Novitas navigatio: Exploring fluorescent reporters of respiratory and photosynthetic poise in the algal Chlorophyte *Eremosphaera viridis*[1].

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OBJECTIVE

To monitor metabolic poise of respiration and photosynthesis in the green unicellular alga *Eremosphaera viridis* by quantifying fluorescence intensities of a fluorescent reporter of mitochondrial potential and chloroplast chlorophyll (autofluorescence).

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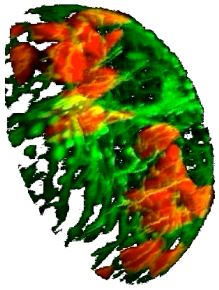
INTRODUCTION

How do the various organelles inside photosynthetic cell function? How do respiration and photosynthesis interact? We need to look at their function *in situ*, inside the cell.



The challenges are to image the 3D structure of the cell and to identify fluorescent reporters of organelle function. Imaging must encompass changes over time in the living cell. We performed several experiments to explore visualizing techniques to investigate and analyze the relationship between mitochondrial and chloroplast function. The spatial complexity of the data required sophisticated visualization and measurement of the imaging data obtained.

To understand the relationship between the chloroplast and mitochondria, we must first understand what each organelle does (photosynthesis and respiration, respectively).

Photosynthesis occurs in the chloroplast, specifically the thylakoid region and is initiated by the absorption of electromagnetic radiation. Plants and other autotrophs convert this light energy into chemical energy through photosynthesis. The chemical energy is used to synthesize carbohydrates and other organic compounds. Heterotrophic organisms obtain energy and other nutritional requirements by consuming the products of photosynthesis, oxidizing them in their mitochondria (which photosynthetic organisms also have!).

Photosynthesis is a phenomenon performed by plants, algae, and some species of bacteria. It consists of a series of oxidation and reduction reactions (Lawlor, 2001). The basic chemical reactions are shown below.

For the light reactions:

$$2H_2O + \text{Light Energy} \rightarrow O_2 + NADPH + ATP$$

For the dark reactions:

$$4H^+ + CO_2 + 2NADPH + 3ATP \rightarrow (CH_2O)_n + H_2O$$

Photosynthesis would not be possible without the energy derived from light. Light behaves like waves that travel in packets of energy called photons. When absorbed by a molecule, the photon energy is transferred to electrons, which are raised to a higher energy level known as an exciton.

The source of the light is the sun. The sun emits electromagnetic radiation as a flux of photons that enter the earth's atmosphere. As shown below in Figure 1, solar irradiance varies as a function of wavelength. Photosynthetic organisms absorb wavelengths between 400 and 800 nm; where maximal photon flux occurs.

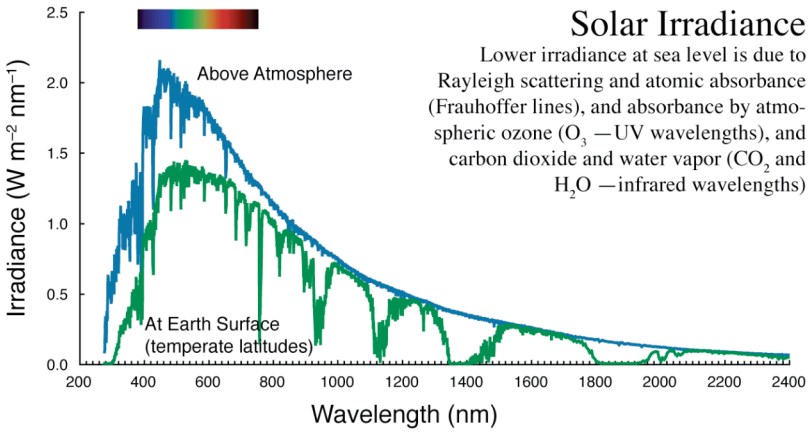


Figure 1. Solar irradiance *versus* wavelength. The data were obtained from http://rredc.nrel.gov/solar/spectra/am1.5/. Solar irradiation above the atmosphere and at sea level are shown.

