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Speeding up enzyme discovery and engineering with ultrahigh-throughput methods

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Abstract

Exploring the sequence space of enzyme catalysts is ultimately a numbers game. Ultrahigh-throughput screening methods for rapid analysis of millions of variants are therefore increasingly important for analyzing sequence-function relationships, searching large metagenomic libraries for interesting activities, and accelerating enzyme evolution in the laboratory. Recent applications of such technologies are reviewed here, with a particular focus on the practical benefits of droplet-based microfluidics for the directed evolution of natural and artificial enzymes. Broader implementation of such rapid, cost-effective screening technologies is likely to redefine the way enzymes are studied and engineered for academic and industrial purposes.

Introduction

Enzymes have found application as efficient and highly selective catalysts in manifold fields of chemistry and biology. The development of successful biocatalytic processes depends on the identification of a suitable catalyst for the reaction of interest and, if necessary, subsequent engineering of its properties. Genomic databases represent a valuable source of enzymes for chemical transformations important in cellular metabolism. Alternatively, viable candidates may be discovered by searching strain collections or metagenomic libraries. If a natural catalyst does not exist, for example for an abiological reaction, other strategies are necessary. One possibility is to re-design an existing enzyme, capitalizing on its inherently promiscuous catalytic abilities. Another is to create an artificial enzyme *de novo*, for example by computational design.

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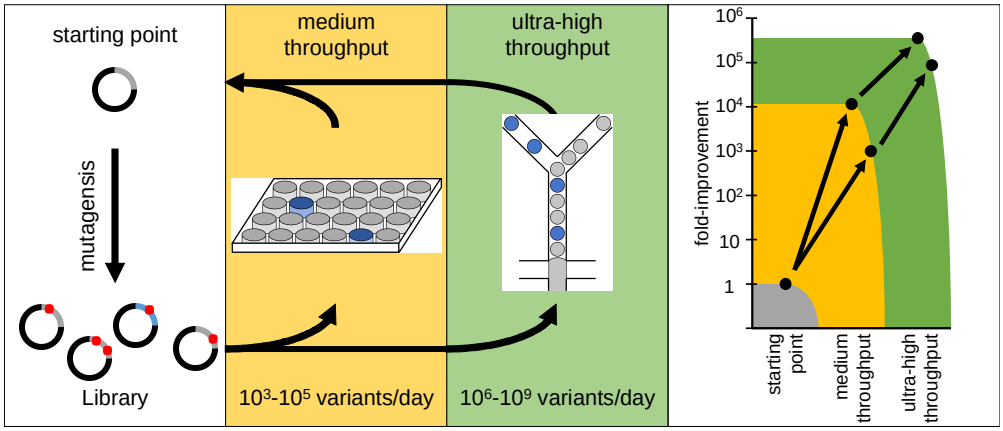
Once an appropriate starting point has been identified, it may require refinement to optimize its catalytic properties or to meet specific process needs. Directed evolution, which entails iterative rounds of genetic diversification and phenotypic screening or selection, has emerged as a particularly powerful tool for shaping protein properties in the laboratory. Specific activity, stability, substrate scope, and stereoselectivity of enzymes can be optimized using this technique.

Enzyme discovery and development both depend critically on efficient methods for identifying variants with desirable properties. Screening methods for monitoring reactions directly or through coupled assays are especially versatile in this regard, and have been used to tailor diverse enzymes for synthetic, pharmaceutical and therapeutic applications [1]. Because such methods are typically time-consuming and laborious, however, they may not match well with the pace of business [2]. In order to speed up screening campaigns, new technologies with substantially enhanced throughput have been developed [3-5]. In this review, we appraise the impact of ultrahigh-throughput screening platforms, particularly droplet-based microfluidics, on the operational study, selection, and engineering of enzyme function (Figure 1).

Figure 1



Medium and ultrahigh-throughput screening assays. The increased throughput of droplet-based microfluidics enables more efficient exploration of fitness landscapes during directed evolution than is possible with conventional microtiter plate assays.

Increasing screening throughput

Successful screening of large pools of variants to identify effective enzymes requires a robust link between genotype and phenotype. The simplest way to establish such a connection is to compartmentalize variants separately in the wells of microtiter plates, which serve as reaction chambers that can be individually monitored by chromatography, spectroscopy or mass spectrometry. Using conventional equipment, hundreds to thousands of variants may be screened per experiment, and throughput can be substantially increased by robotic automatization. Nevertheless, screening is usually the most time- and labor-intensive step in biocatalyst discovery and optimization. The large quantities of reagents and other consumables needed represent a significant cost factor, as well.

