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Letter

Cell-free Directed Evolution of a Protease in Microdroplets at Ultrahigh Throughput

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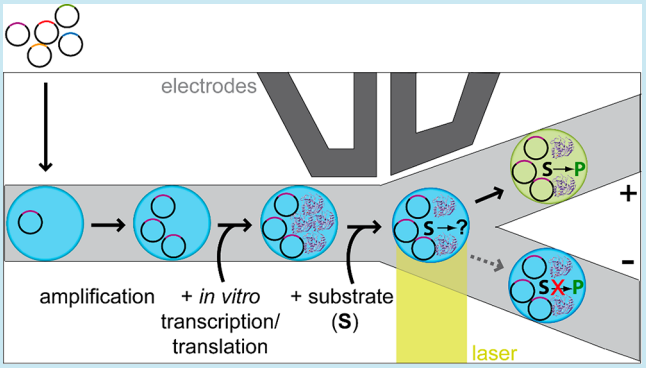
ABSTRACT: Compartmentalization of single genes in water-in-oil emulsion droplets is a powerful approach to create millions of reactors for enzyme library selections. When these droplets are formed at ultrahigh throughput in microfluidic devices, their perfect monodispersity allows quantitative enzyme assays with a high precision readout. However, despite its potential for high quality cell-free screening experiments, previous demonstrations of enrichment have never been successfully followed up by actual enzyme library selections in monodisperse microfluidic droplets. Here we develop a three-step workflow separating three previously incompatible steps that thus far could not be carried out at once: first droplet-compartmentalized DNA is amplified by rolling circle amplification; only after completion of this step are reagents for *in vitro* protein expression and, finally, substrate added via picoinjection. The segmented workflow is robust enough to allow the first *in vitro* evolution in droplets, improving the protease Savinase that is toxic to *E. coli* for higher activity and identifying a 5-fold faster enzyme.



KEYWORDS: *protein engineering, directed evolution, microfluidics, ultrahigh throughput, cell-free protein synthesis*

Directed evolution has emerged as a powerful approach to protein engineering that complements rational design. One key to success in the random combinatorial exploration of protein sequence diversity is the ability to screen large numbers of library members, at low cost and preferably automatically at ultrahigh throughput. Sequence space is vast and only sparsely populated with "islands" of desired function and reaching them relies on chance.[1] *In vivo* selections (e.g., for survival) provide ready access to the testing of large libraries but may be compromised by incompatibility with the host organism and encumbered by possible diversity loss of libraries due to low transformation efficiency. *In vitro* systems are required to improve the intrinsic properties of a protein by overcoming such constraints in order to explore larger fractions of sequence space bias-free without host interference. Compartmentalization of genes into water-in-oil emulsion droplets, akin to artificial single purpose cell-like structures, provides a completely artificial format for combining genotype and phenotype.[2] Highly monodisperse droplets can be generated and analyzed in an automated and highly controlled way in microfluidic devices and allow a massive scale-down to picoliter volumes along with a massive scale-up of screening throughput to $>10^7$ candidates per day.[3−6] However, a demonstration of its utility in an actual library selection in this quantitative *in vitro* format is still lacking.

Here we address the conspicuous absence of a complete *in vitro* directed evolution campaign in monodisperse microfluidic droplets. Early *in vitro* compartmentalization directed evolution campaigns in polydisperse droplets had been successful,[7] but often relied on modification of the coding DNA (or conjugates) as the readout of activity and were limited to improvement of already rather active enzymes. Systems for microfluidic droplet evolution on chip using *in vitro* expression systems have been developed, but only enrichment experiments have been documented.[8,9] Enrichment experiments are often used to validate a workflow, but do not require the selection and recovery of single catalyst genes (in a sea of genes that are not selected), that is, circumstances typical for the low hit rates in a library experiment. Also, enrichment experiments using an *in vitro* workflow, in which each droplet is filled with multiple copies of a gene at the outset[9] circumvent key challenges of a complete directed evolution that starts with Poisson-distribution of single gene copies of library members. Such a true library evolution experiment in droplets has indeed not been reported thus far. However, *in vitro* expression is possible from single genes,[10]

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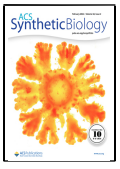

