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Use of photoswitchable fluorescent proteins for droplet-based microfluidic screening

Adilya Dagkesamanskaya[a,*,1], Krzysztof Langer[b,1], Alexandra S. Tauzin[a], Catherine Rouzeau[a], Delphine Lestrade[c], Gabrielle Potocki-Veronese[a], Laurent Boitard[b], Jérôme Bibette[b], Jean Baudry[b], Denis Pompon[a], Véronique Anton-Leberre[a]

[a] LISBP, Université de Toulouse, CNRS, INRA, INSA, Toulouse, France
[b] Laboratoire Colloïdes et Matériaux Divisés, the Institute of Chemistry, Biology and Innovation (CBI) - ESPCI ParisTech, CNRS – UMR 8231, PSL* Research University, 10 rue Vauquelin, 75005 Paris, France
[c] TWB, Université de Toulouse, CNRS, INRA, INSA, Toulouse, France

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ABSTRACT

Application of droplet-based microfluidics for the screening of microbial libraries is one of the important ongoing developments in functional genomics/metagenomics. In this article, we propose a new method that can be employed for high-throughput profiling of cell growth. It consists of light-driven labelling droplets that contain growing cells directly in a microfluidics observation chamber, followed by recovery of the labelled cells. This method is based on intracellular expression of green-to-red switchable fluorescent proteins. The proof of concept is established here for two commonly used biological models, *E. coli* and *S. cerevisiae*. Growth of cells in droplets was monitored under a microscope and, depending on the targeted phenotype, the fluorescence of selected droplets was switched from a "green" to a "red" state. Red fluorescent cells from labelled droplets were then successfully detected, sorted with the Fluorescence Activated Cell Sorting machine and recovered. Finally, the application of this method for different kind of screenings, in particular of metagenomic libraries, is discussed and this idea is validated by the analysis of a model mini-library.

1. Introduction

Droplet-based microfluidics is an innovative and powerful approach to assess cellular properties at the cell and micro-colony level. It has been successfully applied to metagenomics in the past three years (Colin et al., 2015; Gielen et al., 2018; Najah et al., 2014). Diverse, specific features can be detected by this method using absorbance- (Gielen et al., 2016) or fluorescence-activated droplet sorting (Baret et al., 2009) as well as microscopy: enzyme activity (Hosokawa et al., 2015), growth rate (Boitard et al., 2012), colony shape, variations of gene expression, cellular responses to different agents (antibiotics and toxic molecules), biological molecule production and secretion (Mazutis et al., 2013) etc. This method gives the possibility to screen large numbers of individually growing cells and to reveal heterogeneity within a cell population. Applying this technique for screening requires that cells with desired features are recovered following droplet observation in order to perform DNA sequencing or further phenotypic analysis. However, direct and on-the-spot access to a chosen droplet is not possible for the majority of systems used, since the microfluidic channels or chambers are usually well sealed in order to prevent evaporation of continuous and dispersed phases. Therefore, to manage cell recovery from any droplet of interest, cells from selected droplets have to be labelled during observation, thus allowing their subsequent sorting. One of the approaches that is used to discriminate droplets with cells producing specific target molecules (e.g., enzymes and antibodies) from non-producing ones is co-encapsulation with a reporter molecule such as a fluorogenic substrate (Agresti et al., 2010; Hosokawa et al., 2015; Sjostrom et al., 2014; Wang et al., 2014; Zinchenko et al., 2014) or with fluorescent microbeads allowing to capture secreted products (Mazutis et al., 2013). In these cases, fluorescence-based sorting can only be performed at the droplet level, since the fluorescent particles are present in the media surrounding the cells in the droplet and not within the cells themselves. Droplets recovered from the observation chamber can be sorted either by special droplet sorters developed by microfluidics-specialized laboratories (Baret et al., 2009), or by easier and more available commercial fluorescence-activated cell sorting

* Corresponding author.
E-mail address: dagkesam@insa-toulouse.fr (A. Dagkesamanskaya).
[1] These authors contributed equally.

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(FACS) devices (Terekhov et al., 2017; Zinchenko et al., 2014). The latter technique requires conversion of a single, aqueous droplets of water-in-oil emulsion into a double, water-in-oil-in-water emulsion, involving additional steps in droplet preparation. For some types of screening based on contact between the droplets ("droplet shrinking" due to yeast growth, for example, described by (Boitard et al., 2012)) an aqueous layer around the droplets must be added after observation, which can lead to the loss of some positive hits.

