Exploring sequence space in search of functional enzymes using microfluidic droplets

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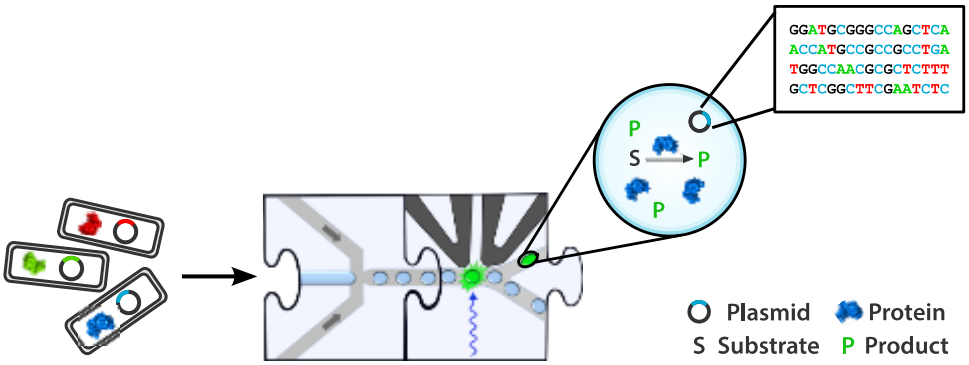
Graphical Abstract



Abstract

Screening of enzyme mutants in monodisperse picoliter compartments, generated at kilohertz speed in microfluidic devices, is coming of age. After a decade of proof-of-principle experiments, workflows have emerged that combine existing microfluidic modules to assay reaction progress quantitatively and yield improved enzymes. Recent examples of the screening of libraries of randomised proteins and from metagenomic sources suggest that this approach is not only faster and cheaper, but solves problems beyond the feasibility scope of current methodologies. The establishment of new assays in this format – so far covering hydrolases, aldolases, polymerases and dehydrogenases – will enable the exploration of sequence space for new catalysts of natural and non-natural chemical transformations.

Highlights

• Droplets made at kHz speed in microfluidic devices constitute a new assay format
• Ultrahigh-throughput (>10^7 per day) makes it possible to identify rare events
• Compartmentalisation uniquely allows evaluation of multiple turnover catalysts
• Detection modes include fluorescence, absorbance and fluorescence anisotropy
• A key current challenge is to establish a wider range of assays in droplets, to search for more types of functional proteins

Introduction

Exploring sequence-space to discover and evolve enzymes requires methods capable of assaying a very large number of mutants in the shortest possible amount of time. Ultrahigh-Throughput Screening (UHTS) in microfluidic droplets has emerged as a new tool with the potential to identify even very rare events from large libraries (with $\sim 10^6$-10^8 members) at low cost. The economic imperatives of downscaling assay volumes, increasing the speed of analysis, and automating the handling of library members are obvious drivers of this research and together will make combinatorial exploration of sequence space easier.

The basic workflow to screen enzyme libraries in microfluidic droplets is illustrated in Figure 1. A gene library is first transformed and expressed by a host organism. Single host cells are encapsulated together with a chosen substrate and a lysis agent into water-in-oil droplets. The cell lyses inside the droplet causing the release of the expressed enzyme. Crucially, the droplet boundary ensures retention of the linkage of genotype and phenotype. Depending on the activity of the enzyme variant, a certain amount of substrate is turned into product. A bespoke microfluidic device sorts the most active enzymes using high-voltage electric pulses to drag droplets towards a sorting channel. The genes encoding the selected catalysts can be recovered, followed by further rounds of evolution or, ultimately, sequencing, expression and characterisation.

Droplet sorters using fluorescence [1] or absorbance [2••] as a read-out are available. Selections are based on exceeding a chosen threshold of product formed. The operator can determine the stringency of the selection regime. Selection pressure can be exerted based on catalytic activity under freely chosen conditions, but indirectly also on protein stability or solubility. The precision of the optical readout allows excellent control over the selection decision and distinguishes this *in vitro* assay system from *in vivo* selections based on survival or growth (typically characterised by a narrow dynamic range) [3]. Continuous evolution scenarios that have recently become popular and proceed *via* largely non-adaptive, neutral pathways may be accommodated by future circular devices that embody repetitive evolution cycles to allow many rounds [4-6].