Miniaturization of the reaction chambers can increase throughput substantially, and thus the speed of a screening campaign, while reducing costs. Microtiter plates in 96-, 384- and 1536-well formats, which are compatible with standard liquid handling systems, are available. By replacing wells with microcapillaries, further miniaturization is possible. The GigaMatrix, for instance, has 100,000 capillaries per standard-sized plate and can be readily loaded through capillary forces by soaking in a cell suspension [6]. However, the small volume of each capillary (~200 nL) introduces new challenges associated with evaporation, aeration, and extraction of active clones from specific wells. Sample recovery becomes even more problematic upon further miniaturization. For lithographic microchips containing up to 340,000 wells per cm^2, for example, variants are manually extracted with toothpicks under a microscope [7].

Capillary-based approaches have been recently improved through the development of microcapillary single-cell analysis and laser extraction (μSCALE) [8●●]. The μSCALE platform utilizes chips containing 10^6 microcapillaries that can be readily loaded with a cell suspension and read out by fluorescence microscopy. The aforementioned extraction challenges are solved by accurate draining of individual capillaries with a laser pulse. In addition, volumes as small as 100 pL improve the detection limit by increasing the effective concentration of single cells in the capillaries. However,

μSCALE, like other miniaturization approaches, is limited by the inability to manipulate individual reaction chambers once they are loaded, for example to supply additional reagents. Nonetheless, several promising applications, including evolution of a fluorescent protein biosensor and screening of alkaline phosphatases libraries, have been reported using a μSCALE prototype [8●●,9●], highlighting the potential of this emerging technique.

Although wells and capillaries are the conceptually simplest way to link genotype and phenotype, assay throughput typically does not exceed ~10^6 variants. Higher numbers can be analyzed if screening is moved from a solid support to solution. For instance, intact cells can serve as sortable reaction compartments that are readily analyzed by fluorescence-activated cell sorting (FACS) [10,11]. Flow cytometers, which are widely available in many institutes, enable screening of up to 10^8 variants per day. FACS has been successfully employed to switch enantioselectivity in esterases [12], improve catalytic efficiency of peptidases [13,14] and a glutathione transferase [15], and alter the substrate scope of aminoacyl-tRNA synthetases [16] and non-ribosomal peptide synthetases [17]. Though efficient, this approach requires non-toxic fluorogenic substrates that either readily diffuse into the cell and become trapped upon reaction, or that are tethered to the cell surface to link genotype and phenotype. As an alternative, it is possible to sort enzymes encapsulated together with their encoding DNA and a fluorogenic substrate in hydrogel beads stabilized by a polyelectrolyte shell [18●●]. This approach has been successfully employed to evolve a phosphotriesterase in the laboratory.

Droplet-based screening platforms

A different approach to in-solution miniaturization relies on *in vitro* compartmentalization (IVC) of mutant libraries and reagents within surfactant-stabilized micron-sized droplets in emulsions [19]. Here, the droplet serves as the reaction chamber, and its integrity ensures the genotype-phenotype link. Active variants can be enriched by sorting water-oil-water double emulsions with conventional FACS instruments. Thiolactonases [20], galactosidases [21], cellulases [22], and other hydrolases

