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Novel Fiber Optic Detection Method for *in Situ* Analysis of Fluorescently Labeled Biosensor Organisms

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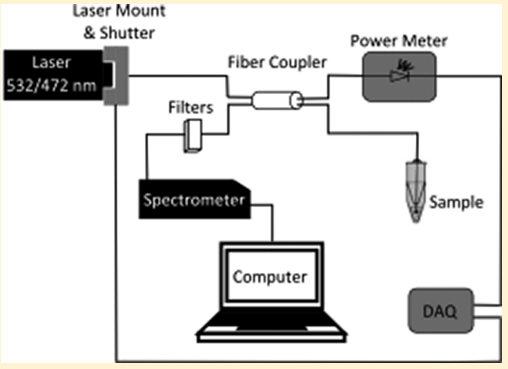
ABSTRACT: This research presents a novel, time-resolved fiber-optic "Optrode" system for accurate real-time in situ detection of fluorescent proteins produced by biosensor organisms. The Optrode fluorescence detection system was able to identify, characterize, differentiate, and quantify red and green fluorescently labeled organisms, individually and in mixed aqueous cultures. Detection was also possible in sand systems, where a consistent reduction in signal intensity indicates that signal collection volume was reduced by one-third. The optrode was shown to be sensitive enough to detect fluorescently labeled cell concentrations of 1.9×10^4 CFU/mL, indicating it is suitable for detecting typical concentrations of degrader organisms reported in bioremediation trials. The effect of fluorophore photobleaching was characterized for different fluorescent proteins and demonstrated a linear relationship to cell concentration, meaning the effect can be accounted for within methods of fluorescence collection and analysis. Proof of concept is provided for all aspects of this research, which represents an important step toward the goal of achieving a complete, nondestructive, in situ monitoring system to characterize all aspects of microbial activity and gene expression.



1. INTRODUCTION

The use of microbial biosensors with fluorescent or bioluminescent reporter genes is a well-established approach for monitoring living systems. Biosensor technology has developed rapidly over recent decades in response to an increasing need for methods to accurately describe microbiological activity, particularly in laboratory modeling scenarios. Biosensor organisms have been applied in a wide range of microbiological scenarios for the identification and quantification of toxic substances, factors influencing gene expression, and the spatial distribution of bacterial cells.[1−4] Highly sensitive biosensor organisms may now be engineered through a variety of novel microbiological techniques to produce reporter proteins simultaneously to the expression of a specific gene.[5] There is no ideal reporter gene for application to all biosensor monitoring scenarios; however reporters have been developed that do not require exogenously applied substrates, can be assayed nondestructively, can be expressed by diverse bacterial species, give strong signals with low background (a high dynamic range and a high signal-to-noise ratio), and can be quantified in the test tube or detected by microscopy.[6] Both bioluminescence and fluorescence based reporter genes have found widespread application within biosensor microorganisms; as a result a wide variety of versatile reporter proteins are now available with varying characteristics and advantages.[7] Bioluminescence systems, where light is produced independently by luciferase-catalyzed reactions, do not require an excitation source for signal response and can give a more real-time representation of biosensor activity due to the relative instability of the Luciferase (lux) proteins in comparison to fluorescent proteins.[8] Fluorescent reporter proteins require an independent source of excitation; however, these systems are encoded by much simpler gene pathways and offer a wider variety of signal wavelengths, including green fluorescent protein (Gfp, with blue light excitation peak emission 509 nm for purified protein)[9,10] and red fluorescent protein (DsRed, with blue light excitation peak emission at around 580 nm and green light excitation emission peak around 600 nm).[11] Fluorescence systems can also be quantified in terms of

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Table 1. Isolates Used in This Research Including Modifications, Reporters, and Antibiotic Resistance

name	description	reporter gene	antibiotic resistance	abbreviated name
P. putida NZRM 852	wild type	n/a[a]	n/a	*P. putida* 852
P. putida NZRM 852-Tn7::*gfp*::*term*	chromosomally integrated transposon cassette from pAKN137 constitutive promoter	*gfp*	Gm, Cm	*P. putida* 852−137
E. coli 536 -gfp	transformed with pfdhL::gfp	*gfp*	Er	*E. coli*-Gfp
E. coli MG1655 - pAKN133	transformed with pAKN133	*DsRed Express*	Km, Sm, Cm	*E. coli*-DsRed

[a]n/a, not applicable.

a single cell response, whereas systems of bioluminescence produce a signal that is at the present time measurable only as a population response.

The use of biosensor technology is crucially limited by the accuracy and applicability of reporter protein detection systems. Traditionally, approaches for fluorescence and bioluminescence detection and quantification have been limited to ex-situ strategies for sample analysis; such as epifluorescence microscopy, fluorescence scanning, and flow cytometry. More recently, Ivis technology has allowed luciferase and fluorescence tagged pathogens to be detected in a range of light penetrable systems including experimental animals.[12] Advances in imaging also allows for spatial distribution and the use of distinguishable dual signals for multiple applications such as bacterial dissemination and immune response. Ivis technology has found widespread acceptance within the pharmaceutical industry; however, it has limitations in that it is expensive and can only be applied to scenarios where microbes are in light penetrable systems. The application of reporter gene technology to monitoring microbial activity requires novel strategies for nondestructive signal detection, particularly in light-impenetrable environments such as soils and similarly heterogeneous environments. The use of fiber-optic probes to deliver excitation wavelengths and collect emission spectra from reporter gene products in situ is an attractive possibility. In particular, the analysis of emission spectra by spectrophotometry may facilitate the quantification of multiple labels,[13] where the use of fiber-optic probes may allow for automated sample collection and remote sensing in situ.

