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A microsphere-based rolling circle amplification microarray for the detection of DNA and proteins in a single assay

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Abstract

We describe a high-density microarray for simultaneous detection of proteins and DNA in a single test. In this system, Rolling Circle Amplification (RCA) was used as a signal amplification method for both protein and nucleic acid detection. The microsphere sensors were tested with synthetic DNA and purified recombinant protein analytes. The target DNA sequence was designed from a highly conserved gene that encodes the outer membrane protein P6 (OMP-P6) of both typeable and non-typeable strains of *Haemophilus influenzae*. The proinflammatory mediators IL-6 and IL-8 were selected as target proteins. Capture antibodies were first immobilized on fluorescently-encoded microspheres. The microspheres were then loaded into the etched microwells of an imaging optical fiber bundle. A sandwich assay was performed for target proteins IL-6 and IL-8 using biotin-labeled secondary antibodies. Biotinylated capture DNA probes were then attached to the detection antibodies via an avidin bridge. A padlock probe, complementary to the target sequence, was subsequently hybridized to the capture probe. In the presence of the target sequence, the padlock probe was ligated and this circular sequence was used for RCA. Following RCA, multiple fluorescently-labeled signal probes were hybridized to each amplified sequence and the microarray was imaged using an epi-fluorescence microscope. With this assay, detection limits down to 10 fM and 1 pM were achieved for proteins and target DNA, respectively. In addition to this new approach for detecting both protein and DNA in a single test using RCA, the limit of detection for IL-8 and IL-6 was improved by three orders of magnitude compared to similar microsphere-based assays.

Introduction

Microsphere assays have become an important format for performing multiplexed analyses including genotyping, gene expression, and protein immunoassays.[1] Different microsensors can be analyzed simultaneously by encoding each probe-functionalized microsphere type via chemical,[2] spectrometric,[3, 4] or physical means.[5] Our group has previously developed microsphere arrays in which the microspheres were functionalized with different oligonucleotide or antibody probes.[1, 6] In this paper, we describe a high-density microsphere-based rolling circle amplification (RCA) microarray forsimultaneous detection of proteins and DNA in a single test. With this new approach for detecting both a protein and a target DNA associated with the protein, medical conditions could be diagnosed by a single rapid analysis, rather than testing for different disease markers with distinct tests. For instance, increased airway obstruction in patients with chronic obstructive pulmonary disease (COPD) or asthma can be associated with bacterial respiratory infections, such as *Haemophilus influenzae (H. influenzae)*, which can lead to the secretion of proinflammatory cytokines, such as IL-8 and IL-6. [7-12] The assay presented here responds to the combined presence of IL-8 or IL-6 and bacterial DNA in a sample using fluorescence amplified via RCA.

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Microarray technologies coupled to enzymatic reactions, such as PCR[13-15] and immuno-PCR[16-21], have been used to improve detection sensitivity via target amplification. However, PCR and immuno-PCR are considered to be too complicated for a diagnostic setting as they require sophisticated equipment and skilled operators to perform the assays.[22-24] RCA is an alternative amplification method used to increase sensitivity in DNA quantitation,[24-32] DNA mutation detection,[32] and array-based sandwich immunoassays.[22, 23] RCA is a simple amplification method with high sensitivity and specificity owing to the stringent strand matching requirement for ligation and its high amplification efficiency.[25, 32] In RCA, a circular template or padlock is isothermally amplified by DNA polymerase, which creates a long, single-stranded DNA product. The circular template can be synthesized from a padlock probe in which the 5′- and 3′-termini hybridize precisely to the target DNA and are joined by a DNA ligase.[25, 32] The RCA product contains thousands of repeat sequences that are complementary to the fluorescently-labeled signal probes.[25, 32]

This detection method is also compatible in a sandwich immunoassay format, since RCA can be used as the signal generation method for a detection antibody.[22, 23] The isothermal amplification process preserves the integrity of the antibody-antigen complexes and the amplified DNA product can be attached covalently or via an avidinbiotin bridge to the detection antibody. Furthermore, the RCA signal can be confined locally if the amplification primer is immobilized onto a solid support,[33, 34] such as on microspheres or glass slides.

