Screening of new or improved enzymes by microfluidic chips

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The functional annotation of novel protein sequences, as well as the large libraries generated through directed evolution to improve (newly found) proteins are in need for ultra-high throughput screening. Discovery and subsequent improvement of enzymes could pave the way for a broader application of enzymes in fields ranging from the chemical industry, pharmaceutics, and biotechnology to food and oil processing. Where old screening methods were time consuming and/or expensive, microfluidic chips are cheap to prepare and allow for a library size with a throughput limit of 10^7mutants/novel enzymes. Although the throughput limit is still one factor lower compared to fluorescent-activated cell sorting (FACS), microfluidic chips are capable of sorting water-in-oil droplets. The combination of biomimetic water-in-oil droplets with fluorescent-activated droplet sorting (FADS) makes it possible to assay a wider range of expression systems like lysed cells or excreted enzymes contained in the oil compartment. This essay looks at the development of microfluidic chips, how they work, and examples of its application. Furthermore, its limitations and what its prospects are for future research are discussed.

Introduction

Enzymes are of great interest for commercial and industrial processes,[1] and are already being applied in many different industries. In the food industry examples include isolated α-amylase which aids in the production of corn syrup, and trypsin which is used to pre-digest baby food.[2] For cleaning products like detergents, enzymes are optimized to broaden their reaction scope to various stains.[1] In the biofuel industry, cellulases are used to degrade biomass to sugars which can be fermented to produce biofuels.[3] For pharmaceutical use, enzymes can be used as a drug, but also to produce highly specific antibiotics.[4] The highly specific catalysis of enzymes is also applied in the chemical industry as an alternative to synthetic chemistry for the production of fine chemicals. Examples include the production of amino acids, amides, amines, peptides, organic acids and epoxides.[5] Because of their unique architecture, enzymes are able to produce highly specific chemicals in an aqueous environment under low temperatures whereas producing the same chemicals by synthetic chemistry efforts are often considered harmful to the environment. Furthermore, and most important is the chirality of enzymes, making these catalysts capable of producing enantiopure compounds. However, there are also many limitations for the use of enzymes dependent on the industry of interest. General issues include instability, solubility in desired media and low levels of expression.[6]

Novel proteins and engineering of known enzymes are ways to solve these limitations. Because of huge improvements in DNA sequencing, millions of novel protein sequences are being discovered from environmental DNA (eDNA).[7] Although this gives an overview about genetic diversity, functional annotation (attaching biological information to genomic data) of these genes is challenging.[8] The function of 30-40 % in newly sequenced genomes is unknown, and initial

annotations of these genes are often proven incorrect.[7] This results in a need for a reliable ultra-high throughput screening for novel enzymes derived from eDNA.

Engineering of proteins relies mostly on either rational protein design or directed evolution.[9] The latter mimics Darwinian evolution, improving enzymes through random mutations and selecting for the ´fittest´. Directed evolution also gives insight in the relationship between protein sequences and the structure and function of proteins, facilitation better functional annotation in the future. Directed evolution can select for mutants with an increase in specificity, activity or acceptance of other substrates.[10] While directed evolution circumvents the need for structural information of the protein, it creates huge libraries of mutants (10^6-10^9) which are dependent on reliable ultra-high throughput screening.[10]

Functional annotation of novel protein sequences can be done by heterologous expression[11], whereas selection of successful mutants in directed evolution can be done by for example microtiter plate screening.[10] Both methods are reliable but time consuming, with an average throughput limit of 10^2-10^4 for microtiter plate screening. This limits the amounts of new genes which can be annotated and the library size of directed evolution, resulting in mutational studies limited to the surroundings of the active site.[10] The use of fully automatic robotic systems can increase the throughput limit significantly (10^6) but are expensive as initial expense and to use because of the large reaction volume. The reaction volume of one well can be reduced to 100 µl but reaches its limit due to uncontrolled liquid evaporation.[12] FACS allows for an even larger throughput limit of 10^8. Although very fast, it is limited to fluorescent assays in which the fluorescent marker stays inside of the cell or is bound to its surface.

While relevant for multiple fields ranging from physics to evolutionary biology[13], only quite recently microfluidics found its application as a high throughput screening method. As of now, both directed evolution[14] and functional annotation[15] have been done with microfluidics as high throughput screening tool. Microfluidics, the precise manipulation of liquids in channels smaller than 1 mm, is an ever growing multidisciplinary field.[13] This technique allowed for the increase in throughput speed by a 10 to a 100-fold (10^7) while decreasing the reaction volume by more than a 10-million-fold, thereby also decreasing the costs by 4-million-fold compared to the use of robots.[14] Compared to FACS, this method is also dependent on fluorescent screening, but can make use of a larger range of expression systems, and efforts are on its way to broaden the range of screening methods.

In the next chapters the concept of microfluidics and its application in biotechnology will be discussed. First the genotype phenotype linkage will be described as this is essential for screening. *In vitro* compartmentalization (IVC), is one way in which this can be done, allowing a single cell to be stored in one drop of oil.[16] These drops were first analyzed by fluorescence-activated droplet sorting (FACS),[17] but were later combined with microfluidics as droplet-based microfluidics.[10] How the current method (Microfluidics on-chip, droplet method) works and how this technique is being applied in the discovery of new enzymes and in directed evolution will be discussed next.

Finally the limitations of this technique will be reviewed, as well as the future directions in which microfluidics can prove its worth.

Genotype – Phenotype linkage

When screening for new or improved enzymes, co-localization of both genotype and phenotype is essential.[18] In this way, any sort of improvement or newly found functional enzyme can be directly related to the sequence and/or the mutations which were needed to obtain this improvement. Coupling genetic information to functional traits can be done in several ways. SELEX[19] and phage display[20] are well known examples, but are not fully suitable for testing catalysis.[18] Cells of course compartmentalize both the protein and the relevant gene by itself and can be screened by FACS. [13] This method can sort up to 7 x10^4 cells a second, making it one of the most widely used screening platforms in use today. The technique of interest for this essay, *in vitro* compartmentalization (IVC), creates an artificial oil based compartment surrounding the gene and the protein, or whole cells.

