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High-throughput screening technologies for enzyme engineering

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Emerging technologies are enabling ultra-high-throughput screening of combinatorial enzyme libraries to identify variants with improved properties such as increased activity, altered substrate specificity, and increased stability. Each of these enzyme engineering platforms relies on compartmentalization of reaction components, similar to microtiter plate-based assays which have been commonly used for testing the activity of enzyme variants. The technologies can be broadly divided into three categories according to their spatial segregation strategy: (1) cells as reaction compartments, (2) *in vitro* compartmentalization via synthetic droplets, and (3) microchambers. Here, we discuss these emerging platforms, which in some cases enable the screening of greater than 10 million enzyme variants, and highlight benefits and limitations of each technology.

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Introduction

The ability of enzymes to catalyze a diverse set of reactions with often exquisite specificity makes them both fascinating subjects for biochemical study and also promising catalysts for reactions useful to humankind. Indeed, many naturally occurring enzymes have the potential to accelerate a variety of reactions for applications such as the production of pharmaceuticals, fuels, and materials through scaled-up biocatalysis or fermentation processes [1]. However, natural enzymes have evolved under physiological conditions for the benefit of the organisms in which they reside, and thus generally require modification for industrial and research use. Protein engineering is the effort to optimize a protein's sequence to generate a desired phenotype, and has been applied to enzymes to study their biochemistry [2,3,4•] and to make them more useful industrial catalysts [5•]. Enzyme engineering involves screening mutants to identify variants with improved properties such as increased activity, altered substrate specificity, increased stability, or tolerance to changes in pH or temperature. These efforts require an assay where an enzyme variant's catalytic activity is coupled to a biochemical readout, such as a change in optical properties (*e.g.*, fluorescence or absorbance), usually via either substrate depletion or product formation [6].

While it is possible to rationally design and test a specific set of mutant enzymes using existing biochemical information [7], there is great interest in evaluating large numbers of enzyme variants. Combinatorial engineering strategies mimic the algorithm of natural evolution by subjecting the enzyme to iterative rounds of genetic diversification with DNA mutagenesis followed by phenotypic selection based on an enzyme activity assay. These approaches benefit from bulk PCR-based methods that enable the creation of libraries of thousands to millions of mutated enzymes produced using cell free protein expression or in host cells such as bacteria or yeast, where each individual cell expresses one distinct protein variant [6,8]. While library synthesis using established methods is relatively facile, the creation of large numbers of enzyme mutants results in a screening challenge: the variant yielding a particular phenotype must be able to be traced back to the genotype that encoded it so that beneficial gene mutations can be identified [9]. This connection between genotype and phenotype can be difficult to achieve with enzyme engineering since the activities being measured usually rely on substrates and products that naturally diffuse away from the enzyme after catalysis. Genotype-to-phenotype linkages can be reliably achieved by spatial separation of library members in microtiter plate wells or on agar plate colonies. Although advancements in sophisticated robotic plate-based platforms have been made [10], the throughput of these methods is typically limited to 10^3–10^4 variants per screen [8].

Advances in computational modeling in recent years have greatly improved the efficiency of enzyme engineering by

using *a priori* biochemical and structural information to focus library design and inform engineering strategies [11–13]. Although smaller, focused libraries and microtiter plate-based screening methods have been sufficient for a number of enzyme engineering projects, there are many examples where larger libraries paired with higher throughput screening methodologies are required or desirable. First, when limited biochemical or structural data is available, a broader library of random mutations is often necessary, at least initially, to find beneficial mutations or key 'hot spot' functional residues. Moreover, a more extensively mutated enzyme library, created through error-prone PCR or saturation mutagenesis of multiple residues, yields the greatest probability of finding epistatic interactions between mutations which are neutral or deleterious alone but beneficial when paired [14•].

To screen larger enzyme libraries with acceptable coverage, one must employ a protein engineering platform that can achieve higher throughputs than microtiter plates while maintaining the genotype-to-phenotype connection required for directed evolution. With the exception of strategies that connect enzyme activity directly to survivability or infectivity [15], these engineering platforms generally have three discrete components, operating together to achieve a directed evolution workflow (Figure 1). First, a compartmentalization strategy is employed to spatially segregate the enzyme genotype (*e.g.*, a cell harboring a plasmid encoding the variant) with an optically detectable proxy for enzyme activity (*e.g.*, a fluorescent product). Next, an optical technology for measuring the assay signal of the reaction compartments is used to assess enzyme function in high-throughput. Lastly, a strategy for isolating desirable enzyme mutants from the rest of the library members is employed. This review will highlight emerging protein engineering platforms available for screening enzyme libraries that expand beyond the throughput capabilities of microtiter plates while retaining the ability to assess enzyme activity as the phenotypic readout.

