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Development of GFP-based high-throughput screening system for directed evolution of glucose oxidase

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Glucose oxidase (GOx) mutants with higher activity or stability have important role in industry and in the development of biosensors and biofuel cells. Discovering these mutants can be time-consuming if appropriate high-throughput screening (HTS) systems are not available. GOx gene libraries were successfully screened and sorted using a HTS system based on GOx activity dependent fluorescent labeling of yeast cells with tyramids and quantification of the amount of expressed enzyme by yeast enhanced green fluorescent protein (yGFP) tagging and flow cytometry. For this purpose, we expressed wild type and a mutant GOx as a chimera with the yGFP to confirm differences in catalytic activity between wild-type and mutant GOx. Fluorescence of yGFP is preserved during expression of chimera, and also after the oxidative enzymatic reaction. We have obtained a 2.5-fold enrichment in population of cells expressing active enzyme, and percentage of enzyme variants with enzymatic mean activity higher than wild type activity was increased to 44% after a single round of GOx gene library sorting. We have found two mutants with 1.3 and 2.3-fold increase in V_{max} values compared to the wtGOx. By simultaneous detection of protein expression level and enzyme activity we have increased the likelihood of finding GOx variants with increased activity in a single round of flow cytometry sorting.

[**Keywords:** Glucose oxidase; Yeast surface display; High-throughput screening; Yeast enhanced green-fluorescent protein; Directed evolution]

Glucose oxidase (GOx, β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) is industrially important enzyme, used in food preservation, textile bleaching, and as an analytical reagent for glucose level determination (1). Recent developments led to the construction of GOx based logical gates (2), amperometric biosensors and biofuel cells (3–5). GOx is a glycoprotein, consisting of two identical subunits and each subunit has non-covalently bound FAD co-factor. The enzyme catalyzes the oxidation of β-D-glucose to D-gluconolactone, using molecular oxygen as electron acceptor and producing hydrogen-peroxide (1,6).

Making enzyme more fit for industrial applications is a great challenge, and the knowledge about structure–function relation is an advantage. However, sometimes the structure of the enzyme is not available or the property we want to alter is not easily correlated to the known structure. In those instances directed evolution through random mutagenesis is a tool that enhances probability of finding novel variant with desired property or variant with several improved ones. For enzymes most important property is its activity and there are many reported enzymes with improved activity, obtained through directed evolution and high-throughput screening methods (7–13).

A breakthrough in the screening process has been the in vitro compartmentalization (IVC) where fluorescent enzyme assay takes place in microdroplets that are then sorted according to their fluorescence intensity by fluorescent activated cell sorting (FACS) (14–16). Most important aspect of directed evolution is to maintain connection between genotype and phenotype of the target enzyme in order to isolate the desired mutant. In previous work for GOx library screening this connection is obtained through covalent staining of cells with enzymatic activity with fluorescein-tyramide that binds to tyrosine residues of cell-surface proteins (17). With this method it is possible to quantitatively determine cells with higher activity enzyme variant, but fluorescence intensity also depends of the amount of expressed enzyme on cell surface, which can lead to false selection. Further development in high-throughput screening of GOx libraries has been the introduction of yeast surface display of GOx and a substrate delivery system, using β-D-octyl-glucoside and β-glucosidase for precise control of the start of the reaction as well as glucose concentration. In this work another novelty was normalization of fluorescence intensity using fluorescent antibodies for c-myc epitope for quantification of the expressed protein on the cell surface (9). Even though antibody binding is highly specific, labeling of cells can be time consuming as well as inconsistent (18).

Green fluorescent protein (GFP) is a 27 kDa monomer first time isolated form jellyfish *Aequorea victoria* (19,20), with the tendency to form dimers at the high concentrations (21). The GFP chromophore is a result of autocatalytic cyclization and air-mediated oxidation (20), and recently it has been shown that chromophore formation affects biological pathways associated with oxidative

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stress (22). Wild-type GFP absorbs light at two wavelengths, 395 nm (excitation maximum) and 475 nm, which can be explained by existence of the two GFP chromophore forms, neutral and anionic (23). In order to expand application of GFP in research, Cormack and co-workers found a GFP mutant (S65T, F64L, known as EGFP) with shifted excitation maximum to 488 nm (24), which is one of the most common wavelengths used for FACS.

Because of its small size, GFP is easily expressed as fusion protein at either N- or C-terminus, making it widely used in biochemical and biological research (19,25). When working with fusion proteins it is important for both entities to retain their functions as well as proper folding. As it was shown in the work of Wu and co-workers, they have correlated activity of the organophosphorus hydrolase with the GFP fluorescence expressed in *Escherichia coli* culture; even though the activity of the enzyme was reduced while fused to the GFP, it was recoverable after separation of the GFP with enterokinase (19). Using split-GFP technology, normalization of the protein expression level was employed in the microtiter-plate screening of small transaminase library (approximately 600 clones) expressed in *E. coli*, in which they identified the variant with improved activity and variant with improved expression level (26). Along with the use as a fusion protein marker, GFP can also be employed as an indicator of successful cloning, with either non-recombinants that emit fluorescence (27) or recombinants that do (28).

