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High-efficiency recovery of target cells using improved yeast display system for detection of protein–protein interactions

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Abstract We constructed a high-throughput screening (HTS) system for target cells based on the detection of protein–protein interactions by flow cytometric sorting due to the improvement in the yeast cell surface display system. Interaction model proteins, which are the ZZ domain derived from *Staphylococcus aureus* and the Fc part of human immunoglobulin G (IgG), were displayed on the yeast cell surface. We achieved a rapid and enhanced expression of these proteins as a result of adopting an appropriate yeast strain and a suitable promoter. The displayed ZZ domain had an ability to bind to rabbit IgG and the displayed Fc part to protein A. These were confirmed by flow cytometry and fluorescence microscopy. Furthermore, the cells displaying the ZZ domain or Fc part were isolated from the model libraries constructed by mixing the control yeast cells with the target yeast cells. The ratio of the target cells was increased from 0.0001% to more than 70% by two cycles of cell sorting. These results indicate that we can achieve a rapid and highly efficient isolation method for the target cells with FACSCalibur and that this method will further extend the application of flow cytometric sorting to library selections.

Keywords Improved yeast display system · Flow cytometric sorting · High-efficiency recovery · Protein-protein interactions

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Introduction

The functional screening of a combinatorial library is an extremely attractive method because of its potential for improving molecules. Combinatorial screening as represented by a phage display system has been reported by numerous researchers. Following reports on typical systems, a large number of new analogous systems have been developed, and various libraries have been screened (Arap et al. 2002; Giordano et al. 2001). The screenings of displayed peptide libraries have been successful owing to the use of various anchoring motifs in several microbial hosts (Boder and Wittrup 1997; Lofblom et al. 2005). Host fitting for surface display may depend on its intended purpose. In the case of library screening for mammalian proteins, *Saccharomyces cerevisiae* is a highly favorable host because its protein

folding and secretory machineries are similar to those of mammalian.

In this study, we improved the yeast display system by selecting an appropriate strain and a suitable promoter, resulting in a rapid and high-level expression of displayed proteins. Flow cytometric sorting has the advantage of being a reliable quantitative high-throughput screening (HTS; Yeung and Wittrup 2002). We have demonstrated the significant effect of expression levels of target proteins on the recovery rates and performed highly efficient recovery of target cells based on the detection of protein–protein interactions by flow cytometric sorting with fluorescent-probe labeling. The ZZ domain and Fc part that bind to each other (Kronvall and Williams 1969; Nilsson et al. 1987) but originate from different organisms were used as models of protein–protein interactions (Shibasaki et al. 2006). The ZZ domain has a repetitive structure of a small Z domain (58 amino acids) derived from staphylococcal protein A, which consists of two α-helical domains (Nakamura et al. 2001). Bacterial proteins such as the Z domain efficiently fold in different milieus (Lofblom et al. 2005). The affinity selection of the ZZ-displaying yeast by magnetic separation has been successfully carried out (Furukawa et al. 2003). Therefore, we tried the high-efficiency recovery of target cells displaying the ZZ domain in a cell mixture with an excessively large number of control cells by flow cytometric sorting. Moreover, we displayed the Fc part of human immunoglobulin G (IgG) on the yeast cell surface and attempted the recovery of the cells displaying the Fc part as well as the ZZ domain. The high-efficiency recovery using the improved yeast display system will allow us to obtain the protein of interest without loss of the precious target from a protein display library.

Materials and methods

Strains and media Escherichia coli NovaBlue (Novagen, Madison, WI, USA) was used for genetic manipulation. *S. cerevisiae* BY4741 (Brachann et al. 1998) was used for cell-surface display. *E. coli* was grown in Luria–Bertani (LB) medium [containing 0.5% yeast extract (Nacalai Tesque, Kyoto, Japan), 0.5% sodium chloride, and 1% tryptone; *w*/*v*] with 100 μg/ml ampicillin.

Plasmid construction The plasmids used for the cell-surface display of ZZ or Fc in *S. cerevisiae* are listed in Table 1.