Microarrays that can provide both genomic and proteomic information for several biological targets offer a significant advance as they provide more complete and reliable information while simplifying the overall assay protocol. [35, 36] In the experiments described here, a two-layer sandwich assay is performed to test for the combined presence of a protein and DNA using RCA for signal amplification. Capture antibodies conjugated to microspheres are first used to detect antigens using biotin-labeled secondary antibodies (Figure 1A). Biotinylated capture DNA probes are then attached to the detection antibodies via an avidin bridge (Figure 1B). These capture probes also act as amplification primers for RCA. A padlock probe is then hybridized onto the primer(Figure 1B), and in the presence of a target sequence (Figure 1C), the padlock is ligated by a DNA ligase (Figure 1D). Following RCA (Figure 1E), multiple fluorescently-labeled signal probes are hybridized to each amplified sequence (Figure 1F). The readout can then be performed using an epi-fluorescence microscope. Since the microspheres are spatially resolved in an array, the assay is readily multiplexed by using an array of microspheres with different capture antibodies.

Experimental

Materials

Optical fiber bundles (1.4 mm-diameter) containing ~50,000- 3.1 micron diameter fibers were obtained from Schott Fiber Optics, Inc. (Southbridge, MA). Lapping films for fiber polishing were purchased from Allied High-Tech Products (Rancho Dominguez, CA). Amine-modified methylstyrene-divinylbenzene polymer microspheres (3.1-μm diameter) were purchased from Bangs Laboratories, Inc. (Carmel, IN). All oligonucleotide probes used in this study were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Europium (III) theonoyltrifluoroacetonate-$3H_2O$ (Eudye) was obtained from Acros Organics (Morris Plains, NJ). Sterile water used to reconstitute oligonucleotide probes was purchased from Abbott Laboratories (North Chicago, IL).Tetrahydrofuran (THF), methanol (MeOH), glutaraldehyde (8% aqueous solution) and avidin were purchased from Sigma-Aldrich (St. Louis, MO). Tris-Starting Block, PBS Starting Block Tween-20, and Protein free (PBS) blocking buffers were purchased from Pierce Biotechnology (Rockford, IL). All reagents were used without further purification. Mouse monoclonal capture antibodies (Abs) directed against human IL-6 (clone 6708) and IL-8 (clone 6217), recombinant human IL-6 and IL-8, and biotinylated polyclonal

anti-human IL-6 and IL-8 antibodies were obtained from R&D Systems, Inc. (Minneapolis, MN). *E.coli* DNA ligase was purchased from Invitrogen (Minneapolis, MN). RepliPHI Phi29 Reagent Set and Phi29 DNA polymerase (100 U/μL) were purchased from Epicentre Biotechnologies (Madison, WI).

Microsphere encoding

Encoded microspheres were prepared from 50 μL (5 mg) aliquots of a 3.1 μm amine-functionalized microsphere stock. The aliquots were washed in triplicate with 200 μL PBS and were then washed in triplicate with 200 μL THF. A 200 μL solution of 0.1 M Eu-dye in THF was then added, and the microsphere suspension was shaken in the dark for 2 h at room temperature (RT). The reaction vessel was centrifuged, and the microsphere pellet was washed six times with 200 μL MeOH and then washed six times with 300 μL PBS (0.154 M NaCl, 2.7 mM KCl, 10 mM sodium phosphate and 1.7 mM potassium phosphate, pH 7.4). The encoded microspheres were then suspended in 500 μL PBS with 0.01% Tween-20 and stored at 4 °C in the dark. An identical procedure was followed for a second set of encoded microspheres; however, the europium dye concentration in THF solution was increased to 0.5 M.

Preparation of sensors

A 100 μL aliquot (1 mg) of the encoded microsphere suspension was centrifuged, the supernatant was removed, 1 mL of 8% glutaraldehyde in PBS was added, and then the mixture was shaken at RT for 2 h in the dark.[37] The microspheres were then washed three times with 300 μL of PBS. Subsequently, 45.3 μg of IL-6 or IL-8 monoclonal capture antibodies were added to 1 mg of the glutaraldehyde-activated microspheres (encoded with 0.1 and 0.5 M Eu-dye, respectively) suspended in 500 μL PBS.

