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Measurement of Bacterial Bioluminescence Intensity and Spectrum: Current Physical Techniques and Principles

Kun Jia and Rodica Elena Ionescu

Abstract Bioluminescence is light production by living organisms, which can be observed in numerous marine creatures and some terrestrial invertebrates. More specifically, bacterial bioluminescence is the "cold light" produced and emitted by bacterial cells, including both wild-type luminescent and genetically engineered bacteria. Because of the lively interplay of synthetic biology, microbiology, toxicology, and biophysics, different configurations of whole-cell biosensors based on bacterial bioluminescence have been designed and are widely used in different fields, such as ecotoxicology, food toxicity, and environmental pollution. This chapter first discusses the background of the bioluminescence phenomenon in terms of optical spectrum. Platforms for bacterial bioluminescence detection using various techniques are then introduced, such as a photomultiplier tube, charge-coupled device (CCD) camera, micro-electro-mechanical systems (MEMS), and complementary metal-oxide-semiconductor (CMOS) based integrated circuit. Furthermore, some typical biochemical methods to optimize the analytical performances of bacterial bioluminescent biosensors/assays are reviewed, followed by a presentation of author's recent work concerning the improved sensitivity of a bioluminescent assay for pesticides. Finally, bacterial bioluminescence as implemented in eukaryotic cells, bioluminescent imaging, and cancer cell therapies is discussed.

Keywords Bioluminescence · Genetically engineered bacteria · Luminometer · Pesticide toxicity · Whole-cell biosensor

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1 Introduction

Bioluminescent bacterial sensor-reporters, which use genetically engineered microorganisms to produce a light signal proportional to toxicant content, have offered an interesting simple alternative for monitoring the environmental pollution since the 1990s [1]. Because of the diversity of sensing parts (promoter gene) [2] and reporting elements (fluorescent protein or luciferase) [3], as well as the intrinsic properties of living cells, whole-cell bioassays using engineered bacterial cells are able to detect the effects of bioavailable parts of toxicants and also quantify the total toxicity of several toxicant mixtures to living cells. The possible evaluation of bioavailability via these bacterial bioreporters is therefore regarded as the most prominent advantage over other traditional biosensing techniques using biomolecules (e.g. antibodies, enzymes, nucleotides) as sensing elements [4]. Moreover, bacterial bioluminescence, enabled by the *lux* genes, is one special kind of chemiluminescence that occurs in living microbial cells without the requirement of external optical excitation; thus, the absence of background luminescent signals from host cells indicates that bacteria bioluminescent detection efficiency is solely determined by the optical detector. Consequently, a bioluminescent assay can achieve extremely high sensitivity due to changes in the self-illumination of bioluminescence in the presence of any physical or chemical stress. Therefore,

genetically modified microorganisms have been widely used in the screening of environmental toxicants, such as organic pollutants [5, 6], heavy metals [7–9], pesticides [10–13], and antibiotics [14, 15].

Often, recombinant bacterial cells have been constructed by transforming the plasmids fused with promoter probes (such as *recA* or *grpE*) and reporter genes (*luxCDABE*, *luxAB* or *luc*) into host cells (e.g. *Escherichia coli*). The promoter probe is responsible for the specific recognition of the analyte, while the expression of the reporter gene leads to the production of bioluminescence via the synthesis of luciferase and its corresponding substrate [16]. Depending on the toxicants to be detected, a large number of engineered bacteria strains harboring different promoter genes have been constructed, as summarized in review articles [17–21]. The most frequently used bioreporter *lux* genes responsible for encoding the luciferase enzyme are the *luxCDABE* gene [22–24]. For example, *E. coli* TV1061 (*grpE*::*luxCDABE*) is sensitive to cellular metabolism changes, which is attributed to promoter *grpE*, one of about 20 promoters that is activated under specific conditions when the cells experience protein damage (e.g. upon addition of ethanol or phenol). Following this activation, the reporter gene is transcribed into the necessary components that are responsible for light production [25]. Another frequently used bacterial strain is based on the fusion of a DNA-damage inducible promoter *recA* and reporter gene (*luxCDABE*) and is responsive in the presence of genotoxical materials [26].

The first part of this chapter presents some background information on bioluminescence in terms of the optical spectrum, followed by the introduction of various physical platforms where the bacterial bioluminescent signal is detected and analyzed. Moreover, several protocols to improve the analytical performance of bacterial bioluminescent bioassays or biosensors is discussed based on the literature.

The temperature for monitoring bacterial bioluminescence evolution is usually 25 °C. Based on this limitation, the authors investigated different temperatures and reported their results through two independent protocols that drastically improved the sensitivity of the bacterial bioluminescent biodetection of pesticides. Finally, some perspectives and emerging applications of bacterial bioluminescence beyond biochemical compound detection and toxicity are briefly discussed.

2 Bioluminescence Background

2.1 Intensity and Spectrum of Bioluminescence

Bioluminescence occurs due to the presence of a specific enzyme named luciferase. Biochemically, all known luciferases are oxygenases that oxidize their corresponding substrates (generically named luciferin, and literally meaning “light-bearing”

molecules) in the presence of oxygen. The spectra properties of this bioluminescent emission are dependent on various parameters, such as luciferase/luciferin structures, organism habitat, optical biological filters, and accessory lumipores [e.g. green fluorescent protein (GFP) and yellow fluorescent protein (YFP)]. Taking GFP, in the bioluminescent marine hydroid *Obelia* as an example, it has been reported that light emissions from the living organism are green, whereas blue light is emitted during in vitro reaction. Such results indicate that the different emitted colors can be attributed to the different enzyme conformations under in vivo and in vitro conditions [27]. The color of the bioluminescent signal is strongly dependent on an organism's habitat. Thus, deep-sea species have blue emissions (450–490 nm), the bioluminescence of coastal marine species is green (490–520 nm), whereas terrestrial and fresh water species are red-shifted to 550–580 nm [28].

Although bioluminescence has been observed in various organisms, bacterial bioluminescence occurs naturally in 11 bacterial species from four genera (*Vibrio*, *Photobacterium, Shewanella, and Photorhabdus*). In terms of bioluminescent spectra, the majority of bacteria cells emit blue-green light with a single peak located around 490 nm, which is driven by a complex biochemical reaction involving a luciferase enzyme, aliphatic aldehyde, reduced flavin mononucleotide, and oxygen. However, experimentally, two bioluminescent peaks have been observed using a sensitive photodetector [29]. Thus, the first peak appeared at 490 nm when detected by a conventional spectrophotometer based on photomultipliers, whereas the second peak was visualized in the yellow range of 585–595 nm when using a more sensitive Raman CCD camera (Fig. 1). More interestingly, the emission spectra from different types of bacteria (nature vs. recombinant cells, Gram-positive vs. Gram-negative strains) shared similar properties; these emission spectra were also found to be independent of bacterial cell growth media and conditions. Thouand et al. [29] claimed that the second peak in the yellow range could be attributed to the presence of an accessory emitter protein inside bacterial cells and/or the autofluorescence of the luciferase enzyme.

