SUPPLEMENTARY INFROMATION

Materials and Methods

Materials

RAW264.7 (ATCC TIB-71) cell line, *Shigella sonnei* (*S. sonnei*; ATCC 29930), *Staphylococcus aureus* (*S. aureus*; ATCC 25923), and *Listeria monocytogenes* (*L. monocytogenes*; ATCC 19117) were purchased from America Type Culture Collection (ATCC; Manassas, VA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) with high glucose were obtained from Thermo Fisher Scientific (South Logan, UT). CO_2-independent medium (1×) was from Life Technologies (Carlsbad, CA). Penicillin-streptomycin (50×), casein (from bovine milk), Tween 20, mineral oil (molecular biology grade), and biocytin (> 98%) were from Sigma-Aldrich (St. Louis, MO). Tryptic soy broth (TSB) was from Merck (Darmstadt, Germany). 96-well culture plates (flat bottom) and 100 mm Ø culture dishes were from Corning (Tewksbury, MA). The 'Dip and Read' optical fiber sensor (coated with streptavidin) and Kinetics Buffer (10×) were from ForteBio (San Francisco, CA). Mouse TNF-α cytokine ELISA kit, standard mouse TNF-α recombinant protein, rabbit antibody specific to anti-mouse TNF-α (K0113001), and biotinylated rabbit antibody to anti-mouse TNF-α (K0113002) were from Koma Biotechnology (Seoul, Korea). Other reagents were of analytical grade.

Preparation of Mammalian Cell and Bacterial Samples

Cultivation of mammalian cell. The RAW 264.7 cells, as the sensing element of the biosensor, were maintained and stored according to standard protocols [1]. The cells were suspended in DMEM with 10% FBS and 1% penicillin-streptomycin, and dispensed in 100-mm cell culture dish (10 mL each). They were then cultured in an incubator maintained at 5% CO_2 atmosphere and 37°C until the cells reached 90% confluency (approximately, 1×10^9 cells/mL). The medium was changed every 2 days. For cell stocking, the cells were diluted to 3×10^5 cells/mL with the culture medium containing 5% FBS and 10% DMSO, and snap-frozen in liquid nitrogen and kept until used.

In a bacterial contamination test, the cells were harvested from the 100-mm culture dish (10^9 cells/mL) and diluted to 10^4 cells/mL with 20% FBS-containing CO_2-independent medium without antibiotics (Analysis Medium). The diluted cells were then dispensed into a 96-well culture plate as the sensor container (200 μL each well; see below for details). For stable attachment, the animal cells were cultivated at 5% CO_2 atmosphere and 37°C for 48 h. The used medium was then changed for the bacterial analysis to Analysis Medium containing a titer of bacteria defined in each experiment below.

Preparation of bacterial samples. Three bacterial cell stocks were maintained for the supply of infectious agent: *S. sonnei*, *S. aureus*, and *L. monocytogenes*. They were grown separately in TSB medium shaken at 150 rpm and 37°C for 16 h. The medium was prepared by dissolving 30 g of TSB powder in 1 L of deionized water and then autoclaving for 15 min at 121°C. After cultivation, the bacterial cells were harvested by centrifugation (7000 rpm, 10 min). The supernatants were removed and the bacterial pellets were resuspended in 10 mM phosphate buffer, pH 7.4, containing 140 mM NaCl (PBS). For storage, the bacterial cell stocks (1×10^8 cells/mL; 500 μL each) were prepared in the TSB medium with 50% glycerol, snap-frozen in liquid nitrogen, and kept at -80°C until used. The live bacterium density was determined by colony counting according to a conventional protocol [2].

Analysis of Bacteria based on Animal Cell Stimulation

Detection of secreted TNF-α via conventional ELISA. The animal cells prepared above were infected with *S. sonnei* (1×10^2 CFU/mL) diluted in Analysis Medium and the secreted TNF-α was then measured. After inoculation, the medium was covered with sterilized mineral oil (80 μL) to prevent evaporation during cultivation [3]. The 96-well plate was mounted on the heating block (set to 36°C) of the Octet Red sensor system (ForteBio; San Francisco, CA). The density of the grown bacteria was determined by colony counting every 2-3 h. At the same time, the TNF-α secreted by the stimulated host cells was quantified using a commercial mouse TNF-α ELISA kit. The same experiment was also repeated for comparison except that the incubation in a standard incubator maintained at a 5% CO_2 atmosphere and 37°C. Mineral oil was not used in this case. These experiments were

conducted in triplication.

