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Reviews

Bioluminescent Bioreporter Integrated Circuits: Sensing Analytes and Organisms with Living Microoranisms

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Whole cell bioluminescent reporter bacterial strains containing *lux* CDABE fusions to catabolic operons have been broadly developed for organics chemical pollutants. The use of these reporter strains has been to determine the presence and bioavailability of the pollutants in environmental samples, and as endpoint determinants and monitoring and control tools in bioremediation processes. Research applications have been concluded for *in situ* large-scale analysis of *nah*G-*lux* bioreporters in subsurface soils, *on site* groundwater contaminant distributions for naphthalene (*nah*G-*lux*) and BTEX (*tod* C1-*lux*) bioreporter, and analytical sensing of 2,4, D in Agent Orange contaminated soils (*tfd*R-*lux*) and PCB's in bioremediation analysis (*bph*A-*lux*). Bioreporter strains can approach instrumental methods in terms of sensitivity and reproducibility with significant cost and timesavings and certain applications can provide new information for which no analytical counterparts exist. However, careful consideration of standardizing the cellular assay is fundamental to reproducible unambiguous extraction of information from complex environmental systems. To accomplish this later objective, the bioreporters are being fabricated as part of a Bioluminescent Bioreporter Integrated Circuit (BBIC).

BBIC's are new analytical biosensors that include a self-contained, optical application specific integrated circuit with genetically engineered bioluminescent bacteria that respond specifically to a certain analyte(s) and an interface between the biotic and abiotic components. A prototype system utilizing a circuit board, a power supply, computer hardware/software, and three different types of light-tight enclosures was developed. *Pseudomonas fluorescens* 5RL, containing a salicylate inducible *luxCDABE* gene cassette was selected as the test bioreporter. Salicylate was chosen as a test molecule because it is water-soluble, nontoxic and not degraded by 5RL. Using the prototype system, we have shown that 4×10^5 *P. fluorescens* 5RL cells grown in LB containing 10 ppm salicylate are required for light detection with a linear dynamic range of three orders of magnitude. These data provide a foundation for further development of application specific biosensors.

Key words: Biosensor, bioluminescence, integrated circuit, environmental contamination, bioremediation

Introduction

In developing bioremediation technology or assessing the effectiveness of natural attenuation processes, it is ultimately necessary to provide quantitative *in situ* measures of the active microbial biodegradative processes involved. This necessity is determined by several factors including:

- □ Proving that the bioremediation technology or microorganisms are effective in the contaminated environment
- □ Optimizing the bioremediation technology through process monitoring and control to maximize or sustain biodegradation processes under dynamic *in situ* conditions
- □ Evaluating the reduction in chemical contaminant levels for endpoint determination to evaluate exposure to bioavailable contaminants and metabolic transformation products

The need to provide evidence documenting these factors can be met in some cases by conventional microbiological assessments or analytical chemical techniques, such as GC/MS, or with newer geohydrological methods, such as stable isotope ratios and push-pull well tests. However, often times these methods may lead to ambiguous results or are costly and time consuming in their environmental implementation.

Recently new molecular biology based technology focusing on the level and activity of specific organisms and genes involved in the biodegradation process have been developed and applied for *in situ* analysis of bioremediation technology and natural attenuation processes. Among these technologies is the analysis of messenger

RNA (mRNA) levels indicative of expression of biodegradative genes *in situ*, and the use of bioluminescent (*lux*) reporter gene technology for on-line real-time analysis of biodegradative expression and chemical sensing in contaminated environments. RNA analysis is a powerful tool for environmental assessment and, with development of microarray methods, can significantly enhance our understanding of *in situ* microbial biodegradation processes. Gene expression monitoring using mRNA has been recently reviewed[1,2] and, while the technology continues to advance, its complexity in application has somewhat limited its environmental utility.

However, *lux* based bioreporter gene expression technology has moved forward as a significant analytical tool in environmental chemical sensing and bioremediation process monitoring. It is the objective of this report to describe the recent developments and applications of this technology in bioremediation. These developments include the real-time *in situ* analysis of chemical contaminants, as well as biodegradation process analysis. Most recently living cell bioreporter technology has been incorporated into microelectronic integrated circuits sensor technology ultimately to provide wireless real-time monitoring technology for bioremediation.

