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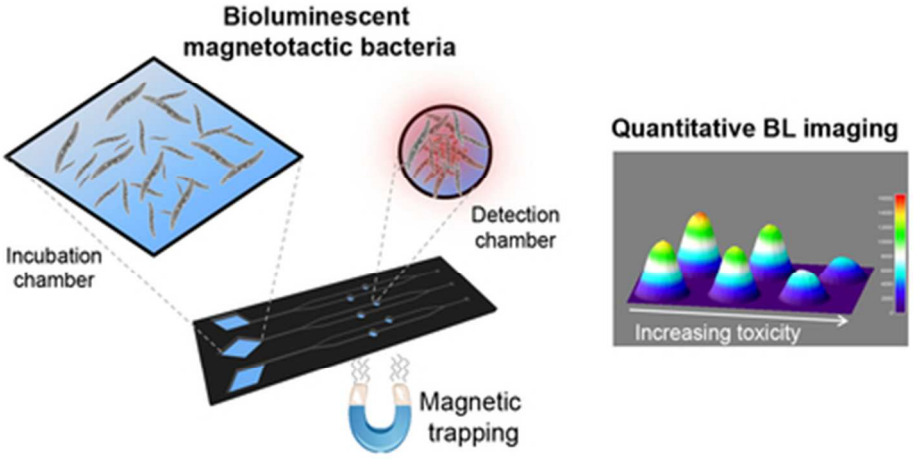

Schematic view of the MAGNETOX biosensor
40x20mm (300 x 300 DPI)

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Bioengineered bioluminescent magnetotactic bacteria as powerful tool for chip-based whole-cell biosensors

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Abstract

This paper describes the generation of genetically engineered bioluminescent magnetotactic bacteria (BL-MTB) and their integration into a microfluidic analytical device to obtain a portable toxicity detection system.

Magnetospirillum gryphiswaldense strain MSR-1 was bioengineered to constitutively express a red-emitting click beetle luciferase whose bioluminescent signal is directly proportional to bacterial viability. The magnetic properties of these bacteria have been exploited as "natural actuator" to transfer the cells in the chip from the reaction to the detection area, optimizing its analytical performance.

A robust and cost-effective biosensor for the evaluation of sample toxicity, named MAGNETOX, based on lens-free contact imaging detection, has been developed. A microfluidic chip has been fabricated using multilayered black and transparent polydimethyl siloxane (PDMS) in which BL-MTB are incubated for 30 min with the sample, moved by microfluidics, trapped, and concentrated in detection chambers by an array of neodymium-iron-boron magnets. The chip is placed in contact with a cooled CCD through a fiber optic taper to perform quantitative bioluminescence imaging after addition of luciferin substrate. A model toxic compound (dimethyl sulfoxide, DMSO) and a bile acid (taurochenodeoxycholic acid, TCDCA) were used to investigate the MAGNETOX analytical performance. The incubation with DMSO and TCDCA drastically reduces the bioluminescent signal in a dose-related manner.

The generation of bacteria that are both magnetic and bioluminescent combines the advantages of easy 2D cells handling with ultra sensitive detection, offering an undoubted potential to develop cell-based biosensors integrated into microfluidic chips.

Keywords: magnetotactic bacteria, bioluminescence, biosensor, magnetic actuator, microfluidic chip, toxicity

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Introduction

The increasing demand for robust and cost-effective portable analytical devices for on-site environmental toxicity screening has prompted the development of miniaturized whole-cell biosensors able to provide information about potential in vivo toxicity, thus predicting risks for human and animal health.[1] Advances in molecular biology techniques offer the opportunity to enable cells to express specific recognition elements such as receptors or regulatory proteins that trigger intracellular signaling events as result of a specific interaction with target analyte(s). Reporter gene technology is based on a cascade of signaling events that ultimately results in the expression of a reporter protein, such as green fluorescent protein (GFP) or luciferase, whose expression can be measured by fluorescence or bioluminescence (BL).[2] Thanks to the high signal/noise ratio and no need for external light source or special sample geometry, BL represents one of the most powerful detection principles for miniaturized devices.[3] Besides, the availability of several luciferases with different BL emission properties allowed to develop cell-based BL assays in multiplex formats or with an internal control to correct the analytical signal according to cell viability.[4] Whole-cell biosensors are amenable to be implemented into miniaturized and/or microfluidic devices, with the advantages of low sample and reagent consumption, portability, and short analysis time. Several examples have been reported for the detection of different classes of analytes, ranging from heavy metals to endocrine disruptors.[5-6] Nonetheless, the majority of previously reported devices lack adequate analytical performance for real-life applications, thus failed to reach the market.[7-8] To increase robustness of such devices, several cell immobilization strategies have been investigated aimed at keeping cell alive and responsive to the target analyte. However, analyte and/or reagents (e.g., oxygen, BL substrates…) diffusion through the immobilization matrix may result in prolonged analysis time and reproducibility issues.

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A promising strategy to improve the sensitivity of BL-based devices is the possibility to move the cells towards different areas of the chip appointed to carry out specific functions such as incubation with the sample, reagent(s) addition, washing, and detection. To this end the exploitation of magnetotactic bacteria (MTB) magnetism as "natural actuator" could represent a valuable approach.

MTB have the innate ability to produce magnetosomes (or bacterial magnetic nanoparticles, BacMPs), i.e. nanoparticles of magnetite (Fe_3O_4) or greigite (Fe_3S_4) enveloped in a 3-4 nm thick lipid membrane, which are aligned in a well-ordered chain to achieve the maximum magnetic moment. Owing to the presence of magnetosomes, MTB orient and migrate along geomagnetic field lines. In the past years, extensive research has been focused on the elucidation of the mechanisms regulating magnetosome biosynthesis and on the optimization of culture techniques for the production and purification of BacMPs.[9-10]

Two major strategies can be envisaged to exploit MTB for bioanalytical applications: (i) the use of genetically engineered MTB as living biosensors, and (ii) the use of BacMPs as an alternative to chemically synthesized magnetic nanoparticles (MNPs). So far, the latter has been explored more extensively. Thanks to their size, ranging from 30 to 120 nm[11] and their biocompatibility, due to the presence of a surrounding lipid bilayer membrane, BacMPs are highly advantageous for developing several kinds of biosensors and more generally for binding assays[12,13] and drug delivery systems.[14]

Although significant advances in genomic studies of MTB have been reported,[15] the genetic engineering of MTB to obtain magnetic bioluminescent whole-cell biosensors (BL-MTB) has not been accomplished. In addition, a miniaturized device integrating BL-MTB and a light detector has not been reported in scientific literature.