Using different yeast signal peptides, both GFP and EGFP have shown low secretion levels in yeast cells (29,30). With codon optimization and introduction of new mutations (S65G, S72A) yeast-enhanced GFP (yGFP) has been developed and used as an efficient reporter in *Saccharomyces cerevisiae* and *Candida albicans* (31–33). It has been successfully secreted to culture media (31,34), and displayed on the yeast cell surface, where it can be easily visualized using fluorescent microscopy (35) and quantified using flow cytometry (36–38). Yeast surface display (YSD) has been proved as a powerful technique for protein engineering by directed evolution in terms of improved affinity, specificity, stability (39–41) and catalytic activity (9). Some of the most significant features of this system include eukaryotic biosynthetic apparatus for efficient protein folding, rapid library screening by FACS and characterization of isolated mutants directly without need for purification of individual clones (39,42). Enzymes expressed on the yeast surface are showing great potential for use in the biofuel cells as a whole cell biocatalysts, because of easy production and simple genetic manipulation (43,44).

In this study we have improved quantification of the amount of expressed GOx on the yeast surface using yGFP as a fusion partner. With simultaneous detection of protein expression level with yGFP tagging and GOx activity dependent fluorescent labeling of yeast cells with tyramids, we have developed flow cytometry HTS systems for finding GOx variants with increased activity.

MATERIALS AND METHODS

Fluorescent tyramide synthesis DyLight650-tyramide was synthesized as previously described (9). Tyramine-HCl (20 mM), triethylamine (25 mM) and *N*-hydroxysuccinimide-DyLight650 (Thermo Fisher Scientific, Waltham, MA, USA) (20 mM) were dissolved in dimethylformamide (DMF) and incubated for 2 h at room temperature. Solution was diluted in ethanol to final concentration of 2 mM DyLight650-tyramide and stored in dark at 4°C until use.

Construction of yGFP-pCTCON2 vector Using standard PCR protocol with sense primer (ATT***GGATCC***TCTGGTATGTCTAAAGGTGAAGAATT) and antisense primer (AAT***CTCGAG***TCATTTGTACAATTCATCCATAC) we amplified yeast enhanced GFPM (monomeric form of the yGFP) from pCT-GFPM (provided by Eric Shusta, University of Wisconsin). This version of GFP is codon optimized for yeast expression and contains three mutations (S65G, S72A, A206K) that enhance fluorescence and solubility (33). Using these primers we introduced Gly-Ser-Ser-Gly linker (underlined sequence in forward primer in combination with *Bam*HI restriction site) before start codon of the yGFP. The yGFP gene was inserted in pCTCON2 vector (provided by Dane Wittrup, Massachusetts Institute of Technology; Addgene reference no. 41843) between *Bam*HI and *Xho*I restriction sites, leading to excision of *c-myc* epitope from the vector.

Glucose oxidase library First, we inserted wild-type GOx (wtGOx; accession no. P13006) gene from wtGOx-pCTCON2 plasmid (45) in yGFP-pCTCON2 vector using sense (ATC***GCTAGC***AGCAATGGCATTGAAGC) and antisense (ATC***GGATCC***TCCCTGCATGGAAGC) primers with *Nhe*I and *Bam*HI restriction enzymes. With the same primers we created GOx library using GeneMorph II Random Mutagensis Kit (Agilent Technologies, Santa Clara, CA, USA) and *E. coli* XL10Gold competent cells. PCR mix contained 45 ng/μL of template (wtGOx-yGFP-pCTCON2, for low mutation frequency, 2 to 5 nucleotide mutations per gene), 2.5 ng/μL of each primer, and the rest of the components from the kit. The reaction was heated to 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 2 min, followed by final extension at 72°C for 10 min. Reaction products were digested with *Dpn*I enzyme for 2 h at 37°C. Purified PCR products were digested with *Bam*HI and *Nhe*I restriction enzymes and used as megaprimers for PCR with Phusion High-Fidelity polymerase (New England Biolabs, Ipswich, MA, USA). Megaprimer PCR mix contained 450 ng of megaprimers, 50 ng of template (wtGOx-yGFP-pCTCON2), 3% DMSO and other components from the kit. Reaction was heated to 98°C for 1 min, followed by 25 cycles of 98°C for 30 s, 60°C for 1 min, 72°C for 4 min, followed by final extension at 72°C for 10 min. The reaction products were digested with *Dpn*I enzyme for 2 h at 37°C and transformed to ultracompetent *E. coli* XL10Gold. Plasmid DNA was isolated using Macherey–Nagel Plasmid DNA kit (Macherey–Nagel, Düren, Germany).

Transformation of *S. cerevisiae* EBY100 cells Plasmid DNA was introduced into *S. cerevisiae* EBY 100 cells (provided by Dane Wittrup, Massachusetts Institute of Technology) as described previously (46), using a 42°C heat-shock step for 1 h. Cells were grown in YNB-CAA (yeast nitrogen base–casaminoacid) glucose medium (2% (w/v) glucose) for 48 h at 27°C, 160 rpm, then induced to express GOx-yGFP construct by transferring to YNB-CAA galactose (2% (w/v) galactose) medium (final OD_{600} ~ 1 in galactose medium) under the same conditions for 18 h.

Flow cytometry analysis Cells expressing GOx-yGFP construct were washed three times with PBS (phosphate buffered saline) and adjusted to concentration of 2 x 10^8 cells/mL. Fluorescent cell labeling was done by slightly modified procedure reported previously (9,17). A 25 μL of suspension containing 2 x 10^6 cells, 20 μM DyLight650-tyramide, 10 U/mL horseradish peroxidase (HRP, AppliChem, MT, USA) and 50 mM glucose was added to 500 μL 1.5% Abil Em 90 (Evonik, Essen, Germany) in light mineral oil (Sigma–Aldrich, Darmstadt, Germany) and emulsified using MICCRA D-1 dispenser (Miccra, Heitersheim, Germany) at 8000 rpm for 3 min on ice. For the negative control, reaction mix contained all components except glucose and was prepared in the same way. Reaction mixture was incubated on ice for 30 min and stopped by adding 100 μL 0.5 M ascorbic acid and 100 μL 1% BSA in PBS, and vortexed. Cells were recovered from emulsions as described (17) and resuspended in 1 mL of PBS.

