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Flow Cytometer-Based High-Throughput Screening System for Accelerated Directed Evolution of P450 Monooxygenases

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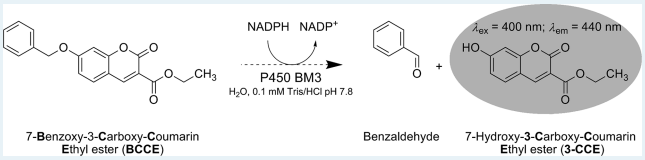
Supporting Information

ABSTRACT: Flow cytometry-based screening systems have successfully been used in directed evolution experiments. Herein, we report the first whole-cell, high-throughput screening platform for P450 monooxygenases based on flow cytometry. O-dealkylation of 7-benzoxy-3-carboxycoumarin ethyl ester (BCCE) by P450 BM3 generates a fluorescence coumarin derivative. After one round of directed evolution, P450 BM3 variants with up to 7-fold increased activity (P450 M3 DM-1: R255H) could be identified at a sampling rate of 500 events s^{-1}. The reported screening platform can likely be applied to directed evolution campaigns of any P450 monooxygenase that catalyzes the O-dealkylation of BCCE.



KEYWORDS: *directed evolution, coumarin, flow cytometer, P450 monooxygenase, high-throughput screening*

Enzymes provide a broad variation of catalytic activities enabling reactions that are highly desirable for organic chemists, such as regio- and stereoselective hydroxylation of nonactivated carbon atoms by cytochrome P450 monooxygenases.[1] Tailoring catalyst properties by directed evolution to specific application demands in terms of stability, enantioselectivity, or stereoselectivity became a standard approach in biocatalysis and medical science as well as in synthetic biology.[2−4] Rational and evolutive protein engineering strategies employ focused mutant (OmniChange,[5] CASTing[6]) or random mutant libraries.[4,7] The theoretically generated diversity exceeds the possible throughput of high-throughput screening platforms with respect to the number of variants that can be screened.[4] For instance, in focused mutant libraries with five simultaneously NNK-randomized codons, 10^8 clones have to be sampled for 99% coverage (PEDEL AA and GLUE-IT[8,9]). A few thousand clones are usually screened in directed evolution experiments employing a microtiter plate screening format[10] in which costs as well as time requirements are commonly high.[11] The availability of reliable and efficient prescreening systems (>10^4 clones/day) is the main limitation to identify beneficial variants in diverse mutant libraries.[12]

In the past decade, innovative ultrahigh-throughput screening platforms based on flow cytometry[13−15] or microfluidic devices[11,16−18] have been developed. Flow cytometer-based screening systems enable sampling of more than 10^8 clones/day with excellent accuracy.[13,19] The throughput is 4 orders of magnitude higher than with commonly employed microtiter plate-based screening platforms, and accuracy is usually higher than in agar plate-based prescreening systems.[21,22] In general, fluorescent screening systems have a high sensitivity that ensures that active and inactive clones can be reliably separated, even in very small reaction volumes (femtoliter droplets).[23]

A key requirement in every directed evolution campaign is to keep the link between phenotype and genotype, which is usually ensured by compartmentalization of mutant libraries; for instance, in emulsions or whole cells.[13,17−19,23,24] In the case of whole cell screening systems, the challenge lies in entrapping the product within the cell and thereby avoiding cross-contamination.[20] In the case of emulsion systems, a main limitation often lies in generating double emulsions (water/oil/water) so that sorting in aqueous solution with a flow cytometer becomes possible. In double emulsions, often more than one single emulsion droplet is entrapped, reducing the fraction of active variants that should be sorted. Nevertheless, flow cytometry-based screening systems have successfully been used to enrich the active population in mutant libraries[13,20,25] and to identify in successive rounds of sorting or diversity generation better variants with improved specificity,[26] inhibitor resistance,[27] and activity.[28]