Within the chloroplast, pigments and other molecules work together to capture light energy and convert it to chemical energy at the light reaction center. The core components of the light reaction center are found in two functionally distinct units called Photosystem I and Photosystem II —named after the order of their discovery— which are provided with excitons by light harvesting complexes. The light is first absorbed by the Light Harvesting Complex (LHC), which is comprised of hundreds of molecules of antenna chlorophylls and accessory pigments. The exciton is then transferred from one molecule to another until it eventually reaches the reaction center.

The reaction center contains a special pair of chlorophylls. These are the final destination of the exciton. The excited chlorophyll molecule donates the 'excited' electron to a donor and becomes positively charged. The primary electron acceptor is quinone Q.

The electron is immediately transferred from Q into the electron transport chain to fuel ATP production and NADP reduction.

Back to the reaction center, the positively charged chlorophyll needs an electron. The electron is provided by water. In the end, for every molecule of water that is split, the net products are 2 e^-, 2 H^+, and 1/2 O_2. Therefore, for every 4 photons of light absorbed by the PSII reaction center, two molecules of water are split, one molecule of diatomic oxygen is created, and four protons are pumped into the lumen of the thylakoid.

Oxygen, the waste product of photosynthesis, is vital for heterotrophs (and the mitochondria of autotrophs) in the process of cellular respiration.

When light intensity increases, so do absorption events and the generation of excited state chlorophylls, leading to downstream electron transport. If there are too many excitations, some of the excitons will be de-excited, releasing fluorescence. Therefore, fluorescence is a reporter of the light reactions of photosynthesis. Besides fluorescence, excessive light (and thus absorption events) can overwhelm the reaction centers and lead to 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)

Monitoring chloroplast fluorescence is one way to indirectly quantify the poise of the photosynthetic process.

We also need a way to monitor mitochondrial activity. For this we used Rhodamine 123. Rhodamine 123 is a cationic fluorescent dye that is known to accumulate in mitochondria and is sensitive to the *transmembrane* potential during mitochondrial respiration. It is good indicator for mitochondria energization —the membrane potential— which is used to drive ATP production by ATP synthetase (along with the pH gradient across the mitochondrial membrane) (Scaduto and Grotyohann, 1999). In their study, Scaduto and Grotohann showed that the addition of ADP caused a decrease in Rhodamine 123 fluorescence. This is consistent with functional monitoring of the potential, since ADP would cause the ATP synthetase to 'run', resulting in H^+ translocation into the lumen, depolarizing the potential. In this work, we validated the use of Rhodamine 123 by examining the effect of cyanide on *in situ* Rhodamine 123 fluorescence. Cyanide is a potent inhibitor of cellular respiration, acting on mitochondrial cytochrome c oxidase, which effectively blocks oxidative phosphorylation. Cyanide also affects photosynthesis at multiple sites (see discussion).

Our research objective —to monitor metabolic poise of respiration and photosynthesis in the green unicellular alga *Eremosphaera viridis* by quantifying fluorescence intensities of a fluorescent reporter of mitochondrial potential and chloroplast chlorophyll (autofluorescence)— first involved validation of the use of fluorescent reporters, and then examined the effect of high intensity light that would overwhelm exciton transfer.

MATERIALS AND METHODS

The algal *Eremosphaera viridis* de Bary strain was collected at Plastic Lake (Ontario) in 1987 and is 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). The light intensity was 50-μmol m^{-2} sec^{-1}.

