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The Laboratory in a Droplet

In this issue of *Chemistry & Biology*, the groups of Tawfik [1] and Griffiths [2] present fluorescence-activated cell sorting of double emulsions as a generally applicable screen for enzyme activity. This novel methodology increases the throughput of a typical enzyme screen by two orders of magnitude.

Numbers are all important in the world of high-throughput protein technology, as the size of the throughput can make the difference between success (striking gold with a rare positive event) and failure (not digging deep enough!). Today's biological questions often concern scales of millions and billions: the human interactome is currently estimated at 280,000 functional interactions from a total of 2.4×10^8 possible pairwise combinations, whereas screening for tomorrow's antibody pharmaceuticals is performed on libraries of 10^9–10^{13} molecules (www.cambridgeantibody.com). Current techniques for assaying enzyme activity, however, fall far short of the mark. Typical high-throughput screens are performed in microplates at scales of 10^3–10^5 reactions, and the investment of handling time and reagent costs fixes a ceiling of 10^6–10^7.

Microplates permit parallel reactions of soluble molecules without diffusion, cross contamination, or loss of identity of each well, but trends toward miniaturization and increased throughput will eventually reach their limits. Nature suggests an alternative compartmentalization, namely the cell, which retains the advantages of the microplate, but at drastically reduced size. Cells keep together the genes, the RNAs, and the proteins that they encode, and the products of their activities, thus linking genotype to phenotype. Inspired by this property, an in vitro system of cell-like compartments was developed by Tawfik and Griffiths in 1998 [3]. An aqueous in vitro transcription/translation reaction is emulsified to give a water-in-oil (w/o) emulsion of 10^{10} droplets. Since DNA is added at limiting dilution, each aqueous droplet contains a single gene, and acts as a unique, independent reaction vessel. Initially the method was demonstrated by in vitro expression of DNA methyltransferases, which then methylated their own genes, providing a selection criterion for active enzymes.

In the last few years, activity-based selections of other DNA-associated enzymes have been developed, including polymerases [4] and restriction enzymes [5]. Heat-stable w/o emulsion formulations allow PCR in emulsion as a method for superior unbiased amplification of complex samples of genomic DNA or RNA [6], and the generation of libraries of DNA on beads as a viable alternative to cloning [7–9]. In vitro expression in emulsion is an efficient method to generate large libraries of proteins physically linked to their encoding genes either by direct covalent attachment or via beads [10]. Microfluidic channels offer improved methods to generate and manipulate emulsions of precisely controlled droplet size, and the contents of droplets can be interrogated by confocal microscopy [11]. Recently the massively parallel nature of emulsified reactions was exploited by two independent groups to sequence an entire bacterial genome in a single reaction [12, 13]. Each group developed an innovative method of sequencing by synthesis, and in both cases, the role of the emulsion is in single-molecule "cloning" and template preparation on beads.

Emulsion technology offers unprecedented high throughput of compartmentalized reactions and will undoubtedly be taken up, adapted, and applied for diverse new methods, some as yet unimagined. Nevertheless, it has suffered from one serious drawback so far: the lack of a general method to assay the contents of each droplet and retrieve those of interest. Directed evolution of enzyme function in emulsions was either obligately linked to gene survival (selection rather than screening), with the inherent compromises in scope of reaction and dynamic range, or was reliant on bead capture and subsequent screening of the beads.

To overcome this limitation, the groups of Tawfik and Griffiths have turned back to the analogy of cells. Fluorescence-activated cell sorting (FACS) is a cornerstone technique in biology, as single cells can be selected at a rate of 10^7 per hour on the basis of one (or several) fluorescence properties. Fluorescence assays are extremely sensitive, and the display of proteins on the surface of yeast, followed by capture of fluorescent ligands, is becoming an important screen for binding affinity [14]. Although fluorogenic substrates exist for many enzymes, FACS has not been widely applied to screen enzyme activity because of the difficulty in capturing the fluorescent products after the reaction. For individual enzymes, particular strategies have been devised to capture products on the cell surface [15], or on beads [16], but the need to design and synthesize a new substrate for each enzyme severely limits the scope of FACS for application to new enzymes, and limits the accessibility to specialist laboratories.