Here we describe a new screening strategy that combines the simplicity of water-in-oil droplet generation with that of FACS utilization. This workflow, which allows to specifically label the droplet during microscopy-based phenotype screening and to recover the living cells after FACs sorting, is based on the use of a photoconvertible fluorescent protein (PCFP). PCFPs are fluorescent proteins whose fluorescence properties can be switched on or off by a pulse of light of a specific wavelength. In the last ten years, many new PCFPs were described and novel applications in cell imaging have been developed based on their photo-switching properties, such as the study of the redistribution of a protein of interest inside a cell (Miyawaki et al., 2003), organelle labeling and tracking (Molina and Shirihai, 2009), cell labelling (Lukyanov et al., 2005), protein degradation studies (Zhang, 2007), and super-resolution microscopy (Chudakov et al., 2007). We propose to use switchable fluorescent proteins produced inside the cells of a library for labelling droplets, and we demonstrate the suitability of this method for high-throughput screening of cell growth for both yeasts and bacteria. The potential of this highly generic method is discussed in the context of microbiome functional exploration.

2. Material & methods

2.1. Plasmids, strains and mini-library construction

The pET3-Dendra2 plasmid expressing the Dendra2 (Gurskaya et al., 2006) protein was constructed by cloning of the Dendra2 gene-coding fragment in Nde1 and BamH1 sites of the pET3-a vector (NOVAGEN) under the control of strong T7 promoter. The BL21 strain transformed with this plasmid was used in *E. coli* model experiments.

For mini-library studies, the fluorescent BL21 *E. coli* strain, carrying the pET3-Dendra2 plasmid was additionally transformed with the fosmids extracted from the metagenomic library clones. Transformants were selected and further grown on LB medium with 100 mg L^{-1} ampicillin (Amp) and 12.5 mg L^{-1} chloramphenicol (Cm).

Yeast vectors were constructed by insertion of the mEos2p coding sequence either between the Xba1 and BamH1 restriction sites of the pUG36 (Niedenthal et al., 1996) plasmid (MET25 promoter controlled expression), or between the BamH1 and Pst1 restriction sites of the pCM185 plasmid containing tetO-CYC1 promoter to control expression (Garí et al., 1997). A yeast strain with a deleted TRP1 gene from the YKO collection (MAT alfa hi3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp1::KANMX) was transformed by the resulting plasmids pUG36-mEos2 and pCM185-mEos2.

2.2. Hit clone identification by PCR

DNA inserts of clones obtained during metagenomic library screening were identified by PCR amplification using Phusion High fidelity DNA polymerase (NEB) classical protocol. The following primer pairs were used for amplification of specific regions:

Clone F5 GCGGTCTGCCGATAGCATC and GATGAGTGGCTTGTCC CTG

Clone F3 GAGGTTCGCAGCGGCCAGA and GACGGCGCAAACATG CTGG

Clone I7 CGCACAATACAGAGTCGCG and CGTGCCGGAGGCTG GGG

Clone F4 TGACCATCGAACTGGTCGCC and TCCGGAGACCAAGCA GCC

2.3. Media and growth conditions

The *E. coli* strain carrying the pET3-Dendra2 plasmid was cultivated in LB liquid or solid media with 100 mg L^{-1} Amp at 37 °C.

Yeast strains were grown at 30 °C in YNB selective medium without uracil or tryptophan for the pUG36-mEos2 and pCM185-mEos2 constructs, respectively.

For mini-library growth and for microfluidics screening, minimum synthetic media (M9) with 100 mg L^{-1} Amp and 12.5 mg L^{-1} Cm containing 0.5% xylo-oligosaccharides (XOS) (Wako chemicals, Japan) as the only carbon source was used. Growth of the mini-library strains was performed as described by (Tauzin et al., 2016) with some modifications. Cells grown in LB medium supplemented with 12.5 mg L^{-1} Cm and 100 mg L^{-1} Amp were inoculated in M9 with the same antibiotics and 0.5% of xylose. Overnight cultures from this medium were used to inoculate 0.5 mL of M9 XOS medium at OD_{600} 0.05 into 48-well microplates. The growth was followed by measuring the OD_{600} over 48 h at 37 °C using the FLUOStar Optima (BMG Labtech).

2.4. Droplet generation with the microfluidic device

E. coli or *S. cerevisiae* cell suspensions used for the droplets generation were prepared in the corresponding medium just prior the emulsification procedure.

Droplet generation chips and observation chambers were developed and provided by the Colloïdes and Matériaux Divisés Laboratory (LCMD) from the Ecole Supérieure de Physique et Chimie Industrielles of Paris (ESPCI). The water in oil emulsion with the droplets size of approximately 50 μm was made by flow-focusing the cell suspension stream with two streams of HFE7500 fluorinated oil (3 M) containing 2% (*w*/w) 008-FluoroSurfactant (RAN Biotechnologies) (Boitard et al., 2012).