The plasmids for inducible expression controlled by the *UPR-ICL* promoter were constructed as follows: The fragment containing the *UPR-ICL* promoter and the transcriptional terminator *TERM-ICL* derived from *Candida tropicalis* was prepared by digesting pWI3 (Kanai et al. 1996) with *Pvu*II and inserted into the *Pvu*II site of pUM (Katahira et al. 2004), yielding the plasmid pUI3. The fragment containing *UPR-ICL*, the secretion signal sequence of the glucoamylase derived from *Rhizopus oryzae*, the gene encoding synthetic EZZ consisting of the first six amino acids of domain E and the dimer of the Z domain derived from *Staphylococcus aureus* protein A, the 3′-half of the region encoding 320 amino acids of α-agglutinin and *TERM-ICL*, was prepared by digesting pMWIZ1 (Nakamura et al. 2001) with *Sal*I, and inserted into the *Sal*I site of pUM, yielding the plasmid pMUIZ1. The fragment containing the gene encoding the Fc part of human IgG was amplified from pUC19Fc (Shibasaki et al. 2006) by polymerase chain reaction (PCR) with the primers 5′-AGTGACGT CATGGGGGGACCGTCAGTCTTCCTCTTC-3′ and 5′-GGCTCTCGAGAGTTTACCCGGAGACAGGGAGA-3′

Table 1 Characteristics of strains and plasmids used in this study

Plasmid or strain	Relevant feature	Reference source
Plasmid		
pUI3	*URA3*, no display controlled by *UPR-ICL* promoter	Present study
pMUIZ1	*URA3*, display of ZZ controlled by *UPR-ICL* promoter	Present study
pUF318-Fc	*URA3*, display of Fc controlled by *UPR-ICL* promoter	Present study
pUMGP	*URA3*, no display controlled by *GAPDH* promoter	Present study
pUMGPZZ	*URA3*, display of ZZ controlled by *GAPDH* promoter	Present study
pUMGPFc	*URA3*, display of Fc controlled by the *GAPDH* promoter	Present study
pESC-URA	*URA3*, no display controlled by the *GAL1* promoter	Stratagene
pGUZZ	*URA3*, display of ZZ controlled by the *GAL1* promoter	Shibasaki et al. (submitted)
pGUFcM	*URA3*, display of Fc controlled by the *GAL1* promoter	Present study
S. cerevisiae yeast strain		
BY4741	*MAT α his3 Δ1 leu2 Δ0 met15Δ0 ura3 Δ0*	Brachann et al. (1998)
Bacterial strain		
E coli NovaBlue	*endA1 hsdR17*($r^-_{K12}m^+_{K12}$)*supE44 thi-1 recA1 gyrA96 relA1 lac* F′[*proA*$^+$ *B*$^+$ *lacI*^{q}*ZΔM15*:: Tn*10*(Tc^R)]	Novagen

(*Aat*II-*Xho*I fragment). The *Aat*II-*Xho*I fragment was inserted into the *Aat*II-*Xho*I site of pIF318 (Sato et al. 2002), yielding the plasmid pIF318-Fc. The fragment containing *UPR-ICL*, the secretion signal sequence of the α-factor, the gene encoding the Fc part of human IgG, the region encoding the C-terminal 318 amino acids of Flo1p and *TERM-ICL*, was prepared by digesting pIF318-Fc with *Bss*HII and inserted into the *Bss*HII site of pUM, yielding the plasmid pUF318-Fc.

The plasmids for constitutive expression controlled by the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) promoter were constructed as follows: the fragment containing *GAPDH* promoter and terminator was amplified from pWGP3 (Takahashi et al. 2001) by PCR with the primers 5′-TTTGCGCGCACCAGTTCTCACACGGAA CAC-3′ and 5′-CCCGCGCGCTCAATCAATGAATC GAAAATG-3′ (*Bss*HII-*Bss*HII fragment) and inserted into the *Bss*HII site of pUM, yielding the plasmid pUMGP. The fragment containing the secretion signal sequence of glucoamylase, the gene encoding synthetic EZZ and the 3′-half of the region of α-agglutinin, was prepared by digesting pMUIZ1 with *Eco*RI and *Sal*I and inserted into the *Eco*RI/*Sal*I site of pUMGP, yielding the plasmid pUMGPZZ. The fragment containing the secretion signal sequence of the α-factor, the gene encoding the Fc part of human IgG and the region encoding the C-terminal 318 amino acids of Flo1p, was amplified from pUF318-Fc by PCR with the primers 5′-CCCCGAATTCATGAGATTTCCTTCAATTTT-3′ and 5′-CCCCGGATCCTTAAATAATTGCCAGCAATA-3′ (*Eco*RI-*Bam*HI fragment).

The *Eco*RI-*Bam*HI fragment was inserted into the *Eco*RI/*Bam*HI site of pUMGP, yielding the plasmid pUMGPFc.

