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carbohydrate sequences should be isolated. These highly specific lectins would be ideal as molecular probes for the assessment of biofilm sorption properties as well as for the structural examination of microbial biofilm communities.

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[11] Spatially Resolved, Quantitative Determination of Luciferase Activity by Photon-Counting Microscopy

By ROBERT J. PALMER, JR. and DAVID C. WHITE

Luciferase activity offers some distinct advantages as a bioreporter. It can be selected for using relatively simple equipment (bioluminescent bacterial colonies can be seen in a dark room, whereas early experiments used photographic film as a detection/recording method), and signal (light output) ceases after the translation of target gene/luciferase gene fusion stops[1] (in contrast to fluorescent molecules such as unmodified Green Fluorescent Protein, which remains fluorescent for extended periods). One major advantage for biofilm researchers is that with the type of equipment described in this article, the origin of luciferase activity can be spatially resolved and quantitated in two dimensions, thereby allowing quantitative discrimination at the level of the single bacterial cell.[2] Several quantitative detection techniques are used to determine luciferase-mediated light production (scintillation counters, luminometers, photomultipliers) but none of these offers the spatial resolution provided by photon-counting camera setups. Hamamatsu Photonics (Bridgewater, NJ) and Science Wares (East Falmouth, MA) market turnkey photon-counting camera/microscope systems, and at least one laboratory has developed a cruder, yet effective, system "in house."[3] Such systems are designed to operate at photon fluxes

[1] R. J. Palmer, Jr., B. Applegate, R. Burlage, G. Sayler, and D. C. White, *in* "Bioluminescence and Chemiluminescence: Perspectives for the 21st Century" (A. Roda, M. Pazzagli, L. J. Kricka, and P. E. Stanley, eds.), p. 609. Wiley, Chichester, 1999.

[2] R. J. Palmer, Jr., C. Phiefer, R. Burlage, G. S. Sayler, and D. C. White, *in* "Bioluminescence and Chemiluminescence: Molecular Reporting with Photons" (J. W. Hastings, L. J. Kricka, and P. E. Stanley, eds.), p. 445. Wiley, Chichester, 1997.

[3] J. Elhai and C. P. Wolk, *EMBO J.* 9, 3379 (1990).

0076-6879/99 $30.00

of $<10^{-5}$ lux and are at least 1 order of magnitude more sensitive than "intensified" or cooled charged-coupled devices (CCD), ISIT cameras, etc. Although much of the methodology presented in this article is hardware specific, the principles are those which must be applied to any quantitative, spatially resolved method.

Hardware

The Hamamatsu camera consists of a CCD camera attached to an image intensifier (Fig. 1). The image intensifier contains a photocathode, anodes, microchannel plate, and a phosphor plate, all located within an evacuated