Reporter Genes Systems

Bioreporters refer to intact, living microbial cells that have been genetically engineered to produce a measurable signal in response to a specific chemical or physical agent in their environment (Fig. 1). Bioreporters contain two essential genetic elements, a promoter gene and a reporter gene. The promoter gene is turned on (transcribed) when the target agent is present in the cell's environment. The promoter gene in a normal bacterial cell is linked to other genes that are then likewise transcribed and then translated into proteins that help the cell in either combating or adapting to the agent to which it has been exposed. In the case of a bioreporter, these genes, or portions thereof, have been removed and replaced with a reporter gene. Consequently, turning on the promoter gene now causes the reporter gene to be turned on. Activation of the reporter gene leads to production of reporter proteins that ultimately generate some type of a detectable signal. Therefore, the presence of a signal indicates that the bioreporter has sensed a particular target agent in its environment.

Originally developed for fundamental analysis of factors affecting gene expression, bioreporters were early on applied for the detection of environmental contaminants[2] and have since evolved into fields as diverse as medical diagnostics, precision agriculture, food-safety assurance, process monitoring and control, and bio-microelectronic computing. Their versatility stems from the fact that there exist a large number of reporter gene systems that are capable of generating a variety of signals. Additionally, reporter genes can be genetically inserted into bacterial, yeast, plant, and mammalian cells, thereby providing considerable functionality over a wide range of host vectors.

Several types of reporter genes are available for use in the construction of bioreporter organisms, and the signals they generate can usually be categorized as either colorimetric, fluorescent, luminescent, chemiluminescent or electrochemical. Although each functions differently, their end product always remains the same – a measurable signal that is proportional to the concentration of the unique chemical or physical agent to which they have been exposed. In some instances, the signal only occurs when a secondary substrate is added to the bioassay (*luxAB*, Luc, and aequorin). For other bioreporters, the signal must be activated by an external light source (GFP and UMT), and for a select few bioreporters, the signal is completely self-induced, with no exogenous substrate or external activation being required (*luxCDABE*). The following sections outline in brief some of the reporter gene systems available and their existing applications.

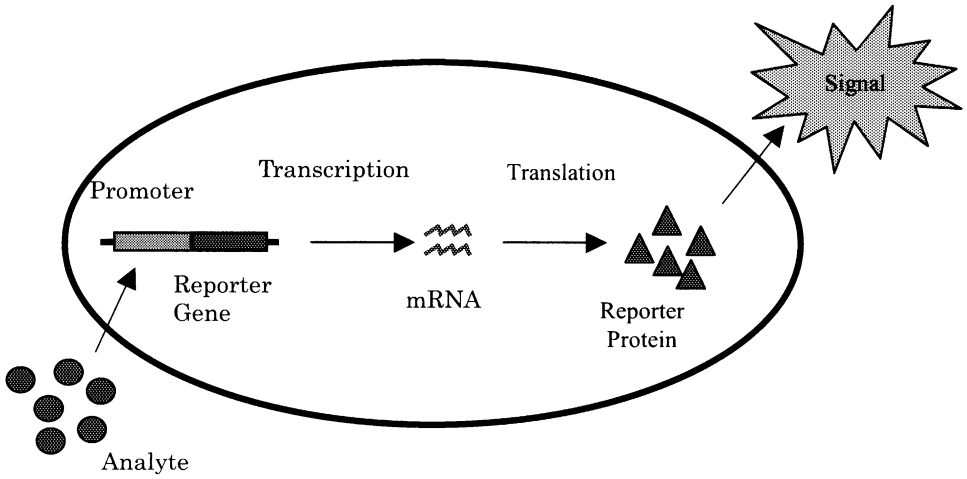

Fig. 1. Anatomy of a bioreporter organism. Upon exposure to a specific analyte, the promoter/reporter gene complex is transcribed into messenger RNA (mRNA) and then translated into a reporter protein that is ultimately responsible for generating a signal.

