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生物技术与方法

微流控芯片监测绿色荧光蛋白在枯草芽孢杆菌中的表达

董惠钧[1*], 付景林[2*], 李永泉[3], 姜俊云[1]

1 鲁南制药集团股份有限公司研发中心, 临沂 276005
2 浙江大学微分析系统研究所, 杭州 310058
3 浙江大学生命科学学院, 杭州 310058

摘 要: 目前主要使用激光共聚焦扫描显微镜观察绿色荧光蛋白的表达, 但需要昂贵的仪器并耗费大量时间。本研究开发了一种新型激光诱导的微流芯片检测系统来监测绿色荧光蛋白在枯草芽孢杆菌中的表达。该系统主要由激光装置、光路系统、微流控芯片、光电倍增管和计算机处理系统等 5 部分组成。对该系统的测试结果显示, 随着诱导强度的增强监测信号峰也随之增强, 并且与激光共聚焦显微镜观察的结果一致。利用该芯片系统能够快速准确地筛选和鉴定用绿色荧光蛋白作为标记的细胞克隆, 可以替代 PCR 鉴定方法。但该系统仅仅能够监测表达强度, 不能够满足蛋白定位等高水平研究, 因此, 该系统适合应用于环境的微生物监测、药物筛选和其他无需观察蛋白定位等研究。

关键词: 微流控芯片, 绿色荧光蛋白, 激光诱导荧光, 枯草芽孢杆菌

Microfluidic chip for detecting the expression of green fluorescent protein in *Bacillus subtilis*

Huijun Dong[1*], Jinglin Fu[2*], Yongquan Li[3], and Junyun Jiang[1]

1 *Research and Development Center, Lu'nan Pharmaceutical Group Inc. Ltd., Linyi* 276005, *China*
2 *Institute of Microanalytical Systems, Zhejiang University, Hangzhou* 310058, *China*
3 *College of Life Sciences, Zhejiang University, Hangzhou* 310058, *China*

Abstract: Laser scanning confocal microscope (LSCM) is currently the only equipment to observe fluorescence. However, this technique has disadvantages such as high cost and long test process. In this study, we developed a new system of laser-induced fluorescence (LIF) for microfluidic chip applied to detecting the expression of green fluorescent protein (GFP) in *Bacillus subtilis*. This novel system was comprised of laser device, optics unit, microfluidic chip, photomultiplier and computer treatment unit. The tests indicated that microfluidic chip could detect the expression of GFP as sensitively as LSCM in *Bacillus subtilis*. Moreover, this LIF detection system could instead of PCR to identify the positive clone in this special case. Nevertheless, the LIF system only was suitable to detect the fluorescent strength of GFP, and could not meet the request of some cases for example protein location. Therefore, this system will be applied in environmental detection with microbe, drug discovery and other cases.

Keywords: microfluidic chip, green fluorescent protein (GFP), laser-indced fluorescence (LIF), *Bacillus subtilis*

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Corresponding author: Huijun Dong. Tel: +86-539-8378557; Fax: +86-539-5030678; E-mail: donghuijun_747@yahoo.com.cn.
* These authors contributed equally to this work.
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Introduction

Microfluidic chips have become an attractive tool for the analysis of single cell because its dimensions are comparable with the size of single cell and because of the potential for performing rapid analyses on a massively parallel scale. Recently, several reports have appeared on the separation of the contents of individual cell by using microfluidic chip[1–3]. The green fluorescent protein (GFP) from the jellyfish Aequorea victoria has become a widely used marker in molecular and cell biology due to its strong intrinsic visible fluorescence, which is easily detectable by fluorescence spectroscopy[4]. This protein of around 27 kD can be fused to many other proteins, allowing their visualization in living cells without interfering with their function. Therefore, it becomes possible to follow signaling and trafficking in cells[5] and to study protein-protein interactions[6]. Some GFP variants are also used as noninvasive intracellular pH biosensor[7] or fluorescent indicator for local Ca^{2+} concentration[8]. As yet, the fluorescent microscopy has still been regarded as the unique technique for detecting GFP although it actually has some inconveniences such as pre-treating cell sample, cell fixation and focusing when only need to know whether the GFP expresses normally. In this study, a new detection technique for GFP was developed based on laser-induced fluorescence for microfluidic chips, which has advantages of micro-volume analysis, convenience and high sensitivity.