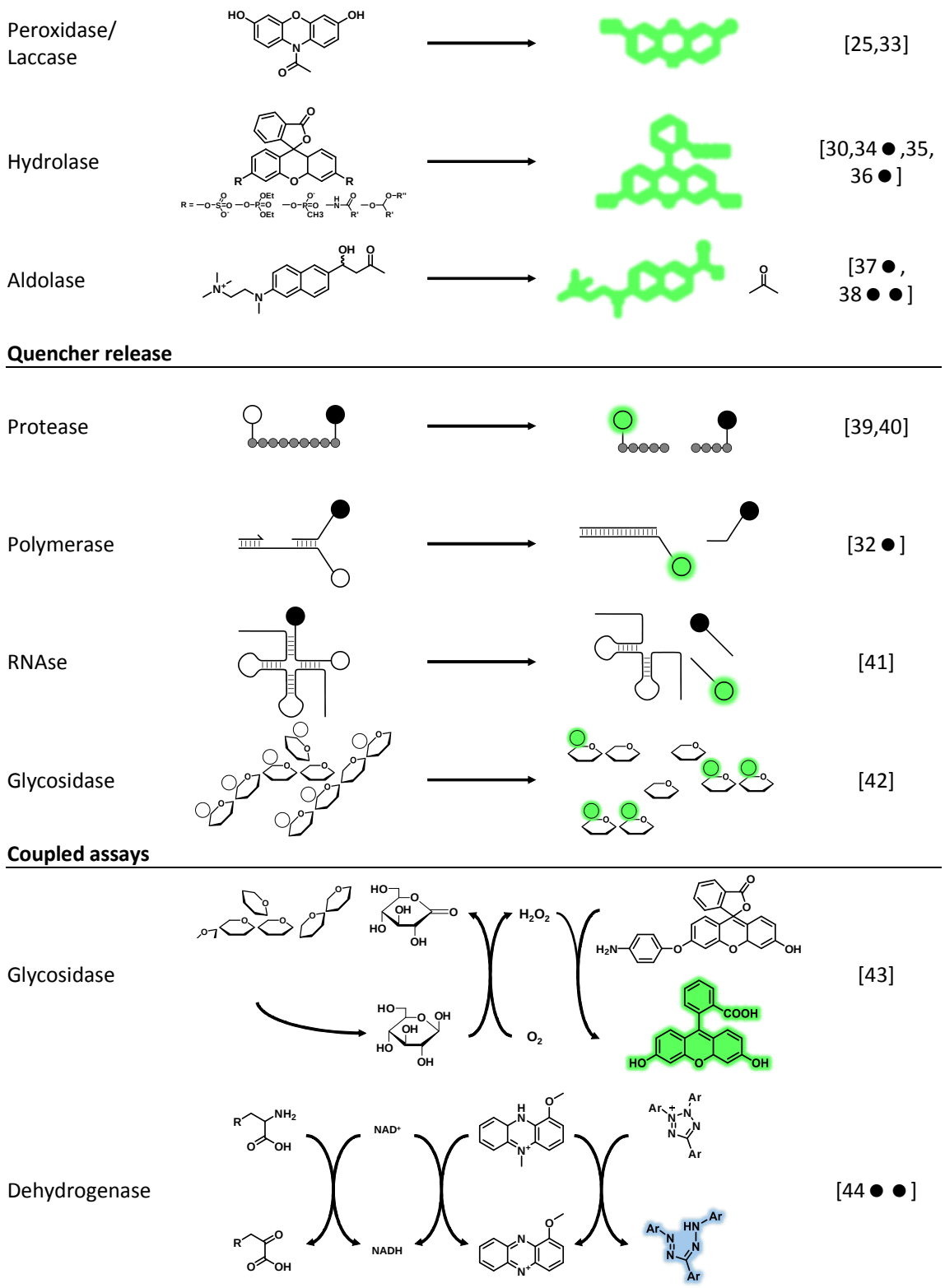
[23●,24] have been successfully evolved in this way. Because common emulsification strategies lead to polydisperse droplet populations, the relationship between activity and readout is variable and the detection of hits is therefore problematic.

The advent of microfluidic technologies has greatly expanded the utility of IVC. Droplet-based microfluidics enables production of large numbers ($\sim 10^8$) of monodisperse droplets at very high rates by continuous flow on chip [25]. Sophisticated manipulations such as droplet fusion, incubation, mixing, splitting, and sorting are also possible [26]. As a consequence, droplet-based microfluidics has become a powerful tool for analyzing and manipulating enzymes encapsulated within pico- or nanoliter droplets, combining the versatility of traditional microtiter plate-screening with the high throughput accomplished by FACS. Moreover, the tiny volumes involved reduce the costs of screening a single clone, as well as the waste generated, by as much as a million fold compared to automatized microtiter-plate screening [26].

Various methods are available for compartmentalizing enzyme libraries in droplets. In one modality, the catalyst variants are biosynthesized directly from their encoding genes by *in vitro* transcription and translation [27], eliminating bottlenecks associated with cellular transformation efficiencies. Alternatively, they may be produced *in vivo* by encapsulated host organisms. Depending on the protein and the assay, the enzymes can be secreted, displayed on the cell surface, or produced cytosolically. The substrate is either co-encapsulated with the host cell or added at a later time point by droplet fusion [28] or picoinjection [29]. If the cells do not take up the substrate, they can be lysed enzymatically [30], electrically [31] or by heating [32●] directly in the droplets to free the catalyst.

