Comparison of the Photoresponses of the Anoxygenic Purple Non-Sulfur Bacteria *Rhodospirillum centenum* and *Rhodobacter sphaeroides*

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Introduction

Photoresponses of bacteria were first noted in 1883 by Engelmann when the cells he was observing "took fright" from specific regions of lower light intensity. The cells were trapped within the lighted region by continually reversing their swimming direction back into the light at the light/dark interface (Engelmann, 1883). This photoresponse has been termed scotophobia to refer to the bacterium's apparent fear of darkness. The scotophobic response does not imply that the cells move directionally toward the light source, but rather that they preferentially stay within the lighted area (Ragatz *et al.*, 1995). Movement toward or away from a light source is termed positive or negative phototaxis, respectively (Häder, D.-P., 1987).

Rhodospirillum centenum and *Rhodobacter sphaeroides* are two anoxygenic photosynthetic bacteria from the purple non-sulfur bacteria group whose mechanisms of photoresponse have been described in some detail (for overview, Armitage *et al.* 1995). Both of these organisms display a photoresponse when passing through a light/dark boundary or a light intensity gradient, but have some distinct differences in their modes of response. Liquid grown *R. centenum* displays a scotophobic photoresponse by reversing its direction at the light to dark boundary by switching the direction of its flagellar rotation from clockwise to counterclockwise so that the cells swim back into

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the region of higher light intensity (Ragatz *et al.*, 1995) (Figure 1A). On the other hand, *R. sphaeroides* only rotates its flagellum unidirectionally (clockwise), so in order for the cell to change direction upon reaching a light to dark boundary it stops rotating its flagellum, allows Brownian motion to reorient the cell and then begins to swim forward in a new direction (Armitage and Macnab, 1987) (Figure 1B). This stopping of flagellar rotation as the cells pass from a light to dark region is called a step-down response (Packer *et al.*, 1996). This response occurs only when *R. sphaeroides* is moving down a gradient, but does not occur when the cells move up a light intensity gradient.

Another difference in the photoresponse behavior between *R. centenum* and *R sphaeroides* is at the macroscopic level of colony movement on agar plates. Ragatz *et al.* (1995) noted that the macroscopic phototactic movement of *R. centenum* colonies is to swarm directly toward a light source even when the intensity of light decreased as the colony moved toward the light source (Figure 1C). Such a photoresponse to the direction of light is true phototaxis. Colonies of *R. centenum* are able to display both positive and negative phototactic movement depending upon the wavelength of light used (Ragatz *et al.*, 1994). For instance, the presence of infra red light at 800-880 nm wavelength acts as an attractant so that the cells display positive phototaxis by moving *en mass* toward the light source. However, in the presence of visible light from 580-600 nm in wavelength, the cells display negative phototaxis and the entire colony moves away from the light. This positive and negative phototactic activity of *R. centenum* directly correlates with the spectrum of its photosynthetic pigments (Ragatz *et al.*, 1995). The positive phototactic response occurs in the wavelength region which corresponds to the light-harvesting and reaction center absorbency peaks (800 and 875 nm) while negative phototaxis is induced in the wavelengths of light equivalent to the carotenoid peaks and the Qx bacteriochlorophyll peak (Ragatz *et al.*, 1995).

Swarm plate grown *R. sphaeroides* cells do not display macroscopic photoresponse to a variety of light sources (J. P. Armitage, personal communication) (Figure 1D). One possible explanation for this is that lateral flagellar production is not induced in *R. sphaeroides* when grown on an agar surface.

In this work, we demonstrate that neither *R. centenum* nor *R. sphaeroides* display true phototaxis. Both respond to a beam of light with *R. centenum* becoming "trapped" within the light beam produced by a fiber optic cable and with *R. sphaeroides* becoming "trapped" outside of the light beam.

Materials and Methods

Bacterial strains, media and growth conditions

Rhodospirillum centenum wild type strain (gift of Dr. Carl Bauer) used in this study (*R. centenum* strain SW; ATCC 51521) is a derivative of the original *R. centenum* strain isolated from Thermopolis Hot Springs, WY (Favinger *et al.*, 1989). Liquid cultures were grown photosynthetically in PYVS medium (0.3% peptone, 0.3% yeast extract, 0.4% soytone, 20 ng/mL vitamin B_{12} and 1.5 µg/mL biotin) in a screw cap tube illuminated by a 75 W halogen bulb at 27°C-42°C (Ragatz *et al.*, 1995). *R. centenum* swarm cells with lateral flagella were grown as described (Ragatz *et al.*, 1995). Briefly, 1 mL of early stationary phase liquid culture cells was centrifuged and resuspended in 50 µL of spent medium. 8 µL of this concentrated sample was spotted onto an 0.8% PYVS swarm plate (9 cm X 9 cm square), allowed to air dry briefly, and then incubated in the dark at 42°C for approximately 4 hours or until a dense growth of cells was present at the original spot. The cells were then induced for positive phototaxis by placing the swarm plate cultures in the light of a single tungsten light bulb (60 W), shielding the plate from ambient light, with the temperature maintained at 37°C - 42°C. The cells were incubated in this fashion until phototaxis had begun and lateral flagella were evident by staining.

The *R. sphaeroides* WS8NΔ*op* strain used in this analysis is a spontaneous nalidixic acid resistant mutant of WS8 (wild-type isolate from W. Sistrom). *R. sphaeroides* was grown as described with 5 mM sodium succinate as the carbon source (see Appendix I for medium recipe) at 25°C under high light illumination (Armitage *et al.*, 1985).

Flagellar Staining

Flagella were stained using Flagella Stain (Carr-Scarborough Decatur, GA) which contains 0.6% crystal violet, 2 % tannic acid, 2.5% phenol, and 5.7% aluminum potassium sulfate. The protocol from the manufacturer was modified so that wet mounts of cells were stained. 3 μL of liquid medium, sterile deionized water or liquid suspension of cells was placed on a microscope slide. If medium or sterile deionized water was used, a colony was picked and gently resuspended in the liquid. A coverslip was then placed over the sample and 2-3 μL of flagella stain was placed next to the coverslip and allowed to move under the glass by capillary action. The cells were stained for approximately 5 minutes (and up to 2 hours) before being visualized under 100X objective. Longer periods of staining allow for better visualization of difficult to stain flagella such as the lateral flagella of *R. centenum* cells.

Sample Preparation for Microscopic Photoresponse Assay

Approximately 150 μL liquid grown cells were placed directly into the sample chamber. Plate grown cells were placed in the sample chamber by first resuspending the cells in approximately 250 μL of sterile deionized water and then place approximately 150 μL of the suspension under the microscope slide.

Microscopic Photoresponse Assay

The main premise of this assay utilizes the upward light scattering of the bacteria present in the light beam path produced by a fiber optic cable to analyze the photoresponse of motile photoheterotrophic cells. If cells entering the beam are scotophobic, they will become trapped in the beam leading to an increase in the number of cells present in the beam. The increased number of cells in the light beam is proportional to the amount of light scattered by the cells which leads to a more detectable intensity of light. Also, if cells are attracted to the light source (as in positive phototaxis), then the amount of light scattering at the end closer to the end of the fiber optic cable will be greater than at the other end of the light beam. A schematic of the analytical set-up is present in Figure 2 in

which the white light from a 100 W halogen bulb is collected using a Zeiss 20X objective and sent through a fiber optic cable to the sample chamber. The sample chamber is composed of a slide and three coverslips in which two of the slides are fixed to the microscope slide with Apiazon L high vacuum grease in order to be a platform for the third coverslip. Both the sample (see "sample preparation" below) and the fiber optic cable are placed under the raised coverslip and placed on a Zeiss Axioplan2 microscope (Figure 3). The light scattering from the bacteria present in the light beam produced by the fiber optic cable is captured by a CCD camera (MTI VE1000) after passing through an intensifier composed of a 512 X 512 array of photomultiplier tubes (Hamamatsu Photonics K. K.) and recorded on a Sony Video Cassette Recorder EV-C100. See Figure 4 for an example of the resulting light scattering recorded image.