For P450 monooxygenases (CYPs) (>5000 cloned members[29]), colorimetric and fluorescence-based high-throughput screening platforms based on microtiter plates (MTP) are available with a reported throughput of 10^3−10^5 clones/day.[30,31] Fluorescence-based screening systems in MTP format were reported for monooxygenases with excellent sensitivities (e.g., 900-fold increase activity; detection limit in the nanomolar range).[32] Among the fluorescence-based screening systems for P450 monooxygenases, coumarin derivatives are used preferentially.[33−35] Coumarin derivatives have furthermore been employed successfully in directed evolution campaigns of cellulases,[36] lipases,[37] and phosphatases.[38,39]

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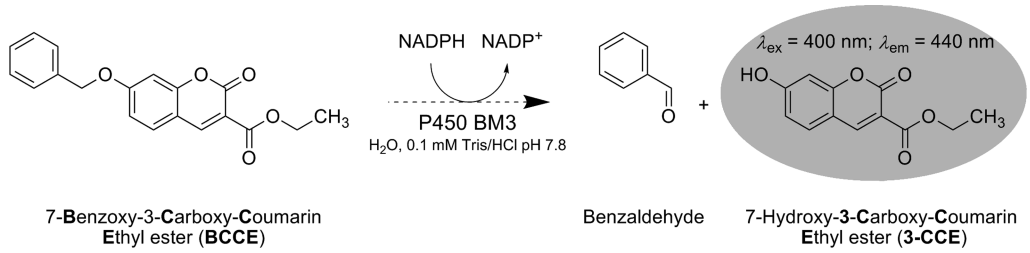

Figure 1. Scheme of the 7-*b*enzoxy-3-carboxy*c*oumarin *e*thyl ester (BCCE) conversion catalyzed by P450 BM3 variants.

In this Letter, we report the first whole-cell, high-throughput screening system for P450 monooxygenases employing a flow cytometer that allows efficient sorting and enrichment of active variants from random mutant libraries with a throughput of 500 events s^{-1}. The developed flow cytometry screening system does not require double emulsions for sorting since the converted fluorogenic substrate 7-benzoxy-3-carboxycoumarin ethyl ester (BCCE) is retained within the *Escherichia coli* BL21(DE3) Gold expression host. The ethyl ester is cleaved to the corresponding carboxylic acid in *E. coli*. Because of the negative charge the product cannot penetrate easily by diffusion through E. coli membranes, in contrast to the corresponding ester.[40,41] The monooxygenase P450 BM3 (CYP102A1) (variant F87A)[42] was selected for development of the screening system as the model monooxygenase because of its industrial attractiveness and broad substrate profile.[31,43,44]

BCCE was synthesized (see Supporting Information) and fluorescence was recorded upon hydroxylation (see Figure 1) via O-dealkylation, as previously described for 7-benzoyloxytrifluoromethyl coumarin by Cheng et al. using P450 3A4.[35] The BCCE flow cytometry screening system was developed in two steps. In the first step, the variant P450 BM3 F87A was employed in investigating the signal-to-noise ratio in crude cell extracts and in a whole-cell flow cytometry setup. In the second step, the mutant M3 DM (R47F F87A M354S D363H R471C N543S) was randomly mutated (epPCR) and sorted to identify more active variants. The mutant M3 DM was evolved for improved electron transfer via mediators starting from the reported variant P450 BM3 M3 (R47F F87A M354S D363H).[45,46] The BCCE flow cytometry screening system was used as a prescreen to sort out the most active variants and enrich the population of active P450 BM3 variants. The sorted variants were subsequently screened in a 96-well microtiter plate to identify P450 BM3 variants with higher activity toward hydroxylation of BCCE and to validate the developed BCCE flow cytometer screening system.

