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Ultra-High-Throughput Screening Method for the Directed Evolution of Glucose Oxidase

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SUMMARY

Glucose oxidase (GOx) is used in many industrial processes that could benefit from improved versions of the enzyme. Some improvements like higher activity under physiological conditions and thermal stability could be useful for GOx applications in biosensors and biofuel cells. Directed evolution is one of the currently available methods to engineer improved GOx variants. Here, we describe an ultra-high-throughput screening system for sorting the best enzyme variants generated by directed evolution that incorporates several methodological refinements: flow cytometry, in vitro compartmentalization, yeast surface display, fluorescent labeling of the expressed enzyme, delivery of glucose substrate to the reaction mixture through the oil phase, and covalent labeling of the cells with fluorescein-tyramide. The method enables quantitative screening of gene libraries to identify clones with improved activity and it also allows cells to be selected based not only on the overall activity but also on the specific activity of the enzyme.

INTRODUCTION

The *Aspergillus niger* enzyme glucose oxidase (GOx) is used in the food processing and pharmaceutical industries, as a component of biosensors in the medical diagnostics field (Bankar et al., 2009; Rossi et al., 2004; Wong et al., 2008) and to manufacture miniature biofuel cells that can power biomedical implants such as insulin pumps (Mano et al., 2002, 2004). The output of these devices is limited by the performance of GOx within the anodic compartment (Heller, 2004; Mano and Heller, 2003), but this can be enhanced by increasing the enzyme activity under physiological conditions (pH 7.4 and 5 mM glucose). Directed evolution, involving iterative cycles of mutation and screening, is one approach that allows such improved enzyme variants to be produced (Arnold and Volkov, 1999). The limiting step has been the low-throughput screening process, but breakthroughs in this field include in vitro compartmentalization technology, where fluorescent enzyme assays take place in microdroplets that are then sorted according to their fluorescence intensity by fluorescence-activated cell sorting (FACS).

During directed evolution, it is necessary to maintain a connection between the genotype and phenotype. This is easily achieved in the microtiter plate (MTP) screening format by constraining reactions within individual wells, but a different approach is required with FACS. One solution is to use a fluorescence assay that covalently stains the cells containing the enzymatic activity. For example, the tyramide-fluorescein assay stains yeast cells directly by binding fluorescence-labeled tyramine to tyrosine residues on cell-surface proteins. Such an assay has been used to select horseradish peroxidase variants without compartmentalization (Lipovsek et al., 2007). However, the GOx assay is a coupled reaction so there would be too much crosstalk between cells without an initial compartmentalization phase. Even so, compartmentalization takes ~3 min and if the glucose is supplied at the beginning of the reaction, substantial crosstalk between the cells occurs during emulsification. It has been impossible to increase the purity of the reference mix to more than 40% positive cells (expressing the active enzyme) after sorting using this method (Prodanovic et al., 2011).

We overcame the challenges and limitations discussed above and further expanded the GOx screening system by adding significant improvements to the tyramide fluorescein assay. These include (1) covalently linking the expressed enzyme molecules to the yeast surface (preventing the migration of protein molecules from one cell in the vicinity of another), (2) determining the number of GOx molecules per yeast cell by antibody staining (allowing the measurement of specific activity), and (3) delivering glucose through the oil phase after the emulsification step is completed (starting the reaction only after the compartments are formed around each cell). The last modification is probably the most important because it allows precise control of the start of the reaction and the amount of glucose delivered to the system. Because glucose itself is insoluble in the oil phase, it was delivered as β-octylglucoside (β-OG), which has a detergent-like structure and accumulates at the oil-water interface. The presence of β-glucosidase in the aqueous phase results in the hydrolysis of β-OG, thus releasing glucose into the water and making it available to GOx reaction (Figure 1). This article is reporting the use of an auxiliary enzyme to provide an enzyme substrate in situ although a more universal approach involving the delivery of substances to emulsions by nanodroplets has been reported (Bernath et al., 2005). Our modifications allow the quantitative selection of improved enzymes based on their