In situ bioremediation has emerged as a promising technique for contaminated soil and groundwater restoration, where the microbial communities enhance pollutant removal through catabolism of target compounds.[14] Bioremediation is governed by biological systems and physicochemical factors (including contaminant solubility and sorption/desorption), the limitations to which must be addressed in establishing effective remedial strategies. Developing models for microbial contaminant degradation activity, distribution and biomass production is a key aspect pertaining to the success of this environmental engineering application. In this study we describe the development of a time-resolved fiber-optic spectroscopic probe ("Optrode") system and evaluate its limits of detection with the intention of assessing its application for real-time monitoring of fluorescent protein activity within bioremediation trial applications. Data analysis algorithms are described which may remove the effects of background noise and photobleaching in fluorescence signal detection.

2. EXPERIMENTAL SECTION

2.1. Bacterial Strain and Culture Conditions. *Pseudomonas putida* NZRM 852 (NZ Reference Culture Collection, Medical Section) was chromosomally labeled with the *gfp* gene under the control of a growth dependent promoter using the mini-Tn7 transposon system as previously described.[15] Transformation of *P. putida* NZRM 852 with helper plasmid pAKN68 (pUX-BF13) and plasmids containing the *gfp* and *DsRed-Express* genes, pAKN137 (miniTn7(Gm)P_{rrnB1} −*gfp*-a) and pAKN133 (miniTn7(Km, Sm)$P_{A1/04/03}$ −DsRedExpress) respectively[15] was achieved through electroporation using the previously described protocol for the transformation of *Pseudomonas aeruginosa*[16] (settings: 25 μF, 200 Ω, 1.8 kV using the Bio-Rad Gene Pulser II).[16] The fluorescent isolates were confirmed to contain the chromosomal transposon insert within the *att*Tn7 site and in the correct orientation using primers designed to the Tn7 right element (Tn7R109; CAG CAT AAC TGG ACT GAT TTCAG) and the *glmS* gene (Tn7*glmS*; AAT CTG GCC AAG TCG GTGAC) (15). This isolate was incubated at a temperature of 28 °C and cultured in L-broth media (LB; contains 30 g/L tryptone, 15 g/L yeast extract and 15 g/L NaCl − supplied by Difco Media) shaking at 200 rpm for liquid culture. Solid media was created via addition of Difco Agar to 1.5% (wt/vol) to Difco LB prior to autoclave sterilization. Transformed colonies were selected on spread plates following electroporation recovery using A.B. triton citrate minimal media (ABTC; A media contains: 2 g/L $(NH_4)_2SO_4$, 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 3 g/L NaCl, 10 mM NaH $(C_3H_5O(COO)_3)$; B media contains: 0.05% Triton, 0.005 mM FeEDTA, 0.01 mM $CaCl_2$, and 1 mM $MgCl_2$ with the appropriate selection antibiotics (For pAKN137 transformants Gentamicin 10 mg/L; and for pAKN133, Kanamycin 50 mg/L was used).[17]

E. coli 536[18] and *E. coli* MG1655[19] were transformed with pfdhL::gfp[20] and pAKN133[15] respectively using a standard electroporation protocol. Liquid cultures were grown at 37 °C, with shaking at 200 rpm, in LB containing Erythromycin (300 mg/L) and Kanamycin (50 mg/L) for pfdhL::gfp and pAKN133 transformants respectively. Solid media was made for colony isolation and storage through the addition of Agar (1.5% wt/vol) to the broth formulations described above.

2.2. Cell Culture Preparation. For the purposes of Optrode trials, *P. putida* 852−137 isolates were grown overnight in sterile 50 mL centrifuge tubes with 10 mL of LB media at 28 °C, with shaking at 200 rpm. *E. coli*-DsRed and *E. coli*-Gfp were cultured overnight in LB media and appropriate selection antibiotics at 37 °C, with shaking at 200 rpm. Cells were harvested by centrifugation (2316 *g*, 5 min) and resuspended twice in equal volumes of saline. 600 μL of these cells were transferred to 1.5 mL amber microcentrifuge tubes (Eppendorf, Inc.). Bacterial cell concentration of each culture was measured using standard (Miles and Misra) cell count protocol[21] and expressed as colony forming units (CFU)/mL.

2.3. Fluorescence Collection and Detection. The Optrode, illustrated in Figure 1, uses 100 mW diode pumped solid state lasers at 473 and 532 nm (Changchun New Industries Optoelectronics Tech. Co., Ltd.) to excite Gfp

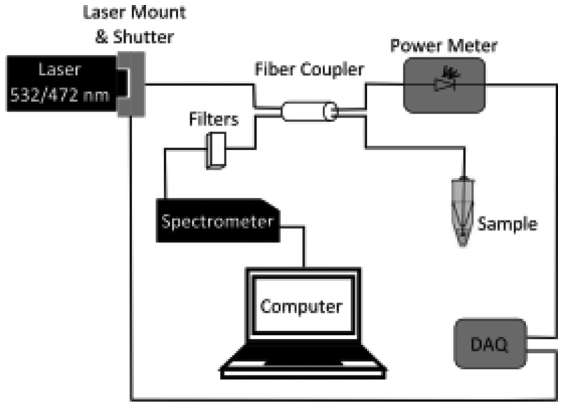

Figure 1. Optrode setup for the detection of fluorescent proteins. Diode pumped solid state lasers are used for excitation. The light is directed to the sample via a multimode fiber coupler, the collected spectrum is then measured by a spectrometer. The laser mount includes a shutter to control excitation timing and duration, and a data acquisition card (DAQ) synchronizes the shutter with the spectrometer.

