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Ultrasensitive DNA Chip: Gene Expression Profile Analysis without RNA Amplification

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We have developed a new DNA chip whose substrate has a unique minute columnar array structure made of plastic. The DNA chip exhibits ultrahigh sensitivity, up to 100-fold higher than that of reference DNA chips, which makes it possible to monitor gene expression profiles even with very small amounts of RNA (0.1–0.01 μg of total RNA) without amplification. Differential expression ratios obtained with the new DNA chip were validated against those obtained with quantitative real-time PCR assays. This novel microarray technology would be a powerful tool for monitoring gene expression profiles, especially for clinical diagnosis.

Key words: agitation, columnar array structure, DNA chip, microarray, sensitivity.

The Completion of the Human Genome project has significantly accelerated functional genomic studies. Nowadays, DNA microarrays, such as a DNA chip, are being used for high-throughput analysis. Microarray technology has recently been shown to be the most useful among many functional genomic approaches (*1–5*). Moreover, the DNA chip is promising to be a powerful tool not only for genetic diagnosis (*6–10*) but also for personalized therapy (*3*).

Generally speaking, there are two dominant methods for immobilizing oligonucleotides on a substrate for DNA chips; one is the direct synthesis of nucleic acids step by step on the solid substrate, and the other is the immobilization of synthesized oligonucleotides on the solid substrate using a high speed robot. The advantage of the photolithography method is the mass production of high-density DNA chips on which high-density oligonucleotides are immobilized. On the other hand, it requires special facilities and many photomasks to prepare the DNA chips, resulting in high cost. Taking genetic diagnosis with DNA chips into account, made-to-order DNA chips containing selected DNA probes for individual patients will be required for personalized therapy. From the viewpoint of personalized therapy, the DNA chips prepared by the second method are preferable.

However, most current DNA chips are not applicable for clinical use because of their low sensitivity. Assersohn *et al.* (*11*) reported that the mean recovery of breast fine needle aspirate was 202,500 cells, which corresponds to approximately 0.1 μg of total RNA. In general, a large amount of total RNA (1–100 μg) is required for a DNA chip (*12*). For example, the Affymetrix GeneChip prepared by the photolithography method operates with 1–15 μg of total RNA with a single round cDNA synthesis. Analysis of the gene expression profile in a small amount of sample using current DNA chip technology requires RNA amplification (*15–17*), which may lead to biased results (*18*, *19*). If only a small amount of sample, such as a biopsy one, is available for the assay, gene expression analysis of the sample is difficult using the existing DNA chips.

Here, we succeeded in highly sensitive detection of hybridization signals using our newly developed DNA chip having a unique structure made of plastic. The level of nonspecific adsorption of target DNA was reduced and the signal intensity of hybridization was increased on the DNA chip, hence the signal/noise (S/N) ratio was remarkably improved. Moreover, our DNA chip makes it possible to perform gene expression profiling with 0.1 μg of total RNA without any amplification. This performance strongly suggested that our new DNA chip would be useful for genetic diagnosis.

MATERIALS AND METHODS

Oligonucleotides—In most experiments, 64 different 70 bases oligonucleotides selected from a commercially available oligonucleotide set (QIAGEN, Human sample set, ver. 3.0) were used as DNA probes. In some experiments (Table 1 and Fig. 8), a 60 bases oligonucleotide (sequence, 5′-ACATTTTGAGGCATTTCAGTCAGTTGCTCAATGTACCTATAACCAGATCGTTCATCTGGA) complementary to plasmid pkF3 (Takara Bio) was used. A 20 bases oligonucleotide (sequence, 5′-TGGAGAACTTGATCGACAAG) was also used (Fig. 7). All oligonucleotides were chemically modified with an amino group at the 5′-terminal.

New DNA Chip—Substrate: A poly (methyl methacrylate) (PMMA) substrate, 76 × 26 × 1 mm, was used as the substrate for our newly developed DNA chip. This substrate has a unique structure, as shown in Fig. 1. A depressed structure with 256 arrayed pillars is located at the center of the substrate. The diameter of the top of the pillars is 0.15 mm, and their height is 0.2 mm. The DNA

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Table 1. **Effect of bead agitation on hybridization signal intensities with the new DNA chip.** The target DNA originated from the pKF3 plasmid.