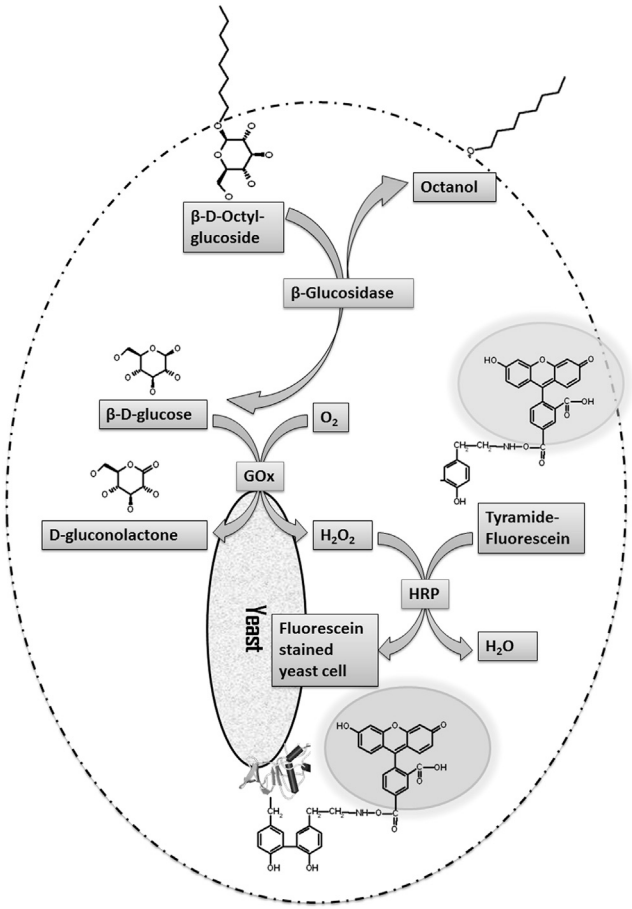


Figure 1. General Overview of the Tyramide-Fluorescein FACS Screening System

Yeast cells expressing GOx enzyme variants are encapsulated in water-in-oil single emulsions together with the components needed for the enzymatic reaction and β-glucosidase. After the compartments are formed around the cells, the substrate is delivered through the oil phase in the form of β-octyl-glucoside. Because of the detergent-like structure, this compound will go to the oil-water interface where, due to β-glucosidase activity, glucose is released into the water phase and becomes available to the GOx reaction. This starts the enzymatic cascade that leads to the staining of the surface of yeast cells with tyramide-fluorescein. After the reaction is complete, the emulsions are removed and the cells can be further stained with antibodies and analyzed by FACS.

activity rather than the plus/minus screening capabilities of other systems. With these improvements, we were able to increase the purity of our reference mixes to 92% positive cells and identify mutants with up to 5.8-fold higher activity than wild-type GOx.

RESULTS AND DISCUSSION

Reference Mix Sorting

We set out to develop an ultra-high-throughput screening platform for the quantitative selection of improved enzymes (as opposed to the plus/minus capabilities of other screening systems), based on our previously described tyramide-fluorescein assay. We used the developed platform to identify improved versions of GOx with higher activity at pH 7.4 and/or greater thermostability, reflecting the anticipated operational environment for recombinant GOx enzymes.

One significant drawback of current GOx screening systems based on FACS is the presence of glucose in the initial reaction mixture before emulsification. This causes the enzymatic reaction to start before the emulsion has been formed and the cells compartmentalized, resulting in cross-staining and reduced accuracy. For this reason, it has never been possible to achieve more than 30%–40% purity when sorting reference mixes (Prodanovic et al., 2011). The inclusion of glucose in the reaction mix also makes it impossible to control how much substrate is available in the assay, because the yeast cells consume glucose (Ozcan et al., 1996).

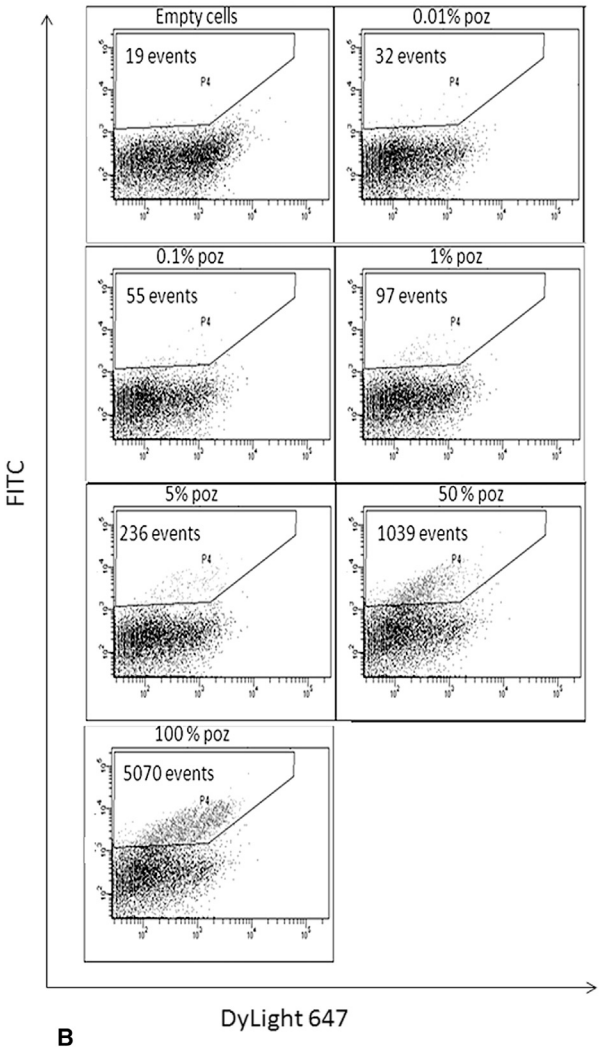
We addressed this challenge by delivering exogenous glucose through the oil phase. The cells were premixed with all the assay components except glucose, which was delivered after the compartments within the emulsion were formed. The glucose was delivered in the form of β-octylglucoside (β-OG), which because of its detergent-like structure accumulates at the oil-water interface. We also included β-glucosidase in the water phase to catalyze the hydrolysis of β-OG, thus releasing glucose into the water and making it available to GOx. The GOx reaction then produces hydrogen peroxide, which in the presence of horseradish peroxidase (HRP) oxidizes the phenolic rings of tyramine (from the tyramide-fluorescein substrate) and tyrosine (from the proteins on the surface of the yeast cell), creating phenolic radicals that polymerize among themselves and stain the cell surface. The process is summarized in Figure 1. When the reaction was complete, the oil phase was removed and the cells analyzed and sorted by FACS.

