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Droplet-based microfluidics and enzyme evolution

Ankit Jain, Stavros Stavrakis and Andrew deMello

Enzymes are widely used as catalysts in the chemical and pharmaceutical industries. While successful in many situations, they must usually be adapted to operate efficiently under nonnatural conditions. Enzyme engineering allows the creation of novel enzymes that are stable at elevated temperatures or have higher activities and selectivities. Current enzyme engineering techniques require the production and testing of enzyme variant libraries to identify members with desired attributes. Unfortunately, traditional screening methods cannot screen such large mutagenesis libraries in a robust and timely manner. Droplet-based microfluidic systems can produce, process, and sort picoliter droplets at kilohertz rates and have emerged as powerful tools for library screening and thus enzyme engineering. We describe how droplet-based microfluidics has been used to advance directed evolution.

Address
Institute for Chemical and Bioengineering, Department of Chemistry and Applied Biosciences, ETH Zürich, Vladimir Prelog Weg 1, 8093 Zürich, Switzerland

Corresponding author: deMello, Andrew (andrew.demello@chem.ethz.ch)

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Introduction

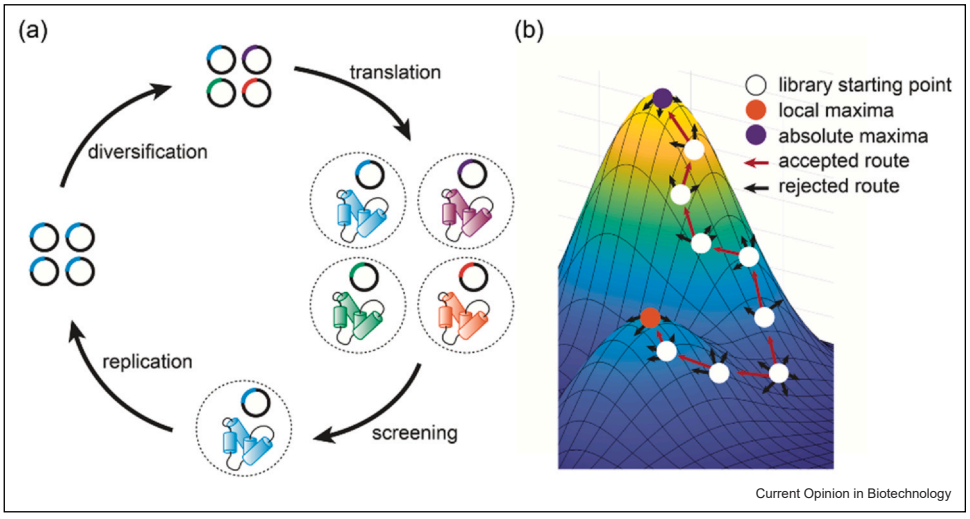
Enzymes are natural catalysts that drive the chemistry of life. They facilitate almost all the biochemical reactions that are necessary to sustain life on earth. Owing to their high selectivity, high turnover frequencies under mild reaction conditions (10^3–10^7 s^{-1}), and their environmental-friendliness, they are effective biocatalysts in a wide range of industrial processes [1]. Biocatalysts are normally used under mild conditions, for example, in aqueous solvents at pressures and temperatures close to ambient. This enables the relatively simple coupling of biocatalytic steps in a cost-effective and environmentally friendly manner. Unfortunately, the use of biocatalysts in the chemical and pharmaceutical industries can often be problematic since industrial processes commonly require conditions, such as temperatures, pressures, solvents, and substrate-load, that differ drastically from the optimum physiological conditions for a given enzyme.

In recent years, various methods have been developed to generate and identify new enzymes able to efficiently drive reactions under nonphysiological conditions [2–5]. If an enzyme exhibits activity for a certain reaction but does not meet the required performance criteria (e.g. thermostability), protein engineering technologies can be used to adapt or enhance performance when using particular solvents, temperatures, or substrate-loads. Directed evolution is a bioengineering technique that can be used to modify and tailor the properties of proteins or nucleic acids by performing iterations of gene diversification and selection of functional gene variants [6,7]. The importance of directed evolution in protein engineering was recognized in 2018 through the award of the Nobel Prize in Chemistry [8].