The concurrent presence of two emission peaks at 490 and 540 nm from a single bacterial strain has been previously reported [30]. The YFP, which carries a flavin moiety, was found to be responsible for the appearance of a second peak and was temperature sensitive (+4 °C) both in vivo and in vitro, with changed in the color of bioluminescent emissions and a reaction rate that increased up to tenfold. Thus, the concomitant modification of emission spectra and reaction kinetics indicated that the emitter YFP should interact with *Lux* intermediate to form the excited state, which finally produces the second peak located in yellow range [31].

2.2 Instrumental Optics for Bacterial Bioluminescence

There are several methodologies and commercial instruments available for measurement of bacterial bioluminescence, generically named *luminometers*. A luminometer is used to record the optical signal from a bioluminescent assay in liquid

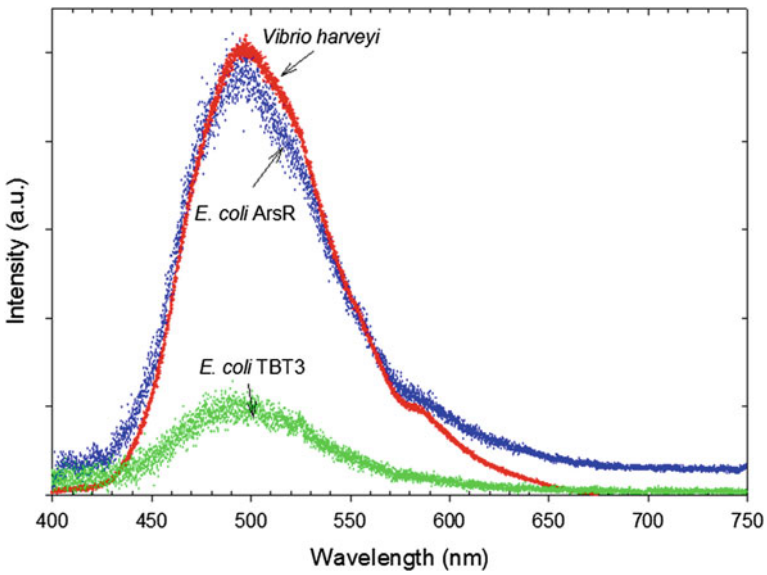

Fig. 1 Emission spectra of two engineered microorganisms strains (*E. coli* and *Vibrio harveyi*) cultivated in batch conditions in DSMZ6904 medium. Besides the normal peak at 500 nm, an additional peak around 585 nm was recorded for both strains (*E. coli* ArsR and *Vibrio harveyi*) using a more sensitive Raman CCD camera

phase. An imaging device is normally required if the optical signal is produced from two-dimensional sources, such as membranes used in blotting and tissue sections [32]. Specifically, a luminometer is designed for the measurement of a very small amount of light in the visible range. The photon detector frequently used is either a photomultiplier (PMT) or solid-state photodiode, where the detected signal may be either current or photon counts.

Different models of luminometers are available, either as a single-sample fitted with a PMT or solid-state detector or as multi-sample and automatic luminometers dedicated for microtiter plate measurement [33]. On the other hand, the imaging device permitting the location and measurement of light from a two-dimensional source is usually constructed by using a CCD camera as the detector and specialized software for imaging signal processing. There are also some hybrid instruments, such as a camera luminometer [33], where the samples in the microtiter plates are exposed to high-speed film. Semi-quantitative results can be obtained very quickly from this inexpensive instrument.

The following sections briefly discuss the various physical platforms that are available for bacterial bioluminescent detection.

3 Platforms for Bacterial Bioluminescent Detection

3.1 Photomultiplier Tube for Bacterial Bioluminescent Detection

One typical example of a vacuum phototube is the PMT tube, which is an extremely sensitive detector for light in a wide spectrum, ranging from ultraviolet to near-infrared wavelengths. Bacterial bioluminescence, enabled by the *lux* reporter, normally appears as a blue-green light with a center wavelength at 490 nm; thus, it can be easily detected by the PMT tube. For instance, optical fibers have been developed based on bioluminescent bioreporters for online water pollutant detection and air toxicity monitoring [34–37]. An illustration of the homemade setup for air toxicity monitoring is shown in Fig. 2. In this biosensor setup, the sensing element is a self-contained unit, with a disposable feature concerning the immobilization of engineered bacterial on the optical fiber tip cores via a calcium mediated gelation of alginate. The signal detection element is represented by a PMT tube. Upon exposure to a given toxicant, a light signal is created and transmitted through an optic fiber to the selected PMT. Thus, the analytical performances of the above system are optimized by investigating different experimental parameters: immobilized bacterial cells density, numerical aperture of optical fiber core, working temperature, etc., to detect 25 μg/L of mitomycin C [37]. Unlike this online biosensor configuration, others researchers [38] have immobilized the bacterial cells onto a nondisposable optical fiber-optical. For experiments, the genetically engineered *E. coli* bacteria cells containing firefly luciferase gene fused to *TOL* plasmid have been immobilized on optical fibers for benzene derivative detection at the ppm level with a Hamamatsu C2310 PMT [38].

A multiplexing bacterial luminescent bioassay using a bundle of 22 optical fibers was simultaneously prepared to detect various heavy metal pollutants [39]. Interestingly, it is reported that the disadvantages result from the cross-reaction among the presence of different metals. Furthermore, a portable optical biosensor based on immobilized bacterial cells, namely Lumisens2, was constructed by fixing the bacterial cells in a disposable card. The resulting bioluminescent signal was transmitted through an optical fiber to a PMT that enabled the detection of 2 μM for organotin compounds (tributyltin) in 400 min [40].

Although the bacterial bioluminescent signal can be readily detected by a conventional PMT with high sensitivity [37], the high working voltage, cost, large size, and the limited linear response range hinder its application in compact integrated biosensing systems. As a promising alternative, the solid-state silicon photomultiplier (SPM) shows some advantages over conventional PMT, such as small size, low bias voltage, and insensitivity to magnetic interference. Additionally, the SPM is more compatible with modern standard microelectronics technology containing a complementary metal oxide semiconductor (CMOS), which offers better integration of the optical signal detection element (SPM) and signal postprocessing unit on board [41, 42]. Followed by the initial work using the SPM to detect bacterial