We tested the efficiency of the assay for plus/minus screening by sorting a series of reference mixes constructed artificially by mixing different concentrations of yeast cells expressing the wild-type enzyme with cells lacking the enzyme (empty vector transformants). The cells were washed three times with PBS to remove unbound GOx molecules and mixed in different concentrations according to their optical density and combined with the assay components and emulsified. When the emulsions had formed, we added 185 nmol of β-OG through the oil phase. The cells were further stained with a mouse anti-c-*myc* primary antibody followed by a goat anti-mouse DyLight 647 secondary antibody and analyzed by FACS.

As shown in Figure 2A, there was a clear correlation between the percentage of positive cells added to the mix and the number of positive cells observed after the reaction. The 0.01% and 0.1% mixes were sorted using the yield mode because only a small number of cells were present in gate P4, whereas the 1% and 5% mixes were sorted using the single-cell mode. The yield mode increased the sorting speed and reduced the number of aborted events compared to the single-cell mode, but the overall purity of the final sorted population was lower. The single-cell mode was slow and there were many aborted events. The sorted cells were regrown and their activity was tested using the ABTS agar plate assay (Supplemental Experimental Procedures and Figure S1 available online). Figure 2B summarizes the enrichment factors for the four reference mixes and confirms that the sorting method was efficient.

The purity of the enriched populations depended on both the purity of the reference mix and the sorting mode, with relatively

A



B

Reference mix name	Before sorting	After sorting	Sorting mode
Ref1	0.01%	6.89%	yield
Ref2	0.1%	7.1%	yield
Ref3	1.4%	46.34%	yield
Ref4	3.3%	92.6%	purity

Figure 2. Reference Mixes

(A) FACS recordings of different combinations of reference mixes: various concentrations of cells expressing wt-GOx activities were mixed with cells transformed with the empty vector. The different mixes of cells were combined with the assay components (fluorescein-tyramide, HRP, and β-glucosidase) and emulsified to form single emulsions. We then added β-octylglucoside to the mix and the emulsions were incubated at room temperature for 30 min. The emulsion components were removed, and GOx molecules on the cell surface were stained using fluorescent antibodies before 10^4 cells were analyzed by FACS. The bivariate histograms show the correlation between FITC (representing GOx activity) and DyLight 647 fluorescence B (abundance of GOx molecules on the surface of the yeast cells). The percentages indicated above each graph represent the theoretical concentration of positive cells present in each mix.

(B) Enrichment of reference mixes by FACS. The positive cells were gated (P4) by comparing 0% and 100% positive mixes. Approximately 1,000 cells were sorted from the P4 gate of each mix and 300–600 cells were recovered on agar plates (see also Figures S1B and S1C). We then transferred 100 cells from each mix to induction plates and the number of positive cells was assessed using the ABTS agar plate assay (see also Figure S1A).

low purity achieved for the 0.01% and 0.1% libraries. This probably reflected the reduced stringency of the yield sorting mode, which allows the sorting of some events with suboptimal parameters. Alternatively, the weak signal generated by the small number of positive cells could be so close to the background level that the population becomes highly exposed to measurement errors. Even the 1% population was partially affected, with a final purity of ~50%. It appears that optimal sorting requires a 3%–5% positive starting population, achieving a final purity of 92%. The best strategy for sorting any type of library containing less than 5% positive cells would be to carry out a prior enrichment step using the yield mode to reach at least the 5% threshold, followed by a second round of sorting using single-cell mode to achieve an overall purity greater than 90%.

Consensus Library Sorting

The experiments with reference mixes confirmed that our screening system is suitable for plus/minus-type sorting, which is important for directed evolution experiments based on "neutral drift" theory where enormous libraries must be subjected to multiple rounds of sorting to identify large numbers of active enzyme mutants (Amitai et al., 2007). However, a quantitative screening platform would be even more valuable because it would allow directed evolution by the application of selection pressure to recover enzyme variants with higher activity.

We therefore created a *GOx* gene library using the consensus approach by site directed mutagenesis at 16 positions (Figure 3A). Five random clones that were chosen after the first transformation and sequenced showed a wide distribution of mutations. This led us to assume that all possible combination of the 16 positions are represented in the library (Figure 3B). The library contained 10^5 different GOx mutants represented 100 times each to ensure all clones were accessible for screening. This was necessary because testing revealed a 70% survival rate under reaction conditions and a 60% survival rate during FACS (see Supplemental Experimental Procedures and Figure S1). This is probably due to the presence of detergent and oil during the emulsification process and the electrostatic forces present during FACS sorting which can destabilize cellular membranes.

To determine the optimal substrate concentration and provide evidence for quantitative screening, the consensus libraries were tested at different glucose concentrations. More cells can be detected at higher glucose concentrations because under these conditions the system can detect even low-activity clones, whereas at lower substrate concentrations only the most active clones appear as positive (Figure 4A).