The plasmid for inducible expression controlled by the *GAL1* promoter was constructed as follows: The fragment containing the secretion signal sequence of the α-factor, the gene encoding the Fc part of human IgG and the region encoding the C-terminal 318 amino acids of Flo1p, was amplified from pUF318-Fc by PCR with the primers 5′-GCGGAAGCTTATGAGATTTCCTTCAATTTT-3′ and 5′-CGGGGCTAGCTTAAATAATTGCCAGCAATA-3′ (*Hin*dIII-*Nhe*I fragment). The *Hin*dIII-*Nhe*I fragment was inserted into the *Hin*dIII/*Nhe*I site of pESC-URA (Stratagene, CA, USA), yielding the plasmid pGUFc. An ATG sequence, which is an initiation codon derived from the myc-epitope before the desired initiation codon under the control of *GAL1* promoter, was present in pGUFc. To prevent translational frameshift, a point mutation from the ATG sequence to the TTG sequence was achieved by PCR from pGUFc with the primers 5′-CCCGGGCGTCGACTTGGAACAGAAGTT GAT-3′ and 5′-ATCAACTTCTGTTCCAAGTC GACGCCCGGG-3′, yielding the plasmid pGUFcM. pGUZZ, which was used as the plasmid for display of the ZZ domain under the control of the *GAL1* promoter, was constructed by Shibasaki et al.

Yeast transformation The plasmids listed in Table 1 were introduced into *S. cerevisiae* BY4741 by the lithium acetate method.

Cultivation conditions for cell-surface expression Yeasts were grown in SDC medium [containing 0.67% yeast nitrogen base without amino acids (YNB; Becton Dickinson, Franklin Lakes, NJ, USA), 2% (for *GAPDH*) or 0.5% (for *UPR-ICL*) glucose, and 2% casamino acids; *w*/*v*], or SGC medium [containing 0.67% YNB, 2% galactose (for *GAL1*), and 2% casamino acids; *w*/*v*].

Quantification of expression levels of target proteins on yeast cell surface The yeast cells grown in the respective medium were harvested and washed with phosphate-buffered saline (PBS; 50 mM phosphate and 150 mM sodium chloride; pH 7.4). The cells were resuspended in 100 μl of PBS to give an optical density of 5.0 at 600 nm (OD_{600}=5.0), and quantitative immunofluorescence staining was performed as follows. In suspension, cells displaying the ZZ domain were stained with 10 μg/ml anti-protein A antibodies labeled with fluorescein isothiocyanate (FITC; Immune System I.M.S AB, Uppsala, Sweden); on the other hand, those displaying the Fc part were stained with 10 μg/ml anti-human IgG (Fc) antibody labeled with FITC (Kirkegaard Perry Laboratories, Gaithersburg, MD, USA). The fluorescence intensity of the immunostained cells was measured on FACSCalibur equipped with a 488-nm air-cooled argon laser (Becton Dickinson), and the data were analyzed using CELLQuest software (Becton Dickinson). Each parameter was set as follows: The amplifiers were set in the linear mode for forward scattering (FSC) and in the logarithmic mode for the green fluorescence detector (FL1) and orange fluorescence detector (FL2); the amplifier gain of FSC was set at 1.00; the detector voltage of FSC was set at E00, that of FL1 at 700 V and that of FL2 at 500 V; and the threshold of the FSC was set at 52. The fluorescence signal from yeast cells was collected through a 530/30-nm band-pass filter (FL1) and a 585/21-nm band-pass filter (FL2). The geometric mean of FL1 height (FL1-H geo mean) was determined by analyzing 10,000 cells. Fluorescence intensity was determined by subtracting the FL1-H geo mean of unstained cells from that of stained cells.

Functional evaluation of displayed proteins Cell suspensions (OD_{600}=5.0, 100 μl) were prepared as in the previous section. Aliquots containing various concentrations of rabbit IgG labeled with FITC (Vector Laboratory, Burlingame, CA, USA) and protein A labeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) were respec-

tively prepared by diluting them with PBS. One microliter of the diluted rabbit IgG was added to the cell suspension displaying the ZZ domain, and 1 μl of the diluted protein A was added to those displaying the Fc part. The treated cells were analyzed with FACSCalibur under the same conditions described above. Moreover, the yeast cells stained with 10 μg/ml fluorescence-labeled proteins were observed under a microscope.