As a first proof-of-concept of this new approach, *M. gryphiswaldense* MSR-1 strain was genetically engineered to constitutively express a red-emitting luciferase. A microfluidic

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chip prototype has been fabricated using multilayered polydimethylsiloxane (PDMS) constituted by three diamond-shaped incubation chambers connected with detection areas. After incubation with the sample, bacteria can be magnetically trapped and concentrated in the detection areas which are placed in contact with a CCD sensor thought a fiber optic taper to maintain an adequate spatial resolution. Quantitative BL imaging is performed for few minutes, upon substrate addition (D-luciferin). This prototype was used as rapid and sensitive biosensor for the evaluation of sample toxicity.

Results and discussion

In this study we genetically engineered magnetotactic bacteria to obtain BL magnetic biosensors. Some so-called “Magnetically labeled biosensors” have been previously obtained by chemical functionalization of cells (mammalian cell lines, yeast and bacteria) with synthetic MNPs.[16,17] Indeed this approach has some technical limitations requiring functionalization steps that may create standardization problems. The cells will divide during the incubation time and the amount of MNPs on cell’s surface decreases, thus negatively affecting the reproducibility of an assay relying on the movement or trapping of biosensing cells within a microfluidic chip via magnetic fields. Our strategy exploits the intrinsic capability of magnetotactic bacteria, such as Magnetospirillum gryphiswaldense, to produce a chain of magnetic nanoparticles (magnetosomes) within the cell which confers the ability to orient and migrate along magnetic field lines. The number and shape of genetically encoded magnetic particles is strictly regulated by the bacterial genome. When grown in microaerobic conditions the majority of the cells are magnetic and retain magnetic properties through cell divisions. In the purpose of microfluidic chip integration we choose magnetotactic bacteria in order to have an homogenous population of magnetic cells.

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The biosensors were integrated into a newly designed magnetic microfluidic chip, named MAGNETOX, that allows to perform rapid, sensitive and direct toxicity screening without the need for sample pre-treatment steps.

The use of a magnet array to separate the biosensing cells from the sample allows to simultaneously remove interferents and concentrate the BL-MTB in front of the CCD device, improving BL detection in terms of light output and sensitivity.

Preparation and characterization of bioluminescent magnetotactic bacteria

M. gryphiswaldense has been selected as host strain because it is well characterized and its genetic toolbox is well developed.[18]

M. gryphiswaldense strain MSR-1 was genetically engineered to express the red-emitting click beetle luciferase (CBR, λ_{max} = 615 nm) under the control of the constitutive $P_{mamDC45}$ minimal promoter which ensures high expression levels of heterologous proteins (unpublished data). To this end, CBR luciferase, a mutant of a yellow-green luciferase from *Pyrophorus plagiophthalamus,* was selected as reporter protein because of its thermostability, pH-insensitiveness and glow type emission.[19]

When expressed in bacterial cells, CBR has an half-life much longer than that of wild-type *P. pyralis* luciferase (5 hrs vs 0.26 hrs).[20] Since CBR requires endogenous bacterial ATP for the chemical reaction any change in light output truly reflects alterations in viability and metabolic state of the cell. A shuttle vector containing the cDNA encoding for CBR under the regulation of a constitutive promoter was used to transform an *E. coli* donor strain and transferred to MSR-1 strain by conjugation.

The BL emission of the BL-MTB cells has been investigated in terms of kinetics and spectral emission properties to obtain information useful for the subsequent implementation of the cells in the microfluidic chip. For this reason we measured BL signals emitted by intact living cells in 96-well microplates after addition of the BL substrate

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D-luciferin at pH 5.0. BL-MTB emission kinetics showed that after injection of the BL substrate the signal reaches a peak within few seconds followed by a rapid decay and a stabilization to a signal corresponding to about 50% of the maximum BL emission [see Fig. 1(a)]. Therefore a suitable temporal window from 5 to 15 min was identified for BL measurements. This glow-type emission is particularly interesting for imaging applications since it allows to integrate the BL signal for several minutes thus increasing sensitivity. BL emission spectrum showed a λ_{max} at 615 nm with a half bandwidth of 53 nm [see Fig. 1(b)], being consistent with CBR expression in other bacterial systems such as *E. coli* strains (data not shown).

As known from other proteins previously expressed in MSR, cytoplasmic expression of the luciferase enzyme is not likely to influence magnetosome formation. This was confirmed by transmission electron microscope (TEM) characterization which revealed magnetosome number and morphology in BL-MTB indistinguishable to those of MSR-1 WT strain [see Fig. 1(d)].

The investigation of time course luciferase expression and cell magnetism allowed to select 3 day-old cultures for inclusion in the MAGNETOX biosensor. Fig. 1(c) shows both the BL signal and cell magnetism (C_{mag}) of BL-MTB cultured in medium containing Fe(III) citrate. Luciferase BL reached a maximum intensity at 72 hrs after inoculum, when microaerobic conditions prevail and cell magnetism is 1.40 ± 5% (corresponding to 90% of maximum C_{mag} obtained after 6 days incubation. Magnetic properties were also macroscopically confirmed by moving BL-MTB with a permanent magnet (see movie M1).