Bacterial luciferase (Lux): Luciferase is a generic name for an enzyme that catalyzes a light-emitting reaction. Luciferases can be found in bacteria, algae, fungi, jellyfish, insects, shrimp, and squid, and the resulting light that these organisms produce is termed bioluminescence. In bacteria, the genes responsible for the light-emitting reaction (the *lux* genes) have been isolated and used extensively in the construction of bioreporters that emit a blue-green light with a maximum intensity at 490 nm (Fig. 2)[4]. Three variants of *lux* are available, one that functions at $<30°C$, another at $<37°C$, and a third at $<45°C$. The *lux* genetic system consists of five genes, *luxA*, *luxB*, *luxC*, *luxD*, and *luxE*. Depending on the combination of these genes used, several different types of bioluminescent bioreporters can be constructed.

luxAB bioreporters: *luxAB* bioreporters contain only the *luxA* and *luxB* genes, which together are responsible for generating the light signal. However, to fully complete the light-emitting reaction, a substrate must be supplied to the cell. Typically, this occurs through the addition of the chemical decanal at some point during the bioassay procedure. Numerous *luxAB* bioreporters have been constructed within bacterial, yeast, insect, nematode, plant, and mammalian cell systems.

luxCDABE bioreporters: Instead of containing only the *luxA* and *luxB* genes, bioreporters can contain all five genes of the *lux* cassette, thereby allowing for a completely independent light generating system that requires no extraneous additions of substrate or any excitation by an external light source. So in this bioassay, the bioreporter is simply exposed to a target analyte and a quantitative increase in bioluminescence results, often within less than one hour. Due to their rapidity and ease of use, along with the ability to perform the bioassay repetitively in real-time and on-line, makes *luxCDABE* bioreporters extremely attractive. Consequently, they have been incorporated into a diverse array of detection methodologies ranging from the sensing of environmental contaminants to the real-time monitoring of pathogen infections in living mice. Table 1 illustrates the widespread application of *luxCDABE* bioreporters.

Detecting the Optical Siganature

Using light as the terminal indicator is advantageous in that it is an easily measured signal. Optical transducers such as photo-multiplier tubes, photodiodes, microchannel plates, or charge-coupled devices are readily available and can be easily integrated for environmental research. For example, a "down-hole" photomultiplier system was built to insert into subsurface bore holes to directly measure light from soils originally inoculated with *Pseudomonas fluorescens* HK44[1,5]. Figure 3 provides a diagram representation of the photomultiplier devise with a direct photographic recreation of luminescing soil from HK 44 *in situ* biodegradation process activity. As these usually consist of large, table-top devices, demand for smaller, portable light readers for remote monitoring has resulted in the development of battery-operated, hand-held photomultiplier units. Recently, The Center for Environmental Biotechnology and Oak Ridge National Laboratory have taken steps towards genuine miniaturization of optical transducers and have successfully developed integrated circuits capable of detecting bioluminescence directly from bioreporter organisms (Fig. 4)[6].

These bioluminescent bioreporter integrated circuits (BBICs) consist of two main components; photodetectors for capturing the on-chip bioluminescent bioreporter signals and signal processors for managing and storing information derived from bioluminescence (Fig. 5, 6). Remote frequency (RF) transmitters can also be incorporated into the overall integrated circuit design for wireless data

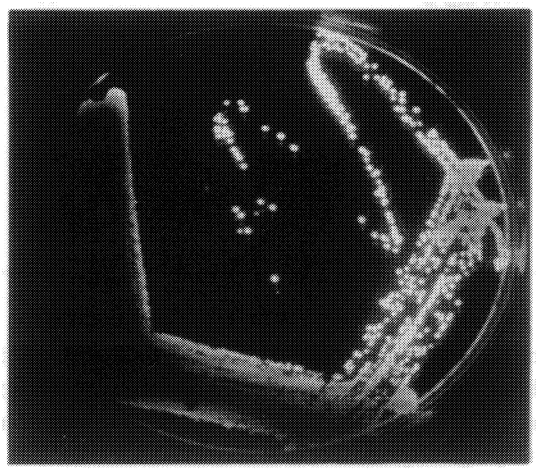

Fig. 2. Bioluminescence emitted from individual colonies of microbial cells containing the genes for bacterial luciferase.