In its most basic form, a directed evolution workflow involves the identification of a starting protein, genetic diversification, an expression method, and a screening/selection strategy (Figure 1a). This process is repeated with rediversification and rescreening until a desired or optimal enzymatic activity, binding affinity, or specificity is reached. In simple terms, this is analogous to ascending a fitness landscape within a mutagenesis space (Figure 1b), where each mutagenesis step explores the proximal surface, while selection signifies ascension toward a global (or local) fitness maximum. Accessing this maximum in a time-efficient manner requires an effective strategy for mutagenesis (increasing the likelihood of active variants within the library) and a rapid screening strategy that combines both genotype and phenotype characterization. The screening strategy becomes especially important if random mutagenesis is employed, since library sizes can often exceed 10^6 variants. Such large libraries are needed when the information relevant for creating focused libraries, such as enzyme structure and/or substrate–enzyme interaction mechanism, is unavailable. Given the vast mutant library size that can result from a direct evolution campaign, tools and technologies that allow for the creation of high-throughput screening (HTS) platforms are invaluable.

HTS is an experimental approach commonly used in the pharmaceutical and biotechnology industries, and a

Figure 1



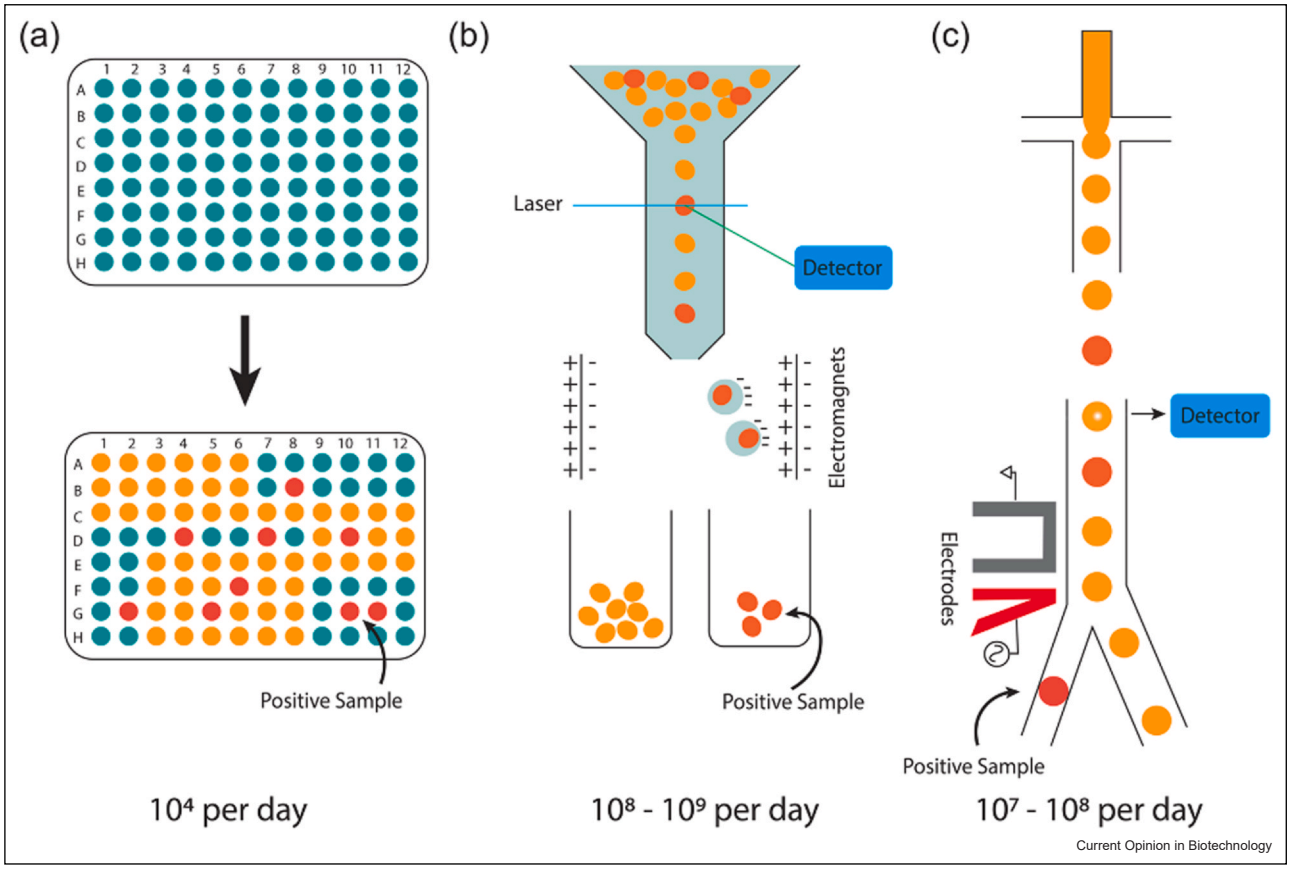
The essential steps involved in the directed evolution cycle. **(a)** A directed evolution workflow comprises genetic diversification, translation, and screening, and maintains the gene and enzyme linkage. The selected gene goes through multiple rounds of rediversification and reselection until the desired results are obtained. **(b)** A representative fitness landscape within mutagenesis space. Depending on the path chosen, evolution of a protein progresses toward a local maximum or a global maximum. Such variability highlights the need for correct diversification and high-throughput selection methods.