Data analysis was performed using Metamorph (Universal Imaging Corporation, West Chester, PA). A 16-bit sum image of 30 frames was obtained every 10 or 20 seconds over the period of data collection for each assay. The intensity of the beam on either the side of (closest to or farthest away from) the light source was determined by integrating the area under the curve of the intensity as obtained by a 45 pixel width scan of each image.

Results

Rhodospirillum centenum - Liquid Grown Cells

Liquid grown *R. centenum* cells with their single polar flagellum were placed within the sample chamber and analyzed using the microscopic photoresponse assay. Within one minute a large number of cells had accumulated within the beam (Figure 5). These cells appeared to be "trapped" by the beam due to the steady increase in the intensity of the light scattering from zero to 100 seconds (Figure 6).

Rhodobacter sphaeroides - Liquid Grown Cells

A sample of a liquid culture of *R. sphaeroides* that had been grown under high light intensity, so that cells have a low number of reaction centers and light harvesting complexes, was placed within

the sample chamber and analyzed for a photoresponse. Figure 7 illustrates that there was a steady decrease in the light intensity within the beam. This indicates that there was a corresponding decrease in the number of bacteria present within the path. When the sample chamber was visually inspected, it was noted that the cells had actually cleared an area corresponding to the path that the light path followed through the sample (Figure 8).

Rhodospirillum centenum - Swarm Plate Grown Cells

Swarm plates of *R. centenum* were set up as described in the Material and Methods. Once the cells had begun to swarm towards the halogen light, they were tested for the presence of lateral flagella. If lateral flagella production had been induced in the cells by the presence of the agar in the medium (Ragatz *et al.*, 1995), a small amount of cells from the front, moving portion of the swarm colony were picked and resuspended in sterile deionized water. The suspension was placed within the sample chamber and were adapted to the new environment for 5 minutes under constant light illumination. By allowing the cells to adapt for 5 minutes they are able to swim in the less viscous environment of water versus 0.8% agar and assays of movement are best completed within 15 minutes from the time the cells were diluted in water (Carl Bauer, personal communication).

To ensure that the cells maintained their lateral flagella during the time course for the photoresponse, aliquots were taken of the remaining aliquot of resuspended cells every 5 minutes for 25 minutes (the time course observed was 15 minutes in duration) and flagella stained (Figure 9). The cells did not shed within this time. Out of curiosity, a separate time course was performed to determine when the cells actually dropped their lateral flagella (Figure 10). The cells in this time course were resuspended in PYVS medium and it was noted that the lateral flagella were intact on the cells up to the 40 minute time point. Between the 40 and 50 minute time points, the cells lost their lateral flagella and only cells with either a single polar flagellum or no flagella could be found on the slide.

The microscopic photoresponse assay of these cells indicates that the cells neither accumulated within the light path nor those present in the light beam migrate toward the light source (Figure 11).

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To ensure that their was not a bias in the intensity of light scattering within the area of the beam, the intensity of the beam at each horizontal point (in a 45 pixel width) within the path was averaged over the time course of the experiment (Figure 12). This graph is a linear line which indicates that the average intensity of each point along the line through the middle of the beam was not greater on one side versus the other.

Discussion

Bacterial response to light is one of many environmental cues that motile photosynthetic bacteria respond to in order to move to areas of optimal conditions for their survival. Two anoxygenic photosynthetic, purple non-sulfur bacteria *Rhodospirillum centenum* and *Rhodobacter sphaeroides* have very similar yet distinct photoresponse behaviors. *R. centenum* in liquid medium displays a scotophobic response when passing through a light to dark boundary by reversing the direction of the rotation of their single polar flagellum. This reversal of the motor causes the bacterium to move backward into the lighted area. In the case of liquid grown *R. sphaeroides*, the photoresponse to a decreasing light intensity is to increase the number of step-down responses in which the cell stops swimming and is reoriented by Brownian motion. This increase in step-down response has recently bee shown to directly correlate with the change in the rate of electron transport through the photosystem (Grishanin *et al.*, 1996).

The photoresponses of these organisms varies depending upon how the cells are grown. When *R. centenum* cells are grown on an agar medium, the cell produce numerous lateral (peritrichous) flagella and is able to display macroscopic phototaxis by the colony moving forward toward a light source even when the intensity of the light decreases the closer to the source (Ragatz *et al.*, 1995). *R. sphaeroides* does not show a macroscopic photoresponse which may be due to the fact that *R. sphaeroides* does not induce the expression of lateral flagella when grown on a solid agar surface (J. P. Armitage, personal communication).

We have developed a microscopic photoresponse assay to compare in detail the responses of these two photosynthetic bacteria *R. centenum* and *R. sphaeroides*. The liquid cultures of these two

bacteria display very distinct responses to the light produced by a 75 W halogen lamp focused through a fiber optic cable. The *R. centenum* cells displayed a scotophobic response by collecting within the area of the path of the light beam (Figure 5). Integrating the intensity of the light scattering, which is proportional to the number of cells present in the beam, on both sides of the beam indicates that the cells do not cluster on either side of the beam. If the cells displayed phototaxis they would enter the path of the light and then travel toward the light source which would lead to an increase in intensity on the side of the beam closest to the light source. No difference in intensity was noted between the two sides of the beam indicating that the cells that were trapped within the beam path due to a scotophobic response were not displaying phototaxis.

The photoresponse of high light grown *R. sphaeroides* produced a rather striking result. The cells did not cluster in the beam as in a scotophobic response and did not cluster towards the light source as expected for a phototactic response; instead, the cells cleared the area where the beam of light was present (Figures 7 and 8). The most drastic result was visualized when the *R. sphaeroides* cells were grown under high light so that they produced very few light harvesting complexes and reaction centers which makes them exquisitely sensitive to changes in light intensity (Grishanin *et al.*, 1996). These cells may be so sensitive to the change in light intensity between the path of light and the surrounding dark area that they may have a prolonged or even permanent step-down response in which the rotation of their flagella is stopped. Such a prolonged or permanent step-down response decreases the already low probability that they will reenter the higher light area.

Since phototaxis of *R. centenum* has been described at the macroscopic level as a colony movement on an agar surface of cells with peritrichous flagella, we also analyzed the photoresponse of these cells on a microscopic level. Cells were picked from a plate and resuspended in sterile deionized water for the analysis of their photoresponses. The microscopic photoresponse of these cells did not mimic the macroscopic photoresponse of colonies of cells. The cells in the sample chamber neither collected in the beam nor migrated toward the light source. These data indicate the swarm plate *R. centenum* cells display neither scotophobia nor phototaxis.

A flagella staining time course was performed simultaneously to the microscopic photoresponse assay to ensure that plate grown *R. centenum* cells retained their lateral flagella and the loss of flagella was not the reason for the lack of a photoresponse in these cells (Figure 9). The cells retained their lateral and polar flagella throughout the time course of the experiment and beyond. In order to determine at which point the cells dropped their lateral flagella, a longer time course was performed by resuspending the cells in PYVS medium and taking aliquots and flagella staining at specific time points from zero to 60 minutes (Figure 10). Between the 40 and 50 minute time points, the cells shed all of their flagella except for the single polar flagellum indicating that the adaptation period for the solid surface swimming state to the state with only the polar flagellum occurs after 40 minutes of resuspension in liquid medium. Thus, a loss of flagellum cannot be attributed to the loss of photoresponse of plate grown cells.