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
$CaCl_2$ • $2H_2O$ (0.25 g/100 ml)	147	0.01	0.17
$MgSO_4$ • $7H_2O$ (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
$Na_2EDTA•2H_2O$ (1.0 g/100 ml) KOH (0.62 g/100 ml)	372.24 56.11	0.001	0.027 0.11
$FeSO_4$ • $7H_2O$ (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**), $MnCl_2•4H_2O$ (0.181, *197.92*, **9.1**), $ZnSO_4•7H_2O$ (0.0222, *287.54*, **0.77**), $Na_2MoO_4•2H_2O$ (0.039, *241.95*, **1.6**), $CuSO_4•5H_2O$ (0.0079, *249.7*, **0.31**), $Co(NO_3)_2•6H_2O$ (0.00494, *291.03*, **0.17**)			
Vitamins (g/10 ml): Thiamine•HCl (0.1), Vitamin B_{12} (0.002), Biotin (0.001)			

Pre-Treatment with Rhodamine 123. The fluorescent cationic dye Rhodamine 123 was used to visualize mitochondria and assess whether it 'reports' on the membrane potential of mitochondria in *Eremeosphaera viridis*. The cells were pre-incubated at room temperature in the dark for 1 hour with a mixture comprised of 40 μL of 5 mM Rhodamine 123 in methanol and 5 mL of Bolds medium. The cells were washed twice to remove extracellular dye. The cells were pelleted at 1000 rpm in a tabletop centrifuge and re-suspended in 15 mL of Bolds medium. The centrifugation was repeated. After a third centrifugation, the pellet of cells was re-suspended in about 2 mL of Bolds medium. The cells were used for imaging for 1–2 hours, and then fresh cells were prepared. About 0.5 ml of cells was added to 2 mL of Bolds medium in the lid of a small (30 mm) culture dish.

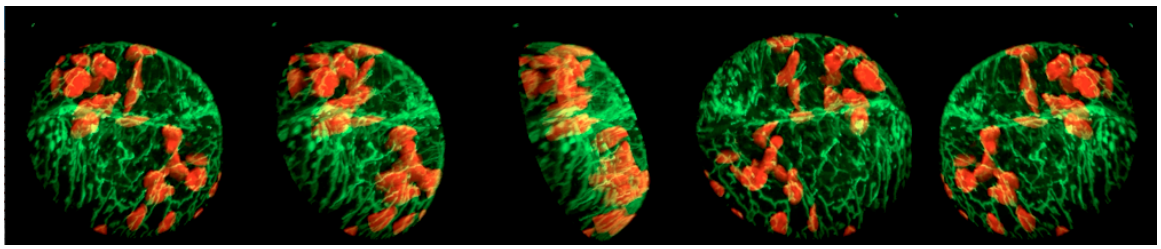
Dual Fluorescent Imaging on the Confocal Microscope. To determine the events occurring within both the mitochondria *and* chloroplast of *Eremosphaera viridis*, both need to be imaged simultaneously. Two lasers were used to detect the fluorescence of Rhodamine 123 and chlorophyll autofluorescence. For Rhodamine 123, the 488 nm laser line on a multi-argon laser was used. For chlorophyll autofluorescence, we used the 633 nm laser line on a helium-neon laser. A bandpass emission of 505–535 nm was used to

image Rhodamine 123 (effectively blocking chlorophyll autofluorescence). A long-pass emission (>660 nm) was used to image chlorophyll autofluorescence.

In preliminary experiments, it was found that an excessive laser intensity photobleached the fluorescence of the mitochondrial dye Rhodamine 123. To tackle this problem, the confocal aperture was initially set to smallest opening (1). Additionally, the intensity of the lasers was adjusted based on initial experiments to effect a compromise between too high an intensity (which could cause photobleaching) and a good fluorescence signal to noise ratio. Later the aperture was increased to 2, to maximize signal, with good results and minimal photobleaching. This may be because the photo-bleaching effect could be minimized with more than 3 washes in the centrifuge when preparing of cells. Using 4 washes decreased photo-bleaching effects because it seemed that less florescence dye accumulated in the cytoplasm.

