Lucido motivus: Photonic dependence of chloroplast translocation (systrophe) in the algal Chlorophyte *Eremosphaera viridis*[1].

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OBJECTIVE

To determine the wavelength and photon flux dependence of systrophe in the green unicellular alga *Eremosphaera viridis*.

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INTRODUCTION

Electromagnetic radiation is the driving force for photosynthesis. Plants and other autotrophs convert the energy of light into chemical energy used for synthesizing carbohydrates and other organic compounds, which heterotrophic organisms use for energy and other nutritional requirements. Photosynthesis is a unique process performed by plants, algae, and some species of bacteria. It consists of a series of oxidation and reduction reactions; the basic chemical formula is shown below:

$$6CO_2 + 6H_20 + \text{Light Energy} \rightarrow C_6H_{12}O_6 + O_2$$

Photosynthesis would not be possible without the energy derived from light. Electromagnetic radiation enters the earth's atmosphere from the sun. A plot of solar irradiance as a function of wavelength is shown in Figure 1. Peak irradiance occurs at wavelengths between 400 and 500 nm, absorbance by atmospheric gases (especially O_3, CO_2 and H_2O) results in lower intensities below the atmosphere. Wavelengths between 400 and 800 nm are used by photosynthetic organisms.

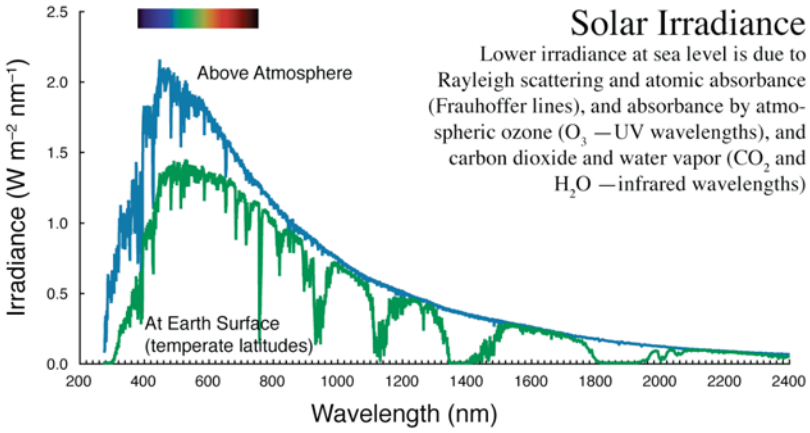


Figure 1. Solar irradiance *versus* wavelength. The data were obtained from http://rredc.nrel.gov/solar/spectra/am1.5/. Solar irradiation above the atmosphere and at sea level are shown.

The sun can be approximated as a near perfect blackbody with a surface temperature of 5800 K. Figure 2 shows some examples of blackbody curves, described by Planck's Black Body Radiation Law for different temperatures. Not only does intensity increase with increasing temperature, but the maximal wavelength becomes shorter (corresponding to higher photonic energy).

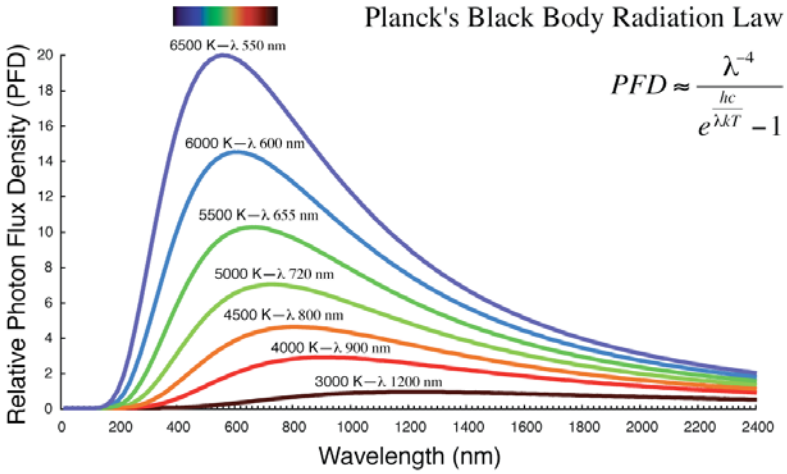


Figure 2. Black Body Radiation. Relative photon flux density is shown *versus* wavelength for a black body. It's expected that the tungsten halogen bulb on a microscope exhibits a similar photon output, and that the maximal photon flux wavelength will vary dependent on the amperage used to power the 12 Volt lamp.