Design and fabrication of the MAGNETOX microfluidic chip

Exploiting multilayer PDMS casting, a microfluidic chip comprising incubation and detection chambers in which BL-MTB can be loaded and trapped into specific positions for the analysis has been fabricated. The chip comprises three 60 µl-volume diamond-shaped

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incubation chambers, each of them connected to two detection areas where the BL-MTB are navigated via a microfluidic system and trapped and accumulated by permanent magnets [see Fig. 2]. Subsequently the BL substrate is delivered to the 6 detection chambers allowing simultaneous imaging. Lens-free BL imaging was performed using a CCD camera interfaced with a fiber optic taper to keep an adequate sample resolution.[21] This prototype configuration was designed to concentrate the cells in a reduced volume, thus improving light collection in the detection chambers. This strategy will be pursued to further miniaturize the MAGNETOX chip and increase the number of detection areas. Another peculiarity of the MAGNETOX is that, after the analysis, cells are washed out of the chip that can be re-used. Since incubation of cells with the analyte lasts only 30 min no sterility is required and the chip can be simply washed with 70% ethanol for reuse. This makes the device suitable for applications in low-resource settings.

As we previously reported, CCD contact lens-less imaging does not require any optical system, thus simplifying the fabrication of compact and miniaturized analytical devices.[22] In addition lens-less imaging allows the achievement of higher detectability taking advantage of light collection efficiency of the system.

The Sony ICX285 monochrome CCD sensor has been selected since it has a quantum efficiency higher than 50% in the range of 420-680 nm which covers the whole emission of the CBR reporter. A fiber optic mosaic taper which transmits the emitted light directly towards the CCD sensor was used to increase the sensing surface 2.3 times compared to the actual size of the CCD sensor (i.e., from 9.0x6.7 mm^2 to 20.7x15.4 mm^2), still maintaining a good resolution. A double Peltier cooling system reduced the thermal noise of the CCD thus improving the signal/noise ratio and a lid provided shielding from ambient light.

The main significant improvements of the device are: i) the implementation of a straightforward strategy to cast black and transparent PDMS; ii) the integration of a

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magnet array in the chip; iii) the design and fabrication of a microfluidics platform optimized for magnetic biosensors.

Although the use of black PDMS (a suspension of charcoal in PDMS) to fabricate microfluidic devices has been already reported elsewhere,[23] to the best of our knowledge the present work represents the first proof-of-concept of a black PDMS microfluidic chip that includes clear bottom detection chambers for BL imaging detection. In addition this approach may have a more general application for optical detection.

We first investigated the capability of neodymium-iron-boron (NdFeB) magnets to rapidly and efficiently trap BL-MTB within the chip. Fig. 3(a) shows BL imaging of the MAGNETOX chip obtained after D-luciferin addition. Signal is homogeneously distributed in the incubation chambers. As expected, after magnetic trapping, the BL signal is mostly concentrated in the detection chambers (approximately 85% of total light emission) [see Fig. 3(b)]. Weak signals still appear along the microfluidic channels, indicating that the magnetic trapping can be further optimized to increase reproducibility. The prototype was on purposely designed to disregard signal emitted by BL-MTB in the microfluidic channels or in the incubation chamber. In fact the background signal of non trapped cells does not interfere with the CCD detection since the channels are made with black PDMS.

In addition, thanks to the light-reflecting surface of the magnets, BL emitted towards the opposite side of the sensor can be detected as well, thus increasing the sensitivity of the analysis. The use of a reflecting surface increases the light signal by about 30%, with respect to that obtained with black tape covered magnets (data not shown).

Evaluation of sample toxicity with MAGNETOX

The feasibility of the MAGNETOX platform as general toxicity biosensor was assessed using standard solutions of DMSO as model toxic compound and TCDCA, a detergent bile acid that causes structural and dynamic effects on membranes. The toxic effect of DMSO

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is mainly due to its activity on cell membranes[24], thus affecting cell metabolism and viability. The alteration of cell energy systems dramatically alters the intracellular ATP levels, which can be monitored with an ATP-dependent luciferase. Therefore only metabolically active cells are able to provide the bioluminescent signal, which parallels their viability.

Concentration-response curve using DMSO were obtained and compared to those obtained using BL-MTB in a miniaturized 6-well cartridge [see Fig. S1(a)]. This microwell cartridge, holding 6 wells of 60 µL volume each, was on purposely designed for integration in the same portable device. This allowed to assess the actual advantage of concentrating BL-MTB via microfluidics and adding the BL substrate after a washing step in comparison to a conventional microwell format using the same CCD detector. The BL recombinant cells were incubated for 30 min at room temperature with increasing concentrations of toxic compounds inside the MAGNETOX chip or inside the microwells. For the MAGNETOX assay, after incubation, cells were moved toward the detection chamber, and trapped by applying the magnet array. Upon addition of the D-luciferin substrate, images were acquired with the CCD and analyzed to quantify BL emission [see Fig. 4(a)]. Fig. 4(b) shows that BL signal is strongly affected by DMSO with an LC_{50} of 7.5% v/v DMSO obtained with both the configurations. The two curves showed a similar sensitivity with slight differences at low DMSO concentrations (e.g., in the MAGNETOX a 2.5% v/v DMSO produces a signal of 75±12% and of 85±5% in the microwell configuration) while at DMSO concentrations higher than 40% v/v a complete cell death is observed in both the configurations.

We then used the bile acid TCDCA (0.001-10mM). An increasing toxic effect can be detected (see Fig. 5) while approaching TCDCA critical micelle concentration (CMC 3mM).[25] Micelles can modify the membrane constituents, thus resulting in a change in physicochemical properties causing adverse effects on cell viability.[26,27]

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The response of MAGNETOX was quite reproducible with an intra-assay variability of 15% calculated by considering the 6 detection chambers as replicates and an inter-assay variability of 18% with four replicates. We also compared the results with the closest widely recognized assay, the Microtox® system, which measures the light output of luminescent bacteria (V*ibrio fischeri*) after they have been exposed to a sample. Using 1 hour incubation with standard solutions containing different concentrations of DMSO we observed a LC_{50} of 8.3% v/v DMSO with the first toxic effects appearing at 2.5% v/v of DMSO (BL signal 81±9%) and complete cell death at DMSO concentrations higher than 40% v/v, (see Fig. S2), showing consistency with the MAGNETOX assay.

These preliminary results highlight that it is possible to exploit magnetic concentration to increase the light output and reduce assay volume. Despite the reproducibility of the MAGNETOX could be improved by optimizing the microfluidic chip design and fabrication, these results supports the use of BL-MTB as a powerful tool suitable for microfluidic (bio)sensors.

