THE SCIENCE AND APPLICATIONS OF SYNTHETIC AND SYSTEMS BIOLOGY

Workshop Summary

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Forum on Microbial Threats

Board on Global Health

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The serpent has been a symbol of long life, healing, and knowledge among almost all cultures and religions since the beginning of recorded history. The serpent adopted as a logotype by the Institute of Medicine is a relief carving from ancient Greece, now held by the Staatliche Museen in Berlin.

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"Knowing is not enough; we must apply.
Willing is not enough; we must do."
—Goethe

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SYNTHETIC BIOLOGY AND THE ART OF BIOSENSOR DESIGN

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Introduction

The term "biosensor" refers to a wide variety of devices. The common element is that a biological component provides highly specific recognition of a certain target analyte, and this detection event is somehow transduced to give an easily detectable, quantifiable response, preferably one that can be easily converted to an electrical signal so that the result can be fed to an electronic device

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for signal processing, data storage, etc. The biological component in many biosensors is either an enzyme, as in the glucose-oxidase–based biosensors used for blood glucose monitoring, or an antibody, as in most optical biosensors. Another class of biosensor, sometimes also referred to as a bioreporter, uses living cells as a component. These cells detect the target analyte via some more-or-less specific receptor and generate a detectable response, most commonly by induction of a reporter gene. Many such devices have been reported in the scientific literature, with detection of mercury and arsenic in the environment being particularly common applications. However, very few such devices are commercially available, the best-known examples being the mutagen-detecting devices such as the SOS-Chromotest (Environmental Bio-Detection Products, Inc.) system. Here we discuss the reasons for this gap between promise and delivery, and ways in which the emerging discipline of synthetic biology may lead to a new generation of whole-cell biosensors.

Whole-Cell Biosensors

Whole-cell biosensors, or bioreporters, are living cells that indicate the presence of a target analyte. The most commercially successful by far are nonspecific toxicity sensors based on naturally occurring luminescent bacteria such as *Vibrio harveyi*, *Vibrio (Photobacterium) fischeri*, and *Photobacterium phosphoreum*. Examples include MicroTox (Strategic Diagnostics, SDIX) and BioTox (Aboatox). In these mainly marine organisms, above a certain population density, light is produced continuously by the action of bacterial luciferase (LuxAB), which oxidizes a long-chain aldehyde such as tetradecanal in the presence of $FMNH_2$ and oxygen. Regeneration of the reduced flavin (catalyzed by LuxG) and the aldehyde substrate (catalyzed by LuxCDE) requires NADPH and ATP, so any toxic substance that interferes with metabolism will reduce light emission, which is easily detected using a luminometer.

However, these systems are nonspecific and are only useful for preliminary screening of environmental samples to determine whether or not a toxic substance is present. The potentially more useful class of bioreporter consists of genetically modified microorganisms in which the presence of a specific target analyte is linked to a detectable response. The genetic modification involved in these cases consists of linking the receptor for the target analyte to induction of an easily detectable reporter gene. Commonly used reporter genes are shown in Table A5-1. For recent reviews of such systems, see Belkin (2003), Daunert et al. (2000), Tecon and van der Meer (2008), and van der Meer and Belkin (2010).

Synthetic Biology and Whole-Cell Biosensors

Like “biosensor,” the term “synthetic biology” is widely used by different authors to mean different things. In this context, we are using it to refer to a

TABLE A5-1 Reporter Genes Commonly Used in Whole-Cell Biosensors

Reporter	Characteristics
lacZ (ß-galactosidase)	Chromogenic (X-gal, *o*-nitrophenyl galactoside) and chemiluminescent substrates are available. In *E. coli* host strains with the *lacZΔM15* mutation, only a small peptide representing the missing N-terminus, designated *lacZ′α*, is required.
luxAB (bacterial luciferase)	Blue bioluminescence in the presence of added substrate (a long-chain aldelhyde, usually decanal).
luxCDABE (bacterial luciferase)	As above; presence of *luxCDE* allows biosynthesis of the substrate so that it need not be added to the reaction.
Firefly or click-beetle luciferase	Bioluminescence in the presence of added substrate (D-luciferin). Quantum yield is higher than for bacterial luciferase, but the substrate is much more expensive. Luminescence is normally green, but color variants are now available.
Fluorescent proteins	Fluorescence when stimulated by ultraviolet or visible light. The original green fluorescent protein (GFP), still widely used, is stimulated best by ultraviolet; enhanced green fluorescent protein responds well to blue light, and numerous color variants are now available.

systematic approach to rationalizing genetic modification to make it more like other engineering disciplines, in terms of the use of standardized parts that can be assembled in a modular way to make a variety of different constructs. Since whole-cell biosensors are intrinsically modular, consisting of a recognition element coupled to an arbitrarily chosen reporter, synthetic biology seems well suited to the development of such devices.

This approach to synthetic biology is associated particularly with MIT, BioBricks, the Registry of Standard Biological Parts,[36] and iGEM (the International Genetically Engineered Machine competition).[37] BioBricks (Knight, 2003) are a type of standardized biological "part," consisting of pieces of DNA which conform to a certain standard (defined by a document known as RFC10, available

[36] See http://partsregistry.org/Main_Page.

[37] For more information, see the following: iGEM 2006, University of Edinburgh, http://parts.mit.edu/wiki/index.php/University_of_Edinburgh_2006; iGEM 2007, University of Cambridge, http://parts.mit.edu/iGEM07/index.php/Cambridge; iGEM 2007, University of Glasgow, http://parts.mit.edu/igem07/index.php/Glasgow; iGEM 2007, University of Science and Technology, China, http://parts.mit.edu/igem07/index.php/USTC; iGEM 2008, Harvard University, http://2008.igem.org/Team:Harvard; iGEM 2009, University of Cambridge, http://2009.igem.org/Team:Cambridge; iGEM 2010, Bristol Centre for Complexity Studies, http://2010.igem.org/Team:BCCS-Bristol; iGEM 2010, Imperial College, London, http://2010.igem.org/Team:Imperial_College_London; and iGEM 2010, Peking University, http://2010.igem.org/Team:Peking.

from the BioBricks Foundation[38]) specifying certain characteristics of the ends. Each BioBrick may be a protein-coding region, or some other component such as a promoter, ribosome binding site, transcription termination sequence, or any other piece of DNA that may be useful in making genetic constructs. The essential point of this format is that any BioBrick can, through a standardized procedure, be combined with any other BioBrick to form a new BioBrick, which can then be combined with any other BioBrick, and so on. In this way, quite large and complex constructs can be built up fairly quickly. The Registry of Standard Biological Parts, currently hosted at MIT, was established to store both the DNA of these parts and also associated information such as DNA sequence, performance characteristics, and user experience. The intention was, and is, that this library of BioBricks and associated information should become a valuable resource for synthetic biologists.

