Enzyme Engineering in Biomimetic Compartments

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Highlights

• successful experiments demonstrate ultrahigh throughput enzyme evolution in microdroplets

• water-in-oil emulsion droplets compartmentalize genotype and phenotype

• microfluidic devices produce monodisperse droplets at rates >1 kHz for quantitative assays

• droplets are sorted on-chip and double emulsions and gel-shell beads by flow cytometry
Abstract

The success of a directed evolution approach to creating custom-made enzymes relies in no small part on screening as many clones as possible. The miniaturisation of assays into pico- to femtoliter compartments (emulsion droplets, vesicles or gel-shell beads) makes directed evolution campaigns practically more straightforward than current large scale industrial screening that requires liquid handling equipment and much manpower. Several recent experimental formats have established protocols to screen more than 10 million compartments per day, representing unprecedented throughput at low cost. This review introduces the emerging approaches towards making biomimetic man-made compartments that are poised to be adapted by a wider circle of researchers. In addition to cost and time saving, control of selection pressures and conditions, the quantitative readout that reports on every library members and the ability to develop strategies based on these data will increase the degrees of freedom in designing and testing strategies for directed evolution experiments.
Introduction

The cellular compartment plays a crucial role in evolution, by demarcating the boundary of one individual ‘evolutionary unit’ and distinguishing it from many other cells that are in competition during Darwinian evolution. Directed enzyme evolution \[1, 2\] tries to adapt Darwinian principles, to complement or go beyond rational design of catalysts. Instead of general cell survival in natural evolution, artificial evolution is directed towards improvement of a specific function \((e.g.\) thermostability or catalytic efficiency) under non-natural conditions. Avoidance of \textit{in vivo} survival selections with cells widens the scope of reactions that can be evolved beyond metabolically relevant processes and bypasses complications that arise when cells evade selection pressure in unexpected ways. Biomimetic compartments maintain the key feature of cells, the linkage of genotype and phenotype \[3\], and thereby allow relating the functional trait of a protein (such as catalytic activity) to the nucleic acid sequence encoding it (thus providing access to the identity of a library member after selection).

Figure 1 shows the archetype of one such reductionist compartment that contains the DNA and the catalytic protein expressed from it: a substrate is co-compartmentalised with the protein catalyst, multiple turnovers occur and selections can be based on product detection. When selected for a phenotype, the DNA coming with it reveals the identity of the catalyst. After the compartment is broken up, the DNA of the selected clones is recovered and analyzed - or fed into further rounds of randomization and selection. Just as cells survive environmental changes or competition in evolution, compartments that contain more reaction product will be selected and ‘survive’. However, the survival criterion is molecularly defined \((e.g.\) by product concentration – allowing direct selections for catalytic turnover), only one chosen protein \((\text{as opposed to a whole organism})\) is stringently put under selection pressure and the selection threshold is deliberately adjusted by the experimenter.

Such \textit{in vitro} compartments are easily made by dispersing an aqueous solution in an oil phase and can be stabilised by surfactant molecules. Large numbers of such droplets (~\(10^7\)-\(10^9\) in one experiment) can now be produced in a variety of formats (Table 1). Not only do larger numbers of experiments
become possible in highly parallelised fashion, but the cost per assay is also dramatically reduced by ~10⁶-fold [4], as they are carried out on the femto- to picoliter scale in droplets. Directed evolution campaigns carried out at higher throughput are more likely to be successful, making droplet technologies a promising tool for accelerated library selections. After more than a decade devoted to establishing protocols to generate a variety of compartments, this approach is now poised to become more widely used.

**Polydisperse Emulsion Compartments**

(i) **Water-in-oil emulsions.** The potential of emulsion compartments for molecular evolution was first explored by Tawfik and Griffiths [5]. Simple emulsion droplets can be prepared from an oil/water mixture using a stirring bar [5], an emulsifier [6] or simply by using custom-made tips with filters (~10 µm) and straightforward up-and-down pipetting [7] (Table 1). Such polydisperse compartments (i.e. with wide variety of sizes) are suitable for engineering DNA polymerases or DNA-modifying enzymes, as the “readout” is obtained from amplified DNA coding for improved variants (that are then gradually enriched over iterative selection rounds). For example, polymerases expressed in *E. coli* were evolved for higher thermostability or enhanced resistance to inhibitors [8], or to synthetize polymers containing unnatural nucleotides [9]. The development of compartmentalised partnered replication (CPR) broadened the target activities to tRNA synthetases (*e.g.* to genetically incorporate non-natural amino-acids) and may be used in the future for selecting other traits that can be linked to DNA polymerase expression [10].

