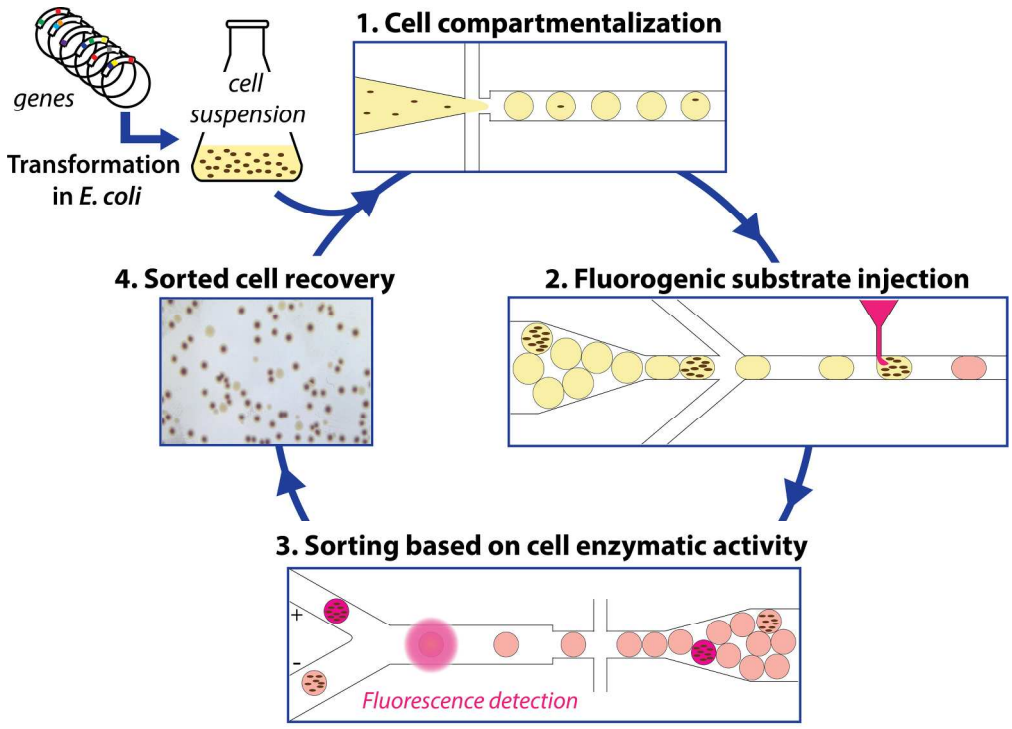
Analyst

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/analyst



106x76mm (600 x 600 DPI)

A high-throughput cell analysis and sorting platform using droplet-based microfluidics is introduced for directed evolution of recombinant CotA laccase expressed in *E. coli*.

Analyst

RSCPublishing

ARTICLE

CotA laccase: high-throughput manipulation and analysis of recombinant enzyme libraries expressed in *E. coli* using droplet-based microfluidics

Cite this: DOI: 10.1039/xoxxoooooox

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

www.rsc.org/

Thomas Beneyton[ab], Faith Coldren[a], Jean-Christophe Baret[cd], Andrew D. Griffiths[ab] and Valérie Taly[ae]

We present a high-throughput droplet-based microfluidics analysis/screening platform for directed evolution of CotA laccase: droplet-based microfluidics modules were combined to develop an efficient system that allows cell detection and sorting based on enzymatic activity. This platform was run on two different operating modes: an "analysis" mode allowing the analysis of enzymatic activity in droplets at very high rates (>1000 Hz) and a "screening" mode allowing sorting of active droplets at 400 Hz. The screening mode was validated for the directed evolution of the cytoplasmic CotA laccase from *B. subtilis,* a potential interesting thermophilic cathodic catalyst for biofuel cells. Single *E. coli* cells expressing either the active CotA laccase (*E. coli CotA*) or an inactive frameshifted variant (*E. coli ΔCotA*) were compartmentalized in aqueous droplets containing expression medium. After cell growth and protein expression within the droplets, a fluorogenic substrate was "picoinjected" in each droplet. Fluorescence-activated droplet sorting was then used to sort the droplets containing the desired activity and the corresponding cells were then recultivated and identified using colorimetric assays. We demonstrated that *E. coli CotA* were enriched 191-fold from a 1:9 initial ratio of *E. coli CotA* to *E.coli ΔCotA* cells (or 437-fold from a 1:99 initial ratio) using a sorting rate of 400 droplets.s^{-1}. This system allows the screening of 10^6 cells in only 4h, compared to 11 days for screening using microtitre plate-based systems. Besides this low error rate sorting mode, the system can also be used at higher throughputs in an "enrichment" screening mode to make an initial purification of a library before further steps of selection. The analysis mode, without sorting, was used to rapidly quantify the activity of a CotA library constructed using error-prone PCR. This mode allows the analysis of 10^6 cells in only 1.5 h.

1. Introduction

Directed evolution is a powerful biotechnological tool that mimics and accelerates natural evolution at the laboratory scale. It relies on inducing artificially mutations to create populations of new variants and selecting the rare ones which exhibit improved functionality under a user-defined and controlled selection pressure. It has been intensively used this last decade to improve enzymes[1] or microorganism strains[2] for industrial applications or, for example, to study the fundamentals of evolution itself[3]. The impact of directed evolution is however limited by its need for efficient high-throughput screening methods, especially when selection occurs on enzymatic activity. Indeed, efficient directed evolution requires screening of very large libraries under conditions that closely match the desired activity. As genetic information is encoded in the genotype and the selection is based on phenotypic activities, genotype and phenotype have to be linked. Ideally, they must be physically linked by compartmentalization as it is in cells. Nowadays, the most controlled and flexible high-throughput enzymatic activity screening technique is the robotized compartmentalization of assays in microtitre plates, allowing to process up to 1 assay a second. This technology has even reached its physical limits as reducing assay volumes to below 1 µL is problematic due to evaporation and capillarity forces[4]. One way to miniaturize the reaction vessels exploits microarrays and microfluidic lab-on-chip technology, decreasing the reaction volume to ~250 pL[5]. Nevertheless, these screening techniques are still limiting the applicability of directed evolution[6]. Another way is to use *in vitro* compartmentalization (IVC): genotype and phenotype are linked by compartmentalizing individual components of a library in water-in-oil droplets[7]. Firstly used with bulk emulsion, IVC is suitable for *in vitro* evolution, reducing the reaction volume below 1 pL and allowing screening of large libraries (up to 10^{11})[8]. However, the range of activities that can

be screened and the flexibility of the method is rather limited because of the lack of control of droplets volume and timing. Moreover, most of assays require the addition of new reagents at defined times to initiate or terminate a reaction. As bulk emulsions are hardly manipulated, the applicability of IVC is markedly limited in that case. These limitations can be overcome by using droplet-based microfluidics technology, which offers the possibility to use IVC in a highly controlled manner.

Indeed, droplet-based microfluidics allows the production of highly monodisperse water-in-oil droplets and the manipulation of those ones at the single level and very high frequencies[9]. During recent years, this technology was subject to an increasing interest for screening applications, which has led to intensive developments of efficient tools to handle single droplets within an emulsion. Different droplet-based microfluidics modules have been developed to perform controlled droplet manipulation, such as fusion[10-12], picoinjection[13, 14], incubation[15, 16] or sorting based on fluorescence[17], especially in the droplets volume range of 10 pL to 100 pL, suitable for biological assay and cells handling. This technology has come to the point where single modules can be combined to create efficient integrated screening platforms[18]. However, very few directed evolution platforms have been described in the literature. An integrated encapsulation/fusion/sorting platform has been developed for complete *in vitro* directed evolution[19]. *In vivo* screening platforms have also been described but their flexibility is limited as they only consist in encapsulation/sorting techniques[17, 20-22]. A flexible and efficient *in vivo* screening platform for directed evolution would allow the growth of cells within droplets and then the addition of the substrate to initiate the enzymatic reaction at a controlled time prior to sorting.

