined the contribution of eyespot and cell body to this shading. In the first approach we employed two confocal cones of light, one being focused directly onto the eyespot by the upper water immersion objective, and the second cone being collected by the lower water immersion objective facing the first one. The measuring geometry is shown in Fig. 4 *a*, while the results are depicted in Fig. 4 *b*. The extinction (this term was chosen in order to indicate that light attenuation was not solely due to absorption, but also due to scattering) changed little when the measuring focus was placed slightly off the eyespot. This seems to indicate that both absorption and reflexion of the eyespot contribute significantly less to the shading than the absorption of the cell body. Shading reached a maximum around 480 nm (an attenuation of

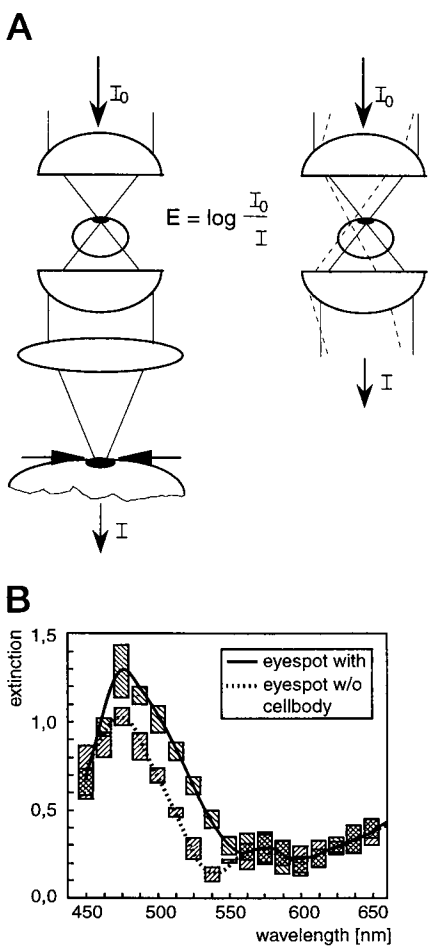


FIGURE 4 (*a*) Optical set-up for the confocal measurement of the extinction of eyespot-apparatus and cell body. (*b*) Wavelength dependence of the extinction properties of cell body and eyespot, determined with a confocal cone of light.

20-fold is suggested by the spectral data) and declined steeply toward the red end of the spectrum, reaching a value of ~2 at 550 nm. The fact that the difference between the "through the eyespot" value and the "near the eyespot" value was almost not noticeable at 550 nm, i.e., in the maximum of reflectivity, suggests that the difference between the two spectra, with its broad maximum between 480 and 540 nm, is mostly due to the absorbance of the eyespot and not due to its reflectivity. We attribute it to the carotenoids in the interference reflector (Kreimer and Melkonian, 1990; Grung et al., 1994). The Cramers-Kronig relationship for anomalous dispersion predicts a maximal refractive index at a wavelength at which the absorbance change with wavelength is the steepest (Hecht, 1987), and this appears to be the case at 550 nm, the maximum of reflectivity. Spectra shown by Crescitelli et al. (1992) lend further support to this notion, even though they were interpreted differently by the authors (see below). Moreover, the spectral properties of isolated eyespot apparatuses of *Spermatozopsis similis* (Kreimer et al., 1991) suggest the same wavelength-dependence of the refractive index and hence the reflectivity.

The extinction values obtained by the confocal approach have to be taken as an upper limit. The spherical cell body, with its higher refractive index than the surrounding saline, acts like a lens which introduces a significant degree of spherical aberrations. These aberrations cause a blurring of the image of the eyespot and lead to a diminished light intensity being picked up from the eyespot region and hence to an overestimation of the cell absorptivity. This, and the fact that in the confocal measuring geometry it was not possible to determine the angular dependence of the cell extinction, prompted us to try a different measuring geometry, using a parallel measuring beam (Fig. 5 *a*). The results are shown in Fig. 5 *b*. The structure of the spectrum is virtually the same, i.e., there is a maximum at 480 nm, a steep decline toward the red, a very low shading at 550 nm, and only a small contribution of the eyespot itself. The maximal values for the attenuation were significantly lower, however, reaching a value not much above 4 in the maximum at 480 nm. This time we have reason to believe that the value is a severe underestimation, since the cell is a highly scattering object which causes many rays scattered inside the cell to look as if they originated from the eyespot (Fig. 5 *a*). This out-of-focus haze reduces the contrast, and hence the determined absorption value. It is therefore safe to assume that the actual contrast (shading) ratio lies between the "confocal" and the "parallel" value, i.e., between 4 and 20. Harz and Hegemann (1991) have determined a contrast-value of 8 from electrophysiological experiments; however, these authors used a different cell mutant. In Fig. 6 it is shown that the cell extinction depends on the angle of the measuring beam relative to the eyespot, but only to a minor extent.

