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## SINGLE-CELL BIOLUMINESCENCE AND GFP IN BIOFILM RESEARCH

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### Introduction

In recent years, it has become apparent that bioluminescence is one of several metabolic processes in bacteria that are controlled by population dynamics, and that the population-size trigger (quorum sensing) is widespread throughout the bacterial kingdom (1). Thus, quorum sensing is a "global" regulation mechanism demonstrated by many different genera and species, and bioluminescence is the quorum-sensing-regulated metabolic process about which we have the most information. In concert, rapid advances in understanding gene regulation have arisen from the development of bioreporter systems in which the synthesis of a foreign biomolecule, often an enzyme, is linked to the transcriptional activity of a particular "gene-of-interest". The enzyme is produced when the gene is activated, and the enzyme activity can be monitored in various ways that are less laborious than direct probing for the product of the gene-of-interest. Luciferase activity (bioluminescence) is frequently used as a reporting system in eukaryotic and in prokaryotic cells, although the eukaryotic reporter systems normally rely on luciferase of eukaryotic origin. Certain fluorescent proteins can also be used as bioreporters when their sequences, inserted into the operon of the gene-of-interest, are transcribed and translated along with the natural gene product. Bioluminescence and fluorescent proteins are therefore powerful tools for detection of gene expression in living cells in real time (e.g., 2).

Attachment of bacteria to substrata (formation of biofilms) is a trigger that, like quorum sensing, can induce changes in physiology and thus in gene regulation (3,4); the additional trigger of population size could become important in the developmental biology of biofilms as single cells develop into microcolonies. We are therefore interested in the development and application of reporters to bacteria in biofilms - from single cells to multiple layers of cells tens-of-microns in thickness. The important criteria in selection of these systems are spatial resolution (in x, y, and z) and temporal resolution (rapid response to induction and rapid response to cessation of gene activity). For reporter systems based on fluorescence, (e.g., GFP-linked reporters), spatial resolution criteria have been largely fulfilled through the development of confocal microscopy and of digital deconvolution microscopy. However, most fluorescence based systems have poor temporal response, at least when one considers downshifts in gene activity. Luciferase-based reporters have good temporal response but, to our knowledge, have not yet been applied in a three-dimensional manner.

There have been several reports that include the terminology "single bacterial cells" in connection with luciferase as a reporter. With few exceptions (e.g., 5), these papers deal with detection of light from colonies grown on plates (inferring that the colony arose from a single cell), with non-quantitative detection (unprocessed CCD data), or with detection of light not conclusively demonstrated to be colocalized with a single bacterial cell. The present contribution demonstrates the types of applications in which we have interest and shows how true single-cell bioluminescence and GFP bioreporters can be used in bacterial biofilm research.

### Materials and Methods

Bacterial Strains: *Vibrio fischeri* (ATCC 7744) and *Vibrio harveyi* (ATCC 14126) were maintained, respectively, on Seawater Complete and Marine agar media. Frozen

stock cultures are maintained as reserves should dark mutants arise. *Escherichia coli* DH5 $\alpha$  was maintained on LB agar in the presence of ampicillin.

**Establishment of Bacterial Biofilms in Flowcells:** For all microscopy work, bacterial cells were allowed to adhere and to grow attached in glass flowcells. The flowcells (6) are essentially perfusion chambers constructed of microscopy coverslips; the volume of each chamber is 200  $\mu$ L and the thickness (from top to bottom) is 1 mm. Normally, an inoculum is injected and allowed to adhere for 10-30 minutes prior to initiation of flow through the chamber. Medium flowrates are low (< 5 mL/hour) except when the chambers are cleared of unattached organisms.

**Instrumentation:** Photon-counting was performed using a Hamamatsu VIM-CCD camera mounted on a Zeiss Axioplan microscope. A 100 x Plan-Apo oil-immersion lens (NA 1.4) delivered light directly to the camera. Hamamatsu Argus 50 controller hardware (including center-of-gravity board) and software ran on a P90 IBM-compatible with 32 MB RAM and a 1.2 GB removable optical drive. Standard transmitted-light images were captured using the Argus software and a Dage MTI 70 camera.

## Results

**Photon-counting Demonstrates Variations in Bioluminescence Within and Between Strains:** Figure 1A shows a transmitted-light image, and Figure 1B a photon-counting image ("slice" image), of *Vibrio harveyi* cells soon after (20 minutes) attachment inside a flowcell. It is clear that not all cells are producing light and, among those that are, a large variation in light output exists. The arrows mark identical cells in each image; the marked cells are those producing high amounts of light.

