Ultra-high throughput detection of single cell β -galactosidase activity in droplets using micro-optical lens array

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We demonstrate the use of a hybrid microfluidic-micro-optical system for the screening of enzymatic activity at the single cell level. *Escherichia coli* β -galactosidase activity is revealed by a fluorogenic assay in 100 pl droplets. Individual droplets containing cells are screened by measuring their fluorescence signal using a high-speed camera. The measurement is parallelized over 100 channels equipped with microlenses and analyzed by image processing. A reinjection rate of 1 ml of emulsion per minute was reached corresponding to more than 10^5 droplets per second, an analytical throughput larger than those obtained using flow cytometry. 2013 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4830046>]

Droplet-based microfluidics holds an enormous potential for high-throughput screening applications.¹ By isolating and manipulating reagents in discrete, monodisperse, picoliter to nanoliter volume droplets, experiments are parallelized. This technology found applications in biochemical sciences for directed evolution of proteins,² drug screening,³ quantitative molecular diagnosis,^{4,5} or cell screening^{6–9}—all applications requiring ultra-high throughput manipulation of small volumes of compounds for quantitative assays. Key elementary modules for droplet production,^{10,11} incubation,^{12–14} fusion,^{15,16} and sorting^{17,18} have been developed over the past years to provide parallelized droplet manipulation required for these applications¹⁹ as well as specific emulsification materials.^{20,21} Detection systems have so far been poorly parallelized, setting up a bottleneck for the increase of throughput.^{7,22} Schonbrun *et al.*²³ have proposed a system based on the integration of zone plate arrays over microfluidic channels to parallelize the measurements. We have recently proposed an alternative based on micro-optical lens array which is shown to result in similar ultra-high throughput.²⁴

In this Letter, we show that microlens arrays can efficiently be used to detect β -galactosidase activity of single *Escherichia coli* cells in droplets at an ultra-high throughput. Throughputs larger than 10^5 droplets per second are achieved, larger than those obtained with flow cytometry.²⁵ The method is based on a wide-field measurement with a high-speed camera and an integrated microlens array, resulting in a versatile alternative to the laser-based screening methods.^{2,7,17,22}

We have designed a microfluidic device with 100 parallel channels spaced by a distance of 100 μm , fabricated by standard soft-lithography using PolyDiMethylSiloxane (PDMS).²⁶ The microlens array was fabricated using thermal reflow process^{24,27} on a 2-in. cover glass with a thickness of 150–160 μm (Figure 1). In brief, a pedestal structure was fabricated from positive photoresist (AZ9260, MicroChemicals GmbH) with a height of 11.8 μm . Subsequently, it was heated

up to 150 $^\circ\text{C}$ for 1 min to form a spherical cap with a sag of 20 μm . The diameter and sag height of the microlenses were designed to be 120 μm and 20 μm , respectively, to allow a focal point in the corresponding microchannel. The pitch of microchannels and lenses should be identical, despite the lateral shrinkage during polymerization. The extent of lateral shrinkage (typically $\sim 1\%$) is dependent on many processing parameters, such as baking temperature and time, as well as the applied amount of cross-linker. To avoid alignment failure caused by the pitch difference, the microlens array was designed with five different pitches ranging from 98 μm to 100 μm . To increase the density of microfluidic channels and lenses on the chip, the channel pitch was smaller than the microlens diameter, and the array was designed as a zigzag pattern to avoid interconnection of the microlenses (Figure 1). A metallic layer, composed of gold and silver with a cumulative thickness of approximately 100 nm, was coated on the PDMS structure by thermal evaporation. This layer acts as a mirror structure to improve reflectance at the wavelengths of excitation and emission. To allow plasma bonding of PDMS and glass, the metallic layer was removed using adhesive tape (3M), leaving metal only in the microchannels. Imaging and

FIG. 1. Photograph of the fabricated chip with 100-parallel channels harboring the micro-optical elements. The droplets reinjected through the three inlets are introduced to the wide channel (~ 15 mm width), and are then separated into 100 microchannels (50 μm width) for detection.

