ULTRA-MULTIPLEXED BEADS SYSTEM WITH IN SITU DNA PROBE SYNTHESIS

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ABSTRACT

New suspension array system was studied in terms of multiplicity, productivity and costs. Microbeads contain dot-codes for high multiplicity and are designed for image processing at high speeds. The encoded beads were fabricated on a wafer from SU-8 by photolithography. Due to optimizing a sacrificial layer under the beads, corresponding DNA probes were synthesized *in situ* on the wafer by standard phosphoramidite chemistry, facilitating the preparation of beads with a wide variety of customized probes at a reasonable cost. In addition the dedicated beads holder we developed enables efficient observation of beads for the following evaluation by image processing.

KEYWORDS: Suspension array, Microfabrication, Encoded particles, DNA, Multiplexing

INTRODUCTION AND THEORY

In the fields of drug discovery or genetic analysis, efficient multiplexed assay technologies are required, which allow for the simultaneous assay of many items, but they necessitate an encoding scheme for identification of molecular items at issue. The most popular technology is the planar microarrays, such as DNA and protein chips, which use positional encoding. But many groups also study suspension array technologies using encoded microparticles because they offer several advantages over microarrays, including solution-like kinetics, ease of assay modification, and small sample volume. However, some of these suspension array approaches contain complicated or expensive processes for encoded particle fabrication, functionalization, or detection and decoding of active particles, and often yield a limited number of codes.

Recently Doyle's group presented a new method using continuous-flow lithography that combines particle synthesis, encoding, and incorporation of DNA probes into a single process [1]. A dot-coding scheme was used to generate particles bearing over a million codes, but the particles were relatively large, which limits the detection throughput during flow-through. It will not take advantage of the dot-coding multiplicity enough. And Luminex Corporation developed a suspension array technology based on flow cytometry using 5.6-μm polystyrene microspheres with color-codes [2]. They launched the latest multiplexing system called FLEXMAP 3D, which increased multiplexing up to 500 items per well. But in both cases, DNA probes have to be attached with their corresponding particles individually, which increases costs and time as the number of kinds of probes increases. For more practical applications, it is important to prepare thousands or tens of thousands kinds of encoded particles or beads bearing customized DNA probes at a reasonable cost.

In this report, we study a new suspension array system to overcome those multiplexing limitations. Microbeads fabricated by photolithography have dot-codes that are suitable for high multiplicity and productivity. The microbeads that have 16 dots in their inner area can generate more than 65,000 codes. The dot-alignment and the round shape of beads are designed to be recognized by image processing at high speeds (Figure 1). Although DNA probes can be attached to the beads individually, numerous DNA probes bearing a linker modifier need to be prepared in advance and reacted with the corresponding beads through a time-consuming process. However, due to optimizing sacrificial layer under the beads depending on chemicals used in the processes, DNA probes can also be synthesized *in situ* on wafers from phosphoramidite monomers (Figure 1). It will save costs and time as the number of kinds of probes increases.

After assays with the beads, they are collected in the dedicated beads holder for efficient observation, which has pillar structures within a flow channel. The channel thickness is slightly thicker than that of the beads, and the beads are filtrated or rearranged at the pillars under reduced pressure for bright field and fluorescence imaging. The former images are converted to binary images, and a bead-sized circle is scanned onto them to distinguish between and extract beads for decode. The latter images are used to evaluate the fluorescence of the extracted beads. Both the design of beads and a developed algorithm reduce processing task for recognition, decode and fluorescent evaluation of beads.

EXPERIMENTAL

At first, 16 kinds of encoded microbeads were fabricated from SU-8 photoresist on a Si wafer with an appropriate sacrificial layer by photolithography. The beads on the wafer were activated by O_2 plasma treatment and derivatized with 3-glycidoxypropyltrimethoxysilane by vapor deposition. The wafer was immersed in tetraethylene glycol containing a catalytic amount of concentrated sulfuric acid to yield an alkyl hydroxyl derivatized surface. To each encoded beads, corresponding 16 kinds of DNA probes were synthesized on it by standard phosphoramidite chemistry, or the each phosphoramidite monomer solution was spotted onto each area of the encoded beads in a stepwise manner. The synthesized DNA probes had the length of 20mer and random sequences. After deprotection of the synthesized DNA, the beads were removed from the wafer by dissolving the sacrificial layer and collected by filtration. Then the beads were washed on the filter and dispersed in the following buffer solution.

Hybridizations were conducted using Cy3 or Alexa488 labeled targets complementary to two of the 16 DNA probes. The targets were mixed with the collected beads in a microtube and diluted to a final concentration of 2 μM in 35 mM

Tris buffer (pH 8.0), 2 M TMAC, 3 mM EDTA, and 0.05% Sarkosyl (references cited in [1] and [2]). The beads were incubated for 20 minutes at 50℃ after denaturation, and moved from the tube to the dedicated beads holder for efficient observation (Figure 2). Pillar structures and side walls in a flow channel of the holder were fabricated on a glass slide from the dry film resist, which thickness is slightly thicker than that of the beads. Onto the structures was mounted the coverslip that consists of an acrylic resin and has a set of inlet and outlet port. Under reduced pressure, the beads were collected and washed at the pillars inside the holder, then bright field and fluorescence images were obtained by a microscope equipped with a cooled CCD camera.