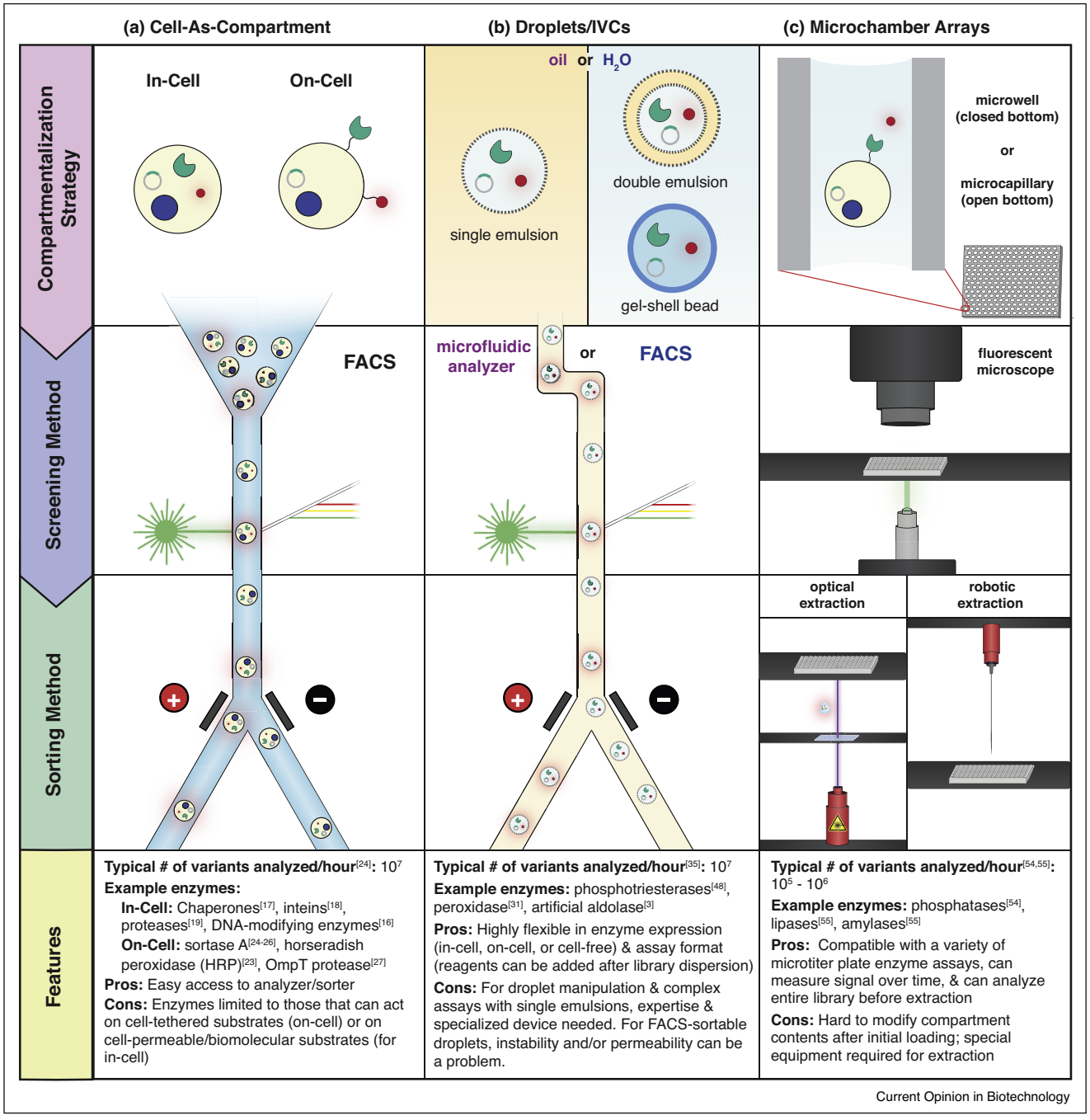
Cell-as-compartment platforms for enzyme engineering

Cells provide several natural compartments that can serve as enzyme reaction vessels to couple genotype to phenotype. Using the cell itself as the measurable, sortable compartment in an engineering screen is attractive because fluorescence activated cell sorting (FACS) provides both a screening and a sorting technology for the directed evolution workflow with broad device availability and ease of use. The challenge for enzyme engineering using individual cells as compartments comes in developing a strategy for keeping an assay signal tethered to or contained within the cell.