critical component of drug discovery and development pipelines. HTS is normally performed using multiwell plates, which comprise between 96 and 3456 sample wells arranged in a 2:3 rectangular matrix (Figure 2a). Despite the obvious throughput advantages associated with compartmentalization within multiple, sub-mL volumes, microtiter plates can only screen at rates up to 10^4 samples per day, even when combined with state-of-the-art fluid-handling systems.

Flow-based systems present a powerful and attractive alternative to multiwell plates for HTS. A good example in this regard is flow cytometry. Contemporary flow cytometers are able to analyze several thousand cells per second, classifying cells on the basis of scattering and fluorescence signals. Fluorescence-activated cell sorting (FACS) is a specialized form of flow cytometry that integrates a sorting functionality and allows cells to be separated into pure subpopulations based on their optical or electrical properties (Figure 2b). In theory, cells may be sorted at throughputs of between 10^4 and 10^5 cells/s, although practical constraints limit sorting rates to between 10^3 and 10^4 cells/s. While powerful, the use of FACS in enzyme engineering is mainly restricted to live cells containing fluorogenic moieties [9] or surface-enzyme-displaying cells [10,11].

Droplet-based microfluidic systems combine the high-throughput nature of flow cytometry with the physical compartmentalization features of multiwell plate platforms [12]. Such systems allow the creation of extremely large numbers of pL-volume compartments (droplets), by uniting two or more immiscible fluids within a microfluidic channel [13,14]. In screening- or evolution-based experiments, individual cells (expressing individual enzymes) can be encapsulated within such droplets (Figure 2c). They can then be lysed to bring an expressed enzyme in contact with a chemical substrate located within the droplet, maintaining the genotype–phenotype linkage. Genetic information is encoded in the genotype, while selection is based on phenotypic characteristics. After selection, the genetic information of the cell can be recovered for analysis. In general, droplet-based microfluidic platforms are desirable since they consume extremely small amounts of reagent, operate at exceptionally high speeds, are able to manipulate single cells in a robust manner, and can be directly integrated with a variety of optical, electrical, or magnetic modalities [15]. Taken together, these capabilities ensure that droplet-based microfluidic systems are efficient and versatile tools for analyzing complex biological samples at the single-cell level. Of particular interest to enzyme engineering is fluorescence-activated droplet sorting (FADS) [16]. Fluorescence-activated droplet sorters probe the fluorescence signature of rapidly moving droplets and subsequently select 'positive' droplets based on user-defined criteria. Compartmentalization in such platforms allows screening of a wide variety of phenotypic characteristics, ranging from surface-bound and internal proteins to secreted antibodies and metabolites at rates in excess of 10^8 variants per day [17,18]. This makes them well-suited for processing large drug or mutant libraries in the pharmaceutical and biotechnology industries.

