Anal Bioanal Chem (2011) 401:201–211
DOI 10.1007/s00216-011-5091-3

ORIGINAL PAPER

Analytical strategies for improving the robustness and reproducibility of bioluminescent microbial bioreporters

Aldo Roda · Barbara Roda · Luca Cevenini · Elisa Michelini · Laura Mezzanotte · Pierluigi Reschiglian · Kaisa Hakkila · Marko Virta

Received: 12 March 2011 / Revised: 4 May 2011 / Accepted: 9 May 2011 / Published online: 21 May 2011

Abstract Whole-cell bioluminescent (BL) bioreporter technology is a useful analytical tool for developing biosensors for environmental toxicology and preclinical studies. However, when applied to real samples, several methodological problems prevent it from being widely used. Here, we propose a methodological approach for improving its analytical performance with complex matrix. We developed bioluminescent *Escherichia coli* and *Saccharomyces cerevisiae* bioreporters for copper ion detection. In the same cell, we introduced two firefly luciferases requiring the same luciferin substrate emitting at different wavelengths. The expression of one was copper ion specific. The other, constitutively expressed, was used as a cell viability internal control. Engineered BL cells were characterized using the noninvasive gravitational field-flow fractionation (GrFFF) technique. Homogeneous cell population was isolated. Cells were then immobilized in a polymeric matrix improving cell responsiveness. The bioassay was performed in 384-well black polystyrene microtiter plates directly on the sample. After 2 h of incubation at 37 °C and the addition of the luciferin, we measured the emitted light. These dual-color bioreporters showed more robustness and a wider dynamic range than bioassays based on the same strains with a single reporter gene and that uses a separate cell strain as BL control. The internal correction allowed to accurately evaluate the copper content even in simulated toxic samples, where reduced cell viability was observed. Homogenous cells isolated by GrFFF showed improvement in method reproducibility, particularly for yeast cells. The applicability of these bioreporters to real samples was demonstrated in tap water and wastewater treatment plant effluent samples spiked with copper and other metal ions.

Electronic supplementary material The online version of this article (doi:10.1007/s00216-011-5091-3) contains supplementary material, which is available to authorized users.

A. Roda · L. Cevenini · E. Michelini · L. Mezzanotte
Department of Pharmaceutical Sciences, University of Bologna,
Via Belmeloro 6,
40126 Bologna, Italy

A. Roda · B. Roda · E. Michelini · P. Reschiglian
INBB, Istituto Nazionale di Biostrutture e Biosistemi,
Viale delle Medaglie d'Oro 305,
00136 Rome, Italy

B. Roda · P. Reschiglian
Department of Chemistry "G. Ciamician", University of Bologna,
Via Selmi 2,
40126 Bologna, Italy

M. Virta
Department of Food and Environmental Sciences,
University of Helsinki,
PO Box 56, 00014 Helsinki, Finland

K. Hakkila
Department of Biochemistry and Food Chemistry,
University of Turku,
20014 Turku, Finland

A. Roda (✉)
Laboratory of Analytical and Bioanalytical Chemistry,
Department of Pharmaceutical Sciences,
Alma Mater Studiorum-University of Bologna,
Via Belmeloro 6,
40126 Bologna, Italy
e-mail: aldo.roda@unibo.it

Keywords Whole-cell bioreporters · Bioluminescence · Field-flow fractionation · Cell immobilization · Biosensor

Introduction

Bioluminescent whole-cell bioreporters are genetically engineered living microorganisms that produce a specific analytical signal in response to targeted analytes. Usually, they combine a molecular recognition element for the targeted analyte with a reporter gene, which encodes for a bioluminescent protein (usually luciferase). The response, which may include activation or inhibition of signal transduction pathways, is converted into the expression of the reporter luciferase enzyme. The reporter's activity gives a signal that is proportional to the concentration of the bioavailable fraction of analyte, i.e., able to cross the cell membrane [1, 2]. This information could be highly valuable in environmental toxicology for risk assessment and for the prompt selection of suitable remediation options. One example would be pollutants released by electronic waste (e-waste), which includes end-of-life electronic products like computers, mobile phones, and televisions. Most electronic products contain hazardous chemicals, such as heavy metals (lead, copper, cadmium, chromium, and mercury), phthalates, and polychlorinated biphenyls. The lack of standardized methodologies hinders the appropriate management and disposal of these substances [3].

For first-level screening purposes, it would be very helpful to be able to use whole-cell bioreporters that can rapidly detect these pollutants directly in environmental or biological samples, down to submolar concentration and without any sample clean-up [4–6].

Several bioreporters have been developed to detect heavy metals. Most are based on engineered bacteria, such as *Escherichia coli* and *Pseudomonas putida* [7–10]. Many efforts have been made to evaluate the effect of experimental conditions such as temperature, pH, and ionic strength on bioluminescence emission properties. This is in order to improve the analytical performance of these bioreporters [11]. Recently, "hyper-sensitive" bioreporters have been obtained by disrupting metal homeostasis in the host cell to remove heavy-metal efflux pumps [12]. Unfortunately, many methodological problems must be solved before whole-cell bioreporters can be considered as robust biosensing tools for environmental analyses [13].

The main shortcoming is that, when living cells are in direct contact with real complex samples, the sample's constituents can aspecifically affect cell viability, producing false uncontrolled analytical signals. Examples of such real complex samples include wastewater from treatment plants, seawater, and river water, even if highly diluted when analyzed. A cell viability control must, therefore, be introduced to monitor cell health, correct the analytical signal where appropriate, and, thus, increase the dynamic range and robustness of the assay [14]. Another weak point is the relatively long response time. This is usually numbered in hours because of the time needed for the RNA to express the minimum detectable amount of luciferase. With such a long exposure, the potentially toxic sample matrix constituents can affect cell viability, even when present at a very low concentration. An ideal bioreporter should be able to provide accurate and sensitive results in a few minutes. It would, thus, be suitable for a portable format. To date, however, the shortest times still take more than a few minutes, despite improvements in the available molecular biology tools.

To use bioreporters for biosensing applications or high-throughput screening, researchers also require a ready-to-use device where cells can be immobilized or trapped in a solid matrix.

Here, we have sought to improve the analytical performance of bioreporters by developing a working procedure for obtaining a robust and reproducible whole-cell biosensor format. First, we introduced an internal viability control by engineering *E. coli* and *Saccharomyces cerevisiae* cells with two reporter genes coding luciferases emitting at different wavelengths. The expression of one was copper ion specific. The other, constitutively expressed, was used as a cell viability control.