(excitable at 473 nm) and DsRed (excitable at both 473 and 532 nm) fluorescent proteins. A laser mount (OZ Optics), which includes an electronic shutter to control timing and duration of the excitation, launches the excitation light into an all-silica pyrocoat high OH-, multimode, non-Hermetic optical fiber with core and cladding diameters of 200 and 220 μm respectively. The light from the laser is then split with a 50/50 coupler and directed to the sample to excite biosensor bacteria within the immediate environment or "collection volume" around the optical fiber output or probe. The emitted fluorescence within the collection volume of the probe is collected by the same fiber and measured by the spectrometer (QE65000, Ocean Optics). A long-pass filter is placed before the spectrometer to ensure that the fluorescence signal is measured solely without any residual pump signal. A data acquisition card (DAQ) (USB-1608FS, Measurement Computing) is used to synchronize the shutter with the spectrometer. Illumination power is monitored using a photodiode (10530DAL, Integrated Photomatrix Ltd.). The Optrode is housed in a light proof box for ease of setup and transport. A custom computer acquisition program was designed using LABVIEW 8.2 (National Instruments) to control the hardware and present data in an end-user-friendly format for user analysis.

2.4. Sample Collection Volume. The contribution to fluorescence activity made by an individual biosensor organism at a particular population concentration is determined by the collection volume, which is defined within a cone projection of distance Z (axially) from the fiber tip.[22] The Z_{80} is the distance Z at which 80% of total fluorescence is collected. The full width half minimum (fwhm) at Z_{50} represents the radial diameter of the intensity profile at a distance of Z_{50}. Collection volume is defined as a cone with length Z_{80}, diameter Z_{50}. The fiber used in this investigation had a Z_{80} of 900 μm and fwhm at Z_{50} of 200 μm in liquid media. The collection volume in different soils can be influenced by variations in factors such as soil particle size, particle packing, and organic matter composition. However, as long as the scale of inhomogeneities is not of the order of magnitude of the core fiber, we can correct for the effect of these factors by measuring the background contribution using untreated soil samples. The errors introduced in the analysis are small as long as the effects do not extinguish or overwhelm the response from biosensor organisms. Additionally, the influence of the external factors can be minimized by using the signal from portions of the spectrum where background is minimal. Similarly, spatial data variability can be addressed by taking measurements from a higher density of sampling locations.

2.5. Optrode Trial Protocol. Following a setup stabilization period of 20 min, the power of the specific laser was measured from free end using the photodiode and adjusted to 10 mW. A 1.5 mL amber microcentrifuge tube provided a light proof environment while simultaneously supporting the sample tube. The fiber end was rinsed in pure ethanol and allowed to air-dry before and after each reading.

Substrate microcosms were created using 1.6 g of size 0.0625−2 mm sand, which was sterilized by autoclaving and mixed by inverting 10 times with 400 μL of washed cells resuspended in 0.85% saline at the original concentration or desired dilution, yielding an unsaturated substrate environment consistent with damp sand. For solution readings the probe was submersed in 600 μL of resuspended washed cells at the original concentration or predetermined dilution. Samples were prepared in triplicate.

Background elimination was achieved by taking readings of blank samples containing 600 μL of saline, or in the case of sand as a simplified representative model system for substrate, 1.6 g of sterile sand with 400 μL of saline. Six blank samples were prepared and checked for consistency in terms of emission spectra intensity and normalized spectra characteristics. Custom Labview software was developed to remove the background spectrum from the fluorescent microbe emission spectrum in real time during sample measurement.

2.6. Deconvolution of Multiple Fluorescence Spectra. The use of an Optrode setup with a single laser excitation source offers increased economy, simplicity and lower potential for photobleaching in trial applications. However, in samples which contain multiple biosensor organisms with different reporter genes, fluorophores have specific excitation requirements which reduce the choice of excitation source. Further, maximum emission peak of different fluorescent proteins must be discernible when excited at the same wavelength. Two methods were investigated in order to fit and deconvolute a mixed fluorophore sample into individual components, as described below.

Method 1, the Curve fitting method,[23] involves resolving multiple spectra from different fluorescent proteins (for example a green fluorescent or protein or "GFP" such as Gfp, and a red fluorescent protein or "RFP" such as DsRed) by fitting known spectra curves to the measured spectrum (RAW). The measured raw spectrum is assumed to be made entirely of the known spectra so that it can be broken down into their individual components as

$$\mathrm{RAW}^* = [\mathrm{GFP}^* + \alpha\mathrm{RFP}^*]^* \quad (1)$$

Where the asterisk denotes that the spectra are normalized. The coefficient α is chosen to best fit the measured spectrum by maximizing their goodness of fit. The goodness of the fit is given by the inverse of the standard deviation (σ) of the difference between the fitted curve of GFP + αRFP to the raw spectrum:

$$\text{goodness of fit} = \frac{1}{\sigma(\mathrm{RAW}^* - [\mathrm{GFP}^* + \alpha\mathrm{RFP}^*])^*} \quad (2)$$

Assuming that the concentration, $[GFP]_{ref}$ and $[RFP]_{ref}$, of the reference spectra for GFP and RFP is known, and that the optimal value of α is obtained, the concentration of the measured spectra is given by:

$$[GFP]_{meas} = \frac{\beta \int GFP^*}{\int GFP}[GFP]_{ref}$$

$$[RFP]_{meas} = \frac{\beta \int RFP^*}{\int RFP}[RFP]_{ref} \quad (3)$$

where β = RAW/[GFP* + αRFP*]* is the factor used to convert the normalized spectra back to their original form. This method can be applied to any arbitrary spectral shape as long as information is available for the individual spectra prior to resolving, and only one spectrum is required for deconvolution. However, fitting error may occur due to the assumption that the measured spectrum is comprised entirely of the known reference spectra.

