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Directed Evolution in Drops: Molecular Aspects and Applications

Aitor Manteca,^{*,‡} Alejandra Gadea,[‡] David Van Assche,[‡] Pauline Cossard, Mélanie Gillard-Bocquet, Thomas Beneyton, C. Axel Innis, and Jean-Christophe Baret^{*}



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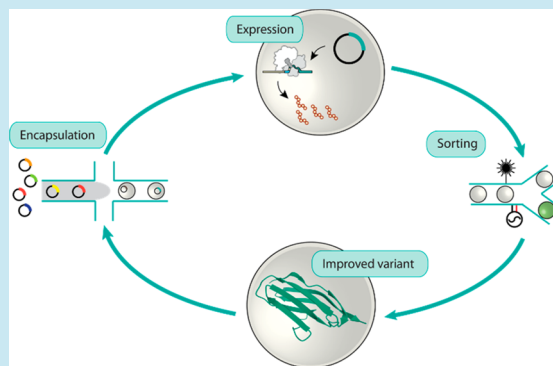
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ABSTRACT: The process of optimizing the properties of biological molecules is paramount for many industrial and medical applications. Directed evolution is a powerful technique for modifying and improving biomolecules such as proteins or nucleic acids (DNA or RNA). Mimicking the mechanism of natural evolution, one can enhance a desired property by applying a suitable selection pressure and sorting improved variants. Droplet-based microfluidic systems offer a high-throughput solution to this approach by helping to overcome the limiting screening steps and allowing the analysis of variants within increasingly complex libraries. Here, we review cases where successful evolution of biomolecules was achieved using droplet-based microfluidics, focusing on the molecular processes involved and the incorporation of microfluidics to the workflow. We highlight the advantages and limitations of these microfluidic systems compared to low-throughput methods and show how the integration of these systems into directed evolution workflows can open new avenues to discover or improve biomolecules according to user-defined conditions.

KEYWORDS: droplet microfluidics, directed evolution, enzyme, antibody, aptamer, molecular engineering



The compartmentalization of phenotype and genotype in living cells is a key requirement for natural evolution. Since the first experiments using oil and aqueous phases to generate cell-like compartments in the late nineties,¹ the marriage between droplet-based microfluidics and directed evolution has become a key technique in the field of protein and nucleic acid engineering. Droplet-based microfluidics encompasses a set of microelectromechanical systems able to generate, steer, manipulate, and analyze water-in-oil droplets inside a microfluidic chip. Molecular engineering refers to biomolecule optimization methods to improve a specific property of a protein or a nucleic acid, such as its catalytic activity, ability to bind a ligand, or thermostability. From the first experiments carried out in the late 1960s² to the Nobel Prize awarded to Frances Arnold in 2018,³ research in this field has experienced exponential growth. Directed evolution consists of three well-defined steps, variant generation, production, and selection, performed iteratively until a biomolecule with a set of desired properties is obtained. In this Review, we highlight the advantages of incorporating microfluidics into the directed evolution workflow. Additional information on the directed evolution of novel catalytic functions and improved enzymes in drops can be found in several excellent recent reviews from the Hilvert⁴ and Hollfelder⁵ groups, among others. Here, we focus on the versatility of the microfluidics technology and how it allows the

use of multiple strategies for assay design, protein expression, and selection.

■ FROM NATURAL TO DIRECTED EVOLUTION

Biomolecular evolution can be described as a path from one functional biomolecule to another in the space of all possible biomolecular variants, where each variant has an assigned fitness⁶ (Figure 1). In nature, the fitness is the ability of an organism to reproduce in a particular environment and consequently spread its genes. In the laboratory, the selective pressure and therefore the fitness are set by the experimenter. Directed evolution is a growing field in synthetic biology and has the capacity to provide new proteins or nucleic acids on the basis of predefined industrial or biomedical needs. It relies on the Darwinian principle of mutation and selection, where the probability of success is determined by the ability to find rare optimal variants within a large pool of sequences. All directed evolution experiments require a measurable activity that acts as a fitness indicator to drive the selection process

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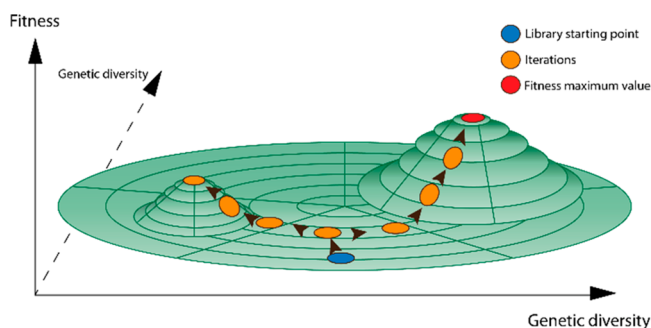


Figure 1. Directed evolution fitness landscape. A predefined library is used as a starting point to navigate the genetic diversity landscape, ideally reaching a local or global fitness maximum after several iterations of mutagenesis, expression, and selection.

“uphill” within the protein or nucleic acid sequence space, ultimately resulting in a biomolecule with a desired set of properties. As a result, high-throughput screening techniques are necessary to achieve directed evolution, and droplet-based microfluidics now provides a way of overcoming the limiting step of screening, making it possible to achieve ~ 1000 -fold higher throughput than microtiter plate screening.⁷ Droplets act as a picoliter-volume reaction vessel to perform biochemical assays at the single variant level, linking the activity of the functional molecule often made of amino acids (phenotype) to its corresponding encoding molecule in the form of nucleic acids (genotype). This compartmentalization is as crucial for directed evolution as it is for natural evolution since the encoding molecules and the functional molecules are distinct with the exception of DNA- and RNA-based molecules (ribozymes, DNAzymes, and aptamers). Furthermore, working at a picoliter scale not only allows for higher throughput but also decreases reagent consumption by a million-fold, thus greatly impacting the cost of molecular engineering.⁸ In the following sections, we present the various elements that make up a directed evolution workflow with an emphasis on aspects that are unique to droplet-based microfluidic setups.