1 Materials and methods

1.1 Plasmids, strains and cultural conditions

Strain *B. subtilis* 168 (trpC2) and plasmid pSG1729 (Bla amy E3' spc Pxyl-gfpmut1' amyE5') were presented by Dr. Daniel R from Bacillus Genetic Store Center in USA. The *B. subtilis* 168 was grown at 30°C in TY (Tryptone/Yeast extract) broth containing Bacto-Tryptone (10 g/L), Bacto-Yeast extract (5 g/L) and NaCl (10 g/L). When required, media for *B. subtilis* 168 was supplemented with 100 μg spectinomycin/mL[9].

1.2 Transformation and expression

Transformations were carried out according to the description by Cutting *et al*[10]. To confirm that insertions had occurred by double cross-over at the *amyE* site, transformations were patched onto TY medium plates containing 1% (*W*/*V*) dissoluble starch, grown overnight and stained by a few iodine liquid. Correct constructs were selected on their amylase deficient phenotype. The positive constructs were cultivated in TY medium and induced with xylose to express GFP.

1.3 Western blotting

Western blotting analysis is applied to confirm the expression of GFP. Antibody against GFP and anti-antibody were bought from Beoytime Institute of Biotechnology in China. The detail process referred to Cold Spring Harbor Protocols.

1.4 GFP detection with microfluidic chips

This innovative laser-induction fluorescent detection system for microfluidic chips is developed by Fu *et al*[11]. In this system, argon ion gas laser generator with 4 mW power emits laser ray of 488 nm which is reflected by concavity mirror and ejects to the objective site on chip as 45°. The fluorescent substance flowed through the microchannel on the chip are excited and generates fluorescence that is collected by 2 mm diameter optical fiber equipped at the bottom of chip. The collected optical signals are multiplied and translated to electronic information by photomultiplier. Finally, the electronic signals are analyzed by computer program (Fig. 1). In this study, the 50 μL xylose-induced *B. subtilis* cells are injected into the injection pool, and flow through the microchannel driven by capillary action, and analyzed by computer.

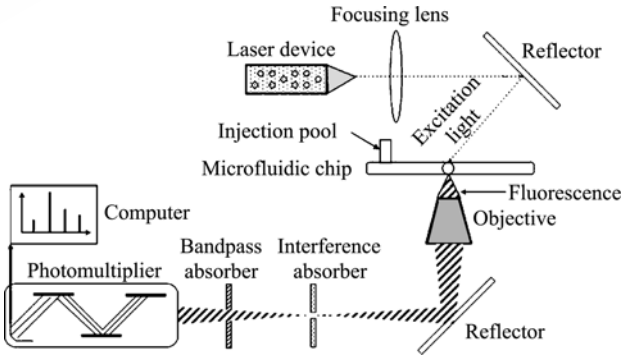

Fig. 1 Diagram of the non-confocal LIF detection system.

1.5 GFP detection with LSCM

The reconstructed *B. subtilis* cells are induced with xylose to express GFP when cells have been cultivated in TY medium at 30°C. Secondly, cells were transferred onto the slide to be observed by LSCM (Carl Zeiss LSM 510 NLO, Germany) after washing with PBS (phosphate-buffered saline) buffer. The observation conditions were 488 nm of the emission wave and 510 nm of the absorption wave [12].

1.6 DNA manipulation and PCR

Genome DNA of *B. subtilis* is isolated and purified as the method described in Molecular Cloning[13]. The

gfp gene located in the genome of *B. subtilis* is attained by PCR with proper primers. The forward primer is 5′-ATGGTGAGCA AGGGCGAGGAGC-3′, and the reverse primer is 5′- TTACTTGTACAGCTCG TCCATG-3′. All kinds of kits used in this research were purchased from Takara Company of China.

2 Results

2.1 Transformation and selection

Plasmid pSG1729 carries the homologous *amyE* fragment that could make itself to insert into the *amyE* locus on the genome of *B. subtilis* by double cross-over. The expression of the *gfp* fusion is controlled by the xylose-inducible Pxyl promoter (Fig. 2A). The successful insertions would show the amylase deficient phenotype and be detected on plate through iodine. On the contrary, the transparent circle around single cell indicated the abortive recombination (Fig. 2B).

