INSTITUTE OF PHYSICS PUBLISHING NANOTECHNOLOGY
Nanotechnology **13** (2002) 756–762 PII: S0957-4484(02)53241-4

In situ synthesis of oligonucleotide arrays by using soft lithography

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Received 9 September 2002
Published 22 November 2002
Online at stacks.iop.org/Nano/13/756

Abstract

This paper describes the *in situ* synthesis of oligonucleotide arrays on glass surfaces by using soft lithography. In this method, based on the standard phosphoramidite chemistry protocol, the coupling was achieved by the glass slide being printed with a set of polydimethylsiloxane (PDMS) microstamps, on which was spread nucleoside monomer and tetrazole mixed solution. The elastic characteristic of PDMS allowed it to make conformal contact with the glass slide in the printing coupling. With regard to the efficiency of the printing coupling, the hybridization microscope images of 20-mer oligonucleotide synthesized via the directly drip-dropped coupling and the contact coupling were compared; the fluorescence intensities of the two methods showed no significant differences. The coupling efficiency was also investigated via an end-labelled fluorescence nucleotide method and a stepwise yield of 97% was obtained. A high-quality, high spatial resolution and large-scale PDMS stamp, which was developed by integrating 168 different microstamps on one glass substrate for synthesizing oligonucleotide arrays. The stamp was modified to improve the surface wettability by plasma discharge treatments, so that microstamps could be used to fabricate oligonucleotide arrays. A motional printing head was developed to improve the contact effect of the glass slide with different microstamps. A higher boiling point solvent was used in the printing coupling to inhibit solvent volatilization and to maintain the consistency of reagents on different features of the microstamp. An effective method was used to eliminate residual reactive nucleosides on chips with small molecules containing a hydroxyl group. A specific oligonucleotide array of four probes both matched and mismatched with the target sequence was fabricated to identify the perfect match and mismatch sequences.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

In biological systems, genetic information is read, stored and translated using the rule of molecular recognition. Every nucleic acid strand carries the capacity to recognize complementary sequences through base pairing. Genechip, or DNA microarray, anchors tens of thousands of closely arrayed oligonucleotide probes on the surface of solid substrates, on which the process of recognition can be highly parallel. This has many applications in environmental genomic and pharmacogentics research. Oligonucleotide arrays are proving to be powerful tools for monitoring gene expression, and for resequencing genes to screen for mutations and polymorphism [1–8]. Several on-chip synthesis methods of oligonucleotide arrays have been reported in the past ten years [9–14]. For example, Fodor *et al* [1–8] developed light-directed synthesis for the construction of high-density DNA probe arrays by using photolithography and solid-phase DNA synthesis. Affymetrix Corporation has achieved DNA probe arrays with high spatial resolution [1–8]. In this

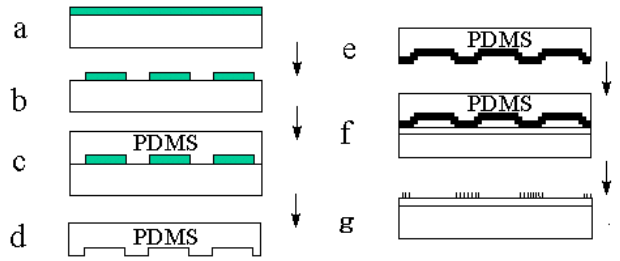


Figure 1. Schematic illustration of the fabrication of microstamp (a → d) and the printing coupling (e → g). (a) A photoresist coated on the glass plate, (b) a pattern formed by lithography, (c) precursor solution of PDMS, (d) PDMS microstamp, (e) spreading chemical reagents on the surface of the stamp, (f) delivering reactants onto the modified glass slide by printing and then conducted coupling, (g) a new monomer bonded on the substrate.

