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High-throughput single-cell analysis of low copy number β-galactosidase by a laboratory-built high-sensitivity flow cytometer

Lingling Yang [1], Tianxun Huang [1], Shaobin Zhu, Yingxing Zhou, Yunbin Jiang, Shuo Wang, Yuqing Chen, Lina Wu, Xiaomei Yan *

The Key Laboratory of Analytical Science, The Key Laboratory for Chemical Biology of Fujian Province, Department of Chemical Biology, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen, Fujian 361005, China

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ABSTRACT

Single-cell analysis is vital in providing insights into the heterogeneity in molecular content and phenotypic characteristics of complex or clonal cell populations. As many essential proteins and most transcription factors are produced at a low copy number, analytical tools with superior sensitivity to enable the analysis of low abundance proteins in single cells are in high demand. β-galactosidase (β-gal) has been the standard cellular reporter for gene expression in both prokaryotic and eukaryotic cells. Here we report the development of a high-throughput method for the single-cell analysis of low copy number β-gal proteins using a laboratory-built high-sensitivity flow cytometer (HSFCM). Upon fluorescence staining with a fluorogenic substrate, quantitative measurements of the basal and near-basal expression of β-gal in single *Escherichia coli* BL21(DE3) cells were demonstrated. Statistical distribution can be determined quickly by analyzing thousands of individual cells in 1–2 min, which reveals the heterogeneous expression pattern that is otherwise masked by the ensemble analysis. Combined with the quantitative fluorometric assay and the rapid bacterial enumeration by HSFCM, the β-gal expression distribution profile could be converted from arbitrary fluorescence units to protein copy numbers per cell. The sensitivity and speed of the HSFCM offers great capability in quantitative analysis of low abundance proteins in single cells, which would help gaining a deeper insight into the heterogeneity and fundamental biological processes in microbial populations.

1. Introduction

Single-cell analysis is of fundamental importance in quantitative biochemical analysis of cells (Huang et al., 2007; Lin et al., 2011; Ryan et al., 2011; Schmid et al., 2010; Wang and Bodovitz, 2010; Zhao et al., 2012). The heterogeneity in molecular content and phenotypic characteristics of genetically identical cell populations has been mainly ascribed to the stochastic nature of gene expression and protein synthesis and has been suggested to offer a survival advantage to cells subjected to the stress of environmental perturbations or changes (Kaern et al., 2005; Longo and Hasty, 2006). Global analysis of protein expression in *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Escherichia coli* (*E. coli*) revealed that many essential proteins and most transcription factors are produced at a low-copy number (fewer than one thousand molecules per cell) (Ghaemmaghami et al., 2003; Guptasarma, 1995; Taniguchi et al., 2010). Flow cytometry and fluorescence microscopy are the two most commonly used techniques to perform single-cell measurements. While microscopic imaging enables continuous monitoring of protein expression dynamics in single live cells (Cookson et al., 2005; Elowitz et al., 2002; Locke et al., 2011; Rosenfeld et al., 2005), flow cytometry provides a rapid snapshot of the expression pattern at a particular moment in time by analyzing a large number of individual cells in 1–2 min (Blake et al., 2003; Gardner et al., 2000; Newman et al., 2006; Ozbudak et al., 2002). Although the distribution profile of protein abundance can be obtained with both techniques, applications have been limited to moderate or high expression levels (Taniguchi et al., 2010).

β-galactosidase (β-gal), which is encoded by the *lacZ* gene of the *E. coli lac* operon, has been the standard cellular reporter for gene expression in both prokaryotic and eukaryotic cells (Jiang et al., 2008; Nolan et al., 1988). In its tetrameric form, a single β-gal enzyme is capable of generating a large number of fluorescent product molecules by hydrolyzing synthetic fluorogenic substrates. Yet the marked leakage of the fluorescent hydrolysis products from the cells makes fluorescence microscopic analysis of β-gal molecules at a low copy number a considerable challenge. To circumvent the problem of low intracellular retention of the

* Corresponding author. Tel.: +86 592 218 4519; fax: +86 592 218 9959.
E-mail address: xmyan@xmu.edu.cn (X. Yan).
[1] These authors contributed equally to this work.