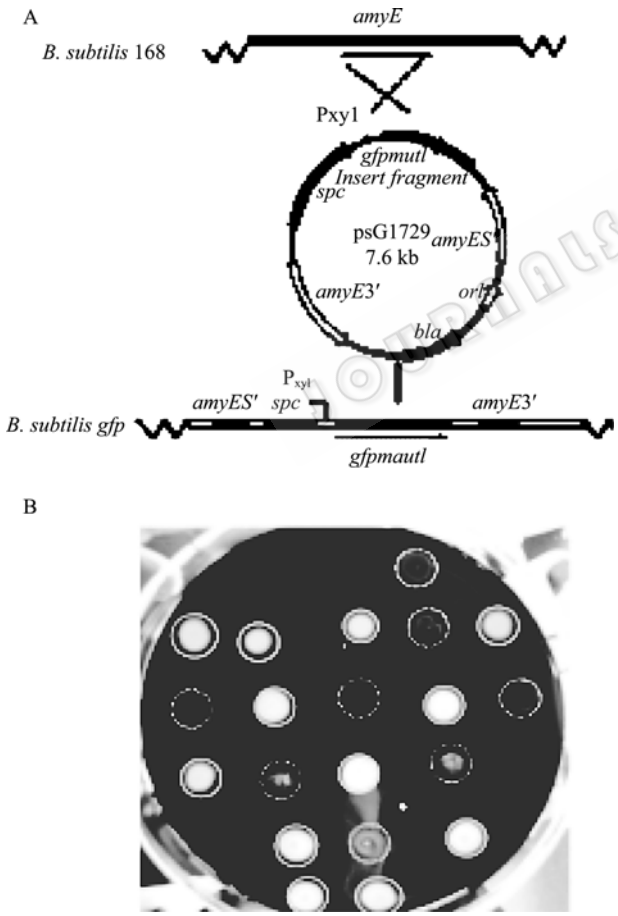

Fig. 2 Recombination mechanism and selection for the correct transformations. (A) Diagram of the recombination mechanism. (B) Screening the correct recombination on plates with iodine treatment. Solid line circles indicate the negative recombinants, and dash line circles indicate the positive recombinants.

2.2 Expression and detection of GFP

The constructed *B. subtilis* could express GFP under the induction of xylose. In this experiment, three different detecting methods including western blotting, microfluidic chips and LSCM were respectively used to investigate the GFP expression. For microfluidic chip detection, the micro-volume culture, which required no pre-processing, was injected into the sample pool on chip and passed through the detecting locus at the proper velocity with the promotion of capillary action. The excitation ray of 488 nm generated by laser device radiated cells to generate the light signals of green fluorescence that would be collected, dealt and transformed to the electronic signals in order. Finally, a computer program analyzed the information and gave a precise plot result. To investigate the novel system's sensitivity the different strength green fluorescence were produced by induction with different concentration xylose. The results shown in Fig. 3B indicated that the fluorescence intensity increased remarkably with the raise of xylose from 0% to 1.0% (*W*/*V*). Notably, the signal peaks reflected the fluorescence intensity of the majority cells and no single cell. There is an emerging doubt whether the detecting results from the microfluidic chips system could really indicate the fluorescence intensity of single cell. Therefore, the further necessary detection was exerted to exam the fluorescence of single cell by LSCM. The excitable results revealed that the single cell presented the fluorescence stronger and stronger

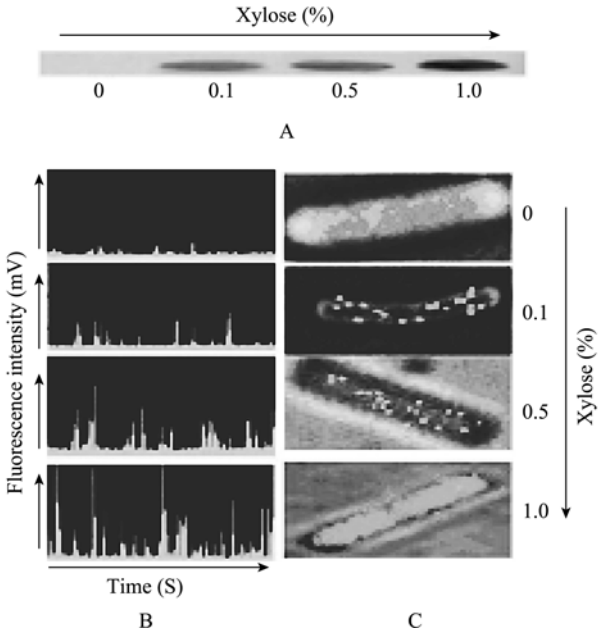

Fig. 3 GFP detections with different methods. (A) Western blotting for GFP protein. (B) LIF detection for microfluidic chip. (C) LSCM pictures.

with the increase of xylose (Fig. 3C), which consisted with the results of the microfluidic chips. Moreover, the western blotting for GFP protein also confirmed these results (Fig. 3A).