IVC

The use of biomimetic compartments started by encapsulating *in vitro* transcription and translation (IVTT) machinery by Griffiths and Tawfik in 1998.[21] This involves the compartmentalization of an aqueous solution containing a gene coding for a single enzyme in a drop of oil (water-in-oil emulsion) (Figure 1)[22]. In this way, each drop functions as an independent micro reactor, being the equivalent of one well on a microtiter plate. Because one milliliter of *E. coli* culture contains more than 10^8 cells, 1 milliliter of culture is the equivalent of roughly 10^4 1536-well plates.[16] Each droplet contains both the gene and the protein, and it thus fit for the linkage of genotype and phenotype. Aside from the expression system IVTT, cell lysates, whole cells with surface exposed proteins, and whole cells with secreted proteins can also be compartmentalized in oil droplets. (Figure 1)[22]

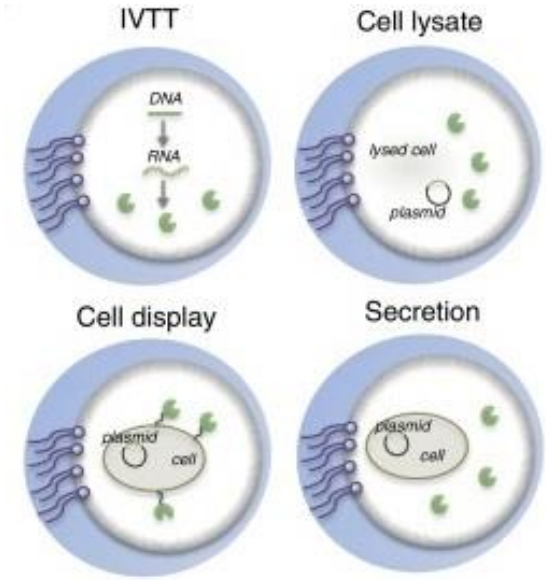

Figure 1. The 4 different expression systems which can be compartmentalized in droplets.

The screening of these oil droplets is mostly based on fluorescence. The first fluorescence based screening method used for biomimetic compartments was FACS. As mentioned, this method does not need cells in a drop of oil to be effective. IVC can however broaden the scope of FACS to screen secreted proteins and lysed cells. Moreover, because IVC provides high enzyme concentrations, low-signal to noise ratios can also be obtained.[16] With IVC, proteins which are normally toxic to cells can also be screened by FACS, where this is not possible for cells without an oily biocompartment. One requirement for the use of FACS is the use of a double emulsion, producing water-in-oil-in-water-droplets.[22] This is because FACS is only capable of sorting aqueous solutions. These so called bulk emulsion droplets have been used in directed evolution.[23-25] There is however a big disadvantage because the size of double emulsified droplets vary a lot i.e. are polydisperse.[22] Quantitate essays to test product formation of (improved) biocatalyst are therefore unreliable. The size of the biocompartment prohibits direct correlation between improved product formation and improved catalytic abilities by the biocatalyst as the size difference changes the concentration of the product. Improved biocatalysts in larger biocompartments could result in false negatives, whereas diminished catalytic efficiency in smaller biocompartments could result in false positives. Efforts to decrease the polydispersity of water-in-oil-in-water double emulsions are so far unsuccessful.[23] Monodisperse water-in-oil droplets containing either of the four mentioned expression systems cannot be screened by FACS but are essential for ultra-high throughput screening for both directed evolution and the discovery of new enzymes.

A successful screening method for biomimetic, monodisperse water-in-oil droplets was achieved with microfluidic chips.[26] These small channels can separate an emulsion of monodisperse droplets, and screen them on the microfluidic chip with a fluorescence assay similar to FACS. Also called lab-on-a-chip (microfluidic chip) this method is capable of ultra-high throughput screening for directed evolution and the discovery of new enzymes.

Microfluidic chips for ultra-high throughput screening of water in oil droplets

General generation of microfluidic chips

Even though originally, for any sort of application, microfluidic chips were made out of glass or silicon, nowadays most microchips are made out of poly(dimethylsilocane) (PDMS).[13] This polymer is inexpensive, can create channels by molding instead of etching, is reproduced with high fidelity and is nontoxic. Production of microfluidic chips with PDMS is mostly done with soft lithography, in which the elastomeric structure of PDMS covers a mold (Figure 2, right)[13]. Because the size and shape of microfluidic chips depend on one's research goals, different molds or masters need to be created[27] (Figure 2, left)[13].

The design of a master is done with a computer-aided design (CAD) program. The resulting image of the microfluidic chip specific for your research is printed on a transparency. From this transparence, a so-called photomask is created by a method called photolithography. The areas which you want affected are now resistant to the chemical treatments, which engrave the mold

pattern (developing). This produces the master mold, on which PDMS can be placed. After polymerization, and sealing of the bottom, the microfluidic chip is ready for use.[13,27]