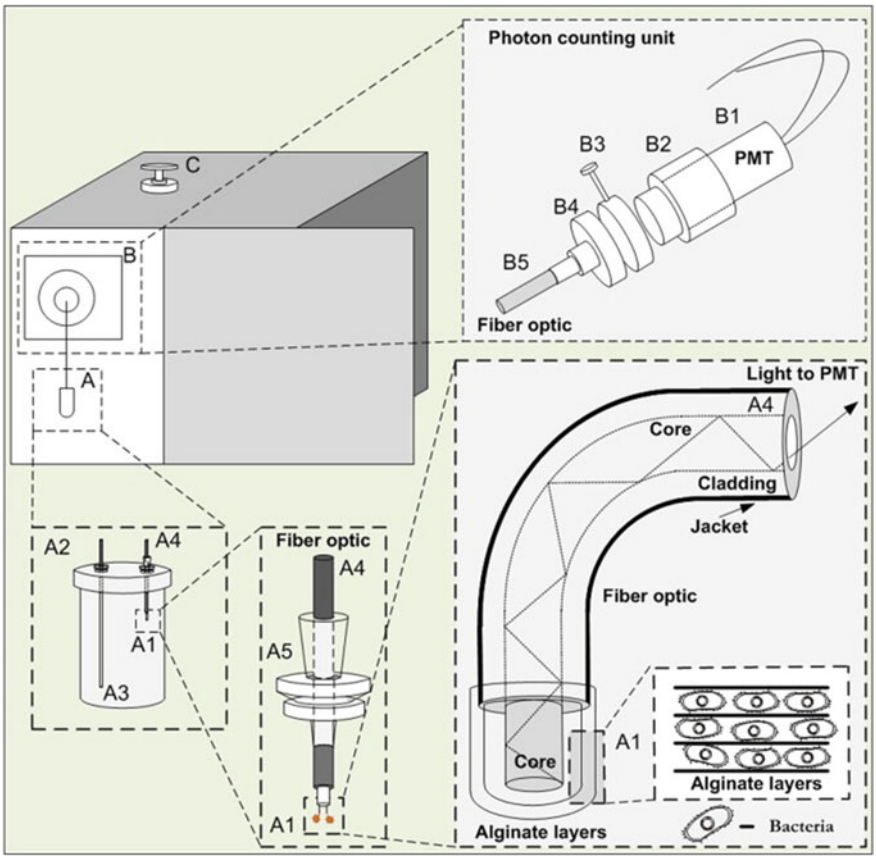

Fig. 2 Fiber-optic setup for the real-time monitoring of toxicity in the air. *A* detection unit; *A1* hermetic chamber; *A2* alginate matrix with bacteria immobilized on the fiber-optic core; *A3* needle for air pollutant entrance; *A4* Fiber optic; *A5* fiber-optic holder; *B* photon counting unit; *B1* Hamamatsu HC135-01 PMT sensor module; *B2* PMT fixation ring; *B3* manual shutter (71430, Oriel); *B4* fiber holder that prevents the movement of the fiber inside the photon counting unit; *B5* fiber optic; *C* outside handle of manual shutter that enables light access to the PMT. Reprinted with permission from [35], copyright 2011 Elsevier

bioluminescent signals [43], a more detailed study involving SPM detection of low-level bioluminescent signals was reported [44]. Based on these results, the authors claimed that the photo-counting readout properties and signal-to-noise ratio of SPM can be enhanced by using a thermoelectric cooler and digital filter, respectively. For model toxicant (salicylate) detection, the lower limit of detection of 250 ppb was obtained by using a digital filter to improve the signal-to-noise ratio. However, the implication of SPM or PMT in a fully integrated applicable biochip platform is still questionable, as the power consumption, size, and additional electronics need to be optimized.

3.2 CCD Detection of Bacterial Bioluminescence

A charge-coupled device (CCD) is widely used in the digital image sensing field by converting incoming photons into electrical signals; therefore, it is used as a sensitive photon detector for recording bacterial bioluminescence. For real-life applications, multi-channel bioluminescent biosensors based on genetically engineered bacteria immobilized in a disposable card have been designed for environmental heavy metal detection [8, 45]. This device contains 64 wells (3 mm in depth) arranged in an 8 × 8 array, where the various different bacterial cells can be locally immobilized. The bioluminescence images produced by the immobilized bacteria in agarose hydrogel arrays were recorded and quantified with a CCD cooled camera in a dark chamber, followed by image processing via customized software. This device can be used for 7 days in laboratory conditions, although its application in field has been limited to only 2 days because of oxygen shortage.

An updated prototype built on the basis of freeze-dried bacteria shows confident bioluminescent detection (with 3 % reproducibility) for 10 days in both laboratory conditions and environmental conditions [8]. An overview of the latest CCD-based bioluminescent bacteria biosensor arrays is shown in Fig. 3.

Because of the addressable configuration of the *Lumisens IV* instrument working in *online* mode, the "fingerprint" of a given pollutant or the total toxicity of various pollutants can be simultaneously investigated by using different populations of bacterial cells placed in a specific array positions. Considering the non-specificity of promoter genes in the engineered bacterial cells, the use of multi-strains is a promising alternative to rapidly improve the selectivity of biosensors in a real time.

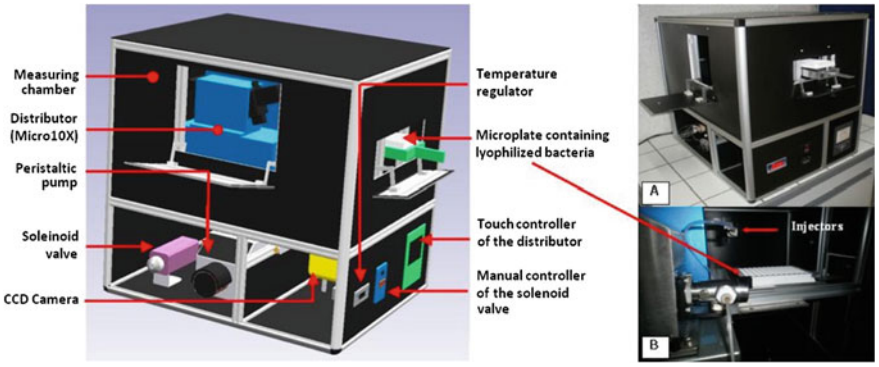

Fig. 3 Three-dimensional modeling performed by computer-aided design (*left*) and photos (*right*) of the Lumisens IV instrument (**a** global view; **b** inside of the measuring chamber). Reprinted with permission from Jouanneau et al. [8], copyright 2012 ACS

3.3 Electrochemical Detection of Bacterial Bioluminescence

A microfluidic whole-cell bacterial biosensor has been developed based on the electrochemical detection of water pollutants [46]. The biochip was fabricated by MEMS technology on a silicon substrate that contains four microchambers of different volumes and electrode radii (see Fig. 4). When engineered bacterial cells are exposed to the toxicant, a specific enzyme is produced, which catalyzes the enzymatic reaction of an exogenous substrate, leading to the generation of electrochemical active materials. Consequently, the concentration of toxicants can be directly correlated with the quantity of electroactive species created over cellular biochemical reactions. The authors assumed that a biotic-micro electro mechanical (biotic-MEMS) system would allow the fast screening of unknown analytes in a high-throughput manner.

Based on the above application of micro-electrochemical biochips in bacterial bioluminescent assays, the optimization of analytical performances has been reported [47]. Specifically, the authors developed two independent protocols for the modification of a working electrode integrated in an electrochemical biochip. Thus, one protocol used Cu–Au micropillars to increase the electrode's effective area, while the second one used conductive polypyrrole (PPy) films. It was noticed that,

