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A portable bioluminescence engineered cell-based biosensor for on-site applications

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ABSTRACT

We have developed a portable biosensing device based on genetically engineered bioluminescent (BL) cells. Cells were immobilized on a 4 × 3 multiwell cartridge using a new biocompatible matrix that preserved their vitality. Using a fiber optic taper, the cartridge was placed in direct contact with a cooled CCD sensor to image and quantify the BL signals. Yeast and bacterial cells were engineered to express recognition elements, whose interaction with the analyte led to luciferase expression, via reporter gene technology. Three different biosensors were developed. The first detects androgenic compounds using yeast cells carrying a green-emitting *P. pyralis* luciferase regulated by the human androgen receptor and a red mutant of the same species as internal vitality control. The second biosensor detects two classes of compounds (androgens and estrogens) using yeast strains engineered to express green-or red-emitting mutant firefly luciferases in response to androgens or estrogens, respectively. The third biosensor detects lactose analogue isopropyl β-D-1-thiogalactopyranoside using two *E. coli* strains. One strain exploits the lac operon as recognition element for the expression of *P. pyralis* luciferase. The other strain serves as a vitality control expressing *Gaussia princeps* luciferase, which requires a different luciferin substrate. The immobilized cells were stable for up to 1 month. The analytes could be detected at nanomolar levels with good precision and accuracy when the specific signal was corrected using the internal vitality control. This portable device can be used for on-site multiplexed bioassays for different compound classes.

1. Introduction

Interest in developing portable analytical devices for on-site analysis has risen sharply in recent years. Many areas, including medical diagnostics, environmental toxicology and bioterrorism controls, would benefit from devices that can perform a rapid first-level screening without the need for equipped laboratories or skilled personnel (Aravanis et al., 2001). The ability to detect multiple analytes in a single run would offer the advantage of a more accurate diagnosis when used in clinical areas.

Owing to their ability to exploit highly specific biomolecular recognition mechanisms integrated within the detection system, biosensors can satisfy many of the analytical requirements related to on-site analysis. These include sensitivity, selectivity, robustness, rapidity, and contained costs. As a branch of biosensors, engineered bioluminescent (BL) cells are now emerging as sensitive analytical tools (Struss et al., 2010; Misawa et al., 2010; Banerjee and Bhunia, 2010; Michelini et al., 2010; Merkoçi, 2010; Roda et al., 2006). They are obtained by introducing a reporter gene into the host cell. The reporter gene's expression is controlled by the analyte recognition element (e.g., a receptor or a regulatory protein) and promoter (O/P) sequences. The recognition element interacts with the analyte, activating the expression of a reporter protein (e.g., luciferase), whose activity can be measured after addition of a BL substrate (Roda et al., 2004). Since the analyte has to cross cell membrane before interacting with the specific recognizing element, these biosensors provide information about bioavailability, toxicity, and speciation (Ivask et al., 2004; Leskinen et al., 2005). Although there has been great progress in developing BL cell-based biosensors, many issues continue to hamper their use in on-site applications. The main problem is how to integrate cells in a robust and portable analytical device. Various different approaches, including freeze-drying, use of spores, and immobilization in biocompatible polymers, have been reported, each with its own advantages and disadvantages (Amoura et al., 2007; Avnir et al., 2006; Date et al., 2010; Choi and Gu, 2002). Yet the issue

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of long-term vitality and responsiveness of whole-cells remains unresolved (Alvarez et al., 2009).

In addition, many uncontrolled factors affect the analytical performance of whole-cell biosensors. These include temperature, pH, and toxic components of the sample. All have the potential to aspecifically influence cell vitality, giving unreliable analytical signals (Mirasoli et al., 2002).

With regard to the portability of biosensors, we note the unsuitability for on-site applications of conventional photon imaging instrumentation, such as ultrasensitive luminographs based on an intensified charge-coupled device (CCD) video cameras. This is due to their high cost and benchtop dimensions.

Lensless imaging refers to an approach in which the area to be imaged is in contact with the imaging sensor (e.g., CCD or complementary metal oxide semiconductor, CMOS). It has recently been suggested that this approach, with its high photon collection efficiency, could be used to develop compact portable devices. Various lensless imaging configurations have already been successfully applied to light transmission or fluorescent detection (Li et al., 2010; Coskun et al., 2010) for real-time and high-content analysis of single cells (Tanaka et al., 2010).

In the present work, we propose possible solutions to three key issues. First, lensless contact imaging could be combined with BL detection to develop a compact portable device. Second, cells could be immobilized in a new polymeric matrix to increase cell shelf-life and to provide ready-to-use disposable biosensor cartridges. Finally, an internal vitality control could be introduced into the same sensing cell to correct the analytical signal.

Herein, we report the development of a portable biosensing device relying on lensless contact imaging. The device comprises a disposable cartridge containing immobilized BL whole-cell biosensors coupled with a CCD detector via a fiber-optic-based taper.

