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ABSTRACT: Lipopolysaccharide (LPS), a characteristic component of the outer membrane of Gram-negative bacteria, can be used as an effective biomarker to detect bacterial contamination. Here, we reported a 293/hTLR4A-MD2-CD14 cell-based fluorescent biosensor to detect and identify LPS, which is carried out in a 96-well microplate with nondestructive, user-friendly and high efficiency. The promoter sequence of the critical signaling pathway gene *ZC3H12A* (encoding MCPIP1 protein) and Enhanced Green Fluorescence Protein (EGFP) were combined to construct a recombinant plasmid, which was transferred into 293/hTLR4A-MD2-CD14 cells through lipid-mediated, DNA-transfection way. LPS was able to bind to TLR4 and co-receptors-induced signaling pathway could result in green fluorescent protein expression. Results show that stable transfected 293/hTLR4A-MD2-CD14 cells with LPS treatment could be directly and continually observed under High Content Screening imaging system. The novel cell-based biosensor detects LPS at low concentration, along with the detection limit of 0.075 μg/mL. The cell-based biosensor was evaluated by differentiating Gram-negative and Gram-positive bacteria and detecting LPS in fruit juices as well. This proposed fluorescent biosensor has potential in sensing LPS optically in foodstuff and biological products, as well as bacteria identification, contributing to the control of foodborne diseases and ensurance of public food safety with its high throughput detection way.

KEYWORDS: LPS; cell-based fluorescent biosensor; 293/hTLR4A-MD2-CD14 cells; TLR4; green fluorescent protein; High Content Screening imaging system

■ INTRODUCTION

A major group of pathogens are Gram-negative bacteria and severely affect food/environmental safety and threaten public health. Lipopolysaccharides (LPS), the bacterial endotoxin, forms a major structural element of the outer membrane of Gram-negative bacteria. Therefore, LPS is a useful biomarker for Gram-negative bacterial contamination detection.[1] LPS leads to an imbalanced, dysregulated immune response, triggering septic shock, and causing multiple organ failure, and can threaten human health, even in small quantities.[2,3] Therefore, recognizing the biological activity of LPS and evaluating LPS toxicity are as important as identification of the bacteria itself, which may serve as an early warning sign of pathogenic Gram-negative bacterial contamination.

Limulus Amoebocyte Lysate (LAL) assay is the most popular FDA-approved gold standard LPS assay.[4,5] The assay relies on an enzymatic reaction that is based on clotting of Limulus and LPS, which usually takes several hours to perform and is not reliable owing to non-specific reactions with other LAL-reactive compounds, such as β-(1,3)-D-glucan.[6,7] Moreover, the unstable results from LAL assay happened due to the environmental variations, especially in pH and temperature.[8] Silver staining offers an inexpensive detection method for LPS, and the result can be separated by polyacrylamide gel electrophoresis. Nonetheless, potentially hazardous formaldehyde must still be used in order to obtain the gel-separated LPS, as it is an indispensable silver reductant.[9] Other sensing assays depending on natural LPS binding proteins or

peptides, and artificial affinity-recognizing molecules need complicated preparation and detection processes, often suffer from narrow dynamic detection ranges and high detection limitations.[10-14]

The development of cell-based biosensors is a promising strategy for screening, monitoring, and measuring toxic and harmful substances.[15] Rider *et al*. reported the first cell sensor that used B lymphocytes to recognize specific bacteria with the help of membrane-bound IgM antibodies.[16] Wang *et al*. developed a mouse neuroblastoma cell-based impedance biosensor (CIB) and used it successfully to detect PSP toxins.[17] Although these studies have examined the gross effects (e.g., viability, proliferation) of analytes on cells,[18] the assays lack investigations on particular phenotypes of interest, such as the activation of signaling pathways.

Because LPS can enter the circulatory system, causing a systemic inflammatory response that is detrimental to the host, using whole living cells for LPS recognition would be a best choice and enable direct functional information to be obtained regarding the effects of it on a living system. Methods based on reporting of analytes by fluorescence reactions in engineered cells have the potential to be reagent-free, simple, and nondestructive.[19-21] Living cells used as biosensors are typically propagated with a plasmid containing the genes that code for the bio-reporter are placed under control of a promoter that recognizes the analytes of interest, inducers activate the promoter genes, providing a genetic signal transducer that triggers and regulates the bio-reporter expression.[22] What is more, mammalian cell-based

biosensors for LPS have a significant advantage in reflecting cellular physiological action rather than quantitative detection, because external stimuli or changes in cellular microenvironment can disturb the "normal" physiological activities of mammalian cells, and can provide insight into mechanism of action of LPS. At last, the use of the High Content Screening (HCS) measurement of fluorescence in microplates allows visualization, automatic operation, and high-throughput data acquisition for LPS detection.

LPS is composed of lipid A, a core polysaccharide chain, and a serotype-specific O-antigenic oligosaccharide.[2] Lipid A is a potent bacterial effector that promotes activation of the innate immune system after binding to the CD14 complex, MD-2 and TLR4.[23] LPS specifically binds to TLR4, which functions as the transmembrane component of the LPS receptor complex and transduces the LPS signal, alerting the host to infection by Gram-negative bacteria,[24] thus LPS recognized by TLR4 provided an unduplicated pathway for the detection of endotoxin. Besides, TLR4 recruits myeloid differentiation protein (MyD88) to the cytoplasm, where MyD88 activates JUK, which combines with transcription factors, such as c-Jun and Elk-1, phosphorylating them and inducing gene expression later.[25] Phosphorylated Elk-1 can then combine with the *ZC3H12A* (MCPIP1) promoter region to initiate its transcription directly,[26] and the stimulation of TLR4 by LPS induces the release of critical proinflammatory cytokines that are necessary to activate potent immune responses.[27,28]

Based on the above LPS signaling pathway described, a novel, cell-based

fluorescent biosensor was developed for visual and nondestructive LPS detection. Specifically, the key target gene MCPIP1 promoter (-76 bp to + 60 bp) of the LPS toxicity pathway, was combined with Enhanced Green Fluorescent Protein (EGFP) to construct a recombinant plasmid. The plasmid was transformed into 293/hTLR4A-MD2-CD14 cells to obtain genetically stable fluorescent expression. The engineered cells exposed to LPS were regarded as a model for fluorescence detection in 96-well microplates by High Content Screening imaging system. The fluorescence signal of the transfected cells treated with LPS was measured, and the relationship between different LPS concentrations and relative fluorescence intensity was analyzed. The novel 293/hTLR4A-MD2-CD14 cell-based biosensor successfully detected LPS optically with high efficiency and accuracy, which is good for high-throughput detection for samples in large numbers and provide opportunity for on-site assay.