Figure 2



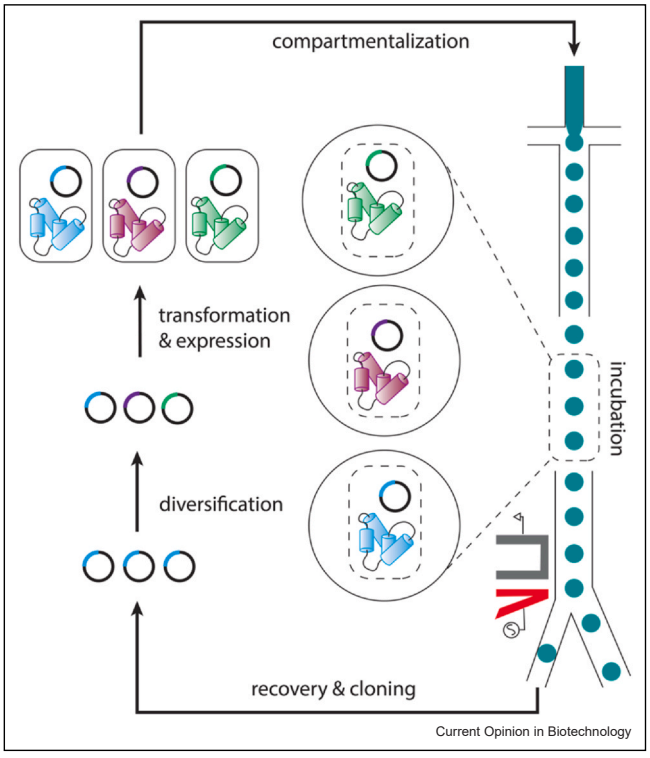
Comparison of methods for high-throughput experimentation. **(a)** Microwell plates, **(b)** FACS, and **(c)** droplet-based microfluidic sorting. While FACS can operate at rates in excess of 10^9 cells per day, analysis requires labeling of the cells with fluorescence reporters and cannot link cells to their secreted metabolites or lysed-cell content. Conversely, droplet-based microfluidic systems are able to link cells to their secreted metabolites or cellular contents and operate at throughputs at rates in excess of 10^8 cells per day. Multiwell plate systems screen at much lower rates and require significantly larger volumes of sample and reagent.

Recent highlights in droplet-based sorting platforms

A typical enzyme evolution workflow comprises library creation and transformation and bacteria encapsulation in droplets with lysis agents and enzyme substrates. Droplets are then incubated for a period of time sufficient to allow for the formation of detectable amounts of product. Subsequently, droplets containing cells of interest are sorted, based on the detected signal, and collected. Plasmid DNA is then recovered from the sorted bacteria through polymerase chain reaction amplification and cloning. The recovered DNA is used for further rounds of mutagenesis and screening. This process is illustrated in Figure 3.

Most droplet-based sorters incorporate fluorescence detection and dielectrophoretic (DEP) droplet actuation [19]. DEP forces are generated when droplets are subject to nonuniform electric fields, with their magnitude depending on the dielectric constant contrast between the aqueous droplet and the immiscible continuous phase. Briefly, fluorescence emanating from a flowing droplet is detected by a photodetector and converted into an electrical signal. A microcontroller interprets this signal and based on user-defined thresholds, produces a voltage pulse that generates a DEP force to actuate a droplet of interest toward the positive channel. Such sorters typically operate at rates between 1000 and 2000 droplets per second and have been used extensively in enzyme engineering [20] and antibody discovery [21–23]. Interestingly, Sciambi and Abate showed that by manipulating the geometry of a divider between the 'positive' and 'negative' outlets, FADS systems can operate at throughputs of up to 30 000 droplets/s under certain conditions [24]. Many variations to the traditional FADS structure exist. For example, Caen and coworkers presented a multiplexed droplet sorting chip capable of sorting five different droplet populations through modulation of the applied voltage on electrodes located on either side of the sorting region [25]. While

Figure 3



A droplet-based microfluidic workflow for directed evolution and HTS. After library generation, bacterial transformation, and enzyme expression, bacteria are encapsulated in droplets. The droplets are then incubated for a period of time that is sufficient to yield a detectable amount of product. Droplets are subsequently reinjected into a microfluidic sorter for screening and selection. Genes are recovered from the sorted droplets, analyzed, and used for further rounds of screening.

electrical actuation is the most widely used method for droplet sorting, other actuation mechanisms have been used to good effect, most notably, those based on acoustics [26,27]. Compared with electrical actuation, the maximum throughput achieved by acoustic sorting is 3000 droplets/s [26].

