BIOSENSORS

ADVANCED BIOREPORTER TECHNOLOGIES FOR TARGETED SENSING OF CHEMICAL AND BIOLOGICAL AGENTS

Introduction

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 (Figure 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.

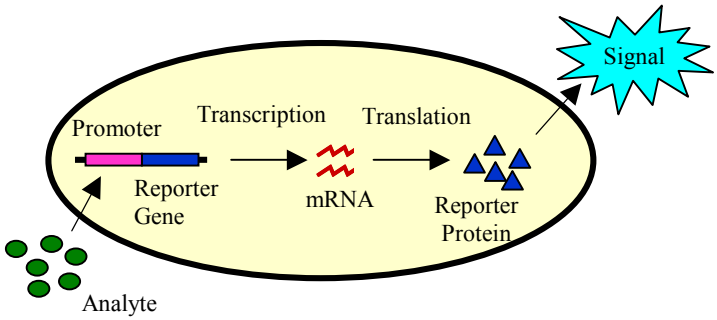

Figure 1. Anatomy of a bioreporter organism. Upon exposure to a specific analyte, the promoter/reporter gene complex is transcribed into messenger RNA (mRNA) and then translated into a reporter protein that is ultimately responsible for generating a signal.

Originally developed for fundamental analysis of factors affecting gene expression, bioreporters were early on applied for the detection of environmental contaminants[1] 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.

Reporter Gene Systems

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.

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 (Figure 2)[2]. Three variants of *lux* are available, one that functions at < 30ºC, another at < 37ºC, and a third at < 45ºC. 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.

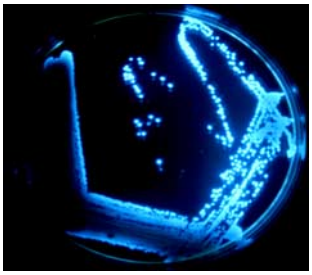

Figure 2. Bioluminescence emitted from individual colonies of microbial cells containing the genes for bacterial luciferase.

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. Table 1 lists some of the chemical and biological agents capable of detection by *luxAB*-based bioreporters.

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 nor 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 2 illustrates the widespread application of *luxCDABE* bioreporters.

Nonspecific lux bioreporters: Nonspecific *lux* bioreporters are typically used for the detection of chemical toxins. They are usually designed to continuously bioluminesce. Upon exposure to a chemical toxin, either the cell dies or its metabolic activity is retarded, leading to a decrease in bioluminescent light levels. Their most familiar application is in the Microtox® assay where, following a short exposure to several concentrations of the sample, the decreased bioluminescence can be correlated to relative levels of toxicity[3].

Firefly luciferase (Luc): Firefly luciferase catalyzes a reaction that produces visible light in the 550 – 575 nm range. A click-beetle luciferase is also available that produces light at a peak closer

to 595 nm. Both luciferases require the addition of an exogenous substrate (luciferin) for the light reaction to occur. Numerous *luc*-based bioreporters have been constructed for the detection of a wide array of inorganic and organic compounds of environmental concern. Their most promising application, however, probably lies within the field of medical diagnostics. Insertion of the *luc* genes into a human cervical carcinoma cell line (HeLa) illustrated that tumor-cell clearance could be visualized within a living mouse by simply scanning with a charge-coupled device camera, allowing for chemotherapy treatment to rapidly be monitored on-line and in real-time[4]. In another example, the *luc* genes were inserted into human breast cancer cell lines to develop a bioassay for the detection and measurement of substances with potential estrogenic and antiestrogenic activity[5].

Aequorin: Aequorin is a photoprotein isolated from the bioluminescent jellyfish *Aequorea victoria*. Upon addition of calcium ions (Ca^{2+}) and coelenterazine, a reaction occurs whose end result is the generation of blue light in the 460 - 470 nm range. Aequorin has been incorporated into human B cell lines for the detection of pathogenic bacteria and viruses in what is referred to as the CANARY assay (Cellular Analysis and Notification of Antigen Risks and Yields)[6]. The B cells are genetically engineered to produce aequorin. Upon exposure to antigens of different pathogens, the recombinant B cells emit light as a result of activation of an intracellular signaling cascade that releases calcium ions inside the cell.