To demonstrate the potential of this approach to synthetic biology, the iGEM competition was established in 2005. Each year, interdisciplinary teams of undergraduates consisting of a mixture of biologists, engineers, and computer scientists compete over the summer vacation period to conceive, design, mathematically model, construct, and test novel genetically modified systems made using BioBricks from the Registry, as well as new BioBricks created specifically for the project. All new BioBricks made are deposited in the Registry and are available for use by future teams. iGEM projects, completed on a short timescale by undergraduate students, are generally not nearly as well characterized as systems reported in the peer-reviewed literature; however, they are often based on highly creative ideas and generally include a mathematical modeling component far in excess that usually found in biological publications. They can therefore be a very interesting way to follow possible future application areas in synthetic biology.

Since biosensors are conceptually simple devices with a clear real-world application, and many opportunities for elaboration in terms of novel input and output modalities, in vivo signal processing, and other aspects, they are a popular choice of project, and a number of interesting innovations have been reported as a result of iGEM projects. We refer to a number of these later in this report. All information relating to previous iGEM projects is available via the relevant websites.

Arsenic Biosensors

Arsenic is a particularly attractive target for whole-cell biosensors, in that it is a major groundwater contaminant in Bangladesh, West Bengal, and a number of other regions (Meharg, 2005; Smith et al., 2000). This only came to light in the 1980s, and it is a major and increasing public health issue. The problem initially arose when, to combat waterborne diarrheal diseases caused by consumption of contaminated surface water, nongovernmental organizations drilled some millions of tube wells to supply clean drinking water. Unexpectedly, it was

[38] See http://www.biobricks.org/.

discovered some years later that many of these produced water with unacceptably high levels of arsenic. The current recommended World Health Organization (WHO) limit for drinking water is 10 ppb arsenic, while Bangladesh and some other countries maintain an earlier limit of 50 ppb, but many wells exceed this by a large margin. Chronic consumption of water with high arsenic concentrations leads to arsenicosis, resulting in skin lesions and various cancers. Thus, there is a clear and present need for cheap and simple tests for monitoring arsenic levels in drinking water. Current field test kits are based around the Gutzeit method, which involves reduction of arsenate to toxic arsine gas, and the detection of this as a color spot following reaction with mercuric salts. Such tests reportedly are unreliable at low but still significant arsenic concentrations, and disposal of mercuric salts poses its own environmental issues. Thus there is a clear potential niche for a simple arsenic biosensor device (Diesel et al., 2009; M. Owens, Engineers Without Borders, personal communication).

Most early arsenic biosensors were based on the arsenic detoxification operons of *Staphylococcus* plasmid pI258 and *Escherichia coli* plasmid R773. The former consists simply of the *ars* promoter controlling genes *arsR*, *arsB*, and *arsC*, encoding the repressor, arsenite efflux pump, and arsenate reductase, respectively, whereas the latter has a relatively complex structure, *arsRDABC*, with two separate repressors, ArsR and ArsD, which control the operon with different affinities (Oremland and Stolz, 2003). ArsD is also reported to act as a metallochaperone, carrying arsenite to the efflux pump formed by ArsAB (Lin et al., 2006). Later systems were based on the simpler *E. coli* chromosomal arsenic detoxification operon (Cai and DuBow, 1996; Diorio et al., 1995), which consists of the *ars* promoter followed by *arsR*, *arsB*, and *arsC*, and the similar operon of *Bacillus subtilis* (Sato and Kobayashi, 1998), which is discussed further below. In either case, the preparation of the biosensor organism is straightforward—the reporter gene is simply inserted adjacent to the controlled promoter so that induction of the promoter results in expression of the reporter gene, giving an easily detectable signal (usually a color change, luminescence, or fluorescence). A number of systems are reportedly at or near commercialization; for example, the Aboatox BioTox Heavy Metal Assay kits, developed at the University of Turku, use *E. coli* as host, with firefly luciferase as the reporter gene. Stocker et al. (2003) described production of a set of *E. coli*–based arsenic bioreporters using ß-galactosidase with a chromogenic substrate, bacterial luciferase, or green fluorescent protein (GFP) as reporter; one of these, based on the luciferase reporter gene, has been field tested in Vietnam (Trang et al., 2005) and was reported to give good results in comparison to chemical field tests. Whole-cell arsenic biosensors have been reviewed recently by Diesel et al. (2009).

The Edinburgh Arsenic Biosensor

One example which we consider in some detail is the first biosensor project to be submitted to iGEM: the arsenic biosensor submitted for iGEM 2006 by the

team from the University of Edinburgh, under the supervision of C. French and A. Elfick. (K. de Mora was a student member of this iGEM team.) The intention was to develop a device that would be suitable for field use in developing countries. It should therefore be cheap, simple to use, and should deliver an output that could be assessed by eye, without requiring expensive electrical equipment, but should also give a quantifiable response using cheap equipment where this was required. For this reason the standard reporters based on luminescence and fluorescence were not considered appropriate. Instead, it was decided that the output should be in the form of a pH change. This would give a bright and easily assessed visible response using a pH indicator chemical, and it could also give a quantitative electrical response using a cheap glass pH electrode or similar solid-state device (ISFET).

For practical reasons, it was decided to use a standard laboratory host strain of *Escherichia coli*. Such organisms are easy to manipulate, carry multiple disabling mutations that make them harmless to humans and unable to propagate in the environment, and grow well at temperatures up to 45°C. *E. coli* and related organisms naturally ferment a variety of sugars, including lactose, via the mixed acid pathway, resulting in the production of acetic, lactic, and succinic acids, which can rapidly lower the pH of the medium below 4.5. Many laboratory strains carry a deletion in the gene *lacZ*, encoding ß-galactosidase, the initial enzyme of lactose degradation, so that a nonfunctional truncated LacZ protein, missing the first few amino acids, is produced. This can be complemented by a short peptide known as the alpha peptide, consisting of the first 50 to 70 amino acids of LacZ. This is encoded by a short open reading frame known as *lacZ′α*. Thus, to generate an acid response, it was only necessary to use such a lactose-defective host strain, such as *E. coli* JM109, and place the *lacZ′α* gene under the control of the *ars* promoter in a standard Registry multicopy plasmid, pSB1A2. Thus, in the presence of arsenate or arsenite, expression of the LacZ alpha peptide would be induced, complementing the truncated LacZ and allowing rapid fermentation of lactose to acids, lowering the pH. To generate an alkaline response, the urease genes, *ureABC*, of *Bacillus subtilis* (Kim et al., 2005) were chosen. (Uropathogenic strains of *E. coli* also possess urease genes, but these are longer and more complex than those of *B. subtilis*.) Expression of these genes allows conversion of urea to ammonia and carbon dioxide and can raise the pH of the medium above 10. Both acid- and alkali-producing systems were tested and were found to work well (Aleksic et al., 2007).

