Viridae Flourescence: Fluorescence intensity measurements of mitochondrial and chloroplast activity in *Eremosphaera viridis*[1].

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OBJECTIVE

To assess the use of Rhodamine 123 and chlorophyll autofluorescence to monitor the physiological states of mitochondrial respiration and chloroplast photosynthetic activity in *Eremosphaera viridis*.

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INTRODUCTION

The alga *Eremosphaera viridis* has been used for a variety of physiological measurements over the past 30 years. Much of this work has taken advantage of the large size of the unicellular alga to characterize the electrophysiological properties of the plasma membrane (Köhler et al., 1983, 1985, 1986), vacuole (Bethmann et al., 1995; Linz and Köhler, 1994) and ion fluxes (Lew, 2010) in the context of light-induced signals.

The large size of the cells also makes it possible to explore in detail the cell biology of the mitochondria and chloroplasts by using potential-sensitive fluorescence dyes that accumulate in the mitochondria (Rhodamine 123) and the autofluorescence of chlorophyll (which 'reports' on the poise of the light-driven electron transport in the chloroplasts).

MATERIALS and METHODS

Algal Strain. The algal *Eremosphaera viridis* de Bary strain originates from a 1987 collection from Plastic Lake (Ontario) (Dillon et al., 1987). The strain (CPCC 127, formerly UTCC 127) is maintained at the Canadian Phycological Culture Centre. The strain is designated LB (live bacteria). The cultures used for the fluorescence experiments were grown under T8 fluorescence lamps (photon flux 50 μmol m^{-2} s^{-1}) on a rotary shaker (120 rpm) in 125 or 250 ml Ehrlenmeyer flasks containing 25 or 50 ml of Bold's basal medium (BBM) supplemented with vitamins (Table I). Samples or transfers were taken from cultures that were one to two weeks old (that is, in log phase —actively dividing).

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 (0.25 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)			

Rhodamine 123 loading. A 40 μl aliquot of Rhodamine 123 stock (5 mM in methanol) was added to a 15 ml plastic tube to which APW6 (3.5 ml) (Table II), and 0.5 ml of cells were added. The tube was wrapped in aluminum foil and incubated at room temperature

for 1–3 hours. The cells were washed by centrifuging at 1000 rpm for a few seconds to settle the cells to the bottom of the tube; the supernatent was carefully poured off to avoid dislodging the pelleted cells; APW6 (10 ml) (Table II) was added to the tube and the cells re-suspended. The wash was repeated. The cells were resuspended in 1 ml of APW6 and kept in the aluminum foil wrapped tube at room temperature.

TABLE II: APW Ion and Buffer Concentration

	KCl	$CaCl_2$	$MgCl_2$	NaCl	Buffer
Final APW Concentrations (mM)	0.5	0.1	0.1	0.5	1
pH adjusted to 6.0 with 1 N NaOH					

Laser confocal fluorescence imaging. An aliquot of cells (0.1 ml) was added to APW6 (3 ml final) in the center of a lid of a 30 mm culture dish and placed on the specimen stage of the confocal microscope (Olympus). Dual excitation was used to fluoresce the Rhodamine 123 (488 nm line on an multi-argon laser) and chlorophyll (633 nm laser line on an helium-neon laser). Bandpass emissions of 505–535 nm were used to image Rhodamine 123. Long-pass emission (>660 nm) was used to image chlorophyll autofluorescence.

Laser confocal z-slicing. The FITC dye setting was used for Rhodamine 123 imaging. Digital gain was set between 1.0 and 2.5. The black offset was set between 3 and 5. The Cy5 dye setting was used for chlorophyll autofluorescence. The photomultiplier gain was between 400 to 550 Volts. Digital gain was set between 1 and 2. The black offset was set between 3 and 5. In both instances, care was taken to assure that the fluorescence signal was not saturated. Scans were Kalman filtered (2 slices). Zoom was X2 to X2.5 to fill the image with the whole cell. Zooms of between X5 and X8 were used for scanning internal regions of the cell. Z-sectioning used 48 slices with a step distance of 0.3 μm to image to a total depth of 14 μm. Normally the z-sectioning was initiated near the cell surface at the top of the cell, and extended into the cell to capture peripheral regions of the cell.

Three-dimensional reconstruction. The z-section optical slices were used in three-dimensional reconstructions using the Fluoview software. The initial rotation angle was 22° for X, 22° for Y, and 0° for Z (initial view). The rotation angle increments were –4° for ΔX, –4° for ΔY, and 0° for ΔZ (sequence view). The total rotation angle was –92° for X, –92° for Y, and 0° for Z. The stretch factor was set to 1.3 (to minimize 'squashing' of the three-dimensional structure). Figure 1 shows example of images from a three-dimensional reconstruction.