In addition, the generation of BL-MTB with a codon-optimized version of the luciferase integrated into the bacterial chromosome will surely result in a more robust biosensor. The use of a codon optimized luciferase coding sequence could reduce the time required for its expression. Indeed MAGNETOX assay performed using overnight and 36h-old cultures resulted in lower analytical performance (e.g., no significant bioluminescent signal at concentrations higher than 20% DMSO v/v and an increased CV% (CV%=20%).

To circumvent this limitation, lyophilized BL-MTB will be obtained providing a ready-to-use suspension of organisms to be used in the chip. The implementation of BL-MTB into field-deployable devices could be exploited for direct analysis of environmental or clinical samples, in which matrix components that may interfere with the BL detection, could be easily removed from detection areas. As an alternative a microelectromagnetic pad

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actuator could be used to precisely control the movement and positioning of BL-MTB within the microfluidic chip and further miniaturize the system.

Experimental

Chemicals and reagents

All chemicals used for cell culture media preparation and toxic compounds were purchased from Sigma (St. Louis, Missouri, USA). The enzymes required for cloning were from Fermentas (Vilnius, Lithuania). The kits for plasmid extraction and purification were from Qiagen (Hilden, Germany). Sylgard 184 (Dow Corning, USA) was used to create PDMS chip. D-luciferin solution,1 mM at pH 5.0, 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. Taurochenodeoxycholic acid sodium salt (TCDCA) dilutions were prepared in FSM medium.

Organism and growth conditions

M. gryphiswaldense strain (MSR-1 R3/S1; Rifr Smr spontaneous mutant)[28] was cultured at 28°C in 10 mL hungate tubes (GPE Scientific UK) in microaerobic conditions (1% O_2 in the headspace). The oxygen concentration in the gas phase was reduced to less than 1% O_2 by repeated flushing with N_2. MSR-1 medium with 50 μM Fe(III) citrate was used as described by Heyen and Schüler.[29]

Obtainment of bioluminescent magnetotactic bacteria (BL-MTB) and characterization of emission properties

M. gryphiswaldense strain was genetically engineered to constitutively express the red-emitting click beetle luciferase (CBR, λmax = 615 nm). Briefly, the cDNA encoding for CBR was PCR amplified from vector pCBRbasic (Promega, WI, USA) using the primers

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CBR Fw AGT**GGATCC**TTACTAACCGCCGGCCTT and CBR Rev CAG**CATATG**GTAAAGCGTGAGAAAAAT, adding BamHI and NdeI restriction sites, shown in bold, respectively. These restriction enzymes were then used to digest and insert the luciferases into the pAP150 vector under the control of the $P_{mamDC45}$ constitutive promoter. The resulting vector pAP150_CBR was used to transform *E. coli* donor strain (BW29427 [thrB1004 pro thi rpsL hsdS lacZ_M15 RP4-1360_(araBAD)567 _dapA1341::(erm_ pir_)] (K. Datsenko and B. L. Wanner, unpublished) via heat shock[30] and transferred to MSR strain by conjugation.[28] The obtained strain (MSR_CBR) was routinely grown microaerobically at 28°C in selective MSR medium containing 5 µg/mL kanamycin. MSR_CBR emission kinetics and bioluminescence emission spectra were obtained in 96-well plate using 100µL overnight liquid cultures; BL signal was acquired for 10 minutes (300 ms integration time) with a Varioskan Flash spectral scanning multimode reader (Thermo Scientific, Whaltham, MA, USA) after automatic injection of 100 µL D-luciferin 1mM, pH 5.0. Bandwidths (nm) of emission spectra were measured at 50 and 20% of the intensity at the maximum wavelength. Data were analyzed with GraphPad Prism v5.02 (Software, Inc., San Diego, CA, USA). All light measurements were performed in triplicate.

TEM micrographs of MSR-1 expressing CBR luciferase were taken at 18kx and 71 kx with a Morgagni 268 at 80 kV.

Evaluation of luciferase time course and magnetic orientation

The magnetic orientation (C_{mag}) of MSR_CBR cells was evaluated spectrophotometrically using the optical density at 565 nm as previously described.[31] Briefly, at given time intervals, cell suspensions of 1.0 mL were withdrawn from the culture for C_{mag} measurement. Cell density was set to an OD of 0.1 and an external magnetic field was applied to align the cells in different angles towards the light beam of the spectrometer.

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This results in maximum and minimum light extinction and the ratio of both correlates with the average number of magnetosomes per cell. This method is used to semiquantitatively assess the magnetism of a culture (non-magnetic cells have a C_{mag} value of 0).

Design and fabrication of the MAGNETOX microfluidic chip

The microfluidic chip fabrication process is based on multiple layer casting of black and transparent PDMS on a home made master mold.

Transparent PDMS was prepared using a monomer:curing agent in a weight ratio of 5:1 and 25mg/mL of activated charcoal powder was added to obtain black PDMS. The solutions were then centrifuged at 4000 rpm for 10 min to remove bubbles and stored at -20°C until use.

Black PDMS was first poured up to fill the edge of the relief structures on the mask, which creates the diamond-shaped chambers (7.0 x14.0 mm diagonals, height 1.5 mm), the microfluidics channels (1 mm width) and detection areas (3 mm diameter, height 1.5 mm). To avoid mixing with transparent PDMS, black PDMS was let to harden for 1h at 60 °C; then a thin layer of transparent PDMS was poured on black PDMS layer, to create the clear bottom of the wells, and let to harden for 2h at 60°C. During the curing process the black and transparent PDMS layers are fused together.

A separate layer of transparent PDMS was casted on a different mold, comprising inlets and outlets, to create the top of the chip and let to harden as previously described. The two partially-cured PDMS layers are then removed from their mask, superimposed and hardened overnight at 70°C to obtain the final chip [see Fig. 2(a)].

An array of Neodymium-Iron Boron circular disc magnets (NdFeB; NeoDeltaMagnet NE32, 3 mm diameter, L=2 mm, remanence 1170-1250 mT, IBS Magnet, Berlin, Germany) was placed on the detection chambers of the PDMS chip.