As for all miniaturized reaction chambers, sensitive detection methods are the key to success. These are usually based on changes in fluorescence (Figure 2). For instance, fluorophores have been activated by enzymatic oxidation [25,33], hydrolysis [30,34●,35,36●], and retro-aldol cleavage [37●,38●●]. In addition, removal or displacement of a quencher by proteases [39,40], polymerases [32●], RNAses [41], or glycosidases [42] has afforded fluorescent readouts. Finally, coupled assays can be employed to detect the activity of enzymes such as glycosidases [43] and dehydrogenases [44●●] in droplets. To maintain the link between genotype and phenotype, the fluorophore must be retained

Figure 2



Reactions targeted with droplet-based microfluidics. The assays typically rely on direct activation of the fluorophore, release of a quencher, or coupled assays. Reactions can be monitored via a fluorescence (green) or absorbance (blue) readout.

in the droplet where it is produced [45,46]. Equipping it with charged or hydrophilic groups is generally an effective means to minimize leakage [47,48]. Because droplets are quite stable, enzymatic reactions can be monitored over timescales ranging from minutes to hours on-chip [49], and up to days off-chip [50]. As a consequence, enzymes that vary by at least five orders of magnitude in catalytic efficiency are assayable with this technique [25,37●].

Library enrichment is performed with efficient microfluidic fluorescence-activated droplet sorters (FADS) that sort single cells compartmentalized in water-in-oil emulsion droplets by dielectrophoresis at rates between 1,000 and 30,000 droplets per second [51]. The ultra-high throughput of such devices enables screening of up to 10^9 variants in a day, outcompeting the efficiency of robotic microtiter plate screening by several orders of magnitude. The sorted populations are generally isolated in bulk, and the genetic information propagated either by regrowth of the isolated active variants, if intact cells are assayed, or amplified by PCR and recloned, if cell lysates are analyzed. To minimize false positives resulting from variable single-cell expression levels or encapsulation of multiple cells per droplet, sorting is usually repeated two to three times to ensure sufficient enrichment before individual variants are analyzed via conventional plate assays. Owing to the efficiency of these procedures, screening no longer limits the pace of discovery in favorable cases. Instead, DNA recovery after sorting, subsequent genetic diversification, and downstream data analysis constitute more significant bottlenecks.

The recent development of a microfluidic absorbance-activated droplet sorter (AADS) has the potential to extend the range of assays amenable to high-throughput screening considerably [44●●]. Although AADS currently has lower throughput (300 droplets per second) than FADS, and requires more advanced chip manufacture, successful enrichment of active NAD^+-dependent amino acid dehydrogenases using a miniaturized coupled assay convincingly established the feasibility of implementing widely used chromogenic assays in microfluidic workflows. Much effort is currently invested in coupling droplet-based microfluidics with other label-free detection methods such as electrochemical detection, mass spectrometry, and IR, Raman and NMR spectroscopy [26]. Rapid

158 determination of enzymatic stereoselectivity is of particular interest for the identification and
159 development of synthetically useful biocatalysts. Recent work has shown that the selectivity of
160 enzymatic chiral resolutions conducted in droplets containing a few hundred cells can be analyzed in
161 chip-integrated electrophoretic systems, paving the way towards microfluidics-based evolution of
162 stereoselective enzymes [52,53]. Successes in this arena promise to make droplet-based microfluidics
163 a universally applicable screening platform.
164
165 **Discovery, characterization and optimization of natural enzymes**
166 By making rapid and efficient searches of sequence space possible, ultrahigh-throughput screening
167 systems are uniquely suited to aid the discovery, characterization and engineering of enzymes
168 (Figure 1). Enrichment of rare enzymes present in large populations of predominantly inactive
169 proteins illustrates the practicality of such approaches. In one recent example, droplet-based
170 microfluidics facilitated isolation of proficient natural sulfatases and phosphatases from a
171 metagenomic library at a hit rate of only one in a million variants [34●]. In another, bacteria
172 possessing elevated cellulase activity were similarly identified in libraries of uncultivated
173 microorganisms [36●]. Encapsulation of the microorganisms in droplets circumvented the need for
174 cumbersome cultivation of individual clones prior to assay. Moreover, the microfluidics screen
175 afforded hits with broader diversity and higher activity than traditional plate assays.

