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Review

(R)evolution-on-a-chip

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Billions of years of Darwinian evolution has led to the emergence of highly sophisticated and diverse life forms on Earth. Inspired by natural evolution, similar principles have been adopted in laboratory evolution for the fast optimization of genes and proteins for specific applications. In this review, we highlight state-of-the-art laboratory evolution strategies for protein engineering, with a special emphasis on *in vitro* strategies. We further describe how recent progress in microfluidic technology has allowed the generation and manipulation of artificial compartments for high-throughput laboratory evolution experiments. Expectations for the future are high: we foresee a revolution on-a-chip.

Highlights

High-throughput droplet generation using on-chip microfluidics allows for screening genetic expression libraries in monodisperse compartments in which a genotype to phenotype linkage is maintained.

Synthetic compartments are compatible with *in vitro* expression of genes, making them ideal containers for laboratory evolution.

Fluorescence-independent screening methods increase the scope of microfluidics-assisted *in vitro* compartmentalization (μIVC).

μIVC is progressing rapidly from pilot stage to industrial applications.

Laboratory evolution in artificial compartments

Laboratory evolution is based on consecutive cycles of differentiation and selection. To trace a desired phenotype back to its genetic origins, phenotype to genotype linkage is a key requirement. It can be achieved by physically linking the gene and gene-encoded product (e.g., DNA display [1], mRNA display [2], ribosome display [3]) or by compartmentalizing the gene and gene-encoded product within the same physical space. Unicellular microorganisms (e.g., bacteria or yeast [4,5]; or viral particles (phage-display by bacteriophages M13 and lambda [6,7])] can serve as *in vivo* microcompartments that separate each gene variant of the population pool. However, despite several successful studies [8–10], *in vivo* approaches can suffer from transformation bottlenecks, host genome mutations, and in the case of a fluorescent reporter, it needs to be cell-constrained [11]. Alternatively, compartmentalization can be obtained *in vitro* from artificial compartments [12]. For example, microtiter well-plates are commonly used for this purpose, but even in combination with sophisticated robotics systems, this system is low-throughput, typically allowing the screening of libraries consisting of 10^4–10^5 members [11,13]. Moreover, microtiter plates have already reached their physical barrier because assays with volumes less than 1 μl are problematic due to capillarity and evaporation [14].

A more promising solution is provided by ***in vitro* compartmentalization (IVC)** (see Glossary) [15]. In IVC, libraries of gene variants are generated, after which single gene copies are engulfed into (sub)picolitre-sized artificial compartments such as water-in-oil droplets. Gene expression inside artificial compartment is catalyzed by ***in vitro* transcription and translation (IVTT)** machinery [16]. A subset of enzyme variants successfully catalyzes the desired substrate-to-product conversion, after which enrichment for the product (and the associated gene variants) is performed [17]. The process of replication–variation–selection is repeated, until enzyme variants reaching the desired level of performance are obtained. The advantage of IVC is that billions of such droplets can be produced using simple and rather crude techniques allowing effective selection of huge libraries (10^8–10^{11}) [18]. A drawback of bulk droplet generation, however, is the inevitable variation in container size. This polydispersity issue complicates accurate quantification of enzymic activity, often resulting in unreliable screening outcomes [19]. Another challenge concerns the chemical synthesis required for generation of a physical link between the encoding gene and the enzyme substrate [20]. As a result, IVC applications have been mainly restricted to DNA-interacting proteins [12]. Recent progress in microfluidic technology (Box 1) has allowed the

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Box 1. Microfluidics

Since sample quantity is often a limiting resource in biological experiments, reducing sample volumes is highly favorable. Microfluidics enables handling small fluid volumes (in the microliter range or less) and makes use of the predictable behavior of fluids at low-enough flow rates within microchannels of desired geometries. Microfluidic technology has revolutionized biological analysis tremendously by downscaling the laboratory-based systems into, as they are popularly called, lab-on-a-chip devices. These miniaturized systems allow for a substantial reduction of required reagents as well as the analysis time. For example, a typical microtiter plate (96 wells) generally requires 100 µl of sample per well, whereas a typical water-in-oil droplet of 20 µm diameter produced during on-chip experiments corresponds to a volume of merely ~4 pl (i.e., more than seven orders of magnitude smaller).

How do fluids flowing at smaller length scales and low-enough flow velocities differ from fluid flows that we experience in daily life, such as stirring your morning black coffee? The main difference is a predictable laminar flow against an unpredictable turbulent flow, depending on whether the viscous forces or the inertial forces dominate the system. To determine which of these forces get an upper hand, one can calculate the Reynolds number (*Re*) for a system, which is defined as the ratio of inertial forces to viscous forces (Equation I).

$$Re = \frac{\rho v L}{\eta} \quad [I]$$

Here, ρ is the fluid density, υ is the velocity, L is the characteristic linear dimension of the system, and η is the dynamic viscosity. Typically, for Re <2000, viscous forces dominate, resulting in laminar flow [81] Thus, while mixing of an ink drop in water is obviously a turbulent phenomenon (Figure IA), two water streams meeting each other in a microfluidic device will simply flow parallel to each other (although they will mix in a diffusive manner further downstream) (Figure IB). Calculating *Re* for a typical microfluidic system makes this clearer. For a water stream flowing through a microfluidic channel ($\eta \approx 10^{-3}$ Pa.s, $\rho \approx 103$ kg/m^3, $\upsilon \approx 1$ mm/s, $L \approx 100$ µm), $Re \approx 0.1$, confirming a laminar flow. The laminar nature of the fluid flow, combined with competing effects of the deformation of the interface by a local shear stress and the resistance to this deformation by interfacial forces, leads to production of µIVC containers (single/double emulsions, liposomes, etc.) in a highly controlled manner [84].

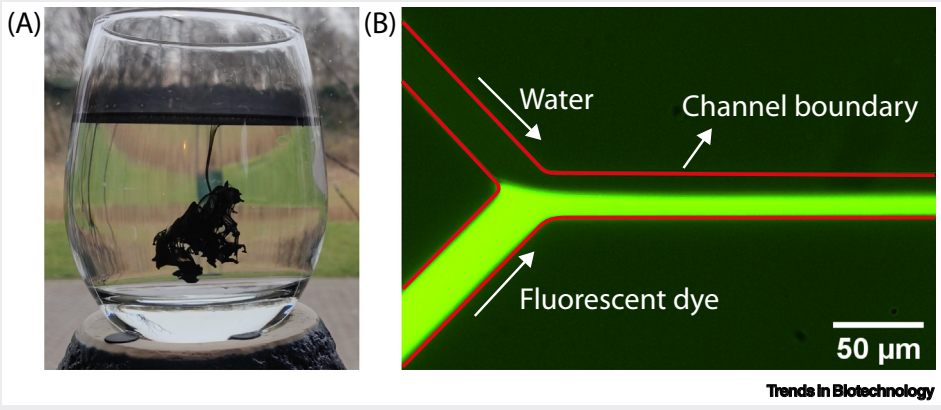


Figure I. Turbulence and laminar flow. (A) An ink drop mixing in water in a turbulent manner. (B) Demonstration of laminar flow in a lab-on-a- chip device by co-flowing two streams of water (with and without a fluorescent dye) parallel to each other without mixing.

production of highly monodisperse droplets, widely extending the application scope of IVC and giving birth to **microfluidics-assisted *in vitro* compartmentalization (µIVC)** [21].

