

Combinatorial Bioengineering: Review

Photoimmobilization for Microarrays

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A photoimmobilization method has been developed for the preparation of microarray biochips. This photoimmobilization method makes it possible to easily covalently immobilize various types of organic molecules and cells on a chip. In addition, by using hydrophilic polymers as matrixes, it is possible to reduce nonspecific interactions with biological components. Various proteins, antibodies, and cells have been microarrayed using this technique, and interactions between these proteins, antibodies, and cells have been investigated. This type of microarray biochip will be important for academic applications such as genomics, proteomics, and cellomics, and clinical analyses.

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Introduction

Recent progress in the life sciences has been significantly enhanced by microfabrication technology. DNA microarray technology is a typical result of the combination of biotechnology and microfabrication technology. Now, targets that can be analyzed are expanding, with applications in genomics, proteomics, glycomics, cellomics, and metabolomics, and along with progress in bioinformatics, the importance of microarray technology is increasing in clinical analysis. The integrity of microarrays has increased in comparison with that of the integrated circuit (IC) of computer technology, in which the number of transistors per square inch has doubled every year since the IC was invented, in accordance with Moore's Law. The clinical use of DNA microarrays began in 2004, and some antibody and protein microarray chips have recently become commercially available.

Microarray technology is a crucial tool for large-scale and high-throughput biological science and technology (1). It allows

Table 1. Attachment Strategies for Microarray Production

functional groups	attachment		references
	non-covalent	covalent	
nitrocellulose	physical adsorption		15, 27, 68, 29, 69, 82, 83
polystyrene, silanized glass	physical adsorption		81, 84
self-assembled hydrogel	encapsulation		14
poly(L-lysine), poly(ethylene imine), amino	electrostatic adsorption		18–20, 86
cyano groups		amino groups	2, 17
aldehyde groups		amino groups	16
amino/bifunctional		amino groups	
N-hydroxysuccinimide			
mercapto groups		amino, thiol groups	
epoxy groups		amino, thiol, hydroxy	26, 30
Au		thiol groups	23, 24, 28
solid synthesis			25
nickel coating	His-tag		3
neutravidin	biotinylated		
avidin	biotinylated		
phenyl nitrene		organic groups	32–38, 44–46, 51–61
phenyl carbene		organic groups	39–43, 48–50

fast, easy, parallel detection of thousands of addressable elements in a single experiment under identical conditions. The technology has provided scientists with various types of multiple analyte assay systems, which are likely to transform medical diagnosis in the future. Various types of protein microarrays and a variety of other methodologies have been developed to immobilize biological molecules, as shown in Table 1 (1–11). Snyder's group synthesized proteins carrying oligohistidine, immobilized the proteins on nickel-coated glass slides, and then investigated protein interactions using protein microarrays (3). Espejo et al. (12) fused protein domains with glutathione S-transferase for the preparation of microarrays, and Newman and Keating (13) have identified new protein–protein interactions using protein microarrays. Recently, Kiyonaka et al.

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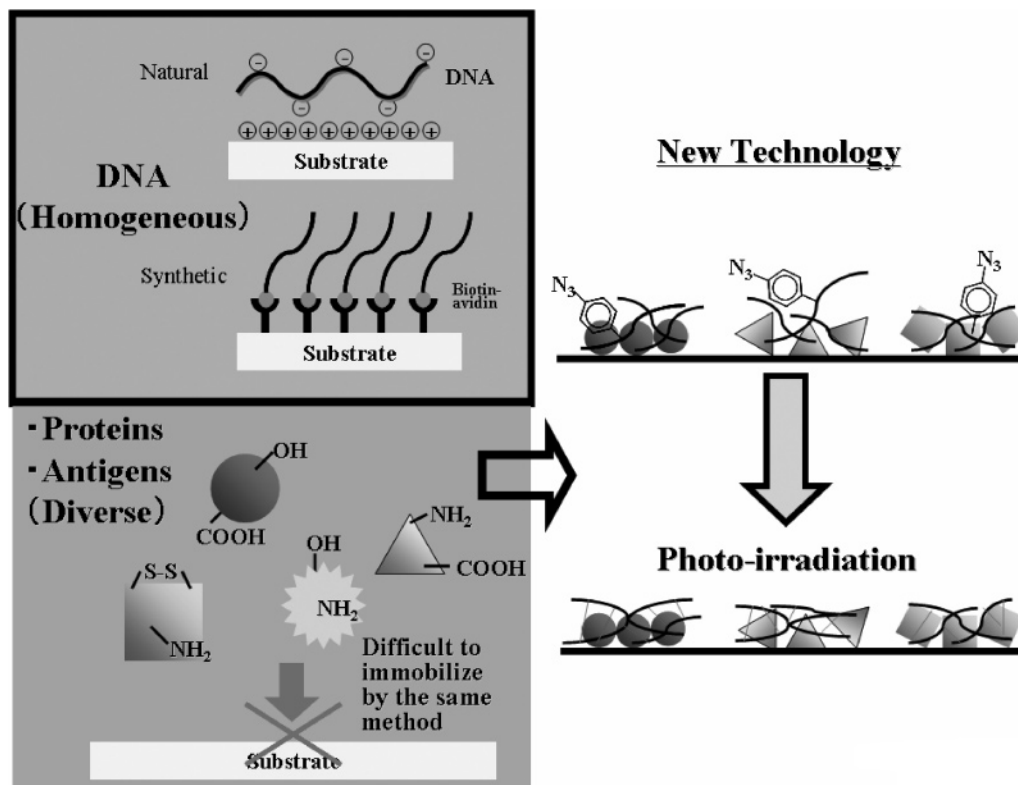