Concentration of DNA (ng/μl)	0.15	0.3	0.75
With agitation	2,020	2,470	4,510
Without agitation	660	770	1,400
Ratio of (with agitation)/ (without agitation)	3.1	3.2	3.2

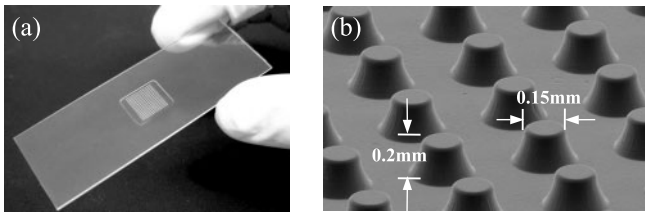

Fig. 1. **(a) Photograph of the new DNA chip and (b) magnified SEM image of pillars at the center of the substrate.**

chip substrate is manufactured by injection molding. Figure 1 (a and b) shows a photograph and a SEM image of this chip substrate, respectively. The oligonucleotides were covalently immobilized on the upper surface of the pillars according to the following procedure.

Surface treatment and oligonucleotide immobilization: The side chain of PMMA was hydrolyzed in an aqueous 1 N sodium hydroxide solution to produce carboxyl groups on the surface. 5′-Amino–modified oligonucleotides, dissolved in an aqueous solution at 30 μM, were spotted onto the upper surface of the pillars robotically using Gene STAMP II (Nippon Laser & Electronics Lab.). The solution ("spotting solution") comprised 100 mM 2-morpholinoethanesulfonic acid, pH 7.0 (MES, Sigma), 500 mM NaCl, 50 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Dojindo), and 0.005% (w/v) sodium dodecylsulfate (SDS, Sigma). After spotting, the substrate was incubated for 16 h in a 100% humidity chamber at 37°C and then washed with Milli-Q water. The oligonucleotides were immobilized the entire tops of the pillars through amide bonds. Figure 7b shows the reaction scheme.

Reference DNA Chip—Reference DNA chips were fabricated using commercially available glass slides (SDA0011; Matsunami Glass Industries, Ltd.). The 5′-amino–modified oligonucleotides described above were dissolved in Solution-I (Takara-Bio) at 30 μM and then spotted robotically. After spotting, the glass slides were incubated for 16 h in a humid chamber and then immersed for 20 min in a blocking solution (DBL0500; Matsunami Glass Industries, Ltd.). The glass slides were washed twice with Milli-Q water and once with ethanol, and then dried.

Target DNAs—In most experiments, target DNAs were prepared as described below: Total RNAs extracted from human brain and liver were purchased from BD Biosciences Clontech. Cy3 (Brain)- and Cy5 (liver)-labeled cDNAs were synthesized by reverse transcription from total RNAs using a CyScribe First-Strand cDNA Labeling Kit (RPN6200; Amersham Biosciences), according to the manufacturer's instructions.

In some experiments (Fig. 7), a chemically synthesized 20-mer oligonucleotide (sequence, 5′-CTTGTCGATCAAGTTCTCCA; Cy3-labeled at 5′-terminal) was used. A labeled DNA originating from pKF3 was also used in place of the target DNA (Table 1 and Fig. 8). The latter was prepared as follows: A pKF3 template was amplified by PCR (primer sequences: 5′-GGGCGAAGAAGTTGTCCATA-3′ and 5′-GCAGAGCGAGGTATGTAGGC-3′). The PCR conditions were as follows: initial 94°C for 4 min, and them 94°C for 40 s, 59°C for 1 min, 72°C for 1 min; 35 cycles. All PCR experiments were conducted with PCT-200 (MJ Research). The PCR products were purified by ethanol precipitation, dissolved in 40 μl of water and then heat-denatured. Next, 2 μl of a random 9 bases primer (6 mg/ml), 5 μl of 10× Klenow buffer, 2.5 μl of a dNTP mixture (2.5 mM each dATP, dTTP and dGTP, and 400 μM dCTP), 2 μl of Cy3-dCTP (Amersham Bioscience), and 10 U of Klenow fragment (2140A; Takara Bio) were added to the DNA solution. The mixture was incubated for 4 h at 37°C and the labeled product was purified by ethanol precipitation.