BCCE conversion of P450 BM3 variants displayed a detectable fluorescence (λ_{ex} = 400 nm and λ_{em} = 440 nm), and the control samples (pET28a(+)/lacking P450 BM3 gene) (Figure 2A) revealed a low noise-to-signal ratio in MTP. No fluorescence could be detected for *E. coli* cells harboring the pET28a(+) vector without the P450 BM3 gene. Figure 3 shows a control population lacking P450 BM3 F87A (left) and a population expressing P450 BM3 F87A in *E. coli*. The flow cytometer analysis illustrates that both populations can clearly be distinguished (Figure 3; fluorescence intensity and distribution) with a final signal-to-noise ratio of about 10:1 (Figure 3; λ_{ex} 350 nm and λ_{em} 450 nm). Interestingly, a whole-cell conversion of BCCE by P450 BM3 F87A could be achieved in *E. coli* (5×10^6 cells/μL), and microscopic analysis proved entrapment of the coumarin derivate in *E. coli* (see Supporting Information Figure S1). Figure 2A shows that conversion in

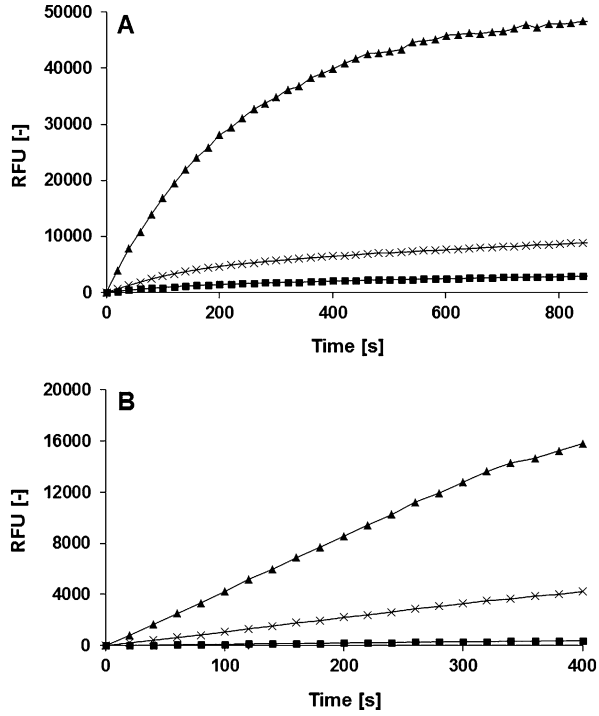

Figure 2. Conversion over time of BCCE using cell free lysates with adjusted protein concentration by CO titration[50] (A) and whole cells (B). pET28a(+) (♦) as background signal, variant P450 BM3 M3 DM (R47F F87A M354S D363H R471C N543S) (■) and the obtained mutant P450 BM3 M3 DM-2 (x) (R47F F87A M354S D363H R471C N543S ***R203H I401 V F423L***) and P450 BM3 M3 DM-1 (▲) (R47F F87A M354S D363H R471C N543S ***R255H***). Novel substitutions in bold and fluorescent signals were measured in a 96-well microtiter plate reader.

crude cell extracts follows the same trend as in whole cells for the variants DM, DM-1, and DM-2 (Figure 2B, P450 M3 DM = R47F F87A M354S D363H R471C N543S, P450 M3 DM-1 = R47F F87A M354S D363H R471C N543S *R255H*, P450 M3 DM-2 = R47F F87A M354S D363H R471C N543S *R203H I401V F423L*).

For further evaluation, mixed populations consisting of varied ratios of active (P450 BM3 F87A) to inactive populations (pET28a(+) without P450 gene) were merged (ratios of 9:1 and 1:1) and sorted (threshold >50-fold pET28a(+) RFU). After sorting of both mixed populations, cells were plated without further concentration on LB_{kan} agar plates and incubated overnight (37 °C). Sixty clones were picked and investigated for whole-cell BCCE conversion in 96-well microtiter plates (Supporting Information Figure S2). The ratio 9:1 yielded a 100% active population, and the ratio 1:1 resulted in an increase from 50 to 82% of active variants after only one round of sorting. This demonstrates that the BCCE-