Intensity measurements using z-slices. Single mitochondria or chloroplast were selected and cropped out for measurement over all z-slices for a single imaging time of the imagined cell. A linear montage of the cropped z-slices was created in ImageJ. This was used to create an intensity plot of the fluorescence intensity. This measurement process was repeated for numerous mitochondria or chloroplasts within the same cell, and for all cells that were imaged.

Intensity measurements using three-dimensional reconstructions. The three dimensional image was rotated to select single mitochondria or chloroplast lying flat in

the field of view. The mitochondria or chloroplast was cropped out for intensity measurements for all imaging times of particular cell. Direct fluorescence intensity measurement made using cropped region. This measurement process was repeated for numerous mitochondria or chloroplasts within same cell, and for all cells that were imaged.

After image processing in Fluoview to create the three-dimensional reconstructions, all further image processing, measurement and analysis was performed with ImageJ (Rasband, 2009). Data were transferred to Excel for final compilation.

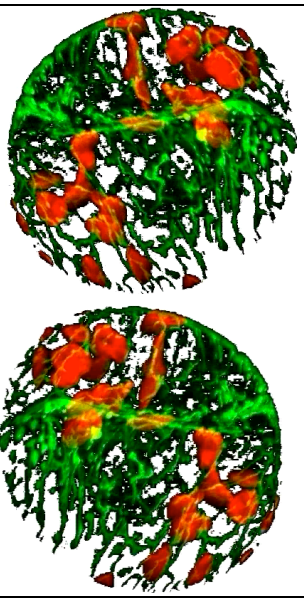

Figure 1: Dual imaging of mitochondrial fluorescence (Rhodamine 123, green) and chloroplasts (chlorophyll autofluorescence, red). Images are taken from three-dimensional reconstructions. Overall width of the algal cell is about 100 microns.

RESULTS

Analysis of fluorescence intensity. The large size of the algae and its spherical geometry simplifies analysis of fluorescence intensity. With confocal fluorescence z-sectioning, it is possible to collect scans throughout the three dimensional structure of organelles: either mitochondria (Rhodamine-123) or chloroplasts (chlorophyll autofluorescence). Once z-sections had been collected and converted into three-dimensional images, two methods were tried to quantify the fluorescence intensity of the organelles. In one, intensities through multiple optical slices were measured and summed. In the other technique, the three dimensional reconstruction was rotated so that the organelle could be viewed *in toto* and intensity measured. It is difficult to assess which offers the best approach. So, both were used on the same organelle and the relative intensities compared (Figure 2).

For both mitochondria and chloroplasts, there was a strong correlation between the two techniques; more so for mitochondria ($R = 0.97$) than for chloroplasts ($R = 0.82$). Intensity measurements of the three-dimensional reconstructions are less tedious, taking far less time, and is the measuring technique of choice.

Effect of cyanide on fluorescence intensity of mitochondria and chloroplasts. To validate the use of fluorescence intensity as a measure of the activity of respiration and photosynthesis, the cells were treated with sodium cyanide (5 mM final) or control additions of APW6 and fluorescence monitored over a period of 20 minutes. Unpublished results show that *both* respiration (oxygen consumption in the dark) and photosynthesis (oxygen production in the light) are rapidly inhibited (within 1–2 min) after cyanide addition at 5 mM (oxygen electrode measurements, Lew, unpublished). Thus, changes in fluorescence intensity are expected for both Rhodamine 123 (which is sensitive to the mitochondrial electrical potential) and chlorophyll autofluorescence (which should be affected by blockage of carbon dioxide fixation after cyanide treatment). The results do not show a clear effect of cyanide on fluorescence of either Rhodamine 123 or chlorophyll (Figure 3).