CotA laccase is an oxidoreductase (EC 1.10.3.2) that catalyzes the one-electron oxidation of phenols, polyphenols and anilines with the concomitant four-electron reduction of O_2 to water[23]. Due to their broad substrate specificity and few requirements, laccases such as CotA have a high interest for green industrial applications, such as delignification of wood, biosensors or biofuel cells[24]. We designed a flexible droplet-based microfluidics platform that can be used for high-throughput screening of *E. coli* cells for CotA enzymatic activity (Fig.1). This platform involves the following steps: (i) encapsulation of cells in aqueous droplets; (ii) incubation for cell growth and protein expression in droplets; (iii) picoinjection of the fluorogenic substrate; (iv) incubation for enzymatic reaction; (v) sorting of the droplets based on enzymatic activity according to fluorescence intensity; (vi) recovery of the cells from sorted droplets. We demonstrated that this high-throughput screening platform allows the enrichment of specific cells according to CotA enzymatic activity and should be a powerful tool to screen large libraries for CotA directed evolution experiments. Furthermore, the droplet-based microfluidics platform provides also a powerful high-throughput cell analysis tool to quickly quantify the distribution of enzymatic activity within a library. We created a library of CotA variants by error-prone PCR and analyzed the library of cells obtained by transformation of the library. In general, such screening tools could be extended to many other laccases or oxidoreductases, enzymes of high industrial interest.

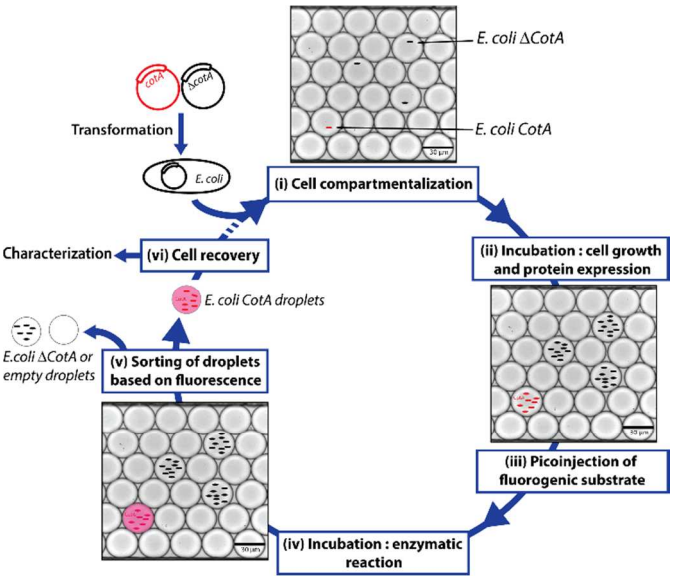


Fig. 1 Schematic of the droplet-based microfluidics model screening platform. Wild type *cotA* gene and frameshifted inactive variant *ΔcotA* gene were transformed into *E. coli* cells to give *E. coli CotA* and *E. coli ΔCotA* strains. **(i)** A mixture of *E. coli CotA*/*E. coli ΔCotA* cells suspensions was encapsulated in droplets containing induction medium. **(ii)** Emulsion was incubated off-chip for cells growth and CotA or ΔCotA enzymes expression within the droplets. **(iii)** Fluorogenic substrate was picoinjected in every droplet. **(iv)** Emulsion was incubated off-chip for enzymatic reaction. **(v)** Droplets were sorted based on enzymatic activity according to fluorescence intensity. **(vi)** Cells were recovered from sorted droplets and could be either characterized to determine enrichment in *E. coli CotA* cells or engaged in another round of selection.

2. Experimental

2.1 Reagents

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonicacid) (ABTS), ampicillin, *D*-glucose, ethylenediaminetetraacetic acid (EDTA), copper (II) chloride were purchased from Sigma-Aldrich. Isopropyl β-*D*-1-thiogalactopyranoside (IPTG), LB medium and LB agar were obtained from Roth. Dextran Texas Red (10 000 MW), Dextran fluorescein (10 000 MW) and fluorescein were purchased from Life Technologies.

2.2 Fabrication of Microfluidic devices

Microfluidic devices were fabricated from poly-(dimethylsiloxane) (PDMS) using standard soft-lithography methods[25]. A mold of SU8-2015 negative photoresist (MicroChem Corp.) was prepared on a silicon wafer (Siltronix)

by UV exposure (MJB3 contact mask aligner; SUSS MicroTec) through a photolithography mask (Selba SA) and subsequent development (SU-8 developer; MicroChem Corp.). A curing agent was added to the PDMS base (Sylgard 184 elastomer kit; Dow Corning Corp.) to a final concentration of 10% (w/w), mixed, and poured onto the mold. After degassing under vaccum to remove air bubbles, the mold was incubated several hours at 65°C for PDMS cross-linking. PDMS was then peeled off and inputs and outputs were punched with a 0.75 mm-diameter biopsy punch. The structured side of the PDMS slab was bound to a 75 x 50 x 1 mm glass microscope slide (Corning) by exposing both parts to an oxygen plasma (Plasma system FEMTO; Diener Electronic). The device was incubated several minutes at 65°C. Finally, an additional hydrophobic surface coating was applied to the microfluidic channel walls by injecting channels with 1% (w/w) 1H, 1H, 2H, 2H-perfluorodecyltrichlorosilane (97%; ABCR) in HFE7100 fluorinated oil (3M).

Electrodes were included in the microfluidic device as additional channels which were filled with metal: the device was heated at 110 °C and a 51In/32.5Bi/16.5Sn low-temperature solder (Indium Corp.) was melted inside the electrode channels[26]. Electrical connections with the solder electrodes were made with short piece of electrical wire (Radiospares) and strengthened with Loctite 3526 UV glue (Epotecny).

2.3 Optical setup, data acquisition and control system

The optical setup consisted of an Axiovert 200 inverted microscope (Carl Zeiss SAS) mounted on a vibration-dampening platform (Thorlabs GmbH). A 20 mW, 488 nm solid-state laser (LAS; Newporte-Spectraphysics) and a 50 mW, 532 nm solid-state laser (CrystaLaser) were focused through the objective lens (40x/0.6; Carl Zeiss SAS) across a channel within the microfluidfic device. Light emitted from fluorescing droplets was captured by the objective, channeled back along the path of the lasers. Emitted light was separated from the lasers beam and split between two photomultiplier tubes (PMT, H5784-20; Hamamatsu), which captured the light through a 510 nm bandpass filter (510/20-25; Semrock Inc.) for the green PMT and through a 617 nm bandpass filter (617/73-25, Semrock Inc.) for the orange PMT. The signal output from the PMT was analyzed using a PCI-7831R Multifunction Intelligent data acquisition (DAQ) card (National Instruments Corporation) executing a program written in LabView 8.2 (FPGA module, National Instruments Corporation, Fig. S1, ESI), which allowed the identification of droplets by peaks in fluorescence, as well as the width of each droplet. The data acquisition rate for the system was 100 kHz. A Phantom v4.2 high speed digital camera (Vision Research) was mounted on the top camera port of the microscope to capture digital images during droplet manipulation.

Liquids were pumped into the microfluidic devices using standard-pressure infusion-only PHD 22/2000 syringe pumps (Harvard Apparatus Inc.). Syringes (Omnifix-F®; BBRAUN) were connected to the microfluidic devices using 0.6 x 25 mm needle (Terumo) and PTFE tubing with an internal diameter of 0.56 mm and an external diameter of 1.07 mm (Fisher Scientific).

2.4 Description of microfluidic devices

We used aqueous droplets in HFE7500 fluorinated oil (3M) stabilized against coalescence by block-polymer homemade surfactant, named KryJeffa900 in this report. KryJeffa900 surfactant was prepared from the commercially available carboxylic acid Krytox157-FSH (Dupont) and Jeffamine® polyetheramines (ED 900, Huntsmann) following a synthesis route based on the one described in Holtze *et al*[27].

All the microfluidics devices were fabricated with a channel depth of 25 µm. Four different modules were used to compose the platform.