We genetically engineered yeast and bacterial whole-cell biosensors, which express luciferases that emit at different wavelengths or require different BL substrates. These were integrated into the device to expand its multiplexing capability. In this paper, we report and critically discuss the analytical performances of the developed biosensors in three different representative assay formats.

2. Materials and methods

2.1. Chemicals and reagents

Testosterone, 17-β estradiol, ampicillin, and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Sigma (St. Louis, MO). All the enzymes required for cloning were from Fermentas (Vilnius, Lithuania). The kits for plasmid extraction and purification were from Qiagen (Hilden, Germany). Luciferin solution, 1 mM at pH 5, was prepared by dissolving 28.3 mg D-luciferin sodium salt (Synchem, Kassel, Germany) into 35 mL of 0.1 M citric acid and 65 mL of 0.1 M trisodium citrate solution. Native coelenterazine was from Sigma (St. Louis, MO, USA).

2.2. Design and development of the device

The BL cell-based biosensor comprised two main parts: a CCD sensor and a multiwell cartridge with immobilized cell. These were in direct contact via a fiber optic taper (Fig. 1(A)).

The detector was a sensitive low-light double Peltier cooled CCD, 83 mm wide, 83 mm deep, and 52 mm high (MZ-2PRO, San Marco Optical Centre, Pordenone, Italy), with a $9.0 \times 6.7\ mm^2$ Sony ICX285 sensor and 1.4 MP resolution. A round fiber optic taper (FO TAPER, 25/11 mm size, EO Edmund Optics, United States) was used to maintain high 2D resolution without an optical system and to magnify the CCD sensor's light sensitive area from 85 to 200 mm^2 (Fig. 1(B)). The taper was put in direct contact with the sensor surface in a fixed position by an aluminium adapter with a neoprene o-ring. This provided thermal insulation and avoided fogging phenomena when the CCD sensor was cooled.

A customized 36 mm × 32 mm cartridge containing an array of 4 × 3 wells with 3 mm diameter and 70 μl volume was obtained by cutting black 384-well polystyrene microtiter plate with transparent bottom (Labsystems, Helsinki, Finland). The cartridge was placed on the fiber optic taper surface, which allowed simultaneous imaging of the light from each well. Disposable cartridges with different whole-cell biosensors can be easily replaced (Fig. 1(C)). The device was connected to a laptop and the CCD-Cap software V2.3.1 was used for parameter setup and image acquisition (Fig. 1(D)).

A mirror was inserted under the lid that covered the cartridge to increase light collection efficiency by back reflection. When needed, filters could be inserted between the cartridge and the optical taper surface to spectrally resolve the light emission at different wavelengths. A set of filters was designed according to the RGB values obtained with the Spectra application (www.efg2.com/Lab/ScienceAndEngineering/Spectra.htm), printed with an HP Color LaserJet 2605dn (Hewlett-Packard Company, Paolo Alto, USA) on transparency films (3 M, St. Paul, MN, USA). The filters were previously characterized with the Cary 50 Eclipse spectrophotometer (Varian, Palo Alto, CA) by measuring their transmittance from 400 to 800 nm. In addition, the device was designed to incorporate a temperature control system to keep the cell cartridge thermostated.

2.3. Genetically engineered cells

Yeast (*Saccharomyces cerevisiae* BMA64-1A) and bacterial (*Escherichia coli* JM109) cells were genetically engineered by introducing a reporter gene. The reporter gene's expression was either constitutive or regulated by the activation of a specific recognition element (e.g., a receptor or a regulatory protein) introduced into the same cell. The following recombinant strains were used:

(a) A yeast strain expressing the green-emitting *P. pyralis* wild-type luciferase (PpyWT, λ_{max} = 557 nm) regulated by the human androgen receptor, and a red *P. pyralis* mutant (PpyRE8) luciferase (λ_{max} = 618 nm) used as internal vitality control.
(b) A yeast strain expressing PpyRE8 luciferase regulated by human estrogen receptor (red estrogen responsive strain);
(c) A yeast strain expressing PpyWT regulated by human androgen receptor previously developed by us (Michelini et al., 2005);
(d) An *E. coli* strain in which expression of PpyWT is regulated by the lac promoter induced by the addition of IPTG (lactose responsive strain);
(e) An *E. coli* strain with a blue-emitting luciferase (λ_{max} = 490 nm) from *Gaussia princeps* (Gluc) controlled by a non-inducible CMV promoter, which was used as an internal vitality control strain.

