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Simultaneous quantification of the fluorescent responses of an ensemble of bacterial sensors

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ABSTRACT

Bacterial bioreporters are genetically engineered microbial strains capable of detecting specific chemicals, groups of chemicals or global biological effects such as toxicity or genotoxicity. A scheme for simultaneous selective detection of the fluorescent signals emitted by a bacterial biosensor array, able to detect four different types of toxicants, using a single photodetector (photomultiplier) is presented. The underlying principle of the scheme is to convert the spatially distributed signals from all the elements in the array to temporally distributed frequency multiplexed signals at the output of the photodetector. Experimental proof of this concept is demonstrated in a four-channel system, in which low power (a few tens of picowatts) fluorescent signals produced by the bacterial sensors are measured, while maintaining a wide dynamic range of detection (more than 3 orders of magnitude). Simultaneous monitoring of concentrations down to a few mg/l of different chemicals in a liquid sample is demonstrated.

1. Introduction

Whole-cell biosensors harness the ability of living cells to continuously monitor their microenvironment, and to respond to local environmental changes by expressing specific gene sets. Among whole-cell sensor systems, bacterial-based ones are particularly attractive, since such cells can be genetically "tailored" to respond to diverse types of chemical and physical or biological stress. Consequently, in recent years, a considerable effort has been devoted to genetically engineer bacteria that sensitively detect trace quantities of specific chemicals or groups of chemicals (Van der Meer and Belkin, 2010). The response of the bacteria is dose-dependent and may be manifested in different ways including the generation of an electrical charge, a luminescent signal and, most often, by fluorescence.

In most cases, the engineered bacterial sensors harbor a plasmid-borne fusion between a sensing element that detects the presence of the target compound(s), and a reporter element the expression of which yields a quantifiable output. As a result of this fusion, a dose-dependent response is generated upon exposure to the target chemical. The most common sensing elements used are gene promoter regions which are activated in response to the target chemical. Of several available reporter elements, the most commonly employed are the *lacZ*, *gfp or lux* genes, yielding colorimetric, fluorescent or bioluminescent signals respectively.

Bacterial biosensor arrays provide an accurate, simple and in situ method for parallel monitoring of several chemicals (Elad et al., 2008). Each site (element) in the array is loaded with a bacterial strain genetically engineered to respond to a specific chemical by activating its responding scheme (*i.e.* fluorescence, bioluminescence or electrical charge emission). Following the exposure of the entire array to the sample under examination, sites at which a recognition event occurs will respond by emitting their reporting signal. Whole-cell bacterial sensors are easy to genetically manipulate, and can be engineered to detect almost any desired chemical; the number of potential array combinations is thus practically unlimited. The most optimal manner by which the signals of such arrays can be collected is in parallel, thus allowing the simultaneous monitoring of diverse substances in the same sample, e.g. different pollutants in water.

Parallel sampling may be achieved by assigning a separate detector to each element of the array, and sampling these detectors simultaneously. Using this principle, an increasingly large number of applications are being developed in diverse areas, ranging from environmental monitoring to homeland security and food safety.

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In fluorescent bacterial sensors the reporting scheme involves, in most cases, the synthesis of fluorescent proteins (e.g. *GFP*), the quantification of which is carried out by measuring the fluorescence they emit when excited at the appropriate wavelength. In a typical detection event the signal is initially very weak, and in time builds up and may become very strong if the concentration of the inducer is high. In order to detect a recognition event as early as possible and monitor its evolution until it saturates, the detector is expected to manifest both high sensitivity and a wide dynamic range; these characteristics are particularly important for bacterial biosensor arrays, each element of which may fluoresce at a different intensity. The quintessential detector under these conditions is the PMT; however, PMTs are cumbersome vacuum tubes and are not available in arrays. Hence, selective sampling of the signal from each element in a fluorescent biosensor array are carried out using a single PMT by serial sampling of the array elements, employing mechanical scanning (Kenneth et al., 2009).

2. Materials and methods

2.1. System underlying principle

In the sampling scheme presented henceforth, each element is illuminated by an excitation beam (Ex-B) modulated at a separate frequency. As fluorescent proteins respond to the excitation within a few nanoseconds (Lakowicz, 2006), and the modulation frequency is well below 1 MHz, the fluorescent light emitted by each element in the array is modulated in phase with its respective Ex-B. The fluorescent light emitted by the entire array is focused onto one photodetector.