Firstly, a dropmaker module (Fig. S2, ESI) was used to generate 14 pL droplets (coefficient of variance (CV) = 3%) at 1600 Hz by flow-focusing of the aqueous stream (80 µl/h) with two streams of HFE7500 fluorinated oil (3M) (190 µl/h) containing 1% (w/w) of KryJeffa900 surfactant. A dual-dropmaker module (Fig. S3, ESI) was used to generate two 14 pL droplets emulsions simultaneously (same condition of operation). The generated emulsions flowed off-chip through PTEF tubing to a syringe.

The second module was a picoinjection device (Fig. S4 and supplementary movie "picoinjection.avi", ESI) in which droplets were reloaded (70-160 µl/h) and spaced-out at a flow-focusing junction (Fig.3b) with HFE7500 fluorinated oil (3M) (200-600 µl/h) containing 1% (w/w) of KryJeffa900 surfactant. The picoinjected phase was loaded into a 250 µL gas-tight syringe (Hamilton Company) and pumped into the device (Fig.3b). A continuous AC field (20 KHz; 200 V) was applied across the electrodes and more than 99.5% of the droplets were picoinjected (~ 1 pl; estimated by video analysis) at 1500-3000 Hz (working range of the module) to give droplets of ~15 pL (CV = 4.5%). The generated emulsions flowed off-chip through PTEF tubing to a glass vial immerged in ice to slow down the enzymatic reaction. The off-chip incubation time at room temperature (25°C) was determined by the length of the PTFE tubing connected from the chip to the glass vial: droplets were incubated at 25°C while they were flowing to the vial (collection) and from the vial (reinjection).

Under screening mode operation, the last module was a fluorescence-activated droplet sorting (FADS)[16] (Fig. S5, ESI) in which droplets were reloaded (20-70 µl/h) and spaced-out at a flow-focusing junction (Fig.3c) with HFE7500 fluorinated oil (3M) (500-1000 µl/h). The droplets were analyzed by the optical setup and fluorescent droplets were sorted at 400 Hz by applying an AC field pulse (30 kHz; 1000-1400 V; 0.5 ms). Sorted droplets were collected in a 1.5 mL microcentrifuge tube (Axygen Inc.).

Under analysis mode operation, the last module was a reloading module (Fig. S6, ESI) in which droplets were reloaded (50-150 µl/h) and spaced-out at a flow-focusing

junction with HFE7500 fluorinated oil (3M) (200-400 µl/h). The droplets were detected by the optical setup and analyzed at ~ 1000 Hz.

2.5 Plasmids construction

The plasmid pLOM10 coding for the wild type CotA laccase was kindly provided by Prof. L. O. Martins (Universidade Nova de Lisboa). The plasmid pLOMP3 coding for the inactive frameshifted variant ΔCotA was constructed starting from pLMO10. A mixture of 44 µL of pLOM10 (35 $ng.\mu L^{-1}$), 5 µL of NEB4 buffer 10x (New England Biolabs) and 1 µL of aatII enzyme (20 $U.\mu L^{-1}$; New England Biolabs) was incubated 15 h at 37 °C. T4 DNA Polymerase (3U; New England Biolabs) and 200 µM of dNTP (New England Biolabs) were added and the mixture was incubated 15 min at 12 °C. The reaction was quenched adding 10 mM of EDTA and the mixture was incubated 20 min at 75 °C. After purification using PCR purification kit (Qiagen), 10 µL of DNA (18 $ng.\mu L^{-1}$) was mixed with 2 µL of T4 DNA ligase buffer 10x (New England Biolabs), 1 µL of T4 DNA ligase (400 $U.\mu L^{-1}$; New England Biolabs) and 7 µL of ultrapure water. The mixture was incubated 3 h at 25 °C. The plasmid was then transformed into Xl10 gold *E. coli* competent cells (Stratagene). 12 colonies were isolated and sequenced.

2.6 Preparation of cell suspensions for encapsulation

Plasmids pLOM10 and pLOMP3 were transformed into *E. coli* C41(DE3)[28] using standard molecular biology protocols to give an active stain (*E. coli CotA*) and an inactive strain (*E. coli ΔcotA*) in which the CotA or ΔCotA protein could be produced under the control of the T7*lac* promoter. 2 mL of LB medium containing ampicillin (0.1 $mg.mL^{-1}$) was inoculated with a single colony of the desired strain. This aliquot was grown for 14 h at 37 °C (230 rpm). The following day, 50 mL of fresh LB medium containing ampicillin (0.1 $mg.mL^{-1}$) and $CuCl_2$ (0.25 mM) was inoculated with 500 µL of preculture. The culture was grown under the same conditions to reach an OD_{600} of 0.6. 5 mL of culture was centrifuged (10 min; 2000 g; 4 °C) and the pellet was resuspended in 5 mL of fresh LB medium containing ampicillin (0.1 $mg.mL^{-1}$), $CuCl_2$ (0.25 mM) and IPTG (1 mM) for induction of protein expression. OD_{600} was measured and the culture was diluted to have desired number of cells per droplet volume (OD_{600} of 1 corresponded to 5.10^8 $cells.mL^{-1}$).

2.7 CotA and ΔCotA enzymatic activity in droplets

The dual-dropmaker module (Fig. S2, ESI) was used to produce simultaneously two emulsions. *E. coli CotA* cells suspension was encapsulated in 14 pL droplets containing 1 µM of Dextran fluorescein while *E. coli ΔCotA* cells suspension was encapsulated in 14 pL droplets containing 5 µM of Dextran fluorescein. The binary emulsion was incubated 15 h at 30 °C and reloaded into the picoinjection module (Fig. S3, ESI). LB medium containing Amplex®UltraRed (0.5µM; Life Technologies) was used as the picoinjected phase. After 10 min of incubation at room temperature (25°C), the emulsion was then reloaded into the reloading module (Fig. S5, ESI) for analysis.

2.8 Screening mode: model selection

E. coli CotA and *E.coli ΔCotA* cells suspensions were mixed together in the desired ratio prior to encapsulation using the dropmaker module (Fig. S1, ESI). The emulsion was incubated 15 h at 30 °C and reloaded into the picoinjection module (Fig. S3, ESI). LB medium containing Amplex®UltraRed (0.5µM; Life Technologies) was used as the picoinjected phase. After 10 min of incubation at room temperature (25°C), the emulsion was then reloaded into the FADS module (Fig. S4, ESI) for sorting. Sorted droplets were recovered by cutting the collection PTFE tubing and draining last droplets into the collection microcentrifuge tube. To dislodge any droplets remaining in the tubing, it was flushed with 200 µL of fresh LB medium. All the contents of the collection microcentrifuge tube was added to 10 mL of fresh LB medium containing ampicillin (0.1 $mg.mL^{-1}$) and grown for 15 h at 37°C. As a reference, 250 µL of initial emulsion was grown under the same conditions.

Cells before and after sorting were then identified using two different techniques. On one hand, sorted cells were plated on LB Agar containing ampicillin (0.1 $mg.mL^{-1}$), $CuCl_2$ (0.25 mM), IPTG (1 mM) and ABTS (5 mM) and incubated for 15 h at 30°C. Plates were then kept at 4°C during 48 h for coloration to occur. *E. coli CotA* colonies were colored in dark brown while *E.coli ΔCotA* colonies remained colourless (Fig.$3d_2$).

On the other hand, sorted cells were plated on LB Agar containing ampicillin (0.1 $mg.mL^{-1}$) and incubated for 15 h at 37°C. 87 colonies were transplanted in a 96-deepwells microtitre plate (Nunc) in 1 mL of fresh LB medium containing ampicillin (0.1 $mg.mL^{-1}$), $CuCl_2$ (0.25 mM) and *D*-glucose (20 mM) with 3 positive controls (*E. coli CotA*), 3 negative controls (*E.coli ΔCotA*) and 3 blanks (growth medium). The microtitre plate was covered by a gas-permeable membrane (Thermofisher) and incubated 15 h at 37°C (250 rpm). The microtitre plate was then centrifuged (10 min; 1000 g) and the medium was replaced with fresh LB medium containing ampicillin (0.1 $mg.mL^{-1}$), $CuCl_2$ (0.25 mM), IPTG (1 mM). The microtitre plate was covered by a gas-permeable membrane (Thermofisher) and incubated 15 h at 30°C (250 rpm). The CotA enzymatic activity of each wells was tested in a standard 96-well microtitre plate by mixing 50 µL of cells with 50 µL of acetate buffer (50 mM; pH 4.5) containing ABTS (5 mM). CotA enzymatic activity resulted in green coloration of the solution (Fig.$3d_1$).

