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Microstructured Arrays with Pre-synthesized Capture Probes for DNA Detection Based on Metal Nanoparticles and Silver Enhancement

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DNA microarrays exhibiting highly defined squared spot geometry and increased homogeneity in capture DNA distribution were prepared by a combination of microfabrication and DNA spotting. The microfabricated substrates were tested using metal nanoparticles as markers. The optical absorption signal is improved by a silver enhancement step. Experiments demonstrated the specificity and the concentration-dependency of the signal.

KEY WORDS: DNA microarray; gold nanoparticles; micro structures.

INTRODUCTION

Affinity DNA chip detection requires solid substrates equipped with immobilized capture DNA molecules. These capture molecules are complementary to the DNA of interest (target DNA), so that the target DNA will be captured from the solution. Then, the presence of target DNA on the substrate surface is sensed either by physical effects or by labels. Labeling by fluorescent dyes is the standard technique for DNA chip detection today. It provides a highly sensitive and rather well established detection method, with the ability to use differently colored dyes in one experiment to compare the signal from two states. This one experiment can address up to several thousands of individual DNA sequences, such as the complete gene set of a given organism. For a quantification of the signal as required for most applications, the distribution and the intensity of the dyes are determined for each spot. Because the intensity is influenced by the chemical environment of the dye molecule, a chip-to-chip comparison is hampered. Moreover, irreversible destruction of dye molecules by light-induced "bleaching" effects complicates the needed calibration. Another problem is the different vulnerability of the dyes to other influences, such as e.g. low ozone concentrations in the laboratory air during stringency washes [1].

Gold nanoparticles promise to overcome the problems of signal instability. Their signal does not depend on the chemical environment, and it is long-term stable (for a review see [2]). There is easy access to individual marker particles by AFM measurements, providing in principle single molecule sensitivity [3]. For routine applications, optical readout is preferred due to ease of use and the fast readout of even large numbers of spots [4]. A silver enhancement procedure known from electron and optical microscopy [5] allows for simple detection by office desktop scanners [6] or even single nanoparticle sensitivity [7].

Quantification of the experiments requires the visualization of the spots with their respective intensity values prior to image processing (for a review see [8]). In order to automate the process, an image-processing program identifies and isolates the spots. After a signal-based background correction, the number of bound target molecules is estimated, and this value is used for an estimation of the concentration in the solution. The isolation of the spot

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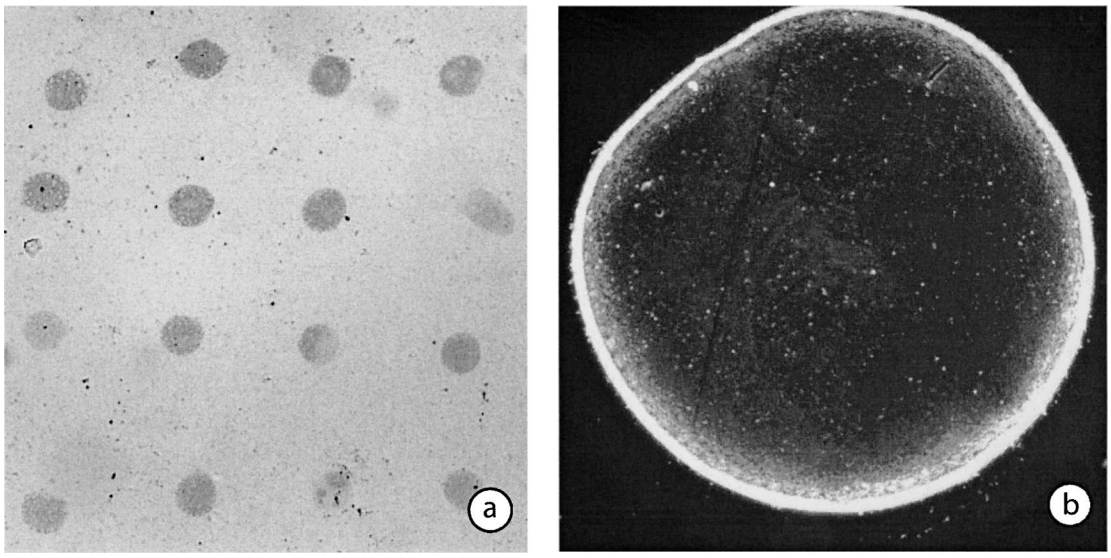

Fig. 1. Typical problems with standard spotting techniques are irregular positions of the spots (a) and an inhomogeneous, doughnut-shaped distribution of the signal in the spot (b).

area from the background (segmentation) is a key step for a successful quantification, but it is usually hampered by irregular shape and inhomogeneous intensity distribution inside the spots.

