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# 1 Use of photoswitchable Fluorescent Proteins for droplet-based microfluidic screening.

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10

## 11 **Abstract**

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

24 **Key words:** Droplet-based microfluidics; library screening; photoconvertible fluorescent protein;  
25 *Saccharomyces cerevisiae*; *Escherichia coli*; Fluorescence Activated Cell Sorting.

## 26 **Introduction**

27 Droplet-based microfluidics is an innovative and powerful approach to assess cellular properties at the  
28 cell and micro-colony level. It has been successfully applied to metagenomics in the past three years  
29 (Colin et al., 2015; Gielen et al., 2018; Najah et al., 2014). Diverse, specific features can be detected by  
30 this method using absorbance- (Gielen et al., 2016) or fluorescence-activated droplet sorting (Baret et  
31 al., 2009) as well as microscopy: enzyme activity (Hosokawa et al., 2015), growth rate (Boitard et al.,  
32 2012a), colony shape, variations of gene expression, cellular responses to different agents (antibiotics  
33 and toxic molecules), biological molecule production and secretion (Mazutis et al., 2013a) etc. This  
34 method gives the possibility to screen large numbers of individually growing cells and to reveal  
35 heterogeneity within a cell population. Applying this technique for screening requires that cells with  
36 desired features is recovered following droplet observation, in order to perform DNA sequencing or  
37 further phenotypic analysis. However, direct and on-the-spot access to a chosen droplet is not possible  
38 for the majority of systems used, since the microfluidic channels or chambers are usually well sealed  
39 in order to prevent evaporation of continuous and dispersed phases. Therefore, to manage cell  
40 recovery from any droplet of interest, cells from selected droplets have to be labelled during  
41 observation, thus allowing their subsequent sorting. One of the approaches that is used to  
42 discriminate droplets with cells producing specific target molecules (e.g., enzymes and antibodies)

43 from non-producing ones is co-encapsulation with a reporter molecule such as a fluorogenic substrate  
44 (Agresti et al., 2010; Hosokawa et al., 2015; Sjostrom et al., 2014; Wang et al., 2014; Zinchenko et al.,  
45 2014) or with fluorescent microbeads allowing to capture the secreted products (Mazutis et al.,  
46 2013b). In these cases, fluorescence-based sorting can only be performed at the droplet level, since  
47 the fluorescent particles belong to the media surrounding the cells in the droplet and not to the cells  
48 themselves. Droplets recovered from the observation chamber can be sorted either by special droplet  
49 sorters developed in microfluidics specialized laboratories (Baret et al., 2009), or by easier and more  
50 available commercial fluorescence-activated cell sorting (FACS) devices (Terekhov et al., 2017;  
51 Zinchenko et al., 2014). The latter technique requires conversion of aqueous droplets of water-in-oil  
52 emulsions into a double, water-in-oil-in-water emulsion, involving additional steps in droplet  
53 preparation. For some types of screening based on contact between the droplets (“droplet shrinking”  
54 due to yeast growth, for example, described by (Boitard et al., 2012a) a aqueous layer around the  
55 droplets must be added after observation, which can lead to the loss of some positive hits. Although  
56 equipment for direct water-in-oil droplet sorting exist in several microfluidics laboratories, these  
57 instruments cannot be routinely used due to limited access to this not yet commercialized material.

58 Here we describe a new screening strategy that combines the simplicity of water-in-oil droplet  
59 generation with that of FACS utilization. This workflow, which allows to specifically label the droplet  
60 during microscopy-based phenotype screening and to recover the living cells after FACS sorting, is  
61 based on the use of a photoconvertible fluorescent protein (PCFP). PCFPs are fluorescent proteins  
62 whose fluorescence properties can be switched on or off, by a pulse of light of a specific wavelength.  
63 In the last ten years, many new PCFPs were described and novel applications in cell imaging have been  
64 developed, such as the study of the redistribution of a protein of interest inside a cell (Miyawaki et al.,  
65 2003), organelle labeling and tracking (Molina and Shirihai, 2009), cell labelling (Lukyanov et al., 2005),  
66 protein degradation studies (Zhang, 2007), and super-resolution microscopy based on their photo-  
67 switching properties (Chudakov et al., 2007). We propose to use switchable fluorescent proteins  
68 produced inside the cells of a library for labelling droplets, and we demonstrate the suitability of this  
69 method for high-throughput screening of cell growth for both yeasts and bacteria. The potential of this  
70 highly generic method is discussed in the context of microbiome functional exploration.