The images were processed using the software we developed. The bright field images were converted to binary images, and a bead-sized circle was scanned onto them to extract beads for decode. Markers for bead orientation around a code were recognized to distinguish two sides of the extracted bead and to decide a rotation angle of it. And a code inside the bead was decoded by coordinate matching of the dot-pattern. Then fluorescence images were used to evaluate fluorescent intensities of the extracted beads.

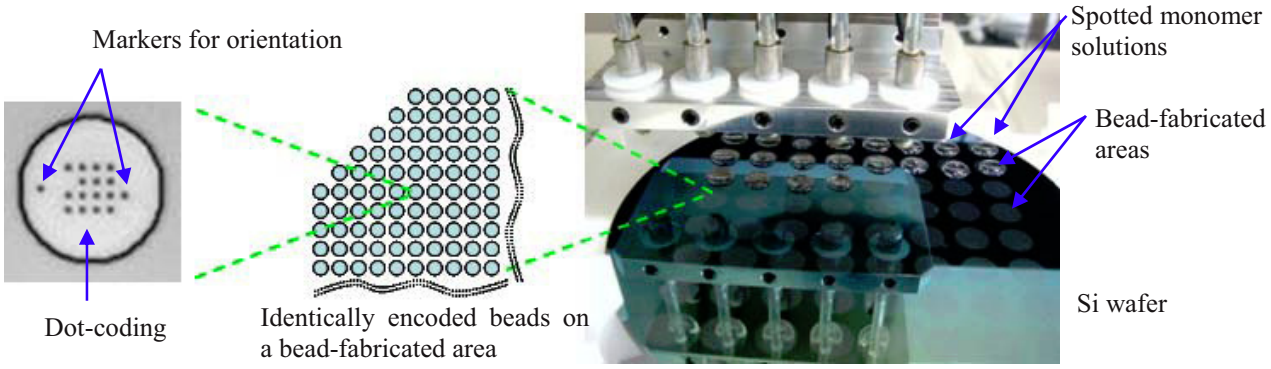

Figure 1: A bright field image of our encoded microbeads used in this study (left), and a part of the DNA synthesizer we developed (right). The microbeads were designed with dot-coding scheme along with their orientation markers. In order to synthesize DNA probes to the beads on a wafer, each DNA monomer solution is spotted to the each bead-fabricated area that contains thousands of identically encoded beads. The adjacent areas are differently encoded.

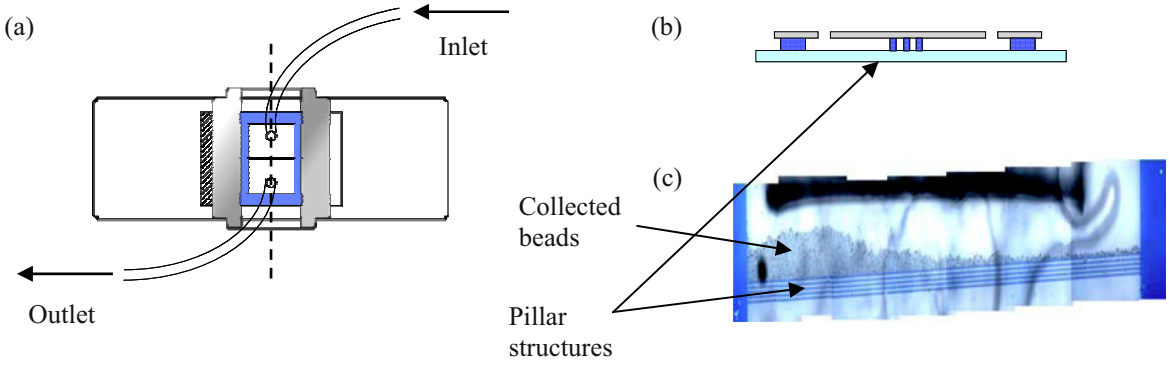

Figure 2: (a) Schematic illustration of the dedicated beads holder that has pillar structures within a flow channel to collect beads efficiently for the following observation. (b) A cross-sectional diagram of the holder at the broken line shown in (a). (c) A magnified view of the collected beads at pillar structures.

RESULTS AND DISCUSSION

Microbeads, measuring about 40 μm in diameter and about 8 μm in thickness, were fabricated from SU-8. The beads had 16 coding dots in their inner area, which can produce more than 65,000 codes, along with another 2 dots outside the area to distinguish their orientation (Figure 1). To demonstrate a multiplexed assay, 16 kinds of encoded microbeads were fabricated on a Si wafer by photolithography and 16 kinds of DNA probes were synthesized *in situ* on the wafer from phosphoramidite monomers. To each area of the encoded beads on it, the corresponding DNA probes were synthesized in a stepwise manner by automated synthesizer we developed. This method will enable the preparation of beads with a wide variety of custom-made DNA probes at a reasonable cost. In this method, a sacrificial layer must have stability for chemicals used in the synthesis and alkaline deprotection, and have enough adhesive ability to the beads not to fall away during those fabrication processes. But when necessary, it can be dissolved to remove the beads from the wafer. Besides these features, the sacrificial layer in this study works as a barrier to avoid monomer solutions spotted adjacently from mixing each other. So it is important to optimize the sacrificial layer carefully according to the processes.