The most straightforward of these strategies employs the cytoplasm or another compartment within the cell as a reaction vessel. This approach can be reliably applied to enzymes that use biomolecules as substrates, since they naturally reside inside of the cell. For example, in-cell enzyme assays have been successful for engineering DNA recombinases [16], protein chaperones [17], inteins [18], and proteases [19] by linking enzyme activity to the expression, folding, or trafficking of a fluorescent protein. It is also possible to use in-cell enzyme engineering with external substrates provided that the substrate is cell-permeable and that the enzyme activity can be linked to the generation of in-cell fluorescence. As one example, glycosyltransferases have been engineered in the cytoplasm of *E. coli* since fluorescently-labeled versions of many sugar substrates can gain access into the cell via dedicated transporters, and subsequent enzymatic activity results in a fluorescent product that is unable to leave the cell [20]. Alternatively, if the reaction has no cell permeable fluorescent substrate, activity can instead be coupled to production of a detectable reporter protein. For example, a three-hybrid chemical complementation system couples enzymatic processing of a cell-permeable small molecule substrate to reporter transcription by using DNA binding and regulatory domains that are bridged by the substrate [21,22]. Although technologies like chemical complementation can be generalized to other enzymes, the requirement for substrate permeability, the challenges of coupling enzyme activity to an intracellularly-confined fluorescent readout, and the rigid chemical conditions inside the cell still limit the enzymes and substrates that are amenable to in-cell engineering approaches.

A more generalizable strategy uses cell, virus, or particle display to provide the enzyme with access to a wider range of substrates. To maintain the genotype-phenotype linkage, enzyme engineering methods based on surface display must tether the assay signal resulting from the enzymatic activity to the outside of the cell or entity harboring the variant genotype. In the case of bond-forming enzymes, one substrate can be labeled and in solution while the other substrate is physically tethered to the cell or particle surface such that the ability of the displayed enzyme to attach the labeled substrate to its membrane-tethered partner is proportional to the activity of the enzyme variant displayed on the same cell. This approach has been used, for example, to engineer horseradish peroxidase [23] and the bioconjugation enzyme sortase A (srtA) [24,25••,26]. For bond-breaking enzymes like proteases, a similar strategy can be employed with the addition of fluorescence resonance energy transfer (FRET) probes on either side of a cell-tethered substrate such that FRET activity is lost after enzyme processing [27]. In general, if an enzyme's activity can act on a cell tethered version of a substrate on the surface where it is displayed, then an on-cell technology may be amenable for combinatorial enzyme engineering.

Figure 1



There are three main categories of high throughtput platforms that are commonly used for enzyme engineering. These platforms differ in their technique for keeping the phenotype (enzyme activity assay signal, depicted as a red circle) and genotype (depicted as a green gene/enzyme) connected, and they differ in the available methods for library analysis and sorting. **(a)** In cell-as-compartment platforms, the enzyme variant is expressed within or displayed on the surface of a cell and the assay signal is tethered to the surface or captured in the cell interior. The cell library can then be analyzed and sorted by FACS. **(b)** *In vitro* compartmentalization (IVC) is a strategy where spatially segregated droplets are formed around the library member to keep the assay signal contained with the enzyme genotype. Single emulsion (water-in-oil) droplets can be manipulated in complex ways and are analyzed and sorted, usually by dielectrophoresis or sophisticated microfluidic devices. Double emulsions (water-in-oil-in-water) and gel-shell beads place the droplet in an aqueous carrier fluid and can be sorted with a standard flow cytometer. **(c)** Microchamber arrays include microwell and microcapillary arrays and use micron-scale chambers to separate enzyme variants. The arrays are analyzed with a fluorescence microscope and sorted by optical extraction (*e.g.*, laser disruption of capillary action) or robotic extraction (*e.g.*, aspiration).

Droplet-based platforms for enzyme engineering

While *in vivo* compartments are attractive engineering units, the requirement for a cell-tethered assay signal presents a significant limitation for the types of enzymes that can be engineered. To overcome these limitations, engineering platforms have been developed that allow freedom of product and substrate diffusion by using a man-made compartment to spatially segregate the assay for enzyme variant activity (phenotype) with the corresponding DNA or cell (genotype). These artificial compartments fall into two broad categories: droplets (*i.e.*, *in vitro* compartmentalization, or IVC) and microchambers.