paper, DNA microarray synthesis *in situ* by soft lithography is reported. Soft lithography was initially developed by G M Whiteside's group [15] and has been widely applied in biology, chemistry and materials science [16–22]; it is simple, reliable and can achieve sub-microstructure. The basic strategy for the oligonucleotide synthesis method is in accord with the standard phosphoramidite chemistry protocol but the coupling is replaced by soft lithography (see figure 1). The mixed solution with nucleoside monomer and tetrazole as reactants is spread on features of the modified microstamp, then transferred onto the modified glass slide surface by machine alignment printing. Thus the nucleoside monomer on features of the microstamp is coupled with the predefined regions on the slide. A first microstamp with adenosine will cover those sites which are destined to contain an oligonucleotide beginning with adenosine; the next microstamp allows other sites to couple thymidine; a third allows the coupling of cytosine, and a final one, the coupling of guanosine. Next, oxidation, capping and detritylation are conducted successively in a sealed cell after four printing couplings are completed. Thus, by using different stamps and their corresponding monomers the attachment of the first-layer nucleotides is completed on their corresponding predefined regions. The nucleotide of the second layer is then added to each site with four other stamps. Thus, in order to synthesize N-mer nucleotide on the chip, $N \times 4$ stamps were usually required, each stamp exhibiting specific features, although an alternative combinatory optimized design of microstamps has been considered [23].

2. Experimental details

5′-DMT-2′-deoxynucleoside phosphoramidites (thymidine, N^4-isobutyryl-2′-deoxycytosine, N^2-isobutyryl-2′-deoxy-guanosine, N^6-phenoxyacetyl-2′-deoxy-adenosine; abbreviated T, C, G, A respectively) and the other synthesis reagents were purchased from PE Biosystems. The fluorescence group hexachloro-6-carboxyl-fluorescein (HEX) or 6-carboxyl-fluorescein (FAM) was purchased from Shanghai Biological Produce Corporation. The glass substrates used for the coupling reaction were standard 'precleaned' soda lime microscope slides. The commercially available polydimethylsiloxane (PDMS) was obtained from Hangzhou silicone rubber plant. Other chemical reagents were of analytical grade and purchased locally.

The modification of the glass slide

A common sodium silicate glass slide was treated in H_2SO_4–$K_2Cr_2O_7$ solution for 24 h, and strongly washed with water, distilled water, then immersed in 5% aminopropyltrimethoxysilane in CH_3Cl for 5 min, washed successively with ether, acetone, and anhydrous ethanol, and dried at 110 °C for 30 min. The slide was treated with 5% glutaradehyde in phosphate-buffered saline (pH = 7.4) for 2 h, 10% aminoethyl alcohol for 2 h, and then $NaBH_4$ solution for 15 min at ambient temperature. The modified glass slide was dried at 110 °C for 30 min for oligonucleotides synthesis.

Preparation of the PDMS stamps

A master stamp was made by lithography in the usual way. A mixed precursor including catalyst, ethylene silicate and dimethylsiloxane was cast onto the master whose surface had predesigned patterns. Air bubbles were removed from the precursor, which was then covered with a silanized glass plate. The sandwiched precursor was left to cure. Then, the master was peeled away from the elastic cured polymer, and so a stamp was fabricated.

The above-mentioned stamps were modified to improve the surface wettability by microwave plasma treatment. The microwave plasma treatment chamber used here was a sealed cylinder, and the PDMS stamp plate was put horizontally in the cylinder with a microwave plasma inducing ring around the outside wall, then sealed to allow gas introduction above and evacuation below by vacuum. The plasma treatment procedure involved initial evacuation to a pressure of about 6 Pa. Then the pressure was purged with argon back to atmospheric pressure, followed by evacuation to 6 Pa again. In the case that the argon was introduced in a flow rate of 30 SCCM to induce plasma, and then hydrogen was gradually adjusted up to a flow rate of 15 SCCM to mix with the argon while a stable plasma discharge remained for 5 min. During the microwave plasma exposure pre-treatment for the PDMS stamp surface, the chamber pressure stayed under 60–70 Pa and the output power was 800 W. After plasma exposure, the PDMS stamp board was transferred into acrylonitrile solution bearing additives and catalysts for 4 h immersion. To extract some absorbed impurities during wet grafting for the PDMS stamps, the acetone, ethanol, detergent solution, water and deioned water rinses were carried out in sequence, followed by a drying process of 1 h at 100 °C and cooling down to room temperature. A Rame-Hart 100R contact angle instrument was used to measure the contact angle of the modified surface of the PDMS stamps.