The absorbance spectra in Figs. 4 and 5 show a small but reproducible difference between a cell with and without eyespot in the measuring beam. We attribute it to the sum of

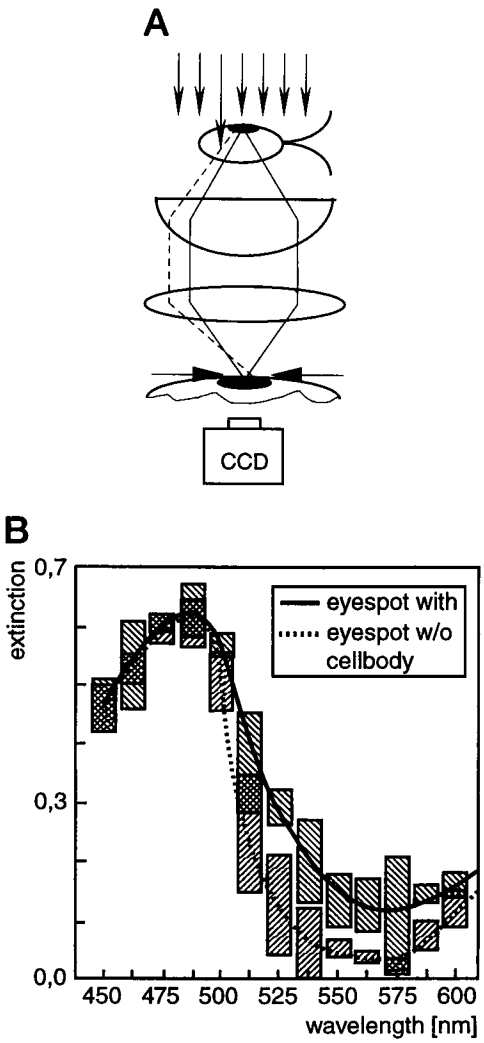


FIGURE 5 (*a*) Optical set-up for the measurement of the extinction properties of eyespot-apparatus and cell body using parallel light. Note the beam (*long arrow*) which is scattered within the specimen such that the scattered beam appears to originate from within the eyespot. This "out-of-focus haze" increases the apparent transmittance. (*b*) Wavelength dependence of the extinction properties of eyespot-apparatus and cell body, determined with parallel light.

absorbance and reflectance of the stigma. Crescitelli et al. (1992) have recently reported microspectrophotometric experiments from eyespots, and they have assigned the peak around 500 nm to rhodopsin molecules. This, however, cannot be correct since the absorbance they measure (0.067 OD units) is much too high to be accounted for by the 20,000–30,000 rhodopsin molecules in a single eyespot (Smyth et al., 1988; Beckmann and Hegemann, 1991; Deininger et al., 1995). These rhodopsins are presumably spread out in a single layer over a surface of 1–3 $\mu m^2$ (Melkonian and Robenek, 1981; Kreimer and Melkonian, 1990), so with an absorption cross-section of $1.5 \times 10^{-20}$ $m^2$ (Smyth et al., 1988; Uhl and Hegemann, 1990; Beckmann and Hegemann, 1991), one would expect that only 1.5–4.5 of $10^4$ photons get absorbed during passage through a single layer. This