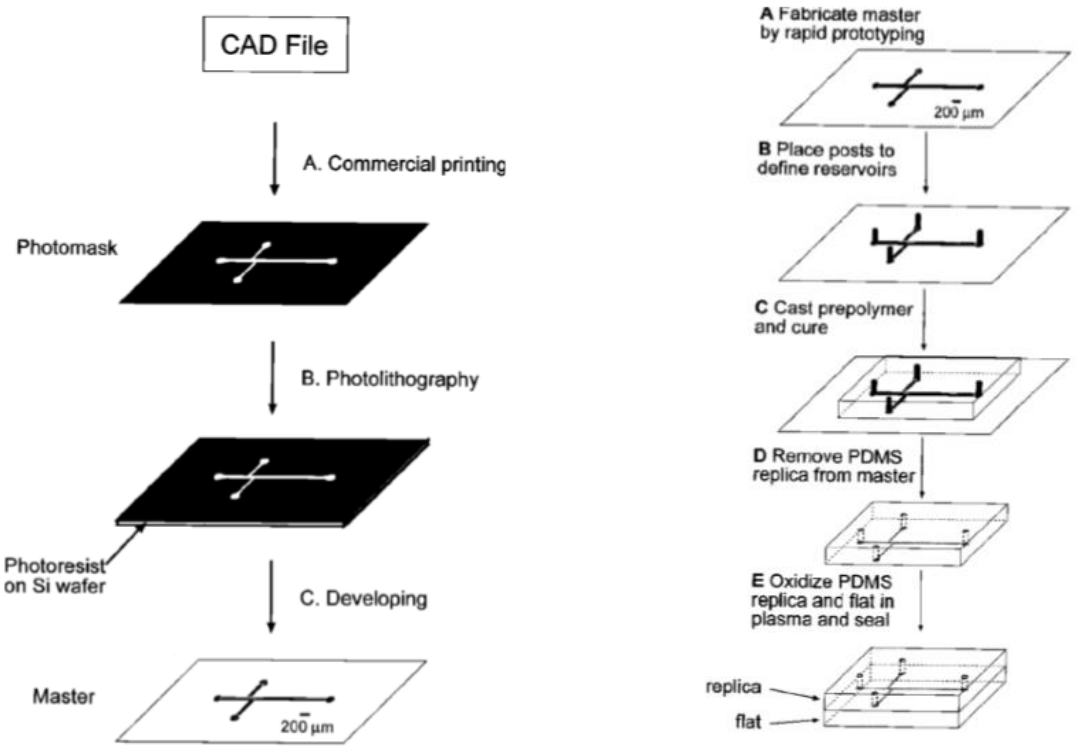

Figure 2. Fabrication of a microfluidic chip. (Left) The photomask is created by CAD. This protects certain areas of the Si wafer from UV exposure which molds the channels, creating the master. Afterwards the PDMS is molded on the master creating a PDMS slab. This mold is then attached on a glass slide and the channels are pretreated (oxidized) to allow for aqueous streams.

Preparation of cells

Before the microfluidic chip can prepare and sort droplets, the cells have to be grown, and more importantly, an estimation must be made about the number of cells in a droplet. After creation of the library with all different mutants and or different enzymes, the cells can be grown with each cell containing a unique gene. Growing cells must be done carefully, as cells which are lysed before droplet creation can clog the microfluidic device. For the preparation of droplets it is important that the majority of droplets contain only one cell (or lysed cell/ IVC). The number of cells inside each droplet can be estimate by a Poisson distribution[27] (Eq. 1)

Eq. 1 $$P(X = x) = e^{-\lambda} [\lambda^x/x!]$$

In this equation, $P(X = x)$ denotes the probability of finding x cells per droplet and λ represents the mean number of cells in the volume of each droplet. By changing the cell density of the culture which is put in the microfluidic chip, predictions can be made on the amount of droplets which contain zero, one or more cells after encapsulation. For example, when the concentration is altered in such a way that λ=0.1, 90% of the droplets with contain 0 cells, and 10% of the droplets will

contain 1 cell. At λ=0.5, 60% will contain 0 cells, ~30% will contain 1 cell, ~6% will contain 2 cells and ~3% will contain 3 cells. (Figure 3)[27]

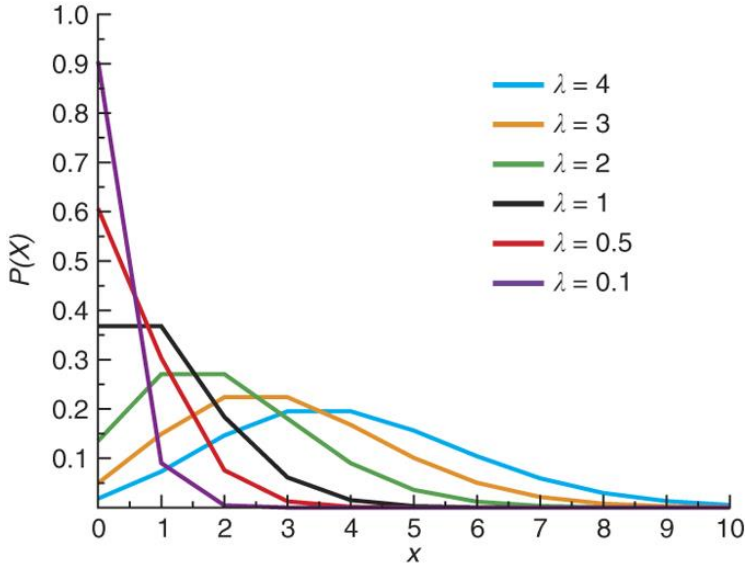

Figure 3. Probability P(X) of having a certain number of cells in (*x*) in a droplet with alternating (λ) related to the concentrations of cells before encapsulation.

Creation and sorting of droplets on microfluidic chips

A microfluidic chip for library screening needs to have three important features. The creation of monodisperse droplets, incubation time with the substrate and a mechanism for sorting. This can be done by two separate microfluidic chips, in which one produces the droplets and the other separates them after incubation in a tube or reservoir. (Figure 4, and Figure 6)[27] Other microfluidic chips, in which incubation is done in a channel between the two chips are nowadays also considered.[14,28] In figure 4 we see a typical microfluidic chip made from PDMS for the production of monodisperse droplets. Two separate inlets, one for the continuous stream of (fluorinated) oil, and two for the aqueous phases; cells and a separate inlet for the substrate which is to be catalyzed. The three streams meet in a so-called flow-focusing junction.