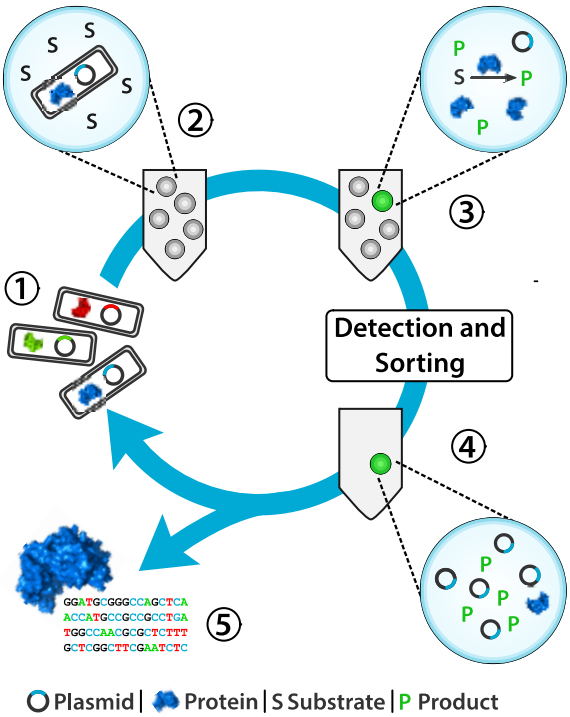


Figure 1. A typical workflow for the screening of an enzyme library using microfluidic droplets. **1:** A gene library is first transformed and expressed by a host organism. **2:** Individual cells are encapsulated into picoliter water-in-oil droplets together with a chosen substrate and lysis reagents. **3:** After cell lysis, the expressed enzymes are released and, if active, convert the substrate into product. **4:** If the fluorescence or absorbance of a droplet exceeds a set threshold it is physically sorted using a bespoke microfluidic device. **5:** After sorting, the genetic material can be recovered and the phenotypically superior mutants expressed for further characterization.

Microfluidic technology is inherently modular [7]. The basic microfluidic operation modules replicate standard laboratory operations such as mixing of two reagents and other subsequent handling steps on the microscale. The capability of the detection module determines which enzymes are amenable to experiments in droplets; the speed of the sorting module determines the throughput. The units of this microfluidic toolbox can be integrated to create specific workflows which typically include a combination of droplet generation, incubation, reagent addition followed by sorting. The assay formats that have been used recently are summarized in Table 1. A workflow for fluorescence-activated droplet sorting (FADS) was initially validated in enrichment experiments [1,8], in which the success of selection of positive hits over negatives is quantified. This sorting mode is analogous both in name and principle to fluorescence-activated cell sorting (FACS). FACS cannot be used to sort water-in-oil emulsion droplets, unless they are turned into 'double emulsions' (i.e. water-in-oil-in-water emulsions) [9] or immortalised as gel-shell beads [10]. Both of these formats allow the sorting of hits by FACS, which will enable users without device building experience to implement selections based on a commercial instrument rather than on a do-it-yourself chip.

After a decade dominated by proof-of-principle experiments in droplets, the utility of this new technology was shown in several library screening campaigns [2••,11-14••]. The first directed evolution campaign in microfluidic droplets was targeting horseradish peroxidase, using a coupled assay and lead to over 10-fold improved catalysts [15]. In addition to such highly active enzymes, even slow reactions can be screened, as exemplified for a sulfatase:

the thermodynamically most challenging biochemical reaction is amenable to the droplet format [13,16]. These experiments demonstrate the range of possible timescales with incubations between 5 minutes and 48 hours, suggesting that fast and slow reactions can be monitored.

A range of chemical transformations can be assayed in droplets

The range of chemistries for which droplet assay formats are available is increasing rapidly, with screening formats for several classes of enzymes being available.