Acknowledgments

Figure Legends

Figure 1. <u>Schematic of the modes of swimming of *R. centenum* and *R. sphaeroides*.</u> **A)** Liquid grown *R. centenum* displays a scotophobic response when passing through a light to dark boundary by reversing the rotation of its flagellum so that the cell reenters the lighted area. **B)** Liquid grown *R. sphaeroides* displays a step-down response when passing into an area of decreased light intensity. The flagellum stops rotating and curls up next to the cell. The non-swimming cell is then reoriented by Brownian motion before the flagellum begins to rotate again. **C)** Agar plate grown *R. centenum* cells induce the production of numerous lateral flagella in addition to the polar flagellum. Colonies of these cells are able to display phototaxis toward or away from sources of light depending upon the wavelength of light. **D)** Agar plate grown *R. sphaeroides* colonies do not respond to any type of light source.

Figure 2. <u>Schematic of the experimental set-up for the microscopic photoresponse assay.</u> White light from a 75 W halogen lamp is collected through a 20X objective prior to passing through a fiber optic cable. The opposite end of the fiber optic cable is placed into the sample chamber where the bacteria for analysis are present. The resulting light beam is perpendicular to the intensifier and CCD camera (not shown) which detect the light that is scattered upward by bacteria within the light beam.

Figure 3. <u>Bright field image of the end of the fiber optic cable within the sample chamber.</u> The cylinder in the end of the fiber optic cable within the sample chamber that was first filled with liquid grown *R. centenum*.

Figure 4. <u>Recorded image of light scattering.</u> Upward light scattering produced by bacteria present within the horizontal beam of light as detected by CCD camera with intensifier.

Figure 5. <u>Series of images over 1 minute of collection of *R. centenum* in the path of the light beam.</u> Serial images of the average of 30 frames of 16-bit images every 5 seconds from zero to 60 seconds.

Figure 6. <u>Liquid grown *R. centenum* photoresponse.</u> The intensity of the light scattering produced by the collection of *R. centenum*. Intensity was calculated for each end of the beam within the field of the image.

Figure 7. <u>Liquid grown *R. sphaeroides* photoresponse.</u> The intensity of the light scattering produced by the presence of *R. sphaeroides* on either side of the beam within the field of the image.

Figure 8. <u>Photograph of the photoresponse of *R. sphaeroides*.</u> Photograph of the clearing of *R. sphaeroides* within 20 minutes from the path of the beam of light. The black mark indicates the end of the fiber optic cable with the zone of clearing immediately above it and extending nearly to the end of the sample chamber.

Figure 9. <u>Flagella stain in sterile deionized water.</u> Flagella were stained at 5 minutes time points for 25 minutes. The lateral flagella were not shed within this time frame.

Figure 10. <u>Flagella stain in PYVS.</u> Time course to determine at which point the cells shed their lateral flagella.

Figure 11. <u>Swarm plate (0.8% agar) grown *R. centenum* photoresponse.</u> The intensity of the light scattering produced by *R. centenum* present within the path of the light beam.

Figure 12. <u>Average light intensity horizontally through the beam.</u> The intensity horizontally across the beam was averaged and plotted.
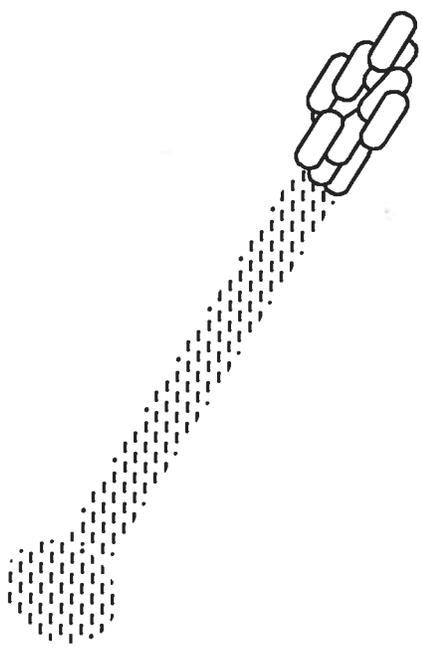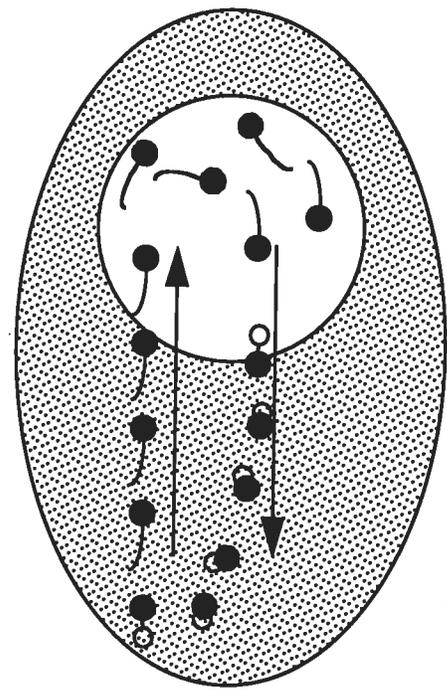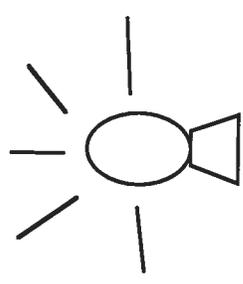
A — *R. centenum* liquid culture

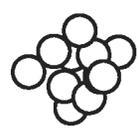**B** — *R. sphaeroides* liquid culture

C — *R. centenum* swarm plate culture

D — *R. sphaeroides* swarm plate culture

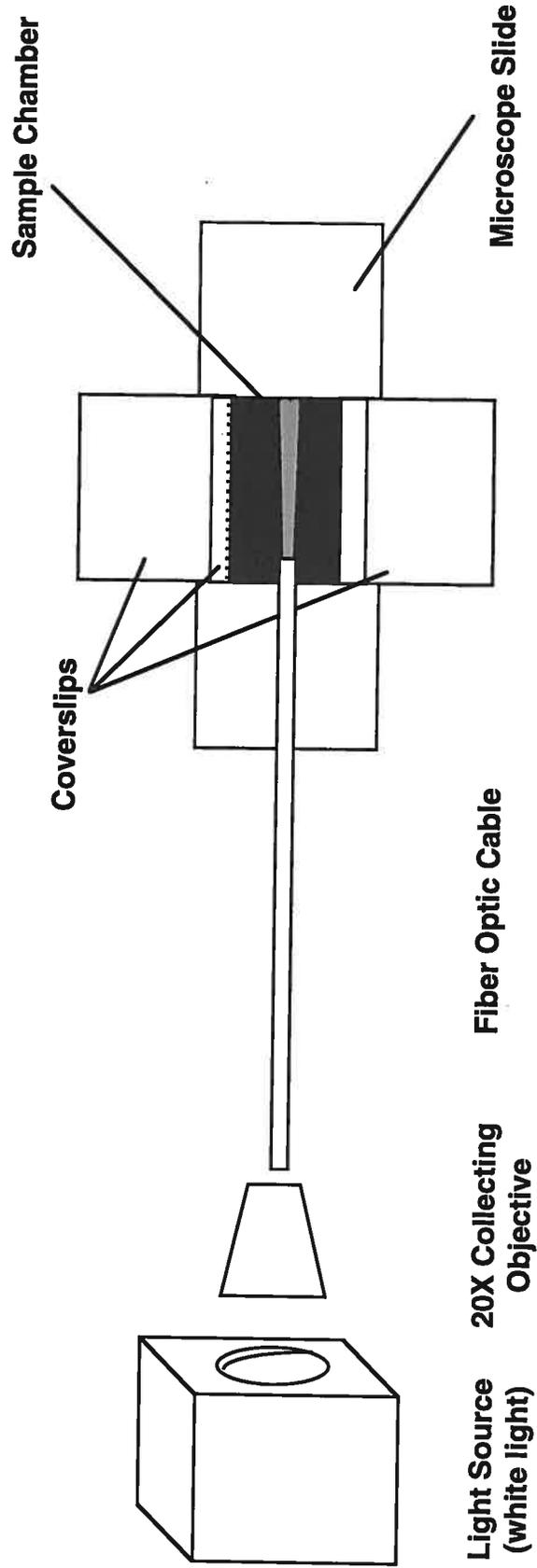**Figure 1.** Schematic of the modes of swimming of *R. centenum and R. sphaeroides*