(ii) **Double emulsions.** For enzymes that do not modify DNA, but process small molecules, other assays have to be implemented, *e.g.* based on an optical readout. Enzymes that turn over substrates generating a fluorescent product trapped inside the compartment can be analyzed by flow cytometry [11]. However, a second emulsification has to be brought about to generate water-in-oil-in-water double emulsions with overall aqueous character, so that they can be sorted by fluorescence-activated cell sorter (FACS) [11]. This set-up has been used to increase catalytic efficiencies of β-galactosidase [12], thiolactonases [13] or to improve catalytic detoxification of nerve agents by a phosphotriesterase [14]. However, as a result of two subsequent
emulsification steps these compartments are highly polydisperse in size. Since the droplet volume has a cubic dependence on its radius, already small variations in droplet size can result in large concentration differences between compartments, even though the same amount of product is produced by a compartmentalised catalyst. Under these circumstances it will be difficult to carry out quantitative assays (even when normalization is attempted by co-expression of a fluorescent protein [13]), especially when only incrementally improved mutants are contained in the library under selection, requiring precise distinction as the basis for the selection decision.

**Monodisperse Emulsion Compartments**

To enable quantitative measurements control over concentration measurements is essential. To this end microfluidic techniques were explored to generate compartments with precisely defined, uniform size. The notion of combining unit operations (*e.g.* mixing, diluting, adding, sorting) provided a framework for implementing classical laboratory sample handling steps *en miniature* and at ultrahigh throughput, and forms the basis for the design of complex workflows on-chip [15].

(i) *Water-in-oil emulsions.* Water-in-oil emulsion droplets (Table 1) are generated in poly(dimethylsiloxane) (PDMS) microfluidic chips [16, 17], in which an oil flow emulsifies an aqueous stream (Figure 2). Several chip designs (*e.g.* T-junction or flow focusing, reviewed in [18, 19]) have been described to generate compartments with minimised variations in size distribution. Microdroplets are generally produced at kHz frequencies but generation of femtolitre drops by jetting was demonstrated in the MHz range [20]. Emulsions are usually generated in oil but are incompatible with FACS instruments that employ aqueous sheath fluids. Therefore, to separate droplet populations according to product fluorescence on-chip devices for fluorescence-activated droplet sorting have been developed (Figure 2). Microdroplets are deflected by dielectrophoresis [21] or acoustic waves [22] and are generally operated at frequencies around ~2 kHz (even though accurate sorting has also been demonstrated at 40 kHz [23]).

(ii) *Double emulsions.* To limit drop-to-drop volume differences, monodisperse water-in-oil-in-water double emulsions can be produced in capillary devices
or PDMS chips [25] (Table 1). As above, such double emulsions are first produced as water-in-oil emulsion in hydrophobically modified channels, followed up by a second emulsification step in channels with hydrophilic surfaces. Hydrophobic or hydrophilic device coating promotes wetting with the carrier fluid and prevents droplets adherence to the channel walls. Two different channel coatings can be difficult to implement when double emulsions are produced in a single PDMS chip [25]. Using two separate chips (one hydrophobic-modified and a second hydrophilic-modified) instead facilitates the workflow and allows integration of additional operations (e.g. stopping enzymatic reactions by an off-chip heating step) [26].

(iii) Gel-Shell Beads (GSBs). Agarose microspheres were recently adapted to selections for catalysis: single bacteria were encapsulated with substrate in microdroplets and lysed to liberate the protein of interest. Upon lowering the temperature, additional droplet components, agarose and alginate, solidify creating agarose microspheres (∅ ~25 µm) in droplets and ‘immortalising’ the monoclonal nature of the original droplet. The deposition of layers of polyelectrolytes on the surface of these microspheres creates a size-selective shell (with permeability only for molecules <2 kDa [27]), so that reaction product can be captured together with enzyme and its encoding plasmid DNA, when GSBs are selected by flow cytometry. The semi-permeable boundary of GSBs presents advantages over emulsions: (i) it allows buffer exchange (e.g. for successive incompatible reactions) or addition of small molecules (e.g. triggering subsequent reactions), facilitating multistep systems such as cascade reactions; (ii) substrates and products can diffuse in and out, while enzymes are retained within the compartments making GSBs an alternative for enzyme immobilization that may replace covalent surface attachment of the catalyst by ‘caging’.