2.5. Microscope observation and "switching"

For cell growth observation and blue light illumination ("switch") of selected droplets, fluorescent Leica DM4000B microscope and Leica EL6000 light source was used. Pictures were taken using a LEICA DFC300FX camera.

2.6. Cytometry and sorting

Analysis of cell populations extracted from the original or "switched" emulsion was performed on a MACS Quant VYB cytometer from Miltenyi Biotec. For red and green fluorescent cells, a 488 nm laser with a 500–550 nm filter (GREEN state) and 561 nm laser with 605–626 filter (RED state) combinations were used. The cell sorting experiments were performed on the MoFlo Astrios EQ cell sorter using the Summit v6.3 software (Beckman Coulter).

De-emulsification was performed by adding 100 volumes of TBS buffer, and cells recovered in the aqueous phase were stored at 4 °C prior to further analysis. Cell sorting was carried out with a 70 μm nozzle and 60 psi operating pressure. The sorting speed was kept around 30,000 events per second.

To sort cells with the red and green fluorescence state of the Dendra2 (or mEos2) protein simultaneously, the red (560 nm laser, 614/20 filter) and green (488 nm laser, 526/52 filter) level of fluorescence of the strains with non-switched protein was first measured. The level of red fluorescence of the resulting plot was used as a background value. The cells with red fluorescence higher than this background was considered as "switched" cells and sorted.

3. Results

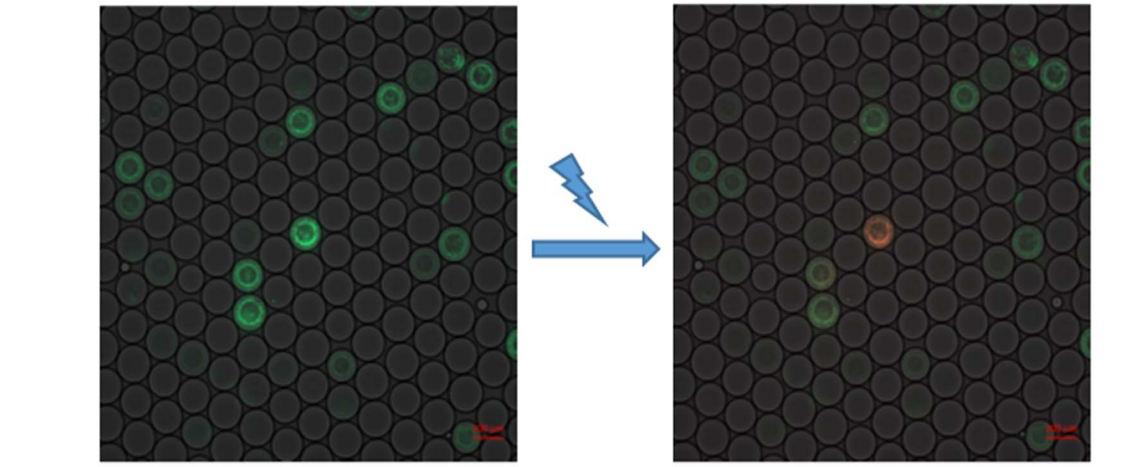
In order to develop a workflow for ultra-high throughput screening of cell growth in droplets, that is compatible with fluorescent activated cell sorting and living cell recovery, we tested the potential of switchable fluorescent

proteins for the labelling of encapsulated growing cells. Two model microorganisms have been chosen for their generic usage in synthetic biology and as screening hosts for protein engineering and activity-based functional metagenomics: the bacterium *E. coli* and the yeast *S. cerevisiae*. In both cases, cells were rendered fluorescent by plasmid-based expression of PAFPs. These fluorescent strains were used to monitor cell growth and to label selected droplets into a microfluidic chamber.

3.1. Selective cell labelling, sorting and recovery with two model microorganisms

3.1.1. The E. coli DENDRA2p model

A BL21 *E. coli* strain expressing the green/red switchable fluorescent protein Dendra2 was used for the proof of concept in bacteria. Green fluorescent cells were re-suspended in LB Amp medium at OD_{600nm} 0.001, which corresponds to a theoretical cell concentration of 10^6 cells/mL. After droplet generation, the cell-containing emulsion was incubated in Eppendorf tubes at 37 °C overnight and observed the next day in a monolayer under the fluorescent microscope. Using the ×100 objective, fifteen droplets filled by overnight grown cells were illuminated with blue light (405 nm) in order to change their fluorescence from the initial green color into red. This magnification allowed precise light targeting of a selected droplet without switching the fluorescence of neighboring droplets. Fig. 1A shows the result of illumination of one of the green droplets that led to the appearance of red fluorescence. After the "switch", all the droplets from the observation chamber were collected, immediately broken and released cells were kept in TBS buffer. The cell suspension was then analyzed by cytometry and the appearance of a new, red fluorescent cell population was



