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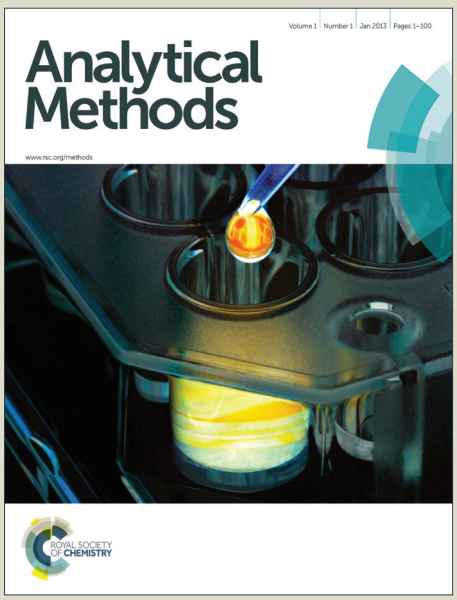

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FACS-based immunoassay of troponin-I using *E. coli* cells with autodisplayed Z-domains

Min Park[1,2], Ji-Hong Bong[1], Gu Yoo[1], Joachim Jose[2], Min-Jung Kang[3], Jae-Chul Pyun[1,*]

[1]Department of Materials Science and Engineering, Yonsei University

50 Yonsei-Ro, Seodaemun-Gu, Seoul, 120-742, Korea

Tel: 82 2 2123 5851 Fax: 82 2 312 5375 Email: jcpyun@yonsei.ac.kr

[2] Institute of Pharmaceutical and Medical Chemistry, University of Muenster

Muenster, Germany.

[3] Korea Institute of Science and Technology (KIST), Seoul, Korea

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*To whom correspondence should be addressed

Abstract

Fluorescence-activated cell sorter (FACS)-based immunoassays using *E. coli* cells with autodisplayed Z-domains were performed to (1) improve the sensitivity of immunoassay through orientation control of antibodies with autodisplayed Z-domains and (2) minimize the required amount of analyte by using FACS to measure the fluorescent signal from individual *E. coli* cells. The expression (autodisplay) of Z-domains on the outer membrane of *E. coli* was confirmed by fluorescence image analysis, and the homogeneous distribution of autodisplayed Z-domains was presented by SEM image analysis after treatment with antibodies labeled with gold nanoparticles (diameter of 15 nm). As FACS measures the fluorescent signal from individual *E. coli* cells, the optimal FACS parameters for the effective detection of fluorescently labeled *E. coli* cells was determined, and the minimum amount of analyte required for the *E. coli* cell-based immunoassay was identified using two model immunoassay configurations. Finally, the medical diagnosis of heart infarction with a biomarker called troponin-I using the *E. coli* cell-based immunoassay with FACS analysis was demonstrated.

Key words: immunoassay, autodisplay, Z-domain, *E. coli*, FACS

1. Introduction

The term “autodisplay” was initially introduced to refer to the use of the auto-transporter domain of a protein called adhesin involved in diffuse adherence (AIDA-I) from *E. coli* for outer membrane translocation of a recombinant protein in combination with the signal peptide of the cholera toxin-subunit (CTB) and an artificial promoter [1]. As shown in Fig. 1(a), the recombinant passenger protein is transported as a fusion protein with AIDA-I to the outer membrane of *E. coli* cells simply by introducing its coding sequence in-frame between the signal peptide and the translocating domain of the autodisplay vector [2-4]. In this work, the autodisplayed protein was the Z-domain, which is known to be the antibody (IgG)-binding domain of protein-A, and it has been used for the orientation control of antibodies by using its binding activity to the F_c regions of antibodies [5-8]. *E. coli* cells with such autodisplayed Z-domains were used as the solid support for the antibodies of the immunoassay in order to improve the sensitivity of the immunoassay through the orientation control of antibodies [9-10]. In addition, the outer membrane with the autodisplayed Z-domains could be isolated and layered on microplates [11] and SPR (surface plasmon resonance) biosensor surfaces for immunoassays [12-13].

Immunoassays have been widely used for the detection of target analytes in complicated mixture samples for medical diagnosis, environmental monitoring, and other applications [14]. For the detection of a target analyte in complex mixtures, immunoassays use the specific interactions between antigens and antibodies, and various formats have been used

according to the application field [15-16]. In particular, immunosorbent assays have been the most frequently used among various immunoassays using microplates or magnetic beads as solid supports for the antibodies [17-18]. For immunoassay applications, the sensitivity for the detection of a target analyte and the required amount of analyte are important factors that could be improved [19].

In this work, FACS-based immunoassays are presented that use *E. coli* cells with autodisplayed Z-domains (1) to improve the sensitivity of the immunoassay through orientation control of antibodies with autodisplayed Z-domains and (2) to minimize the required amount of analyte by measuring the fluorescent signal from the individual *E. coli* cells with FACS [10, 13]. In this work, FACS was used for the quantification of analyte because FACS can be used to measure the fluorescent signal from individual *E. coli* cells to minimize the amount of analyte required as shown in Fig. 1(b). For the statistical validation of the fluorescent signal measured by FACS, the minimum number of *E. coli* cells was estimated by calculating the relative deviation of measured values at standard analyte concentrations. For this work, the FACS threshold parameters were also optimized for the effective measurement of the fluorescent signals from *E. coli* cells, and the minimum amount of analyte for the *E. coli* cell-based immunoassays was estimated. Finally, the medical diagnosis of heart infarction by the detection of a biomarker called troponin-I was demonstrated by FACS-based immunoassay of the *E. coli* cells. For this work, the sensitivity of the *E. coli* -based immunoassay was compared with a magnetic bead assay based on the orientation control of antibodies by immobilized protein-A [20-21].

