ARTIFICIAL DARWINIAN SELECTION TECHNOLOGY ON MICROARRAY CHIPS TOWARDS DIRECTED EVOLUTION USING SINGLE MOLECULE PROCESSING

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ABSTRACT

On-chip artificial Darwinian selection technology using a microarray was developed as one of the most promising approaches to efficiently obtaining the genetic codes of superior proteins from a large number of mutant libraries. A first-generation mutant GFP (green fluorescent protein) library in which random mutation was introduced to a chromophore region was translated on a chip. Subsequently, a second-generation protein chip arraying only the brightest GFPs was successfully obtained using the Darwinian selection technology. This selection technology is expected to become an essential tool for directed molecular evolution.

KEYWORDS

Directed evolution, Mutants, Microarray, Emulsion PCR, Selection

INTRODUCTION

In the field of high-speed protein molecular evolution technology, the high-throughput selection of genetic codes of mutants is a challenging issue, and an integrated microarray chip with directed evolution is one of the most efficient approaches to achieving the evolution of superior proteins [1]. We have been developing an ultra large scale (1.44×10^8 wells/chip) microwell array chip as a platform to enable the screening and one-step synthesis of mutant proteins [2]. In this paper, we report a simple and robust technology for selecting superior genetic codes from a mutant protein library on a microarray chip.

EXPERIMENT

A schematic of the selection technology is shown in Fig. 1. Each molecule of a random mutant GFP-DNA was amplified and immobilized onto a 2.8 μm diameter magnetic bead using the BEAMing method that was applied to emulsion PCR [3]. Subsequently a DNA microarray chip (6.9×10^5 beads/cm^2) arraying random mutant DNAs was fabricated using our developed automatic magnetic bead arrangement system, which enables an array of DNA-immobilized beads to be formed in a PDMS microwell (4.0 μm diameter and height) with the assistance of an external dynamic magnetic force. A cell-free translation reagent was poured onto the DNA microarray chip. Subsequently, the entire surface of the chip was covered with a synthetic oil to isolate each microwell. After incubation, translated random mutant GFPs on the chip were observed under a confocal microscope at 488 nm excitation, and the mutant DNAs indexed to the brightest GFPs were recovered selectively. Finally, the recovered mutant DNAs were amplified.