We analyzed the cells using BD Influx flow cytometry system (Becton Dickinson, Franklin Lakes, NJ, USA). The analysis rate was 1000–5000 events/s and the sorting speed was 10–100 events/s. We used the 488 and 642 nm laser excitation wavelengths for detection, and emissions were detected using the 530 and 670 nm filters. The positive cells were gated on a fluorescence double plot for higher ratio of red versus green fluorescence and sorted in single-cell mode on the YNB-CAA glucose chloramphenicol (25 μg/mL chloramphenicol) media agar plates.

Agar plate and microtiter plate assay After FACS sorting, cells on plates were incubated for 3 days at 27°C. Around 60 colonies were transferred from glucose plates to YNB-CAA galactose plates and incubated for another 24 h. Cells on galactose plates were overlaid with 1% agar medium containing 250 mM glucose, 2 U/mL HRP and 2 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), AppliChem). Green halos around cells are indication of the GOx activity.

Single colonies from glucose plates were inoculated in the micro-titer plate (MTP) wells containing 150 μL of YNB-CAA glucose chloramphenicol medium and were grown for 48 h at 25°C, 500 rpm. A volume of 5 μL of the cell culture was transferred to a new MTP well containing 30 μL YNB-CAA glucose media and grown under the same condition for 24 h. Expression of GOx-yGFP was induced adding 80 μL of YNB-CAA galactose medium, culturing cells under the same conditions for 18 h before measuring GOx activity. In each MTP five colonies with wtGOx-yGFP construct were inoculated for standardization. To measure the GOx activity, 5 μL of the yeast cell culture was transferred to MTP wells containing 95 μL of PBS pH 7.4. Subsequently, 100 μL of the ABTS solution (500 mM β-D-glucose, 2 mM ABTS and 2 U/mL HRP) was added and the absorbance was followed at 405 nm, every 30 s, for 5 min. A 1 U of GOx activity is defined as the amount of enzyme that oxidizes 1 μmol of glucose in 1 min at 25°C. For each measurement the slope of the linear region was calculated and normalized to the OD_{600} of the cells in each well.

Kinetic analysis of GOx expressed on YSD and as a soluble protein Cells expressing best performing GOx variants were sequenced, and kinetic analysis was performed. Yeast cells were grown in YNB-CAA glucose medium for 48 h at 27°C, 160 rpm, than induced to express GOx by transferring to YNB-CAA galactose medium (final OD_{600} ~ 1 in galactose medium) under the same conditions for 18 h. Cells were washed with PBS and set to OD_{600} = 10. To determine relative kinetic constants, MTP-based ABTS assay described in previous paragraph was

used to measure activity with glucose concentrations ranging from 10 mM to 500 mM in PBS buffer pH 7.4. For each measurement the slope of the linear region was calculated and normalized to the OD_{600}. Results were fitted onto Michaelis–Menten hyperbola using Origin 8.0.

The kinetic constants of purified soluble GOx variants without yGFP were determined by measuring the activity of GOx in different concentrations of glucose (from 5 to 500 mM) in PBS buffer pH 7.4. Michaelis–Menten hyperbola was fitted directly using the Origin 8.0 software. The V_{max} values were calculated from the protein concentration by measuring absorbance at 280 nm (the absorption of 1 mg/mL GOx is considered equivalent to 1.5 AU based on the sequence, as calculated using ProtParam).

Cloning, expression and purification of GOx variants from *Pichia pastoris* KM71H Selected mutants were amplified using corresponding GOx-yGFP-pCTCON2 plasmid as a template and sense (ATCT***CTCGAG***AAAAGAAGCAATGGCATT GAAG) and antisense (GAAGC***TCTAGA***GCTCACTGCATGG) primers for PCR. With these primers only GOx genes were amplified, and following restriction of PCR products and vector with *Xho*I and *Xba*I restriction enzymes, they were inserted in pPICZαA vector for extracellular expression in *Pichia pastoris*. Transformation of *P. pastoris* KM71H (Invitrogen), expression and purification of expressed GOx variants, using a linear gradient of 10–500 mM sodium phosphate buffer (pH 6) over 10 column volumes for the ion-exchange chromatography, was performed as we previously described (47).

RESULTS

Expression of GOx-yGFP construct In this study we have used yGFP as a marker of GOx expression level on the yeast surface for high-throughput screening of GOx library. Introducing yGFP as an expression level marker for YSD, *c-myc* epitope from the pCTCON2 vector was excised (Fig. 1A), and fusion protein of GOx and yGFP was made (Fig. 1B) (48).

In order to achieve successful screening for improved GOx specific activity, at first we have tested activity in MTP of wild type and A2 mutant (9) GOx expressed as a chimera with yGFP on yeast surface (Fig. S1). We have seen that even though the activity of GOx-yGFP chimera decreased compared to the activity of GOx without yGFP, the ratio in activity between the wild type enzyme and more active A2 mutant is maintained during expression as chimera with yGFP on the yeast surface (Fig. S1).

