ISSN 1995-0780, Nanotechnologies in Russia, 2008, Vol. 3, Nos. 9–10, pp. 622–632. © Pleiades Publishing, Ltd., 2008.
Original Russian Text © S.E. Peltek, T.N. Goryachkovskaya, V.M. Popik, V.F. Pindyurin, V.S. Eliseev, B.G. Gol'denberg, M.A. Shcheglov, N.V. Tikunova, T.M. Khlebodarova, N.B. Rubtsov, G.N. Kulipanov, N.A. Kolchanov, 2008, published in Rossiiskie nanotekhnologii, 2008, Vol. 3, Nos. 9–10.

EXPERIMENT

Microfluidics in Biology and Genosensor Construction

S. E. Peltek[a]*, T. N. Goryachkovskaya[a], V. M. Popik[b], V. F. Pindyurin[b], V. S. Eliseev[b], B. G. Gol'denberg[b], M. A. Shcheglov[b], N. V. Tikunova[a], T. M. Khlebodarova[a], N. B. Rubtsov[a], G. N. Kulipanov[b], and N. A. Kolchanov[a, c]

[a] *Institute of Cytology and Genetics, Russian Academy of Sciences, Novosibirsk, 630090 Russia*
[b] *Budker Institute of Nuclear Physics, Russian Academy of Sciences, Novosibirsk 630090 Russia*
[c] *Novosibirsk State University, Novosibirsk 630090 Russia*

**e-mail: peltek@bionet.nsc.ru*

Abstract—We describe key stages in the development of a genosensor-based bioanalytical complex capable of functioning in a micro/nanofluidic system (MNFS), which is intended for detecting damaging agents in liquid media. Using the LIGA technology, we have developed an MNFS with a channel width of a few microns or more. Methods of (i) processing microfluid channels for positioning bacterial cells–genosensors and (ii) detecting fluorescent signals in a microfluid module are developed. A search for promoters ensuring the response of genosensor structures to a broad range of damaging factors of the external environment is carried out using a bioinformatics approach (based on GenSensor database). An experimental genosensor has been constructed based on the *dps* gene promoter of *E. coli* and the pRS-GFPvaa vector plasmid. It is shown that an *E. coli*/pDps-gfp genosensor in a microfluid module responds by GFPvaa protein synthesis to the presence of oxidative stress agents and agents capable of penetrating membranes in the same range of concentrations as those outside the module. The results of our investigation show that the proposed interdisciplinary approach can be used for obtaining bacterial cells-genosensors capable of functioning in MNFSs and detecting damaging factors of the external environment.

DOI: 10.1134/S1995078008090127

INTRODUCTION

At present, there is a revolution taking place in world science and technology that consists in passage to small and ultrasmall (so-called "lab-on-a-chip") devices intended for determining the functions of biological macromolecules, genomes, cells, and cell structures with a view to clinical diagnostics and biochemical investigations. The new approach is based on the use of micro/nanofluidic system (MNFSs).

Micro/nanofluidic bioanalytical systems are integrated devices comprising advanced, sophisticated high-tech elements of hydraulics, micromechanics, electronics, optics, biochemistry, molecular biology, gene and cell design, and bioinformatics. The importance of R&D in micro/nanofluidic technology is confirmed by snowballing growth in the number of publications in this field. In particular, more than 3000 articles devoted to MNFSs and related subjects have been published in 2004–2006 (see, e.g., an insightful series of articles in *Nature* [1–7]). In Russia, this direction of research is being developed, for example, at the Institute of Physicochemical Medicine (Moscow) and institutions of the Siberian Branch of the Russian Academy of Sciences [8, 9].

The basic element in an MNFS is a glass or polymer plate with a multilevel system of channels, microreactors, valves, and pumps manipulating pico- and nanoliter volumes of liquids [10–14]. The fabrication of MNFSs is based on the use of technologies such as LIGA, laser ablation, lithography, diamond powder processing, stamping, and molding. Such systems ensure preset characteristics of microscopic flows, exact dosing of dissolved substances, optimum computer-aided regulation of flows, and conjugation with external macroscopic devices.

The most important properties of MNFSs are (i) the laminar flow in microfluidic channels and (ii) the possibility of exactly calculating the transported amounts of substances to various elements of the system with allowance for the geometry of various channels, distribution of pressures, and properties of liquids. By virtue of their laminar character, flows in MNFSs are mixed in the diffusion regime. Laminar flow and diffusion mixing are combined so as to provide the necessary gradients of dissolved substances in microfluidic channels. In addition, MNFSs are characterized by an increased ratio of the liquid surface to the volume, which can provide optimum conditions for chemical reactions.

Using MNFSs, it is possible to implement on a qualitatively new level methods and facilities of modern analytical chemistry such as flow injection analysis and high-efficiency capillary electrophoresis, to use the advantages of laminar flows, to optimize the ratio of the liquid volume to the reaction surface in microreactors, etc. [15–20]. An important advantage of MNFSs is the

ability to handle individual cells at various stages of their development and to deal with noncellular systems.

The radical miniaturizatiuon of experimental devices achieved by means of micro/nanofluidic technologies opens the way for novel, extremely low-cost but highly effective methods of solving a broad range of both basic and applied problems in molecular and cell biology, biotechnology, and medicine. The ability of MNFSs to operate with pico- and nanoliter volumes of liquids leads to a radical (by several orders of magnitude) decrease in the amounts of analyzed biological samples such as macromolecules, cells, and biologically active substances. Accordingly, there is drastic decrease in the consumption of high-cost reagents and in the cost of experiments and analyses carried out in micro/nanofluidic bioanalytical systems.

Highly sensitive methods available for the analysis of substances and monitoring of processes, such as fluorescent microscopy and spectroscopy, makes it possible to detect desired components at a trace level (up to individual molecules). The volumes of liquids that can be handled in microfluidic devices are comparable with cell dimensions or even several orders of magnitude smaller, which opens basically new research possibilities in the fields of molecular and cell biology, biotechnology, and medicine.

The present article describes the key stages in the development of a genosensor-based bioanalytical complex functioning in an MNFS, which is intended for detecting damaging agents in liquid media. The genosensor represents a recombinant *E. coli* cell modified with a plasmid containing the *gfp* reporter gene (encoding green fluorescent protein (GFP)), the transcription of which is controlled by a promoter sensitive to damaging factors of the external environment. The level of reporter gene activity can be monitored by measuring the level of induced fluorescence. The main stages of this study are (i) the fabrication of an MNFS by means of the LIGA technology, (ii) construction of a base vector for cloning genosensor promoters, (iii) computer-aided design and experimental construction of a vector plasmid containing the *gfp* reporter gene, whose transcription is activated from the *E. coli dps* gene promoter, (iv) investigation of the reporter gene activation in response to the presence of hydrogen peroxide and phenol (model oxidative stress agents), (v) selection of conditions for positioning the genosensor bacterial cell in the microfluidic channel, and (vi) development of a method for fluorescent signal monitoring in pico- and nanoliter volumes of the microfluidic module.