In this review, we focus on µIVC as a platform to perform laboratory evolution. As µIVC has started moving from simple **enrichment assays** to actual directed evolution campaigns, we consider reviewing this approach promptly. The structure of our review follows the structure of a classic laboratory evolution scheme (Figure 1, Key figure): gene differentiation, gene expression, and selection and high-throughput screening strategies. However, we are not addressing the subject of gene differentiation as it has been already extensively covered [11]. Additionally,

Glossary

Cell-surface display: allows peptides and proteins to be displayed on the surface of microbial cells by fusing them with membrane-anchoring protein or motifs.
Dielectrophoresis: a physical phenomenon that refers to the movement of dielectric particles as a result of the force they experience when placed in a nonuniform electric field.
Double emulsions: microscopic confinements obtained by emulsifying a single emulsion. An example is dispersion of water-in-oil droplets in an aqueous phase to obtain water-in-oil-in-water droplets (see: Single emulsions).
Enrichment assay: usually two variants of genotypes (active/inactive) are mixed into known ratios and, after a screening or a selection procedure, the enrichment of the desired genotype is quantified.
***In vitro* compartmentalization (IVC):** encapsulation of single gene copies into artificial compartments. The gene-of-interest is delivered either as linear/circular DNA, or as part of a cellular genome. The term is usually used to describe compartmentalization into water-in-oil droplets, generated by bulk techniques (see: µIVC).
***In vitro*transcription and translation (IVTT):** cell-free transcription and translation of genes by crude cell extracts or reconstituted IVTT components.
Liposomes: aqueous microscopic confinements whose boundary is composed of a semi-permeable, self-assembled lipid bilayer. Liposomes can be crudely compared with double emulsions, where a continuous lipid bilayer, instead of an oil phase, forms the interface between the inner and the outer aqueous phase.
Microfluidics-assisted *in vitro* compartmentalization (µIVC): encapsulation of single gene copies into artificial compartments generated using microfluidic technology (see: IVC).
Mimic library: also referred to as a model library, it contains specific gene variants of known activity mixed into known ratios in order to test the enrichment capabilities of a screening or a selection approach (see: Enrichment assay).
On-chip microfluidics: a versatile technology developed for manipulating small fluid volumes (in the µl range or less) within microchannels, ranging from

noteworthy examples of μIVC campaigns are discussed throughout the review. Finally, we present a perspective on future developments in the field.

Microcompartments for laboratory evolution

Evolution of genes in a laboratory setting strictly requires a genotype to phenotype association. This is normally achieved by ensuring a single gene variant per compartment (Box 2). Although cells function as natural compartments, their use is restricted by transformation bottlenecks, interference of host-cell proteins with target protein, and toxic product-encoding genes. In the following section we discuss the most common artificial compartments currently used in μIVC: **single emulsions**, **double emulsions**, and **liposomes**, how they are generated, their advantages and disadvantages, and their applicability.

Single emulsions: water-in-oil confinements

Emulsion-based compartments can serve as an excellent alternative to cells, of which, single emulsions (hereby simply referred as emulsions) are most frequently used. Depending on the volume ratio of water to oil and on the surfactant type, either oil-in-water or water-in-oil emulsions are formed, with the droplet size ranging from a few nanometers to hundreds of micrometers [22]. Since oil-in-water emulsions are unsuitable to be used as compartments for the described biochemical reactions, emulsions in the context of laboratory evolution basically refer to water-in-oil emulsions (Table 1). To prevent emulsion droplets from fusing, surfactants are routinely added to the oil and sometimes also to the aqueous phase [21]. Emulsions were initially generated in large numbers using straightforward bulk approaches(i.e., by mixing aqueous buffer with oil in presence of surfactants). However, this poorly controlled process generally resulted in highly polydisperse emulsion populations, seriously affecting the uniform encapsulation of reagents. Despite the polydispersity, numerous evolutionary campaigns have been successful [23–26]. Nonetheless, polydispersity tends to hamper downstream screening, as small volumetric deviations can substantially affect the concentration of enzyme-derived products [27]. Incremental evolution campaigns will significantly benefit from uniform volumes as well as constant reagent concentrations and stoichiometry, highlighting the importance of monodisperse emulsions.

Highly monodisperse water-in-oil droplets are generated using **on-chip microfluidics**. For example, droplets 45 μm in diameter, have been produced at a rate of 1–10 kHz [27]. Production rate can be substantially increased by operating multiple parallel production channels in a single microfluidic device, or by using a serial droplet-splitting technique, subsequently resulting in smaller droplets. Frequency up to 1.3 MHz was achieved by running multiple microfluidic devices in parallel [28]. Mechanical splitting of large droplets into smaller vesicles by introducing splitter array can also increase the throughput, preferably >1000 droplets/hour [29]. The high throughput nature, the excellent encapsulation, and the feasibility of subsequent gene expression within emulsions make this approach a highly suitable platform for laboratory evolution. Yet, one of the major limitations of these emulsions is their incompatibility for downstream screening using fluorescence-assisted cell sorting (FACS). This is due to the external oil phase, which is not suitable for commercial cell-sorting machines.

Double emulsions: water-in-oil-in-water confinements

This limitation of single emulsions can be resolved by using double emulsions. A typically used double emulsion for biological applications is a surfactant-stabilized water-in-oil-in-water emulsion, that is, an aqueous droplet containing the gene of interest along with the IVTT machinery, surrounded by an oil shell, and dispersed in the outer aqueous phase (Table 1). The presence of this external aqueous phase makes double emulsions compatible with commercially available

sub-100 nm to hundreds of μm in diameter. A typical on-chip microfluidic device comprises of a network of mutually connected user-defined channels, through which multiple fluids are flown in a highly controlled manner.

Poisson distribution: a probability distribution that reflects the probability of a given number of events occurring within a specified time period, provided that the events occur independently and at a constant mean rate. In this review, Poisson distribution is referred to in context of encapsulating a single gene per confinement.

Sequential evolution of ligands by exponential enrichment (SELEX): a molecular biology technique for generating oligonucleotides [single-stranded DNA (ssDNA) or RNA] that tightly bind to a specific molecular target (usually a protein).

Single emulsions: microscopic confinements obtained by dispersing one fluid phase in another immiscible fluid phase. An example is an aqueous phase dispersed in an oil phase to obtain water-in-oil droplets (see: Double emulsions).