Green fluorescent protein (GFP): Green fluorescent protein (GFP) is also a photoprotein isolated and cloned from the jellyfish *Aequorea victoria*[7]. Variants have also been isolated from the sea pansy *Renilla reniformis*. GFP, like aequorin, produces a blue fluorescent signal, but without the required addition of an exogenous substrate. All that is required is an ultraviolet light source to activate the fluorescent properties of the photoprotein. This ability to autofluoresce makes GFP highly desirable in biosensing assays since it can be used on-line and in real-time to monitor intact, living cells. Additionally, the ability to alter GFP to produce light emissions besides blue (i.e., cyan, red, and yellow) allows it to be used as a multianalyte detector. Consequently, GFP has been used extensively in bioreporter constructs within bacterial, yeast, nematode, plant, and mammalian hosts. Table 3 lists some examples of GFP applications in mammalian cell systems, where its use has revolutionized much of what we understand about the dynamics of cytoplasmic, cytoskeletal, and organellar proteins and their intracellular interactions.

Uroporphyrinogen (Urogen) III Methyltransferase (UMT): UMT catalyzes a reaction that yields two fluorescent products which produce a red-orange fluorescence in the 590 - 770 nm range when illuminated with ultraviolet light[8]. So as with GFP, no addition of exogenous substrates is required. UMT has been used as a bioreporter for the selection of recombinant plasmids, as a marker for gene transcription in bacterial, yeast, and mammalian cells, and for the detection of toxic salts such as arsenite and antimonite.

Detecting the Optical Signature

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 into high-throughput readers. As these usually consist of large, table-top devices, demand for smaller, portable light readers for remote monitoring has

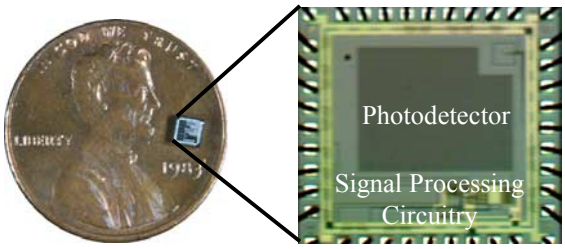

Figure 3. The integrated circuit photoluminometer. Actual size is 2 mm x 2 mm.

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 (Figure 3)[9]. 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 (Figure 4). Remote frequency (RF) transmitters can also be incorporated into the overall integrated circuit design for wireless data 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.

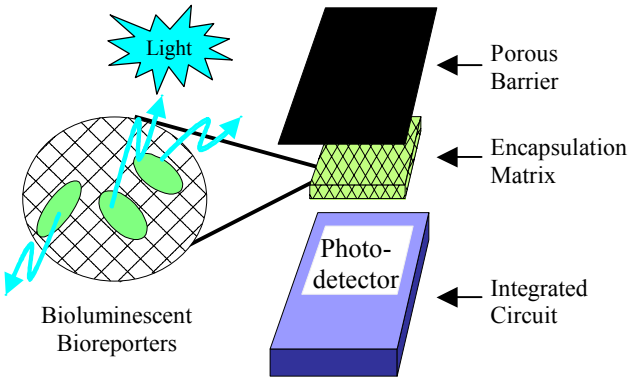

Figure 4. Assembly of a bioluminescent bioreporter integrated circuit (BBIC) sensor.

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.

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[10]. 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[11,12]. 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 (Figure 5). 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.

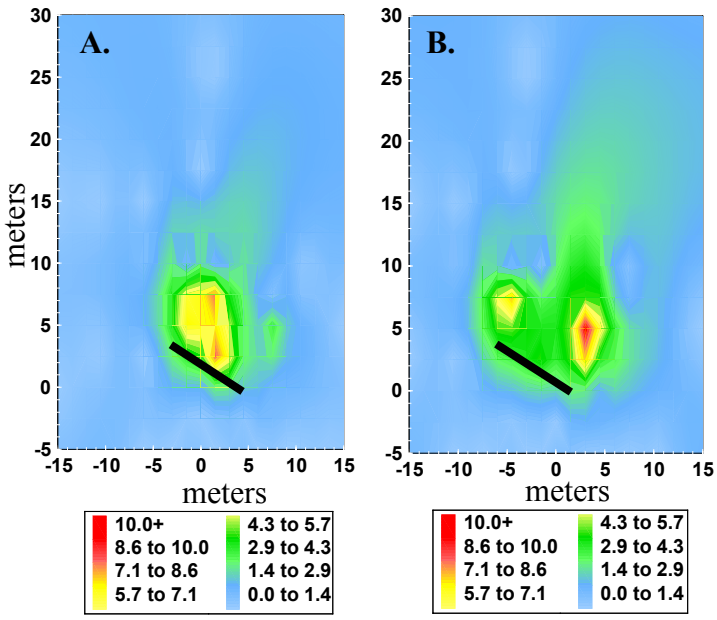

Figure 5. Distribution and dispersal of toluene contamination in the Columbus Air Force Base aquifer. Concentrations (parts-per-million) were determined using the bioreporter *P. putida* TVA8 (A) or using standard GC/MS techniques (B). While not being highly quantitative, the bioreporter could predict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

Benefits

Bioreporter technology will provide a robust, cost-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.