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fluorescent products, microfabricated devices consisting of a large array of enclosed chambers are used to confine the fluorescent products and restore the fluorescence increase upon enzymatic amplification. Using this approach, detection of single β-gal molecules (Cai et al., 2006; Rondelez et al., 2005) have been demonstrated using fluorescence imaging. Bearing a resemblance to microfluidic chambers, microfluidic-based droplets have been applied to encapsulate single cells for the detection of β-gal activity in mammalian cells and for the sorting of induced *E. coli* cells (Baret et al., 2009; He et al., 2005).

Compared with microscopic measurement, flow cytometry provides a much higher throughput method for the generation of statistical distributions. Nevertheless, the analysis of β-gal has been mainly limited to induced bacterial cells and mammalian cells due to the inadequate sensitivity of the instrument (Miao et al., 1993; Nolan et al., 1988). Designed based on the techniques for single-molecule fluorescence detection in a sheathed flow (Ambrose et al., 1999; Castro et al., 1993; Chen and Dovichi, 1996; Dovichi et al., 1983; Keller et al., 1996), we have developed a high-sensitivity flow cytometer (HSFCM) for the analysis of synthetic and biological nanoparticles (Yang et al., 2010, 2012, 2009; Zhu et al., 2010, 2011). Particular, the ability of the HSFCM to resolve dimly fluorescent populations from each other is significantly improved. Compared to a state-of-the-art commercial flow cytometer, the fluorescence detection efficiency was improved 62-fold and the measured background light intensity is 12 FITC equivalents (Zhang et al., 2012). Herein, we describe the development of a high-throughput method for the single-cell analysis of low copy number β-gal using the laboratory-built HSFCM. Quantitative measurements of the basal and near-basal β-gal expression in single *E. coli* BL21(DE3) cells were demonstrated. Combined with the quantitative fluorometric assay and rapid bacterial enumeration by HSFCM, the β-gal expression distribution profile could be converted from arbitrary fluorescence units to protein copy numbers per cell.

2. Experimental section

2.1. Reagents and chemicals

The *E. coli* BL21 Star™(DE3) (F^-, *ompT*, $r_B^-m_B^-$) strain was purchased from Invitrogen (Carlsbad, CA). Isopropyl-β-D-thio-galactoside (IPTG), ortho-nitrophenyl-β-galactoside (ONPG), and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) were obtained from Sangon Biotech (Shanghai, China). IPTG and X-Gal were stored frozen as 200 mM and 20 mg/mL stock solutions in water, respectively. ONPG was freshly dissolved in PM2 assay medium at a concentration of 4 mg/mL. 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG) was purchased from Invitrogen and stored frozen as a 20 mM stock in 98% H_2O, 1% dimethylsulfoxide (DMSO), and 1% ethanol. Nucleic acid stain dye SYTO 9, 200 nm yellow–green FluoSpheres beads, and 210 nm orange FluoSpheres beads were obtained from Molecular Probes. Lysozome and lyophilized β-gal purified from *E. coli* were purchased from Sigma (St. Louis, MO). They were reconstituted at 1 mg/mL and 250 μg/mL in PBS containing 0.1% BSA (pH 7.0), respectively, and stored at 4 °C. 4-Methylumbelliferyl β-D-galactopyranoside (MUG) and phenylethyl β-D-thiogalactoside (PETG) from Sigma were stored as a 50 mM stock solution in DMSO at 4 °C and −20 °C, respectively. PopCulture Reagent was purchased from Novagen (Madison, WI).