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The MAGNETOX device consists of the microfluidic device connected to a CCD camera modified for lens-free CL imaging detection. The CCD imaging detector was built from a MZ-2PRO CCD camera (MagZero, Pordenone, Italy) equipped with a Sony ICX285 monochrome CCD image sensor (1360X1024 pixels, pixel size 6.45 X 6.45 μm^2) and a 16 bit analog-to-digital (A/D) converter. To reduce thermal noise, CCD sensor was thermoelectrically cooled by a double Peltier cell. A round fiber optic taper (25/11 mm size, Edmund Optics, Barrington, NJ) was placed in contact with the CCD sensor as previously described by us.[22] The MAGNETOX is computer controlled via a USB 2.0 interface by a software (EZ Cap, v3.13) that allows to perform data acquisition and parameter settings.

Design of a microwell cartridge

A custom made PDMS 6-well cartridge was obtained using black and transparent PDMS as previously described. The mask for PDMS casting has been designed in order to obtain an array of 2x3 wells of 4mm diameter and 4.5mm deep each.

First black PDMS was poured up to fill the edge of the mask followed by addition of a thin (<200µm) layer of transparent PDMS to create the bottom of the wells [see Fig. S1(b)]. After overnight incubation at 70° C the cartridge was carefully separated from the mask.

Toxicity evaluation with MAGNETOX

Different concentrations of dimethyl sulfoxide (DMSO) (in the range 2-50 % v/v) and TCDCA (in the range 0.001-10mM) were used as model toxic compounds to evaluate the feasibility of using BL magnetotactic bacteria as a toxicity biosensor. All serial dilutions of compounds were performed using FSM medium as diluent.

Different experimental conditions were optimized to improve the biosensor performance (e.g., incubation temperature and time, volumes and ratio of cell suspension and sample).

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Under optimized conditions, an analysis with the MAGNETOX chip includes the following steps: i) a volume of 40 µL of 3 day-old liquid culture is driven into the chip by air-displacement pipette; ii) cells are incubated for 30 min at room temperature with 20 µL of sample (medium is used as blank); iv) cells are moved and concentrated to the detection chambers by adding the array of permanent magnets; v) BL imaging measurements after addition of 1mM D-luciferin pH 5.0. Images are acquired for 5 min and analyzed with ImageJ software v.1.46 (National Institutes of Health, Bethesda, MD). Images are recorded in the FITS (Flexible Image Transport System) format. Regions of interest (ROIs) corresponding to detection chambers are selected and light emissions quantified as raw integrated densities.

For toxicity experiments, normalized BL signals (BL emission of untreated control was set as 100%) were plotted against toxic compound concentration. Lethal concentration (LC_{50}) of each compound was calculated as the concentration producing a 50% reduction in light. All experiments were performed in duplicate and repeated at least three times.

The toxic effects of DMSO and TCDCA solutions (in the range 2-50 % v/v and 0.001-10mM, respectively) were also assessed by Microtox® assay with *Vibrio fischeri*.[32] Different exposure times were tested (10, 30 and 60 min at 25° C) in 96 microplate format using 90 µL of cell suspension and 10 µL of analyte or control (medium) and results analyzed as described for the MAGNETOX assay.

Conclusions

Here, for the first time, the use of bioengineered bioluminescent magnetotactic bacteria in combination with microfabrication technologies is reported for biosensing applications. A novel concept of black and transparent PDMS microfluidic chip has been developed which could find broad use in the opto-fluidic field. The chip has been integrated with a portable CCD sensor for lens-less imaging detection of light signal emitted by the BL magnetotactic

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bacteria used as biosensing “living actuator”. Differently from other whole-cell biosensors, BL-MTB can be easily moved and concentrated in specific detection chambers, where the sample matrix is removed and bacteria are washed, thus increasing the analytical signal and performance of the system. The interaction of BL-MTB with the analyte is facilitated in the detection chamber since this interaction takes place in a dispersed suspension, resulting in a shorter incubation time. In this regard, the MAGNETOX allowed to rapidly (30 min) measure the toxicity of a sample with the non negligible advantage of chip re-usability.

This is the first attempt to integrate bioengineered magnetotactic bacteria into an analytical device and several optimizations regarding both the cell and the chip design will be addressed. An array of electromagnets or a microelectromagnetic pad actuator will be included to better control BL-MTB within the chip, by tuning the magnetic trapping or continuously directing their swimming to the detection area.

Albeit many improvements are surely required before applying BL magnetic biosensors to real-life needs, we are confident that they represent forerunners of a new concept of whole-cell biosensing.

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References

1.S. K. Checa, M. D. Zurbriggen, F. C. Soncini, *Curr. Opin. Biotechnol.,* 2012, **23**, 766-772.

2.A. Roda, P. Pasini, M. Mirasoli, E. Michelini, M. Guardigli, *Trends Biotechnol.,* 2004, **22**, 295-303.

3.E. Michelini, L. Cevenini, L. Mezzanotte, A. Roda, *Methods Mol. Biol.,* 2009, **574**, 1-13.

4.A. Roda, B. Roda, L. Cevenini, E. Michelini, L. Mezzanotte, P. Reschiglian, K. Hakkila, M. Virta, *Anal. Bioanal. Chem.,* 2011, **401**, 201-211.

5.S. Melamed, T. Elad, S. Belkin, *Curr. Opin. Biotechnol.,* 2012, **23**, 2-8.

6.A. Rothert, S. K. Deo, L. Millner, L.G. Puckett, M.J. Madou, S. Daunert, *Anal. Biochem.,* 2005, **342**, 11-9.

7.E. Michelini, L. Cevenini, M. M. Calabretta, S. Spinozzi, C. Camborata , A. Roda, *Anal. Bioanal. Chem.*, 2013, **405**, 6155-6163.

8.I. Barbulovic-Nad, H. Yang , P. S. Park, A. R. Wheeler, *Lab Chip*, 2008, **8**, 519-26.

9.D. Faivre and D. Schüler, *Chem. Rev.*, 2008, **108**, 4875-4898.

