Detection of bioluminescence from individual bacterial cells: a comparison of two different low-light imaging systems

Claus Sternberg, Leo Eberl,† Lars Kongsbak Poulsen‡ and Søren Molin*
Department of Microbiology, Technical University of Denmark, DK-2800 Lyngby, Denmark

Detection of very low light levels arising from individual cells of the naturally bioluminescent bacterium Vibrio fischeri as well as from a luminescence-marked Pseudomonas putida strain was achieved by the aid of two different camera systems. Using a liquid nitrogen-cooled slow-scan CCD (charge-coupled device) camera we were able to detect single-cell bioluminescence within 1 min, and the pictures obtained were of good resolution. In contrast, employing a photon-counting video camera we were able to detect bioluminescent cells within 10 seconds, but at the expense of spatial resolution. This study demonstrates the feasibility of microscopic single cell analysis employing bioluminescence as reporter system. © 1997 John Wiley & Sons, Ltd.

INTRODUCTION

Bacterial bioluminescence is now widely used in microbiology as a reporter for measuring gene expression and as a highly sensitive marker for bacterial detection. One major attraction of bioluminescence-based systems is the ability to monitor light emission in a real-time and non-invasive manner (for reviews see (10, 16)). Such systems have been successfully employed for in situ analyses of bacteria in their natural habitats, since luminescent organisms are rare in terrestrial and freshwater environments. Bioluminescence-marked strains were used to follow phytopathogenic bacteria during the infection process (14), to monitor root nodule formation (9) and to detect bacteria in soil, on leaves and in root rhizospheres (3, 11, 13).

With the development of low light imaging systems the visualization of bioluminescence in two dimensions, and thus detection of the spatial distribution of luminescence-marked bacteria in natural samples, have become feasible, and the sensitivity of commercially available camera systems has now reached a level that allowed the detection of light emitted from a single bacterial cell (4, 5, 11, 15). In previous
reports, sufficient luciferase expression for single cell detection was only observed in connection either with the use of powerful promoters in combination with a high gene dosage, or when genetic amplification systems were used (17, 19). In addition, relatively long exposure times (more than 10 min) were needed for single-cell detection, independent of the camera type used—CCD (charge-coupled device) or photon-counting video cameras. While the sensitivity of these systems is sufficiently high for bacterial tagging, in many cases it may not be satisfactory for real-time gene expression studies. A new generation of cameras now provide a hitherto unprecedented sensitivity.

In this communication we report the detection of single-cell bioluminescence from both the naturally bioluminescent bacterium Vibrio fischeri MJ-1 and a luciferase-marked strain of Pseudomonas putida KT2442. The latter strain contains a single copy of the Vibrio harveyi luxAB genes inserted into the chromosome, such that its expression is under the control of a strong constitutive, yet unidentified, P. putida promoter. Bioluminescence was visualized using either a liquid nitrogen-cooled slow-scan CCD camera or a photon-counting video camera. We demonstrate that with the photon-counting device, light signals arising from individual bacterial cells can be detected unequivocally within less than 1 min.

EXPERIMENTAL PROCEDURES

Strains, plasmids and media

Pseudomonas putida KT2442 is a rifampicin-resistant, TOL plasmid-cured derivative of P. putida mt-2 (1). The brightly bioluminescent strain Vibrio fischeri MJ-1 (12) was kindly provided by Dr G. S. A. B. Stewart (University of Nottingham, UK). For mutagenesis the promoter probe transposon plasmid pCK218, containing the promoterless luxAB genes derived from Vibrio harveyi, was used (7). P. putida was grown at 30°C in AB minimal media (2), supplemented with 10 mM citrate. V. fischeri was

Figure 1. Detection of Vibrio fischeri MJ-1 using a cryogenically-cooled CCD camera. 100 × microscope objective and 2.5 X optovar magnification. A, Differential interference contrast (DIC) image. Bioluminescence: B, 1 min exposure time. Bar represents 3 μm

cultivated at 25°C in a 70% sea water nutrient medium (SWT) containing (per litre): 5 g tryptone, 3 g yeast extract and 3 ml glycerol. For detection of cells and micro-colonies, appropriately diluted inocula were placed on nutrient agar plates or directly onto microscope slides.