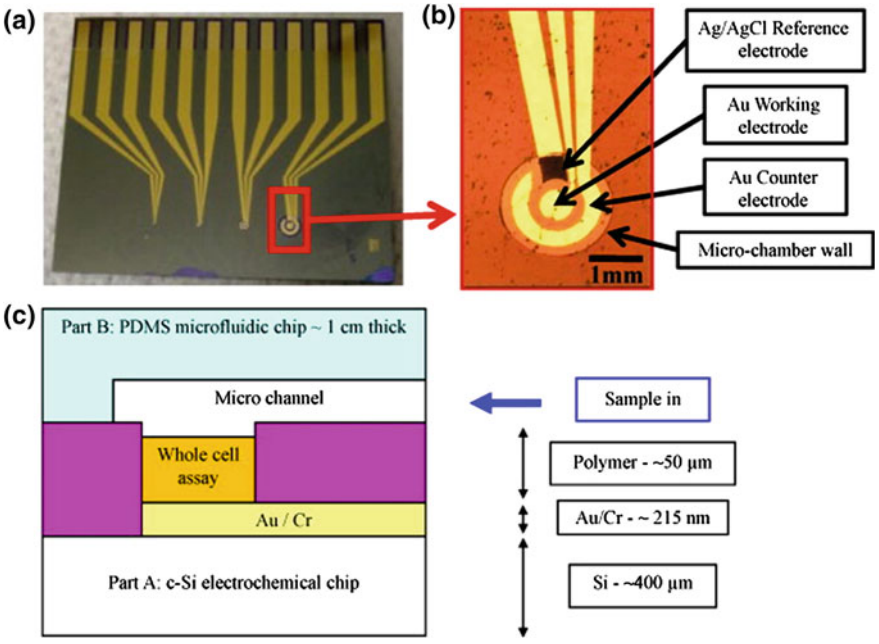

Fig. 4 A silicon-based micro-chip with four differentially sized electrochemical micro-chambers (**a**). Internal view of a single three-electrode electrochemical micro-chamber (**b**). Schematic layout of the electrochemical unit (**c**). Reprinted with permission from Ben-Yoav et al. [46], copyright 2009 Elsevier

in the presence of bioluminescent bacteria, both protocols produced a higher current signal when compared to the nonmodified working electrodes. Concerning the efficiency of the biodetection, the Cu/Au micropillar structured electrode provided a 24 % increase compared to the nonmodified electrode. However, the PPy modification of the working electrode resulted in an overall decrease of efficiency, which could be attributed to a high background signal.

3.4 CMOS Detection of Bacterial Bioluminescence

As discussed previously, although bacterial bioluminescence can be sensitively collected by different photon detectors, it is still a big challenge to design a compact and field-portable bacteria biosensor that includes all requested elements, such as incubation, detection, and signal processing. Despite these constraints, some research groups have designed field-deployable bioluminescent whole-cell biosensors based on the advanced CMOS technique [48, 49]. A highly compact integrated circuit CMOS micro-luminometer of low power (<100 μW), small size (1.5 × 1.5 mm), and on-board signal processing was reported [50, 51]. This micro-luminometer was fabricated by the standard 0.35-μm CMOS process, containing integrated photodiodes for bioluminescent detection and a signal processing unit to convert the current from photodiode arrays to a digital signal bearing frequency information modulated by the target toxicant concentrations (Fig. 5). Both the photodiode arrays and the signal processing electronic circuits have been optimized to provide sensitive detection ability of pollutants (salicylate and naphthalene, 0.1 ppm), both in the gas phase and liquid phase.

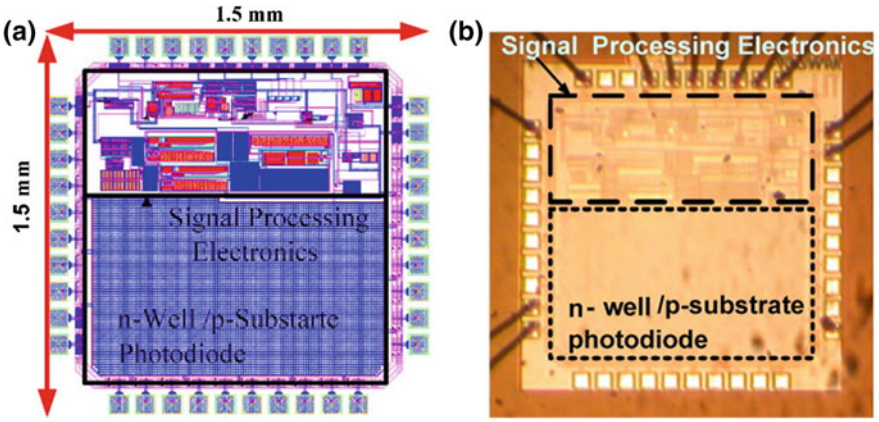

Fig. 5 Layout and microphotograph of the CMOS-based micro-luminometer. The chip measures 1500 × 1500 μm (2.25 mm^2). Reprinted with permission from Vijayaraghavan et al. [51], copyright 2007 Elsevier

3.5 Bacterial Bioreporter Arrays for High-Throughput Measurements

An important aspect in the bioluminescent field is the development of appropriate protocols for the immobilization of living bacterial cells onto different solid transducers, (optical fibers, electrochemical biochips, lab-on-chip total microanalysis), which will be of potential interest for designing high-throughput and sensitive detection of analyte(s). Therefore, a wide range [17, 19, 20] of bacteria array biosensors have been created by assembling a panel of bacterial cells harboring different promoter:reporter fusions. For instance, bacteria sensor arrays were fabricated by printing various engineered bacteria cell spots onto glass surfaces using an adapted noncontact robotic printer [52]. Experimentally, the bacterial cell immobilization efficiency can be improved either by chemical modification of the substrate surface with amino group functionalized self-assembled monolayers or biological modification of bacteria cells resulting in an overproduction of external cellular surface protein by gene mutation. It has been reported that the viability and bioluminescent activity of the immobilized bacterial cells can be preserved for at least 2 months when kept at 4 °C. The optical microscope images of bacteria arrays on different glass supports are shown in Fig. 6.

Generally, bacterial bioluminescence is derived from the luciferase-catalyzed bioreaction involving three substrates: oxygen, reduced riboflavin 5'-phosphate ($FMNH_2$), and long-chain aliphatic aldehyde. It is known that the emission spectrum of natural bacterial bioluminescence has a wide peak, ranging from 400 to 650 nm with a maximum absorption at 490 nm. However, bacterial bioluminescent color can present fluctuations. For instance, the bacterial bioluminescent peak can be red-shifted to 530 nm or blue-shifted to 470 nm by replacing the original $FMNH_2$ substrate with 2-thiol FMN and iso-FMN, respectively [53]. Another method to affect the bacterial spectrum is by using random mutation [54] or site-directed mutagenesis [55]. However, the bacterial activity is substantially reduced and only a small shift of bioluminescent spectrum is obtained by the above-mentioned methods. Thus, under in vivo and in vitro conditions, bacteria bioluminescence can be affected by the formation of a non-covalent complex between luciferase and specific fluorescent proteins (e.g. lumazine protein from *Photobacterium phosphoreum* [56], yellow- and blue-fluorescent proteins from *Alivibrio fischeri* strain Y1 [57, 58]. Due to the limited formation of luciferase-bacterial fluorescent protein complex [59] and its non-covalent nature, the bioluminescent spectrum is very sensitive to external perturbation factors, such as temperature and concentration. Besides these procedures, one robust and versatile protocol for investigating the bioluminescent resonant energy transfer (BRET) effect between *Vibro harveyi* luciferase (energy donor) and coral *Discosoma sp.* fluorescent protein mOrange (energy receptor) has been reported [60], where an

