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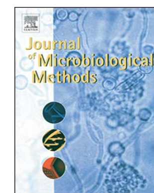

Use of photoswitchable fluorescent proteins for droplet-based microfluidic screening

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A B S T R A C T

Application of droplet-based microfluidics for the screening of microbial libraries is one of the important on-going 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

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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 GATGAGTGGCTTGTCCCTG

Clone F3 GAGGTTCGCAGCGGCCAGA and GACGGCGCAAACATGCTGG

Clone I7 CGCACAATACAGAGTCGCG and CGTGCCGGAGGCTGGGG

Clone F4 TGACCATCGAACTGGTCGCC and TCCGGAGACCAAGCAGCC

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

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