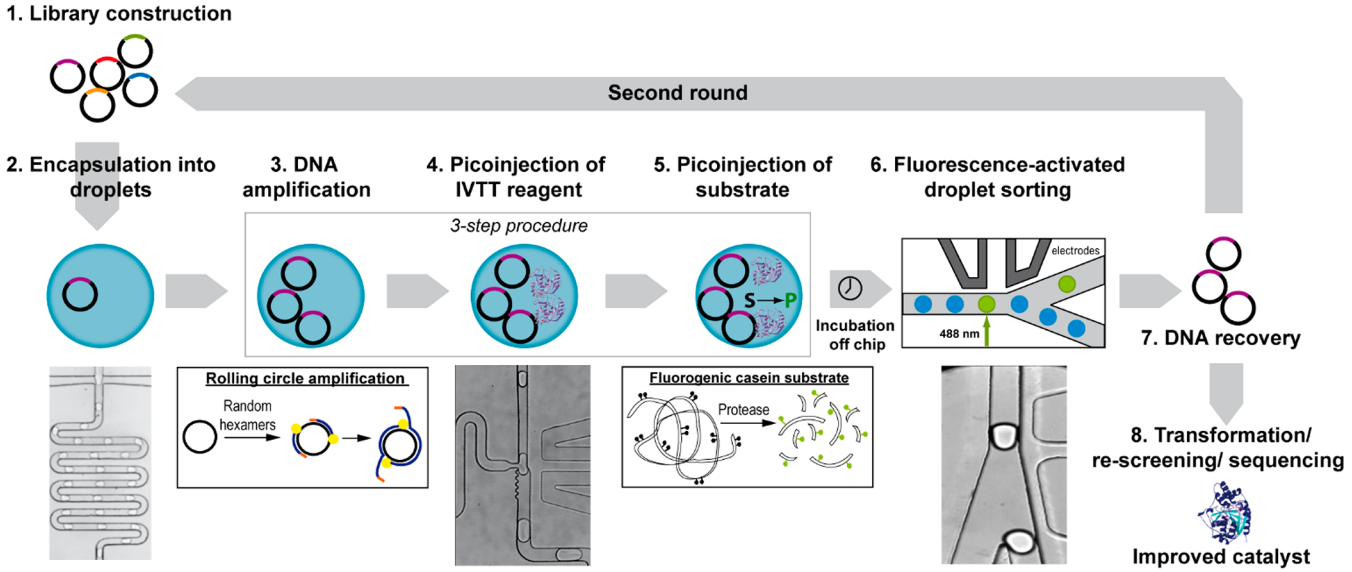

Figure 1. Functional screening for protease activity using microfluidic droplets. Single plasmids of a randomized Savinase library (1) were compartmentalized in picoliter water-in-oil droplets (2) together with reagents to perform rolling circle amplification (RCA, 3). After an off-chip incubation of emulsion droplets, reagents for *in vitro* transcription and translation were added stepwise (4). Droplets were incubated off chip for another 4 h at 37 °C before the fluorogenic casein substrate was picoinjected (5). Emulsion droplets were then reinjected into a sorting device and strongly fluorescent droplets were collected (6). Selected droplets were de-emulsified and RCA product was recovered followed by restriction and ligation (7). Plasmids containing DNA sequences of active catalysts were transformed into *B. subtilis*, rescreened, and sequenced (8) to identify improved catalysts. For further enrichment, iterative selections led to improved enzyme variants. The three key steps that had to be separated in this work to achieve successful selection of proteases with increased activity (overcoming previous limitations) are highlighted by the central box.

and DNA amplification by PCR[8] or by isothermal amplification[11] increases the number of expression templates, leading to more enzyme molecules and a more detectable product. Amplification will also make it easier to recover selected hits. Coordinating DNA amplification, *in vitro* expression, and enzymatic reaction is tricky, because of droplet stability and device complexity in a PCR-based protocol that requires heating/cooling steps in thermocycling, or—in the case of isothermal amplification—because of potential cross-inhibition among more than 70 components, that are present at once in droplet compartments after all addition steps (shown in Figure 1).

