Bead-emulsion technology for directed evolution of enzymes

Directed evolution, a technology to improve proteins towards novel desirable properties, is based on introducing mutations on the genetic level, subsequent screening for improved variants, and iteration. With enzymes, assays usually require reaction vessels which limits the number of clones that can be screened with conventional methods. Using microfluidic, picoliter-sized emulsion droplets for assays enables directed evolution experiments to have a much higher throughput and thus improved chances of success. We developed two novel formats for emulsion-based directed evolution campaigns, the first based on transforming monodisperse water-in-oil (w/o) emulsion droplets into water-in-oil-in-water (w/o/w) double emulsions, the second transforming emulsions into gel-shell beads. Both approaches allow to analyze and sort the resulting microcompartments with fluorescence-activated cell sorting (FACS). The resulting workflows, due to their exceptional high throughput, provide novel possibilities for research while being simple enough to be adapted by laboratories not specialized in microfluidic technology.

From enzyme assays in emulsion droplets to library screens

Monodisperse emulsion droplets are produced in microfluidic chips with high throughput (up to 10 kHz) so that single cells (containing enzyme alongside encoding plasmid) are compartmentalized together with substrate. For a lysate assay, cells are destroyed directly after droplet formation, starting the enzymatic reaction. Monodispersity of the sample allows precise and quantitative readout reflecting the amount of substrate turned over to fluorescent product. Typically the level of stringency in our selections is adjusted with heat inactivation: the assays are stopped where the "parent"- enzyme shows little or no measurable turnover yet, so that hits can be confidently distinguished. Currently, we can screen libraries of about one million clones per experiment.

Sorting of droplets with Flow Cytometry

In contrast to using sophisticated microfluidic chips for sorting (*Agresti J et al., PNAS, **2010**, 4004*) we transform emulsions into double emulsions or gel-shell beads without losing the required genotype-phenotype linkage. FACS can then be used to screen for improved variants. After sorting the encoding plasmids of selected clones are extracted, amplified and recloned for being applied to another round of screening, mutation or in-depth monoclonal analysis using 96-well plates. In this way, the microfluidic tools necessary for directed evolution experiments are reduced to operating a microfluidic droplet generator; all equipment is inexpensive and commercially available. Flow cytometers are widely used for sorting cells and sorting with exceptional high throughput (about 10^8 events/h) is routine with this mature technology.

Double emulsion compartments

The initial emulsion containing enzymatic assays of single library members is transformed into a double emulsion by processing with a hydrophilic flowfocusing chip. This second droplet generator transforms the w/o emulsion into w/o/w double emulsion without decoupling the genotype-phenotype linkage. Size and monodispersity of the first emulsion is preserved. These samples can then be sorted with FACS.

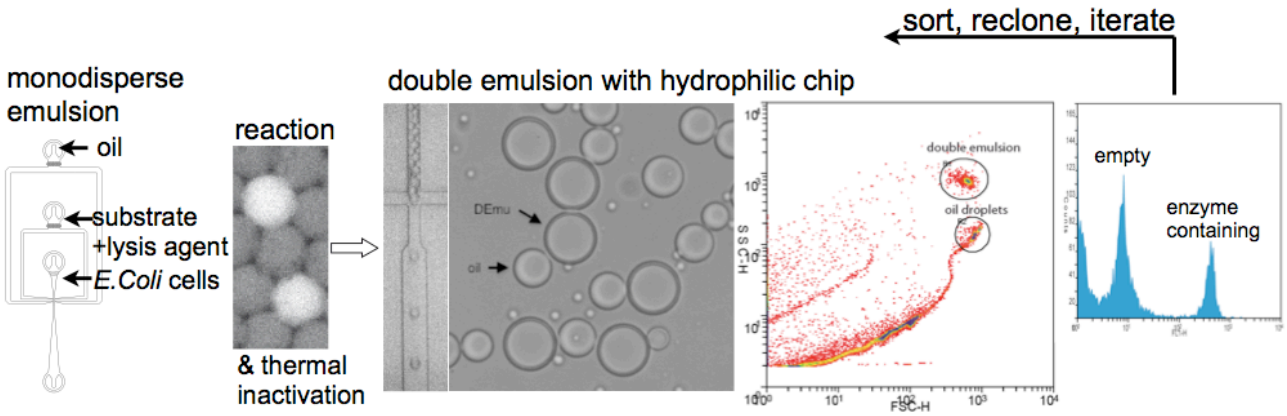

Figure 1: Workflow for directed enzyme evolution using double emulsions. First, a lysate assay based on single cells in emulsion is conducted. The emulsion sample is then processed with a hydrophilic emulsion generator producing monodisperse w/o/w double emulsions containing the enzymatic assays and smaller, not monodisperse droplets containing oil only. These two emulsion populations separate in FACS forward-sidescatter plots and can be gated. The last image on the right shows a fluorescence-histogram of gated double emulsion. The difference in fluorescence from "empty" double emulsions and "full" double emulsion droplets (containing one lysed *E.Coli* cell with expressed enzyme, here enzyme-wildtype) demonstrates the signal range that can be used for gating in a library selection.