■ MATERIALS AND METHODS

Material and Regents. LPS (*E.coli* O111:B4), Dulbecco's Modified Eagle's Medium (DMEM) and Fetal Bovine Serum (FBS) were obtained from Gibco Laboratories (Gaithersburg, MD). Glass-bottomed dishes (35 mm) were purchased from Shengyou Biotechnology Co., Inc. (Hangzhou, China). Other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All solutions were prepared with deionized water, and all reagents were of analytical grade.

Apparatus. Fluorescence signals were recorded by Confocal Laser Scanning

Microscopy (CLSM, LSM 710, Carl Zeiss Microscopy GmbH, Göttingen, Germany). 293/hTLR4A-MD2-CD14 cells were incubated in a CO_2 incubator (Thermo Scientific Forma Series II Water Jacket, Thermo Fisher Scientific, Inc., Waltham, MA). The fluorescence response of the biosensor cells was determined at different time points after exposure by High Content Screening (HCS) (ImageXpress Micro XLS, Molecular Devices, USA).

Construction of a *pGL3-ZC3H12A-EGFP* Expression System. The homo *ZC3H12A* (MCPIP1) promoter was obtained from the reported research, and there existed binding sites of Elk-1 between the -76 bp and + 60 bp sequence.[29] Thus, that sequence was inserted into the *pGL3-EGFP* vector and the recombinant plasmid *pGL3-ZC3H12A-EGFP* was constructed. The sequence of the promoter was amplified by PCR with specific primers: P-ZC3H12A-S-KI: 5'-GGGGTACCAGCAGGAAGGGGCGAGGCA-3'; P-ZC3H12A-AS-EI: 5'-CCGGAATTCGGGTAAGGACGGCGGCGCCTTTAT-3'. The PCR product and *pGL3-EGFP* vector were digested with *KpnI* and *EcoRI*. The purified plasmid was subjected to quality control using agarose gel electrophoresis.

Transfection of the Plasmid and Cell Viability Evaluation. Approximately, 293/hTLR4A-MD2-CD14 cells were seeded at 2×10^5 cells/well in 2 mL growth medium without antibiotics, respectively, in a 6-well plate 24 h prior to transfection, such that the density of cells was 80-90% at the time of transfection. Cationic liposome transfection was used, and according to the Lipofectamine®3000 transfection instruction, some methods were optimized. Specifically, a total of 7.5 μL

of Lipofectamine®3000 (Invitrogen) was diluted into 125 μL Opti-MEM without serum (Gibco Invitrogen, Life Technologies). 5 μg *pGL3-ZC3H12A-EGFP* plasmid was diluted in 250 μL of Opti-MEM without serum (Gibco Invitrogen, Life Technologies), and 5 μg *pGL3-RFP* plasmid was diluted in 250 μL of Opti-MEM without serum (Gibco Invitrogen, Life Technologies) as well, then 5 μL P3000^{TM} was added and gently mixed. After that, diluted DNA was added to the above diluted Lipofectamine® 3000 (1:1 ratio) and were incubated for 5 min to form DNA-lipid complexes, then they were added to each well containing cells and complete growth medium (medium without penicillin and streptomycin). After 6 h of plasmid transfection, the medium was replaced with 1 mL fresh complete culture medium. Assessment of the transfection efficiency was obtained from the intracellular fluorescence intensity of Red Fluorescence Protein (RFP) (Excitation wavelength: 587 nm, Emission wavelength: 610 nm) and Enhanced Green Fluorescence Protein (EGFP) (Excitation wavelength: 488 nm, Emission wavelength: 525 nm).

Cell Counting Kit-8 (CCK-8) colorimetric assay was used to estimate the cytotoxicity of the transfected cells. Briefly, the transfected 293/hTLR4A-MD2-CD14 cells and the untransfected cells were incubated at 2×10^5 cells/well with 100 μL of culture medium. At the end of the incubation, 10 μL CCK-8 was added to each well and the cells were incubated at 37 °C under 5% CO_2. After 3 h, the ultraviolet absorbance at a wavelength of 450 nm was measured with a microplate reader.

The levels of Ca^{2+} were measured by Fluo-3/AM, a visible wavelength calcium probe. The dye was added to the transfected 293/hTLR4A-MD2-CD14 cells and

179 untransfected cells at 2×10^5 cells/well for 1 h at 37 ℃ under 5% CO_2 in the dark.
180 Next, the cells were washed with PBS (pH 7.4), and the fluorescence was measured.
181 The intracellular generation of ROS was investigated using 6-carboxy-2,
182 T-diclorodihydrofluorescence diacetate, di (acetoxy ester) (DCFH-DA) (Molecular
183 Probes, Eugene, OR). The transfected and untransfected cells at 2×10^5 cells/well were
184 loaded with 10 mM DCFH-DA and incubated for 20 min at 37□. The cells were then
185 washed three times with serum-free medium to remove the extracellular DCFH-DA.
186 Intracellular ROS levels in the transfected and untransfected cells were detected by
187 HCS with an excitation of 488 nm and an emission of 525 nm.

188 **Western Blot Analysis for EGFP Protein Expression Stimulated by LPS.** The
189 expression of EGFP in transfected 293/hTLR4A-MD2-CD14 cells after stimulation
190 by LPS was determined using Western Blot. The transfected
191 293/hTLR4A-MD2-CD14 cells were seeded at 2×10^5 cells/well in a 6-well cell
192 culture plate, after adherence, 2 mL cell culture medium containing 100 ng/mL, 1
193 μg/mL and 10 μg/mL LPS was added into the well, while the sample without LPS was
194 used as a control group. After cultivation for 8 h, the total protein extraction was
195 obtained. 30 μg of the total protein and the loading buffer were thoroughly mixed, and
196 denatured in the boiling water for 5 min. Then, 10 μL of the above mixture was added
197 into a pre-gel to begin electrophoretic transfer. The PVDF film was washed with
198 deionized water then and balanced for 10 min in TBS, followed by sealing and
199 beginning the incubation process. After washing the film, the ECL Plus hypersensitive
200 liquid was used for coloring, and the Tanon-2500 gel imager was used to perform

CCD photo-imaging.