Recovery of target cells from model libraries Model libraries were prepared by mixing the target cells with the control cells (BY4741 haboring the control plasmids, pUI3, pUMGP, or pESC-URA) in the initial rates shown in Table 2 and Fig. 5. The cell mixture was stained with 10 μg/ml labeled rabbit IgG or protein A. Cell sorting was performed with FACSCalibur under the same conditions described in the previous section. In the case of the cell mixtures containing 0.1–50% of the target cells, 1 ml each of the cell suspensions was applied to SDC plates containing 2% agar and incubated at 30°C for 2 days. Twenty colonies were picked up and separately grown in SDC medium overnight. The plasmids were extracted, and the coding region was amplified by PCR with the primers 5′-ATTTTCGGTTTGTATTACTTC-3′ and 5′-GCGTTATCCCCTGATTCTGT-3′. Recovery rate was calculated by determining the number of colonies that retain the target genes. In the case of the cell mixtures containing 0.0001–0.01% of the target cells, cell sorting was repeated twice. In the first cycle, the target cells were sorted and grown in SDC medium with 100 μg/ml ampicillin at 30°C for 2 days. These culture solutions were appropriately diluted, and 1 ml each of the diluted solutions was applied to SDC plates containing 2% agar and incubated at 30°C for 2 days. Furthermore, the yeast cells grown after the first cycle of cell sorting were cultivated for expressional induction. After washing with PBS and staining with the fluorescence-labeled rabbit IgG or protein A, the second cycle of cell sorting was performed in the same way as the first cycle, and 1 ml each of the recovered cell suspensions was applied to SDC plates containing 2% agar and incubated at 30°C for 2 days. At each stage, ten colonies were picked up and separately grown in SDC medium. The plasmids were extracted, and the coding region was amplified by PCR with the same primers described above. Recovery rate was also calculated by determining how many colonies retain the target genes.

Table 2 Rates of recovery from model libraries by one cycle of flow cytometric sorting: the targets of the sorting were yeast cells displaying the ZZ domain and Fc part

Initial rate of target cells in model libraries (%)	Recovery rate after single cycle of sorting (%)
ZZ domain	
0.1	80
1	95
10	95
50	100
Fc part	
0.1	90
1	90
10	100
50	95

Results

Quantitative analyses of expression levels of displayed proteins controlled by different promoters To determine the promoter suitable for HTS, the expression levels of the ZZ domain or Fc part displayed on the cell surface under the control of the *UPR-ICL* promoter (induced by glucose depletion), *GAPDH* promoter (expressed constitutively), and *GAL1* promoter (repressed by glucose and induced by galactose) were examined (Fig. 2). As shown in Fig. 2a (ZZ) and b (Fc), there was no difference in expression trend between the ZZ domain and the Fc part, except in relative fluorescence intensity. The expression levels were lower in the cells harboring plasmids with the *GAPDH* promoter than in those harboring plasmids with the other two promoters. On the other hand, those of the cells controlled by the *UPR-ICL* promoter were the highest, although it required considerable time to attain high expression levels. In contrast, the cells induced by the *GAL1* promoter achieved high expression levels of the proteins after 12 h of cultivation. It was determined that the *GAL1* promoter is suitable for HTS systems. Hence, *GAL1*-inducible cells (BY4741/pGUZZ and BY4741/pGUFcM) were examined in the subsequent analyses.

Functional analyses of ZZ domain and Fc part To confirm the functions and examine the performance of the displayed proteins, the adsorptions of fluorescent-labeled rabbit IgG (for ZZ) and protein A (for Fc) on the cell surface were quantified. Figure 3 shows the adsorptions of rabbit IgG (FITC labeled) and protein A (Alexa Fluor 488 labeled), whose fluorescence intensities were measured by flow cytometry. The detection ranges of the rabbit IgG and protein A concentrations plateaued at about 10 μg/ml, although the fluorescence intensity of the cells displaying the ZZ domain was nearly twice that of the cells displaying the Fc part. Furthermore, evaluation of association constants between the displayed proteins and fluorescently labeled proteins was performed by Scatchard plot. The association constant of the displayed ZZ domain for rabbit IgG was estimated to be 2.1×10^{8} M^{-1}, and that of the displayed Fc part for protein A was estimated to be 6.6×10^{7} M^{-1}.

Microscopy of displayed ZZ domain and Fc part on yeast cell surface To confirm that the ZZ domain or Fc part is displayed on the cell surface, immunostaining with 10 μg/ml labeled rabbit IgG or protein A was performed, and then, these cells were observed under a fluorescence microscope. As shown in Fig. 4, the fluorescences of the immunostained ZZ domain and Fc part are clearly observed along the outline of both cells harboring pGUZZ and pGUFcM. In contrast, no cells harboring the control plasmid pESC-URA were immunostained. These results suggest the cell surface displays of the ZZ domain and Fc part.