Key figure

A starter gene is randomly differentiated into a library of variants

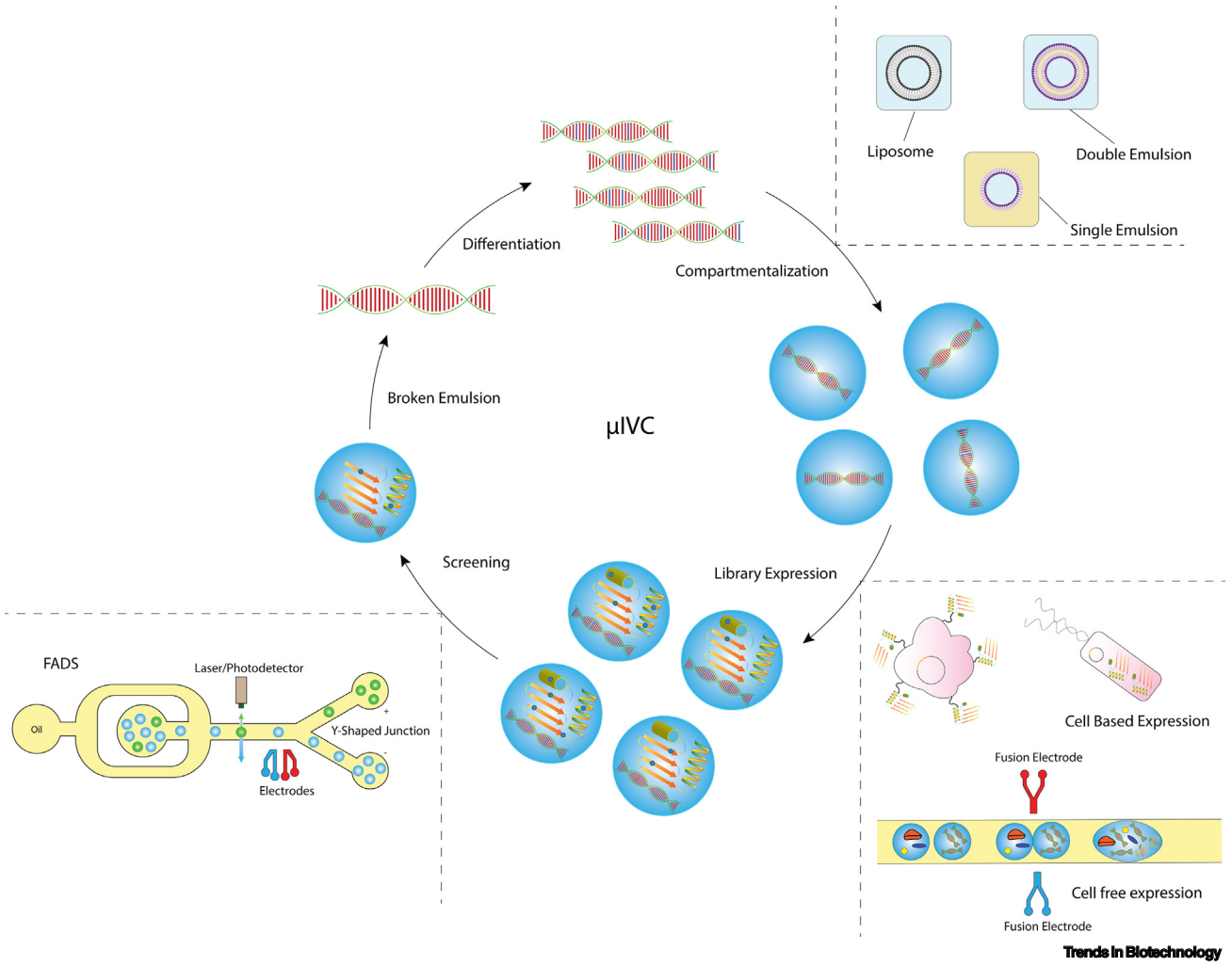


Figure 1. A single gene variant is compartmentalized into artificial compartments (single/double emulsions, liposomes, etc.). Gene variants are cell-based or cell-free expressed. Clonal amplification of gene variants usually precedes cell-free expression and *in vitro* transcription and translation (IVTT) components are provided by droplet fusion. High-throughput screening of droplets is based on fluorescence-activated droplet sorting (FADS). Active gene variants that are selected by the screening process serve as the starter genes for the next round of laboratory evolution. Abbreviation: μIVC, microfluidics-assisted *in vitro* compartmentalization.

droplet-sorting techniques like FACS. Double emulsions can be made in bulk by further emulsifying water-in-oil single emulsions, with the same key disadvantage of polydispersity. This limitation can be overcome using a microfluidic approach to obtain monodisperse double emulsions with efficient encapsulation. While double emulsions have their own merit owing to the compatibility with downstream sorting processes, the on-chip production rate is relatively low but good enough to be used for laboratory evolution campaigns [30]. Indeed, double emulsions produced on-chip have has been successfully applied to screen and evolve soluble proteins [31,32].

Box 2. Poisson distributions: genotype to phenotype linkage

Independently of the compartment type, the genotype to phenotype link is essential for a successful laboratory evolution scheme. It is achieved by compartmentalizing a single gene variant per compartment. The gene variant can be encoded by 'naked' DNA templates or by single cells transformed with a gene library. In both cases, encapsulation of a gene variant in an artificial compartment is a stochastic process and obeys **Poisson distribution**, where the probability of finding a droplet with k encoding templates follows Equation I, with λ being the average number of encoding templates per droplet [48,55].

$$P(k,\lambda) = \frac{e^{-\lambda}}{k!}\lambda^{k} \quad [I]$$

Thus, in order to ensure the monoclonal nature of each compartment and prevent the simultaneous sorting of an active variant with an inactive variant, most of the compartments are better left empty. For example, at λ = 0.3, 74% of the droplets will be left empty, 22% will have one template, and only 3% will have two [85]. Once inside the compartment, cells or an IVTT system will drive gene expression.

Liposomes: semi-permeable membranous confinements

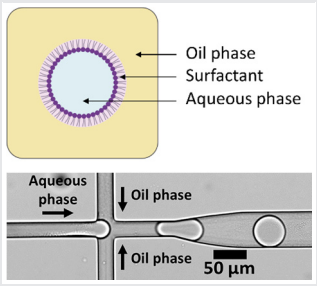
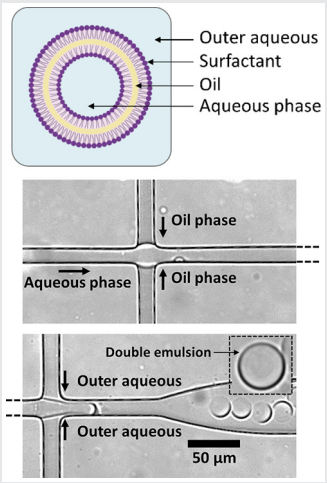
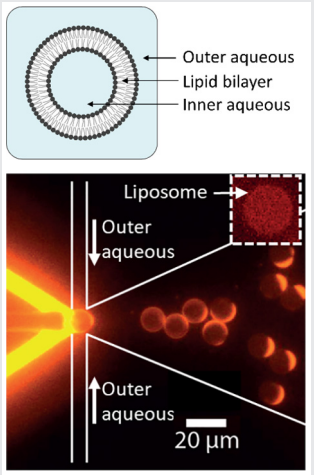
An alternative to emulsions is liposomes, whose boundary is composed of a continuous lipid bilayer. The lipid bilayer in turn is composed of phospholipids, which are surfactant-like amphiphilic molecules with a hydrophilic head and two hydrophobic chains, allowing them to self-assemble in aqueous environments to form such 3D containers (Table 1). Liposomes have gained tremendous interest in the context of building synthetic cells [33,34] and they are suitable compartment candidates, especially for membrane proteins. Their applicability has already been demonstrated by engineering an α-hemolysin variant, a membrane-bound toxin from *Staphylococcus aureus*, with enhanced pore-forming activity [35].

Liposomes can be produced in various sizes (tens of nm to hundreds of μm in diameter) and different lamellarities [unilamellar or multilamellar,having a single or multiple lipid bilayer(s), respectively]. For IVC applications, unilamellar liposomes of a few micrometers in diameter, also known as giant unilamellar vesicles (GUVs), are preferred partly due to the detection limit of FACS measurements. GUVs can be generated via bulk techniques such as thin film hydration, rehydration of freeze-dried empty liposome (FDEL), and inverted emulsion transfer [36]. However, similar to emulsions, GUVs produced by bulk techniques suffer from polydispersity and an additional size-based sorting step in FACS is required. Two other drawbacks originating from bulk liposome production are the formation of multiple compartments [liposome(s) within a liposome] and multilamellarity. Multicompartment liposomes tend to trap components between different subcompartments, causing inaccurate evaluation of enzymic activity. They also tend to yield low protein expression, which is undesirable for laboratory evolution [37]. Fortunately, several effective on-chip production techniques have been developed over the years, which could prove to be very suitable for evolution experiments. Octanol-assisted liposome assembly is a process akin to bubble-blowing, where the initially produced double emulsions give rise to unilamellar liposomes within minutes [38,39]. A similar technique was developed using a glass capillary-based approach and cell-free, GFP expression was carried out inside the liposomes [40]. Spatz and coworkers recently presented another approach, starting with copolymer-stabilized droplets to generate liposomes, termed droplet-stabilized GUVs [41]. When combined with a droplet splitting technique, an impressive production rate of $>10^6$ GUVs/min was reported [42]. Overall, on-chip GUV production is a promising approach, especially in terms of sample monodispersity and encapsulation efficiency.