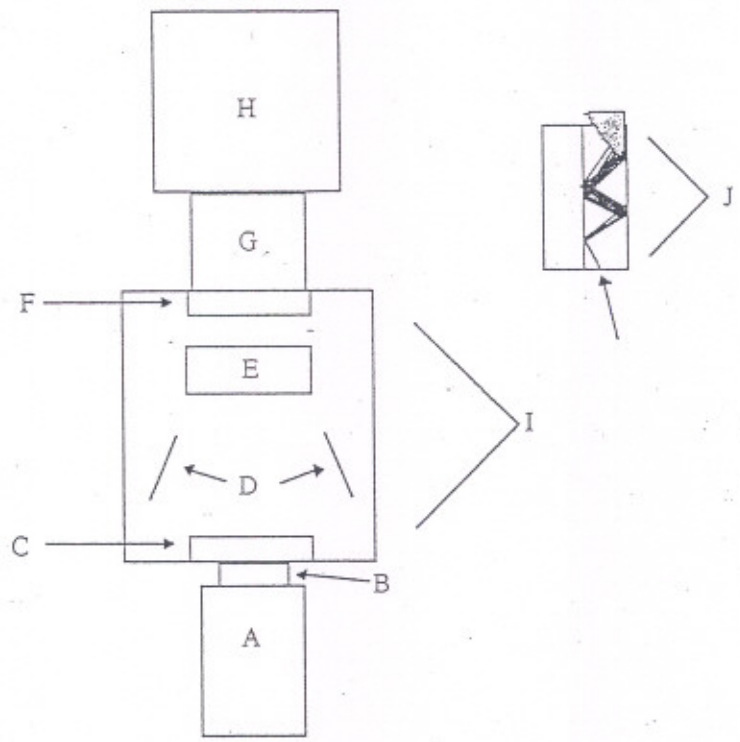

FIG. 1. Diagram of a turnkey photon-counting device (Hamamatsu Photonics). (A) Microscope phototube or adapter to C-mount, (B) C-mount, (C) photocathode, (D) anodes, (E) microchannel plate, (F) phosphor plate, (G) relay lens, (H) CCD camera, and (I) evacuated chamber. (J) Representation of photon multiplication within the microchannel plate. Two microchannels are shown. The photon enters one channel (arrow) and strikes the walls of the chamber, thereby generating additional electrons that exit the top of the channel in a burst (gray).

chamber. Photons strike the photocathode and are converted to photoelectrons and accelerated past the anodes into the microchannel plate. Within the microchannel plate, the photoelectrons strike the walls of the channel, resulting in the generation of additional electrons up to a multiplication of approximately 10^6 for each photoelectron that initially enters (see inset J in Fig. 1). A burst of electrons thus exits the microchannel plate, strikes the phosphor plate, and is converted back to photons that then strike the CCD camera. Data are collected as a 16-bit image that can be pseudocolored (blue, lowest intensity; green, yellow, red, white, highest intensity) and displayed on a video monitor. The voltage applied across the microchannel plate is controlled in two places: a button is used to turn the high voltage on and off and the voltage is increased/decreased using the sensitivity knob. Both of these controls are located on an interface box between the camera and a computer used for data storage. The camera/intensifier unit is connected to a microscope via a C-mount. For use as a macro camera (e.g., to record bioluminescence of bacterial colonies on plates), a photographic lens can be attached to the C-mount and the camera/intensifier/lens unit held by a tripod or other support.

Software

The burst of photons that illuminate the CCD exposes more than one pixel. In order to obtain higher spatial resolution, a software/hardware combination called the "center of gravity" board remaps the likely origin of the original photon to one pixel based on the size and shape of the multipixel illuminated spot. This feature is required for quantitative work. In addition, several algorithms and macros are included that allow the determination of the number of exposed pixels within user-defined areas, automatic collection of time-resolved data, and graphs of pixel intensity across user-defined lines within an image. The software package includes rudimentary image processing hardware and software (Argus 50) that are used to operate many different types of cameras. This software can be used to acquire transmitted light images with the camera (see later).

Dark Box

For true photon-counting applications, the camera must be isolated from background light. Clearly, a windowless room with a well-sealed door is a prerequisite. Even with this precaution, a dark box that encloses the microscope or the entire microscope/camera assembly is ordinarily required. When designing a dark box, careful consideration should be given to the types of experiments to be conducted. For example, flow-through

(perfusion) chambers are routinely employed in this laboratory (see Chapter 12 of this volume); therefore, ports for tubing are required. If ambient room light is kept to a minimum (e.g., computer monitors only), it is usually sufficient to have these ports constructed out of 5 to 8 cm of black-painted 1-cm-diameter polyvinyl chloride pipe. Clearance for the operator's hands (for focusing the microscope) and any external light sources or cameras (such as might be required for correlative work) must be incorporated into the design, otherwise frequent (and cumbersome) removal of the box will be necessary. In this laboratory, because upright and inverted microscopes are used in conjunction with the photon-counting system, two different dark box designs were required. In both designs, consideration was given to mounting a laser confocal scan head to allow sequential photon-counting/confocal microscopy on the same field of view (correlative microscopy, see later). The front of the boxes (the front of the microscopes) is open; during photon counting, the opening is covered with photographer's dark cloth that seals to the edges of the box with Velcro strips. All microscopy light sources (transmitted, UV) must be turned off completely (not just turned down) prior to photon counting. Many modern microscopes, particularly those with electronic focus or lens switching, have lighted information panels built into the body of the microscope that are operational whenever the microscope is turned on. These panels are a light source within the dark box; mask them with a piece of aluminum foil.