2.9 Library construction

Primers cotAprom (5'-TGTGACTCAGATCTCGATCCCGCGAAATTAATAC-3') and cotAterm (5'-GGTTATGCTAGTTATTGCTCAGCGG3'), biotinylated in 5', were used to PCR-amplify a 1808-bp

fragment containing the *cotA* gene under mutagenesis conditions using GeneMorph® II Random mutagenesis kit (Stratagene). A mixture of 1µL of plasmid PLOM10 (100 ng.µL^{-1}), 2.5µL of cotAprom (10µM), 2.5µL of cotAterm (10µM), 1µL of dNTP (5mM each, New England Biolabs), 1µL of Mutazyme® II (2.5 U.µL^{-1}), 5µL of buffer (10x) and 37.5µL of RNAse/DNase free water (Roth) was submitted to PCR reaction in a MT Research thermocycler. The thermal cycle included 240s initial denaturation at 94°C followed by 30 cycles of thermal cycling and 420s of final extension at 72°C. The thermal cycling protocol consisted of 30s at 94°C, 30s at 55°C, and 120s at 72°C. The PCR product was captured using streptavidin coated magnetic beads (Dynabeads® M-280, kilobaseBinder™ kit, Invitrogen) and released from the magnetic beads using the two cloning sites *BglII* and *NotI*.

An "easy-purification" acceptor vector pET-TB was constructed by modifying pET-21a(+) vector (Novagen) : pET-21a(+) was digested using *BglII*/*NotI* cloning sites and a small oligonucleotides cassette was inserted using $K7_1$ (5'-GGCCGCAAGCTTCA-3') and $K7_2$ (5'-GATCTGAAGCTTGC-3') oligonucleotides phosphorylated in 5'. This plasmid pET-TB was easily purified prior to cloning step as its digestion using *BglII* and *NotI* cloning sites generates a small 14-bp fragment, easily eliminated using a PCR purification kit (Qiagen).

The purified mutagenesis PCR product was inserted into the acceptor vector pET-TB using *BglII* and *NotI* as cloning sites to give the library plasmid pLOM10mut.

2.10 Analysis mode: library analysis

The library cells suspension was produced as followed: 4 µL of plasmid pLOM10mut was transformed by electroporation into 480 µL of *E. coli* C41(DE3) competent cells (~ 2.10^6 cells per µg of DNA) using standard molecular biology protocols. Cells were then grown for 15 h at 37°C (230 rpm) in 500 mL of fresh LB medium containing ampicillin (0.1 mg.mL^{-1}) and *D*-glucose (20 mM). 500 mL of fresh LB medium containing ampicillin (0.1 mg.mL^{-1}) and $CuCl_2$ (0.25 mM) was inoculated with 500 µL of preculture. The culture was grown at 37°C (230 rpm) to reach an OD_{600} of 0.6. 5 mL of culture was centrifuged (10 min; 2000 g; 4 °C) and the pellet was resuspended in 5 mL of fresh LB medium containing ampicillin (0.1 mg.mL^{-1}), $CuCl_2$ (0.25 mM) and IPTG (1 mM) for induction of protein expression. OD_{600} was measured and the culture was diluted to 0.2 cells per 14 pL.

The dual-dropmaker module (Fig. S2, ESI) was used to produce simultaneously two emulsions. *E. coli CotA* cells suspension was encapsulated in 14 pL droplets containing 10 µM of Dextran fluorescein while the library cells suspension was encapsulated in 14 pL droplets without any marker. The binary emulsion was incubated 15 h at 30 °C and reloaded into the picoinjection module (Fig. S3, ESI). LB medium containing Amplex®UltraRed (0.5µM; Life Technologies) was used as the picoinjected phase. After 10 min of incubation at room temperature (25°C), the emulsion was then reloaded into the reloading module (Fig. S5, ESI) for analysis.

3. Results and discussion

3.1 Screening mode

3.1.1 CotA laccase model

CotA laccase, a 65-kDa protein, is an abundant component of the outer coat layer of endospores produced by the Gram-positive soil bacterium *B. subtilis*[29]. It shows a particular thermostability with a maximal activity at an optimal temperature of 75°C[30]. Due to this thermal resistance, CotA is an interesting catalyst for O_2-based biofuel cells (BFC), devices that use enzymatic catalysts to convert chemical energy into electrical energy[31]. BFC are especially limited by the lifetime and the catalytic activity of theirs biocatalysts. A way to increase BFC efficiency is to use protein engineering to improve biocatalysts[32]. Ideally, a BFC should work under physiological pH and temperature within a single compartment, which can be possible because of the high specificity of cathodic and anodic biocatalysts. The inherent thermostability of CotA laccase makes it attractive as a cathodic catalyst. It has already been shown in previous works that CotA can be used in a BFC[33, 34]. However, its catalytic activity at pH7.0 is reduced by 80% compared to pH4[35]. It would be very interesting to evolve CotA laccase for BFC applications by improving its catalytic activity under physiological pH conditions while preserving its thermostability.

This work reports a microfluidic screening platform to evolve recombinant CotA laccase expressed in *E.coli*. We used the following fluorescent assay based on laccase enzymatic activity: CotA laccase converted the fluorogenic substrate Amplex®UltraRed (AUR) into a fluorescent dye analogue to resorufin (λ_{max} excitation: 568 nm / λ_{max} emission: 581 nm). In order to validate the droplet-based microfluidic screening platform, we built a frameshifted inactive variant *ΔCotA*.

3.1.2 CotA/ΔCota activity in droplets

The activity of both *E. coli CotA* and *E. coli ΔCota* strains was studied within droplets. Each strain was encapsulated in 14 pL droplets containing LB inductive medium (LB_{ind}; LB medium + 0.1 mg.mL^{-1} ampicilin + 1 mM IPTG + 0.25mM $CuCl_2$). The distribution of cells between droplets during encapsulation followed the Poisson distribution[17]. Cell suspensions were diluted to 0.1 cells per 14 pL to minimize co-encapsulation events. Under these conditions, the emulsions were composed of 91% of empty droplets, 9% of droplets contained single cell and less than 1% of droplets were double or multiple encapsulation events. This distribution was confirmed experimentally by encapsulating *E. coli* cells expressing Green Fluorescent Protein (data not shown). The encapsulation rate was also controlled for each experiment by imaging the emulsion after cells growth and counting droplets containing bacteria colonies.