An ideal arrangement would be a highly regular array of spots with fixed positions and fixed geometries, and exhibiting a highly homogeneous distribution of capture DNA. The process of assigning coordinates to each of the spots could be easily automated in order to permit high-throughput analysis. Moreover, also the segmentation procedure (the classification of pixels as signal or background) would be straightforward [8].

The irregular shape of the spots in microarrays (Fig. 1) is caused by the applied immobilization technique. In general, two approaches can be differentiated based on the oligonucleotide synthesis: Immobilization of pre-synthesized DNA and on-chip synthesis. In the first case, DNA is prepared by standard techniques with high efficiency and minimal costs. The transfer of the DNA on the chip is usually done by droplet incubation ("spotting"); the droplets are generated using capillary, needle or ink-jet based instrumentation. To enhance binding and achieve a more defined terminal coupling, covalent bonds between a functionalized surface and a complementary functional group on the DNA are used. Typical examples are the use of amino-modified DNA and either epoxy (e.g., 3′-glycidoxypropyl-trimethoxy-silane GOPS silanization, [9]) or amino (e.g., 3′-aminopropyl-trimethoxy-silane APTES silanization using PDC mediated binding, [10]) modified substrates. However, the droplets applied onto the substrate vary considerably in their deviation from the intended position, their dimension (influenced by hydrophobicity of the substrate and the composition of the solution) and their shape (Fig. 1a). Moreover, the droplet boundaries usually exhibit a much higher density of capture molecules, leading to a "doughnut-" appearance (Fig. 1b).

The other approach for DNA chip fabrication is on-chip synthesis. Thereby, the spot locations and dimensions can be highly defined by microfabrication techniques. Another advantage is the much higher integration density compared to standard droplet incubation techniques. Although recent developments in scanning-probe based microarray production also address smaller arrays, the serial character of these techniques does not allow mass production. The applicability of on-chip synthesized DNA chips for nanoparticle-labeled detection was demonstrated [4]. It was shown that even small (lower micrometer range) spots could be detected using standard optical microscopes, and that exposures in the millisecond range were sufficient to visualize whole arrays [11]. The rectangular spots would be advantageous for an automatic image processing. Moreover, due to the immobilization protocol, the DNA distribution is quite homogeneous and not influenced by the shape of the spots. However, these demonstrations exhibited only one kind of DNA in every spot on a given chip. For a real chip, the sequences vary from spot to spot. Such an arrangement requires sophisticated technologies for the local DNA synthesis [12], which are not accessible as in-house technology for research institutions with the need for flexible chip design.

This paper presents a technical development that combines advantages form both sides of immobilization approaches: the flexibility from the spotting technology, and the highly defined geometry from on-chip synthesis. The technology merges microstructured arrays with pre-synthesized capture probes and produces signals with high precision regarding position, shape and homogeneity.

MATERIALS AND METHODS

Preparation of Microstructured Substrates

Microscope slides were cleaned prior to activation in a solution of $HCl:H_2O_2:H_2O$ (1:1:1) for 10 min, a further cleaning step in hot water, and drying. An incubation in 1 mM octadecyltrichlorsilane (OTS) in dried toluene for 4 hr at 40°C was followed by washing and drying. Then the slides were ready for micropatterning using resist and photolithography as described elsewhere [13]. For GOPS silanization, the substrates were put in a 10 mM solution of 3-glycidyloxypropyl-trimethoxysilane (GOPS) in dried toluene and incubated for 6 hr at 70°C. The washing steps were the same as for the octadecyltrichlorsilane silanization.