Bacterial analysis using different animal cell-based biosensor models. Two experimental biosensor models with different medium volumes (200 μL or 50 μL) based on the surface area, on which the animal cells were grown to 50-70% confluency, were constructed using a standard 96-well plate. After allowing cell to attach to the surfaces, the bacterial analysis for *S. sonnei* was initially conducted by adding medium containing a pre-determined titer of the bacterium (desired final titers: order of 10 to 10^5 CFU/mL). The final titer transferred into each sample was determined by colony counting using a traditional method [2]. Each solution also contained the unmodified rabbit antibody specific to mouse TNF-α (1 μg/mL) as the detection binder for signal enhancement. After dipping the label-free immunosensor into the solution, the air-aqueous medium interface was covered with 80 μL of mineral oil to prevent evaporation and then analyzed for the bacterium as described below.

Immunosensor preparation and label-free biosensing protocol. Using the label-free sensor system, Octet Red, a biosensor sensitive to TNF-α was constructed by combining the biotinylated rabbit antibody (1 μg/mL) immobilized on the optical fiber sensor via a streptavidin-biotin linkage. To prepare the immunosensor, the optical fiber sensor was dipped into a well containing Kinetics Buffer diluted 10 times with PBS for 35 min. The sensor was transferred to deionized water with biocytin (1 mg/mL) and reacted for 15 min, and the residual surfaces were blocked in 0.5% casein in PBS (Casein-PBS) for the same period. The prepared sensor was finally equilibrated in Analysis Medium for 20 min. Each step was conducted in a microwell holding a volume of 200 μL maintained at 30°C. The immunosensor was then used to analyze samples containing a defined titer of bacteria in real time up to 16 h. The kinetic data were collected and recorded using the Data analysis 6.3 program provided by the manufacturer, and further processed for corrections as described in detail below.

Performance correlation between label-free immunosensing and ELISA. The performance of label-free immunosensor for TNF-α was validated by comparing the cellular response time with that measured via sandwich ELISA. The mammalian cells coated in 96-well culture plate (200 μL volume) as described were infected with *S. sonnei* (order of 5×10^3

CFU/mL) diluted in Analysis Medium. After the liquid surfaces were covered with mineral oil (80 μL), the plate was placed within the Octet Red system and then incubated at 36°C without agitation. The label-free biosensing for TNF-α was conducted according to the protocol described above, and the same experiment was simultaneously carried out without infection as control. The measurement for the cytokine in the medium was also accomplished using the commercial-version mouse TNF-α ELISA kit under the same conditions. To this end, the medium (150 μL) was hourly harvested from each well of the manifold under cultivation for total 12 h, and stored at 4°C until analyzed. The experiments were accomplished in duplicated manner. The mean of the quantified data for the cytokine was plotted against culture time together with the label-free sensing data in the same graph. The initial response time was determined from each response curve as described in detail below.

Medium pH measurement. The pH change, which may occur during sample analysis, was monitored using a hand-held pH meter (pH Spear, Vernon Hills, IL) every hour. To this end, *S. sonnei* was diluted in Analysis Medium to different titers (10^1 to 10^5 CFU/mL), and each standard solution was dispensed in microtiter plates (total, 24 wells). After covering the liquid surfaces with mineral oil (80 μL), the plate was mounted on the Octet Red heating block and incubated at 36°C without agitation. At predetermined times, the pH probe, calibrated prior to use, was inserted into the culture medium for 1 min and then recorded at steady state. The experiment was carried out in triplicate manner, which was continued for 12 h after inoculation. The mean was plotted against the cultivation time, and the variation for each measurement was also indicated.

Manipulation of Real-time Monitoring Data from the Animal Cell-based Biosensor

Reconstruction of time-response curve via baseline correction. Problems with baseline drift in the real-time monitoring of secretory TNF-α were settled by subtracting the background signal from that of the negative control and then by correcting the baseline reflecting the initial steady state. As the cytokine is also present under 'normal' conditions (Esposito et al. 2002), the basal-level signal measured without infection was first subtracted from the signal detected during sample analysis. A default baseline was then found in the

initial time period between 1 and 2 h, during which the mean slope of drift in the corrected sensorgram was not changed significantly, i.e., the steady state was maintained. To this end, the signal intensities fluctuating for the time period were linearly regressed using the least squares method [4], and the slope was obtained as drift for the time span. The default baseline was then corrected by multiplying the time by the slope, and by subtracting the product from the signal measured against time. Because the reconstructed time-response curve showed some variation against time, a moving average method [5] was used to calculate to a series of averages for different subsets (every 100 measurements) of the full data set [6]. Such corrected data were used to draw the final time-response curve, which was conducted throughout the study.