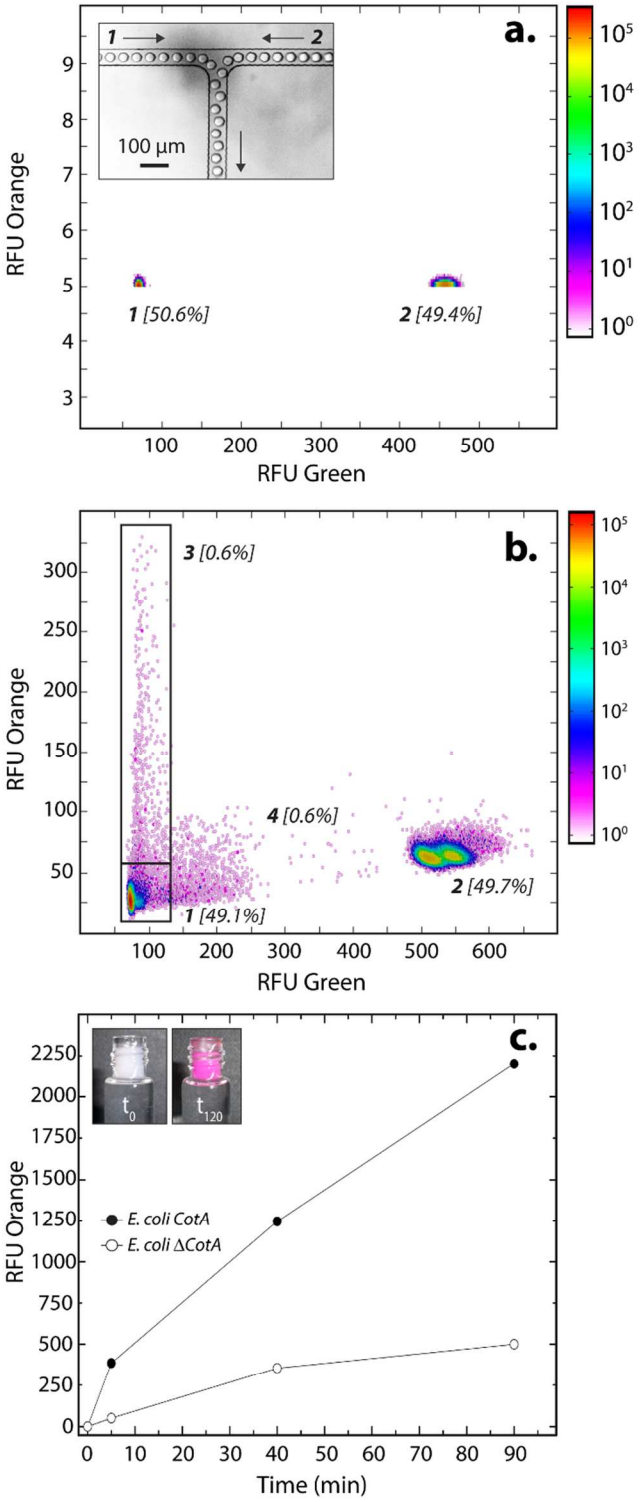


Fig. 2 *E. coli CotA* vs *E. coli ΔCotA* laccase activity in droplets **(a)** 2D contour plot representing the green fluorescence (corresponding to barcode) and the orange fluorescence (corresponding to CotA laccase activity) of two populations of 14 pL droplets containing LB inductive medium (LB medium + 0.1 mg.mL^{-1} ampicilin + 1 mM IPTG + 0.25mM $CuCl_2$) after incubation 15 h at 30°C. Population 1 contained *E.coli CotA* and 1µM of Dextran-fluorescein and population 2 contained *E.coli ΔCotA* and 5 µM of Dextran-fluorescein. Insert shows an image of the dual-dropmaker during encapsulation, focalizing on the area where the two emulsions combined. **(b)** 2D contour plot representing the green fluorescence and the orange fluorescence of the droplets after picoinjection of the AUR substrate and 10 min of incubation at 25°C. Two new populations were detected: population 3 showing CotA laccase activity (3) and a small population 4 due to droplets coalescence. **(c)** Evolution of the average fluorescent signal of *E. coli CotA* (dark) and *E. coli ΔCotA* (white) droplets function of incubation time after picoinjection. Insert shows the emulsion in the collection vial after picoinjection of the AUR substrate and 0 (t_0) or 120 min (t_{120}) of incubation at 25°C.

E. coli CotA and *E. coli ΔCotA* strains were simultaneously encapsulated using dual-dropmaker module and a green fluorescent barcode to tag the two populations (Fig.2a insert). The binary emulsion was incubated 15 h at 30 °C and then reloaded into the picoinjection device for fluorogenic substrate injection. The fluorescent signal was measured before injection (Fig.2a) and after injection and incubation 10 min at 25°C by reloading the emulsion in a reloading module (Fig.2b). *E. coli CotA* strain showed laccase activity with apparition of orange fluorescent active droplets population (Fig.2b, 3 [0.6%]; theoretically 1%) while fluorescent signal of *E. coli ΔcotA* droplets remained unchanged. It also can be noticed that the droplets are efficiently stabilized by surfactant, as only 0.6% of emulsion is coalesced (Fig.2b, population 4). This is mainly due to the fact that our dropmaker devices insured enough stabilization time after droplets formation and before the droplets first come into contact[36].

The evolution of the fluorescent signal of the two populations over incubation time was evaluated by reloading the emulsion after 0, 5, 40 and 90 min incubation after picoinjection (Fig.2c). *E. coli CotA* active droplets showed a significant increase of fluorescent signal over time while *E. coli ΔCota* droplets showed no activity with a slight increase of fluorescent signal. This slight increase of inactive droplets would be due to the leakage of the enzymatic reaction product, a resorufin analogue, from active droplets mediated by the surfactant[37]. The activity ratio *E. coli CotA: E. coli ΔCotA* increased to reach 8 at 10 min and then decreased to remain constant (~3) while equilibrium between enzymatic kinetic and leakage kinetic would be reached. According to those results, the model selection was validated using an incubation time of 10 min prior to sorting.

3.1.3 Model selection

The droplet-based microfluidics screening platform we developed is described on Fig.3. Mixtures of *E. coli CotA / E. coli ΔCotA* strains cell suspensions were encapsulated in 14 pL droplets containing inductive medium LB_{ind} using the dropmaker device (Fig.3a). Emulsions were incubated at 30°C

for 15h to allow cell growth and protein expression within droplets.