■ VARIANT GENERATION

Any evolutionary process requires genetic diversity as a starting point from which an improved variant can emerge. In directed evolution, genetic diversity is obtained by introducing mutations within the gene of interest to yield genetic libraries with up to tens of thousands of variants. Methods to generate genetic diversity are broadly divided into two groups: random and semirational. Random mutagenesis methods⁹ are based on the use of an error-prone polymerase chain reaction (epPCR),¹⁰ a process whereby PCR reaction conditions are altered to facilitate the misincorporation of nucleotides, resulting in randomly incorporated variations along the sequence. This approach is widely used when the key residues responsible for a given function are not known. In contrast, semirational design relies on *a priori* biochemical or structural knowledge of the system to create constraints in the design of the mutant library,^{11,12} such that residues that influence a biomolecule’s function are preferentially targeted and consequently the resulting library is more likely to contain variants with enhanced properties. The most widely used technique for semirational library design is saturation mutagenesis, where the targeted residues are randomized to introduce all possible amino acids or nucleotides in the final product. For protein

evolution experiments, codons are often not fully randomized and more restricted codon sets are used, such as NNK (N = A/C/G/T, K = G/T), which covers all possible amino acids and one stop codon. In addition, one can also use codons to encode a minimal set of amino acids, representing the main chemical types.¹³ The method of choice for generating mutants will depend on prior knowledge of the key residues of a biomolecule, the size of the gene, and the screening capacities. As an example, in a NNK library, all 20 amino acids will be represented at each mutated codon, and the theoretical number of full-length protein variants is 20^n , where n indicates the number of targeted sites. Therefore, if this is the chosen strategy, one should carefully choose the residues to mutate, as it will be virtually impossible to test all of the possible amino acid variants at each position for most proteins. If the key residues responsible for the function of a biomolecule are not known or the researcher simply wishes to explore different evolutionary paths, it may therefore be more effective to use a randomized library, bearing in mind that, the bigger the biomolecule, the lower the likelihood that a key residue will be targeted.

■ VARIANT PRODUCTION

Protein variants may be expressed either inside a host or within a cell-free system. The use of a host for protein expression is well established in research and in the pharmaceutical industry¹⁴ and has historically been the most used method to amplify and produce candidates for directed evolution.¹⁵ The two most common prokaryotic and eukaryotic hosts for protein production are the model bacterium *E. coli* and the yeast *S. cerevisiae*, respectively. In both cases, a plasmid carrying the gene of interest is transformed into the host cells for expression by the translation machinery. Cells are then propagated and induced to express the protein in a variety of ways, most commonly inside the cytoplasm (Figure 2) but also

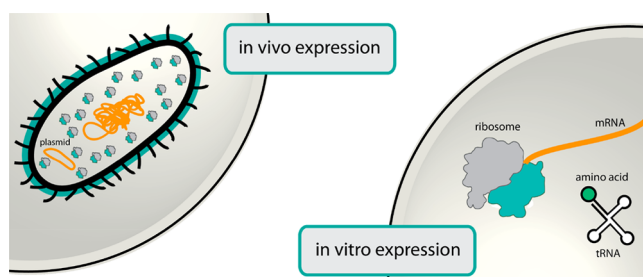


Figure 2. Cell and cell-free protein expression in drops. *In vivo* expression takes advantage of a host organism (bacteria, yeast) for the heterologous expression of a gene or a DNA fragment. *In vitro* transcription and/or translation systems use the basic machinery of the cells to produce the protein of interest without the cell wall.

inside the membrane or secreted into the periplasm or into the surrounding medium. The advantages of using a cellular expression system lie in its simplicity and in the reliance on the cell environment and translational machinery, which enable the correct folding and post-transcriptional modification of the target proteins. Moreover, cell expression provides a tighter genotype–phenotype linkage, which is useful in the context of directed evolution.

An alternative to protein expression inside a host is a cell-free system, in which an *in vitro* transcription and translation system (IVTT) processes the information encoded in a DNA

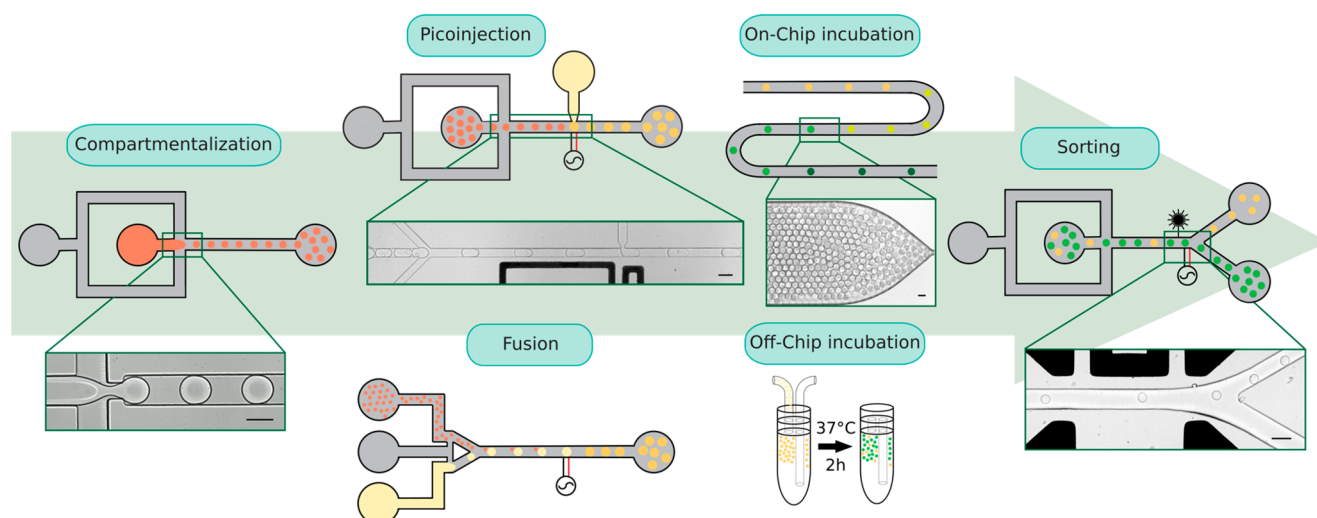


Figure 3. Typical microfluidic workflow and description of the main microfluidic modules. An initial compartmentalization is followed usually by one or more picoinjection, fusion, and/or incubation events. Finally, a sorting step is needed to select the biomolecules of interest. Scale bars are 50 μm in all the pictures. Insets are reprinted with permission from open access papers: Beneyton *et al.* Out-of-equilibrium microcompartments for the bottom-up integration of metabolic functions. *Nat. Commun.* **2018**, *9*, 1–10;⁴⁷ Beneyton *et al.* High-throughput synthesis and screening of functional coacervates using microfluidics. *ChemSystemsChem.* **2020**, *2*, e2000022;⁴⁸ Schütz, S. S. *et al.* Rational design of a high-throughput droplet sorter. *Lab Chip* **2019**, *19*, 2220–2232.⁶⁰

