ANALYTICAL SCIENCES MARCH 2013, VOL. 29

Notes

Parallel Real-Time PCR on a Chip for Genetic Tug-of-War (gTOW) Method

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A microchip-based real-time polymerase chain reaction (PCR) device has been developed for the genetic tug-of-war (gTOW) method that provides quantitative data for research on biorobustness and systems biology. The device was constructed of a silicon glass chip, a temperature controlling Peltier element, and a microscope. A parallel real-time amplification process of target genes on the plasmids and the housekeeping genes in a model eukaryote *Saccharomyces cerevisiae* were detected simultaneously, and the copy number of the target genes were estimated. The device provides unique quantitative data that can be used to augment understanding of the system-level properties of living cells.

(Received December 24, 2012; Accepted January 13, 2013; Published March 10, 2013)

Introduction

Intracellular biochemical parameters, such as the gene expression level of gene products, are considered to have been optimized during the evolutionary process to allow biological activities to be carried out in an organism's habitat. These parameters should have some permissible range so that the systems have robustness against perturbations. However, little is known about the permissible ranges of parameters in real cells because there has been no experimental technique to comprehensively measure the limits of intracellular parameters. Gene knockout experiments are used to reduce the expression level of target genes.[1,2] These experiments provide phenotypical information that reveals the functions of target genes. However, such experiments do not provide quantitative information associated with the limit of expression of the target genes in order to maintain function of the biological systems. Promoter-swapping experiments, in which the promoter of the target gene is changed into a strong promoter, are used to increase the expression level of the target genes, and this method also has provided much useful information for predicting the functions of target genes, as well as genetic interactions between target genes.[3-5] However, it is also difficult to determine the upper limit of the expression of the target genes because this method ignores the native expression level and regulation of the target genes.[6-9] Genetic screening by a genetic tug-of-war (gTOW) method allows the upper limit of each target gene in living cells to be measured by increasing the copy number of that gene.[10,11] Each target gene and its native regulatory DNA elements (promoter and terminator) are used as a unit so that the increased copy number of that gene can be determined quantitatively, and the gene expression level is expected to increase according to the copy number. Knowledge obtained from the gTOW method is important for understanding of biology at the system level.[12-14]

The gTOW method analysis is useful exploring the foundation of biological systems such as biological robustness.[15-17] Biological robustness is proposed as the main concept for the application of systems biology in cancer research. Biological robustness is a fundamental feature of evolvable complex systems that must be robust against environmental and genetic perturbation to be evolvable. At the same time, robust systems face fragility and performance setback as an inherent trade-off.

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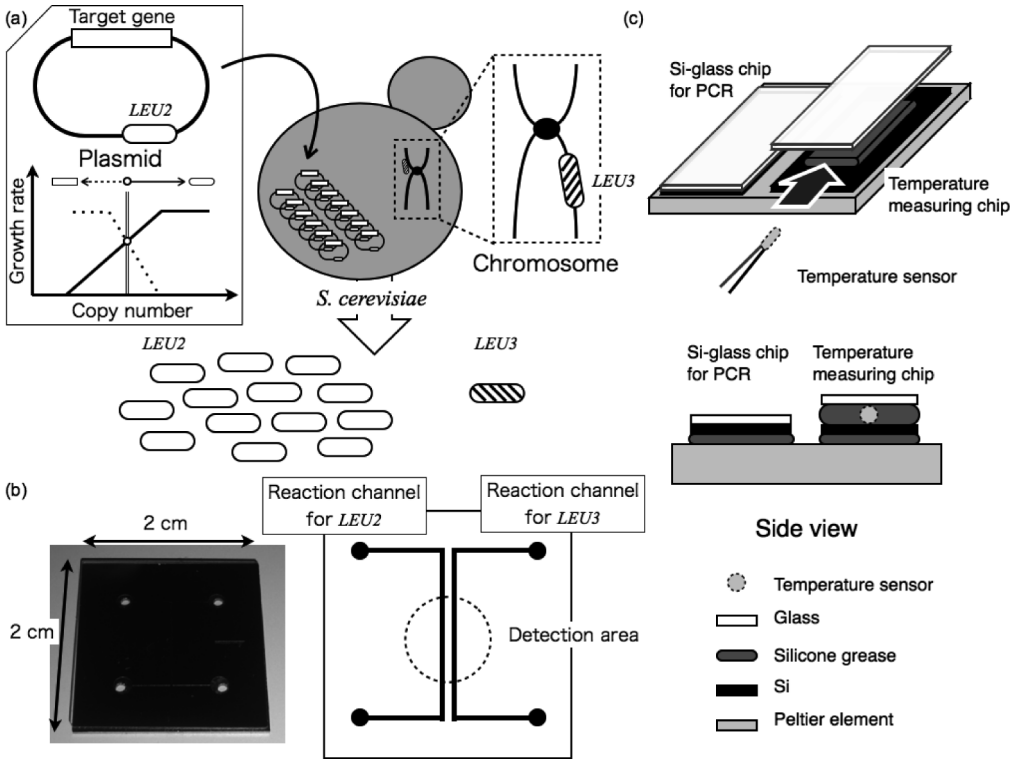