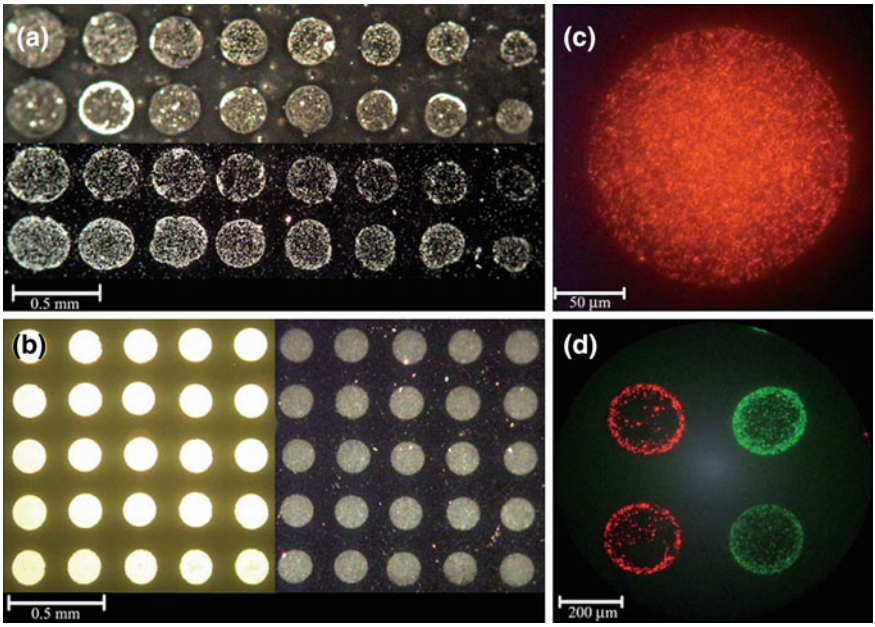

Fig. 6 Light microscope image of fluorescent reporter strains SM110 and SM111 printed (0.5–2.5 nL) on a non-modified glass slide. Printing (*upper* 2 rows) and 24 h incubation at 30 °C were followed by the removal of non-adhered cells (2 *bottom* rows) (**a**). Strain SM111 (1 nL) was printed on APTES-coated glass (5 *left* columns) and incubated for 24 h at 4 °C, followed by the removal of non-adhered cells (5 *right* columns) (**b**). Epifluorescence microscope images of strains SM110 (**c**) and SM111 (**d**) were printed (2 nL) on APTES-coated glass. Reprinted with permission from [52], copyright 2011 RSC

obvious modification of the bacterial bioluminescent spectrum could be observed (Fig. 7). This protocol has some advantages: the fluorescent protein is not directly exposed to the laser excitation that is usually used in conventional fluorescence and the emission from the bacterial luciferase-fluorescent protein fusion is derived from an enzymatic reaction [61].

In summary, due to a specific bioluminescent response signal in the presence of a particular toxicant, the bioreporter whole-cell chips are easy-to-handle tools for identification of a wide spectrum of toxicants [62] and compounds from unknown mixtures [63] that help in the classification of their toxicity [64, 65]. However, only a few studies have investigated the change of bioluminescent light color from wild-type luciferase systems for future applications in the development of multiplexing whole-cell biosensors [66].

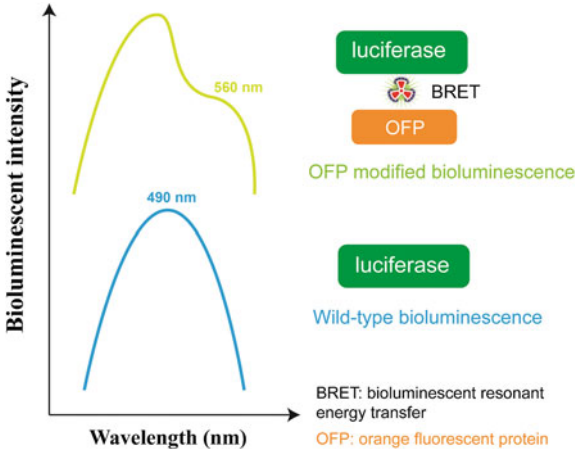

Fig. 7 The bioluminescence spectra from wild-type luciferase (from *Vibrio harveyi*) is displayed as the *bottom blue curve* containing a single peak at 490 nm, while an additional shoulder peak at 560 nm is detected for the luciferase covalently modified by orange fluorescent protein (OFP, from coral *Discosoma sp.*), shown as the *top green curve*

4 Protocols for Improving The Analytical Performances of Bacterial Bioluminescence

4.1 Protocol Based on The Optimization of Bacterial Culture Medium

Since the early stages of bioluminescence investigations, the optimization of bacterial cell medium has been considered as a principal parameter responsible for high signal-response events. Thus, by comparing the toxicant-induced bioluminescent signals from bacterial cells grown in four mediums containing different carbon sources (acetate, pyruvate, glucose, and citrate), Thouand's group found that bacteria cultivated in a medium with acetate exhibited the highest sensitivity, which was attributed to the alternation of bacteria's metal exchange and adsorption via changing energy sources and bacterial cellular membrane states [67]. The induction ratios of bacterial bioluminescence are also dependent on the growth phase and the genetic construction of bacterial cells, as different promoters are expressed in varying degrees under different growth phases.

Even though the promoter gene of most genetically modified bacteria is responsive to a range of heavy metals, the selectivity of the whole-cell bioluminescent assays could be improved by exposing various bacterial cells to the same samples at the same time. Thereby, the combination of different bacterial responses will decrease the detection time of targets. In other cases, the bacterial cells need immobilization and the choice of substrate is an important issue. An ideal

"bioluminescent" substrate should have strong adhesion ability for the bacterial cells, have no chemical interaction with the target compound(s), and be transparent and biocompatible.

4.2 Protocol Based on Electrochemical Measurements

Since most bacterial cells are surface charged, the external electric field can also be used to enhance the bioluminescent response in the whole-cell biosensors using electrochemical platforms. For instance, a portable bacteria whole-cell biochip was fabricated by controlling the adhesion of genetically modified bacterial cells onto the ITO-based electrochemical substrate (Fig. 8). The exposure of bacterial cells to target toxicants initiates a cascade of cellular biochemical reactions, leading to the luciferase enzyme that catalyzes a specific added substrate with the production of electrochemical active species detected by a chrono-amperometric technique [68, 69].

4.3 Protocol Based on Electrophoretic Effects

An interesting procedure for the immobilization of bacterial cells on conductive ITO surfaces using the electrophoretic deposition (EPD) technique has recently been reported [70, 71]. The EPD technique consists of depositing charged particles