The original design of the arsenic biosensor submitted for iGEM 2006 was a complex system, with a multistage output. This is discussed further below. However, the practical demonstration provided consisted only of the acid-generating system, which was found to give robust and reliable responses to arsenic concentrations as low as 2.5 ppb, with the time of pH change being related to the arsenic concentration in a simple and reproducible way (Aleksic et al., 2007; Joshi et al., 2009). This construct is available from the Registry of Standard Biological Parts (BBa_J33203), as are its components, the *ars* promoter and associated *arsR* gene

(BBa_J33201) and the *lacZ′*α reporter gene (BBa_J33202). Interestingly, during early testing to determine whether buffer ions expected to be present in groundwater might interfere with the pH response, we found that bicarbonate ions actually increase the sensitivity of the response, leading to induction at much lower arsenate levels (Joshi et al., 2009). The reason for this is not clear, but it may be due to altered speciation or uptake of arsenate (de Mora et al., 2011).

The original concept for this biosensor system involved use of a universal pH indicator solution which gives a strong color response—blue in alkaline conditions, green in neutral conditions, and red in acidic conditions—coupled with quantitation via a glass pH electrode. However, it became apparent that the red component of the universal pH indicator, as well as pure methyl red, were rapidly bleached in the presence of living cells under the conditions used. This was therefore replaced with bromothymol blue, which is blue under alkaline conditions and yellow under acid conditions, with pKa around 7.3 (Figure A5-1).

For quantitative monitoring of multiple samples simultaneously, as might be useful in a local or regional testing laboratory, an inexpensive system was developed based on the use of freeze-dried cells together with a webcam; following aseptic addition of groundwater samples to freeze-dried cells and sterile medium, the webcam would monitor the color of multiple tubes simultaneously, and software would extract the pixels representing the tubes and monitor the color of each over time. From these data, the time of color change could be extracted, and this was found to correlate well with arsenic levels in model groundwaters

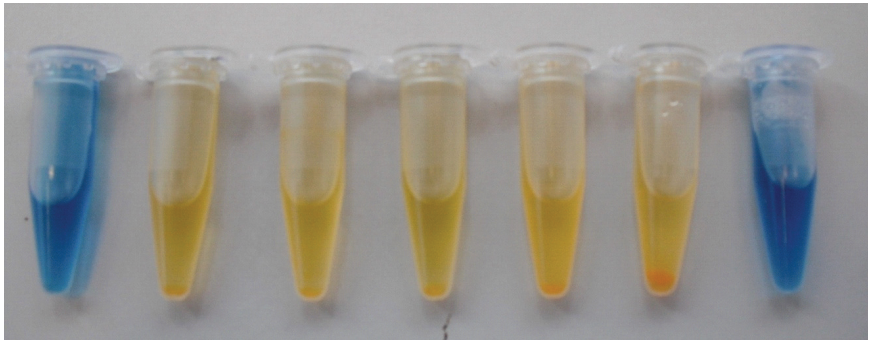

FIGURE A5-1 Demonstration of the Edinburgh pH-based arsenic biosensor, *Escherichia coli* JM109/pSB1A2-BBa_J33203 with bromothymol blue as pH indicator, following static overnight incubation. From left to right: arsenic-free control; 5, 10, 25, 50, and 100 ppb arsenic as sodium arsenate; and cell-free control with 100 ppb arsenate. Note the increasing size of the cell pellet in tubes with increasing arsenic concentrations. Color change occurs more rapidly in samples with increasing arsenic concentration (not shown). SOURCE: C. French, unpublished.

and also in real arsenic-contaminated groundwater samples from Hungary (de Mora et al., 2011).

This system seems well suited for use in water quality laboratories at a local or regional level. However, our original aim was to develop a system that could be used by relatively unskilled users in the field, so that local people could easily monitor the quality of their own well water. This poses several further issues. One technical issue is that any contamination with lactose-degrading bacteria will lead to false-positive results. To avoid this, water samples must be sterilized prior to introduction into the test device. To overcome this problem, we envisage a disposable plastic device containing freeze-dried cells and medium components, with an integral sterile filter through which the water sample is introduced. A second potential complicating factor is the temperature dependence of the rapidity of color change. This requires further investigation. Storage lifetime under relevant conditions also requires further research. However, we are confident that these issues can be overcome, and that this system can form the basis of a simple, cheap, sensitive, and reliable field test for arsenic concentration in groundwater.

One nontechnical issue which must first be addressed is the regulatory and safety issues associated with use of live genetically modified bacteria outside of a contained laboratory context. As noted above, the host strains used are disabled and are unable to colonize humans or to propagate in the environment in competition with wild-type bacteria (Chart et al., 2001). Nevertheless, depending on the jurisdiction, there are many regulatory hurdles associated with the use of genetically modified mciroorganisms in poorly contained applications. This is discussed further below. In the meantime, we are focusing our attention on development of a device suitable for use in local or regional laboratories. A company, Lumin Sensors, has been formed to explore these possibilities (www.luminsensors.com).

In the remainder of this paper, we consider some of the ways synthetic biology can improve the performance of whole-cell biosensors and lead to the development of a new generation of devices with improved performance.

Better Biosensors Through Synthetic Biology

Alternative Host (Chassis) Organisms

Synthetic biology generally involves the introduction of a new genetic system into a host organism, which in the context of synthetic biology is often called a "chassis." The great majority of whole-cell biosensors reported in the literature have used *E. coli* as a host organism, due to familiarity, ease of manipulation, and availability of a wide variety of vector systems and other tools. However, *E. coli* has a number of characteristics which may mean that it is not necessarily the ideal chassis for any given purpose. For example, as a Gram-negative bacterium, it produces and sheds lipopolysaccharide (endotoxin), a powerful activator of the innate immune system; hence, it is generally unsuitable for any in vivo uses. Its