During FACS analysis of yeast cells expressing yGFP on its surface, we observed two distinctive populations of cells; one population showing fluorescence corresponding to the yGFP and second population of non-fluorescent cells (Fig. S2). In an attempt to determine if the lack of fluorescence in the second population is consequence of yGFP misfolding or cell death, we sorted separately both populations in tubes. Sorted cells were re-analyzed in order to confirm population purity, and from each population 1000 events were sorted directly on YNB-CAA glucose chloramphenicol agar plates. After two days of growth on selective media, we observed that survival rate is 35% for fluorescent cells, and only 1% for non-fluorescent cells.

Fluorescent tyramides assay optimization In screening systems previously developed for GOx (9,17), tyramide-fluorescein was used as a labeling agent for the active variants of GOx, and since its excitation and emission wavelengths overlap with the ones from the yGFP, new fluorescent tyramide was synthetized. We synthesized and tested two different fluorescent tyramides, CC-tyramide (3-carboxy-coumarin-tyramide, ex 355 nm, em 460 nm) and DyLight650-tyramide (ex 642 nm, em 670 nm), in reaction with cells expressing wtGOx-yGFP construct and glucose as substrate (data not shown). From this initial fluorescent tyramide test, we decided to continue screening of the GOx library with DyLight650-tyramide, because of its higher quantum yield and fluorescence stability compared to the CC-tyramide. Using DyLight650-tyramide we have set enzymatic reactions in emulsions with yGFP and wtGOx-yGFP expressed on YSD. In order to determine optimal assay conditions, one set of reactions was with β-D-octyl-glucoside/β-glucosidase (9) and the other was with glucose (17). Analyzing cells on FACS we haven't observed any difference in the number of stained cells between cells expressing yGFP (negative control) and wtGOx-yGFP (positive control) when β-D-octyl-glucoside/β-glucosidase system was used (Fig. 2A). When glucose was used as a substrate we were able to clearly identify cells with GOx activity in P1Q2 field (Fig. 2B).

GOx library screening With established assay parameters for GOx screening, we created low frequency mutation library of GOx mutants using random mutagenesis method with Mutazyme II enzyme and analyzed yeast cells by FACS. Negative control where we omitted glucose from reaction mix was also included. From the cell analysis we marked P3 gate to sort cells from GOx library (Fig. 3). The chosen gate isolates GOx mutants with highest ratio of red fluorescence (which is the result of GOx enzyme activity) over green fluorescence (which corresponds to the amount of the expressed GOx-yGFP protein). Sorting cells from this gate should lead to the selection of the most active GOx mutants from the library.

Around sixty sorted cells from glucose agar plates were regrown on galactose agar plates and tested for activity using ABTS agar plate assay (Fig. 4A). Agar plate assay showed enrichment in positive cells from approximately 35% before sorting to 88% after a single round of sorting. In order to quantify number of sorted GOx variants with activity higher than wtGOx, we picked 100 random clones and tested them with MTP assay for enzyme activity (Fig. 4B). In a single round of sorting, the population of statistically

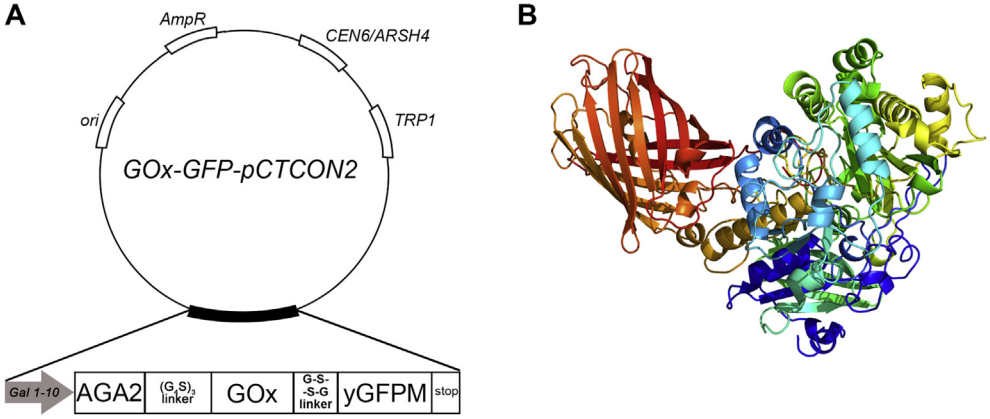

FIG. 1. (A) Schematic of GOx-yGFPM-pCTCON2 plasmid construct. (B) Three-dimensional structure of GOx-GFP fusion protein modeled on Phyre2 server (48).