The amplification on the photomultipliers for sensing the fluorescence emission was 620 V and 480 V for Rhodamine 123 and chlorophyll, respectively. The gain and offset setting were normally less than 5% for both channels. The images were usually zoomed by 2.5

Three Dimensional Imaging on Confocal Microscope. To obtain three-dimensional images of chloroplast and mitochondria in *Eremosphaera viridis*, a ×63 water immersion objective lens was used. Normally, the cell was z-sectioned, from the medial optical section to the top of the cell, creating about 64 slices with a z-step of about 0.3 micron. After z-sectioning was complete, the software Fluoview was used to create a 3D reconstruction of image by specifying a degree of rotation of about 120 degrees and a stretch of factor of approximately 2.5 for best visualization.



The three-dimensional images were used in preliminary experiments trying to determine the best way to measure fluorescence intensity of mitochondria and chloroplasts in the cell, since they did not lie in a flat plane within the optical sections of the cell.

Time Series Imaging on Confocal Microscope. An optical section of the cell was selected on the basis of a good representation (with zoom ranging from values of 2–3.5). The time series was comprised of 64 pictures taken every 10 seconds. The images were Kalman-filtered. Between pictures 20 and 21, cyanide (0.5 mL of 100 mM NaCN in Bolds) was added to 3 mL of Bold's in the dish (final cyanide concentration of 14 mM). As a control, Bolds medium was added instead of cyanide.

In these experiments, it was observed that chlorophyll fluorescence did not change (see results). We explored cyanide effects on chlorophyll autofluorescence using a more quantitative method to continuously monitor fluorescence, as described below

Continuous Monitoring of Chlorophyll Autofluorescence during Cyanide Treatments. The light paths of a Zeiss Axioskop II was modified so that high light irradiances could be used, while monitoring chlorophyll autofluorescence with a radiometer probe placed on the camera mount.

The cells were irradiated with light between 400 and 575 nm by placing a $CuSO_4$ filter (10% [w/v] in a flat culture flask (1.65 cm depth) in the light-path below the condenser, with a short-pass filter (cut-off 590 nm). Emission was monitored at the camera port by placing a long-pass (cut-on 670 nm) in one of the filter cube slots. Thus, the cell was irradiated at wavelengths shorter than 590 nm, but the radiometer probe measured only fluorescent light greater than 670 nm. Leakage was assessed by measuring the radiometer output when cells were in the light-path *versus* no cells present: Leakage was about 10% at all irradiation intensities.

Different irradiation intensities were used. To calibrate light intensity: First, with the ×10 objective, Kohler illumination was setup, and the field diaphragm set to completely fill the field of view. The area of the field of view was determined to be $2.8502\ 10^{-6}\ m^2$. Then the radiometer probe was placed on the specimen stage and the wattage monitored while adjusting the lamp intensity to produce photon flux densities of 20, 50, 200, or 1000 $\mu mol\ m^{-2}s^{-1}$ (based on a photon energy of 3.46×10^{-19} Joules/photon for 575 nm wavelength light). When moving cells into the field of view, neutral density filters were used to minimize light intensity prior to the beginning of the experiment.

The same protocol was used for imaging on the confocal microscope. The light irradiance was set to 2000 $\mu mol\ m^{-2}s^{-1}$. The cell was z-sectioned (12 slices) 120, 240, and 600 sec after onset of the high light treatment.

Quantifying Fluorescence. The image analysis software ImageJ was used for quantization (Rasband, 2011). The organelle was outlined with a tracing tool and the pixel intensity (and area) measured (Figure 2). The background fluorescence of cytoplasm near the measured mitochondria was measured with the same outline, to allow correction (by subtraction) for background fluorescence. A similar protocol was followed for measurements of chlorophyll autofluorescence. Normally, 3–4 mitochondria and chloroplasts were sample from each cell. The date were averaged and normalized to maximal fluorescence intensity.