The assay requires the same luciferin substrate. Light emission can, thus, be simultaneously measured using two appropriate filters.

To obtain homogeneous cell populations, BL-engineered cells underwent a soft separation technique suitable for cell isolation. This was based on their morphology during their growth phase.

The gravitational field-flow fractionation technique (GrFFF) was used [15, 16]. In this technique, the separation mechanism is based only on the morphological properties of cells, such as their dimension, shape, and surface properties. Due to the absence of a stationary phase, the fractionation mechanism is "soft." That is, cell viability is preserved during fractionation, and sorted cells can be collected and reused in their native form [17, 18], still suspended in the culture medium. Cell fractionation was achieved through a simple device (an empty capillary channel) by the combined action of a transporting laminar flow and the gravitational field perpendicular to the flow.

The selected BL cell fractions were immobilized and trapped, as previously described, in a polymeric matrix that preserved their viability and ensured that the analyte could access the molecular recognition site in the cell [19].

The copper-specific bioreporters were used to measure the Cu^{2+} concentration in tap and wastewater, which had been spiked with copper and other heavy metals.

Materials and methods

Chemicals and reagents

Conventional chemicals of analytical grade used for bacterial and yeast cultures and cell immobilization were purchased from Sigma-Aldrich (Deisenhof, Germany). All the enzymes required for molecular cloning were from Fermentas (Vilnius, Lithuania) while the kits for plasmid extraction and purification were from Qiagen (Hilden, Germania). The plasmid pPROTet.E was purchased from BD Biosciences Clontech (Palo Alto, CA).

Luciferin solution of 1 mM at pH 5 was prepared by dissolving 28.3 mg D-luciferin sodium salt (Synchem, Kassel, Germany) into a 35-ml 0.1 M citric acid monohydrate and 65-ml 0.1 M trisodium citrate solution.

All media and metal ion solutions were prepared using deionized distilled water (Milli-Q water purification system, Millipore, Bedford, MA).

Genetically engineered cells

Two whole-cell bioreporters for copper ion detection were obtained using bacterial (*E. coli* MC1061) and yeast (*S. cerevisiae* BMA64-1A) strains as host cells. Both strains were genetically engineered with the introduction of two reporter genes: the *Photinus pyralis* wild-type luciferase (PpyWT, λ_{max}=557 nm) and the *P. pyralis* red-emitting mutant thermostable luciferase (PpyRE8, λ_{max}=618 nm) with a thermal inactivation time (defined as time for the maximum initial activity to decay to 50%) of 3.5 h at 37 °C with respect to the 0.26 h of wild-type enzyme [20]. All the DNA manipulation procedures were performed as described by Sambrook et al. [21]. Plasmid constructs were confirmed by restriction enzyme digestions and DNA sequencing.

Bacterial bioreporter for copper ion with internal viability control

The copper sensor strain *E. coli* MC1061 (pSLcueR/pDNPcopAluc) harbors the gene for the regulatory protein *cue*R and the promoter of copA, driving the expression of the PpyWT luciferase [22]. It was engineered to express the thermostable PpyRE8 luciferase under the regulation of a promoter inducible by anhydrotetracyclin (ATc). Briefly, the cDNA encoding for PpyRE8 was inserted into the multiple cloning site of pPROTet.E133 vector by blunt ligation.

The copper strain was made chemically competent with the one-step transformation and storage solution method [23]. Plasmid pPROTet.E-PpyRE8, carrying chloramphenicol resistance, was transformed into this strain to obtain the new strain *E. coli* MC1061 (CuGreen/Red). This was routinely grown in selective Luria Bertani (LB) medium containing 100 μg/mL ampicillin, 12.5 mg/mL tetracycline, and 25 μg/mL chloramphenicol.

Yeast bioreporter for copper ion with internal viability control

The dual-color copper-responsive strain was obtained by introducing the red-emitting luciferase PpyRE8, under the regulation of the CUP1 promoter, into the pSal1 [24] vector, and the PpyWT into pVTU260 vector (Euroscarf Collection, Frankfurt, Germany) as a control reporter gene.

The PpyRE8 cDNA was amplified by PCR from plasmid pGEX-PpyRE8 with forward and reverse primers carrying *Xho*I and *Nco*I sites, respectively. Both vector and insert were cut with *Xho*I and *Nco*I and ligated with T4 DNA ligase. The resulting plasmid was named pSal-PpyRE8.

The yeast expression vector pVTU260 was cut with *Nco*I while pGEX-PpyWT with *Bam*HI and *Xba*I to isolate the wild-type luciferase gene, and then, vector and insert were blunted and ligated. The vectors pSal-PpyRE8 and pVTU-PpyWT were sequentially transformed into the BMA64-1A *S. cerevisiae* yeast strain by the LiAc/SS-DNA/PEG (lithium acetate, single-strand carrier DNA, and polyethylene glycol, MW 3350) method [25]. The dual-color copper-responsive yeast strain, named *S. cerevisiae* BMA64-1A (CuRed/Green), was routinely grown in synthetic complete (SC) medium lacking uracil and leucine.

Fractionation of engineered cell populations by GrFFF

The GrFFF device

The fractionation device is a plastic, biocompatible, flat channel (GrFFF channel) based on two parallel plates made of polyvinylchloride. These are separated by a spacer from which the channel volume is removed [15]. Channel dimensions are: 4.0 cm in breadth, 30 cm in length, and 0.025 cm in thickness. A peristaltic pump (Miniplus 3, Gilson, Middleton, WI, USA) was used to generate the mobile-phase flow inside the GrFFF channel. The sample volume was injected using an HPLC syringe into the GrFFF inlet tube (in PEEK; L=7 cm, I.D.=0.750 mm, O.D. 1/16″). An UV–vis diode-array detector equipped with a 5-cm light-pipe cell (ThermoQuest, Austin, TX, USA) was connected to the channel outlet to monitor the elution process. The absorbance at 600 nm was recorded. A fraction collector was connected to automatically collect cell fractions at given retention times.

The GrFFF method

Aliquots of 50 μL of bioreporters cell suspension (approximately, 1×10^7 cells/mL of the CuRed/Green yeast

strain and 0.5×10^9 cell/mL of the CuGreen/Red bacterial strain) were injected into the GrFFF system using an HPLC syringe introduced into the inlet tube with a flow rate of 0.25 mL/min for 15 s. Flow was then interrupted for 6 min to allow sample relaxation. After this step, flow was reactivated at a rate of 2 mL/min for the elution. Cell culture media were used as mobile phase.

