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A Picoliter Chamber Array for Cell-Free Protein Synthesis

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The completion of human genome sequencing has shifted the focus of research from genes to proteins. In this regard, a protein library chip has become a useful tool for cell-free protein synthesis. In this study, we attempted to make a highly-integrated protein chip from a DNA library using *in vitro* protein synthesis on a microchamber array fabricated by using PDMS (polydimethyl siloxane), a hydrophobic surface, and glass, a hydrophilic bottom substrate. These structural properties prevented cross-contamination among the chambers. The minimum volume capacity of the smallest chamber was about 1 pl. The total number of chambers per chip was 10,000 on one chip (capacity 150 pl) and 250,000 on two others (1 and 5 pl). Next, we attempted *in vitro* protein synthesis using this microchamber array. The fluorescence of Green Fluorescent Protein (GFP) expressed on the chamber was rapidly detected (within just 1 h). GFP expression was also successful using immobilized DNA molecules on polymer beads. DNA immobilized beads were added as the source to each microchamber. Protein was successfully synthesized from DNA immobilized beads, which allowed easy handling of the DNA molecules.

Key words: cell-free/*in-vitro* protein synthesis, green fluorescent protein, picoliter chamber, polydimethoxyl siloxane (PDMS), protein chip.

Abbreviations: PDMS, polydimethyl siloxane; GFP, green fluorescent protein; RIE, reactive ion etching; anti-HAS-scFv, anti-human serum albumin single-chain antibody.

The progress made so far in analyzing the human genome has shifted the focus of research from genes to proteins (*1–5*). Although the number of human genes is reported to be 28,000–38,000 (*6*), the functions of most remain unknown. A rapid and easy method for synthesizing gene products has yet to be developed. Thus, an *in vitro* protein synthesis system to make a protein library chip was designed in this study. The chip has proteins arranged in an array, and can detect target molecules. Gene cloning and expression is widely used in the preparation of proteins. However, some kinds of proteins often cannot be expressed well in host cells. Our cell-free protein synthesis system could be suitable for expressing such proteins. This synthesis system has other advantages as well, such as labelling proteins with isotopes for detection by NMR spectroscopy (*7*), easy purification of the synthesized protein, and short protein synthesis time.

A highly-integrated protein chip is a powerful tool for accelerating post-genomic research. Our aim is to prepare protein chips directly from a DNA library using the *in vitro* protein synthesis system described here. Recently, a cell-free protein synthesis system from *Escherichia coli*, rabbit reticulocytes, and wheat germ was commercialized (*8*). In this study, a rapid translation system from *E. coli* was used for protein expression.

Previously, we reported the development of large-scale integrated picoliter microchamber arrays for PCR (*9*), the introduction of a novel nanoliter dispensing system suitable for DNA amplification on microchamber array chip (*10*), the development of a simultaneous multianalyte immunoassay method for detecting human immunoglobulins based on a protein chip and imaging detection (*11*), and the development of a new approach for manufacturing encoded microstructures used as versatile building blocks for miniaturized multiplex bioassays (*12*). Others have reported the construction of protein chips (*13*, *14*) or cell-free protein synthesis in small chambers (*15*). Kukar and co-workers detected 8 samples simultaneously on one chip (*13*), and Kojima *et al*. (*14*) constructed an electrochemical immunochip including an assembly of 36 electrodes.

There have been reports of high-throughput screening of a mutated anti-human serum albumin single-chain antibody (anti-HSA-scFv) using an *in vitro* protein synthesis system (*16*, *17*). In these reports, 2 amino acids were mutated randomly, and over 600 mutations were screened on 96-well plates. Our newly developed chip could also be a powerful tool in similar applications.

High-throughput screening is required for the rapid elucidation of protein functions. Microscale reactions have the advantages of short reaction time and the use of a small amounts of samples and reagents. Especially in a high-throughput screening system, numerous samples must be analyzed simultaneously and, if possible, eco-