The microcentrifuge tube containing the mixture was covered with aluminum foil and shaken at RT for 4 h. The microspheres were washed once with 300 μL Tris-Starting Block (blocking buffer), and then were suspended in 300 μL blocking buffer. The suspension was shaken at RT for 30 min in the dark, and then washed once with 300 μL blocking buffer. The microsphere probes were suspended and stored in 100 μL blocking buffer at 4° C, protected from light.

Microarray Fabrication

The ends of the optical fiber bundles were sequentially polished with 30, 15, 6, 3, 1, 0.5, and 0.05 μm lapping films. The fiber bundles were then sonicated in water for 10 s to remove residue on the finished ends. One end of the polished fiber bundle was chemically etched to form microwells as described previously.[8] Etched fibers were thoroughly rinsed with Nanopure water. The etched end of the fiber bundle was blocked with 200 μL Protein free (PBS) blocking buffer for 30 min. Anti-IL-6 (encoded with 0.1 M Eu-dye) and anti-IL-8 (encoded with 0.5 M Eu-dye) microsphere types were combined to form a microsphere stock solution. This microsphere stock was loaded into the array by pipetting a 0.5 μL aliquot onto the etched end of the fiber, and allowing the solution to dry for 10 min. The end of the fiber containing the microspheres was then blocked a second time with 200 μL Starting Block Tween-20 (PBS) blocking buffer for 30 min at RT. The fiber was incubated in 100 μL of Starting Block Tween-20 (PBS) buffer containing various concentrations (0-10 nM) of IL-6, IL-8, or both (Figure 1A), for 2 h, and then rinsed with 1mL Starting Block (PBS) Tween-20 buffer. The fiber was subsequently incubated with 100 μL of a solution containing a mixture of 3 μg/mL of each biotinylated detection antibody (anti-IL-8 and anti-IL-6) for 30 min (Figure 1A) and then rinsed with 1 mL of Starting Block Tween-20 (PBS) buffer. After incubation in PBS solution containing avidin (20 μg/ml) for 45 min, the fiber was rinsed with 1 mL of PBS (Figure 1B). Subsequently, the fiber was incubated in PBS solution containing 10 μM of biotinylated capture DNA probes for 45 min at RT and then rinsed with 1 mL of PBS (Figure 1B).

The oligonucleotide target of interest (0-50 nM) was hybridized to the 5′and 3′ termini of a linear padlock probe (50 nM) in 50 μL of ligation buffer (18.8 mM Tris-HCl, pH 8.3, 4.6 mM $MgCl_2$, 90.6 mM KCl, 0.15 mM NAD, 10 mM $(NH_4)_2SO_4$, 3.8 mM DTT) during a 1h incubation at 37 °C (Figure 1C). Subsequently, the optical fiber microsphere array was immersed in ligation buffer with 0.1 U/μL of *E.coli* DNA ligase (Figure 1D). After incubation at RT for 1h, the fiber bundle end was washed once using 1 mL of PBS.

RCA

After ligation, polymerization was carried out at 37°C for 30 min in 50 μL of Phi29 reaction buffer (40 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 4 mM DTT) with 1 U/μL Phi29 polymerase and 625 μM dNTP (Figure 1E). Fibers were rinsed with 1 mL of PBS and then incubated with 1 μM of the detection probe in 50 μL of PBS for 30 min at RT (Figure 1F). After incubation, the fiber bundle end was washed once using 1 mL of PBS. Finally, the hybridization of DNA target to the array was imaged using an epi-fluorescence microscope.

Imaging system

A custom-built epi-fluorescence imaging system was used to acquire all fluorescence images. The system included a mercury light source, excitation and emission filter wheels (Chroma, Rockingham, VT), microscope objectives (Olympus, Center Valley, PA), and a CCD camera (Orca-ER, Hamamatsu). Filter wheels and shutters were computer-controlled and analysis was performed with IPlab software (Scanalytics, Fairfax, VA). The system was equipped with a chuck to immobilize the fiber-optic bundle. After the microspheres were randomly loaded onto the fiber optic bundle, their locations on the array were registered using their unique optical barcodes that consisted of a defined level of Eu-dye (excitation 360 nm/emission 600 nm). Observing the encoding fluorescence enabled the two microsphere types to be distinguished (Figure 2, 4 and S-1A). Cy3 fluorescence (excitation 550 nm/ emission 570 nm) (Figure 2, 4C and S-1B) was monitored to evaluate the microsphere assay. Fluorescence intensity values were acquired for encoding, background, and signal images using a 200 ms image exposure time and 20 × magnification. The images were analyzed with IPlab software.