Cell Viability and Fluorescent Response of the Cell-based Biosensor Exposure to LPS. CCK-8 colorimetric assay was used to estimate the cytotoxicity of LPS. Briefly, the transfected cells were seeded at 2×10^5 cells/well in a 96-well plate containing 100 μL DMEM, with LPS at various concentration of 0.1, 0.5, 1, 5, 10, 20, 50, 100, 150 μg/mL. After 24 h cultivation, 100 μL cell culture medium with 10 μL CCK-8 was added to each well and the cells were incubated at 37 ℃. After 3 h, the ultraviolet absorbance at a wavelength of 450 nm was measured with a microplate reader to verify the toxic effect of LPS on the cells.

Based on the LPS cytotoxic pathway, the capability for LPS toxicity evaluation was tested by observing the fluorescence changes in 293/hTLR4A-MD2-CD14 cells. Firstly, the transfected cells were seeded at 2×10^5 cells/well in a 96-well plate, and subsequently adhered to the wells. 100 μL DMEM containing 1 μg/mL and 10 μg/mL of LPS was added into each well respectively, and the DMEM without LPS was served as a control group. Then, the fluorescence intensity in 293/hTLR4A-MD2-CD14 cells was monitored every 2 h under HCS, and the stable early expression time of EGFP protein was determined through MetaXpress image software analysis.

Lastly, the transfected cells at 2×10^5 cells/well were seeded into Confocal Dish, after adherence for 24 h, 1 mL of DMEM containing 0.1, 0.5, 1, 5, 10, 20, 50 ,100, 150 μg/mL of LPS were added into each well, respectively. After incubation for 8 h, the fluorescence detection was conducted using Laser Confocal Microscope at the

223 excitation wavelength of 488 nm.

224 **Fluorescence Analysis in Response to Pathogenic Bacteria.** Inactive *E.coli*
225 O111:B4 (G^-) and *S.aureus* (G^+) were used to stimulate the transfected cells. The
226 transfected 293/hTLR4A-MD2-CD14 cells were seeded at 2×10^5 cells/well in a
227 96-well plate for 24 h. *E.coli* O111:B4 (G^-) and *S.aureus* (G^+) at Multiplicity
228 of Infection (MOI) = 50 were added into each well then, and the fluorescence
229 intensity in 293/hTLR4A-MD2-CD14 cells was monitored every 4 h under HCS.

230 In order to detect the relationship between different MOI value of *E.coli*
231 O111:B4 (G^-) and intracellular fluorescence signal, the transfected
232 293/hTLR4A-MD2-CD14 cells were seeded at 2×10^5 cells/well in a 96-well plate,
233 and the *E.coli* O111:B4 (G^-) of MOI= 5, 10, 20, 50, 100, 200 were added into it,
234 respectively, and incubated for 16 h. The fluorescence detection was conducted using
235 HCS at the excitation wavelength of 488 nm, and the fluorescence data were analyzed
236 by MetaXpress image software.

237 **Real Sample Assay.** Detection of LPS using the developed cell-based
238 fluorescent biosensor was carried out in fruit juices (apple, mango and orange juice)
239 pre-treatment. Firstly, the fruit juices were centrifuged 10000 rpm for 5 min to remove
240 the particulate matters, then the supernatant was diluted 10 times and autoclaved for
241 analysis. The concentration of LPS for all spiked samples was kept in the linear range
242 of detection. Then, the transfected 293/hTLR4A-MD2-CD14 cells were seeded at
243 2×10^5 cells/well in a 96-well plate containing 100 μL DMEM, after adherence, 100
244 μL DMEM containing fruit juices with LPS (2 μg/mL and 15 μg/mL, respectively)

was added into each well respectively. At last, the fluorescence intensity in 293/hTLR4A-MD2-CD14 cells was monitored after incubation for 8 h under HCS, and EGFP protein expression was determined through MetaXpress image software analysis.

■ RESULTS AND DISCUSSION

Schematic Illustration of Cell-based Biosensor. As shown in Scheme 1, the recombinant plasmid *pGL3-ZC3H12A-EGFP* was first constructed by inserting the specific *ZC3H12A* gene promoter sequence into the *PGL3-EGFP* vector. Then, lipid-mediated plasmid-transfection of 293/hTLR4A-MD2-CD14 cells was fused with the membrane by endocytosis. Finally, the fluorescent protein gene diffused across the intracellular membranes into the nucleus and stable expression of the fluorescent cell-based biosensor was successfully established.

When the cells were exposed to LPS, TLR4 and co-receptors on the cell membrane would recognize LPS and activate the relevant cell toxicity pathway then, along with the successive expression of EGFP upon the *ZC3H12A* gene promoter activation. In this way, the cell toxicity levels for LPS could be directly evaluated by monitoring the intracellular fluorescence signal under HCS. In addition, the visualization of fluorescence intensity changes is a more straight-forward and sensitive detection method than those offered by traditional toxicity evaluation methods.

Successful Construction and Expression of Fluorescent Protein. To achieve the expression of EGFP in 293/hTLR4A-MD2-CD14 cells, the

pGL3-ZC3H12A-EGFP plasmid was constructed (Figure 1A) by the PCR product of *ZC3H12A* gene promoter, along with the vector of *pGL3-EGFP* being digested with the *KpnI* and *EcoRI* and ligated with T4 DNA ligase. This recombinant plasmid was verified by 2% agarose gel electrophoresis. As shown in Figure 1B, band c showed two fragments of 136 bp and 4818 bp, corresponding to the molecular weights of the *ZC3H12A* promoter and the *pGL3-EGFP* vector.

RT-PCR and Western Blot were used to verify *ZC3H12A* gene and MCPIP1 protein expression of 293/hTLR4A-MD2-CD14 cells that were exposed to LPS standard. As shown in Figure 2A, different concentrations of LPS used to treat cells (100 ng/mL, 1 μg/mL and 10 μg/mL) enhanced *ZC3H12A* gene transcription to 5.4 times, 6.7 times and 7.1 times, which was higher than the control group, respectively. Western Blot analysis (Figure 2B), with GADPH serving as an internal control, confirmed that the intensity of the MCPIP1 bands following 10 μg/mL of LPS stimulation was higher than that detected for lower LPS concentrations, and no band present in the control. These results indicated that LPS was able to promote *ZC3H12A* gene and MCPIP1 protein expression through signaling pathway, and further proved in principle that using the recombinant plasmid containing the *ZC3H12A* gene (MCPIP1) promoter for sensing LPS was practicable.