Oligonucleotide synthesis

Oligonucleotide automation synthesis on the modified slide was conducted as follows: a pre-designed groove was chiselled out on a flat PDMS film; a synthesis region on the glass slide was jointed with the groove. A Teflon plate with entry and exit plastic tubes was covered on the carved PDMS film. The sandwich was fixed with iron clamps, and formed a closed fluidic system. Oligonucleotide synthesis was carried out on the glass slide. The printing process for oligonucleotide was carried out in a glove box, and other reactions were

conducted as the above-described closed fluidic system. Two kinds of oligonucleotide sequences were synthesized: one was poly-T with its final nucleoside fluorescent deoxynucleoside phosphormidite; the other was 5′-AGG AGG CTA AGT CTC TCA GG-3′.

Oligonucleotide array fabrication

The mechanical device used for printing was controlled by computer. X–Y–Z axes with a setting accuracy of 3 μm controlled the motion of the glass slide, using a CCD camera as monitor. In order to improve the contact of the glass slide with the stamp, we developed a printing set in which a motional printing head helped parallel adjustment between the glass slide and the PDMS stamps. In this experiment, the feature size of synthetic probe was 9.0×10^{-5} m diameter and 1 cm^2 had 2500 features for four different probe arrays.

The deprotection, hybridization and detection of synthetic oligonucleotide arrays

Following the synthesis, the glass slide was treated in a mixed solution of ethanol and aminoethyl alcohol (vol/vol = 1) in a sealed box at 75 °C 2 h for deprotection. Then it was washed with distilled water and dried by cold blowing before hybridization. The fluorescence signals of poly-T sequences were immediately imaged by a scanning laser confocal fluorescence microscope (TCS/SP, Leica, Germany). As for the 5′-AGG AGG CTA AGT CTC TCA GG-3′sequence, oligonucleotide arrays were hybridized with HEX-CCT GAG AGA CTT AGC CTC CT-3′ probe solution at 55 °C in the hybridization chamber, and then rinsed with 0.1% sodium dodecyl sulfate in 6xSSC (sodium chloride/sodium citrate buffer), 0.1% SDS in 0.1x SSC respectively. The probe array was scanned using the Scanarray Microarray system (Packard BiochipTechnologies, USA). Once the scan was completed, a grid was aligned on the scanned image and a digitized intensity table was generated for each of the probe features on the chip.

3. Results and discussion

In the standard phosphoramidite chemistry, any extraneous nucleophiles will compete with the slide-bound 5′hydroxyls for the activated phosphoramidites and will decrease coupling efficiency (e.g. water contamination of even 1% will lead to a 2% decrease in coupling efficiency [25]). However, high-precision locating equipment is needed for repeated overlapping between the glass slide and different microstamps. So an operation system was designed and constructed to meet the above-described conditions for fabricating DNA microarrays. The system included a glove box to provide an oxygen- and water-free environment, a computer-controlled three-dimensional stage for high-precision pressing and location of microstamps, and a reaction cell connected to a DNA automatic synthesizer. The volume concentrations of water and oxygen in the glove box were kept within 1.5×10^{-4} and 2×10^{-4}%, respectively. The motion extensions of the stage are 220 mm × 290 mm × 70 mm, with a location precision of about 3 μm. The printing couplings were conducted on the three-dimensional stage, and the other

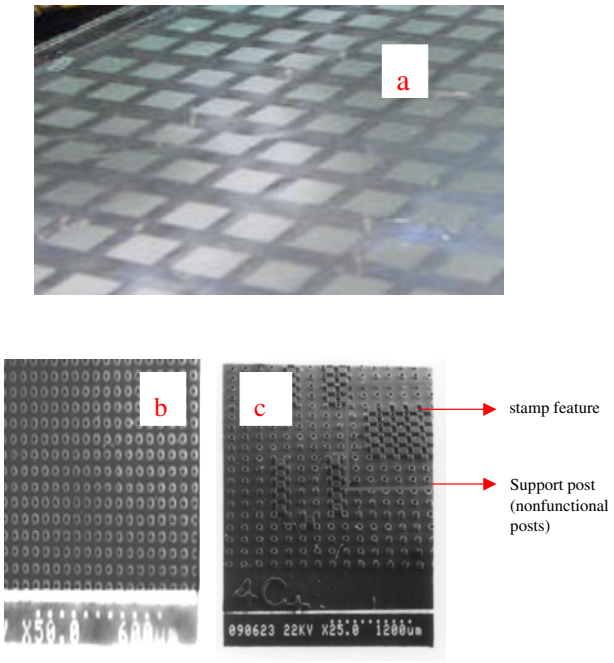


Figure 2. (a) PDMS stamps on the modified glass plate. (b), (c): scanning electron micrographs of PDMS stamp.

chemical reactions or washing steps were conducted in the reaction cell. A glass slide was placed on the reaction cell to constitute a sealed reaction cell. The sealed reaction cell eliminated interference by the different chemical reagents and maintained the favourable conditions of the glove box to fabricate oligonucleotide arrays.