Gel-shell beads

With gel-shell beads, monodispersity and colocalization of genotype and phenotype of emulsions containing enzyme assays is maintained by substituting the oil-water interface with a polyelectrolyte shell around the particles. The initial emulsion contains agarose and alginate alongside cells, substrate and lysis agent. Cooling of the sample after heat inactivation of the enzymatic assay gelates the agarose core and provides a spherical structure. Breaking the emulsion sample into a solution containing a polycation leads to a diffusion-based formation of a polyelectrolyte complex at the former droplet interface. This approach, in contrast to double emulsions, facilitates processing of a multitude of samples, because shell fabrication can be performed in parallel in tubes rather than using microfluidic chips.

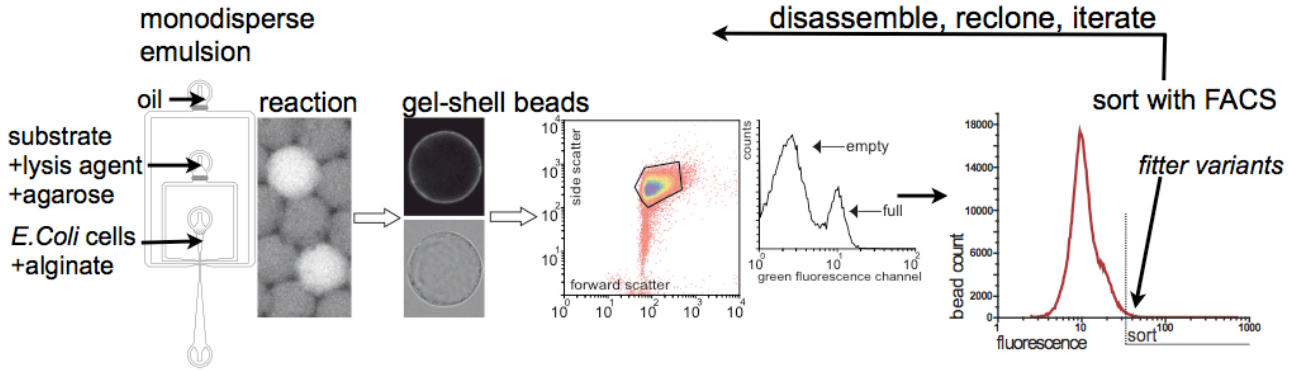

Figure 3: Workflow for directed enzyme evolution using gel-shell beads. *E.coli* cells with expressed enzymes are introduced into droplets following a poisson distribution. After droplet formation cell lysis takes place and the enzyme starts to turn over the substrate allowing for the fluorescent product to accumulate. After incubation, emulsion samples are heated to 95°C to inactivate the enzymes. This decouples enzyme reaction from further treatment of samples. Subsequently the droplets are put on ice to allow for gel-bead formation. These samples, which contain the polyanion alginate are then deemulsified together with emulsion containing a polycation to form beads composed of an agarose core equipped with a compartmentalizing polyelectrolyte complex at their interface. The image shows a brightfield microscopy image of a bead together with a fluorescence image highlighting the polyelectrolyte shell using a fluorescein-labelled polycation. Flow cytometry measurements show a population of single beads in forward-sidescatter plots. In the case of an emulsion sample harboring a monoclonal enzyme assay, the fluorescence histogram of single beads splits up the signal into two distinct populations: one population of empty beads and a second population initially equipped with a single cell and therefore expressed enzyme at the assay endpoint. The graph on the right shows a fluorescence histogram of a library screen. The assay-emulsion has been inactivated at a point where only a small fraction of samples is near the assay endpoint. Beads obtained from this region of the activity landscape show improved catalysis. After sorting, the polyelectrolyte interface is removed, the DNA-plasmids recovered and recloned for the next iteration.

Engineering phage M13 for directed enzyme evolution

Phage display, for example based on filamentous phage M13, is highly developed and routinely used in high throughput library technologies for finding specific antibodies or other binders. Working with a virus particle instead of plasmids in droplet-based directed evolution experiments would have benefits: Iterative cycles could be shortened because only infection of host cells is required after sorting rather than extraction of plasmids from samples following cloning. Protocols for making large libraries (up to 10^9 library members) are in the public domain. We genetically engineered a phage M13-based phagemid system such, that phagemid containing *E.coli* cells expressed soluble enzyme intracellularly. Upon infection with helper phage, infectious phages are produced containing phagemid ssDNA. We applied this system to emulsion-based enzyme assays. Superinfected cells (*E.coli* containing phagemid and helper phage) were introduced into emulsions, incubated to produce infectious phage particles monoclonally in droplets and enzymatic assays were started by freeze-thawing emulsion droplets to induced cell lysis and substrate turnover. Alternatively, we introduced superinfected cells into agarose containing emulsion droplets and incubated. After production of monoclonal infectious phage particles inside the droplets, agarose particles (containing immobilized phage progeny together with the corresponding enzyme-containing *E. coli* cells) were prepared. Further work is in progress to demonstrate the utility of this type of genotype-phenotype linkage for directed evolution.