2. Materials and methods

2.1. Materials

Purified troponin-I (troponin-I), Cy-5 labeled anti-human antibodies (goat polyclonal), fluorescein-conjugated anti-goat antibodies (rabbit polyclonal), anti-troponin-I antibodies (mouse monoclonal), and fluorescein-labeled anti-troponin-I antibodies (goat polyclonal) were purchased from AbCam (Cambridge, UK). Luria–Bertani (LB) broth was bought from Duchefa (Haarlem, Netherlands) and magnetic beads with protein A on their surfaces were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Autodisplay of Z-domains

The autodisplay of Z-domains was carried out as in previously reported works [1-2]. *E. coli* cells transformed with Z-domain autodisplaying plasmid, PET-Z-18-3, were routinely cultured at 37°C overnight in LB broth with 10 μM EDTA, 10 mM 2-mercaptoethanol, and ampicillin at a concentration of 100 mg/l. Then, *E. coli* cells with pET-Z-18-3 were grown until reaching a 100-fold increase in freshly prepared medium at 37°C with vigorous shaking until the optical density (OD) reached 3.0 at a wavelength of 578 nm. After the *E. coli* cells were harvested, they were washed three times with PBS and then resuspended in PBS to a final optical density of 1.0 at a wavelength of 578 nm.

2.3. Scanning electron microscope (SEM) analysis

The *E. coli* samples for the SEM analysis were prepared by the following procedure: 1) freshly cultured *E. coli* was incubated in 1% glutaraldehyde and 1% formaldehyde (in PBS) at 4C° overnight, 2) *E. coli* was immobilized on a membrane by filtering with a PVDF membrane with pore sized of 0.2 μm, 3) the membrane with *E. coli* was treated with 0.1% osmium tetroxide for 60 min after three times of washing with PBS, 4) the membrane with *E. coli* was dehydrated by sequential incubation in 25%, 50%, 75%, 95%, 100% ethanol, 5) finally, the membrane was freeze-dried.

2.4. Flow cytometry analysis

E. coli cells transformed with pET-Z-18-3 plasmid were grown overnight and diluted (1:20) in freshly prepared medium. The *E. coli* cells were grown at 37°C with vigorous shaking until the optical density reached a value of 3.0 at a wavelength of 578 nm [10,12]. The *E. coli* cells were harvested, washed three times with PBS, and then resuspended in PBS/3% fetal calf serum to a final OD value of 1.0 at a wavelength of 578 nm. Then, fluorescein-labeled antibodies were added to 1 ml of cell suspension. After incubation for 1 hour at room temperature, the *E. coli* cells were washed three times with 1 ml of PBS and then resuspended in filter-sterilized PBS to an OD value of 0.5 at a wavelength of 578 nm. For each experiment, *E. coli* cells were analyzed with a FACSCalibur flow cytometer (Becton–Dickinson, Franklin Lakes, USA), using an excitation wave length of 488 nm and filter-sterilized PBS as a sheath fluid. The threshold trigger was set on side scatter to eliminate background noise and to ensure that only intact cells are analyzed [13].

2.5. Troponin-I detection using *E. coli* with autodisplayed Z-domains

For the immobilization of anti-troponin-I-antibodies, *E. coli* cells with autodisplayed Z-domains were treated with anti-troponin-I antibodies at a concentration of 10 μg/ml for 1 h at room temperature. For the comparison of assay results, anti-troponin-I antibodies were also immobilized to protein A-coated magnetic beads. Anti-troponin-I antibody solution at a concentration of 10 μg/ml was incubated with protein-A-coated magnetic beads (100 μl, 10^8 beads/ml) from Invitrogen (Carlsbad, CA, USA) for 1 h at room temperature. The immunoassay of troponin-I was carried out using standard solutions of troponin-I which were diluted 10-fold in bovine serum for 1 h at room temperature. The amount of bound troponin-I was estimated by treatment with fluorescein-labeled anti-troponin-I antibodies for 1 h at room temperature, and the fluorescent signal was measured using FACS.

3. Results and discussion

3.1 Autodisplay of Z-domains on *E. coli* cells

For the application of *E. coli* cells in a FACS-based immunoassay, the detection antibodies should be bound to autodisplayed Z-domains on the outer membrane, and the bound antibodies must have a homogeneous distribution on the outer membranes of the *E. coli* cells for effective fluorescence measurement by FACS. The expression of Z-domains was confirmed by testing the binding activity on the outer membrane of E. coli cells. As shown in Fig. 1(c), fluorescent Cy-5-labeled antibodies were added to both the *E. coli* cells with

autodisplayed z-domains and the intact *E. coli* cells. The fluorescence images show that *E. coli* cells with autodisplayed Z-domains have a significantly higher fluorescent signal in comparison with intact *E. coli* cells. The confocal image of *E. coli* cells with autodisplayed Z-domains shown in Fig. 1(c) illustrates that the intensity of fluorescence was significantly higher at the outer membrane of *E. coli* cells in comparison with the cytosol. This result showed that the autodisplayed Z-domains were positioned on the outer membrane of *E. coli* cells and that they could effectively bind the antibodies used in the immunoassays. The distribution of autodisplayed Z-domains was analyzed by treatment with antibodies labeled with gold nanoparticles (diameter of 15 nm). As shown in Fig. 1(d), SEM images revealed many objects in the shape of nanoparticles with the same diameter of 15 nm bound to the outer membranes of *E. coli* cells, and EDX analysis revealed that the gold content increased in comparison with that of the intact *E. coli* cells. Furthermore, the gold nanoparticles observed in SEM image analysis were homogeneously distributed over the whole surface of the *E. coli* cells. In the case of the intact *E. coli* cells, gold nanoparticles were not observed to bind to the outer membrane. These results demonstrated that the outer membranes of *E. coli* cells can be effectively used as an immunosorbent material for immunoassays.