Fig. 1 Schematic illustrations of the PCR device for gTOW method. (a) Samples used in the gTOW method. Two kinds of genes were used, one is *LEU2* gene coded on plasmid introduced to *S. cerevisiae* and the other gene is *LEU3* coded on a chromosomes of *S. cerevisiae*. After a tug-of-war between the increasing activity of the copy number from *LEU2* and the decreasing activity of the copy number from *LEU3*, we could define the copy number of the plasmid. (b) Design of the PCR chip. Two channels were aligned in a single microscopic field to allow direct comparison of the relative quantities of *LEU2* and *LEU3*. (c) Schematic illustration of the temperature measurement system. To measure the temperature at the microfluidic channel, a temperature sensor was sandwiched between the silicon wafer and glass cover next to the chip for PCR.

Identification of the basic architecture for a robust system and the associated trade-off is essential for understanding their faults and countermeasures against fatal diseases. Although the gTOW method is the only way to provide quantitative data that can be used to determine the system-level properties of living cells and to quantitate biorobustness against gene overexpression, it needs high-throughput, comprehensive, and accurate measurement devices to obtain precisely quantified information.

Microchip devices are one of the most suitable technologies to be introduced to design and build next-generation experimental devices. It may drastically improve the speed and accuracy of measurement through integration and automation of experimental procedures. Especially, real-time polymerase chain reaction (PCR) is an important procedure to understand biological systems through the gTOW method. Although several real-time PCR devices have been reported,[18,19] they were not optimized for the gTOW method. In this paper, we describe development of a microchip-based device that realizes parallel real-time PCR on a chip for the gTOW method.

Experimental

The gTOW method can estimate the upper limit of the gene expression level for each target gene by increasing the copy number of that gene. Two kinds of genes were amplified in the gTOW method to determine the upper limit copy number of target genes; one was leucine biosynthesis gene *LEU2* coded on the plasmid DNA with a target gene and the other was transcriptional regulator of leucine *LEU3* coded on the chromosome of *Saccharomyces cerevisiae* (Fig. 1(a)). By comparing the relative quantity of *LEU2* and *LEU3* genes, we could estimate the copy number of the plasmid per haploid genome. A design of our device is shown in Fig. 1(b). The temperature was controlled by the Peltier element and reaction channels were fabricated on a silicon wafer. The thermal control system was comprised of a power supply (HLE, Nihontecmo Co., Ltd., Fukuoka, Japan), a controller (SP5R7-576, Nihontecmo Co., Ltd.), the Peltier element (TEC-12708, Nihontecmo Co., Ltd.) and LabVIEW-based temperature control and data-logging software. The reaction mixture contained intercalating dye, SYBER Green1, which allowed DNA detection with a fluorescence microscope. In this device design, the amplification process of *LEU2* and *LEU3* genes could be detected simultaneously in a single observation view.