Example: Photon Counting of Attached *Vibrio fischeri* Cells

As a preface, it should be emphasized that the high voltage on the photon-counting camera should be turned off (or, in dimly lit rooms, the sensitivity dial should be set at its lowest setting) before sending light to the camera. This avoids the sudden delivery of a high photon flux to the microchannel plate. The voltage should always be increased slowly while watching the monitor to assure that the system does not saturate. If saturation is reached, the system shuts off automatically, but these accidents can reduce the effective life span of the instrument.

Test for Light Leaks

Before an experiment is begun, it is useful to check for light leaks in the system, particularly if the dark box is removed frequently. All microscope light sources are switched off, the dark box is sealed, and the photon-counting camera is turned on. The voltage is turned up slowly until the display shows saturation (indicative of a large light leak) or maximum voltage is reached (photon-counting mode). If photon-counting voltage is

reached, a count is made over 1 min accumulation time. Our system has very low counts when no light leaks are present (80–100 counts per field per minute) and we attempt to control leakage if counts of >200 per field per minute are reached.

Background Light Levels

Background light levels should be established under exactly the same conditions as those for the experiment. Changes in the optics (numerical aperature of lens, magnification, oil immersion vs dry lens) will affect the background; the procedure just described for light leak detection should be repeated with a "blank" (nonluminescent sample preparation similar to that to be examined in the experiment) prior to recording photon-counting data. The blank sample should be brought into focus using the transmitted light illumination, the light turned off, and the photon-counting procedure initiated. Once the investigator becomes sufficiently experienced with the system, the leak detection step will be replaced by the background determination step. Our background levels are very close (100–120 counts per field per minute) to the levels recorded during the procedure described earlier.

Collection of Data

It is almost always necessary to collect two types of data. First, a transmitted light image should be acquired. This image is useful for the correlation of light output (the .IMA image) with cellular location. We usually acquire these data using the photon-counting camera as the detector in the following manner.

a. bring the specimen into focus in the oculars using transmitted light, turn off the light, switch the light path from oculars to camera, check that the photon-counting sensitivity (voltage) dial is set to the lowest (counter-clockwise) position, and depress (turn on) the high-voltage button.

b. within the MONITOR window, set DISPLAY (image source) to RAW (= live) and uncheck the "color" box to produce a pseudocolored image.

c. turn on the transmitted light illumination at its lowest voltage

d. the transmitted light image may be visible at this point or the transmitted light voltage may have to be increased to make the image visible. If no color is visible after reaching the maximum voltage of the transmitted light source, slowly turn up the camera sensitivity (voltage) dial. Readout on the voltage is from 1 to 10. If no image is visible on reaching 3, it is likely that problems with the light path (i.e., no light is reaching the camera)

or camera electronics exist. It is not advisable to turn the voltage higher. Once a color image is visible, the pseudocolor should be turned off (uncheck the "color" box to obtain a gray scale image). Some refocusing may be required to compensate for differences in light paths between the binocular (view) port and the camera port of the microscope; this can be accomplished without saturating the camera if the room is dark and the sensitivity knob is set relatively low. The focused image can be acquired using the Argus software commands "freeze" or "integrate" located under the IMAGING menu. "Freeze" stores a single video frame in the memory, whereas "integrate" stores the sum of several (number is user defined) frames. The latter function is generally preferable because lower transmitted light and sensitivity levels can be used. Once the transmitted light image is acquired to memory, it must then be saved to disk using the Argus software "save as TIFF" command under the FILE menu. Figure 2a shows a transmitted light image of bacterial cells obtained in this manner.