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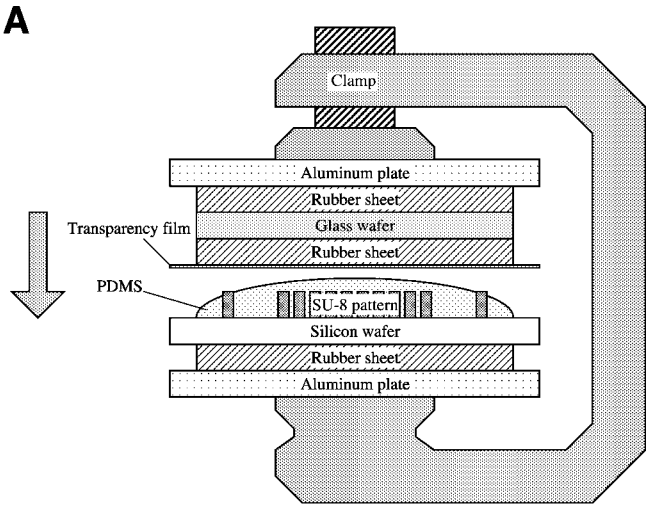

Fig. 1. **Schematic images of the fabrication of micro chamber chips.** (A) Sandwich molding process: A master pattern was formed on a silicon wafer using SU-8 photoresist. PDMS prepolymer mixture poured onto the master is covered with transparency film. A multilayer stack of aluminum plates, the master pattern, PDMS, a transparency film, a glass wafer, and rubber sheets are clamped tightly and crosslinked the PDMS prepolymer is crosslinked at 80°C, 2 h. (B) Glass and PDMS fabrication process; a) alignment between slide glass and PDMS sheet in acetone; b) PDMS sheet on the slide glass was treated with oxygen plasma (200 W, 8.8 Pa, 10 s); c) cross section image of a chamber. The PDMS sheet has holes and a hydrophobic surface.

nomically. Thus, we made a highly-integrated microchamber array chip by using micro-fabrication techniques and PDMS. PDMS micro-molding techniques have been used to fabricate micro-fluidic systems (*18*, *19*). Unlike traditional micro-fabrication materials, such as silicon and glass, PDMS can be bonded and manufactured easily and efficiently (*20*). In addition, PDMS has some properties that are advantageous for biochemical applications such as high transparency in the 230–700 nm wavelength range, and high permeability to gases.

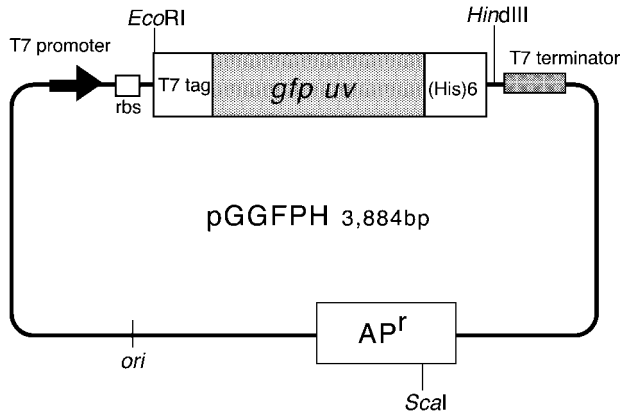

Fig. 2. **Constitution of the vector pGGFPH.** This vector contains the *gfpuv* gene. The PCR primers used for immobilization of the *gfpuv* gene are located upstream of the T7 promoter and downstream of the T7 terminator.

MATERIALS AND METHODS

Fabrication of PDMS Chambers—A sandwich molding process (*20*) was used to fabricate thin PDMS sheets, as out lined in Fig. 1. A master pattern was formed on a silicon wafer using SU-8 photoresist (MicroChem, USA). The PDMS prepolymer (Sylgard® 184: Dow Corning, USA) mixture was poured onto the master, and covered with a transparency film. A multilayer stack of aluminum plates, the master pattern, PDMS, a transparency film, a glass wafer, and rubber sheets were clamped tightly and the PDMS prepolymer was crosslinked at 80°C for 2 h.

The resulting thin PDMS sheet was put on a slide glass in acetone and treated with oxygen plasma to bind the sheet to the glass. A parallel-plate reactor Reactive Ion Etching (RIE) system (RIE-10NR: SAMCO, Japan) was used for the oxygen plasma treatment. The conditions for bonding were: RF power, 200 W; oxygen partial pressure, 8.8 Pa; treatment time, 10 s. The PDMS sheet had holes, and a hydrophobic surface. Thus, only the bottoms of the chambers were hydrophilic and the solution easily remained in the chambers.

Three different types of chips were designed and fabricated. Table 1 shows the specifications of these chips.

