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Colorimetric Silver Detection of DNA Microarrays

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Development of microarrays has revolutionized gene expression analysis and molecular diagnosis through miniaturization and the multiparametric features. Critical factors affecting detection efficiency of targets hybridization on microarray are the design of capture probes, the way they are attached to the support, and the sensitivity of the detection method. Microarrays are currently detected in fluorescence using a sophisticated confocal laser-based scanner. In this work, we present a new colorimetric detection method which is intented to make the use of microarray a powerful procedure and a low-cost tool in research and clinical settings. The signal generated with this method results from the precipitation of silver onto nanogold particles bound to streptavidin, the latter being used for detecting biotinylated DNA. This colorimetric method has been compared to the Cy-3 fluorescence method. The detection limit of both methods was equivalent and corresponds to 1 amol of biotinylated DNA attached on an array. Scanning and data analysis of the array were obtained with a colorimetric-based workstation.

Microarrays represent one of the main new breakthroughs in molecular analysis through miniaturization of the assay and the ability to permit the monitoring a large number of genes simultaneously (1, 2).

Despite other alternatives like the electronic support, the two main DNA array supports are the nylon filters and the glass slides.

High-density filter arrays are easily accessible to the scientific community due to their compatibility with commonly used hybridization methods and equipment. Their major drawback is the use of radioactive DNA labeling (3).

Glass slides are now becoming the standard support for microarrays mostly because they are compatible with fluorescence labeling. Target DNA sample is directly labeled either by incorporation of fluorophore-labeled nucleotides or by using fluororophore-labeled streptavidin which binds to biotinylated nucleotides (1, 4). Since several fluorochromes are available, simultaneous and distinct analyses of samples and controls can be performed. Two-color analysis allows competitive hybridization between a sample target DNA and a reference standard DNA on the same array (5). The analysis is performed by specific excitation of each fluorophore by the appropriate laser wavelength. This working protocol reduces experimental analytical variations of the analysis but introduces bias because efficiency of fluorescent emission is not equivalent for both fluorophores. Although such method perfectly suits the requirements for gene expression analysis, the cost of the equipment restricts its use to the research field and makes it unsuitable for the reading of microarray in a wide clinical and daily-based practice.

Accordingly, alternative methods for the reading of hybridization results on microarray have been proposed. Chemiluminescent detection has been proposed for filter arrays (6). Sensitivity seems to equivalent to fluorescence and radioactive detection but the spatial analysis is limited by the diffusion of light emitted on X-ray photographic film. Colorimetric detection appears to be another choice. Chen and colleagues (7) proposed a two-color labeling using peroxidase and alkaline phosphatase detection. The method is however limited by its lower sensitivity.

In this work, we reviewed many different colorimetric detection methods and found that the photographic-based silver precipitation gave unexpected sensitive detection of DNA on microarray compared to other precipitates.

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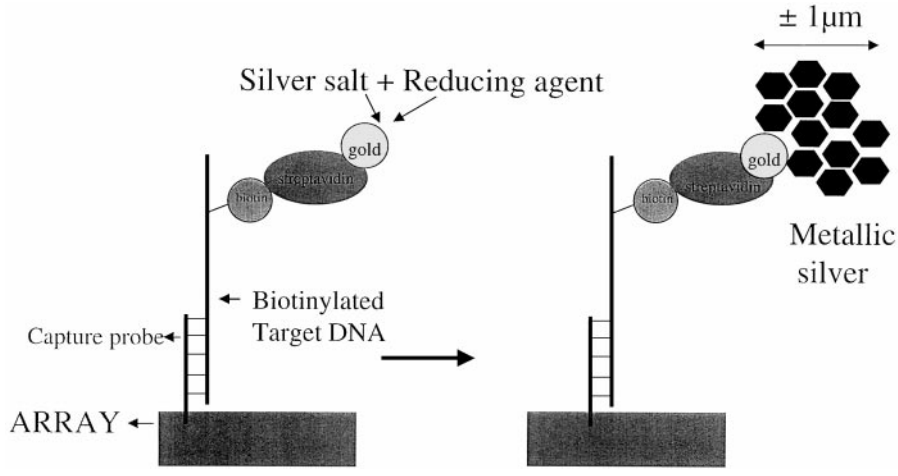

FIG. 1. Schematic representation of the colorimetric silver staining detection of hybridized target DNA on a microarray. The detection system is based on the use of streptavidin–nanogold particles and silver precipitation.