Bacterial and yeast cells immobilization procedure

Bacterial and yeast bioreporters for copper detection were immobilized in a polymeric matrix composed of 0.5% agarose, 1% polyvinylpyrrolidone, and 0.05% collagen solution final concentration as described in detail elsewhere [19]. Bacterial or yeast cell cultures containing nutrients were mixed with an equal volume of the freshly prepared immobilization matrix solution. A volume of 30 μL of the cell–matrix mixture was poured into the wells of a black 384-microwell plate at room temperature, hardened for 30 min, and stored at 4 °C. Before use, cells were left to equilibrate at room temperature for 15 min.

Copper detection with free and immobilized bacterial and yeast dual-color bioreporters

Single colonies from an agar plate of bacterial or yeast bioreporters were used to inoculate 3.0 mL of LB or SC medium for bacterial or yeast growth, respectively. These cultures were grown overnight at 30 °C with orbital shaking at 250 rpm. The day after, bacterial or yeast cultures were diluted with respective fresh medium and OD_{600} was monitored until it reached the value of 0.8.These cultures were injected into the GrFFF system. Fractionated cells were used to perform cell-based assays in liquid medium or to immobilize them, as described in the previous section.

Briefly, the assay procedure was performed by incubating 30 μL of cell suspension, transferred to a sterile black 384-well microtiter plate, with 5 μL of $CuSO_4$ dilutions. These were prepared in ultrapure Milli-Q water at pH 5.5, ranging from 10^{-8} to 10^{-2} M (5 μL of sterile water were used as blank control) and incubated for 2 h at 30 °C. For the bacterial bioreporters only, we added a 1×10^{-3} M ATc solution to the cell culture. This was to induce the expression of the control PpyRE8 luciferase before incubation with $CuSO_4$ dilutions.

For assays with immobilized cells, 5 μL of $CuSO_4$ dilutions and a blank control (5 μL of sterile water) were added in triplicate to the wells of a 384-well microplate containing 30 μL of the immobilized cells and incubated for 2 h at 30 °C.

After incubation, 30 μL of D-luciferin (1 mM) were injected and bioluminescence measurements were performed using the Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific, Whaltham, MA, USA), equipped with green (band pass 520–540 nm) and red (band pass 610–650 nm) hard-coated filters. Light emissions (1 s integration time) were taken 5 min after the addition of D-luciferin and expressed as relative light units. Light emissions were spectrally resolved using the Promega Chroma-Luc Calculator.

Dose–response curves were corrected by the viability control light emission by reporting for each measurement the ratio of the analyte-specific light signal to internal control signal (BL_s/BL_c) against log [Cu^{2+}].This allows to normalize the intensity of the specific signal according to the cell viability evaluated by their BL signal which could be aspecifically affected by sample matrix constituents or by a too high copper concentration in the sample which became toxic to cells.

GraphPad Prism software v5.02 (Software, Inc., San Diego, CA 92121) was used to fit the data for dose–response curves. The equation for the best-fit line, produced by the program, was used to calculate the amount of Cu^{2+} present in the sample. The detection limit (LOD) was defined as the concentration of copper giving a BL_s/BL_c value corresponding to that of the blank plus three times its SD.

Dose–response curves with simulated cell toxicity

Dose–response curves for Cu^{2+} in the range 10^{-6}–10^{-4} M were produced with both immobilized bacterial- and yeast-based bioreporters as described above. Spiked samples containing 10^{-5} M Cu^{2+} and ethanol or dimethyl sulfoxide (DMSO) at 5% and 10% (*v*/*v*), as model toxic compounds, were evaluated. Assays, bioluminescence measurements, and data analysis were performed as described above. Each assay was performed in triplicate.

Real sample analysis

Wastewater treatment plant effluent and tap water samples from Bologna municipal water supply were analyzed without any pretreatment and spiked with copper at concentrations ranging from 0.0630 to 63.5 mg/L. The bioreporters' copper selectivity was evaluated by incubating the cells with tap water, also spiked with copper sulfate (10^{-5} M) and other metals ions including Zn^{2+}, Fe^{2+}, Ag^{2+}, Pb^{2+}, Cd^{2+}, and Ni^{2+} at 10^{-3} and 10^{-5} M. Assays, bioluminescence measurements, and data analysis were performed as described above. Each assay was performed in triplicate. The copper content of all the test samples was also determined by flame atomic absorption spectrometry (FAAS, PerkinElmer, AAnalyst 400).

Results and discussion

As outlined in a recent review book [26], many obstacles prevent whole-cell bioreporters from being considered

efficient bioanalytical tools, especially for real sample analysis. These include limited shelf life, low robustness, and low dynamic range. In the present work, we improved their analytical performance by introducing an internal viability control. We did this by engineering a cell with two reporter genes, one under the control of copper and the other constitutively present. We also developed a cell-sorting technique for selecting a homogenous BL cell population, and we introduced cell immobilization.

Bacterial and yeast bioluminescent bioreporters with internal control

One major drawback encountered in the analytical application of whole-cell bioreporters is the high variability of the response caused by the sample matrix, which has nonspecific effects on the analyte-dependent reporter protein synthesis. Cells stay in contact with the sample solution for a relatively long time. Toxic substances or inhibitory effects such as pH variation and ionic strength may, thus, lead to a decrease in the reporter signal. Alternatively, the nonspecific stimulation of cells' metabolism may also lead to overestimations (i.e., the presence of nutrients in the sample).

To take into account the nonspecific effects of the sample, the analyte-specific signal must be corrected according to cell viability. Most methods use a separate cell population, which is exposed to the same sample to test its toxicity (external control) [26–28].

Alternatively, BL reporter proteins emitting at different wavelengths can be introduced in bacteria and mammalian cells as an internal control system for a faster, simpler, and more reliable evaluation of the analytical response [14, 29, 30].

We developed dual-color BL bacterial and yeast bioreporters for the same model analyte, Cu^{2+}, with internal correction for nonspecific stimuli. A second BL reporter gene, emitting at a different wavelength, was integrated into the same bacterial and yeast cells. Its expression, thus, occurred simultaneously and in the same cell as the analyte-induced reporter gene.