2.4. Cell engineering

We began with yeast cells (*S. cerevisiae* BMA64-1A strain, Baudin-Bailleu et al., 1996) constitutively expressing the human androgen receptor (hAR) and PpyWT regulated by the androgen responsive element (ARE) sequences. These cells were transformed with plasmid pSal1-PpyRE8, obtained by cloning the cDNA for PpyRE8 (Branchini et al., 2010) into the shuttle vector pSal1 (Mascorro-Gallardo et al., 1996). For this, we used the lithium acetate method (Gietz and Schiestl, 1995). Restriction digestion and sequencing confirmed the correct insertion of PpyRE8. The recombinant strain was grown in a selective synthetic complete

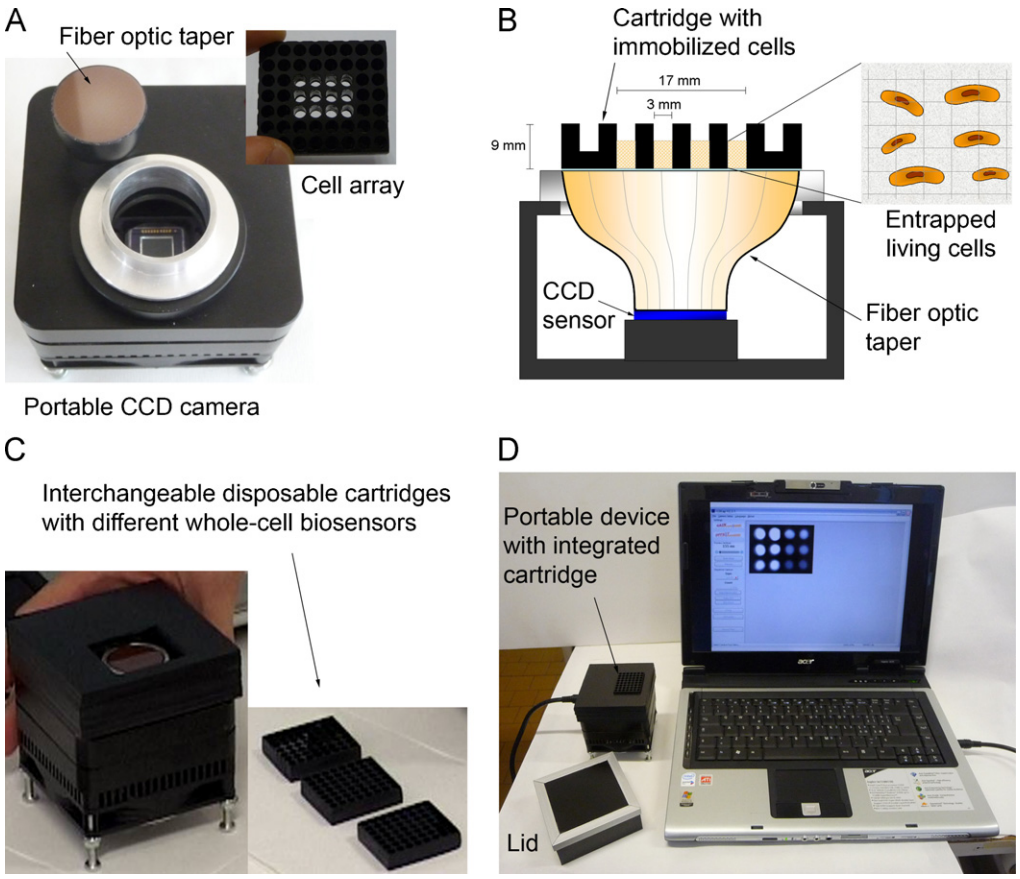

Fig. 1. (A) Picture of the device components: a Sony MZ-2PRO CCD cooled camera, the cartridge with immobilized cells and the fiber optic taper. (B) Schematic view of the clear-bottom cartridge with immobilized cells in contact with the CCD sensor through the fiber optic taper. (C) Picture of the device with interchangeable cartridges in which different whole-cell biosensors are immobilized. (D) Overview of the portable device connected to a laptop via USB cable.

(sc) medium containing 6.7 g/L yeast nitrogen base without amino acids, 1.4 g/L yeast synthetic drop-out media supplement, and 40 mL/L of a 50% (w/v) D-glucose w/o uracil, tryptophan, and leucine. The estrogen responsive strain was obtained as previously described (Leskinen et al., 2005), except that the PpyWT was replaced by PpyRE8.

The bacterial control and lactose responsive strains were obtained by transforming chemically competent BL21 *E. coli* cells (Sambrook et al., 1989), respectively, with the plasmid pcDNA3-hGluc for the expression of humanized *G. princeps* luciferase (a kind gift from Dr. Bruce Bryan, NanoLight Technologies, Pinetop, AZ) and the plasmid pGEXPpyWT, expressing the *P. pyralis* WT luciferase under the control of the lac promoter (Michelini et al., 2008a). Bacterial strains were cultured in LB broth with 100 μg/mL ampicillin final concentration.