Figure 1. Comparison of DNA- and protein-immobilization and photoimmobilization. Because DNA is chemically homogeneous and can be specifically modified by organic synthesis it is easy to immobilize on a chip (top left). However, because proteins are chemically different, it is difficult to immobilize them with the same methods used to immobilize DNA (bottom left). The photoimmobilization method was invented to covalently immobilize various biomolecules, including proteins (right).

invented a microencapsulation method for microarrays using hydrogels (14). Various immobilization methods, including protein microarrays (15–17), membrane–protein microarrays (18–21), antibody microarrays (22), peptide microarrays (23–25), small-molecule microarrays (26), and sugar microarrays (27–29), have been developed by many research groups (Table 1).

Although many types of biopolymer microarray have been investigated, the practical technology has not yet been developed. One of the reasons why biopolymer microarray technology has not been developed as rapidly as DNA microarray technology is the difficulty in immobilizing proteins. Although DNA contains varied genetic information, its structure is composed of four chemically similar bases. Therefore, DNA is considered to be a homogeneous biopolymer. In addition, the DNA biopolymer is conveniently synthesized by the solid phase method. In principle, different DNAs and peptides can be immobilized or synthesized on one plate by the same method (Figure 1, top left panel). In contrast, the chemical structure of individual proteins is different. Proteins have different functional groups, and they also differ in quantity and location. Therefore, it is very difficult to covalently immobilize different proteins, polysaccharides, antigens, and cells on the one chip using the same method (Figure 1, bottom left panel). Fall et al. (30) reported some difficulties in screening of some allergen-specific IgEs, although they did not attribute it to difficulties in the immobilization method. It is easy to understand why covalent immobilization is difficult, because biological molecules may not have enough functional groups or their recognition sites may be used in the immobilization process. To overcome the difficulties of covalent immobilization of various types of biocomponents by the same method, we have invented a new

photoimmobilization technique (Figure 1, right panel) for the preparation of microarrays (31).

The photoimmobilization method uses photogenerated radical cross-reactions. Because radical reactions occur on every organic material, including biological molecules, the polymer matrix, and the chip surface, the method does not require any special functional groups, such as amino, carboxyl, hydroxyl, or thiol groups, unlike other conventional immobilization methods. Therefore, different biocomponents can be immobilized by the same method. In addition, as shown in Figure 2, it is possible to immobilize biological materials without molecular orientation. Some orientation usually occurs when the conventional immobilization method is used because of the uneven distribution of functional groups on biological molecules. In contrast, there is no particular orientation of immobilized molecules when the photoimmobilization method is used. This method is suitable for multiple interactions of immobilized molecules with analytes because recognition sites on the immobilized molecules are not as limited.

To date, we have used this technique to immobilize growth factor proteins on a solid matrix to show the effect of immobilized protein on cell function (32–38). Using micro-pattern-immobilization, the effect of the immobilized growth factor proteins was visualized by observing cellular behavior on immobilized and nonimmobilized regions of the matrix (32–38). Because the immobilized proteins retain their biological activity, even after immobilization, we considered that photoirradiation does not significantly destroy the 3D structure of the immobilized biomolecules and applied this immobilization technology to the preparation of microarray biochips. In the present article, the photoimmobilization method is introduced and its applications in cell and antibody analysis are described.