Bacterial cells were engineered to express the green-emitting firefly PpyWT luciferase (λ_{max}=557 nm) under the regulation of a copper-inducible promoter and the thermostable red-emitting luciferase PpyRE8 (λ_{max}=618 nm) [20], used as internal viability control. Yeast cells were engineered to express PpyRE8 in a copper-inducible manner and PpyWT luciferase as internal viability control. The two luciferases require the same D-luciferin substrate and the light emission is measured simultaneously by spectral resolution. The citrate-buffered D-luciferin substrate is added at the end of incubation with copper or other metal ions to trigger the emission of the reporter proteins already produced avoiding any changing in the speciation of the added metal ions.

BL assays were optimized in the 384-well microplate format using liquid cultures or immobilized cells. Several experimental parameters were optimized to obtain the best analytical performance of the bioreporters including incubation time of the bioreporter with analyte and incubation temperature. In addition, we also optimized the time of bioluminescence measurements upon addition of D-luciferin; kinetic measurements showed we had a temporal window of about 5 min (ranging from 5 to 10 min), in which the intensity of the BL signal was maintained at a steady-state level allowing to detect the green and red BL signals sequentially with optical filters without changes due to the emission kinetics.

Dose–response curves for Cu^{2+} were produced. Using the internal viability control allowed us to correct the signal with a consequent increase in the dynamic range, particularly at high Cu^{2+} concentrations when cell toxicity was observed (Fig. 1; Fig. S1 in the Electronic supplementary material). The bioluminescence decrease due to the analyte itself is a frequent phenomenon observed in previous works. The dose response curves at a high concentration where analyte is toxic for cells shows an inverse relationship with concentration, thus producing ambiguous results [26].

A LOD of 1.5×10^{-7} and 5.0×10^{-7} M was obtained with bacteria and yeast, respectively. These values are slightly higher than those obtained with whole-cell bioreporters for more toxic heavy metals such as lead, cadmium, and

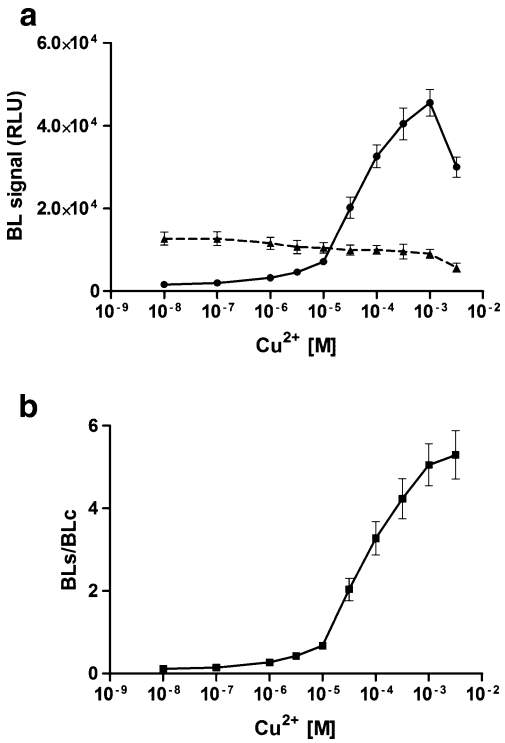

Fig. 1 Noncorrected dose–response curve for Cu^{2+} obtained with yeast bioreporter that express PpyRE8 luciferase (*solid line*, *circles*) under the regulation of a copper-inducible promoter and the PpyWT as internal viability control (*dashed line*, *triangles*) (**a**). Corrected dose–response curve for Cu^{2+} (**b**). Data are the average±one standard deviation (n=3)

mercury [31]. This is because of the presence of homeostatic mechanisms that avoid cell deprivation of essential trace elements, such as copper and zinc. In addition, bacteria and yeast have different copper-recognition systems and metal homeostasis mechanisms. This influences metal bioavailability and the sensing ability of the bioreporters. It also explains the slight differences between bacterial and yeast Cu^{2+} bioreporters. The EU Directive on the quality of water intended for human consumption (98/83/EC) establishes a recommended Cu^{2+} maximum limit of 30 μM [32]. As such, both bioreporters, according to their LOD and with a dynamic range of four orders of magnitude (10^{-7}–10^{-3} M), are suitable for real samples analysis.

GrFFF of cellular bioreporters

Sorting of cellular bioreporters

When using living BL cells for biosensing, cells that are more viable and responsive (i.e., in the optimal growth phase) must be chosen and characterized in order to obtain more precise and sensitive biosensors. This is one of the most overlooked and underestimated aspects of using this approach. The usual experimental procedure is to grow a liquid cell culture until a given optical density is reached. This should correspond to a particular growth phase (mid-exponential phase). Then, aliquots of cells are incubated with the analyte for a given period of time and bioluminescence is measured to correlate light emission with the analyte concentration. Since batch cultures grown to a given optical density do not allow the selection of a synchronized cell population with specific features, we therefore used GrFFF to characterize cell populations and select cells with homogeneous and appropriate properties. Due to the effect of the applied gravitational field, larger micrometer-sized analytes (like whole cells) can thus be eluted quicker than smaller particles [33].

First, we evaluated the best conditions for cell fractionation and collection. The main issue was to choose a mobile phase that retained cell viability, with the absence of cell growth or modifications during the elution process, which usually takes about 30 min. As liquid carriers, we used either SC medium for yeast cells and LB broth for bacteria or phosphate-buffered saline at pH 7.4. To prevent temperature from influencing cell growth, all fractionations were performed at room temperature. Since no significant differences were reported using the two carriers, the culture medium was selected as mobile phase. Fractographic profiles of engineered yeast cells for copper detection are reported in Fig. 2a.

The fractographic profile of yeast cell suspension was obtained from a liquid culture grown to an $OD_{600\ nm}$ of 0.8. It showed a heterogeneous population comprising asynchronously growing cells with different metabolic activity. Using this heterogeneous population for biosensing could affect the robustness of the assay, because Cu sensitivity is affected by the cell cycle stage [34].