outer membrane prevents large analytes such as peptides from approaching the cell membrane. Unlike many other bacteria, it generally does not secrete most proteins effectively into the medium, unless specific modifications are introduced to allow it. Most significantly in terms of biosensor applications, *E. coli* does not naturally produce dormant states such as spores, meaning that freeze drying is likely to be required for storage and distribution. While freeze drying of bacterial cells is a well-understood process, this adds an extra level of complexity and expense to the manufacturing process. Some other potential hosts have much better characteristics in this regard. Most attention has been paid to *Bacillus subtilis*, a low-GC Gram-positive soil bacterium. *B. subtilis* is used as a model Gram-positive bacterium; its physiology is therefore well studied, and a number of vector systems and other tools are available, though in both characteristics it lags well behind *E. coli*. In contrast to *E. coli*, *B. subtilis* is naturally competent during a certain stage of its life cycle, and, unlike *E. coli*, will happily take up large pieces of linear DNA and integrate them onto its chromosome by homologous recombination. Also, and more importantly, *B. subtilis* naturally forms a dormant resting state known as endospores (Errington, 2003). When conditions become unfavorable for growth, each cell undergoes an asymmetrical cell division resulting in a large "mother cell" and a small "forespore." The mother cell engulfs the forespore and produces layers of protein coats to surround it, while the forespore produces small acid-soluble proteins and calcium dipicolinate, which act to protect its nucleic acids. When this process is complete, the mother cell lyses to release the mature endospore. The spores can simply be harvested and dried for storage and distribution. Endospores are extremely tolerant to heat, drying, and other stresses. There are well-attested reports of endospores surviving for hundreds and even thousands of years in dry conditions (Nicholson et al., 2000), as well as more controversial reports of survival for many millions of years in unusual contexts such as in the guts of insects preserved in amber (Cano and Borucki, 1995).

B. subtilis possesses an arsenic detoxification operon similar to that found on the *E. coli* chromosome (Sato and Kobayashi, 1998); thus, arsenic biosensors can be prepared in a similar way to that described above, either by adding the *ars* promoter and *arsR* regulatory gene to a reporter gene on a multicopy plasmid, or by introduction of such a reporter gene to the chromosome downstream of the *ars* promoter. As a demonstration of principle, we have constructed such a system, designated a "Bacillosensor," using the plasmid vector pTG262 and the reporter gene *xylE* of *Pseudomonas putida*, which encodes catechol-2,3-dioxygenase. This enzyme acts on the cheap substrate catechol to produce a bright yellow compound, 2-hydroxy-*cis,cis*-muconic semialdehyde. This system was found to be sensitive to arsenic levels well below the WHO recommended limit of 10 ppb (Figure A5-2). Spores could be boiled for 2 minutes prior to use in the assay; this would not only kill competing organisms but also activate the spores for rapid germination. The ability to remove most contaminating organisms from

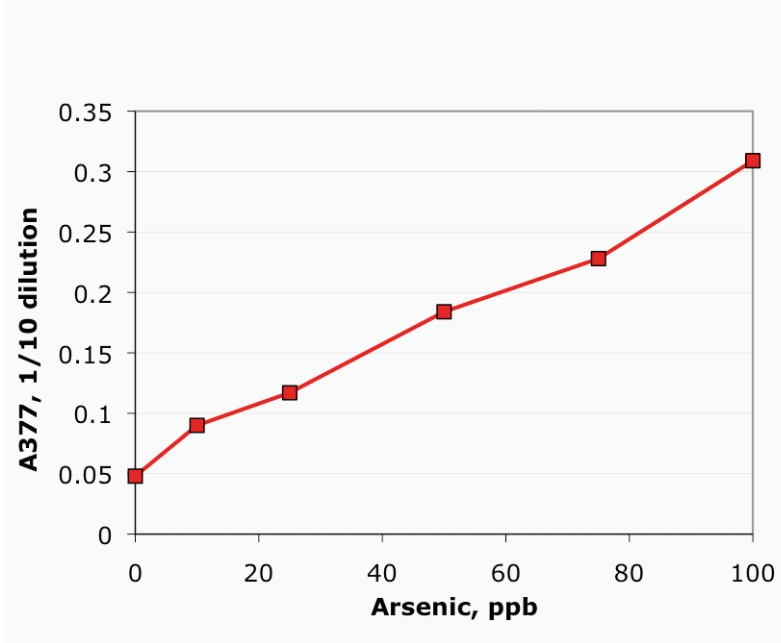

FIGURE A5-2 Detection of arsenic by *B. subtilis* 168/pTG262-*arsR-xylE*: absorbance at 377 nm vs. arsenic concentration (ppb arsenic as sodium arsenate). The vector and BioBrick components used to make this device (BBa_J33206, BBa_J33204) are available from the Registry of Standard Biological Parts. Conditions of the assay were as described by Joshi et al. (2009).
SOURCE: L. Montgomery and C. French, unpublished.

the sample simply by heat treatment offers a considerable potential advantage for field use of such devices, eliminating the need for filter sterilization or similar treatments.

Several reports in the literature have also described bioreporters based on *B. subtilis* and related organisms. Tauriainen et al. (1997, 1998) reported the use of *B. subtilis* as a host for firefly luciferase-based bioreporters for arsenic, antimony, cadmium, and lead but did not specifically describe the use of endospores in the assays. More recently, Date et al. (2007) reported the construction of bioreporters for arsenic and zinc based on endospores of *B. subtilis* and *Bacillus megaterium*, with ß-galactosidase plus a chemiluminescent substrate, or enhanced green fluorescent protein, as reporter genes. The genetically modified spores could be stored at room temperature for at least 6 months. The same authors later described incorporation of such endospore-based bioreporters into microfluidic devices (Date et al., 2010, discussed further below). Fantino et al. (2009) reported the construc-

tion of a device designated "Sposensor," incorporating *B. subtilis* endospores engineered to produce ß-galactosidase in response to the target analyte. Systems responsive to zinc and bacitracin were demonstrated using a chromogenic substrate with spores dried on filter paper discs.

Another potentially interesting host is the yeast *Saccharomyces cerevisiae*. Again, this is a model organism, well studied, and for which numerous vector systems and other genetic modification tools are available. It can be stored and distributed in a "dry active" state (Baronian, 2003). However, as a eukaryote, *S. cerevisiae* has more complex regulatory systems than bacteria such as *E. coli* and *B. subtilis*, and to date there have been relatively few reports of its use as a host for bioreporter applications. One example is a nonspecific toxicity reporter described by Välimaa et al. (2008), using *S. cerevisiae* modified to produce firefly luciferase; as in the MicroTox system described above, toxic substances reduced the level of luminescence observed. *S. cerevisiae* has also been used as a platform for analyte-specific biosensors. For example, Leskinen et al. (2003) reported construction of a yeast-based bioreporter for copper ions, with firefly luciferase under control of the copper-responsive CUP1 promoter. This was used in environmental analysis for bioavailable copper (Peltola et al., 2005). Some further examples are described by Baronian (2003). With further development, analyte-specific yeast-based biosensors could be a useful addition to the biosensor toolkit.