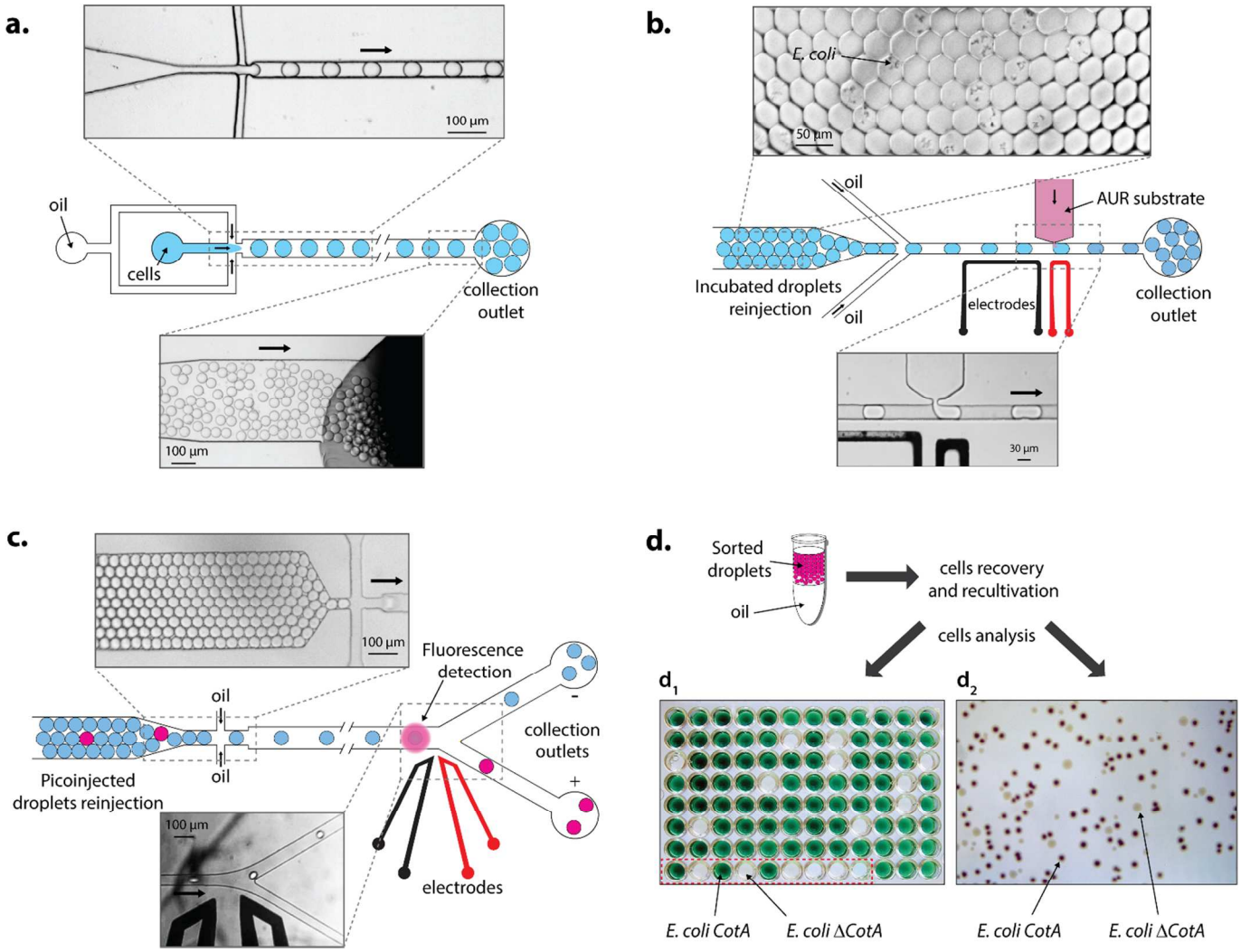


Fig. 3 Description of the droplet-based microfluidic screening platform **(a)** Cell encapsulation: a mixture of *E. coli CotA* / *E. coli ΔCotA* cells in LB_{ind} medium was compartmentalized in 14 pL droplets at 0.1 cell/droplet using HFE-7500 fluorinated oil containing 1% (w/w) KryJeffa900 surfactant. Once collected, the emulsion was incubated 15 h at 30°C for cell growth and protein expression. **(b)** Picoinjection of substrate: incubated droplets were reloaded and spaced using HFE-7500 fluorinated oil containing 1% (w/w) KryJeffa900 surfactant. AUR substrate in LB medium was injected each time a droplet passed in front of the picoinjection head by application of an AC field (20 kHz, 200 V). Emulsion was collected in ice. **(c)** Fluorescence-activated droplets sorting: after 10 min of incubation at 25°C, picoinjected droplets were reloaded and spaced using HFE-7500 fluorinated oil. Fluorescent-activated AC field pulses (30 kHz, 1400 V, 0.5 ms) were used to deflect fluorescent droplets in the positive thinner collection outlet. **(d)** Cell recovery and characterization: sorted droplets were placed in 10 mL of LB medium containing 0.1 $mg.mL^{-1}$ of ampicilin and incubated 15h at 37°C. Two techniques were then used: (d1) cells were plated on LB Agar medium plate. After growth, colonies were then cultivated separately in a microtitre plate. Protein expression was induced and ABTS substrate was added. Wells showing CotA laccase activity became green while others stayed colourless. Wells in the red square are controls with 3 *E. coli CotA* (green) and 3 *E. coli ΔCotA* (colourless) and then 3 without inoculation (colourless). (d2) cells were directly plated on a LB Agar medium plate containing IPTG and ABTS substrate. After growth and coloration, colonies showing CotA laccase activity became dark while others stayed colourless.

Droplets were reloaded in the picoinjection module (Fig. 3b) where AUR fluorogenic substrate was picoinjected in every droplet (~ 1 pL). After 10 min of incubation at 25 °C, droplets were reloaded in a FADS module for sorting based on enzymatic activity according to fluorescence intensity (Fig. 3c). The active droplets population, defined with a fluorescence sorting threshold, was sorted at a rate of 400 Hz and recovered. Sorted cells were grown and characterized to determine sorting efficiencies (Fig. 3d). Two colorimetric assays were developed to identify sorted cells. In both cases, cells were firstly grown in

LB medium. Then, cells were either recultivated separately in 96-deep wells microtitre plates where the colorimetric assay was then performed (Fig.3d$_1$) or directly plated on colorimetric inductive agar medium (Fig.3d$_2$). The second identification methods was more suitable to analyze large number of colonies than the first one, which was limited to 87 colonies and 9 controls per microtitre plate.

In a previous work, the fluorescence-activated droplet sorting module (FADS) has been reported to work with a low error rate within a specific operating range[17]. In that case, the main factor limiting the sorting enrichment is the co-encapsulation events of *E. coli ΔCotA* cells with *E. coli CotA* cells. A model for theoretical enrichment was previously described based upon the Poisson distribution during encapsulation[17]. The theoretical enrichment (η_t) is then given by the equation:

$$\eta_t = \frac{1}{1 - exp(-\frac{\varepsilon_0 \lambda}{1+\varepsilon_0})}$$

where ε_0 is the initial ratio of *E. coli CotA* to *E. coli ΔCotA* and λ is the initial mean number of cells per droplet. The enrichment is defined as the ratio of ε after sorting (ε_1) to ε before sorting (ε_0). λ was determined for each emulsion by imaging the emulsion and using the equation (in the limit of Poisson encapsulation):

$$\lambda = -\ln(1-k)$$

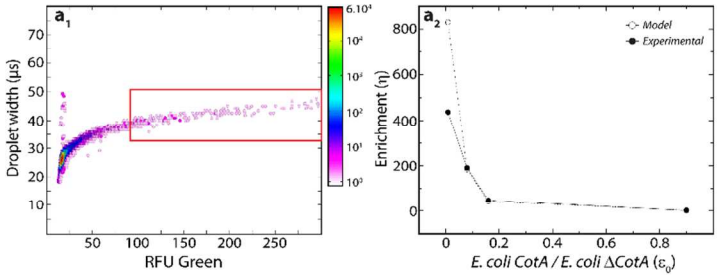
where $k \leq 1$ is the fraction of droplets occupied by colonies. A series of sorts was performed at a rate of 400 Hz using different ε_0 and λ values to demonstrate that our screening platform was following this model. The results are shown in the table of Figure 4. For each emulsion, the CotA active droplets population, defined by the red box sorting threshold, was sorted (Fig. 4a$_1$). This sorting threshold was defined as referred to the average fluorescent signal of the inactive droplets population: in the case of a sorting threshold of 8, droplets showing a fluorescence signal 8 times higher than the average fluorescent signal of inactive droplets population were sorted. ε before sorting (ε_0) and after sorting (ε_1) were determined using the colorimetric assays we developed based on CotA laccase activity (Fig.4b). Experimental enrichments η_{exp} were calculated and plotted against the theoretical model (Fig. 4a$_2$).

As expected, experimental data were closely fitting the theoretical model: η_{exp} was observed to increase decreasing ε or decreasing λ. Sorting at very low ε (0.009) lead to high enrichment. η_{exp} is only half η_t in that case, which can be explain by the lower sorting threshold (2) implying that more false positive droplets were caught. Those results highlighted the fact that this screening technique offers two operation modes. Working at high λ imply more co-encapsulation events (26% for λ = 1) but higher throughput (250 cells.s^{-1} for λ = 1) and is suitable for fast enrichment of a library before further finest selection steps. Working with low λ (λ < 0.2) imply less co-encapsulation events (1.8%) and lower throughput (70 cells.s^{-1}) and is suitable to sort cells to high purities for fine selection steps.

The major advantage of this droplet-based microfluidics platform relies on the flexibility brought by the injection of the substrate at a defined time. This allows to adapt the incubation time prior to sorting to the kinetic of the enzymatic reaction and therefore make the selection occurs in the linear range of the kinetic

a.

λ	ε_0	number of droplets analyzed	number of droplets sorted	Sorting threshold	ε_1	η_t	η_{exp}
0.17	*0.16*	*1.10^5*	*1500*	*8*	*7.68*	*43*	*48*
0.135	0.009	1.10^5	150	8	3.93	831	437
0.071	**0.08**	**1.10^5**	**150**	**8**	**15.28**	**191**	**191**
0.1	0.9	1.10^5	50	2	1.12	2.65	1.24



b.