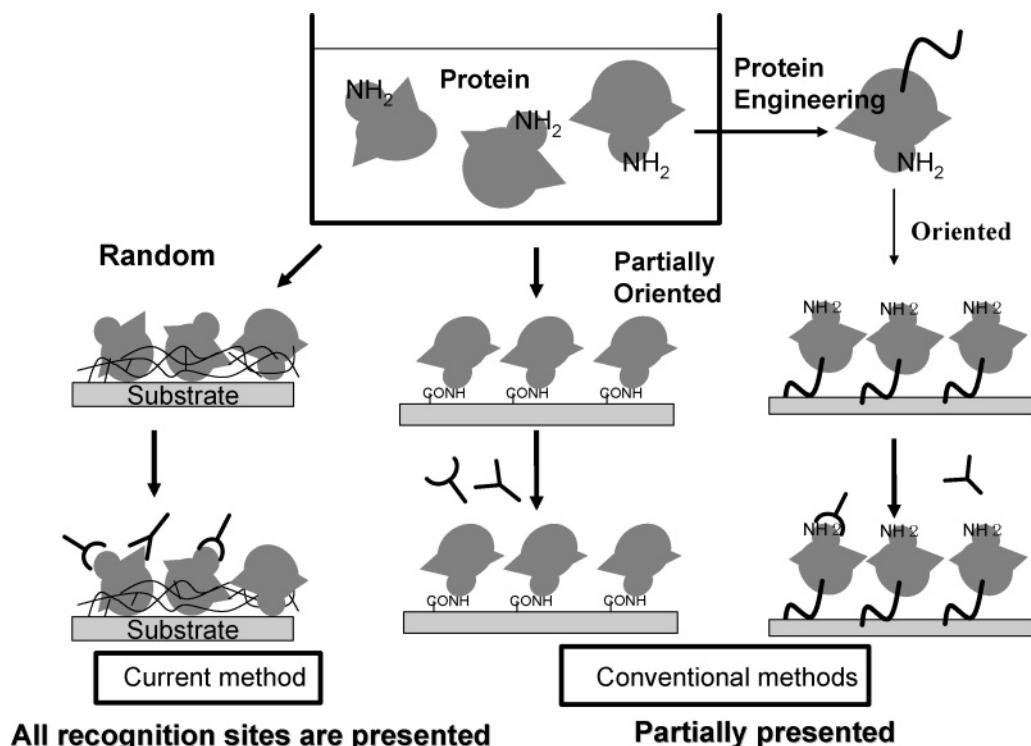


Figure 2. Comparison of immobilization methods. Photoimmobilization leads to the random orientation of immobilized molecules, whereas other covalent immobilization methods lead to some orientation of immobilized molecules because of the uneven distribution of functional groups on the molecules being immobilized. In genetically engineered proteins, which have adhesive peptide sequences on the end chain, the recognition site is limited to the remaining part of the molecule.

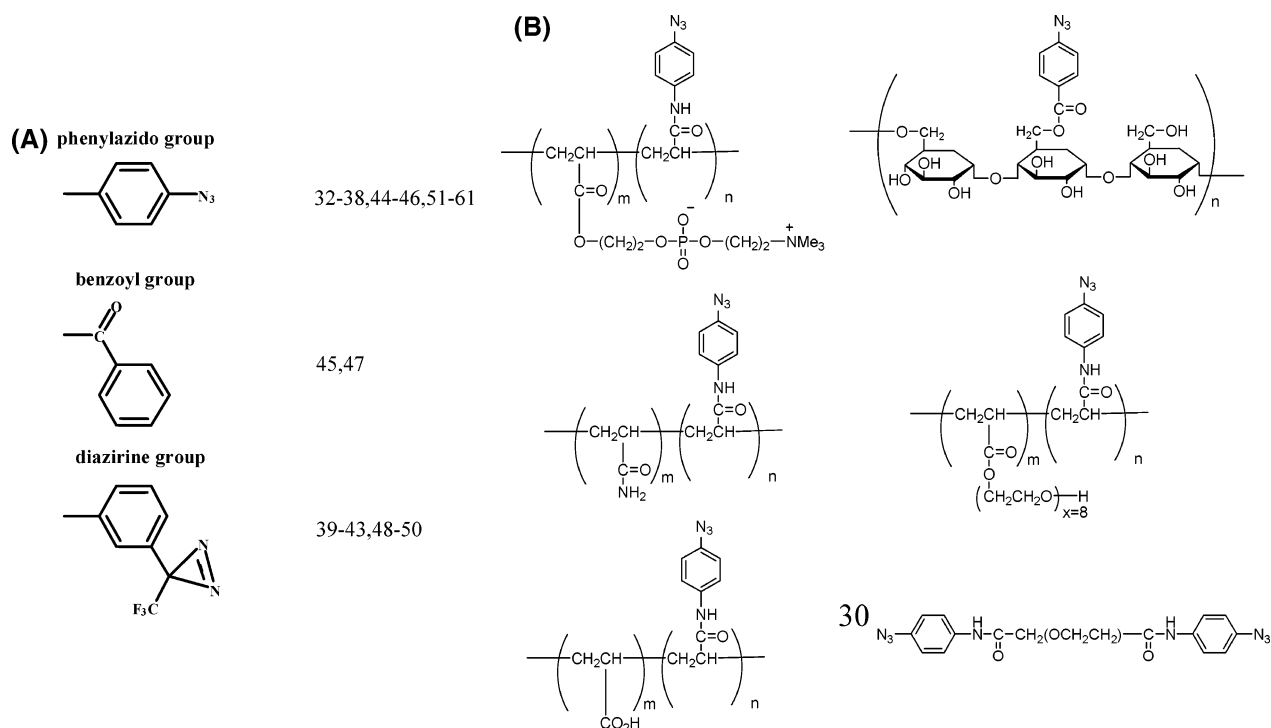


Figure 3. (A) Examples of photoreactive functional groups used in photoimmobilization. The numbers on the right indicate the references for photoimmobilization studies that have been conducted on each photoreactive functional group. (B) Photoreactive polymers synthesized by our group's current work.