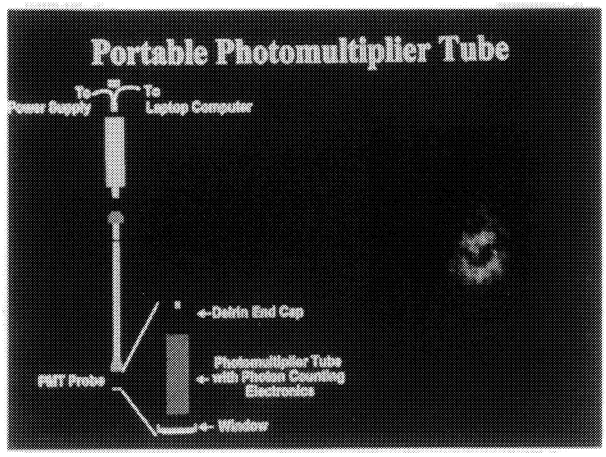

Fig. 3. Down hole photomultipler used to measure *P. fluorescens* HK44 bioluminescence during naphthalene bioremediation in subsurface soil. Right inset is photograph of bioluminescing soil at a depth of app. 2m.

Table 1. *luxCDABE*-based bioreporters.

Analyte	Time for induction	Concentration	Ref.
2,3 Dichlorophenol	2 h	50 mg/L	10
2,4,6 Trichlorophenol	2 h	10 mg/L	10
2,4-D	20–60 min	2 μM–5 mM	11
3-Xylene	Hours	3 μM	12
4-Chlorobenzoate	1 h	380 μM–6.5 mM	13
4-Nitrophenol	2 h	0.25 mg/L	10
Aflatoxin B1	45 min	1.2 ppm	14
Alginate production	1 h	50–150 mM NaCl	15
Ammonia	30 min	20 μM	16
Antibiotic effectiveness against *Staphylococcus aureus* infections in mice	4 h	100 CFU	17
BTEX (benzene, toluene, ethylbenzene, xylene)	1–4 h	0.03–50 mg/L	8
Cadmium	4 h	19 mg/kg	18
Chlorodibromomethane	2 h	20 mg/L	10
Chloroform	2 h	300 mg/L	10
Chromate	1 h	10 μM	19
Cobalt	n.s.	2.0 mM	20
Copper	1 h	1 μM–1 mM	21
DNA damage (cumene hydroperoxide)	50 min	6.25 mg/ml	22
DNA damage (mitomycin)	1 h	0.032 μg/ml	23
Gamma-irradiation	1.5 h	1.5–200 Gy	24
Heat shock	20 min	Various, depending on chemical inducer used	25, 26
Hemolysin production	n.s.	5 mM cAMP	27
Hydrogen peroxide	20 min	0.1 mg/L	28
in vivo monitoring of *Salmonella typhimurium* infections in living mice	4 h	100 CFU	29
Iron	Hours	10 nM–1 μM	30
Isopropyl benzene	1–4 h	1–100 μM	31
Lead	4 h	4036 mg/kg	18
Mercury	70 min	0.025 nM	32
N-acyl homoserine lactones	4 h	Not specified	33
Naphthalene	8–24 min	12–120 μM	34
Nickel	n.s.	0.3 mM	20
Nitrate	4 h	0.05–50 μM	35
Organic peroxides	20 min	Not specified	28
PCBs	1–3 h	0.8 μM	36
p-chlorobenzoic acid	40 min	0.06 g/l	13
p-cymene	$<$30 min	60 ppb	37
Pentachlorophenol	2 h	0.008 mg/L	10
Phenol	2 h	16 mg/L	10
Salicylate	15 min	36 μM	34
Tetracycline	40 min	5 ng/ml	38
Trichloroethylene	1–1.5 h	5–80 μM	39
Trinitrotoluene	n.s.	n.s.	40
Ultrasound	1 h	500 W/cm^2	41
Ultraviolet light	1 h	2.5–20 J/m^2	42
Zinc	4 h	0.5–4 μM	43

n.s. = not specified

relay. Since the bioreporter and biosensing elements are completely self-contained within the BBIC, operational capabilities are realized by simply exposing the BBIC to the desired test sample.