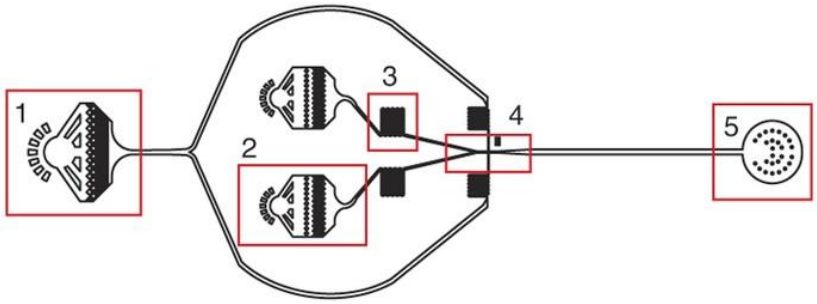

Figure 4. Example of an droplet creating microfluidic chip. (1) Oil inlet. (2) Aqueous phase inlet, two are shown as is normally the case, one for the substrate/surfactant and one for the cells. (3) Fluid Resistor, lowers the flow rate before arriving at the flow-focussing junction (4). After a short incubation line for the surfactant and cells to stabilize the droplets are collected (5), and incubated.

This junction was first described by Anne et al,[29] and a close up can be seen in figure 5, left. The outer phases of oil force the combined aqueous phase into a narrower channel, creating water in oil droplets which are subsequently collected in a reservoir. The formation of these droplets is dependent on the flow rates of the aqueous (Q_i) and oil phase (Q_o). In figure 5, right, the effect of the ratio of flow Q_i over Q_o plotted against the flow of Q_o. Changing the flows can result in polydisperse and monodisperse droplets, and is an important variable to assess when starting a microfluidic chip experiment.

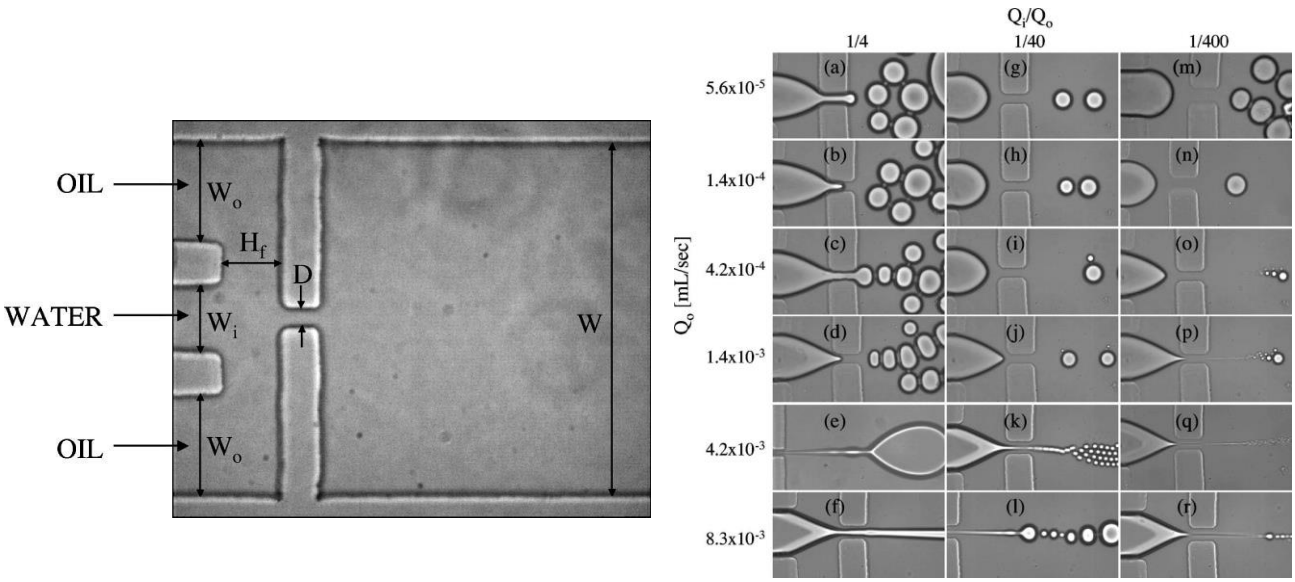

Figure 5. (Left) The flow-focussing junction to produce monodisperse droplets. The oil puts pressure on the aqueous phase in the middle, producing droplets through the narrow channel. (Right)

Even when the flow rates are properly set, coalescence i.e. the merging of cells can still occur in the collection tube. This is inhibited by surfactants[30], which populate the water-oil interface. Because it takes time for the surfactant and the droplets to stabilize, there must be a small piece of channel between the flow-focusing junction and the collection tube or syringe.[27] When an assay uses screening by fluorescence (see below) a fluorinated carrier oil must be used with biocompatible fluorosurfactants.

After droplet formation, the cells are typically incubated in a tube or syringe, or alternatively run along a long channel to the other microfluidic chip capable of sorting. The length of the channel together with the flow rate predicts the incubation time.[14] The cells are then sorted by a second microfluidic chip (Figure 6). The cells are re-injected in the aqueous reservoir, and with a similar junction the oil phase is added. This time the oil functions to separate the already monodisperse droplets. Next in line is the screening device. For efforts concerning directed evolution and functional annotation using microchips, each article so far used the same fluorescence based essay called fluorescence-activated droplet sorting or FADS[31] (Figure 6, blue electrodes and box 9).[27] This system is highly similar to FACS, bus has small alteration to make it compatible with microfluidic chips. Both methods irradiate the cell or droplet with a laser, after which the fluorescence is measured on basis of which the cells/droplets are sorted.