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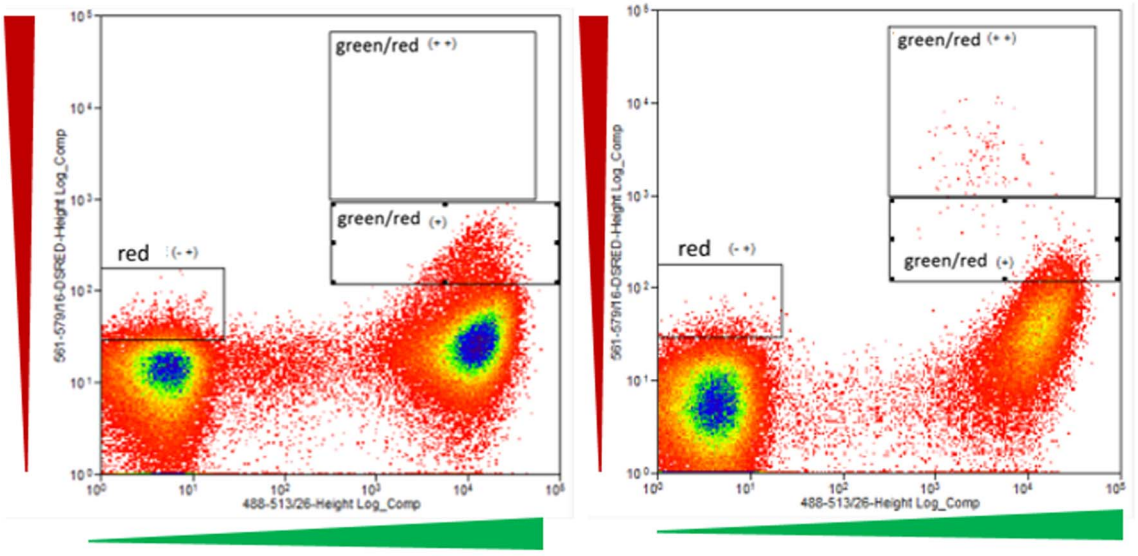

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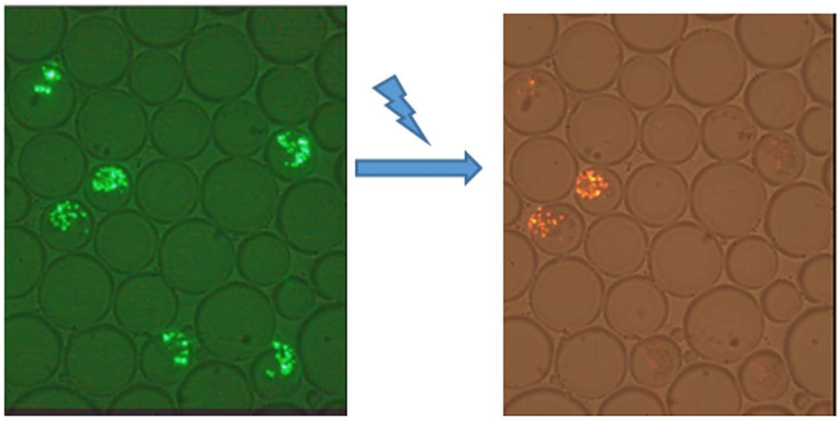
Fig. 1. A. Switching of Dendra2p fluorescence in *E. coli* cells grown in droplets green/red overlay images. *E. coli* BL21 strain expressing Dendra2 fluorescent protein were grown during 24 h at 37 °C in LB + Amp medium. Selected droplets were zoomed with 100× objective and illuminated during 30 s by blue light using EL6000 light source and 435/40 Bright Line HC filter. Images were taken with 20× objective before (1A left) and after (1A right) illumination. B. FACS images of cells populations before and after the "switch" of Dendra2 protein. X axes correspond to the green fluorescence level, Y – to the red. Cells recovered from the emulsion illuminated (right) or not (left) with the blue light in observation chamber were analyzed and sorted by FACS. New red fluorescent cells population appearing in the upper right corner of the plot is the result of fluorescence switch of Dendra2 protein in few selected droplets of the chamber. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

detected (Fig. 1B). Subsequently, the green and red populations were separated using FACS. In order to check the effect of the illumination and fluorescence switch on viability, the survival of these two sorted populations was compared: 4000 red and 4000 green FACS sorted cells were plated on LB + Amp medium and grown at 37 °C. After 2 days of incubation 1127 (28% of the sorted ones) red cells formed colonies. Survival of green (non-switched) cells after the sorting procedure was 43%, meaning that our "switching" parameters were slightly harmful for the cells, but did not dramatically reduce their survival after sorting. Considering that several living cells were recovered from one single droplet after overnight cell growth, we presume that even with a survival rate of 28%, the whole "switch + FACS" procedure applied to *E. coli* should allow us to recover enough cells from each labelled droplet during the screening procedure.