Total DNA used as a template DNA sample was extracted from wild type yeast cells (BY4741) with pTOW that is a 2-micron based plasmid with *leu2d* for the gTOW method.[10] Yeast cells, collected from 200 μL of saturated culture, were suspended in lysis solution (10 mM Na-phosphate (pH 7.5), 1.2 M sorbitol, and 2.5 mg/mL Zymolyase 100T) (Seikagaku, Tokyo, Japan) and incubated with a block incubator (BI-525, ASTEC, Fukuoka, Japan) for 10 min at 37°C to digest the cell wall. Then the cell suspension was heat-shocked at 94°C for

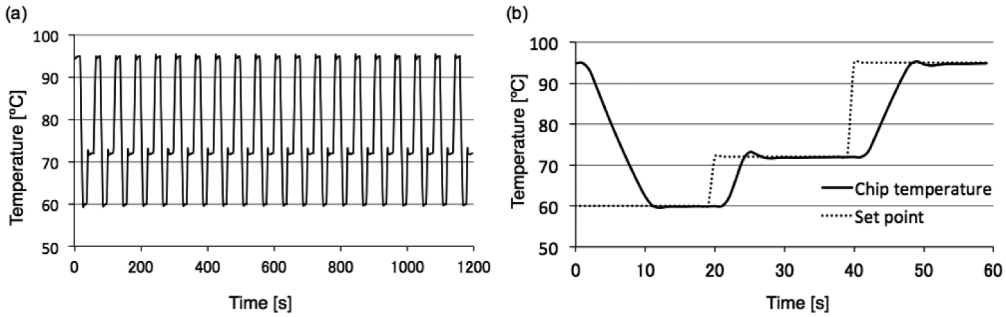

Fig. 2 (a) Temperature profile for 20 cycles: one cycle consisted of heating at 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s. (b) Comparison between chip temperature and set point for 1 cycle.

15 min, –80°C for 5 min, and then 94°C for 15 min. After the cell suspension was chilled and centrifuged, supernatant (containing total DNA) was used for the following two real-time PCRs: LightCycler FastStart DNA MasterPLUS SYBER Green I (Roche Diagnostics K.K., Tokyo, Japan) with LightCycler 2.0 instrument (Roche Diagnostics K.K.) was used for the kinetic PCR. Supernatant (2 µL) was mixed with each reaction mix (18 µL) containing 0.5 µM of *LEU2* primer set (F: GCTAATGTTTTGGCCTCTTC, R: ATTTAGGTGGGTTGGG-TTCT) and *LEU3* primer set (F: CAGCAACTAAGGACAAGG, R: GGTCGTTAATGAGCTTCC) using 0.1% bovine serum albumin (Sigma Aldrich Japan K.K., Tokyo, Japan). The genes were amplified using the following thermal cycling profile; initial denaturation at 95°C for 10 min, followed by 30 cycles with one cycle consisting of 95°C for 10 s, 60°C for 10 s and 72°C for 7 s.

Reaction channels were fabricated on silicon wafers by using standard photolithography and deep reactive ion etching (MUC-21, Sumitomo Precision Products Co., Ltd., Hyogo, Japan). The reaction channel had a rectangular cross section of 100 µm width and 20 µm depth. After dicing the wafers with an automatic dicing saw (DAD522, DISCO, Tokyo, Japan), inlet and outlet holes were made using ultrasonic drilling (SOM-121, Shinoda K.K., Tokyo, Japan). A glass cover (SD-2, Hoya Candeo, Saitama, Japan) was anodically bonded to the silicon substrate at 400°C, 1 kV with the handmade anodic bonding machine consists of a hot plate (MSA Factory, Tokyo, Japan) and a power supply (31601-5N, Apple Electronics Co., Ltd., Shanghai, China). A silicon-glass chip for PCR and silicon-glass plate sandwiching a temperature sensor for temperature measurements were put into silicone grease (TCOH-1002, Taica, Tokyo, Japan) on the Peltier element (Fig. 1(c)). As for real-time PCR on a chip, the same reaction mixture was used with different thermal cycling; initial denaturation at 95°C for 2 min, followed by 30 cycles (95°C for 27 s, 60°C for 28 s and 72°C for 15 s). Fluorescent intensity was measured by a fluorescent microscope (Eclipse T*i*, Nikon, Tokyo, Japan) and open source image analysis software (ImageJ).