**Luminometry**

Light emission from strains with an integration of luxAB was dependent on addition of the long-chained aldehyde n-decanal. For liquid culture analysis this substrate (1/100 vol) was added directly to the bacterial suspension. When analysing cells on agar surfaces, a 5 μL drop of substrate was placed next to the bacterial cells. Quantification of light emission from pure cultures was measured using a TD-20e luminometer (Turner Designs, Sunnyvale, CA, USA). Specific light intensities were measured from exponentially growing cells.

**Microscopy**

For microscopic examination, an Axiovert 100 TV (Carl Zeiss) was used, with a photo tubus at the bottom of the microscope facilitating a minimum loss of light. The Axiovert was equipped for bright field and differential interference contrast microscopy (DIC) and an Optovar option for additional 2.5 x magnification, facilitating up to 250 x magnification when using the photo tubus. A 40 x/0.75 Plan-Neofluar (Carl Zeiss) was used for bright field examination of agar plates, and an oil-immersion 100 x/1.3 Plan-Neofluar (Carl Zeiss) for DIC examinations of liquid bacterial cultures.

Two cameras were used; a cryogenically cooled slow-scan charged coupled device (CCD) and a double-intensified camera. The CCD camera was CH260 (Photometrics) with a thinned back-illuminated TK512CB/AR chip. The chip was operated at -110 °C and read out at 40 kHz in 16-bit resolution which offered approximately 70% quantum efficiency at 490 nm. Pixel size was 27 x 27 μm. The camera was controlled by the PMIS v2.11 software (Photometrics) running on a DOS 486 computer. The double-intensified camera was a Hamamatsu C2400-47 equipped with a Controller II control unit and an ARGUS-50 image processor. The camera was in part controlled by a DOS 486 computer and the images were saved in 16-bit format using the ARGUS-50 software (Hamamatsu). When set for photon counting, the Controller II automatic amplification circuit was employed for maximum sensitivity. For presentation, images from either camera were printed using a video printer or transferred as scaled Tiff images into Adobe Photosho (Adobe, Mountain View, CA, USA) software.

**Figure 2.** Detection of Vibrio fisheri MJ-1 using a photon-counting camera. A, DIC image.Bioluminescence; B, 30 s exposure time. Bar represents 5 μm
All microscopic operations were done in complete darkness and at room temperature (22–24°C).

RESULTS AND DISCUSSION

Selection of strains

V. fisheri is naturally bioluminescent and does not require the addition of external substrate but is dependent on high cell density for light induction (8). To obtain a bioluminescence-marked derivative of P. putida KT2442, the strain was mutagenized using three-factor conjugation, as previously described (7). A promoterless luxAB cassette was thereby randomly integrated into the bacterial chromosome. Colonies of random insertion mutants (approximately 5000) grown on selective plates were screened for strong light emission after n-decanal was provided in the lid of the Petri dish. One particularly strong mutant, P1, was selected. The light signal from this mutant was

Figure 3. Detection of Pseudomonas putida P1 using a cryogenically-cooled CCD camera. 40 × microscope objective. A, DIC image. Exposure times for bioluminescence; B, 3 min; C, 10 min; D, 30 min. Bar represents 2 μm.
independent of growth. By luminometry, \textit{V. fisheri} was shown to have a specific light intensity approximately 5–10 times stronger than \textit{P. putida} P1 at OD\textsubscript{450} 0.6 (data not shown).