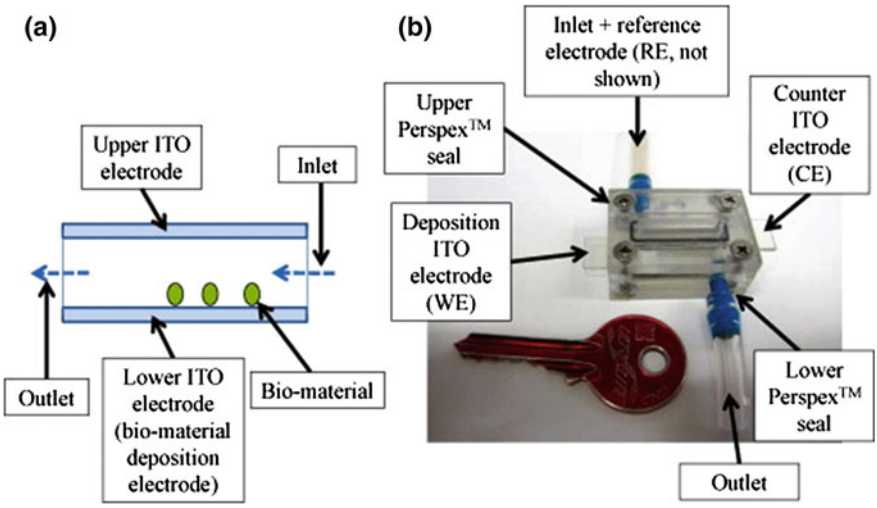

Fig. 8 Electrochemical-based flow chamber designed for microscopic observations and electrochemical measurements with the flow chamber *side view* (**a**) and the entire assembled flow chamber (**b**). Reprinted with permission from Ben-Yoav et al. [69], copyright 2011 Elsevier

in the liquid phase onto a solid conductive surface with an opposite charge under a DC or AC electric field. For cells, the adhesion phenomenon is attributed to the random attachment of cells on supports followed by nucleation and growth of cell cluster biological events. Thus, the efficiency of electrophoretic bio-deposition (EPBD) is highly dependent on the zeta potential and the electrophoretic mobility of bacterial cells. Practically, even though the electrostatic forces between negatively charged bacterial cells and positively charged ITO electrodes exist, the electrophoretic force established between two parallel electrodes will be the driving force for manipulation of bacterial cells [68].

Because the sensitivity of a biosensor is mainly determined by the diffusion rate of analytes inside the bacterial cells [72], the electroporation approach will greatly contribute to the deformation of bacterial membranes, thus increasing permeability to external molecules [73, 74].

4.4 Protocol Based on Molecular Manipulation

The essential ingredient of genetically engineered bioluminescent bacteria is the fusion of bacterial bioluminescent genes (*lux*) and promoter genes. The latter is responsible for the specific recognition of target toxicants or stress effects exposed to bacterial cells, whereas the expression of the former produces dose-dependent light emission. The promoter gene is a non-coding DNA sequence preceding the actual gene coding section, while the transcription of downstream genes is only initiated after this promoter gene is recognized by the RNA polymerase. Therefore, the appropriate molecular manipulation of promoter region should improve the total analytical performance of bacterial bioluminescent sensors.

An interesting approach that used four independent strategies to manipulate the promoter gene has been reported by Yagur-Kroll et al. [75]. Specifically, the first strategy was to modify the length of the DNA segment containing the promoter region. The second approach introduced a random gene mutation via a directed evolution process, whereas the third approach introduced more specific site mutations in the promoter sequence (−35 and −10 regions). The final strategy was the duplication of the promoter sequence to increase the binding sites for RNA polymerase. As a consequence, the bioluminescent sensitivity, kinetic response (earlier response time), and intensity of emitted light were significantly improved. Belkin's group claimed that these molecular manipulation protocols were applicable to almost all of the promoter:reporter fusions of genetically engineered bacterial cells.

In a typical promoter:reporter fusion, the emitted optical signal is derived from the expression of the *lux* gene, which is regulated by the upstream promoter sequence. Thus, besides molecular manipulation of promoter region to improve bioluminescent efficiency, another genetic method has been reported to update the reported bacterial bioluminescence performance by splitting the *lux* gene [76]. Specifically, the *luxCDABE* cassette from *Photorhabdus luminescens* was divided

into two smaller subunits of *luxAB* and *luxCDE*, with the former encoding the enzyme luciferase and the latter encoding an enzymatic complex responsible for synthesizing the long-chain aldehyde substrate of the bioluminescent reaction. These two smaller subunits can be genetically modified to be under control of an inducible stress-responsive promoter or a synthetic constitutive promoter. The authors tested the different combinations of inductive/constitutive *luxAB* and inductive/constitutive *luxCDE*; they found that the optimized configuration was an inducible *luxAB* and a constitutive *luxCDE*, which indicated that the stronger intensity and faster response of bacterial bioluminescent reporter in this case may be attributed to the improved bioavailability of the aliphatic aldehyde substrate. The other possible reason is that the transcription and/or translation of short DNA segments may be faster and more efficient than that of long ones after lux operon splitting. This study clearly proved the previously published results that aliphatic aldehyde substrate is the rate-limiting factor of bioluminescent reactions when the entire *luxCDABE* gene is used [77].

In addition to the molecular manipulation of the *lux* bioreporter and its promoter sequence, other methodologies based on molecular biology have been developed. For instance, a faster bioluminescent response was recorded either by using different plasmid-bearing host strains [78]. Also, the background signal of an *arsR*::*lacZ* bioreporter was greatly reduced by placing another *ArsR* binding site downstream of *arsR* [79].

To conclude, the bioluminescent sensitivity can be increased by certain methods, such as integration of the promoter:reporter fusion into the bacterial chromosome instead of plasmid, modifying the cell permeability towards the target compound, and decreasing the plasmid copy number [80, 81].

4.5 Protocol Based on Numerical Modelling

The specificity of a bacterial bioluminescent assay is dictated by the biorecognition element of a promoter sequence inside the cells, although this response circuit can be tightly controlled to only respond to a single compound [79, 82]. It is known that most bioluminescent bacteria bioreporters are normally responsive to a broad spectrum of chemicals sharing similar structures [83]. To improve the analytical specificity of a bacterial bioluminescent assay, a simple alternative is to use a panel of several bacteria reporters containing the *luxCDABE* operon fused with different stress promoters. When various bacteria strains are exposed to target compounds, "fingerprint" bioluminescent responses of each target are produced by the corresponding bioreporter, which are analyzed by a special computing program based on different algorithms. Some examples of algorithms for identification of antibiotics and heavy metals are the Bayesian decision theory, nonparametric nearest-neighbor technique [84, 85], chi-squared automatic interaction detector type [7], and support vector machine classifier [86].

4.6 Protocol Based on Overnight "Cold Incubation"

To improve the sensitivity of a bioluminescent assay for the detection of water pollutants (e.g. atrazine), a new protocol was created using a two-stage bioluminescence reading approach (both were required): one for continuous monitoring of light evolution for 5 h (fresh stage investigation) and a second for another 5 h (after an "overnight" cold incubation at 4 °C) [11]. In both phases, the bioluminescence responses were acquired at 25 °C. The "overnight" cold incubation condition was tested for different periods of times, such as 3, 6, 9, and 12 h, respectively. From the obtained results, 9 h at 4 °C gave the best sensitivity for atrazine bioluminescent detection.