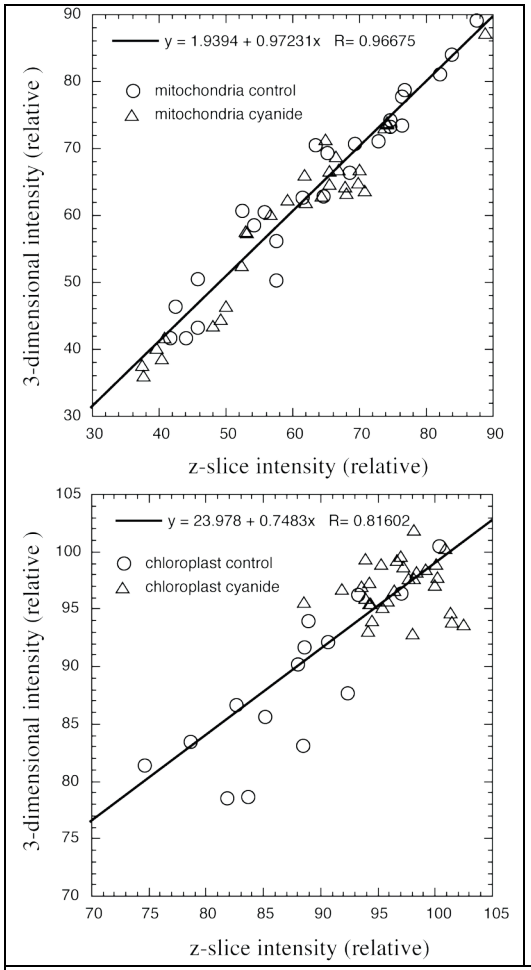

Figure 2: Comparisons of three-dimensional intensity measurements and summed z-slice intensity measurements for mitochondria (upper panel) and chloroplasts (lower panel). For both scatter plots, all data (controls and cyanide treatments) are compiled. Linear regressions demonstrate strong correlations between the two techniques for measuring organelle fluorescence intensity.

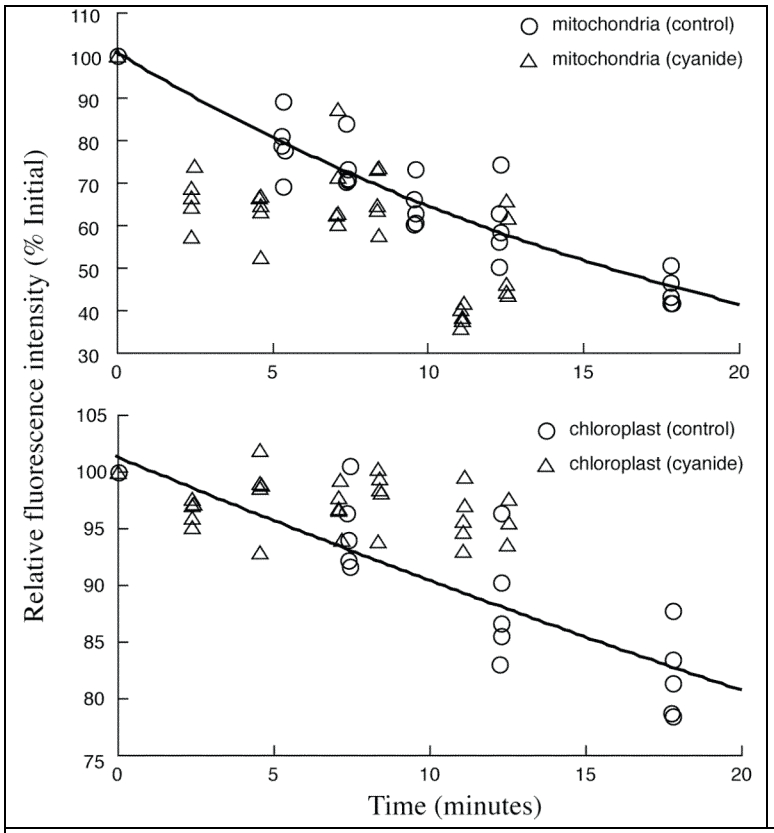

Figure 3: Effect of cyanide on three-dimensional intensity measurements of mitochondria (upper panel) and chloroplasts (lower panel). Cyanide was added at time zero (triangles), or, as a control APW6 (circles).

DISCUSSION

The experiments were exploratory in nature. First, we needed to assess different techniques for measuring fluorescence intensity in the complex three-dimensional structure of the large spherical uni-cell of *Eremosphaera viridis*. Either technique (z-slices or three-dimensional reconstructions) resulted in similar measures of intensity, but it was much easier to reconstruct the three-dimensional image of the alga and select mitochondria or chloroplasts in a 'flat' view. Validation of the use of fluorescence as a metric of the physiological status of respiratory and photosynthetic activity was more problematic. Chlorophyll fluorescence was unaffected by cyanide treatment, while rhodamine 123 fluorescence declined similarly in controls (APW6) and cyanide treatments. It was noticeable that Rhodamine 123 fluorescence may have declined rapidly after cyanide addition (within 5 minutes), but we did not measure that quickly in controls. Short-term effects of cyanide on Rhodamine 123 fluorescence should be explored in future work.

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