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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.