The first step in the conversion of the energy of photons to chemical energy is absorption by photosynthetic pigments, principally chlorophyll. Chlorophyll is located inside the thylakoid vesicles in the inner membrane of the chloroplast. After absorbing the photon, an electron in chlorophyll transitions to an excited state. The excited state electron (*exciton*) is transferred to a photosynthetic reaction center to begin the flow of electrons through the electron transport chain, eventually to produce ATP (from a transmembrane H^+ gradient) and reducing equivalents ($NADP + 2e^- + 2H^+ \rightarrow NADPH + H^+$). Chlorophyll, now with one less electron, is chemically unstable and receives an electron from a water molecule (H_2O). With the loss of 4 electrons from $2H_2O$, molecular oxygen (O_2) is produced, as well as $4H^+$ used for ATP synthesis. Oxygen, the waste product of photosynthesis, is vital for heterotrophs in the process of cellular respiration.

As light intensity increases, so do absorption events and the generation of excited state chlorophylls, and downstream electron transport. At a high enough light intensity, these can cause the formation of a variety of undesirable oxidative products that can damage the photosynthetic apparatus. Examples include triplet state chlorophyll, and various reactive oxygen species (through direct reduction of O_2 to O_2^-, and subsequent formation of H_2O_2). The general term photo-oxidation is used to describe this damaging process. There are protective mechanisms to avoid oxidative damage (Li et al., 2009), one of these may be to decrease the absorptive cross-sectional area of chloroplasts by changing their location in the cell (Kasahara et al., 2002).

The phenomenon known as systrophe is described as the accumulation of cytoplasmic organelles around the nucleus, (Weidinger, 1980, 1982; Weidinger and Ruppel, 1985). Systrophe of the chloroplasts is observed in the unicellular green algae *Eremosphaera viridis* when the cell is exposed to intensities of light greater than it would experience in its natural environment. The objective of our research was to determine the photonic flux dependence of organelle translocation and determine if this response depends on the wavelength of light as well. In addition we explored whether the intracellular signals mediating systrophe are global or localized within the irradiated area of the cell. Finally, we tested whether systrophe can protect the cell from photo-oxidation.

MATERIALS AND METHODS

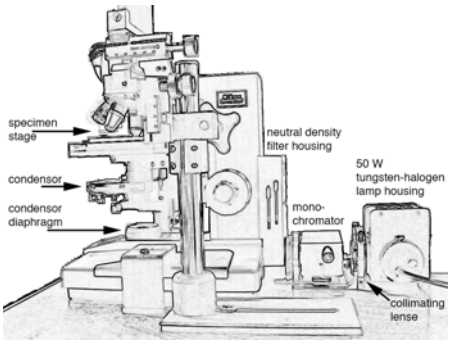
The algal *Eremosphaera viridis* de Bary strain used in this project was collected at Plastic Lake (Ontario) in 1987 and maintained at the Canadian Phycological Culture Centre (CPCC 127, formerly UTCC 127). The strain is designated LB (live bacteria). It was grown under T8 fluorescence lamps on a rotary shaker (120 rpm) in 125 or 250 ml Ehrlenmeyer flasks containing 25 or 50 ml of Bold's basal medium supplemented with vitamins (Table I).

Table I: Bold's basal medium composition.

Salt (stock)	MW	Dilution	Final concentration
KH_2PO_4 (1.75 g/100 ml)	136.09	0.01	1.29 mM
$CaCl_2 \cdot 2H_2O$ (0.25 g/100 ml)	147	0.01	0.17
$MgSO_4 \cdot 7H_2O$ (0.75 g/100 ml)	246.5	0.01	0.30
$NaNO_3$ (2.5 g/100 ml)	84.99	0.01	2.94
K_2HPO_4 (0.75 g/100 ml)	174.2	0.01	0.43
NaCl (1.75 g/100 ml)	58.44	0.01	2.99
$Na_2EDTA \cdot 2H_2O$ (1.0 g/100 ml) KOH (0.62 g/100 ml)	372.24 56.11	0.001	0.027 0.11
$FeSO_4 \cdot 7H_2O$ (0.498 g/100 ml) H_2SO_4 (conc. 18 M) (0.1 ml/100 ml)	278.0 98.08	0.001	0.018 0.00018
Trace Metal Solution	*see below*	0.001	*see below*
H_3BO_3 (1.15 g/100 ml)	68.13	0.0007	0.012
Vitamins		0.000025	
Trace Metals (g/100 ml, *MW*, final concentration [**µM**]): H_3BO_4 (0.286, *68.13*, **42.0**), $MnCl_2 \cdot 4H_2O$ (0.181, *197.92*, **9.1**), $ZnSO_4 \cdot 7H_2O$ (0.0222, *287.54*, **0.77**), $Na_2MoO_4 \cdot 2H_2O$ (0.039, *241.95*, **1.6**), $CuSO_4 \cdot 5H_2O$ (0.0079, *249.7*, **0.31**), $Co(NO_3)_2 \cdot 6H_2O$ (0.00494, *291.03*, **0.17**)			
Vitamins (g/10 ml): Thiamine•HCl (0.1), Vitamin B_{12} (0.002), Biotin (0.001)			