template into an RNA transcript that is subsequently translated into protein without the environment of a cell¹⁶ (Figure 2). Cell-free systems allow the user to have direct control over the transcription and translation machinery of the cell without the constraints of the cell envelope. In this way, it is possible to co-translationally produce and solubilize membrane proteins,^{17,18} translate toxic or difficult-to-express proteins,¹⁹ introduce site-specific labels, or incorporate nonstandard amino acids^{20–23} or other monomers²⁴ into the polypeptide chain. However, the use of these systems results in lower yields of protein compared to host organisms. The first effective cell-free transcription–translation system dates back to 1961,²⁵ when Nirenberg and Matthaei successfully synthesized proteins with the translational machinery of *E. coli* in order to decipher the genetic code, but it was not until the 2000s when the applications of this technology began to be exploited with notable advances in the field of protein synthesis,²⁶ production of pharmaceutical compounds,^{27,28} or screening of protein and peptide libraries.²⁹

Finally, DNA variant libraries for directed evolution are typically synthesized by solid-phase synthesis and/or amplified by PCR, while RNA libraries are generally produced enzymatically from DNA templates using T7 RNA polymerase.

Once the choices for generating diversity and expressing variants are made, the microfluidics workflow must be adapted accordingly (Figure 3). How microfluidics technologies are implemented into each directed evolution scenario mainly depends on the expression system used. Common modules included in a droplet-based microfluidic system are droplet makers³⁰ and sorters, on the basis of either dielectrophoresis³¹ or acoustic waves.³² In addition to these key modules, various modules allow droplet manipulation, such as droplet fusion,³³ splitting,³⁴ and picoinjection,³⁵ making microfluidics a versatile and adaptable tool. Commonly, droplets are collected and incubated off-chip and later reinjected for an end-point measurement. Alternatively, incubation can take place on-chip in incubation channels when shorter incubation times are needed.³⁶ The latter allows precise control on the reaction's incubation time, as it is possible to precisely control flow

parameters, making it possible to measure the reaction at an end point or at a controlled time point, such as in kinetics measurements. To illustrate this, let us consider a typical microfluidic workflow for the directed evolution of enzymes with enhanced properties. In cases where the enzyme of interest is to be produced in an *E. coli* host, individual cells expressing a single enzyme variant are coencapsulated with a substrate for the chosen assay. If the protein is set to remain within the cytoplasm, protein induction will be performed off-chip, and the cells will later be encapsulated together with a lysis agent to provide enzyme accessibility to the assay. In the case of protein secretion, the order would be to first encapsulate the cells and later induce them by picoinjection once they are inside the drops to maintain phenotype–genotype linking. Alternatively, cells can also be encapsulated in inductive medium. Finally, if the protein is displayed at the cell surface or targeted to the periplasm, fewer steps are required, as there is no need to lyse the cells, and the induction can be carried out prior to the encapsulation. If, on the other hand, the enzyme is to be produced using an IVTT system, a DNA library encoding a large number of variants will be diluted and encapsulated together with a PCR mixture, such that each drop contains no more than a single DNA molecule. After performing in-drop PCR amplification, each droplet can be fused with another drop containing IVTT reagents and incubated, making these kind of processes more complex than cell-based experiments. After the enzyme of interest has been expressed, substrate is added to perform the enzymatic assay. Finally, droplets containing cells or the IVTT mixture are (re)injected into a sorting module to select the desired variants.

■ VARIANT SELECTION

The assessment of the performance of individual variants is achieved through an assay that produces a readout signal proportional to the fitness of the variant. The choice of the assay is crucial in determining the success of the experiment and in most cases the restraining factor on the possibility to

perform directed evolution. First of all, a minimal starting activity is required to proceed with a directed evolution workflow. Second, the readout must be fast and sensitive enough for the high-throughput screening of the droplets, which are flown at high velocity through microfluidic channels. Third, the diffusion of the assay components between droplets and into the oil should be limited.^{37,38}

Most assays performed in droplets rely on a laser-induced fluorescence readout, such as fluorescence-activated droplet sorting (FADS)^{31,32} or adapted versions of commercial fluorescence-activated cell sorting (FACS).^{39,40} This allows for highly sensitive measurements to be performed in the sub-millisecond time scale down to 1 nM of product,⁴¹ thus enabling the sorting and selection of variants in the kilohertz range. However, the implementation of a fluorogenic assay is not always trivial and has so far been limited to a narrow range of highly specific reactions that result in the activation of a fluorophore directly, by using a fluorogenic substrate,^{41–43} through a coupled assay,^{44,45} or by the release of a quencher.^{40,46} In the first case, typically, the natural substrate of the enzyme must be chemically modified with a fluorophore, which could potentially lead to the identification of an enzyme with improved activity toward the modified fluorogenic substrate rather than the native substrate. To minimize this risk, a coupled assay with a fluorophore may be used to keep the substrate unmodified. This may require an additional enzymatic cascade reaction to be included in the assay, though it must be ensured that the side reactions do not interfere with the assayed enzyme. Certainly, these additional reactions should not be rate-limiting in order to make sure that the selection pressure is applied to the enzyme of interest. In the final case, the assay relies on the activation of a fluorophore by displacing or removing a quencher from the donor–quencher pair and in this way increasing fluorescence intensity. Finding a good donor–quencher pair for a substrate is not straightforward. The substrate needs to be chemically modified with the donor–quencher pair, which can alter enzymatic activity toward the native substrate, similar to the use of a fluorogenic substrate. Finally, with this method, the background signal tends to be higher, which makes the assay less sensitive.^{47,48}

Recent developments in detection systems compatible with microfluidic chips have been made in order to extend the range of assays amenable to droplet microfluidics. Absorption-based methods are more universal but remain challenging because of the reduced optical path length of the microfluidic channels, which impacts the sensitivity and reliability of the measurements. However, successful absorption-based directed evolution has been achieved at a reduced throughput (300 Hz)⁴⁹ with a detection limit of 10 μ M of product through a coupled assay. Another method was recently developed for high-speed absorbance measurements. The method relies on the phase shift of light due to the photothermal effect and allows single-point absorption measurements at rates similar to those used in FADS.⁵⁰ Recently, the first directed evolution screen based on electrochemical measurements⁵¹ and other label-free screening methods have been demonstrated in droplets. Moreover, light scattering⁵² and image processing⁵³ could successfully be applied to the screening of populations based on cell growth with promising capabilities when coupled to artificial intelligence.^{54–56} Finally, efforts were made recently to integrate Raman spectroscopy⁵⁷ and mass spectroscopy^{58,59} into the droplet format.