2.5. Cell immobilization

Both yeast and bacterial cells were immobilized onto a polymeric matrix comprising natural and synthetic polymers. An aqueous 1% (w/v) solution of agarose and 2% (w/v) polyvinylpyrrolidone (PVP) was sterilized by autoclaving at 121 °C for 15 min. Then, sterile 0.1% (v/v) collagen solution was added. The cartridge was prepared as follows: yeast and bacterial cells were cultured in selective medium to mid-log phase (optical density at 600 nm [OD 600] ~0.6–0.8 depending on the strain). Then, cell cultures were mixed 50% (v/v) with the freshly prepared immobilization matrix solution. A volume of 30 μL of the cell-matrix mixture was poured into the 70 μL-wells of the cartridge at room temperature and left to harden for 30 min. Immobilized cells were stored at 4 °C.

The effect of matrix thickness on substrate diffusion and/or oxygen limitation was investigated using matrix layers with a thickness of 1, 2, and 3 mm. After 1 day storage at 4 °C, BL acquisitions were performed with a NightOwl LB 981 luminograph (EG&G Berthold, Pforzheim, Germany), 30 s and 5 min after substrate (D-luciferin 1 mM at pH 5.0) addition. To compare the performance of the new matrix with that obtained with previously used immobilization procedures, yeast cells were immobilized into calcium alginate at 1:2 alginate 3% (w/v): 5×10^6 cell/mL.

2.6. Whole-cell viability and light emission kinetics in immobilized cultures

Vitality of immobilized cells stored at 4 °C was monitored once a week for 1 month. BL emissions were acquired for 1 s with a Varioskan Flash spectral scanning multimode reader (Thermo Scientific, Whaltham, MA, USA) after automatic injection of 30 μL D-luciferin.

Kinetic profiles were compared with those obtained with liquid cultures and with yeast cells immobilized into calcium alginate. For each strain, BL emissions were acquired for 30 min with a 1 s integration time. Emission kinetics were analyzed with GraphPad Prism software v5.02 (Software, Inc., San Diego, CA, USA). The percentage of living cells after 1 month was evaluated. We dissolved 30 μL of matrix in 1 mL of growth medium by heating at 40 °C in water bath, and followed with a 10^6-fold serial dilution. A volume of 100 μL of bacterial or yeast suspension was spread on three replicas of selective agar plates and the colony-forming units (CFU/well) were counted after 24 h or 48 h incubation, respectively.

In addition, we tested sample pH effects on cell vitality. To do this, 30 μL of immobilized bioluminescent bacterial and yeast cells, expressing a thermostable luciferase (PpyRE8), were incubated with 5 μL of 0.1 M PBS solutions at pH ranging from 2 to 12.

2.7. Detection of androgenic compounds using a whole-cell biosensor with an internal vitality control

We used an immobilized yeast strain, which expressed green PpyWT in response to activation of the human androgen receptor and used red *P. pyralis* mutant PpyRE8 as internal vitality control. The results were compared with those obtained by using cells in liquid cultures and conventional instrumentation. Testosterone stock dilutions were prepared in sterile water with 10% ethanol final concentration. In the assay with the portable device, 5 μL of different testosterone solutions ranging from 10^{-11} to 10^{-6} M and a negative control (5 μL of sterile water with 10% ethanol) were added in duplicate to the wells containing the immobilized cells and incubated for 2 h at 30 °C. Then, the cartridge was placed in contact with the CCD sensor equipped with filters. The BL signals were acquired for 30 s after the addition of 30 μL of D-luciferin 1 mM. Images were acquired using a 30 s exposure time and the BL signal of each well was measured by integrating the photon emission over the well area. The background signal was subtracted from all the wells.

The procedure used for liquid cultures is a slight modification to the 384-well microplate format of a previous assay (Michelini et al., 2008b). Briefly, 30 μL of cell suspension (at OD_{600nm} 0.8) were transferred to a sterile black 384-well microtiter plate with 5 μL of the analyte in solution. After a 2 h incubation at 30 °C, 30 μL of 1 mM D-luciferin were injected and luminescence measurements (1 s integrations) were performed using the Varioskan Flash spectral scanning multimode reader equipped with green (band pass 530–570 nm) and red (band pass 610–650 nm) hard-coated filters.

Light emissions were spectrally resolved using the Promega Chroma-Luc Calculator. For both immobilized and liquid cultures, testosterone dose–response curves were corrected to account for the internal vitality control. GraphPad Prism software v5.02 was used to fit the data for the testosterone dose–response curves with a four parameter non-linear regression curve. The detection limit was defined as the testosterone concentration that corresponded to the blank plus three times the standard deviation.

2.8. Simultaneous detection of two analytes using estrogen and androgen responsive strains

Section 2.3 describes two yeast populations marked (b) and (c). These both express luciferases from the same species (*P. pyralis*) requiring the same D-luciferin substrate but emitting at different wavelengths. These two yeast populations were combined and immobilized on the cartridge. Dose–response curves for testosterone and 17β-estradiol were obtained in the range 10^{-11}–10^{-6} M and negative controls were included as described in Section 2.6. In addition, we evaluated mixtures of different androgens and estrogens (Supplementary material Table S1).