Just prior to light treatments, an aliquot of cells was placed on a microscope slide and a cover slip gently placed over the cells. To avoid problems with drying out during the long time course of the light treatment, small pieces of tissue (Kimwipes) were placed on either side of the cover slip, and wetted with dH_2O.

Microscopy Setup for Light Treatments. A Nikon Optiphot was used for most of the light treatments. The microscope was modified for spectral dependence experiments. The lamp housing with collimating lens was set back to allow space for a Mini-chrom monochromator (Optometrics Corp., Ayer, MA 01432 USA) to be positioned in the light path as required. The lamp housing used a tungsten-halogen bulb (50 Watt), that was powered by a constant voltage power supply with adjustable current (0–6 amperes) to change the wattage output. This was used to adjust the light intensity of the bulb in concert with neutral density filters.



For time lapse imaging of chloroplast translocations, some experiments were performed

on a Zeiss Axioskop II microscope using a x40 (water immersion) objective. The camera was a digital CCD camera (model C-4742-95, Hamamatsu Photonics KK). The software program Openlab (Improvision Inc.) was used to control the camera and acquire digital images. Normally, images were taken every 20 seconds for up to 60 minutes.

Standardizing Light Treatments. To standardize light intensity and/or photon flux, the light at the focusing stage was adjusted to be Kohler illumination, and the diaphragm size adjusted to fill the field of view with a X10 objective. The diameter was measured based on calibrations with a micrometer slide. It was 1750 microns, yielding an area of $2.41 \bullet 10^{-6}$ μm^2 ($2.41 \bullet 10^{-6}$ m^2). The light energy was measured with a radiometric probe (Model S471 Portable Optometer, UDT Instruments). Knowing the size of the irradiated area, the photon flux was calculated. The first step was to calculate the intensity of light penetrating the cell; this was done by dividing the power (measured with the radiometric probe) by the illuminated area. This gives intensity in units of $W \bullet m^{-2}$, which is equivalent to $J \bullet m^{-2} \bullet s^{-1}$ using the fact that one Watt is equivalent to one Joule per second. To determine how many photons were penetrating the cell every second, the intensity was divided by the energy of a single photon, using the Planck relation:

$$E = \frac{hc}{\lambda}$$

where h is Planck's constant ($6.626 \bullet 10^{-34}$ Joules sec), c is the speed of light in air ($3 \bullet 10^{17}$ nm sec^{-1}), and λ is the wavelength of the photon (nm). For broad bandpass irradiations, the photon flux was calculated using the photon energy for an intermediate wavelength (494 nm; $4.014 \bullet 10^{-19}$ Joules/photon). Note that the wavelength changes when the bulb wattage is adjusted (Figure 3), as expected based upon the Planck black body radiation law (Figure 2), since the bulb wattage —and therefore temperature— varies with amperage.

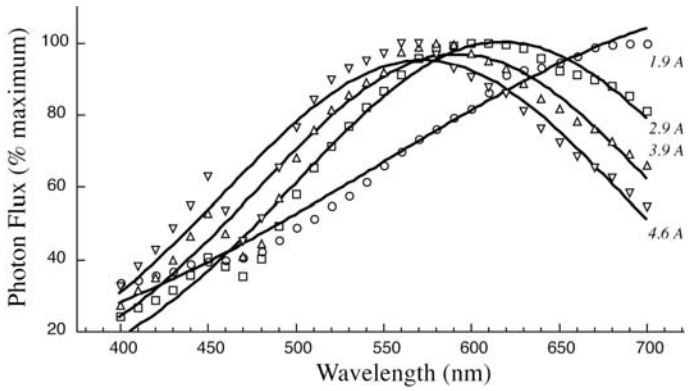


Figure 3. Lamp output dependence of maximal wavelength.
The tungsten halogen bulb output was adjusted by changing the current (A) with voltage constant at 12V. Note that the wavelength shifts dramatically to longer maxima as the bulb wattage is lowered. These spectra were obtained *without* a cupric sulfate filter.