Figure 2. Schematic of experimental set-up for the microscopic photoresponse assay
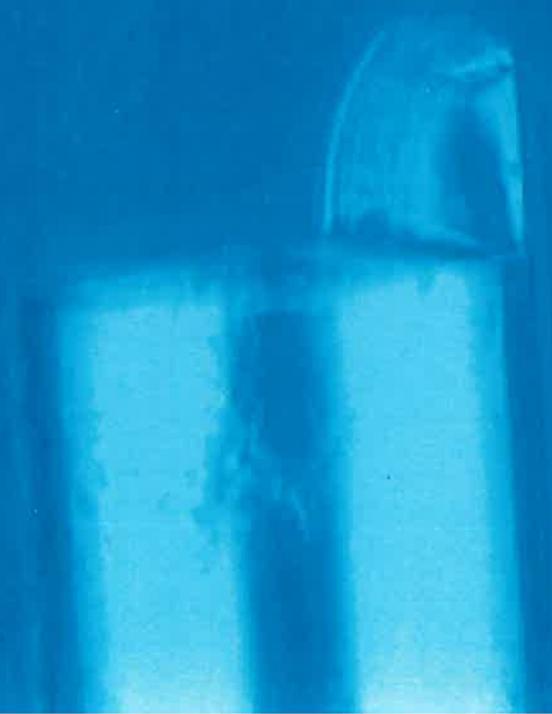
Sample Chamber

Microscope Slide

Coverslips

Fiber Optic Cable

Light Source (white light)

20X Collecting Objective

Fig. 3. Bright field image of end of fiber optic cable + cells.
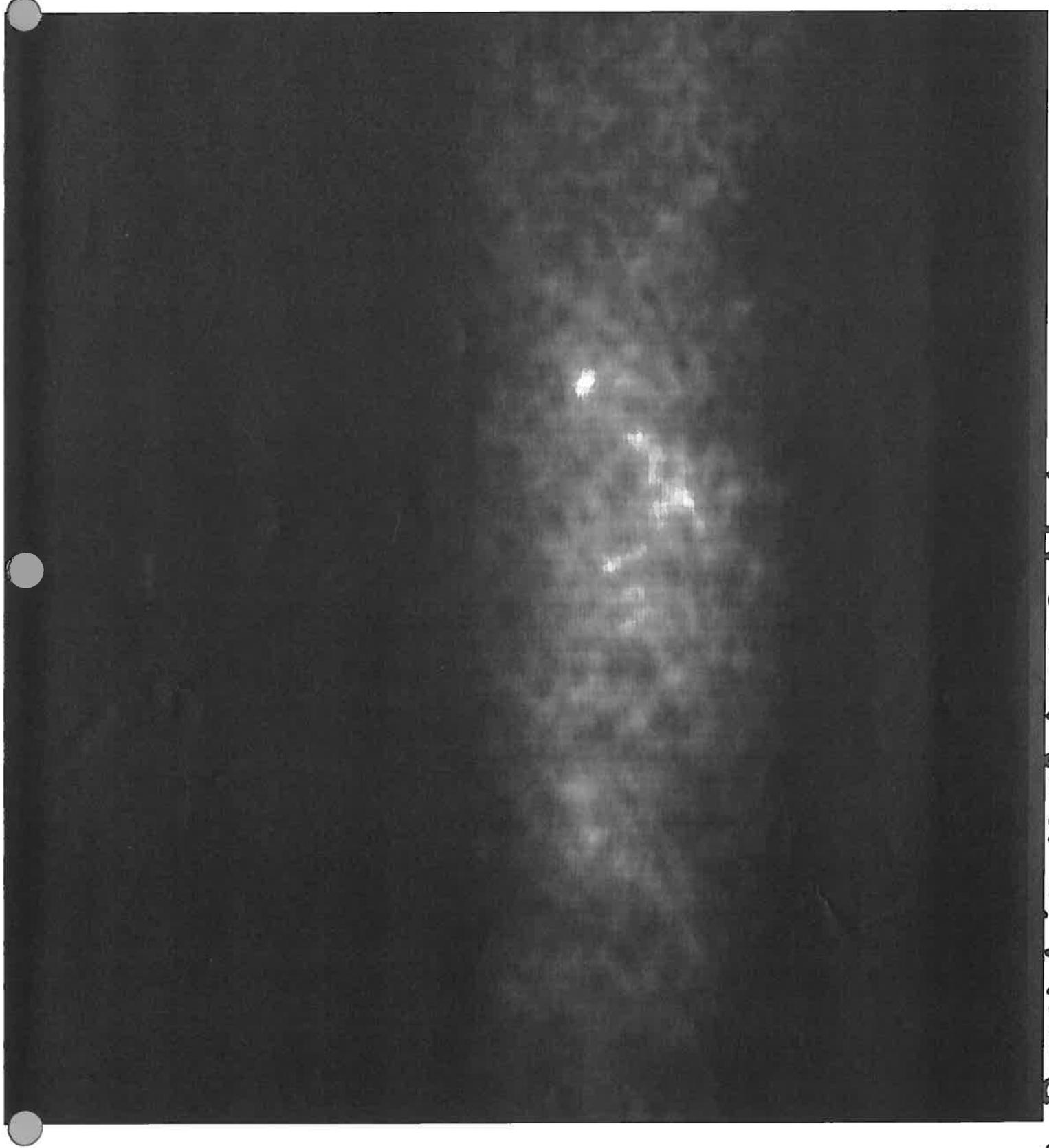
Fig. 4. Recorded image of light scattering