3.2 FACS analysis of *E. coli* cells by fluorescence

For the immunoassay based on *E. coli* cells with autodisplayed Z-domains, the fluorescent signal from the *E. coli* cells is measured by the binding of fluorescently labeled antibodies. When fluorescence (FITC)-labeled antibodies were reacted with the *E. coli* cells with autodisplayed Z-domains, the dot plot (FSC channel vs. SSC channel) of FACS

measurement showed the fluorescent signals from the *E. coli* cells, as shown in Fig. 2(a). The binding efficiencies of *E. coli* cells with fluorescent signals in the low intensity range of the histogram plots (FITC channel vs. count) were estimated to be less than 50% without FACS threshold adjustment (threshold=0). Such fluorescent signal in the low intensity range of the histogram plots (FITC channel vs. count) was considered to be produced by signal artifacts that were irrelevant to *E. coli* cells. In this work, the gating parameter of the FACS threshold was optimized to decrease the noise level during the fluorescence measurement of *E. coli* cells. As shown in Fig. 2(a), the FACS threshold level was increased to eliminate fluorescence signals from objects with sizes smaller than that of *E. coli* cells. As shown in Fig. 2(b), the percentages of *E. coli* cells with fluorescent signals increased to 42.5%, 67.2%, 77.6%, 86.5%, 94%, and 98% when the FACS threshold level was increased to 0, 100, 200, 300, 400, and 450 (AU), respectively. In this work, the optimal threshold level was set to 450 (A.U), since the influence of artifact signals on the immunoassay results can be clearly eliminated at this level.

By using the optimized gating parameters, the cut-off value to discriminate *E. coli* cells with fluorescence from intact *E. coli* cells without fluorescence was established. As shown in Fig. 2(c), intact *E. coli* cells without fluorescent signals were observed to be positioned in the low fluorescence range and the *E. coli* cells with autodisplayed Z-domains before treatment with fluorescently labeled antibodies were also observed to be positioned at the same fluorescence range as the intact *E. coli* cells. However, when the *E. coli* cells with autodisplayed Z-domains were reacted with FITC-labeled antibodies, the *E. coli* cells were observed to be positioned in the high intensity range of more than 3×10^3 (A.U). From these

results, the *E. coli* cells could be discriminated from intact *E. coli* cells at the FITC channel after the binding of fluorescently labeled antibodies.

3.3 Evaluation of the effective event number of immunoassays

As FACS analyzes the fluorescent signal from each single particle, the immunoassay results are obtained by accumulating the fluorescent signal measurements from individual *E. coli* cells with autodisplayed Z-domains. The fluorescent signals from the outer membrane of individual *E. coli* cells are expected to be different within a certain range of error, even if the same concentration of antigens are analyzed with *E. coli* cells prepared at the same time. In this work, the influence of the number of *E. coli* cells on the final assay results was analyzed by estimating the relative deviation of measurements [22]. In this work, *E. coli* cells with autodisplayed Z-domains that were treated with FITC-labeled antibodies were used as a model analyte. As shown in Fig. 2(d), the fluorescence intensities were observed to increase with the concentration of reacted antibodies. However, the deviation between intensity measurements was observed to decrease when a larger number of events, that is, number of *E. coli* cells were used for the FACS analysis. The deviation of measurements was plotted according to the number of events, as shown in Fig. 2(e), which was observed to be correlated with both of the number of events and the concentration of FITC-labeled antibodies. In order to correlate the deviation of measurement to the number of events, the relative deviation was evaluated by dividing the deviation of measurement by the concentration of analyte. As shown in Fig. 2(f), the relative deviations were decreased to 23,

8, 2.9, 1.7, 1.6, and 0.5% for event numbers of 10, 100, 1000, 10^4, and 5 x 10^4, respectively. These results indicate that an immunoassay using 5 x 10^4 *E. coli* cells can achieve a relative deviation of less than 1%. Usually, immunoassays based on *E. coli* cells with autodisplayed Z-domains have made use of an *E. coli* solution of an $OD_{570\ nm}$ value of 1.0 at a concentration of 10^8 cells/ml, and the calculated number of *E. coli* cells (5 x 10^4 cells) corresponds to a volume of 0.5 μl of this concentration of *E. coli* solution. These results indicate that a stable FACS-based immunoassay with a deviation of less than 1% can be achieved by resuspending the *E. coli* cells with only 0.5 μl of FITC-labeled antibody solution (Fig. 2(a)) within a concentration range of a few ng/ml to 1 μg/ml. As another model of immunoassay, *E. coli* cells with immobilized antibodies were used for the analysis of antigens. After detection antibodies against human immunoglobulin G (hIgG) were immobilized on autodisplayed Z-domains, the *E. coli* cells were used in the detection of hIgG in samples, and the amount of bound hIgG was then quantified using Cy-5-labeled antibodies. As in previous experiments, the fluorescent signals from individual *E. coli* cells were analyzed using FACS. As shown in Fig. 3(a), the fluorescence intensity was observed to increase with the concentration of hIgG in the samples. The deviation of measurements was plotted according to the number of events, as shown in Fig. 3(b), which was also observed to be correlated with both the number of events and the concentration of analyte. As shown in Fig. 3(c), the relative deviations were decreased to 24.5%, 8.4%, 2.5%, 1.6%, 1.1%, and 0.7% for event numbers of 10, 100, 1000, 10^4, and 5 x 10^4, respectively. These results indicate that an immunoassay for the detection of antigens using 5 x 10^4 *E. coli* cells and with detection antibodies that are immobilized to the autodisplayed Z-domains can

achieve a relative deviation of less than 1%.