Immunogold staining has been used over the past 20 years in order to detect tissue antigens (8, 9). It is based on the use of gold nanoparticles coated with antibodies. The colloidal gold particles are electron dense and they allow high resolution for the localization of the antigen with the electron microscope.

To enhance the detection signal of gold particles, an additional silver staining enhancement can be used (10–12).

This method has several advantages. The metallic deposition is an autocatalytic process whose amplification power can exceed that for catalytic deposition of chromogenes by enzyme-labeled reagents. Second, silver deposit strongly reflects light in the visible spectrum, a feature allowing the use or development of light reflection-based readers.

Recently, a similar method of silver precipitation based on the use of DNA-gold particles has been described (13). The use of DNA probe bound to colloidal gold particles is followed by silver reduction as assessed here.

MATERIALS AND METHODS

Amplification of Cytomegalovirus DNA

Human cytomegalovirus DNA was used as target for the optimization of the detection. Target DNA and capture probes were synthesized by PCR within exon 4 of the major immediate early gene of human cytomegalovirus as described by Zammatteo *et al.* (14).

Two primers, MIE-4 and MIE-5, were used to amplify the target amplicon of cytomegalovirus (14). For PCR amplification, 1 ng of cytomegalovirus template was supplemented with PCR buffer (0.075 M Tris, pH 9, 50 mM KCl, 2 mM $MgCl_2$, 20 mM $(NH4)_2SO_4$) containing 50 μM dATP, dCTP, and dGTP; 25 μM dTTP; and biotin-11–dUTP (Roche, Indianapolis, IN), 0.1 μM each primer, and 2.5 units of DNA polymerase (Biotools, Madrid, Spain). Amplification was performed in a DNA PE 9600 thermocycler (Perkin–Elmer, Foster City, CA). An initial denaturation step (94°C for 3 min) was followed by 40 cycles (94°C for 30 s, 65°C for 30 s, and 72°C for 30 s) and a final extension step at 72°C for 7 min.

The capture probes of cytomegalovirus were amplified using the same protocol as above except that 5′ aminated primer MIE-4 was substituted to MIE-4 and that 50 μM dTTP was substituted to the mix dTTP and biotin–dUTP.

Amplification of femA Gene from Staphylococcus

The use of *femA* gene for specific identification of the *Staphylococci* based on consensus and species-specific sequences was proposed by Vannuffel *et al.* (15). Accordingly, *femA* gene from *S. epidermidis* has been used in this work. Amplification was based on the use of degenerated primers, consensus for the genus *Staphylococcus:* APcons3-1 (5′-TAAYAAARTCACCAACATAYTC-3′)–APcons3-2 (5′-TYMGNTCATTTATGGAAGATAC-3′) (where Y is C or T, R is A or G, M is A or C, and N is A, G, C or T) (Eurogentec, Seraing, Belgium) as described by Hamels *et al.* (16). The amplification of the *femA* genetic marker sequence was performed in a final volume of 50 μl containing 2.5 mM $MgCl_2$, 75 mM Tris–HCl, pH 9.0; 50 mM KCl; 20 mM $(NH_4)_2SO_4$; 0.5 μM each degenerated primer; 200 μM dATP, dCTP, and dGTP; 150 μM dTTP; 50 μM biotin-16–dUTP (Roche); 0.5 U of uracil–DNA–glycosylase (Roche); 1.25 U of *Taq* DNA polymerase (Biotools); 1 ng of *femA* DNA template. The reagents were first incubated at 94°C for 5 min and then cycled 40 times in a

DNA PE 9600 thermocycler (Perkin–Elmer) using the following temperatures and cycle times: 94°C for 30 s, 49°C for 45 s, 72°C for 30 s. A final extension step of 10 min at 72°C was performed.

Array Hybridization

Glass slides carrying aldehyde groups on their surface (Diaglass, AAT, Namur, Belgium) have been spotted with various aminated capture probes according to the manufacturer. The spots had diameter of 400 μm, the distance between two spots (center to center) being 600 μm.

Hybridization of CMV Target Amplicons

Five microliters of PCR product was added to 60 μl of hybridization solution and this solution was loaded on the array framed by an hybridization chamber (MJ Research Inc., Watertown, MA).

The hybridization solution was made of 2× SSC, 5× Denhardt, pH 7, and contained 100 μg/ml DNA from salmon sperm. After heating at 98°C, the hybridization was performed during 2 h at 65°C.