The electrical signal at the output of the photodetector is digitized and computationally phase locked (Horowitz and Hill, 1989) to reference signals from the oscillators that drive the Ex-B modulators. This yields a data vector, each component of which corresponds to the fluorescence signal emitted by one specific element of the FBSA, *i.e*. one bacterial sensor type, engineered to sense a specific group of substances (Wu et al., 2006).

2.2. Architecture of the measurement system

This detection scheme was demonstrated in a four-channel measurement system, illustrated in Fig. 1b. The excitation light source was a 50 mW laser beam at λ=488 nm. The laser output beam was split into four excitation beams modulated at 30 kHz, 32 kHz, 40 kHz and 48 kHz, respectively, by electrooptic crossed-polarizers modulators (Yariv and Yeh, 2007). The modulators employed a potassium lithium tantalate niobate (KLTN) crystal (Agranat et al., 1992) as their electrooptic medium.

The core of the system is an array of four 10 μl cylindrical cuvettes, each illuminated along its symmetry axis by one of the excitation beams. The light emitted radially from the cuvettes was collected and collimated by an optical system, and notched filtered to remove residual excitation photons scattered toward the detector. The remaining fluorescent light was focused onto a PMT (Hamamatsu H9307-04) as demonstrated in Fig. 1a. The output signal of the PMT was digitized by a NI PCI-4472 data acquisition card, which also digitized in parallel the modulation driving voltages of the four modulators.

Four digital lock-in amplifiers were implemented in the data acquisition card, and were locked on the respective frequency and phase of each excitation channel. Thus, the output signal of each lock-in amplifier represents the light emitted by its respective cuvette. This method, in addition to providing a clear separation between individual channels, yields a significantly improved signal to noise ratio (SNR).

2.3. Bacterial reporter strains

Four characterized fluorescent bacterial sensors were selected to comprise a model 4-sensor array for the detection of different chemical classes. Each of the bacterial sensors was an *E. coli* K12 MG1655 strain harboring a plasmid with a genetic fusion between a stress-specific sensing element and a fluorescent reporter gene (*GFPmut2*) (Zaslaver et al., 2006): the *recA::GFP* sensor is induced by DNA damaging agents (Vollmer et al., 1997), *zntA::GFP* by heavy metals (Kessler et al., 2012), *marR::GFP* by various antibiotics (Melamed et al., 2012) and *yqjF::GFP* sensor by nitro-aromatic compounds (Yagur-Kroll et al., 2013). The 4 bacterial sensors are listed in Table 1 along with their model inducers.

2.4. Bacterial growth and induction

A strict protocol was implemented to ensure repeatability of the results. Fresh bacterial colonies were grown overnight in TGA medium (10 g/l bacto trypton, 5 g/l NaCl, 2 g/l D-(+) glucose, 11.9 g/l HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, $C_8H_{18}N_2O_4S$), pH 7.0) supplemented with 30 μg/ml kanamycin at 37 °C with shaking (200 rpm). The bacteria cultures were diluted 70-fold in fresh TGA medium and regrown under the same conditions to the mid-logarithmic growth phase ($OD_{600\ nm}$≈0.2, approximately 10^8 cells/ml). Then 10 μl from each bacterial culture was loaded into a different 10 μl cuvette and exposed either to the test inducer or to an inducer-free control medium, and the fluorescent signal emitted by both induced and non-induced bacteria was monitored. Several series of measurements were repeated on different dates with satisfactory reproducibility. Deviations between independent experiments did not exceed 20%.

3. Results

3.1. System characterization

A preliminary characterization of the optoelectronic system was carried out by measuring fluorescein, selected due to the similarity of its fluorescence spectra to that of *GFP*. The sensitivity of the system was evaluated by filling the four cuvettes in the system with fluorescein solutions at different concentrations. Fig. 2a and b display the fluorescent signal intensity vs. time for the different fluorescein concentrations. It can be seen that the system distinguishes between pure water and 0.1 ng/ml of fluorescein with sufficient SNR, while simultaneously measuring in other channels samples that emit a fluorescent signal over 3 orders of magnitude stronger. A previously reported fluorescein detection threshold in a PMT-based fluorescent biosensing system was approximately 40 ng/ml only (Miyaki et al., 2005).