Hybridization—For the new DNA chip, we devised a special hybridization method to enhance the hybridization fluorescence signal. Briefly, a 20 × 20 mm transparent plastic cover with two through-holes, 0.8 mm in diameter and at opposite corners, was bonded to the center portion of the substrate with double-coated adhesive tape (No. 532; Nitto Denko Co., Ltd.). Figure 2 shows illustrations of the new DNA chip. The target DNA was dissolved in 40 μl of hybridization solution; this solution was applied to the center of the new DNA chip through the holes using a micropipette. About 0.5 mg of micro-glass beads, 125 μm in diameter, suspended in the hybridization solution, was also applied to the center of the new DNA chip in the same way. The hybridization solution comprised 5× SSC (saline-sodium-citrate; Sigma), 0.1% (w/v) SDS, 1% (w/v) BSA (bovine serum albumin; Sigma), and 0.01% (w/v) salmon sperm DNA (Sigma). The holes were sealed with adhesive tape. Beads added to the hybridization solution were driven between the convex-concave structures of the new DNA chip, which agitated the hybridization solution. The beads moved in the concave portion of the new DNA chip without scratching the upper surface of the pillars whereon the oligonucleotides were immobilized.

For reference DNA chips, the target DNA was dissolved in 30 μl of hybridization solution. A 50 × 20 mm cover glass (CG00014; Matsunami Glass Industries, Ltd.) was placed on the spotted area of the slide glass, and then the target DNA solution was applied.

Both the new and reference DNA chips were incubated for 16 h at 42°C. For the new DNA chip, the hybridization solution was agitated by movement of the beads during the hybridization. After the hybridization, the covers were removed, and the chips were washed with 3× SSC containing 0.1% SDS, 1× SSC and 0.1× SSC sequentially at room temperature, and then dried with a spin drier. Hybridization signals were scanned using a DNA chip scanner (GenePix 4000B; Axon Instruments).

Quantitative Real-Time PCR Analysis—Real-time RT-PCR (TaqMan) analysis was carried out using an ABI Prism 7000 Sequence Detector System (Applied Biosystems), according to the manufacturer's instruction. TaqMan probes (Assay-on-Demand) were purchased from Applied Biosystems.

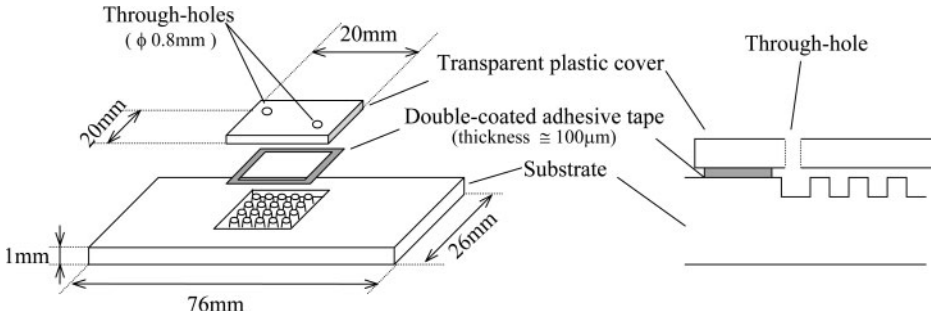

Fig. 2. **Schematic illustrations of the new DNA chip; the right one shows a side view of the chip**.

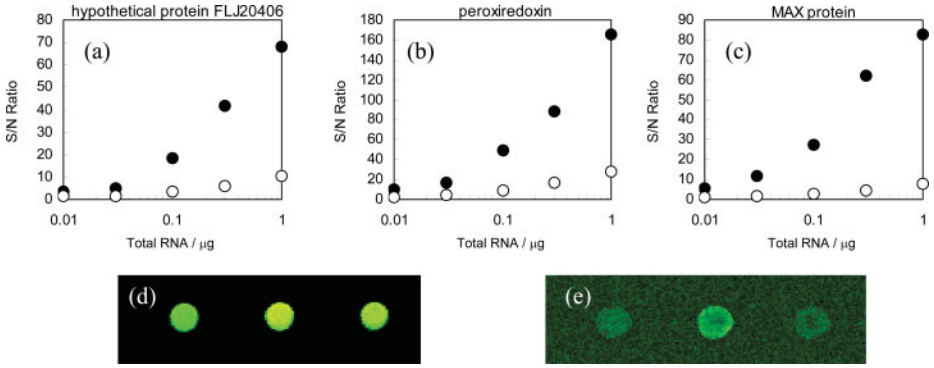

Fig. 3. **S/N ratios and fluorescence images of the new and reference DNA chips after hybridization.** The S/N ratios for the new (solid circles) and reference (open circles) DNA chips for three selected genes are shown in (a), (b) and (c). Fluorescence images of the new (d) and reference (e) DNA chips (amount of total RNA, 0.1 μg) are also shown. In each image, the spots represent hypothetical protein FLJ20406, peroxiredoxin and MAX protein from the left.