Flow cytometric sorting by protein–protein interaction with FACSCalibur To achieve a high-efficiency recovery in a small population of target cells, flow cytometric sorting from model libraries was performed using FACSCalibur. The model libraries in varying proportions were prepared by mixing the cells displaying the ZZ domain or Fc part (target cells) controlled by the *GAL1* promoter with the cells harboring pESC-URA (control cells). After the immunostained libraries were sorted using the R_1 gate (Fig. 5a), the collected cells were grown on SDC plates, and the retention rate of the plasmid containing the target genes was examined (Table 2 and Fig. 5). The rates of recovery from the libraries including 50–0.1% of the target cells through only one cycle of sorting are shown in Table 2. The target cells were recovered from the libraries with a frequency of 1/1,000 cells with more than 80% recovery rate. Then, we attempted the recovery of the target cells from an extremely small population of libraries possessing 0.01–0.0001% of target cells through two cycles of sorting (Fig. 5). In the case of the ZZ-domain-displaying cells that showed high fluorescence intensity in immunostaining (see Fig. 3), the first cycle of sorting from the library with a frequency of 1/10,000 or 1/100,000 achieved a recovery rate of more than 90% (Fig. 5b). The Fc-part-displaying cells whose fluorescence intensity was lower than that of the ZZ domain-displaying cells were recovered with 50% of the rate of recovery from the library of 1/10,000 through the first cycle of sorting (Fig. 5c). Moreover, we attempted to recover cells displaying the Fc part controlled by the *UPR-ICL* promoter, which induced a stronger expression at 48 h or later, and those displaying the Fc part controlled by the *GAPDH* promoter, which induced a weaker expression (Fig. 2b), to examine the effect of expression level on the recovery rate of the target cells (Fig. 5d,e). As a result, we obtained higher recovery rates due to the higher expression levels of the target proteins in cells displaying the Fc part controlled by *UPR-ICL* (Fig. 5e), although it required a substantially longer time for cultivation. Meanwhile, a significant decrease in recovery rate was observed using the *GAPDH* promoter (Fig. 5d). All the cells displaying the ZZ domain or Fc part controlled by the *GAL1* and *UPR-ICL* promoters were effectively enriched through two cycles of flow cytometric sorting from the model libraries with a frequency of 1/1,000,000 (Fig. 5b,c, and e).

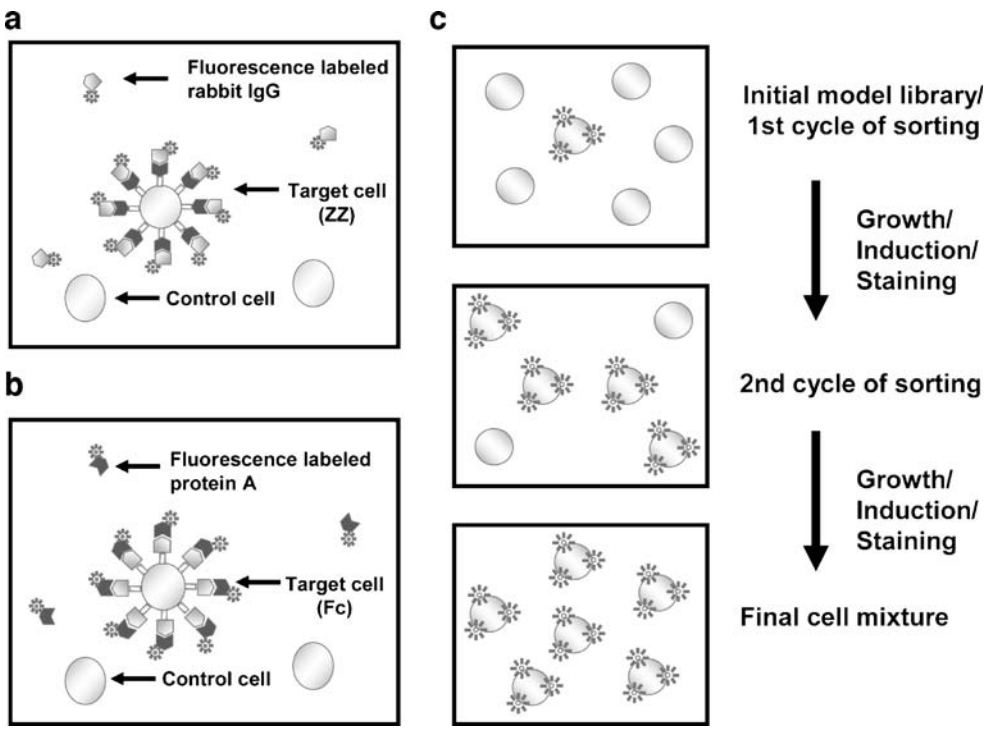

Fig. 1 Schematic illustrations of **a** staining for yeast cells displaying the ZZ domain with fluorescence-labeled rabbit IgG, **b** staining for yeast cells displaying the Fc part with fluorescence-labeled protein A. **c** Flow diagram for recovery of target cells using flow cytometric sorting