Hybridization of femA Gene Amplicons

Five microliters of PCR product was denatured with 5 μl of 0.1 N NaOH for 5 min at room temperature then 60 μl of hybridization solution (AAT, Namur, Belgium) was added. This hybridization mix was loaded on the array framed by an hybridization chamber.

We added 10 nM CMV[2] biotinylated DNA as positive control for hybridization. The hybridization chambers were sealed with a plastic coverslip and slides were incubated during 2 h at 53°C.

Detection of Hybridized DNA

For fluorescent detection, slides were washed four times (1 min per wash) with a 10 mM maleate buffer containing 15 mM NaCl and 0.1% Tween, pH 7.5. The slides were incubated for 45 min with streptavidin–Cyanin 3 conjugate diluted 500× (Amersham, Buckinghamshire, UK). The slides were then washed five times in the same washing buffer. Then slides were dried for 5 h and scanned with a fluorescent confocal scanner GMS 418 (Genetic Microsystem, Woburn, MA).

The colorimetric Silver Blue detection kit was purchased from AAT and used according to the manufacturer's instructions. A streptavidin–gold conjugate replaced the streptavidin–cyanin 3. After 45 min of streptavidin–gold incubation, glass slides were washed five times in the washing buffer and then incubated at room temperature for 15 min in 800 μl of the Silver Blue solution. This Silver Blue solution is the combination of 1/1 vol of two solutions, the first one

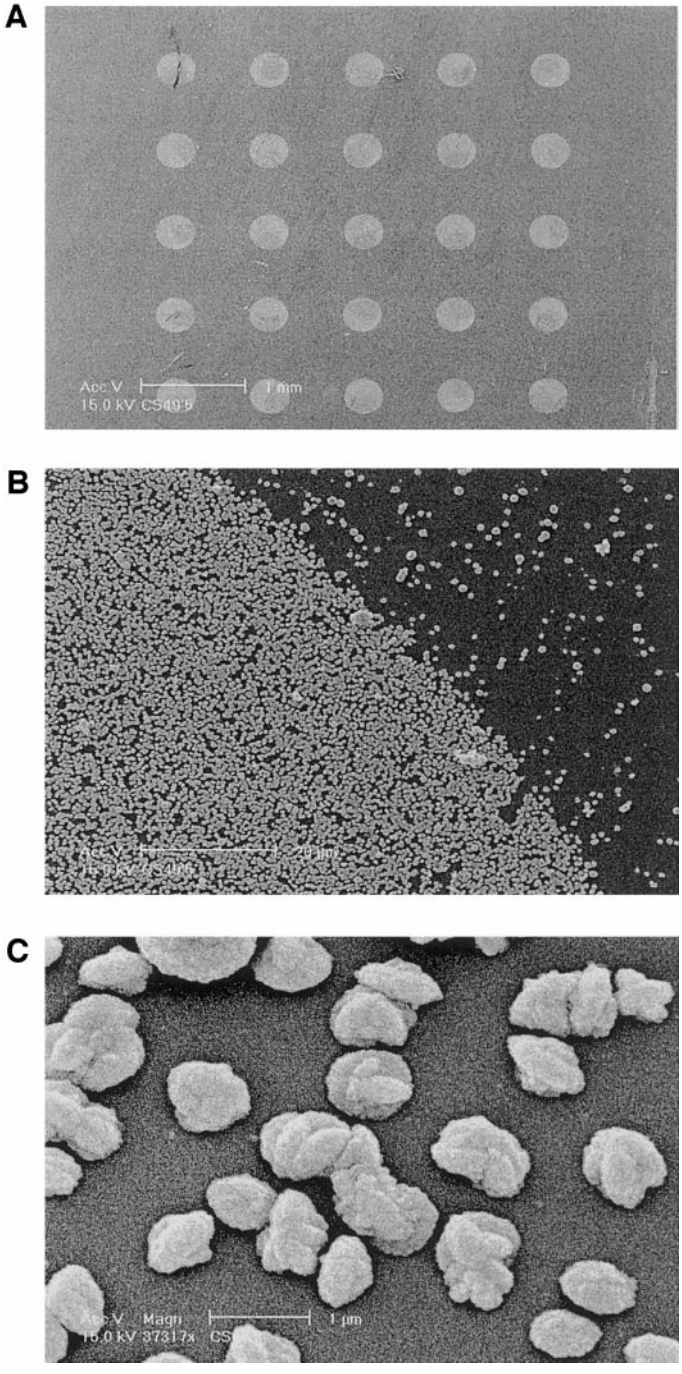

FIG. 2. Electron scanning microscope visualizations at different magnifications of a silver deposit after a 15-min incubation of Silver Blue solution on 10-nm gold particles on glass surface. (A) Visualization of 25 spots on the array. (B) Spatial limit between specific signal and background. (C) Visualization of individual silver precipitates.