Based on these indications, the consensus libraries were screened (two rounds of sorting) using 1.85 nmol β-OG and under stringent gating conditions in order to increase the probability of selecting the most active clones. Screening was carried out at pH 7.4 to ensure the resulting enzymes were suitable as components of miniature biofuel cell devices operating in human blood. After each round of sorting, ~100 clones from the sorted

A

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1   SNGIEASLLTDPKDVSGRTVDYIIAGGGLTGLTTAARLTE 40
1   .....................................K... 40
41  NPNISVLVIESGSYESDRGPIIEDLNAYGDIFGSSVDHAY 80
41  ............F........................... 80
81  ETVELATNNQTALIRSGNGLGGSTLVNGGTWTRPHKAQVD 120
81  ........................I.............. 120
121 SWETVFGNEGWNWDNVAAYSLQAERARAPNAKQIAAGHYF 160
121 ........................................ 160
161 NASCHGVNGTVHAGPRDTGDDYSPIVKALMSAVEDRGVPT 200
161 ............................T........... 200
201 KKDFGCGDPHGVSMFPNTLHEDQVRSDAAREWLLPNYQRP 240
201 ........................................ 240
241 NLQVLTGQYVGKVLLSQNGTTPRAVGVEFGTHKGNTHNVY 280
241 ......................................D.. 280
281 AKHEVLLAAGSAVSPTILEYSGIGMKSILEPLGIDTVVDL 320
281 ............I.................D.......... 320
321 PVGLNLQDQTTATVRSRITSAGAGQGQAAWFATFNETFGD 360
321 ........................................ 360
361 YSEKAHELLNTKLEQWAEEAVARGGFHNTTALLIQYENYR 400
361 ............D........................... 400
401 DWIVNHNVAYSELFLDTAGVASFDVWDLLPFTRGYVHILD 440
401 ..L.........................I............ 440
441 KDPYLHHFAYDPQYFLNELDLLGQAAATQLARNISNSGAM 480
441 ................................L...... 480
481 QTYFAGETIPGDNLAYDADLSAWTEYIPYHFRPNYHGVGT 520
481 ............................V..N.......... 520
521 CSMMPKEMGGVVDNAARVYGVQGLRVIDGSIPPTQMSSHV 560
521 .......L........K.................V.... 560
561 MTVFYAMALKISDAILEDYASMQ 583
561 ....................... 583
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B

Mutations	Clone1	Clone2	Clone3	Clone4	Clone5
R37K			X	X	
S53F			X		X
V106I		X			
A192T			X	X	
N278D		X			
V293I			X		
E310D			X		
E374D	X		X		X
I403L		X	X		
L429I			X		X
I474L			X		
H510N				X	
M528L					
R537K	X		X		X
M556V					X
I597V					

Figure 3. *GOx* Mutant Library

(A) Sequence of the parental *GOx* used for library construction. The possible mutations present in the gene library are shown.

(B) Five random mutants were sequenced after the first transformation showing the distribution of mutations.

population were tested in the MTP assay for enzyme activity. The remaining clones (10^4) were regrown and GOx expression was induced before the next round of sorting. The results of the MTP assay showed that the percentage of active clones increased after each round of sorting, reaching 100% after the second round (Figure 4B).

In addition, the proportion of higher-activity clones also increased after each round, showing that the assay is suitable for screening the most active enzyme variants. These results were also supported by the FACS recordings, which confirmed that the number of cells with the highest GOx activity (sorting gate P1) increased after each round of sorting (Figure 4C).

Characterization of GOx Variants

Clones representing the most active mutants were analyzed in MTP-based 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay. We selected the 50 best-performing clones and repeated the analysis using fresh MTP cultures (five replicates per clone in separate wells to minimize deviations caused by variations in cell growth and transgene expression). The SD of all measurements was 2.4%–17%. The five most active mutant clones were sequenced (Table 1), subcloned in pICZalphaA to remove the N- and C-terminal tags and used to prepare pure protein following expression in *Pichia pastoris*.

The best-performing cultures representing each mutant were scaled up and the fermentation conditions were optimized to produce large amounts of protein. The recombinant GOx proteins were purified by ultrafiltration and DEAE chromatography yielding ~30 mg of each enzyme variant with sufficient purity (demonstrated by SDS-PAGE) for kinetic analysis (Supplemental Experimental Procedures and Figures S2A and S2B).

The kinetic parameters of the enzymes were investigated so that the resulting data could be fitted to the Michaelis-Menten equation (kinetic curves are provided in Supplemental Experimental Procedures and Figures S2C and S2D). All the mutants had a higher intrinsic activity than the wild-type enzyme (tested at pH 5.5) but the difference was more pronounced at pH 7.4. The improved kinetic constants of the mutants are summarized in Table 2. The best-performing mutant was A2, with a 1.5-fold lower K_M and a 2.6-fold higher k_{cat}, giving an overall 4-fold increase in activity at pH 5.5 and a 5.8-fold increase at pH 7.4.

We also found that the thermal stability of all the mutants was greater than the wild-type enzyme (Table 3). In this case, the best-performing mutant was F9-1 with double the half-life at 60°C compared to the wild-type enzyme. The fitting of the thermal stability data are presented in the Supplemental Experimental Procedures and Figure S3.