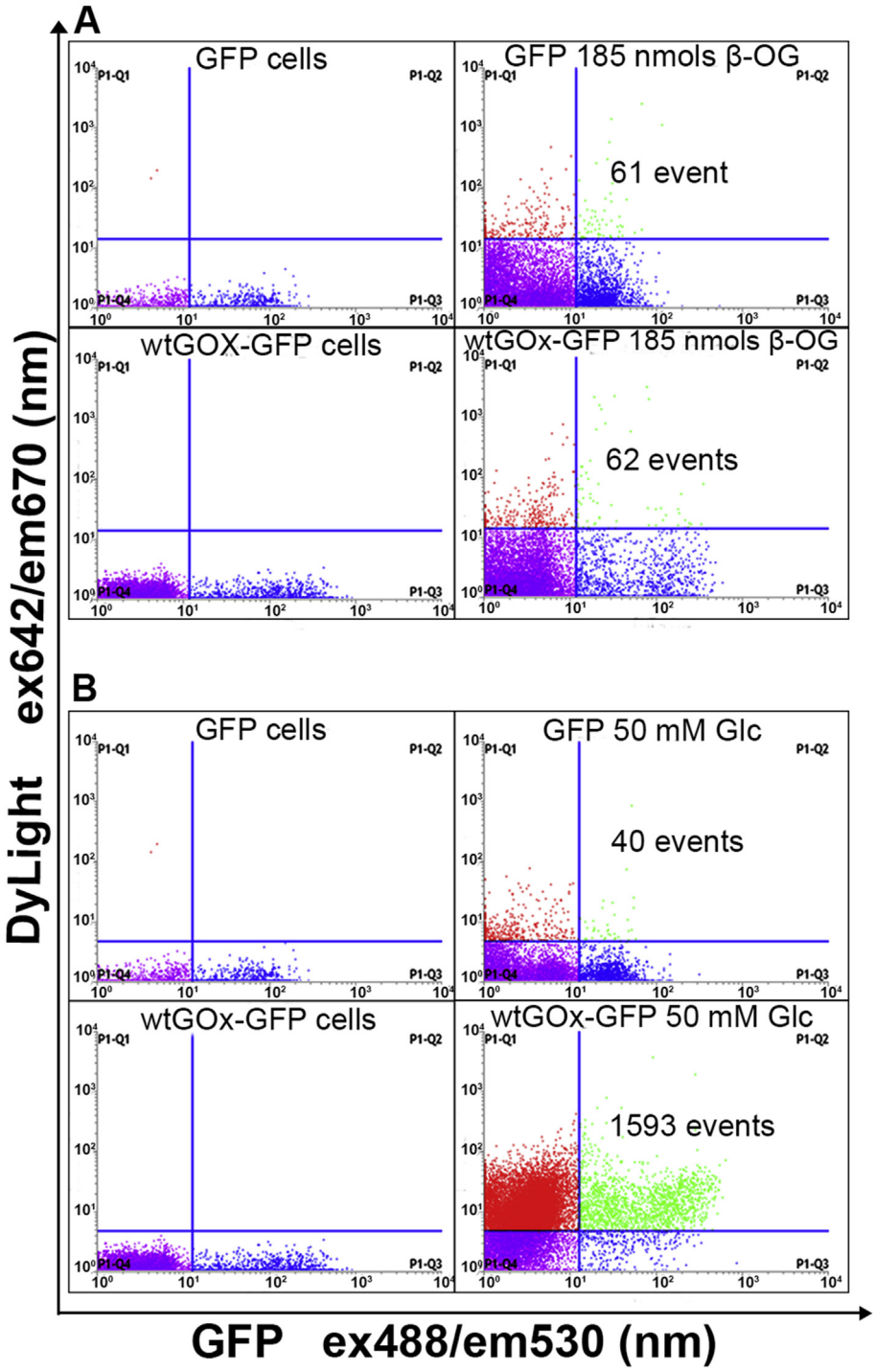

FIG. 2. FACS recordings (10^4 recorded events) of yGFP and wtGOx-yGFP in different DyLight650-tyramide assays performed in water-in-oil emulsions; double plots of yGFP fluorescence representing abundance of GOx-yGFP molecules on cell surface versus DyLight 642 fluorescence corresponding to GOx activity. (A) yGFP and wtGOx-yGFP expressing cells in reaction with 185 nmol β-D-octyl-glucoside and β-glucosidase. (B) yGFP and wtGOx-yGFP expressing cells in reaction with 50 mM glucose.

significant more active clones compared to the wtGOx-yGFP was increased, from around 10% before sorting to 30% after sorting (activity higher for more than one standard deviation of the mean GOx activity).

Characterization of best performing GOx variants Three most active clones from population of sorted cells have been sequenced, and relative kinetic constants V_{max} and K_m between wtGOx, wtGOx-yGFP and mutants expressed on yeast surface were compared (Fig. S3). As was expected, wtGOx expressed on yeast surface is showing 2.1-fold higher relative V_{max} than wtGOx-yGFP, which confirms our initial finding that GOx activity is decreased when expressed as fusion protein with yGFP. Two out of three selected clones have mutation at the same position (A292T), and as it can be seen in Table 1, they have relative V_{max} values 1.3-fold higher compared to the starting wtGOx-yGFP enzyme variant when expressed on yeast surface, and also when expressed soluble without yGFP and compared to the wtGOx. Third clone