Coupling microfluidics to protein expression

Cell-based expression is the most straightforward way to achieve protein production. To make use of this technique in a microfluidic platform, single microbial cells are encapsulated in water-in-oil droplets where substrate-to-product catalysis takes place. However, as protein expression occurs intracellularly and the substrate for the enzyme is provided extracellularly, enzyme–

Table 1. Synthetic microcompartments used for laboratory evolution

Compartment	Schematic and example of on-chip production process[a]	Production rate	Advantages	Limitations
Single emulsions		Up to 10 kHz (>10^7 droplets/h) [28]	• Extremely high production rate • Very robust technique	• Incompatible with FACS • Requires sophisticated techniques to modify the inner content • Incompatible with oil-soluble and membrane proteins
Double emulsions		Up to 1 kHz (>10^6 droplets/h) [31]	• Compatible with FACS • Robust technique	• Relatively low production rate • Incompatible with oil-soluble and membrane proteins • Relatively more complicated set up and generally requires a two-step production process.
Liposomes		Not applicable since only bulk techniques have been used so far.	• Biocompatible membrane • Compatible with membrane-interacting proteins • Compatible with FACS • Semi-permeable interface allows small molecule diffusion	• Much lower production rate • Relatively less robust

[a]Bright-field and fluorescence images captured at the Laboratory of Physical Chemistry and Soft Matter, Wageningen University and Research.

substrate interaction must be ensured for a successful activity assay. Hydrophobic substrates can freely diffuse through cell membrane and interact with target enzyme. However, diffusing substrates are not available for every target enzyme. **Cell-surface display**, enzyme secretion, and cell lysis are common alternatives to achieve enzyme–substrate interaction.

Surface display was applied by Agresti and colleagues in the first demonstration of a directed evolution campaign using μIVC [43]. Yeast cells were transformed with a randomly generated variant of horseradish peroxidase (HRP) that were fused with the membrane anchoring peptide (Aga2) which allowed surface display. Each yeast cell displayed ~10 000 copies of a single HRP mutant in its surface [43]. Single cells were compartmentalized into emulsions along with a nonfluorescent substrate (Figure 2A) and active HRP variants converted the substrate to a fluorescent product, allowing bright droplet sorting. Surface display was recently shown with *Escherichia coli* cells too [45]. By exploiting the autotransporter protein system, the target enzyme takes the place of the passenger and it is surface displayed by fusion with the β-barrel through the

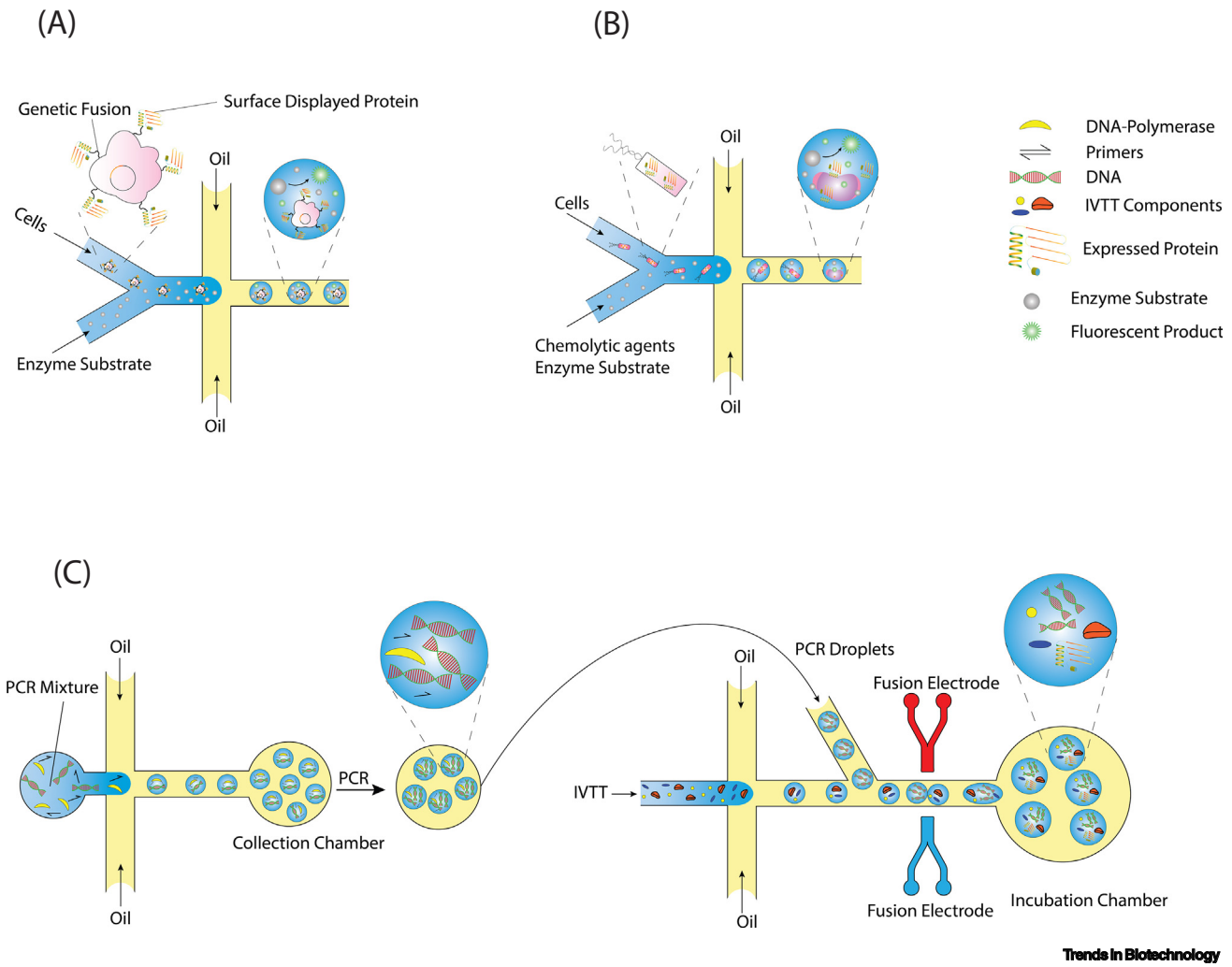


Figure 2. Protein expression and enzyme interaction in on-chip compartments. (A) The target protein is programmed to be displayed on the microbial cell surface through a genetic fusion with a membrane-anchoring peptide. Each cell displays thousands of copies of the same protein variant. Single cells are compartmentalized into water-in-oil droplets along with appropriate enzyme substrate. Upon encapsulation, active protein variants catalyze the conversion of the substrate to its fluorescent product. (B) Target protein expression happens intracellularly. Single cells are compartmentalized into water-in-oil droplets along with cellulolytic agents and the enzyme substrate. After encapsulation, cells are lysed and their proteinaceous content is released inside the compartment. Enzymes encounter the substrate and active variants catalyze its conversion to a fluorescent product. (C) For *in vitro* gene expression inside artificial compartments, an initial DNA amplification step may boost the expression of each variant gene. To do so, single DNA templates are encapsulated along with a PCR mixture (top). After DNA amplification, the DNA-containing droplets are transferred to a new microfluidic chip (bottom), where it is combined with compartments that contain *in vitro* transcription and translation (IVTT) components for gene expression. The two types of droplets are fused through electrocoalescence, after which expression of the PCR-amplified DNA templates results in a detectable phenotype.

linker domain. The advantage of using surface display is that after droplet sorting, live cells can be retrieved and recultured [43,45].

Yarrowia lipolytica is a non-model yeast species that made a case in μIVC for its biotechnological significance and secretion abilities [44]. Efficient secretion of heterologous proteins in *Y. lipolytica* is directed by signal sequences fused upstream of the protein of interest [46]. To screen for thermostable mutants of endo-β-1,4-xylanase, *Y. lipolytica* cells were transformed with two randomly generated xylanase gene libraries generated with high and low mutation rates [44]. Eight clones were found to have up to tenfold higher residual activity than the wild type, following a heat shock at 90°C [44]. However, small library sizes (<1000 clones) could indicate a transformation bottleneck.