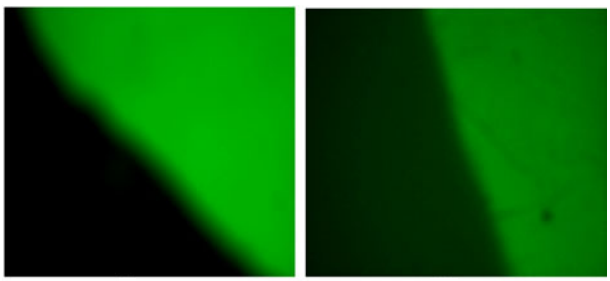
In order to overcome shrinkage and deformation of the elastic stamp, $(CH_3)_2SiCl_2$ was used to form a self-assembled monolayer (not double or multiple layer)—formed in a spontaneous, self-assembly process. The short-range Van der Waals force between the self-assembled monolayer and the PDMS guarantees excellent adherence of the PDMS layer to the glass plate. The shrinkage of a single feature of PDMS microstamp could be controlled to within 0.3% in area, while the shrinkage of the whole PDMS stamp film was only 0.0427% in linearity due to its very strong adherence to the glass plate. The adhesion of PDMS stamp to glass plate was of significant importance in the accurate overlapping of features of different microstamps in the *in situ* synthesis of oligonucleotide microarrays (see figure 2(a)). The master surface was modified with silver or aluminium film to inhibit structural damage of the microstamp; the master could be peeled easily from the large-scale stamp, and perfectly negative patterns were replicated, as shown in figure 2(b).

Sometimes, features in microstamps were not distributed evenly, and then the aspect ratios were too low, and the PDMS features were not able to withstand the compressive forces typical of printing. This would result in sagging of features. To solve this problem, nonfunctional features were introduced into the designs to support the noncontact regions (see figure 2(c)): an additive nucleotide is coupled to all synthetic regions on the slide by an additive microstamp that includes all features but not nonfunctional features. And nonsynthetic regions on the slide (corresponding to nonfunctional posts

Table 1. Contact angle data and their retrogression with time.

PDMS sample surface	H_2O (deg)	CH_3CN (deg)
Original without washing	100 ± 5	90 ± 5
PDMS washed and dried	90 ± 5	90 ± 5
Not grafting (in an hour)	70 ± 10	76 ± 8
Not grafting (in a week)	85 ± 5	84 ± 10
Grafted (in an hour)[a]	28 ± 12	17 ± 7
Grafted (in a week)[a]	35 ± 15	25 ± 10
Grafted (in a month)[a]	35 ± 15	23 ± 13

[a] The oriented acrylonitrile grafting.



(a) (b)

Figure 3. Laser confocal fluorescence microscopy images measured under the same conditions for *in situ* synthesized oligonucleotides. (a) TT-FAM; (b) 5′-AGG AGG CTA AGT CTC TCA GG.

of stamps) were capped with the capping reagents after the additive nucleotide had been coupled.

The microstamps were modified to improve the surface wettability by microwave plasma treatment. Through H_2 and Ar mixed gas pre-treatment in a large-volume microwave plasma generator under optimized conditions, the PDMS surface was oriented/grafted with acrylonitrile molecule when post-immersed in acrylonitrile grafting solution. It was found that the cyano functional groups resulted in hydrophilic surface formation on PDMS stamps (see table 1). The modified surface applied to DNA microarray synthesis promised much and the results implied a way of controlling surface chemistry.