Determination of response time and standard curve of the biosensor. To measure the earliest response time to bacterial stimulation, an algorithm of high-threshold setting, widely used in real-time PCR analysis [7], was used. The earliest response time was determined as the time at which the signal exceeded the high-threshold value. This value was calculated by multiplying the standard deviation in the range of the default baseline by three. The response curve was further obtained by averaging the calculated response times for triplicate measurements and plotting the mean against the bacterial titer initially inoculated in the sample. The *x*,*y*-axis standard deviation [8] was also indicated in the same graph. The data in the plot was regressed linearly and the correlation coefficient (R^2) between the experimental values and estimates was calculated. The bacterial titer was determined by colony counting.

Statistical analysis. To test significance between data obtained under different conditions, statistical analysis was performed using one-way analysis of variance followed by Tukey's *post hoc* multiple range tests [9]. The SPSS software was used (SPSS Inc., Chicago, IL). A value of $P < 0.05$ was considered to indicate statistical significance.

Characterization of the Sensor Performance with Real Sample

A milk product (200 mL capacity, Maeil Dairy; Seoul, Korea) was purchased in the market to use it as real sample matrix. The milk was centrifuged at a centrifugal force of 2450 times of the gravity for 5 min and the lipid layer on the top was removed. The fat-free fraction was mixed with Analysis Medium in the identical volume. This mixture was immediately frozen

at -20°C for storage, and melted at 4°C immediately prior to use. To prepare standard samples, three different bacteria, *S. aureus*, *L. monocytogenes*, and *S. sonnei*, were recovered from each frozen stock and separately grown in a TSB medium according to the procedure mentioned above. After harvest, each bacterium was diluted with the real sample mixture to standard concentrations mentioned earlier in the bacterial analysis. The standard samples were separately added in the small-volume system (0 rpm, 50 μL). The rest of the procedure was remained the same as that described for the biosensor. For each bacterial analyte, the response time was plotted with respect to the initial titer and the x-y standard deviation was also expressed. After linear regression for the data, the correlation coefficient was determined. These experiments were conducted in triplication. To compare the standard curves for the different analytes, the statistic analysis was further carried out as stated earlier. The identical experiment was repeated except for the use of bare Analysis Medium as the reference sample matrix.

Results

Real-time Infection Monitoring using the In vitro Innate Immune System

Secretory cytokine detection. The detection capability of the label-free immunosensor was tested as to whether it could detect TNF-α secreted from the animal cells on infection. The RAW264.7 cells were initially grown on the culture container surfaces to about 50% confluency. Such density may protect the cell-to-cell contact signaling from inhibition which may occur under crowding conditions [10]. The cell confluency could also provide an escape space for the bacterium (e.g., *S. sonnei*; [11]), infected particularly in a low titer (e.g., about 100 CFU/mL; [12]), from the macrophage phagocytosis [13]. Upon survival, the bacteria will effectively stimulate the host cells.

In this experiment, *S. sonnei* (234±34 CFU/mL) was used to infect the animal cells placed within the sensor system (maintained at 36°C), Octet Red, and the microorganism and cytokine produced were determined quantitatively in duplicate fashion by sampling at predetermined cultivation times (Fig. S1, a). The bacteria and mouse TNF-α concentrations were measured using the traditional colony assay technique and a commercial ELISA kit, respectively. After inoculation, the bacterial growth initially had a lag phase for about 2 h and, thereafter, the bacterium expanded in a logarithmic pattern (the red solid line). The doubling time was estimated to be about 30 min, consistent with the time reported for *S. sonnei* grown in bacterial medium [14]. The result also matched the growth curve obtained by cultivating it in a standard incubator (the blue solid line). On the other hand, the TNF-α concentration (dotted lines) began to increase significantly at 6 h after infection regardless of the incubation means, reaching a maximum at 10 h, and decreasing thereafter. At the earliest stage of increase, the cytokine concentration in the culture container was approximately 1.28±0.11 ng/mL, consistent with a result reported previously [15].

Detection of such secretory TNF-α level using the label-free technique was essential to support the analytical concept although we further described optimal conditions for the novel sensor in the main text. We, thus, initially examined the feasibility of detection by adding a defined amount of cytokine into an identical culture container except for the absence of

animal cells. The signal was then monitored in the sandwich complex-based manner on the label-free immunosensor against time (Fig. S1, b). The binding signals obtained at different doses of TNF-α revealed that the minimum detection limit of the sensor was about < 1.25 ng/mL. This result supported the proposed concept for label-free biosensing of the cytokine secreted from the host cells on infection.