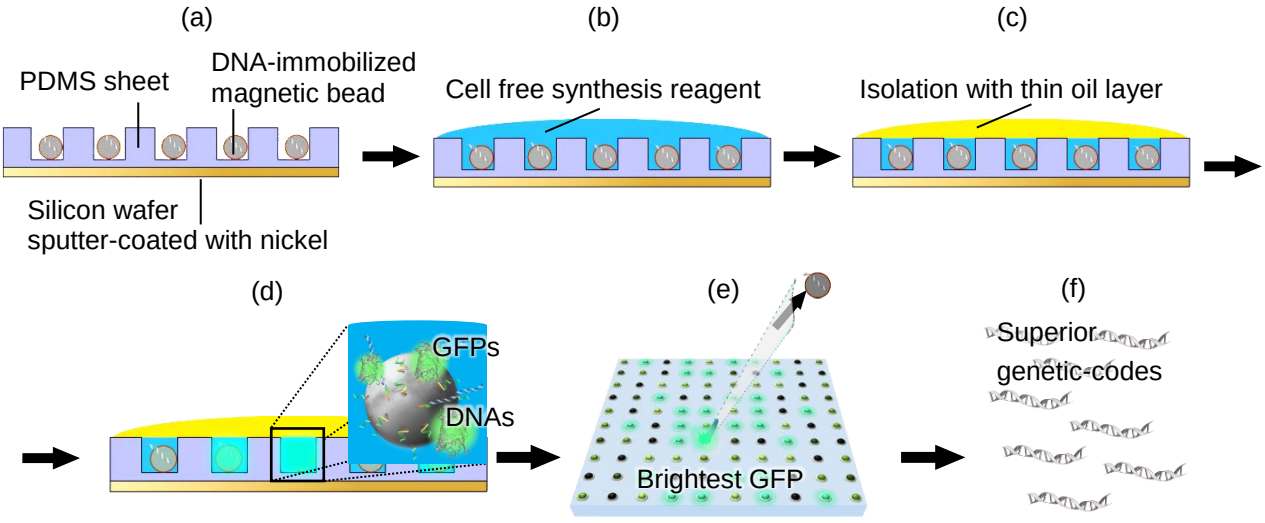

Figure 1 Schematic of artificial Darwinian selection technology. a) Mutant DNA microarray chip. b) Pouring a cell-free translation reagent onto the microarray array chip. c) Isolation of all wells with thin oil layer. d) Cell-free translation from mutant DNAs to GFPs. e) Recovery of the beads immobilizing mutant DNA including genetic codes with brightest mutant GFPs. f) Amplification of the recovered mutant DNAs.

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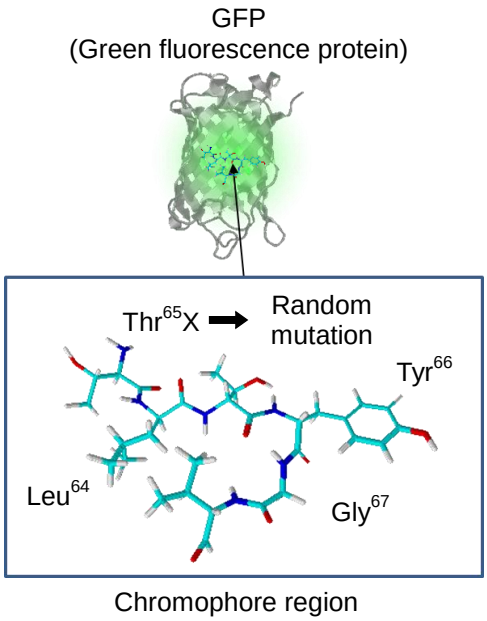
RESULTS AND DISCUTION

As shown in Fig. 2, the mutant GFPs introduced random mutation into threonine at position 65 in the chromophore region of wild-type GFP. On the other hand, the cell-free translation reagent was perfectly dispensed into all the wells only by flowing of a thin oil layer (See Fig. 3 for the simulation result). Hence, as shown in Figs. 4(a) and (b), the DNA microarray chip was converted into a protein array chip with various fluorescence intensities. The synthesis of mutant GFPs was also confirmed by SDS-PAGE (Fig. 4(c)).



5'ATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTC
CCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGC
ACAAATTTTCTGTCAGCGGAGAGGGTGAAGGTGATG
CAACATACGGAAAACTTACCCTTAAATTTATTTGCACT
ACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCA
CTACTCTGNNNTATGGTGTTCAATGCTTTTCCCGTTA
Chromophore region
N = A or T or C or G
CCGGATCACATGAAACGGCATGACTTTTTCAAGAGTG
CCATGCCCGAAGGTTATGTACAGGAACGCACTATATC
TTTCAAAGATGACGGGAACTACAAGACGCGTGCTGA
AGTCAAGTTTGAAGGTGATACCCTTGTTAATCGTATC
GAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACA
TTCTCGGACACAAACTCGAGTACAACTATAACTCACA
CAATGTATACATCACGGCAGACAAACAAAAGAATGG
AATCAAAGCTAACTTCAAAACTCGCCACAACATTGAA
GATGGCTCCGTTCAACTAGCAGACCATTATCAGCAAA
ATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGA
CAACCATTACCTGTCGACACAATCTGCCCTTTTGAAA
GATCCCAACGAAAAGCGTGACCACATGGTCCTTCTTG
AGTTTGTAACTGCTGCTGGGATTACACATGGCATGGA
TGAGCTCTACAAA3'

Figure 2 Schematic of a position of introduced random mutation to wild-type GFP. a) A position of random mutation (threonine65X (Thr^{65}X)) introduced into amino-acid sequence of the GFP. b) Position of random mutation (NNN) introduced into DNA sequence of the GFP.