Our initial objective was to explore the feasibility of the molecular printing method. Poly-sequence TT-FAM was synthesized by contact coupling and the strong fluorescence intensity was checked via Scanarray (figure 3(a)). The effect of contact printing on the synthetic efficiency of the contact coupling in synthesizing one nucleoside was also investigated. It was shown that the fluorescence intensity and uniformity of double contact was better than for single contact, while for contacting three times or more, the fluorescence intensity and uniformity was not improved further significantly. Therefore, every nucleoside lengthening was carried out with contact coupling twice.

As a challenging test of the generality of this synthesis procedure, we attempted the preparation of a 20-mer DNA, 5′-AGG AGG CTA AGT CTC TCA GG-3′. As judged by hybridization, the synthesis was successful (figure 3(b)).

Two methods were used to investigate the printing coupling efficiency. One was to compare the hybridization results of the same sequence oligonucleotides synthesized on different regions of the same slide using the printing coupling and a directly drip-dropped coupling, respectively. Their fluorescence intensity values showed no significant differences and this result indicated that the effect of printing to the synthetic yield was little. The other was the end-labelled fluorescence intensity method. A series of different poly-T compounds whose bases increased one by one were synthesized on different regions of the same slide. Their terminal nucleosides were labelled with HEX. Provided the coupling efficiency of automatic synthesis was 98%, thus that of the printing coupling was 97.4% through formulating the relation between the fluorescence intensity and fluorescence molecules of poly-T compounds (figure 4). Also, a series of failed poly-T compounds were labelled with FAMs. In this special experiment, the terminal nucleoside was coupled (to 5′-hydroxyl groups) and oxidized without subsequent capping and detritylation, and then an FAM-labelled nucleoside was coupled via directly drip-dropped coupling to the uncoupled 5′-hydroxyl groups. Naturally, the fluorescence signal could be observed and measured with the addition of FAM-labelled nucleoside as long as the former coupling was not fully completed. The stronger the fluorescence intensity, the poorer the former contact coupling efficiency was. The fluorescence intensity ratio of synthetic regions to non-synthetic regions was defined as the relative fluorescence intensity (RFI) to provide a measure of the coupling efficiency or synthesis yield of this method. As shown in figure 5, the RFI values were equal to 1 after the sixth nucleoside was coupled and this result indicated that the coupling efficiency was nearly equivalent to that of phosphoramidite chemistry protocol from the sixth nucleoside being coupled. All results in the above-described methods proved the high coupling efficiency of soft lithography. This was because those reactants of the printing coupling were quasi-solid phase and their consistencies were more concentrated, thus the speed was faster than that of the direct coupling although reactants of the latter had more capacity.

As in our previous processes of on-chip fabrication of oligunucleotide microarray by the molecular printing method, the glass slide was washed with anhydrous acetonitrile to clean traces of the residual coupling reagents after the printing coupling step was completed. The hybridization experiment showed that a weak fluorescence signal could be detected in the non-synthetic region besides a strong signal detected in the synthetic region. The RFI was 1:0.12 for 20-mer oligunucleotide sequence hybridized at 55 °C. This result indicated that the activated nucleoside would react with hydroxyl groups of the non-synthetic regions when the traces of residual reactants were washed with anhydrous acetonitrile. Accordingly the non-synthetic region fluorescence intensity was attributed to contamination by traces reactants. Some investigations have [24, 25] revealed

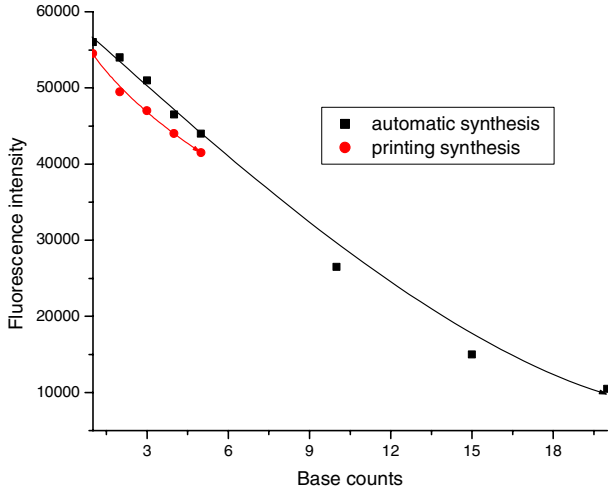


Figure 4. Fluorescence intensity of poly-T oligonucleotides.