176 When combined with next-generation sequencing, ultrahigh-throughput assays represent a
177 powerful strategy for comprehensively analyzing sequence-function relationships in biopolymers
178 [54●●,55-60]. This approach has been used to map the activity of millions of glucosidase variants
179 generated by error-prone PCR [54●●]. The resulting fitness landscapes not only revealed patterns
180 of mutational tolerance, but also identified previously unknown sites crucial for glycosidase function
181 and facilitated discovery of mutations that enhanced enzyme thermostability. In addition to insights
182 into intrinsic protein properties, such mutational scanning experiments can shed light on how
183 proteins behave in cells and the effects of genetic variation in higher organisms [55]. They also aid

enzyme engineering by identifying potential mutagenesis hotspots as shown by the conversion of 4-oxalocrotonate tautomerase into two enantiocomplementary 'Michaelases' [56]. In addition, mutational scanning by microfluidics offers a rapid means of generating neutral drift libraries [61]. Neutral drift libraries may in turn lead to discovery of novel activities and selectivities, as was shown in studies on serum paraoxonase [62] and a cytochrome P450 [63].

The ability to screen large libraries of variants is particularly valuable for refinement of enzymatic activities and selectivities by directed evolution. The number of possible protein variants is essentially unlimited, so strategies that increase the likelihood of identifying rare but desirable clones constitutes a major advantage. Exhaustive screening of libraries in which multiple residues are simultaneously randomized increases the chances of discovering both epistatic and synergistic mutations that are typically missed in medium-throughput assays [64]. Although significant rate accelerations have been achieved by iterative saturation mutagenesis using small subsets of proteinogenic amino acids to reduce combinatorial complexity [65], four to five residues can be completely randomized and exhaustively assayed with droplet-based microfluidics [37●,38●●]. Iteration of this process can be expected to lead to large evolutionary leaps. In principle, alternative evolutionary trajectories from one or more starting points can also be explored by this approach.

Identifying beneficial mutations usually becomes increasingly difficult in later rounds of directed evolution. For example, efforts to evolve a promiscuous arylsulfatase to cleave a fluorogenic phosphonate ester using a conventional microtiter plate assay failed to yield any improved variants [30]. Increasing the throughput and screening millions of variants by microfluidics can sometimes rescue such stalled evolutionary trajectories. In the case of the arylsulfatase, two rounds of mutagenesis and microfluidic screening afforded variants with 6-fold higher phosphatase activity and a comparable improvement in expression. Other catalysts have similarly benefited from this approach, including a nearly diffusion-limited variant of horseradish peroxidase (10-fold improvement) [25] and a ribozyme with RNase activity (28-fold improvement) [41]. In the latter case,

screening under multiple turnover conditions using droplet-based microfluidics proved superior to conventional SELEX (systematic evolution of ligands by exponential enrichment) experiments.

Optimizing computationally designed enzymes

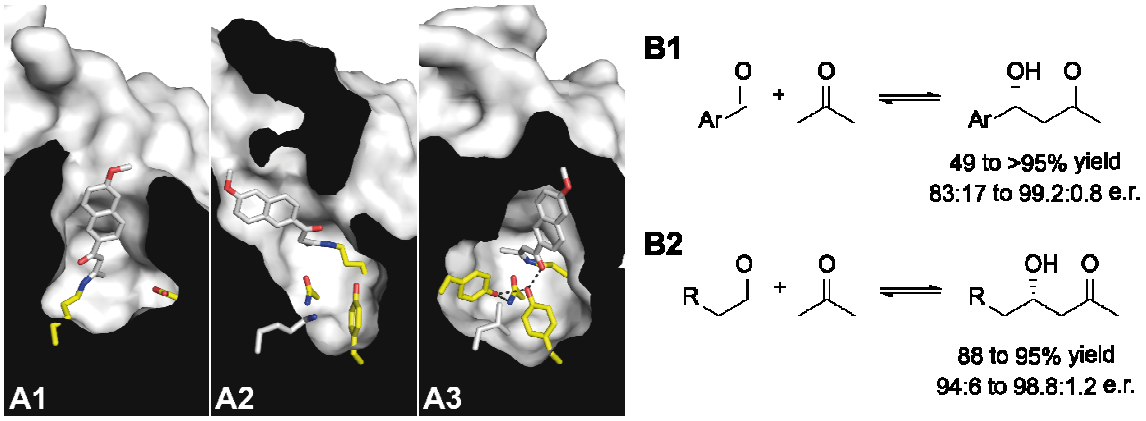
Enhancing promiscuous activities by directed evolution has produced a broad repertoire of enzymes with abiological function. Creating artificial enzymes *de novo* with made-to-order activity is, in contrast, a far more challenging task. Over the last decade, biocatalysts for several abiological reactions have been created using computational tools to design enzymatic active sites and embed them in protein scaffolds [66]. Although the starting catalysts typically exhibit only modest activities, they are excellent starting points for directed evolution [67]. In favorable cases, computationally designed and experimentally optimized enzymes have achieved proficiencies matching those of natural enzymes [38●●,68].