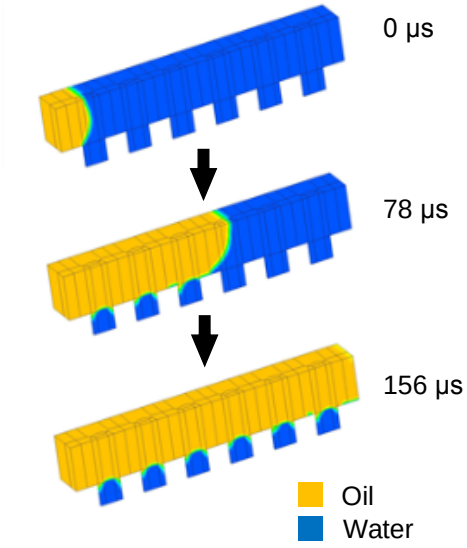


Figure 3 Volume of fluid (VOF) simulation for the isolation of all PDMS microwells sinply by the flow of a thin oil layer.

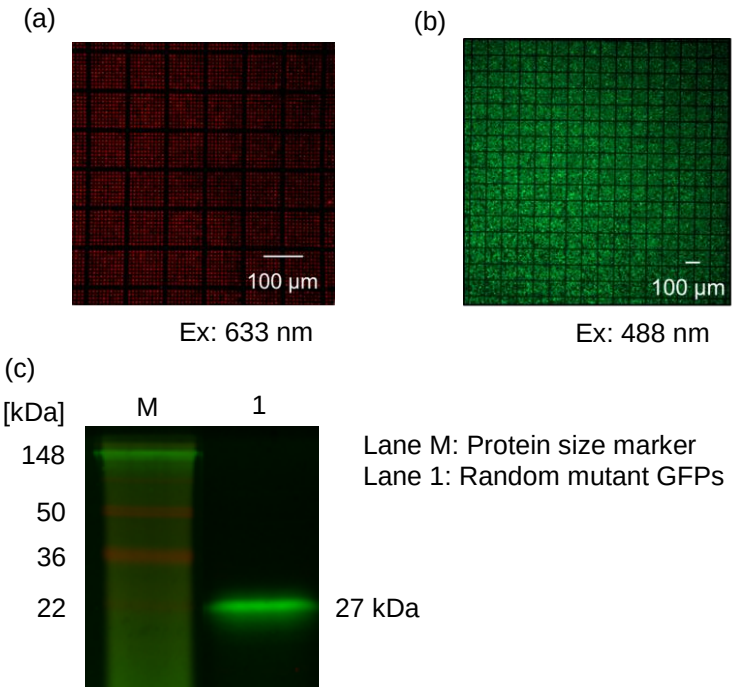


Figure 4 a) Fluorescence microscopy image of a mutant DNA microarray chip. Cy5-labeled DNA-immobilized beads were arrayed on the microwell array chip with high density. b) Fluorescence microscopy image of first-generation GFPs with various fluorescence intensities on the microarray chip. c) SDS-PAGE analysis of the mutant GFPs.

Five of mutant-DNA-immobilized beads indexed to the brightest GFPs (Fig. 5(a)) were successfully recovered using a glass manipulator with 4 μm diameter (Fig. 5(b)). Subsequently, the recovered DNAs were amplified using PCR (Fig. 5(c)). The amplified mutant DNAs were again subjected to emulsion PCR, DNA microarray chip fabrication, and on-chip cell-free translation; then, the mutant GFPs on the chip that exhibited the brightest uniform fluorescence were observed (Fig. 6). Consequently, first-generation mutant GFPs with various fluorescence intensities were successfully shifted to second-generation brightest mutant GFPs (Fig. 7).