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fluorescence recording were performed on an inverted microscope (Olympus IX71) equipped with a 130 W mercury vapor short arc lamp (U-HGLGPS light guide-coupled illumination system, Olympus) for fluorescence excitation, a filter set adapted to fluorescein splitting excitation and emission signal, and a high-speed camera (Phantom v210) for image acquisition. The high-speed camera was operated at a rate of 20 000 frames/s and 50 μ s exposure time (Figure 2). For each lens

FIG. 2. (a) Droplet production by flow focusing on a microfluidic chip in the case of a mixed emulsion. The droplets are collected in a reservoir where they are incubated for 48 h. (b) Upon reinjection, the droplets flow under the lens array at a rate of 1 ml/min under fluorescence illumination (snapshot recorded on the high-speed camera for a droplet passing through one of the lenses). (c) Fluorescence histogram obtained after image processing for a reinjection throughput of $\sim 120\,000$ droplets/s. (d) Bright field illumination of the droplet reinjection in the presence of β -galactosidase and (e) when flowing under the lens array.

and each frame, the mean intensity on the overall lens area is computed using Hough circle detection. Having access to the intensity distribution over time allows characterizing noise floors and their amplitudes (supplementary Figure 1, Ref. 29). Noise floors are computed for each lens independently, in order to compensate for illumination variation over the lens array. The noise amplitude is computed over the entire lens array, as it is consistent over the entire camera sensor. These noise characteristics, along with the intensity as a function of time, are then fed into a finite-state-machine to detect droplet sequences.

We first performed control experiments with two types of droplets, all containing phosphate buffered saline (PBS). Droplets of about 100 pl were produced on a first microfluidic flow-focusing device by coflowing two aqueous phases with fluorinated oil (HFE 7500, 3M Novec), containing 0.5 wt. % Pico-Surf 2 (Dolomite) surfactant (Figure 2(a)). Droplets containing 250 μ M fluorescein were produced at a frequency of 2600 droplets/s flowing the aqueous phase at 15 μ l/min and the oil at 40 μ l/min in one nozzle of the chip. Droplets containing PBS only were produced at a frequency of 250 droplets/s flowing the aqueous phase at 2 μ l/min and the oil at 15 μ l/min in the second nozzle of the chip. The resulting emulsion therefore contains a mixture of 9% of fluorescent droplets (~ 100 pl) and 91% of PBS droplets (~ 130 pl) and is stored in an external reservoir, fabricated from a 5 ml plastic syringe (B. Braun AG), and subsequently reinjected at a flow rate of 1 ml/min into the second microfluidic screening chip, equipped with the lens array. The flow rates were controlled by syringe pumps (neMESYS, Cetoni). The fluorescent signals were recorded on the high speed camera (Figure 2(b)) and the fluorescence histogram resulting from the analysis of the series of images (Figure 2(c)). Since the empty droplets are not fluorescent leading to no signal, we estimated the droplets reinjection frequency by measuring the frequency of fluorescent droplets in the reinjection experiment over the 100 channels. We obtained 1.6×10^4 droplets per second. Since the fraction of fluorescent droplets is ~ 0.09 , we obtain a total reinjection frequency of 1.4×10^5 droplets per second. This value is consistent with the reinjection flow rate of 1 ml/min: The reinjection of 100 pl droplets at 1 ml/min would be of order 1.6×10^5 droplets per second if only droplets are reinjected. Assuming an oil volume fraction of 20% in the emulsion—which is reasonable for the packing of soft objects leads to 1.3×10^5 droplets per second, close to the total reinjection frequency estimated from the fluorescence measurement. The values are compatible with those we obtained previously²⁴ and correspond to throughputs larger than those reached in flow cytometry.²⁵ In addition, a fraction of coalescence estimated to $\sim 10\%$ was observed and revealed by the presence of droplets with approximately half-fluorescent signal and doubled size (Figure 2(c) inset).

We used our chip for the detection of β -galactosidase activity at the single cell level as a model biological reaction. The β -galactosidase activity is revealed by a fluorogenic assay where the non-fluorescent substrate Fluorescein Di- β -D-Galactopyranoside (FDG) is hydrolyzed by β -galactosidase in two successive steps, yielding free fluorescein (highly fluorescent) and two galactose

molecules. Figures 2(d) and 2(e) show the typical fluorescence intensity during the reinjection step. The *E. coli* C41(DE3) strain (Lucigen, Wisconsin, USA) was used for the expression of endogenous β -galactosidase. The cells were grown in standard Lysogeny Broth (LB) medium overnight at 37 °C, and subsequently used to inoculate LB medium containing additionally 4 mM lactose in order to induce the Lac operon, and consequently, the expression of β -galactosidase. After incubation for 6 h at 37 °C, the cells were spun down at 12 000 rpm for 5 min, washed 3 times with PBS to remove any β -galactosidase from the surrounding medium, and resuspended in PBS to a final optical density $OD_{600} \approx 0.2$. We prepared three cell suspensions by diluting the solutions to $OD_{600} \approx 0.02$, 0.004, and 0.0008, respectively. Under such conditions, the average number of entrapped cells per 100 pL droplet is smaller than 0.1, and the number of occupied droplets should vary as 25:5:1.