**Options for expression of the protein of interest**

Directed-evolution relies on iterative cycles of gene diversification, selection and gene recovery (Figure 2). In contrast to FACS analysis where only intact natural cells can be used for enzymatic reaction detections, protocols for man-
made droplets can be integrated with different protein expression systems (Figure 2).
Display of the protein of interest on yeast or bacteria provides a robust solution for delivery of protein and coding gene into droplets. Each cell expresses a different enzyme variant on their surface and single species are compartmentalised. Here the droplet boundary retains reaction product resulting from turnover by the displayed protein (Figure 2) and marks clones encoding successful catalysts, which would be impossible in bulk. Successful directed-evolution in microfluidic droplets containing a yeast-displayed enzyme was used to increase the activity of horseradish peroxidase (HRP) towards Amplex UltraRed by 10-fold [4]. Microfluidic droplets were also shown to be suitable for monitoring the activity of secreted enzymes (Figure 2), e.g. for metabolite consumption (xylose) or production (L-lactate) by yeasts [28] or the screening of microorganisms secreting α-amylase [29].

As the majority of proteins cannot be displayed on cells or secreted efficiently, screening of cytoplasmically expressed proteins as cell lysates is a widely used alternative. Lysis ensures that the enzyme encounters its substrate, even if it cannot cross the cell membranes. A workflow to miniaturise this process in a single-cell lysate protocol was implemented [30]. Single cells (each cell representing one library member) were compartmentalised with lysis reagents and substrate, so that after cell disintegration (that occurs within minutes after droplet formation) compartmentalised enzymatic reactions catalyzed by the protein produced by a single cell can be monitored and subsequently sorted. Catalysts can be incubated in a delay line (with several point measurements) [30] or – for slow reactions – after offline storage for several days [31]. This procedure was exemplified by the successful evolution of a promiscuous hydrolase [30] in two rounds of genetic diversification and selection which led to improve expression and activity by an order of magnitude each. The genotype-phenotype linkage provided by the droplet boundary was maintained until de-emulsification after selection. The use of a high copy plasmid enabled efficient “storage” genes coding for improved variants by directly transforming plasmids into highly competent E.coli (avoiding PCR amplification). If microcolonies are grown in droplets prior substrate addition (e.g. by picoinjection [32]) bacteria can be directly
recovered after de-emulsification [33]. However this protocol requires either substrates able to cross the cell membrane or that at least a fraction of the in the microcolony lyse within the droplet. In both cases the lack of control over concentration of catalysts encountering substrate may bias selections in directed-evolution experiments.

*In vitro* transcription/translation (Figure 2c) is particularly attractive when overexpression in cellular hosts is toxic, for incorporation of non-natural amino acids/cofactors or to avoid biological background [34]. Expression can be performed from single genes in droplets [5, 6, 35], typically yielding $>10^4$-$10^5$ molecules [6, 36]. *In vitro* expression can be boosted by generating multiple copies of the DNA template (~30,000 after emulsion PCR [37]), which also avoids large variations in expression efficiency from a unique DNA molecule [37]. Such complete *in vitro* systems require assemblies of multiple microfluidic operations, i.e. amplification, expression and sorting [37, 38].

**What can go wrong? – and ways to fix it**

*(i) Leakage.* The boundary of the droplet compartment is never ideally impermeable: hydrophobic molecules eventually leave the aqueous environment and partition into the surrounding oil phase. Even when mineral oils are replaced by fluorous oils - designed to act as a ‘third phase’ [39] with hydrophobic and lipophobic properties to prevent leaking of assay reagents [40, 41] - small molecules escape [19, 42, 43]. Leakage of e.g. product molecules compromises faithful measurement of turnover and makes hits harder or impossible to detect and separate from undesired clones. Chemical modification of fluorescent leaving groups without affecting their fluorescence properties can improve their retention, *e.g.* adding a charged sulfate group to a coumarin leaving group [44, 45]. Addition of BSA [43] or small molecules (e.g. β-cyclodextrin) to the aqueous phase, adjustment of pH, variation of the surfactant concentrations and choice of the fluorous carrier oil are parameters that can improve the retention of fluorophores by several orders of magnitude [46].