When enzyme substrates can freely diffuse through cell membrane, enzyme–substrate interaction is ensured without any additional prerequisites. Amplex UltraRed (AUR) was used as freely diffused substrate in an enrichment assay for CotA laccase, which is a periplasmic enzyme from *Bacillus subtilis* [47]. Single *E. coli* cells expressing either CotA laccase or a frameshifted ΔCotA variant were compartmentalized into single emulsion [47]. AUR was later picoinjected (Box 3) into the droplets, allowing fluorescence-based droplet sorting, reaching enrichment up to 437-fold [47]. Like hydrophobic substrates, secondary metabolites can also diffuse through cell membrane. In another enrichment test, *E. coli* cells producing either D-lactate or L-lactate were mixed in a **mimic library** along with reagents for L-lactate reaction assay [48]. Enrichment for L-lactate-producing cells reached up to 5800-fold [48].

Even though living cells efficiently drive protein expression, cellular barriers can obstruct enzyme–substrate interactions. To overcome this issue, compartmentalization can be followed by cell lysis. This allows the proteinaceous content of the cell to be released into the synthetic compartment, greatly facilitating the interaction of the target enzyme with its substrate. In case of evolution of a thermostable enzyme, *E. coli* cells can be lysed by exposure to near-boiling temperatures. Heat lysis was successfully applied for engineering and enriching xeno nucleic acid-incorporating DNA polymerases [32,49]. However, as high temperatures tend to compromise the functionality of most mesophilic enzymes, chemical lysis is the preferred method. Chemolytic agents and *E. coli* cells are mixed on-chip just before they are compartmentalized into droplets, to ensure cell lysis after encapsulation (Figure 2B). This approach has been successfully tested by enrichment assays for arylsulfatase enzymes reaching 2500-fold enrichment [31]. Chemical lysis has been successfully applied for the laboratory evolution of an esterase with altered enantioselectivity [50], for remodeling the active site of cyclohexylamine oxidase [51], and for the laboratory evolution of an artificial aldolase [52].

Box 3. Compartment content modification

Droplet modification can be defined as the process of manipulation of droplet content by merging two or more droplets. In droplet-based laboratory evolution, it is a common practice to first express the gene of interest in the host droplet and then add the substrate required for enzyme catalysis, which is present in another droplet. This is often achieved by fusing the substrate-containing droplet to the host droplet. The governing principle to induce droplet fusion is to temporarily destabilize the oil–water interface. Destabilization through electrocoalescence(i.e., electric field-induced droplet merging) [86] has gained most ground in the μIVC field (Figure IA).

Building upon electrocoalescence, the idea of picoinjection was developed. A second microfluidic device allows reagent delivery to existing droplets by applying an electric field as they pass a pressurized channel containing reagents (Figure IB) [87]. With this design, fluid delivery was attained at high precision, up to sub-picoliter volumes. Picoinjection-based droplet modification has tremendously helped droplet modification in laboratory evolution experiments. Wang and colleagues utilized picoinjection to deliver AUR assay reagents with enzymes (oxidase and HRP) into pre-existing droplets to detect metabolites of interest [48]. Subsequently, new versions of picoinjectors have been developed, eliminating the requirement for electrodes by using dissolved electrolytes in solution [88].

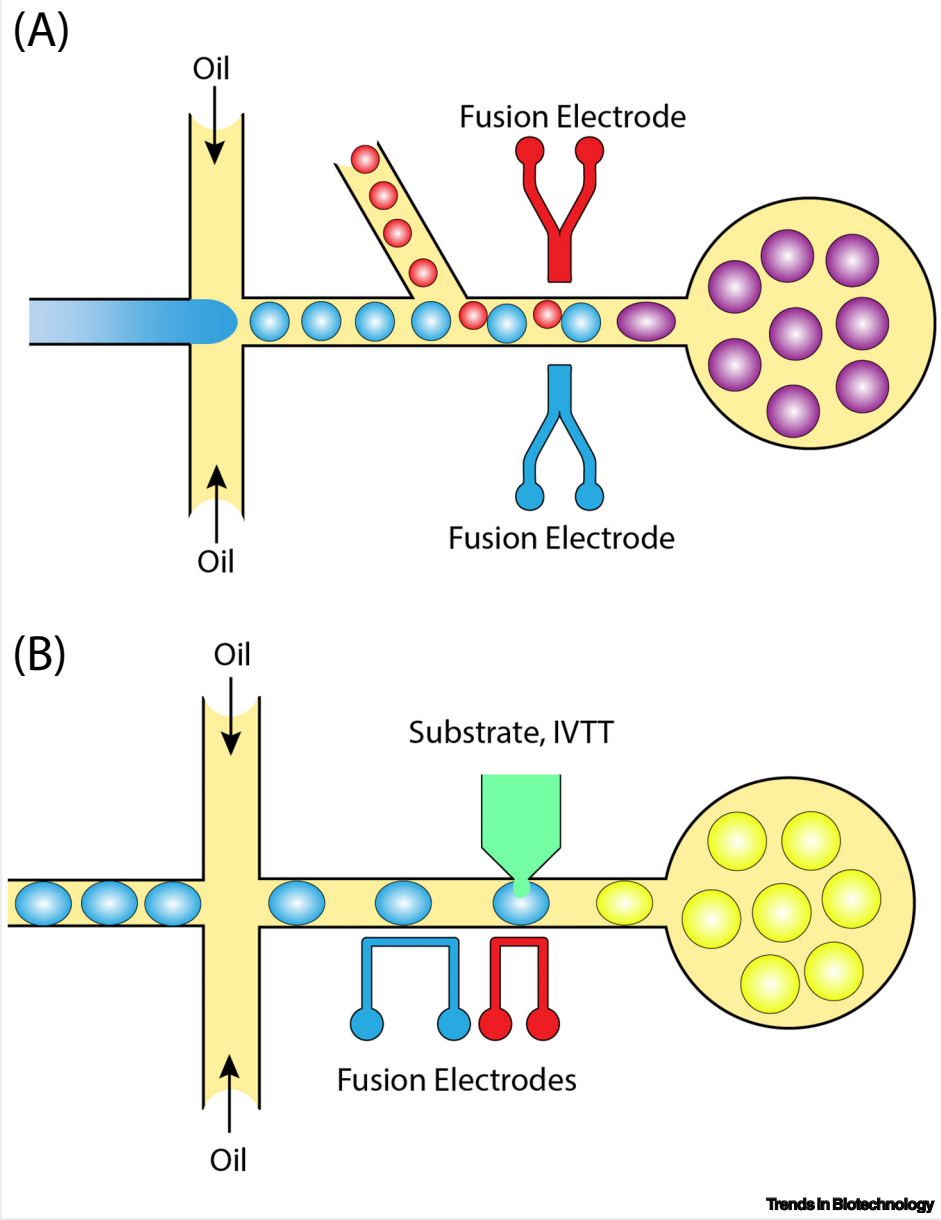


Figure I. Droplet content modification. (A) In droplet fusion, droplets are fused by temporal destabilization through electric current application. (B) In picoinjection, reagent delivery [substrate, *in vitro* transcription and translation (IVTT)] is applied through an additional microfluidic channel through temporal destabilization by electric current.