(i) **Hydrolases.** Hydrolytic reactions in which water displaces a fluorescent leaving group are perhaps the reactions that can be assayed most simply, as the reaction product itself is detectable. Directed evolution of triesterases [10], phosphonate hydrolases [13] and enrichment experiments for glycosidases [17] or sulfatases [9] have been successful. Screening of natural gene repertoires for glycosidases [12], amylases [18] and triesterases [14••] have also been demonstrated.

(ii) **Aldolases.** Obexer *et al.* accomplished the directed evolution of a computationally designed aldolase which had previously been evolved in a microtiter-plate screening assay [19,20••]. Notably, in this study the enzyme evolved a strong preference for the (S)-enantiomer of the substrate. In a similar study, the same authors achieved (R)-selectivity by choosing an evolutionary trajectory which had not been accessible in the classic screening format [21].

(iii) **Polymerases.** The above studies monitored formation of a fluorescent product directly. In a different approach, Larsen *et al.* used a molecular beacon to evolve a non-natural threose nucleic acid (TNA) polymerase [22•]. This assay was performed in a double emulsion, which is compatible with commercial FACS machines circumventing the need for a custom-built FADS setup [9]. Using a molecular beacon and the multistep format, Ryckelynck *et al.* achieved the implementation of a full SELEX workflow in order to evolve a ribozyme [23].

(iv) **Proteases.** Although no library screening was achieved, protease activity has been successfully detected in droplets. A single-cell secretion assay in droplets employing multiple protease substrates and multi-colour analysis was implemented based on fluorescence quenchers [24]. Price and Paegel developed a droplet assay to detect inhibition of HIV-1 protease activity by UV-induced release of defined amounts pepstatin A from beads [25].

(v) **Amino-acid dehydrogenases.** To select improved L-phenylalanine dehydrogenases, a coupled reaction in which the reduction of the cofactor NAD^+ to NADH leads to the formation of a strongly absorbing water-soluble formazan salt has been implemented. This study established the first absorbance-activated droplet sorting (AADS) module, broadening the scope of droplet microfluidics to include a new class of assays [2••]. The coupling of cofactor reduction with production of the tetrazolium dye enabled a 25-fold signal amplification for product detection compared to direct detection of NADH. Similar formazan assays are available for dehydrogenases and reductases and should make these reactions amenable to screening in droplets [2••].

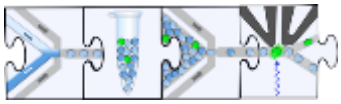
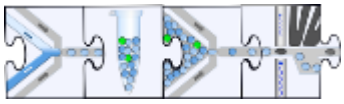
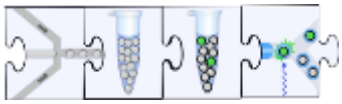
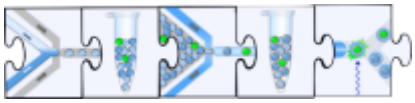
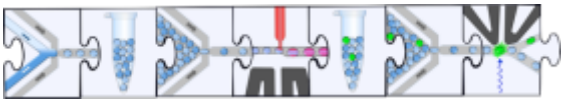
Workflow	Method	Enzyme Assay		Ref.
	FADS	Aldolase	Product Formation	[20••,21]
	FADS	Aldolase, Glycosidases, Phosphotriesterease, Phosphonate Hydrolase, Sulfatase	Product Formation	[13,14••, 17,18, 20••,21]
	AADS	Amino Acid Dehydrogenase	Product Formation	[2••]
	FACS	Sulfatase	Product Formation	[10]
	FACS	Sulfatase, TNA Polymerase	Molecular Beacon	[9,22•]
	FADS	X-motif Ribozyme	Molecular Beacon	[23]

Table 1. Workflows for enzyme assays that have recently been performed in droplet microfluidics and their respective enzymatic target reactions. The jigsaw pieces represent modules for unit operations which can be combined at will to miniaturise macroscopic workflows.

Just better or different - can ultrahigh-throughput screening meet unprecedented challenges?

In addition to experiments that can be carried out with conventional methodology, albeit at higher cost and on longer timescales, microdroplets will find use in experiments, in which the odds of success are so small that only the larger throughput of UHTS in droplets will deliver a successful outcome.