Definition of Experimental Biosensor Models

To optimize performance, the biosensor configuration using a standard 96-well microtiter plate was varied to hold different medium volumes (Fig. S2). As the standard system usually contained a large culture volume (i.e., 200 μL) relative to the surface area immobilized with the host cells, the secretory cytokine accumulation could be delayed until a certain level detectable by the sensor is reached. To alleviate such potential delay, we adopted the standard well size with a volume reduced to a quarter of the original (50 μL). The ratio of volume to surface area was 6.25 and 1.56 μL per mm^2 for the two models, respectively (refer to Fig. S2). The optical fiber sensor placed close to the bottom of the container in each model (indicated in the figure), which, however, was limited by the preset arrangement of the commercial system. The liquid-air interfacial area was covered with mineral oil to prevent the culture medium from evaporation, which gradually decreased the volume otherwise.

Reconstruction of Real-time Response Curves

Baseline correction of time-response curve. As the time-response sensorgram obtained with each standard sample was seen to drift against the monitoring time, the chart was reconstructed via baseline corrections (Fig. S3). Each curve was first normalized by subtracting the basal-level signals measured under the normal conditions without infection (Fig. S3, 1→2). We then determined the default baseline [6] for the initial time period at steady state, particularly between 1 and 2 h (Fig. S3, 2). After linear regression for the period, the slope was then corrected by multiplying the time by the slope and subtracted it from the normalized signal (Fig. S3, 3). Such a reconstructed sensorgram was used to calculate moving averages (Fig. S3, 4) [16], plotted against the incubation time, to obtain a trend curve (Fig. S3, 5). The trend curves obtained with different numbers of bacterial samples were

graphed in overlaid pattern for each condition regarding medium volume and stirring rate (Fig. S4). We then determined the earliest response time for the sensor signal exceeding the threshold value (Fig. S3, 6; see main text for results) and used the parameters representing the sensor performance.

Correlation between the Analytical Performances: Label-free Immunosensing vs. ELISA

To validate the label-free immunosensing for TNF-α, the cellular response to the stimulation was compared with that measured via sandwich ELISA. The bacterium, *S. sonnei* (5×10^3 CFU/mL), was used to infect the RAW264.7 cells and the secretory cytokine was then measured employing the real-time monitoring technique during the cultivation (0 rpm, 200 μL) for 12 h. The cytokine was also measured using the commercial-version mouse TNF-α ELISA kit under the same conditions by harvesting the supernatant from each container of the manifold under cultivation every hour. The whole time responses were produced in duplication and plotted in the same graph (Fig. S5). The results showed about the same response pattern, and the initial response times separately determined from each curve were well matched (approximately, 6 h). However, the quantitative data between the two measurements at an identical time revealed some differences.

Monitoring of Chemical Environmental Change of the Biosensor

Bacterial growth rates. The growth rates of *S. sonnei* in the biosensor model containing a large or small medium volume were tested according to the initially inoculated concentration in the order of 10 to 10^3 CFU/mL of the bacteria. After inoculation, the bacterial titers were measured every 2 h using a conventional colony counting method [2] and plotted against the culture time (Fig. S6). Comparison of the growth curves indicated that a high inoculum level shortened the initial lag phase and accordingly made the growth enter the exponential phase earlier regardless of the medium volume in the biosensor.

Medium acidity. To check the chemical environmental change of the medium, the mean pH change in the 200-μL volume model was monitored in parallel with the sensor response to

TNF-α (Fig. S7). The pH and cytokine concentration were measured using a commercial pH meter and the label-free Octet Red sensor system, respectively. For sample including a low titer of bacterium (e.g., ~10^1 CFU/mL), the initial pH, 7.4, was kept constant during the monitoring period. The sensor signal change was also not significant and barely increased at about 12 h after inoculation. As the inoculum density was increased, the medium pH and sensor signal began to profoundly decrease and increase, respectively, at almost the same time. Further study with a larger density showed that the time for such changes was inversely proportional to the bacterial density initially inoculated. These trends were identical to those shown for the 50-μL volume model described in the main text.

References

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Figure Legends

Fig. S1 Production of TNF-α, a proinflammatory mediator secreted from a murine macrophage cell line, RAW264.7, with bacterial stimulation using *S. sonnei* (a) and cytokine detection on a label-free sensor system, Octet Red (b). After inoculation at a density of 234±34 CFU/mL, the concentrations of bacterium and TNF-α produced from the host cells were measured at predetermined times using the colony assay and ELISA, respectively (a). These experiments were conducted in triplication. Both analytes began to increase significantly after 6 h, at which the TNF-α level was about 1.28±0.11 ng/mL. This level or higher could be detected on the label-free sensor, as shown in the dose response range for the cytokine (b).

Fig. S2 Different experimental models of animal cell-based biosensing system for bacterial contamination via the innate immune response. The 96-microwell plate was used as a culture container with a 200-μL volume. For a reduced-volume model, we maintained the same well with a smaller volume (50 μL). Each surface area and volume used for the cell cultivation was indicated, and the sensor position adoptable for the Octet Red system was also specified by the distance from the bottom of container. The surfaces of the culture medium were covered with mineral oil to maintain the volume constant.