3.4 FACS-based immunoassay of troponin-I

E. coli cells with autodisplayed Z-domains were used in the FACS-based immunoassay of troponin-I, which is known to be a biomarker of cardiovascular diseases. Typically, the cut-off value used for troponin-I is a concentration between 0.1 and 1 ng/ml for the early diagnosis of cardiac diseases [23-25] and between 25 – 35 ng/ml for the medical prediction of early postoperative course after pediatric cardiac surgery in intensive care units [25]. As previously determined, the threshold level of FACS analysis were set to 450 (AU) and the number of *E. coli* cells was adjusted to 5 x 10^4 cells to achieve immunoassay results with a relative deviation of less than 1%. A sandwich-type immunoassay of troponin-I was carried out by immobilizing detection antibodies to the autodisplayed Z-domains and the amount of bound troponin-I was then quantified by additional treatment with secondary antibodies labeled with FITC. Troponin-I standard samples were prepared in human serum by spiking with known concentrations of troponin-I. As shown in Fig. 4(a), the fluorescence intensity at the FITC channel was increased according to the concentration of troponin-I. In the case of intact *E. coli* cells, the fluorescence intensity at the FITC channel remained at baseline levels. These results show that sandwich-type immunoassay of troponin-I can be performed using the specific interaction between the immobilized detection antibodies on the outer membranes of *E. coli* cells and troponin-I.

In order to demonstrate an improvement in sensitivity by the orientation control effect of

autodisplayed Z-domains, the immunoassay results of troponin-I were compared with those from intact *E. coli* cells used as a negative control and magnetic beads coated with protein-A. As shown in Fig. 4(b), the *E. coli* cells showed a steady baseline signal over the whole concentration range of analyte, which indicated a very low level of non-specific binding of troponin-I to the outer membrane of *E. coli* cells. For the estimation of the selectivity of anti-troponin I antibodies which were bound to the autodisplayed Z-domains, human serum albumin (HSA) was treated as blank samples. As shown in Fig. 4(b), HSA samples at the comparable concentration to troponin-I presented only baseline level signals. These results showed that the immunoassay based on the anti-troponin I antibodies bound to the autodisplayed Z-domains had a suitable selectivity for the detection of troponin-I at the corresponding concentration range. As previously mentioned, we tried to keep the relative deviation of the *E. coli* cell-based immunoassay to less than 1% by using 5×10^4 *E. coli* cells. When the concentrations of troponin-I were 10.0 μg/ml, 3.3 μg/ml, 1.1 μg/ml, 370.4 ng/ml, 123.5 ng/ml, 41.2 ng/ml, 13.7 ng/ml, 4.6 ng/ml, and blank, the relative deviations were estimated to be 1.43, 1.10, 1.08, 1.64, 2.42, 2.94, 3.69, 2.34, and 1.40%, respectively. Such a slight increase in the relative deviation was considered to have occurred from the sandwich-assay configuration, which required one additional step to be carried out during the immunoassay of troponin-I. These results show that the relative deviation of the FACS-based immunoassay can be effectively controlled by adjusting the number of *E. coli* cells. The magnetic beads were selected to have the diameter of 1 μm so that they would have a surface area similar to that of a single *E. coli* cell. However, the sensitivity of the *E. coli* cell-based immunoassay was estimated to be higher than that of the magnetic-bead-based

immunoassay for a dynamic analyte concentration range that did not reach signal saturation. The limits of detection were measured to be 9 ng/ml and 60 ng/ml for the *E. coli* -based immunoassay and the magnetic bead-based immunoassay, respectively. The signal saturation levels were estimated to be 480 (AU) and 320 (AU) for the *E. coli* -based immunoassay and the magnetic bead-based immunoassay, respectively. The surface area of *E. coli* and magnetic beads with a diameter of 1.0 μm was calculated to be 3.4 μm^2 and 3.0 μm^2, respectively. The calculation was carried out with an assumption that *E. coli* was a cylindrical shape with a diameter of 0.5 μm and a length of 2.0 μm [26,27]. From these results, the surface area of *E. coli* was calculated to be only 10 % larger than that of magnetic beads. Considering the similar surface areas of the two materials, these results show that the binding capacity of *E. coli* cells was far larger than that of the magnetic beads. Such results were considered to have occurred because the protein-A protein molecules must be well-orientated on the magnetic beads for the orientation control of antibodies. However, the autodisplayed Z-domains on the outer membrane always have the same orientation. These results showed that the *E. coli* cell-based immunoassay could be applied in the medical diagnosis of troponin-I through the optimization processes.

Conclusions

A FACS-based immunoassay using *E. coli* cells with autodisplayed Z-domains as a solid support for antibodies was developed. The expression of Z-domains on the outer membranes of *E. coli* was confirmed by testing binding activity with Cy-5-labeled antibodies,

and confocal images of *E. coli* cells with autodisplayed Z-domains showed that the autodisplayed Z-domains on the outer membranes of *E. coli* cells could effectively bind the antibodies for immunoassays. The distribution of autodisplayed Z-domains was analyzed by treatment with antibodies labeled with gold nanoparticles (diameter of 15 nm), and the gold nanoparticles were observed on the SEM images to be homogeneously distributed over the surfaces of the *E. coli* cells. For the immunoassay based on *E. coli* cells with autodisplayed Z-domains, the fluorescent signal from the *E. coli* cells is measured by the binding of fluorescently labeled antibodies. In this work, the gating parameter of the FACS threshold was optimized to decrease the noise level during fluorescence measurement using *E. coli* cells. For the application FACS in *E. coli* -based immunoassays, the influence of the number of *E. coli* cells on the final assay results was analyzed by estimating the relative deviation of measurements. By using two model configurations of the immunoassays, the relative deviations of measurements were estimated to be less than 1% for the detection of antigens when using 5 x 10^4 *E. coli* cells with detection antibodies that were immobilized to the autodisplayed Z-domains. Finally, the medical diagnosis of heart infarction with a biomarker called troponin-I was demonstrated using the *E. coli* cell-based immunoassay with FACS analysis. The sensitivity of the *E. coli* cell-based immunoassay was estimated to be higher than that of the magnetic bead-based immunoassay over the dynamic analyte concentration range before signal saturation. The limit of detection was also measured to be 9 ng/ml and 60 ng/ml for the *E. coli*-based immunoassay and the magnetic bead-based immunoassay, respectively. From these results, the E. coil cell-based immunoassay was determined to be feasible for the medical diagnosis of troponin-I.