*(ii) Compatibility between the droplet formation protocol and the in vitro expression system.* Compatibility of fluorescent molecules or enzymatic reactions with various oil/surfactant conditions can rapidly be tested by image
analysis of polydisperse droplets produced in bulk. Better oil/surfactant compatibilities with fluorescent molecules show stronger size/fluorescence dependences [47].

Towards synthetic cells

Droplet compartments can be seen as minimalist versions of a natural cell, maintaining the elements necessary for the directed evolution of an encapsulated protein [48]. This analogy can be extended, when compartments can be made even more cell-like by replacing the emulsion interface with a lamellar phase lipid bilayer in liposomes. To make liposomes, water is first emulsified in oil (containing lipids as surfactants), resulting in inverted micelles. The micelles are added to a lipid-saturated oil phase, poured on top of an aqueous solution, forming a lipid monolayer at the interface. Liposomes are simply formed when inverted micelles pass through the interface by sedimentation [49]. Now proteins that require embedding in hydrophobic environment of cell membranes to be functional become amenable to engineering (receptors, membrane transporters, pore-forming proteins) [50, 51].

Beyond the representation of a genotype-phenotype linkage droplets would become fully ‘alive’, if the ability to self-replicate can be passed on to daughter droplets. A step towards such a system has been taken using Qβ replicase that can reproduce its own coding single-stranded RNA in compartments [52]. Fusion of droplets containing monoclonal replicated RNA with in vitro translation mixture droplets enabled translation before “droplet division” by filtering and transmission of genetic information. An increase of fitness (defined as replication ability) and the successive fixation of mutations along the evolutionary trajectory demonstrates that Darwinian features emerge spontaneously in synthetic compartments that mimic cell division [52].

Future directions

(i) Smaller is better. Microdroplet volumes are in the range of $10^{-12}$ – $10^{-15}$ L, so incredibly small amounts or reagents are needed. Micro- to millimolar concentrations of product are reached with few hundred thousands of
turnovers of enzyme, making this platform also extraordinarily sensitive, so that even weak activities can be detected. Miniaturisation to the pico- or femtoliter level in droplet compartments provides access to much larger numbers of experiments, with relatively simple means: up to \( \sim 10^9 \) when droplets are produced in bulk or \( \sim 10^7 \), when microfluidics are used to improve the precision for more quantitative studies. Even at the lower end, \( >10^3 \)-fold more single reactions can be carried out than by colony screening or robotic liquid handling technologies.

In the future this highly economical approach will become especially powerful, when connected to high-throughput sequencing to draw up sequence-function relationships on the basis of deep mutational scanning [53]. Classical mutational studies are usually limited to few residues around the active site, deep mutational scanning will enable a more complete survey, by identifying distant mutations that have dramatic effect on protein fitness (e.g. by stabilization [54]) and by characterizing ‘fitness landscapes’ [55]. Protocols are so far limited to display methods or assays based on survival [56, 57] but the types of target proteins could be expanded (i.e. to biocatalysts) by using compartmentalization formats. Finally droplet-on-demand formats in which enzymatic reactions are monitored as a function of substrate concentration or other parameters can be used to determine biophysical or steady-state kinetic parameters fully unsupervised much give much more rapid access to data that is cumbersomely obtained in current formats [69, 70].

(ii) *Entertaining parallel trajectories for evolution in high throughput systems.* Directed-evolution in the laboratory mimics natural evolution and must be based on similar concepts (selectionism and neutralism [58], recognised to have shaped present organisms). Even though there are many successful examples of directed-evolution [2] laboratory enzyme evolution remains highly constrained [59]. Epistasis (i.e. the effects of mutations’ interactions on protein functions) can restrict the number of mutational paths leading to fitter protein variants [60] and often resulting in ‘rugged’ fitness landscapes, where mutations are mutually interdependent and dependent on their context [55]. Some functionally silent or neutral mutations (i.e. permissive mutations - including stabilizing mutations - that are not selected) may be necessary to
allow the subsequent fixation of highly adaptive mutations that would otherwise have either neutral or deleterious effects [61]. Such permissive mutations introduce another dimension of historical contingency to the evolution of new functions.