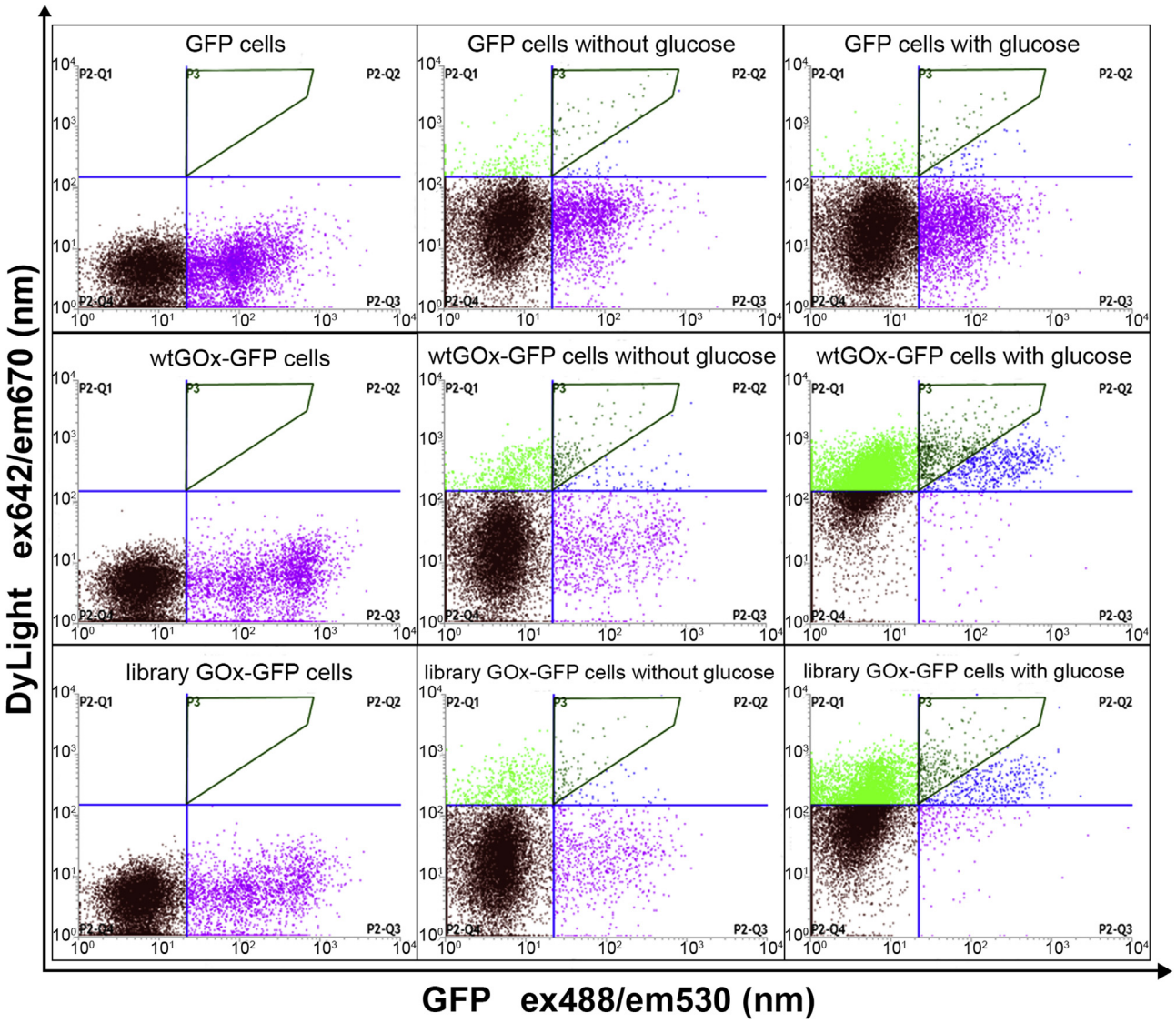

FIG. 3. FACS recordings (10^4 recorded events) of cells expressing yGFP, wtGOx-yGFP and GOx library without and with 50 mM glucose in reaction assay with DyLight650-tyramide in water-oil-emulsions. Double plots of yGFP fluorescence representing abundance of GOx-yGFP molecules on cell surface versus DyLight 642 fluorescence corresponding to GOx activity. Upper panel represent yGFP cells, middle panel represent wtGOx-yGFP cells, lower panel represent GOx library cells. From left to right panels are organized in the following order: analyzed cells without treatment in emulsion, recovered cells from emulsion after reaction with DyLight650-tyramide in absence of glucose, recovered cells from emulsion after reaction with DyLight650-tyramide in presence of 50 mM glucose. P3-sorted region of GOx library.

(Y249H) when expressed as a soluble protein without fusion with yGFP showed 2.3-fold increase in V_{max} value compared to the soluble wtGOx.

DISCUSSION

Directed evolution is a method of choice for engineering enzymes toward desired properties, and in combination with the high-throughput screening systems based on flow cytometry the probability of finding improved enzyme variants significantly increases (15,16). We have developed a high-throughput screening method for glucose oxidase mutant gene libraries based on yGFP as a marker of protein expression level and flow cytometry sorting. Using yGFP for avoiding labeling of cells with primary and fluorescent secondary antibodies, simplified and improved precision of measuring GOx protein expression level compared to our previous work (9). When working with chimera of GFP and an enzyme, one of the most important aspects is maintenance of function of both proteins. It has been shown that GFP can decrease enzyme activity expressed in *E. coli*, which was restored after cleavage with enterokinase (19). Comparing activites of wild type and mutant GOx expressed as chimera with yGFP on the yeast surface, we have seen that the difference in enzymatic activity of wild type and mutant is maintained, but decreased compared to the activity of the same variants expressed without yGFP. These results are consistent with previously reported ones where wild type GOx and B11 mutant were expressed and purified as chimera with Aga2 protein (45).

Analyzing yeast cells expressing yGFP on FACS, we observed a population of non-green fluorescent cells, that did not grow on selective media after sorting. We also noticed that observed dead non-green fluorescent cell population could be fluorescently labeled by the DyLight650-tyramide, which is probably due to the permeability of the cell membrane that allows dye uptake. These results indicate that using yGFP as a marker of enzyme expression level enables us also to exclude population of dead cells from sorting which increases the sorting speed and efficiency.