71

## 72 **2. Material & Methods**

### 73 *2.1. Plasmids, strains and mini-library construction*

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

78 For mini-library studies, the fluorescent BL21 *E.coli* strain, carrying the pET3-Dendra2 plasmid was  
79 additionally transformed with the fosmids extracted from the metagenomic library clones.  
80 Transformants were selected and further grown on LB medium with 100 mg.L<sup>-1</sup> ampicillin (Amp) and  
81 12.5 mg.L<sup>-1</sup> chloramphenicol (Cm).

82 Yeast vectors were constructed by insertion of the mEos2p coding sequence either between the Xba1  
83 and BamH1 restriction sites of the pUG36 (Niedenthal et al., 1996) plasmid (MET25 promoter

84 controlled expression), or between the BamH1 and Pst1 restriction sites of the pCM185 plasmid  
85 containing tetO-CYC1 promoter to control expression (Gari et al., 1997). A yeast strain with a deleted  
86 TRP1 gene from the YKO collection (MAT alfa hi3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp1::KANMX) was  
87 transformed by the resulting plasmids pUG36-mEos2 and pCM185-mEos2.

88

## 89 2.2. Hit clone identification by PCR

90 DNA inserts of clones obtained during metagenomic library screening were identified by PCR  
91 amplification using Phusion High fidelity DNA polymerase (NEB) classical protocol. Following pairs of  
92 primers were used for amplification of specific regions:

93 **Clone F5** GCGGTCTGCCGATAGCATC and GATGAGTGGCTTGCCCTG

94 **Clone F3** GAGGTTTCGACGCGCCAGA and GACGGCGCAAACATGCTGG

95 **Clone I7** CGACAATACAGAGTCGCG and CGTGCCGGAGGCTGGGG

96 **Clone F4** TGACCATCGAACTGGTCGCC and TCCGGAGACCAAGCAGCC

97

## 98 2.3. Media and growth conditions

99 The *E. coli* strain carrying the pET3-Dendra2 plasmid was cultivated in LB liquid or solid media with  
100 100 mg.L<sup>-1</sup> Amp at 37°C.

101

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

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

112

## 113 2.4. Droplet generation with the microfluidic device

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

116 Droplet generation chips and observation chambers were developed and provided by the Colloïdes  
117 and Matériaux Divisés Laboratory (LCMD) from the Ecole Supérieure de Physique et Chimie  
118 Industrielles of Paris (ESPCI). The water in oil emulsion with the droplets size of approximately 50μM  
119 was made by flow-focusing the cell suspension stream with two streams of HFE7500 fluorinated oil  
120 (3M) containing 2% (w/w) 008-FluoroSurfactant (RAN Biotechnologies) (Boitard et al., 2012b).

121

122 *2.5. Microscope observation and “switching”*

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

126

127 *2.6. Cytometry and sorting*

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

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

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

141

142 **3. Results**

143 In order to develop a workflow for ultra-high throughput screening of cell growth in droplets that is  
144 compatible with fluorescent activated cell sorting and living cell recovery, we tested the potential of  
145 switchable fluorescent proteins for the labelling of encapsulated growing cells. Two model  
146 microorganisms have been chosen for their generic usage in synthetic biology and as screening hosts  
147 for protein engineering and activity-based functional metagenomics: the bacteria *E. coli* and the yeast  
148 *S. cerevisiae*. In both cases, cells were rendered fluorescent by plasmid-based expression of PAFPs.  
149 These fluorescent strains were used to monitor cell growth and to label selected droplets into a  
150 microfluidic chamber.

151

152 *3.1. Selective cell labelling, sorting and recovery with two model microorganisms*

153 *3.1.1. The E. coli DENDRA2p model*

154 A BL21 *E. coli* strain expressing the green/red switchable fluorescent protein Dendra2 was used for the  
155 proof of concept in bacteria. Green fluorescent cells were re-suspended in LB Amp medium at OD<sub>600nm</sub>  
156 0.001, which corresponds to a theoretical cell concentration of 10<sup>6</sup> cells/mL. This cell concentration  
157 allows emulsion to be formed with around 70% of empty droplets and 30% of cell containing droplets.  
158 According to Poisson distribution in this case in majority (around 22%) of populated droplets will be