Photoimmobilization

Photoreactive functional groups that have been used in photoimmobilization are shown in Figure 3A. Many researchers have performed the photoimmobilization of biomolecules to material surfaces, and some types of photogenerated nitrenes, carbenes, and ketyl radicals have been used to form covalent

links with target molecules and materials. Sigrist's group (39–43), for example, used diazirine derivatives to generate carbene groups by photoirradiation. Matsuda's group (44, 45) used azidophenyl, benzophenone, dithiocarbamate, and camphorquinone as UV-reactive groups, and fluorescein, eosin, and Rose Bengal as visible light-sensitive groups. In addition, they used

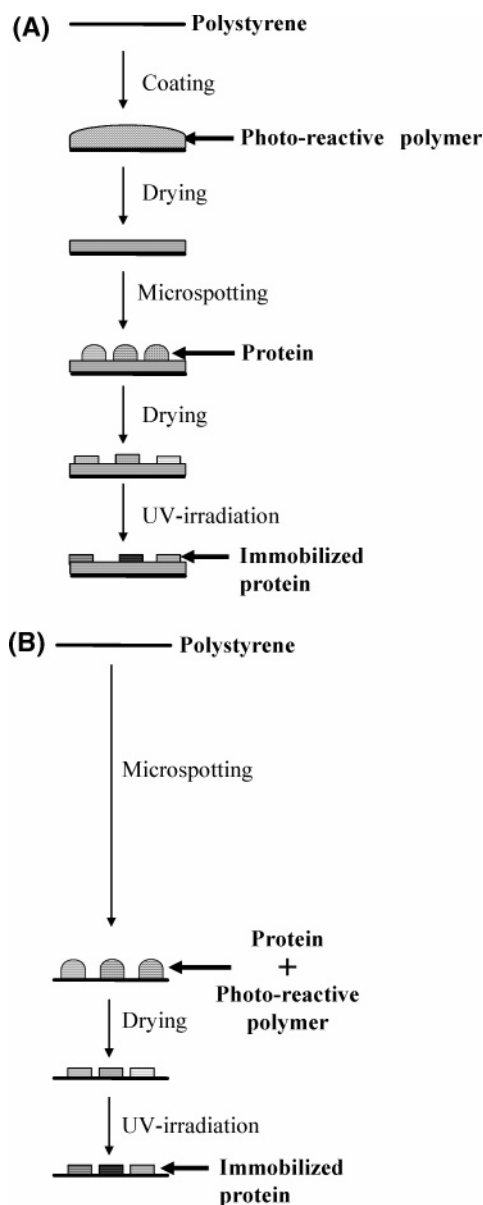


Figure 4. Synthesis of microarray chips by photoimmobilization. (A) After coating with a photoreactive polymer, proteins were microspotted and immobilized by photoirradiation. (B) Proteins were mixed with photoreactive polymer, and the mixture was microspotted and immobilized by photoirradiation.

photocross-linking and photopolymerization methods for the photoimmobilization of target molecules. Recently, Miller et al. (46) used azidophenyl groups for the preparation of an antibody microarray. Prucker et al. (47) incorporated benzophenone on a plate and immobilized organic molecules and macromolecules to investigate binding strength. Recently, Sigrist's group synthesized diazirine-containing dextran to reduce nonspecific interactions and immobilize proteins on a plate (43). Kanoh et al. (48–50) prepared photoaffinity glass slides containing aromatic diazirine through a poly(ethylene glycol) anchor and immobilized low molecular weight molecules on the slide by irradiation with 360 nm light.

We developed some photoreactive polymers in previous work (32–38, 51–56). Figure 3B shows the polymer that was employed in the current work (57–61). Azidoaniline or azido-benzoic acid has been incorporated into water-soluble synthetic polymers, including poly(acrylic acid) (57), poly(vinyl alcohol) (58), poly(phosphatidylcholine methacrylate) (59, 60) and poly-