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Figure legends

Fig. 1. Autodisplay of Z-domains on the outer membrane of *E. coli*. (a) The transformed autodisplaying vector with Z-domains expressed and autodisplayed on the outer membrane of *E. coli* (OM: outer membrane, PP: periplasm, IM: inner membrane, SP: signal peptide). (b) Procedure of troponin-I detection using *E. coli* with autodisplayed Z-domains. Anti-troponin-I antibodies bound to Z-domains reacted with troponin-I. For quantification, anti-troponin-I antibodies conjugated with fluorescein were used, and fluorescent signals were measured using a flow cytometer. (c) Antibody-binding activity of autodisplayed Z-domains. Confocal microscope image of *E. coli* with autodisplayed Z-domains was taken after treatment with Cy-5 labeled antibodies. (d) SEM image of *E. coli* with autodisplayed Z-domains after treatment with 15-nm gold-nanoparticle-labeled antibodies.

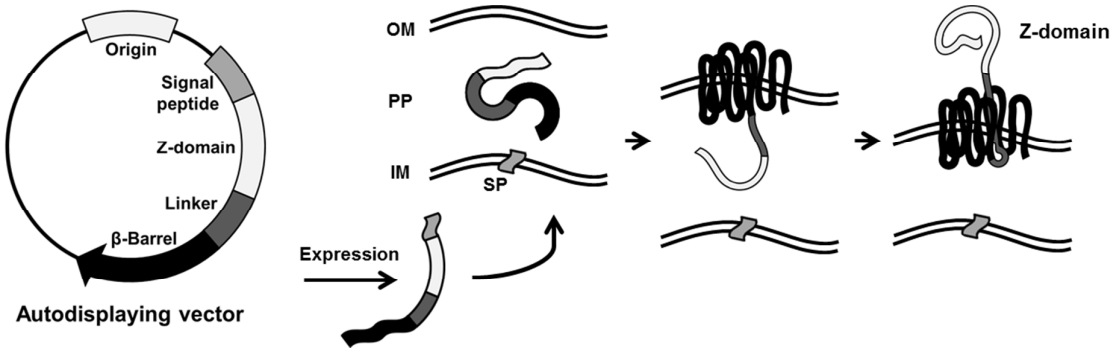
Fig. 2. Optimization of *E. coli* detection with FACS. (a) Dot plots (SSC vs FSC plot) and histogram (count vs. FITC intensity) of *E. coli* cells with fluorescence-labeled antibodies. (b) The signal-to-noise ratio of the FACS signal from *E. coli* cells with fluorescently labeled antibodies according to the change in threshold values. (c) Comparison of histograms of *E. coli* cells with and without autodisplayed Z-domains after treatment with fluorescently labeled antibodies. (d) FACS analysis of *E. coli* cells after reaction with fluorescently labeled antibodies (The parameters of inner coordinate axis were same as outer coordinate axis). (e) Comparison of standard deviations of FACS signals according to the number of analyzed *E. coli* cells. (f) Relative deviation of FACS signal according to analyzed *E. coli* cells.

Fig. 3. FACS analysis for the detection of antibodies in a sample. (a) FACS signal according to the concentration of Cy-5 labeled antibodies (The parameters of inner coordinate axis were same as outer coordinate axis). (b) Comparison of standard deviations of FACS signals according to the number of analyzed *E. coli* cells. (c) Relative deviation of FACS signal according to analyzed *E. coli* cells.

Fig. 4. FACS-based immunoassay of troponin-I. (a) Histograms of *E. coli* cells with autodisplayed Z-domains and intact *E. coli* cells (negative control) according to the concentration of troponin-I. (b) Standard curve of troponin-I in comparison with *E. coli cells* with autodisplayed Z-domains (■) and magnetic beads coated with protein A (●). Intact *E. coli* cells without Z-domains (▲) were used as a negative control for the assay. Human serum albumin was used as samples instead of troponin-I to test the selectivity of the anti-troponin-I antibodies bound to the autodisplayed Z-domains (▼).

Fig. 1.

(a)



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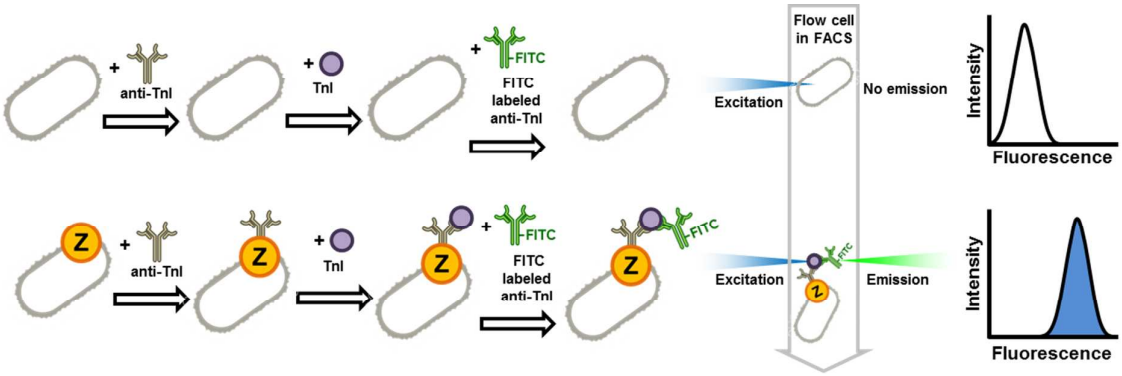