Fig. S3 Reconstruction of time-response sensorgram via baseline corrections to eventually determine the response time. Each raw sensorgram output (1) from the sensor system, Octet Red, was first normalized by subtraction of the basal-level signals measured under normal conditions without infection (1→2). The default baseline shown for the initial time period at the steady state, particularly between 1 and 2 h, was then determined (2). After linear regression for the period, the slope was then corrected by multiplying the time by the slope and subtracted it from the normalized signal (3). Such a reconstructed sensorgram was used to calculate moving averages, which were then plotted against the incubation time (curve in red color; (4)). The curve was reshaped by graphing the average as the dependent variable to show the trend curve (5). Finally, the earliest response time was determined for the sensorgram exceeding the threshold value (3× the standard deviation of the background; (6)).

Fig. S4 Reconstructed, typical real-time dose responses of the cell-based biosensor under different conditions of medium volume and stirring rate. The RAW264.7 cells were stably cultured on the container surfaces, and the optical fiber sensor with the immobilized capture antibody to TNF-α on the tip was immersed in the culture medium. The detection antibody to the cytokine was also contained in the medium. After adding the standard sample of the bacterium, *S. sonnei*, the cytokine secretion was monitored continuously. The time-response curves were corrected and subsequently used to determine the earliest response time for the sensor signal increase as described in Fig. S3. The same experiment was carried out three times.

Fig. S5 Comparison of the performances obtained using the label-free sensor and ELISA. After the bacterial infection for the cells, TNF-α produced in the medium was monitored either using the label-free sensor during the cell cultivation (0 rpm, 200 μL) for 12 h or via sandwich ELISA for the cytokine. The initial response times separately determined were nearly identical, approximately 6 h. Such experiments were carried out in duplicate manner.

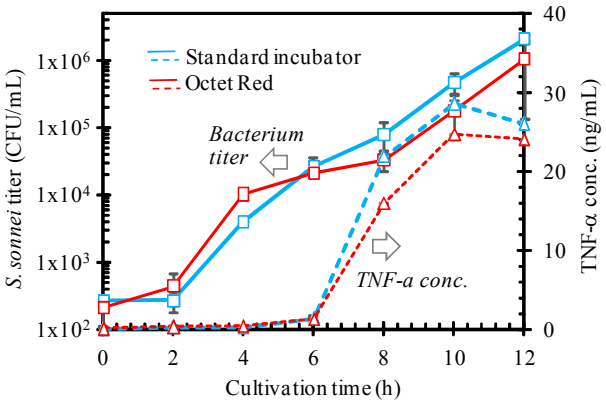
Fig. S6 Effect of the initial inoculum concentration on the growth of *S. sonnei*. The bacterial growth in the biosensor holding a large (a) or small volume (b) was measured every 2 h using a colony counting technique. For the both volume models, the bacteria began to expand after a time lag and the time entering into the log phase was inversely proportional to the initial inoculum concentrations.

Fig. S7 Monitoring of the mean pH and secretory TNF-α for the 200-μL model without agitation under different inoculum densities. The pH was measured discretely using a commercial pH meter while the cytokine was monitored continuously in real time using the Octet Red sensor system. The experiment was carried out in triplicate manner. When the inoculum density was low (a), no significant change in either indicator occurred. As the density was increased, $> 10^3$ CFU/mL (b), the pH was decreased from about 4 h after inoculation while the sensor signal was increased at almost the same time. At a 100-fold further increased density (c), the chemical concentration changes took place in a more profound manner than those occurring at lower densities.

Figures

Fig. S1

(a) Detection of TNF-α secreted from the RAW264.7 cells stimulated with bacterium



(b) Detection of TNF-α using label-free sensor

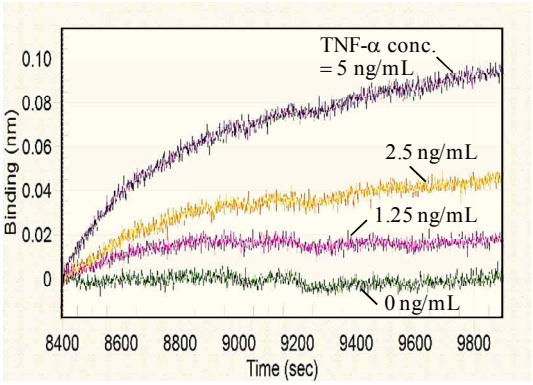
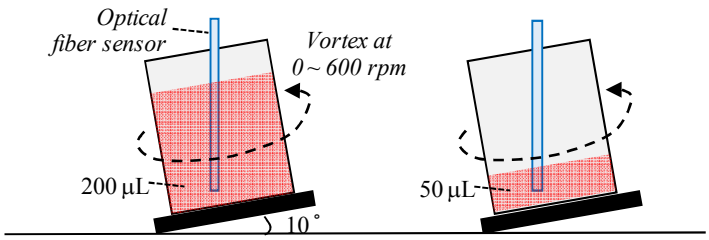