After the transmitted light image is obtained and saved, the photon-counting image is acquired in the following manner.

a. Turn off the transmitted light source.

b. Reset DISPLAY to RAW (display defaults to PROCESSED during acquisition of the transmitted light image) and check (select) the "color" box to produce the pseudocolor image.

c. Slowly turn up the sensitivity knob until the maximum (10) is reached. If the system saturates prior to reaching this setting, the photon flux is too high to count with this setup (it is possible to count with the voltage set below 10; however, these voltage levels are difficult to reproduce accurately and the quantitative data are comparable only with those obtained at exactly the same voltage setting). At this point, centers of high light production should be visible. If no light is being produced, only scattered photon events (noise) will be seen. If no heterogeneity of light production exists, then high (relative to background) but noisy activity will be seen. If very low levels of light are being produced, it may be necessary to do an actual count (light accumulation) to see the light production. To count, select the command "photon counting" under the IMAGING menu. A dialog box appears in which the accumulation time for the image file (.IMA) can be defined (frames, seconds, minutes, hours). In our experience, accumulation times of 30 sec to 20 min are sufficient for bacterial luciferase bioluminescence using 100× oil-immersion optics. "Mode" should be set as "slice/gravity"; this results in storage in memory of both the "slice" image (the image prior to center of gravity calculation) and the "gravity" image (after center of gravity calculation). The slice image is frequently more useful as a visual data presentation than is the gravity image; however, only the gravity image is useful for quantitative purposes. After the acquisition