Results and Discussion

Before demonstrating the combined protein and nucleic assay, we first optimized the individual assays. Optimization was accomplished by varying the protein concentrations while adding a fixed concentration of DNA target. Similarly, the DNA target assay was optimized by varying the target concentration while keeping the protein concentration constant.

Protein microarray system

A microarray-based assay was developed for the detection of IL-8 and IL-6 using capture antibodies immobilized on fluorescently-encoded microspheres (Figure 1A). The microsphere assay was first validated using a single protein target to demonstrate the feasibility of the sandwich assay and to confirm the utility of the antibody—DNA conjugates for RCA. To assess the feasibility of the protein assay, a fixed concentration of target probe DNA (50 nM) was used to enable the ligation step when interrogating various concentrations of protein. A sandwich assay was performed for the target proteins using biotin-labeled secondary antibodies (Figure 1A). The biotinylated DNA capture probes (amplification primers) were then attached to the detection antibody via an avidin bridge (Figure 1B). A padlock probe, complementary to the target sequence, was subsequently hybridized to the capture probe (Figure 1B). In the presence of the target sequence (Figure 1C), the padlock probe was ligated (Figure 1D) and amplified by a DNA polymerase using the RCA mechanism (Figure 1E).

Next, multiple Cy3-labeled probes were hybridized to the single-stranded RCA product, as shown in Figure 1F. The net signal for all microsphere assays was calculated by subtracting the background image signal (Figure 2B), acquired prior to hybridization of the fluorescently-labeled signal probe, from the intensity of each microsphere sensor in the image (Figure 2C), acquired after hybridization of the fluorescently-labeled signal probe. A minimum of 30 beads for each protein was averaged for each measurement. A pre-set signal threshold was defined as the average measurement of three negative control fiber arrays (buffer sample including all assay reagents excluding the target protein (Table S-1, exp.3) plus three times the standard deviation (SD)). Any net signal from a microsphere greater than this threshold was considered a positive response while any net signal below this threshold was considered a negative response.

Figure 3 shows the averaged fluorescence intensity of the IL-8 protein assay obtained by collecting data from three optical fiber arrays at IL-8 concentrations ranging from 10 fM to 10 nM. The range of IL-8 concentrations examined using the duplexed sensor platform demonstrated specific binding with positive signals only from the anti-IL-8 microspheres (Figure 2C) with a low amount of signal due to non-specific binding to the anti-IL-6 microspheres. Using the average SD of the negative control, we calculated the limit of detection for IL-8 to be 10 fM. We also tested the performance of the microsphere-based assay for detection of 10 fM of IL-6 protein using duplexed sensor platform. Using the average SD of the negative control, the positive signal was demonstrated only from the anti-IL-6 microspheres with a low amount of signal due to non-specific binding to the anti-IL-8 microspheres.

In addition, simultaneous detection of both 10 fM of IL-8 and IL-6 proteins using duplexed sensor platforms demonstrated positive signals at the locations of both the IL-8 and IL-6 probe microspheres (Figure 4C). The RCA allowed us to improve on our previously reported sensitivity of fiber-based microsphere arrays for detection of IL-8 and IL-6 from 8 and 64 pM, [38] respectively, to 10 fM. Therefore, this assay exhibits an appropriate sensitivity for analyzing human saliva samples, which contain 4 fM - 250 pM of IL-8 protein.[38, 39]

DNA microarray system

We next tested the performance of the microsphere-based RCA assay for DNA detection. Various concentrations of target DNA were tested using a constant concentration of target protein (10 nM). The target sequence from the coding region of the OMP-P6 DNA and padlock probe sequences (Table S-2) were designed to mimic the detection of bacterial DNA. The 5′- and 3′- ends of the linear padlock probe were designed to hybridize next to each other on the target strand (Figure 1C). Upon perfect hybridization of the padlock to the target probe, the adjacent ends of the oligonucleotides would be joined by DNA ligase (Figure 1D). Subsequently, DNA polymerase extends the primer sequence immobilized on protein conjugated microspheres by repeatedly traveling around the circular DNA template (Figure 1E). The resulting single-stranded DNA product from the RCA reaction consists of a long string of tandem copies complementary to the circularized probe. The amplified products hybridize to the fluorescently-tagged detection probes linked to microspheres (Figure 1F). Hybridization was detected with an epi-fluorescence microscope.