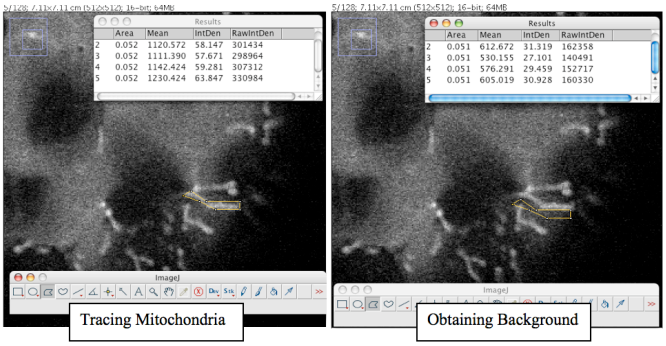


Figure 2. Quantifying fluorescence intensity. The screenshots show the selection of a mitochondria (left panel) and selection of a nearby background (cytoplasmic) fluorescence intensity. The measurements were done in ImageJ (Rasband, 2011).

RESULTS

Cyanide Affects Rhodamine 123 and Chloroplast Fluorescence. Cyanide —as a generic inhibitor— was used to validate the use of Rhodamine 123 and chloroplast autofluorescence as metabolic 'reporters'. The results are shown in Figure 3. After cyanide addition the fluorescence of chloroplast increases (but not in control treatments with Bolds medium). The increase is slight, and not statistically significant. In contrast, cyanide causes a strong and statistically significant decrease in Rhodamine 123 fluorescence.

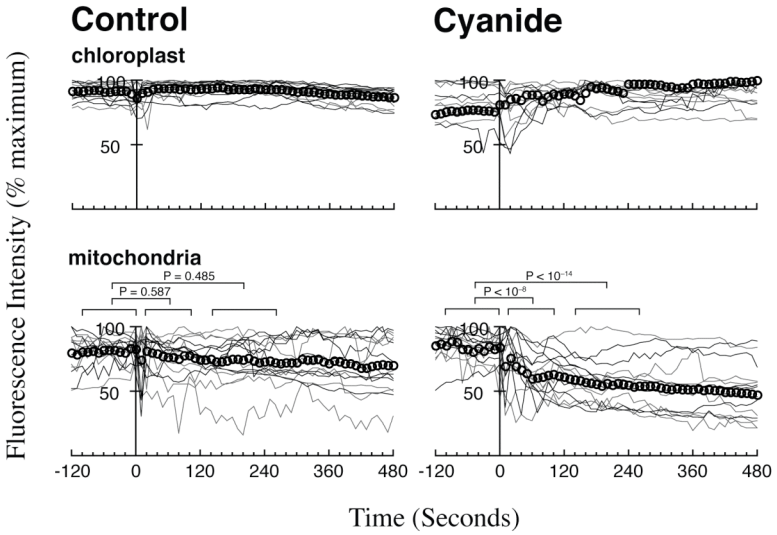


Figure 3. Effect of 5 mM cyanide on fluorescent intensity of chlorophyll auto fluorescence (upper panels, chloroplasts) and Rhodamine 123 (lower panels, mitochondria) in *Eremosphaera viridis*. An optical section of the cell was selected on the basis of a good representation of either chloroplasts or mitochondria. It was imaged every 10 seconds. At time 120 seconds, either 0.5 mL of Bolds medium (control) or 0.5 ml of 100 mM NaCN (in Bolds) was added in drop-wise fashion around the water immersion objective. Individual experiments (lines) and the means (circles) are shown. To account for variations in the pixel intensity, the data were normalized to maximal intensity (set at 100).

We wanted to explore the effect of cyanide on chloroplasts more completely, and did so with continuous quantitative measurements of fluorescence.

Cyanide Changes Fluorescence Intensity of Chloroplasts. The cells were irradiated at various intensities (continuously) and the effect of cyanide on chloroplast fluorescence monitored with a radiometer probe (Figure 4). Cyanide had an effect at all but the lowest intensity (20 μmol $m^{-2}s^{-1}$). At a light intensity similar to growth conditions (50 μmol $m^{-2}s^{-1}$), chloroplast fluorescence increased. At high intensities (200 and1000 μmol $m^{-2}s^{-1}$), fluorescence decreased.