2.3 Application of microfluidic chip in screening clones

The microfluidic chip detection system described in this study was successfully used to screen the clones carrying the *gfp* gene (Fig. 4A). Meanwhile, the PCR method was applied to confirm the detection by microfluidic chip (Fig. 4B). These results indicated that the microfluidic chip detection system could exactly and quickly identify the positive clones containing the fluorescent protein such as GFP protein. However, this system could not give the quantitative data since the roving strains in microchannel presented the colonial reflection but no single cell reflection. If the larger mammalian cell substituted the microorganism cell, this detection system would quantitate the detection as the previous study[14]. Therefore, this novel microfluidic chip detection system could use a tool to detection the expression of fluorescent material qualitatively.

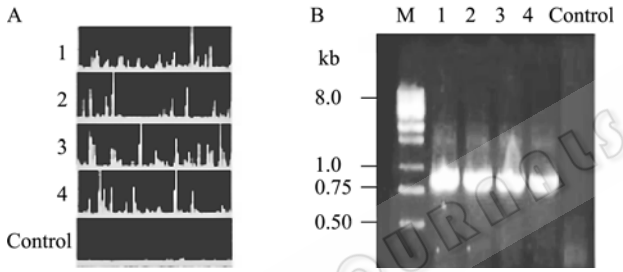

Fig. 4 Identification of the positive clones with PCR and microfluodic chip. (A) Identification of the GFP expression with microfluidic chip. The spectrograms from 1 to 4 present the GFP detection in the positive clones. The control spectrogram indicates the detection for the origin strain. (B) Identification of the *gfp* gene with PCR. Lanes 1–4 indicate the positive clones carrying the 720 bp *gfp* gene in their genome. The control lane indicates the strain without the *gfp* gene.

3 Discussion

Microfluidic chips have been applied in the different scientific domains including clinical diagnostics, environmental monitoring, pharmaceutical drug discovery, and chemical synthesis, which will have the potential to dramatically change the way of biochemical analysis[15–17]. The laser-induced fluorescence for microfluidic chips are usually used to detect the chemical fluorochromes such as fluorescein iso-thiocyanate (FITC) and propidium iodide as the modifier for biomoleculars in researches of cell biology. Although Lewandowski AT developed an approach to fuse the GFP to the C-terminus of the objective protein for detecting the enzymatic reaction by microfluidic device *in vitro*[18], however there have been few reports for the detection of the GFP protein expressed in microbe using microfluidic chips. In this study, the GFP expression in *B. subtilis* was monitored successfully with the LIF system which was designed and constructed by our research team. This novel simple detection system has the advantages of low-cost, portability and sensitivity. However, the system could not give the visibility of GFP as the fluorescence microscopy in cells. Therefore, this detection system only supplies the strength of fluorescence, which has the potentials to assist the environmental monitoring, drug discovery and other cases when it is unnecessary to observe the GFP.

In the further studies, the efforts will be made to separate and monitor the single microbial cell, which could expand the application of the microfluidic chips.

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快 讯

艾滋病的基因疗法

人类首个艾滋病病毒（HIV）感染患者的基因治疗临床试验结果终于出来了。新成果发表在日前在线出版的《自然—医学》期刊上。对艾滋病患者来说，基因治疗是一种颇有吸引力的选择，因为这种疗法的一次性使用就可减少病毒数量、保护免疫系统和避免抗逆转录治疗的终身使用。

Ronald Mitsuyasu 和同事对 74 位 HIV 成人感染者实施了随机、双盲、安慰剂控制的基因转换临床试验，受试者或者是接受安慰剂，或者接受携带有名为 OZ1 分子的血液干细胞，这种分子以 2 种 HIV 蛋白质为靶标从而预防了病毒的复制。在整个临床试验过程中，OZ1 是安全的，没有产生任何副作用。尽管在 47 周和 48 周的试验中，接受 OZ1 和安慰剂的 2 组受试者在病毒数量上没有统计学上的区别，但在 100 周时，OZ1 组的 $CD4^+$淋巴细胞数量要高一些，而在患者体内，这种淋巴细胞被 HIV 去除了。

新研究显示，对 HIV 感染者实施基因治疗是安全有效的，而且这种治疗方法可发展为对付艾滋病的通用方法。

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