[2] Abbreviation used: CMV, cytomegalovirus.

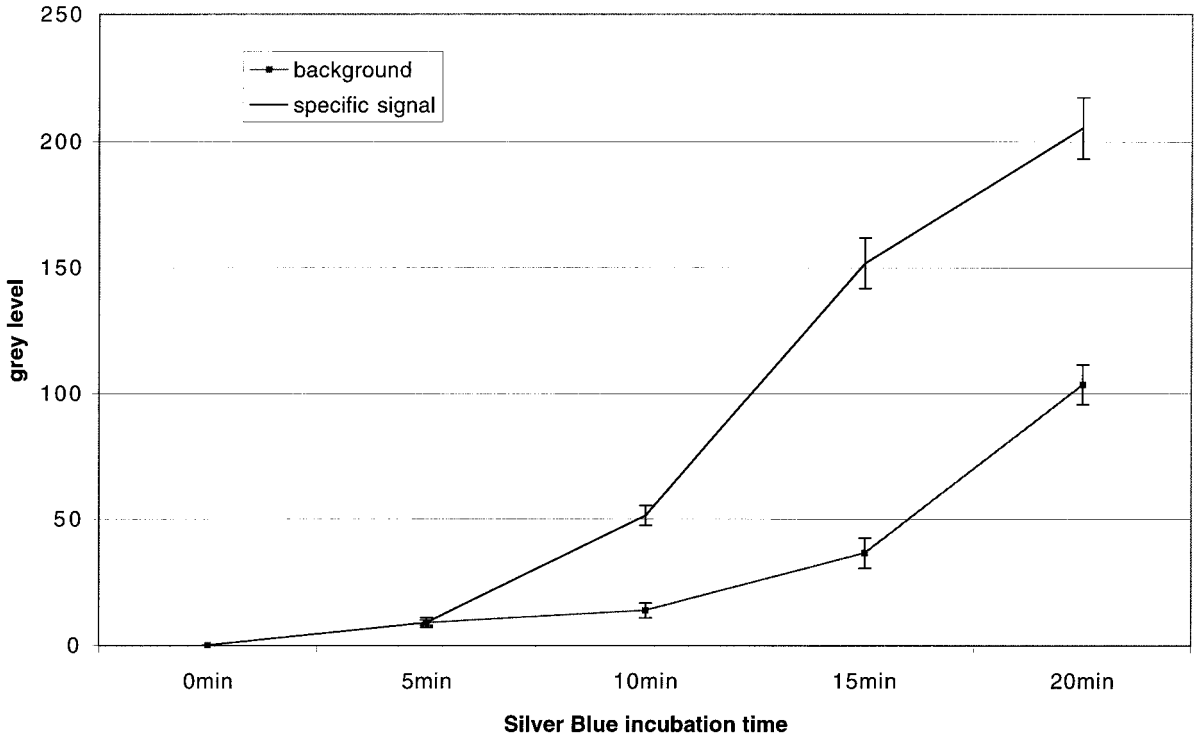

FIG. 3. Kinetics of silver deposition by self-nucleation (background) or on gold particles (specific signal). The biotinylated DNA was spotted at a concentration of 30 nM before reacting with streptavidin–gold conjugate. Results were expressed as the mean of gray level of the pixels inside a spot minus the background calculated as the mean of gray level of pixels around the spot. Results were the mean of four replicates ±2 s.

containing the silver salt ($AgNO_3$) and the second one containing the hydroquinone. Slides were rinsed in water, air-dried 5 h at 37°C, and read with an array colorimetric workstation (AAT). The workstation is a computer tower containing a CCD camera with the appropriate illumination and software for image analysis, quantification of the spots, and statistical treatment of the data.

RESULTS

The principle of the colorimetric method for detecting hybridized DNA on microarray is illustrated in Fig. 1. First, the target DNA is labeled with biotin by the incorporation of biotin–dUTP during amplification. After target hybridization on the array, streptavidin–nanogold particles are added for binding to biotin. Silver labeling relies on the catalytic activity of gold resulting in the reduction of silver ions into metallic silver and a deposition on gold particles. The silver shell around gold particle, in turn, autocatalyzes further silver depositions. At the end of the reaction, silver deposit onto gold particles leads to an increase of particle size going from 10 to about 1000 nm. Silver Blue solution was optimized for applications on microarray. A picture of the silver particles on the array is presented in Fig. 2. The high sensitivity of the method results from an increase of the particles volume of around 1 million times compared to their initial volume. This enhancement allows a direct visual detection of the microarray labeled spots and a straightforward analysis using a colorimetric detector.