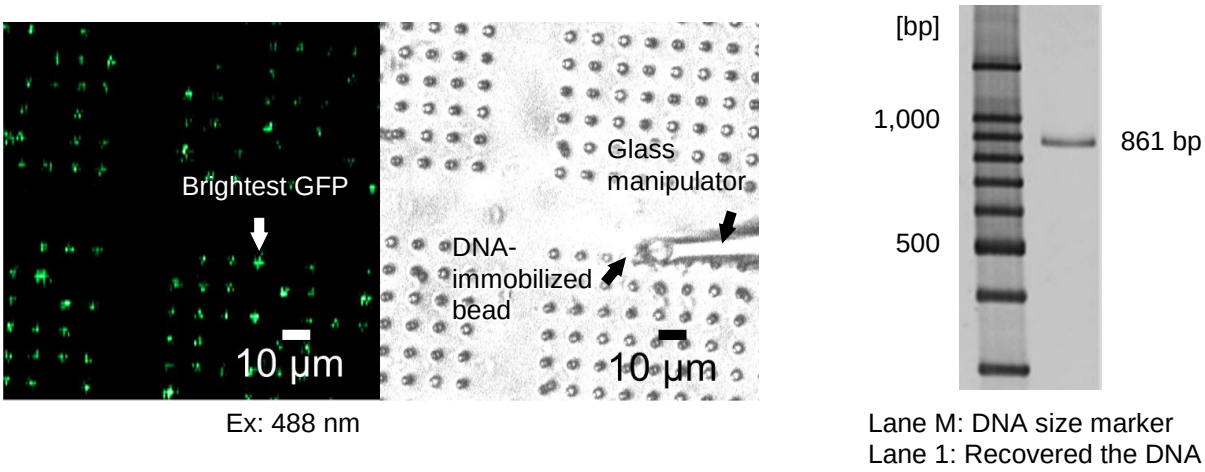


Figure 5 Recovery and amplification of genetic codes of the brightest GFPs on a chip. a) Fluorescence microscopy image of screening of the brightest GFPs. b) Bright-field image of recovered the mutant-DNA-immobilized beads. c) PAGE analysis of an amplified mutant DNA recovered from (b).

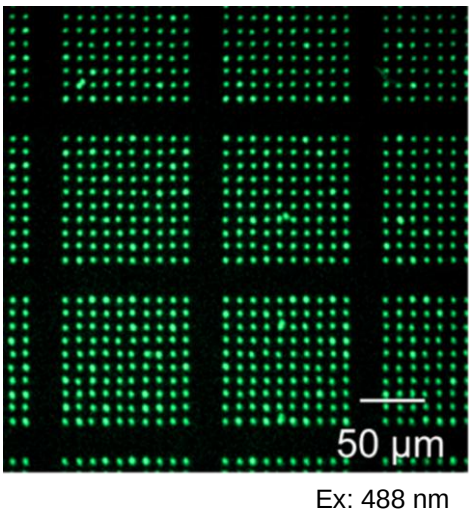


Figure 6 Fluorescence microscopy image of second-generation mutant GFPs.

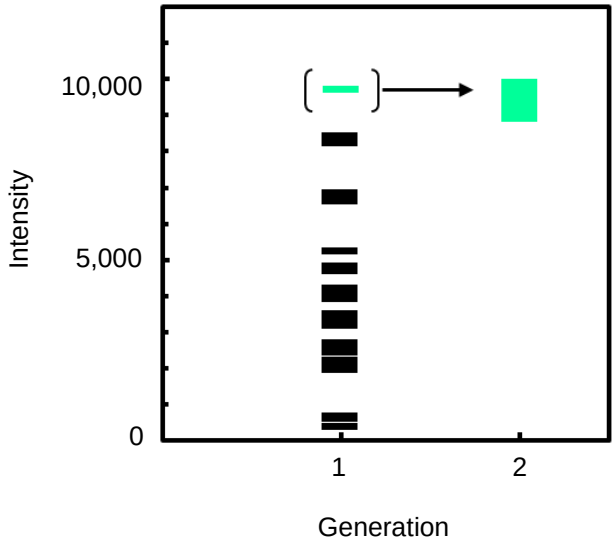


Figure 7 Progression of artificial Darwinian selection from first-generation to second-generation mutants.

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