Detection of Extracellular Analytes

In the examples discussed so far, the signal has been generated internally, either as a stress response (as in the case of the SOS-Chromotest) or else by binding of an intracellular protein, such as ArsR, to an analyte that has been internalized (arsenate is probably taken up in error by the phosphate uptake machinery). For medical applications, it would be advantageous to be able to detect and respond to analytes such as peptides, which do not naturally enter bacterial or fungal cells. However, bacteria are able to sense and respond to extracellular analytes via "two-component" systems. In these cases, one component is a sensor kinase that spans the cell membrane, and the second is a response regulator protein that binds DNA to activate or repress transcription from a given promoter. The extracellular analyte binds to the extracellular domain of the sensor kinase, and this increases or decreases the kinase activity of the intracellular domain. This alters the tendency of the kinase domain to phosphorylate the response regulator protein, which in turn alters its propensity to bind to and activate or repress the promoter(s) in question. Most bacteria possess multiple two-component sensor systems responding to a variety of different stimuli, including osmotic strength of the extracellular medium, extracellular phosphate levels, and the presence of various small molecules such as sugars and amino acids. One interesting subgroup consists of two-component sensor systems in Gram-positive bacteria such as *Bacillus*, *Streptococcus*, and *Enterococcus*, which sense and respond to the pres-

ence of short-peptide pheromones produced by other cells of the same species, a phenomenon analogous to the more familiar N-acyl homoserine lactone–based quorum-sensing systems of Gram-negative bacteria. One might imagine that such systems could be modified, perhaps by rational engineering or directed evolution, to respond instead to some peptide of analytical interest.

There are two interesting points regarding two-component sensor systems. One is that these systems show a surprising degree of modularity. A number of reports have described cases where the extracellular domain of one sensor kinase has been fused to the intracellular domain of another, giving a hybrid protein that responds to the normal stimulus of the first sensor kinase by activating the normal response regulator of the second. One well-known example is the report of Levskaya et al. (2005) describing fusion of the extracellular domain of the light-sensing domain of a cyanobacterial phytochrome, Cph1, to the intracellular domain of an *E. coli* osmotic stress sensor, EnvZ; the hybrid protein, Cph8, responded to red light by activating the response regulator, OmpR, which normally responds to EnvZ. When a promoter controlled by OmpR was fused to a pigment-producing gene, the resulting genetically modified cells responded to red light by producing a pigment, allowing "bacterial photographs" to be made by focusing images onto a plate of the bacteria (Levskaya et al., 2005). Another example is fusion of the extracellular domain of a chemotaxis receptor, Trg, which responds to the presence of ribose, among other chemoattractants, by controlling the cell's motility apparatus, to the intracellular domain of EnvZ (Baumgartner et al., 1994). (In this case, the interaction is indirect: ribose binds to periplasmic ribose binding protein, which then interacts with the extracellular domain of Trg.) In the presence of the hybrid protein, designated Trz, an OmpR-controlled promoter (specifically, the *ompC* promoter) was found to respond to the presence of ribose. Recent structural studies have begun to offer some insight into the basis of communication between extracellular and intracellular domains in two-component sensor kinases (Casino et al., 2010), which may allow more rational engineering of such hybrid proteins.

The second point is that it is possible to engineer the recognition elements of such sensors to respond to nonnatural target molecules. For example, Looger et al. (2003) reported rational reengineering of the ribose binding protein, which binds ribose and interacts with the extracellular domain of the hybrid Trz sensor kinase mentioned above, so that it would respond to lactate or to 2,4,6-trinitrotoluene (TNT). In the presence of these molecules, OmpR-controlled promoters were reported to be activated. This opens the possibility that it might be possible to use such a platform as a "universal bioreporter" by generating a library of reengineered sensor kinases or associated binding proteins to respond to any analyte of interest. If such rational reengineering proved challenging in the general case, another interesting possibility would be to attempt fusion of the extracellular domain of such a sensor to some binding molecule such as a nanobody (variable region of a camelid heavy-chain-only antibody) or scFv fragment of an antibody,

so that any analyte to which an antibody could be raised could be detected by a bioreporter. Other protein-based scaffolds for specific recognition are also under development (Hosse et al., 2006; Nuttall and Walsh, 2008) and might also be suitable for such applications. Alternatively, extracellular domains able to bind a desired target analyte might be selected from a library of mutants by a technique such as phage display or cell surface display (Benhar, 2001); this might also enable simple screening for clones which were able not only to bind to the analyte of interest but also to generate the appropriate response on binding.

An interesting, but rather application-specific, approach to the detection of extracellular analytes via two-component sensing systems was proposed by the 2010 iGEM team of Imperial College, London. In this case, the objective was to detect cercaria (larvae) of the parasite *Schistosoma* in water. It was proposed that a protease produced by the parasite could be detected by cleavage of an engineered substrate to release the autoinducer peptide of *Streptococcus pneumoniae*; this peptide would then be detected by the two-component sensor system ComDE of *S. pneumoniae* expressed in *B. subtilis*. A similar system might be used to detect other proteases or hydrolytic enzymes of biological interest.

Another possible platform for detection of extracellular peptides would be the yeast mating peptide sensing system. In *Saccharomyces cerevisiae*, mating peptides (a and α) are detected by G-protein coupled receptors, Ste2 and Ste3, which initiate an intracellular signalling pathway (Bardwell, 2004; Naider and Becker, 2004). As noted above, *S. cerevisiae* is arguably an underexploited platform for biosensor development, with much potential for investigation. One interesting example of a yeast-based sensor system was reported by Chen and Weiss (2005). In this case, a cytokinin (plant hormone) was detected by yeast cells expressing *Arabidopsis thaliana* cytokinin receptor Atcre1, a two-component-type sensor kinase, which apparently is fortuitously able to interact with the endogenous yeast response regulator Ypd1 in the absence of Ypd1's normal sensor kinase partner, Sln1 (Inoue et al., 2001). The engineered "receiver" cells expressed GFP from a Ypd1-activated promoter in the presence of cytokinin.

Animal cells have numerous and varied extracellular receptors but, with current techniques, probably lack the necessary robustness to be generally useful as a biosensor platform except possibly for specialized clinical uses.