159 found one cell, and two or more cells in the others. After droplet generation, the cell-containing  
 160 emulsion was incubated in Eppendorf tubes at 37°C overnight and observed the next day in a  
 161 monolayer under the fluorescent microscope. Using the X100 objective, fifteen droplets filled by  
 162 overnight grown cells were illuminated with blue light (405 nm) in order to change their fluorescence  
 163 from the initial green color into red. This magnification allowed precise light targeting of a selected  
 164 droplet without switching the fluorescence of neighboring droplets. Figure 1 shows the result of  
 165 illumination of one of the green droplets that led to the appearance of red fluorescence due to  
 166 conformational changes of the Dendra2p molecules in the encapsulated *E. coli* cells (Fig 1A). After the  
 167 “switch”, all the droplets from the observation chamber were collected, immediately broken and  
 168 released cells were kept in TBS buffer. The buffer, and not a culture medium, was used in order to keep  
 169 cells alive, but not growing and not synthesizing *de novo* Dendra2 protein. The cell suspension was  
 170 then analyzed by the cytometer and the appearance of a new, red fluorescent cell population was  
 171 detected (Fig 1B). Subsequently, the green and red populations were separated using FACS. In order  
 172 to check the effect of the illumination and fluorescence switch on viability, the survival of these two  
 173 sorted populations was compared: 4000 red and 4000 green FACS sorted cells were plated on LB+Amp  
 174 medium and grown at 37°C. After 2 days of incubation 1,127 (28% from sorted ones) red cells formed  
 175 colonies. Survival of green (non-switched) cells after the sorting procedure was 43%, meaning that our  
 176 “switching” parameters were slightly harmful for the cells, but did not dramatically reduce their  
 177 survival after sorting. Considering that several living cells were recovered from one single droplet after  
 178 overnight cell growth, we presume that even with a survival rate of 28 %, the whole “switch+FACS”  
 179 procedure applied to *E. coli* should allow us to recover enough cells from each labelled droplet during  
 180 the screening procedure.

181

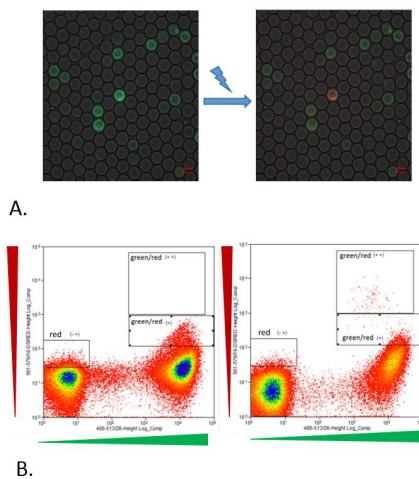


Figure 1A. **Switching of Dendra2p fluorescence in *E. coli* cells grown in droplets green/red overlay images.** *E. coli* BL21 strain expressing Dendra2 fluorescent protein were grown during 24 hours at 37°C in LB+Amp medium. Selected droplets were zoomed with 100X objective and illuminated during 30 sec by blue light using EL6000 light source and 435/40 Bright Line HC filter. Images were taken with 20X objective before (1A left) and after (1A right) illumination.

Figure 1B. **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.

182

183

### 184 3.1.2. The *S. cerevisiae* mEos2p model

185 *S. cerevisiae* strains expressing the mEos2 fluorescent protein were used to demonstrate usefulness of  
 186 this strategy for yeast. In addition, to address the question whether fluorescence intensity influences  
 187 the performance and accuracy of the selective systems, in this model the mEos2 gene was placed

188 under the control of two different promoters: a strong tetO-CYC1 promoter and a weaker - MET25  
 189 promoter. Cells transformed by these plasmids exhibited different levels of fluorescence (Fig 2).  
 190 Similarly to the *E. coli* experiment, 1 cell-per-droplet emulsions were prepared from these different  
 191 strains, by using cell suspension at OD<sub>600nm</sub> 0.05 (which corresponds to a theoretical cell concentration  
 192 of  $5.0 \times 10^5$  cells/mL). Twenty droplets from each emulsion were “switched” by blue light illumination  
 193 (Fig 2A) and the cells recovered from the observation chambers with and without a switch were  
 194 compared. Cytometry plots (Fig 2B) show for both strains expressing mEos2 gene at different level,  
 195 the appearance of a new red cell population after the blue light illumination, clearly distinguishable  
 196 from the background. Fluorescence based sorting of these switched cells was as successful as in the  
 197 case of *E. coli*, although the intensity of fluorescence of both yeast strains was much lower than that  
 198 of Dendra2p expressing bacteria. Testing for viability following the sorting of red and green cells  
 199 showed, as in the case of *E. coli*, that blue light treatment slightly influences the survival of yeast cells  
 200 recovered after FACS : 22% of treated vs 34% of non-treated cells formed colonies on YNB plates.