Previously we have developed high-throughput screening methods for GOx library using fluorescein-tyramide and glucose

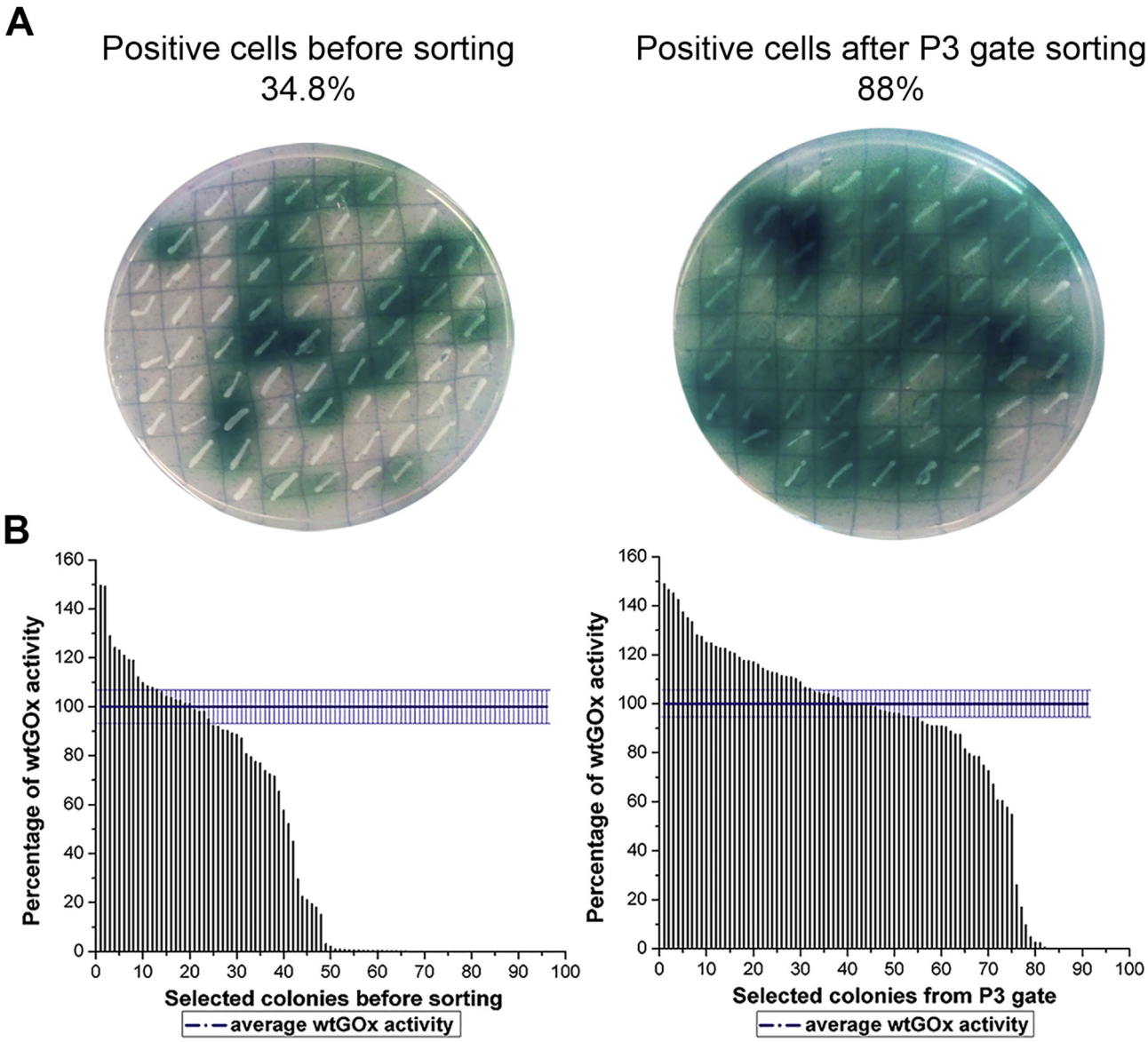

FIG. 4. (A) ABTS agar plate assay of GOx library showing percentage of active colonies before sorting and after P3 gate sorting. (B) MTP analysis of GOx variants activity compared to the average wtGOx-yGFP activity before sorting and after P3 gate sorting.

as a substrate (17), or β-D-octyl-glucoside and β-glucosidase as a glucose delivery system for GOx (9). Because fluorescence of the fluorescein overlaps with the yGFP fluorescence, we have tested new DyLight650-tyramide with both reported glucose delivery systems for GOx-yGFP screening. One of the possible reasons why β-D-octyl-glucoside/β-glucosidase system has shown to be inadequate for screening of GOx fused with yGFP could be decreased activity of the GOx when expressed as a fusion protein. Therefore, the amount of delivered glucose from β-D-octyl-glucoside previously reported (9) is probably less than optimal compared to adding glucose directly (17) for detection of GOx-yGFP activity.

TABLE 1. Activities of best performing variants sorted from GOx library measured at pH 7.4.

GOx variant	Yeast surface display[a]		Purified protein[b]	
	Relative V_{max} (IU mL^{-1}/OD$_{600}$)	Relative K_m (mM)	V_{max} (IU mg^{-1})	K_m (mM)
wtGOx-yGFP	1.55 ± 0.02	18.4 ± 0.6	/	/
E6 (A292T) GOx-yGFP	1.98 ± 0.01	23.5 ± 1.0	56.32 ± 0.27	14.6 ± 0.1
A11 (A292T) GOx-yGFP	2.13 ± 0.07	21.2 ± 1.6	55.08 ± 0.58	16.4 ± 1.0
H8 (Y249H) GOx-yGFP	1.16 ± 0.06	17.5 ± 2.0	98.45 ± 1.07	15.8 ± 0.5
wtGOx	3.29 ± 0.09	16.2 ± 0.8	42.60 ± 0.48	16.2 ± 0.5

[a] Mutants E6, A11, H8 and wtGOx were expressed with yGFP tag and compared on yeast surface, and compared to wtGOx without yGFP tag expressed on yeast surface. Data are the averages ± standard deviation of three measurements.