Fig. S2



Culture volume (Oil volume)	200 µL (80 µL)	50 µL (80 µL)
Bottom surface area for cell immobilization	32 mm^2	32 mm^2
Ratio of volume to bottom area	6.25 µL per mm^2	1.56 µL per mm^2
Mean sensor distance from the bottom	0.25 mm	0.25 mm

Fig. S3

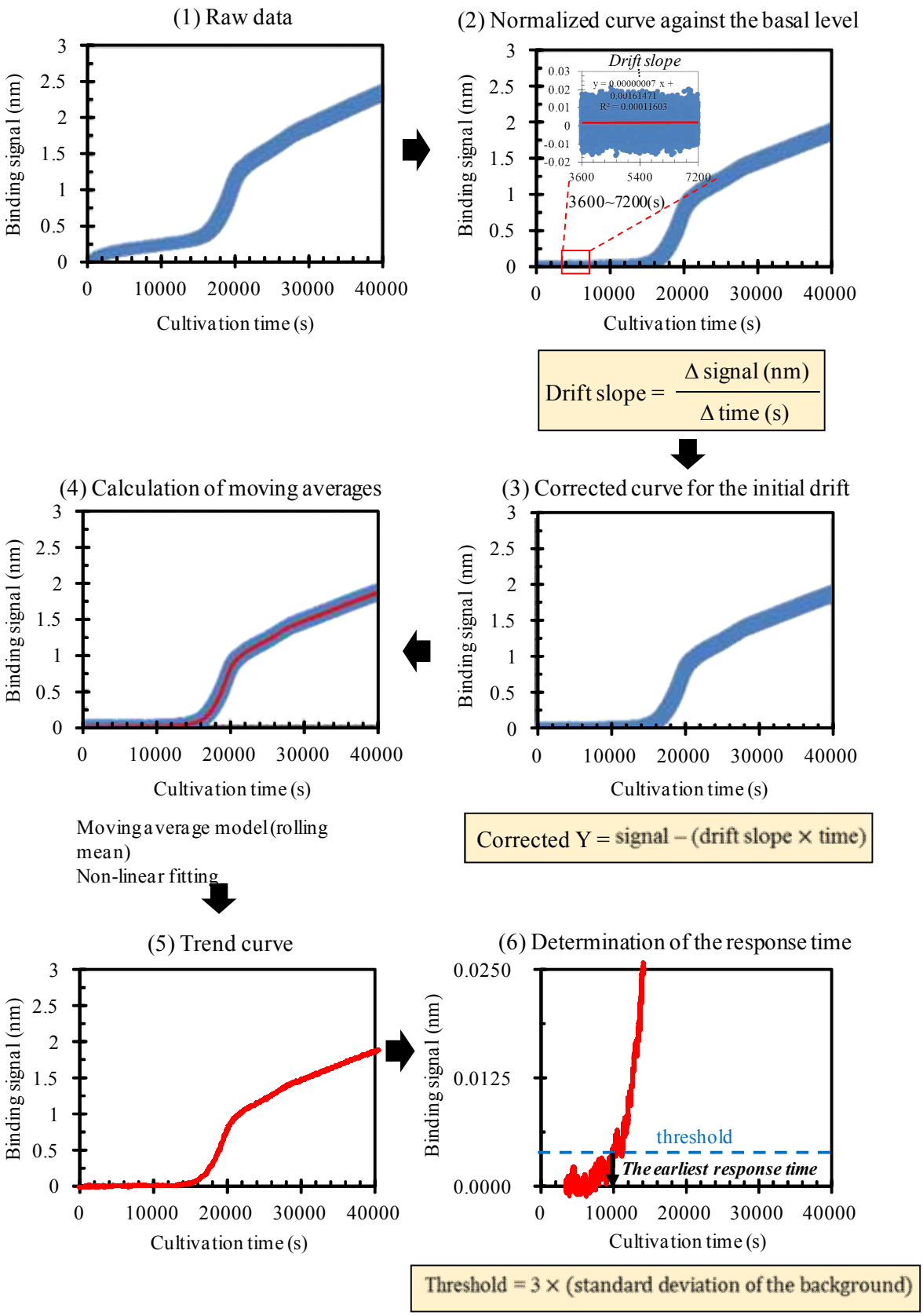

(1) Raw data
Binding signal (nm)
Cultivation time (s)
(2) Normalized curve against the basal level
Drift slope
y = 0.00000007 x + 0.00161471
R² = 0.00011603
3600~7200(s)
Drift slope = Δ signal (nm) / Δ time (s)
(3) Corrected curve for the initial drift
Corrected Y = signal – (drift slope × time)
(4) Calculation of moving averages
Moving average model (rolling mean)
Non-linear fitting
(5) Trend curve
(6) Determination of the response time
threshold
The earliest response time
Threshold = 3 × (standard deviation of the background)