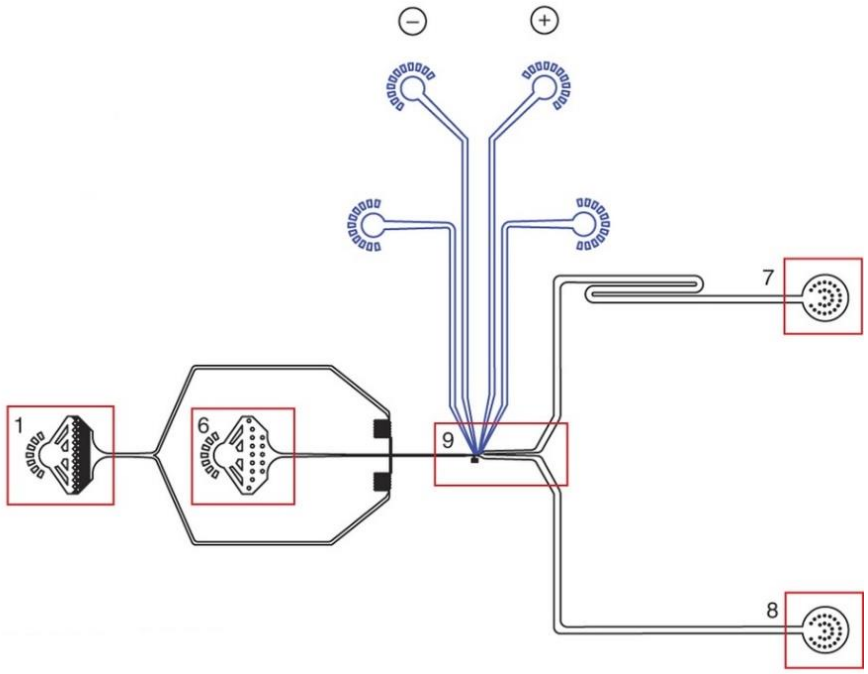

Figure 6. Example of a FADS based sorting microfluidic chip. (1) A second oil inlet, this time for the separation of the existing droplets which are reinjected in (6). (9) The actual FADS cell sorting device. (7) Collection tube for fluorescent cells. (8) Collection tube for waste cells. The blue electrodes cause the alternating current which directs fluorescent cells to the upper channel (discussed below).

A close up of the FADS system (box 9 in Figure 6) can be seen in Figure 7[31]. Each passing droplet is irradiated by a laser (LAS). After irradiation, the subsequent amount of fluorescence of the cell can be measured by a photmultplier tube (PMT). The combination of a microscope´s halogen lamp (LAMP) and a high-speed camera (CAM) make sure that irradiation is only happening when a cell passes. When the PMT detection reaches a certain threshold, electrodes further down the channel, right before the Y-shape junction can be switched on. Normally all cells go down the bottom branch because it is wider and has little flow resistance. When applying a high-voltage alternating current, the positive electric field deflects the droplet into the upper channel. The positive dielectic force pulls the cells towards the high electric gradient region. At the end of both channels following the y-shape junction is a collection outlet.

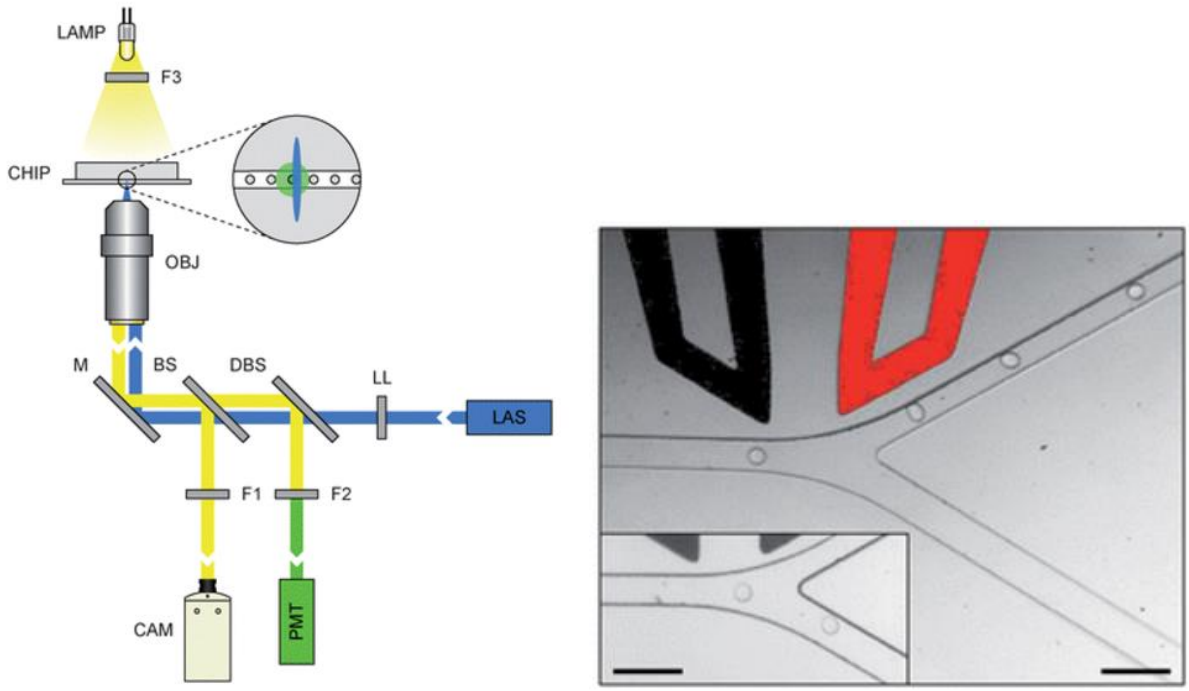

Figure 7. (left) Measurement of fluorescence after initial laser irradiation. After the camera (CAM) detects a cell, the laser (LAS) irradiates it. The fluorescence is measured by the photomultiplier tube (PMT). (Right) When the PMT detects fluorescence above a certain threshold, an alternating positive current is applied to the upper channel, channeling the cells away from the waste branch.