3.1.2. The S. cerevisiae mEos2p model

S. cerevisiae strains expressing the mEos2 fluorescent protein were used to demonstrate usefulness of this strategy for yeast. In addition, to address the question whether fluorescence intensity influences the performance and accuracy of the selective systems, in this model the mEos2 gene was placed under the control of two different promoters: a strong tetO-CYC1 promoter and a weaker - MET25 promoter. Cells carrying these constructs exhibited different levels of fluorescence (Fig. 2). Similarly to the *E. coli* experiment, 1



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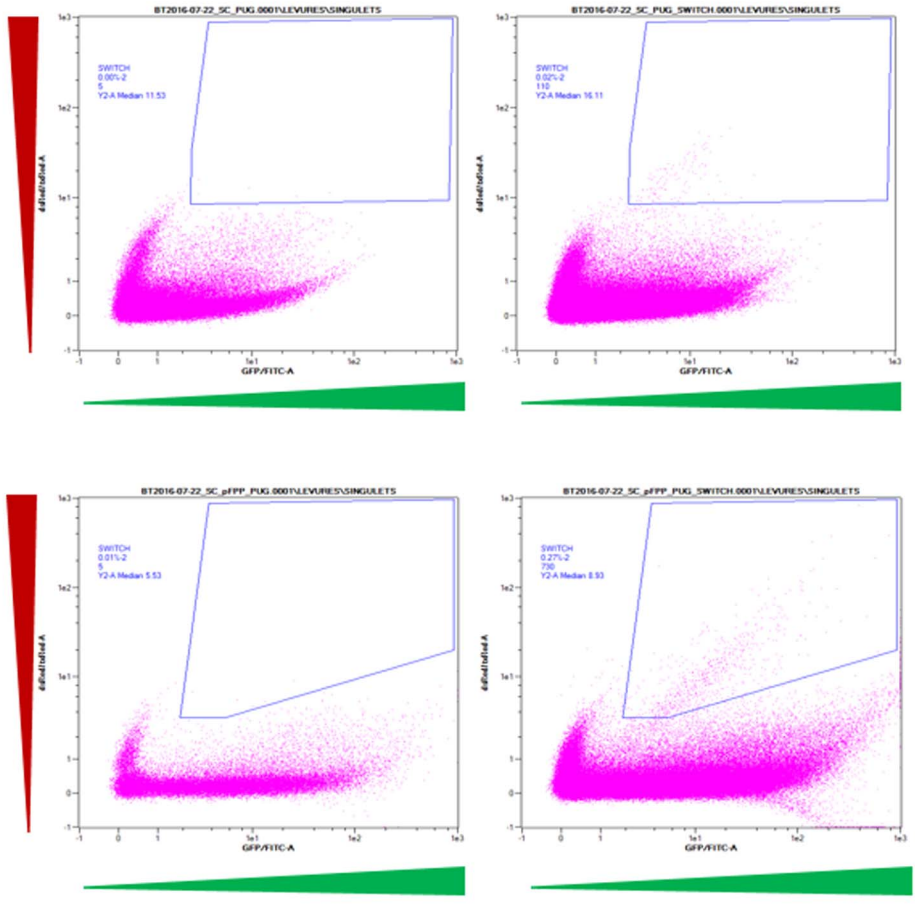

B.

Fig. 2. A. Switching of mEos2 fluorescence in *S. cerevisiae*. Result of fluorescent switch of two droplets containing yeast cells grown in YNB medium. Images are taken in green (left) or in red (right) channels before and after the switch. B. Cytometry plots before and after "switch" of mEos2 protein in *S. cerevisiae*. mEos2 protein expressed from the plasmid at different level in two yeast strains – lower expression from MET25 promoter (upper plots), higher - from tet0-CYC1 (lower plots). After the "switch" (two right plots) for both strains increased amount of "red" cells is detected by cytometer, allowing further sorting by fluorescence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cell-per-droplet emulsions were prepared from these different strains, by using cell suspension at OD_{600nm} 0.05. Twenty droplets from each emulsion were "switched" by blue light illumination (Fig. 2A) and the cells recovered from the observation chambers with and without a switch were compared. Cytometry plots (Fig. 2B) for both strains expressing mEos2 gene at different levels show the appearance of a new red cell population after the blue light illumination, which is clearly distinguishable from the background. Fluorescence based sorting of these switched cells was as successful as in the case of *E. coli*, although the intensity of fluorescence of both yeast strains was much lower than that of Dendra2p expressing bacteria. Testing for viability following the sorting of red and green cells showed, as in the case of *E. coli*, that blue light treatment slightly influences the survival of yeast cells recovered after FACS: 22% of treated vs 34% of non-treated cells formed colonies on YNB plates.