Broad bandpass irradiation was provided using a $CuSO_4$ filter (3.5% [w/v] in a flat culture flask (1.65 cm depth) inserted into the lightpath (Figure 4). Maximal irradiation was at a wavelength of about 570 nm.

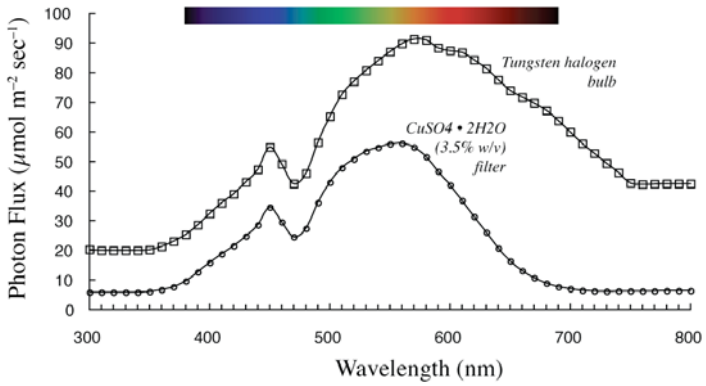


Figure 4. Photon flux *versus* wavelength for the cupric sulfate filter.
A cupric sulfate filter was constructed with a 3.5% (w/v) solution of $CuSO_4 \cdot 2H_2O$ in a flat culture flask (depth 1.65 cm) placed in the light path below the microscope condensor. Radiant energy *versus* wavelength was measured with a monochromator and radiometer probe. Lamp output (without the filter) is also shown.

For finer wavelength resolution, interference filters (Optometrics Corp., Ayer, MA 01432 USA) were used. The spectral properties of the interference filters were measured in a spectrometer; transmission is shown in Figure 5.

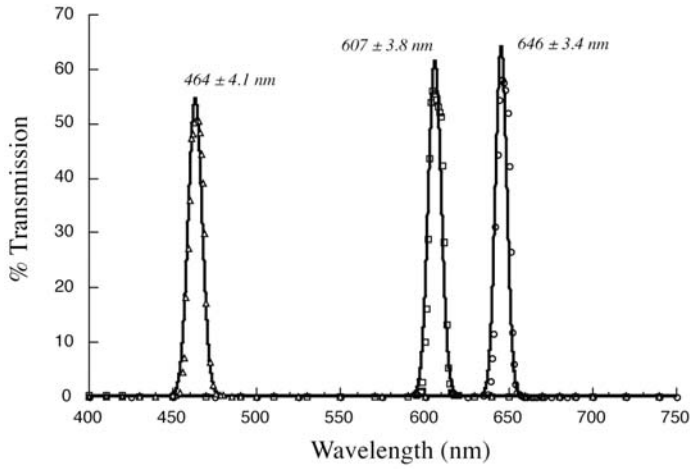


Figure 5. Interference filters: % transmission.
The % transmission of the two interference filters are shown, along with best fits to a Gaussian distribution: Blue (464 ± 4.1 nm), red (607 ± 3.8 nm) and long red (646 ± 3.4 nm).

Light Treatment Protocol. After the microscope condensor diaphragm was adjusted to fill the field of view, the amperage of the lamp power supply was adjusted while measuring the light intensity with the radiometric probe to yield a photon flux of 4.0 mWatts (with the cupric sulfate filter). This was used as the reference point for treatments determining photon flux and wavelength dependence of systrophe. The intensity was adjusted by inserting a combination of neutral density filters and photonegatives in the light path. The neutral density filters and photonegatives are wavelength independent and only change the intensity of light. This protocol —using filters rather than changing the lamp output— meant that the peak wavelength of the light was unchanged.

Under low light intensity (using the red (646 nm) interference filter (to avoid premature chloroplast translocation), a field of cells was selected so that 50–60 cells were in the field of view (to maximize the sample size per light treatment).

Cells were irradiated with light for 60 minutes and observed every 2 minutes for the first 15 minutes, every 3 minutes for the next 30 minutes, and very 5 minutes for the last 15 minutes. At each time interval the cells were scored as being either incipient systrophe (partial chloroplast translocation), complete systrophe (complete chloroplast translocation), or showing no response (no chloroplast movement).

RESULTS

The Nature of the Chloroplast Translocation. During the irradiations, chloroplasts tended to move from the periphery of the spherical cell to the center (Figure 6).