In Vitro Protein Synthesis on the Chip—The PDMS microchamber was used for *in vitro* protein synthesis. The ribosome source was based on a lysate from *E. coli* (RTS-500 kit: Roche, USA). The wild type GFP gene contained in the kit was used as a reporter gene, and expressed on the chips. Cell-free protein synthesis reagents were prepared according to the supplier's directions. The reaction solution was composed of a mixture of 0.25 ml of *E. coli* lysate solution, 0.75 ml of the reconstituted reaction mixture, 50 μl of the enzyme mixture, and the GFP vector at a final concentration of 10 μg/ml.

First, we dripped the *in vitro* protein synthesis solution on the chip, and removed the surplus. Next, the microchamber chip was covered with a gap cover glass (Matsunami, Japan), and sealed to prevent evaporation. There was a 20 μm gap between the chip surface and the cover glass. This gap prevented capillary action among

Table 1. **Specifications of three types of chips.**

Type	Length (μm)	Width (μm)	Depth (μm)	Diameter (μm)	Volume (pl)	Area (cm^2)	No. of wells
I (rectangular)	100	100	15	n/a	150	3	10,000
II (cylindrical)	n/a	n/a	15	20	5	3	250,000
III (cylindrical)	n/a	n/a	15	10	1	3	250,000

the chambers. The chip was held at 30°C, and GFP expression was detected by an optical fluorescence microscope with an FITC filter (excitation: 450–490 nm, emission: 515–565 nm).

Self-Arrangement of DNA Immobilized Beads on the Chip—A DNA primer oligonucelotide labelled with an amino group at the 5′ end, (NH_2-5′-TAACTATTCCCCGCAAATTAATACGACTCAC-3′) was immobilized on Dynabeads® M-270 Carboxylic Acid (Dynal, USA) using 1-ethyl-3-(3-dimetylaminopropyl) carbodiimide. The primers on the beads were elongated by PCR (*21*) with Ampli Taq Gold DNA Polymerase (Roche, USA), a reverse primer (5′-TAACTATTCCTCCGGATATAGTTCCTCCTTTC-3′), and pGGFPH vector (Fig. 2) as a template. DNA primer oligonucleotides were purchased from FASMAC (Japan). After elongation of the GFP gene, the beads were recovered, washed, and digested with *Eco*RI. The digestion caused the gfpuv gene fragment to be released from the bead. The digest solution was separated by agar electrophoresis. The amount of immobilized gene per bead was calculated by the intensity of the gfpuv gene band. In addition, Dynabeads® M-280 Streptavidin (Dynal, USA) were used for DNA immobilization. Template DNA was prepared by PCR with a biotinylated primer (biotin-5′-CCGCGAAATTAATACGACTC-3′), a reverse primer (5′-GGGTTTTTTGCTGAAAGG-3′), and pGGFPH vector as a template. Then, amplified DNA was adsorbed on the streptavidin-conjugated beads. The amount of the immobilized GFP gene per streptavidin-conjugated bead was determined by the same digestion method as described above.

The self-arrangement of beads into the chamber was carried out as follows. A solution containing 1 mg/ml phosphatidylcholine, 40% glycerol, 10 mM HEPES buffer (pH 8.0), and 0.3% beads was dropped and spread on the chip. The chip was then soaked in water, washed once with water, and air dried. The chambers were filled with the *in vitro* protein synthesis solution as described by Nakano *et al.* (*20*). After 1 h of incubation, the fluorescence intensity was measured under an optical microscope (excitation: 400–440 nm, emission: 475 nm). Retention time of fluorescent images was 8 s.

RESULTS AND DISCUSSION

RIE treatment (oxygen plasma treatment) was used to bind PDMS to a slide glass. Three different types of PDMS chips were designed and fabricated (Fig. 3). Since the PDMS and glass construction gives the chamber structure a hydrophobic surface and a hydrophilic bottom substrate, an aqueous solution poured onto the chip enters through the holes of the array, and remains only in these microchambers. This phenomenon prevents the cross-contamination between the microchambers. This chip is also suitable for optical observations because of its transparency over a wide wavelength range.