Method 2, the Alentsev-Fok Method,[24] resolves two unknown spectra from a combined fluorescence spectrum. Here, GFP (λ) and RFP (λ) are individual unknown spectra making up a compound spectrum, f1(λ) = GFP (λ) + RFP (λ), where λ is the wavelength. The individual spectra can be determined if they are such that there are two ranges in λ, (λ_1, λ_2) and (λ_1', λ_2'), in which one of the spectra become zero, whereas the other remains nonzero. In addition, f2(x) must exist that consists of the same spectra but with different weights:

$$f2(\lambda) = a\,GFP(\lambda) + b\,RFP(\lambda) \text{ with } a/b \neq 1 \quad (4)$$

When these two conditions are met, the graph for the ratio $F(\lambda) = f2(\lambda)/f1(\lambda)$ should have two horizontal parts whose ordinates are a for $\lambda_1 < \lambda < \lambda_2$ and b for $\lambda'_1 < \lambda < \lambda'_2$. After a and b are known the individual components of GFP (λ) and RFP (λ) can be determined by solving the simultaneous equation from eq 5 for f1 and f2 such that

$$GFP(\lambda) = \frac{bf_1(\lambda) - f_2(\lambda)}{b - a} \text{ and } RFP(\lambda) = \frac{af_1(\lambda) - f_2(\lambda)}{a - b} \quad (5)$$

To check for consistency, the comparison of Alentsev-Fok to the curve fitting method can be made by substitution of eq 5 into eq 1. Theoretically speaking, the value of the coefficient α obtained using the Alentsev-Fok method should match the value obtained using the curve fitting method.

3. RESULTS AND DISCUSSION

3.1. Optrode Fluorescence Detection in Liquid and Substrate Microcosms. Initial Optrode measurements were performed using liquid cultures in order to characterize signal emission spectra in fluorescent *E. coli* and *P. putida* isolates labeled with the *gfp* and *DsRed-Express* genes. Control measurements of unlabeled *E. coli* and *P. putida* parental strains were made and these measurements were subtracted from raw emission spectra of biosensor strains in order to remove the effect of innate microbial fluorescence. Second, Optrode measurements of *E. coli*-Gfp were carried out in representative substrate microcosms and compared to equivalent cell concentrations of the sample culture measured in solution. Identical spectral characteristics were observed in the normalized emission spectra of isolates in both environments; demonstrating that the Optrode is able to accurately remove sand background fluorescence (autofluorescence) from collected emission spectra to reveal an unaltered fluorescence spectra directly attributed to reporter proteins (data not shown).

Measurements of Gfp using laser excitation of 473 nm (blue light) demonstrated an emission spectrum with peaks at 521 (Shoulder at 545 nm) and 531 (shoulder at 560 nm) for *E. coli*-Gfp and *P. putida* 852−137 isolates respectively (Figure 2);

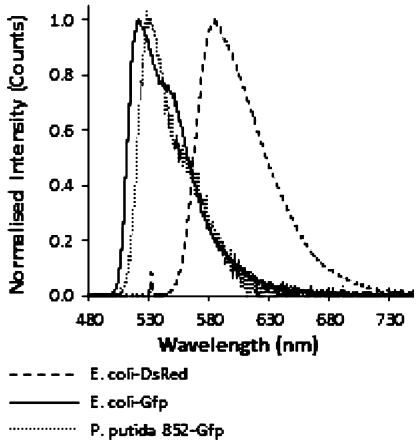

Figure 2. Normalized emission spectra. The emission spectra from *E. coli*-Gfp and *P. putida* 852-Gfp, subjected to excitation at 473 nm, and *E. coli*-DsRed excited at 532 nm following subtraction of matched spectra from unlabeled cells.

higher than generally reported in other research but within the yellow-green classification.[9,10,25] As expected, the Gfp protein emission was not detectable using the 586 nm excitation wavelength (green light) in any tests conducted in this research. Comparisons of all normalized emission spectra collected for *E. coli*-Gfp and *P. putida* 852−137 isolates revealed an upward shift in wavelength for *P. putida* of 8−10 nm across the 500−540 nm wavelength range. This observation has not been reported in the wider literature. The same *gfp* gene was used to transform both isolates, so differences in fluorescence spectra are likely a reflection of minor dissimilarities in intracellular environments between different microbial species. Optrode measurements of the red fluorescence spectrum were taken from *E. coli*-DsRed isolates with excitation at both 473 and 532 nm wavelength laser excitation sources. Both excitation wavelengths demonstrated a characteristic DsRed spectrum, with an excitation peak at 586 nm (Figure 2) which is consistent with previous reports for this fluorescent protein.

3.2. Characterization of Optrode-Induced Photobleaching. All known fluorescent proteins are subject to photobleaching upon extended excitation. There is wide variation in photobleaching rates between different fluorescent proteins, and between fluorescent proteins with similar optical properties. The extent of photobleaching for a reporter protein in a sample is a function of laser power and duration of excitation. The Optrode was used to measure photobleaching rates and characteristics of the fluorescent proteins used in this research in order to validate their direct comparison within trial applications, and to check the assumption that photobleaching rates remain constant at different cell concentrations. Optrode measurements were taken in both liquid and substrate

Table 2. Calculated Parameters of Photobleaching with *E. coli*-Gfp Labelled Isolates in Sand and Saline and the Effect of Dilution on the Photobleaching of *E. coli*-DsRed

sample	dilution factor (dimensionless)	fraction of signal intensity at steady state (dimensionless)	decay constant k (/s)	signal half life $t_{(1/2)}$ (s)	fraction of signal intensity at $t_{(1/2)}$ (dimensionless)
E. coli-Gfp in Saline	neat	0.93	2.38	0.29	0.965
E. coli-Gfp in Sand	neat	0.61	0.37	1.87	0.806
E. coli-DsRed in Saline	neat	0.71	0.72	0.96	0.857
E. coli-DsRed in Saline	1/10	0.72	0.58	1.19	0.860
E. coli-DsRed in Saline	1/100	0.68	0.72	0.96	0.840