In addition to incorporation in a BBIC format, the whole-cell bioreporter matrix can also be immobilized on something as simple as an indicator test strip. In this fashion, a home water quality indicator, for example, could be developed to operate in much the same way as a home pregnancy test kit.

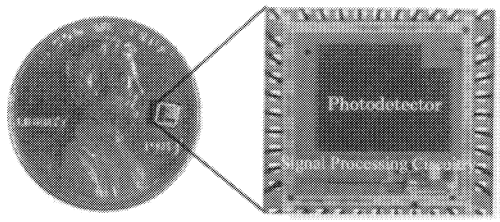

Fig. 4. The integrated circuit photoluminometer. Actual size is 2 mm × 2 mm.

An example of lux-based bioreporters in use: Monitoring environmental contaminants in a groundwater plume

A groundwater research facility at Columbus Air Force Base, Mississippi was contaminated with a simulated jet fuel mixture consisting of naphthalene, toluene, ethylbenzene, and p-xylene[7]. Numerous multilevel sampling wells installed upstream and downstream of the contaminant source allowed for monitoring of the contaminants. Typically, water would be pumped up from designated wells and sent to an off-site laboratory for contaminant analysis using gas chromatography/mass spectrometry (GC/MS) techniques. GC/MS analysis is extremely sensitive and accurate, and is by far the best method available for detecting chemical contaminants in environmental sources. However, it also requires expensive and bulky instrumentation, a trained technician, the use of hazardous chemicals, and a significant allotment of time. As an alternative, we proposed using bioreporters as sensors for the groundwater contaminants. Two bioreporters were used, *Pseudomonas fluorescens* 5RL, a bioreporter for naphthalene, and *Pseudomonas putida* TVA8, a bioreporter for toluene[8,9]. Analysis occurred on-site, where bioreporters were simply combined with groundwater samples and allowed to incubate for a set time. Resulting bioluminescence was measured using a field portable photomultiplier unit interfaced to a laptop computer. Duplicate samples were sent to an off-site laboratory for GC/MS determination of toluene and naphthalene concentrations. Bioluminescent bioreporters consistently predicted contaminant concentrations within 50% of the GC/MS analytic measurements (Fig. 7). Although in this case not highly quantitative, bioluminescent bioreporters did provide a rapid, general assessment of contaminant presence within the groundwater aquifer, and established an overall snapshot of plume dynamics within a few hours of initial sampling at a cost of approximately 1/10 of that of GC/MS analysis.

Conclusions

Bioreporter technology will provide a robust, cost-

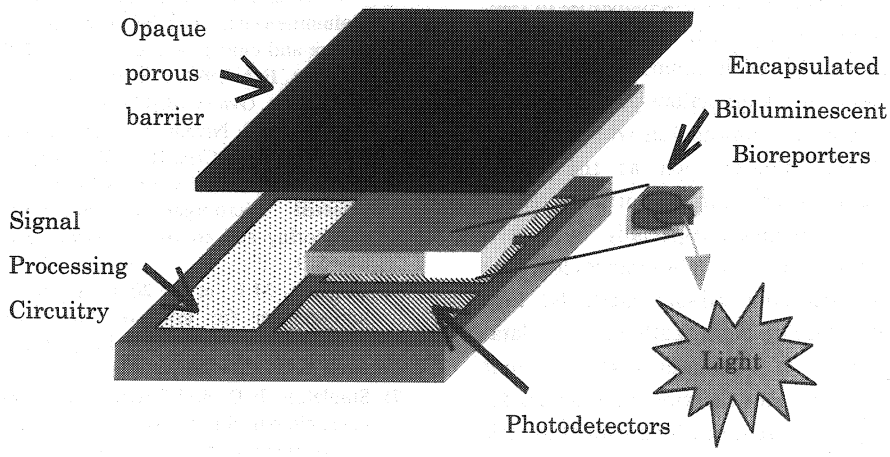

Fig. 5. Assembly of a bioluminescent bioreporter integrated circuit (BBIC) sensor. Right panel, 4 order of magnitude responsivity to cell concentration; Left panel, detection threshold requires a minimum of 3×10^4 cells per BBIC.