After detection, droplets are deflected toward the desired outlet mostly using electric fields. The design of the electrodes plays a key role in the optimization of the electric field gradient and in maximizing droplet displacement.⁶⁰ A second key factor is the size of the droplets to be sorted. The highest sorting throughput reported was 30 kHz,⁶¹ which is achieved for rather small droplets (8 pL). However, this throughput has yet to be reached for biological experiments. The limiting factor at this point was the data acquisition of the electronic system. Nevertheless, when investigating, for example, cell proliferation, larger drops (100 pL to 1 nL) are required to provide a sufficient amount of nutrients for the longer incubation time.⁶² Sorting of such large drops has been performed at decreased throughputs since these require a larger deflective force to be displaced, tend to break easily due to the electric field, or split at the sorting junction when the flow velocity is too high. Recently, a novel method that utilizes an array of electrodes that can be triggered sequentially has been introduced. This method allows one to sort larger drops at increased throughputs⁵⁵ (850 Hz for 1 nL droplets). The relationship between droplet size and throughput is shown in Table 1 using selected studies and their novelties.

Table 1. Achieved Sorting Throughputs for Various Droplet Sizes^a

drop size (pL)	sorting throughput (kHz)	novelty	ref
12	2	fluorescence-activated droplet sorting	31
110	0.2	concentric sorting electrode	63
8	30	layered sorting junction	61
100–1000	0.85–4.4	sequentially addressable electrode array	55

^aDifferent technical advances have been implemented in order to enhance the final throughput.

Several standard analytical techniques have been compartmentalized, such as PCR,⁶⁴ ELISA,⁶⁵ MDA,⁶⁶ and cell transfection,⁶⁷ giving rise to “digital” techniques and resulting in improvements in accuracy, sensitivity, and throughput. Further, droplet microfluidics can be integrated with benchtop flow cytometers for droplet sorting. However, since FACS is not compatible with the oil phase of droplet microfluidics, additional steps are necessary to do so. For example, it is possible to create a double water–oil–water emulsion^{40,68,69} or to create hydrogel beads to compartmentalize the aqueous phase^{39,70} to allow droplet sorting. As described above, directed evolution in droplets is a multistep process and often requires a complex workflow with numerous handling steps where temperature and pressure control are vital. Consequently, efforts to automate these processes have been made, such as developing a platform, based solely on integrating temperature control, to automate transformation, culture, and expression of recombinant proteins inside a host microorganism.⁷¹ Newly, the possibility of full automation and integration of the microfluidic workflow was demonstrated using a system composed of 3 components: (i) a robotic liquid handler; (ii) syringe pumps with valves, which can withdraw and pump fluid; (iii) microfluidic unit operations, such as droplet generation, merging, and sorting.⁷² Such a system can perform all of the steps required for directed evolution and shows a high level of flexibility.

■ ENZYME ENGINEERING BY DIRECTED EVOLUTION IN DROPS

Natural enzymes have been optimized over billions of years of evolution to effectively perform an enormous variety of catalytic reactions in a selective manner. For industrial or therapeutic needs, this process needs to be accelerated and must be adapted to reactions that do not normally occur in nature. Enzyme engineering and directed evolution allows one to improve the natural activity of an existing enzyme, change its preferred substrate toward an activity of interest, or even design enzymes by computational methods.⁷³

Native Substrates. The evolution of enzymes for their activity on their natural substrate is a challenging game. Since the enzyme has already been improved through the multiple cycles of natural evolution, it is reasonable to expect, at best, a low magnitude improvement from the starting molecule in the final hit. However, the high number of variants that can be sorted using microfluidics increases the chances of finding an enhanced variant that will entail an enzyme with improved catalytic activity toward their original substrate. Usually, the generation of genetic variation includes a step of random mutagenesis to cover different evolutionary pathways, as the positions that need to be changed in the protein are not always known to the protein engineer.

One example of an enzyme that has been evolved to improve its activity toward their original natural substrates is the enzyme phenylalanine dehydrogenase. In this case, the enzyme, which catalyzes the NAD⁺-dependent deamination of amino acids, was improved to yield 2.7-fold higher activity than the wild-type.⁴⁹ To do so, genetic diversity was first introduced by epPCR followed by DNA shuffling of the resulting amplicon. The originality of this work, however, lies in the use of an absorbance detection module for the subsequent selection of protein variants in contrast with the FADS approach, which is often used in microfluidics. The protein expression system of choice was the prevalent cytoplasmic expression, where the cell is encapsulated with a lysis agent and the drop is used as a compartment to link the genotype of a variant to its phenotype, usually expressed as the level of fluorescence.

One interesting alternative to cytoplasmic cell-expression involves the generation of hydrogel beads. These beads are surrounded by a polyelectrolyte shell to link phenotype with genotype, where the compartmentalization is robustly preserved. They can therefore function as a compartment containing lysate from a single cell, allowing the use of a benchtop flow cytometer for droplet sorting. The potential of this system was demonstrated using phosphotriesterase (PTE), a bioremediation catalyst, where a single round of mutagenesis by epPCR followed by sorting of the 0.2% most active variants resulted in the identification of a variant presenting an 8-fold improvement in $k_{\text{cat}}/K_{\text{m}}$ for its native substrate, the pesticide paraoxon, and a 19-fold improvement for the substrate tetraethyl-O-fluorescein-diphosphate.³⁹ As expected, the increased activity toward the non-native substrate was more significant than for its natural substrate.