3.2. Biosensors' sensitivity

Sensitivity of the optoelectronic biosensor system was evaluated by measuring the fluorescent signal emitted by the different *E. coli* sensor strains following their activation by different concentrations of their respective model inducers. A typical result is presented in Fig. 2c and d where the *recA:GFP* bacterial sensor was induced by 1.25 mg/l–20 mg/l of Nalidixic acid (NA). These results indicate a typical dose-proportional response of the fluorescent signal. In particular, it can be seen that the optoelectronic biosensor system is able to detect the relatively low induction of *recA:GFP* bacterial sensor at the lowest NA concentration tested (1.25 mg/l).

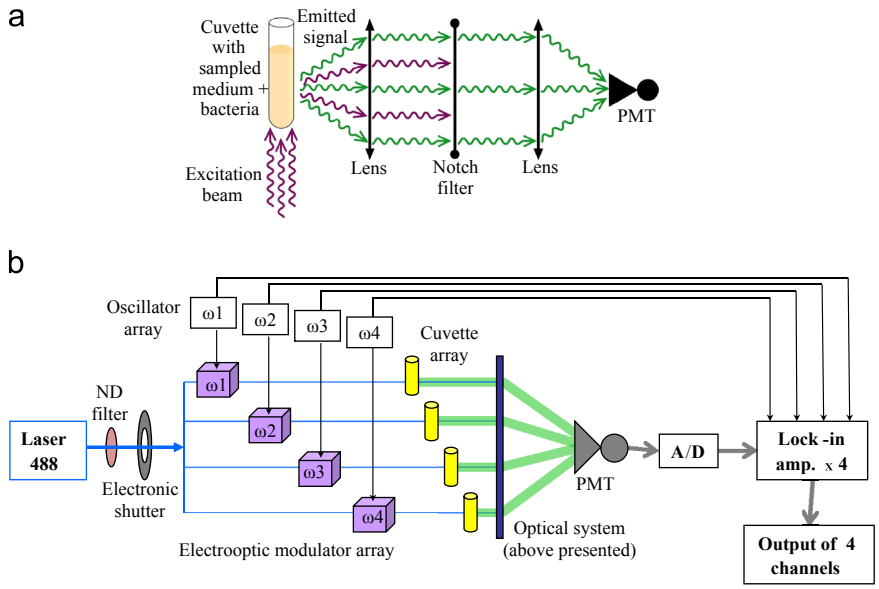

Fig. 1. Schematic presentation of the optical system (a) and the four-channel biosensor sampling system (b).

Table 1
List of bacterial sensors used in this study and their model inducers.

Bacterial sensor	Model inducer (detected substance)	Class of toxicants detected
recA::GFP	Nalidixic acid (NA)	DNA damaging agents
zntA::GFP	Cadmium chloride ($CdCl_2$)	Heavy metals
marR::GFP	Chloramphenicol (CM)	Antibiotics
yqjF::GFP	2,4-Dinitrotoluene (DNT)	Nitroaromatics

3.3. Bacterial sensors' selectivity

The ability of the four different bacterial sensors to operate as an array detecting four different inducers using the optoelectronic biosensor system was analyzed by exposing each of the four classes of bacterial sensors to each of the four inducers (Table 1). The results are presented in Fig. 3. The response difference (*i.e.* the difference between the fluorescence signal intensity emitted by a bacterial sensor exposed to one of the four inducers, and a control signal emitted from an identical bacterial sensor exposed to a toxicant-free medium) is shown as it develops over time for all four inducers.

Each of the bacterial sensors produces a fluorescent signal that is significantly higher than that of the control, only in response to a model toxicant belonging to the class of chemicals it has been molecularly designed to detect. As can be seen, different bacterial sensors manifest different selectivity. For example, the response of the *recA:GFP* sensor to its respective model inducer (NA) is faster and stronger than the response of the *marR:GFP* sensor to its respective model inducer (CM). Nevertheless, the selective response of each bacterial sensor used in this work was tested in several independent measurements in different dates and found to be reproducible. These results demonstrate the potential of the presented optoelectronic biosensor system for simultaneous monitoring of the individual bacterial-array members.