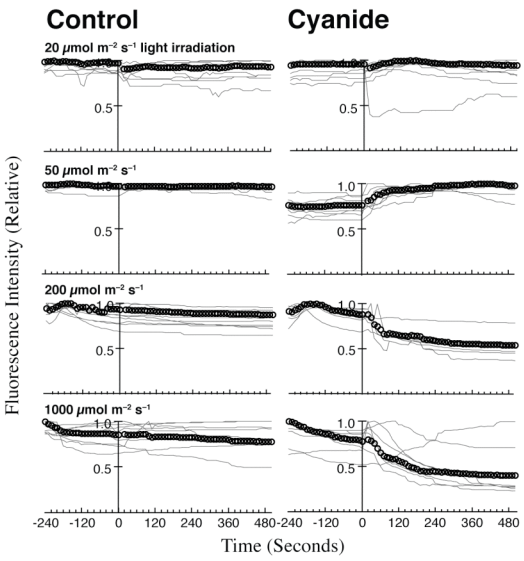


Figure 4. Effect of 5 mM cyanide and light intensity on fluorescence intensity of chlorophyll autofluorescence in *Eremosphaera viridis*. As described in the materials and methods section, the cells were irradiated with various intensities of light at wavelengths between 400 and 590 nm. Chlorophyll fluorescence (at wavelengths greater than 670 nm) was measured with the radiometer probe inserted into the camera port of the Zeiss Axioskop II. Intensities were varied from 20–1000 μmol m^{-2} sec^{-1}, as shown. Cyanide was added at time 0. The data are normalized to maximal output (which varied from 40 to 500 pW with increasing irradiation for the 2-4 cells in the field of view).

Therefore, both Rhodamine 123 fluorescence and chloroplast autofluorescence change when metabolic poise is perturbed with cyanide. We next examined whether either 'reporter' of mitochondrial and photosynthetic activity is affected by high intensity actinic light.

High Intensity Actinic Light Does Not Affect Rhodamine 123 and Chloroplast Autofluorescence. On the confocal microscope, the cells were irradiated at 2000 μmol m^{-2} s^{-1} and imaged with 12 z-sections. During z sectioning, the high light was off (for a period of about 30 second). Otherwise, irradiation was continuous. Neither Rhodamine 123 nor chloroplast autofluorescence were affected by the prolonged irradiation (Figure 5).

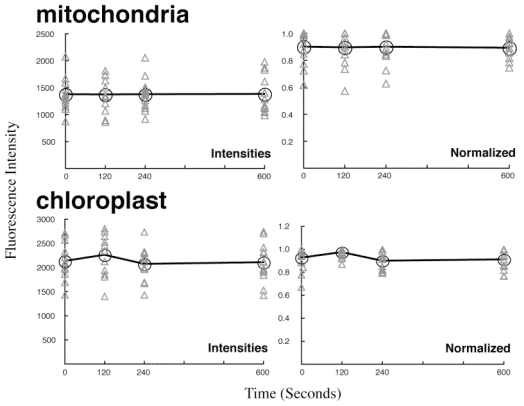


Figure 5. Irradiation with 2000 μmole m^{-2} s^{-1} light on the confocal microscope was started at time 0. The cells were scanned (12 z-sections) at the times shown. Results from Figure 5 shows that meither Rhodamine 123 nor chlorophyll autofluorescence was affected by the high intensity light.

We wanted to confirm whether there was an effect of chloroplast autofluorescence under continuous irradiation (similar to Figure 4).

Chloroplast Autofluorescence Declines During Continuous High Intensity Irradiation. The cells were irradiated with 2000 μmole m^{-2} s^{-1} and values of fluorescent intensities were recorded over a period of 10 minutes. During continuous high irradiation chloroplast autofluorescence declined (Figure 6).