Many enzyme evolution campaigns are frustrated by reaching apparent local fitness plateaus, from where it seems hard to reach further improvements. The limited screening capacity exacerbates such situations, where departure from a fitness plateau may only be achieved by very few sequences. Obexer's aldolase evolution is a case in point: a stalled microtiter plate-based directed evolution experiment could be salvaged by testing orders of magnitude more enzyme members in the droplet format. Beating the odds in this way allowed a million-fold improvement of the catalytic rate constant (k_{cat}) of the previously evolved enzyme [20••]. Furthermore, while the microtiter plate format only allowed access to (R)-enantioselective enzymes, experiments in droplets were steered towards a different evolutionary trajectory by choice of suitable intermediate mutants and brought about enzymes with (S)-enantioselectivity [21].

While previous studies used FADS for directed evolution, a more recent application of FADS is the screening of metagenomic libraries. Metagenomic libraries consist of environmental DNA from microorganisms, whose biochemistry is otherwise inaccessible in the laboratory environment [26]. These libraries hold the promise of containing unknown enzymes, but the hit rates are typically very low - for example, one in 10^3-10^4 even for the ubiquitous lipase function. Hit rates for other reactions are not known, but likely much lower [27]; biotechnologically relevant reactions will often have no direct natural equivalent and thus be especially 'rare'. Despite the low chances, UHTS in droplets has been successfully used to screen a metagenomic library consisting of >1 million members for hydrolytic activities [14••]. To the best of our knowledge, this study represents the largest library screen ever carried out in functional metagenomics save for selections that relied on antibiotic resistance.

Fourteen new promiscuous enzymes were identified for these two thermodynamically challenging reactions. In colony screens, a typical throughput of 10^4 can be achieved, but would have had to be carried out 10-times to capture only one hit. In addition, the low activities of these enzymes would have made it difficult to reliably identify positive variant colonies on an agar plate. Sophisticated liquid handling systems may provide better sensitivity to detect weaker or lowly expressed catalyst, but such assay precision comes at a high price. Experiments that would take too much time with conventional means thus become feasible. Isolating catalysts with utility for industrial processes may often be the primary goal, but also fundamental insights are to be expected from this approach:

(i) *Enzyme annotation.* Currently sequence annotation is based on similarity – unless a sufficient number of enzymes is known to define a pattern, such annotation will be misleading. The functional tests possible in droplets provide data that complement and correct sequence analysis, allowing a better understanding of the existing vast amounts of sequence that would remain meaningless without further input. Indeed, in [14••] the 'native' activities of triesterase hits predicted by sequence similarity and bioinformatics could rarely be validated, while the droplet screening established a functional assignment. Functional metagenomics in droplets thus provides means for more comprehensive annotation based on experiments.

(ii) *New bridgeheads for better extrapolation of functional annotation.* Unpredictable reactions, i.e. those for which so few experimentally characterised examples exist that sequence prediction is not feasible, are only accessible experimentally. One example where sequence comparisons have never been successful, is the prediction of promiscuous reactions of enzymes [28] that can be understood as evolutionary starting points: after gene duplication they can be further refined by adaptive evolution and lead to refunctionalised proteins. The more starting points we know, the more new activities we can evolve. The activities identified in [14••] largely were such promiscuous activities.

(iii) *Simulating early evolution.* The chemical challenge of a metagenome mimics the encounter of 'virginal' environmental microbiota with external compounds that are toxic and have to be degraded (e.g. in the case of phosphate triesters, as in [14••], recapitulating the advent of these man-made chemicals). Finding promiscuous head start activities for their destruction suggests that an initial response already exists even in naïve environments [28,29]. These starting points for the emergence of resistance can be further elaborated by evolution. Such follow-up by directed evolution can of course also take place in droplets.

What can be detected in droplets?