Table 2. **Signal intensities obtained with the new and reference DNA chips (amount of total RNA, 0.1 μg).**

Gene name	Serine/ threonine kinase 24	Peroxiredoxin	MAX protein
Signal intensity of new DNA chip	1,070	4,010	2,220
Signal intensity of reference DNA chip	510	1,500	520
Ratio of (new DNA chip)/ (reference DNA chip)	2.1	2.7	4.3

RESULTS AND DISCUSSION

We first examined the agitation effect on the hybridization efficiency using the new chip. Table 1 shows the concentration dependence of the hybridization efficiency with and without agitation of the solution by beads.

The hybridization signals in both cases decreased with decreasing concentration of the target DNA. This result indicates that the agitation with the beads increased the hybridization signals to more than three times compared with no agitation. In the case of no agitation, the low signal intensity was due to the low diffusion constant (D) of the target DNA in the solution. The diffusion constant of 25 bases oligonucleotides is reported to be 8×10^{-8} cm^2/s (*20*). Therefore, the mean square distance of the target DNA in 16 h is calculated to be 0.0092 cm^2, and the average migration distance is 0.096 cm. This average migration distance indicates that only a 1.5% of the target DNA in the hybridization solution is accessible to the probe DNAs on the pillars without agitation under our conditions, assuming the area of hybridization assay is 4 cm^2.

In the next step, the signal intensities obtained with the new DNA chip were compared with those obtained with the reference DNA chip. Three oligonucleotides were selected from the commercially available oligonucleotide set (hypothetical protein FLJ20406, peroxiredoxin and MAX protein). After synthesizing a Cy3-labeled cDNA from 5 μg of total RNA, 0.1 μg of cDNA was applied to the new DNA chip as well as to the reference DNA chip. As shown in Table 2, the new DNA chip showed a markedly higher signal intensity sensitivity, approximately 2–4 times fold higher, than the reference DNA chip.

Next, we examined the signal-to-noise (S/N) ratio of the new DNA chip compared with that of the reference DNA chip because not only enhancement of the signal but also control of the background noise is important for the development of new DNA chip. In most cases, the noise mainly comes from nonspecific adsorption of target DNA onto the substrate. Figure 3 (a, b and c) shows the S/N values obtained with three kinds of target DNA. The results indicate that the new DNA chip showed a markedly higher S/N ratio, approximately 20–100 fold higher, than the reference DNA chip. That is to say, the new DNA chip required small amounts of target DNA (1/20–1/100) to show an equivalent S/N ratio to in the case of the reference DNA chip.

Figure 3 (d and e) shows fluorescence images of the new and reference DNA chips after hybridization. These images clearly indicate the new DNA chip gives higher signals and lower noise compared to the reference chip. In the case of Fig. 3 (d and e), the S/N ratio for the new DNA chip was approximately 5–10 fold higher than that for the reference DNA chip. The high S/N ratios of the new DNA chip were obtained by enhancing the signal level as well as by reducing the noise level. In the case of the new DNA chip, the

noise level was 60–70% lower than that for the reference DNA chip (data not shown). The enhanced signal level was predominantly due to the bead agitation. The markedly reduced noise was due to three reasons. The first reason is the new chip's unique structure. Table 3 shows the relationship between defocus and noise intensity on untreated substrates. As we expected, the noise decreases in the background area when the focal point is adjusted to the top of the pillar. Practically, the noise decreased to 60% when the defocus distance was 200 μm. The second reason is that PMMA exhibits low autofluorescence. Table 3 shows the comparison of the noise of due to PMMA and the slide glass used for the DNA chips. The noise mainly comes from the autofluorescence of the substrates. The noise of PMMA is less than that of the glass substrate. The last reason is surface charges on the substrate. Unreacted carboxyl groups might remain on the surface after the reaction, as shown Fig. 7. Because the carboxyl group shows negative charge at neutral conditions, repulsive forces might exist between the negatively charged target DNA and the surface, resulting in less nonspecific adsorption of the target DNA during the hybridization assay.