To provide more evidence for quantitative screening and to prove that we can distinguish between clones with different activities, the wild-type clone was compared with mutant A2. The two populations of cells were emulsified with the substrates as described above and different concentrations of β-OG were added (185 nmol, 18.5 nmol, and 1.85 nmol). After incubation for 30 min, the cells were stained with antibodies as discussed above and analyzed by FACS. At high glucose concentrations, there was little difference between the wild-type and mutant clones, whereas significant differences emerged at lower glucose concentrations (Figure 5). This is because the reaction was stopped before it reached a plateau at low substrate concentrations, allowing the enzyme activity to be determined, confirming that it is possible to identify quantitative differences in activity between clones.

SIGNIFICANCE

We have developed an ultra-HTS platform based on tyramide-fluorescein assay, which allows both plus/minus and the quantitative screening of gene libraries to identify

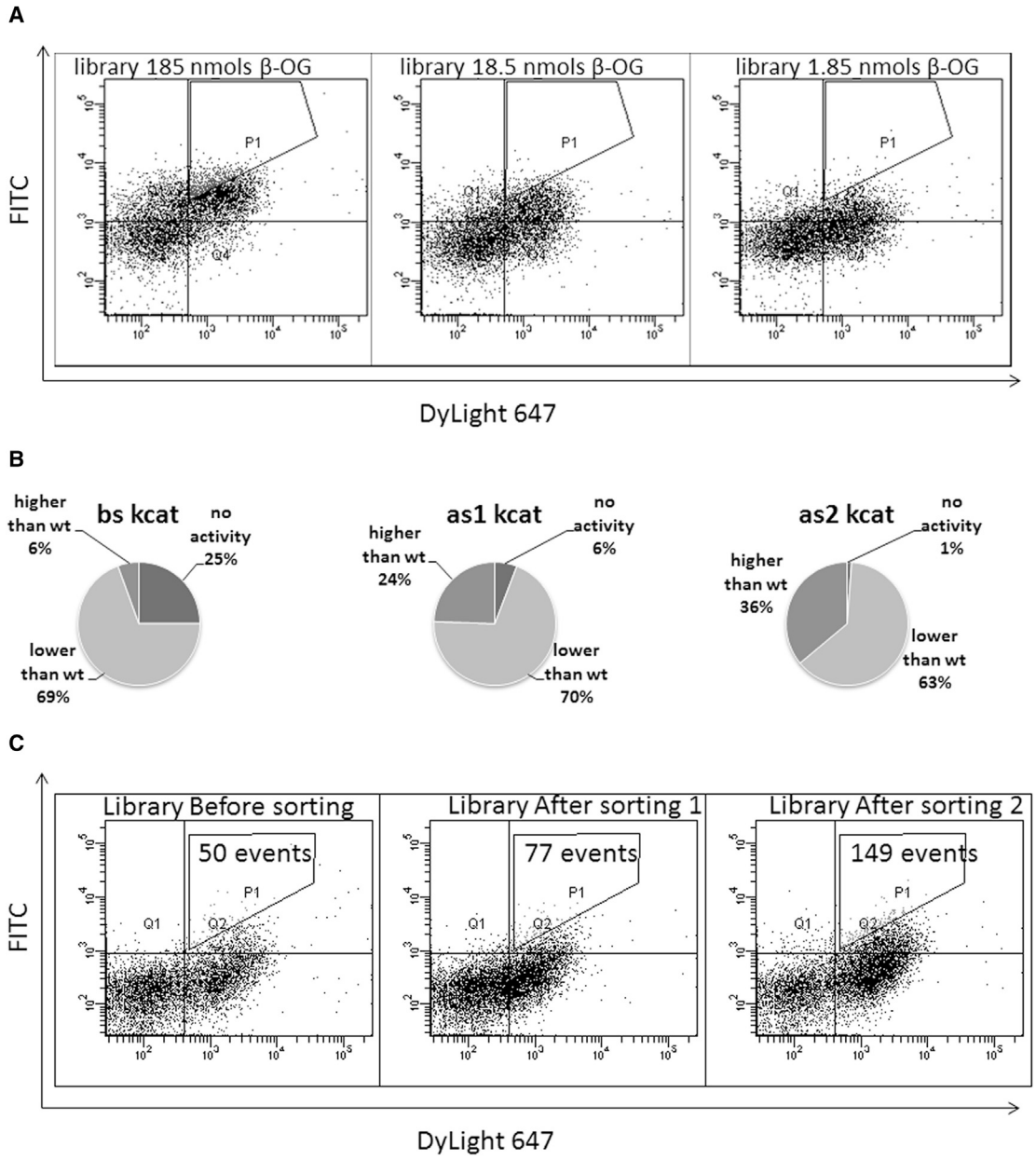

Figure 4. Sorting of the GOx Consensus Library

(A) FACS recordings of the consensus library at three different β-octylglucoside concentrations for assay optimization. Double plots of FITC fluorescence correspond to enzyme activity versus DyLight 647 fluorescence representing the abundance of GOx molecules on cell surface. The lowest concentration of β-octylglucoside was used for sorting at gate P1 because only the most active cells are stained under these conditions. The total number of events presented in each FACS recording is 10^4.

(B) MTP analysis of mutant GOx activity after each round of sorting. We picked 100 random clones before and after each round of sorting. The GOx activity was measured for each clone using the ABTS MTP assay.