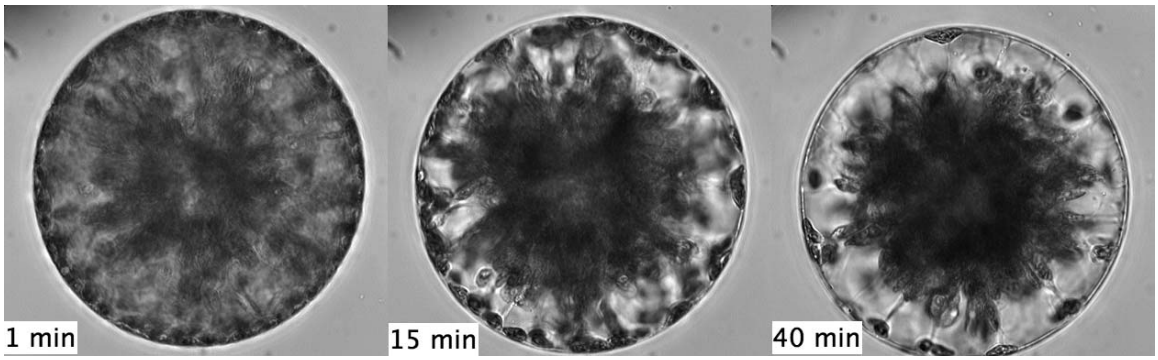


Figure 6. Examples of incipient and near-complete systrophe in *Eremosphaera viridis*. The cell was irradiated with 1840 μmol m^{-2} sec^{-1} for the times shown. Note that chloroplasts, arrayed around the perimeter of the spherical cell migrate towards the center (where the nucleus is located). Cytoplasmic strands connect the central core with the cell perimeter. At 20 minutes, the cell is scored as incipient systrophe. At 40 minutes, the incipient systrophe is more dramatic. With all chloroplasts aggregated in the central core, the cell would be scored as complete systrophe.

Scoring of the Chloroplast Translocations. Movement of the chloroplasts away from the periphery of the cell was considered incipient systrophe. Only after all of the chloroplasts moved to the center was the cell considered to exhibit complete systrophe. Scoring incipient systrophe was challenging, because chloroplasts do tend to move to varying degrees even under low light intensities; thus there is an inherent subjectivity when scoring cells as incipient systrophe. However, the initial chloroplast translocations often leave optically clear regions which improves the accuracy of scoring. Examples of time courses at various light intensities are shown in Figure 7. Initially, cells underwent incipient systrophe. At higher light intensities, the cells transitioned to complete systrophe.

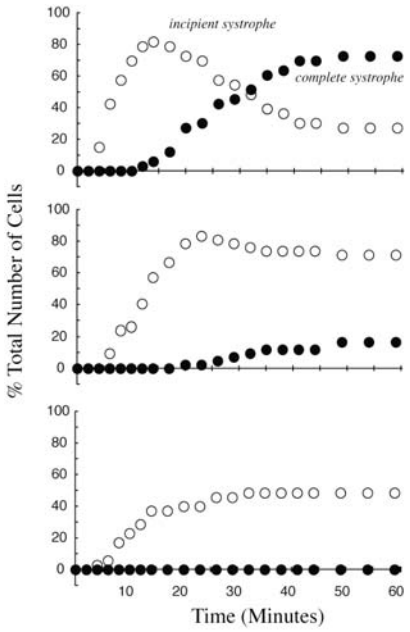


Figure 7. Time dependence of incipient and complete systrophe in *Eremosphaera viridis*. Example of time dependence of systrophe in *Eremosphaera viridis*. The cells were irradiated with 7935 μmol m^{-2} sec^{-1} (upper panel), 3827 μmol m^{-2} sec^{-1} (middle panel), or 223 μmol m^{-2} sec^{-1} (lower panel) for the times shown. All irradiances were performed with the cupric sulfate filter. At high light intensities, incipient cells rapidly transition to complete systrophe, while at lower intensities, cells remain in an incipient state.

Intensity Dependence of Chloroplast Translocations. The photon flux dependence of the incipient and complete systrophe responses are shown in Figure 8. The photon flux received from full sunlight is about 2000 μmol m^{-2} sec^{-1}, (Nobel, 1991). There is a sharp increase in the percentage of complete systrophe at photon flux greater than 4000 μmol m^{-2} sec^{-1} (about 2-fold higher than full sunlight intensity). At intensities lower than full sunlight, cells did not exhibit complete systrophe, instead, incipient systrophe was observed at intensities greater than about 900 μmol m^{-2} sec^{-1}.