10.D. Schüler and R. B. Frankel, *Appl. Microbiol Biotechnol.*, 1999, **52**, 464-73.

11.D. A. Bazylinski and R. B.Frankel, *Nat. Rev. Microbiol.,* 2004, **2**, 217-230.

12.Y. Amemiya, T. Tanaka, B. Yoza, T. Matsunaga, *J. Biotechnol.,* 2005, **120**, 308-314.

13.T. Yoshino, C. Kaji, M. Nakai, F. Saito, H. Takeyama, T. Matsunaga, *Anal. Chim. Acta.* 2008, **626**, 71-77.

14.T. Tang, L. Zhang, R. Gao, Y. Dai, F. Meng, Y. Li, *Appl. Microbiol. Biotechnol.*, 2012, **94**, 495-503.

View Article Online
DOI: 10.1039/C3LC50868D

15.C. T. Lefèvre, D. Trubitsyn, F. Abreu, S. Kolinko, C. Jogler, L.G. de Almeida, A. Tonc Vasconcelos, M. Kube, R. Reinhardt, U. Lins, D. Pignol, D. Schüler, D. A. Bazylinski, N. Ginet, *Environ. Microbiol.*, in press doi: 10.1111/1462-2920.12128.

16.D. Zhang, R. F. Fakhrullin, M. Özmen, H. Wang, J. Wang, V.N. Paunov, G. Li, W.E. Huang. *Microb. Biotechnol.* 2011, **4**, 89-97.

17.M. R. Dzamukova, A. I. Zamaleeva, D. G. Ishmuchametova, Y. N. Osin, A. P. Kiyasov, D. K. Nurgaliev, O. N. Ilinskaya, R. F. Fakhrullin, *Langmuir*, 2011, **27**,14386-14393.

18.K. H. Schleifer, D. Schüler, S. Spring, M. Weizenegger, R. Amann, W. Ludwig, M. Köhler, *Syst. Appl. Microbiol.,* 1991, **14**, 379–385.

19.U. Stolz, S. Velez, K. V. Wood, M. Wood, J. L. Feder, *Proc. Natl. Acad. Sci. USA*, 2003, **100**, 14955-14959.

20.M. I. Koksharov and N. N. Ugarova, *PEDS,* 2011, **24**, 835-844.

21.A. Roda, M. Mirasoli, L. S. Dolci, A. Buragina, F. Bonvicini, P. Simoni, M. Guardigli, *Anal. Chem.,* 2011, **83**, 3178-3185.

22.A. Roda, L. Cevenini, E. Michelini, B.R. Branchini, *Biosens. Bioelectron.,* 2011, **26**, 3647-3653.

23.L. S. Roach, H. Song, R. F. Ismagilov, *Anal. Chem.,* 2005, **77**, 785-796.

24.S.A. Markarian, A.A. Poladyan, G.R. Kirakosyan, A.A. Trchounian, K.A. Bagramyan, *Lett. Appl. Microbiol*., 2002, **34**, 417-421.

25.A. Roda and A. F. Hofmann, *J. Lipid Res.,* 1984, **25**, 1477-1489.

26.Z.Y. Zheng, C. Bernstein, *Nutr. Cancer.,* 1992, **18**, 157-164.

27.P. Chieco, E. Romagnoli, G. Aicardi, A. Suozzi, G.C. Forti, A. Roda. *Histochem. J.*, 1997,29, 875-883.

View Article Online
DOI: 10.1039/C3LC50868D

28.D. Schultheiss and D. Schüler, *Arch. Microbiol.*, 2003, **179**, 89–94.

29.U. Heyen and D. Schüler, *Appl. Microbiol. Biotechnol.,* 2003, **61**, 536–544.

30.J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual; Eds; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989; Vol. 1.

31.D. Schüler, R. Uhl, E. Baeuerlein, *FEMS Microbiol. Lett.,* 1995, **132**,139–145.

32.K.L.E. Kaiser, J.M. Ribo, *Toxicity Assess*., 1988, **3**, 195–237.

View Article Online
DOI: 10.1039/C3LC50868D

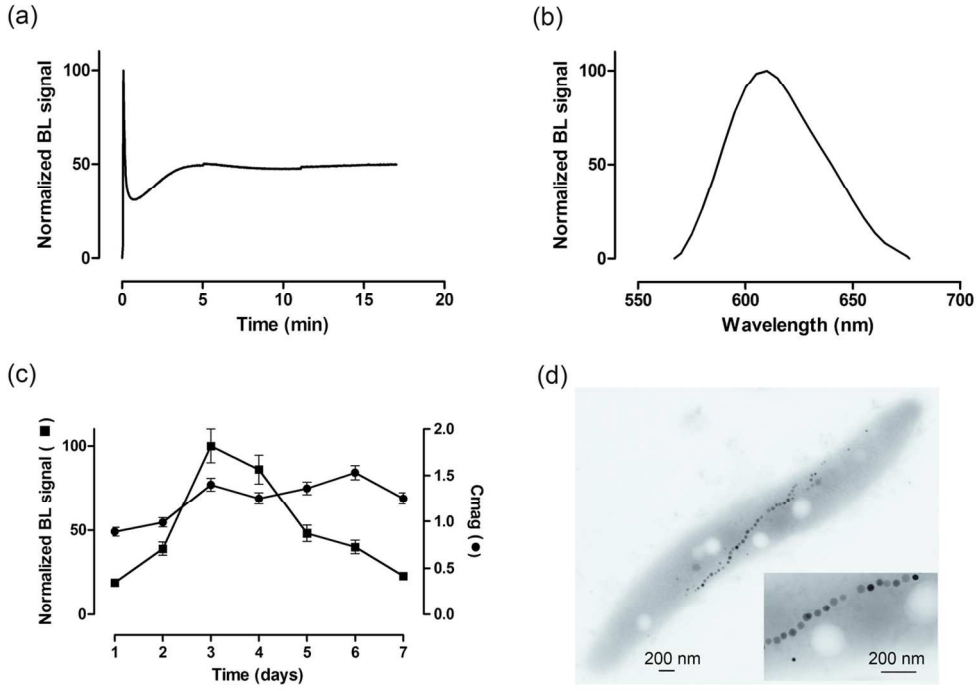

Fig. 1 Characterization of the bioluminescent magnetotactic bacteria BL-MTB. (a) Normalized BL emission kinetics. (b) Normalized emission spectrum (λmax=615nm). (c) Time-course expression of the CBR luciferase and cell magnetism (Cmag) of BL-MTB growing cultures. (d) TEM micrographs of MSR-1 expressing CBR luciferase taken with a Morgagni 268 at 80 kV. Inset shows magnetosome chain magnification.
121x88mm (300 x 300 DPI)