The averaged fluorescence intensity was plotted against the DNA target concentration as shown in Figure 5. The net signal was calculated as for the protein microarray system. A pre-set signal threshold was defined as the average measurement of three negative controls (buffer sample including all assay reagents excluding target DNA analyte (Table S-1, exp.2)) +3× SD. Figure 5 illustrates that a positive sample can be discriminated from the negative control at the 1 pM level. This sensitivity compares well to DNA sequence detection based on single-stranded oligonucleotide-tagged magnetic nanobeads[25] and a microsphere-based assay using a flow

cytometer.[32] For the nanobead and flow cytometer assays, the RCA amplification demonstrated sensitivities of 3 pM[25] and 0.25 pM[32], respectively.

Combined DNA and protein detection

To demonstrate the array's ability to detect low concentrations of both target DNA and IL-8 protein, concentrations were held in the low pM range (Figure S-1). The same pre-set signal threshold defined above was used to distinguish between positive and negative signals. Using this assay, a positive sample could be discriminated from a negative control sample when both target DNA and IL-8 protein were present at the 1 pM and 10 fM, respectively (Figure S-1). In order to show the sensitivity and dose response of the microsphere array we used defined concentrations of the analytes. Our goal was to demonstrate the ability of detecting both protein and DNA in a low concentration range in one test from the same sample. The antigens were inflammatory markers that would be elevated during a bacterial infection. The DNA sequence detected should not be present at any concentration. If needed, unknown concentrations of these analytes could be determined by acquiring calibrations of the array with known concentrations of both analytes.

Conclusions

We have demonstrated a proof-of-concept for the combined detection of a nucleic acid sequence and a protein in a single assay using an optical fiber microsphere array platform. The assay employs RCA and is designed to generate a signal only when both analytes are present. Detection limits of 10 fM and 1 pM were achieved for proteins (IL-6 and IL-8) and DNA, respectively. This sensitivity was achieved mainly due to the high amplification efficiency of RCA and the high binding capacity of the microsphere's surface. Although we have shown duplexed protein detection, this method could easily be applied to multi-target detection, due to the high density of the fiber-optic array. Additional microsensors that bind other protein targets can be easily incorporated in this type of array. The ability to use combinations of analytes for signal generation should enable simplification of diagnostic tests. In the future, this sensor may be useful for analyzing serum or saliva samples collected from patients with COPD and/or asthma. One application of this technology would be to detect both genes and their protein products.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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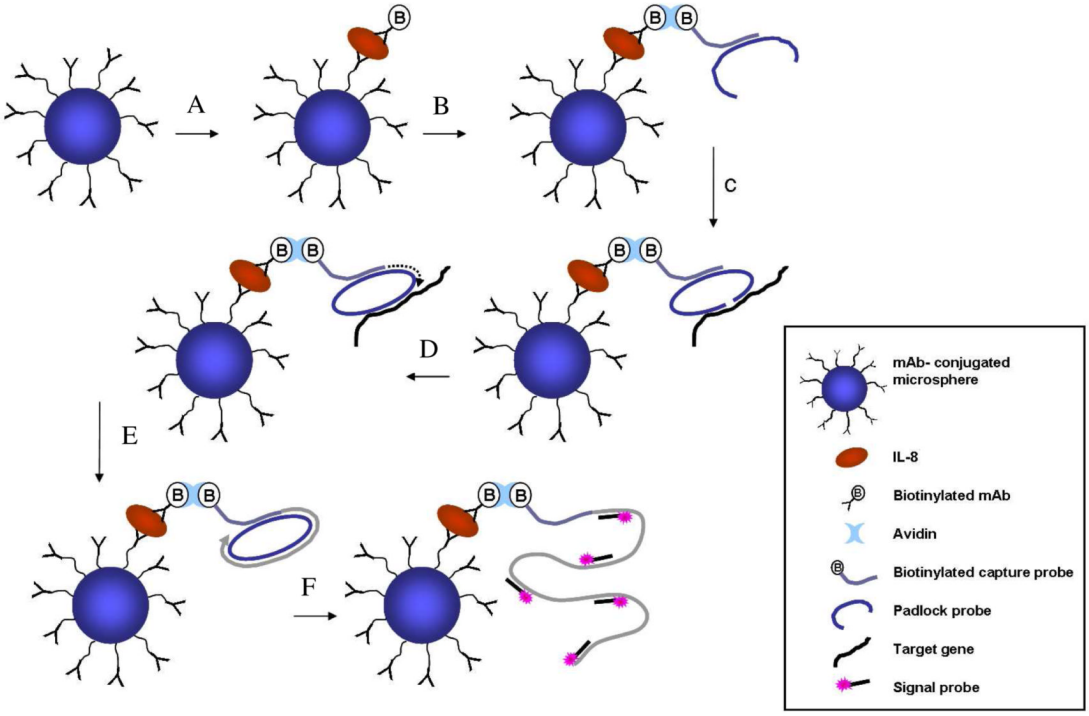