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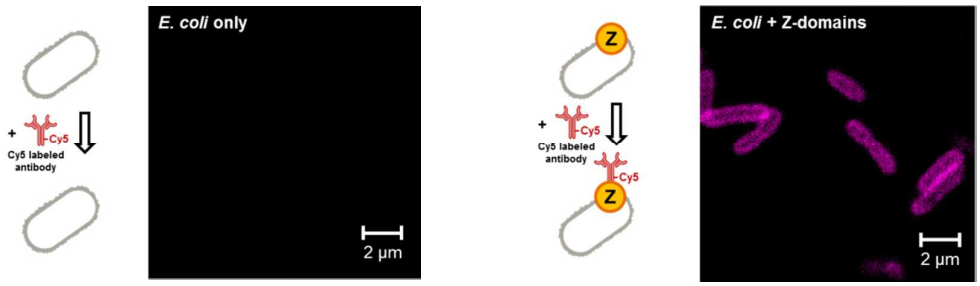

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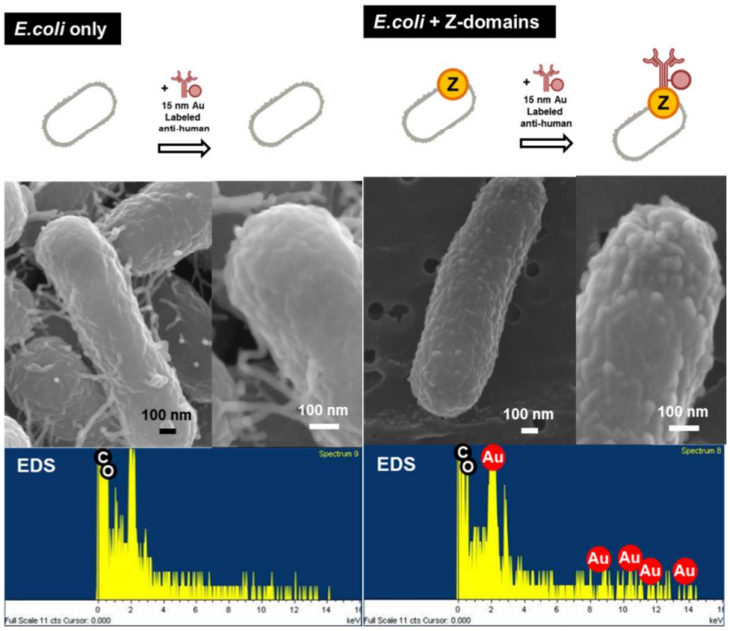
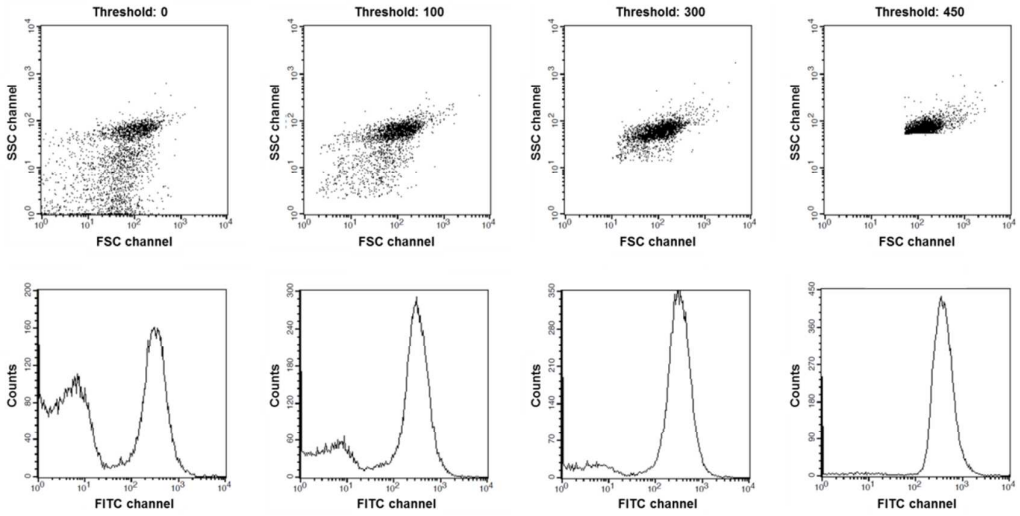


Fig. 2.

(a)



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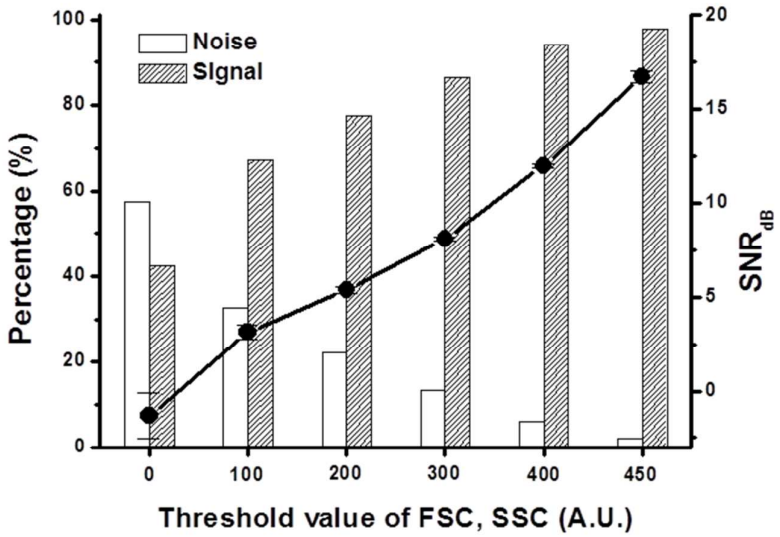