To estimate the cytotoxic interactions of transfected 293/hTLR4A-MD2-CD14 cells, CCK-8 assay was tested (Figure S1). $[Ca^{2+}]i$ production was evaluated, as shown in Figure S2, $[Ca^{2+}]i$ produced the same value between transfected and control cells. Similarly, there were no significant differences in ROS production between the

289 transfected and untransfected cells (Figure S3).

290 **Quantification of the LPS by the Cell-based Sensor.** Furthermore, the time-
291 and dose-dependence of the cell-based biosensor response to LPS standard (1 μg/mL
292 and 10 μg/mL) were investigated.

293 As shown in Figure 3A, green fluorescence signal appeared within 2 h after LPS
294 stimulation of 293/hTLR4A-MD2-CD14 cells and the fluorescence intensity increased
295 with the time of exposure to LPS, and the response of sensors to different LPS
296 concentrations in terms of the fluorescence intensity versus time was quantified as
297 well (Figure 3B). In cells exposed to 1 μg/mL LPS, the measured F_t/F_0 value
298 increased from 6.19-fold after 2 h of exposure to 10.01-fold after 6 h of exposure,
299 11.78-fold after 8 h, and 12.32-fold after 12 h. In cells exposed to 10 μg/mL of LPS,
300 the measured F_t/F_0 value increased from 7.66-fold after 2 h of exposure, to 10.54-fold
301 after 6 h, 13.10-fold after 8 h, and 14.28-fold after 12 h. The resulting curves
302 indicated that the F_t/F_0 values rapidly increased during early stage of incubation,
303 which slowed down after peaking at an LPS exposure time of 8 h. At longer exposure
304 times, cells displayed no additional increase in the induction of green fluorescence,
305 which remained stable thereby reaching a plateau. The induced expression level of the
306 reporter genes was relatively stable over the tested exposure periods, while the control
307 (medium without LPS) cells did not induce EGFP expression over any of the tested
308 time periods.

309 Western Blot analysis was also used to verify the intracellular protein levels
310 when the cells was treated by LPS standard for 8 h, as shown in Figure 3C. GADPH

served as an internal control, and the intensity of EGFP band at an LPS concentration of 10 μg/mL was higher than that in the lower LPS concentration, no significant band present in the control group simultaneously. These results confirmed that the fluorescent cell-based biosensor could be used to detect LPS toxicity.

The dose-dependence of the cell-based biosensor response to LPS was also examined. Based on the above experimental results, a range of concentrations of LPS (0.1, 0.5, 1, 5, 10, 20, 50, 100, 150 μg/mL) was used to treat 293/hTLR4A-MD2-CD14 cells for 8 h, and the fluorescence image was monitored under the Laser Confocal Microscope (Figure 4A). As shown in Figure 4B, low relative fluorescence intensity in cells was observed when the LPS was in the range of 0.1-0.5 μg/mL, but the intracellular fluorescence intensity enhanced upon the addition of increasing concentrations of LPS, when the concentration of LPS was in the range of 1-100 μg/mL, the relative intracellular fluorescence intensity showed a linear relationship (Figure 4B), with the following equation: $y = 0.224 x + 7.097$, $r = 0.996$, LOD was 0.075 μg/mL, and the fluorescence intensity increased slowly when the concentration of LPS reached 150 μg/mL.

For comparison purposes, reports using different recognition elements and approaches for LPS sensing were shown in Table 1. The proposed method showed impressive results with the detection limit lower than other reported works.

Conventional CCK-8 assay was also used to test the cytotoxicity of LPS as a validation. As shown in Figure 4C, when the concentration of LPS was 0.1-20 μg/mL, cell viability was over 90%, whereas when the concentration of LPS was 50 μg/mL,

cell viability decreased significantly. At LPS concentrations of 50, 100, 150 μg/mL, cell viability was decreased to 74.77%, 65.01% and 56.67%, respectively.

Response of Cell-based Biosensor Exposure to LPS from Pathogenic Bacteria. LPS is the main pathogenic factor on the surface of Gram-negative (G^-) pathogenic bacteria. To verify whether the developed cell-based fluorescent biosensor was specific to Gram-negative pathogenic bacteria, inactive *E.coli* O111:B4 (G^-) and *S.aureus* (G^+) bacteria were used to stimulate 293/hTLR4A-MD2-CD14 cells, and intracellular fluorescence was monitored in real-time by HCS. As shown in Figure 5, the fluorescence signal in the cells treated with *E.coli* O111:B4 (G^-) was sharply enhanced, and further plateaued at 16 h. On the contrary, for the cells stimulated with *S.aureus* (G^+), there was no obvious change in fluorescence intensity. These findings indicated that the Gram-negative pathogenic bacteria significantly up-regulated the EGFP expression in transfected cells, and the cell-based biosensor could be flexibly used to differentiate Gram-negative pathogenic bacteria.

The cells were also infected with different Multiplicity of Infection (MOI) values of *E.coli* O111:B4 (G^-). As shown in Figure S4, intracellular fluorescence intensity gradually increased upon additional increase of the MOI value with a good linearity in the range of 5-200, along with the equation $y = 0.014 x + 6.998$, and $r = 0.925$.

LPS Sensing in Real Samples. To assess the utility of the biosensor for recognizing LPS from the fruit juices, we used apple, mango and orange juice to test the performance of this biosensor, different concentrations of LPS were spiked after pre-treatment. The intracellular fluorescence intensity varied with different

concentrations of LPS added into juices (Figure S5A), the detected contents of LPS calculated from the standard curve of the fluorescence intensity (Figure S5B), and the quantitative recovery of 95.00-106.67% was obtained (Table S1). The results demonstrated that the new method described in this study can be applied for the efficient detection of LPS in real samples.

In summary, the living cell-based fluorescence biosensor provides a simple and effective new method to evaluate LPS toxicity with the advantages of visual detection and noninvasive execution. The cell-based biosensor, which exploits the signaling pathway, can be used to observe the morphological and fluorescence intensity changes of cells treated by LPS continuously and dynamically. This LPS assay was also used to analyze drink samples and were found to have an excellent recovery percentage of about 95.00-106.67%. This novel technique has promising future applications in the in-situ, high throughput, early detection and warning of bacterial contamination.