Figure 1.

Microsphere-based RCA assay for the detection of a target DNA sequence and IL-8. (A) Capture of the target protein and binding of secondary biotin-labeled antibodies. (B) Capture of biotinylated DNA probe via an avidin bridge, and hybridization o the padlock probe. (C) Hybridization of the target gene brings padlock probe termini in proximity. (D) Joining of the padlock probe ends by DNA ligase, followed by capture probe extension by polymerase. (E) Rolling circle amplification of the ligated probe. (F) Hybridization of the Cy3-tagged detection probes to the RCA product.

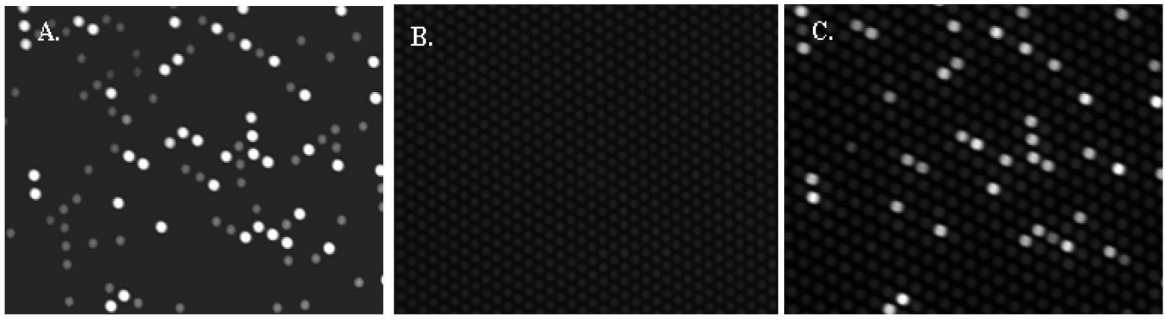

Figure 2.
CCD images of a small section of a fiber-optic array containing two different probe-functionalized microspheres. (A) Duplexed Eu-dye encoding of microspheres: 0.5 M Eu-dye anti-IL-8 antibody-encoded microspheres appear as brighter circles compared to the 0.1M Eu-dye anti-IL-6 antibody-encoded microspheres. (B) Background signal images prior to incubation with DNA signal probe. (C) An image following incubation with 10 nM of IL-8, detection antibody, and RCA with DNA signal probe. Note the perfect correlation between the high intensity encoded microspheres and the microspheres showing fluorescence in the signal image.