Table 3. Sensitivity Data of the Fluorescently Labelled Isolates Used in This Research, Extrapolated Data Is Calculated Based on Dilution Factor and SNR

isolate	excitation wavelength (nm)	detection limit (CFU/mL)	integration time (ms)	signal to noise ratio (SNR)	dilution at SNR of 3
E. coli-Gfp (saline)	473	5.7×10^4	5000	53.7	1/17 900
E. coli-Gfp (sand)	473	3.3×10^5	3000	91.9	1/3060
E. coli-DsRed (saline)	473	3.0×10^6	100	10.7	1/330
E. coli-DsRed (saline)	532	6.0×10^5	100	5.1	1/1700
P. putida 852−137 (saline)	473	4.8×10^5	1000	1.6	1/530
P. putida 852−137 (sand)	473	6.3×10^6	1000	12.0	1/40

microcosms in order to determine the effect of media upon photobleaching characteristics.

Optrode measurements were taken from both *E. coli* Gfp and *E. coli* DsRed in sand and saline microcosms; and compared over 10-fold dilutions (up to 1/100th dilution) of *E. coli* isolates. Between 500 and 900 emission spectra were collected during the excitation period, with the minimum possible integration time to ensure maximum time resolution and minimum photobleaching were achieved. Labview software was programmed to automatically select the minimum acquisition time required for excitation of each sample, in order to minimize the photobleaching effect while retaining an acceptable signal-to-noise ratio (SNR, typically set to 10). To evaluate the photobleaching effect an exponential decay curve was fitted to the acquired emission data over time,[26] their fitted parameters were calculated and are presented in Table 2.

There was significant variance between the steady state of *E. coli*-Gfp isolates in sand and saline (Table 2). The steady state for the freely suspended cells in saline was 0.93, which equates to 93% of the signal intensity remaining and for cells mixed with sand the steady state was much lower at 0.61 or 61% of the signal. While bacteria in solution move in and out of the collection volume, bacteria in sand have limited or no mobility and therefore experience greater photobleaching, resulting in larger attenuation in signal strength. Unlike for sand where the observed decay in emission can be attributed to photobleaching (isolated system), the observed decay rates in solution incorporate the combined effects of decay from photobleaching and the flow in and out of the collection volume (system with continuous inflow and outflow).

In trials which measured *E. coli*-DsRed over a 10-fold dilution series, steady state was achieved with 71, 72, and 68% of the initial signal remaining in samples with dilution factors of 1, 10, and 100, respectively. Similar decay constant values resulted in 86, 86, and 84% signal remaining at the signal half-life, for samples with dilution factors of 1, 10, and 100 respectively (Table 2). From this data the photobleaching of fluorescent *E. coli*-DsRed samples measured by the Optrode was found to be similar and independent of dilution within detectable limits.

3.3. Optrode Limits of Detection. Limits of detection for the Optrode system were quantified in order to validate its potential for monitoring biological activity. Optrode sensitivity can be defined by calculating the signal-to-noise ratio (SNR) of individual samples, where signal is divided by the noise (error) of the integrated acquired data within the limits of set boundary windows. Windows are set from 515 to 550 nm for the Gfp protein; or from 570 to 670 nm for the DsRed protein. At SNR of 1 there is no detectable signal over sample noise; this is the absolute limit. However, in practice it is desirable to maintain a SNR well above this limit to maintain confidence in the collected data, where observed Optrode measurements taken from high concentrations of fluorescently labeled *E. coli* often demonstrated a SNR of over 300. The Optrode SNR calculations for each fluorescent isolate at a particular dilution were extrapolated to determine the maximum dilution that could be achieved to produce an SNR of 3 (refer to Table 3). The specific dilutions tested were 1, 1/10, 1/100, and 1/1000.

E. coli-Gfp cells suspended in saline had a calculated detection limit of 5.7×10^4 CFU/mL (1/17900 dilution) compared to the lower detection limit in sand of 3.3×10^5 CFU/mL (1/3060 dilution). Detection limits for *P. putida* 852−137 in saline were 4.8×10^5 CFU/mL compared to the limit of 6.3×10^6 CFU/mL (approximately 1 log decrease in sensitivity) determined for the same isolate in sand. The reduced detection limit can be attributed to the quenching of light by sand particles; and is consistent with research undertaken by Yolcubal et al., (2000) where a 10-fold reduction in peak luminescence response following chemical induction of luminescence was observed for the bioreporter *P. putida* RB1353 in sand media when compared with liquid cultures.[8]

Upon further investigation, integration of the raw intensity plots between 510 and 550 nm of the *E. coli*-Gfp spectra in sand repeatedly exhibited a 3-fold reduction in intensity compared to solution. The reduction was observed consistently within the full range of detectable cells, and can be explained by a reduction in the collection volume of the optical probe in substrate. This suggests that although the Optrode is able to accurately measure fluorescence in sand, decreased SNR and reduced signal intensity is due to a reduced collection volume

of approximately $^1/_3$ in sand compared to solution. The detection limits observed for the optrode in sand fall within ranges of microbial concentrations commonly reported in a wide range of field trials such as investigations into soil bioremediation. For example, in a study of the natural attenuation and biostimulation of native degrader organisms in diesel oil contaminated soil in an alpine glacier skiing area identified culturable heterotrophic microorganisms within the order of 1×10^7 CFU/g (dry weight) of soil.[27] This value exceeds the detection limit of the Optrode, in some cases by an order of magnitude, indicating that it could potentially be a feasible and accurate option for in situ monitoring of microbial activity in tests aimed to mimic field applications.