(C) FACS recordings (10^4 recorded events) of the consensus library during multiple rounds of sorting. For each round of sorting, 10^7 cells were analyzed (representing more than 100 copies of each variant).

enzymes with improved activity. The concentration of glucose available to the system and the enzymatic reaction can be controlled precisely, permitting the selection of cells based not only on the overall activity but also on the specific activity of the enzyme (ratio of the overall activity and enzyme abundance). The principle of the assay was demonstrated by screening a consensus library containing 10^5 different GOx mutants. The best mutant (A2) was 5.8-fold more active at pH 7.4. Because GOx has many industrial applications (Wong et al., 2008) and the natural substrate was used in this assay, we anticipate that the assay can be used to screen mutant libraries under application-driven

Table 1. Mutation Positions in the *GOx* Variants

	Mutations
Wild-type	No mutations
Parent	T30V, I94V, A162T
A2	T30V, I94V, A162T, R537K,M556V
A21	T30V, I94V, R537K,M556V
F5	T30V, R37K, I94V, A162T, V293I, E310D, R537K,M556V
F9	T30V, R37K, I94V, V106I, A162T, M556V
F91	T30V, R37K, I94V, V106I, A162T

See also Table S1.

conditions based on the principle that "you get what you screen for" (Arnold and Volkov, 1999).

EXPERIMENTAL PROCEDURES

Chemical Synthesis

Tyramide-fluorescein was synthesized as previously described (Hopman et al., 1998). We incubated 20 mM tyramine-HCl, 25 mM *triethylamine* and 20 mM *N-hydroxysuccinimide*-fluorescein (all in dimethylformamide) for 2 hr at 4°C. The solution was diluted 10-fold with ethanol (final concentration 2 mM tyramide-fluorescein) and stored in the dark at 4°C until further use.

Multiple-Site-Directed Mutagenesis

The consensus library was created using the QuickChange multiple-site-directed mutagenesis kit (Agilent Technologies) and *Escherichia coli* XL10Gold ultracompetent cells. We synthesized 16 site-directed primers (Eurofins MWG Operon) annealing at different positions in the *GOx* sequence, which was housed in the $pCTCON_2$ vector and included a c-myc tag. The primer sequences are listed in the Supplemental Experimental Procedures. The PCR mix comprised 400 pg/μl template DNA, 200 nM of the primers, and the remaining components from the mutagenesis kit. The reactions were heated to 95°C for 1 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 65°C for 16.5 min, followed by a final extension at 65°C for 20 min. The reaction products were then digested with DpnI for 3 hr at 37°C and stored at 4°C until required. The single-stranded DNA obtained from the PCR was introduced into the ultracompetent bacteria according to the instructions in the mutagenesis kit, and plasmid DNA was isolated using the Macherey-Nagel Plasmid DNA kit (Düren).

Table 2. Kinetic Constants of the GOx Mutants Compared to the Wild-Type Enzyme

Buffer	K_M Average (mM)	k_{cat} Average s^{-1}	Specificity $mM^{-1} \times s^{-1}$
Acetate (pH 5.5)			
Wild-type	28.26 ± 1.15	189.38 ± 8.94	6.7
Parent	14.98 ± 0.51	291.82 ± 10.10	19.5
A2	18.54 ± 0.57	498.34 ± 15.12	26.9
A21	15.75 ± 0.31	466.18 ± 10.05	29.6
F5	16.05 ± 0.55	368.16 ± 11.91	23.0
F9	19.76 ± 0.54	345.16 ± 14.79	17.5
F91	15.12 ± 0.48	276.35 ± 10.37	18.3
PBS (pH 7.4)			
Wild-type	23.19 ± 0.57	130.16 ± 3.97	5.6
Parent	10.58 ± 0.20	222.63 ± 4.38	21.0
A2	13.08 ± 0.49	432.17 ± 16.38	33.0
A21	10.93 ± 0.27	350.77 ± 7.97	32.0
F5	10.37 ± 0.22	228.36 ± 7.19	22.0
F9	9.53 ± 0.16	266.80 ± 3.53	28.0
F91	9.91 ± 0.25	315.27 ± 7.18	31.8

See also Figure S2.

Table 3. Half-Life of Thermal Stability Calculated from the Residual Activity of the GOx Mutants and Wild-Type Enzyme at 60°C in the Absence of Substrate

	$t_{1/2}$ (min)
Parent	9.00 ± 0.70
Wild-type	10.50 ± 0.71
A2	11.74 ± 0.30
A2-1	13.86 ± 0.18
F5	11.74 ± 0.95
F9	15.75 ± 0.71
F9-1	19.80 ± 0.33

See also Figure S3.

Transformation of *S. cerevisiae* EBY 100 Cells

Plasmid DNA was introduced into *S. cerevisiae* EBY 100 cells (kindly provided by Professor Dane Wittrup, Massachusetts Institute of Technology) as described (Gietz and Schiestl, 2007) using a 2.5 hr 42°C heat-shock step. The cells were cultivated in YNB-CAA glucose medium for 48 hr at 27°C, 160 rpm, then induced to express GOx by transferring to YNB-CAA Gal/Raf medium under the same conditions for 16–18 hr prior to FACS analysis.