3.2. Application to functional metagenomic

In order to exemplify the use of our fluorescence switching based screening for functional metagenomic, we analyzed the growth phenotype of a synthetic mini-library issued from the human ileal and fecal microbiomes. Four *E. coli* clones selected as positives by conventional screening of metagenomic libraries for prebiotic metabolization pathways (Cecchini et al., 2013) were used to construct this mini-library (F3, F4, F5 and I7). These clones all contain 30–40 kb metagenomic DNA fragments cloned in fosmid pCC1FOS, which encode at least one glycoside-hydrolase (GH) acting on xylo-oligosaccarides (XOS). In addition, the metagenomic sequence of clones F3, F5 and I7 also encode XOS transporters that could, with the appropriate intracellular GHs, confer to *E. coli* the ability to both internalize and to fully hydrolyze XOS into xylose, as demonstrated for F5 (Tauzin et al., 2016). In contrast, F4 does not contain any oligosaccharide transporter sequence (Cecchini et al., 2013). Here, we extracted fosmids from these clones and used them separately to transform the BL21 *E. coli* strain expressing the DENDRA2 fluorescent protein.

The resulting fluorescent clones were checked in liquid cultures for the ability to grow on XOS-selective medium in order to assess the influence of Dendra2 expression on that of the metagenomic genes. The growth of all the positive clones issued from the conventional screening on solid medium was confirmed in liquid cultures, except for the clone F4 that does not contain any oligosaccharide transporter sequence (Fig. 3).

The final step of the proof of concept consisted in mixing XOS

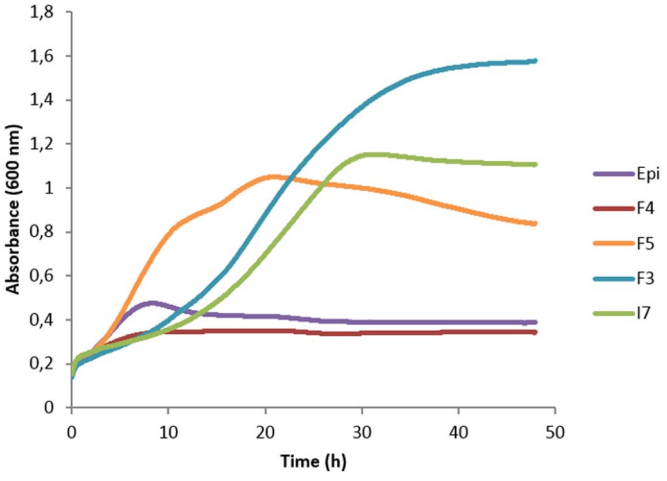

Fig. 3. Growth curves in liquid XOS-selective medium of the fluorescent BL21 pET3-Dendra2 strains carrying metagenomic fosmids. The F5, F3, and I7 metagenomic sequences confer XOS utilization to *E. coli*, through the expression of functional XOS transporters and hydrolytic enzymes, although growth rate differs depending on the metagenomic sequence. Clone with empty fosmid (Epi) and negative clone F4, which only expresses XOS hydrolytic enzymes, didn't grow.

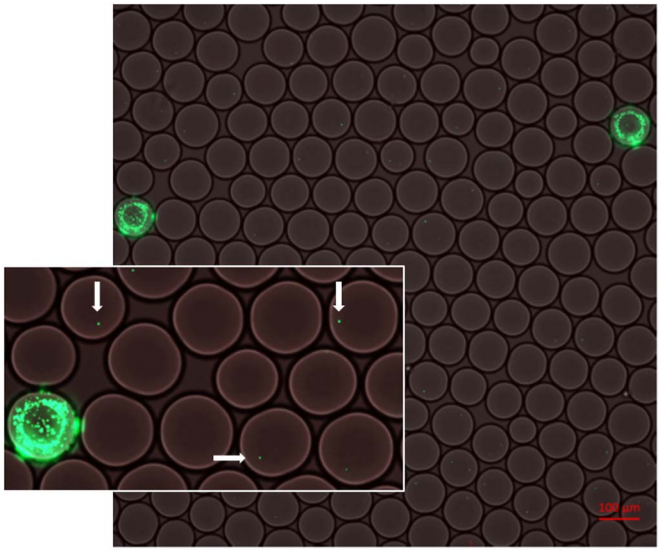

Fig. 4. Mini-library growth in droplets on XOS-selective medium. Mini-library *E. coli* cells at OD_{600nm} 0, 05 in XOS M9 medium was encapsulated for the screening. After 24 h of incubation highly fluorescent droplets corresponding to positive hits, surrounded by many droplets with 1–3 non-grown fluorescent cells (white arrows) are observed.