In this context exploration of (i) multiple starting points (generated by neutral drift [62]) and (ii) multiple trajectories in directed enzyme evolution should avoid evolutionary dead-ends. Indeed if >10 millions of variants can be screened in droplets, there is no need to severely bottleneck evolution by choosing only one ‘winner’ in each round of directed-evolution. Instead of picking ‘the best’ clone in each round (as most directed evolution approaches do now), a larger variety of clones could be selected (Figure 3) based on more tolerant sorting criteria. Tolerant selections will come up with near neutral mutations [63] that may constitute entry points to the next round of variants generating a greater diversity of mutants. Now more starting points for alternative trajectories exist, possibly overcoming the limits of the evolutionary trajectory constrained to a single path. It remains to be seen whether – instead of a single evolutionary trajectory - several trajectories will then emerge (Figure 3), resulting in better coverage and exploration of sequence space.

(iii) Evolution based on multiple traits. It is now well established that enzymes are capable of performing chemically distinct reactions within the same active site (catalytic promiscuity) [64, 65]. The use of two (or more) fluorescent substrates in droplet compartments (with different emission wavelengths) would enable selection based on several activities at the same time. Now the experimenter can make a considered choice between generalists or specialist enzymes. Given the speculation that generalists are more evolvable (or more central to evolutionary trajectories) imposing such a criterion may change the course and outcome of an evolution campaign. Alternatively, highly specific enzymes could be selected based on the largest possible difference between two readouts to deliberately exclude side activities [66].

(iv) Biocatalyst discovery. Enzyme discovery feeds the pipeline of enzyme engineering by identifying novel starting points for directed evolution.
Functional metagenomics is a powerful way to identify novel enzymes without relying on existing homologs [67]. However, heterologous expression and the random fragmentation of genomic DNA (that compromises the position of regulatory elements and enzyme-encoding genes) make expression low and hits very rare (estimated as less than 1 in 10,000 variants) [68]. Overcoming the odds with highly sensitive high throughput microdroplets should make this format suitable for screening of metagenomic libraries of unprecedented size [31].

More types of assays needed. The diversity of tasks that can be fulfilled by droplet-based experiments has expanded during the past few years. However most assays require fluorescent products in directed-evolution experiments. Development of new detection systems will be key to target more activities. Adaptation of other optical detection modes will be useful: absorbance measurements can already be carried out in larger droplets ($\Phi \sim 50 \, \mu m$) [69, 70], but will be difficult with path lengths at the $\mu m$ range (corresponding to the droplet diameter) challenging the Beer-Lambert law. Fluorescence anisotropy [71], Small Angle X-ray Scattering (SAXS) [72], SERS [73], capillary electrophoresis [74] and mass spectrometry [75, 76] are examples of biophysical technics successfully miniaturised in microfluidic droplets. They provide potential additional readouts and enlarge the circle of reactions that can be miniaturised in droplets. Involvement of physicists, chemists and biologists will be required to develop droplet microfluidics further and establish it as a household tool for enzyme engineering.

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Figure 1. A biomimetic compartment maintaining the linkage of genotype and phenotype. An enzyme encoded by a gene is expressed (see Fig. 2 for the expression formats) and turnover of substrate to product is measured. As the compartment links genotype and phenotype (gene and reaction product, respectively), ‘hits’ are readily identified by quantification of product, can be selected and decoded after DNA recovery.
**Table 1** Polydisperse and monodisperse compartments used for protein engineering.

<table>
<thead>
<tr>
<th>Species</th>
<th>Polydisperse</th>
<th>Monodisperse</th>
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<tr>
<td></td>
<td>Bulk emulsion droplets</td>
<td>Water-in-oil emulsion droplets</td>
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<td></td>
<td>Bulk double emulsion droplets</td>
<td>Double emulsion droplets</td>
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<td></td>
<td>Liposomes</td>
<td>Agarose-alginate gel-shell beads</td>
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<td>Schematic representation</td>
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<td><img src="image2.png" alt="Diagram" /></td>
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<tr>
<td>Formation</td>
<td>Stirring bar [5, 77]; Vortex [47, 78]; extruder [12]; homogenizer [6, 79]; filter [7]</td>
<td>Microfluidic chip [80]; jetting [20, 81], on-demand [69-71]</td>
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<tr>
<td></td>
<td>Vortex [78]; extruder [12]; homogenizer [79]; filter [7]</td>
<td>Microfluidic chip [25, 26]</td>
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<td>Microfluidic chip [82]</td>
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<td>Droplet Diameter (μm)</td>
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<td>~4-200 μm</td>
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<td>~4 - 520 flL</td>
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<td>Droplet Volume</td>
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<td>~4-200 μm</td>
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<td>~ 4 – 520 fL</td>
<td>~20-130μm a</td>
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<td>Number of compartments b</td>
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<td>10^{9}-10^{6}</td>
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<td>10^{11}-10^7</td>
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<td>Time required for typical experiment</td>
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* a Diameter of the inner aqueous droplets
  b Emulsion/oil or bead/water mixture in a 1 mL test tube.
Figure 2: Experimental alternatives for directed-evolution cycles using compartmentalised formats.