Modulation of Sensitivity and Dynamic Range

The critical parameters in the performance of a sensor are the sensitivity (the lowest analyte concentration to which a detectable response is seen) and the dynamic range (in this context, the range of analyte concentrations over which the analyte concentration can be estimated based on the response; that is, between the sensitivity limit and the concentration at which the response saturates). In simple whole-cell biosensors such as those described above, a hyperbolic or sigmoidal response is seen, and it may be that the response curve initially obtained is not in

the desired range for the planned application. Obviously, one critical parameter is the affinity of the receptor for the analyte; however, in practice, many other factors are important, making analysis rather complicated (for a more detailed discussion of such issues, see van der Meer et al., 2004). For example, in the case of arsenic biosensors, arsenate is first taken up by the cell (probably mistakenly, via the phosphate uptake system). The characteristics of uptake determine the ratio between extracellular and intracellular arsenate concentrations. It then interacts with the ArsR repressor with a certain affinity, and interferes with the binding of the repressor to the *ars* promoter; both of these interactions have a characteristic affinity. Within a given cell, the numbers of arsenate anions, ArsR repressor molecules, and promoter sites are also all limited, so the relative numbers of these will also strongly affect the interaction. Finally, when the repressor is not bound to the promoter, and the promoter is thus "active," RNA polymerase will bind the promoter with a certain affinity, and begin transcription with a certain efficiency. The actual level of arsenate repressor and reporter protein molecules generated will be strongly affected by the rate of messenger RNA synthesis, the rate of mRNA degradation, the affinity of ribosomes for the ribosome binding sites on the mRNA, and the degradation rate of the proteins. Thus, many steps intervene between the extracellular arsenate concentration and the level of the reporter protein. This means that, in practice, it is possible to modulate the sensitivity and dynamic range of the sensor without actually altering the receptor at all, simply by making minor changes to factors such as the strength of the ribosome binding sites.

This type of experiment is greatly facilitated by the modular, composable nature of BioBricks and the availability of a library of ribosome binding sites of different strengths in the Registry of Standard Biological Parts. To give one trivial example, we assembled an alternative version of the simple arsenic biosensor construct BBa_J33203, the differences being that the reporter gene was moved to a position between the promoter and the *arsR* gene encoding the repressor, and the native ribosome binding site of *arsR* was replaced by a strong synthetic ribosome binding site, BBa_J15001. The response characteristics of this modified construct were quite different from those of the original construct (Figure A5-3). While the mechanism of this was not investigated, one plausible explanation would be an increased level of expression of the ArsR repressor due to the stronger ribosome binding site.

More profound reorganizations of the arsenic recognition system have also been investigated. For example, a second copy of the ArsR-binding site was introduced between *arsR* and the reporter gene, with the aim of decreasing background expression in the absence of arsenic. This led to considerably improved induction characteristics (Stocker et al., 2003). It was further reported that modification of the activity or synthesis rate of the reporter enzyme (cytochrome c peroxidase or ß-galactosidase) led to strong changes in the system response to given arsenic concentrations (Wackwitz et al., 2008), allowing the generation of an array of

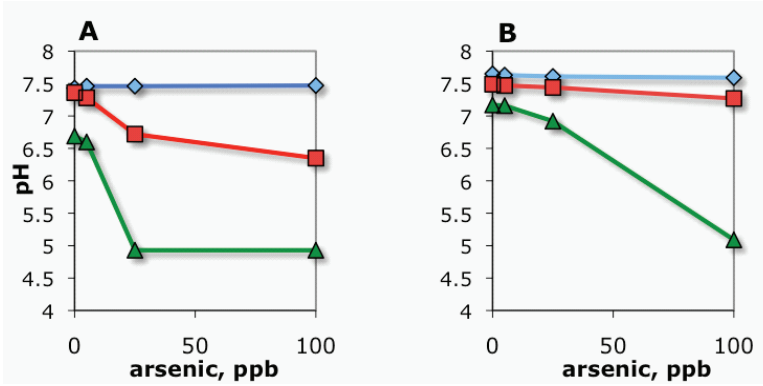

FIGURE A5-3 Altered response characteristics of a whole-cell arsenic biosensor through reassembly of the components. (A) Original Edinburgh arsenic biosensor, consisting of the *E. coli* chromosomal *ars* promoter and *arsR* gene (BBa_J33201) followed by *lacZ′*α (BBa_J33202). Both *arsR* and *lacZ′*α have their native ribosome binding site. (B) Reassembled operon consisting of *ars* promoter (BBa_J15301), strong synthetic ribosome binding site (BBa_J15001), *lacZ′*α coding sequence (BBa_J15103), ribosome binding site (BBa_J15001), and *arsR* coding sequence (BBa_J15101). Diamonds, time zero; squares, 6 hours; triangles, 24 hours. The vector was pSB1A2 and host was *E. coli* JM109 in all cases. Assay conditions were as described by Joshi et al. (2009). All components and assembled constructs are available from the Registry of Standard Biological Parts.
SOURCE: X. Wang and C. French, unpublished.

reporter strains with different response characteristics (discussed further below). In a similar investigation, using mercury biosensors, the 2010 Peking University iGEM team investigated the effects of placing the regulatory gene *merR* under the control of a variety of promoters of different strengths and found that this resulted in a wide variety of different sigmoidal response curves. A similar effect was achieved by screening a library of mutants with altered MerR-binding sites in the mercury-responsive promoter. Thus, a variety of simple modifications to the system can be used to achieve alterations in the sensitivity and dynamic range of such sensors. The composable nature of BioBricks, together with other assembly strategies used in synthetic biology, make it easy to generate and screen a large number of such systems to find a set with the desired characteristics.

It is also possible to use rational design principles to modify the dynamic range of a sensor, for example, by amplifying a weak transcriptional signal. One way to do this is through the use of genetic "amplifiers." One set of such devices, submitted and tested by iGEM teams from the University of Cambridge in 2007 and 2009, consists of bacteriophage activator-promoter pairs. Rather than the

analyte-responsive promoter deriving the reporter gene directly, the promoter drives expression of an activator protein, which activates a second promoter, which controls the reporter gene. This gives a genetic "amplification" effect. A small library of cross-reactive activators and promoters allows a mix-and-match approach to select a pair that gives the desired response characteristics. In these projects, the promoters came from bacteriophages P2 (promoters P_F, P_O, P_P, and P_V) and P4 (promoters P_{sid} and P_{LL}), and the activators from bacteriophages P2 (Ogr), P4 (δ protein), PSP3 (Pag), and ϕR73 (δ protein) (Julien and Calendar, 1996). Fifteen promoter-activator combinations were characterized, allowing the biosensor designer to choose a pair with the desired response characteristics.

The availability of a number of similar biosensors with different response characteristics, as described above, allows the preparation of "bar graph"-like arrays of sensors to obtain a quantitative output over a wider range of analyte concentrations than any single sensor could achieve. This was proposed by Wackwitz et al. (2008), who used the term "traffic light biosensors" to describe such devices. In principle, such devices should not require calibration (van der Meer and Belkin, 2010).