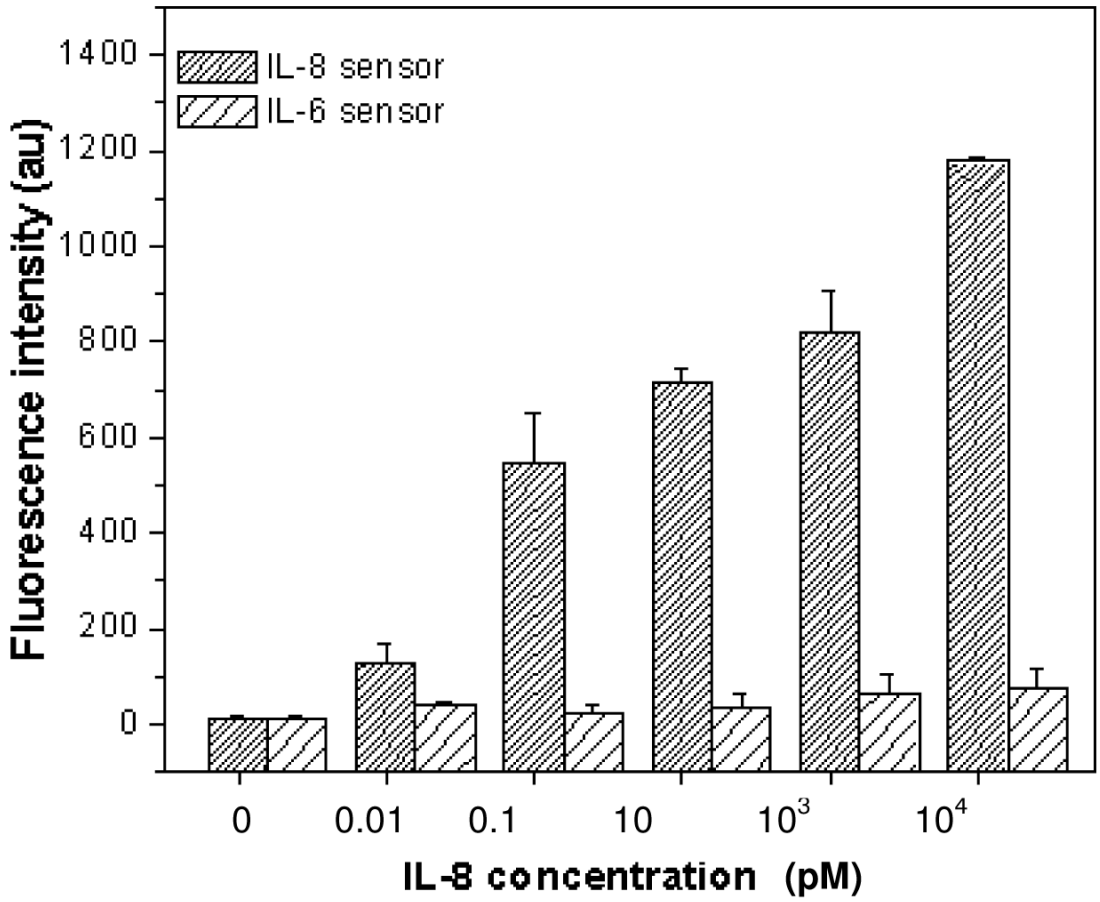

Figure 3.

Averaged fluorescence intensities of the IL-8 protein assay obtained in triplicate for IL-8 concentrations ranging from 10 fM to 10 nM using constant concentration of target probe DNA (50 nM). IL-6 microsphere signals are shown as a control signal at each concentration.

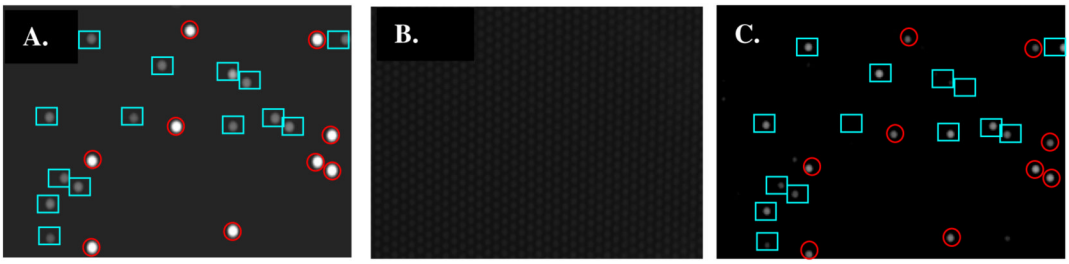

Figure 4.