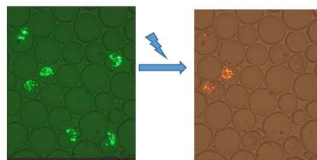


Figure 2A. **Switching of mEos2 fluorescence in *S.cerevisiae*.** Result of fluorescent switch of two droplets containing yeast cells grown in YNB medium. Images are taken in green (left) or in red (right) channels before and after the switch.

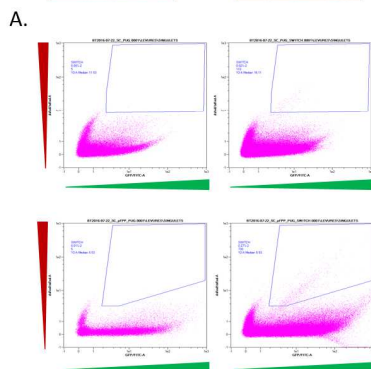


Figure 2B. **Cytometry plots before and after “switch” of mEos2 protein in *S.cerevisiae*.** mEos2 protein expressed from the plasmid at different level in two yeast strains – lower expression from MET25 promoter (upper plots) , higher - from tetO-CYC1(lower plots). After the “switch” (two right plots) for both strains increased amount of “red” cells is detected by cytometer, allowing further sorting by fluorescence.

201 B.

### 202 3.2. Application to functional metagenomic

203 In order to exemplify the use of our fluorescence switching based screening for functional  
 204 metagenomic, we analyzed the growth phenotype of a synthetic mini-library issued from the human  
 205 ileal and fecal microbiomes. Four *E. coli* clones selected as positives by conventional screening of  
 206 metagenomic libraries for prebiotic metabolization pathways (Cecchini et al., 2013) were used to  
 207 construct this mini-library (F3, F4, F5 and I7). These clones all contain 30-40 kb metagenomic DNA  
 208 fragments cloned in fosmid pCC1FOS, which encode at least one glycoside-hydrolase (GH) acting on  
 209 xylo-oligosaccharides (XOS). These GHs are produced intracellularly in *E. coli*. In conventional screening,  
 210 they are released during the spontaneous cell lysis occurring when the clones are cultured during 1 to  
 211 3 weeks on solid medium containing XOS as sole carbon source (Cecchini et al., 2013). This allows the  
 212 enzymes to access the extracellular substrate, and to degrade it into xylose that will boost cell growth  
 213 and result in the apparition of colonies. In addition, the metagenomic sequence of clones F3, F5 and I7  
 214 also encode XOS transporters that could, with the appropriate intracellular GHs, confer to *E. coli* the  
 215 ability to both internalize and to fully hydrolyze XOS into xylose, as demonstrated for F5 (Tauzin et al.,  
 216 2016). In contrast F4 does not contain any oligosaccharide transporter sequence (Cecchini et al., 2013).

217 Here, we extracted fosmids from these clones and used them separately to transform the BL21 *E. coli*  
218 strain expressing the DENDRA2 fluorescent protein.

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

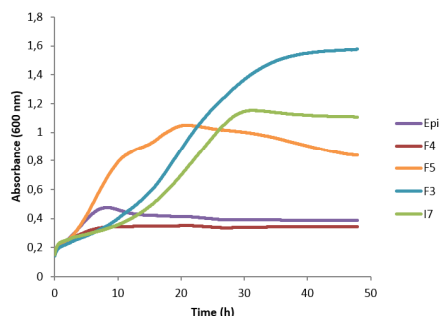


Figure 3. **Growth curves in liquid XOS-selective medium of the fluorescent BL21 pET3-Dendra2 strains carrying metagenomic fosmids.** The F5, F3, and I7 metagenomic sequences confer XOS utilization to *E. coli*, through the expression of functional XOS transporters and hydrolytic enzymes, although growth 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.

224  
225 Then, the growth of each of these fluorescent clones on XOS-selective medium was separately checked  
226 in droplets. As expected, both the Epi negative control containing the empty fosmid and the F4 clone  
227 didn't grow in droplets. Clones F3, F5 and I7 correctly divided, which resulted in a dramatic increase of  
228 the droplet fluorescence.

229 The final step of the proof of concept consisted in mixing XOS metabolizing and not-metabolizing  
230 clones and to use the resulting mini-library for screening in our microfluidic system. Clones F3, F4, F5  
231 and I7 were added at an equal ratio in a mixture and the negative one (Epi) at 1000 times excess to get  
232 the ratio close to a real metagenomic library (Cecchini et al., 2013; Tasse et al., 2010). This cell  
233 suspension in XOS-selective medium at an  $OD_{600nm}$  of 0.05 was used for encapsulation. The resulting  
234 library emulsion was incubated in Eppendorf tubes at 37°C during 24 h. We observed few very  
235 fluorescent droplets with good growth, which presumably contained positive clones (Fig 4).