Optimization of the Silver Reduction

Silver solutions are sensitive to UV or sunlight and spontaneous conversion of silver solution into metallic grains can occur, leading to nonspecific silver deposition. To limit this drawback, at least two requirements have to be met: first the microarray handling has to be as clean as possible and the silver precipitation reaction must be controlled. Several parameters influence the rate of the reaction including temperature, pH, the silver salt type and concentration, and reducing agent concentration. The composition of the Silver Blue solution has been optimized in order to obtain within one incubation the largest silver precipitation on the gold particles with the minimum of nonspecific deposit. However, the background increases with incubation time as illustrated in Fig. 3. The highest signal to noise ratio was obtained after 15 min of incubation. These conditions were used in further experiments.

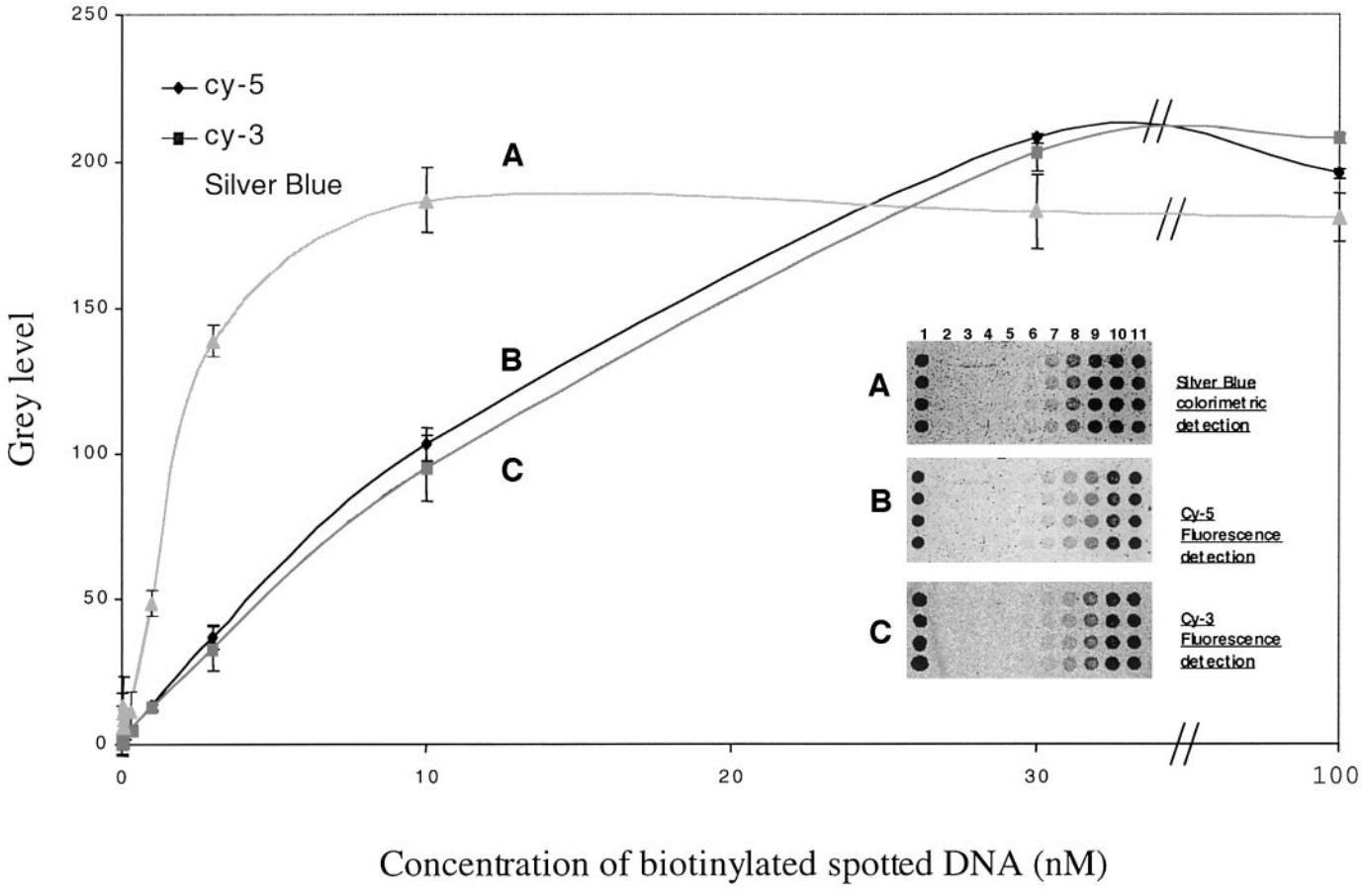

FIG. 4. Comparison of Silver Blue detection (A), fluorescent Cy-5 detection (B), and fluorescent Cy-3 detection (C) of microarray spotted with increasing concentrations of 257-bp CMV DNA 5′ aminated and multibiotinylated. The four rows of the arrays were replicates. The following DNA concentrations were spotted: 100 nM on line 1, 0.01 nM on line 2, 0.03 nM on line 3, 0.1 nM on line 4, 0.3 nM on line 5, 1 nM on line 6, 3 nM on line 7, 10 nM on line 8, 30 nM on line 10, and 100 nM on line 11. (Inset) Quantification of raw data presented in main figure. Results are expressed as the mean of gray level of the pixels inside a spot minus the background calculated as the mean of gray level of pixels around the spot. Results are the mean of four replicates ±2 s.