3.4. Analysis of an unknown sample by a bacterial biosensor array

The simultaneous operation of the four-channel FBSA was demonstrated by exposing all four channels in the array, each containing a different bacterial sensor, to the same polluted water sample at the same time. The fluorescent signals generated in the four channels were monitored simultaneously and are presented in Fig. 4a and b for polluted water samples containing either NA only (20 mg/l) or a mixture of NA and $CdCl_2$ (10 mg/l each). For each measurement, four cuvettes containing identical bacterial sensors were exposed at the same time to an unpolluted control water sample. Since there are only four channels in the current system, the control samples were kept in identical conditions to that of the samples in the system and were measured once after approximately 150 min.

As can be seen, each of the sensor types responds exclusively to the specific inducer to which it was engineered, even when exposed to a mixture of inducers. No extra fluorescence was found in the control samples.

3.5. Signal dependence on excitation power

The dependence of the emitted fluorescence on the excitation power was measured in a two channel array with 100 μl cuvettes. The excitation power of each channel was measured simultaneously by two photodiodes added to the system. The two cuvettes were loaded with *yqjF*-based bacterial sensor targeted to detect nitro-aromatic compounds. A solution containing 50 mg/l of DNT was added to the cuvette in one channel, while the cuvette in the second channel served as the control. After 4 h, the fluorescence intensity emitted from both cuvettes reached a steady state. At that point, the power of the excitation beams was gradually decreased, with a simultaneous measurement of both the excitation and the emission. The results are presented in Fig. 5.

The emitted fluorescent power was found to be linear with the excitation power over a wide range of excitation intensities. These results open the way for optimizing the detection dynamic range and accuracy by using the fluorescent emitted power as a feedback mechanism, *i.e.* for channels in which there are extremely low or high signals, the excitation power can be increased or decreased respectively.

4. Discussion and conclusions

Consider one of the four channels in the system exposed to a sample containing the material to which this channel is engineered to respond. Following exposure, the concentration of the fluorescent