Table 3. **Relationship between defocus and the noise level on untreated substrates.**

Defocus (μm)	0	50	100	200
PMMA substrate	330	250	190	180
Glass slide (SDA0011)	460	–	–	–

Next, we investigated whether our DNA chip is applicable to gene expression analysis with a small amount of target DNA. Cy3- and Cy5-labeled cDNAs synthesized from the total RNA (1 to 0.01 μg) from human brain and liver, respectively, were used as target cDNAs. Fluorescence images of the new and reference DNA chips after hybridization are shown in Fig. 4. As shown in Fig. 4, all the hybridization signals obtained using the new DNA chip were markedly higher and had lower noise compared to with the reference DNA chip. In particular, when the amount of total RNA was 0.1 or 0.01 μg, most signals were difficult to detect for the reference DNA chip, while signals on most spots were clearly observed with the new DNA chip. Figure 5 shows scatter plots of the new and reference DNA chips with different amounts of total RNA. The new DNA chip showed a wide dynamic range even when the amount of total RNA was 0.1 or 0.01 μg.

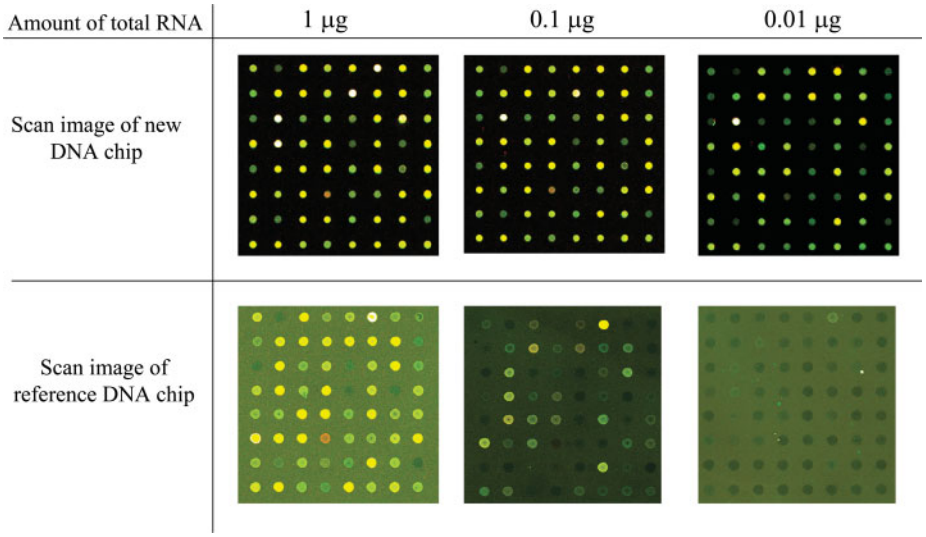

Fig. 4. **Scan images of the new and reference DNA chips.**

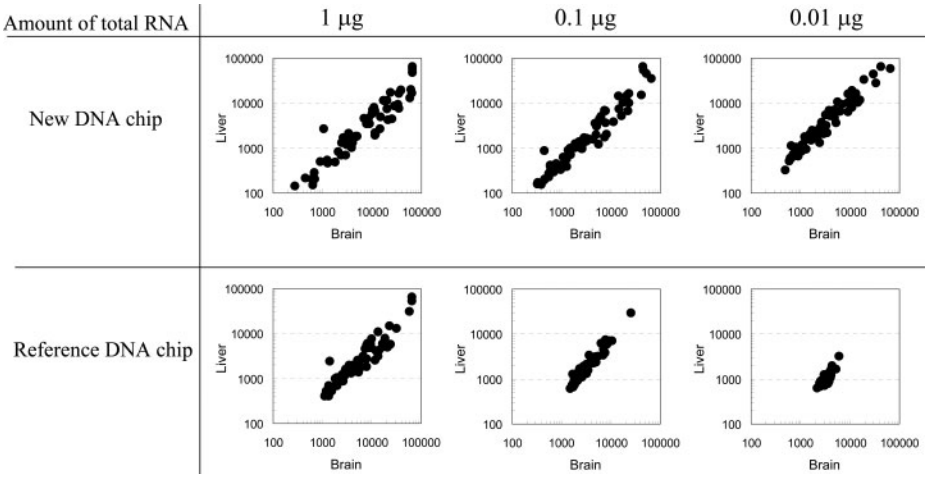

Fig. 5. **Scatter plots of the new and reference DNA chips with different amounts of total RNA.**