Fig. S4

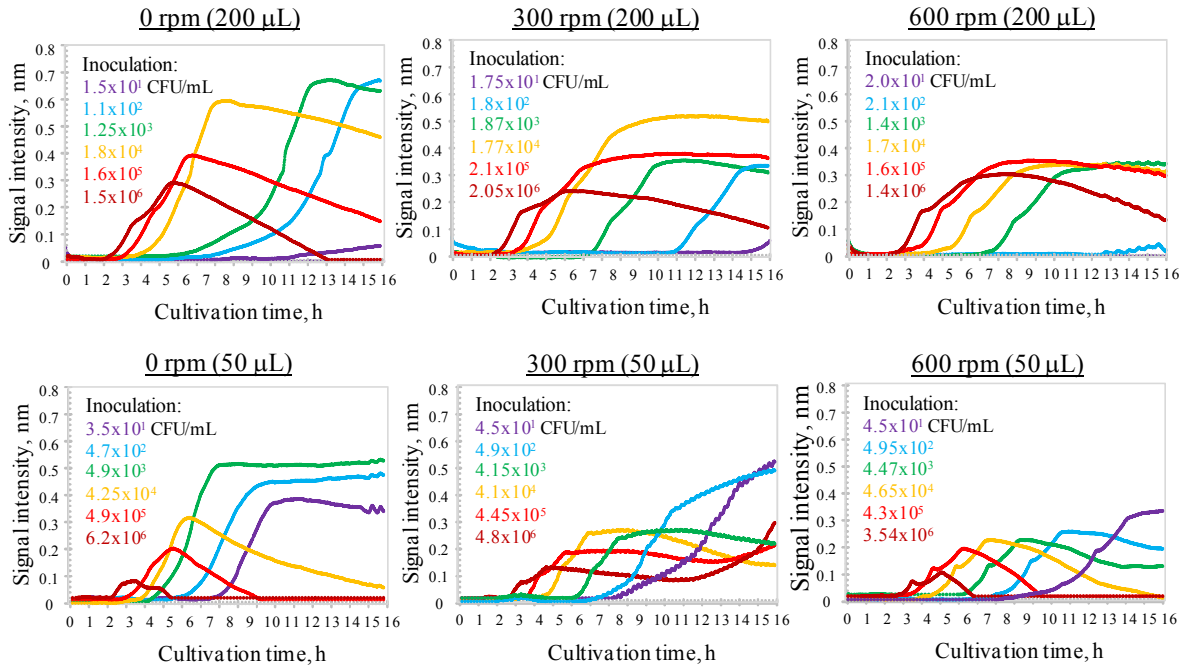

0 rpm (200 µL)
300 rpm (200 µL)
600 rpm (200 µL)
0 rpm (50 µL)
300 rpm (50 µL)
600 rpm (50 µL)
Signal intensity, nm
Cultivation time, h
Inoculation:
1.5x10¹ CFU/mL
1.1x10²
1.25x10³
1.8x10⁴
1.6x10⁵
1.5x10⁶
1.75x10¹ CFU/mL
1.8x10²
1.87x10³
1.77x10⁴
2.1x10⁵
2.05x10⁶
2.0x10¹ CFU/mL
2.1x10²
1.4x10³
1.7x10⁴
1.6x10⁵
1.4x10⁶
3.5x10¹ CFU/mL
4.7x10²
4.9x10³
4.25x10⁴
4.9x10⁵
6.2x10⁶
4.5x10¹ CFU/mL
4.9x10²
4.15x10³
4.1x10⁴
4.45x10⁵
4.8x10⁶
4.5x10¹ CFU/mL
4.95x10²
4.47x10³
4.65x10⁴
4.3x10⁵
3.54x10⁶