metabolizing and non-metabolizing clones and to use the resulting mini-library for screening in our microfluidic system. Clones F3, F4, F5 and I7 were added at an equal ratio in a mixture and the negative one (Epi) at 1000 times excess to get the ratio close to a real metagenomic library (Cecchini et al., 2013; Tasse et al., 2010). This cell suspension in XOS-selective medium at an OD_{600nm} of 0.05 was used for encapsulation. The resulting library emulsion was incubated in Eppendorf tubes at 37 °C during 24 h. We observed few very fluorescent droplets with good growth, which presumably contained positive clones (Fig. 4).

The majority of droplets had one to three small fluorescent spots corresponding to the single initial fluorescent cell encapsulated in the droplet, which was unable to grow on this substrate. Fifteen highly fluorescent droplets were "switched" and 1112 red cells from recovered and broken emulsion were collected by sorting with FACS. All the sorted red cells were plated on solid LB + Cm medium, and resulted in around 300 colonies after 24 h of incubation. We randomly selected 20 colonies for growth validation in liquid XOS-selective medium. All the cells were able to grow, meaning that labelling and sorting allowed to perfectly select the clones that are able to grow in selective conditions. The identity of these 20 positive clones was then checked by PCR using pairs of primers specific to each metagenomic DNA insert. Clones F5, I7 and F3 were present in almost equal amount (9:6:5). No F4 was found confirming that this insertion is not able to confer growth on this substrate in liquid medium.

This whole experiment presented in Fig. 5, demonstrate that the cells representing each positive clone from the library were recovered after random switching of growing droplets, and following cell sorting. No F4 was found, confirming that contrary to conventional screening on solid plates, our method allowed us to discriminate between the clones that encode or do not encode for a complete XOS metabolization pathway involving an active transport system.

4. Discussion

In this paper we present a new droplet-microfluidic workflow to screen microbial libraries for growth phenotypes. The original way of labelling used in this method includes fluorescence color switching in the droplets and sorting of "switched" selected cells, steps which can be easily done using readily available laboratory tools: a fluorescent microscope and a FACS machine. We chose *E. coli* and *S. cerevisiae* strains expressing switchable fluorescent proteins as model examples of