In this work we demonstrate how controlled, stepwise on-chip addition of the components for these three processes leads to a robust workflow that remedies the current impasse in *in vitro* evolution in microfluidic droplets. We set out to evolve Savinase, a subtilisin-like protease naturally secreted by the alkalophilic bacterium *Bacillus lentus* for nutrition and defense[12] and commercially developed for the detergent industry.[13,14] Its multiple uses in fine chemical synthesis, washing powder, and other biotechnological applications explain why it is produced on a multiton-scale. This enzyme class is covered by numerous patents, yet no directed evolution has been reported. The expression of Savinase in *E. coli* is not possible due to its acute cytotoxicity. The possible alternative of using *B. subtilis* as a directed evolution host would limit the library size due to its poor transformation efficiency (of typically only hundreds[15]) compared to the throughput of millions in droplet microfluidics. Therefore, cell-free protein synthesis is necessary to evolve this enzyme. We designed an ultrahigh-throughput screening platform for *in vitro* directed evolution of Savinase that is illustrated in Figure 1. In a first step single genes (as plasmids or circularized genes containing a T7 promotor as well as a ribosome-binding site) were encapsulated on-chip into monodisperse droplets (~14 pL). Starting with these "monoclonal" droplets, single gene copies were amplified by rolling circle amplification (RCA). RCA uses random hexamer primers and can potentially amplify any DNA in a nonspecific manner.[16] This isothermal process is easier to handle in a device compared to PCR that requires heating modules[17] and avoids droplet coalescence at elevated temperatures. While higher Φ29 DNA polymerase concentrations are attractive (*e.g.*, 0.07 pg/μL DNA leads to up to 30 000 copies per gene),[11] strong inhibition of subsequent *in vitro* expression was observed at the highest possible Φ29 concentration (500 nM), along with droplet destabilization (see Supporting Information, Figure S1). A productive balance between optimal DNA amplification and droplet stability was obtained by using a Φ29 DNA polymerase concentration of 100 nM and performing RCA for 6 h at 30 °C. Given RCA inhibition by the reagents used for *in vitro* transcription and translation (IVTT) (Figure S2) and the requirement of different incubation temperatures for optimal performance of *both* processes, these steps were performed separately. To achieve the separation of RCA and IVTT in one microfluidic device, a design that first injected IVTT components (PURE)[18] into droplets was used. Defined volumes (equal to the volume of the droplet; varying between experiments but typically in the range of ~10−15 pL) were introduced into surfactant-stabilized droplets at rates of 1 kHz using an electro-microfluidic picoinjector.[19] After the addition of IVTT, the droplets were incubated off-chip for another 4 h at 37 °C. To start the reaction under controlled conditions (i.e., after completion of amplification and expression) and in a pH range that is not necessarily compatible with the preparatory steps, the fluorogenic substrate (a BODIPY-labeled casein that generates fluorescence after cleavage) was picoinjected (~20−30 pL at 0.5 kHz). A 2-fold higher green fluorescent signal (compared to

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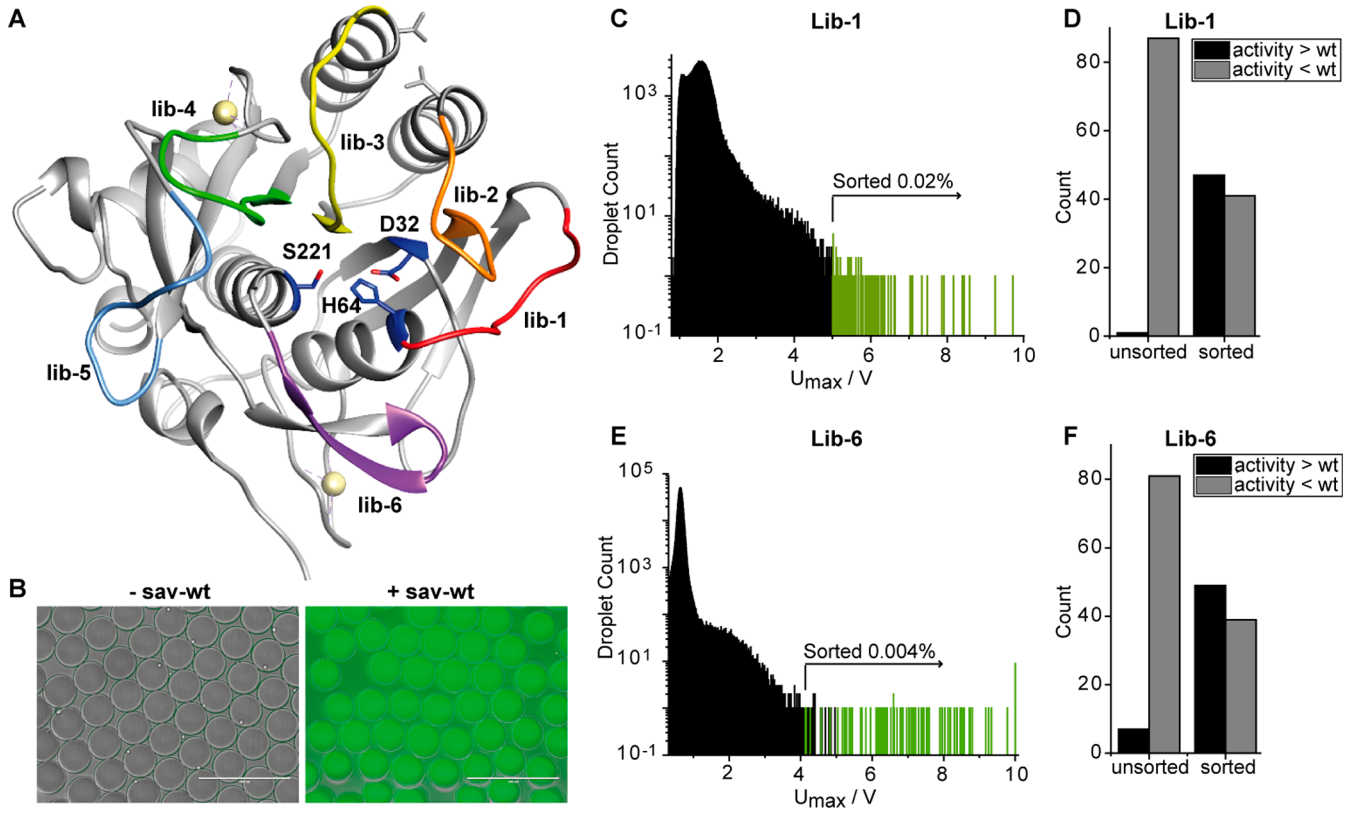