Another issue with induction-based whole-cell biosensors is the time required for induction; few systems in the literature show detectable responses in less than 30 minutes or so, and several hours is more typical. For many applications, it would be advantageous to obtain a faster response. One ingenious approach to this problem was presented by the Imperial College iGEM team of 2010. In this case, the reporter enzyme is presynthesized in the cell in an inactive form. The analyte-responsive promoter drives expression of a protease, which cleaves and activates the reporter. Since each molecule of protease can rapidly activate multiple molecules of the reporter protein, this can potentially give a much faster response. In the case of the Imperial College iGEM project, the reporter, catechol-2,3-dioxygenase (XylE), was synthesised as an inactive fusion with GFP, joined by a linker which could be cleaved by site-specific TEV protease. Detection of the target analyte, in this case a peptide released by a protease of the parasite *Schistosoma*, led to induction of TEV protease expression and consequent cleavage of the linker, allowing rapid formation of active catechol-2,3-dioxygenase tetramers.

In Vivo Signal Processing and Multiplex Output

In addition to tuning of response characteristics, it is possible to introduce more complex forms of in vivo signal processing. One simple example is a genetic inverter, in which a promoter that would normally activate transcription is instead used to repress it. This is accomplished by having the analyte-responsive promoter drive production of a repressor, which represses expression from a second promoter driving the reporter gene. Three well-characterized repressor-promoter pairs are widely used in such systems: the LacI/*lac* promoter pair, bac-

teriophage λ cI/P_L or P_R pair, and the TetR-*tet* promoter pair (see, for example, Elowitz and Leibler, 2000). These repressor-promoter pairs are analogous to insulated wires used in an electronic circuit to communicate between different parts of the device. For complex devices, it would clearly be useful to have more than three such "wires" available. One interesting approach to this was reported by the USTC iGEM team of 2007 (following work described by Sartorius et al., 1989). In this case, mutations were made to the bases of the *lac* operator site involved in binding of the repressor LacI, and also to the amino acids of LacI involved in this binding. The libraries of mutant *lac* promoters and LacI repressors were then analyzed to determine which pairs interacted efficiently. From this experiment, multiple repressor-promoter pairs were chosen that did not crosstalk. Furthermore, these were used to generate biological equivalents of several logic gates. The simplest of these is the NOT gate, in which activation of one promoter leads to repression of another. This is simply achieved by having the first promoter drive expression of a repressor which represses the second, as described above. More complex gates include NAND and NOR. In the former case, binding of two different repressors is required to inactivate the second promoter; in the latter case, binding of either of two repressors is sufficient to achieve this effect. From combinations of such gates, more complex circuits can be assembled. Such logic systems can also be extended to systems consisting of several different types of engineered cell, with the cells communicating via quorum-sensing signals (Tamsir et al., 2010).

The term "traffic-light sensors," discussed above, is also applied to a class of devices that have been proposed and modeled, but, so far as we know, never demonstrated in practice, in which discrete, different outputs are activated at different analyte levels by in vivo signal processing within a single bioreporter organism. The originally proposed iGEM 2006 Edinburgh arsenic biosensor fell into this category, giving an alkaline response at very low arsenate concentrations, a neutral pH at moderate arsenate concentrations, and an acidic response at dangerously high arsenate levels (Aleksic et al., 2007). This was to be achieved through the use of two separate repressors, with different affinities for arsenate, controlling two different reporters: urease for an alkaline response, and ß-galactosidase for the acidic response. Response of the high-affinity repressor-promoter pair was inverted via a repressor, so that the presence of a low concentration of arsenate led to production of a repressor that switched off production of urease, whereas higher levels of arsenate switched on production of ß-galactosidase. A similar arrangement of two different repressor systems can be used to generate a genetic "band detector," which responds only to analyte concentrations within a certain concentration range.

Visual Outputs

While the majority of reported arsenic sensors use luminescence or fluorescence as output, it might be advantageous in some cases to have an output that can be easily detected by eye. The use of enzymes such as ß-galactosidase and catechol-2,3-dioxygenase, together with chromogenic substrates, offers one route to achieving this end. An alternative is the pH-based approach used in the Edinburgh arsenic biosensor described above; this is useful in that pH changes, together with standard pH indicators, give very strong and easily detected color changes, but they can also be quantified using an inexpensive pH electrode. In some cases it might be preferable to have cells produce an endogenous pigment in response to the target analyte. Several such examples have been reported. Fujimoto et al. (2006) described a system in which the photosynthetic bacterium *Rhodovulum sulfidophilum* was engineered to place the endogenous carotenoid pigment gene *crtA* under the control of the *E. coli ars* promoter, so that the presence of arsenite led to a change in cell pigmentation from yellow to red. Subsequently, Yoshida et al. (2008) reported a similar system based on *Rhodopseudomonas palustris*. The combinatorial nature of synthetic biology components, together with the possibility of multistage outputs discussed above, opens the possibility of systems in which a range of discrete colors is produced for different levels or combinations of analytes. To facilitate the construction of such devices, the University of Cambridge iGEM team, 2009, presented a set of modular BioBrick components that could generate a variety of carotenoid and indole-based pigments using different combinations of components from the carotenoid and violacein biosynthetic pathways (Figure A5-4). As with all of the iGEM entries discussed in this paper, these genetic modules are freely available from the Registry of Standard Biological Parts.

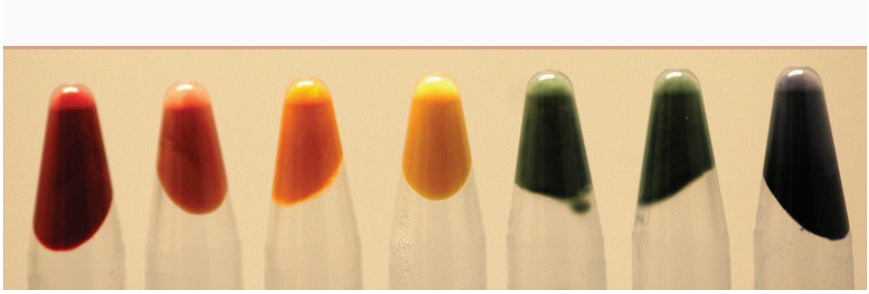

FIGURE A5-4 *Escherichia coli* cells producing a variety of pigments. From left to right, the first four tubes are derived from the carotenoid biosynthesis pathway, the last three from the violacein biosynthesis pathway. All of these pigment-producing pathways are available in BioBrick format from the Registry of Standard Biological Parts.
SOURCE: University of Cambridge, iGEM 2009.