In some cases, the product of interest after an enzymatic reaction can be one of two possible stereoisomers. Enzymes that exhibit high enantioselectivity are seldom found in nature, and their directed evolution has been limited by the requirement of a chiral chromatography step. In order to engineer an esterase with improved enantioselectivity for the production of pharmaceutically important (*S*)-profens, a dual

channel microfluidic droplet screening system was developed.⁴³ This system uses a dual-fluorescence detection/sorting microfluidic device that allows the evaluation of two reaction channels to simultaneously screen for improved catalytic activity and enantioselectivity. Importantly, this system could also be used to select for additional enzymatic properties such as regioselectivity or chemoselectivity. After five rounds of mutagenesis and screening, a variant with 700-fold improved enantioselectivity for the desired (*S*)-profens was selected and identified. In this case, the genetic diversity was generated by both random and rational mutagenesis with a combination of epPCR, DNA shuffling, and saturation mutagenesis.

Alternatively, the display of the protein of interest at the cell surface rather than in the cytoplasm simplifies the whole workflow of directed evolution in drops by bypassing the lysis step and allowing simple DNA recovery by colony regrowth after sorting. In this manner, the link between genotype and phenotype is strengthened, and such an approach is typically chosen when using yeast as a host organism for detection and sorting experiments. In one of the earliest examples of directed evolution using microfluidics, horseradish peroxidase (HRP) was displayed at the surface of *S. cerevisiae* by anchoring it to its cell wall. As mentioned earlier, the improvement of an already highly efficient enzyme can be challenging, but in this case, the final protein was an improved mutant with a 10-fold greater catalytic rate compared to the wild-type enzyme. Notably, the high-throughput screening system was key in this process, since it allowed the identification of ~100 variants at least as active as the wild-type HRP from a population of ~10⁷, discarding the degenerate mutations, which are a majority. Genetic diversity was achieved by combining libraries created by epPCR that target residues along the whole protein with libraries created by saturation mutagenesis that target residues closer to the active site. The most active variants from both libraries were further mutated and screened by a final round of microfluidics.⁴¹

More recently, the same display system was used to enrich a population of cells expressing glucose oxidase mutants with higher activity compared to the wild-type enzyme.⁷⁴ The library of mutants was created using site-directed mutagenesis, where the changes of residues are directed to one specific amino acid, in contrast with the less targeted site-saturation mutagenesis mentioned in previous examples. Interestingly, from the top five mutants, three had previously been discovered by the same group using FACS, while two were isolated for the first time using microfluidics, including a variant with k_{cat} increased by 2.1-fold, demonstrating the robustness, sensitivity, and efficiency of the microfluidics strategy.

Successful microfluidics droplet sorting using surface display in *E. coli* has also been achieved.⁷⁵ In this case, a homodimeric arylsulfatase was evolved for improved sulfatase activity toward two different substrates, since the enzyme shows considerable activity toward phosphoester compounds, apart from its primary activity of catalyzing the hydrolysis of arylsulfates. Two libraries generated by epPCR were screened, initially against the first substrate, fluorescein disulfate, and then also against the second substrate, 4-nitrophenyl sulfate. The experiments resulted in the identification of 25 unique SpAS1 variants with up to 30-fold and 6.2-fold improved activity, respectively, after a single round of mutagenesis.

Non-native Substrates. Since minimum levels of activity must be detected to start the cycle of mutagenesis, expression,

and selection, a common strategy to identify an enzyme variant with activity toward a non-native substrate by directed evolution is to improve an existing promiscuous enzymatic activity. Nearly a decade ago, droplet-based microfluidics was used to successfully screen a promiscuous sulfatase with hydrolytic activities toward the nonnative substrate phosphate. Genetic diversity was generated by epPCR, and the library was expressed in the cytoplasm of *E. coli* with the corresponding cell lysis. The top 4% of the most active clones, displaying at least 4-fold improved activity, was selected in each of a total of 3 rounds of sorting. The final candidate presented a 6-fold increase in k_{cat}/K_m for the desired function after purification of the enzyme.⁴² This work demonstrated that rare variants with small improvements in activity could be detected and selected by microfluidic droplet screening.

When targeting non-native substrates, another common strategy is to apply semirational library design to directly target residues in the substrate binding site. This approach was used to completely remodel the active site of cyclohexylamine oxidase, an enzyme used in the industrial production of chemicals and active pharmaceutical ingredients.⁷⁶ Genetic diversity was generated by targeting 8 residues close to the bound cyclohexanone and randomizing them with either DYT codons (encoding for A, S, T, V, I, and F) or BYT codons (encoding for A, S, P, V, L, and F), followed by selection of the top 0.1% of the most active variants for each of 3 consecutive rounds of directed evolution. Notably, the three most active variants obtained after the third round of sorting had identical amino acid sequences despite having been obtained independently. This variant had five amino acid changes compared to the wild-type and after purification presented an impressive 960-fold improvement in catalytic efficiency for the same substrate.

Non-native substrates also include synthetic substrates, such as xeno nucleic acid (XNA) polymers. As these synthetic polymers increasingly show potential for synthetic biology and future applications in molecular medicine, nanotechnology, and materials science, the development of efficient synthetic polymerases by directed evolution is gradually gaining importance. One example is the evolution of a polymerase that replicates an unnatural genetic polymer composed of repeating units of α -L-threofuranosyl nucleic acid (TNA) sugars. This approach was used to develop a manganese-independent TNA polymerase that functions with 99% template-copying fidelity after making the hypothesis that the presence of manganese was making the polymerization unspecific.⁴⁰ To achieve this, three key residues known to affect substrate specificity were altered by saturation mutagenesis. The resulting enzyme variants were encapsulated in double emulsion droplets and sorted by FACS based on their ability to elongate a full-length product, which produced a fluorescent signal by donor–quencher pair disruption. Presumably, it should be possible to evolve other polymerase functions provided that the optical detection of the product can be achieved.