P1
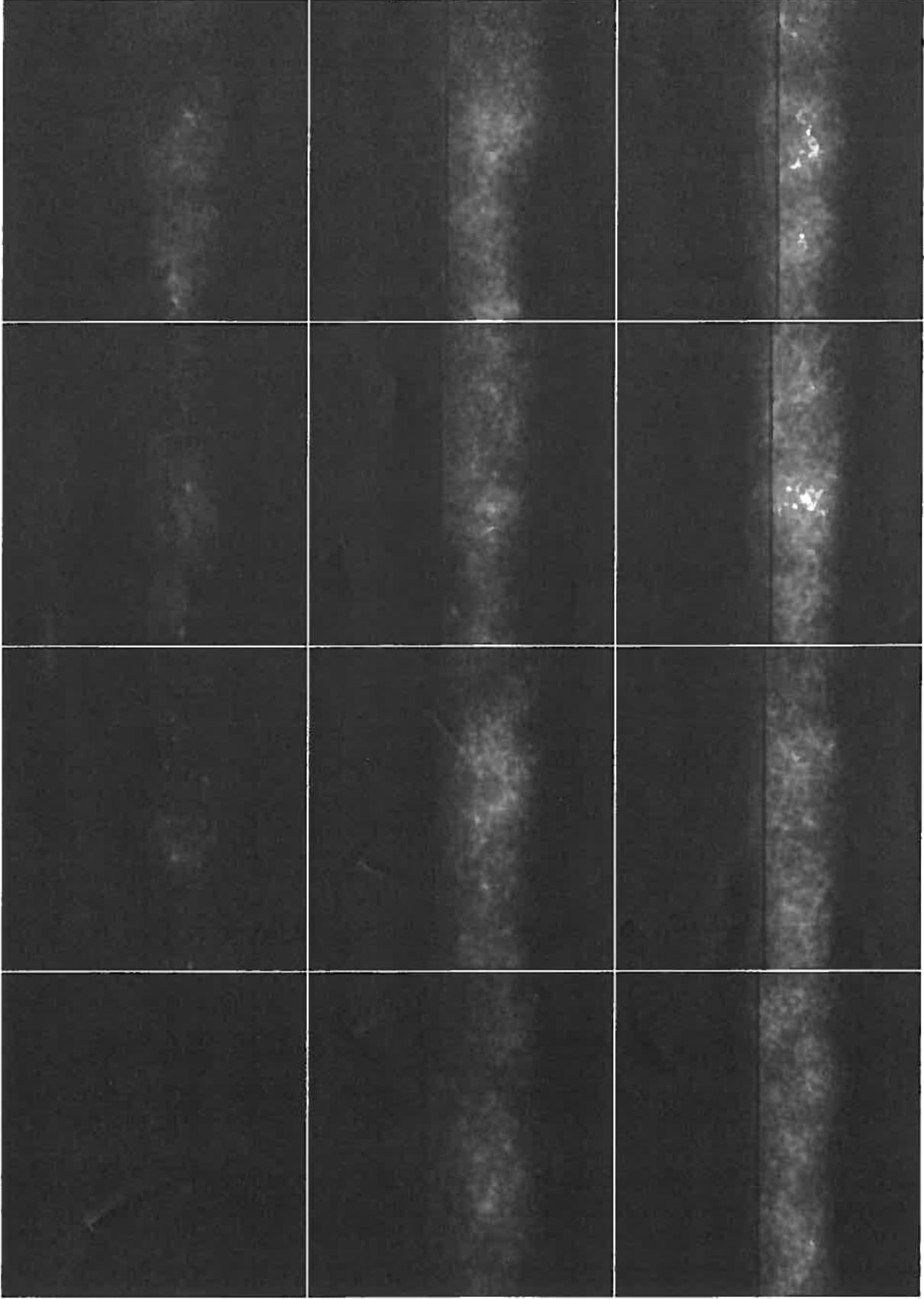
Fig. 5. Series of light scattering images of liquid *L. cantenum* 0 - 60 sec.
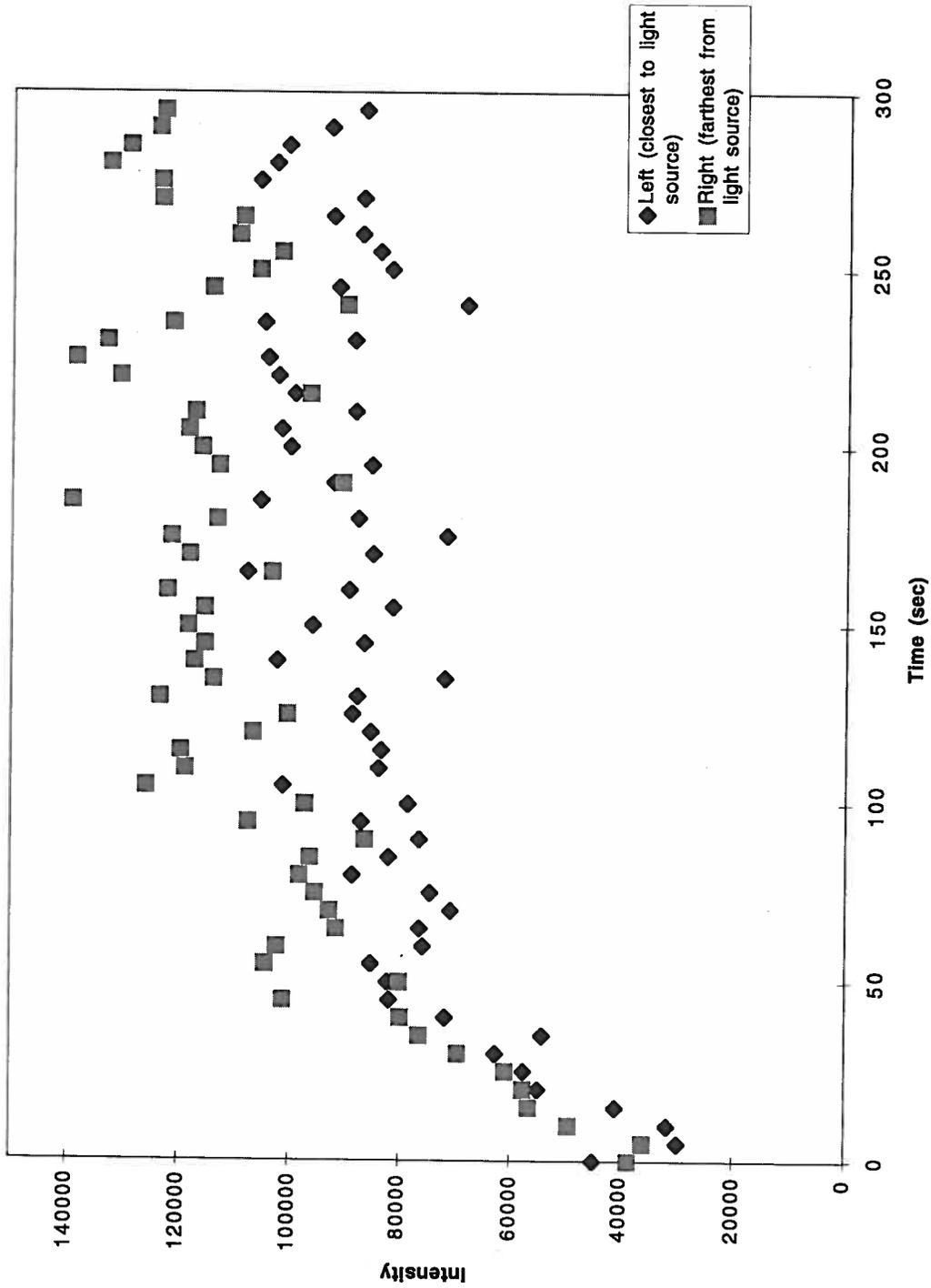
15

7-18-96 A
R. centenum
Liquid

Left (closest to light source)
Right (farthest from light source)

Intensity

Time (sec)