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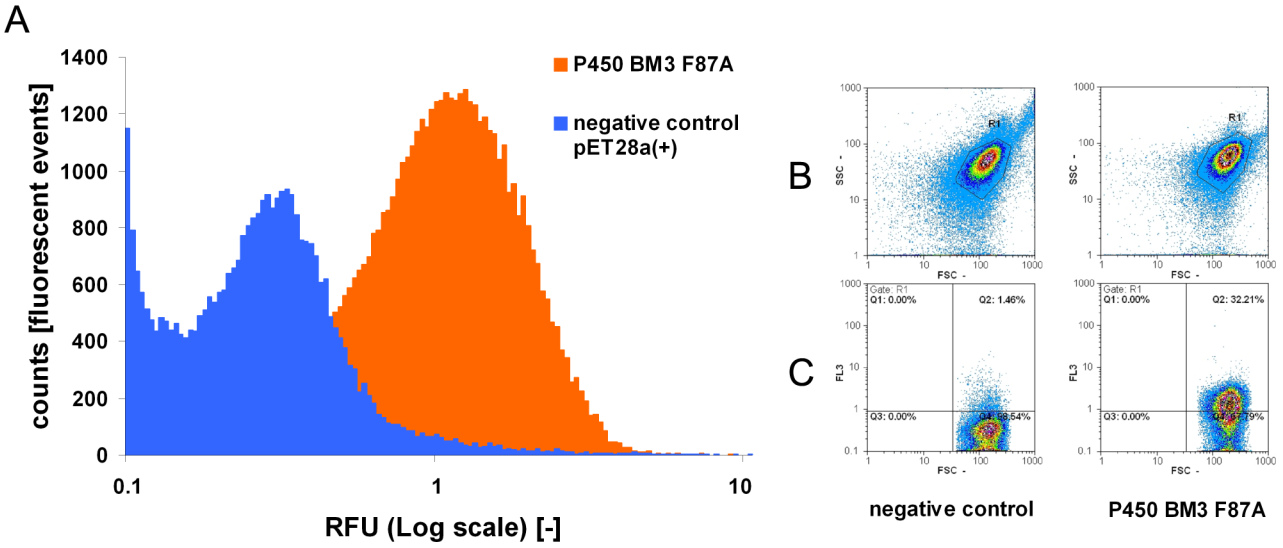

Figure 3. Overlay of the emitted fluorescence from two different cell populations. (A) Flow cytometer analysis: blue cells represent the negative control (*E. coli* BL21 (DE3) Gold and empty vector pET28a(+) and in orange: *E. coli* BL21 (DE3) Gold cells expressing active P450 BM3 F87A). (B) Flow cytometer analysis after conversion of BCCE with P450 BM3 F87A-expressing *E. coli* BL21 (DE3) Gold cells and pET28a(+)-harboring *E. coli* BL21 (DE3) Gold cells (as negative control). R1 (C) shows the gated population; side scatter (SSC; $\log_3$ scale), forward scatter (FSC; $\log_3$ scale), and UV-laser (FL3: λ_{ex} 350 nm and λ_{em} 450 nm; $\log_4$ scale) were recorded.

based flow cytometry screening system can efficiently enrich the population of active variants and thereby fulfills requirements postulated for high-throughput screening systems.[15,20] As a final proof, error-prone PCR libraries (0.05, 0.1, 0.2 mM $MnCl_2$) were generated on the basis of the P450 BM3 M3 DM variant. The P450 M3 DM library (~10^4 variants) was sorted and enriched three times.

After each round of enrichment, the percentage of active clones was determined by measuring the BCCE activity of 90 clones in a 96-well MTP. After the third enrichment, 600 clones were picked, expressed, and subjected to activity measurements in MTP employing BCCE as substrate. The "true" standard deviation of P450 BM3 activity in the 96-well MTP format was determined to be 10%, ensuring a reliable detection of improved variants.[30] Around 8% of the rescreened variants displayed increased P450 monooxygenase activity for BCCE when compared with the starting variant P450 BM3 M3 DM.