Cell-free expression

While cell-based protein production is certainly a valuable approach to perform μIVC, it does not allow for the expression of toxic genes or the introduction of non-natural amino acids, and the biological background can impair screening accuracy. An interesting alternative is cell-free expression by IVTT. IVTT consists of either purified transcription/translation components expressed in *E. coli* (protein synthesis using recombinant elements or PURE) [53], or by cell

lysates provided with additional supplements [54]. As with cells, cell-free expression is obtained by single gene copies engulfed into artificial compartments [55]. Importantly, *in vitro* expression of single genes should result in a detectable phenotype. For example, the expression of GFP has been detected inside droplets by single DNA templates [56]. Moreover, when penicillin-acylase activity was coupled to the expression of a GFP-reporter, it was also detectable from single gene copies encoding for the acylase enzyme and the GFP [57]. Such fluorescence-based detection of enzymatic activity encoded by single DNA templates is, however, not always possible. For example, β-galactosidase and FeFe hydrogenase activity could not be detected when encoded by single DNA molecules [55,58]. To enhance *in vitro* expression in such cases, a clonal population of a gene inside artificial compartments can be achieved by PCR amplification or rolling cycle amplification [55,58]. Nonetheless, DNA amplification (replication) and cell-free expression are incompatible processes because the sensitive IVTT components will be inactivated at the elevated PCR temperatures and rolling cycle amplification is inhibited by IVTT components Therefore, DNA amplification is first performed and components for the cell-free expression and the screening assay are added by droplet fusion (Figure 2C) [59] or picoinjection [60] (Box 3).

A completed and cell-free, protein-directed evolution campaign had long eluded us, as only enrichment assays were making their case [57,59]. However, recently a subtilisin-like protease was evolved to a fivefold more active variant, completely cell-free [60]. Single plasmids were compartmentalized into single emulsion and they were clonally amplified through rolling cycle amplification [60]. IVTT reagent and enzyme substrate were provided by two consecutive steps of picoinjection [60]. It seems that high controllability over the consecutive steps (clonal amplification, protein expression, enzyme catalysis) is the key for a cell-free directed evolution campaign by μIVC.

Apart from optimizing enzymic activity, cell-free expression has been applied for ribozyme engineering. Ribozymes can be expressed by simpler *in vitro*transcription (IVT) machineries because any translation step is unnecessary. X-motif is a ribozyme that catalyzes RNA-cleavage and was initially engineered by **sequential evolution of ligands by exponential enrichment (SELEX)** [61]. After nine rounds of μIVC, a new variant of X-motif demonstrated 28-fold increased catalytic activity [62]. Spinach is another ribozyme that binds the DFHBI dye, acquiring fluorescent properties [63]. Randomly generated variants of spinach were compartmentalized into single emulsions and were clonally amplified by droplet PCR [64]. IVT mixture and DHBFI were later provided by droplet fusion [64]. The most optimal isolated variant was named ISpinach and showed increased fluorescence, enhanced thermostability, and reduced salt sensitivity [64].

Selection and high-throughput screening

A crucial part of a laboratory evolution experiment is selection or screening for the gene variants with the desired characteristics. When selection pressure is applied in a population, only members with the required key mutations will be able to survive and/or reproduce. However, screening is a testing procedure that checks individually each genetic variant for the desired activity. In selection schemes, there is no need for highly monodisperse emulsions because there is no quantification of an assay reaction product. Therefore, selection schemes are well-suited for IVC studies.

Selection pressure in IVC

Selection within artificial compartments is well-suited for DNA-interacting proteins because the protein-encoding gene and its target substrate can easily be combined on a single DNA molecule, linking the genotype and the phenotype. For example, in the first demonstration of IVC, an enrichment assay was performed between methyltransferase encoding genes and non- methyltransferase-

encoding genes [15]. Successful DNA-methylation protected DNA by subsequent restriction digestion. Therefore, only intact DNA molecules could be rescued by PCR [15].

A different approach is followed with DNA polymerases. DNA polymerases can drive PCR inside single emulsions, leading to the over-representation of active genes in the gene library. Compartmentalized self-replication (CSR) has been applied successfully to engineer DNA polymerases capable of unnatural nucleotide incorporation [65]. Building upon CSR, it was shown that gene variants that drive the expression of DNA polymerase more efficiently will be preferentially amplified during the subsequent compartmentalized *in vitro* PCR step. Compartmentalized partner replication (CPR) has been successfully applied for the laboratory evolution of T7 RNA polymerase that drives DNA polymerase transcription from an alternative T7 promoter and of an aminoacyl-tRNA synthetase (ARS):tRNA pair able to incorporate an unnatural amino acid in place of an amber codon, restoring DNA polymerase expression [25].

Screening with FACS

All the aforementioned selection schemes have been only applied to DNA-interacting proteins because the encoding DNA is coupled to a DNA-motif that acts as a substrate, ensuring the genotype to phenotype link. Hence, in case of proteins that do not act on DNA, different screening/selection strategies must be developed. The first example of an IVC approach with an alternative, DNA-independent selection was the evolution of a phosphotriesterase and was based on microbead display [20]. The role of the microbead is to act as the physical link between the genotype and the phenotype. To do so, magnetic microbeads were coated with streptavidin and the gene library was biotinylated by PCR [20]. Due to strong streptavidin–biotin affinity, microbead–gene complexes were formed. Moreover, a biotinylated enzyme substrate was also attached to the microbead [20]. Single microbead–gene–substrate complexes were compartmentalized into droplets, where gene expression took place [20]. After incubation to allow for protein expression and substrate-to-product conversion, the emulsion was resolved. The microbeads were then exposed to an antiproduct antibody and, as a result, only those microbeads carrying functional gene variants were selected [20]. Microbead display is suitably combined with FACS. In this case, microbeads are coated with a fluorogenic substrate that turns to a fluorescent product. Fluorescent microbeads are then sorted by FACS. This approach is gaining some ground and has been positively valuated by enrichment assays for an oxygen-resistant FeFe hydrogenase and HRP activity [58,66]. It has also been applied for screening of a random library of formate dehydrogenase variants and for engineering a protease with post-translational modification specificity [67,68].

Even if useful, microbead display still requires complex biochemistry schemes. Hence, one would like to have artificial compartments that can be sorted by FACS, without the use of microbeads. As indicated earlier, double emulsions are synthetic compartments that can be sorted by FACS [69] and microfluidic technology has made possible the generation of highly monodisperse compartments that allow for high screening accuracy. This was convincingly demonstrated by screening for wild-type arylsulfatase variants from a mimic library containing a low activity arylsulfatase variant in overwhelming numbers. Enrichment was found to reach up to 100 000-fold [31]. The approach has also been applied successfully to engineer a manganese-independent polymerase that can incorporate non-natural nucleic acid building blocks [32]. FACS is expected to be compatible with different artificial compartments if the continuous phase remains aqueous. For instance, a library of ARSs was co-compartmentalized in liposomes with a GFP gene carrying an amber codon [70]. Successful incorporation of an unnatural amino acid by ARSs restored GFP expression, allowing for sorting of liposomes containing active ARS variants by FACS [70]. Alternatively, by adding agarose and alginate in the aqueous solution of water-in-oil droplets and cooling to 4°C, a solid 'gel-shell' bead is formed [71]. By exchanging the oil phase to water, the gel-shell bead

remains stable and can be sorted by a normal FACS facility. After two rounds of sorting, enrichment >100 000-fold has been obtained for gel-shell beads that encapsulated active phosphotriesterase variants [71].

On-chip, high-throughput droplet sorting

μIVC owes its rapid progression to technological advancements that allowed the performance of all the necessary steps to perform laboratory evolution on-chip. FACS is not suitable for sorting the commonest of artificial compartments: the water-in-oil emulsions. Even alternative compartments such as liposomes are more complicated to generate and must be transferred to the FACS facility. Fluorescent-activated droplet sorting (FADS) is a system to sort water-in-oil droplets on-chip by **dielectrophoresis**. If fluorescent and nonfluorescent droplets are to be sorted, they are exposed to a laser. In case of detected fluorescence, a sensor triggers the generation of a nonuniform electric field that polarizes the fluorescent droplet, causing its deflection towards the positive arm of a Y-shaped junction (Figure 3A). Droplets without positive signal spontaneously flow in the negative arm because of the lower hydraulic pressure of the Y-shaped junction. Apart from fluorescence discrimination, the system was found to be suitable for other types of sorting, for example, of a model library of *E. coli* cells expressing β-galactosidase [72]. Based on the enzymatic conversion of a chromogenic substrate (ONP-Gal, releasing a yellow color after cleavage) sorting was performed at ~300 Hz (300 droplets per second) with false positives rates less than 1 in 10 000 [72].