Figure 2. Generation of six different libraries of the serine protease Savinase (pdb 1SVN) and screening results. (A) 9−11 residues of six different loops located around the active site (indicated in red, orange, yellow, green, blue, and purple) were mutated using a Slonomics approach to generate libraries 1−6. The catalytic triad is highlighted in dark blue (D32, H64, and S221). Two calcium ions are shown as yellow spheres. (B) Overlay of fluorescent and bright field microscope images showing droplets exhibiting enzymatic activity. The casein substrate (20 μg/μL) was incubated either with 1 μM of sav-wt (+sav-wt) or without protease (−sav-wt). Scale bars: 100 μm. (C,E) Histograms of the fluorescence signal distribution in fluorescence-activated droplet sorting (FADS) of droplets screened for Savinase activity after 3 days of incubation at room temperature in darkness. The sorting gate was set up so that droplets showing increased fluorescence over the population average were selected. A fluorescence image of a droplet population with typically few hits in a library is shown in Figure S4. (D,F) Enrichment of positive hits after droplet sorting. After DNA recovery and transformation of *B. subtilis*, 88 colonies were randomly picked from the unsorted original library and the sorted samples, followed by an assay against the fluorogenic casein substrate. The data were normalized to the rate of sav-wt. The bar graphs give a comparison of variants showing higher activity (activity > wt) or lower activity (activity < wt) compared to wt.

the simultaneous injection of IVTT mixture and casein substrate) was obtained by adding the substrate after enzyme production (Figure S3). After addition of the substrate, the droplets were incubated off-chip to allow for hydrolytic cleavage. Starting with single genes in monoclonal and monodisperse droplets, followed by the addition of identical volumes of IVTT and substrate, the equal expression and assay conditions in each droplet suggest a level playing field for subsequent screening. At the completion of an incubation period of 3 days, droplets were reinjected into a sorting device and the brightest ones were selectively extracted by fluorescence-activated droplet sorting (FADS)[20] to select active proteases. A control experiment demonstrates that the complex workflow was absolutely necessary to create a viable assay: adding all reagents at once into droplets in a standard flow focusing device design[21,22] produced no measurable product (Figures S1, S2, S3), suggesting that fine-tuning of the concentration, conditions, and order of addition was crucial to overcome the incompatibility of different reagents and bring about the reaction product of Savinase at detectable levels.

The workflow was validated by a sorting experiment to enrich droplets expressing Savinase wildtype (sav-wt) from an excess of droplets producing the esterase EstB, which is an enzyme that is not active on the fluorogenic casein substrate. Both DNA constructs were mixed in a 1:250 ratio and the abundance of sav-wt before and after sorting was analyzed via quantitative PCR (qPCR). The initial content of 0.4% sav-wt plasmid was increased to 99% sav-wt plasmids after sorting, giving an enrichment of 250-fold (calculated according to Zinchenko et al.[23]) or 300 000-fold (calculated according to Baret et al.,[20] Table S1, Figures S5 and S6).