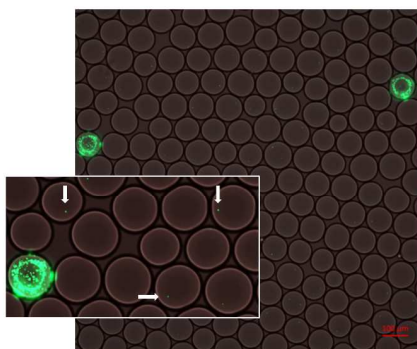


Figure 4. **Mini-library growth in droplets on XOS-selective medium.** Mini-library *E. coli* cells at  $OD_{600nm}$  0, 05 in XOS M9 medium was encapsulated for the screening. After 24h of incubation highly fluorescent droplets corresponding to positive hits, surrounded by many droplets with 1-3 non-grown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

236  
237 The majority of droplets had one to three small fluorescent spots corresponding to the single initial  
238 fluorescent cell encapsulated in the droplet, which was unable to grow on this substrate. Fifteen highly  
239 fluorescent droplets were "switched" and 1112 red cells from recovered and broken emulsion were



240 collected by sorting with FACS. All the sorted red cells were plated on solid LB+Cm medium, and  
 241 resulted in around 300 colonies after 24 hours of incubation. We randomly selected 20 colonies for  
 242 growth validation in liquid XOS-selective medium. All the cells were able to grow, meaning that  
 243 labelling and sorting allowed to perfectly select the clones that are able to grow in selective conditions.  
 244 The identity of these 20 positive clones was then checked by PCR using pairs of primers specific to each  
 245 metagenomic DNA insert. Clones F5, I7 and F3 were present in almost equal amount (9:6:5). No F4 was  
 246 found confirming that this insertion is not able to confer growth on this substrate in liquid medium.

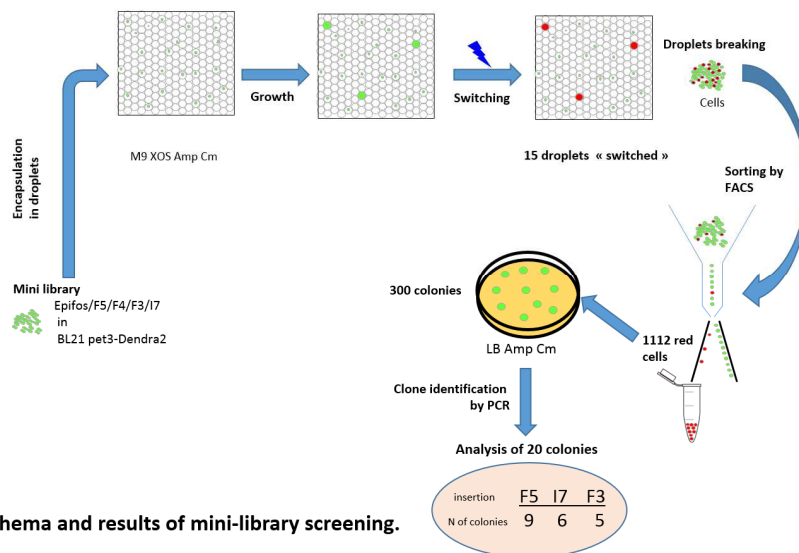


Figure 5. Schema and results of mini-library screening.

247

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

253

#### 254 4. Discussion

255 In this paper we present a new droplet-microfluidic workflow to screen microbial libraries for growth  
 256 phenotypes. Original way of labelling used in this method includes fluorescence color switching in the  
 257 droplets and sorting of “switched” selected cells, steps which can be easily done using readily available  
 258 laboratory tools: a fluorescent microscope and a FACS machine. We chose *E.coli* and *S.cerevisiae*  
 259 strains expressing switchable fluorescent proteins as model examples of commonly used  
 260 microorganisms, and showed that in both systems our method gives reliable results. The estimation of  
 261 cell viability for both microorganisms after sorting showed slightly harmful effect of blue light  
 262 treatment during the “switch”. However, lethality level due to this effect should not significantly  
 263 influence the yield of microfluidic or millifluidic screenings, since each droplet normally contains big  
 264 number of switched cells (100-1000), and 30% of survival is enough for further analysis. If in some  
 265 cases lethality becomes critical for the screening, modulation of exposure to blue light source by the  
 266 time, strength and wavelength (488 instead of 405nm) of the illumination might be considered.