E. coli-DsRed had a detection limit in saline of 2.0×10^5 CFU/mL when using an excitation of 532 nm; approximately 5 times greater than the detection limit when using 473 nm excitation (refer to Table 3). This supports the argument that increased sensitivity can be achieved when using an excitation source that is closer to the peak excitation of the fluorophore.[28] Although sensitivity is greater when laser excitation source is closer to a fluorescent protein's excitation peak, Optrode detection of the same fluorophore using 473 and 532 nm excitation sources is possible even when the excitation wavelength is as far as 83 nm from the reported excitation peak.[11] Potentially, lasers may be used at different wavelengths to identify multiple fluorescent proteins within the same sample, allowing emission spectra of a particular fluorophore to be isolated and measured without the complication of deconvoluting combined emission spectra into different components.

3.4. Fluorescence Intensity Linearity to Cell Concentration. The relationship between dilution and emission signal intensity readings (taken as the integration of the response signal within a window of 510 and 550 nm) was investigated in order to determine the accuracy of the optrode in measuring fluorescence spectra intensity consistently between samples of cells at differing concentrations. Plotted data of the observed spectra in doubling dilutions of a culture to 1/1024 dilution demonstrated an anticipated linear relationship between dilution and signal intensity, with an R^2 value of 0.998 (see Figure 3).

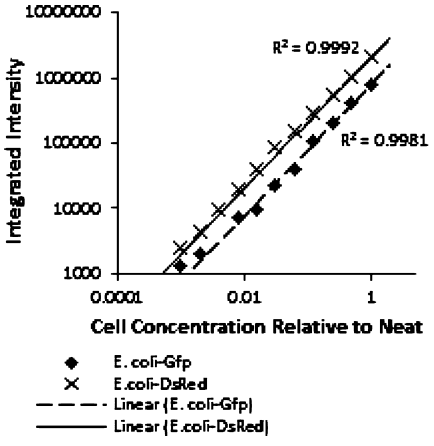

Figure 3. *E. coli*-Gfp integrated spectrum intensities taken from a window of 510–550 nm over doubling dilutions relative to neat concentration (1). A log–log scale is used; concentration of cells is relative to the neat sample (1×10^9 CFU/mL).

3.5. Detection and Deconvolution of Multiple Fluorescence Spectra within Mixed Samples. To demonstrate an accurate method of signal deconvolution, overnight cultures of *E. coli*-Gfp and DsRed were adjusted in concentration to produce identical signal intensity response following excitation with the 473 nm wavelength laser. These samples were mixed using ratios: (Gfp:DsRed) 0:10, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, and 10:0. The normalized and raw spectral information collected was considered for each mixed sample in terms of integrated area within the two predefined windows. The fitted data was broken down into constituent DsRed and Gfp spectra using the two methods described in Section 2.6. As we have demonstrated in Figure 3, the area under a spectrum is directly proportional the cell concentration. Since the area and cell concentration of a reference spectrum is predetermined, the concentration in CFU/mL of each protein's concentration can be obtained by comparing the resolved experimental spectra to the reference spectrum. Two assumptions are made during the calculations: (1) that when the samples are mixed the respective spectra are not altered by the presence of another fluorophore; (2) that the photobleaching for each respective sample stays constant independent of the presence of the other fluorophore. Because fluorescent proteins are produced and sequestered within different bacterial cells, there is unlikely to be direct interaction between different fluorescent proteins.

Figure 4 compares the Alentsev-Fok and curve fitting method by plotting the deconvoluted Gfp and DsRed spectral components side by side. These plots show that the resolved Gfp and DsRed spectra have very similar spectral characteristics and dilution ratio regardless of the methods used. Figure 5 examines the effectiveness of the two methods by plotting the parameter α against the ratio of Gfp to 1 DsRed as the value of α is predetermined by the reference spectra and dilution ratios. The linear relationship in the log–log plot shows that both spectral deconvolution methods are equally effective across the range of the dilution ratio tested.

These results indicate that it is possible to determine the fluorescence of an individual protein within a mixed sample containing multiple reporter proteins. The curve fitting method is advantageous in that it can be easily extended to deconvolute more than two proteins; however, it requires a prior knowledge of each fluorescence spectrum of each protein whereas the Alentsev-Fok Method does not. The Alentsev-Fok method could therefore be particularly applicable to scenarios where biosensor fluorescence signal is not well characterized, or where its expression varies between different strains. There are a number of potential applications for the simultaneous detection and quantification of bacteria within a mixed biosensor population. Potentially, individual fluorophores could be assigned to specific cellular events within the same organism.[13,29] For example, a fluorescence response linked to the promoter activity of a biosurfactant biosynthesis operon could be measured at the same time as a fluorescence response linked to the promoter activity of a target contaminant degradation operon.[30] Alternatively, different fluorescent proteins could be linked to a growth dependent promoter[31] and a contaminant degradation operon promoter; potentially indicating the extent to which cellular growth was attributed to degradation of the target contaminant in a bioremediation trail. The in situ monitoring of biological responses to potential improvements and amendments in trials could provide data that would assist in optimizing the engineering design of bioremediation applications. In practice, the scale of