Protein synthesis was carried out on the microchamber chips with the GFP gene used as a reporter gene. The expression of GFP was detected by fluorescence using an optical microscope. Figure 4 shows the results of cell-free protein synthesis on the chip. Fluorescence intensity was detected within 1 h of incubation, and remained constant. In a batch system, protein synthesis is said to be inhibited by a lack of substrate or accumulated waste within 2 h (*22*). In our system, cell-free protein synthesis stopped within 2 h. This result is in agreement with the

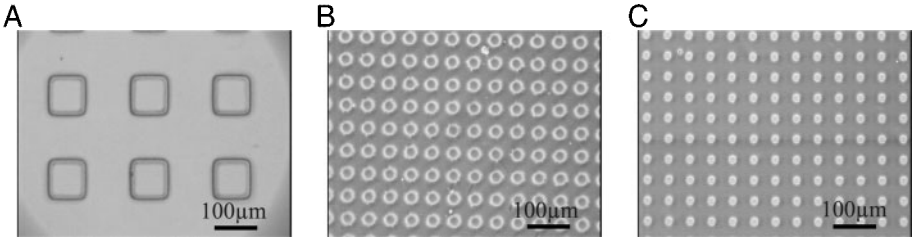

Fig. 3. **Optical images of the PDMS-glass complex chambers.** (A) Rectangular chambers about 100 × 100 × 15 μm; the volume is about 150 pliters. (B) Cylindrical chambers 20 μm in diameter and 15 μm deep; the volume is about 5 pliters. (C) Cylindrical chambers 10 μm in diameter and 15 μm deep; the volume is about 1 pliter.

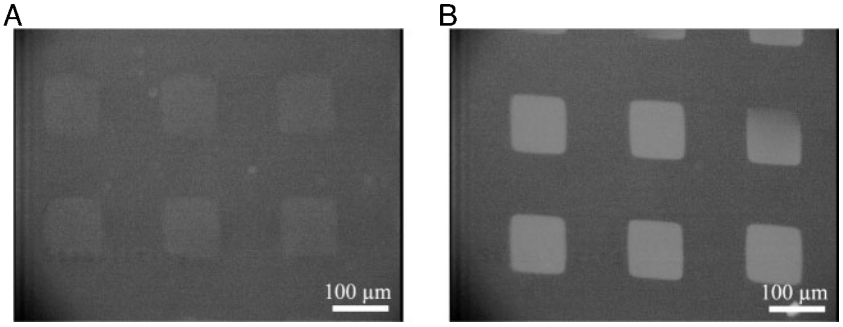

Fig. 4. **Fluorescent images of microchips showing the expressed GFP protein.** The chambers on the chip were filled with the cell-free protein synthesis reagents. (A) Fluorescent image of the chambers after incubation for 0 h; (B) fluorescent image of the chambers after incubation for 1 h; GFP expression was detected under an optical microscope with an FITC filter.

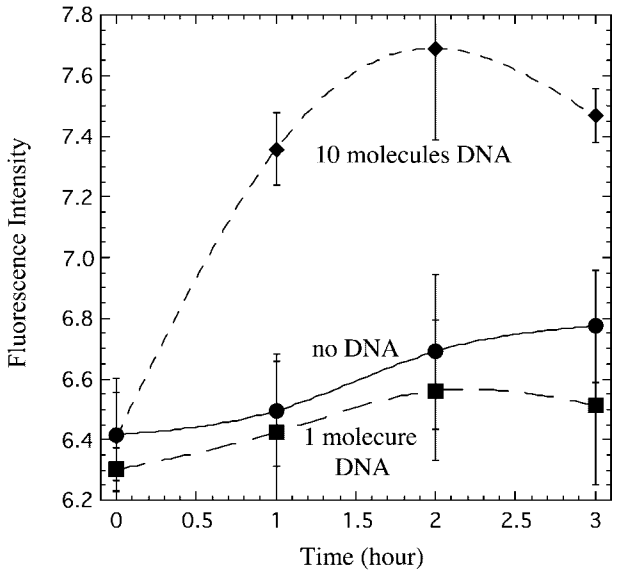

Fig. 5. **GFP fluorescence expressed with no DNA (circles), 1 DNA molecule (squares) and 10 DNA molecules (diamonds) per chamber.** We succeeded in detecting GFP fluorescence from 10 molecules of DNA per chamber. The concentration of 10 molecules of DNA solution is about 4×10^{-5} mg/ml. The chip used for expression had cylindrical chambers 10 μm in diameter. The volume of the chambers was about 1 pliter.