267 In order to confirm the relevance of this new method for metagenomic library screening, we used an  
268 artificially created mini-library consisting of previously characterized positive metagenomic clones  
269 issued from conventional screening on solid medium (Cecchini et al., 2013; Tauzin et al., 2016) . With  
270 this model we demonstrated that after the complete procedure, all the positive clones metabolizing a  
271 specific substrate are successfully recovered. In addition, in our screening we were able to perfectly  
272 discriminate clones that are really able to grow in a selective medium (clones P5, F3 and I7) from those,  
273 like clone F4, which only produce enzymes to hydrolyze the substrate but no transporter to internalize  
274 it. This type of clones, in fact, constitute false positives picked from solid media, on which only  
275 substrate degradation can be specifically screened. This selectivity is an important advantage of  
276 microfluidics compared to conventional high-throughput screening on solid media which is actually  
277 the most extensively used method for the functional exploration of microbial libraries issued from  
278 protein engineering and metagenomic studies (Ufarté et al., 2015). But the most striking interest of  
279 our workflow, as well as of any micro- or millifluidic screen, is the significant reduction of the required  
280 substrate amount, compared to positive selection in liquid media performed by a classical way in  
281 micro-plates. Here, for the generation of more than 10000 droplets we used only 200  $\mu$ L of selective  
282 substrate containing medium, whereas the volume required to screen 1000 individual clones in the  
283 micro-plates would be 250 mL. Even though, for the moment, switching is done manually for each  
284 droplet, it is easily possible to screen in a few minutes a library of several thousands of cells. One  
285 chamber is indeed enough to observe around 10000 droplets and each droplet “switch” takes couple  
286 of minutes. Furthermore, libraries screened for positive growth can be encapsulated with higher  
287 density (more than 1 cell per droplet), allowing to increase positive hits proportion and decrease  
288 screening time.

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

301

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### 307 **References**

308 Agresti, J.J., Antipov, E., Abate, A.R., Ahn, K., Rowat, A.C., Baret, J.-C., Marquez, M., Klibanov, A.M.,  
309 Griffiths, A.D., Weitz, D.A., 2010. Ultrahigh-throughput screening in drop-based microfluidics  
310 for directed evolution. *Proc. Natl. Acad. Sci.* 107, 4004–4009.

311 Baret, J.-C., Miller, O.J., Taly, V., Ryckelynck, M., El-Harrak, A., Frenz, L., Rick, C., Samuels, M.L.,  
312 Hutchison, J.B., Agresti, J.J., Link, D.R., Weitz, D.A., Griffiths, A.D., 2009. Fluorescence-activated  
313 droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity. *Lab Chip*  
314 9, 1850–1858.

315 Boitard, L., Cottinet, D., Kleinschmitt, C., Bremond, N., Baudry, J., Yvert, G., Bibette, J., 2012a.  
316 Monitoring single-cell bioenergetics via the coarsening of emulsion droplets. *Proc. Natl. Acad.*  
317 *Sci.* 109, 7181–7186.

318 Boitard, L., Cottinet, D., Kleinschmitt, C., Bremond, N., Baudry, J., Yvert, G., Bibette, J., 2012b.  
319 Monitoring single-cell bioenergetics via the coarsening of emulsion droplets. *Proc. Natl. Acad.*  
320 *Sci.* 109, 7181–7186.

321 Cecchini, D.A., Laville, E., Laguerre, S., Robe, P., Leclerc, M., Doré, J., Henrissat, B., Remaud-Siméon,  
322 M., Monsan, P., Potocki-Véronèse, G., 2013. Functional Metagenomics Reveals Novel  
323 Pathways of Prebiotic Breakdown by Human Gut Bacteria. *PLoS ONE* 8, e72766.

324 Chudakov, D.M., Lukyanov, S., Lukyanov, K.A., 2007. Tracking intracellular protein movements using  
325 photoswitchable fluorescent proteins PS-CFP2 and Dendra2. *Nat Protoc.* 2, 2024–2032.

326 Colin, P.-Y., Kintsès, B., Gielen, F., Miton, C.M., Fischer, G., Mohamed, M.F., Hyvönen, M., Morgavi,  
327 D.P., Janssen, D.B., Hollfelder, F., 2015. Ultrahigh-throughput discovery of promiscuous  
328 enzymes by picodroplet functional metagenomics. *Nat. Commun.* 6, 10008.