The breakthrough described in this issue of *Chemistry & Biology* [1, 2] lies in the formation of double water-in-oil-in-water (w/o/w) emulsions to provide stable linkage between genes, enzymes, and enzyme products. The oil layer which surrounds each droplet has low, controllable permeability, allowing fully soluble reactions to be performed in complete isolation from each other. The external aqueous phase renders the emulsions nonviscous and amenable to manipulation in a standard flow sorter (Figure 1). These papers pave the way for FACS screening of almost any fluorogenic assay. The two groups demonstrate the efficiency of the method with unrelated enzymes: Tawfik and coworkers assayed thiolactonase activity by coupling of the reaction product to a thiol-reactive fluorogenic dye, whereas Griffiths and coworkers detected β-galactosidase by the release of fluorescein from fluorescein di-β-D-galactopyranoside. Test mixtures of active and inactive genes were screened to demonstrate detection of enzyme activity,

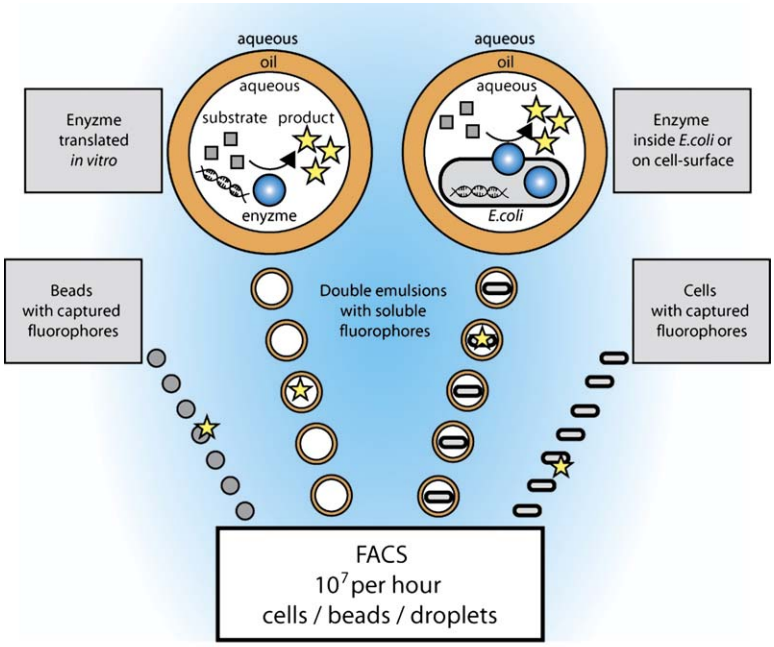

Figure 1. Water/Oil/Water Emulsion for Genotype-Phenotype Linkage during Fluorescence-Activated Cell Sorting

Various methods for screening enzyme activity by FACS are shown in schematic form. Direct screening of cells (lower right) or enzymes on beads (lower left) rely on physical capture of the fluorophore, whereas the novel emulsion-based methods presented in this issue use soluble fluorophores. The in vitro strategy of Griffiths and coworkers is shown on the left: Each gene (of a library) is translated within its own droplet in a water/oil emulsion. Active enzymes (blue balls) convert substrate (gray squares) to product (yellow stars), thus rendering their droplet fluorescent. By contrast, Tawfik and coworkers transform and express the gene library in *E. coli* (shown on the right). Single bacteria are compartmentalized in the aqueous droplets of a water/oil emulsion. The enzyme reaction then takes place inside the emulsion, and droplets containing active enzymes become fluorescent. Finally the strategies converge with a second emulsification to yield an external aqueous phase for FACS separation of the fluorescent and nonfluorescent droplets.

enrichment of genes encoding active enzymes (by 300-fold), and the ability to distinguish different rates of enzyme activity by the difference in fluorescence intensity. Both groups chose an enzyme that had previously been studied by directed evolution, thus safeguarding the success of the experiment while proving the worth of the system by evolving hundred-fold improvements on the low activities of the parent proteins (PON1 [1] and Ebg [2]). In each case, the improved mutants were equal to the best mutants isolated by traditional methods of bacterial colony screening on agar plates. Furthermore, Griffiths and coworkers discovered new beneficial mutations in the Ebg system, which was thought to have been mutated to exhaustion.

An important difference between the two experiments lies in the means of protein expression. Griffiths and coworkers used in vitro translation, which combines the advantages of cloning-free library preparation, lack of bias due to clonal selection, control of the reaction conditions and constituents, and the potential to work with toxic substrates or toxic proteins [2]. Tawfik and coworkers emulsified intact *E. coli*, expressing enzyme variants either in the cytoplasm or on the cell surface, to obtain higher enzyme concentrations [1]. It seems likely that, for future applications, the choice between a cell-based or in vitro approach will be dictated by the activity in question, and that both approaches might be used in turn during the evolution of a single enzyme, perhaps taking advantage of the high enzyme concentrations in bacteria for the initial evolution of activity against a new substrate and then using a cloning-free and more chemically defined in vitro system for iterative improvements to fine tune substrate selectivity. In vitro display technologies such as bead display or ribosome or mRNA display should also be compatible with water/oil/water emulsions, and the technique might also be adapted to screen ribozymes for multiple turnover. The enormous battery of available fluorogenic substrates and coupled assays should allow flow sorting of emulsions to be applied to achieve new goals with many different biocatalysts.