Enzymatic Assay for FACS

Cells expressing GOx were washed three times in PBS and diluted to a concentration of 10^9 cells/ml (where OD_{600} = 1 is equivalent to 2 × 10^7 cells/ml). The yeast cells were compartmentalized for FACS screening as described (Aharoni et al., 2005) (approximately one cell per compartment according to statistical distribution based on the number of cells versus the number of droplets) with the following modifications. A 25 μl suspension containing 10^7 cells, 20 μM tyramide-fluorescein, 10 U/ml HRP (AppliChem), and 5 U/ml almond β-glucosidase (Sigma-Aldrich Chemie) was added to 500 μl 1.5% Abil Em 90 (Tego) in LMO (Sigma-Aldrich Chemie). The mix was emulsified using a MICCRA D-1 dispenser at 8,000 rpm for 3 min on ice and supplemented with octylglucoside (Sigma-Aldrich Chemie) from a 37 mM solution in ethanol, and the emulsion was vortexed for 30 s and incubated for 30 min at room temperature.

Cells were recovered from the emulsions as described (Prodanovic et al., 2011) and stained with a mouse anti-c-*myc* primary antibody (Abcam) followed by a goat anti-mouse Fc IgG Dylight 647 (Abcam) (1:50 dilution) as described (Gai and Wittrup, 2007). Before FACS analysis, the cells were washed three times in PBS containing 0.1% BSA and resuspended in 1 ml PBS.

Flow Cytometry

The cells were analyzed using a BD FACS Diva flow cytometry system with the trigger parameter set on forward scattering. The analysis rate was 1,000–5,000 events/s and the sorting speed was 10–100 events/s. The 488 and 647 nm laser excitation wavelengths were used for detection, and emissions were detected using the 530 and 670 nm filters. The positive cells were gated on a fluorescence double plot as specified in each experiment. The cells were sorted in the single-cell mode in to YNB-CAA glucose chloramphenicol media or into MTP wells containing 100 μl YNB-CAA glucose liquid medium plus 50 μg/ml chloramphenicol. For the multiple rounds of sorting, 10^4 cells were sorted from the specified gate in YNB-CAA glucose chloramphenicol medium, grown for 2 days at 27°C, transferred to YNB-CAA galactose medium, and grown for an additional day. FACS analysis and sorting was repeated using 10^7 of preselected and amplified cells.

Agar Plate ABTS Assay

After FACS, the cells were grown at 27°C for 3 days, replica plated onto YNB-CAA Gal/Raf medium, and cultivated for a further day. Screening medium was