In IVC, the library is partitioned into millions of micron-scale water-in-oil droplets, with each droplet acting as an independent microreactor containing a single library member. Droplets are generated in lithographically-defined microfluidic devices at thousands of droplets per second, with volumes typically ranging from femtoliter to nanoliter and controlled by the channel dimensions and fluid flow rates [28]. Integrated microfluidic sorters are used to screen highly fluorescent droplets at kHz frequencies, typically by dielectrophoresis [29], although other sorting modalities have been developed [30]. In these devices, droplets flowing single file are diverted into a collection or waste chamber according to a user-defined sort gate by an applied electric field. This approach has been used to screen a library of 10^8 yeast surface-displayed horseradish peroxidase enzymes encapsulated in picoliter-sized droplets [31]. In this study, a near diffusion-limited variant was isolated with 10-fold improved kinetics towards a fluorogenic substrate as compared to the parent enzyme.

The major advantage of IVC is that droplets have been shown to be compatible with a wide range of enzyme expression and assay format modalities. IVC is especially useful for engineering enzymes that are not easily displayed on a cell surface or do not have cell-permeable substrates. In fact, completely cell free systems may be used, whereby a single gene is transcribed and translated within the droplet, which are especially useful for engineering enzymes that are toxic to cells [32]. For cell-based enzyme expression, access to intracellularly expressed enzymes can be achieved by cell lysis via co-encapsulation of lysis reagents [33•] or heating [34•] within droplets. Because of this flexibility, examples of enzymes engineered by droplet technologies are many and varied and have been recently reviewed [35].

Moreover, one can leverage droplet technology for enzyme assays that require multi-step processing. Reagents can be added to droplets by picoinjection [36] or droplet merging [37], for example, to initiate or terminate an enzymatic reaction or lyse cells after a growth period. Droplets may also be thermocycled in conventional instruments to amplify genes [32] or can be used to culture cells on- or off-chip [38] and reinjected between different microfluidic devices [37]. Droplets can be split [39], frozen and thawed [40], paired [41], and broken [42] according to the requirements of the assay. As an example, a multistep droplet approach was used to decouple an *in vitro* translation (IVT) and enzyme activity assay that were incompatible with one another [43]. Indeed, droplets are versatile and mobile microreactors that can be used for performing highly-parallel complex assays. However, this complexity comes at the cost of increased expertise needed to integrate sophisticated optics and electronics to utilize the technology. To circumvent this limitation, a double emulsion strategy was developed that is compatible with conventional FACS instruments [44]. This approach was used to engineer a paraoxonase enzyme with 10^5-fold increase in activity towards an *in situ* generated G-type nerve agent [45]. Although double emulsions served to decrease the barrier to entry into droplet-based screens, they are not as robust as single emulsions, requiring carefully balanced parameters (surface chemistry, viscosities, flow rates, etc.) to produce monodisperse droplets, and even then, the integrity of the droplets may be compromised during FACS [46]. Recently, an elegant single emulsion approach to generate polymeric gel-shell beads (GSBs) was developed to produce more robust droplets for FACS [40,47••]. In this method, compartments are constructed from an agarose core surrounded by a polyelectrolyte shell that serves to trap the high molecular weight contents of the agarose core from diffusing out. This approach was used to screen 10^7 phosphotriesterase mutants in one hour to identify a variant with 20-fold faster kinetics.

Microchamber-based platforms for enzyme engineering

Microchambers are the other class of man-made compartments used for high throughput enzyme evolution. These devices maintain each enzyme variant in a physically separated vessel, essentially miniaturizing the microtiter plate and increasing the number of library members that can be screened in parallel. The two formats of microchambers that have been used for enzyme engineering applications are microwell arrays and microcapillary arrays. Microwell arrays are comprised of micron-scale wells fabricated with an open top and closed bottom onto a glass or polymer medium, typically with micromachining or lithography, and are loaded by discontinuous dewetting [48,49]. Microwell arrays often make use of cells or functionalized microspheres to spatially separate single proteins for engineering [50], screening [51], or single molecule analysis [52,53]. Microcapillary arrays, which contain millions of spatially segregated, bottomless high-aspect-ratio microcapillaries (usually in a glass medium), can likewise be used to segregate single cells or particles but can be loaded using simple capillary action. In both microwells and microcapillaries, the

concentration of cells or particles in the library suspension must be controlled such that no more than a single cell or particle on average will occupy a chamber, and this concentration is typically calculated with Poisson statistics [49,54••].