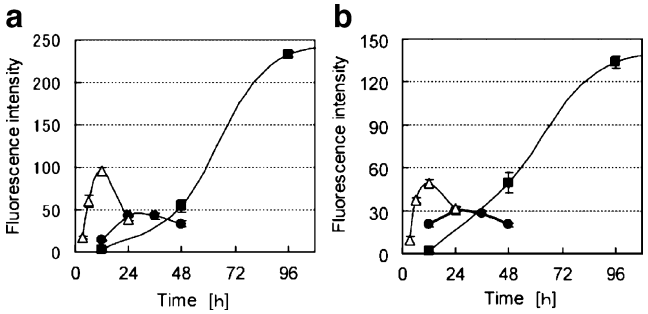

Fig. 2 Quantitative analyses of expression levels of displayed proteins controlled by *UPR-ICL* (*closed square*), *GAPDH* (*closed circle*) and *GAL1* (*open triangle*) promoters during cultivation: **a** Yeast cells displaying the ZZ domain were stained with FITC-labeled anti-protein A antibodies, and **b** yeast cells displaying Fc were stained with FITC-labeled anti-human IgG (Fc) antibody. Each data point and error bar represents the mean and standard error of three independent experiments, respectively

Discussion

Multitudes of successful experiences in the screening of the peptide library by microbial surface display have been reported. *S. cerevisiae* offers the advantage of post-translational modifications and processing of mammalian proteins, making it well suited for library display systems. In recent years, there have been several studies of affinity selection using yeast display systems by flow cytometric sorting or magnetic separation (Boder and Wittrup 1997; Furukawa et al. 2003; Yeung and Wittrup 2002). We attempted a high-efficiency recovery of the target cells on the basis of detection of protein–protein interactions by only flow cytometric sorting. Generally, a yeast display system is used for screening approximately 1×10^6 libraries or more; thus, it is important to recover the target cells from libraries with a frequency of at least 1/1,000,000 cells.

In this study, we report that yeast cells displaying target proteins can be highly efficiently recovered through two cycles of flow cytometric sorting using an improved yeast display system. We adopted the ZZ domain and Fc part as scaffold models for combinatorial screening. Various antibody fragments are most commonly used as scaffolds in protein engineering; however, the cell surface display of correctly assembled and folded antibody fragments is challenging. Bacterial proteins such as the Z domain, which are available for affibody molecules, have therefore been used as a scaffold, and the success in the use of the staphylococcal surface display system for fine affinity discrimination has been reported (Lofblom et al. 2005). Cells of the yeast strain displaying the ZZ domain or Fc part were used as the target cells (Fig. 1), and the recovery of these target cells from the cell mixture was confirmed (Table 2 and Fig. 5).

We compared the expression levels of the ZZ domain and Fc part between the yeast strains BY4741 and MT8-1 (Tajima et al. 1985) induced by the *UPR-ICL* promoter. Although MT8-1 has been commonly used in the surface display of the ZZ domain as a host strain (Nakamura et al. 2001), it was reported that BY4741 and BY4742 enable higher expression levels of the ZZ domain than MT8-1 (Shibasaki et al., submitted). Consistent with these results, BY4741 was able to achieve high expression levels of both proteins (data not shown); hence, we decided to use BY4741 as the host strain. Moreover, we compared the expression levels of the displayed proteins that were controlled by three types of promoter: *UPR-ICL*, *GAPDH*, and *GAL1* promoters (Fig. 2). In these expriments, there were no differences in the growth rates between the ZZ- or Fc-displaying cells and the control cells (data not shown). Although the maximum expression levels of the proteins controlled by the *UPR-ICL* promoter were the highest, the expressional induction required considerable time. Multiple cycles of selection may be required to achieve a high-efficiency recovery of the target cells from the extremely low frequency of the libraries as well as many other displayed screenings (Francisco et al. 1993; Furukawa et al. 2003; Yeung and Wittrup 2002). Indeed, we failed to achieve single-cycle isolation using the strong expression system controlled by the *UPR-ICL* promoter (details described below). Therefore, we determined whether the *GAL1* promoter, which is strongly and rapidly induced in the presence of galactose, is feasible for enhancing the throughput of displayed screening.