CCD images of a small section of a fiber-optic array for IL-8 and IL-6 protein detections containing two different probe-functionalized microspheres. (A) Duplexed Eudye encoding of microspheres: 0.5 M Eu-dye anti-IL8 antibody-encoded microspheres appear as brighter circles compared to the 0.1M Eu-dye anti-IL6 antibody-encoded microspheres. (B) Background signal images prior to incubation with DNA signal probe. (C) Signal image for anti-IL-8 (marked with red circles) and anti-IL-6 (marked with blue rectangles) microspheres following incubation with 10 fM of IL-8 and IL-6, detection antibody, followed by RCA with a DNA signal probe. Since the lowest concentration of both protein analytes was used, some anti-IL-6 encoded microspheres (marked with blue rectangles) had no fluorescent signal.

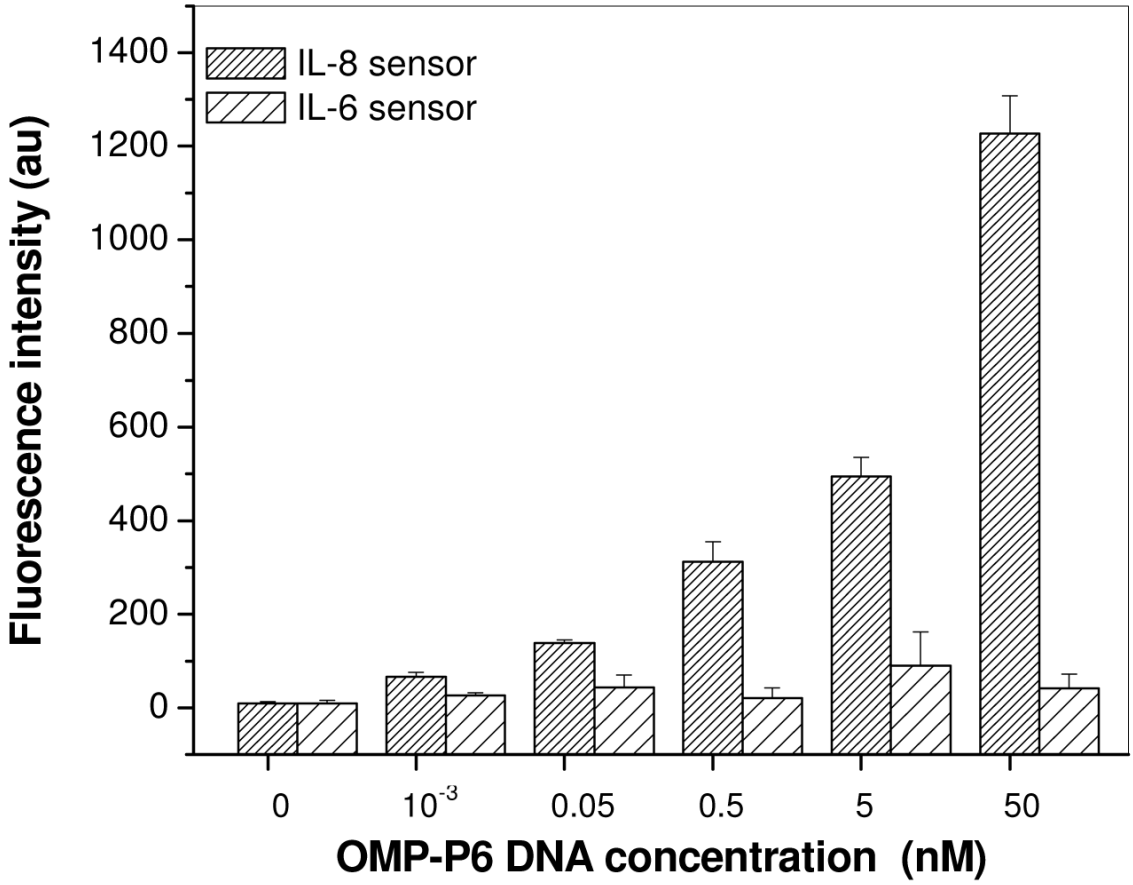

Figure 5.
Averaged fluorescence intensities of the OMP-P6 target DNA assay obtained in triplicate for target DNA concentrations ranging from 1 pM to 50 nM using constant concentration of IL-8 (10 nM). IL-6 microsphere responses are shown as control signals for specificity of the microsphere array.