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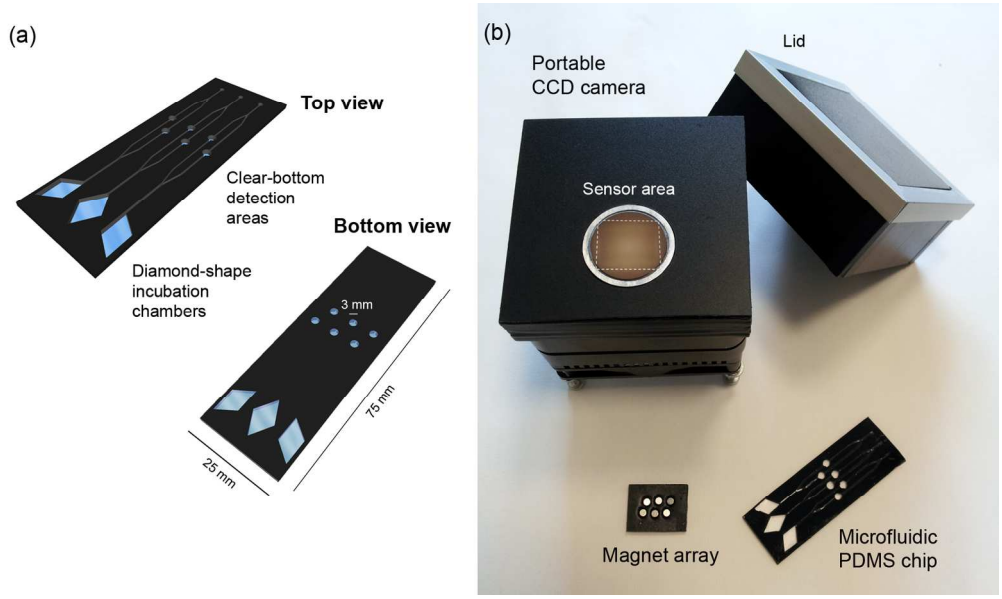

The MAGNETOX biosensor. (a) Schematic representation of the microfluidic chip comprising three diamond-shape incubation chambers and clear-bottom detection areas. (b) Main components of the MAGNETOX biosensor: the microfluidic chip, the magnet array, the portable CCD camera with a fiber optic taper for lens-free BL imaging, and a lid for shielding from ambient light.
160x94mm (300 x 300 DPI)

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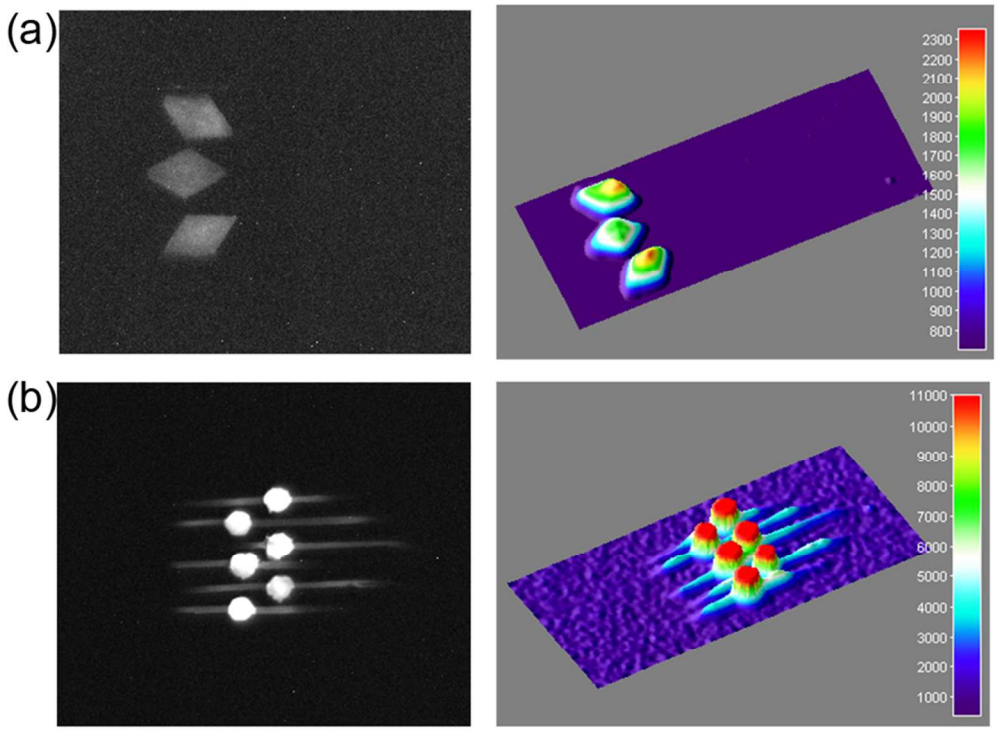

Fig. 3 BL imaging of BL-MTB inside the incubation chambers (a) and obtained after cell movement and magnetic trapping in the detection chambers (b). The MAGNETOX chip was imaged from the top using a Night Owl LB 981 luminograph (EG&G Berthold, Bad Wildbad, Germany) with 5 min integration. 3D surface plot visualization was obtained using ImageJ software.
82x62mm (300 x 300 DPI)