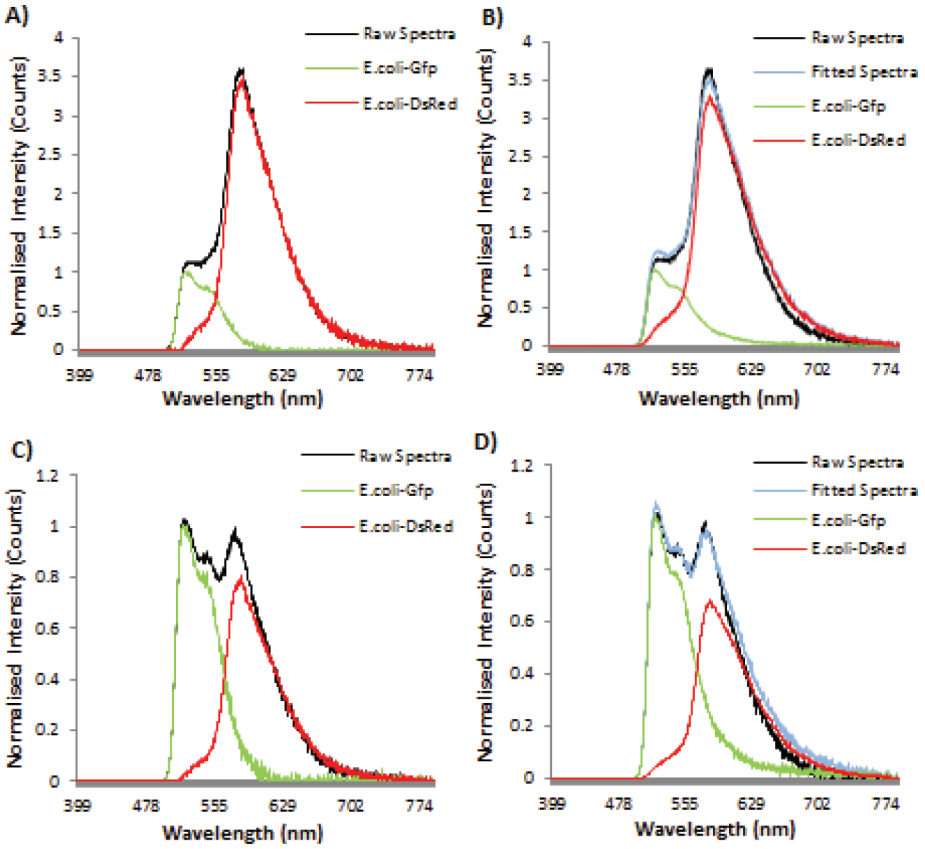

Figure 4. A and C: Deconvoluted *E. coli*-Gfp and *E. coli*-DsRed spectra using the Alentsev-Fok method at dilution ratios of (GFP:DsRed) 3:7 and 7:3 respectively. B and D: Deconvoluted *E. coli*-Gfp and *E. coli*-DsRed spectra using the curve fitting method at dilution ratios of (Gfp:DsRed) 3:7 and 7:3 respectively.

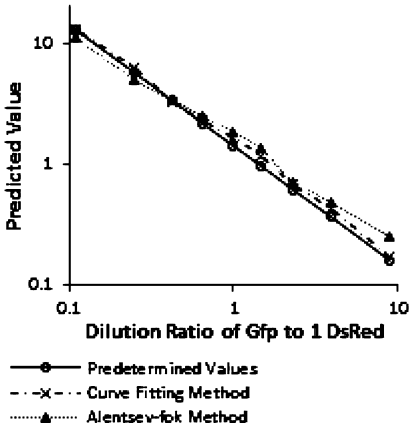

Figure 5. Log−log plot of the parameter α value against the dilution ratio of GFP to 1 DsRed. Both the Alentsev-Fok and curve fitting methods are employed, as well as α predicted by the dilution ratios.

applications will be influenced by the distance to which the fiber optic probe can be introduced into soil and the magnitude of the background fluorescence of the soil matrix. As this technique utilizes measurements of fluorescence (e.g., from biosensor organisms) it will not function with native bacterial populations lacking this property; rather, it is intended use is for obtaining an understanding of in situ system dynamics through the use of fluorescent foreign inoculants (e.g., to assess the efficiency of bioaugmentation). In principle the technique can be extended to any substance that emits measurable fluorescence and potential applications may include the use of fluorescent dyes or fluorophore-tagged chemicals to assess their transport and fate in soils.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Kohler, S.; Belkin, S.; Schmid., R. D. Reporter gene bioassays in environmental analysis. *Fresen. J. Anal. Chem.* **2000**, *366*, 769−779.

(2) Tecon, R.; Wells, M.; Van Der Meer, J. R. A new green fluorescent protein-based bacterial biosensor for analysing phenanthrene fluxes. *Environ. Microbiol.* **2006**, *8*, 697−708.

(3) Burlage, R. S.; Sayler, G. S.; Larimer, F. Monitoring of naphthalene catabolism by bioluminescence with nah-lux transcriptional fusions. *J. Bacteriol.* **1990**, *172*, 4749−4757.

(4) Ford, C. Z.; Sayler, G. S.; Burlage, R. S. Containment of a genetically engineered microorganism during a field bioremediation application. *Appl. Microbiol. Biotechnol.* **1999**, *51*, 397−400.

(5) Horzempa, J.; Tarwacki, D. M.; Carlson, P. E., Jr.; Robinson, C. M.; Nau, G. J. Characterization and application of a glucose-repressible