On the other hand, the dynamic range of the reference DNA chip became narrower as the amount of total RNA decreased. In the next step, we examined the Cy3/Cy5 ratio to confirm the reliability of the new DNA chip. The analysis was performed with different amounts of total RNA (1 and 0.1 μg, 1 and 0.01 μg). The correlation coefficients obtained with using the new and reference chips are summarized in Table 4. The values for the new chip are obviously superior to those for the reference chip. In particular, the correlation coefficient for the new chip was 0.80 even when 0.01 μg of total RNA was used, while it was 0.10 with the reference chip. Additionally, the correlation coefficient between the new and reference DNA chips was 0.88 in the case of 1 μg of total RNA.

These results shows that our DNA chip makes it possible to perform gene expression profiling with only 0.1 μg of total RNA without any amplification. Moreover, it is strongly suggested that our DNA chip has the ability to monitor gene expression profiles even with an amount of total RNA as low as 0.01 μg.

Table 4. **Correlation coefficients of Cy3/Cy5 ratios with different amounts of total RNA.**

Amounts of total RNA (μg)	1 and 0.1	1 and 0.01
New DNA chip	0.87	0.8
Reference DNA chip	0.49	0.1

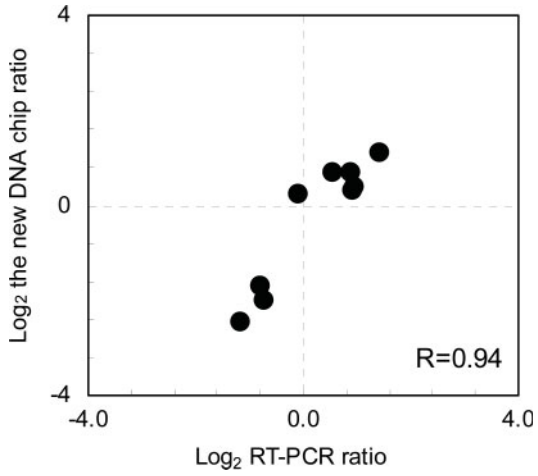

Fig. 6. **Correlation of differential expression ratios (stomach cancer/stomach) for the new DNA chip and RT-PCR (TaqMan).**

To determine the correlation between the signals and the amounts of expressed RNAs, the differential expression ratios obtained with the new DNA chip were compared with those obtained on quantitative real-time PCR analysis. For this analysis, we selected nine genes that are differentially expressed between two human RNA samples (stomach cancer and stomach; BD Bioscience Clontech): HLA-F, PARVB, NR2C1, MDH1, MTI1E, RPL23AP7, BRDT, CD84 and SERPINA1. 5′-Amino–modified 70 bases oligonucleotides originating from the nine genes were chemically synthesized and immobilized on the substrate as described above. The cDNAs hybridized to the new DNA chip were produced from 1 μg of total RNAs. As shown in Fig. 6, the correlation coefficient for both results was 0.94, which is higher than the reported values [0.79–0.92 (*21*)] obtained with a commercially available DNA chip. This result indicates that the new DNA chip can be accurately used to monitor the gene expression ratios in samples.