Revelation Results

A comparison of sensitivity between fluorescence and Silver Blue detection was performed on arrays spotted with various concentrations of CMV-biotinylated DNA. The fixed biotinylated DNA concentrations ranged from 0.01 to 100 nM. Each was made in quadruplicate (Fig. 4). This array was detected with both detection methods.

Results show that in fluorescence as well as in silver detection, gray levels of the spots were well proportional to the concentration of spotted capture probes. The sensitivity of both detection systems was identical with a minimum detection when spotting 1 nM biotinylated DNA (Fig. 4, lane 6). The results obtained with 0.3 nM biotinylated DNA detection were not significatively different from background.

Since the volume of capture probe dispended per spot was estimated to be 1 nl, and assuming that 100% of capture probes were fixed on the array, we calculated that silver detection and Cy-3 and Cy-5 fluorescence allow the detection of 1 amol of multibiotinylated DNA per spot.

Hybridization Results

We then determined the sensitivity of target DNA detection after hybridization on microarray using both detection systems. Unlabeled CMV capture probes were first spotted at a concentration of 150 nM on the array. Various controls were included on the array. Biotinylated DNA capture probes were used as positive control of fixation to indicate the spotting reliability. The negative hybridization controls were capture probes not complementary to target DNA. Different concentrations of biotinylated CMV target DNA ranging from 0.1 to 100 fm in 65 μl were incubated on separate arrays. Silver Blue detection was again com-