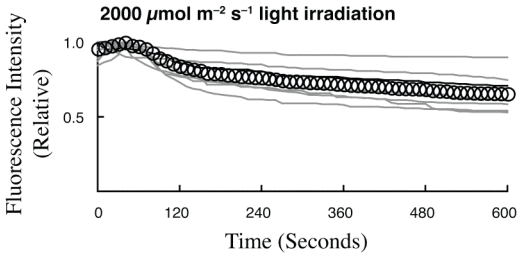


Figure 6. Irradiation with 2000 μmole m^{-2} s^{-1} light on the Zeiss Axioskop II was started at time 0. Values of intensities were recorded every 10 seconds over course of 10minutes. The results showed a decline rate in the fluorescence much similar to what is seen in figure 4 of high intensity irradiation of cells at 1000 μmole m^{-2} s^{-1} prior to addition of bold or cyanide medium. This should be what is expected. The data are normalized to maximal output

DISCUSSION

The research described in this report is an extension of research on the relation between metabolic function and high light irradiation effects on the large spherical cells of *Eremosphaera viridis*. The phenomenon of chloroplast translocation in cells of *E. viridis*, induced by high light irradiation, has been well described (Weidinger, 1980, 1982; Weidinger and Ruppel, 1985). Work from the Lew lab confirmed and extended these findings (Gasumova et al., 2009; Lew, 2010). Chloroplast translocations at high light intensities have often been considered an avoidance reaction, but concurrent measurements of oxygen production revealed photosynthesis is inhibited well before the onset of movement of the chloroplasts to the center of the cell, where they surround the nucleus (Lew, 2010). During the chloroplast translocation, mitochondria remain at the periphery of the cell. Our interest was to monitor chloroplast and mitochondrial function during high light treatments in single cells. To do this, we needed to validate the fluorescence 'reporters': Rhodamine 123 for mitochondrial function and chlorophyll autofluorescence for chloroplast function. We did so using cyanide.

Cyanide is an inhibitor of both respiration and photosynthesis (Yuan and Daniels, 1955). The decline in Rhodamine 123 fluorescence in response to cyanide confirmed that Rhodamine 123 could be used as an *in situ* reporter of mitochondrial metabolic poise.

The inhibitory effect of cyanide on photosynthesis appears to be at multiple sites: inhibition of photosystem I at plastocyanin (Ouitrakul and Izawa, 1973; Berg and Krogman, 1975), near the water splitting activity of photosystem II (Packham et al., 1982), RuBisCO (Wishnik and Lane, 1969) and inhibition of catalase, resulting in H_2O_2 production (Forti and Gerola, 1977). Autofluorescence is not affected in the confocal imaging. This may reflect the nature of confocal scanning, since most of the chloroplast is 'in the dark' as the laser scans the cell. During continuous actinic irradiance, the different responses of chloroplast fluorescence (increase at ambient irradiance and decrease at high irradiance) probably reflect different sites of action. At low irradiance,

backflow of excitons may occur, resulting in elevated fluorescence. At elevated irradiance, the decreased fluorescence could be due to damage caused by the production of oxidative products, including H_2O_2 (Forti and Gerola, 1977). Regardless, the fact that fluorescence is affected indicates the validity of autofluorescence as a reporter of metabolic poise.

Having established that Rhodamine 123 and chlorophyll autofluorescence could be used as reporters, we examined the effect of high light irradiance. In confocal experiments, Rhodamine 123 was unaffected after 10 minutes of irradiation at 2000 μmole m^{-2} s^{-1}. A decline in chloroplast autofluorescence was not observed in the confocal experiments, but could be demonstrated under continuous high light irradiation). Because we were able to observe strong decreases in Rhodamine 123 fluorescence after cyanide addition during confocal measurements, the technique should be able to detect change sin mitochondrial respiratory activity. Therefore, we conclude that high light irradiation does not affect mitochondrial activity under conditions similar to those causing the onset of chloroplast translocation.

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