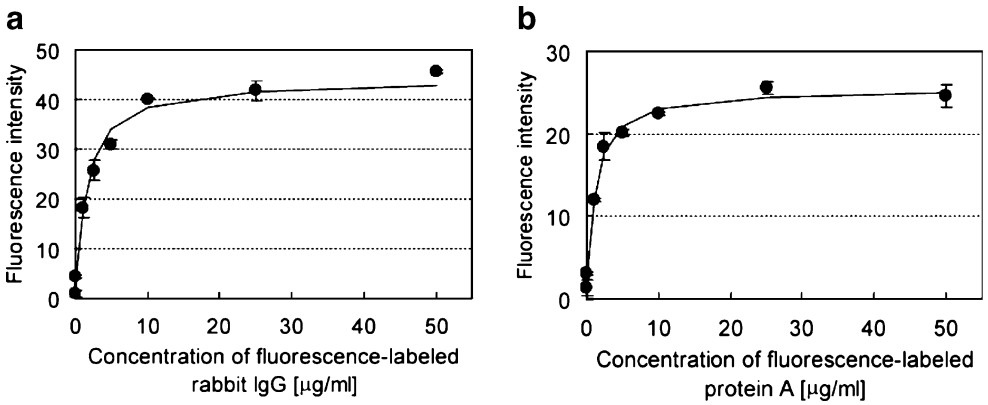

Fig. 3 Functional evaluation of displayed proteins controlled by the *GAL1* promoter: **a** adsorption for cells displaying ZZ estimated with FITC-labeled rabbit IgG and **b** that for cells displaying Fc estimated with Alexa Fluor 488-labeled protein A. Each data point and error bar represents the mean and standard error of three independent experiments, respectively

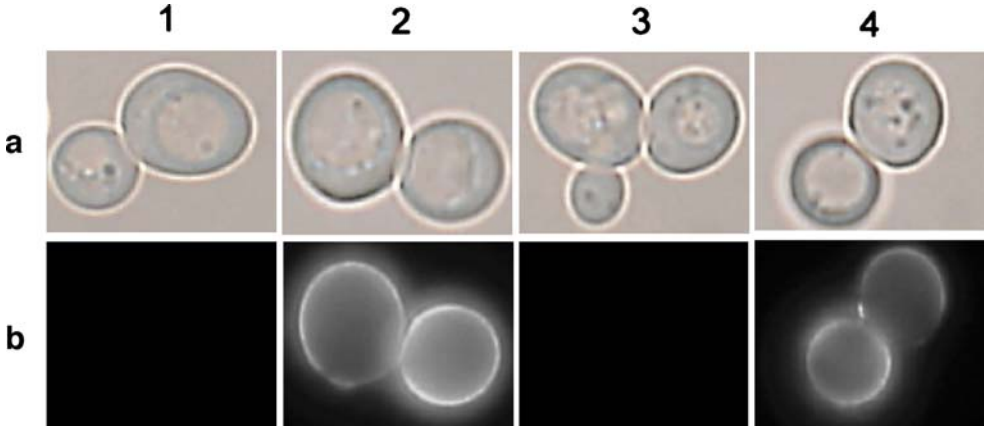

Fig. 4 Visualization of displayed proteins on yeast cell-surface: **a** differential-interference contrast micrographs; **b** fluorescence micrographs. Each column represents the following transformants: BY4741/pESC-URA (*column 1*), BY4741/pGUZZ (*column 2*), BY4741/pESC-URA (*column 3*), and BY4741/pGUFcM (*column 4*). Cells were stained with FITC-labeled rabbit IgG (*columns 1 and 2*) and Alexa Fluor 488-labeled protein A (*columns 3 and 4*)

The functional display of the bacterial ZZ domain or human Fc part on the yeast cell surface was confirmed (Figs. 3 and 4). As shown in Fig. 3, there was a marked difference between the two types of cell displaying the ZZ domain and Fc part. We estimated the association constant of the displayed ZZ domain for rabbit IgG (2.1×10^8 M^{-1}) and that of the displayed Fc part for protein A (6.6×10^7 M^{-1}). The association constant between the ZZ domain and the Fc part has been reported to be 6.8×10^8 M^{-1} (Jendeberg et al. 1995). The difference of the association constants is probably caused by fusion with anchor proteins. The Fc part was probably displayed as a monomeric form and hence showed low affinity for protein A. The difference in fluorescence intensity is useful for comparing the efficiency divergence of screening, although it is difficult to precisely identify the factor that causes this difference without the quantitation of the absolute expression levels of the ZZ domain and Fc part.

When a single cycle of sorting was performed for the libraries with a frequency of 1/1,000, there was only a slight difference in recovery efficiency between the two types of target cell (ZZ domain and Fc part induced by the *GAL1* promoter). With a frequency of 1/10,000 or 1/100,000, there were marked differences in recovery