The three most active variants were sequenced, and two turned out to have identical substitutions (P450 BM3 M3 DM-1 and P450 BM3 M3 DM-3, R255H; P450 BM3 M3 DM-2, R203H I401 V F423L). Variants P450 BM3 M3 DM-1 and P450 BM3 M3 DM-2 were purified and finally used for kinetic characterizations applying varying concentrations of BCCE, as shown in the Supporting Information (Figure S3). Sequencing results revealed that all four identified substitutions in P450 BM3 M3 DM-1 and DM-2 are located in the heme domain of P450 BM3 (R255H and R203H I401 V F423L).[43] To the best of our knowledge, of the four mutated positions, the positions R203H and F423L have not been reported to influence any P450 BM3 property. Position R255S improves alkane hydroxylation,[47] whereas position I401P influences electron transfer and coupling efficiency of P450 BM3.[48] Remarkable improvements could be found for the variant P450 BM3 M3 DM-1 (R47F F87A M354S D363H R471C N543S R255H), where k_{cat} was increased 7-fold and K_m remained unchanged (Table 1).

Characterization of variants P450 BM3 M3 DM (R47F F87A M354S D363H R471C N543S), P450 BM3 M3 DM-1 (R47F F87A M354S D363H R471C N543S R255H), and P450 BM3 M3 DM-2 (R47F F87A M354S D363H R471C N543S R203H I401 V F423L) demonstrates that the developed flow cytometer screening system identifies improved variants in random mutant libraries. A BCCE turnover of >0.07 s^{-1} can be regarded as lower limit and as a prerequisite for flow cytometer sorting of active and nonactive populations. The library of sorted active variants can furthermore be used in standard microtiter plate screening systems for P450 BM3 monooxygenase properties, such as organic solvent resistance,[49] improved selectivity[12] and activity toward nonnatural substrates.[30]

Table 1. Kinetic Characterization of P450 Variants Converting 7-Benzoxy-3-carboxy-coumarin Ethyl Ester[a]

variant	k_{cat}[b]	K_m (μM)	K_{eff}[c]
P450 BM3 M3 DM (**R471C N543S**)[d]	0.07 ± 0.005	25.2 ± 4.2	0.17
P450 BM3 M3 DM-1 (**R255H**)	0.48 ± 0.05	25.3 ± 5.5	1.14
P450 BM3 M3 DM-2 (**R203H I401 V F423L**)	0.24 ± 0.01	34.3 ± 3.4	0.42

[a]Novel substitutions in the parent P450 BM3 M3 DM are highlighted as bold letters. [b]mmol product s^{-1} $mmol^{-1}$ of P450. [c]Catalytic efficiency in min^{-1} μM^{-1}. [d]Starting variant P450 BM3 M3 DM = P450 BM3 M3[39] + N543S R471C

In summary, we developed the first flow cytometer-based high-throughput screening platform that can be applied in directed evolution campaigns of P450 monooxygenases. All requirements for an efficient ultrahigh-throughput screening platform were fulfilled and optimized (reliable fluorescent reporter; compartmentalization in whole cells and, therefore, direct link of geno- and phenotype). In this Letter, we have aimed to prove that the BCCE-based flow cytometer screening can be employed as prescreening to sort out active or activity-improved variants. Sorting and enrichment were possible at an excellent throughput of 500 events/s^{-1}, with an average sampling time of 4 min, corresponding to 1.2 × 10^5 screened events per run (theoretically 3.6 × 10^7 events per day). Variants

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with up to 7-fold increased activity (P450 BM3 DM-1) could be identified from a random mutant library. From our point of view, a main advantage of the BCCE screening system is the direct sorting of fluorescent whole cells (*E. coli*), omitting the preparation of double emulsions and thereby simplifying screening procedures. In general the reported BCCE screening system will very likely be applicable to all P450 monooxygenases that can be expressed in *E. coli* and catalyze an O-dealkylation of coumarin derivatives (e.g., human CYP3A4), and furthermore, it can likely be used in directed evolution of cellulases, lipases, and phosphatases as well as screening of metagenome libraries.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, NMR data, and microscopy images. This information is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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