TECHNOLOGY OF MICRO/NANOFLUIDICS

The fabrication of MNFSs was based on deep X-ray lithography in the photon energy range of 6–40 keV using a VEPP-3 storage ring at the Budker Institute of Nuclear Physics (Novosibirsk). The technological process included the following stages.

Fabricating Templates for X-ray Lithography

Preparation of substrates. Substrates for X-ray lithography templates were SU-900 carbon-reinforced glass composite plates with lateral dimensions of 40 × 40 mm and a thickness of 0.5 mm. This composite possesses a unique combination of properties, including good strength (close to that of glass), the ability of polishing, high chemical and corrosion resistance and electric conductivity, and low absorption in the spectral range of synchrotron radiation (SR). In order to ensure the adhesion of a resist layer, the surfaces of substrates were subjected to special preliminary chemical treatment by exposure in concentrated sulfuric acid for 30 min at room temperature, rinsed in distilled water, and dried in a thermal box at 200°C. Then, each plate was covered with a commercially available negative photoresist (SU-8). The coating technology allows layers with thicknesses of 1–200 μm to be applied. This process was carried out at room temperature in a centrifuge operating at 3000 rpm. The deposited coatings were subjected to soft annealing in a thermal box at 95°C for 16 min.

Etching of templates. In order to obtain X-ray lithography templates, it was necessary to form a relief corresponding to the desired MNFS topology. This topology was patterned in the resist layer by a collimated SR beam with a cross section of 40×40 μm^2 on a LIGA station. For this purpose, the substrates were translated relative to the SR beam by means of a two-coordinate scanner (accurate to within 1 μm) at a velocity of 50 μm/s. As a result, the resist layer acquired a latent image of the desired MNFS topology. The absorbed radiation doses in this stage amounted to 40–60 J/cm^3. After irradiation, the substrates were repeatedly annealed in a thermal box at 95°C for 4 min in order to ensure cross-linking of exposed regions of the resist layer and minimize the residual internal stresses. The nonpolymerized (unexposed) regions of the resist were removed by chemical etching of the plates with propylene glycol methyl ethyl acetate (PGMEA) etchant (MicroChem Co., United States) in an ultrasonic bath for 4 min at room temperature. Finally, the etched plates were washed with isopropyl alcohol. Figure 1 outlines the sequence of stages used for the preparation of substrates for X-ray lithography templates.

Microgalvanoplastics. In order to obtain the template, the plate with a microstructural pattern was galvanically coated with an 8- to 10-μm-thick gold film. As a result, a template with the preset MNFS topography was obtained.

MNFS Reproduction

Poly(methyl methacrylate) (PMMA) plates with a thickness of 5 mm were exposed to white (non-filtered) SR the LIGA station via the X-ray lithography template obtained as described above on. The exposure was performed in a scanning mode in which the sample and template (mounted in front of the sample) were scanned across

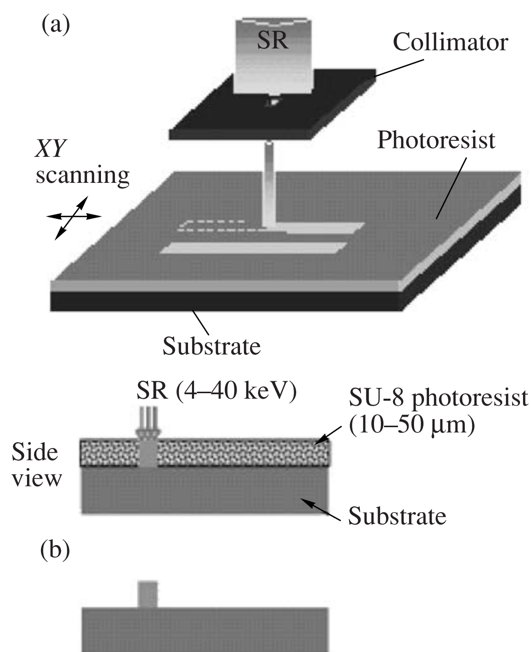

Fig. 1. Preparation of substrates for X-ray lithography templates: (a) carbon-reinforced glass composite substrate covered with photoresist is exposed to collimated SR beam for obtaining a preset MNFS topology pattern; (b) unexposed regions of photoresist are removed by chemical etching.

the SR beam at a velocity of 10 cm/s within amplitude of 10 mm. Then, the exposed regions of samples were removed by etching with GG etchant in an ultrasonic bath. The quality of obtained microchannels was determined by the exposure dose and the conditions of etching.

Figure 2 shows photographs of an MNFS structure on an SU-8 photoresist on a polished carbon-reinforced glass composite substrate, a template obtained upon photoresist removal and galvanic gold plating, and final PMMA plates bearing MNFSs with microfluidic channels of 38 μm width and depth.

The given MNFS (Fig. 3) is comprised of four chambers and three channels, one of which was intended for the arrangement of genosensor cells. Then, a liquid carrying damaging agents is pumped via the channel with these cells. The transition from macroscopic to microscopic level is provided by microsyringes and microdrives.

CONSTRUCTING GENOSENSORS

Bacterial cells contain a large number of genes involved in protective response to exogenous damaging factors. The group of most frequently encountered factors activating the protective response of a bacterial cell includes toxic agents of various types, deficit or excess of cell metabolites, temperature, etc. Genes activated by such detrimental (stress) factors are called susceptible (sensor) genes.