Table 1. Chemical and biological agents detectable by *luxAB*-based bioreporters

Antibiotic effectiveness	Gene expression/regulation
Antimicrobial agents	Growth phase regulation
Bacterial biofilms	Immunoassays
Bacterial biomass	In vivo expression technology (IVET)
Bacterial stress response	Industrial waste runoff
Bacterial transport mechanisms	Metabolic regulation
Bioremediation process monitoring	Mutagenicity tests
Cell viable counts	Plant pathogens
Circadian rhythms	Toxicity assays
DNA damaging agents	Tumor burden
Environmental contaminants	Viral infection
Foodborne pathogens	Xenobiotic detection

Table 2. *luxCDABE*-based bioreporters

Analyte	Time for induction	Concentration	Reference
2,3 Dichlorophenol	2 h	50 mg/ L	13
2,4,6 Trichlorophenol	2 h	10 mg/ L	13
2,4-D	20 – 60 min	2 □M – 5 mM	14
3-Xylene	Hours	3 □M	15
4-Chlorobenzoate	1 h	380 □M – 6.5 mM	16
4-Nitrophenol	2 h	0.25 mg/ L	13
Aflatoxin B1	45 min	1.2 ppm	17
Alginate production	1 h	50 – 150 mM NaCl	18
Ammonia	30 min	20 □M	19
Antibiotic effectiveness against *Staphylococcus aureus* infections in mice	4 h	100 CFU	20
BTEX (benzene, toluene, ethylbenzene, xylene)	1 – 4 h	0.03 – 50 mg/L	11
Cadmium	4 h	19 mg/kg	21
Chlorodibromomethane	2 h	20 mg/ L	13
Chloroform	2 h	300 mg/ L	13
Chromate	1 h	10 □M	22
Cobalt	Not specified	2.0 mM	23
Copper	1 h	1 □M – 1 mM	24
DNA damage (cumene hydroperoxide)	50 min	6.25 mg/ml	25
DNA damage (mitomycin)	1 h	0.032 □g/ml	26
Gamma-irradiation	1.5 h	1.5 – 200 Gy	27
Heat shock	20 min	Various, depending on chemical inducer used	28, 29
Hemolysin production	Not specified	5 mM cAMP	30
Hydrogen peroxide	20 min	0.1 mg/L	31
in vivo monitoring of *Salmonella typhimurium* infections in living mice	4 h	100 CFU	32
Iron	Hours	10 nM – 1 □M	33
Isopropyl benzene	1 – 4 h	1 – 100 □M	34
Lead	4 h	4036 mg/kg	21
Mercury	70 min	0.025 nM	35
N-acyl homoserine lactones	4 h	Not specified	36
Naphthalene	8 – 24 min	12 – 120 □M	37
Nickel	Not specified	0.3 mM	23
Nitrate	4 h	0.05 – 50 □M	38
Organic peroxides	20 min	Not specified	31
PCBs	1 – 3 h	0.8 □M	39
p-chlorobenzoic acid	40 min	0.06 g/l	16
p-cymene	< 30 min	60 ppb	40
Pentachlorophenol	2 h	0.008 mg/L	13
Phenol	2 h	16 mg/L	13
Salicylate	15 min	36 □M	37
Tetracycline	40 min	5 ng/ml	41
Trichloroethylene	1 – 1.5 h	5 – 80 □M	42
Trinitrotoluene	Not specified	Not specified	43
Ultrasound	1 h	500 W/cm^2	44
Ultraviolet light	1 h	2.5 – 20 J/m^2	45
Zinc	4 h	0.5 – 4 □M	46

Table 3. Applications of green fluorescent protein (GFP) in mammalian cells

Application	Example
Gene targeting/expression	Monitoring tumor cells in gene therapy protocols Marking spinal neurons to assess their response to various transducers Monitoring production and release of therapeutic drugs from cells and tissues
Viral infection	Identification of HIV in infected cells and tissues
Fluorescence resonance energy transfer (FRET)	Monitoring of protein-protein interactions in living cells
Time-lapse imaging	Examining the lifetime, sorting, and intracellular pathways of proteins in living cells (i.e., in response to drug treatments, antibodies, chemotherapy)

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