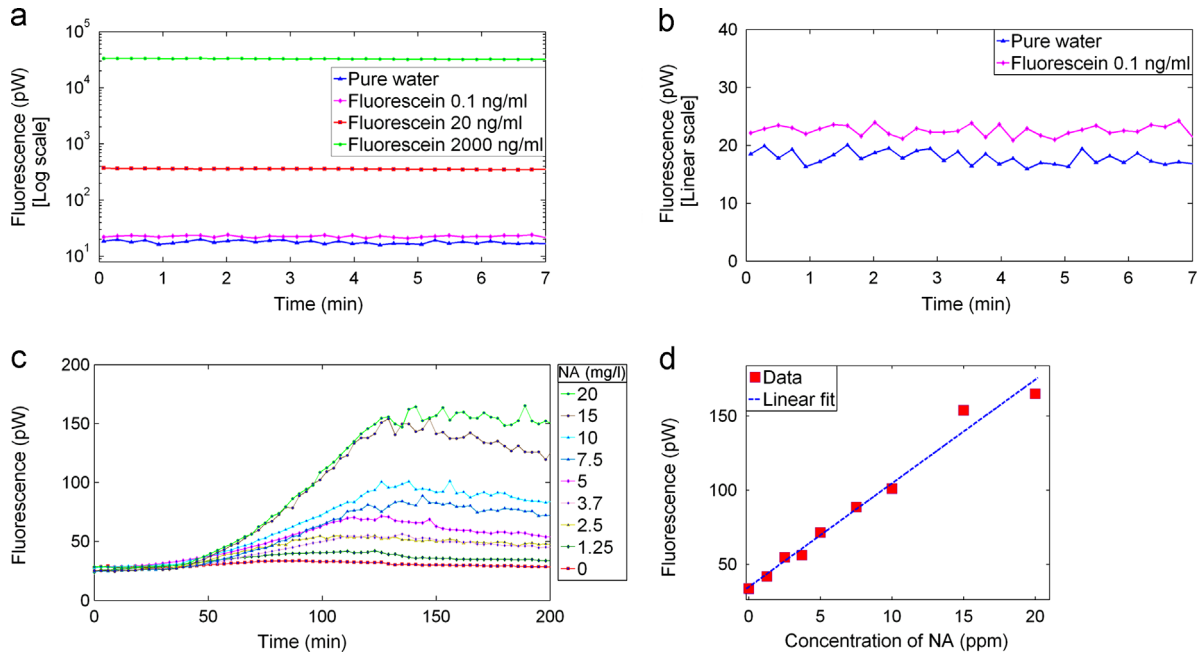

Fig. 2. Detection of fluorescein and *GFP* fluorescence. (a) Simultaneous detection of different fluorescein concentrations; (b) Lower concentration range only; (c) Signal development as a function of time of the *recA*-based bacterial sensor induced by different NA concentrations; (d) Maximal fluorescence of the *recA* bacterial sensor obtained over 200 min as a function of NA concentration.

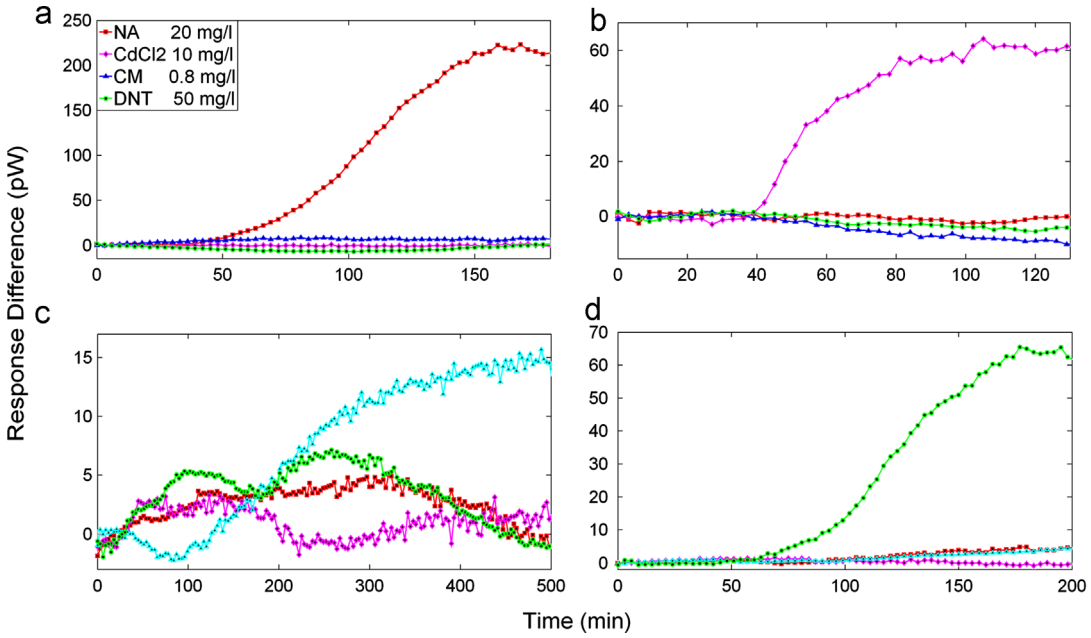

Fig. 3. Selectivity of the bacterial sensors: responses of the *recA:GFP* (a), *zntA:GFP* (b), *marR:GFP* (c) and *yqjF:GFP* (d) bacterial sensors exposed to all four model inducers.

molecules, and accordingly the emitted fluorescent signal, builds up slowly and reaches a maximal level that is proportional to the concentration of the said material in the sample. It is therefore desirable to use a photodetector which combines high sensitivity with a wide dynamic range. Using a PMT, the leading photodetector manifesting these features, enables obtaining accurate results throughout the time evolution of the signal from the first moments of exposure until the maximal signal level is reached. This remains true for both low and high concentrations of the monitored material.

PMTs, however, are not available in arrays. The detection scheme for a FBSA described in this work obviates the need for a detector array by using one PMT only and modulating each element in the array at a different frequency. This enables the parallel selective monitoring of multiple channels using one PMT without a need for scanning. Furthermore, given the fact that the excitation intensity of each channel can be controlled separately, a smart detection scheme can be applied. In this scheme the excitation intensity level of any of the channels can be adjusted continuously so that the signal levels of all channels are approximately the same, so that signals emitted by the weak channels are not masked by the stronger ones.