Results and Discussion

S. cerevisiae lacking the leucine-generating function was used for the gTOW method. The leucine-generating function of the cells was obtained by the plasmids. Cells with a higher copy number of the plasmids can grow faster under the leucine condition because the activity of the leucine-generating function derived from the plasmid is very weak. On the other hand, the copy number of the plasmid is limited by the target gene on the plasmid. The target gene becomes another genetic selection bias toward decreasing the plasmid copy number for inhibition of growth over the upper limit copy number of the target gene. The copy numbers of the plasmids reach an equilibrium between the increasing activity of the copy number from *LEU2* and the decreasing activity of the copy number from *LEU3*, just like a tug-of-war between the two kinds of genes.

First, we evaluated the temperature control device. The Peltier element was computer-controlled with a proportional integral derivative (PID) control algorithm, and we optimized the PID setting to achieve a high temperature ramp rate, high accuracy, and small temperature overshoot. The temperature program was set to 20 cycles (heating at 95°C for 20 s, 60°C for 20 s, 72°C 20 s). As shown in Fig. 2(a), the temperature control device worked reliably throughout 20 thermal cycles and it also functioned for over 30 cycles in subsequent experiments. Total process time could be extended to 3600 s by the temperature control program, which was an adequate time scale for the PCR process. The actual holding time in the reaction channel was 95°C for 13 s, 60°C for 12 s, and 72°C for 17 s, all with the accuracy of ±0.3°C; the rate for changing the temperature was 4°C s^{-1} and it took 2 s to change the heating-cooling mode (Fig. 2(b)). The temperature control system achieved the same level of the temperature ramp rate and accuracy as the commercial product we used, and the temperature overshoot was negligible.

We tried to amplify two kinds of genes simultaneously in a single observation view, and we observed increasing fluorescent intensity of the reaction channel for *LEU2* compared to that of the reaction channel for *LEU3* (Fig. 3(a)). To estimate the relative fluorescent intensity result from amplification of *LEU2* gene to amplification of *LEU3* gene, we measured fluorescent intensity profiles of each image. The relative fluorescent intensity of a *LEU2* channel *versus* a *LEU3* channel was 2.2 times larger after the 30th cycle, whereas there was no change between fluorescent intensities of the channel for *LEU2* and *LEU3* after 10 cycles (Fig. 3(b)). Amplification curves of *LEU2* and *LEU3* genes were obtained using chips (Fig. 3(c)). After fitting with a logistic curve, the relative quantities of *LEU2* and *LEU3* were compared at 40% of their maximum value. The copy number of the plasmid determined by the amplification curves obtained by fabricated devices was 365 copies; this value was quite different from the 107 copies obtained in a bulk experiment (Fig. 3(d)). The difference is attributed to the amplification curves of the on-chip PCR experiment being of low precision due to observation with unfixed focus. Three possible causes for unfixed focus are: non-uniform thickness of