The activation of sensor genes can be mediated by various mechanisms, but all of these are based on a change in the functional state of proteins regulating gene transcription. Such changes can either be mediated by the interactions of regulatory proteins with

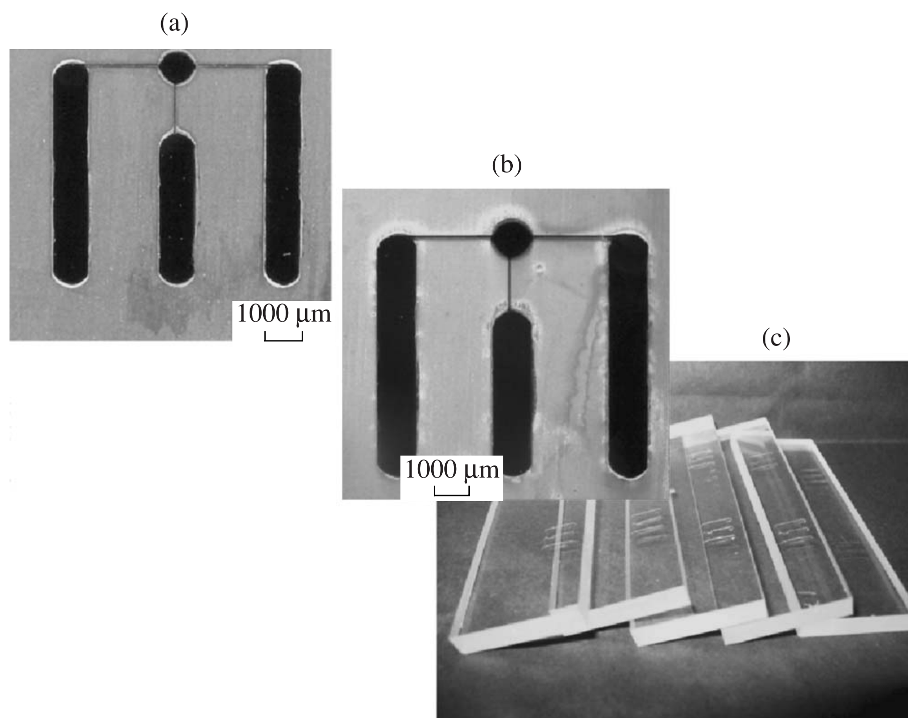

Fig. 2. Photographs of (a) an MNFS structure on SU-8 photoresist on carbon-reinforced glass composite substrate, (b) a template obtained upon photoresist removal and galvanic gold plating (8–10 μm), and (c) PMMA plates (25 × 750 mm) with MNFSs (channel width, 38 μm).

some metabolites or be caused by processes induced by external factors related to oxidation, phosphorylation, or acetylation of regulatory proteins, their interaction with other proteins, etc.

The ability of a cell to activate (via certain mechanisms) the expression of sensor genes under unfavorable conditions was used in this study as a basis for the creation of a genosensor. A genosensor is an artificial genetic system with a gene promoter as a sensitive element, which is naturally activated in response to some metabolic stress action. In genosensor constructions, such gene promoters are related to reporter genes, the role of which is played by genes encoding fluorescent or luminescent proteins [21]. We have used some promoters of sensor genes and the *gfp* reporter gene of *E. coli* (encoding green fluorescent protein (GFP)) to obtain recombinant plasmids. When introduced into a bacterial cell, these plasmids make it possible to determine the level of stressor action, judging by the intensity of induced fluorescence.

Figure 4 shows the scheme of a computer-aided experimental approach to the rational design of artificial molecular-genetic systems (genosensors), which consists of three stages. The first stage involves the search for candidate genes and the computer-aided design of genosensors with desired properties. Using information resources developed and maintained at the Institute of Cytology and Genetics (Novosibirsk), it is possible to select genes with preset properties. In particular, GeneNet and GenSensor databases contain information necessary for the creation of genosensors. The former provides data on the sensor genes and mechanisms of their response to stress factors, while the latter informs on the structure of bacterial promoters whose expression is activated in response to a certain external factor (Fig. 4, left column). At the second stage, experimental genosensors are constructed based on the promoters of selected candidate genes (Fig. 4, central column). The third stage consists in experimental verification of the sensitivity of the obtained genosensor, which is determined by the intensity of induced emission from GFP under the action of particular toxic agents (Fig. 4, right column).

In the present study, these stages have been realized for the creation of a polyfunctional genosensor based on the *yfiA* gene [22] and *dps* gene promoters of *E. coli* as described below. The dynamics of genosensor response was studied in a microfluidic system capable of operating with individual cells, to which the input metabolites were supplied via a microfluidic channel. The production of a fluorescent protein in genosensor cells activated by the metabolite was detected by means of a fluorescent microscope.

Designing a Base Vector for Cloning Sensor Gene Promoters

For designing genosensor structures, we have created a panel of recombinant DNAs, the introduction of which into *E. coli* cells impart them the properties of genosensors. Based on this panel, it is possible to create both specific and polyfunctional genosensors. In order to optimize the process of genosensor construction, the first stage of this study was devoted to the development of a base vector plasmid. This plasmid contains a DNA sequence encoding the reporter protein, which has translation and transcription terminators at the 3' end. At the 5' end, the gene is flanked with a set of sites for restriction exonucleases (restrictases), which ensure rapid and convenient insertion of desired promoter genes. Such a plasmid represents a cassette vector for cloning identical regulatory sequences—in this case, promoters sensitive to various external factors. As the reporter protein, we selected a GFP variant with reduced half-life (GFPvaa) [23].

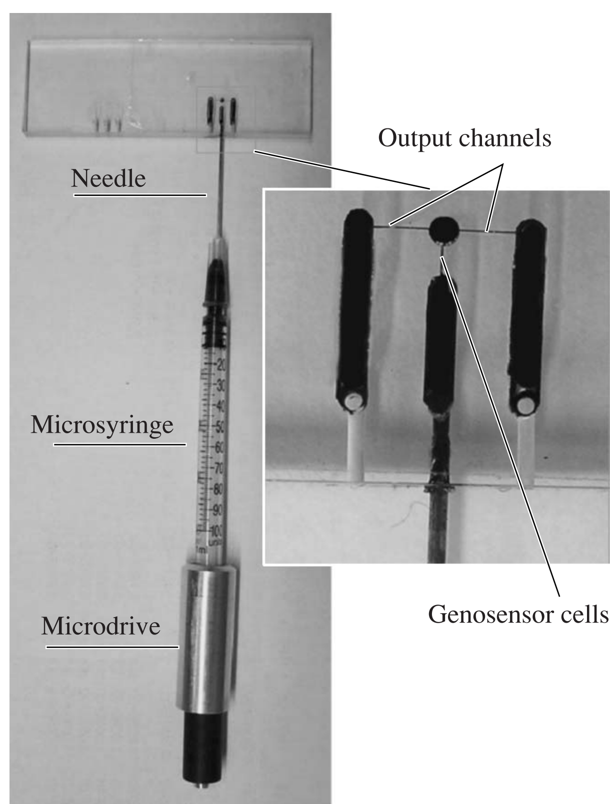

Fig. 3. General view of an MNFS with auxiliary devices ensuring the transition to a macroscopic level. The system of chambers and channels is filled with an ink.