Computer-Designed Enzymes. Computational design can give rise to *de novo* biocatalysts with a function not found in nature. However, newly designed enzymes typically show very low catalytic activity and must subsequently be improved through directed evolution. A striking example of the successful optimization of a computer-designed enzyme is that of retro-aldolase, an enzyme capable of cleaving a specific carbon–carbon bond in a non-natural substrate using amine

catalysis. A retro-aldolase slightly modified from the original computer design⁷⁷ was used as a starting point and reoptimized using a microfluidics-based system able to detect enzyme activities as low as $k_{\text{cat}}/K_m = 0.5 \text{ M}^{-1} \text{ s}^{-1}$. For this optimization, six libraries were generated by saturation mutagenesis using NNK codons. The targeted residues were close to the binding pocket and varied from four to five simultaneously randomized residues. After a first round of selection, two variants with >10-fold improved activity were chosen for DNA shuffling, obtaining a final variant with a 73-fold increase in k_{cat}/K_m compared to the initial enzyme.⁷⁸

The same group had previously evolved by microtiter plate the starting point retro-aldolase.⁷⁹ Notably, the best variant after 13 rounds of directed evolution in microplates was not as active as the one obtained from only two rounds of FADS, highlighting the importance of screening a higher diversity of variants with a high-throughput method. A year later, the microtiter plate-evolved enzyme was reoptimized using FADS, leading to the identification of a new complex catalytic center, which featured a Lys-Tyr-Asn-Tyr tetrad and was 30-fold more active.⁸⁰ When variants with low activity are detected in a high-throughput manner, FADS is a powerful tool for tuning the properties of computationally designed enzymes.

Optimization of Ribozymes. Although there are tens of examples of RNA molecules with catalytic properties (RNAzymes or ribozymes) in nature, scarce attempts have been made to improve these biomolecules using droplet microfluidics. In one of the few studies to date, the catalytic properties of an X-motif capable of RNA cleavage *via* an internal phosphoester transfer reaction were improved using a complex microfluidic workflow.⁴⁶ First, a DNA library and PCR reagents were injected into droplets, and PCR was performed off-chip. Then, the droplets were mixed with a T7 polymerase-based *in vitro* transcription mixture. The authors discovered that T7 RNA polymerase interfered with the fluorogenic nuclease assay. However, picoinjection of the assay mixture with a high NaCl concentration allowed them to inactivate the T7 RNA polymerase and stop the transcription reaction of the ribozyme. Finally, droplets were selected with a FADS device on the basis of a fluorogenic RNA substrate comprising a fluorophore and a quencher at the 5' and 3' ends, respectively. After 9 rounds of selection, the catalytic properties of the ribozyme were enhanced 28-fold. Several mutations could be shown to improve the activity of the ribozyme.

■ NONCATALYTIC BIOMOLECULES

Antibody Optimization with Droplet Microfluidics. Antibodies like immunoglobulin G (IgG) are ~150 kDa, Y-shaped, globular proteins that form an essential component of the immune system used to fight invading pathogens, such as bacteria or viruses. Although their overall structure is very similar, the region used to specifically recognize a given epitope on an antigen varies greatly from one antibody to the next. Substantial advances have been made over the past 20 years in the research, development, and clinical application of therapeutic monoclonal antibodies.⁸¹ Monoclonal antibodies have become one of the fastest growing sectors of human therapeutics for treating various pathologies, such as cancer, inflammation, infections, or autoimmune diseases.⁸² The selection of antibodies using microfluidic systems provides a low-cost and high-throughput approach for disease diagnosis, phenotyping of tumor cells, and biomarker detection.

Although many methods have been devised to screen for specific antibodies, each with its distinctive advantages and limitations, the identification of antibodies that bind to cell-surface receptors or target specific cells remains challenging. Starting from hybridomas producing nonspecific antibodies, more than 80 000 hybridoma-clone secreting antibodies with specific binding properties to the transferrin receptors on leukemic K562 cells could be selected.⁸³ Remarkably, very low amounts of IgG were used per assay (33 fg), and the enrichment of specific hybridoma cells could be achieved thanks to the selection system. This promising work could be transposed to further therapeutic antibody discovery. For instance, a new microfluidic method for single-cell deep phenotyping of IgG-secreting cells was developed, in which thousands of droplet-encapsulated cells arranged as a two-dimensional droplet array were screened using a fluorescence relocation-based immunoassay.⁸⁴ A comprehensive step-by-step description of this method was also published recently.⁸⁵

Historically, one of the most used methods to generate new antibody variants by directed evolution has been yeast display. When these methods were combined with microfluidic tools, a substantial increase in the number of tested variants could be achieved, resulting in a jump from medium (10^2 to 10^3 variants) to high throughput (10^6 to 10^9). In 2017, Adler *et al.*⁸⁶ combined microfluidics, yeast single-chain variable fragment (scFv) display, and deep sequencing to build an alternative to hybridoma-based antibody discovery. With this system, mouse antibody repertoires could be selected against the programmed cell-death protein 1 (PD-1), a checkpoint protein used as a target in cancer immunotherapies. A droplet-based microfluidic system was used to encapsulate B cells from mice with oligo-dT beads and a lysis solution. Polyadenylated transcripts released from cells and bound to the beads were purified from the droplets and injected into a second emulsion with a multiplexed overlap extension reverse transcriptase polymerase chain reaction mix. Finally, DNA amplicons encoding scFv with native pairs of heavy and light chain Ig were generated. These libraries were used for scFv display and screened by FACS, resulting in the identification of high-affinity scFvs against human PD-1 immunogen by deep sequencing. Two rounds of FACS produced populations of scFv with an average enrichment of 800-fold. Seventeen of these anti-PD-1 binders were synthesized as full-length monoclonal antibodies. Among them, 15 specifically bound surface-expressed PD-1 in a FACS assay, while 9 antibodies acted as checkpoint inhibitors. This approach could further be used to screen for other functional monoclonal antibodies.

Aptamer Development by Directed Evolution in Drops. Aptamers are biomolecules that possess the capacity of specifically binding another molecule. Their chemical nature can be proteinogenic or nucleic acid based (both DNA and RNA). RNA aptamers named riboswitches can be found in nature and are involved in the metabolite-dependent control of gene expression.⁸⁷ The discovery and development of aptamers by SELEX methods started in 1990, when Tuerk and Gold identified various RNA ligands against T4 polymerase.⁸⁸ In another study, also focused on discovering ligands against T4 polymerase, the term aptamer was coined by Ellington and Szostak.⁸⁹ Since then, a plethora of new aptamers have been developed with the SELEX methodology.