First, the solution of 500 μ M FDG in PBS was mixed on a microfluidic chip with the β -galactosidase expressing *E. coli* cell dilution at flow rates of 1.5 μ L/min each and co-flow with the fluorinated oil-surfactant mixture (20 μ L/min) to produce droplets at a rate of about 400 Hz (125 pL). The droplets were collected in the reservoir and incubated for 48 h. After incubation, the reservoir was connected to the screening chip, and droplets were subsequently reinjected into the chip at a flow rate of 1 mL/min ($\sim 115\,000$ droplets per second). In order to represent the signal from each lens, we use a space-time diagram where the space index represents the lens and fluorescence intensity is represented by gray levels. Each screening experiment therefore corresponds to a color map (Figure 3). Most of the pixels are black when the field of view under the lens is either filled by oil or by an empty droplet. The presence of a cell is detected by the fluorescent signal since β -galactosidase activity is only present when a droplet contains a cell. The density of white pixels increases with increased cell density as expected (Figures 3(b) and 3(c)): quantitatively, the fluorescence signal over all the lenses is compiled to count the number of positive droplets. We obtain an increased number of positive droplets when the cell density increases (Figure 3(d)). The relationship between cell density measured on chip and the control cell density based on OD measurement is proportional in the first approximation. Discrepancies might arise from inaccuracies when preparing the cell dilutions and the possible cell sedimentation during encapsulation yielding variation in cell density at encapsulation. Coalescence events are detected through droplet size heterogeneity and removed from the analysis, while experimental defects such as the clogging of one channel that might occur during the reinjection can also be detected. The analysis of the data from such a blocked channel can, in principle, be discarded by post-processing.

Based on the current recording capabilities of the camera, a maximum number of images of 1.28×10^5 , a maximum recording time of 6.42 s with the resolution of 1008×64 (px $\times$ px) can be recorded. Taking the maximum recording time into account, this yields a number of 770 000 droplets in a single shot. Beyond the analysis of enzymatic activities, this number would already provide good statistics for the detection of mutant DNA for cancer diagnostics⁴

FIG. 3. Space time diagrams for the fluorescence signal over the array of lenses, with decreasing cell density from top to bottom: (a) $OD_{600} = 0.02$, (b) $OD_{600} = 0.004$, (c) $OD_{600} = 0.0008$. The white stripes (gray level) indicate the fluorescence signals emitted from the droplets in which β -galactosidase generates free fluorescein. The coalescence of droplets can be distinguished as shown in the inset graphs. (d) Measured cell density obtained from the fluorescence signal as a function of initial cell density in droplets. The dashed line corresponds to a linear relationship.

providing an automated system for the analysis of $\geq 10^5$ different DNA sequences in one run of 6 s. At the moment, the main limitation of the system is related to the recording of movies from high-speed camera which requires storage on a buffer memory before saving the data. Here, the programming of Field Programmable Gate Arrays (FPGA) embedded in the camera²⁸ to perform on-the-fly simple image processing routine would be a solution to continuously record signals from large emulsions and directly export from the camera the information relevant to the screening procedure, such as the density of hits, maximum fluorescence signal, fluorescence histograms, thereby directly reducing to the

essential the amount of data generated. The architecture of our micro-lens array is well-suited for such processes as the important information is concentrated over a small number of pixels in the field of view, being always located at the same place.

In summary, we have developed an ultra-high throughput screening platform with a parallelized fluorescent detection method compatible with droplet reinjection at high flow rates on biological targets. We reached with this method a throughput of ~115 000 droplets per second for the detection of enzymatic activity at the single cell level in pl volume droplets. Our method has a great potential for ultra-high-throughput applications of droplet-based microfluidics. We expect that the method will be used for the screening of large population of cells or at a pre-screening stage to determine if a cell population contains variants of interest. It will enable, for example, to analyse mutagenesis strategies in a directed enzyme evolution experiment and provide statistically relevant data on large populations of enzyme mutants.

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