Figure 6. Liquid grown *R. centenum* photoresponse
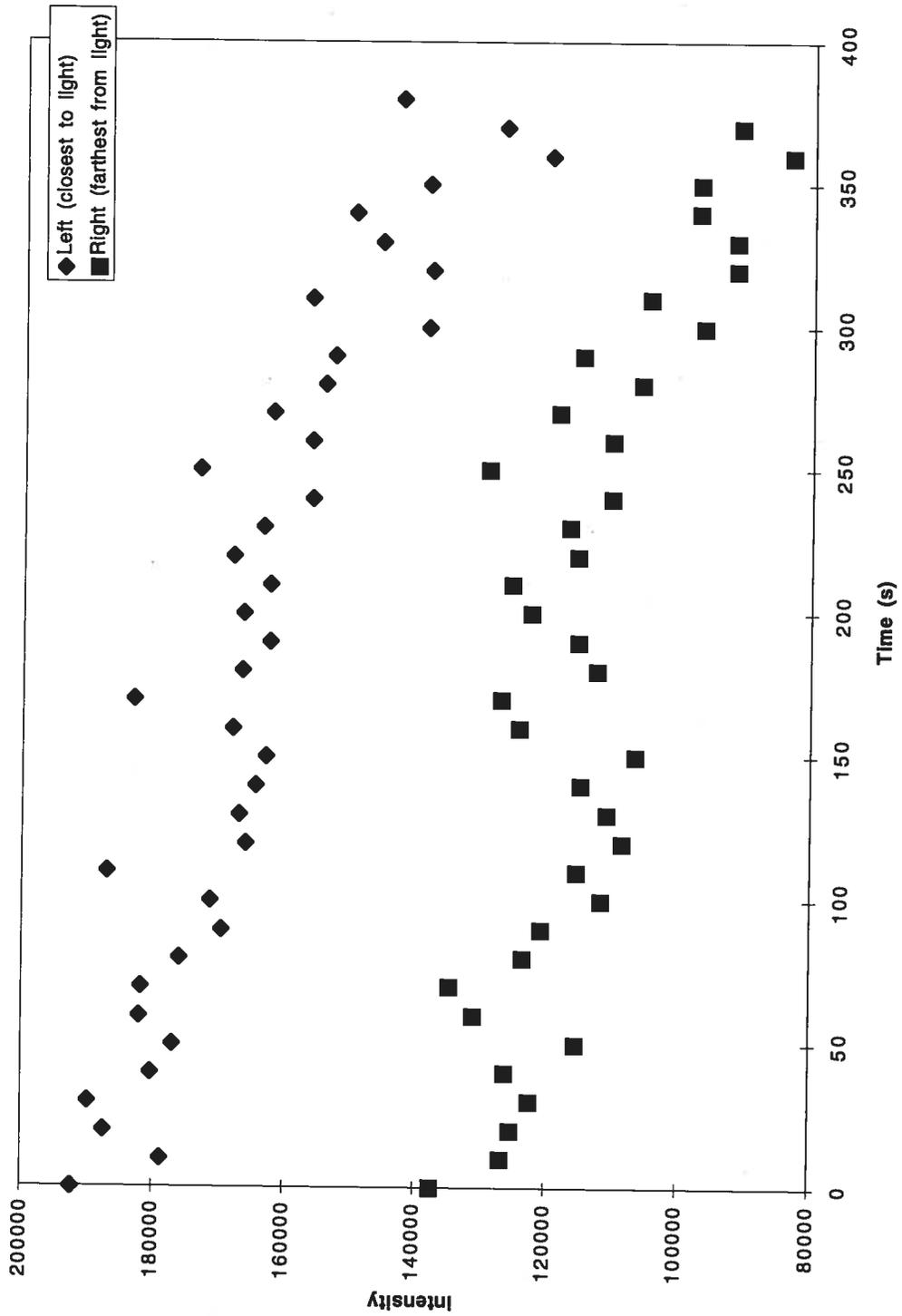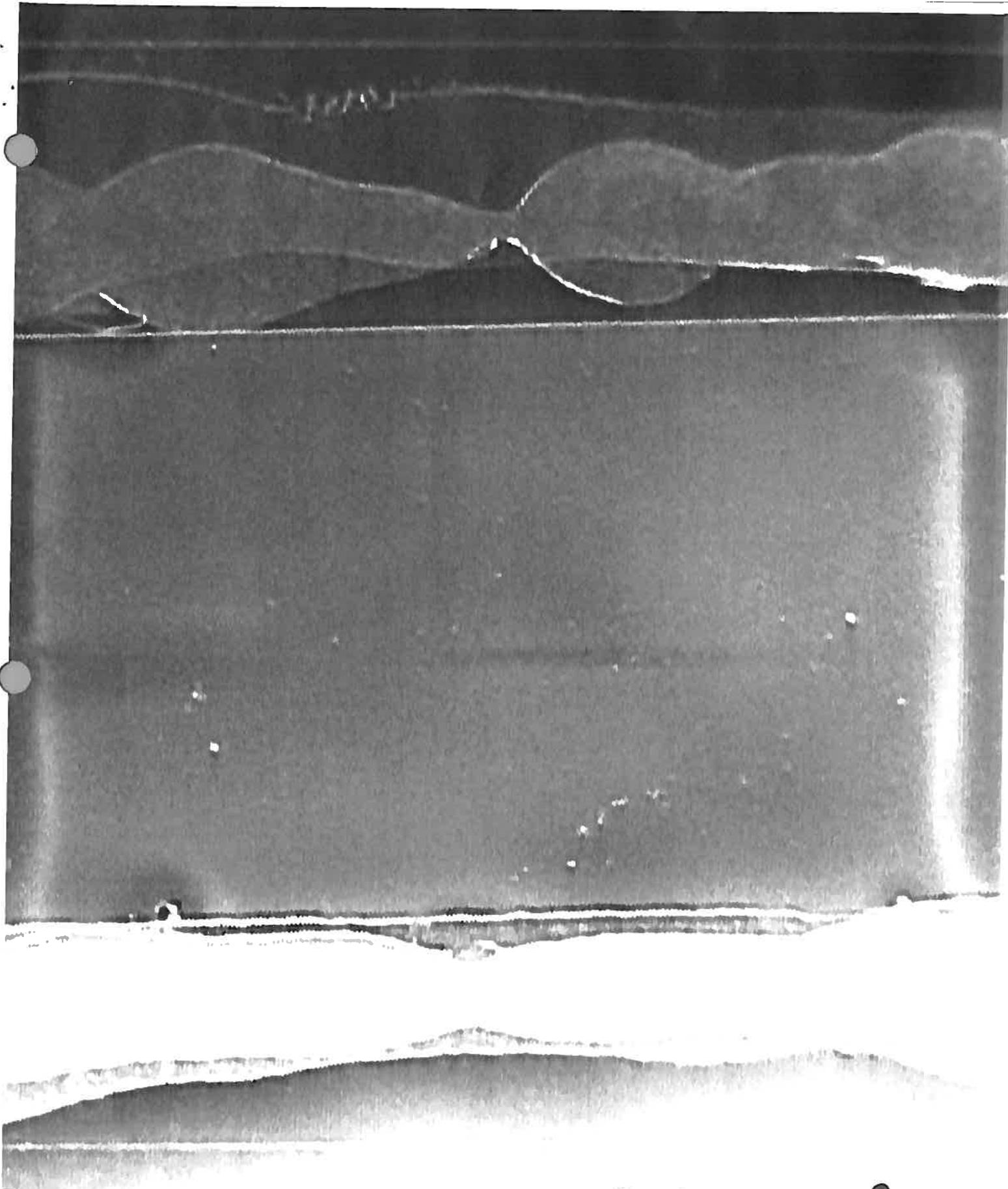
Figure 7. Liquid grown *R. sphaeroides* photoresponse

Fig. 8. Photograph of R. sphaeroides photoresponse.