Next, six Savinase libraries were designed by targeting six active site loops that play a role in substrate binding[24] and mutating 9−11 residues located around the active site (Figure 2A and Figure S7). Randomization was achieved using a Slonomics approach,[25] resulting in approximately three mutations per gene (Figure S8) and library sizes of up to 10^{14} variants. These six Savinase libraries were interrogated (Figure 2C,E and Figure S9): 500 000 to 4 000 000 droplets were screened to yield 34 to 200 hits per library (Table S2). For lib-1 0.02% of the droplets were selected and for lib-6 0.004% of the population, respectively. This value depends on the presence of highly active variants within the library screened and the detection threshold set up at the beginning of the selection. In general, the sorting gate was set up, so that droplets showing increased fluorescence over the population average were selected. The selected droplets were subsequently de-emulsified and DNA (i.e., of long concatemers resulting

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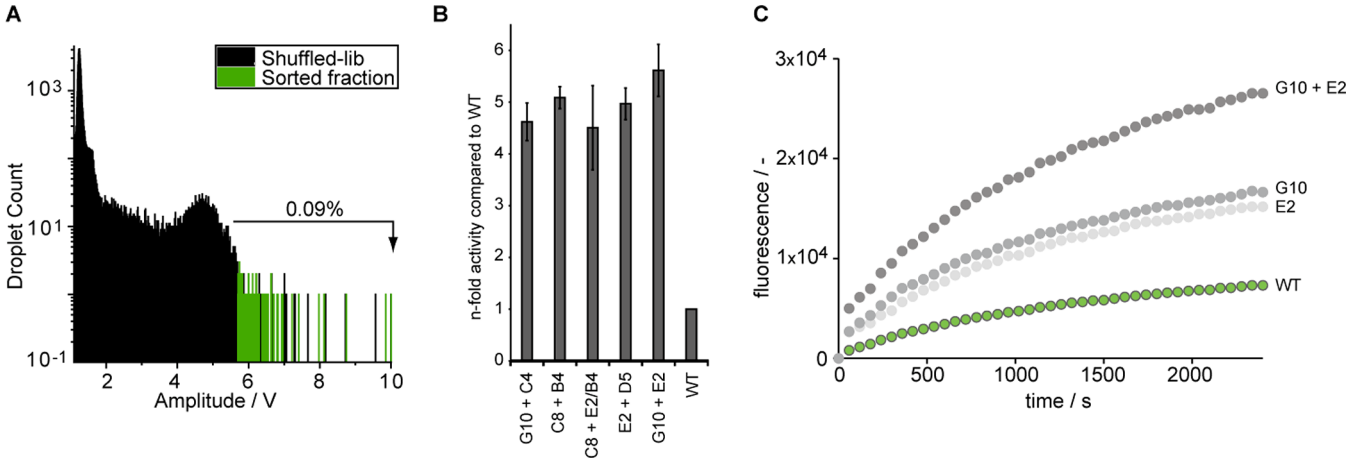

Figure 3. Screening of Savinase library created by reshuffling of mutants obtained in a first screen (Figure S12). (A) Histogram of the fluorescence signal distribution of droplets screened for Savinase activity after 3 days of incubation at room temperature in darkness. The sorting gate was set up, so that droplets showing increased fluorescence over the population average were selected (shown in green). (B) Activities of five identified shuffled variants against the fluorogenic casein substrate are shown. The activity for each shuffled variant is plotted relative to that of sav-wt. (C) Time-course of shuffled variant G10+E2 showing highest activity toward the substrate casein in comparison to parents and wildtype.

from RCA) was recovered. For low-throughput rescreening, these hits were expressed in the original producer, *B. subtilis*. After PCR amplification of the recovered hit DNA and cloning into the expression and secretion vector pCri18a, *B. subtilis* was transformed. Resulting transformants were screened in 96-well plates for hydrolysis of the fluorogenic casein substrate that has been used for the screening in droplets. Rescreening 88 randomly picked colonies of the six Savinase libraries before and after sorting shows an enrichment of variants with higher activity than sav-wt from 1% to 53% for lib-1 and 8% to 56% for lib-6 (Figure 2D,F and Figure S10). Sequencing of the hits from each library identified during the rescreening revealed 58 unique sequences in total. These Savinase variants were recombinantly produced in *B. subtilis*, purified and further tested against the fluorogenic casein substrate (Figure S11). From six libraries, 16 variants were found to be the most active ones, exhibiting up to 3-fold improvements on their initial rates v_0 (Figure S12). The different mutations observed are shown in Supporting Information, Table S3. Overall, two to four mutations per gene were found (matching the expected mutation rates; Figure S8). In 10 out of the 16 variants selected, residues were found to be mutated to arginine or lysine. These two positively charged basic amino acids are mostly exposed to the protein surface and play important roles in protein stability by forming electrostatic interactions.[26] The pI of the substrate casein has been determined to be 4.6, so that its surface at pH values around 8 is negatively charged. Savinase with a pI of 10 has a positive surface charge at this pH, so replacement of negatively charged or neutral residues by positively charged basic amino acids arginine and lysine slightly enhances this charge, potentially promoting enzyme−substrate interaction driven by electrostatic attraction.