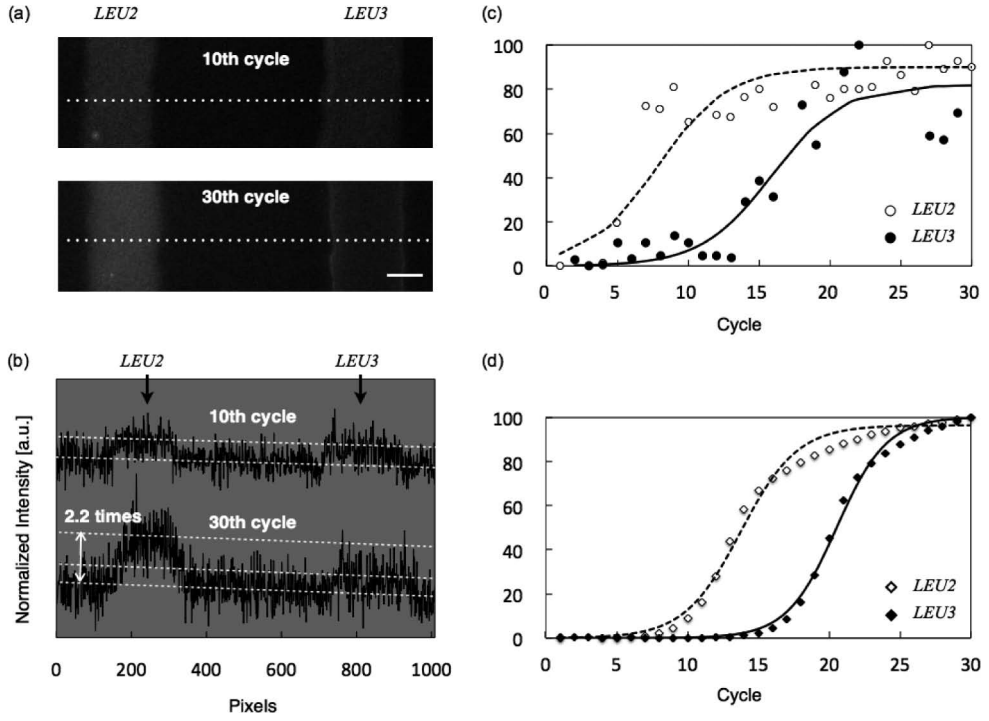

Fig. 3 (a) Fluorescent images of microfluidic channel for directly comparing PCR at the 10th cycle (upper) and 30th cycle (lower). (b) Normalized fluorescent intensity of images from (c). (c) Amplification curves for *LEU2* and *LEU3* genes by the chip. Open and solid circles indicate normalized measured values of *LEU2* and *LEU3*, respectively. The logistic curve was fitted to the measurement values (dotted line, *LEU2*; solid line, *LEU3*). (d) Amplification curves for *LEU2* and *LEU3* genes obtained by a commercial PCR instrument. Open and solid squares indicate the normalized measured values of *LEU2* and *LEU3*, respectively. The logistic curve was fitted to the measurement values (dotted line, *LEU2*; solid line, i*LEU3*).

the silicone grease, an unstable heating system, and thermal expansion of the silicon-glass chip. The non-uniform grease thickness might cause inhomogeneous heating of reaction channels and that would cause slight changes in reaction time and reaction temperature. Although the silicone grease we used was a conductive thermal silicone sheet pad to prepare homogeneous silicone layers, the thickness of grease was changed by manually putting chips on the Peltier element. To obtain higher precision, it is necessary to use special holders with a detector to fix in place the PCR chip with the temperature control system including the Peltier element that is put on the silicone grease under uniform pressure. The problem of thermal expansion of silicon-glass chips was also caused by detection with a manual procedure. To solve the problem we will have to develop a fluorescent capture system that synchronizes with the temperature control software to detect the fluorescent signal of reaction channels periodically. In addition, the PCR device needs significant improvements, that is, microfluidic techniques to integrate experimental procedures and functions into the PCR device for automation and exclusion of caused by manual handling experimental errors. We have developed two key components, a solution mixing device[20,21] and a reaction channel sealing system,[22] for accelerating automation of the procedures to obtain more quantitative data. We will integrate these key components with the PCR device developed here in the near future. There is a possibility that the logistic curve is not suitable for curve fitting in this case. We attempted to make a fitting curve using a logistic curve because DNAs were double a cycle during PCR, and we used the same data analysis method for on-chip and in-bulk PCRs. However, the fitting curve in Fig. 3(d) does not seem to be the best fitting curve. Concentrations of PCR solutions change with increasing number of thermal cycles, which changes the amplification efficiency. We considered that using the best suited curve fitting might reduce the analytical error between on-chip and in-bulk PCRs.

There are several issues that need to be addressed regarding our parallel PCR device. One is that the device used only 2 μL PCR solution, only 10 percent of the sample volume required for a bulk experiment for amplifications of DNA. The device can be extended to an even more parallelized PCR device that can also prepare calibration curves during the amplifications of the genes to determine the upper limit copy number of target genes after fewer thermal cycles with the same or less sample volume for bulk experiments.

Conclusions

We developed a real-time PCR chip for gTOW method and amplified two kinds of genes using the chip. The device could be improved, however, such as by making a special chip holder, changing the channel design and modifying channel surfaces to prevent non-specific adsorption of an enzyme so that the device can be easily used by systems biologists. The device has good potential for promoting studies in systems biology.

Acknowledgements

This research was partially supported by the Japan Society for the Promotion of Science (JSPS) through its "Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program)" and Grant-in-Aid for JSPS Fellows.

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