■ ASSOCIATED CONTENT

Supporting Information

Evaluation on transfection efficiency (Figure S1), Evaluation on cell viability (Figure S2 and Figure S3), Response of cell-based sensor exposure to LPS from *E.coli* O111:B4 (Figure S4), Detection of LPS in real samples using the cell based-green fluorescent biosensor (Figure S5), Concentration of LPS in pre-treated fruit juices (Table S1)

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Author contributions

Xiulan Sun and Jiadi Sun designed the project and contributed to the experimental design. Zhu Pei did some signal pathway investigation. Xiumei Wang and Jian Ji helped do several cell experiments. Jean de Dieu Habimana helped grow bacteria. Jingdong Shao provided the biological samples, such as *E.coli* O111:B4 (G^-) and *S.aureus* (G^+) bacteria. Hongtao Lei gave some suggestions on the plasmid transfection. Yinzhi Zhang took part in the guidance of fluorescent image analysis.

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Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Sattar, A. A.; Jackson, S. K.; Bradley, G. The potential of lipopolysaccharide as a real-time biomarker of bacterial contamination in marine bathing water. *J. Water Health* **2014,** *12*, 105-112.

(2) Shi, J.; Zhao, Y.; Wang, Y.; Gao, W.; Ding, J.; Li, P.; Hu, L.; Shao, F. Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature* **2014,** *514*, 187-92.

(3) Copeland, S.; Warren, H. S.; Lowry, S. F.; Calvano, S. E.; Remick, D. Acute Inflammatory Response to Endotoxin in Mice and Humans. *Clin. Diagn. Lab. Immunol.* **2005,** *12*, 60.

(4) Kakinuma, A.; Asano, T.; Torii, H.; Sugino, Y. Gelation of Limulus amoebocyte lysate by an antitumor (1 leads to 3)-beta-D-glucan. *Biochem. Biophys. Res. Commun.* **1981,** *101*, 434.

(5) Cooper, J. F.; Levin, J.; Jr, W. H. Quantitative comparison of in vitro and in vivo methods for the detection of endotoxin. *J. Lab. Clin. Med.* **1971,** *78*, 138.

(6) Udeni Alwis, K.; Milton, D. K. Recombinant factor C assay for measuring endotoxin in house dust: Comparison with LAL, and (1→3)- β- D- glucans. *Am. J. Ind. Med.* **2006**, *49,* 296–300.

(7) Yao, M.S.; Zhang, H. L.; Dong, S. F.; Zhen, S. Q.; Chen, X. D. Comparison of electrostatic collection and liquid impinging methods when collecting airborne house dust allergens, endotoxin and (1, 3)-β-d-glucans. *J. Aerosol Sci.* **2009**, *40,* 492–502.

(8) Beutler, B.; Rietschel, E. T. Innate immune sensing and its roots: the story of endotoxin. *Nat. Rev. Immunol.* **2003**, *3*, 169

(9) Zhu, Z. X.; Cong, W. T.; Ni, M. W.; Wang, X.; Ma, W. D.; Ye, W. J.; Jin, L. T.; Li, X. K. An improved silver stain for the visualization of lipopolysaccharides on polyacrylamide gels. *Electrophoresis* **2010,** 33, 1220-1223.

(10) 2Nd, J. G.; Jiang, H. Detection of lipopolysaccharide and lipid a employing a spermine-pyrene conjugate. *Bioconjugate Chem.* **2005,** *16*, 621-625.

(11) Zeng, L.; Wu, J.; Dai, Q.; Liu, W.; Wang, P.; Lee, C. S. Sensing of bacterial endotoxin in aqueous solution by supramolecular assembly of pyrene derivative. *Org. Lett.* **2010,** *12*, 4014-7.

(12) Rangin, M.; Basu, A. Lipopolysaccharide identification with functionalized polydiacetylene liposome sensors. *J. Am. Chem. Soc.* **2004,** *126*, 5038.

(13) Rustici, A.; Velucchi, M.; Faggioni, R.; Sironi, M.; Ghezzi, P.; Quataert, S.; Green, B.; Porro, M. Molecular mapping and detoxification of the lipid A binding site by synthetic peptides. *Science* **1993,** *259*, 361.

(14) Little, R. G.; Kelner, D. N.; Lim, E.; Burke, D. J.; Conlon, P. J. Functional domains of recombinant bactericidal/permeability increasing protein (rBPI23). *J. Biol. Chem.* **1994,** *269*, 1865-72.

(15) Bousse, L. Whole cell biosensors. *Sens. Actuators, B* **1996,** *34*, 483-486.

(16) Rider, T. H.; Petrovick, M. S.; Nargi, F. E.; Harper, J. D.; Schwoebel, E. D.; Mathews, R. H.; Blanchard, D. J.; Bortolin, L. T.; Young, A. M.; Chen, J. A B cell-based sensor for rapid identification of pathogens. *Science* **2003,** *301*, 213.

(17) Zou, L.; Wu, C. S.; Wang, Q.; Zhou, J.; Su, K. Q.; Li, H. B.; Hu, N.; Wang, P. An improved sensitive assay for the detection of PSP toxins with neuroblastoma cell-based impedance biosensor. *Biosens. Bioelectron.* **2015**, *67*, 458–464.

(18) Giaever, I.; Keese, C. R. A morphological biosensor for mammalian cells. *Nature* **1993,** *366*, 591.

(19) Fendyur, A.; Varma, S.; Lo, C. T.; Voldman, J. Cell-based biosensor to report DNA damage in micro- and nanosystems. *Anal. Chem.* **2014,** *86*, 7598-605.