The results presented above demonstrate the advantages of the concept of the proposed detection scheme for FBSA in a model four elements array, and highlight the potential of this detection scheme as the basis for a wide range of applications. This brings forward the questions of system scalability, and how it can be implemented in a multi element FBSA. The detection scheme is

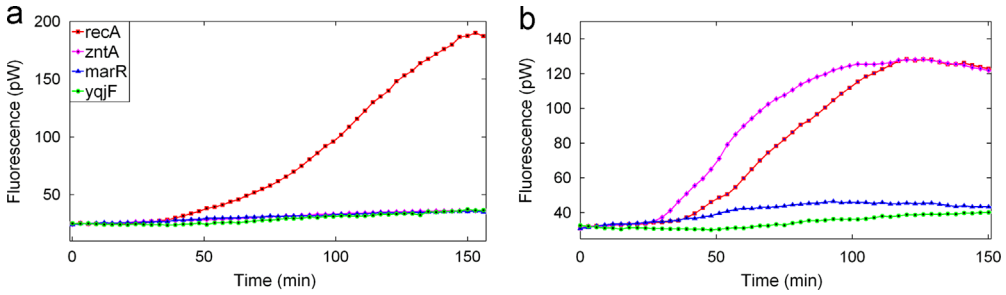

Fig. 4. Responses of all four bacterial sensors exposed to 20 mg/l of NA (a) or to 10 mg/l of NA+10 mg/l of $CdCl_2$ (b).

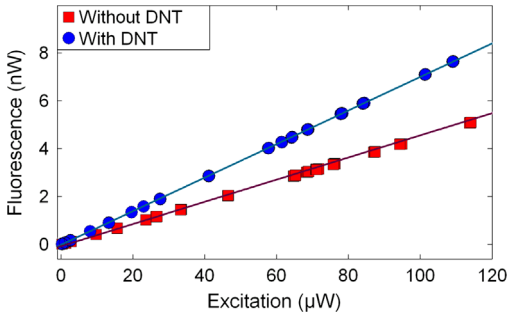

Fig. 5. Emitted fluorescence optical power is linear with the excitation power.

based on exciting each element in the array by an excitation beam modulated at a different frequency. The fluorescent proteins that are produced by the bacteria are excited at visible wavelengths (e.g. *GFPmut2* used in this work is excited with wavelengths between 340 nm and 540 nm). This implies that implementing this detection scheme in a multi element array requires the construction of a photonic integrated circuit that can split the single excitation beam produced by the laser source into a large number of daughter beams, each modulated at a different frequency, and transport it with minimal losses to its respective element in the array. This can be done by employing the methodology of Refractive Index Engineering (RI_Eng) by fast ion implantation that has recently been proposed for constructing electrooptic integrated circuits in KLTN that can be operated at visible wavelengths (Agranat et al., 2010).

RI_Eng is based on the fact that the implantation of light ions at high energies within the depth of oxygen perovskites creates an amorphized layer in which the refractive index (RI) differs significantly from that of the crystalline substrate (Kip, 1998; Chen, 2012). In particular, it was reported that in KLTN substrates, the ion implantation forms an amorphous layer with a reduced RI up to 11%, with respect to the crystalline substrate RI (Gumennik et al., 2007). In addition, post-implantation annealing renders the amorphized regions thermally stable while maintaining high optical quality (Gumennik et al., 2005). This enables to sculpt 3D structures with sub-micron features and reduced RI within the depth of the substrate. RI_Eng combines a series of implantations at different energies, through a respective set of "stopping masks" that govern the entry point of the implanted ions into the substrate (Ilan et al., 2006; Ilan et al., 2008). An important complementary technique is selective etching, using the implantation process to label the etched regions. This technique exploits the fact that the solubility of the amorphized regions created by the implantation is significantly greater than that of the crystalline material (Agranat et al., 2010) and can be used for creating trenches, cavities, and tunnels both on the surface and within the depth of the substrate. In particular it enables the construction of microfluidic systems in the KLTN substrate. Combining RI_Eng and selective etching in KLTN substrates opens the door for constructing an integrated photonic-microfluidic module that can serve as the physical chassis for a large scale FBSA that implements the detection scheme proposed here.

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