Aptamer evolution in droplets took two more decades to become prominent. Droplet-based systems provide the advantage that every mutant can be studied individually inside

drops, in contrast to classical SELEX systems. Fluorogenic aptamers are an alternative to classical fluorescent proteins, such as GFP, and are widely used for biochemistry, cell biology, and biomedical applications, making the exploration of new fluorogenic aptamers a particularly promising area of research. In particular, G-quadruplex RNA aptamers and, in some cases, their corresponding biosensors have been the focus of extensive optimization, resulting in the development of highly fluorescent iSpinach^{90,91} aptamer-based fluorogenic biosensors (Figure 4a), MangoIII^{92,93} aptamer, and the

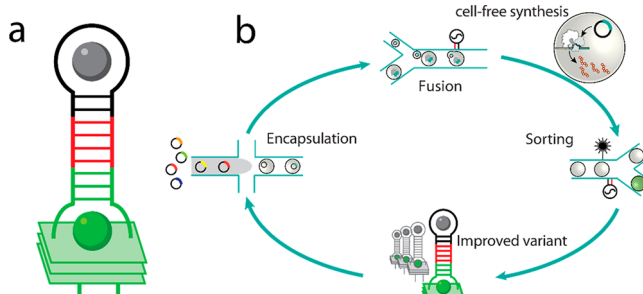


Figure 4. Directed evolution of fluorogenic aptamers in drops. (a) G-quadruplex structure of an iSpinach-based RNA biosensor.⁹¹ The sensor aptamer is in black; the optimized communication module is in red and the fluorogenic G-quadruplex aptamer, in green. (b) Microfluidic workflow of the process: First, a gene library is encapsulated in droplets. After fusion events of all the drops with other drops containing an IVTT system, the aptamer library is generated. Finally, the aptamers of interest are selected by FACS and encapsulated again for another round of selection or analyzed by NGS.

Gemini-o-Coral fluorogenic dimer.⁹⁴ All of these aptamers were improved by directed evolution using the same microfluidic workflow (Figure 4b). First, a variant library was encapsulated in drops with no more than one molecule per drop. Second, the drops were amplified by PCR. Third, the drops containing the amplified variants were reinjected into a microfluidic device and fused with another drop containing *in vitro* transcription reagents. Fourth, the fused drops were collected and incubated to produce RNA, and fifth, the drops were sorted on the basis of their fluorescence and collected for sequencing. Step 2 was performed off-chip in a thermocycler. Step 4 can be carried out either on- or off-chip.

Spinach⁹⁵ is an artificial fluorogenic RNA aptamer whose ligand emits green fluorescence similar to eGFP upon binding. The screening of Spinach gene libraries led to an improved Spinach (iSpinach) aptamer⁹⁰ thanks to microfluidic-assisted *in vitro* compartmentalization. Two pairs of enrichment rounds were separated by a mutagenesis round. The main goal was to develop iSpinach mutants with higher thermal stability and a wider salt tolerance because none of the known DFHBI-binding aptamers are optimal for *in vitro* application. On the basis of this development, a new biosensor capable of detecting theophylline was built⁹¹ by randomizing the communication module of the biosensor. This region links the sensor region with the fluorogenic G-quadruplex aptamer, which in the case of iSpinach binds the fluorophore 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI). After 5 rounds of selection, the fluorescence of the biosensor was improved by 5-fold.

Like Spinach, Mango is another fluorogenic RNA aptamer with a complementary emission wavelength in the red region of the spectrum. A complex was formed with thiazole orange

Table 2. Summary of the Evolved Biomolecules Described in This Review^a

	selection pressure	no. rounds	expression		library design	reference
			<i>E. coli</i>	other		
Enzymes						
phosphotriesterase	fluorescence	1	cytoplasmic		random	39
TNA polymerase	fluorescence	1	cytoplasmic		semirational	40
peroxidase	fluorescence	2		<i>S. cerevisiae</i>	semirational	41
esterase	fluorescence	5	cytoplasmic		semirational	43
dehydrogenase	absorbance	2	cytoplasmic		random	46
oxidase	fluorescence	1	cytoplasmic		semirational	74
sulfatase	fluorescence	1	display		semirational	75
aldolase	fluorescence	2	cytoplasmic		semirational	77
	fluorescence	6	cytoplasmic		random	78
Ribozymes						
X-motif (RNA)	fluorescence	9		PCR	random	46
Antibodies						
anti-transferrin (K562)	fluorescence	1		hybridoma cells		83
anti-PD-1	fluorescence	2		<i>S. cerevisiae</i>		86
Aptamers						
iSpinach	fluorescence	5		IVTT	semirational	90
	fluorescence	5		IVTT	semirational	91
Mango III	fluorescence	9		IVTT	semirational	92
Mango III (A10U), iMango III	fluorescence	4		IVTT	rational	93
Gemini-561, o-Coral	fluorescence	4		IVTT	semirational	94

^aA total of 8 enzymes, 5 aptamers, 1 ribozyme, and 2 antibodies have been reviewed. We have also dissected the key components underlying these directed evolution experiments.

T01-biotin and was selected on the basis of the binding affinity between the aptamer and this ligand by 12 rounds of classical SELEX.⁹⁶ Three new aptamers (Mango II, III, and IV) were developed from Mango I using microfluidic selection. When 9 rounds of directed evolution were applied, the fluorescence of the complex could be increased 6-fold.⁹² Using a structure-guided library in which only key residues are mutated, two novel Mango III mutants were also identified: Mango III (A10U) and iMango III.⁹³ The cocrystal structure of the aptamer and ligand shows a pseudoknot-like base pairing interaction between nucleotides internal and adjacent to a two-tiered G-quadruplex. The novel mutants are 50% brighter than eGFP, making them useful for live cell RNA visualization. The most recent discovery using this method is the complex Gemini–o-Coral,⁹⁴ a cell-permeable fluorogenic dimer of self-quenched sulforhodamine B dyes (Gemini-561) and the corresponding dimerized aptamer (o-Coral). In order to overcome the limitation of the detection of low amounts of RNA with Spinach and Mango, a new strategy using fluorescent quenchers was applied to develop new aptamers with enhanced fluorescence. A recent study shows that this technology could also be applied for the development of the recently discovered double analyte aptamers.⁹⁷ Altogether, microfluidics has had a considerable impact on fluorogenic aptamer research in the past few years and shows great promise for future investigations in this field.

CONCLUSIONS AND FUTURE PERSPECTIVES

Directed evolution is a field of growing importance with an increasing number of successfully improved molecules, from enzymes being improved for therapeutic applications to those used in self-replicating nucleic acids. Droplet microfluidics achieves all these processes in an automated and miniaturized format with a much higher efficiency in time and cost compared to low-throughput methods. The use of droplets

provides an additional compartment in which to perform chemical assays and enzymatic reactions while linking the activity of the molecule of interest to the genetic variant it represents. In this way, as the assay is not limited to the cell, one can broaden the spectrum of analytical tools available compared to classical cell sorting approaches like FACS.