Capture DNA Immobilization

Droplets of a solution of 50 mM oligonucleotides (30 bases long) in 0.1 M KOH were applied on the slides covering the microstructured spots and incubated for 3 hr at 37°C in a humidity chamber. The slides were then washed in 0.1% Triton X-100 solution to remove unbound probes for 10 min and then blocked with 50 mM ethanolamine solution 0.1% SDS in 0.1 M TRIS. Afterwards the slides were washed with water and dried under a stream of nitrogen.

Preparation of Nanoparticle-Labeled Probe DNA

A procedure modified from the literature [14] was used for the preparation of nanoparticle-conjugated DNA as described elsewhere [3]. Briefly, 3′-alkylthiolated oligonucleotides (BioTeZ, Berlin, Germany) were cleaved from a CPG support and incubated with gold nanoparticles (30 nm, British Biocell, UK) for several hours.

Incubation of Substrates with Probe DNA

A droplet of the nanoparticle-labeled probe DNA was applied to the slide and then covered with a cover slip. Then the probes were incubated for 2 hr at 40°C in a humidity chamber and afterwards washed with 0.1 M sodium phosphate buffer and 0.01 M sodium chloride, followed by drying the samples under a stream of nitrogen.

The influence of the solution concentration of DNA labeled with nanoparticle on the signal intensity of hybridized nanoparticles using a sandwich protocol was investigated. Particle concentrations of 0.25, 0.5, 1.0, and 2.0 OD were applied.

Silver Enhancement

For the silver amplification of the slides, two solutions were used. A commercial solution from British Biocell was applied as droplet on the DNA spot for 10 min. Afterwards, the reaction was stopped by washing the slides with water.

For the other silver enhancing solution, 80 mg of silver acetate were dissolved in 40 mL of water and mixed with a solution of 200 mg of hydroquinone in citrate buffer pH 3.8. The slides were put in this solution for 10 min under exclusion of light. Again, washing the samples with water stopped the reaction.

Optical Readout and Image Processing

For the readout the samples were scanned on a flatbed scanner. The images were stored in TIFF format and processed using the image processing software NIH Image 1.61.

RESULTS AND DISCUSSIONS

Substrate Preparation

The goal was the development of DNA microarrays with highly defined spot geometry, location and homogeneity. These properties of microstructured on-chip synthesis are combined with the ease-of-use of spotted arrays: the capture DNA is immobilized by the end user and not by the chip manufacturer. Therefore, microstructured binding spots of a given geometry were defined on a functionalized surface by standard microfabrication technology including photolithography. The scheme in Fig. 2 describes the process: In the first step, cleaned glass substrates are functionalized with OTS, a silane-based passivating layer that later minimizes non-specific DNA binding in between the spots. Using photo resist and patterned exposure, the designed array geometry (squared spots) is transferred into the resist. After development, the OTS layer is exposed in the spot regions. It is removed by etching prior to removal of the resist. Now, the spots are defined on the glass

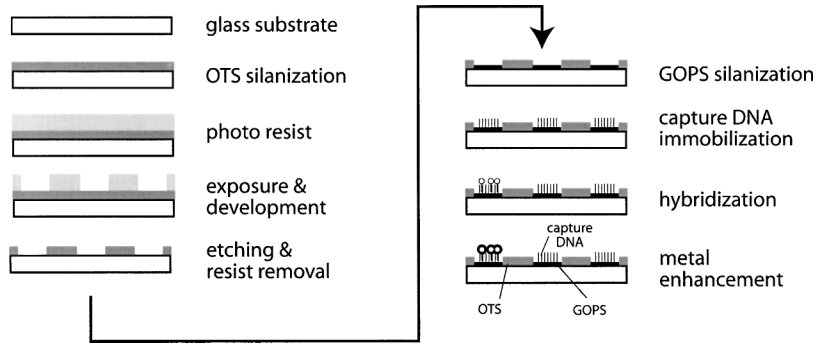

Fig. 2. Scheme of substrate preparation. Left: Microfabrication. Right (upper two): Capture probe immobilization. Right (lower two): DNA hybridization assay.

substrate, surrounded by OTS areas. In order to make the spots accessible for DNA coupling, a functionalization is needed. An established GOPS protocol was applied, resulting in a surface modification of the spot areas with epoxy groups that can covalently couple to amino-modified DNA molecules.