Integration of Biological and Electronic Components

For data processing and storage, it is advantageous if biosensor outputs can easily be converted into electrical signals that can be read by a computer. A number of integrated biological-electrical devices have been reported. One example is the Bioluminescent Bioreporter Integrated Circuit (Nivens et al., 2004; Vijayaraghavan et al., 2007), in which luminescence emitted by bacterial luciferase is detected by a Complementary Metal-Oxide Semiconductor (CMOS) microluminometer incorporated with signal processing circuitry and other relevant components on a "biochip." Several recent reports have described the integration of bioreporter cells into microfluidic devices. For example, Diesel et al. (2009) reported incorporation of an *E. coli* GFP-based arsenic bioreporter into a microfluidic device. Fluorescence from approximately 200 cells within the detection cavity was imaged using a camera. Date et al. (2010) reported the integration of *Bacillus* endospore-based biosensors for arsenic and zinc (described above) into a centrifugal microfluidic platform, in which pumping is provided by rotation of a disc-shaped substrate. Luminescence and fluorescence measurements were performed using separate laboratory instruments with fiber-optic probes held above the detection chambers of the microfluidic device. Storage and germination of endospores, and sensitive detection of the analytes, were reported.

Whereas these devices use "conventional outputs (fluorescence and luminescence), other reporter systems can give an electrical output directly, which may be more convenient for incorporation into simple devices for field use. One example is the Edinburgh arsenic biosensor described above. The pH response can easily be converted to a voltage signal using a standard glass pH electrode, or an equivalent solid-state device (ion selective field effect transistor). An alternative is the generation of an amperometric signal via a device similar to a microbial fuel cell. Bacteria respire by transferring electrons from a donor (such as a sugar) to an acceptor (such as oxygen). This electron transfer occurs at the cell membrane. Many bacteria can instead transfer electrons to or from an electrode, generating an electrical current; others can do so in the presence of electron shuttle molecules known as mediators (Lovley, 2006). An amperometric signal can be generated by placing synthesis of such a mediator, or of an essential component of the electron transfer apparatus, under the control of an analyte-responsive promoter. Both of these approaches have been described by iGEM teams. The iGEM 2007 entry from the University of Glasgow described a system for biosynthesis of pyocyanin, a redox-active metabolite of *Pseudomonas aeruginosa*, which could act as a mediator to transport electrons between the bacterial respiratory chain and an electrode. The iGEM 2008 entry from Harvard University took advantage of a naturally "electricigenic" bacterium, *Shewanella oneidensis*, by controlling synthesis of one of the outer membrane proteins, MtrB, required for efficient transfer of electrons to an electrode. Either of these approaches can allow controllable generation of an electrical current induced by the presence of a target analyte.

Regulatory Issues Related to Field Use of Whole-Cell Biosensors

The Edinburgh arsenic biosensor described above was designed to be used in the field in the form of a contained disposable device. Some other bioreporters have been designed for more direct use in the field. One of the earliest examples to be tested was that of Ripp et al. (2000), reportedly the first genetically modified microorganisms to be approved for field testing for a bioremediation application in the United States, in which a strain of *Pseudomonas fluorescens* modified to produce a luminescent signal in the presence of aromatic hydrocarbons was introduced into the soil in a contaminated site, and its persistence monitored over a 2-year period. While this organism was designed to persist in the environment for a prolonged period, there are other cases where the bioreporter only needs to survive for a few hours—just long enough to report on the conditions in which it finds itself. One well-known example is the Microbial Mine Detection System developed at Oak Ridge National Laboratories. This consisted of a strain of *Pseudomonas* modified to produce GFP in the presence of explosives leaking from land mines (discussed by Habib, 2007). The positions of the land mines could then be mapped using an ultraviolet lamp. This system was reportedly field tested with good results. Another example was reported by the iGEM team of the Bristol Centre for Complexity Studies (BCCS) in 2010. In this case, the bioreporter consisted of *E. coli* cells modified to indicate the presence of nitrate by producing a fluorescent response. These cells, encapsulated in a hydrophilic gel, were to be spread over the soil and left for several hours to express the fluorescent protein, after which the nitrate levels in the field could be mapped by a mobile device, providing information useful in agriculture.

On consideration of the examples described above, we can distinguish between four different cases:

1. bioreporters designed to be used within the confines of a laboratory (e.g., the SOS-Chromotest);
2. bioreporters designed to be used in a contained device, but outside of a laboratory (e.g., the Edinburgh arsenic biosensor);
3. bioreporters designed to be exposed directly to the environment, but not to survive longer than required to report the levels of the target analyte (e.g., the MMDS and Bristol nitrate sensor); and
4. bioreporters designed to survive and persist in the field for long periods (e.g., the bioremediation monitoring system of Ripp et al., 2000).

Unfortunately, current regulatory regimes in the United Kingdom and European Union do not appear to distinguish between cases 2, 3, and 4. Case 1 is unproblematic; such "contained use" applications are dealt with in the United Kingdom by the Health and Safety Executive under the Genetically Modified Organisms (Contained Use) Regulations 2000, whereas "uncontained uses" are dealt with by the Department for the Environment, Food and Rural Affairs un-

der the Genetically Modified Organisms (Deliberate Release) Regulations 2002 (which implement EU Directive 2001/18/EC). Whereas this is wholly appropriate for case 4, where such organisms are designed to persist in the environment, no provision seems to be made for cases 2 or 3, where organisms may be specifically designed not to survive in the environment, thereby minimizing any threat to ecosystems. This is a serious inhibiting factor in the use of genetically modified whole-cell biosensors or bioreporters outside of the laboratory, as expensive field trials with extensive postrelease monitoring are required. We feel that it would be useful if future legislation took this into account. In the United States, the situation is less clear to us; the relevant federal legislation appears to be the Toxic Substances Control Act (Sayre and Seidler, 2005). In the absence of specific legal and regulatory expertise, we will refrain from further comment. However, it is clear that until these regulatory issues are addressed, it will not be possible to use genetically modified bioreporter organisms on a large scale in the field, despite their obvious potential.

Conclusions

Sensitive and highly specific response to various molecules is one of the core functions of biological systems. As such, whole-cell biosensors offer a versatile and widely applicable method for detecting the presence of a wide range of analytes. The techniques of synthetic biology offer numerous possible improvements in terms of response tuning, in vivo signal processing, and direct interface with electronic devices for further signal processing and output. However, regulatory issues will need to be clarified before such devices can fulfill their true potential as highly sensitive, inexpensive sensors for field use.

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