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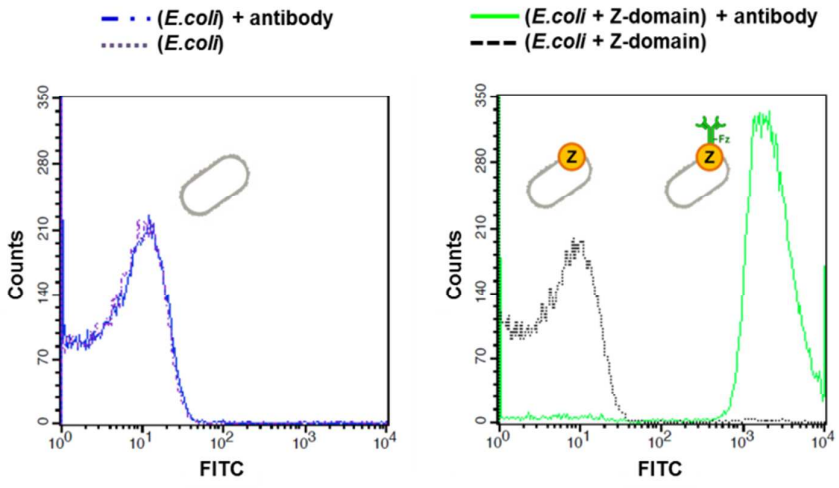

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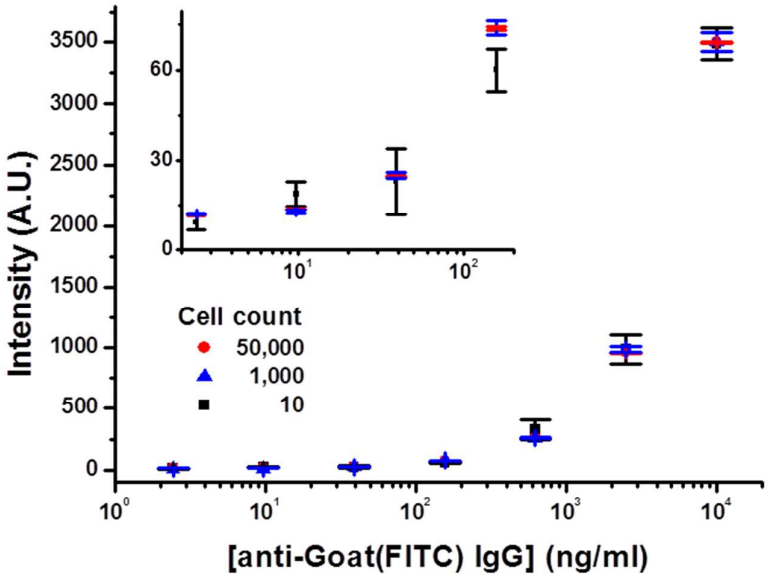

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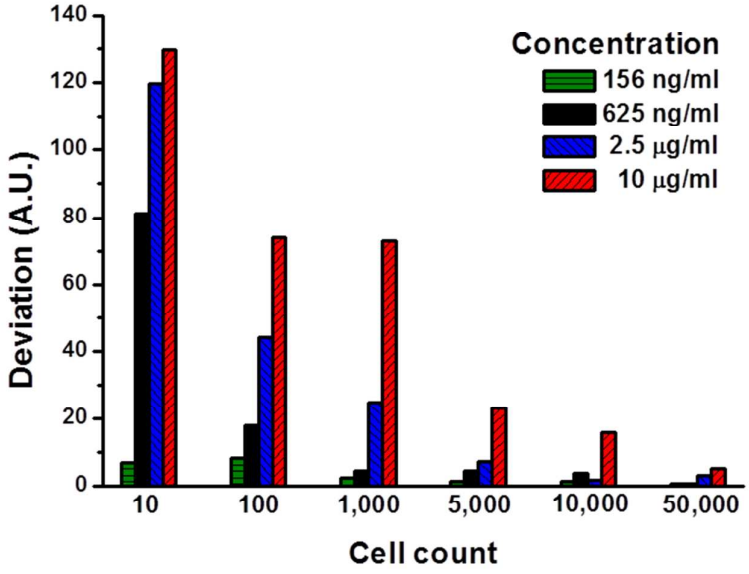

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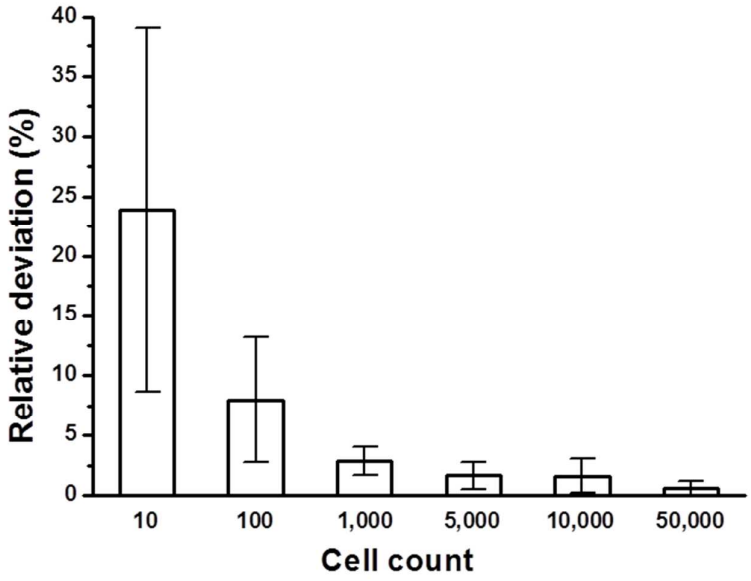
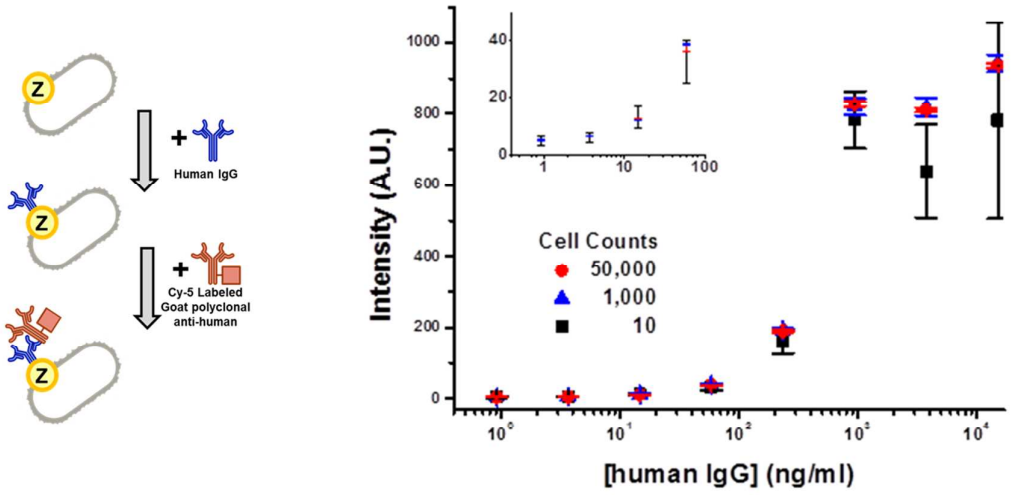