Assay Design

The geometry and lateral arrangement of the spots is shown in the scan of a microarray slide in Fig. 3. Four lanes with each 8 squared spots are defined on the substrate. The slide is shown after the assay that involves the steps given in Fig. 2. The first step in assay preparation includes the immobilization of the required capture probes. Therefore, amino-modified DNA molecules of the chosen sequence are applied to each spot as droplets covering only the desired spot. After incubation and washing, the capture DNA should be covalently coupled to the spots. Now, the substrate is ready for target DNA detection. Thereby, the substrates are incubated with solution containing target DNA that is either directly or indirectly (sandwich assay) labeled with gold nanoparticles. In the presence of complementary capture DNA on the chip, a binding of the particle-labeled DNA due to hybridization will occur. Although this immobilization of gold particles

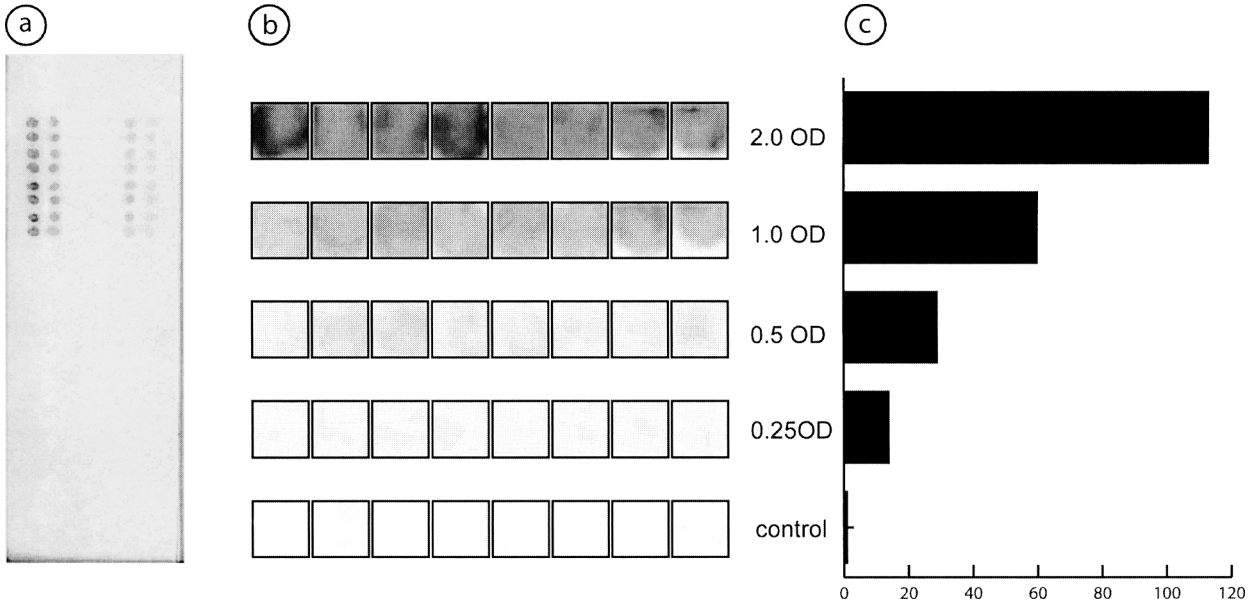

Fig. 3. Quantification of nanoparticle signal. Various concentrations of nanoparticle-labeled DNA (0.25–2.0 OD) were incubated with complementary capture DNA immobilized on the microstructured substrates (slide). After incubation and washing, the coupled nanoparticles were enhanced by specific silver deposition. After visualization (a), the spots were cropped (b) together with a region in-between the spots as negative control (bottom row), and the average density for each row was plotted (c).