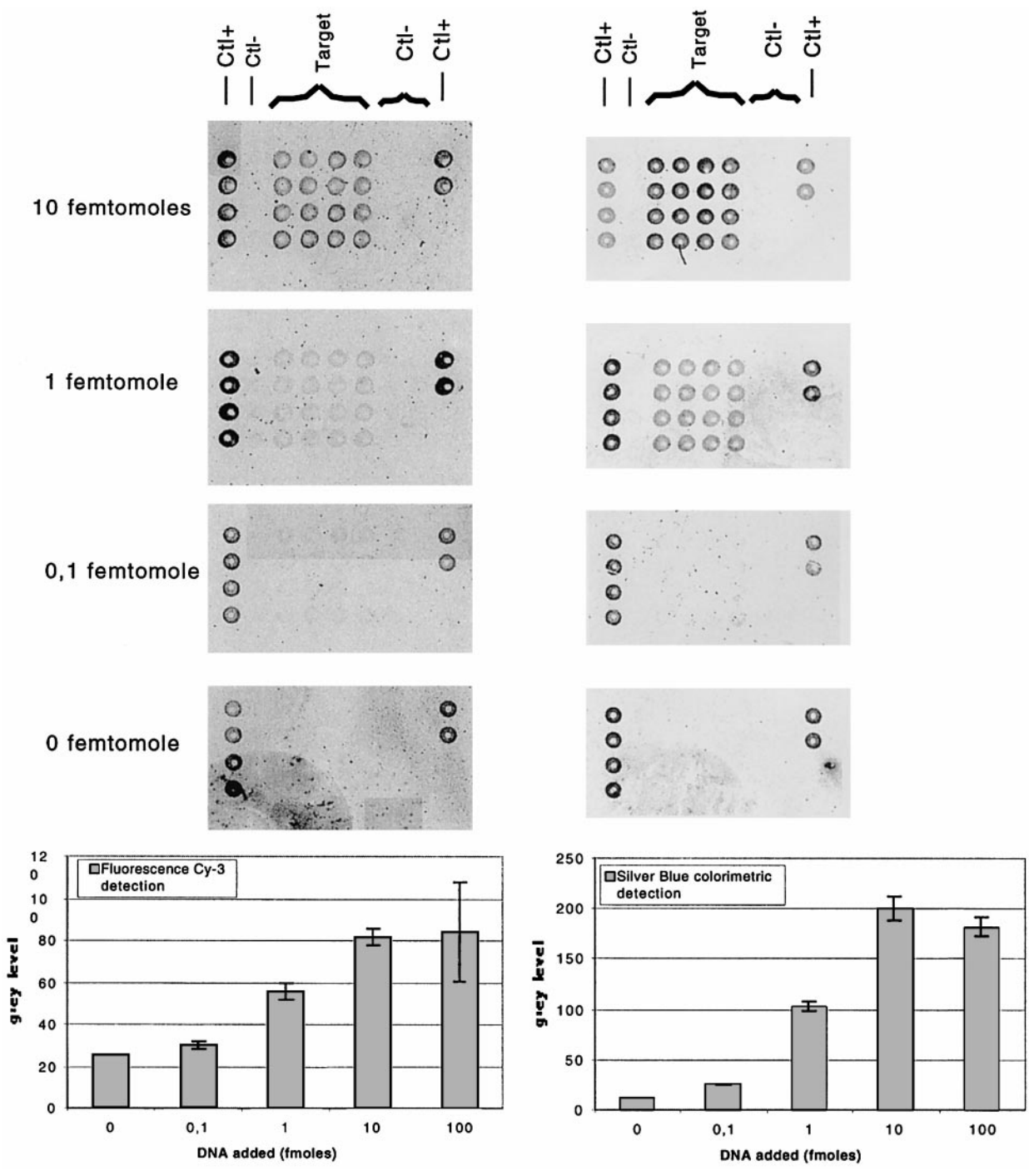

FIG. 5. (Top) Visualization of hybridizations of different concentrations of multibiotinylated HCMV target DNA on the microarrays. Comparison between fluorescent Cy-3 detection (left) and Silver Blue detection (right) methods. The four rows of the array were replicates. The microarray contained the following capture probes: columns 1 and 9, spotted with fixation control; columns 2, 7, and 8, spotted with negative control of hybridization; columns 3–6, spotted with HCMV-specific capture probe. (Bottom) Quantification of raw data presented in top. Comparison between fluorescent Cy-3 detection and Silver Blue detection method. Results are expressed as described in the legend to Fig. 4, inset. Results are the means of 16 replicates ±2 s.

pared to Cy-3 fluorescence detection. The results presented in Fig. 5 show that as few as 0.1 fmol of target DNA amplicons could be detected on the arrays with both detection methods. The detection signal increased steadily between 0.1 and 10 fmol of target DNA and then reached a plateau.