The synthesized DNA probes were deprotected, and the sacrificial layer under the beads was dissolved to collect the beads from the wafer. The beads were dispersed in the buffer solution and incubated with two targets bearing Cy3 or Alexa488 in a microtube. Then the beads were transferred to the developed beads holder for efficient observation, which had pillar structures in a flow channel (Figure 2). The thickness of the channel is about 10 μm, which is slightly thicker than that of the beads, so that the collected beads do not lap over with each other. However, considering the aspect ratio of the beads, this flow channel is thought to be insufficient for smooth and efficient collection of beads. For this reason, it will be required to lower the aspect ratio of them, or to make the channel thickness up to nearly twice as thick as that of the beads.

After washing of the collected beads, the bright field and fluorescence images were taken, which only exhibited fluorescence when each target was hybridized correctly (Figure 3). But the concentration of targets used in this hybridization assay is relatively high, because of low probe density on the beads. Of course it is required to improve the probe density, but also exposure times for fluorescence imaging can be changed appropriately according to the fluorescent intensity. The obtained images were processed to decode and evaluate the beads by the software we developed. The recognition or extract ratio by the software is about 90% because of image-derived problems including dusts or blots on beads, different contrasts among beads, or out of focus. But the decode accuracy for the extracted beads is over 99%. If images are taken at higher magnification, the total accuracy will be improved. However, it means that the number of images needed for the same region increases and that detection throughput decreases. Besides remediation of the software and images, magnification will be balanced with detection throughput. From hybridization to the end of evaluation, it took less than one hour by a manual experiment. Eventually we aim to develop an automated evaluation system that can collect assayed beads to dedicated beads holders and can evaluate their fluorescence by image processing.

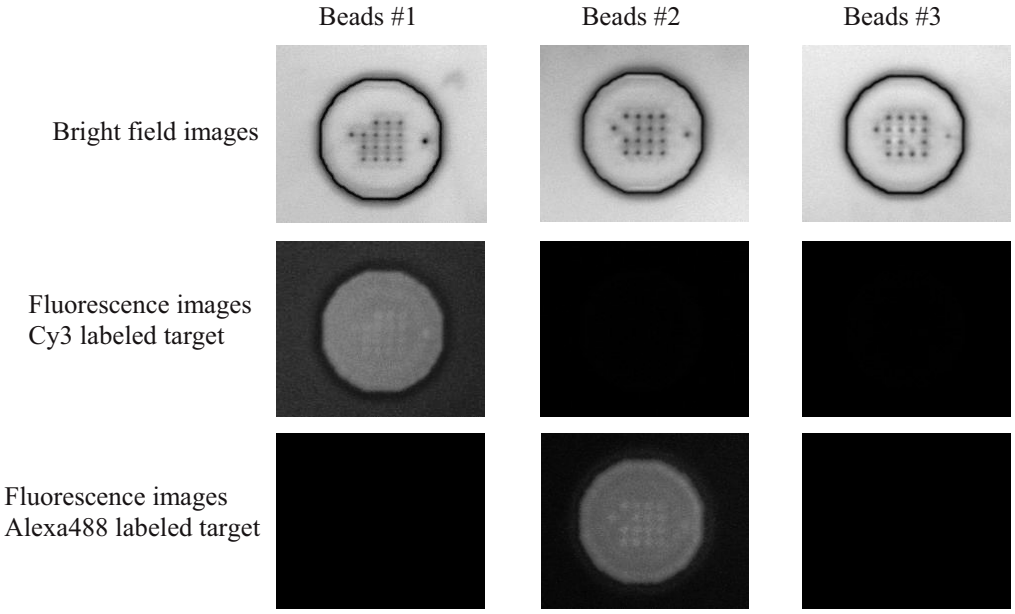

Figure 3: A result of Multiplexed analysis using 16 encoded microbeads. Examples of bright field and fluorescence images of the beads after hybridization with two targets bearing Cy3 or Alexa488.

CONCLUSION

Dot-coded microbeads were fabricated from SU-8 on a Si wafer having the optimized sacrificial layer by photolithography. To each encoded beads on it, the corresponding DNA probes were synthesized *in situ* from phosphoramidite monomers by our developed synthesizer. After hybridization with the beads, they were collected in the dedicated beads holder for efficient observation, which had pillar structures within a flow channel. The bright field and fluorescence images were taken and processed to decode and evaluate the beads by the software we developed. For practical applications, it is important to produce thousands or tens of thousands of different types of encoded beads containing customized probes at a reasonable cost. The method described above will enable the preparation of the beads and allow for a new suspension array system with adequate multiplicity and detection throughput for practical use.

REFERENCES

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