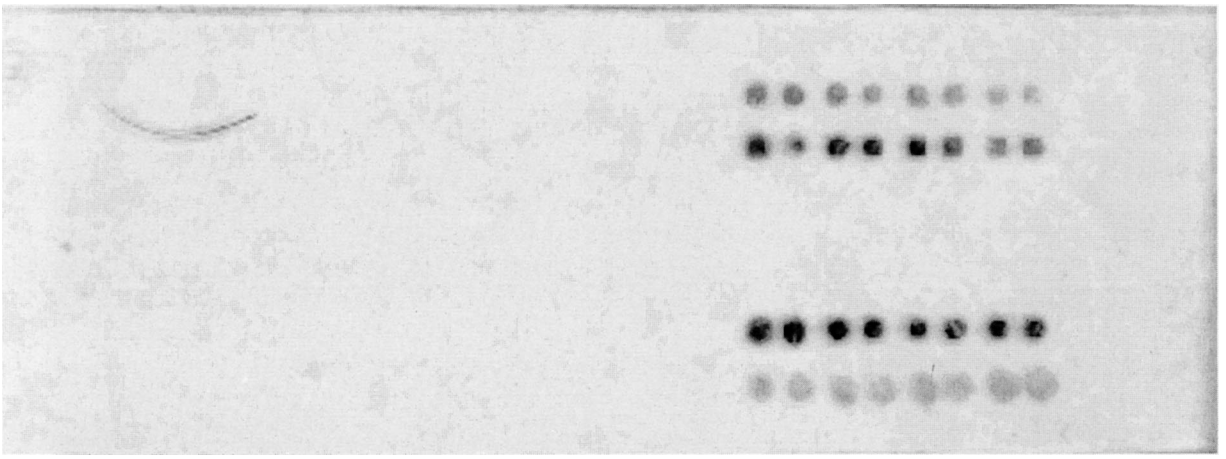

Fig. 4. Specificity of silver-enhanced nanoparticle labeling. The second and third row contain complementary DNA (zero and three mismatches, respectively), the bottom row is a negative control with a non-complementary sequence. The upper row contains an immobilized sequence hybridized with a linker DNA molecule that is complementary to the DNA-nanoparticle complex.

is occasionally detectable by optical means (in the case of high surface densities), the standard protocol includes an additional silver enhancement step. Therefore a silver salt solution is used to deposit specifically silver onto the gold particles. This process results in a growth of the nanoparticles, and thus in a significant increase in optical contrast. As result, the observed absorption correlates with the number (more precisely: the surface density) of immobilized nanoparticles, and therefore with the concentration of target DNA in the solution.

Quantification

The experiment shown in Fig. 3 demonstrates the potential of this method for quantification. Various concentrations of nanoparticle labels were incubated with the capture DNA, and resulted in corresponding signals. In order to evaluate the results, the spots were individually cropped from the image of the processed slide and arranged in rows corresponding to a concentration value (0.25, 0.5, 1.0, 2.0, and background as negative control, Fig. 3b). Then, an integrated density (grey value) was determined for each spot, and an averaged value for each concentration set was plotted in the diagram (Fig. 3c). A clear correlation of particle concentration and signal is observed. A remarkable feature is the low background signal (bottom row) that differs significantly from the measured value for 0.25 OD.

Detection of Specific DNA-DNA Interaction

To demonstrate the developed nanoparticle labeling for the detection of target DNA, experiments were conducted with complementary and noncomplementary capture DNA immobilized on the same substrate (Fig. 4). The second and third rows include complementary sequences with full complement (2nd row) or a three bases mismatch (out of 30, 3rd row). The bottom row contains a non-complementary sequence. The signal is high in the complementary rows, and hardly visible in the non-complementary case (4th row). The upper row contains DNA that requires an additional DNA linker to bind to the

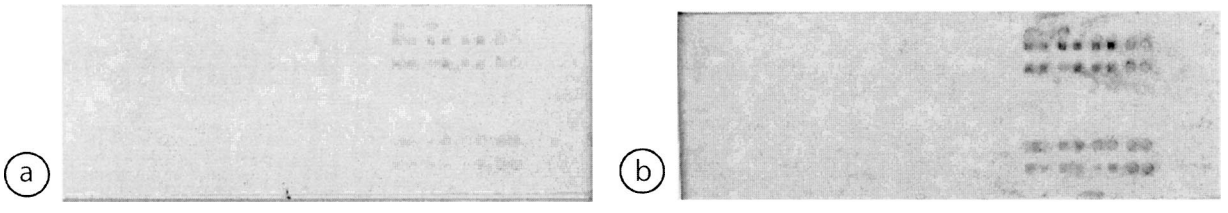

Fig. 5. Signal enhancement by additional silver deposition. a) A substrate was processed using the standard protocol as described in Materials and Methods. b) To further increase weak signals, an additional silver enhancement step is applied. An improvement in signal amplitude is observed.