We have highlighted here some of the most recent advances in the engineering of functional biomolecules (enzymes, antibodies, aptamers, and ribozymes) using droplet microfluidics. Over the past years, experimental procedures have been miniaturized and automated in the droplet format showing the long-term potential of the technology for molecular engineering. Improved variants have already been successfully obtained from a variety of experiments (Table 2). Most of the examples use a cell-based system for the expression of the molecules. Usually, the choice to express the molecule of interest in a single-cell organism is merely economical, since nowadays the cost of using commercial IVTT systems for expressing proteins is orders of magnitude more expensive than using a host organism. In the case of cell display, the choice of this system has an added justification, using the cells not only as a compartment in which to link phenotype and genotype but also as a means to replicate the genetic variants after sorting since lysis is not necessary and the cells can be regrown. Moreover, the related microfluidic workflow is simpler compared to the IVTT-involving platform. In addition, since the scaling up needed for production of a biological protein will usually be done in cell-based systems, it is convenient to use this system from the beginning of the experimental workflow and avoid unexpected difficulties.⁹⁸ However, due to the more generalized use of IVTT systems, one can forecast a progressive lowering of the price. In this sense, the idea of generating in-house methods to produce cell-free systems is gaining importance. Complete methods for the whole process and plasmid designs (One-pot system) are already avail-

able.^{98,99} We foresee that this strategy will be widely deployed in the coming years.

It is interesting to highlight as well that peptides are gaining importance as therapeutic agents. As their use is becoming common to treat acute infections, chronic diseases, and even some types of cancer, droplet microfluidics can help one to discover peptides with novel properties. Droplet-based strategies could be adopted to identify antimicrobial peptides,¹⁰⁰ discover novel antiviral peptides to treat respiratory diseases,¹⁰¹ or find anticancer peptides.¹⁰²

Research in self-replicating nucleic acids is another field that is gaining importance. The search for the molecular origins of life and the construction of a minimal cell have found a versatile toolbox in droplet-based microfluidics. Research in template-directed self-replicating systems or replicators can also lead to the development of artificial ribozymes¹⁰³ or control of protocell compartmentalization and reproduction.¹⁰⁴ Using water-in-oil emulsions, the replication efficiency of an RNA replicating system could be increased 30-fold.¹⁰⁵ Encapsulation of RNA catalysis reactions into droplet coacervates can also have important implications for early Earth chemistry and protocell research.¹⁰⁶

Contrary to protein enzymes or ribozymes, DNA-based enzymes (DNAzymes or deoxyribozymes) are scarce with only a few examples found in nature,¹⁰⁷ acting mainly as ribonucleases and RNA ligases. However, the directed evolution of artificial single-stranded DNA (ssDNA) constructs has been favored due to some of the intrinsic advantages of ssDNA over RNA. For instance, ssDNA generally has higher chemical stability compared to RNA due to the absence of the 2'-hydroxyl group on the ribose moiety. The potential of studying the directed evolution of these molecules could lead to new biomedical and industrial applications due to their different chemical nature and versatility, such as DNA aptamers or DNA-metal nanoclusters for biosensing.

Nonetheless, despite the many examples discussed and the increasing speed in which the field of microfluidics for directed evolution is growing, the technology is far from being fully mature and further improvements are to be expected. For instance, DNA recovery and cell viability are still challenges to take into account and can be the limiting step in biological workflows. Moreover, greater access of nonspecialist laboratories to microfluidic setups is necessary to maximize the impact of this technology. Microfluidic technology will most likely gain in miniaturization, automation, and parallelization capabilities, pushing further the throughputs of selection with the development of new instruments by technology developers. Furthermore, the development of novel surfactant formulations may increase the scope of the assays possible in droplets and solve some of the current limitations such as the leakage of molecules through the drop or the interaction of surfactant with the content of the droplet. Finally, new and emerging approaches in molecular programming will provide interesting functions to further increase the selection throughputs and will provide new methods for the biomimetic selection of improved variants of practical interest.¹⁰⁸ We believe that the combination of these tools will lead in the future to a whole new range of approaches for the discovery and improvement of chemicals of fundamental and practical interest in therapeutics and industrial applications.

AUTHOR INFORMATION

Corresponding Authors

Aitor Manteca — Univ. Bordeaux, Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, ARNA, U1212, UMR 5320, Institut Européen de Chimie et Biologie, F-33600 Pessac, France; Present Address: Center for Cooperative Research in Biomaterials (CIC biomaGUNE), Basque Research and Technology Alliance (BRTA), Paseo de Miramón 194, Donostia-San Sebastián, Spain; orcid.org/0000-0002-8650-0465; Email: amanteca@cicbiomagune.es

Jean-Christophe Baret — Univ. Bordeaux, CNRS, CRPP, UMR 5031, F-33610 Pessac, France; Institut Universitaire de France, F-75231 Paris, France; orcid.org/0000-0003-2048-8317; Email: jean-christophe.baret@u-bordeaux.fr

Authors

Alejandra Gadea — Univ. Bordeaux, CNRS, CRPP, UMR 5031, F-33610 Pessac, France

David Van Assche — Univ. Bordeaux, CNRS, CRPP, UMR 5031, F-33610 Pessac, France

Pauline Cossard — Univ. Bordeaux, Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, ARNA, U1212, UMR 5320, Institut Européen de Chimie et Biologie, F-33600 Pessac, France

Mélanie Gillard-Bocquet — Univ. Bordeaux, Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, ARNA, U1212, UMR 5320, Institut Européen de Chimie et Biologie, F-33600 Pessac, France

Thomas Beneyton — Univ. Bordeaux, CNRS, CRPP, UMR 5031, F-33610 Pessac, France

C. Axel Innis — Univ. Bordeaux, Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, ARNA, U1212, UMR 5320, Institut Européen de Chimie et Biologie, F-33600 Pessac, France; orcid.org/0000-0003-3153-9490

Complete contact information is available at:
<https://pubs.acs.org/10.1021/acssynbio.1c00313>

Author Contributions

[‡]A.M., A.G., and D.V.A. contributed equally.

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Notes

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