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Advances in the directed evolution of proteins

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Natural evolution has produced a great diversity of proteins that can be harnessed for numerous applications in biotechnology and pharmaceutical science. Commonly, specific applications require proteins to be tailored by protein engineering. Directed evolution is a type of protein engineering that yields proteins with the desired properties under well-defined conditions and in a practical time frame. While directed evolution has been employed for decades, recent creative developments enable the generation of proteins with previously inaccessible properties. Novel selection strategies, faster techniques, the inclusion of unnatural amino acids or modifications, and the symbiosis of rational design approaches and directed evolution continue to advance protein engineering.

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Introduction

Synthetic biology describes the engineering of biological parts and whole systems by either modifying natural organisms or building new biosystems from scratch. Till date, most proteins used as parts in synthetic biology are taken from nature. Utilizing naturally evolved proteins has led to numerous successful applications in biotechnology. Nevertheless, these applications invariably benefit from an optimization of the original natural proteins by protein engineering [1]. In contrast, building entirely artificial proteins that do not resemble natural proteins is still a major challenge [2–4] and therefore much less common than the engineering of natural proteins for new or improved properties.

Protein engineering has developed into a multi-faceted field with hundreds of publications in the last two years alone. This field encompasses a variety of approaches for creating desired protein properties, ranging from purely computational design to selecting proteins from entirely random polypeptide libraries. Because of the incredible breadth of the field, and to enable us to focus on recent advances, we will direct the reader to excellent reviews on the fundamentals of directed evolution technologies [5–9] and computational protein design [10–12]. This review will therefore focus on the latest developments in the directed evolution of proteins (Figure 1).

Advancing selection technologies

In any directed evolution experiment, the isolation of the desired protein from a library of gene variants is the crucial step. Many efforts have been made to push the boundaries of evolution schemes, attempting to create better protein libraries, new selection systems with improved features, and faster selection procedures (Figure 2).

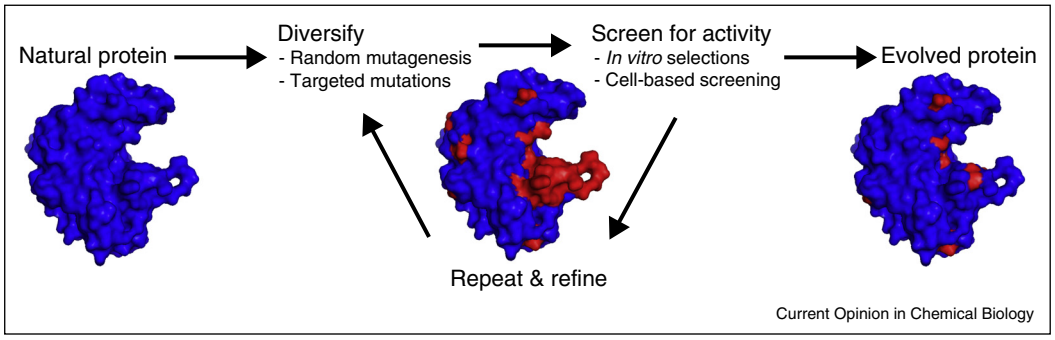
Maximizing library quality

The chance of discovering desired protein variants is directly related to the quality and complexity of the starting library. For example, random mutations that destabilize a protein can be detrimental. Therefore, building libraries with a high potential of containing functional proteins is vital. 'Smarter' libraries have been pursued that are less complex but of high-quality [13]. To build those libraries, targeted mutagenesis guided by structural or phylogenetic information, the use of compensatory stabilizing mutations and other approaches have successfully been applied [14,15]. Alternatively, a library maximizing complexity while enriching for well-folded proteins was constructed based on one of nature's most common enzyme folds, the $(\beta/\alpha)_8$ barrel fold. All residues on the catalytic face of the protein scaffold were randomized and, simultaneously, the library was enriched for protease resistance by an mRNA display selection, which has been correlated with well-folded and therefore more likely functional proteins [16•].