Cable

0' 5' 10' 15' 25'
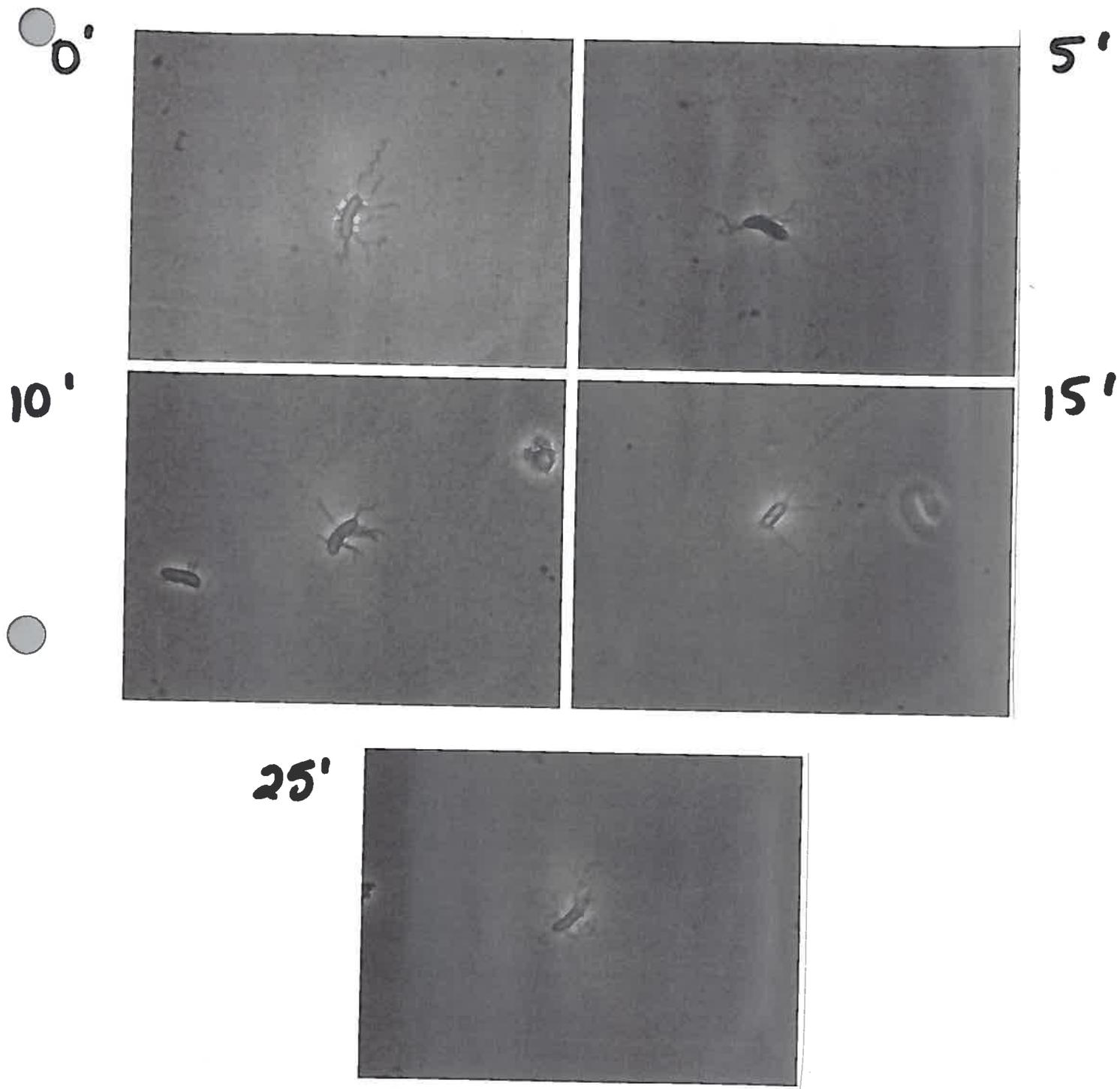
Figure 9. Flagella stain time course in sterile deionized water.
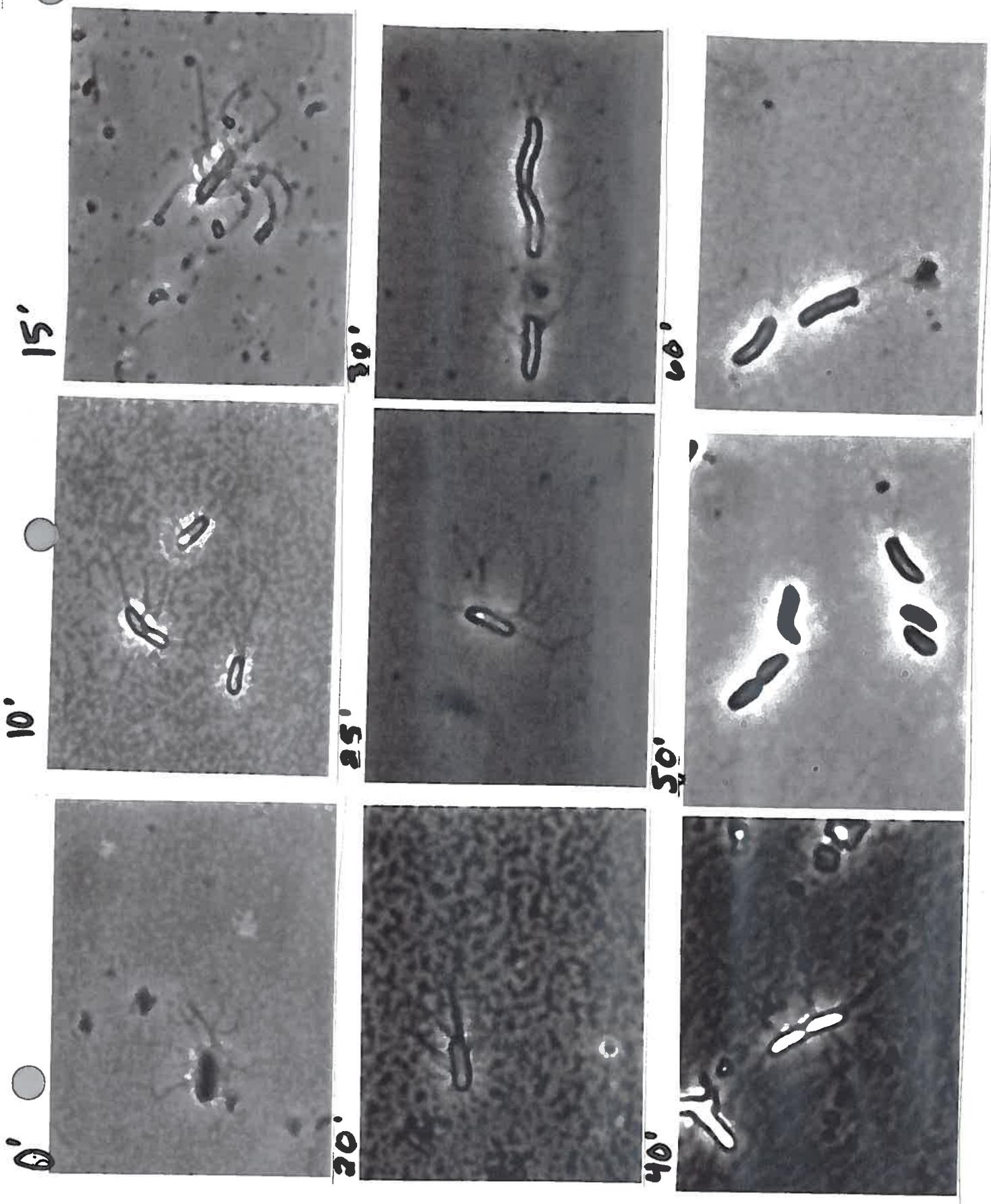
Figure 10. Flagella Stain time course of plate grown R. centenum resuspended in PYVS