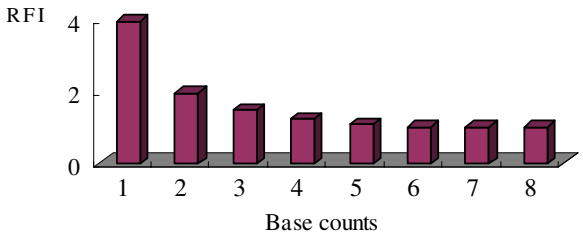


Figure 5. The relation of average RFI of four parallel examples with the number of poly-T.

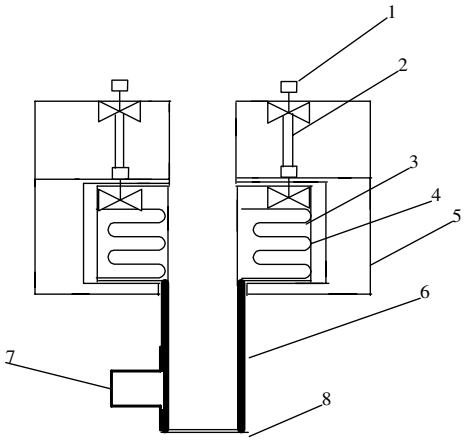


Figure 6. Schematic illustration of the printing set. 1: balance-regulating bolt; 2: screw; 3: spring; 4: fixed cavity of spring; 5: fixed cavity of the printing head; 6: printing head; 7: adjustable screw to fix glass slide; 8: glass slide.

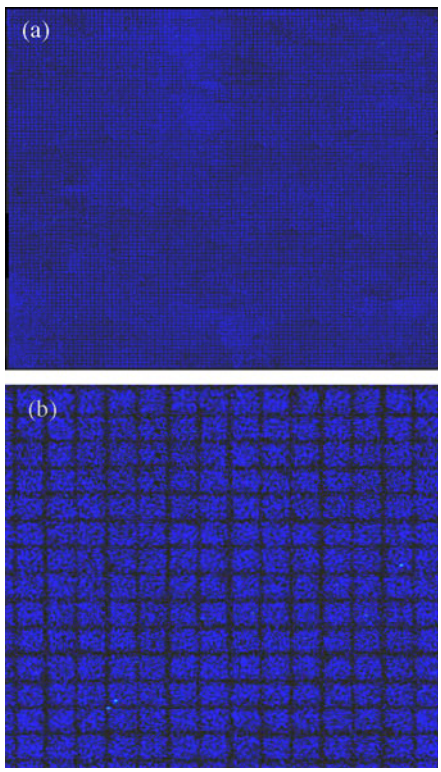


Figure 7. Hybridization to a matrix of 10k oligonucleotides. (a) Fluorescence image of 10k oligonucleotide array following hybridization with 5′-HEX-CCT GAG AGA CTT AGC CTC CT-3′probe solution at 55 °C. (b) Magnification of a portion of (a): the uniform squares represent synthetic region, and the black grid represents nonsynthetic regions.

that small molecules containing activity hydroxyl groups can easily react with the activated nucleoside monomer, and eliminate their contamination in the non-synthetic region. Water and ethanol were good inhibitors. Because the cleanup and disposal procedure of ethanol is much easier than water, ethanol was chosen to remove the excess reagents before the glass slide was washed with acetonitrile. With this new procedure, the fluorescence background has been greatly decreased. The RFI reduced to 1:0.04. The ethanol molecule did not affect the coupling efficiency.

The experiments described above demonstrate the feasibility and high efficiency of oligonucleotide synthesis using soft lithography. In the DNA chips used, the important issue was that the hybridization should be accurate, and that the same sequences should give the same fluorescence intensity. Therefore, on the printing coupling, each feature on the microstamp should contact with the glass slide, and the reaction conditions (including reactant concentration and contacting status) on each feature should remain the same as well. In order to ensure that reaction conditions were consistent on each feature, two measures were implemented in oligonucleotide array fabrication. Firstly, special printing equipment was developed to improve the contact of the glass slide with the microstamp. An adjustable printing head was designed and applied to adjust the parallel status between the glass slide and the PDMS stamps (figure 6). Secondly, a solvent, adiponitrile, with higher boiling point was used as partial substitute for acetonitrile (adiponitrile/acetonitrile = 2/8 (volume)) in the printing coupling. The volatilization of adiponitrile was slow and preserved the consistency of reagents on different features surfaces. Figure 7 shows hybridization fluorescence signals of the same probe 5′-AGG AGG CTA AGT CTC TCA GG-3′with a complementary sequence. The fluorescence intensities of all features on the chip are well proportioned. The fluorescence intensities of synthetic and non-synthetic regions were 6478 and 582. This result indicates that each feature had the same reaction conditions and the same coupling efficiency.