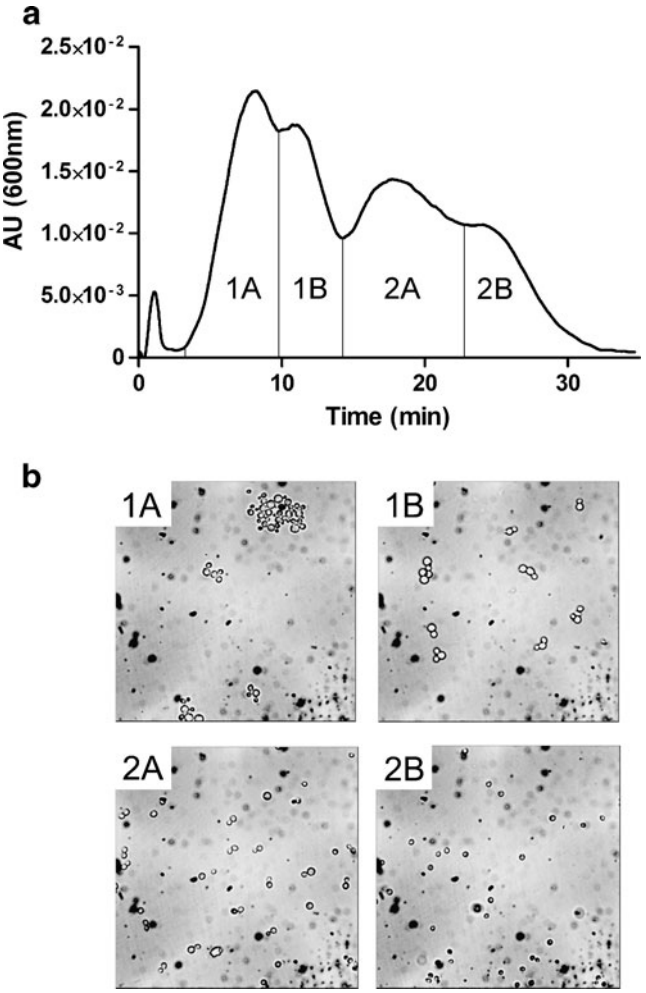

Fig. 2 GrFFF fractographic profile of dual-color copper-responsive yeast strain cell suspension obtained from a liquid culture grown to an $OD_{600\ nm}$ of 0.8 and collected fractions: *1A*, *2A*, *1B*, *2B* (**a**). Live image of four fractions observed with an optical microscope with a ×60 objective (**b**)

We identified two main populations (peak 1 from 3 to 15.5 min and peak 2 from 16 to 30 min), each divided into two subpopulations (band 1A from 3 to 10 min and band 1B from 10.5 to 15.5 min; band 2A from 16 to 21 min and band 2B from 21.5 to 30 min). Four different fractions corresponding to each band were collected and observed with an optical microscope to relate the GrFFF fractogram with cell growth and morphology. Figure 2b shows the live image of the collected fractions with a ×60 objective without any cell suspension treatment.

In the first two fractions (bands 1A and 1B), there were mainly cellular aggregates or cells with multiple buds as a result of growth and gemmation. The last two fractions (bands 2A and 2B) comprised single cells in early growth

stage. The last fraction (2B) was a homogeneous cell population.

Characterization and activity determination of sorted cells

First, we confirmed the total maintenance of viability for fractionated cells with respect to unfractionated cells. Then, we tested the properties of each fraction as biosensing.

Cells were collected at retention times corresponding to fractions 1A, 2A, 1B, and 2B, as reported in Fig. 2. Due to the high reproducibility of the fractionated process, cells collected from three repeated GrFFF runs were pooled. A dose–response curve was prepared for each fraction and for an unfractionated sample, following the procedure for liquid culture described in "Materials and methods." The first fractions show analytical performances worse or similar to the unfractionated cells. However, fraction 2B gives results with a higher precision and higher reproducibility (both run-to-run and day-to-day coefficients of variation (CV) of data points are lower than 10%) with respect to unfractionated samples (CV%=15; n=10). Moreover, higher sensitivity was obtained, with a LOD of 2×10^{-7} M.

These results confirm that the method can be used to sort yeast populations without modifying native properties. A homogeneous biosensing cell population can, thus, be selected to provide higher analytical performance. The GrFFF method requires simple instrumentation and is a useful tool for standardizing yeast cells for this purpose.

Concerning the fractionation of bacterial cells, no significant differences were observed in the different fractions collected. Further studies are needed here to explore the potential use of GrFFF and to understand if cell growth is a critical factor for bacterial cells when using this technique.

Immobilization of bacterial and yeast bioreporters

To increase the shelf life of bioreporter cells, we immobilized the whole-cell bioreporters in a polymeric matrix. This allowed us to maintain cells for up to 1 month at 4 °C. We note that, after long-term storage, not all the cells will be alive or retain their full responsiveness. Moreover, we cannot rule out the possibility that cells will die during the immobilization process itself. The percentage of living cells was estimated by BL emission and cell counting. We reported a 20±5% and 15 ±3% loss in viability after 1-month storage for bacteria and yeast cells, respectively. As we previously reported with other strains, this matrix does not limit the diffusion of oxygen and substrate for bioluminescent reaction [19]. The matrix composition and the cell-to-matrix ratio were optimized to allow free diffusion of different classes of analytes.

The pores of the biopolymer are large enough to allow free diffusion of ions, nevertheless it has been reported that agarose and PVP may form complex with divalent metal ions due to noncovalent forces, thus, limiting metal bioavailability to the cells [35]. Figure 3 shows the corrected dose–response curves for Cu^{2+}, obtained with bacterial (Fig. 3a) and homogeneous yeast cells isolated by GrFFF (Fig. 3b), immobilized in the polymeric matrix, and stored at 4 °C for 0, 15, and 30 days. A LOD of 5×10^{-6} and 2×10^{-6} M was obtained with immobilized yeast and bacterial bioreporters, respectively.