Due to the importance of the bacterial growth phase, intact bacterial cells of different optical density (OD) were first bioluminescent tested at 25 °C (Fig. 9). Interestingly, it was found that for the fresh mode (right-side group), two peaks were always recorded, no matter what OD was selected (0.03, 0.08 or 0.12). Moreover, when the OD increased from 0.03 to 0.12, a significant shift of the peaks from 265 to 210 min was observed. The increase of the OD has also led to an increase of the peak amplitude. By using the overnight mode (left-side group), larger bioluminescent peaks were recorded after 30 min, where the appearances of such peaks were OD independent. However, the intensity of the "overnight" peak

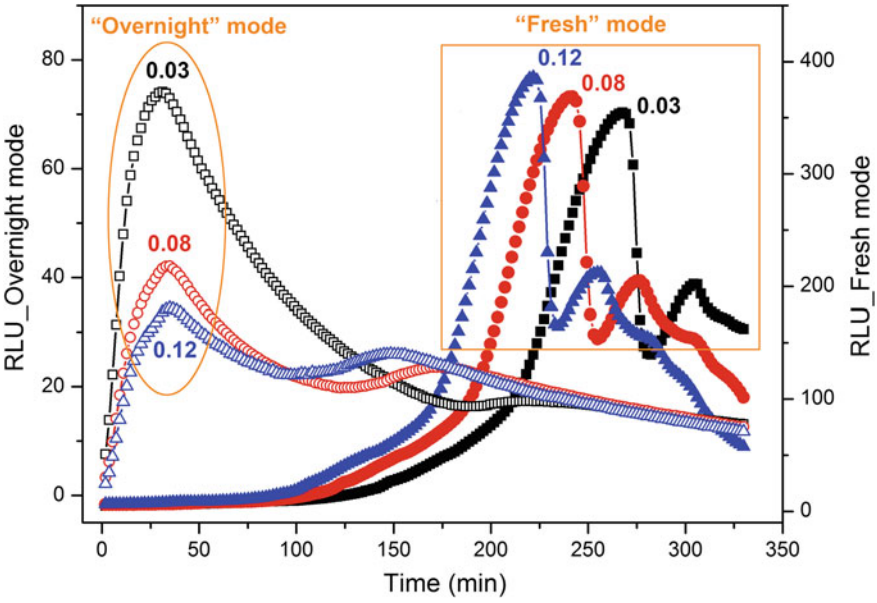

Fig. 9 Bioluminescence of *E. coli* TV1061 bacteria of different cell optical densities (0.03: *black*, 0.08: *red* and 0.12: *blue*) within "fresh" and "overnight" conditions. Both types of bacterial bioluminescence were recorded at 25 °C. An optimized cold incubation (4 °C) was obtained after 9 h

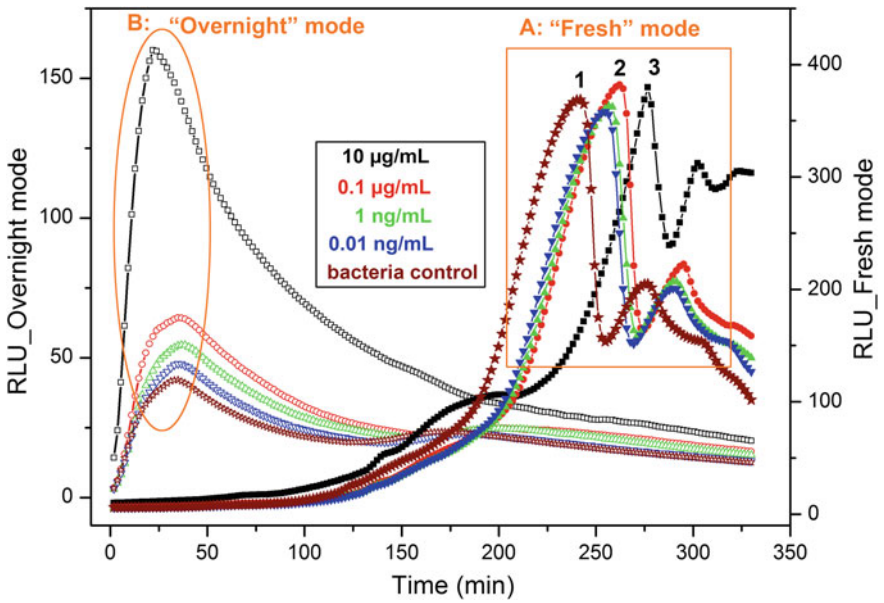

Fig. 10 Bioluminescence behaviors of *E. coli* TV1061 bacteria (OD = 0.08) within the presence of different atrazine concentrations recorded in fresh mode for 5.5 h (group A) and after an overnight incubation of *E. coli* TV1061 at 4 °C (group B). Four atrazine concentrations (10 µg/mL, 0.1 µg/mL, 1 ng/mL and 0.01 ng/mL) were tested for their toxicity to bacterial cells. Bioluminescent measurements for the two modes were recorded at 25 °C

was decreased when compared to the major peak obtained within the fresh mode. Thus, the increase of the OD has led to a decrease of the peak amplitude for the overnight mode.

Once the optimization of the bacterial OD value was acquired, the toxic effect of atrazine compound to *E. coli* TV1061 bacteria was investigated through the proposed "fresh" and "overnight" consecutive tests. Bacteria of 0.08 OD were proven to have the best ability to discriminate between different concentrations of atrazine. Thus, Fig. 10 depicts the variations in bioluminescence signals in the presence of atrazine versus time for both "fresh" and "overnight" tests. For the "fresh" mode (group A), conditions 1, 2, and 3 denote the intact bacteria, bacteria treated with low toxicant (atrazine) concentrations, and bacteria treated with high toxicant (atrazine) concentrations, respectively. Spectra under conditions 1 and 2 exhibited very similar shapes. For condition 3, the toxicity of atrazine is bioluminescent, translated as an important peak shift in time (from 240 to 275 min).

For the "overnight" mode (group B), bacterial bioluminescence was rationally dependent on the atrazine concentration. In contrast to the overlapped bacterial response to lower concentrations of atrazine obtained in "fresh mode," larger distinguished peaks were obtained after only 25 min for various low concentrations of atrazine (from 10 fg/mL to 10 µg/mL). Although it is difficult to elucidate the specific mechanism of bacterial bioluminescence evolution under the overnight

conditions, the use of cold incubation supposedly slows down the bacteria's own metabolisms or may enable the accumulation of the toxicant inside the cells. Consequently, the bacteria are gradually chemical "stressed" by the increase of the atrazine content.

4.7 Protocol Based on "Washing" Bacterial Cells

The generation of bioluminescence is a dynamic process highly dependent on cellular metabolism status [87]. An easy-to-perform protocol has been reported by Jia et al. [10]. The protocol contains three major steps: incubation at 25 °C, centrifugation, and washing of aged cells with fresh LB medium, (Fig. 11). This affects