The base vector plasmid was constructed using a DNA fragment encoding the GFPvaa protein, which was obtained by treating pZE21 plasmid DNA [24] with restriction endonucleases KpndI and HindIII. This DNA fragment was combined by ligation with a pRS2 plasmid [25] hydrolyzed by the same endonucleases. The thus constructed base plasmid (pRS-GFPvaa) carried a gene encoding the reporter protein of GFPvaa and contained an adjacent site for restriction endonuclease Sph1 at the 5' end, which ensured exact connection of any promoter fragment to this gene. The correct

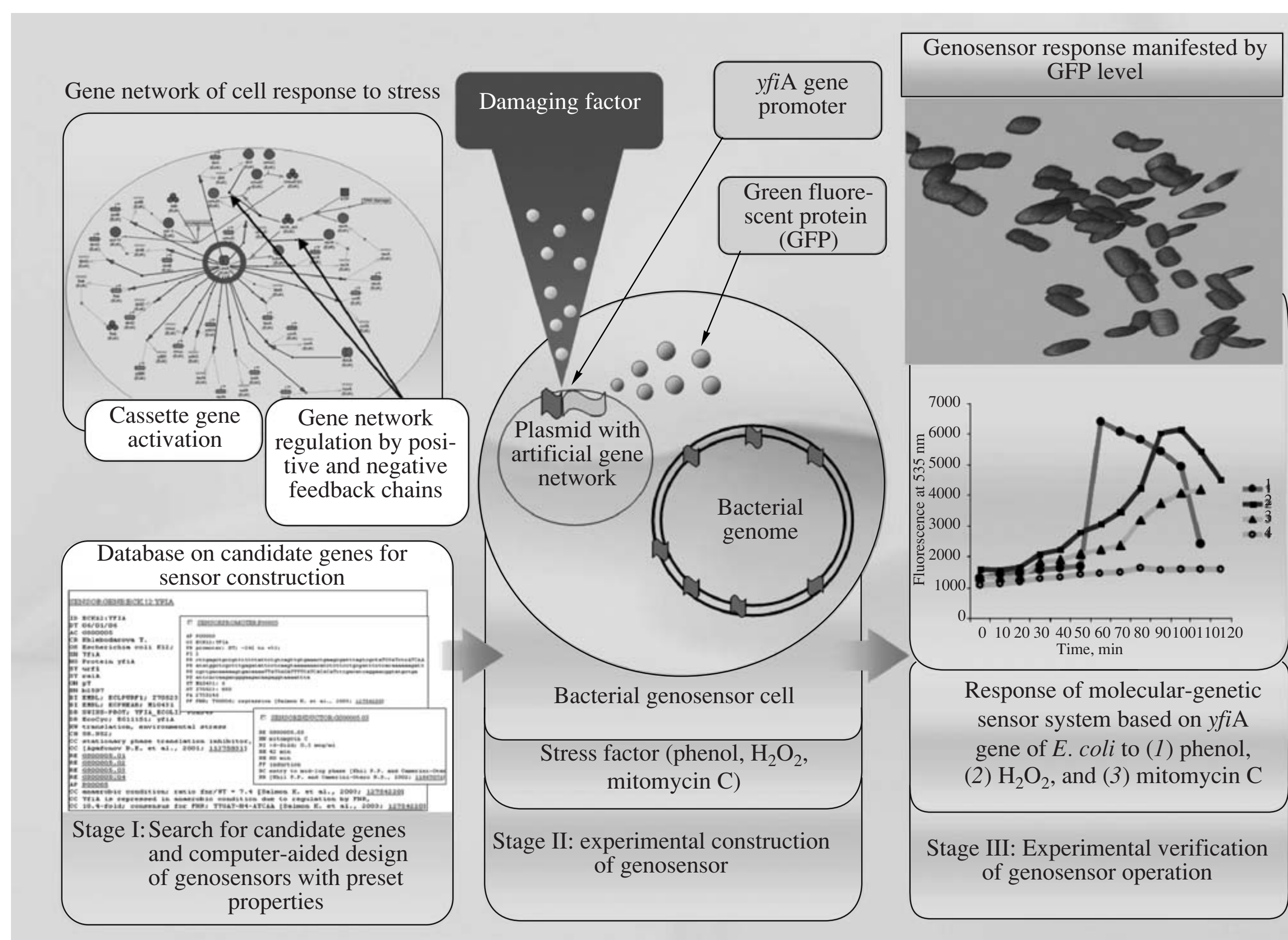

Fig. 4. Computer-aided experimental approach to the rational design of artificial molecular-genetic systems (genosensors) with preset properties.

gene design was confirmed by means of restriction analysis and by sequencing the insert region.

Constructing a Specific Oxidative Stress Sensor

For experimental verification of the correctness of the base vector design, we employed the *katG* gene promoter of *E. coli*, which is a specific sensor for oxidizing agents. Using this promoter and the *lux* reporter gene, we have constructed a genosensor, which is one of the most sensitive to this type of toxic agents [26]. For this purpose we obtained, by means of polymerase chain reaction (PCR) using the DNA of *E. coli* JM109 as the matrix, a DNA fragment containing the *katG* promoter gene region. Primers in the PCR were

5'-CCTCGGTACCAAGCTTAATTAAGATCAATTGATCTAC

and

5'-CCTCGCATGCGTTGCTGACCACGACCCG

polynucleotides. The PCR-produced fragment was hydrolyzed by restriction endonucleases ApaI and SphI and combined by ligation with the pRS-GFPvaa plasmid linearized by the same restrictases.

The intermediate plasmid was grown to a preparative amount and treated with KpnI restrictase and the Klenow fragment of polymerase I for reconstructing the first ATG codon. The correct design was confirmed by means of restriction analysis and by sequencing the insert region. In order to check for the ability of the obtained pKat-gfp plasmid to functioning in the genosensor, *E. coli* JM109 cells transformed with this plasmid were grown overnight, plated on a freshly prepared ampicillin-containing LB medium, and cultivated to a midlogarithmic phase. Since the LB medium possesses

intrinsic fluorescence in the spectral range of GFP protein emission, the cells of *E. coli*/pKat-gfp genosensor were transferred to a minimal M9 medium with the following composition: 0.06% Na_2HPO_4; 0.03% KH_2PO_4; 0.005% NaCl; 0.01% NH_4Cl. The role of the oxidative stress agent was played by hydrogen peroxide. The sensitivity of *E. coli*/pKat-gfp cells to H_2O_2 was evaluated by measuring the intensity of fluorescence at 535 nm (excited at 485 nm by 0.1-s laser pulses) at the cultivation temperature (26°C). The fluorescence was measured (on a relative scale) using a PerkinElmer Victor 3 fluorimeter. The cell activation level was defined as the ratio of the maximum emission intensity to a base fluorescence level (the emission from *E. coli*/pKat-gfp cells not treated with toxic agents).