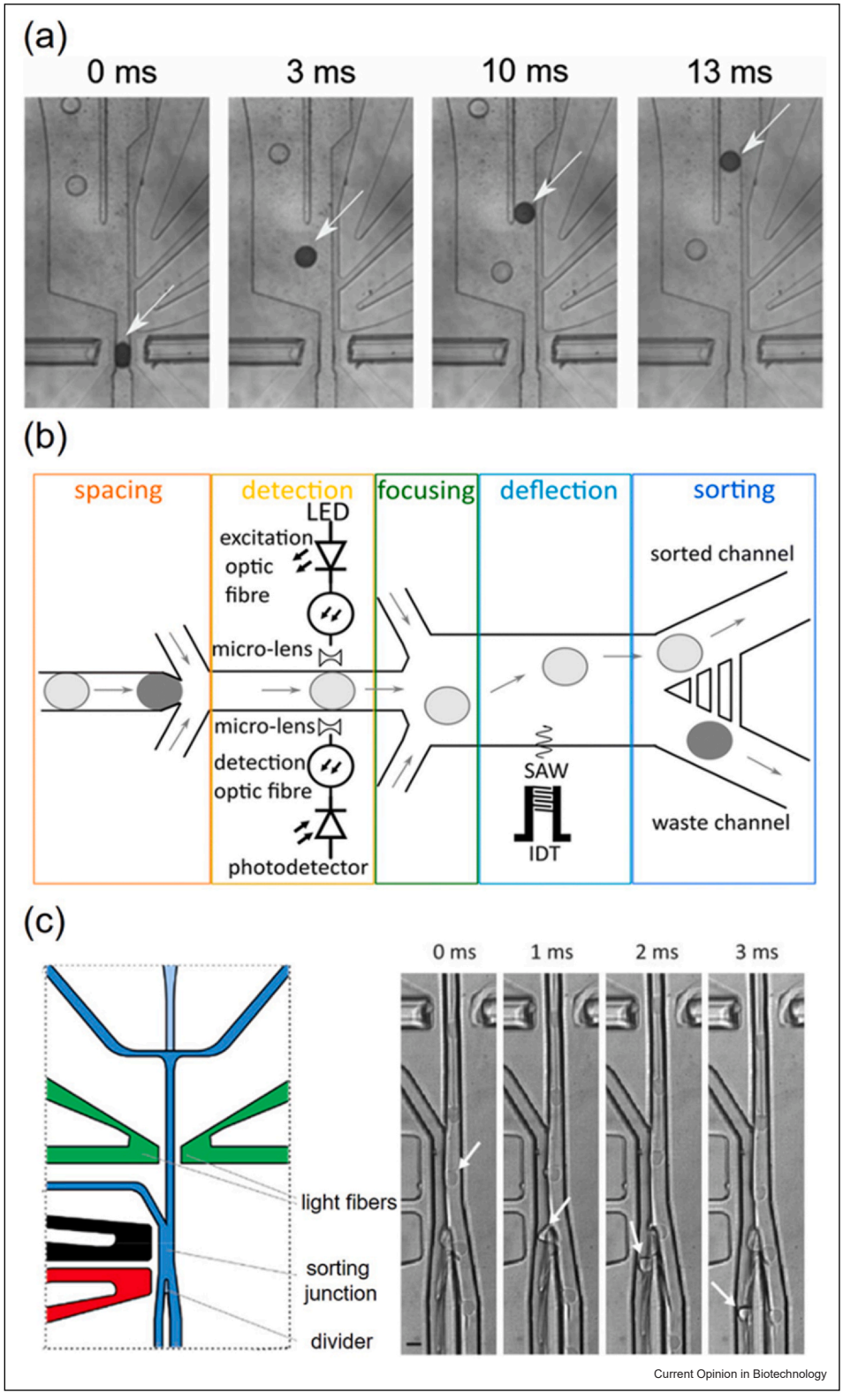
Directed evolution of enzymes using droplet-based microfluidics

As noted, droplet-based microfluidic platforms maintain the genotype–phenotype linkage and are able to screen up to 10^8 mutants per day [17]. Such systems have been successfully used to evolve enzymes such as hydrolases [28•–30], aldolases [31••], and oxidases [32]. Notably, Larsen et al. used a molecular beacon reporter to evolve a manganese-independent α-L-threofuranosyl nucleic acid (TNA) polymerase [33••]. In just one round of selection, the authors identified a Mg-independent TNA polymerase with higher fidelity and a 14-fold improved activity. Interestingly, sorting was performed using a double emulsion, which was compatible with conventional FACS, and thus circumvented the need for a FADS system. Furthermore, droplet-based microfluidic systems have been used to discover rare targets in metagenomic libraries, due to the ultra-HTS capacities. For example, Hollfelder and co-workers uncovered novel β-glucuronidases by FADS-based screening of a million-member metagenomic library [34].