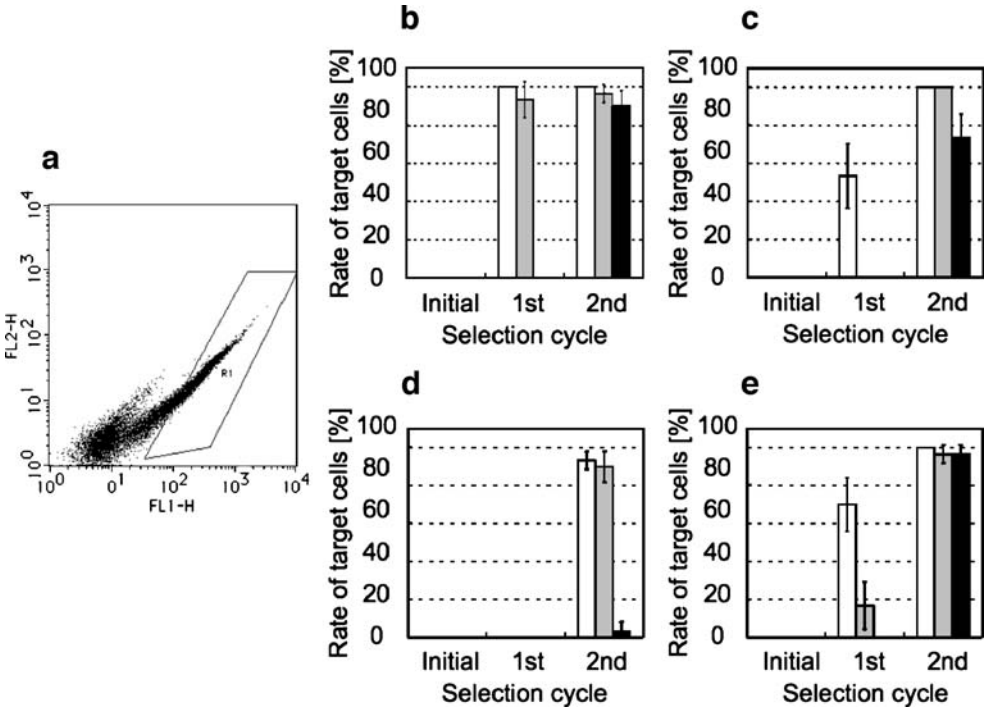

Fig. 5 Recovery from model libraries through two cycles of flow cytometric sorting: **a** the gates R_1 for flow cytometric sorting. Figure shows the data of the model library containing the ZZ-domain-displaying cells controlled by the *GAL1* promoter with a frequency of 1/10. FL1-H denotes the fluorescence intensity of *green*, and FL2-H denotes that of *orange*; **b** recovery rates of yeast cells displaying ZZ domain controlled by *GAL1* promoter; **c** recovery rates of yeast cells displaying Fc part controlled by *GAL1* promoter; **d** recovery rates of yeast cells displaying Fc part controlled by *GAPDH* promoter; **e** recovery rates of yeast cells displaying Fc part controlled by *UPR-ICL* promoter. *Symbols* represent the initial percentage of the target cells relative to the total cells: *open bars* 0.01%; *gray bars* 0.001%; *black bars* 0.0001%. Each data point and error bar represents mean and standard error of three independent experiments, respectively

efficiency between the two types of protein library, which is probably caused by the difference in the fluorescence intensity of the labeled cells. Therefore, we examined the effect of the expression levels of the target proteins on the recovery efficiency of Fc-part-displaying cells using the *UPR-ICL* and *GAPDH* promoters. The *UPR-ICL* promoter induced a strong expression instead of considerable induction time (Fig. 2). The increase in the rate of recovery from the libraries with a frequency of 1/10,000 or 1/100,000 was achieved after the first sorting (Fig. 5e). However, the recovery rate of the target cells significantly decreased with the use of the *GAPDH* promoter compared with that of the recovery system using the *GAL1* promoter (Fig. 5d). These results suggest that the expression levels of the target proteins have significant effects on the recovery rate. In the case of the ZZ-domain-displaying cells, we obtained an enhancement factor of up to 90,000-fold through a single cycle of sorting (Fig. 5b). Although there are several reports on affinity selection by flow cytometric sorting (Boder and Wittrup 1997; Francisco et al. 1993), this enhancement factor obtained through a single cycle of sorting is appreciably high.

In all the types of library with 1/10,000 and 1/100,000 frequencies using the *GAL1* and *UPR-ICL* promoters, two cycles of flow cytometric sorting provided an approximately 100% recovery rate, and a highly efficient sorting from the libraries with a frequency of 1/1,000,000 was finally achieved (Fig. 5b,c, and e). These results suggest that we can highly efficiently recover the target cells from an extremely low frequencies of library through two cycles of flow cytometric sorting using BY4741 as a host strain and using the *GAL1* or *UPR-ICL* promoter and that the expression system using the *GAL1* promoter provides a more rapid recovery of the target cells.

In summary, this study demonstrated the significant effect of expression levels of target proteins on the recovery rates by flow cytometric sorting. We showed the feasibility of high-efficiency recovery from an extremely low frequency of libraries based on protein–protein interactions using an improved yeast display system. We could recover the target cells from a model library with a frequency of 1/1,000,000 through two cycles of flow cytometric sorting. These results indicate that this method will extend the range of its applications to the study of protein–protein interactions and lead to the development of a rapid and highly efficient isolation system.

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