2.9. Multiplexing using cells expressing luciferases requiring different substrates

We examined the possibility of including, in the cartridge, bacterial cell populations expressing BL reporter proteins requiring different substrates. These include Gluc, which uses coelenterazine as a substrate, and *P. pyralis* luciferase, which requires D-luciferin. Briefly, a 15 μL-aliquot of both the vitality control strain (*E. coli* cells expressing Gluc controlled by a non-inducible promoter) and the lactose responsive strain (*E. coli* cells in which the expression of PpyWT is regulated by the lac promoter) were grown to mid-log phase in selective medium, mixed, and immobilized onto the disposable cartridge. Cells were incubated at 30 °C for 2 h with 5 μL of IPTG standard solutions in the range of 10^{-9}–10^{-4} M or 5 μL of sterile water as negative control. Gluc emission was first measured by adding 5 μL coelenterazine (5 μM) (30 s integration); after a delay of 5 min, PpyWT emission was measured with 15 μL D-luciferin 1 mM (30 s integration).

3. Results and discussion

3.1. Design and development of the microwell array cartridge

The device, shown in Fig. 1, included a disposable microwell array cartridge placed in contact with a CCD sensor through a fiber optic taper.

A MagZero MZ2-PRO CCD camera was selected for the development of the portable device. This is because of its simple structure, lack of lens system, light weight (only 400 g), small dimensions, and ability to cool down to −10 °C with a Peltier chamber to decrease dark current. Moreover, it uses an external power supply and control box for the cooling system, which can be powered by a 220 V or 12 V AC/DC power adapter (maximum power consumption 60 W). It is thus suitable for outdoor applications.

Lensless imaging measurements were carried out using a fiber optic taper. This transfers the light emitted by the immobilized cells to the sensor and enlarges the active measurement area of the sensor.

The cooling system reduced thermal noise, thus increasing camera sensitivity. Indeed, the dark current is approximately halved for every 6 °C reduction in chip temperature, thus reducing the shot-noise of CCD (Christensen and Herron, 2009).

With transmission imaging and fluorescence detection applications, CMOS sensors offer sufficient sensitivity with the possibility of obtaining self-contained chips at reduced cost (Rodrigues and Lapa, 2010; Vykoukal et al., 2009). In contrast, bio- and chemiluminescence reactions require more sensitive systems to detect weak signals. A cooled CCD sensor thus offered the best solution.

Images were recorded in the FITS (Flexible Image Transport System) format, which is well suited for most scientific image analysis software.

3.2. Immobilization of the recombinant cells onto the disposable cartridge: whole-cell viability and light emission kinetics

The polymeric matrix was able to keep cells alive in ready-to-use cartridges for up to 35 days at 4 °C. We investigated experimental conditions relating to different growth phases as well as the cells-to-matrix ratio to identify which conditions provided the best mass transfer of chemicals and oxygen, which may be limited by high cell density (Affi et al., 2009).

In terms of reproducibility and BL response, the best results were obtained by immobilizing cells at 50% (v/v) with a mixture of agarose, PVP and collagen. The matrix thickness was critical for BL substrate diffusion and/or oxygen limitation in cell-matrix layers. In a 3 mm cell-matrix layer, 30 s after substrate addition, 60% of the BL signal arose from the uppermost 1 mm-layer. In contrast, the middle and lower layers only contributed to 30% and 10% of the total BL emission, respectively (Fig. 2(A1)). These data suggest that the BL signal intensity was limited by substrate diffusion and that it took 5 min for the BL signal to be almost uniformly distributed across the whole cell-matrix layer (Fig. 2(A2)). Even though 1 and 2 mm cell-matrix layers provided a faster diffusion of substrates,