Refining selection steps

In vivo directed evolution of membrane proteins has been challenging due to toxicity of either the membrane protein or the selection conditions. Liposome display is a new method that has enabled *in vitro* directed evolution of toxic integral membrane proteins [17••]. This approach creates giant unilamellar liposomes and encapsulates a single DNA molecule along with a cell-free translation system. Each liposome will therefore display many copies of a single variant. Coupling protein activity to a fluorescent signal enables subsequent sorting by fluorescence-activated cell sorting (FACS). This approach was applied to evolve an α-hemolysin mutant with pore-forming

Figure 1



Overview of directed evolution.

activity 30-fold greater than wild-type. In addition to membrane protein toxicity, selection conditions can be challenging when using lipid-based barriers, for example when selecting for stability in detergent. To overcome this issue, a cellular high-throughput encapsulation, solubilization, and screening method (CHESS) was developed to screen a library of G-protein coupled receptor (GPCR) variants [18•]. GPCRs are an important group of drug targets. A library of 10^8 variants was expressed in *Escherichia coli* and the cells where then encapsulated in a polymer. The cells were lysed, but the 'nano-container' trapped the GPCR variants along with their encoding DNA while allowing free diffusion of fluorescent ligands and thereby enabling FACS. With this technique, functional receptors were identified in the presence of the detergent of choice.

The use of bead display for directed evolution has been limited by very few copies of DNA or displayed protein [19–23]. Recently, a 'megavalent' bead surface display (BeSD) system was developed to allow the display of protein and its encoding DNA in defined quantities up to a million copies per bead [24]. This method combines advantages of *in vitro* selections with multivalency of *in vivo* display systems, enabling the ranking and sorting of the output variants of an *in vitro* selection by flow cytometry.

Protease enzymes have a tremendous potential in medicine and biotechnology but engineering their activities via directed evolution for altered specificity, instead of simply broadening activity, has been successful until recently in only a few select cases using *E. coli* cell surface display of the *E. coli* outer membrane protease T [25]. This system is limited to the relatively few bacterial proteases that can be displayed and active on the prokaryote's cell surface. To enable the engineering of more complex mammalian proteases, yeast surface display was modified to evolve novel protease specificity. In the revised system, both the protease variants and a yeast adhesion receptor were colocalized inside the endoplasmic reticulum (ER) through attached signal sequences [26••]. Successful proteolytic cleavage of a linker region detached the ER retention signal and enabled the yeast surface display of the adhesion receptor including its FLAG tag, which was then identified by anti-FLAG antibodies. Counter-selection tags were also incorporated to improve the selectivity of resulting protease variants. This method was used to alter the specificity of tobacco etch virus protease, as well as granzyme K and hepatitis C virus protease, and was even modified to demonstrate in principle the selection of kinase activity.

A directed evolution approach was devised to improve the targeting specificity of an engineered methyltransferase. Methylation of only a single site in a target DNA was selected for by digesting with a target site-specific restriction endonuclease and a second, unusual restriction enzyme that digests DNA with two distally methylated sites [27]. This method identified methyltransferase variants that showed 80% methylation at the target site and less than 1% methylation at off-target sites.

Phage assisted continuous evolution (PACE) enables the sustained evolution of protein variants through hundreds of rounds of evolution in a week with little researcher intervention [28]. This method was used to probe evolutionary pathway independence by evolving RNA polymerases for various promoter specificities [29•]. RNA polymerases that initially recognized the T7 promoter were evolved to recognize T3 or SP6 promoters separately, and then a final hybrid promoter of T3 and SP6. The resulting RNA polymerases from the SP6 pathway were ~3–4-fold more active than those from the T3 pathway and further evolution did not diminish this gap. Sequencing at multiple steps along the evolutionary path further illuminated that the divergent populations were unable to converge to the same solution. This suggests that it may be beneficial to evolve through multiple subpopulations instead of a single large population. In additional work, PACE was improved to allow the modulation of selection stringency via engineering phage propagation to be dependent on the small molecule anhydrotetracycline [30•]. Further, the authors enabled

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