The maximum level of activation of *E. coli*/pKat-gfp genosensor cells in the presence of H_2O_2 was observed 40 min after introduction of the oxidizer at a concentration of 8 mM (Fig. 5). Thus, the proposed *E. coli*/pKat-gfp genosensor has a sensitivity comparable with that of the *E. coli*/katG-gfp genosensor described earlier [27], which had a two times lower level of cell activation.

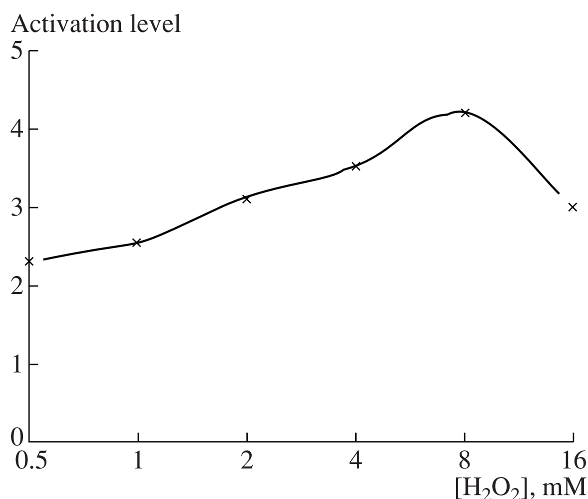

Fig. 5. Plot of the level of *E. coli*/pKat-gfp genosensor activation versus H_2O_2 concentration. The activation level is defined as the ratio of the maximum emission intensity (achieved 40 min after oxidizer introduction) to the base fluorescence level.

Constructing a Polyfunctional Genosensor

Bioinformatics approach. For construction of Genosensors with preset properties, a search was performed for candidate genes in the GenSensor database [18, 28]. This unique database contains information on the structure of promoters whose expression is activated in response to the factors damaging DNA, RNA, cell proteins and membranes, and on the activation mechanisms and conditions corresponding to the maximum response for a given external factor. Using the standard SRS WEB-interface of the GenSensor database, it is possible to search by key words and to submit typical or custom queries. The database is accessible at http://wwwmgs.bionet.nsc.ru/mgs/dbases/genosensor/index.html.html.

The results of our search for candidate genes to be used in the construction of polyfunctional genosensors—that is, sensors capable of responding to stressor factor of different types—are presented in the table. Based on the analysis of these data, we selected the *dps* gene of *E. coli*, for which the character of regulation and the presence of a promoter identified previously [29] suggested the possibility of creating a polyfunctional genosensor.

Experimental design. The experimental construction of a genosensor based on the *dps* gene promoter of *E. coli* and the pRS-GFPvaa vector plasmid was carried out as described for the analogous genosensor based on the *katG* gene. The structure of the promoter region of the *dps* gene is illustrated in Fig. 6. In the PCR reaction, the following nucleotides were used as primers:

5'-CTCTGGGCCCAAATAAGAATTGTTCTTATCAATATATCTAAC

and

5'-GAAGGCATGCATAATTTCATATCCTCTTGATGTTATGTC.

The PCR-produced fragment of the *dps* gene promoter was obtained using the DNA of *E. coli* HB101 as the matrix. A recombinant strain of *E. coli*/pDps-gfp was obtained as described above for *E. coli*/pKat-gfp and then tested for the ability to produce the fluorescent protein GFPvaa in the presence of toxic factors. These were hydrogen peroxide (oxidative stress damage) at concentrations 10, 5, 2.5, 1.25, and 0.68 mM and phenol (cell membrane and protein damage) at concentrations of 3.2, 1.6, and 0.8 mM. The sensitivity of *E. coli*/pDps-gfp to these toxic agents was evaluated as described above for *E. coli*/pKat-gfp.

As can be seen from the data in Fig. 7, the cells of *E. coli*/pDps-gfp responded to the presence of H_2O_2 and phenol in the medium, which demonstrates that this recombinant strain can be used as a genosensor. The maximum level of *E. coli*/pDps-gfp cell activation was observed 50–70 min after the introduction of H_2O_2 and 20–30 min after the injection of phenol. The maximum fluorescence intensity was observed for H_2O_2 at 2.5 mM and phenol at 3.2 mM. With increasing concentration of a toxic agent, the time required for the maximum emission intensity in the case of H_2O_2 increases, while that in the case of phenol decreases, which suggests that

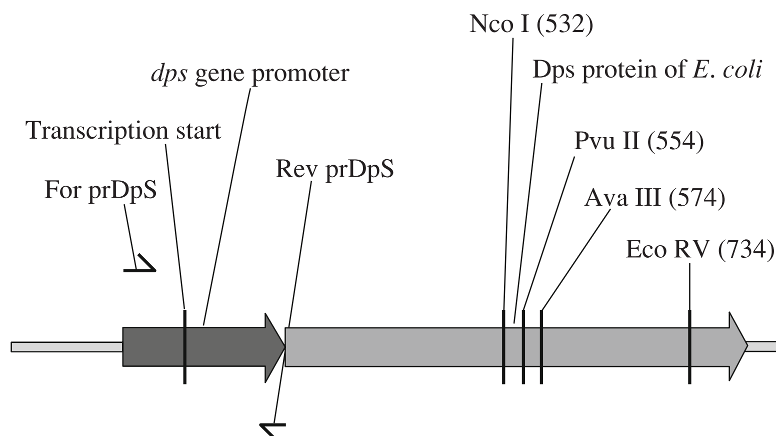

Fig. 6. Schematic diagram of the structure of the promoter region of the *dps* gene of *E. coli*, showing positions of the forward (for prDpS) and reverse (rev prDpS) primers, start of transcription, and restriction sites for NcoI, PvuII, AcaII, and EcoRV restrictases. The size of the synthesized fragment is 830 bp.