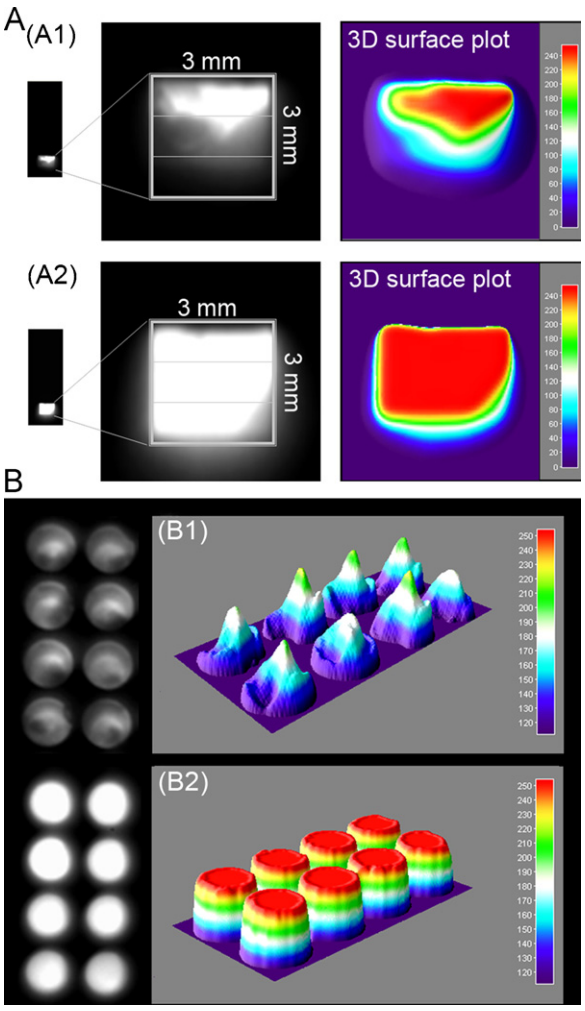

Fig. 2. (A) BL bacteria immobilized in 3 mm cell-matrix layer inside 1 mL cuvettes with a clear window of 3 mm × 3 mm. BL signal acquisitions were performed with NightOwl luminograph, 30 s (A1) and 5 min (A2) upon 1 mM D-luciferin substrate addition. (B) BL signal of the dual-color androgen responsive strain immobilized in Ca^{2+} alginate (B1) and in the polymeric matrix (B2). Images were acquired for 30 s after addition of 1 mM D-luciferin at pH 5.0 and analyzed with ImageJ 3D Surface Plot.

the total BL emissions were lower (data not shown). Therefore, we used 3 mm-thick cell-matrix layers, corresponding to 30 μL of cell-matrix mixture in a 384-well microplate.

We compared this matrix with calcium alginate immobilization using a 1:2 alginate 3% (w/v): 5×10^6 cell/mL. This has been shown to be the optimized ratio for the same yeast strain, as reported by Fine et al., 2006. Fig. 2(B1) and (B2) shows the BL signal from the yeast androgen responsive strain (induced with 10 nM testosterone) immobilized with either the conventional calcium alginate or the reported matrix, respectively. The higher reproducibility obtained with the new matrix (CV < 10% versus CV < 18% with alginate, obtained on 10 replicates) is probably because this immobilization procedure for both yeast and bacterial cells was more precise and reproducible.

The viability of the immobilized yeast and bacterial cells was maintained for up to 35 days at 4 °C. As shown in Fig. 3, a slight decrease in bioluminescence emission was observed. In agreement with bioluminescence emission data, we reported a 20 ± 5% and 15 ± 3% loss in viability after 1 month storage for bacteria and yeast cells, respectively.

Moreover, kinetic measurements with immobilized cells showed we had a temporal window of about 5 min (ranging from 4 to 9 min), in which the intensity of the BL signal was maintained at an approximately constant level (Supplementary information, Fig. S1).

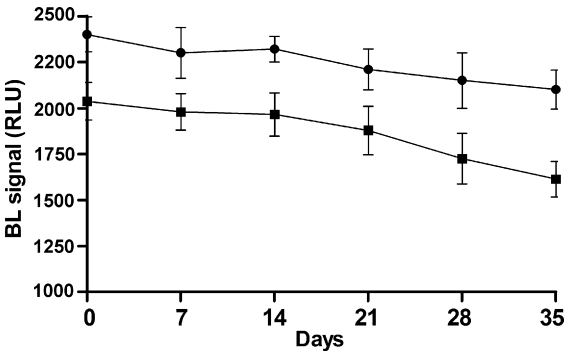

Fig. 3. BL signal of the immobilized yeast (●) and bacterial (■) strains monitored once a week over a period of 35 days.

We also evaluated the performance of the biosensor with samples at different pH values ranging from 2 to 12. This was to investigate its suitability for real samples analysis. Cell vitality was not significantly affected even when using samples with extreme pH values. This is presumably due to their small volume with respect to the volume of the immobilized cells (5 μL versus 30 μL) (data not shown). It was only when exposed to the sample at pH 2 that both bacterial and yeast cells showed a small decrease in cell vitality (approximately 15 ± 8%). We have previously reported similar results with *E. coli* cells expressing the same thermostable luciferase PpyRE8 (Roda et al., 2010).

3.3. Androgenic compound detection using a whole-cell biosensor with an internal vitality control

We began with recombinant yeast cells expressing human androgen receptor (hAR) and containing the sequence androgen response element (ARE), which drives the expression of *P. pyralis* luciferase. These were engineered by introducing a second reporter gene as internal vitality control. This eliminated the use of a reference yeast strain. We selected a novel red-emitting *P. pyralis* luciferase mutant, PpyRE8, which requires the same D-luciferin substrate as the first reporter gene. This choice was due to its red emission and good thermostability at 37 °C (Branchini et al., 2010).