The first application of FADS was to screen a library of HRP variants for enhanced activity [43]. Single yeast cells that expressed HRP on their cell surface were compartmentalized with a fluorogenic substrate in water-in-oil droplets [43]. Droplets were sorted at ~2000 Hz for up to 3 hours and a tenfold faster HRP variant was found [43]. FADS has been successfully modified to screen for enantioselective enzymes using a dual-channel screening platform [50]. The goal was to improve the enantioselectivity of an esterase towards (*S*)-profens rather than (*R*)-profens. Two spatially separated excitation beams monitored the signal readouts of two different fluorogenic substrates corresponding to either (*R*)-profens or (*S*)-profens. After multiple rounds of laboratory evolution, two variants with 700- and 560-fold enhanced enantioselectivity towards (*S*)-profens were obtained [50]. Within a couple of years, FADS has become the main screening approach in μIVC: increased α-amylase production by yeast cells [73] for remodeling the active site of cyclohexylamine oxidase [51]; the laboratory evolution of an artificial aldolase [52]; and a more stable and fluorogenic ribozyme [64] (for more examples and details, see Table 2).

FADS is not always applicable because it is not always possible to link an enzymatic activity to fluorescence. Another issue may be that the engineered enzymatic activity might be optimized to convert the fluorogenic substrate rather than the target substrate ('you get what you screen for'). As an alternative, a screening system based on absorbance was developed. Absorbance activated droplet sorting (AADS) consists of a FADS-like, Y-shaped junction [74]. In the case of an AADS system, however, a light emitter and a photodetector allow for absorbance-based sorting (Figure 3B). When a droplet passes through the interrogation point, light intensity decreases concomitantly with the dye concentration and the droplet, similar to FADS, and is sorted dielectrophoretically (Figure 3B). AADS was first tested on a library of NAD^+-dependent amino acid dehydrogenase-encoding genes [74]. Successful conversion of L-phenylalanine to phenyl-pyruvate by the dehydrogenase was coupled, through NADH oxidation, to the reduction of tetrazolium salt giving a formazan dye. Droplet sorting was performed at ~100 Hz and enrichment reached up to 2800-fold [74]. The utility of the approach was demonstrated by evolving a dehydrogenase variant with improved activity after two rounds of directed evolution [74]. AADS was again applied for the evolution of an amine dehydrogenase [75]. This time best hit droplets were isolated by AADS and their content was sequenced by nanopore sequencing to generate

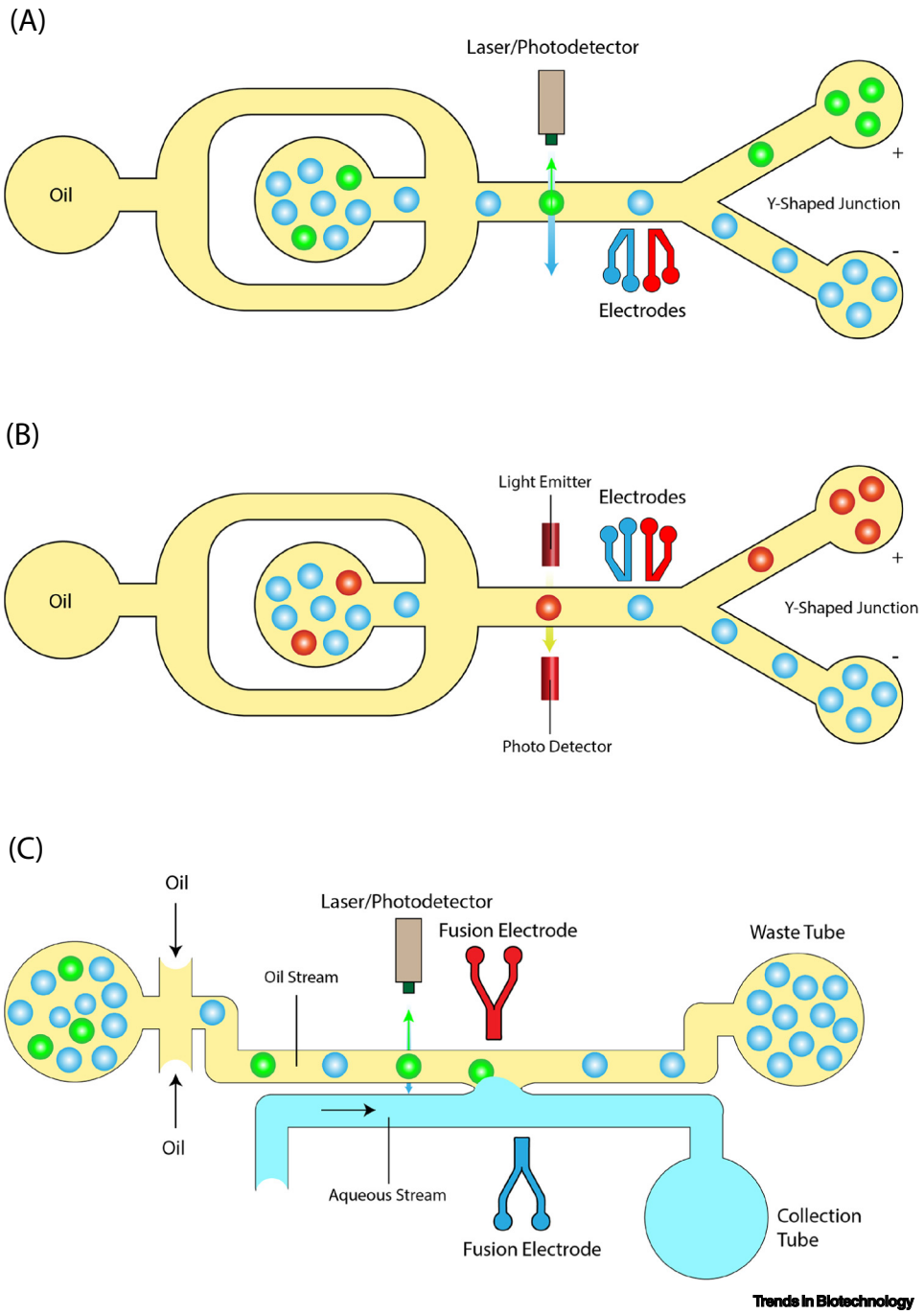


Figure 3. On-chip high-throughput screening based on microfluidics-assisted *in vitro* compartmentalization (μIVC). Of the examples described in the text, three μIVC illustrated approaches have been selected as they are relatively well developed and can be applied for the laboratory evolution of a wide range of enzymic targets. (A) Fluorescent-activated droplet sorting (FADS). Water-in-oil droplets are initially spaced out by an oil phase. Each droplet is interrogated for fluorescence. When fluorescence above a set threshold is detected, a pair of electrodes send nonuniform pulses of electric current that polarize the fluorescent droplet. The polarized droplet is then deflected to the positive arm of a Y-shaped junction. Nonfluorescent droplets passively flow towards the negative arm due to smaller hydraulic pressure.

(Figure legend continued at the bottom of the next page.)

a sequence consensus of beneficial mutations [75]. Long reading sequencing is not possible by next generation sequencing platforms like Illumina (~300 bp sequencing length per base pair), therefore, combining high-throughput droplet screening (e.g., AADS) with long read, deep-sequencing methods facilitates the tracking of beneficial mutations during a directed evolution campaign.