The major advantage of these technologies for enzyme engineering is the ability to readily miniaturize and therefore increase the throughput of a variety of enzyme screening assays that are traditionally performed in the microtiter plate format, provided they use a fluorescent read-out. Notably, 96-well-plate assays that monitor enzyme activity via production of a fluorescent product in many cases can be adapted directly in microchamber array technologies. For example, a microwell-array-based platform used hydrolysis of a fluorescein dioctanate to release fluorescein to engineer a lipase enzyme [50], while microcapillary array-based platforms used hydrolysis of a surrogate substrate to engineer alkaline phosphatase for activity and specificity by tracking conversion to a fluorescent dephosphorylated product [54••]. Reportedly, the GigaMax microcapillary array platform was used to miniaturize a number of assays to engineer enzyme classes ranging from proteases to amylases at throughputs up to two million variants per day [55]. With this method, a bacteriophage library is propagated in bacteria, and lyses them to release the enzyme variant after loading and growth in the microcapillary array. Because microwell and microcapillary arrays are imaged with a microscope, the complete library can be screened at multiple time points and the measurement data can be analyzed before selecting hits, which represents a distinct advantage over microfluidic-based platforms. These technologies have so far only been used to analyze enzyme kinetics of variants but could in principle be used to screen libraries based on kinetic parameters [54••].

A technological challenge for microchambers arises in how to manipulate their contents to add reagents or recover clones in a consistent manner. To extract desired clones, sophisticated robotics have been used to precisely aspirate [56] or introduce air to propel [57•] the contents from the microscopic chambers. Other methods utilize light manipulation with optical tweezers [58] or optical cavitation [54••] to precisely empty the chamber contents. For example, the microcapillary single cell analysis and laser extraction (μSCALE) platform retrieves capillary contents by breaking the surface tension of a capillary with a pulse of laser light, likely by forming a cavitation bubble that disrupts the surface tension [54••].

Considerations and future directions

While traditional microplate screens are still a workhorse in the enzyme engineering field, new methods for performing directed enzyme evolution at higher throughputs have emerged and are already demonstrating promise. Technological advances are enhancing the automation and throughput of enzyme screens, resulting in faster, less labor-intensive, and more efficient workflows. As a result, these technologies are enabling deeper exploration of an enzyme's sequence space in less time.

When evaluating ultra-high-throughput technologies for an enzyme engineering screen, there are advantages and limitations of each method that must be considered. The primary advantage of cell-as-compartment technologies is the convenience of using natural components of a cell to segregate enzymatic assays and the ability to screen with flow cytometry, which can readily analyze and sort libraries upwards of 10^7 variants. However, in-cell technologies depend on substrate access to the cell interior, while on-cell technologies rely on the ability of the enzyme to be displayed and function on cell-surface tethered substrates or products. In contrast, IVC-based technologies are flexible in the mode of enzyme expression and allow for exquisite control of reaction chamber contents, but they require considerable expertise to implement their best features. Additionally, the compartment formation and stability can be sensitive to chemical and physical conditions and may sometimes be undesirably permeable to small molecules [59]. Glass or polymer microchambers represent more rigid and stable compartments, but these platforms are limited by difficulties in controlling reaction contents after initial microwell or microarray loading. Moreover, while microchamber arrays allow for rapid imaging and analysis of a complete library before hits are selected, extraction by mechanical manipulation restricts the sorting throughput to upwards of 120 clones per minute with current instrumentation [54••]. The limitations of each approach are factors to consider for improvement in future iterations of these technologies.

The broader application of high throughput screening technologies involves the continued development of assays to expand the breadth of enzyme classes that can be engineered. Well-validated assays with appropriate signal intensity and dynamic range are critical to effectively differentiate enzyme variants during a library screen. Additionally, modifications in instrumentation hardware to enable optical properties beyond total fluorescence, such as absorbance, would be valuable; efforts have already been made towards this goal [60••]. Interest in these platforms will only continue to increase as they are extended to broader classes of enzymes, as well as other proteins, and thus on-going efforts are also focused on developing opportunities for more widespread use among non-experts in industry and academia.

Conflict of interest

J.R.C is listed as an inventor on pending patent applications owned by Stanford University related to high throughput screening technology, and has financial interests in xCella Biosciences, Inc. which is commercializing

technology related to high-throughput protein analysis and engineering.

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