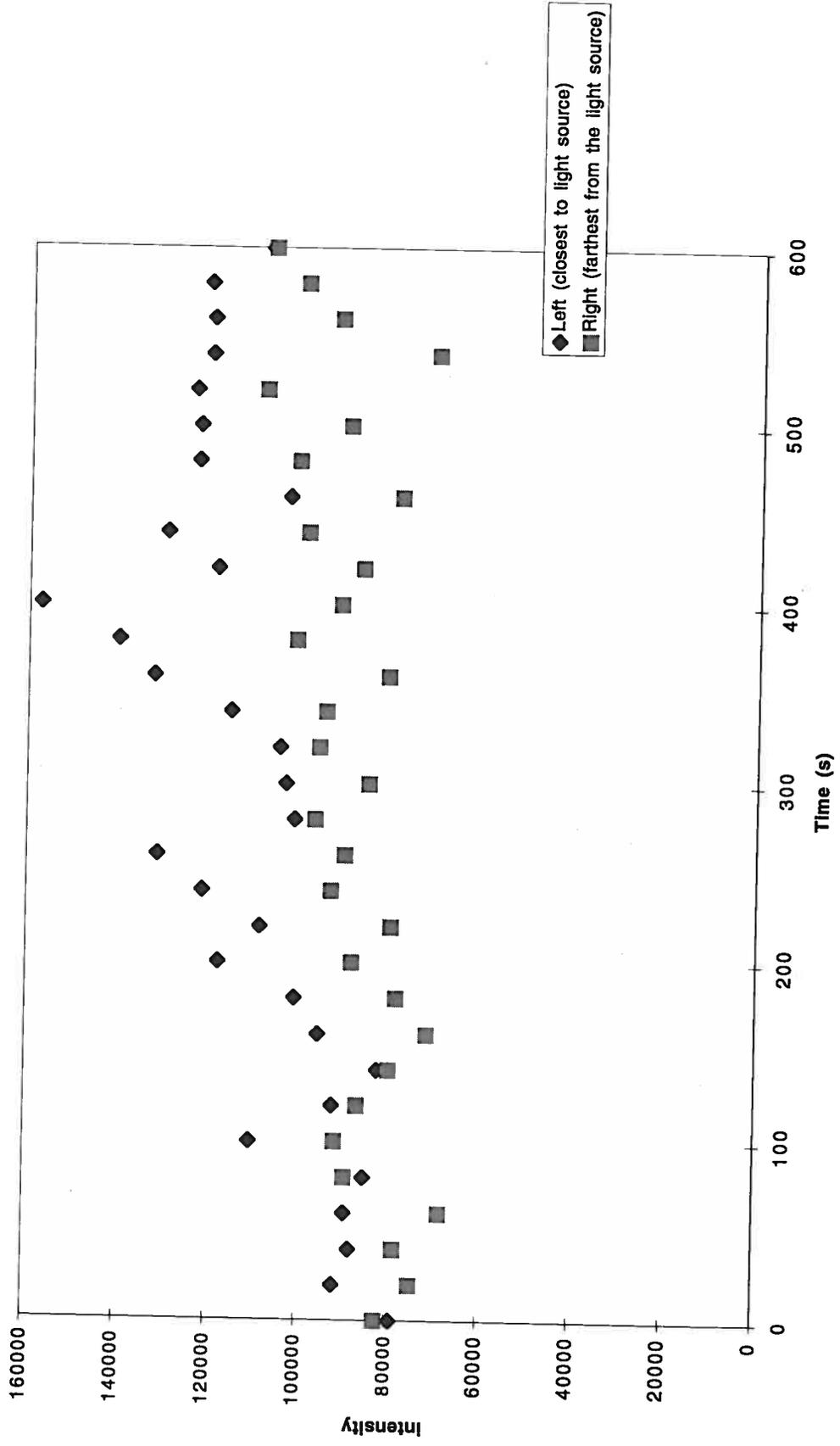**Figure 11.** Swarm plate grown *R. centenum* photoresponse
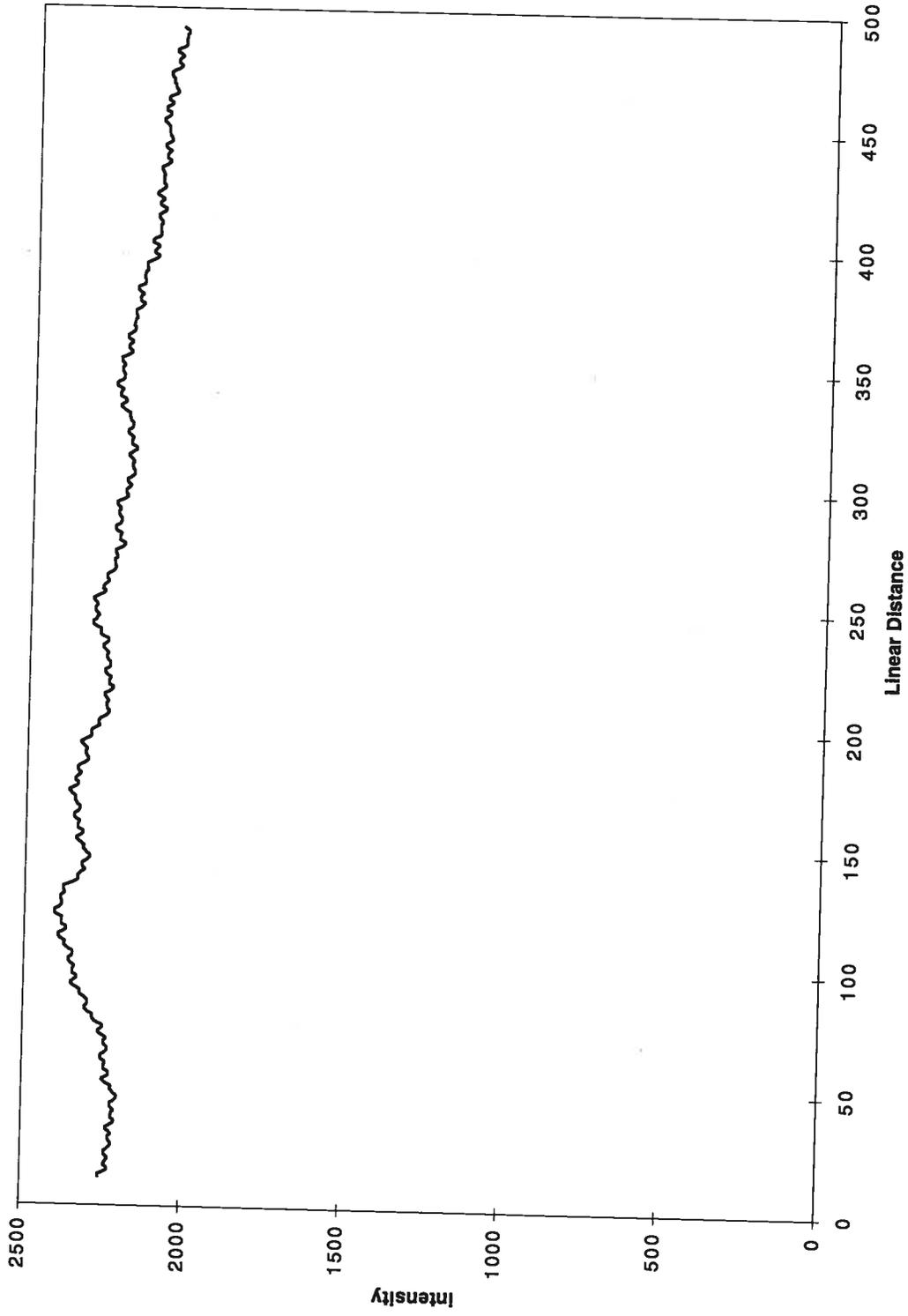
Figure 12. Average light intensity horizontally through the beam

Appendix I:
Rhodobacter sphaeroides Succinate Media

1M Phosphate buffer (see below)	20 mL
"Concentrated Base" (see below)	20 mL
$(NH_4)_2SO_4$	0.5 g
"Growth Factors" (see below)	2 mL
Sodium succinate	2 g
NaCl	0.5 g
Casamino acids	1.0 g

Adjust to pH 7.2 with KOH and bring volume up to 1 liter.

"Concentrated Base"
Nitrilotriacetic acid (diNa salt)	5.94 g
Metals 44 solution (see below)	25 mL
$MgSo_4 \cdot 7H_2O$	14.5 g
$CaCl_2 \cdot 6H_2O$	2.5 g
$FeSO_4 \cdot 7H_2O$	50 mg
Ammonium molybdate ($\cdot 4H_2O$)	4.6 mg

Adjust to pH 6.8 with KOH (20%). Note: NTA should be dissolved in 500 mL dH_2O and any undissolved residue removed prior to pH adjustment to 5.0 with 2 N HCl.

"Metals 44 Solution"
Tetrasodium versenate (EDTA)	2.5 g
$ZnSO_4 \cdot 7H_2O$	11 g
$FeSO_4 \cdot 7H_2O$	5 g
3 M H_2SO_4 (98%)	1.5 mL
$MnSO_4 \cdot 4H_2O$	2 g
$CuSO_4 \cdot 5H_2O$	0.39 g
H_3BO_3 (boric acid)	0.12 g
$COCl_2 \cdot 6H_2O$	0.2 g

Bring volume up to 1 liter with dH_2O and filter sterilize in small aliquots.

1 M Phosphate Buffer, pH 7.0
$K_2HPO_4 \cdot 3H_2O$	136.8 g in 600 mL dH_2O
KH_2PO_4	68 g in 500 mL dH_2O

Add 123 mL K_2HPO_4 to 77 mL KH_2PO_4 - should be pH 7.0 (adjust if necessary). Autoclave.

"Growth Factors"
Biotin	2 mg
$NaHCO_3$	50 mg
Niacin (nicotinic acid)	100 mg
Thiamine HCl	50 mg

Dissolve in dH_2O and make up to 100 mL. Filter sterilize and store at 4°C.

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