Table 2. 16 spot fluorescence intensity export result of figure 8 pane grid.

Array row	Array column	Spot intensity	Background intensity	Background subtracted	Probe sequence
1	1	7999	696	7073	5′-GGACTCTCTATATCGGAGGA
1	2	3587	327	3260	5′-GGACTCTCTATTTCGGAGGA
1	3	3546	325	3221	5′-GGACTCTCTATTTCGGAGGA
1	4	9253	481	8754	5′-GGACTCTCTAAATCGGAGGA
2	1	13864	626	13058	5′-GGACTCTCTGAATCGGAGGA
2	2	7299	589	6710	5′-GGACTCTCTATATCGGAGGA
2	3	3823	295	3528	5′-GGACTCTCTATTTCGGAGGA
2	4	5934	415	5518	5′-GGACTCTCTATATCGGAGGA
3	1	15943	775	14375	5′-GGACTCTCTGAATCGGAGGA
3	2	7829	346	7464	5′-GGACTCTCTATATCGGAGGA
3	3	6066	643	5423	5′-GGACTCTCTATATCGGAGGA
3	4	9687	695	8974	5′-GGACTCTCTAAATCGGAGGA
4	1	15943	403	15540	5′-GGACTCTCTGAATCGGAGGA
4	2	11421	634	10187	5′-GGACTCTCTAAATCGGAGGA
4	3	9802	739	9036	5′-GGACTCTCTAAATCGGAGGA
4	4	12727	405	12322	5′-GGACTCTCTGAATCGGAGGA

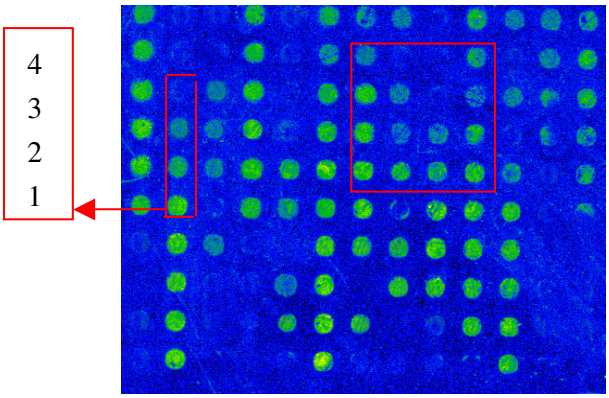


Figure 8. The scanned fluorescent image of hybridization of matched and mismatched oligonucleotide sequences synthesized without capping (1: correctly matched sequence; 2: one base mismatched in the middle of sequence; 3: two bases mismatched in the middle; 4: three bases mismatched in the middle).

The hybridization results of four different probes are shown in figure 8 and in table 2. Although the same probe fluorescence intensity shows different values, perfect match and mismatch had marked difference. The ratio of fluorescence intensity of the matched sequence to that of one middle location base mismatched, that of two middle bases mismatched, and that of three middle bases mismatched is about 1:0.68:0.36:0.19. These results indicate that the oligonucleotide array fabricated through soft lithograph could be used to rapidly screen single-nucleotide polymorphisms. At the same time, the fluorescence intensity of rehybridized oligonucleotide arrays was approximately equal to that of the fresh chip even if the chip was immersed in boiling water for ten hours and then rehybridized many times. This means that oligonucleotide arrays synthesized on the glass substrate are steady and can be reused.

4. Conclusion

This paper describes an approach to preparing oligonucleotide arrays by *in situ* synthesis that used modified PDMS stamps on which surface-spread reactants contacted with the glass slide. This method showed high coupling efficiency and had advantages such as simple and reliable operation.

Acknowledgments

This research is supported by the Chinese 863 High-Tech Project, 973 National Key Fundamental Research Project, Natural Science Foundation of China, and the high-technology programme of Jiangsu province, China.

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