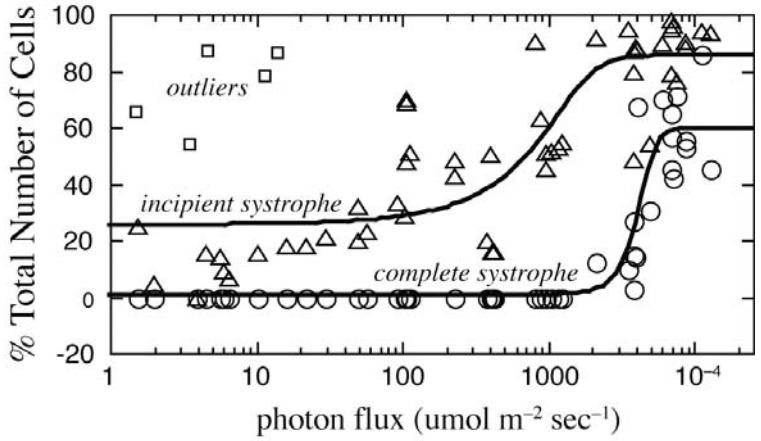


Figure 8. Intensity dependence of incipient and complete systrophe in *Eremosphaera viridis*. Data are fit to a sigmoidal function. Outliers (squares, high proportion of incipient systrophe at very low light intensities) were excluded from the best fits.

Wavelength Dependence of Chloroplast Translocations. To determine the wavelength dependence of chloroplast translocation samples of cells were irradiated under a constant photon flux of 200 μmol•m^{-2}•sec^{-1} under both red and blue interference filters. The blue interference filter had a wavelength peak at 464 ± 4 nm and red interference filters with wavelength peaks at 607 ± 4 nm and 646 ± 3 nm, (Figure 5). Light treatments with blue and red light were interspersed (blue light was used as a positive control). Incipient

systrophe was common in cells irradiated with blue light, but uncommon with red light irradiations at either 610 or 650 nm (Figure 9).

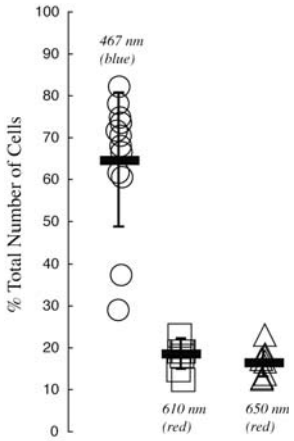


Figure 9. Wavelength dependence of incipient systrophe in *Eremosphaera viridis*. Data are from light irradiations using interference filters (467, 610 or 650 nm). The photon flux was kept constant at 200 μmol m^{-2} sec^{-1}.

Time Dependence of Chloroplast Translocation. The time required for half-maximal incipient systrophe to occur was determined from the time course measurements. There was a slight trend to faster translocation onset at higher light intensities, but the effect was slight (Table II).

Table II: Time dependence of chloroplast translocation

Photon flux range (μmol m^{-2} sec^{-1})	Half-maximal time to highest incipient systrophe (minutes) (mean ± SD [n])
0 – 10	27 ± 10 [9]
10 – 100	22 ± 8 [10]
100 – 1000	20 ± 11 [16]
1000 – 10000	11± 8 [18]

Chloroplast Movements are Localized to the Irradiated Area. To determine if irradiation generated a global signal causing systrophe throughout the cell, localized regions of the cell were irradiated with light intensities from 3 to 1500 μmol m^{-2} sec^{-1} (Figure 10). A chloroplast avoidance response is only seen *out of* the irradiated region of the cell. This implies the signals mediating systrophe are activated only within the irradiated region of the cell. Avoidance responses were observed at intensities of 1500 m^{-2} sec^{-1} or greater. At intensities lower than this there was no movement of chloroplast into irradiated area.