(20) Geng, D.; Zhang, Z.; Guo, H. Development of a Fish Cell Biosensor System for Genotoxicity

441 Detection Based on DNA Damage-Induced Trans-Activation of p21 Gene Expression. *Biosensors* **2012,**
442 *2*, 318-40.
443 (21) Zager, V.; Cemazar, M.; Hreljac, I.; Lah, T. T.; Sersa, G.; Filipic, M. Development of human cell
444 biosensor system for genotoxicity detection based on DNA damage-induced gene expression. *Radiol.*
445 *Oncol.* **2010,** *44*, 42-51.
446 (22) Jiang, H.; Jiang, D.; Shao, J.; Sun, X.; Wang, J. High-throughput living cell-based optical
447 biosensor for detection of bacterial lipopolysaccharide (LPS) using a red fluorescent protein reporter
448 system. *Sci Rep* **2016,** *6*, 36987.
449 (23) K, H.; O, T.; T, K.; H, S.; T, O.; Y, T.; K, T.; S, A. Cutting edge: Toll-like receptor 4
450 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene
451 product. *J. Immunol.* **1999,** *162*, 3749.
452 (24) Beutler, B. Endotoxin, toll-like receptor 4, and the afferent limb of innate immunity. *Curr. Opin.*
453 *Microbiol.* **2000,** *3*, 23.
454 (25) Andreakos, E.; Sacre, S. M.; Smith, C.; Lundberg, A.; Kiriakidis, S.; Stonehouse, T.; Monaco, C.;
455 Feldmann, M.; Foxwell, B. M. Distinct pathways of LPS-induced NF-kappa B activation and cytokine
456 production in human myeloid and nonmyeloid cells defined by selective utilization of MyD88 and
457 Mal/TIRAP. *Blood* **2004,** *103*, 2229.
458 (26) Jura, J.; Skalniak, L.; Koj, A. Monocyte chemotactic protein-1-induced protein-1 (MCPIP1) is a
459 novel multifunctional modulator of inflammatory reactions. *Biochim. Biophys. Acta* **2012,** *1823*, 1905.
460 (27) Lu, Y. C.; Yeh, W. C.; Ohashi, P. S. LPS/TLR4 signal transduction pathway. *Cytokine* **2008,** *42*,
461 145-151.
462 (28) Karren, P.; Danuta, M.; Piotr, T.; Irena, H.; Paulina, W.; Aneta, K.; Hanna, R.; Sharrocks, A. D.;
463 Jolanta, J. Transcription factors Elk-1 and SRF are engaged in IL1-dependent regulation of ZC3H12A
464 expression. *BMC Mol. Biol.* **2010,** *11*, 14.
465 (29) Kasza, A. Signal-dependent Elk-1 target genes involved in transcript processing and cell
466 migration. *Biochim. Biophys. Acta* **2013,** *1829*, 1026-1033.
467 (30) Ong, K. G.; Leland, J. M.; Zeng, K. F.; Barrett, G.; Zourob, M.; Grime, C. A. A rapid
468 highly-sensitive endotoxin detection system. . *Biosens. Bioelectron.* **2006,** *21,* 2270-2274.
469 (31) Artur, R.; Ewa, B.; Sabina, G.; Krzysztof, W.; Andrzej, G.; Slawomir, G. A broadband capacitive
470 sensing method for label-free bacterial LPS detection. *Biosens. Bioelectron.* **2016,** *75*, 328.
471 (32) Bouafsoun, A.; Othmane, A.; Jaffrézic-Renault, N.; Kerkeni, A.; Thoumire, O.; Prigent, A. F.;
472 Ponsonnet, L. Impedance endothelial cell biosensor for lipopolysaccharide detection. *Mater. Sci.*
473 *Eng.,C* **2008,** *28*, 653-661.
474 (33) Zuzuarregui, A.; Arana, S.; Pérez-Lorenzo, E.; Sánchez-Gómez, S.; Tejada, G. M. D.; Mujika, M.
475 Novel fully-integrated biosensor for endotoxin detection via polymyxin B immobilization onto gold
476 electrodes. *J. Sens. Sens. Sys.* **2013,** *2*, 1-8.
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FIGURE CAPTIONS

Scheme 1 A schematic illustration of the working principle of a 293/hTLR4A-MD2-CD14 cell sensor.

Figure 1. Construction of the *pGL3-ZC3H12A-EGFP* plasmid and electrophoretic pattern. (A) Map of the *pGL3-EGFP* plasmid and the insert site of *ZC3H12A* promoter. (B) a: DNA marker, b: the *pGL3-EGFP* vector doubly digested with *KpnI* and *EcoRI*. c: *pGL3-ZC3H12A-EGFP* plasmid doubly digested with *KpnI* and *EcoRI*.

Figure 2. Expression of MCPIP1 gene and protein in 293/hTLR4A-MD2-CD14 cells incubated with LPS. (A) RT-PCR of MCPIP1 gene transcription level. (B) Western Blot of MCPIP1 protein expression. *P<0.05, there is a significant difference compared with the control.

Figure 3. Fluorescence intensity changes of 293/hTLR4A-MD2-CD14 cells exposed to LPS and the expression of fluorescent protein. (A) Time-dependent images of cell fluorescence intensity responses. LPS was employed to stimulate 293/hTLR4A-MD2-CD14 biosensor cells respectively. (B) Quantification of fluorescence intensity in 293/hTLR4A-MD2-CD14 biosensor cells stimulated with LPS respectively. (C) The expression of fluorescent protein of 293/hTLR4A-MD2-CD14 treated with LPS.

Figure 4. Cytotoxicity evaluation of different concentrations of LPS (0.1, 0.5, 1, 5, 10, 20, 50, 100, 150 ug/mL) by 293/hTLR4A-MD2-CD14 cell-based biosensor and CCK-8 assay. (A) Fluorescence image for 293/hTLR4A-MD2-CD14 cells treated with different doses of LPS respectively, (a) Bright field, (b) Fluorescent field; (B)