Fig. S5

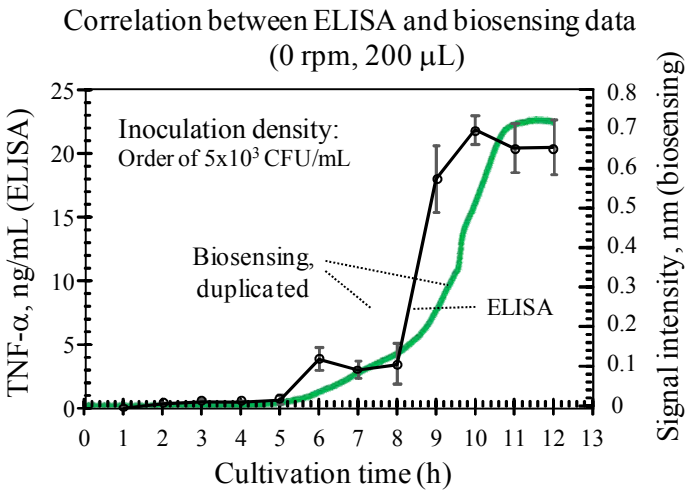

Correlation between ELISA and biosensing data
(0 rpm, 200 µL)
Inoculation density:
Order of 5x10³ CFU/mL
Biosensing, duplicated
ELISA
TNF-α, ng/mL (ELISA)
Signal intensity, nm (biosensing)
Cultivation time (h)

Fig. S6

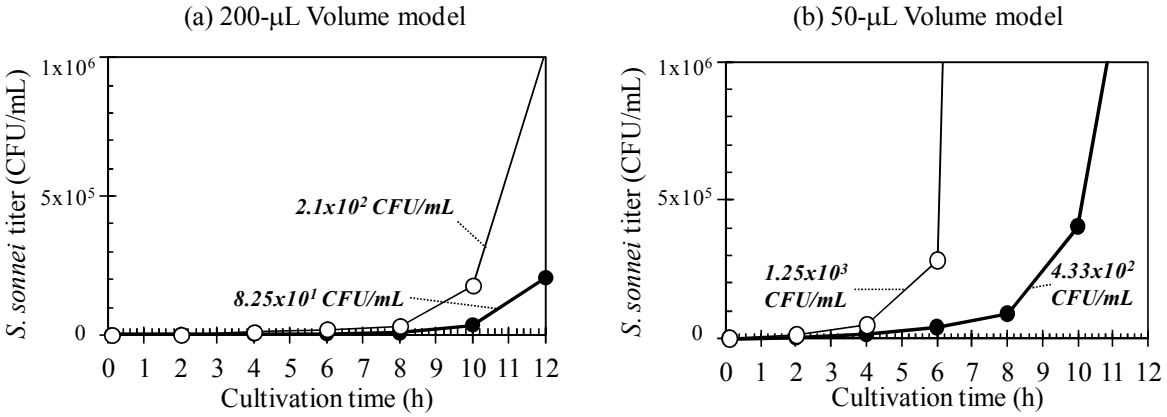
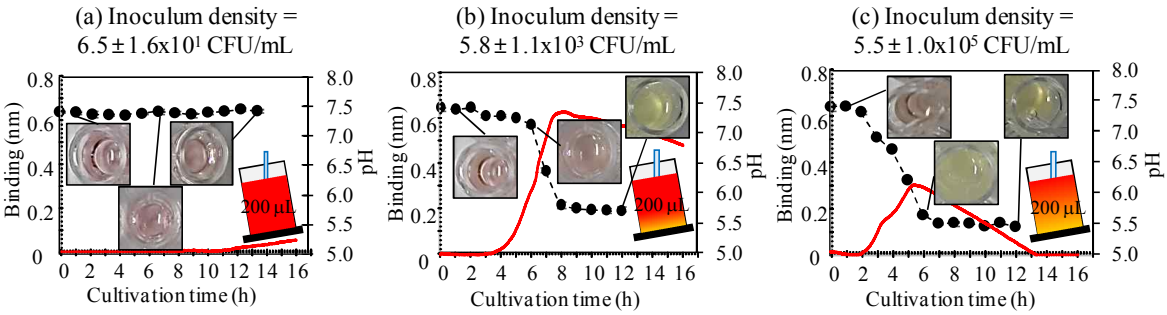

(a) 200-µL Volume model
(b) 50-µL Volume model
S. sonnei titer (CFU/mL)
1x10^6
5x10^5
0
2.1x10^2 CFU/mL
8.25x10^1 CFU/mL
1.25x10^3 CFU/mL
4.33x10^2 CFU/mL
0 1 2 3 4 5 6 7 8 9 10 11 12
Cultivation time (h)

Fig. S7


(a) Inoculum density = 6.5±1.6x10^1 CFU/mL
(b) Inoculum density = 5.8±1.1x10^3 CFU/mL
(c) Inoculum density = 5.5±1.0x10^5 CFU/mL
Binding (nm)
pH
200 µL
0 2 4 6 8 10 12 14 16
Cultivation time (h)