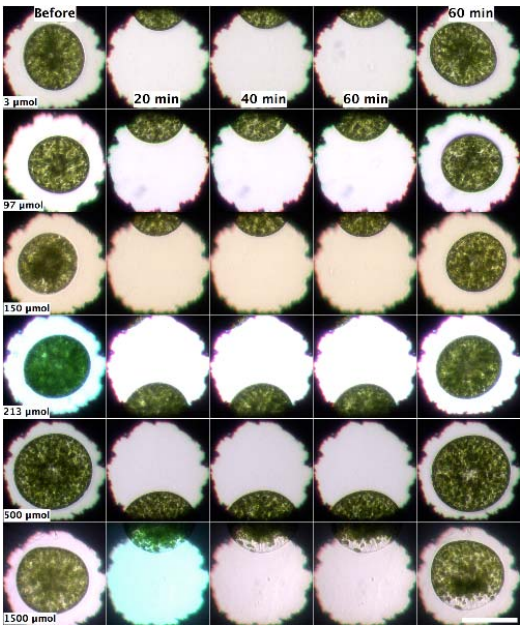


Figure 10. Localized irradiations of *Eremosphaera viridis*.
The cells were irradiated with the light intensities as marked, and images taken every 20 minutes for 60 minutes. Only at very high light intensities (1500 μmol m^{-2} sec^{-1}) was avoidance observed, and only in the localized region of irradiation. It was noteworthy that positive phototropism (movement of chloroplasts from dark to light) was not observed at lower light intensities. Bar, 100 μm.

Chloroplast translocation speeds. From time lapse imaging of cells undergoing systrophe (using the X40 objective), it was possible to determine the speeds of chloroplast translocations to the center of the cell, and, in one experiment, rates of speed during recovery from systrophe (Table III). The translocation rate was about 0.12 microns/second.

Table III: Chloroplast speeds during systrophe (and recovery)

	Speed (microns/second) mean ± SD (sample size)
Cell One	0.131 ± 0.039 (22)
Cell Two	0.122 ± 0.018 (23)
Cell Three	0.115 ± 0.020 (27)
Recovery	0.118 ± 0.016 (12)

Chloroplast Physical Parameters. In a normal chloroplast, there is about $9 \cdot 10^{-13}$ g of chlorophyll (about $6.7 \cdot 10^{8}$ chlorophyll molecules per chloroplast) (Lawlor, 2001). The molecular weight of chlorophyll *a* is about 894; its extinction coefficient is $1.2 \cdot 10^{5}$ M^{-1} cm^{-1} at 430 nm (Lawlor, 2001). An *Eremosphaera viridis* cell has about 400 chloroplasts, based on counting of medial sections of fluorescence images. The amount of chlorophyll in a normal cell of *Eremosphaera viridis* is about $3.6 \cdot 10^{-10}$ g, calculated by multiplying the typical number of chloroplasts by the amount of chlorophyll per chloroplast. These physical parameters can be used to assess whether systrophe would protect the cell from photo-oxidation.

Chloroplast Translocation does not Increase Transmissivity by the Cell. We tested whether systrophe can allieviate photo-oxidative damage by minimizing light absorption by considering a simplified model. A typical cell of *Eremosphaera* can be approximated as a sphere with the chloroplasts distributed peripherally around the perimeter of the

sphere with radial spokes extending into the center of the cell where the nucleus is found. To simplify the geometric problem, only the peripheral chloroplast shell is considered (the spokes are ignored). Thus, the volume can be simplified to a peripheral shell, or, after systrophe, a central shell of smaller diameter surrounding the nucleus. From time lapse imaging of cells, the dimensions of three cells were measured before and after systrophe (Table IV).

Table IV: Cellular Dimensions before and after Systrophe

	Cell One	Cell Two	Cell Three
		(micron)	
Cell Diameter	122.5	108.5	130.5
Systrophe Diameter	80.1	64.7	103.8
Nucleus Diameter	41.8	45.8	61.3
Chloroplast Thickness	4.6	5.5	5.3

The volume of the spherical shell can be calculated by integration over the bounds of inner and outer radii:

$$V = \int_{r'}^{R} 4\pi r^2 dr$$

Where r' is the radius of the cell minus the chloroplast shell and R is the radius of the cell. After integrating the solution is:

$$V = \frac{4}{3}\pi R^3 - \frac{4}{3}\pi r'^3 = \frac{4}{3}\pi\left(R^3 - r'^3\right)$$

The calculated volume was used as an estimate of the volume occupied by chlorophyll. Then, the concentration of chlorophyll within the shell can be calculated by dividing the amount of chlorophyll in a typical cell by the volume containing the chlorophyll. The next step is to consider the absorption of photosynthetically active light. The amount of light absorbed depends upon the Beer-Lambert Law:

$$I = Io\, e^{\varepsilon l c}$$

Where I_o is the intensity of light entering an object, and I is the intensity of light exiting the object (that is, non-absorbed). The extinction coefficient is ε (1.2×10^{16} $mol^{-1}um^2$ for chlorophyll), *l* is the distance through which the photons pass, and c is the chlorophyll concentration. For the three cells (Table IV), their respective volumes, chlorophyll concentrations, and both absorption and transmissivity were calculated in (Table V).