504 The curve of intracellular fluorescence intensity versus various concentrations of LPS;
505 (C) Cell viability of 293/hTLR4A-MD2-CD14 cells treated with LPS was estimated
506 by CCK-8 assay. * $P<0.05$, there is a significant difference compared with the control.
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508 to *E.coli* O111:B4 and *S.aureus*. (A) Bright field: (a) cells infected with *E.coli*
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510 marked with yellow box, the red arrow indicated *E.coli* O111:B4; (c) cells infected
511 with *S. aureus*, MOI = 50; (d) the amplifying image of (c) marked with yellow box,
512 the red arrow indicated *S. aureus*. (B) Time-dependent images of cell fluorescence
513 intensity responses. (a) Cells infected with *E.coli*, MOI = 50; (b) cells infected with *S.*
514 *aureus*, MOI = 50. The images were recorded every 4 h. (C) Quantification of
515 fluorescence intensity at different time.
516 **Table 1.** Comparison of several reported methods for LPS detection
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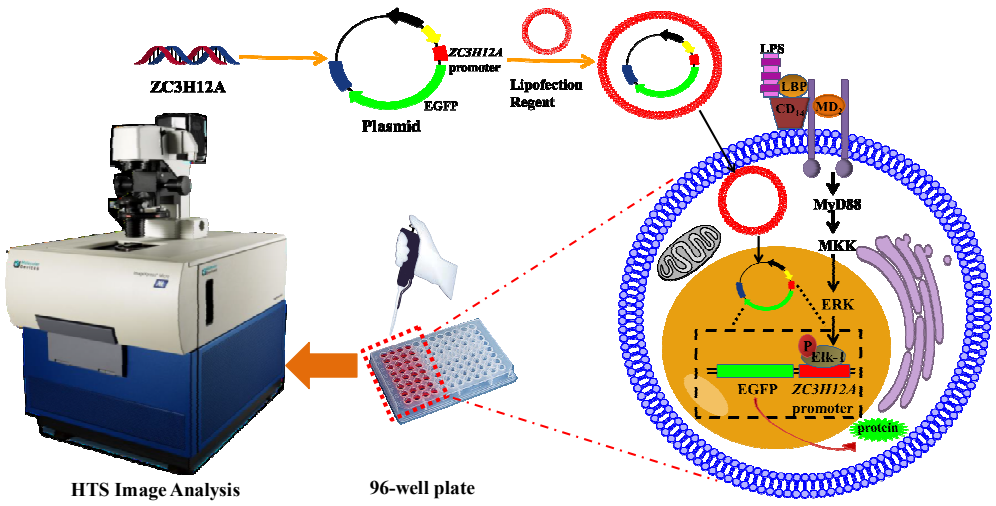

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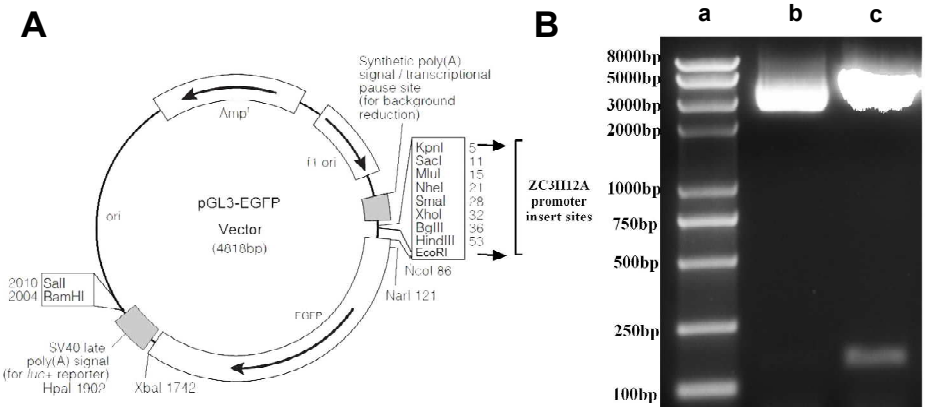
521 293/hTLR4A-MD2-CD14 cell sensor.



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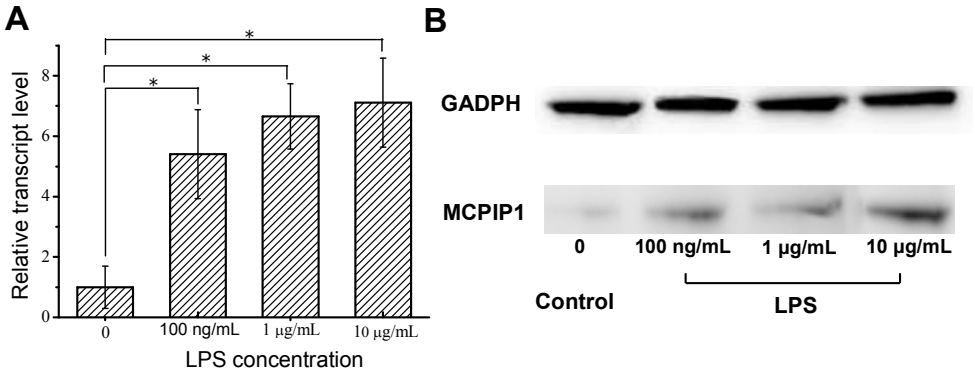

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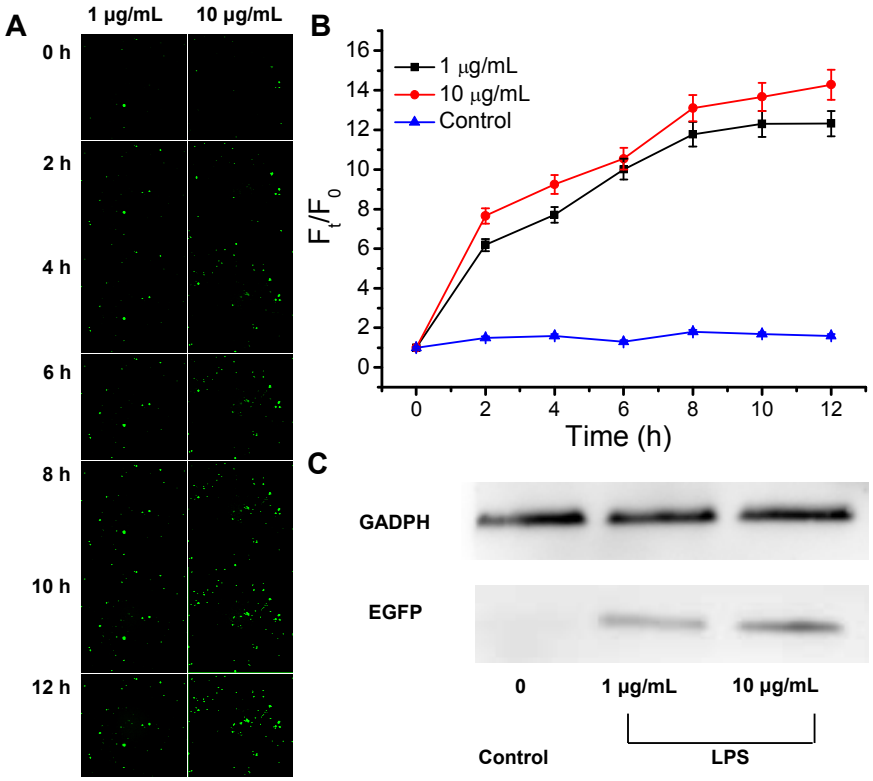