329 Garí, E., Piedrafita, L., Aldea, M. and Herrero, E., 1997. A Set of Vectors with a Tetracycline-Regulatable  
330 Promoter System for Modulated Gene Expression in *Saccharomyces cerevisiae*. *Yeast.* 13: 837–  
331 848.

332 Gielen, F., Colin, P.-Y., Mair, P., Hollfelder, F., 2018. Ultrahigh-Throughput Screening of Single-Cell  
333 Lysates for Directed Evolution and Functional Metagenomics, in: Bornscheuer, U.T., Höhne, M.  
334 (Eds.), *Protein Engineering: Methods and Protocols*. Springer New York, New York, NY, pp.  
335 297–309.

336 Gielen, F., Hours, R., Emond, S., Fischlechner, M., Schell, U., Hollfelder, F., 2016. Ultrahigh-throughput–  
337 directed enzyme evolution by absorbance-activated droplet sorting (AADS). *Proc. Natl. Acad.*  
338 *Sci.* 113, E7383–E7389.

339 Gurskaya, N.G., Verkhusha, V.V., Shcheglov, A.S., Staroverov, D.B., Chepurnykh, T.V., Fradkov, A.F.,  
340 Lukyanov, S., Lukyanov, K.A., 2006. Engineering of a monomeric green-to-red photoactivatable  
341 fluorescent protein induced by blue light. *Nat Biotech* 24, 461–465.

342 Hosokawa, M., Hoshino, Y., Nishikawa, Y., Hirose, T., Yoon, D.H., Mori, T., Sekiguchi, T., Shoji, S.,  
343 Takeyama, H., 2015. Droplet-based microfluidics for high-throughput screening of a  
344 metagenomic library for isolation of microbial enzymes. *Spec. Issue Biosens.* 2014 67, 379–  
345 385.

346 Lukyanov, K.A., Chudakov, D.M., Lukyanov, S., Verkhusha, V.V., 2005. Innovation: photoactivatable  
347 fluorescent proteins. *Nat Rev Mol Cell Biol* 6, 885–891.

348 Mazutis, L., Gilbert, J., Ung, W.L., Weitz, D.A., Griffiths, A.D., Heyman, J.A., 2013a. Single-cell analysis  
349 and sorting using droplet-based microfluidics. *Nat. Protoc.* 8, 870–891.

350 Mazutis, L., Gilbert, J., Ung, W.L., Weitz, D.A., Griffiths, A.D., Heyman, J.A., 2013b. Single-cell analysis  
351 and sorting using droplet-based microfluidics. *Nat. Protoc.* 8, 870–891.

352 Miyawaki, A., Nagai, T., Mizuno, H., 2003. Mechanisms of protein fluorophore formation and  
353 engineering. *Curr. Opin. Chem. Biol.* 7, 557–562.

354 Molina, A.J.A., Shirihai, O.S., 2009. Chapter 16 Monitoring Mitochondrial Dynamics with  
355 Photoactivatable Green Fluorescent Protein, in: *Methods in Enzymology*. Academic Press, pp.  
356 289–304.

357 Niedenthal RK., Riles L., Johnston M., Hegemann JH., 1996. Green fluorescent protein as a marker for  
358 gene expression and subcellular localization in budding yeast. *Yeast.* 30:773-86

359 Najah, M., Calbrix, R., Mahendra-Wijaya, I.P., Beneyton, T., Griffiths, A.D., Drevelle, A., 2014. Droplet-  
360 Based Microfluidics Platform for Ultra-High-Throughput Bioprospecting of Cellulolytic  
361 Microorganisms. *Chem. Biol.* 21, 1722–1732.

362 Sjoström, S.L., Bai, Y., Huang, M., Liu, Z., Nielsen, J., Jönsson, H., Svahn, H.A., 2014. High-throughput  
363 screening for industrial enzyme production hosts by droplet microfluidics. *Lab. Chip* 14, 806–  
364 813.

365 Tasse, L., Bercovici, J., Pizzut-Serin, S., Robe, P., Tap, J., Klopp, C., Cantarel, B.L., Coutinho, P.M.,  
366 Henrissat, B., Leclerc, M., Doré, J., Monsan, P., Remaud-Simeon, M., Potocki-Veronese,  
367 G., 2010. Functional metagenomics to mine the human gut microbiome for dietary fiber  
368 catabolic enzymes. *Genome res.* 11, 1605-1612.