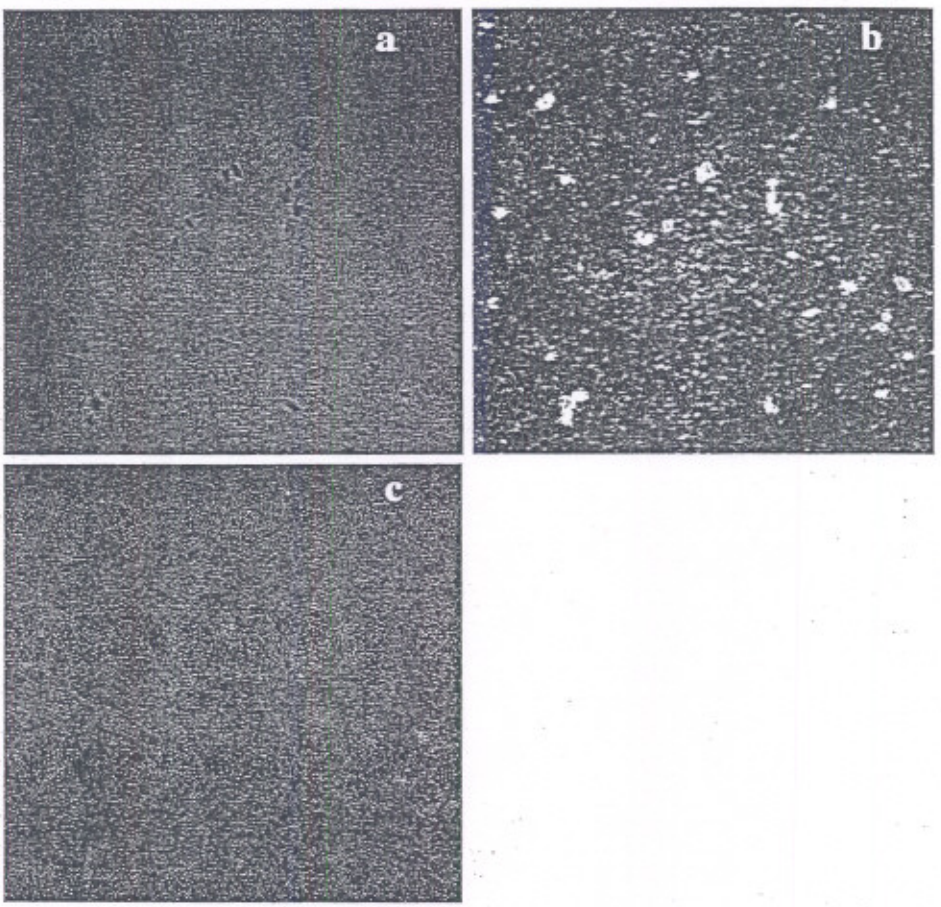

FIG. 2. Images acquired by a photon-counting camera. (a) Transmitted light image of *Vibrio fischeri* cells grown in a microscopy flowcell (perfusion chamber; see Palmer and White, this volume). (b) "Slice" mode image of same field shown in (a). (c) "Gravity" mode image of field shown in (a). Accumulation time for (b) and (c) was 5 min.

parameters have been defined, "start" is selected and the accumulation begins. The process occurs in real time on the video monitor; the remaining accumulation time is displayed on a counter within the dialog box, and, after the user-defined end point is reached, the resulting image(s) is stored in memory. The images must now be saved (as .IMA file) to disk using the "save image" command from the FILE menu. If desired, the acquisition process can be continued (effectively extending the accumulation period) by selecting "continue"; the RAM images are modified and must be saved

as noted earlier. It should also be noted that any images currently in memory are overwritten when a new acquisition process is started. Some warnings appear to inform users of this potential problem; however, images can be overwritten easily. An .IMA file can be reloaded into memory and the "slice" image or the "gravity" image can be saved to disk as a TIFF image for export. Figures 2b and 2c show, respectively, "slice" and "gravity" images of the field in Fig. 2a.

Quantitation and Normalization

Quantitative measurements can be done in many ways; the method employed routinely in this laboratory is to use the "area analysis" command from the ANALYSIS menu. This function permits the user to define a box or circle around the region of light production and query the computer as to how many photons were accumulated within that region. The box is then moved to a region that contains no cells (correlate with transmitted light image) and the computer is queried as to the number of counts within this region (the background noise). It is important to perform the background count on every image as it will vary (generally parallels increase or decrease in overall light production). Background counts are subtracted from the microbially produced counts to give the final result. To obtain numbers comparable from different sized colonies, the final counts can be normalized to colony area. This procedure cannot be performed with the Argus software; a third-party image analysis package is required. The TIFF format-transmitted light image is exported to the image analysis software, spatially calibrated, thresholded to create a binary image encoding the colony only, and the area determined by particle analysis procedures. Difference imagery can be used to determine the area of separate colonies within the same image. A review by Caldwell *et al.*[4] provides examples of these image analysis methods as applied to biofilms, and a book by Russ[5] provides an in-depth theoretical treatment.

Correlative Microscopic Techniques

It is often useful to acquire additional data on colonies from which light production data have been obtained. For example, multiple centers of light production can occur within a single colony. In this case it is useful to know if these centers represent areas in which the thickness of the colony, or the relative activity of cells, within those regions differs from that outside the

[4] D. E. Caldwell, D. R. Korber, and J. R. Lawrence, *Adv. Microb. Ecol.* **12,** 1 (1992)
[5] J. C. Russ, "The Image Processing Handbook." CRC Press, Boca Raton, FL, 1995.

regions. This laboratory has employed confocal microscopy together with photon-counting microscopy to assess just these issues in a study on a *Pludomonas putida* strain with a *tod/lux* fusion (grows on toluene as the sole carbon source and produces light in response to *tod* promoter activity.).[1]

Absolute Photon Flux Values

A drawback of this methodology is that the numbers are relative. Data are not absolute photon flux values because the loss of photons occurs from light scattering, from optical transfer, and from conversions within the intensifier. However, if a calibration method based on objects approximately the size of bacterial cells were to be developed, then an estimate of the efficiency of the camera/microscope combination could be arrived at and used to calculate absolute photon flux values.