Applications of microfluidic chips in biotechnology

Microfluidic chips have been used for directed evolution a number of times, and recently the use of microfluidic chips for the discovery of new functional enzymes has been reported (Table 1). Even though there have been reports in the past of polydisperse droplets in combination with FACS[23-25] being used for directed evolution (Table 1, entry 1) the focus nowadays lays on monodisperse droplets screened by FADS. The cycle for each of these studies is basically the same. A library of mutants or novel protein sequences is created and transformed in a host suitable for expression. The full cells or IVTT are then added to the microfluidic chip and monodisperse droplets are created. When the assay is based on lysed cells, a lysing agent is added in the aqueous form upon formation of the droplets. These droplets are then screened by FADS, and the top performing cells are reinjected for multiple rounds of selection. The final droplets are then de-emulsified and the DNA is sequenced to obtain the genes corresponding to the new or improved proteins.

Agresti et al. (2010)[14] (Table 1, entry 2) reported the first use of microfluidic chips for the directed evolution of horse radish peroxidase (HRP). HRP is already very active towards the substrate tested here, Amplex Ultrared (AUR). The HRP catalyzed reaction of AUR with hydrogen peroxide results in resorufin, a highly fluorescent substance. First they made 2 libraries of 10^7 mutants. One was created by error/prone PCR and the other with saturation mutagenesis. The first creates random mutations throughout the protein whereas the latter creates mutations close to the active

Table 4. Reported use of microfluidic chips for directed evolution or discovery of novel enzymes

Entry	Aim	Compartmentalization	Expression system	Incubation type	Incubation time	Screening method	Screening rate (cells/second)	Original library size	Reference
1	Directed Evolution	Polydisperse droplets	Variable	Variable	Variable	FACS	20000	Variable	23-25
2	Directed Evolution	Monodisperse droplets	Cell display	Channel	5 min.	FADS	2000	10^7	14
3	Directed Evolution	Monodisperse droplets	Lysed cells	Syringe	1 hr.	FADS	1000	10^7	32
4	-	Monodisperse droplets	IVTT	Channel	-	FADS	2000	10^6	28
5	Directed Evolution	Monodisperse droplets	Secreted enzymes	Capillary	2 hr.	FADS	400	3×10^6	33
6	Discovery Enzymes	Monodisperse droplets	Lysed cells	Syringe	48 hr.	FADS	2000-2500	$1{,}25 \times 10^6$	15
7	Directed evolution	Monodisperse GSB´s	Lysed cells	Capillary	5 min.	FACS	20000	5×10^5	34

site where the substrate binds. The randomized DNA was transformed to a suitable host, and grown to get a library of 10^7 tranformants. The cells were grown under selective conditions in which only cells which obtained plasmid DNA are capable of growing. Screening was done by a single microfluidic chip, in which the droplet forming and the droplet screening chips were connected by an incubation tube of 360 µm, corresponding to an incubation time of roughly 5 minutes. After 4 rounds of directed evolution, only a 2 fold increase was observed. From the 18 best scoring clones they made a new library of 10^7 mutants. They only screen for the brightest 5/10% of droplets, and after 5 rounds they found HRP mutants with an 8-fold increase in catalytic efficacy over the wildtype ($2.5 \times 10^7\ M^{-1}\ s^{-1}$).

Kintses et al. (Table 1, entry 3)[32] were the first to use droplets containing lysed cells for directed evolution of arylsulfatase. This enzyme catalyzes the hydrolysis of bis(methylphosphonyl)-fluorescein, the product of which, fluorescein is fluorescent. Because this is not a known substrate of arylsulfatse, this research was mostly aimed to alter specificity, whereas Agresti et al aimed to increase turnover rate. They made 2 libraries based on error prone PCR, one with high mutational rates (3/gene) and one with low mutational rates (1.5/gene). After 4 rounds of selecting for the brightest 4% droplets, they obtained a 6-fold improvement in catalytic efficacy. They lowered the cell/droplet ratio with each round, making sure that there were no droplets with 2 or more cells in the final hits. They found that the increase was mostly due to an increase in stability or better expression of the enzyme, not an actual improvement of the enzyme itself.

Fallah Alagri et al. (Table 1, entry 4)[28] reported the first use of monodisperse droplets containing *in vitro* transcription and translation systems for high throughput screening on microfluidic chips. Linear genes containing either *LacZ*, encoding β-galactosidase, or *LacZmut*, encoding an inactive β-galactosidase were amplified. After amplification the linear genes were directly compartmentalized in droplets. These droplets were later combined with droplets containing the

IVTT system for translation and transcription together with a fluorogenic substrate for β-galactosidase. The droplets were sorted by FADS, and after they recovered the DNA from the fluorescent droplets they tested if the DNA corresponded to the the intact *LacZ* gene. While they did not use the system for directed evolution, they did show that the system could sort 2000 droplets per second while only having <0.004 false positives/negatives. The few false positives or negatives were mostly because of droplets containing 2 or more cells, which can be further decreased by lowering λ in the poisson distribution as seen above. This system has not been applied yet for neither directed evolution nor functional annotation of proteins.

Sjostrom et al. (Table 1, entry 5)[33] used the microfluidic chip to identify yeast mutants which increase the amount of α-amylase produced, not the turnover rate of its substrate. They used starch as the natural substrate with BODIPY attached for a fluorescent read-out. The BODIPY is quenched by the 1,4-α connected glucose chain. When the chain is hydrolyzed by α-amylase, the fluorophore is unquenched. They took the top 5% fluorescent droplets, after random mutations by UV-irradiation mutagenesis. The top hits were tested for production, or yield of α-amylase, of which the top performing clone produced two times as much as the wild type.