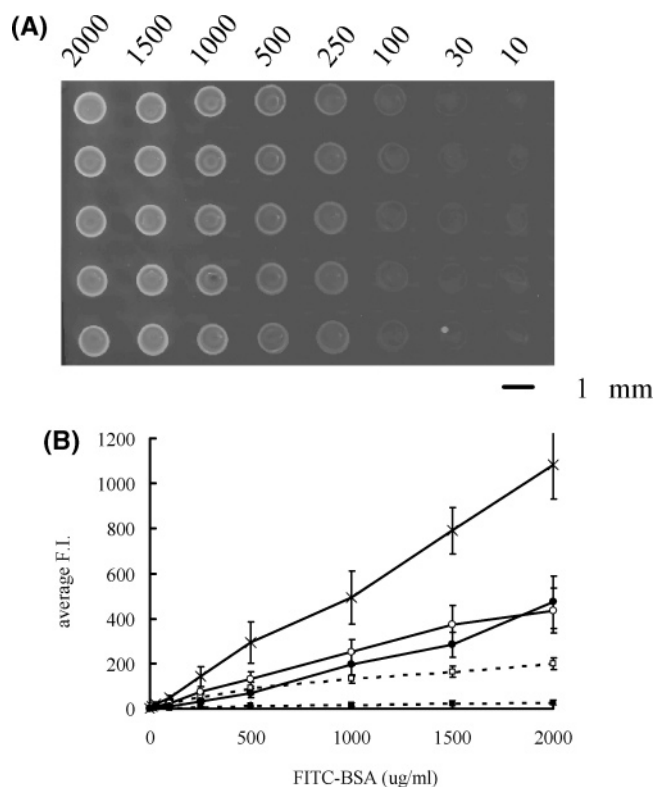


Figure 5. (A) Micrograph of a microarray of fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) with a photoreactive polymer after washing with phosphate buffered saline (PBS). The concentrations of FITC-BSA used in spotting were 2000, 1500, 1000, 500, 250, 100, 30, and 10 $\mu\text{g/mL}$. Bars represent 1000 μm . (B) Fluorescence intensity of cast FITC-BSA (crisscross), FITC-BSA immobilized with photoreactive polymer and washed with 0.1% Tween-20 (●) or PBS (○), and FITC-BSA without photoreactive polymer washed with 0.1% Tween-20 (■) or PBS (□).

(ethylene glycol) (61). Because these water-soluble polymers do not nonspecifically interact with biological components, they have been used to form an immobilization matrix to reduce nonspecific interactions (N) and to enhance specific interactions (S), resulting in a high S/N ratio (57–61).

Two schemata for the synthesis of a microarray chip are shown in Figure 4. Some researchers have adopted the method shown in Figure 4A, and the commercially available “Photo-chip” is made by this method (43). A high amount of protein is considered to be immobilized when using the method shown in Figure 4B because the photoreactive polymers, shown in Figure 3B, are soluble in water, thus allowing proteins or cells to be mixed with them in water (60, 61). The aqueous solution containing the photoreactive polymer and biomolecules of interest is then microarrayed, dried, and photoirradiated.

The photoimmobilization method can be applied to various biological components including proteins, antibodies, and cells. Here, we demonstrate some applications of photoimmobilized microarrays.

Cell Analysis

The modulation of cellular activity through substrate interactions can have a significant effect on biomaterial-based therapies. Material-based control of cellular function is a potentially powerful tool for controlling stem cells, which have the potential to differentiate into many different tissue types. The development of new biomaterials has been an iterative process: polymers are rationally designed and then individually tested for their properties. More recently, attention has focused on the

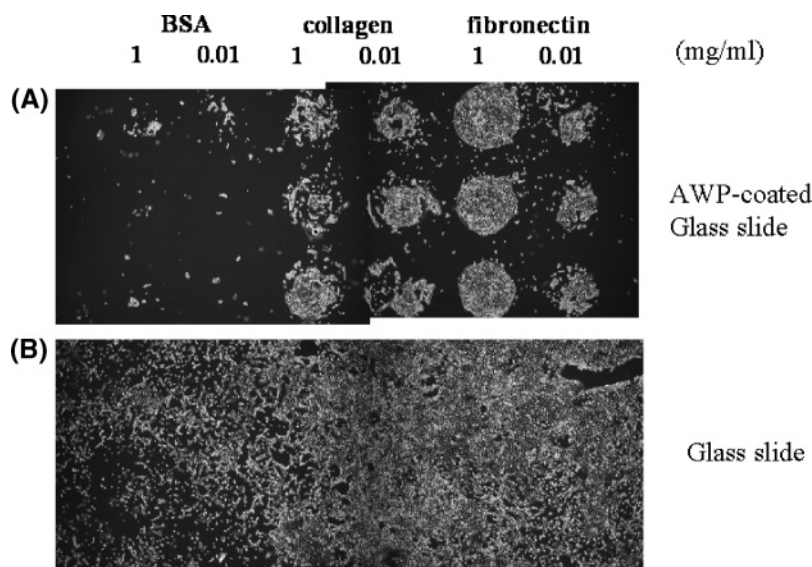


Figure 6. Micrograph of mouse fibroblast STO cells adhered to bovine serum albumin (BSA), collagen, and fibronectin-spotted AWP-coated glass slides (A) or glass slides only (B) over 2 h. The AWP-coated surface reduced nonspecific adhesion of cells to the slide.

development of parallel, combinatorial approaches, and the development of diverse libraries of polymeric biomaterials. Langer's group (62, 63) described a platform that enables the nanoliter-scale synthesis and cell-based screening of thousands of microarrayed biomaterials using human embryonic stem cells. In addition, they prepared an array comprised of blends of well-characterized biodegradable polymers and tested these to examine the ability of this system to efficiently screen biomaterials in a range of cell types.

In our previous work, we demonstrated that some immobilized growth factor proteins enhanced cell growth more than soluble growth factor proteins and that other immobilized growth factors induced effects that were different from the effects induced by soluble growth factors (64–67). In current work, we microarray-immobilized proteins to examine the effect of immobilized proteins on cellular functions, especially cell adhesion.

Protein Microarray. Preparation. One of the photoreactive polymer, poly(acrylic acid) containing a phenylazido group in the side chains, was synthesized by a coupling reaction of poly(acrylic acid) with azidoaniline (57). An aqueous solution containing the photoreactive polymer was microarrayed onto the surface of a polystyrene dish (chip). After drying, protein of various concentrations was spotted onto the polystyrene chip. Finally the chip was irradiated with UV light.

Fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) of different concentrations was then microspotted by a microarrayer onto the polymer cast and photoimmobilized (Figure 5A). Figure 5B demonstrates the binding strength of the immobilized proteins. When BSA alone was microspotted onto the chip and the chip was washed with water or the detergent Tween, the BSA immobilized by the photoreactive polymer was not washed out. This result demonstrated that the proteins were covalently immobilized on the chip.

Using the method shown in Figure 4B, a microarray chip was fabricated using another type of photoreactive polymer, the azide-unit pendant water-soluble photopolymer (AWP; 58). AWP is a poly(vinyl alcohol) with 0.7% phenylazido groups on its side chains and has an affinity to glass surfaces. AWP solution was spin-coated onto a glass slide and pattern-immobilized in the presence of a photomask. When adsorption onto the glass slide was measured, the pattern formed was stable

in organic solvents, including methanol, acetone, and tetrahydrofuran. BSA, collagen, and fibronectin were photoimmobilized on AWP-precoated glass slides.

Assay of Cell Adhesion. Different amounts of BSA or fibronectin were microarrayed, and adhesion of some cell types, including mouse leukemia monocytes RAW264, African green monkey COS-7 kidney cells, and rat pheochromocytoma PC12 cells, was investigated (57). The number of cells that adhered to the photoreactive polymer-immobilized region was the same as the number of cells that adhered to nontreated polystyrene. In contrast, the adhesion of COS-7 and PC12 cells to BSA-immobilized regions was strongly suppressed. Although immobilized fibronectin did not enhance cell adhesion, it did enhance cell spreading for a short time. These cellular behaviors are similar to those observed on protein-adsorbed surfaces. This indicates that the photoimmobilized proteins have the same activity as the adsorbed proteins, which are usually employed in the biological sciences.

Proteins were microarrayed onto glass slides precoated with AWP, and cell adhesion was investigated (58). Figure 6 shows the results of the adhesion of mouse fibroblast STO cells. The STO cells adhered not only to collagen- and fibronectin-spotted AWP-coated glass slides, they also adhered to the glass surface. Thus, when proteins are microspotted onto glass slides, it is difficult to assay for cell adhesion. However, no adhesion was observed on the AWP-coated surface. Therefore, the AWP-coated surface allows proteins to be assayed for cell adhesion. In addition to the STO cells, human hepatocyte HepG2 cells, COS-7 cells, and RAW264 cells did not adhere to the AWP-coated surface. Thus, the AWP-coated surface provided good contrast micrographs for the cell adhesion assay and was useful for profiling cell properties.

Antibody Microarray. Antibody microarray for the detection of cell-surface antigens has also been reported (68–72). Analysis of cell-surface antigens is usually performed by flow cytometry. The principle of this technique is the adsorption of fluorescent-labeled antibody onto a cell-surface antigen and the detection of fluorescence by a laser. It is now possible to detect six different types of fluorescence simultaneously. However, it is difficult to simultaneously detect more than six types of fluorescence. Therefore, to replace and match flow cytometry, 50 different antibodies to anti-cluster of differentiation (CD)

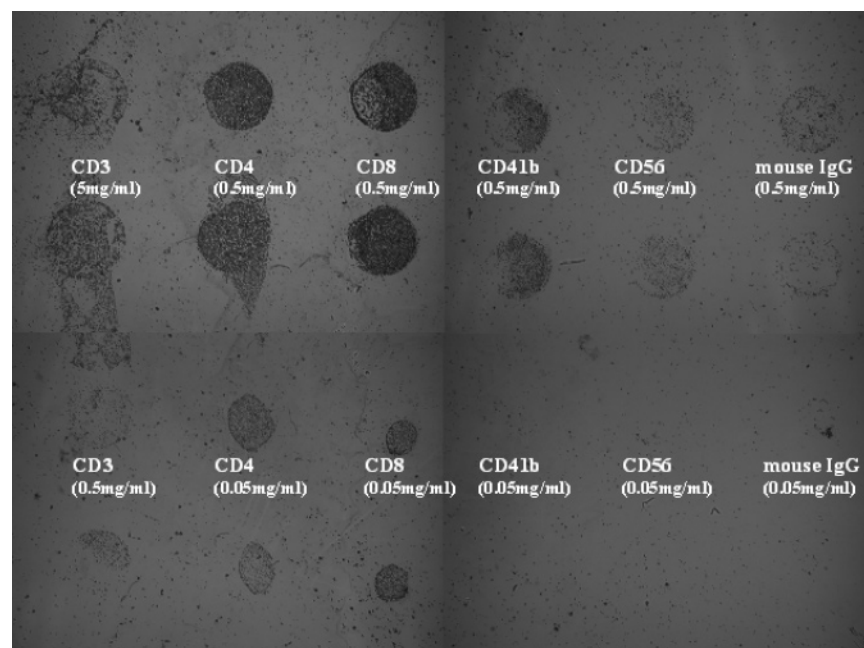


Figure 7. Human leukocyte adhesion to anti-cluster of differentiation (CD) antibody-immobilized surface in the presence of serum. Because the leukocytes have CD4, CD8, and CD41b antigens on their surface, they adhered to the corresponding antibodies immobilized on the chip. The concentrations indicate the antibodies spotted onto the chip.

antigens were microarrayed onto a nitrocellulose membrane to detect CD antigens in human lymphocytes (68, 69). The lymphocytes of healthy individuals were then compared with the lymphocytes of patients who had different levels of expression of CD19, CD20, CD21, CD22, CD23, CD24, CD25, and CD37. These results were confirmed by flow cytometry.