The abovementioned fundamental challenges can only be addressed by a technology that combines ultrahigh throughput with sensitive detection. Typically, promiscuous starting activities in directed evolution are weak. Likewise, catalysts with often low activity need to be harvested in functional metagenomics: problems of cloning DNA fragments randomly without suitably positioned promotors [30] and the limitations of heterologous expression in *E. coli* [31] will undoubtedly reduce hit rates further. These considerations call for detection modes able to monitor low levels of product in individual droplets for a given enzyme reaction. The sensitivity of detection will determine the possible selection regimes (e.g. at saturating (k_{cat}), or subsaturating (k_{cat}/K_M) substrate concentrations). Table 2 summarizes the expected sensitivity of some selected readouts commonly employed in enzymology, including those listed in Figure 2.

The most sensitive readout is fluorescence: 4 nM fluorescein has been detected by Colin *et al.* [14••]. This corresponds to identification of single turnover events [32]. However, the need for fluorogenic substrates in UHTS is limiting the scope of substrates for which catalysts can be found. The size of the typically large fluorogenic moiety may also divert directed evolution away from the structure of the desired substrate. Fluorophores are also typically hydrophobic and often render substrates and products insoluble, with the consequence that solubility effects obscure the readout of enzymatic turnover. Assuming sorting at ~2 kHz, the current throughput achieved with fluorescence-triggered microfluidic sorters is ~$7*10^6$/hour [14••]. Single occupancy of cells in droplets can be achieved by Poisson distribution, which leaves a majority of droplets empty and reduces the effective throughput by ~5-fold [33].

Moving beyond the detection of fluorogenic substrates by employing additional detection modes is therefore attractive. New functional readouts will enable sorting decisions to be based on a wider range of molecular events. A sorting module based on absorbance avoids the complications associated with fluorescence to some extent, but comes at the price of a higher detection threshold: at least 10 µM of the product of a coupled reaction was required [2••]. Yet it was possible to carry out successful selections, as each droplet contained ca. 10^6 enzyme molecules (from one *E. coli* cell). Thus, each catalyst has to turn over only ~10^3-times to reach the detection threshold. Less active enzymes, however, may not reach the required product concentration. In terms of throughput, absorbance sorting is conducted at slower rates compared to fluorescence (up to 300 Hz), because of both the requirement for a higher electric field and the need to avoid splitting of droplets at the sorting junction.

A next challenge for microdroplet research is to further implement optical enzyme assays for additional reactions, paving the way for new applications. In fluorescence anisotropy (FA) the observed signal is brought about by a change in the tumbling rate of a fluorophore-modified portion of the substrate that is released after enzymatic cleavage. FA can be envisioned to be applied to proteases or sugar-degrading enzymes [34], where only a substrate-labelling step is necessary to make a substrate amenable to FA screening. The measurement of FA from single droplets has been achieved recently, albeit for a binding event. In this study, nanoliter droplets containing unique binder-ligand stoichiometries were

measured using a fluorescence anisotropy imaging setup. Dissociation constants down to the nanomolar range could be precisely quantified from high-resolution dose-response curves. The direct droplet-by-droplet assessment of FA will pave the way for the development of an FA droplet sorter [35•].