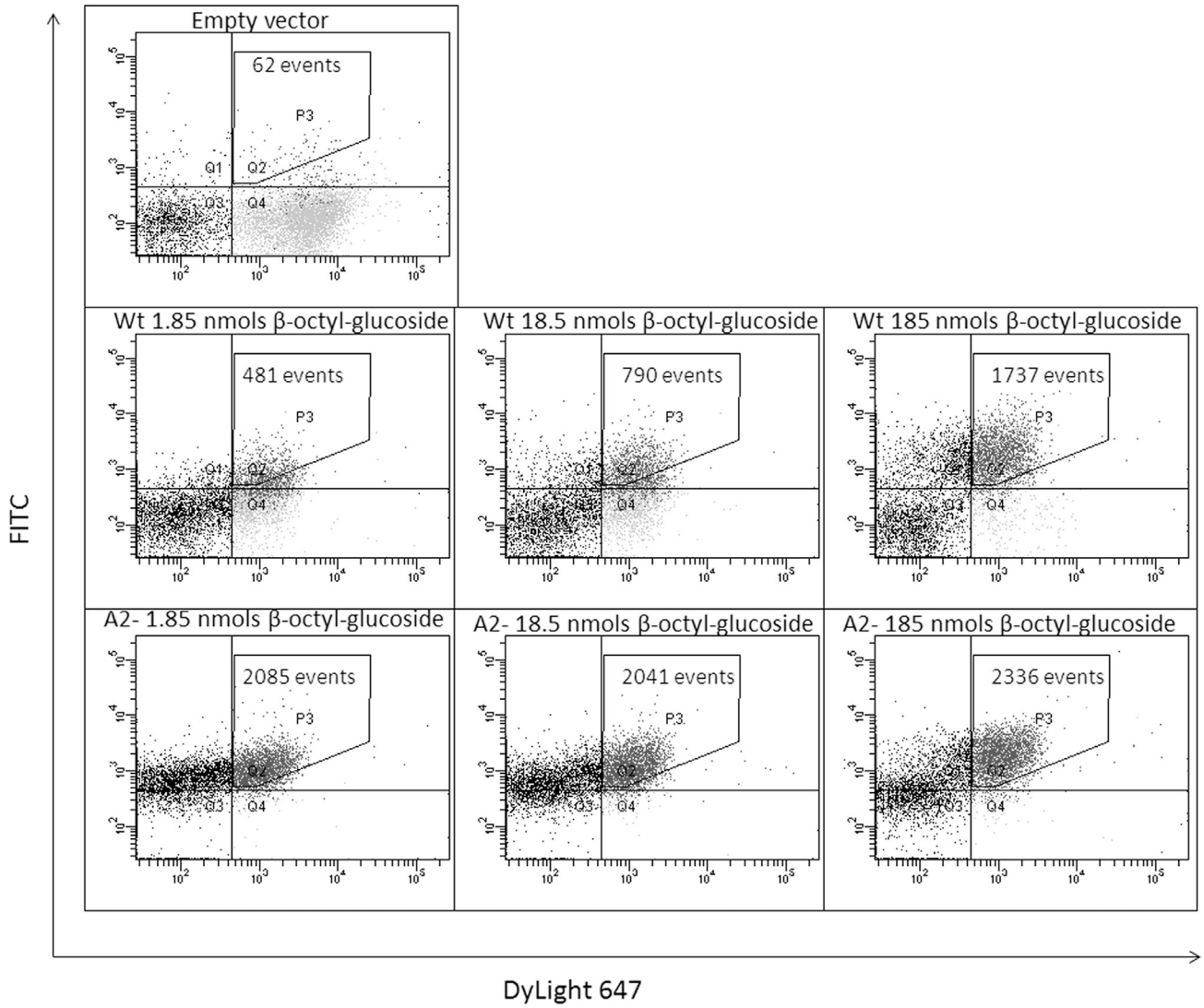

Figure 5. Comparison of Wild-Type GOx and Mutant A2

The tyramide-fluorescein assay was carried out at different β-octylglucoside concentrations to visualize the difference between these variants. Bivariate histograms show FITC fluorescence corresponding to the enzyme activity and DyLight 647 fluorescence corresponding to the enzyme concentration.

prepared by mixing 2% agar with an equal volume of ABTS solution containing 333 mM glucose, 1.75 U/ml HRP, and 7 mM ABTS. This was poured over the agar cell plates. Green halos were observed around colonies with GOx activity.

MTP ABTS Assay

Cells sorted into MTP wells were cultivated as described (Bulter et al., 2003) and 5 μl aliquots were transferred to fresh MTPs for the ABTS assay as described (Baron et al., 1994; Sun et al., 2001; Zhu et al., 2006) with the following modifications. The cells were resuspended in 70 μl PBS and the OD_{600} was determined, then 70 μl ABTS solution was added and the kinetics were measured at 405 nm every 20 s for 10 min. Two measurements were taken from each culture, one using 4 mM ABTS solution containing 250 mM glucose and 1 U/ml HRP and one with the same components but only 5 mM glucose. Three wild-type clones were included in each MTP for standardization. Clones were considered to lack GOx activity if the values were ten times lower than the wild-type enzyme. For each measurement, the slope of the linear region was calculated and normalized to the OD_{600} of the cells in each well. The best-performing mutants were selected and rescreened.

DNA Isolation and Recloning in *Pichia pastoris*

DNA was extracted from the best-performing *S. cerevisiae* mutants as described (Singh and Weil, 2002) and the *GOx* sequence was transferred to the XhoI/XbaI sites of pICZalpha A (Invitrogen) using the appropriate restriction enzymes (New England BioLabs). Competent *P. pastoris* KM71H cells (Invitrogen) were prepared and transformed (Becker and Guarente, 1991), and the best-performing clone representing each mutant was selected.

Protein Purification

After fermentation for 4 days according to Invitrogen recommendations, cells were pelleted by centrifugation at 11,000 × *g* for 10 min using a Beckman Coulter Avanti J26 XP centrifuge. The supernatant was collected and filtered through a 0.22-μm PTFE filter (Carl Roth) and the filtrate was concentrated to 5–10 ml using a Viva Flow 50 system with a 10 kDa membrane (Sartorius AG). The concentrate was dialyzed against 10 mM phosphate buffer (pH 6.0) overnight at 4°C, and loaded onto a 2 ml Fast Flow DEAE Sepharose column (GE Healthcare Europe) using the ÄKTApurifier (GE Healthcare Europe). The protein was purified using a linear gradient from 10 to 250 mM phosphate buffer (pH 6) over 30 column volumes. We tested 50 ml fractions using the

ABTS assay and those with separate peaks of GOx activity were collected and concentrated to 5 ml using 10 kDa ultrafiltration columns (Sartorius AG).

Kinetic Analysis

The kinetic characteristics of each GOx variant were determined using triplicate MTP-based ABTS assays with glucose concentrations ranging from 1.2 to 266 mM, at pH 5.5 and 7.4. The slope of each measurement was calculated over the linear region and fitted onto Michaelis-Menten hyperbola using Origin 8 (OriginLab) to allow the K_M and k_{cat} values to be determined. Lineweaver-Burk, Eadie-Hofstee, and Hanes-Woolf plots were also constructed, and the outliers were identified and removed. We determined k_{cat} values by measuring absorbance at 280 nm (the absorption of 1.5 mg/ml GOx is considered equivalent to 1 AU based on the sequence, as calculated using ProtParam).

Thermal Stability

The thermal stability of GOx mutants was determined by incubating the enzyme in 50 mM acetate buffer (pH 5.5) at 60°C in the absence of substrate (Bhatti and Saleem, 2009) and measuring the residual activity of periodic aliquots using the ABTS assay. We plotted *A(t)*, the percentage residual activity at different time points relative to 100% activity at time 0, on an exponential equation to determine the inactivation rate constants (k_d) as shown: $A(t) = e^{-k_d \times t}$. The half-times for thermal stability were calculated by considering *A(t)* equivalent to 0.5 A(0).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.01.010.

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