Figure 7 shows the immobilization of anti-CD antibodies by our photoimmobilization method for use in an assay of CD antigens in human monocytes. Glass slides were treated with octadecyltriethoxysilane, and the antibodies were microarrayed onto the surface with a photoreactive polymer. Human monocytes were then placed on the microarray glass for 90 min. After staining with Giemsa dye, it was demonstrated that human monocytes did not adhere to mouse IgG; however, they did adhere to CD4 and CD8 antibodies. Therefore, the microarray method is very useful for detecting multiple CD antigens.

Antibody Analysis

Allergen Microarray. The photoimmobilization method not only has the ability to covalently immobilize organic materials on a chip, it also orientates the immobilized materials more randomly than conventional methods, as illustrated in Figure 2. Therefore, the most suitable application for this technique is basic research that investigates various interactions between biological molecules and clinical diagnosis for the detection of polyclonal antibodies in blood. Because a polyclonal antibody has many recognition sites, the immobilized antigen also provides many recognition sites. Oriented immobilization must cover some of these recognition sites. Therefore, the photoimmobilization method is suitable for allergy diagnosis.

Allergy diagnosis using a microarray biochip was performed as follows (31). Various allergens were immobilized on a polymeric chip using the photoreactive polymer, and human serum was added to the chip. After 10 min, the chip was washed, and the bound IgE was detected by anti-IgE-antibody-containing peroxidase. Finally, a substrate for chemiluminescence was added to the chip. Chemiluminescent detection is useful for the construction of a relatively cheap measurement machine. Figure

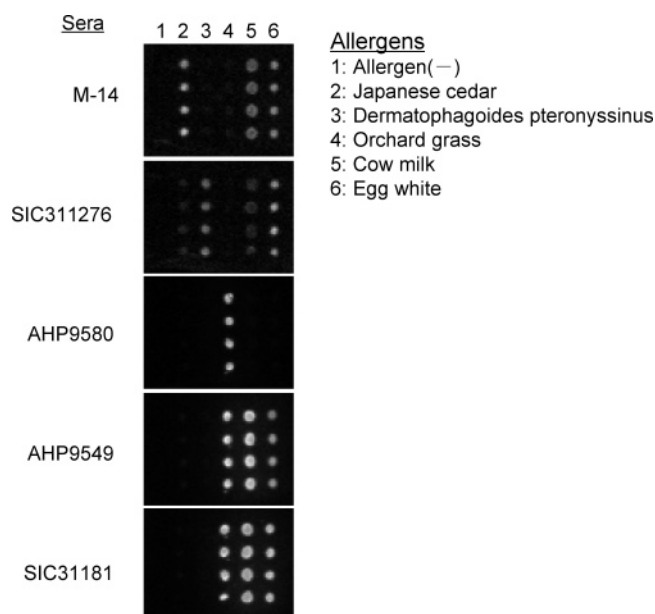


Figure 8. Microarray showing detection of adsorbed IgE from the sera of five different patients (M-14, SIC311276, AHP9580, AHP9549, and SIC31181) to five different allergens (Japanese cedar, *Dermatophagoides pteronyssinus*, orchard grass, cow's milk, and egg white).

8 shows the results of chemiluminescent detection of an allergy test using microarrays (31). Five allergens were microarrayed and the adsorption of IgE was investigated. Serum from five different patients had different reactivities to the five allergens.

To date, various types of allergen microarrays have been prepared (61, 73–83). However, as described above, there are some difficulties with some antigen arrays, as reported by Fall et al. (30). The photoimmobilization method provides a solution for this problem.

Panel Cell Microarray. Cells were also microarrayed using the photoimmobilization method. In addition to molecular immobilization, some pathogens or cells were microarrayed for multiple analyses (84–86). Schwenk et al. (86) prepared a