In addition, intra- and inter-assay precisions determined by analyzing replicates (n=10) of spiked samples at a fixed Cu^{2+} concentration of 1×10^{-4} M in the midrange of the dose–response curve for immobilized bacterial bioreporter and fractionated yeast cells were better (CV%=6) than those obtained with liquid cultures of bacterial cells (CV%=18) and unfractionated yeast cells (CV%=10%). They, thus, produced more reliable results. The immobilized cells had a slightly lower detectability than the nonimmobilized bioreporters. However, the immobilized cells still worked in the environmentally relevant range of concentration. Moreover, they have several advantages, including ease of use, minimal variations between different batches of cells, and the possibility of out-lab applications. Based on these results, immobilized cells obtained from fraction 2B of yeast samples

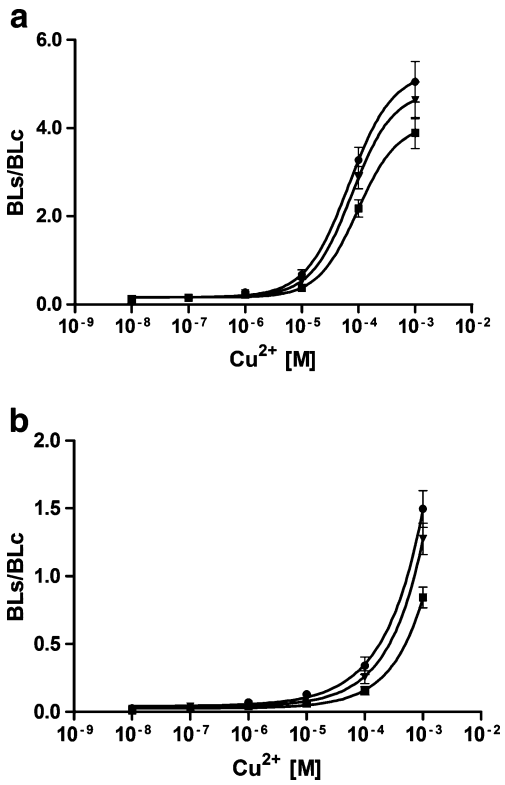

Fig. 3 Corrected dose–response curves for Cu^{2+} obtained with the dual-color yeast (**a**) and bacterial copper bioreporters (**b**) immobilized in the polymeric matrix and stored at 4 °C for 0 (*circles*), 15 (*inverted triangles*), and 30 days (*squares*). Data are the average±one standard deviation (n=3)

and unfractionated bacterial samples were used in assays with toxic simulated samples and real sample analysis, as described in the next paragraphs.

Evaluation of internal correction with simulated cell toxicity

To test the ability of the internal control to correct the analytical signal, we incubated the immobilized whole-cell bioreporters with toxic simulated samples. These comprised a fixed Cu^{2+} concentration (1×10^{-5} M) spiked with different amounts of ethanol or DMSO, which were chosen as model toxic compounds for their well known toxic effect on microbial cells. Table 1 shows the results obtained with immobilized bacterial and yeast bioreporters. Uncorrected Cu^{2+} recovery was calculated by interpolating the copper-induced BL signals directly on the uncorrected dose–response curve for Cu^{2+}. The corrected Cu^{2+} recovery was obtained by interpolating the ratio of copper-induced to internal control BL signals on a corrected dose–response curve. As expected, the presence of ethanol or DMSO dramatically reduced the viability of bacteria and, to a lower extent, the viability of yeast. This causes a significant underestimation of the Cu^{2+} concentration without any signal correction. For example, bacteria exposed to 10% DMSO show a strong reduction in viability, which reduces recovery to 8±2%. By correcting the signal, however, a recovery of 92±5% can be obtained.

In spite of this, thanks to the internal correction of the copper-induced BL signal, we have reliably evaluated the copper content, even in highly toxic samples.

Real sample analysis

Wastewater effluent and tap water samples were fortified with Cu^{2+} and other metal ions so that we could investigate the sample matrix effect and the specificity of the developed bioreporters.

There was not much difference in the bioluminescence responses of the developed Cu^{2+} bioreporters obtained in distilled deionized and tap water samples. This indicates that there is little or no sample matrix effect of tap water on the determination of copper ions.

For the dose–response curve of the wastewater effluent sample, we observed just a slight decrease in cell viability (about 13±3%). This was appropriately corrected by the internal control. The wastewater treatment plant produces water that can be used for irrigation and therefore relatively cleaned.

The specificity of the microbial bioreporters was evaluated by incubating the cells with tap water samples, which were also spiked with copper sulfate and other metals including iron, lead, nickel, zinc, silver, and cadmium as shown in Table 2.

Bacterial bioreporter was induced only by Cu^{2+} and Ag^{2+} as previously reported [22] and the minimum inducible concentrations were respectively 1.5×10^{-7} and 1×10^{-6} M.

Yeast bioreporter was able to respond also to silver ions as demonstrated by the overestimation of the copper content in the sample containing Ag^{2+} concentration between 1×10^{-6} and 1×10^{-5} M with respect to the Cu^{2+} added. Moreover, compared with Cu^{2+}, Ag^{2+} became toxic to the cells at relatively low concentration (loss in viability higher than 90% at 5×10^{-5} M) above which the internal control was not able to properly correct the specific signal.

In addition, only a slight increase of the BL signal (10±2%) was obtained with zinc at concentrations approaching 5×10^{-4} M while no appreciable amount of luciferase was expressed in both bioreporters in presence of nickel, iron and lead, even at high concentrations. We noted that cadmium ions negatively interfere, in a concentration-dependent manner, with the copper-recognition element of the yeast bioreporter, without affecting cell's viability. In the presence of cadmium ions at 1×10^{-5} and 1×10^{-3} M, the measured Cu^{2+} was 0.240 and 0.00440 mg/L, respectively, which is

Table 1 Analysis of samples containing known Cu^{2+} concentrations and various toxic compounds

Added interfering compound	Added Cu^{2+} (μm)	Bacteria (recovery±standard deviation (%))[a]		Yeast (recovery±standard deviation (%))[a]	
		Uncorrected	Corrected	Uncorrected	Corrected
5% ethanol	10	43±4	91±6	68±4	94±5
10% ethanol	10	18±2	89±5	55±3	92±5
5% DMSO	10	18±3	93±5	75±4	95±5
10% DMSO	10	8±2	92±5	69±5	94±6

Uncorrected Cu^{2+} recovery was calculated by interpolating copper-induced BL signals directly on the uncorrected dose–response curve for Cu^{2+}. Corrected Cu^{2+} recovery was obtained by interpolating the ratio of copper induced to internal control BL signals on a corrected dose–response curve, produced in standard conditions

[a] Recovery (%)=(measured Cu^{2+})/(added Cu^{2+})×100

much lower with respect to the 0.635 mg/L added and confirmed by atomic absorption spectroscopy.

Taken together, these results suggest that the Cu^{2+} bioreporters developed could be effectively used for copper detection. This is because the aspecific responses to other metals only occur at irrelevant environmental concentrations.

Nevertheless, since bioreporters are intended to complement traditional analytical techniques, not to replace them, researchers can overcome the lack of specificity of microbial sensors by using a panel of several strains with different specificities and sensitivities combined with a statistical approach. This has been nicely proposed by Jouanneau et al. [36].