\section*{Detection of single bacterial cells by microscopy}

When grown on nutrient broth plates, colonies of \textit{V. fisheri} cells are easily detected using the naked eye or X-ray film. However, for microscopy of individual cells the light is too faint to be detected by conventional equipment. We equipped a microscope with two different digital cameras, which operate with either a liquid nitrogen-cooled CCD chip (C-CCD) or by a two-stage photon-counting photo-multiplier tube connected to a standard CCD camera (PC-CCD). \textit{V. fisheri} MJ-1 is naturally bioluminescent. Light is induced via a cell-density sensing cascade reaction when a culture of \textit{V. fisheri} reaches an OD\textsubscript{450} of 0.1 in SWT media without the addition of an external enzyme substrate. This allowed us to cover a 5 \textmu L sample of an induced culture applied on a standard microscopy slide with a cover slip. We examined the cells using a 100 X oil immersion objective and a 2.5 X Optovar zoom to give an effective magnification of 250 times when captured through the phototubes. Consequently, the light efficiency of this setup is very low and therefore the need for the utmost sensitivity is even more important. \textit{V. fisheri} single cells were detected using both cameras (Figs. 1 and 2). The C-CCD was superior, due to the better resolution of the low signal from the cells despite a longer exposure time compared to the PC-CCD (Fig. 1). Using the PC-CCD we obtained almost equally good images (Fig. 2), but the very long exposure times required to allow the apparent resolution to the level of the C-CCD (see the following section) was not possible, since the oxygen limitation imposed by the cover slip made bioluminescence fade rather rapidly.

To induce light production from \textit{P. putida} P1, the addition of \textit{n}-decanal renders examination of cells under cover-slips unfeasible and an alternative approach was used. To inoculate the cells we diluted appropriately and spotted droplets onto dried nutrient-broth plates. Immediately after the drops had dried the cells were examined under the microscope, using a

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\caption{Detection of \textit{Pseudomonas putida} P1 using a photon-counting camera. 40 \times microscope objective. A, DIC image. Exposure times for bioluminescence; B, 10 s; C, 1 min; D, 2 min; E, 4 min; F, 10 min. Bar represents 10 \mu m}
\end{figure}
40 X air-interface microscopy lens. Using the C-CCD a relatively clear image was obtained after 3 min exposure (Fig. 3(B)). Longer exposure times resulted in a roughly proportional increase in signal intensity without improving picture resolution (Fig. 3(C, D)). The C-CCD accumulates all incoming photons in the chip while exposing and the entire chip is read out after exposure is completed. Cells could be visualized only after applying non-linear image-enhancement in the computer after image capture. With exposure times of less than 3 min signals could not be differentiated from the background noise.

Using the PC-CCD camera unequivocal single-cell signals were detectable after 10 s of exposure (Fig. 4(B)). However, the resolution was rather poor. In the PC-CCD system, data is read out from the camera in real time and accumulation is performed in the computer. Due to the algorithm used in photon-counting imaging, the images apparently become more and more detailed after longer exposure as the noise is gradually averaged over the entire image and consequently becomes less and less apparent. After less than 10 min the cell shapes could be clearly defined (Fig. 4).

In a previous study by Wick et al. (18), it was demonstrated when comparing a C-CCD with a PC-CCD camera that with very low light levels the signal/noise ratio was higher with the C-CCD compared with the PC-CCD, while with higher light levels the signal/noise ratio was found to be better with the C-CCD (18). Due to the higher resolution image quality is better with the C-CCD. However, when exposure times are short this system fails to detect very low light signals, which can be imaged using a photon-counting device (6,18).

Using a low-light imaging camera of either type, visualization of bioluminescent single bacterial cells is now possible. The choice of camera must depend on the application. While the photon-counting camera is superior with regard to sensitivity, the cryogenically-cooled CCD camera provides more detailed images. In biological scenarios involving living cells, however, exposure times of several minutes are often undesirable or unfeasible, which makes the PC-CCD a better choice. With more static systems allowing longer exposure times, the C-CCD might be preferable due to the higher resolution of this system.

Acknowledgements

Cayo Ramos is thanked for construction of P. putida P1. The authors wish to thank Paul Cohen for critical reading of the manuscript. C.S. and S.M. were supported by the EEC BIOTEC Contract No. BIO2-CT92-0084. L.E., L.K.P and S.M. were supported by the Danish Center for Microbial Ecology.

REFERENCES


J. BIOLUMIN. CHEMILUMIN. 1997; 12: 7–13