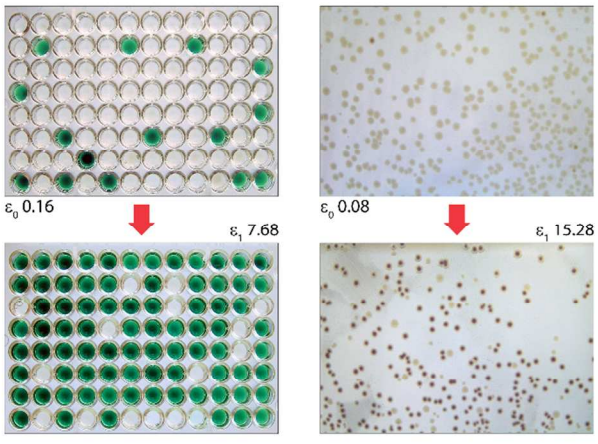


Fig. 4 Model selection of CotA laccase activity **(a)** Table summarizing the results for different sorts: λ, ε_0, ε_1, theoretical (η_t) and experimental (η_{exp}) enrichments. (a$_1$) 2D contour plot representing the width of the droplets (transit time of the droplet passing the detector; µs) and the orange fluorescence (corresponding to CotA laccase activity) of 14 pL LB$_{ind}$ droplets after compartmentalization of a mixture of *E.coli CotA* / *E.coli ΔCotA*, incubation 15 h at 30°C, picoinjection of AUR fluorogenic substrate and incubation 10 min at 25°C. Population defined in the red box sorting threshold was sorted. Cells mixtures before and after sorting were analyzed to compare the initial (ε_0) and final (ε_1) ratio of *E. coli CotA* to *E. coli ΔCotA* cells. (a$_2$) Evolution of sorting enrichment as function of ε_0 for theoretical model (white dots) and experimental data (black dots). **(b)**

Picture of the colorimetric assays performed to determine ε ratio before (ε_0) and after sorting (ε_1). Microtitre plates on the left refer to italic data in the table while Petri dishes on the right refer to bold data in the table.

3.2 Analysis mode

The droplet-based platform can also operate in an analysis mode in which the droplets fluorescence is measured in a reloading module without any sorting. In that case, as FADS is the limiting module in terms of throughput, the analysis of droplets can be performed at very high rates up to 2000 Hz. We constructed a library of 10^6 variants using error-prone PCR. Amplified fragment was purified using a biotin-streptavidin binding and magnetic beads and then cloned into an acceptor vector constructed from pET-21a(+). The library characteristics were evaluated by sequencing twenty variants. It showed, in average, 9-10 mutations per gene that is roughly 2-4 amino acids mutations per variant.

The enzymatic activity distribution of this library was analyzed using CotA wild type as an intern reference: *E. coli CotA* and the library cell suspensions were separately and simultaneously encapsulated (λ = 0.2) using dual-dropmaker module. The binary emulsion was incubated 15 h at 30 °C and then reloaded into the picoinjection device for fluorogenic substrate injection. After 10 min of incubation at 25°C, the enzymatic activity was measured at 1000 droplets.s^{-1} in a reloading module (Fig. 5). A green fluorescent barcode was used to tag the two populations: the CotA intern reference (1 [52%]) and the library (2 [45%]). The encapsulation rate was measured to be 15% of occupied droplets and 85% of empty one. For each both sub-population, the active population was defined using a 250 RFU threshold (red line in Fig. 5): the wild type reference with an average fluorescence signal of 625 RFU (11%) and the library with an average fluorescence signal of 425 RFU (0.25%). This high-throughput analysis (75 variants.s^{-1}) showed that the library contained approximately 2.5% of active mutants or unmutated CotA. As the mutation rate is rather high in this library, it is worth to consider that the proportion of unmodified wild type is quite low. Those 2.5% active variants were in average 1.5 times less actives than wild type CotA. This activity distribution within the library is not surprising as the mutation rate is rather high. The platform allows to analyze libraries using high throughputs. In that example, 150 occupied droplets are analyzed in one second, half referred to wild type and half to the library. In that case, 75 variants are analyzed per second, 10^6 in less than 4 hours. It is possible to work at higher λ values that will increase the throughput: λ = 0.5 allows to analyze 10^6 variants in 1.5 hours with only 9% of co-encapsulation events. Moreover, the intern wild type reference could be used in a smaller ratio than 1:1 with the library, which would increase the throughput of variants analysis. It is then also possible to increase the analysis rate up to 2000 Hz. In that case, with an intern reference/library 1:9 ratio, 10^6 are analyzed in less than 1 h (λ = 0.2) or 10^6 in 20 min (λ = 0.5). Additional gain in analytical throughput might be obtained by parallelization[38]. The efficiency and the throughput could also be theoretically improved by organizing the cells during encapsulation to avoid the Poisson distribution and screen fully loaded emulsion with single cell per droplets[39].

This high-throughput analysis droplet-based microfluidic platform offers a precious tool for directed evolution experiments based on enzymatic activity. It allows the possibility to rapidly study the activity distribution of large libraries with a tremendous saving of time and reagents. Using this new technology, it is possible to follow the distribution of activity after each round of mutagenesis or selection. That could be very helpful for choosing mutagenesis methods and directed evolution strategies to follow a desired evolution path and avoid dead ends.

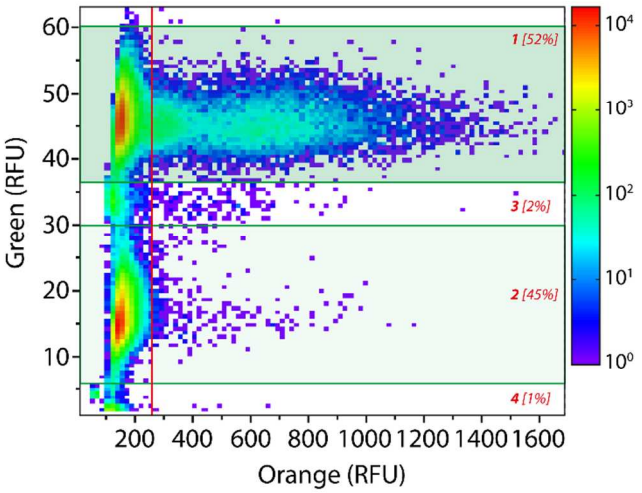


Fig. 5 High-throughput library analysis**:** 2D contour plot representing the green fluorescence (barcode) and the orange fluorescence (enzymatic activity) of a wild type vs. library binary emulsion after picoinjection of the AUR substrate and 10 min of incubation at 25°C. Population [1] refers to wild type CotA intern reference droplets (10 µM Dextran-fluorescein barcode) and population [2] refers to the library droplets (2 µM Dextran-fluorescein barcode). Populations [3] and [4] corresponds respectively to coalesced droplets and unpicoinjected droplets.

4. Conclusions

We have demonstrated a flexible and efficient droplet-based screening technique able to analyze and sort cells based on a recombinant enzymatic activity. The main merit of this screening platform is the injection of the fluorogenic substrate at a defined time, which offers the possibility to tune the incubation time prior to selection depending on the kinetic of the enzymatic reaction. This high-throughput droplet-based microfluidics platform for directed evolution experiments is used to: (i) evaluate the distribution of enzymatic activity within a large library at high-throughput (analysis mode; up to 10^6 variants in 1h). (ii) fast enrich a library in active variants in a high-throughput rate (enrichment mode; up to 10^6 variants in

1h). (iii) select finely variants depending on their enzymatic activity (selection mode; up to 10^6 variants in 4h).

The screening platform is flexible and could be applied to many kinds of recombinant systems implying ideally the secretion of the enzyme or the display of the enzyme at the cell surface, but also, as in the case of CotA laccase, periplasmic or cytoplasmic enzymes (the lysis of a part of the cells within the droplets would be required for the substrate to access the enzyme). Moreover, the incubation time prior sorting could be adapted according to the enzyme kinetics. This platform also gives access to a wide range of controlled selection pressures (pH, temperature…) as the growth of cells and the enzymatic assay are two distinct steps.

It is worth noting that incubating droplets off-chip for such enzymatic reaction time scale (0 - 60 min) introduces a small heterogeneity within the incubation time of each droplet. We are developing an integrated device that can perform picoinjection, incubation on-chip and sorting to attempt higher degrees of control of time and temperature incubation. For longer enzymatic reaction time scale (>60 min), incubation off-chip is necessary and imply that the product of the enzymatic reaction is not leaking out from droplets (which could be overcome using chemical modification of the substrate to make the product more hydrophilic[40]). Such developments should lead to very robust and universal high-throughput screening techniques for directed evolution.

Acknowledgements

T.B. thanks the ADEME (Agence de Développement de l'Environnement et la Maîtrise de l'Energie) and the Region Alsace for financial support. This work was also supported by the Ministère de l'Enseignement Supérieur et de la Recherche, the Centre National de la Recherche Scientifique (CNRS). F.C. was supported by FP-7 FET OPEN Project E-Flux. The authors would like to thank Jean-François Bartolo for surfactant synthesis, Chaouki Ben Salem for technical assistance and Antoine Drevelle for useful scientific discussions.