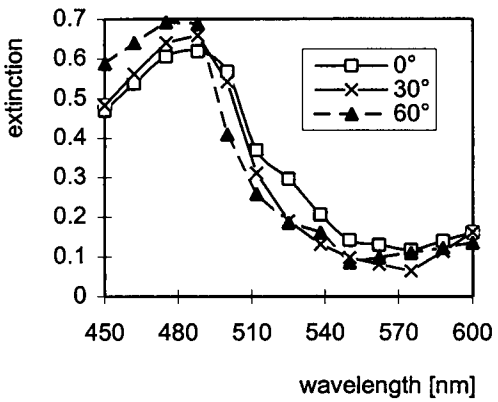


FIGURE 6 Angular dependence of the extinction properties of eyespot-apparatus and cell body. In order not to obscure the subtle wavelengths-shifts error bars have been left out. They would be comparable to the ones shown in Fig. 5 *b*. Measuring configuration as in Fig. 5 (*a*).

corresponds to an absorbance of 0.00035–0.001, a value roughly 100× smaller than the value reported by Crescitelli et al. (1992). Even when a correction is made for the fact that all rhodopsins are thought to have a transition dipole moment lying within a single plane (this will lift the effective absorption cross-section by a factor of 1.5 for unpolarized light), the discrepancy is still much too high. It therefore appears reasonable to assume that what Crescitelli et al. have measured was the absorbance spectrum of the many layers of carotenoids in the interference reflector. Further support for this notion comes from the shape of the absorption band measured by these authors. The long wavelength side of the absorbance band exhibits its steepest slope around 545 nm, exactly where we measure the highest reflectivity, and where the Kramers-Kronig relationship predicts the highest refractive index.

The above measurement clearly demonstrates that the original concept of Foster and Smyth (1980) is correct, i.e., the eyespot indeed acts as an interference reflector. However, there are two major points which question the relevance of this phenomenon for the mechanism of phototaxis: reflectivity is maximal at 550 nm, at a wavelength range where we find hardly any orientation [in contrast to Foster et al. (1984), but in agreement with earlier data by Nultsch et al. (1971)]. At 490 nm, on the other hand, where phototaxis and *chlamy*-rhodopsin exhibit peak sensitivity (Foster et al., 1984, Uhl and Hegemann, 1990) respectively peak absorbance (Beckmann and Hegemann, 1991), the reflectivity is very small in our hands. Foster's previous suggestion of a maximum reflectivity at 480 nm (Foster and Smyth, 1980; Smyth et al., 1988) was derived from calculations based on distances measured electron-microscopically. The distance between refractive layers was found to be 120 nm, and since an interference reflector works best at $\lambda/4$, Foster concluded that maximum reflectivity must be around 480 nm, close to the peak of *chlamy*-rhodopsin. The actual distances, however, appear to be larger than measured in the dehydrated electron micrograph, and there is agreement now (Foster, personal communication) that the reflectivity peaks around 540–550 nm. The second point is that even at the peak of reflectivity the contribution of the eyespot—being comprised of reflectivity and absorbance—is small compared to the absorbance of the remainder of the cell. There are, however, other green algae which have a relatively narrow cell body, and in these the relative contribution of the cell body to the shielding is probably much smaller. It should be kept in mind, however, that while a contrast ratio of 8–10, as found in strain 806 $mt^-$, provides optimal shielding, some orientation is still achieved with contrast ratios as low as 1.5–2 (see Schaller et al., 1997) and this is sufficient for a directed movement.

The authors express their gratitude to Drs. H. Harz and G. Kreimer for critical reading of the manuscript and numerous valuable suggestions. The skillful technical assistance of J. Schiessl and his machine shop is also gratefully acknowledged.

This work was supported by a grant of the Deutsche Forschungsgemeinschaft.

## REFERENCES

Beckmann, M., and P. Hegemann. 1991. In vitro identification of rhodopsin in the green alga *Chlamydomonas. Biochemistry.* 30:3692–3697.

Buder, J. 1917. Zur Kenntnis der phototaktischen Richtungsbewegungen. *Jahrb. wiss. Bot.* 58:105–220.

Crescitelli, F., T. James, J. Erickson, E. Loew, and W. McFarland. 1992. The eyespot of *Chlamydomonas reinhardtii*: a comparative microspectrophotometric study. *Vision Res.* 32:1593–1600.

Deininger, W., P. Kröger, U. Hegemann, F. Lottspeich, and P. Hegemann. 1995. Chlamyrhodopsin represents a new type of sensory photoreceptor. *EMBO J.* 14,23:5849–5858.