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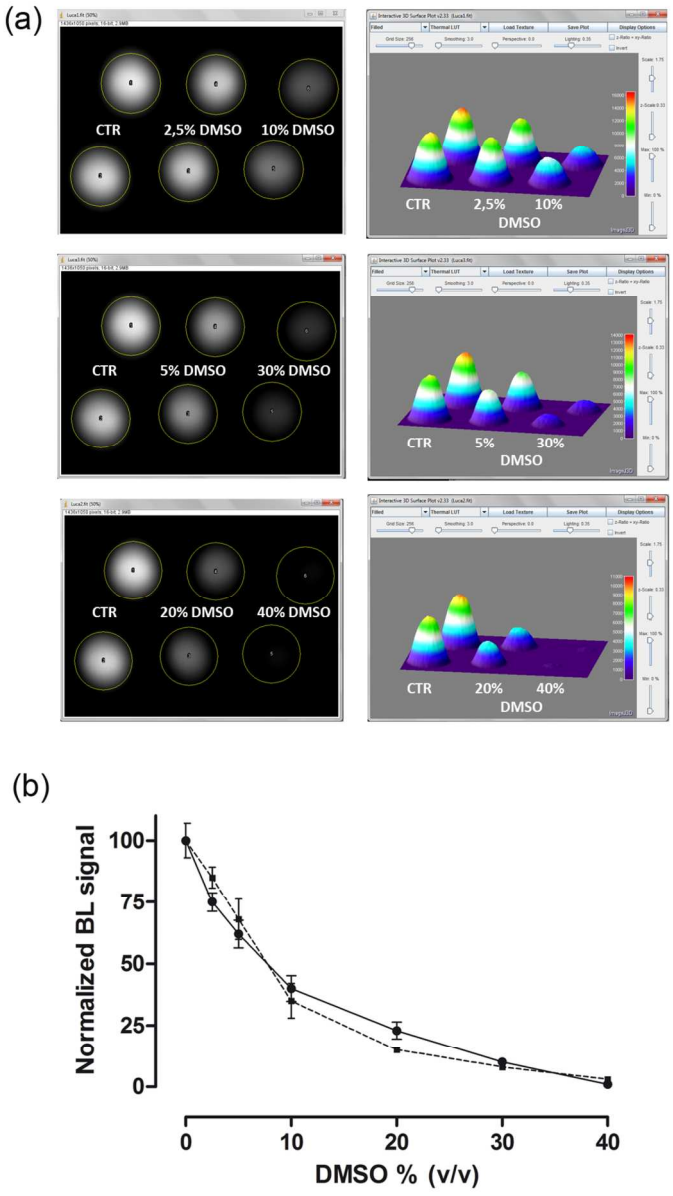

Fig. 4 (a) BL images obtained with the MAGNETOX and 3D surface plot visualization using ImageJ software. Circular ROI were selected and BL signals quantified. (b) Normalized toxicity curves for DMSO (30 min incubation) obtained with the BL-MTB in the microwell cartridge (dashed line) and with the MAGNETOX chip (solid line)

144x251mm (300 x 300 DPI)

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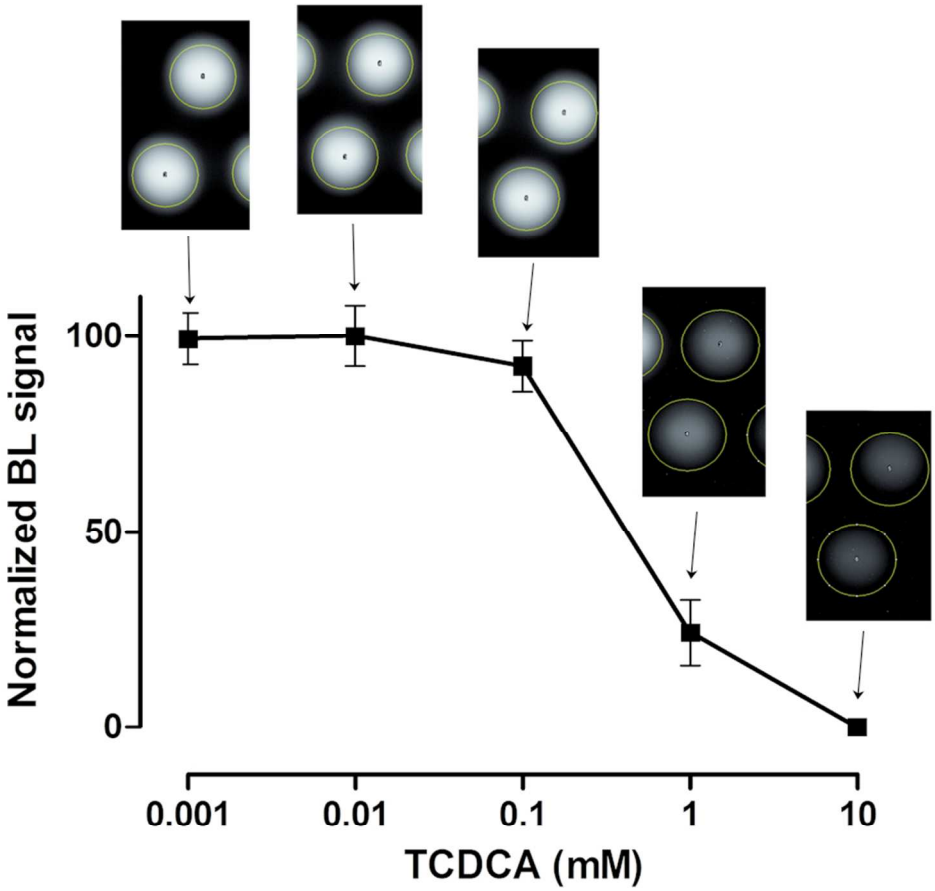

Fig. 5 Toxicity curve for TCDCA (0.001-10mM) obtained with the MAGNETOX chip. Row BL images corresponding to each duplicate are shown in the inset. The BL-MTB cells are incubated for 30 min at room temperature with increasing concentrations of TCDCA inside the MAGNETOX chip. After incubation cells are moved toward the detection chamber, and trapped by applying the magnet array. Upon addition of the D-luciferin substrate, images are acquired with the CCD sensor and analyzed to quantify BL emission.
82x81mm (300 x 300 DPI)