2.2. Laboratory-built high sensitivity flow cytometer

A laboratory-built high-sensitivity flow cytometer (HSFCM) was used for β-gal activity analysis in single bacterial cells and for bacterial enumeration (Yang et al., 2012). Briefly, a solid state 488 nm continuous-wave laser was used as the excitation source. Both the side scattering and green fluorescence signals emitted from a single nanoparticle or bacterial cell can be measured at an analysis rate up to 100–200 events/s. For each bacterial sample, 60 s of data acquisition time was used. Defined by the overlap of the focused laser beam (~8.9 μm) and the sample stream (~4.2 μm in diameter), the detection volume was calculated to be ~0.13 pL. Based on Poisson statistics, when the concentration of bacteria is $\sim 1 \times 10^9$/mL, the probability that two bacterial cells coincidently occupy the probe volume is 0.7%.

2.3. Bacterial sample preparation

The *E. coli* BL21 Star™(DE3) strain were grown overnight at 37 °C in Luria-Bertani (LB) (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter) agar plates containing 0.4 mM IPTG and 40 μg/mL X-Gal. The hydrolysis of colorless X-gal by the β-gal expressed in the cells causes a characteristic blue color in the colonies. Cells from single blue colonies were grown overnight (~16 h) with shaking at 37 °C (250 rpm) in LB broth. On the following day, 300 μL of the culture grown overnight was inoculated into 30 mL of LB and grown under the same conditions until the OD_{600} value reached 0.4–0.5 (~2 h). The culture was divided into 2 mL aliquots, and IPTG was added to each aliquot. The cells were grown under the same conditions for another 6 h. The harvested cells were washed twice by PBS via centrifugation at 10000 rpm for 5 min and resuspended in 2 mL PBS. The bacterial sample was adjusted to OD_{600} ~1.75 (concentration $\sim 5 \times 10^9$ cells/mL) and stored at 4 °C for future use.

2.4. Beta-gal activity analysis and bacterial enumeration on the laboratory-built HSFCM

The harvested bacterial cells were stained with 50 μM of C_{12}FDG described follows: (1) 500 μL of the harvested bacterial cells were centrifuged and resuspended in 500 μL of M63 medium; (2) the cells were transferred to a glass test tube to which 7.5 μL of toluene and 7.5 μL of 0.1% SDS were added; (3) after shaking at 37 °C for 40 min, the cells were transferred to a 1.5 mL eppendorf tube, centrifuged, and resuspended in 500 μL PBS; (4) 20 μL of the treated sample was exposed to hypotonic conditions via mixing with 20 μL of 100 μM C_{12}FDG prepared in water; (5) after a 60 min incubation at 37 °C, the mixture was brought back to isotonicity by the addition of 360 μL of ice-cold PBS; (6) after 30 min incubation on ice, the mixture was washed once with PBS and resuspended in 100 μL ice-cold 2 mM PETG, a competitive inhibitor of β-gal, to stop the hydrolysis of C_{12}FDG. The sample was kept on ice and analyzed on the HSFCM within 2 h. Unstained samples of *E. coli* BL21 Star™(DE3) and *E. coli* JM109 were also subjected to toluene/SDS treatment except for C_{12}FDG staining.

For bacterial enumeration on the HSFCM, 50 μL of 500 nM SYTO 9 prepared in PBS was added to 50 μL of a 50-fold dilution of the harvested cells. After 5 min of incubation, the sample was analyzed on the HSFCM.

2.5. β-gal enzyme activity measurement by ONPG colorimetric assay

After being treated by toluene/SDS (shaking at 37 °C for 40 min), the bacteria cells were transferred to a 1.5 mL eppendorf tube and centrifuged. The supernatant was carefully pipetted into a centrifuge tube and the precipitate was resuspended in 500 μL M63 medium. Then 100 μL of the precipitate suspension and 100 μL of the supernatant were mixed with 900 μL PM2 buffer (70 mM $Na_2HPO_4 \cdot 12\ H_2O$, 30 mM $NaH_2PO_4 \cdot H_2O$, 1 mM $MgSO_4$, 0.2 mM $MnSO_4$, pH 7.0) separately and assayed with the ONPG colorimetric assay on a DU-800 spectrophotometer (Beckman