Ultra-high Throughput Screening for Lipase Enantioselectivity

A Novel Approach for the Screening of Enzyme Libraries by Flow Cytometry

With modern molecular biology methods gene libraries can be generated that upon expression in a bacterial host may result in over $10^9$ different enzyme variants. Using classical microtiter plate screening formats, only a tiny fraction, normally not exceeding several 100,000 clones can be analyzed. Hence, there is an inherent high probability that an enzyme variant with particularly interesting properties remains undiscovered. We have devised a methodology that allows one to screen the whole set of clonal candidates of a library of lipolytic enzymes for activity or enantioselectivity in short time.

Enzyme Display on the Bacterial Cell Surface

When using *E. coli* cells for the recombinant production of an enzyme of interest, it would be highly desirable, if the microbial host would not only serve as a producer of the protein but would also act as a living microparticle that displays the protein under consideration in functional form anchored on the cell surface. As a consequence, the desired reaction could take place under defined buffer conditions with a direct access of the substrate to the enzyme.

We have established two formats for the display of proteins on the surface of *E. coli* cells. Both rely on the N- or C-ter-minal fusion of the protein of interest to an outer membrane protein (fig. 1). Several proteins and enzymes have been success-fully displayed on the cell surface, among them interleukin 2, an immunoglobulin va-riable domain, and several lipolytic enzymes [1, 2].

Single Cell Screening for Esterase Activity

Lipases and esterases are an interesting class of enzymes with broad applications
for the biocatalytic synthesis of chemical compounds or pharmaceuticals. Conceptually, the identification of cell-surface-exposed lipase variants that are able to catalyze a particular reaction can be simplified by activity-dependent cell labeling. A cell that is able to convert a given substrate to a product can be marked for that ability if one takes care that the reaction product - but not the educt - becomes covalently attached to the surface of the bacterial cell from which it was generated.

This product label on the cell surface can then be used to isolate the enzymatically active cell.

An enzyme reaction that is particularly useful for this type of screen is the tyramide signal amplification (TSA) reaction. It is based on the oxidative formation of a tyramide radical from biotin-tyramide by horseradish peroxidase (HRP). This highly reactive tyramide radical is able to react with tyrosyl side chains of proteins in proximity, eventually leading to covalent attachment of biotin. We have established a coupled reaction cascade to occur on the surface of living bacterial cells where an ester substrate is used that requires hydrolysis by a lipolytic enzyme to release a biotin tyramide. This molecule subsequently can be activated by HRP and becomes covalently coupled to the cell surface (fig. 2) [3, 4]. Since lipolytically active cells carry a biotin label, they can easily be identified and isolated by flow cytometry after labeling with a fluorophor. Using state of the art flow cytometry approximately 30,000 individual cells can be screened per second, which opens up the possibility to screen over 10^8 bacterial clones.

**Case Study: Enantioselectivity Screen**

We have applied this technology in a model experiment to the screening of an error prone PCR library of esterase variants. As a model enzyme the P. aeruginosa esterase EstA was chosen, that also serves as a transporter for passenger enzymes
in the EstA display format described above. For enantioselectivity screening, a 1:1 mixture of two different tyramide esters of (R)- or (S)-2-2-methyldecanoate were used that carry different indicator groups (fig. 3). Cells that preferentially hydrolyze the (R)-2-MDA ester substrate release and covalently attach 2,4 dininitrophenyl tyramide to their surface which can be detected by labeling the cells with a Alexa Fluor 488 conjugated anti dinitrophenyl antibody (green cellular fluorescence). Accordingly, cells cleaving the (S)-2-MDA ester obtain a red fluorescent label since ester hydrolysis results in cellular biotin labeling which can be detected using a red-fluorescent streptavidin, R-phycoerythrin conjugate. Wild type EstA has only a slight preference for the (S) enantiomer of the substrate ($E_{\text{app}} = 1.2$). A library of approximately 108 EstA variants was screened for enhanced green and reduced red fluorescence aimed at isolating variants with enhanced (R)-enantioselectivity, with the best candidate obtained having a strong preference for the hydrolysis of the (R)-2-MDA ester ($ER_{\text{true}} = 15.5$). An inherent problem of many enantioselectivity screens lies in the fact that often enzyme variants are obtained that display enhanced enantioselectivity for the cost of enzyme activity. With our type of experimental setup only enzyme variants were obtained that retained wild type activity [4].

**Summary**

Through combination of *E. coli* enzyme display and covalent attachment of the enzyme reaction product to the cell surface followed by fluorescence labeling and FACS isolation of fluorescent cells, libraries exceeding $10^8$ candidate clones can be screened for activity and enantioselectivity in a single working day. The method is currently limited to the activity screen of lipases and esterases and will be expanded to the screening of biotechnologically relevant oxidases and dehydrogenases in the near future.

**References**


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