Each double emulsion approach offers massively parallel, yet individual and identifiable, femtoliter reaction compartments, combined with minimal handling time. High-throughput enzyme screening is a limiting factor, not just in directed evolution, but in other areas such as enzyme discovery by function-based cloning, functional genomics, and drug discovery. For selection of enzymes to catalyze commercially valuable transformations, or for assays of small amounts of natural products or drug leads, the small reaction volume (50 microlitres of aqueous phase forms 10^{10} droplets), and high local concentrations, may be particularly important. This breakthrough increases the throughput of a typical enzyme screen by over two orders of magnitude, and application to other biocatalysts promises to be simple. Apart from a flow sorter, the process does not require specialized equipment and could become as widely adopted as microplate screening.

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Acyclic Peptide Inhibitors of Amylases

In this issue of *Chemistry & Biology*, a library screening approach reveals a linear octapeptide inhibitor of α-amylases reached by de novo design [1]. The selected molecule shares characteristics with naturally occurring protein inhibitors—a result that suggests general rules for the design of peptide-based amylase inhibitors may be achievable.

Amylase proteins, part of the broader class of hydrolytic enzymes called glycosidases, cleave the glycosidic linkages of starch into disaccharide fragments that are subsequently broken down into glucose (Figure 1). Inhibitors of amylases have already demonstrated their utility in aiding diabetics [2]. The glucose levels of diabetics can be controlled after meals by administration of an amylase inhibitor such as acarbose. Acarbose is a natural product obtained by fermentation and is structurally related to the amylase oligosaccharide substrate, as up to five glucose residues are known to be accommodated in the amylase active site [3].

Interestingly, some plants and microorganisms produce amylase inhibitors that are based on protein rather than carbohydrate motifs. These inhibitory proteins, which range in size from 32 amino acids with 3 disulfide bonds to over 19 kDa, serve to regulate endogenous amylase activity, for example in plant seeds, as well as to defend against digestive amylases from other organisms such as insects [4].

Although X-ray structures for five of the seven proteinaceous inhibitor family members are known [5–9], the complex nature of the interaction has made rational design of smaller versions of these inhibitors challenging. Phage display methods have been used to produce altered proteins to serve as amylase inhibitors [10, 11]. However, in the last decade, the use of peptides rather than whole proteins to mimic carbohydrates has emerged as a strategy for vaccine design as well as for the design of glycosidase inhibitors [12]. Although metabolic stability is an issue, smaller peptides usually are easier to synthesize than carbohydrates and less likely to be immunogenic than large proteins. Smaller peptides are also more amenable to computational modeling to correlate properties such as charge distribution with activity [13]. The 74 amino acid protein Tendamistat has only 15 amino acids that actually interact with amylase and therefore has served as a good model for the rational design of a variety of linear and cyclic peptide inhibitors [13–16]. However, the discovery of unrelated peptides has been a challenge. Unrelated peptides have the potential for improved properties such as solubility, stability, and selectivity.

In this issue of *Chemistry & Biology*, the Mares group reports the generation of a random combinatorial peptide library for the discovery of an octapeptide inhibitor of the reaction catalyzed by porcine pancreatic α-amylase [1]. Structure/function relationship studies of the resulting octapeptide found that addition of a tosylate group by chemical means to one arginine residue generated an even tighter binding inhibitor (Figure 1). The new peptide inhibits the porcine α-amylase more strongly than the clinical drug acarbose. Interestingly, the modified octapeptide appears to use some of the same binding motifs as the natural protein-based inhibitors, namely aromatic and arginine moieties, but arranges these motifs in a simple linear scaffold. In fact, the flexibility of the linear scaffold is crucial for effective binding, as the cyclic version of the same peptide is completely inactive [1].

Selectivity among varies glycosidase families [17] is the next issue that has to be addressed in the discovery of inhibitors. Although other α-amylases and α-glucosidases are also inhibited by the octapeptide, the new compound does appear to be selective for glycosidases found in family 13. No evidence for inhibition of enzymes from seven other families was seen. Therefore, the octapeptide likely will inhibit amylases without fear of also shutting down other structurally unrelated glycosidases with important cellular functions.

The work by Mares and coworkers is an important first step in the de novo design of amylase inhibitors, but