Colin et al. (Table 1, entry 6)[15] were the first to report the use of microfluidic chips for the discovery of promiscuous enzymes. Adding to the complexity of functional annotation, they added that *"Functional annotation is further complicated by catalytic promiscuity (that is, the ability of enzymes to process more than one type of substrate)."* With their assay using lysed cells in droplets, they tested if a library of eDNA contained proteins which could function as hydrolases of sulfate monoesters and phosphate triesters. By doing so, they were also able to test for promiscuous activities of the enzymes. Overcoming this obstacle which is deemed impossible by sequence analysis alone. After two screening rounds they obtained 500 hits for a potential sulfate monoester hydrolase and 300 hits for potential phosphate triester hydrolases. Even though, estimated by poisson distribution, the second screening cycle had only 0.1 cells per droplet, there were still a lot of false positives. A low cut-off for the fluorescence detection by FADS was deemed as being detrimental for a high accuracy in screening. Even so, the top performing hits resulted in hydrolases with catalytic efficiencies ranging from 54 $M^{-1} s^{-1}$ to 9 $x10^5$ $M^{-1} s^{-1}$, showing the capacity of this technique to be used to discover new enzymes even with low turnover rates.

Gell-shell beads in combination with FACS

It is worth noting that a new type of encapsulation by polyelectrolyte shells has also been described (Table 1, entry 7)[34] This encapsulation can be used by FACS but without double emulsion as is necessary with oil based droplets. These so called gel shell beads (GSBs) are produced in a similar way (Figure 8)[34] The cells are emulsified in a flow-focusing junction, but contain the agarose and alginate. After incubation, the cells are cooled so that the agarose and alginate form a gel shaped bead. After disruption of the emulsion, the cell is only surrounded by the bead and can be used by FACS. This results in a monodisperse droplet, capable of entrapment of IVTT and lysed cells which can screened by FACS on a rate even higher than with FADS. After sorting, an increase in pH disupts the GSBs and the DNA can be rescued for sequencing. Like Colin et al., they were interested in a phosphotriesterase. They created a relatively small library with 5 $x10^5$ mutants through error prone PCR. Because FACS is capable of screening 20000 cells, or in this case GSBs

per second, the screening was done in less than an hour. They selected the top 10% of fluorescent GSBs and tested the kinetics with microtiter plate based assays. The top hit produced a phosphotriesterase with a 20-fold increase in catalytic efficacy.

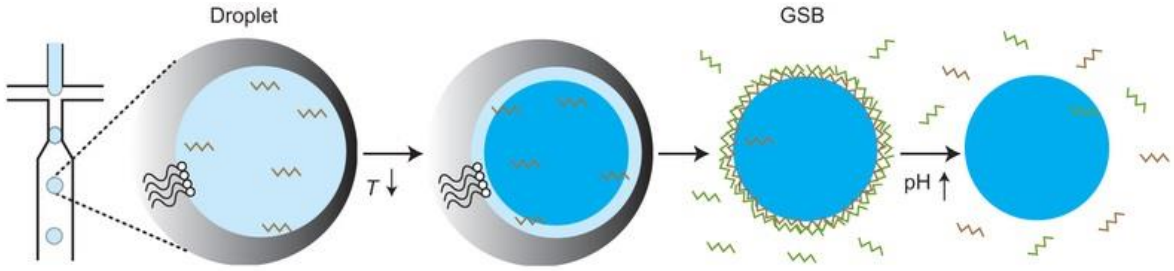

Figure 8. Production of gell-shell beads

Discussion

Microfluidic chips offer a lot of advantages for ultra-high throughput screening. They are cheap to produce, can detect very low turnover numbers and are capable of screening large libraries in a matter of hours. With all these clear cut advantages one might ask why this method has only been applied a handful of times for directed evolution, and only once for the screening of proteins from eDNA. First of all, microfluidic chips seem to cover a niche in which they are outperforming FACS, robotics and microtiter plates. Only when the essay cannot be done with fluorescent markers which stay inside or attached to the cells it has a clear advantage over FACS, aside from the lack of aerosols produced in the process. Furthermore, because detection is at this moment only done with FADS, an essay has to be designed in such a way that the product of catalysis is fluorescent. A famous saying in directed evolution is that "you get what you screen for". Here this would mean that even though you observe an increase for the fluorescent substrate, its natural substrate could be affected to a lesser extent or not at all. Robotics and microtiter plates allow for screening with GC, HPLC, NMR, Massa Spectrometry and other detection methods for product formation. Efforts are being done to increase the screening methods for microfluidic chips with Raman spectrometry,[35] mass spectrometry[36], and absorbance.[37] If these efforts are proven to be successful for directed evolution, microfluidic chips could grow from its niche to a more commonly used high throughput screening tool.

Even though the screening itself is very fast, the whole process is still very complex and time consuming. Finding optimal flow conditions, cell densities, and incubation processes have to be assessed before starting the experiment. Furthermore, producing a new microfluidic chip can also be considered as an endeavor which requires some expertise. Therefore, laboratories which already invested in robotics for handling multiple microtiter plates might want to spend little time on this alternative method.

With new screening methods, microfluidic chips have the possibility to become the method of choice for ultra-high throughput screening. The million-fold reduced costs in reagents is appealing for every laboratory which handles even moderate amounts of experiments in this field. It will be interesting to see if the gel-shell shaped beads in combination with FACS or the microfluidic chips which rely on FADS will become the method of choice in the future. While the latter is more direct

in the sense that the cells do not need to contain agarose and alginate for the production of the GSB, it offers all the advantages of oil droplets and can be screened by FACS which is still 10x faster compared to current microfluidic devices. Either way, the combination of artificial compartments and either method can be expected to have a significant effect on the application of enzymes in several industries.

References

1. Aehle, W. *Enzymes in industry: production and applications*. (John Wiley & Sons, 2007).

2. Choct, M. Enzymes for the feed industry: past, present and future. *Worlds. Poult. Sci. J.* **62,** 5–16 (2006).

3. Himmel, M. E. *et al.* Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science (80-.).* **315,** 804–807 (2007).

4. Vellard, M. The enzyme as drug: application of enzymes as pharmaceuticals. *Curr. Opin. Biotechnol.* **14,** 444–450 (2003).

5. Panke, S., Held, M. & Wubbolts, M. Trends and innovations in industrial biocatalysis for the production of fine chemicals. *Curr. Opin. Biotechnol.* **15,** 272–279 (2004).