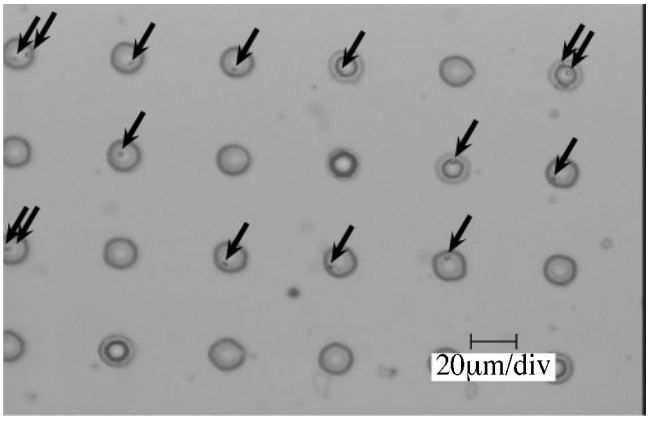

Fig. 6. **Optical image of the micro chamber chip arrayed DNA-immobilized beads.** Arrows indicate the presence of a bead in a chamber. About 60 % of the chambers had one bead. The cylindrical chambers shown in the figure had a diameter of 10 μm.

report of Spirin et al (*22*). However, the formation of the GFP fluorescent group is known to take 1–2 h (*22*); thus, it may be considered that protein synthesis stopped before the GFP fluorescence became constant. However, a similar shift in GFP fluorescence was shown in chambers with 10 (Figs. 6 and 7) or 20 μm i.d.

The lowest concentration of DNA template necessary for the detection of the GFP signal was determined to be only 10 molecules of DNA per chamber (Fig. 5). A type III chip with cylindrical chambers 10 μm wide and 15 μm deep was used in this experiment. The volume of this chamber is about 1 pl. Thus, the concentration of a solution containing 10 molecules of DNA is about 4×10^{-5} mg/ml. This concentration is about 1/100 to 1/500 compared with the DNA concentration utilized in conventional cell-free protein synthesis protocols. The use of a small volume chamber increases the possibility of contact between DNA and reagents making it possible to express a protein using a trace amount of DNA.

The distribution of over 10,000 samples using a DNA spotter would take a very long time; therefore, self-layout of samples containing the DNA library was used in this study. DNA-immobilized beads were used as DNA carriers. The amount of DNA immobilized on one bead was about 200 molecules on Dynabeads® M-270 Carboxylic Acid, and 10,000 molecules on Dynabeads® M-280 Streptavidin (Dynal, USA). A Type III chip with a 10 μm i.d. chamber was used for bead arrangement. About 60% of the chambers contained one bead; however, some chambers had multiple beads (Fig. 6) because the diameter of a bead, which is 2.8 μm, is much smaller than that of the chamber. The design and fabrication of a new chip with smaller chambers to allow the entry of only one bead into each chamber are currently underway in our laboratory.

With the aid of lipid, it was possible to disperse the beads into the chambers. Rhodamine-modified lipid was used instead of phosphatidylcholine, and the chip covered with lipid solution was observed both in air and in water. Interestingly, the lipid moved into the chamber when the chip was soaked in water (data not shown). A chip with beads containing the GFP gene was used for in vitro protein synthesis. As shown in Fig. 7, fluorescence was observed after 1 h of incubation only in chambers that contained DNA beads. As a positive control, pGGFPH vector solution was added to the *in vitro* protein synthesis reagents at 10 fg/pl; no template DNA was added to the *in vitro* protein synthesis reagents as a negative control. The results of these control experiments are shown in Fig. 7 and Table 2. A comparison between DNA-immobilized beads and DNA in solution suggests that the amount of protein per DNA molecule in solution is greater than that on DNA-immobilized beads. Nevertheless, easy and fast manipulation of DNA immobilized beads prompted us to use this method. In the experiments shown in Fig. 7 and Table 2, DNA immobilized beads with biotin-streptavidin conjugate were used. Similar results were observed when primer-immobilized beads were used (data not shown). The concentration of GFP solution was about 10 μg/ml, estimated from the intensity of the fluorescence. The amount of GFP protein per chamber was about 10 fg. The GFP solution did not diffuse from the chambers, indicating that the solution in

Table 2. **Fluorescence intensity of GFPuv under the different conditions.**

Condition	Fluorescence intensity
One bead in a chamber (10,000 molecules per bead)	112.5
pGGFPH vector (10 fg/pl, 2,500 molecules per chamber)	122.7
No template DNA	92.1