Conclusions

In this work, we have critically evaluated and significantly improved the development of BL whole-cell bioreporters for biosensing applications.

We have developed genetically engineered BL bacterial- and yeast-based bioreporters for copper ion detection (as a model analyte) with an internal viability control. We introduced a second luciferase emitting at a different wavelength and requiring the same substrate. This simplified the assay procedure and allowed us to correct the sample matrix effects, leading to increased robustness and a wider dynamic range.

We used GrFFF techniques to select a cell population in which the majority of cells are in the optimal growth phase for biosensing.

To the best of our knowledge, this is the first report in which GrFFF techniques are used not only to characterize and sort cells but also to select the most suitable population for biosensing applications. For yeast cells, we demonstrated that GrFF can be used to sort cells, without affecting their viability and/or metabolism, in order to obtain a homogeneous and well-characterized population. Further studies are needed regarding a similar application to bacterial cells. The integration of the GrFFF system into the biosensor preparation

Table 2 Response of the yeast and bacterial copper bioreporters in wastewater treatment plant effluent samples spiked with different amounts of Cu^{2+} alone or in combination with other metals

Added Cu^{2+} [M] (mg/L)	Other metals [M]	Measured Cu^{2+}±standard deviation (mg/L)		
		Yeast bioreporter (fractionated)	Bacterial bioreporter	FAAS
[10^{-3}] (63.5)	–	57.8±6.2	66.2±7.3	65.4±2.8
[10^{-4}] (6.35)		6.5±0.5	5.9±0.4	6.22±0.31
[10^{-5}] (0.635)	–	0.69±0.06	0.57±0.07	0.615±0.031
[10^{-6}] (0.0635)	–	0.072±0.009	0.055±0.008	0.0658±0.0033
[10^{-7}] (0.00635)	–	0.0069±0.0008	0.0073±0.0009	0.00674±0.00045
[10^{-8}] (0.000635)	–	n.d.[a]	n.d.	0.000588±0.000043
Mixtures				
Cu^{2+} [10^{-3}]	Fe^{2+} [10^{-3}]	58.5±7.4	70.6±7.2	62.3±3.1
Cu^{2+} [10^{-4}]	Fe^{2+} [10^{-5}]	6.9±0.5	8.1±0.7	6.42±0.27
Cu^{2+}[10^{-5}]	Pb^{2+} [10^{-3}]	0.65±0.04	0.55±0.08	0.615±0.031
Cu^{2+}[10^{-7}]	Pb^{2+} [10^{-5}]	0.0057±0.0006	0.0067±0.0005	0.00669±0.00037
Cu^{2+} [10^{-7}]	Ni^{2+} [10^{-3}]	0.0067±0.0006	0.0061±0.0005	0.00615±0.00029
Cu^{2+} [10^{-8}]	Ni^{2+} [10^{-5}]	n.d.	n.d.	0.000594±0.000052
Cu^{2+} [10^{-5}]	Zn^{2+} [10^{-4}]	0.85±0.04	0.79±0.04	0.609±0.036
Cu^{2+} [10^{-8}]	Zn^{2+} [10^{-5}]	n.d.	n.d.	0.000582±0.000057
Cu^{2+} [10^{-8}]	Ag^{2+}[10^{-5}]	0.73±0.05	0.58±0.07	0.000642±0.000048
Cu^{2+} [10^{-3}]	Ag^{2+} [10^{-6}]	115.5±10.2	95.8±11.4	62.9±2.2
Cu^{2+} [10^{-5}]	Ag^{2+} [10^{-4}]	n.d.	n.d.	0.639±0.029
Cu^{2+} [10^{-5}]	Cd^{2+} [10^{-3}]	0.0044±0.0008	0.52±0.05	0.618±0.041
Cu^{2+}[10^{-5}]	Cd^{2+} [10^{-5}]	0.24±0.03	0.75±0.06	0.622±0.035
Cu^{2+} [10^{-5}]	Cd^{2+} [10^{-7}]	0.68±0.04	0.61±0.05	0.640±0.023
Cu^{2+}[10^{-8}]	Cd^{2+} [10^{-5}]	n.d.	n.d.	0.000607±0.000061

n.d. not detected

Data are the average of three replicates

was simple and fast. However, the throughput of the system could still be improved. For example, channel rays could be used to fractionate a higher number of cells per run.

By immobilizing whole-cell bioreporters in a biocompatible polymeric matrix, we maintained cell viability with a good responsiveness, even after a lengthy storage time (1 month) at 4 °C.

This opens up the possibility of using cells as ready-to-use reagents, like antibodies or other biomolecular recognition elements. The possibility of immobilizing whole-cell bioreporters specific for different analytes is also important. This is because it paves the way for developing a multiplex platform to monitor, for example, a panel of heavy metals leaching from e-waste landfill sites.

Acknowledgments The authors gratefully acknowledge the financial support provided by the Italian Ministry of University and Research MIUR with the project FIRB 2009 "Development of bioremediation procedures and novel detection biotechnologies for Endocrine Disruptors" (prot. RBFR08SZTR), and the Istituto Superiore di Sanità (ISS) through the project "Progetto Strategico Salute della donna". We are very grateful to Prof. Bruce Branchini for providing the cDNA encoding for PpyRE 8 and to Dr. Jorma Lampinen for the opportunity to test the Varioskan® Flash instrument. We thank Grace Fox for editing and proofreading the manuscript.