Notes and references

[a] *Laboratoire de Biologie Chimique, Institut de Science et d'Ingénierie Supramoléculaires (ISIS), CNRS UMR 7006, 8 allée Gaspard Monge, F-67083 Strasbourg*

[b] *Laboratoire de Biochimie, Ecole Supérieure de Physique et de Chimie Industrielles de la ville de Paris (ESPCI ParisTech), CNRS UMR 7084, 10 rue Vauquelin, F-75231, Paris*

[c] *Droplets Membranes and Interfaces, Max Planck Institute for Dynamics and Self-Organization, Am Fassberg, 17, D-37077 Goettingen*

[d] *Université de Bordeaux, CRPP-CNRS, 115 Avenue Schweitzer, F-33600 Pessac*

[e] *Université Paris Sorbonne Cité, INSERM UMR-S775, 45 rue des Saints-Pères, F-75270, Paris.*

† Electronic Supplementary Information (ESI) available: one picoinjection movie, scheme of the LabView program and detailed description of microfluidic modules. See DOI: 10.1039/b000000x/

1 S. Bershtein and D. S. Tawfik, *Curr. Opin. Chem. Biol.*, 2008, **12**, 151

2 J. D. Keasling, *ACS Chem. Biol.*, 2008, **3**, 64

3 S. G. Peisajovich and D. S. Tawfik, *Nat. Methods*, 2007, **4**, 991

4 L. M. Mayr and P. Fuerst, *J. Biomol. Screen.*, 2008, **13**, 443

5 T. Thorsen, S. J. Maerkl and S. R. Quake, *Science*, 2002, **298**, 580

6 J. Lay, R. Liyanage, S. Borgmann and C. L. Wilkins, *TrAC Trend Anal. Chem.*, 2006, **25**, 1046

7 D. S. Tawfik and A. D. Griffiths, *Nat. Biotechnol.*, 1998, **16**, 652

8 A. D. Griffiths and D. S. Tawfik, *Trends Biotechnol.*, 2006, **24**, 395

9 R. Seemann M. Brinkmann, T. Pfohl and S. Herminghaus, *Rep. Prog. Phys.*, 2012, **75**, 016601

10 L. Mazutis, J-C. Baret and A. D. Griffiths, *Lab Chip*, 2009, **9**, 2665

11 K. Ahn, J. Agresti, H. Chong, M. Marquez and D. Weitz, Appl. Phys. Lett., 2006, **88**, 264105

12 L. Frenz, A. El Harrak, M. Pauly, S. Bégin-Colin, A. D. Griffiths and J-C. Baret, *Angew. Chem. Int. Ed.*, 2008, **47**, 6817

13 A. R. Abate, T. Hung, P. Mary, J. J. Agresti and D. A. Weitz, *Proc. Natl. Acad. Sci*, 2010, **107**, 19163

14 S. L. Sjostrom, H. N. Joensson ans H. Andersson Svahn, *Lab Chip*, 2013, **13**, 1754

15 L. Frenz, K. Blank, E. Brouzes and A. D. Griffiths, *Lab Chip*, 2009, **9**, 1344

16 D. Pekin, Y. Skhiri, J-C. Baret, D. Le Corre, L. Mazutis, C. Ben Salem, F. Millot, A. El Harrak, J. B. Hutchison, J. W. Larson, D. R. Link, P. Laurent-Puig, A. D. Griffiths and V. Taly, Lab Chip, 2011, 11, 2156

17 J-C. Baret, O. J. Miller, V. Taly, M. Ryckelynck, A. El Harrak, L. Frenz, C. Rick, M. L. Samuels, J. B. Hutchison, J. J. Agresti, D. R. Link, D. A. Weitz and A. D. Griffiths, *Lab Chip*, 2009, **9**, 1850

18 M. T. Guo, A. Rotem, J. A. Heyman and D. A. Weitz, *Lab Chip*, 2012, **12**, 2146

19 A. Fallah-Araghi, J-C. Baret, M. Ryckelynck and A. D. Griffiths, *Lab Chip*, 2012, **12**, 882

20 S. L. Sjostrom, Y. Bai, M. Huang, Z. Liu, J. Nielsen, H. N. Joensson and H. Andersson Svahn, *Lab Chip*, 2014, DOI: 10.1039/c3lc51202a

21 J. J. Agresti, E. Antipov, A. R. Abate, K. Ahn, A. C. Rowat, J-C, Baret, M. Marquez, A. M. Klibanov, A. D. Griffiths and D. A. Weitz, *Proc. Natl. Acad. Sci*, 2010, **107**, 4004

22 B. Kintses, C. Hein, M. F. Mohamed, M. Fischlechner, F. Courtois, C. Lainé and F. Hollfelder, *Chemistry & Biology*, 2012, **19**, 1001

23 F. J. Enguita, L. O. Martins, A. O. Henriques and M. A. Carrondo, *J. Biol. Chem.*, 2003, **278**, 19416

24 M. Fernández-Fernández, M. Ángeles Sanromán and D. Moldes, *Biotechnology Advances*, 2013, **31**, 1808

25 D. C. Duffy, J. C. McDonald, O. J. A. Schueller and G. M. Whitesides, *Anal. Chem.*, 1998, **70**, 4974

26 A. C. Siegel, D. A. Bruzewicz, D. B. Weibel and G. M. Whitesides, *Adv. Mater.*, 2007, **19**, 727

27 C. Holtze, A. C. Rowat, J. J. Agresti, J. B. Hutchison, F. E. Angile, C. H. J. Schmitz, S. Koester, H. Duan, K. J. Humphry, R. A. Scanga, J. S. Johnson, D. Pisignano and D. A. Weitz, *Lab Chip*, 2008, **8**, 1632
28 B. Miroux and J. E. Walker, *J. Mol. Biol.*, 1996, **260**, 289
29 M. F. Hullo, I. Moszer, A. Danchin and I. Martin-Verstraete, *J. Bacteriol.*, 2001, **183**, 5426
30 L. O. Martins, C. M. Soares, M. M. Pereira, M. Teixera, T. Costa, G. H. Jones and A. O. Henriques, *J. Biol. Chem.*, 2002, **277**, 18859
31 A. Zebda, C. Gondran, A. Le Goff, M. Holzinger, P. Cinquin and S. Cosnier, *Nat. Commun.*, 2011, **2**, 370
32 F. Durand, C. Stimes-Chaumiel, V. Flexer, I. André and N. Mano, *Biochem. Biophys. Res. Commun.*, 2010, **402**, 750
33 T. Beneyton, A. El Harrak, A. D. Griffiths, P. Hellwig and V. Taly, *Electrochem. Commun.*, 2011, **13**, 24
34 T. Beneyton, I Putu Mahendra Wijaya, C. Ben Salem, A. D. Griffiths and V. Taly, *Chem. Commun.*, 2013, **49**, 1094
35 P. Durao, Z. Chen, C. S. Silva, C. M. Soares, M. M. Pereira, S. Todorovic, P. Hildebrandt, I. Bento, P. F. Lindley and L. O. Martins, *Biochem. J.*, 2008, **412**, 339
36 J-C, Baret, F. Kleinschmidt, A. El Harrak and A. D. Griffiths, *Langmuir*, 2009, **25**, 6088
37 T. Chen, A. Wijaya Gani and S. K. Y. Tang, *Lab Chip*, 2012, **12**, 5093
38 J. Lim, J. Vrignon, P. Gruner, C. S. Karamitros, M. Konrad and J-C. Baret, *Appl. Phys. Lett.*, 2013, 203704
39 J. F. Edd, D. Di Carlo, K. J. Humphry, S. Köster, D. Irimia, D. A. Weitz and M. Toner, *Lab Chip*, 2008, **8**, 1262
40 M. Najah, E. Mayot, I Putu Mahendra Wijaya, A. D. Griffiths, S. Ladame and A. Drevelle, *Anal. Chem.*, 2013, **85**, 9807