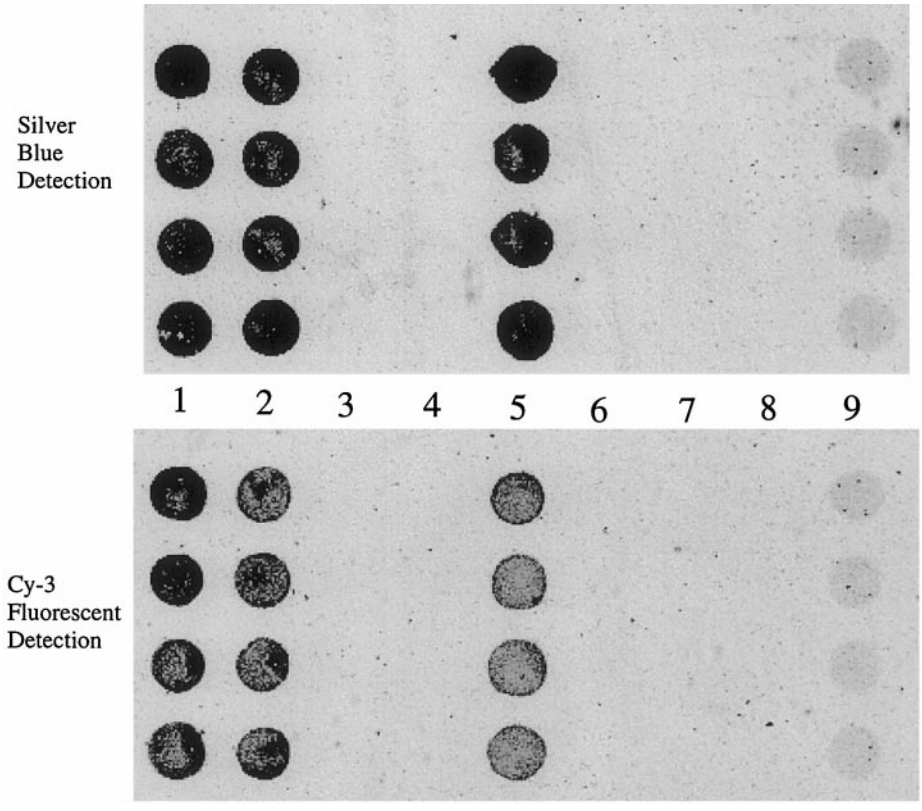

FIG. 6. Detection of the *Staphylococcus epidermidis femA* amplicons on a commercial microarray. Comparison between Silver Blue colorimetric method and fluorescent Cy-3 detection. The four rows of the array were replicates. The microarray contained the following capture probes: columns 1 and 9, fixation controls; column 2, negative control of hybridization; column 3, HCMV-specific capture probe used as positive hybridization control; and columns 4 to 8, five specific capture probes for five *Staphylococcus* species, *S. aureus* (line 4), *S. epidermidis* (line 5), *S. haemolyticus* (line 6), *S. hominis* (line 7), and *S. saprophyticus* (line 8).

Application

The colorimetric Silver Blue detection system was then tested on a DNA microarray developed for the determination of *Staphylococci* species (Staphychips, AAT). The microarray contains five capture probes specific for the *femA* gene of five *Staphylococcus* species: *S. aureus, S. epidermidis, S. haemolyticus, S. hominis,* and *S. saprophyticus* (16). The *femA* gene from *S. epidermidis* was amplified by PCR and the biotinylated amplicons were incubated on the microarray. Hybridization on the array was revealed in fluorescence as well as in colorimetry (Fig. 6). The results were identical with both methods and gave correct identification of the species *S. epidermidis.*

DISCUSSION

Colorimetric detection using silver precipitation has been shown here to be well adapted for detection and analysis of hybridization on microarray. Its sensitivity is comparable to detection in fluorescence using a confocal scanner. This high sensitivity is the result of an amplification step that brings small nanoparticles to grow up 1 million times in volume. With a controlled background, this new technology allows a very good detection sensitivity of hybridized DNA onto glass microarrays which is equivalent to fluorescence. Like in fluorescence, the detection limit is 0.1 fmol of biotinylated target DNA hybridized on the array. The metallic silver deposition specifically occurs on hybridized target DNA, a feature compatible with miniaturization required by the arrays technology (Fig. 2). Since the combination of this gold label and silver enhancement is electron dense, deposits can be read by a simple device such as the colorimetric array workstation.

Another advantage of the method is that silver deposit is stable and the array can be stored for a very long period of time. The Silver Blue colorimetric detection method can be used in similar way on protein arrays using antibodies labeled with nanogold particles. In parallel to this method, Taton *et al.* (13) proposed to use nanogold particles coated with DNA probes to detect DNA hybridized on the array. In this case, the labeling of the target DNA with nanogold particles alters the melting temperature profile of tar-

gets on the array and thus allows the discrimination of targets with single nucleotides mismatches. The limitation of the method is that for each application, nanogold particles must be prepared with the specific probes while in the present paper, standard reagents such as streptavidin–gold and Silver Blue solutions can be prepared and made in a reproducible form.

This colorimetric detection method is also well suited for supports others than glass such as plastic supports, for instance, acrylic layer or polycarbonate. These new supports for microarrays will be interesting for automation since it will be possible to use array on plastic supports which we can mold to create sealed chambers inside and which are adaptable to automate handling. Fluorescent detection is not applicable on plastic arrays because of the strong autofluorescence of these polymers.

The current results indicate that this new silver colorimetric detection method compares favorably with conventional fluorescence detection and could be a cheap, versatile, and as sensitive alternative.

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