To accumulate beneficial mutations identified during the screening campaign and select for synergistic effects, a shuffled library was generated via staggered extension process (StEP).[27] The shuffled library was screened on our new ultrahigh-throughput droplet screening platform (Figure 3A). The platform shows that 0.09% of the population passed the selection threshold and were selected, resulting in 100 hits (Table S2). Rescreening in microtiter plates and sequencing identified five shuffled variants with up to 5.5-fold increased activity compared to the wildtype (Figure 3B,C and Figures S13 and S14). Previous directed evolution campaigns and engineering of subtilisin-like proteases[28] had only achieved improvements in biophysical properties, namely, temperature stability,[29] cold-adaptation,[30,31] tolerance to cosolvent,[32] or specificity changes at the expense of activity.[33,34]

In conclusion, a cell-free ultrahigh-throughput screening platform for the directed evolution of subtilisin Savinase in droplets has been established. Stepwise DNA amplification, IVTT, and substrate conversion in droplets have provided the basis for *in vitro* evolution in droplets, overcoming the previous inability to carry out *in vitro* evolution campaigns in this format, by removing cross-inhibition effects of reagents and careful choice of reaction conditions. The *integration* of multiple steps into a robust workflow with automated processing and screening of "monoclonal" droplets (each representing one library member) and the efficient recovery of hits has been key to the success of this approach. A cytotoxic protease that could not be evolved in *E. coli* or yeast was substantially improved in two rounds of screening, with a large number of >50 hits recovered to allow further improvement by reshuffling.

Using *B. subtilis* as an alternative host for secretion of proteases (e.g., in microtiter plates) would be limited by the poor transformation efficiency of this host: merely a few hundred transformants per μg DNA are possible,[15] effectively reducing library size. By contrast, the complete absence of a transformation barrier when working in droplets makes multiple rounds of mutation/recombination and screening with larger libraries possible. *In vitro* droplet screening may also prove advantageous for functional screening of environmental libraries. Previous discovery campaigns of metagenomic proteases[35−37] were based on agar plate screenings. Using our cell-free workflow the throughput would be improved 100-fold and the absence of potential incompatibility with host organisms should lead to more hits.

Practically this strategy is highly economical: Compared to screening in microtiter plates RCA and IVTT reagents are 150 000-fold reduced, allowing the screening of 10^6 genes using only 140 μL of RCA mix and IVTT components. This approach will help to put currently impossible experiments in

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directed evolution and metagenomic screening into reality, enlarging the list of practically discoverable or evolvable candidates to other cytotoxic or membrane proteins. Based on the degrees of freedom enabled by this approach non-natural reaction conditions (including the introduction of non-natural amino acids or cofactors) can be explored and freely chosen selection pressures can be applied in droplet screening.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00538.

Experimental procedures (chip design, device operation, screening workflow, DNA recovery), and results (sequencing results, hit analysis) (PDF)

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Author Contributions

J.M.H., C.G., and F.H. designed the research; J.M.H. and C.G. performed the research; J.M.H., C.G., and F.H. analyzed data; and J.M.H. and F.H. wrote the manuscript. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare the following competing financial interest(s): Christian Gylstorf is an employee of Novozymes and was seconded to the University of Cambridge (using funding from the EU FP7 network HotDrops).

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■ ABBREVIATIONS

FADS, fluorescence-activated droplet sorting; IVTT, *in vitro* transcription and translation; sav-wt, Savinase wildtype; RCA, rolling circle amplification

■ REFERENCES

(1) Romero, P. A., and Arnold, F. H. (2009) Exploring protein fitness landscapes by directed evolution. *Nat. Rev. Mol. Cell Biol. 10* (12), 866−76.

(2) Tawfik, D. S., and Griffiths, A. D. (1998) Man-made cell-like compartments for molecular evolution. *Nat. Biotechnol. 16* (7), 652−6.

(3) Mair, P., Gielen, F., and Hollfelder, F. (2017) Exploring sequence space in search of functional enzymes using microfluidic droplets. *Curr. Opin. Chem. Biol. 37*, 137−144.

(4) Colin, P. Y., Zinchenko, A., and Hollfelder, F. (2015) Enzyme engineering in biomimetic compartments. *Curr. Opin. Struct. Biol. 33*, 42−51.