(1) Directed-evolution experiments typically start from a diversified parent (made e.g. by error-prone PCR, DNA shuffling or other methods [83]) and the library based on this parent is subsequently screened. (2) Emulsions can be produced in bulk (with high variation in sizes) or “on-chip” using microfluidic devices for highly monodisperse droplets. (3) Emulsions are suitable for a variety of expression systems: in vitro transcription translation (IVTT); miniaturised cell lysate; cell surface display; secretion. (4) Amongst the assay readouts that have been successfully employed for droplet-based selections are (a) self-modification of the coding DNA and, as in (b) and (c), screening for optical properties of the reaction product. (b) Commercial flow cytometers can sort double emulsions based on fluorescence. (c) Sorting in custom-made chips reduces the capital expenditure (but requires specialised skills to operate). In the future sorting chips based on other optical detection techniques (such as absorbance or anisotropy measurements) will become
available, enlarging the arsenal of possible assays (and thus the number of enzymes that are amenable to directed evolution in this format).
Figure 3: Will ultra-high-throughput screening enable emergence of multiple evolutionary trajectories?

(1) In the majority of published examples only the one most improved variant is carried into the next round of evolution (for reasons of work economy), diversified and the resulting library screened again. This bottleneck leads to narrow genetic diversity in subsequent rounds. (2) The highly parallelised nature of a droplet-based ultra-high throughput experiment makes it possible to carry out more permissive selections, with little additional work compared to processing a single selected mutant: instead of just one, many variants can be selected (b'), diversified (a') and screened in subsequent rounds. Now much larger, but more diverse libraries need to be screened. The lower hit rate (as result of the more permissive initial selection) and the desire to cover that larger diversity require the use of ultra-high throughput of droplet-like compartments (~10^7 variants) to identify improved clones.

The lower panel compares two hypothetical scenarios as a result of the experimental protocols (1) or (2): (i) screening throughput and choice of one (or few) mutant(s) in each round limits exploration of sequence space to one evolutionary trajectory at a time; (ii) several evolutionary trajectories are
explored, resulting in better coverage of sequence space. A specific trajectory that ‘takes off’ only in later rounds is shown. Under stringent selections this trajectory would be cut short in round 1 (as the mutant did not stand out and others would be chosen in its stead), while this lineage persists under a more permissive selection regime. Such a scenario is relevant when the sequence context changes the contribution of individual residues (epistasis [60]) that could be brought about by near-neutral drifts [84, 85].

*Fitness may be any combination of the following properties that are assayed in each round of directed evolution: activity, specificity, thermostability, solvent stability.*
References


This paper describes the first directed evolution experiment carried out on-chip in monodisperse emulsion droplets by integrating several unit operation in one device.

Holliger's compartmentalized self-replication [8] is conceptually extended by linking a circuit for the evolution of another molecule to compartmentalized PCR amplification. In the future regulatory parts for synthetic biology (e.g. transcription factors, repressors or riboswitches) may be evolved in this way, if their function can be coupled to a DNA polymerase production readout.


Xylose-overconsuming *Saccharomyces cerevisiae* and L-lactate–producing *Escherichia coli* clones compartmentalised in droplets are enriched $10^3$-$10^4$-fold as examples of screening for extracellular metabolite levels. Here two steps, cell culture and activity assay, were integrated in one device.


The most widespread directed evolution format, cell lysate assays, has been miniaturised to the droplet format and the utility of this system is shown by successful directed evolution experiments for both higher activity and protein expression.


Membrane proteins are hard to work with and have rarely been subjected to directed evolution. This method makes such proteins amenable to high-throughput screening, demonstrated by selection for pore-forming activity of α-hemolysin.


The quantitative characterisation of selected enzymes is the necessary follow-up of any directed evolution campaign. Here (and in [69] kinetic assays with varying reagent concentrations are miniaturised into droplets and carried out in semi-automated fashion - to give data of excellent quality. The implementation of absorbance assays shows that this detection mode can also be used in droplets, paving the way for absorbance-based selections in the future.


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