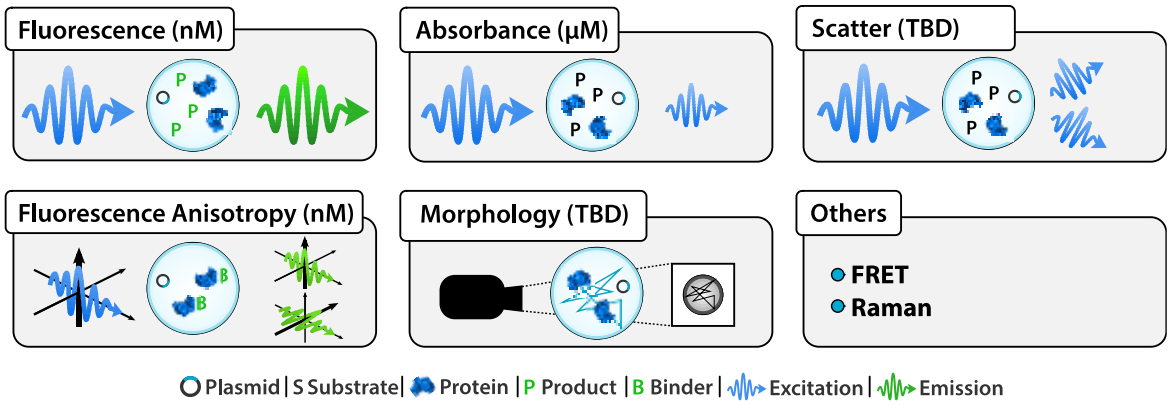


Figure 2. Established and envisioned readouts for droplet microfluidics. Assays based on fluorescence and absorbance have been applied to evolving enzymes. Future detection modes will include fluorescence based approaches (anisotropy, FRET, lifetime) and label-free approaches based on light scatter (including Raman scattering) or droplet morphology. The concentrations shown in brackets give an indication of the minimal analyte concentration that must be present to identify and sort droplets containing reaction product. The detection threshold for screening based on morphology or scatter has not been experimentally determined and is therefore left unspecified (TBD: to be determined).

Detection Method	Sensitivity	Integration time
Fluorescence Intensity	High	µs
Fluorescence Anisotropy		
Fluorescence Lifetime	nM	ms
Resonance Energy Transfer		
Fluorescence Microscopy		
Chemiluminescence		
Electrochemistry		
Surface Enhanced Raman Scattering	µM	s
Light Scattering		
Absorbance		
Brightfield Microscopy	mM	
	Low	µs (MHz), ms (kHz), s (Hz)

Table 2. Sensitivity of selected detection methods assuming standard instrumentation and required time for signal acquisition (e.g. in a droplet sorter). The inverse of the integration time indicates the upper limit of possible throughput for a given detection method, irrespective of limitations imposed by droplet dynamics.

The expected throughput for new analytical readouts like FA depends on the required integration time to obtain a sufficiently high signal-to-noise ratio as well as the ability to sort single droplets in synchrony. In the case of dielectrophoretic sorting, rates as high as 30 kHz have been achieved for 8 pL droplets [36]. Larger droplets (>100 pL) may be needed to push

up the detection limit, for instance in absorbance measurements, where the signal is proportional to the path length, leading to reduced sorting rates. Future derivatives of fluorescence-based detection such as fluorescence anisotropy and fluorescence lifetime will likely be implemented with similar sensitivity, although low lifetime detection speed in classical formats will probably result in lower sorting rates.

New physical readouts are needed to access new chemistries in droplet microfluidics

Ideally, droplet assays would reflect biotechnologically relevant substrates – few of which carry fluorophores or chromophores. If the principal rule of library screening ('*you get what you select for*') holds, such direct resemblance of bait substrate and downstream application is crucial for screening success. Several label-free assays can be envisioned: scatter and morphology detection in droplets have been demonstrated in single droplets in the context of cell growth, albeit not yet as a proxy for catalysis [37,38]. Surface-enhanced Raman scattering would be sensitive enough to be used at the single cell level as shown by studies using single phase devices [39,40]. Electrochemical detection could be achieved via local adsorption of droplets passing over electrodes although fouling of the electrodes is likely to be a challenge. Chemiluminescence only requires a single detector but will require precise timing between reaction initiation and detection as the signal is likely to decay within seconds. Microscopy will require fast computing and efficient algorithms to rapidly detect morphological elements of interest (e.g. presence of aggregates).

Conclusions

The evolution and discovery of catalysts in microdroplets is now well under way. The push towards more sensitive detection schemes will pave the way for the screening of weak, previously undetectable enzymes. In parallel, new detection modules will enable access to an increasing number of possible assays, moving away from classical fluorophores. Limitations of microdroplet screens still to be addressed include the minimization of leakage between droplet compartments [41,42], the efficiency of DNA recovery after sorting, or the number of substrates compatible with current droplet detection modes. Future commercial availability of droplet sorters will become a springboard for a vast research community lacking resources to implement self-made in-house droplet sorting. Notwithstanding such developments, microfluidic droplets are already a simple-to-use platform technology that will make exploration of biological diversity affordable for a much wider circle of scientists in academia and industry.

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