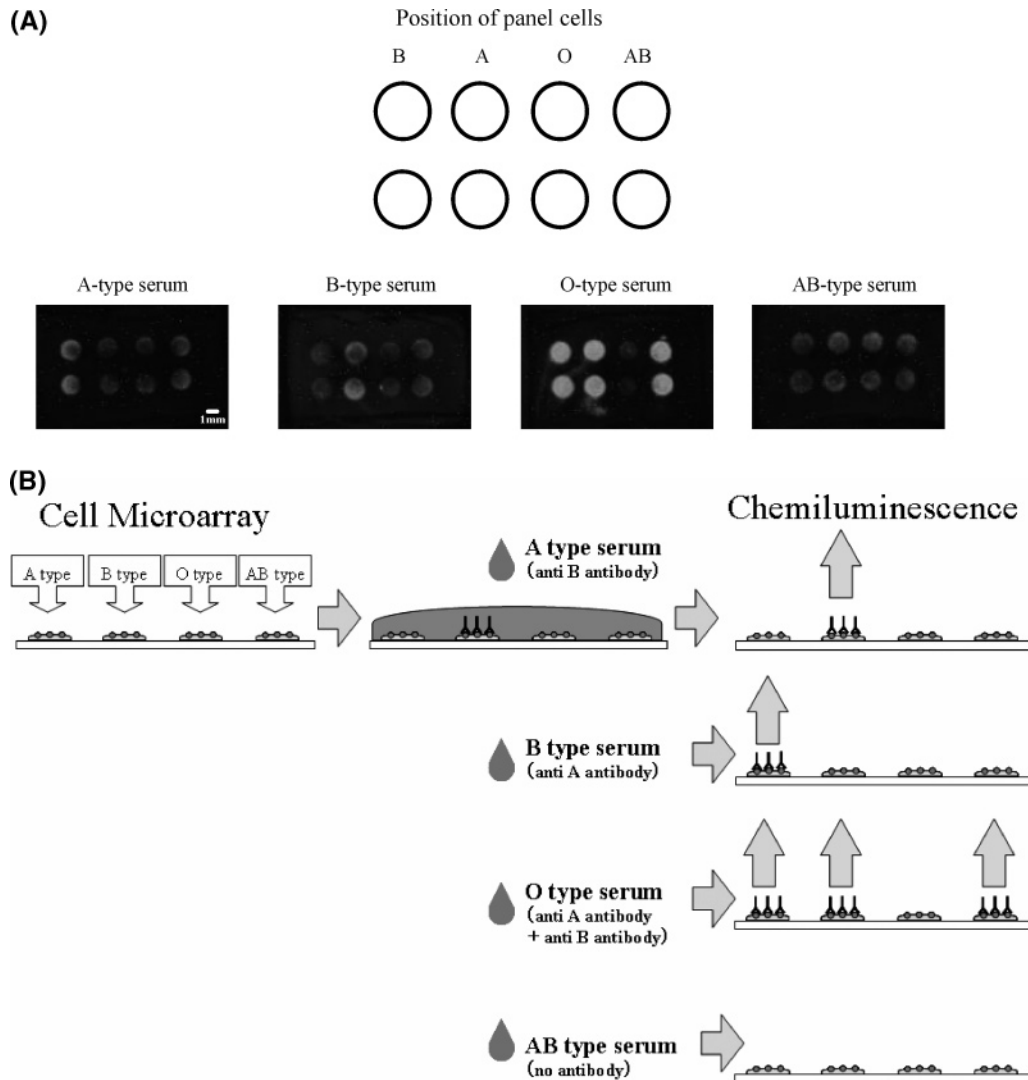


Figure 9. (A) Image of antibodies adsorbed onto blood cells microarrayed on a chip with photoreactive poly(phosphatidylcholine methacrylate). (B) Diagram of the image shown in (A). A-Type serum contained anti-B antibodies that adsorbed onto B-type blood cells, whereas B-type serum contained anti-A antibodies that adsorbed onto A-type cells. O-Type serum contained anti-A and anti-B antibodies that adsorbed onto A, B, and AB cells, and AB-type serum contained no antibodies to any of the cells.

microarray of cultured cell lines for antibody detection. Recently, a cell microarray for clinical analysis was performed (60); the target was the assay of antibodies in blood.

Usually, A-type blood does not have anti-A-type antibodies present; however, irregular antibodies are sometimes produced by previous transfusion or pregnancy. Blood containing irregular antibodies cannot be used for transfusion because the irregular antibodies aggregate with cells of the patient receiving the transfusion. Therefore, the presence of irregular antibodies is usually checked before transfusion. However, because the check is performed by an aggregation assay of blood cells, it is a time-consuming process and skill is required to interpret the results. If a cell array method was realized for detecting irregular antibodies, the assay system could be automated, reducing the time required to obtain results; therefore, we microarrayed a panel of cells that expressed the A, B, O, and AB antigens (60). The cells were mixed with photoreactive polymers and microspotted onto a polystyrene dish that was irradiated with UV light.

Antibody solution or serum was added to the microarray plate and incubated for some time. After washing, the array assay was used for human serum. Figure 9 shows the result of interactions of the cell microarray with antibodies. The O-type

human serum reacted with A-, B-, and AB-type cells, and the A- and B-type sera reacted with B- and A-type cells, respectively, although the reaction intensity was relatively low. This is because A- and B-type sera contain anti-B and anti-A antibodies, respectively. AB-type serum did not react with either type of cell, because it contains no antibodies to these antigens. Because O-type serum contains anti-A and anti-B antibodies, this serum reacted with the panel of cells, except for OD+ cells. These phenomena correspond to the results of an aggregation test for conventional blood types.

Conclusion

The possibility of miniaturizing and carrying out biological assays in parallel has had a great impact on the development of biomedical technologies. Here, we discuss how the photoimmobilization method can be employed for the microarray immobilization of various biological components such as proteins, antibodies, antigens, and cells. Microarray technology will increase in importance with the progress of bioinformatics and clinical informatics, and this progress will produce new applications for these technologies. Further efforts to combine chemistry, biotechnology, and nanotechnology are required for additional progress in this interdisciplinary field.

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