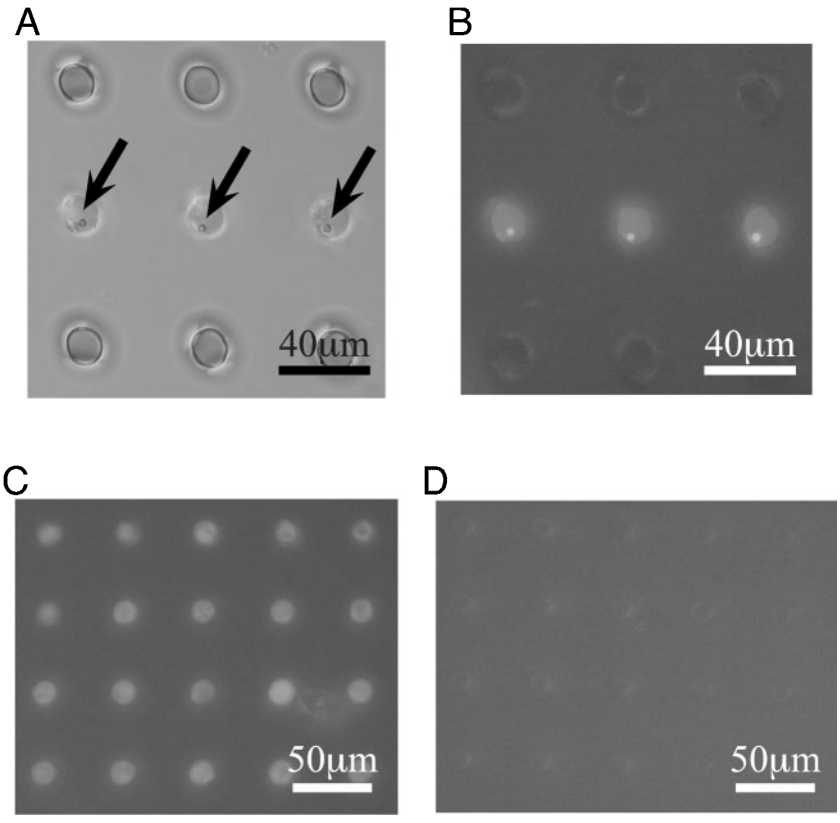

Fig. 7. **Optical images of GFPuv expression from DNA-immobilized beads.** (A) Optical image under white light; the arrows indicate the presence of beads; (B) Fluorescence image of the chamber, Ex.: 400–440 nm, Em.: 475 nm. (C) Fluorescence image of a positive control containing cell-free protein synthesis reagents with 10 fg/pl pGGFPH vector. (D) Fluorescence image of a negative control containing only cell-free protein synthesis reagents (no template DNA). The cylindrical chambers shown in the figure were 10 μm in diameter.

each chamber is physically separated from the solution in the others.

Tabuchi *et al*. (*15*) made a microchamber chip with a chamber volume per chip of 10 μl. In our case, the chamber number is much larger and the chamber volume is much smaller, which is advantageous for high-throughput applications. However, the analysis of over 10^4 chambers takes a long time. Therefore, a scanner-type analyzer for our system is being developed to enable automatic screening in the near future.

A new method for making a highly-integrated protein chip from a DNA library using *in vitro* protein synthesis on a microchamber array is demonstrated. The chambers are of three types based on their volume capacity: 1, 5, and 150 pl, and the total number of chambers per chip is 10,000 (150 pl), and 250,000 (both 1 and 5 pl). The array has a hydrophobic surface of PDMS and a hydrophilic glass bottom. These structural properties provide the advantage of preventing cross-contamination among the chambers. *In vitro* protein synthesis using these chambers was achieved. The fluorescence of GFP expressed on the micro chamber was rapidly detected. GFP expression was also achieved using immobilized DNA molecules on polymer beads, which allows easy handling of the DNA molecules. Brenner *et al.* (*23*) described a method for cloning nucleic acid molecules onto the surfaces of 5 μm microbeads rather than in biological hosts. A unique tag sequence was attached to each cDNA molecule, and the tagged library was amplified. A unique tag was also attached to each bead, and the tagged library was conjugated with the tagged beads. This method allows the immobilization of one kind of DNA on a bead. Because such clones are segregated on microbeads, they can be manipulated simultaneously and then assayed separately. If this method can be applied to the chip described in this report, it will be possible to analyse easily a whole DNA library on a chip in a short time. In the future, this system will be used for the exhaustive expression of proteins included in target cells, the functional analysis of proteins expressed from unknown genes, and the screening of artificially mutated proteins.

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