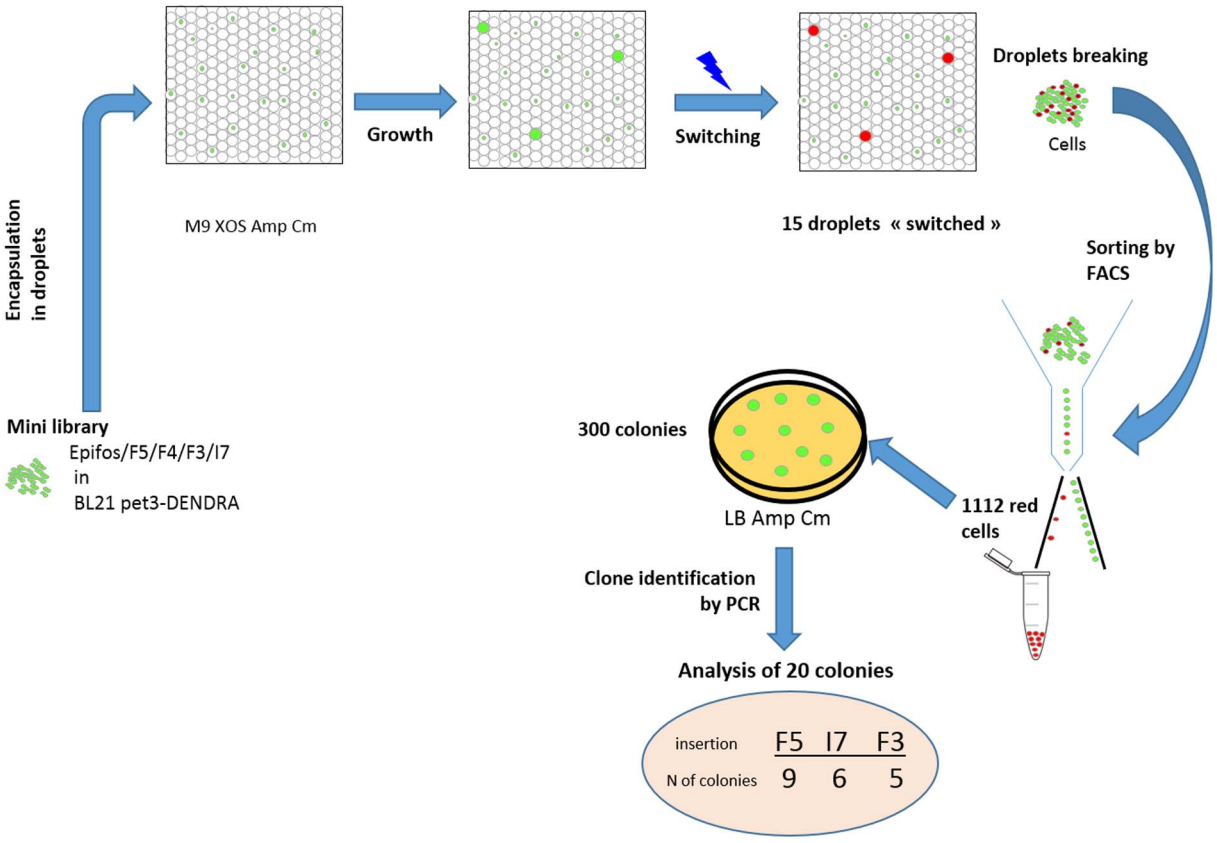

Fig. 5. Schema and results of mini-library screening.

commonly used microorganisms, and showed that in both systems our method gives reliable results. The estimation of cell viability for both microorganisms after sorting showed slightly harmful effect of blue light treatment during the "switch". However, lethality level due to this effect should not significantly influence the yield of microfluidic or millifluidic screenings, since each droplet normally contains a big number of switched cells (100 − 1000), and 30% of survival is enough for further analysis. If in some cases lethality becomes critical for the screening, modulation of exposure to blue light source by the time, strength and wavelength (488 instead of 405 nm) of the illumination might be considered.

In order to confirm the relevance of this new method for metagenomic library screening, we used an artificially created mini-library consisting of previously characterized positive metagenomic clones issued from conventional screening on solid medium (Cecchini et al., 2013; Tauzin et al., 2016). With this model we demonstrated that after the complete procedure, all the positive clones metabolizing a specific substrate are successfully recovered. In addition, our screening was able to perfectly discriminate clones that are really able to grow in a selective medium (clones P5, F3 and I7) from those, like clone F4, which only produce enzymes to hydrolyze the substrate but no transporter to internalize it. These types of clones, in fact, constitute false positives picked from solid media, on which only substrate degradation can be specifically screened. This selectivity is an important advantage of microfluidics compared to conventional high-throughput screening on solid media, which is actually the most extensively used method for the functional exploration of microbial libraries issued from protein engineering and metagenomic studies (Ufarté et al., 2015). But the most striking interest of our workflow, as well as of any micro- or millifluidic screen, is the significant reduction of the required substrate amount, compared to positive selection in liquid media performed by a classical way in micro-plates. Here, for the generation of more than 10,000 droplets we used only 200 µL of selective substrate containing medium, whereas the volume required to screen 1000 individual clones in the micro-plates would be 250 mL. Even though, for the moment, switching is done manually for each droplet, it is easily possible to screen a library of several thousands of cells in a few minutes. One chamber is indeed enough to observe around 10,000 droplets and each droplet "switch" takes a couple of minutes. Furthermore, libraries screened for positive growth can be encapsulated with higher density (more than 1 cell per droplet), allowing to increase the proportion of positive hits and decrease screening time.

Another interesting application of our method is related to the implication of a microscopy observation step, could be phenotypes targeting that cannot be easily screened on solid medium. For example, variation of cell form due to genomic mutations, changed colony development, such as hyphal or pseudo hyphal growth induced for some microorganisms by specific conditions. For this type of screenings, fluorescent switchable proteins serve as a cell labelling switchable marker and also facilitate visualization of the growth in droplets. However, we can conceive that for screening other features PSFP may have an additional function: it can be used for expression monitoring if placed under the investigated promoter or can help for following the protein localization if expressed in fusion with the protein of interest. In those cases, droplets where changed expression or localization phenotype is observed, can be later switched and sorted using the same method as in the examples shown in this work. We believe that the labelling tool described in this study will expand the application of microfluidic systems in molecular biology, protein engineering and functional metagenomics.

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