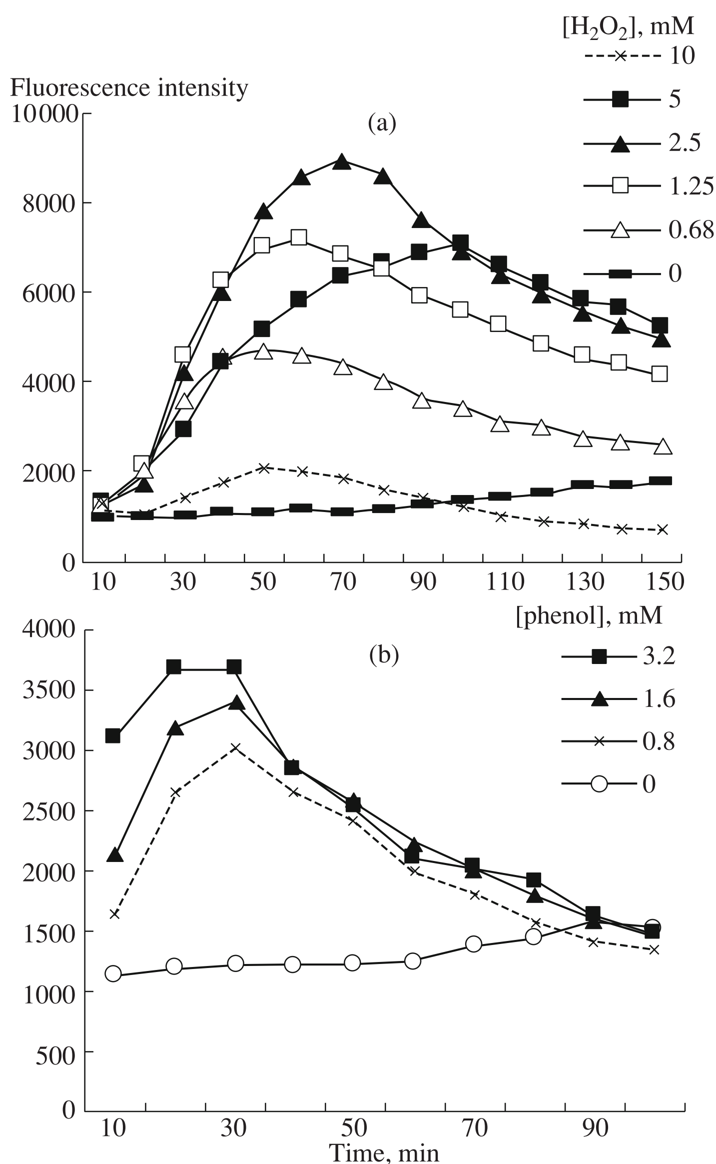

Fig. 7. Fluorescence of *E. coli*/pDps-gfp genosensor cells (in comparison to the control cells) in response to various concentrations of (a) H_2O_2 and (b) phenol. The cell cultures were prepared as described for *E. coli*/pKat-gfp genosensor.

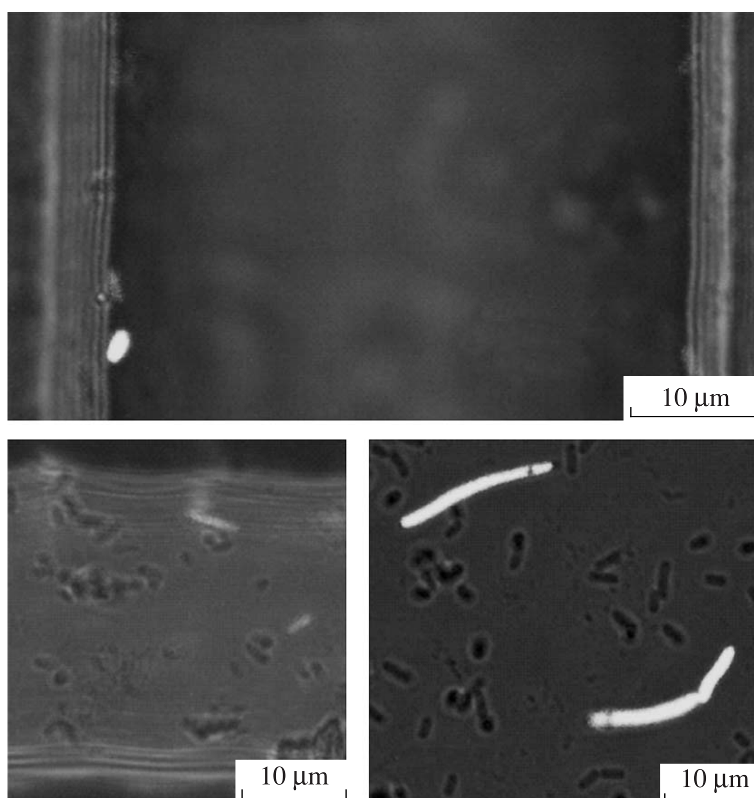

Fig. 8. Cells of recombinant *E. coli*/pDps-gfp strain in an MNFS. Bright signals show the GFPvaa fluorescence in response to the activator injection. The results of confocal microscopy are matched to the image obtained using the conventional optical microscopy in the differential contrast mode (dark signals).

mechanisms responsible for genosensor response to these agents are different. Using the results of these tests, it is also possible to evaluate the maximum coefficient of activation of the fluorescence of *E. coli*/pDps-gfp cell, which amounts to ~8.5 for H_2O_2 and ~3 for phenol. This is evidence of the high sensitivity of this genosensor, at least with respect to peroxide compounds. In comparison to *E. coli*/pKat-gfp, which is a genosensor specific to oxidizing agents, the sensitivity of *E. coli*/pDps-gfp to this type of compounds is three times as large, while the maximum level of activation is twice as large.

STUDYING THE SENSITIVITY OF GENOSENSORS TO ENVIRONMENTAL DAMAGING AGENTS IN A MICROFLUIDIC SYSTEM

The genosensor constructed as described above was used as a biological detector of oxidative stress in an MNFS fabricated in a PMMA matrix using the LIGA technology. PMMA is a solid transparent material convenient for fabricating microfluidic systems and monitoring biochemical processes in these systems by optical methods. This polymer exhibits no fluorescence under irradiation by an Ar laser, which has been used to excite emission from the GFPvaa reporter protein.