Fig. 3.

(a)



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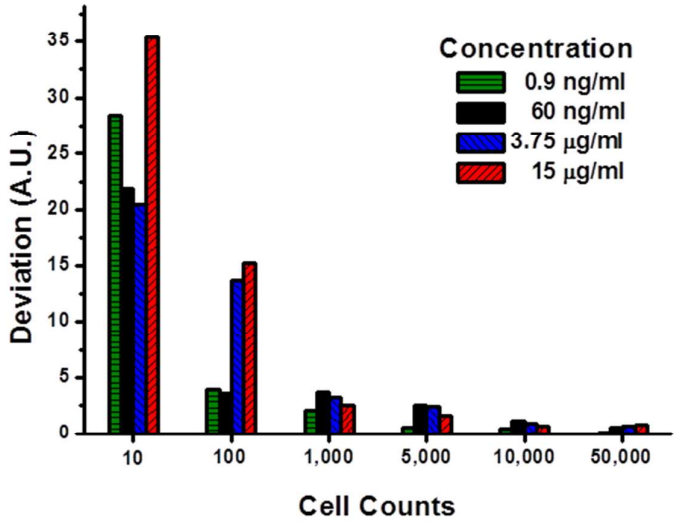

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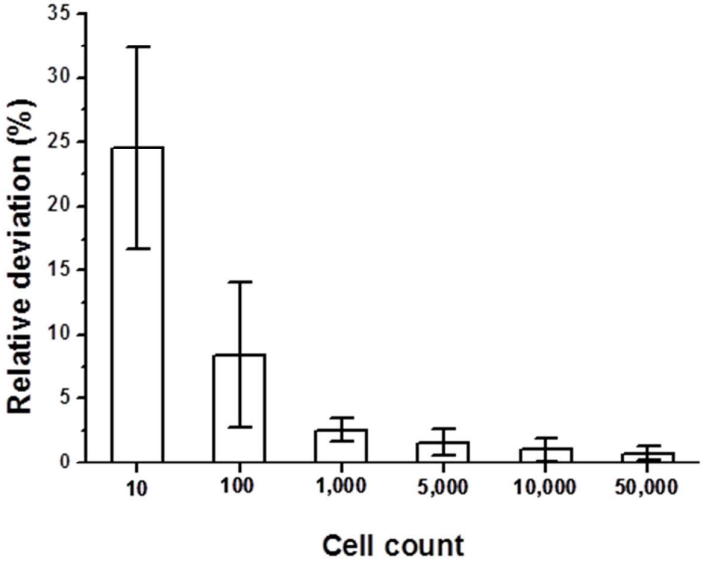
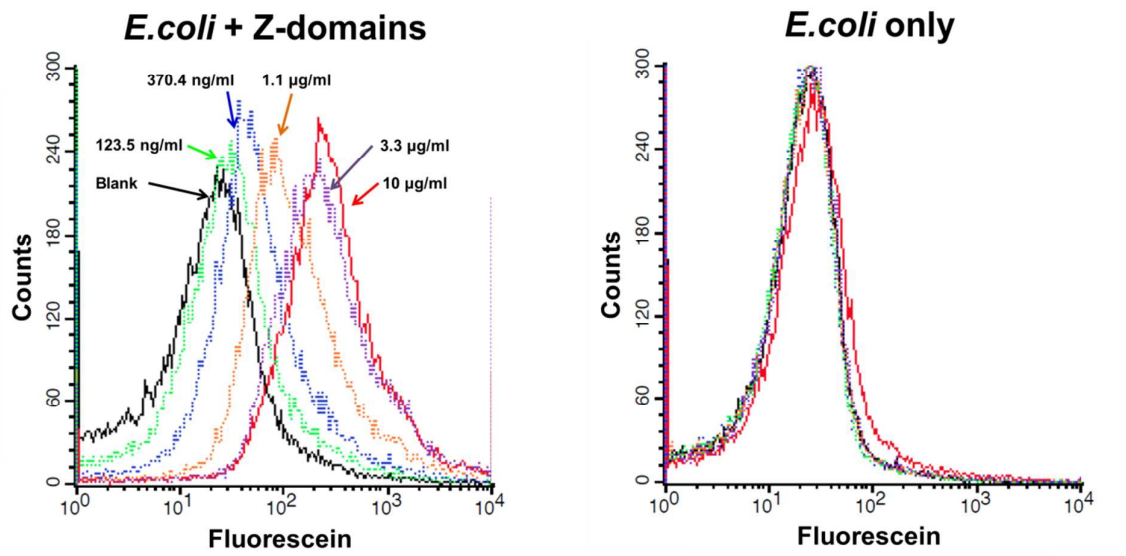


Fig. 4.

(a)



(b)