(5) Markel, U., Essani, K. D., Besirlioglu, V., Schiffels, J., Streit, W. R., and Schwaneberg, U. (2020) Advances in ultrahigh-throughput screening for directed enzyme evolution. *Chem. Soc. Rev. 49* (1), 233−262.

(6) Neun, S., Zurek, P., Kaminski, T., and Hollfelder, F. (2020) Ultrahigh throughput screening for enzyme function in droplets. *Methods Enzymol. 643*, 317−343.

(7) Lu, W. C., Levy, M., Kincaid, R., and Ellington, A. D. (2014) Directed evolution of the substrate specificity of biotin ligase. *Biotechnol. Bioeng. 111* (6), 1071−81.

(8) Fallah-Araghi, A., Baret, J. C., Ryckelynck, M., and Griffiths, A. D. (2012) A completely in vitro ultrahigh-throughput droplet-based microfluidic screening system for protein engineering and directed evolution. *Lab Chip 12* (5), 882−91.

(9) Holland-Moritz, D. A., Wismer, M. K., Mann, B. F., Farasat, I., Devine, P., Guetschow, E. D., Mangion, I., Welch, C. J., Moore, J. C., Sun, S., and Kennedy, R. T. (2020) Mass Activated Droplet Sorting (MADS) Enables High-Throughput Screening of Enzymatic Reactions at Nanoliter Scale. *Angew. Chem., Int. Ed. 59* (11), 4470−4477.

(10) Courtois, F., Olguin, L. F., Whyte, G., Bratton, D., Huck, W. T., Abell, C., and Hollfelder, F. (2008) An integrated device for monitoring time-dependent in vitro expression from single genes in picolitre droplets. *ChemBioChem 9* (3), 439−46.

(11) Mazutis, L., Araghi, A. F., Miller, O. J., Baret, J. C., Frenz, L., Janoshazi, A., Taly, V., Miller, B. J., Hutchison, J. B., Link, D., Griffiths, A. D., and Ryckelynck, M. (2009) Droplet-based microfluidic systems for high-throughput single DNA molecule isothermal amplification and analysis. *Anal. Chem. 81* (12), 4813−21.

(12) Betzel, C., Klupsch, S., Papendorf, G., Hastrup, S., Branner, S., and Wilson, K. S. (1992) Crystal structure of the alkaline proteinase Savinase from Bacillus lentus at 1.4 A resolution. *J. Mol. Biol. 223* (2), 427−45.

(13) Li, Q., Yi, L., Marek, P., and Iverson, B. L. (2013) Commercial proteases: present and future. *FEBS Lett. 587* (8), 1155−63.

(14) Vojcic, L., Pitzler, C., Korfer, G., Jakob, F., Ronny, M., Maurer, K. H., and Schwaneberg, U. (2015) Advances in protease engineering for laundry detergents. *New Biotechnol. 32* (6), 629−34.

(15) Vojcic, L., Despotovic, D., Martinez, R., Maurer, K. H., and Schwaneberg, U. (2012) An efficient transformation method for Bacillus subtilis DB104. *Appl. Microbiol. Biotechnol. 94* (2), 487−93.

(16) Fire, A., and Xu, S. Q. (1995) Rolling replication of short DNA circles. *Proc. Natl. Acad. Sci. U. S. A. 92* (10), 4641−5.

(17) Schaerli, Y., Wootton, R. C., Robinson, T., Stein, V., Dunsby, C., Neil, M. A., French, P. M., Demello, A. J., Abell, C., and Hollfelder, F. (2009) Continuous-flow polymerase chain reaction of single-copy DNA in microfluidic microdroplets. *Anal. Chem. 81* (1), 302−6.

(18) Shimizu, Y., Inoue, A., Tomari, Y., Suzuki, T., Yokogawa, T., Nishikawa, K., and Ueda, T. (2001) Cell-free translation reconstituted with purified components. *Nat. Biotechnol. 19* (8), 751−5.

(19) Abate, A. R., Hung, T., Mary, P., Agresti, J. J., and Weitz, D. A. (2010) High-throughput injection with microfluidics using picoinjectors. *Proc. Natl. Acad. Sci. U. S. A. 107* (45), 19163−6.

(20) Baret, J. C., Miller, O. J., Taly, V., Ryckelynck, M., El-Harrak, A., Frenz, L., Rick, C., Samuels, M. L., Hutchison, J. B., Agresti, J. J., Link, D. R., Weitz, D. A., and Griffiths, A. D. (2009) Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity. *Lab Chip 9* (13), 1850−8.