The surface of PMMA is hydrophobic and exhibits low adhesion with respect to *E. coli* cells. In order to provide for the immobilization of genosensor cells, a special pretreatment of the microfluidic channel surface is necessary. We processed this channel by means of open oxygen plasma at 0.1 bar, which led to the appearance of negative charges fixed on the channel surface. Then, the plasma-modified surface was coated with polylysine, which was retained on the surface due to

Results of GenSensor database search for genes responding to various external damaging factors (including complex)

External factor	List of genes
H_2O_2	*dps*, *hemH*, *sufA*, *grxA*, *yfiA*, *ahpC*, *trxC*, *frp*, *spp*, *ibpA*, *ibpB*, *tnaA*, *cysK*, *uxuA*, *soxS*, *dsdX*, *dsdA*, *katG*, *manX*, *rcN*, *iscR*, *phoP*, *yaaA*, *yaiA*, *clpS*
Sodium salicylate	*manX*, *fumC*, *yfiA*, *dps*
Mitomycin C	*dps*, *polB*, *dinG*, *dinH*, *yfiA*, *uvrB*, *dinB*, *dinC*, *ssb*, *lexA*, *recA*, *ydiO*, *yhaZ*, *yhjD*, *xkdA*, *yneA*, *ymaC*, *yobU*, *uraH*, *ydjM*, *recX*, *rmuC*
H_2O_2 + sodium salicylate + mitomycin C	*yfiA*, *dps*

electrostatic interactions. This was achieved by treating the channel with a 1 mg/ml aqueous solution of polylysine, followed by drying in a flow of sterile air. In order to introduce living cells of the *E. coli*/pDps-gfp genosensor, a culture of these cells in a minimal medium was passed through the central channel of the microfluidic system (Fig. 3). The cells stuck to the surface due to the interaction with polylysine molecules. Nonbonded cells were washed from the channel surface by minimal M9 medium.

After fastening genosensor cells in the microfluidic channel, a minimal medium containing hydrogen peroxide (oxidative stress agent) at a concentration of 2 mM was introduced into the channel. The MNFS with recombinant *E. coli*/pDps-gfp cells immobilized in the channel was studied by fluorescent microscopy 40 min after the onset of activation. The genosensor response to the introduction of oxidative stress agents was studied using an LSM500 Meta (Carl Zeiss) laser scanning microscope. GFPvaa molecules were excited by Ar laser radiation with a wavelength of 488 m. Laser scanning microscopy is capable of measuring a signal originating from the narrow optical layer (about 0.7 μm thick) in which the biological objects are situated, and discriminating background signals from below and above of this layer. The response signal was detected in a wavelength range of 505–525 nm via a ×63 objective. The data were processed by LSM Image Examiner software (Carl Zeiss). In order to determine the positions of cells in the microfluidic channel, the MNFS was also examined by a method providing an image analogous to that in the differential contrast mode. Using the aforementioned software, it is possible to fit the images obtained for fluorescence sections and by optical microscopy. This makes it possible to detect individual cells that produce GFPvaa (Fig. 8).

CONCLUSIONS

We described the key stages in the development of a genosensor-based bioanalytical complex capable of functioning in a micro/nanofluidic system (MNFS), which is intended for detecting damaging agents in liquid media. This approach has an interdisciplinary character and makes use of methods from the three fields: mechanotronics, bioinformatics, and molecular genetics.

Using the LIGA technology, we have developed an MNFS with a channel width of a few microns and above. The high quality of channels of the MNFS was confirmed by laser scanning microscopy using an LSM 500 Meta instrument.

A base vector plasmid was constructed for cloning genosensor promoters, which contains a reporter gene with the 5' end containing restriction sites for inserting target promoters.

Computer-aided design of genosensors was carried out using the GenSensor database, which makes it possible to search for stress-sensitive promoters suitable for the creation of genosensors responding to environmental damaging agents. Bioinformatics analysis allowed us to select the *dps* gene promoter of *E. coli*. According to the GenSensor database, this is a potential promoter for the construction of polyfunctional genosensors; it is capable of activating the transcription of reporter genes in response to a broad range of toxic environmental factors.

An experimental genosensor was constructed based on the *dps* gene promoter of *E. coli* and the pRS-GFPvaa vector plasmid. It was found that the cells of the recombinant *E. coli*/pDps-gfp strain activate transcription from the indicated promoter, which can be detected by measuring the fluorescence intensity.

Methods have been developed for processing the microfluidic channel to ensure immobilization of bacterial genosensor cells and for detecting fluorescent signals from the microfluidic module. It is established that *E. coli*/pDps-gfp genosensor cells in the microfluidic module respond by GFPvaa synthesis to the presence of agents producing oxidative stress and falling into the same range of concentrations as those outside the module.

Thus, the results of our investigation show that the proposed interdisciplinary approach can be used for obtaining bacterial cells–genosensors capable of functioning in MNFSs and detecting environmental damaging factors. Using the proposed methods, it is possible to construct multiposition MNFSs with a large number of channels, each containing a certain genetically modified cell (or an artificial molecular-genetic structure) capable of responding, by the expression of a specific sensor gene, to the presence of a certain damaging factor in the analyzed liquid.

Such bioanalytical systems capable of operating with ultrasmall (pico- and femtoliter) volumes of analyzed liquids can help us in solving a broad range of both basic and applied problems in various fields such as medicine, ecology, pharmacology, biotechnology, and biosafety. In particular, it can be used for (i) multiparametric monitoring of the environment aimed at revealing mutagenic factors; (ii) detecting biologically active, toxic, and immunoactive substances in the ambient medium, food products, water, air, and soil; (iii) large-scale screening of existing and new pharmacological preparations; (iv) detecting dangerous technological products; etc.

Based on the bioinformatics approach (GenSensor database), it is possible to seek gene promoters for use in genosensor structures responding either to a broad range of environmental damaging factors (as in the case of *E. coli*/pDps-gfp cells) or to particular damaging agents (toxins, heavy metal ions, detergents, etc.). Genosensors can also reveal the presence of unknown substances in analyzed liquids, leading to particular kinds of cell damage (e.g., damage of DNA, protein, or membrane structure).

The proposed approach also provides a starting point for the development of more complicated, artificial molecular-genetic structures based on the use of certain sets of gene promoters, which activate the expression of both reporter and regulatory proteins. On this basis, it is possible to study the functions of unknown genes and the dynamics of complicated gene networks.

The MNFS fabrication and application technologies have good prospects in basic science and in solving practical tasks. At present, new-generation miniature bioanalytical complexes (lab-on-a-chip) based on MNFSs are under development, which can even replace analytical laboratories [1–7, 30]. The unique possibilities of creating microchannels of complicated configurations in multiposition MNFSs, incorporating various types of microreactors, and providing optimum computer-aided control (ensuring preset characteristics of micro- and nanoscopic flows), as well as effective means of conjugating MNFSs to external macroscopic devices, make it possible to implement sophisticated bioanalytical systems with multistage functioning mechanisms, capable of solving a broad spectrum of problems in various fields.

ACKNOWLEDGMENTS

This study was supported by the Russian Federal Agency for Science and Innovations, project nos. 01.164.122.0018 and 02.512.11.2165.