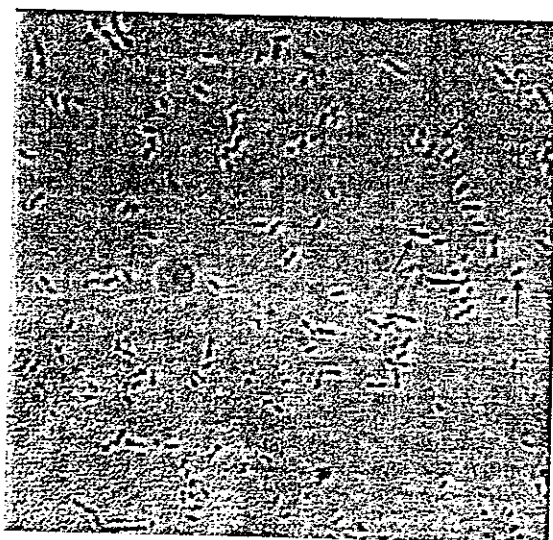
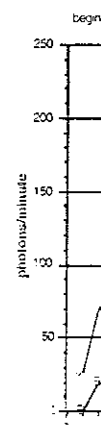


Figure 1A. Transmitted light image of *V. harveyi* cells attached in a glass flowcell.



Figure 2. Presence of the brightest proportion of *V. fischeri* (V. fischeri).



Expression of a plasmid that controls light emission in *V. fischeri* (Figure 3A). Expression of the plasmid in *V. fischeri* (Figure 3B).

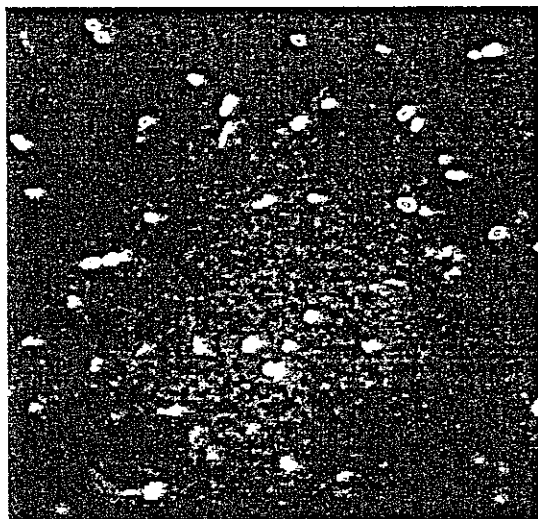


Figure 1B. Photon-counting ("slice") image of field shown in 1A. Arrows indicate identical cells. These cells are also the brightest cells in the photon-counting image (appear red in the original pseudocolored image created by the program).

Figure 2 shows that microcolonies and single cells of *V. fischeri* respond to the presence of autoinducer when attached in the flowcell. Absolute light levels from the brightest *V. fischeri* and *V. harveyi* cells were similar. However, a much lower proportion (roughly 20%) of *V. harveyi* cells emit high levels of light than in *V. fischeri* (roughly 60%; data not shown).

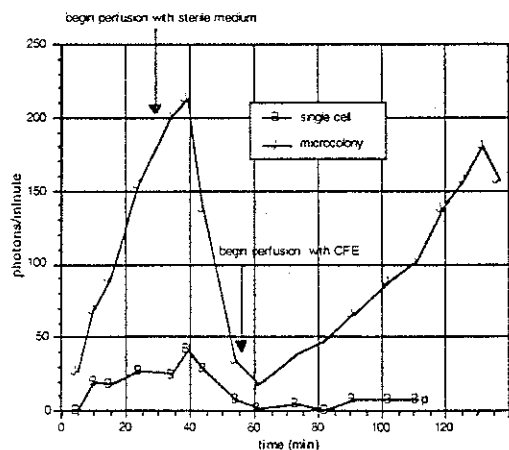


Figure 2. Timecourse of light emission from a microcolony and a single cell of *V. fischeri* grown in a flowcell. Composition of the perfusion medium was changed at times indicated by arrows. CFE = Cell Free Extract (filtered medium containing autoinducer).

Expression of lux and of GFP may be incompatible in the same cell: We have inserted a plasmid containing the lux cassette (under control of an Hg-detoxification promoter; light emitted in the presence of Hg) and a plasmid containing the GFP sequence (under control of the lacZ promoter; GFP formed in the presence of IPTG) into *E. coli* (7). Figure 3A shows a transmitted-light image of cells in the presence of both inducers. Figures 3B and 3C show, respectively, GFP-containing cells and light-emitting cells. Expression of one of the two reporters was seen in several cells. However, no cell expressed both reporters.