369 Tauzin, A.S., Laville, E., Xiao, Y., Nouaille, S., Le Bourgeois, P., Heux, S., Portais, J.-C., Monsan, P.,  
370 Martens, E.C., Potocki-Veronese, G., Bordes, F., 2016. Functional characterization of a gene  
371 locus from an uncultured gut *Bacteroides* conferring xylo-oligosaccharides utilization to  
372 *Escherichia coli*. *Mol. Microbiol.* 102, 579–592.

373 Terekhov, S.S., Smirnov, I.V., Stepanova, A.V., Bobik, T.V., Mokrushina, Y.A., Ponomarenko, N.A.,  
374 Belogurov, A.A., Rubtsova, M.P., Kartseva, O.V., Gomzikova, M.O., Moskovtsev, A.A., Bukatin,  
375 A.S., Dubina, M.V., Kostryukova, E.S., Babenko, V.V., Vakhitova, M.T., Manolov, A.I.,  
376 Malakhova, M.V., Kornienko, M.A., Tyakht, A.V., Vanyushkina, A.A., Ilina, E.N., Masson, P.,  
377 Gabibov, A.G., Altman, S., 2017. Microfluidic droplet platform for ultrahigh-throughput single-  
378 cell screening of biodiversity. *Proc. Natl. Acad. Sci. U. S. A.* 114, 2550–2555.

379 Ufarté, L., Potocki-Veronese, G., Laville, É., 2015. Discovery of new protein families and functions: new  
380 challenges in functional metagenomics for biotechnologies and microbial ecology. *Front.*  
381 *Microbiol.* 6, 563.

382 Wang, B.L., Ghaderi, A., Zhou, H., Agresti, J., Weitz, D.A., Fink, G.R., Stephanopoulos, G., 2014.  
383 Microfluidic high-throughput culturing of single cells for selection based on extracellular  
384 metabolite production or consumption. *Nat Biotech* 32, 473–478.

385 Zhang, L., 2007. Method for real-time monitoring of protein degradation at the single cell level.  
386 *Biotechniques* 42.

387 Zinchenko, A., Devenish, S.R.A., Kintsjes, B., Colin, P.-Y., Fischlechner, M., Hollfelder, F., 2014. One in a  
388 Million: Flow Cytometric Sorting of Single Cell-Lysate Assays in Monodisperse Picolitre Double  
389 Emulsion Droplets for Directed Evolution. *Anal. Chem.* 86, 2526–2533.

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392

### 393 **Figure legends**

394 **Figure 1A. Switching of Dendra2p fluorescence in *E.coli* cells grown in droplets green/red overlay**  
395 **images.** *E.coli* BL21 strain expressing Dendra2 fluorescent protein were grown during 24 hours at 37°C  
396 in LB+Amp medium. Selected droplets were zoomed with 100X objective and illuminated during 30 sec  
397 by blue light using EL6000 light source and 435/40 Bright Line HC filter. Images were taken with 20X  
398 objective before (1A left) and after (1A right) illumination.

399 **Figure 1B. FACS images of cells populations before and after the “switch” of Dendra2 protein.** X axes  
400 correspond to the green fluorescence level, Y – to the red. Cells recovered from the emulsion  
401 illuminated (right) or not (left) with the blue light in observation chamber were analyzed and sorted  
402 by FACS. New red fluorescent cells population appearing in the upper right corner of the plot is the  
403 result of fluorescence switch of Dendra2 protein in few selected droplets of the chamber.

404 **Figure 2A. Switching of mEos2 fluorescence in *S.cerevisiae*.** Result of fluorescent switch of two  
405 droplets containing yeast cells grown in YNB medium. Images are taken in green (left) or in red (right)  
406 channels before and after the switch.

407 Figure 2B. **Cytometry plots before and after “switch” of mEos2 protein in S.cerevisiae.** mEos2 protein  
408 expressed from the plasmid at different level in two yeast strains – lower expression from MET25  
409 promoter (upper plots) , higher - from tet0-CYC1(lower plots). After the “switch” (two right plots) for  
410 both strains increased amount of “red” cells is detected by cytometer, allowing further sorting by  
411 fluorescence.

412

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

418 Figure 4. **Mini-library growth in droplets on XOS-selective medium.** Mini-library E.coli cells at OD<sub>600nm</sub>  
419 0, 05 in XOS M9 medium was encapsulated for the screening. After 24h of incubation highly fluorescent  
420 droplets corresponding to positive hits, surrounded by many droplets with 1-3 non-grown fluorescent  
421 cells (white arrows) are observed.

422 Figure 5. **Schema and results of mini-library screening.**

423