(21) Colin, P. Y., Kintses, B., Gielen, F., Miton, C. M., Fischer, G., Mohamed, M. F., Hyvonen, M., Morgavi, D. P., Janssen, D. B., and Hollfelder, F. (2015) Ultrahigh-throughput discovery of promiscuous

enzymes by picodroplet functional metagenomics. *Nat. Commun. 6*, 10008.
(22) Kintses, B., van Vliet, L. D., Devenish, S. R., and Hollfelder, F. (2010) Microfluidic droplets: new integrated workflows for biological experiments. *Curr. Opin. Chem. Biol. 14* (5), 548−55.
(23) Zinchenko, A., Devenish, S. R., Kintses, B., Colin, P. Y., Fischlechner, M., and Hollfelder, F. (2014) One in a million: flow cytometric sorting of single cell-lysate assays in monodisperse picolitre double emulsion droplets for directed evolution. *Anal. Chem. 86* (5), 2526−33.
(24) Brode, P. F., Barnett, B. L., Rubingh, D. N., and Ghosh, C. K. (1994) Subtilisin 309 variants having decreased adsorption and increased hydrolysis. WO Patent 1995030011A2.
(25) Van den Brulle, J., Fischer, M., Langmann, T., Horn, G., Waldmann, T., Arnold, S., Fuhrmann, M., Schatz, O., O'Connell, T., O'Connell, D., Auckenthaler, A., and Schwer, H. (2008) A novel solid phase technology for high-throughput gene synthesis. *BioTechniques 45* (3), 340−3.
(26) Sokalingam, S., Raghunathan, G., Soundrarajan, N., and Lee, S. G. (2012) A study on the effect of surface lysine to arginine mutagenesis on protein stability and structure using green fluorescent protein. *PLoS One 7* (7), No. e40410.
(27) Zhao, H., Giver, L., Shao, Z., Affholter, J. A., and Arnold, F. H. (1998) Molecular evolution by staggered extension process (StEP) in vitro recombination. *Nat. Biotechnol. 16* (3), 258−61.
(28) Bryan, P. N. (2000) Protein engineering of subtilisin. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol. 1543* (2), 203−222.
(29) Zhao, H., and Arnold, F. H. (1999) Directed evolution converts subtilisin E into a functional equivalent of thermitase. *Protein Eng., Des. Sel. 12* (1), 47−53.
(30) Tindbaek, N., Svendsen, A., Oestergaard, P. R., and Draborg, H. (2004) Engineering a substrate-specific cold-adapted subtilisin. *Protein Eng., Des. Sel. 17* (2), 149−56.
(31) Pulido, M. A., Koga, Y., Takano, K., and Kanaya, S. (2007) Directed evolution of Tk-subtilisin from a hyperthermophilic archaeon: identification of a single amino acid substitution responsible for low-temperature adaptation. *Protein Eng., Des. Sel. 20* (3), 143−53.
(32) You, L., and Arnold, F. H. (1996) Directed evolution of subtilisin E in Bacillus subtilis to enhance total activity in aqueous dimethylformamide. *Protein Eng., Des. Sel. 9* (1), 77−83.
(33) Legendre, D., Laraki, N., Graslund, T., Bjornvad, M. E., Bouchet, M., Nygren, P. A., Borchert, T. V., and Fastrez, J. (2000) Display of active subtilisin 309 on phage: analysis of parameters influencing the selection of subtilisin variants with changed substrate specificity from libraries using phosphonylating inhibitors. *J. Mol. Biol. 296* (1), 87−102.
(34) Bonneau, P. R., Graycar, T. P., Estell, D. A., and Jones, J. B. (1991) Alteration of the specificity of subtilisin BPN' by site-directed mutagenesis in its S1 and S1′ binding sites. *J. Am. Chem. Soc. 113* (3), 1026−30.
(35) Pushpam, P. L., Rajesh, T., and Gunasekaran, P. (2011) Identification and characterization of alkaline serine protease from goat skin surface metagenome. *AMB Express 1* (1), 3.
(36) Pessoa, T. B. A., Rezende, R. P., Marques, E. L. S., Pirovani, C. P., Dos Santos, T. F., Dos Santos Goncalves, A. C., Romano, C. C., Dotivo, N. C., Freitas, A. C. O., Salay, L. C., and Dias, J. C. T. (2017) Metagenomic alkaline protease from mangrove sediment. *J. Basic Microbiol. 57* (11), 962−973.
(37) Neveu, J., Regeard, C., and DuBow, M. S. (2011) Isolation and characterization of two serine proteases from metagenomic libraries of the Gobi and Death Valley deserts. *Appl. Microbiol. Biotechnol. 91* (3), 635−44.

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