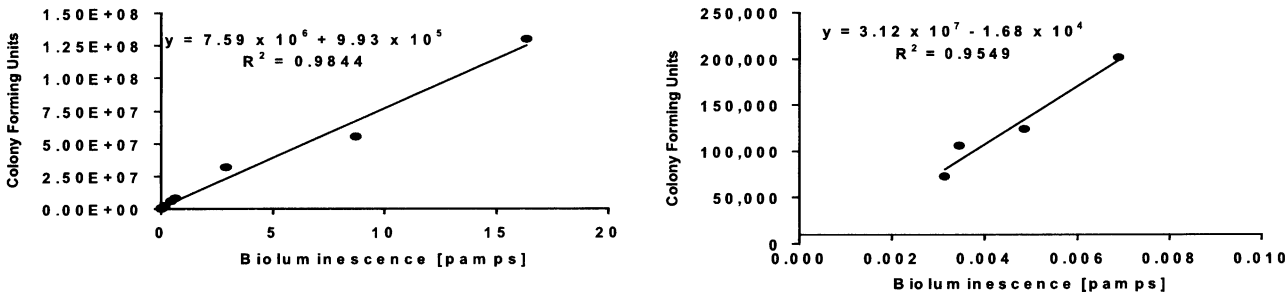

Fig. 6. Bioluminescent Bioreporter Integrated Circuit response to salicylate induction (10 mgl-1) of bioluminescence in *P. fluorescens* 5RL.

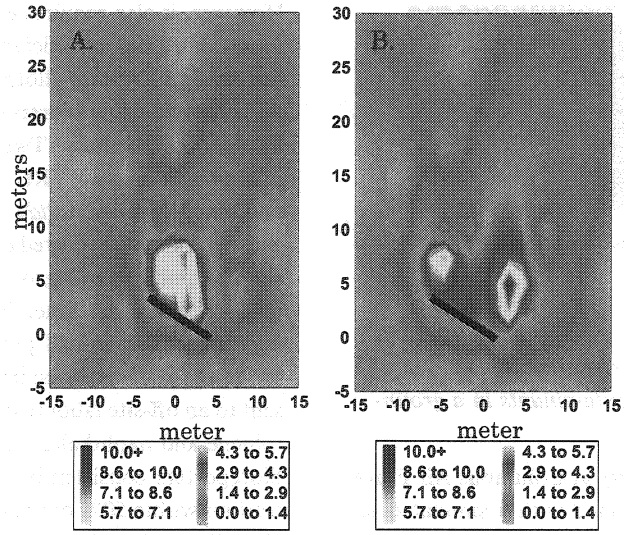

Fig. 7. Distribution and dispersal of toluene contamination in the Columbus Air Force Base aquifer. Concentration (parts-per-million) were determined using the bioreporter *P. putida* TVA8 (A) or using standard GM/MS techniques (B). While not being highly quantitative, the bioreporter could predict toluene concentrations that fell to within 50% of values determined by GC/MS analysis. Plume dynamics in relation to naphthalene concentration were similarly determined using the bioluminescent bioreporter *P. fluorescens* 5RL (data not shown).

effective, quantitative method for rapid and selective detection and monitoring of chemical and biological agents in applications as far ranging as medical diagnostics, precision agriculture, environmental monitoring, food safety, and process monitoring and control. Their attractiveness lies in the fact that they can often be implemented in real-time, on-line bioassays within intact, living cell systems, thus providing a unique and revolutionarily new perspective on bacterial, plant, and mammalian physiology and intracellular interactions. In conjunction with advanced photonic detection technologies such as the BBIC, bioreporters are increasingly becoming important tools for noninvasive monitoring regimes, especially in animal model systems. The monitoring of light requires less time and fewer animals than conventional methods, thus reducing the cost of obtaining biologically relevant data. Consequently, the study of infectious disease, tumor progression and metastasis, gene therapy, mammalian development, and many other areas in which animal models are used as predictors for the human response to therapy can be greatly simplified and accelerated. The same ideals apply in cases of environmental monitoring and food safety, where rapid and remote monitoring using BBIC devices can strategically pinpoint areas of biological hazard, whether in the form of biological warfare agents or pathogenic *E. coli* presence. Further advances in bioreporter genetics and miniaturized optics will clearly impact future monitoring and detection strategies in these fields as well as a host of others.

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