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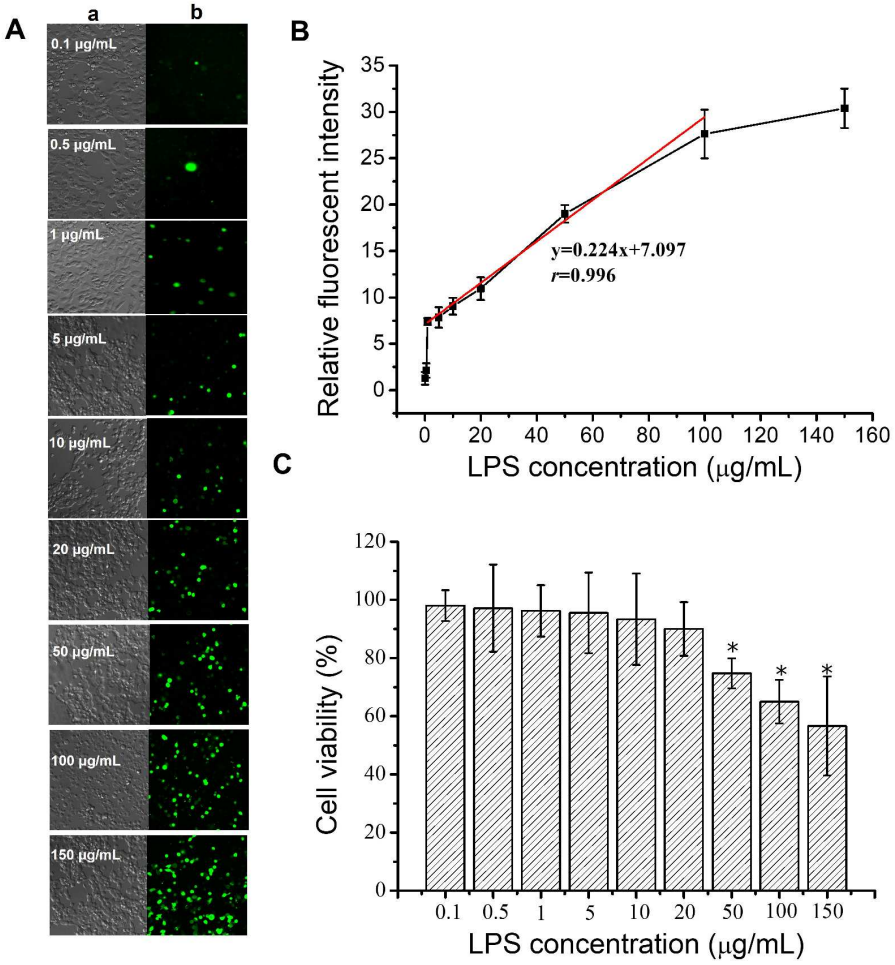

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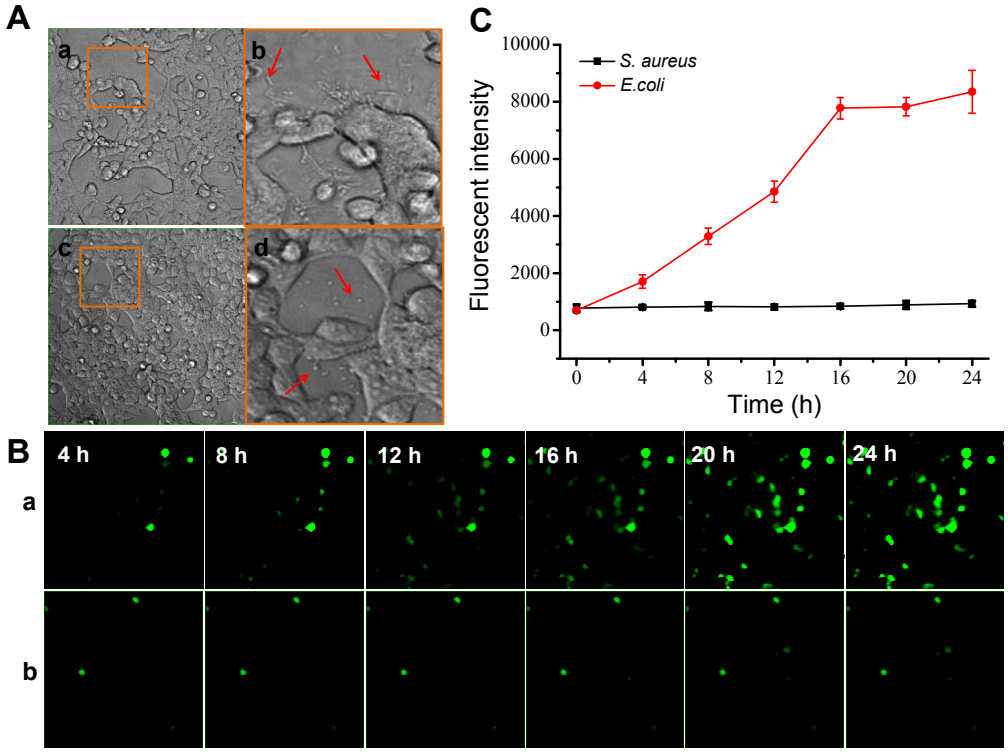

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558 fluorescence intensity at different time.

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562 **Table 1.** Comparison of several reported methods for LPS detection

Recognition element	Detection method	Sensitivity	Reference
Limulus Amoebocyte Lysate	Magnetoelastic sensor	0.0105 EU/mL	30
T4 phage adhesin	Microwave	100 pg/mL	31
Endothelial cells	EIS	0.5 μg/mL	32
Polymyxin B	CV	1 μg/mL	33
293/hTLR4A-MD2-CD14 pGL3-ZC3H12A-EGFP cells	Fluorescent	0.075 μg/mL	Prepared method in the text

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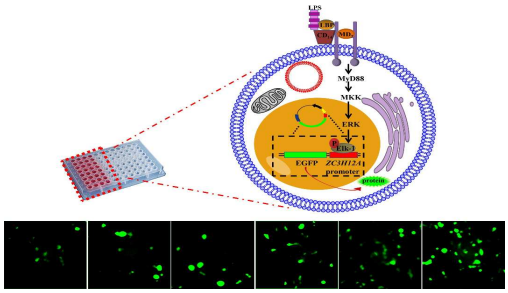
565 **TOC Graph:**



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