6. Porter, J. L., Rusli, R. A. & Ollis, D. L. Directed Evolution of Enzymes for Industrial Biocatalysis. *ChemBioChem* **17,** 197–203 (2016).

7. Bornscheuer, U. T. Protein engineering: Beating the odds. *Nat. Chem. Biol.* **12,** 54–55 (2016).

8. Marchler-Bauer, A. *et al.* CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res.* **39,** D225–D229 (2011).

9. Bornscheuer, U. T. & Pohl, M. Improved biocatalysts by directed evolution and rational protein design. *Curr. Opin. Chem. Biol.* **5,** 137–143 (2001).

10. Packer, M. S. & Liu, D. R. Methods for the directed evolution of proteins. *Nat. Rev. Genet.* **16,** 379–394 (2015).

11. Handelsman, J. Metagenomics: application of genomics to uncultured microorganisms. *Microbiol. Mol. Biol. Rev.* **68,** 669–685 (2004).

12. Du, G., Fang, Q. & den Toonder, J. M. J. Microfluidics for cell-based high throughput screening platforms—A review. *Anal. Chim. Acta* **903,** 36–50 (2016).

13. Anderson, J. R., Chiu, D. T., Wu, H., Schueller, O. J. & Whitesides, G. M. Fabrication of microfluidic systems in poly (dimethylsiloxane). *Electrophoresis* **21,** 27–40 (2000).

14. Agresti, J. J. *et al.* Ultrahigh-throughput screening in drop-based microfluidics for directed evolution. *Proc. Natl. Acad. Sci.* **107,** 4004–4009 (2010).

15. Colin, P.-Y. *et al.* Ultrahigh-throughput discovery of promiscuous enzymes by picodroplet functional metagenomics. *Nat. Commun.* **6,** (2015).

16. Griffiths, A. D. & Tawfik, D. S. Miniaturising the laboratory in emulsion droplets. *Trends Biotechnol.* **24,** 395–402 (2006).

17. Huebner, A. *et al.* Quantitative detection of protein expression in single cells using droplet microfluidics. *Chem. Commun.* 1218–1220 (2007).

18. Paegel, B. M. & Joyce, G. F. Microfluidic compartmentalized directed evolution. *Chem. Biol.* **17,** 717–724 (2010).

19. Stoltenburg, R., Reinemann, C. & Strehlitz, B. SELEX—a (r) evolutionary method to generate high-affinity nucleic acid ligands. *Biomol. Eng.* **24,** 381–403 (2007).

20. Scott, J. K. & Smith, G. P. Searching for peptide ligands with an epitope library. *Science (80-.).* **249,** 386–390 (1990).

21. Tawfik, D. S. & Griffiths, A. D. Man-made cell-like compartments for molecular evolution. *Nat. Biotechnol.* **16,** 652–656 (1998).

22. Colin, P.-Y., Zinchenko, A. & Hollfelder, F. Enzyme engineering in biomimetic compartments. *Curr. Opin. Struct. Biol.* **33,** 42–51 (2015).

23. Mastrobattista, E. *et al.* High-throughput screening of enzyme libraries: in vitro evolution of a β-galactosidase by fluorescence-activated sorting of double emulsions. *Chem. Biol.* **12,** 1291–1300 (2005).

24. Aharoni, A., Amitai, G., Bernath, K., Magdassi, S. & Tawfik, D. S. High-throughput screening of enzyme libraries: thiolactonases evolved by fluorescence-activated sorting of single cells in emulsion compartments. *Chem. Biol.* **12,** 1281–1289 (2005).

25. Bernath, K. *et al.* In vitro compartmentalization by double emulsions: sorting and gene enrichment by fluorescence activated cell sorting. *Anal. Biochem.* **325,** 151–157 (2004).

26. Duffy, D. C., McDonald, J. C., Schueller, O. J. A. & Whitesides, G. M. Rapid prototyping of microfluidic systems in poly (dimethylsiloxane). *Anal. Chem.* **70,** 4974–4984 (1998).

27. Mazutis, L. *et al.* Single-cell analysis and sorting using droplet-based microfluidics. *Nat. Protoc.* **8,** 870–891 (2013).

28. Fallah-Araghi, A., Baret, J.-C., Ryckelynck, M. & Griffiths, A. D. A completely in vitro ultrahigh-throughput droplet-based microfluidic screening system for protein engineering and directed evolution. *Lab Chip* **12,** 882–891 (2012).

29. Anna, S. L., Bontoux, N. & Stone, H. A. Formation of dispersions using 'flow focusing' in microchannels. *Appl. Phys. Lett.* **82,** 364–366 (2003).

30. Baret, J.-C. Surfactants in droplet-based microfluidics. *Lab Chip* **12,** 422–433 (2012).

31. Baret, J.-C. *et al.* Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity. *Lab Chip* **9,** 1850–1858 (2009).

32. Kintses, B. *et al.* Picoliter cell lysate assays in microfluidic droplet compartments for directed enzyme evolution. *Chem. Biol.* **19,** 1001–1009 (2012).

33. Sjostrom, S. L. *et al.* High-throughput screening for industrial enzyme production hosts by droplet microfluidics. *Lab Chip* **14,** 806–813 (2014).

34. Fischlechner, M. *et al.* Evolution of enzyme catalysts caged in biomimetic gel-shell beads. *Nat. Chem.* **6,** 791–796 (2014).

35. Cecchini, M. P. *et al.* Ultrafast surface enhanced resonance Raman scattering detection in droplet-based microfluidic systems. *Anal. Chem.* **83,** 3076–3081 (2011).

36. Chen, D. *et al.* The chemistrode: a droplet-based microfluidic device for stimulation and recording with high temporal, spatial, and chemical resolution. *Proc. Natl. Acad. Sci.* **105,** 16843–16848 (2008).

37. Deal, K. S. & Easley, C. J. Self-regulated, droplet-based sample chopper for microfluidic absorbance detection. *Anal. Chem.* **84,** 1510–1516 (2012).