promoter in *Francisella tularensis*. *Appl. Environ. Microbiol.* **2008**, *74* (7), 2161−70.
(6) Malone, C. L.; Boles, B. R.; Lauderdale, K. J.; Thoendel, M.; Kavanaugh, J. S.; Horswill, A. R. Fluorescent reporters for Staphylococcus aureus. *J. Microbiol. Methods* **2009**, *74*, 2161−70.
(7) Shcherbo, D; Merzlyak, E. M.; Chepurnykh, T. V.; Fradkov, A. F.; Ermakova, G. V.; Solovieva, E. A.; Lukyanov, K. A.; Bogdanova, E. A.; Zaraisky, A. G.; Lukyanov, S.; Chudakov, D. M. Bright far-red fluorescent protein for whole-body imaging. *Nat. Methods* **2007**, *4*, 741−746.
(8) Yolcubal, I.; Piatt, J. J.; Pierce, S. A.; Brusseau, M. L.; Maier, R. M. Fiber optic detection of in situ lux reporter gene activity in porous media: system design and performance. *Anal. Chim. Acta* **2000**, *422*, 121−130.
(9) Chalfie, M. Green fluorescent protein. *Photochem. Photobiol.* **1995**, *62*, 651−656.
(10) Chalfie, M.; Tu, Y.; Euskirchen, G.; Ward, W. W.; Prasher, D. C. Green fluorescent protein as a marker for gene expression. *Science* **1994**, *263*, 802−805.
(11) Bevis, B. J.; Glick, B. S. Rapidly maturing variants of the Discosoma red fluorescent protein (DsRed). *Nat. Biotechnol.* **2002**, *20*, 83−87.
(12) Andreu, N.; Zelmer, A.; Wiles, S. Noninvasive biophotonic imaging for studies of infectious disease. *FEMS Microbiol. Reviews* **2011**, *35*, 360−394.
(13) Tecon, R.; Binggeli, O.; van der Meer, J. R. Double-tagged fluorescent bacterial bioreporter for the study of polycyclic aromatic hydrocarbon diffusion and bioavailability. *Environ. Microbiol.* **2009**, *9*, 2271−2283.
(14) Kosaric, N. Biosurfactants and Their Application for Soil Bioremediation. *Food Technol. Biotechnol.* **2001**, *39*, 295−304.
(15) Lambertsen, L.; Sternberg, C.; Molin, S. Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. *Environ. Microbiol.* **2004**, *6*, 726−732.
(16) Choi, K. H.; Kumar, A.; Schweizer, H. P. A 10 min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. *J. Microbiol. Methods* **2006**, *64*, 391−397.
(17) Clark, D. J.; Maaløe, O. DNA replication and division cycle in Escherichia coli. *J. Mol. Biol.* **1967**, *23*, 99−112.
(18) Brzuszkiewicz, E.; Bruggemann, H.; Liesegang, H.; Emmerth, M.; Olschlager, T.; Nagy, G.; Albermann, K.; Wagner, C.; Buchrieser, C.; Emody, L.; Gottschalk, G.; Hacker, J.; Dobrindt., U. How to become a uropathogen: Comparative genomic analysis of extraintestinal pathogenic *Escherichia coli* strains. *Proc. Natl. Acad. Sci.. U.S.A.* **2006**, *103*, 12879−12884.
(19) Blattner, F. R.; Plunkett, G., 3rd.; Bloch, C. A.; Perna, N. T.; Burland, V.; Riley, M.; Collado-Vides, J.; Glasner, J. D.; Rode, C. K.; Mayhew, G. F.; Gregor, J.; Davis, N. W.; Kirkpatrick, H. A.; Goeden, M. A.; Rose, D. J.; Mau, B.; Shao, Y. The complete genome sequence of *Escherichia coli* K-12. *Science* **1997**, *277*, 1453−1462.
(20) Earl, E. A.; Altaf, M.; Murikoli, R. V.; Swift, S.; O'Toole, R. Native New Zealand plants with inhibitory activity towards *Mycobacterium tuberculosis*. *BMC Complementary Altern. Med.* **2010**, *10*, 25.
(21) Miles, A. A.; Misra, S. S.; Irwin, J. O. The estimation of the bactericidal power of the blood. *J. Hygeine* **1938**, *38*, 732−749.
(22) Tai, D. C. S.; Hooks, D. A.; Harvey, J. D.; Smail, B. H.; Soeller, C. Illumination and fluorescence collection volumes for fiber optic probes in tissue. *J. Biomed. Opt.* **2007**, *12*, 034033.
(23) Hu, Y.; Liu, J.; Li, W. Resolution of overlapping spectra by curve-fitting. *Anal. Chim. Acta* **2005**, *538*, 383−389.
(24) Gusev, E. V.; Turoverov, K. K. Alentsev-fok method of resolving complex spectra. *J. Appl. Spectrosc.* **1978**, *29* (1), 844−849.
(25) Tsien, R. Y. The green fluorescent protein. *Annu. Rev. Biochem.* **1998**, *67*, 509−544.
(26) Dittrich, P. S.; Schwille, P. Photobleaching and stabilization of fluorophores used for single-molecule analysis with one- and two-photon excitation. *Appl. Phys. B: Lasers Opt.* **2001**, *73*, 829−837.
(27) Margesin, R.; Schinner, F. Bioremediation (natural attenuation and biostimulation) of diesel-oil-contaminated soil in an alpine glacier skiing area. *Appl. Environ. Microbiol.* **2001**, *67*, 3127−3133.
(28) Shaner., N. C.; Steinbach, P. A.; Tsien, R. Y. A guide to choosing fluorescent proteins. *Nat. Methods* **2005**, *2*, 905−909.
(29) Wan, H.; He, J.; Ju, B.; Yan, T.; Lam, T. J.; Gong, Z. Generation of two-color transgenic Zebrafish using the green and red fluorescent protein reporter genes *gfp* and *rfp*. *Mar. Biotechnol.* **2002**, *4*, 146−154.
(30) Holden, P. A.; LaMontagne, M. G.; Bruce, A. K.; Miller, W. G.; Lindow, S. E. Assessing the role of *Pseudomonas aeruginosa* surface-active gene expression in hexadecane biodegradation in sand. *Appl. Environ. Microbiol.* **2002**, *68*, 2509−2518.
(31) Ahn, Y. B.; Beaudette, L. A.; Lee, H.; Trevors, J. T. Survival of a GFP-labelled polychlorinated biphenyl degrading psychrotolerant *Pseudomonas* spp. in 4 and 22°C soil microcosms. *Microb. Eco.* **2001**, *42*, 614−623.