Evolving designed enzymes by conventional microtiter plate-based assays is a long, arduous process. For instance, seventeen rounds of evolution were necessary to create an artificial Kemp eliminase that accelerates a proton transfer reaction 6 x 10^8 fold [68]. Optimization of computationally designed Diels-Alderases [69], phosphoesterases [70] and (retro)aldolases [71,72] proved similarly difficult. Much speedier optimization is possible with high-throughput screening methods, as exemplified by a weakly active retro-aldolase [37●]. By exhaustively interrogating a significant fraction of the $(\beta\alpha)_8$-barrel cavity by cassette mutagenesis, two different catalytic arrangements were identified in a single experiment that enhanced retro-aldolase activity up to 80-fold. The complementary stereoselectivity of these improved variants was also notable.

The large dynamic range of microfluidics-assisted assays makes improvement of already highly active variants possible as shown by the evolution of an artificial retro-aldolase with efficiency rivaling that of natural class I aldolases [38●●]. The activity of the starting computational design was increased 4000-fold using a medium-throughput microtiter plate assay, but further gains proved elusive as beneficial mutations became increasingly rare after over thirteen rounds of evolution [71].

Subsequent microfluidic screening of large libraries prepared by targeted mutagenesis and error-prone PCR led to the identification of sixteen additional substitutions that boosted activity 30 fold to give a >10^9-fold rate enhancement (Figure 3A) [38●●]. In addition, unlike its progenitors, the resulting enzyme is a practical synthetic catalyst for asymmetric aldol additions of acetone to diverse electrophilic aldehydes (Figure 3B). Structural and mechanistic analyses suggest that its high activity and stereoselectivity can be ascribed to a constellation of four catalytic residues that emerged, one residue at a time, over the course of evolution [73]. Microfluidics screening will likely be useful for diversifying the promiscuous activities of this catalyst [74-77] and other *de novo* designed enzymes as well.

Figure 3



Directed evolution of a computationally designed aldolase using a droplet-based assay. A: Crystal structures of the starting design (A1), an evolutionary intermediate (A2), and the evolved aldolase RA95.5-8F (A3) represented in equivalent orientations. Key residues and a covalently bound mechanistic inhibitor are shown in stick representation. The catalytic residues of each variant are highlighted in yellow. B: Asymmetric aldol additions of acetone and aromatic (B1) or aliphatic (B2) aldehydes catalyzed by RA95.5-8F.

Perspective and future challenges

High-throughput strategies are revolutionizing the biological sciences. Today it is possible to synthesize and sequence DNA on a massively parallel scale. Large metagenomic libraries of microbial genomes found in nature are being created by directly extracting and cloning DNA from

environmental samples. And methods are available to diversify, in whole or in part, genes encoding specific natural and designed proteins to probe evolutionary relationships and alter or optimize biochemical function. Not surprisingly, comparable methods capable of exploring and exploiting this diversity, efficiently and in high-throughput, are increasingly vital. Powerful screening platforms based on microcapillaries and microfluidic devices have tellingly illustrated how new technologies can complement and extend traditional approaches for analyzing phenotype based on genetic selection or microtiter plate assays. In the future, commercialization of μSCALE devices, droplet sorters and the like, as well as development of more flexible detection systems and invention of completely new ways to survey biological function on large scale will only enhance the accessibility, reliability, and affordability of such systems. High-throughput screening is destined to become an essential tool for any investigation of protein function.

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