References

1. Yagi K (2007) Applications of whole-cell bacterial sensors in biotechnology and environmental science. Appl Microbiol Biotechnol 73:1251–1258
2. Eltzov E, Marks RS (2010) Whole-cell aquatic biosensors. Anal Bioanal Chem. doi:10.1007/s00216-010-4084-y
3. Chen A, Dietrich KN, Huo X, Ho SM (ANNO) developmental neurotoxicants in E-waste: an emerging health concern. Environ Health Perspect doi:10.1289/ehp.1002452
4. Hynninen A, Virta M (2010) Whole-cell bioreporters for the detection of bioavailable metals. Adv Biochem Eng Biotechnol 118:31–63
5. Nivens DE, McKnight TE, Moser SA, Osbourn SJ, Simpson ML, Sayler GS (2004) Bioluminescent bioreporter integrated circuits: potentially small, rugged and inexpensive whole-cell biosensors for remote environmental monitoring. J Appl Microbiol 96:33–46
6. Gu MB, Mitchell RJ, Kim BC (2004) Whole-cell-based biosensors for environmental biomonitoring and application. Adv Biochem Eng Biotechnol 87:269–305
7. Blouin K, Walker SG, Smit J, Turner R (1996) Characterization of in vivo reporter systems for gene expression and biosensor applications based on luxAB luciferase genes. Appl Environ Microbiol 62(6):2013–2021
8. Dorn JG, Frye RJ, Maier RM (2003) Effect of temperature, pH, and initial cell number on luxCDABE and nah gene expression during naphthalene and salicylate catabolism in the bioreporter organism *Pseudomonas putida* RB1353. Appl Environ Microbiol 69(4):2209–2216
9. Neilson JW, Pierce SA, Maier RM (1999) Factors influencing expression of luxCDABE and nah genes in *Pseudomonas putida* RB1353(NAH7, pUTK9) in dynamic systems. Appl Environ Microbiol 65(8):3473–3482
10. Robbens J, Dardenne F, Devriese L, De Coen W, Blust R (2010) *Escherichia coli* as a bioreporter in ecotoxicology. Appl Microbiol Biotechnol 88(5):1007–1025
11. Sørensen SJ, Burmølle M, Hansen LH (2006) Making bio-sense of toxicity: new developments in whole-cell biosensors. Curr Opin Biotechnol 17:11–16
12. Hynninen A, Tõnismann K, Virta M (2010) Improving the sensitivity of bacterial bioreporters for heavy metals. Bioengineered Bugs 1(2):132–138
13. Harms H, Wells MC, van der Meer JR (2006) Whole-cell living biosensors: are they ready for environmental application? Appl Microbiol Biotechnol 70:273–280
14. Mirasoli M, Feliciano J, Michelini E, Daunert S, Roda A (2002) Internal response correction for fluorescent whole-cell biosensors. Anal Chem 74:5948–5953
15. Reschiglian P, Zattoni A, Roda B, Michelini E, Roda A (2005) Field-flow fractionation and biotechnology. Trends Biotechnol 23:475–483
16. Roda B, Zattoni A, Reschiglian P, Moon MH, Mirasoli M, Michelini E, Roda A (2009) Field-flow fractionation in bioanalysis. A review of recent trends. Anal Chim Acta 635:132–143
17. Roda B, Reschiglian P, Zattoni A, Tazzari PL, Buzzi M, Ricci F, Bontadini A (2008) Human lymphocyte sorting by gravitational field-flow fractionation. Anal Bioanal Chem 392:137–145
18. Roda B, Reschiglian P, Alviano F, Lanzoni G, Bagnara GP, Ricci F, Buzzi M, Tazzari PL, Pagliaro P, Michelini E, Roda A (2009) Gravitational field-flow fractionation of human hemopoietic stem cells. J Chromatogr A 1216:9081–9087
19. Roda A, Cevenini L, Michelini E, Branchini BR (2011) A portable bioluminescence engineered cell-based biosensor for on-site applications. Biosens Bioelectron 26:3647–3653
20. Branchini BR, Ablamsky DM, Davis AL, Southworth TL, Butler B, Fan F, Jathoul AP, Pule MA (2010) Red-emitting luciferases for bioluminescence reporter and imaging applications. Anal Biochem 396:290–297
21. Sambrook EF, Fristch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor
22. Hakkila K, Green T, Leskinen P, Ivask A, Marks R, Virta M (2004) Detection of bioavailable heavy metals in EILATox-Oregon samples using whole-cell luminescent bacterial sensors in suspension or immobilized onto fibre-optic tips. J Appl Toxicol 24:333–342
23. Chung CT, Niemela SL, Miller RH (1989) One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc Natl Acad Sci USA 86:2172–2175
24. Mascorro-Gallardo JO, Covarrubias AA, Gaxiola R (1996) Construction of a CUP1 promoter-based vector to modulate gene expression in *Saccharomyces cerevisiae*. Gene 172:169–170
25. Gietz RD, Schiestl RH (1995) Transforming yeast with DNA. Methods Mol Cell Biol 5:255–269
26. Belkin S, Gu MB (2010) Whole cell sensing systems. Advances in biochemical enginering. Springer, Heidelberg
27. Ivask A, Rõlova T, Kahru A (2009) A suite of recombinant luminescent bacterial strains for the quantification of bioavailable heavy metals and toxicity testing. BMC Biotechnol 9:41
28. Michelini E, Leskinen P, Virta M, Karp M, Roda A (2005) A new recombinant cell-based bioluminescent assay for sensitive androgen-like compound detection. Biosens Bioelectron 20:2261–2267
29. Michelini E, Cevenini L, Mezzanotte L, Ablamsky D, Southworth T, Branchini BR, Roda A (2008) Combining intracellular and secreted bioluminescent reporter proteins for multicolor cell-based assays. Photochem Photobiol Sci 7:212–217
30. Michelini E, Cevenini L, Mezzanotte L, Ablamsky D, Southworth T, Branchini BR, Roda A (2008) Spectral-resolved gene technology for

multiplexed bioluminescence and high-content screening. Anal Chem 80:260–267
31. Ivask A, Hakkila K, Virta M (2001) Detection of organomercurials with sensor bacteria. Anal Chem 73:5168–5171
32. Council Directive 98/83/EC on the quality of water intended for human consumption as amended by Regulations 1882/2003/EC and 596/2009/EC
33. Roda B, Lanzoni G, Alviano F, Zattoni A, Costa R, Di Carlo A, Marchionni C, Franchina M, Ricci F, Tazzari PL, Pagliaro P, Scalinci SZ, Bonsi L, Reschiglian P, Bagnara GP (2009) A novel stem cell tag-less sorting method. Stem Cell Rev 5:420–427
34. Howlett NG, Avery SV (1999) Flow cytometric investigation of heterogeneous copper-sensitivity in asynchronously grown *Saccharomyces cerevisiae*. FEMS Microbiol Lett 176:379–386
35. Aksu Z, Egretli G, Kutsal A (1998) A comparative study of copper(II) biosorption on Ca-alginate, agarose and immobilized *C. vulgaris* in a packed-bed column. Process Biochem 33:393–400
36. Jouanneau S, Durand MJ, Courcoux P, Blusseau T, Thouand G (2011) Improvement of the identification of four heavy metals in environmental samples by using predictive decision tree models coupled with a set of five bioluminescent bacteria. Environ Sci Technol 201145:2925–2931