Although the aforementioned cases of μIVC-based sorting have shown success, there are more variations that are worth mentioning. The first one is an approach in which, rather than collecting individual droplets, only their content is retrieved. This technique, introduced as fluorescent activated electrocoalescence (FAE), consists of an aqueous stream and the droplet-containing oil stream that co-flow between two electrodes (Figure 3C) [59]. When fluorescence is detected, an electric pulse is applied, resulting in the coalescence(i.e., merging of the aqueous content of the passing droplet with the aqueous stream below). The droplet disintegrates and its contents are collected. Yet another sorting approach that uses a magnetic field has been developed to separate droplets encapsulating a different number of microalgal cells, reflecting different growth rates between cell strains [76]. All generated droplets contain iron oxide nanoparticles and are attracted by the same magnetic force. According to Newton's second law of motion, differences in mass/cell density of the microdroplets results in different acceleration and hence distinct lateral displacement of each droplet, allowing sorting [76]. A third case is also label-free and uses electrochemical principles. Electrical current passing through droplets can be quantified by a boron-doped diamond electrode, accurately reflecting the NADH concentration [77]. Droplets above a given threshold are then sorted dielectrophoretically using the Y-shaped junctions. Isocitrate dehydrogenase reduces NAD^+ to NADH and, after two rounds of laboratory evolution, five variants showing higher activity than the wild type were isolated [77]. Finally, it was shown that droplet content can be analyzed by mass spectrometry in combination with electron spray ionization (ESI-MS) [78,79]. However, in these cases the droplet was destroyed in the process, thus the beneficial genotype could not be tracked down. Mass-activated droplet sorting (MADS) is based on an ingenious chip design and a programmable sorting algorithm [80]. Droplets injected for sorting are first split into two parts. One part flows through a short line to the mass spectrometry inlet for subsequent analysis and the other part flows through a long delay line that concludes in a Y-shaped sorting device. While the second part travels through the delay line, content analysis of the first part takes place and the sorting algorithm decides, above a given threshold, if the second droplet part corresponds to a positive hit. MADS has been tested over a range of enzyme–substrate concentrations and sorting accuracy was found at 98% [80]. Although promising, MADS performed at 0.7 Hz, which falls greatly behind FADS [80]. However, the universality that comes with its label-free nature leaves expectations for further development.

Concluding remarks and future perspectives

While microfluidics has not yet been a true game changer in the field of life sciences as it was expected to be [81], the strength of droplet microfluidics has been clearly recognized and aptly utilized to progress the field of laboratory evolution. One major disadvantage in the current state of μIVC is that in most cases microfluidic modules are generated by specialized laboratories.

Outstanding questions

The genotype to phenotype linkage confers a strict rule of a single gene per confinement. Assuming a Poisson distribution, this implies that the majority of the containers are left empty. This decreases the experimental efficiency and also results in wasting valuable reagents. Would it be possible to achieve controlled confinement of one gene variant per droplet by dedicated single gene/cell encapsulation?

The process of laboratory evolution is a multistep process that requires extensive human intervention and skilled personnel. Can it be standardized to a more accessible technology?

Apart from currently preferred confinements (single emulsions, double emulsions, and liposomes), what other synthetic confinements (e.g., polymersomes) could prove useful for laboratory evolution?

A typical microfluidic assay comprises three steps: droplet generation, incubation (usually off-chip), and droplet sorting. Could second generation chips integrate all three steps into a single device?

Will droplet-mass spectroscopy reach high enough throughput to perform laboratory evolution in a label-free manner?

(B) Absorbance-activated droplet sorting (AADS). Water-in-oil droplets are once again spaced out with an oil phase. Then they are interrogated for alterations in absorbance of their content. Positive hits are polarized by electrodes and, similar to FADS, are deflected to the positive arm of a Y-shaped junction. Negative-hit droplets passively flow to the negative arm of the junction. (C) Fluorescent-activated electrocoalescence (FAE). FAE is a screening system that collects only the content of the droplets. Water-in-oil droplets flow parallel to an aqueous stream. Detection of fluorescence above a set threshold triggers a voltage that causes the droplets to coalesce with the aqueous stream. The droplet is dissolved and its contents are collected. Droplets with a negative signal are separately collected in a waste tube.

Table 2. Laboratory evolution examples

Enzyme	Differentiation[a]	Compartmentalization	Screening method	Evolved property	Refs
Alpha hemolysin	epPCR	Liposome	Fluorescence FACS	Membrane pore formation	[35]
Aminoacyl- tRNA synthetase	epPCR	Liposome	Fluorescence FACS	Incorporation of unnatural amino acids	[70]
X-motif ribozyme	epPCR	Single emulsion	Fluorescence FADS	Enhanced catalysis activity	[62]
Spinach ribozyme	epPCR	Single emulsion	Fluorescence FADS	Brightness, thermostability, salt tolerance	[64]
Phenylalanine dehydrogenase	epPCR, STEP PCR	Single emulsion	Absorbance AADS	Enhanced catalysis activity	[74]
DNA-polymerase	epPCR	Double emulsion	Fluorescence FACS	Manganese independence	[32]
Aldolase	epPCR, DNA shuffling	Single emulsion	Fluorescence FADS	Stereoselectivity, enhanced substrate range	[52]
Esterase	epPCR, DNA shuffling, Saturation mutagenesis	Single emulsion	Fluorescence FADS	Enantioselectivity	[50]
Oxidase	Saturation mutagenesis	Single emulsion	Fluorescence FADS	Non-natural substrate catalysis	[51]
Savinase protease	Target amino acid randomization by Slonomics, STEP PCR	Single emulsion	Fluorescence FADS	Increased Activity	[60]

[a]Abbreviations: epPCR, error-prone PCR; STEP, staggered extension process.

Standardization and commercialization of microfluidics devices will allow for more consistent assays and give higher accessibility to non-specialists. While the simplest, 'hand-made' version of IVC is an appropriate method for selection of DNA-interacting proteins, μIVC has allowed for high-throughput screening of different enzyme classes. The level of control of microfluidics-based compartment generation allows for standardized, accurate, downstream screening of different compartment types. In addition, its compatibility with both cell-based and cell-free expression systems offers flexibility in terms of gene expression, allowing screening of protein features under desired conditions. Finally, on-chip, fluorescence-activated droplet screening has resulted in obtaining a variety of enzymes with desired properties, meaning that μIVC is now moving from the development stage to real life applications.

It is anticipated that more developments will occur as μIVC gains more space as a laboratory evolution scheme. Starting from screening, FADS has become the norm but there is a well-placed desire for label-free screening methods. AADS has already come into play but droplet-MS could be the next big novelty. However, throughput must be increased substantially to compare with the current screening ratios already achieved by FADS, which can reach up to 30 kHz [82]. Because water-in-oil emulsions may cause problems in sorting devices (FACS), on-chip sorting methods (FADS, AADS) and alternative containers (double emulsions, liposomes) have been sought. In addition, commercial systems have recently become available that use disposable cartridges for sorting (https://www.miltenyibiotec.com/NL-en/products/macs-flow-cytometry/cell-sorter.html, https://on-chipbio.com/product-onchip_sort/). For example, the On-Chip sorter,that successfully sorted single emulsions engulfing GFP expressing *E. coli* cells (https://on-chipbio.com/emulsion-sorting/).

High-throughput, accurate, on-chip sorting of huge libraries is the holy grail of laboratory evolution. As every μIVC module is generated in-house, the approach is highly characterized by controllability and flexibility. However, this fact comes in hand with significant complexity. Some specific points (see Outstanding questions) need to be addressed to expand and simplify μIVC.

As described in this review, the combination of evolution principles with the emerging microfluidics technology allows for unprecedented possibilities with respect to high-throughput screening and selection to obtain biocatalysts (proteins, ribozymes) with desired optimal features. As Darwin concludes in his famous book: 'There is grandeur in this view of life, (...) from so simple a beginning endless forms most beautiful and wonderful have been and are being evolved' [83]. This is not only true for biological creatures, but also for proteins, nucleic acids, as well as inorganic compounds. Hence, expectations for the future are high, we foresee a revolution-on-a-chip.

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Declaration of interests

No interests are declared.

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