When compared to previously published androgenic assay (Michelini et al., 2005), this red and green androgen responsive strain showed higher reproducibility and was therefore immobilized in the microwell cartridge as the first application of the device. Since a black-and-white CCD sensor was used, a set of color filters were printed on transparency film and placed between the cartridge and the fiber optic taper. Filters were characterized to select the best pair for spectral unmixing of the red and green luciferases used in this work. According to emission spectra of PpyWT and its red mutant PpyRE8, the best separation of the two BL signals was obtained with the green filter PF520 (bandpass filter centered at 520 nm with bandwidth 60 nm) and red filter PF590 (cut-off filter at 590 nm). The use of these filters allowed us to spectrally resolve the emissions of the two reporter proteins and thus correct the specific signal according to cell vitality. The dose–response curve for testosterone obtained with the device equipped with these filters showed an LOD of 0.5 nM. This is higher than that obtained with the same recombinant cells using conventional benchtop instrumentation (LOD: 0.05 nM testosterone) and hard-coated filters, but still represents a very promising result for developing low-cost multiplex bioluminescence portable devices (Fig. 4). This androgen assay was more precise, robust, and fast when compared to previously

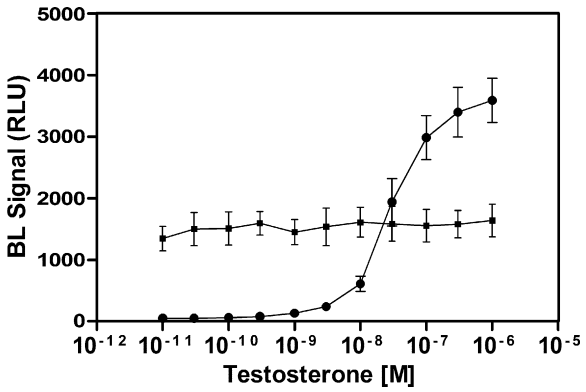

Fig. 4. Non-corrected dose–response curve for testosterone (●) and red BL vitality control signal (■) obtained with the dual-color androgen responsive strain immobilized in the device equipped with printed filters. Values are the mean ± standard deviations of three experiments performed in duplicate.

published cell-based assays for androgenic compounds which need liquid cell cultures, require from 1 to 5 days (Roy et al., 2004), show high variability (Sanseverino et al., 2009) and require an external reference strain to monitor cell vitality.

3.4. Simultaneous dual-analytedetection combining estrogen and androgen responsive strains

We combined and immobilized, in the cartridge, two yeast populations expressing a green-emitting PpyWT and a red luciferase mutant (PpyRE8), requiring the same substrate (D-luciferin) but emitting at different wavelengths. This was to investigate the suitability of the device for multiple analyte detection. Two cell populations, a green-emitting androgen-responsive strain and a red-emitting estrogen-responsive strain, were combined in the same well and dose–response curves for testosterone and 17β-estradiol were simultaneously obtained.

As shown in Fig. 5, 17β-estradiol also activated the human androgen receptor (EC_{50} 1×10^{-8} M), and testosterone activated the human estrogen receptor (EC_{50} 1×10^{-7} M). The information provided by this dual analyte biosensor is thus related to the "hormonal activity" of a sample. It does not identify a single analyte. To mimic the analysis of complex mixtures containing (anti)-androgenic and (anti)-estrogenic compounds, we measured the total androgenic and estrogenic activities of samples containing testosterone, 17β-estradiol, and the xenoestrogen bisphenol A (BPA), in different relative amounts (Supplementary material, Table S1). The results were in agreement with those obtained with separate assays (Michelini et al., 2005), demonstrating the potential use of the device for rapid screening of endocrine disruptors in environmental or food samples.

3.5. Multiplexing using cells that express luciferases requiring different substrates

The multiplexing capacity of the device was further investigated using immobilized cells expressing luciferases from different species and whose emissions require different BL substrates. We combined *E. coli* cells expressing Gluc under the control of a non-inducible promoter used as internal control and a lactose-responsive *E. coli* in which the expression of PpyWT is regulated by the lac promoter. Gluc emission (λ_{max} = 490 nm) is characterized by flash-type kinetics (Tannous et al., 2005) with a half-life of about 30 s after coelenterazine substrate addition. The signal extinguishes completely after 5 min when Gluc is expressed in bacterial cells. This allows subsequent detection of the second reporter gene, firefly luciferase, by adding D-luciferin. We incubated increasing