Foster, K. W., J. Saranak, N. Patel, G. Zarilli, M. Okabe, T. Kline, and K. Nakanishi. 1984. A rhodopsin is the functional photoreceptor for phototaxis in the unicellular eukaryote *Chlamydomonas. Nature.* 311: 756–759.

Foster, K. W., and R. D. Smyth. 1980. Light antennas in phototactic algae. *Microbiol. Rev.* 44:572–630.

Grung, M., G. Kreimer, M. Calenberg, M. Melkonian, and S. Liaaen-Jensen. 1994. Carotenoids in the eyespot apparatus of the flagellate green alga *Spermatozopsis similis*: adaptation to the retinal based photoreceptor. *Planta.* 193:38–43.

Harz, H., and P. Hegemann. 1991. Rhodopsin regulated calcium currents in *Chlamydomonas. Nature.* 351:489–491.

Hecht, E. 1987. *Optics*, Addison-Wesley, Reading, MA.

Kreimer, G., U. Brohnsonn, and M. Melkonian. 1991. Isolation and partial characterization of the photoreceptive organelle for phototaxis of a flagellate green alga. *Eur. J. Cell Biol.* 55:318–327.

Kreimer, G., and M. Melkonian. 1990. Reflection confocal laser scanning microscopy of eyespots in flagellated green algae. *Eur. J. Cell Biol.* 53: 101–111.

Kreimer, G., C. Overländer, O. Sineshchekov, H. Stolzis, W. Nultsch, and M. Melkonian. 1992. Functional analysis of the eyespot in Chlamydomonas reinhardtii mutant ey 627, mt-. *Planta.* 188:513–521.

Kröger, P., and P. Hegemann. 1994. Photophobic responses and phototaxis in *Chlamydomonas* are triggered by a single rhodopsin photoreceptor. *FEBS Lett.* 341:5–9.

Lawson, M. A., and P. Satir. 1994. Characterization of the eyespot regions of "blind" *Chlamydomonas* mutants after restoration of photophobic responses. *J. Euk. Microbiol.* 41:593–601.

Mast, S. O. 1927. The structure and function of the eyespot in unicellular and colonial organisms. *Arch. Protistenkd.* 60:197–220.

Melkonian, M., and H. Robenek. 1981. The eyespot apparatus of green algae: structure, development and function. *Phycologia.* 20:110–130.

Messler, P., H. Harz, and R. Uhl. 1996. Instrumentation for multiwavelengths excitation imaging. *J. Neurosci. Meth.* 69:137–147.

Morel-Laurens, N., and M. E. Feinleib. 1983. Photomovement in an "eyeless" mutant of *Chlamydomonas*. *Photochem. Photobiol.* 37: 189–194.

Nultsch, W., and D.-P. Häder. 1979. Photomovement of motile microorganisms. *Photochem. Photobiol.* 29:423–437.

Nultsch, W., G. Throm, and I. v. Rimscha. 1971. Phototaktische Untersuchungen an *Chlamydomonas reinhardtii* Dangeard in homokontinuierlicher Kultur. *Arch. Microbiol.* 80:351–369.

Rüffer, U., and W. Nultsch. 1985. High speed cinematographic analysis of the movement of *Chlamydomonas*. *Cell Motil.* 5:251–263.

Schaller, K., R. David, and R. Uhl. 1997. How *Chlamydomonas* keeps track of the light once it has reached the right phototactic orientation. *Biophys. J.* 73:1562–1572.

Sineshchekov, O., E. Govorunova, and F. Litvin. 1989. Role of photosynthetic apparatus and stigma in the formation of spectral sensitivity of phototaxis in flagellated green algae (In Russian with English abstract). *Biofizika*. 34:255–258.

Smyth, R., J. Saranak, and K. W. Foster. 1988. Algal visual systems and their photoreceptor pigments. *Progr. Phycol. Res.* 6:255–286.

Uhl, R., and P. Hegemann. 1990. Probing visual transduction in a plant cell. Optical recording of rhodopsin-induced structural changes from *Chlamydomonas*. *Biophys. J.* 58:1295–1302.