REFERENCES

1. G. M. Whitesides, “The Origins and the Future of Microfluidics,” Nature (London) **442**, 368–373 (2006).
2. D. Janasek, J. Franzke, and A. Manz, “Scaling and the Design of Miniaturized Chemical-Analysis Systems,” Nature (London) **442**, 374–380 (2006).
3. D. Psaltis, S. R. Quake, and C. Yang, “Developing Optofluidic Technology through the Fusion of Microfluidics and Optics,” Nature (London) **442**, 381–386 (2006).
4. H. Craighead, “Future Lab-on-a-Chip Technologies for Interrogating Individual Molecules,” Nature (London) **442**, 387–393 (2006).
5. A. J. deMello, “Control and Detection of Chemical Reactions in Microfluidic Systems,” Nature (London) **442**, 394–402 (2006).
6. J. El-Ali, P. K. Sorger, and K. F. Jensen, “Cells on Chips,” Nature (London) **442**, 403–411 (2006).
7. P. Yager, T. Edwards, E. Fu, et al., “Microfluidic Diagnostic Technologies for Global Public Health,” Nature (London) **442**, 412 (2006).
8. N. A. Kolchanov, S. E. Pel’tek, N. V. Tikunova, et al., “Microfluidic Systems in Biology and the Design of Genosensors,” in *Science and Nanotechnologies* (Siberian Branch of the Russian Academy of Sciences, Novosibirsk, 2007), pp. 147–154 [in Russian].
9. K. T. Momynaliev, V. N. Lazarev, E. S. Kostryukova, et al., “Microfluidic Technologies for Detection of Intracellular Processes in a Bacterial Cell,” Ross. Nanotekhnol. **2** (5–6), 126–132 (2007).
10. P. P. P. M. Lerou, G. C. F. Venhorst, C. F. Berends, et al., “Fabrication of a Micro Cryogenic Cold Stage Using MEMS-Technology,” J. Micromech. Microeng. **16** (10), 1919–1925 (2006).
11. C. Zhang, D. Xing, and Y. Li, “Micropumps, Microvalves, and Micromixers within PCR Microfluidic Chips: Advances and Trends,” Biotechnol. Adv. **25** (5), 483–514 (2007).
12. A. Plecis and Y. Chen, “Fabrication of Microfluidic Devices Based on Glass–PDMS–Glass Technology,” Microelectron. Eng. **84**, 1265–1269 (2007).
13. R. M. Tiggelaar, F. Benito-López, D. C. Hermes, et al., “Fabrication, Mechanical Testing, and Application of High-Pressure Glass Microreactor Chips,” Chem. Eng. J. (Amsterdam) **131**, 163–170 (2007).
14. Z. Zhang, G. Perozziello, P. Boccazzi, et al., “Microbioreactors for Bioprocess Development,” JALA, No. 12, 143–151 (2007).
15. Y. Marcy, T. Ishoey, R. S. Lasken, et al., “Nanoliter Reactors Improve Multiple Displacement Amplification of Genomes from Single Cells,” PLoS Genetics **3** (96), 155 (2007).
16. T. Yasukawa, M. Suzuki, T. Sekiya, et al., “Flow Sandwich-Type Immunoassay in Microfluidic Devices Based on Negative Dielectrophoresis,” Biosens. Bioelectron. **22**, 2730–2736 (2007).
17. M. H. Sun, G. V. Casquillas, S. S. Guo, et al., “Characterization of Microfluidic Fuel Cell Based on Multiple Laminar Flow,” Microelectron. Eng. **84**, 1182–1185 (2007).
18. F. Y. H. Lin, M. Sabri, J. Alirezaie, et al., “Development of a Nanoparticle-Labeled Microfluidic Immunoassay for Detection of Pathogenic Microorganisms,” Clin. Diagn. Lab. Immunol., 418–425 (2005).
19. C. Crozatier, I. Tapsoba, L. P. Xu, et al., “Microfluidic Modulus for Convenient Cell Culture and Screening Experiments, Microelectron. Eng. **84**, 1694–1697 (2007).
20. M. Lounaci, P. Rigolet, C. Abraham, et al., “Microfluidic Device for Protein Crystallization under Controlled Humidity,” Microelectron. Eng. **84**, 1758–1761 (2007).
21. K. Hakkila, M. Maksimow, M. Karp, and M. Virta, “Reporter Genes *lucFF*, *luxCDABE*, *gfp*, and *dsred* Have Different Characteristics in Whole-Cell Bacterial Sensors,” Anal. Biochem. **301**, 235–242 (2002).
22. N. V. Tikunova, T. M. Khlebodarova, A. V. Kachko, et al., “A Computational–Experimental Approach to Designing a Polyfunctional Genosensor Derived from the *Escherichia coli* Gene *yfiA* Promoter,” Dokl. Akad. Nauk **417** (6), 835–839 (2007) [Dokl. Biochem. Biophys. **417**, 357–361 (2007)].
23. J. B. Andersen, C. Sternberg, L. K. Poulsen, et al., “New Unstable Variants of Green Fluorescent Protein for Studies of Transient Gene Expression in Bacteria,” Appl. Environ. Microbiol. **64**, 2240–2246 (1998).

24. M. Elowitz and S. Leibler, “A Synthetic Oscillatory Network of Transcriptional Regulators,” Nature (London) **403**, 335–338 (2000).

25. I. V. Frolov, A. A. Kolykhalov, E. V. Agapov, et al., “Study of the Prospects for Creating Vector Systems Based on the Alpha-Virus Genome,” Dokl. Akad. Nauk **326**, 738–741 (1992).

26. B. C. Kim and M. B. Gu, “A Bioluminescent Sensor for High Throughput Toxicity Classification,” Biosens. Bioelectron. **18**, 1015–1021 (2003).

27. C. Lu, C. R. Albano, W. E. Bentley, and G. Rao, “Quantitative and Kinetic Study of Oxidative Stress Regulons Using Green Fluorescent Protein,” Biotechnol. Bioeng. **89**, 574–587 (2005).

28. T. M. Khlebodarova, I. L. Stepanenko, and N. L. Podkolodnyi, “Database: Genes–Sensors (GenSensor),” RF Inventor’s Certificate No. 2 006 620 197 (2006).

29. S. Altuvia, M. Almiron, G. Huisman, et al., “The *dps* Promoter Is Activated by OxyR during Growth and by IHF and sigma S in Stationary Phase,” Mol. Microbiol. **13**, 265–272 (1994).

30. J. M. Kohler and T. Henkel, “Chip Devices for Miniaturized Biotechnology,” Appl. Microbiol. Biotechnol. **69**, 113–125 (2005).