[b] Mutants E6, A11, H8 and wtGOx were expressed without yGFP tag in *Pichia pastoris* KM71H and purified from fermentation liquid. Data are the averages ± standard deviation of three measurements.

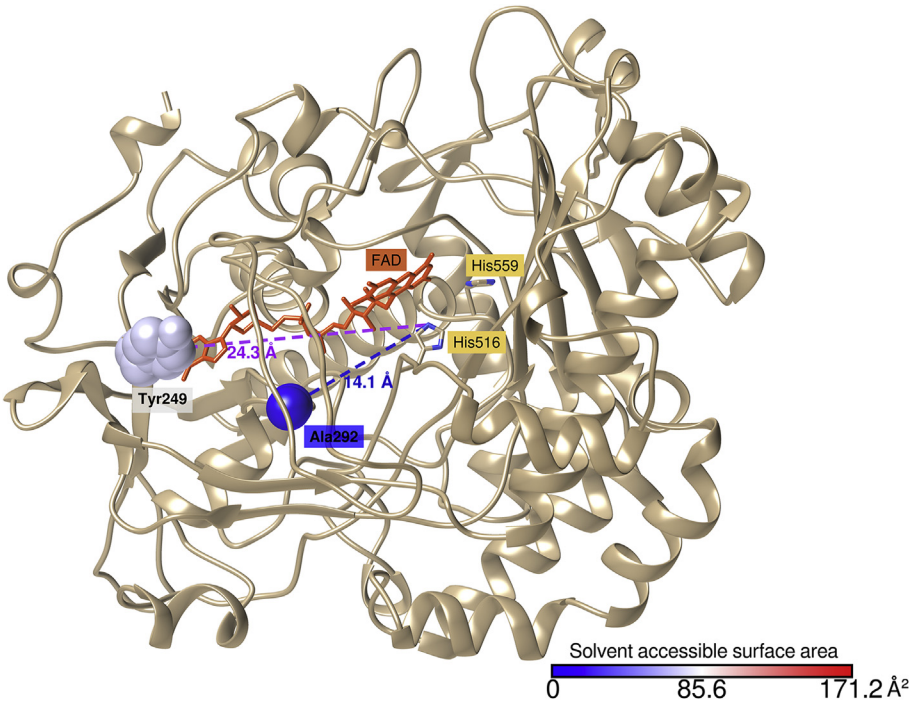

FIG. 5. Three-dimensional structure of the GOx protein (PDB: 1CF3) showing positions of the residues A292 and Y249 colored by the solvent accessible surface area (deep blue color represents less solvent accessible residue, and the light grey represents more solvent accessible residue). Structure also presentes GOx active site (consisting of FAD co-factor, His516 and His559), and calculated distances between Cα atom of the A292 and Y249 residues and N5 atom of the His516 (49).

After sorting of yeast cells expressing GOx-yGFP gene library with highest ratio of red over green fluorescence, we obtained a 2.5-fold enrichment of positive cells in a single round of sorting. Also the percentage of enzyme variants with enzymatic mean activity higher than wild type activity was increased from 21% to 44% after one round of sorting, which is better result compared to increase from 24% to 36% we obtained in our previous work (9). Screening one library with a low mutational load, we have found a mutant with single mutation A292T not previously reported that had 1.3-fold increase in relative V_{max} compared to the wtGOx-yGFP when expressed on the surface of yeast cells and as a soluble protein without yGFP tag. Residue A292 is located in the inner unstructured part of the GOx, which makes it less accessible to the solvent, and is relatively close to the active site (Fig. 5) (49). Another mutant, Y249H, showed 2.3-fold increase in V_{max} value when expressed soluble without the yGFP tag, but not as high increase when expressed on the yeast surface with yGFP tag. Residue Y249 is more solvent accessible when looked on a single GOx subunit (Fig. 5), but it is oriented toward dimer interface (Fig. S4). Since this mutation changes hydrophobic residue with the charged polar one, it is possible that yGFP tag influences dimerization and that this mutation has positive effect on the activity of the soluble GOx dimer. Although it is well known fact that immobilized enzymes have decreased activity compared to soluble ones due to the diffusional limitations, relative ratio of activities of wild type and mutants was proved to remain similar whether they are expressed as soluble or on the surface of yeast cells (45).

Both mutants have slightly increased K_m value compared to the wtGOx when expressed on the yeast surface, which is result of the screening conditions in 50 mM glucose where enzyme activity is mostly influenced by k_{cat} and not by K_m, thus increasing the likelihood of finding mutants with increased k_{cat} than finding mutants with decreased K_m.

There are differences in kinetic parameters for GOx when expressed in an immobilized form on the yeast surface with yGFP tag and when expressed in a soluble form without yGFP tag, like decreased V_{max} and increased K_m, but this is a very well known effect of diffusional limitations on immobilized enzymes. Nevertheless, increased activity of GOx mutants versus wtGOx was retained when they were expressed in a soluble form in *Pichia* expression system which was similar to the results previously obtained for other GOx mutants (45).

We have shown that yGFP tagging for detection of protein expression level by flow cytometry and DyLight650-tyramide labeling for detection of enzyme activity could be applied as a high-throughput screening system for finding enzyme variants with improved activity in one round of flow cytometry sorting.

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