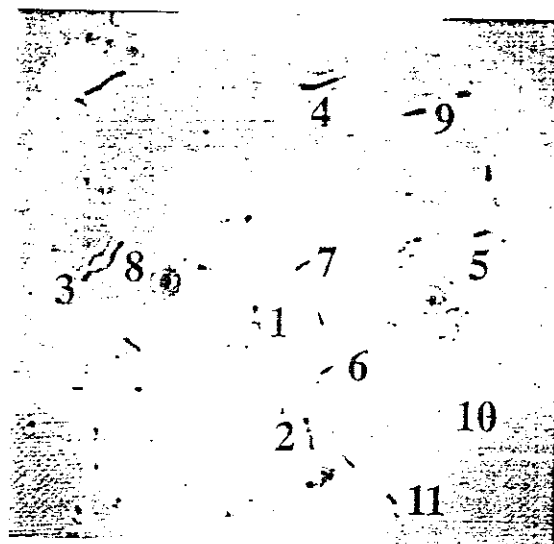


Figure 3A. Transmitted-light image of *E. coli* cells containing a GFP-bearing plasmid and a lux-bearing plasmid. Both bioreporters were induced.

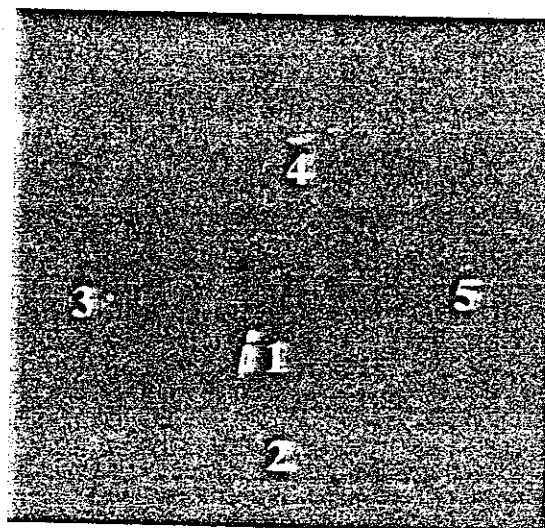


Figure 3B. Epifluorescence micrograph of field shown in 3A. Numbers indicate identical cells in both images. GFP fluorescence was detected using the photon-counting camera in slice mode.

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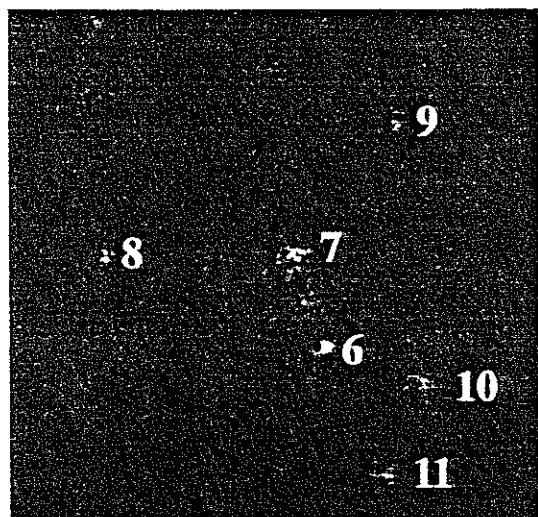


Figure 3C. Photon-counting image (slice mode) of field shown in 3A. Numbers indicate identical cells in both fields. Note that no GFP-containing cells (Fig. 3B) appear in the photon-counting image or vice versa.

### Discussion

Using flowcells and a combination of microscopy techniques, we can unequivocally identify single bacterial cells that express bioluminescent and fluorescent bioreporters, and we can quantitate the light produced by these cells. At the present time, our methodology is limited to detection in two dimensions (x and y). We are currently extending these techniques by incorporating the dimension of depth (z) and time (t) to create four-dimensional detection systems useful for study of gene expression in bacterial biofilms.

We have shown that, for attached cells, bioluminescence output within a bacterial strain can vary greatly from cell to cell. Similar non-quantitative data for *Photobacterium phosphoreum* has been presented (8), although those bacterial cells were grown in liquid culture then transferred to an agar-coated slide plate for imaging; we have seen that such transfer severely inhibits bioluminescence in *V. harveyi*. We suggest that a difference in bioluminescence from cell to cell also occurs in batch culture and that the wide range of output from cell to cell in *V. harveyi* is a simple explanation for the empirical observation that batch cultures of *V. harveyi* are much dimmer than those of *V. fischeri*. Furthermore, these data indicate that careful interpretation is required for bioluminescence data normalized to factors such as optical density or cell number.

To date, we have been unable to detect GFP and luciferase activity in single cells that theoretically should produce both; such double labeling would be desirable from the standpoint of having a positional marker as well as a gene-expression indicator in cells in a biofilm. The two processes (synthesis and post-translational processing of GFP; synthesis, post-translational processing, and supplemental requirements for light production by luciferase) may be incompatible in a single cell. Both processes require significant amounts of cellular energy to be directed toward synthesis of proteins not required for normal cellular metabolism. The concentration of GFP required for epifluorescent detection using standard cameras is quite high and may in some way preclude normal cellular metabolism. Use of extremely sensitive photon-counting methods will allow fluorescent protein bioreporters to be detected at concentrations lower than those currently thought to be necessary.

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