As noted, the most common detection methods in microfluidic-based enzyme evolution are based on fluorescence. Fluorescence detection is fast, exceptionally sensitive, and routinely provides for sub-nM concentration detection limits [28,35]. Despite its utility, fluorescence detection often requires the use of surrogate substrates able to generate a fluorescent (and thus detectable) product [36,37••]. This means that experiments optimize for the surrogate substrate and not the native substrate [38]. Additionally, interdroplet transport of pH-mediating molecules makes pH-based assays impractical [39,40]. To address both issues, cascade reactions (where one reaction product or cofactor drives another reaction) may be used [32,41]. Such an approach allows the decoupling of signal generation from the enzymatic reaction being optimized. While such methods have been demonstrated for a small number of enzymes, they have yet to be extended to other classes of enzymes. A more universal strategy involves the use of optical absorption as the detection method. Significantly, absorbance-based methods can be used to probe a wide range of enzymatic reactions with native substrates. Unfortunately, absorbance signals are pathlength-dependent. Since optical pathlengths are normally below 100 μm within microfluidic systems [42,43], concentration detection limits can often be unacceptably high and analytical sensitivities low. An obvious route to improving both sensitivity and limit of detection is to use larger droplets. While this has some advantage, such structural changes limit sorting throughput, since larger droplets may break into smaller droplets when actuated. Additionally, photothermal detection methods offer an alternative way to extract absorbance signals in a pathlength-independent manner at droplet frequencies exceeding 10 kHz [44].

Until recently, only one example of an absorbance-activated droplet sorter (AADS) could be found in the literature [45•]. As shown in Figure 4a, this system incorporated a fiber assembly across the channel to measure droplet absorbance. Here, a 70-μm droplet diameter was chosen to provide for longer optical pathlengths and extended detector residence times. Using this AADS, the authors evolved a dehydrogenase enzyme from a half-a-million-member library. While interesting, the platform was only able to screen at rates of 100 droplets per second, which is over an order of magnitude lower than typical FADS sorting rates. To address this limitation, Richter et al. presented a 1-kHz AADS that leverages an acoustic

Figure 4


(a)
0 ms
3 ms
10 ms
13 ms
(b)
spacing
detection
focusing
deflection
sorting
LED
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micro-lens
detection
optic fibre
photodetector
SAW
IDT
sorted channel
waste channel
(c)
0 ms
1 ms
2 ms
3 ms
light fibers
sorting
junction
divider
Current Opinion in Biotechnology

Absorbance-activated cell sorting methods. **(a)** Images of the AADS platform developed by Gielen et al. in sorting 70-μm-diameter droplets at 100 Hz [45]. The image sequence shows a 'positive' droplet being directed toward the sorting channel by triggering embedded electrodes. **(b)** The acoustic AADS developed by Richter et al. leverages acoustic actuation to sort 70-μm-diameter droplets at 1 kHz [46]. **(c)** Left: Schematic of the sorting junction in the AADS platform developed by Medcalf et al. [47]. The two-layer chip features a 'bias oil' flow for better spacing of reinjected droplets and a divider at the sorting junction to minimize droplet fragmentation. Right: An image sequence shows a 'positive' droplet being directed toward the sorting channel when the sorting electrode is triggered.

sorting scheme (Figure 4b) [46]. Additionally, Medcalf et al. improved the performance of DEP-based AADS by matching the refractive index of the continuous and dispersed phases (Figure 4c) [47]. While providing for improvements in throughput, the use of a complex system of microlenses and optical fibers somewhat limits applicability. Accordingly, there still remains a need for AADS platforms that are simple to implement and can sort droplets at rates equivalent to FADS-based systems.

Conclusions

Directed evolution, which mimics the process of natural selection, is a potentially powerful enzyme engineering method. Enlarging the size of the involved mutant libraries allows for superior sampling of sequence space and thus improves the probability of finding high-performing variants. As discussed, a significant challenge encountered in directed evolution is access to an effective screening method. Traditional screening methods are unable to screen the large mutagenesis libraries needed for enzyme evolution. Although FACS-based methods provide for excellent sensitivity and throughput, the need to couple the target enzyme with a fluorescent protein for phenotyping poses obvious limitations. To address these limitations, droplet-based microfluidic systems have emerged as powerful tools for ultra-HTS within the directed evolution workflow. While they have shown significant utility in a number of applications, the dependence on fluorescence or absorbance detection has limited their use to select natural substrates and enzyme classes. Accordingly, novel technologies for droplet-based detection need to be developed to allow the efficient screening of a wide array of natural substrates. Promising developments in advanced detection methods, such as those based on mass spectrometry [48], hold potential to significantly enhance the utilization of droplet-based microfluidics in screening of large mutant libraries.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare no competing interests.

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- • of special interest
- •• of outstanding interest

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