In this study, we used PMMA as the substrate. PMMA has following the advantages: Firstly, substrates with unique structures can be easily manufactured by means of injection molding. Secondly, it exhibits low autofluorescence. Thirdly, a simple surface modification is available, as shown in Fig. 7b. Although some PMMA surface modification methods have been reported (*22–25*), these methods have a disadvantage, *i.e.*, the PMMA surface tends to be adversely affected by organic solvents or reagents. Here, the question arose as to whether the PMMA substrate contributed to the ultrahigh sensitivity of the new DNA chip. To address this question, the following experiment on the material effect on the sensitivity was carried out. To introduce carboxyl groups onto a glass surface, aminosilane–coated slides (S8111; Matsunami Glass Industries, Ltd.) were immersed in 1-methyl-2-pyrrolidone containing 1.6% (w/v) succinic anhydride (Sigma) and 2.3% (v/v) borate buffer (100 mM, pH 8.0) for 20 min, rinsed with Milli-Q water and then dried (*26*). A 60-mer oligonucleotide composed of a sequence complementary to the target DNA, dissolved in the spotting solution at 30 μM, was spotted robotically and immobilized on glass and PMMA plates with amide binding. The procedures for the reactions are shown in Fig. 7. The target DNA was synthesized from the pKF3 template by the random priming method. The size of the target DNA was estimated to be 200–300 bases on electrophoretic analysis. The hybridization signals on glass and PMMA are shown in Fig. 8, which clearly indicates that the signal intensity on PMMA is superior to that on glass. Although it is not clear why the higher signal intensity was observed in the case of PMMA, we propose

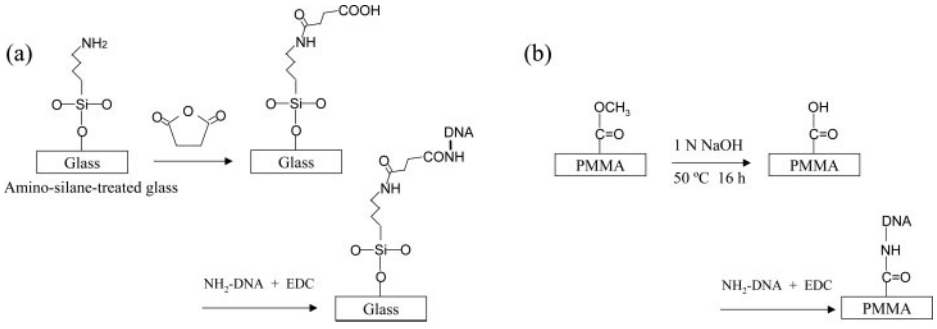

Fig. 7. **Schematic representation of the procedures for immobilization of oligonucleotide on glass (a) and PMMA (b).**

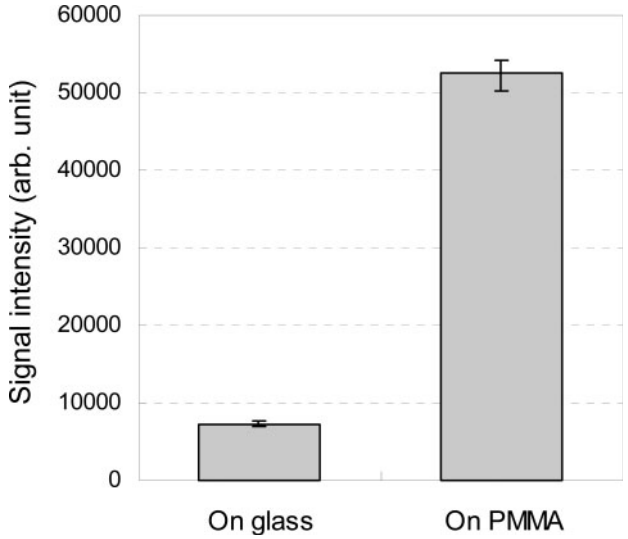

Fig. 8. **Comparison of the hybridization signal intensities of oligonucleotides immobilized on glass and PMMA.** The target DNA was synthesized from the pKF3 template by the random priming method and the oligonucleotide comprised 60 bases.

two possibilities for the signal increase. One possibility is that the PMMA surface might be much rougher than that of glass. Hence, the surface area of a plastic chip might be larger than that of a glass chip. The other possibility is that the influence of steric hindrance might be smaller when a large target DNA hybridizes with an immobilized oligonucleotide on PMMA. When a 20 bases oligonucleotide was immobilized on glass and PMMA plates, and a fluorescent-dye–labeled complementary oligonucleotide (20 bases) was used as the target DNA, interestingly, the signal intensity of PMMA was 0.7–1.2 times as high as that of glass (data not shown). This result implies that a large target DNA can efficiently hybridize with an oligonucleotide on PMMA in comparison with glass. The reason PMMA shows higher hybridization efficiency might be the uneven height of the exposed side chains (carboxyl groups) on PMMA surfaces. This might lead to a lower three-dimensional density of immobilized oligonucleotides on PMMA, resulting in higher hybridization efficiency of a large target DNA.

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