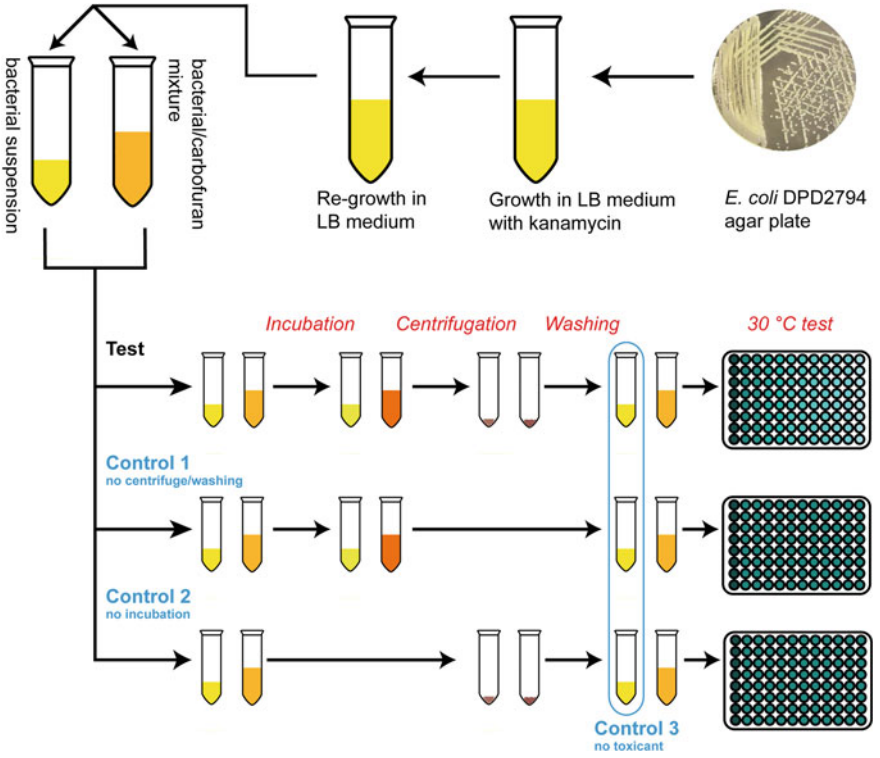

Fig. 11 A bioluminescence-enhancement protocol based on three steps: incubation of bacterial cells with a toxicant solution at room temperature for different periods of time, centrifugation of the resulted bacterial/toxicant suspension, and replacement of aged supernatant with a fresh LB medium free of toxicants. Finally, the bacterial bioluminescence was investigated at 30 °C. Three independent control experiments were prepared: first without the centrifugation/washing step, second without the incubation step, and third without any toxicants

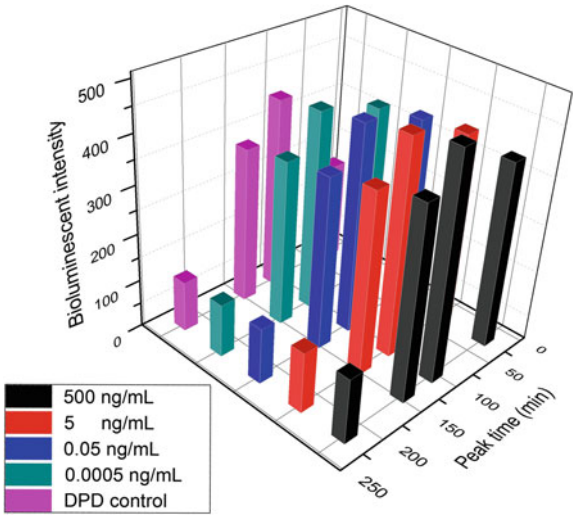

Fig. 12 Bioluminescent signals of DPD2794 bacterial cells induced by the presence of different carbofuran content (0.5 μg/mL, 5 ng/mL, 50 pg/mL, 0.5 pg/mL) and the signal of control cell samples (named DPD control). The incubation time of control bacteria and bacteria with toxicants was 6 h at room temperature, followed by independent centrifugation and washing steps with fresh LB medium before the final bioluminescent investigation at 30 °C

the *E. coli* DPD2794 cellular physiological response, for sensitive bioluminescent detection of a specific toxicant (carbofuran) at an optimized temperature (30 °C).

The precise incubation time of bacterial cells with toxicants and their subsequent washing with fresh LB medium after centrifugation were investigated as a function of the resulting bioluminescent signal in terms of its shape and intensity. Thus, from the five tested times of 0 h (no toxicant incubation), 2, 4, 6, 9, and 12 h at room temperature, 6 h proved to have the highest influence on the ability of *E. coli* DPD2794 cells to differentiate between different carbofuran concentrations (0.5 μg/mL, 5 ng/mL, 50 pg/mL, 0.5 pg/mL) after the bioluminescent test at 30 °C (Fig. 12).

Concerning the bioluminescent shape-response signal at two independent temperatures, a remarkable evolution was noted using the fresh-LB medium washing protocol, demonstrated by the appearance of broadened peaks after 250 min by using 30 °C and only after 350 min by using 26 °C [26, 88–90].

5 Emerging Bacterial Bioluminescence Applications Beyond Biosensing/Assays

Because of the extensive applications of whole-cell bacterial bioluminescence in biochemical and toxicity detection, the ongoing research in *lux*-based systems has been pushed from the original prokaryotic bacterial *luxCDABE* gene cassette further

to eukaryotic cells. The first breakthrough of expressing *lux* genes in eukaryotic hosts used autonomous bioluminescence from *Saccharomyces cerevisiae* yeast [91], where more stable luciferase proteins from terrestrial bacterium *P. luminescens* were expressed instead of marine strains such as *Vibrio harveyi* or *Alivibrio fischeri*. Since this discovery, the expression of bacterial *lux* genes in eukaryotic cells has been under investigation. The development of a robust bioassay based on luminescent yeast, with the potential for *lux* gene expression in human cell lines [92, 93], has been reported.

In addition, the bioluminescent imaging (BLI) approach is becoming a powerful tool for the real-time and in vivo monitoring of various biological processes in living systems [61]. Thus, many bacterial cells can be genetically engineered with *lux* reporter genes to induce the production of light, dramatically enlarging the spectrum of in vivo bacterial investigations with this non-aggressive and easy-to-perform technique. Moreover, due to the fact that bacterial cells possess a natural ability to grow preferentially within tumor(s), engineered bioluminescent bacterial cells can be designed as theranostic probes for the identification of cancer cells. For instance, *Salmonella typhimurium* bacterial cells were genetically engineered to carry cytotoxic proteins (cytolysin A) and luminescent reporter (*lux*) genes for monitoring the cancer therapy process inside mice [94]. Fortunately, the resolution of in vivo bioluminescent imaging from tumor-targeted bacterial cells can be greatly enhanced by using a combinational three-dimensional diffuse optical tomography (3D BLI) and micro-computed tomography (μCT), as reported by Cronin et al. [95].

Besides cancer therapy and imaging investigations, bacterial bioluminescence can also be used in other scenarios, such as the study of intestinal colonization by *E. coli* in mice [96], as well as the monitoring and elucidation of antimicrobial photodynamic inactivation mechanisms [97, 98].

6 Conclusions and Perspectives

This chapter discussed several existing physical platforms used within bioluminescence measurements, such as PMT tubes, CCD cameras, electrochemical biochips, and CMOS-integrated circuits. Then, several published works were introduced on the improvement of the analytical performances of bacterial bioluminescence biosensing/assays. Moreover, the authors' recent work on the highly improved sensitivity of bacterial bioluminescent assays based on two protocols for the fine-tuning of bacterial physiology and application of *lux* genes in eukaryotes cells by Sayler's group were discussed as well.

Although bioluminescence bioassays for toxicant detection based on genetically engineered bacterial cells have been studied since the 1990s [1], much work remains to be done for their real-world application in different scenarios. The most challenging question is how to obtain applicable selectivity towards specific target pollutants, given the fact that the promoter gene used in most engineered bacteria

normally is responsive to many toxicants. It is believed that the solution will be given by advancements in molecular biology, which will help us to understand the cellular interaction with exogenous compounds. Meanwhile, the fine tuning of bacterial metabolism by modulating various experimental parameters has already been proven as an economical option to enhance the analytical performance of bioassays.

Finally, the combination of a bioluminescent signal with other advanced devices, such as micro-/nano-fluidics, or advanced optical materials, such plasmonic gold nanoparticles, to further improve the efficiency of a whole test system will definitely push the current laboratorial bioluminescent research to real-time applications.

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