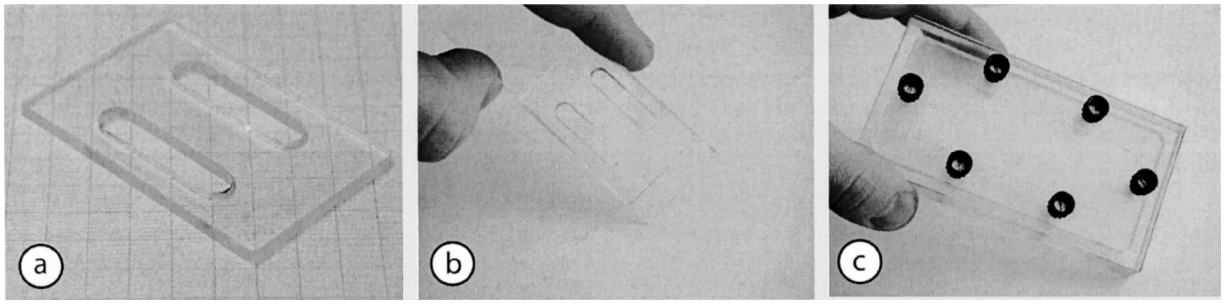

Fig. 6. In order to minimize the required volume for incubation, a hybridization chamber was designed and realized using an elastomeric part (a). The openings in this part fit exactly on the microarray at a surface of a glass substrate (b). After filling with incubation solution, the setup is fixed in the hybridization chamber (c).

DNA-conjugated nanoparticles. By incubation of linker and the respective nanoparticle-DNA complexes, a weak signal is observed (Fig. 4, upper row). The low signal points to a low efficiency of this sandwich-type assay, which requires a protocol optimization for an optimal signal.

Signal Enhancement

The silver enhancement step makes the presented method for DNA detection widely applicable, because it increases sensitivity and allows straightforward detection without the need for spectral filtering. A standard protocol was developed based on the typical concentration range of target DNA in the demonstration systems. However, the nanoparticle-based detection allows for additional enhancement steps. When the dynamic range has to be extended towards higher sensitivity, an additional silver enhancement can be applied (Fig. 5). Therefore, standard protocols can be established and applied routinely, and if needed improved by the addition of extra enhancement steps. Although the background signal is increased, the significant improvement in signal is clearly visible in the shown example (Fig. 5b). In general, the background signal due to non-specific ally deposited silver will be the limiting factor. The experiments pointed to repeated incubation and air-drying as an important factor for increased background. Explanations for this background could include buffer salt or other amorphous residues on the surface, or nucleation inside the silver solution after extended incubation.

Hybridization Chamber

The newly developed system for microstructured microarrays is only applicable when the required standard procedures, such as hybridization, washing, silver enhancement etc. can be easily realized. An important factor is the hybridization stage, with special temperature requirements and the need for decreased sample volume. The latter point is addressed by the development of a hybridization chamber that allows hybridization with total volumes below 200 μL. The inner chamber is defined by a part made of the elastomer poly-dimethyl-siloxane (PDMS), known from micro contact printing (Fig. 6a). This part is connected to the glass substrate (Fig. 6b), and an external hybridization chamber (Fig. 6c) holds the whole construction.

CONCLUSION

The presented approach combines the highly defined geometry of microstructured DNA spots with the ease of use of spotted DNA arrays. It provides a foundation for a highly automated image processing based on fixed positions of square shaped DNA spots. By separating the micro patterning steps from the DNA immobilization, the end-user decides the actual composition of the DNA array. Moreover, a defined spot geometry is provided for work with pre-synthesized capture DNA using standard protocols.

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