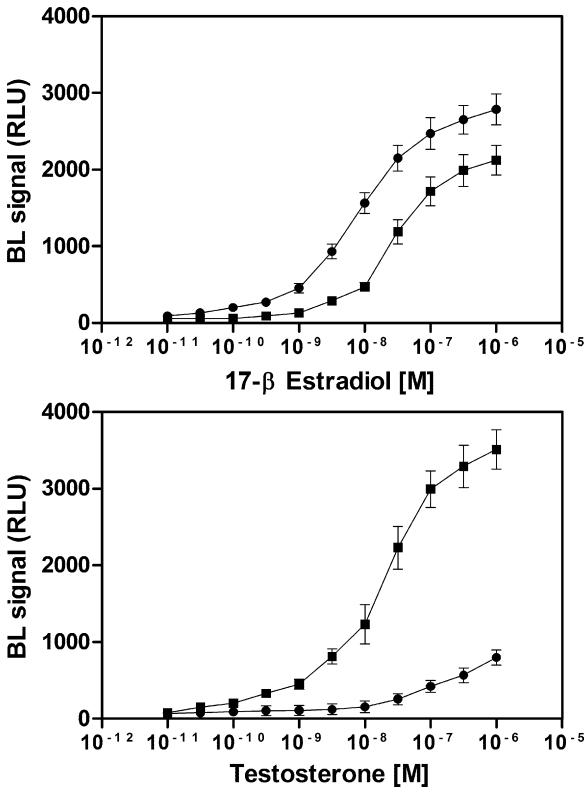

Fig. 5. Dose–response curve for 17β-estradiol (A) and testosterone (B) obtained with mixed population of androgen (■) and estrogen (●) responsive strains. Values are the mean ± standard deviations of three experiments performed in duplicate.

concentrations of IPTG with mixed populations of cells that constitutively expressed Gluc and cells with firefly luciferase under lactose regulation. A dose–response curve was thus obtained for the lactose analogue IPTG, and a stable Gluc emission was obtained as a result of optimal cell viability (Fig. 6). The limit of detection for IPTG, 1.5 nM, is similar to that obtained with liquid cultures (Michelini et al., 2008a).

By combining sequential detection of *G. princeps* and *P. pyralis* luciferases as reporter genes in immobilized whole-cell biosensors, we produced a complete absence of interference between the BL signals, thus facilitating the multiplexed-assays format.

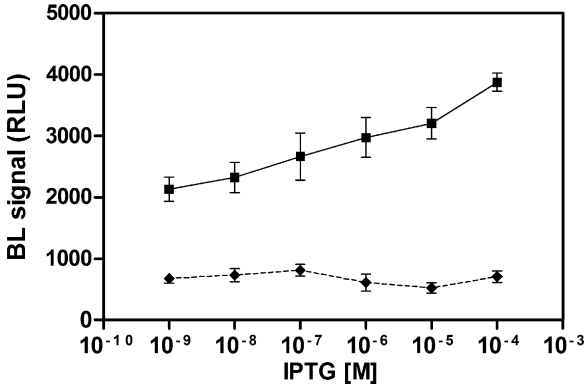

Fig. 6. Dose–response curve for IPTG obtained using BL21 *E. coli* cells harboring plasmid with *lac* promoter driving the expression of *P. pyralis* wild-type (■) and BL21 cells harboring plasmid with constitutive promoter for the expression of Gluc luciferase (■). Data are the mean ± standard deviation of at least three independent experiments each performed in duplicate.

4. Conclusions

In this work, we developed and optimized a new portable device comprising a disposable microwell cartridge with immobilized whole-cell biosensors placed in contact with a compact CCD sensor through a fiber optic taper. We used an aqueous mixture of agarose, PVP and collagen to immobilize cells, thus obtaining ready-to-use reagents, which kept their vitality for at least 1 month at 4 °C.

We demonstrated the suitability of the cellular biosensor for a multiplex detection that measures two resolved BL emission wavelengths using the same substrate. To do this, we used a new yeast strain for androgens (green-emitting luciferase) with an internal vitality control (red-emitting luciferase). We also used a mixed population of green-emitting androgen and red-emitting estrogen responsive yeast strains. We successfully obtained light signal separation of luciferases emitting at different wavelengths. We did this using printed filters that allowed us to quantify testosterone after correction for cell vitality.

As a proof-of-concept, we used whole-cell biosensors that express luciferases requiring different substrates, thus confirming the multiplexing potential of the device.

The assay requires just the sample and BL substrate addition. Therefore, a completely self-contained biodevice could be set up by including microfluidics for sample and reagent handling, and user-friendly software. This device could be used as a rapid screening tool for environmental or clinical applications just by replacing the cartridge. It could also be used in combination with other sensing elements such as cells, antibodies, and nucleic acids. Up to 4–6 BL signals can be managed in a single analytical run by using the same luciferase species with color-shifted mutants and different luciferases triggered by different luciferin substrates. We can thus achieve a large multiplexing capacity.

The proposed device could also be used as a sensitive screening alternative to animal-based tests in managing the potential health and environmental risks of chemicals in accordance with the European Regulation for Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.02.022.

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