Table V: Absorptive Properties of Cells before and after Systrophe						
	Cell One		Cell Two		Cell Three	
	Before	*After*	*Before*	*After*	*Before*	*After*
Volume Occupied by Chlorophyll	$1.9915 \cdot 10^5$ μm^3	$2.3111 \cdot 10^5$ μm^3	$1.5486 \cdot 10^5$ μm^3	$9.1492 \cdot 10^5$ μm^3	$2.6084 \cdot 10^5$ μm^3	$4.655 \cdot 10^5$ μm^3
Chlorophyll Concentration	$2.0212 \cdot 10^{-18}$ mol μm^{-3}	$1.7424 \cdot 10^{-18}$ mol μm^{-3}	2.6003×10^{-18} mol μm^{-3}	$4.40126 \cdot 10^{-18}$ mol μm^{-3}	$1.5438 \cdot 10^{-18}$ mol μm^{-3}	$8.6505 \cdot 10^{-19}$ mol μm^3
Path Length	9.114 um	38.297 um	10.954 um	18.915 um	10.5826 um	42.544 um
Absorption	0.2211	0.8007	0.3418	0.9990	0.19605	0.4416
Transmissivity	0.8016	0.4490	0.7105	0.3682	0.8220	0.6430

In all three cells, the absorption after systrophe is lower (that is, transmissivity was higher). But, in addition to photo absorption, the area of the cell intercepting the light must also be considered, and would be different for the initial cell, and cell that has undergone systrophe.

When a single cell of *Eremosphaera* is in the light path we can approximate the shape of the object absorbing the light to be a circle. The size of this circle diminishes when the cell is undergoing systrophe, the relative cross-sectional areas of the three cells considered above were calculated (Table VI) as well as their effect on transmissivity.

Table VI: Absorption Properties of Cells before and after Systrophe						
	Cell One		Cell Two		Cell Three	
	Before	*After*	*Before*	*After*	*Before*	*After*
Cross-Sectional Area	$1.178 \cdot 10^4$ μm^2	$5.044 \cdot 10^3$ μm^2	$9.25 \cdot 10^3$ μm^2	$3.286 \cdot 10^3$ μm^3	$1.338 \cdot 10^4$ μm^3	$8.466 \cdot 10^3$ μm^3
Transmitted Intensity of Original	0.8016	0.4490	0.7105	0.3682	0.8220	0.6430
Absorbed Intensity of Original	0.1984	0.5511	0.2895	0.6318	0.178	0.357

Because transmissivity is lower after systrophe, it does not protect the cell from photo-oxidation by minimizing absorption.

DISCUSSION

There is no doubt that systrophe in the unicellular alga *Eremosphaera viridis* occurs, and quite rapidly under extremely high light irradiations. The wavelength dependence suggests that photosynthesis is not the light sensor, since red light (active in photosynthesis) does not induce systrophe. This confirms previous work by Weidinger (1980). Only the irradiated area is affected, suggesting that the signal is quite localized. Positive phototropism, that is, movement of chloroplast into the irradiated area at low light intensities (to maximize photosynthetic activity), was not observed, suggesting that only a phototropic avoidance mechanism exists in this alga. When systrophe is observed, it is natural to assume that it provides a protective mechanism, to avoid excessive photo-oxidation.

It was surprising to discover that the *intuitive* explanation of systrophe, to minimize photon absorption, is not borne out by a simplified model that accounts for absorption as a function of chlorophyll concentration and distance of the light path and the cross-sectional intercept area. The reason for this unexpected result is the strong effect of the longer path-length in the systrophic condition. If systrophe plays a role in protection from photo-oxidation, that role remains to be discovered.

There is good evidence that chloroplast translocation can play a role in mitigating the harmful effects of photo-oxidation (Kasahara et al., 2002). In the higher plant species *Arabidopsis thaliana*, mutants lacking the chloroplast avoidance movement, suffer greater photo-oxidative damage. It should be noted that there may be significant differences in the photo-oxidation protective mechanisms of alga and higher plants, because of differences in their habitat (algae are aquatic; *A. thaliana* is terrestrial); that is, water absorbs more light than air.

What was previously assumed about the systrophe as a defense mechanism for the alga by means of minimizing the harmful effects of high intensity radiation has now become debatable in light of the research outlined in this paper. However, what is missing in out research is a direct measure of the photo-oxidation. Systrophe may play some, as yet, undiscovered role in protection from photo-oxidation.

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