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Silver nanoparticle-enhanced fluorescence in microtransponder-based immuno- and DNA hybridization assays

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Abstract

The aim of this study is to improve assay sensitivity in common solid-phase bioassay configurations as the result of using silver nanoparticles. The solid phase was provided by numerically indexed, silicon-based electronic chips, microtransponders (p-Chips) that have previously been used in multiplexed assays. Assay configurations investigated included an ELISA-type immunoassay and a DNA hybridization assay. The surface of p-Chips was derivatized with the silver island film (SIF) and a polymer, and then characterized with AFM and SEM. Silver nanoparticle sizes were in the range of 100 to 200 nm. Four fluorophores were tested for fluorescence enhancement; namely, green fluorescent protein, phycoerythrin, Cy3 and Alexa Fluor 555. We consistently observed significant fluorescence enhancement and sensitivity improvement in the p-Chip-based assays: the sensitivity in the cytokine IL-6 immunoassay was 4.3 pg/ml, which represented a 25-fold increase over the method not involving a SIF; and 50 pM in the hybridization assay, a 38-fold increase. The greatest enhancement was obtained for p-Chip surfaces derivatized first with the polymer and then coated with SIF. In conclusion, we show that the SIF-p-Chip-based platform is a highly sensitive method to quantify low-abundance biomolecules in nucleic acid-based assays and immunoassays.

Keywords

ELISA; Multiplex; Molecular diagnostics; Hybridization; Surface plasmon resonance; Microchip

Introduction

Fluorescent labeling techniques are widely applied in nucleic acid and protein assays [1,2] as they are easy to use, adaptable to different analytes, highly sensitive, and can be part of multiple labeling strategies. Given their widespread use, there is a desire to further improve the limit of

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detection in order to determine low-abundance biomolecules. In this paper, we report on a silver island film (SIF)-based approach that greatly improves the sensitivity of both a fluorescence-based immunoassay and a fluorescence-based DNA hybridization assay.

The effects of silver nanostructures on fluorescence enhancement have been theoretically and empirically studied during the past two decades [3–7]. A significant enhancement of fluorescence signal has been shown by many groups when fluorophores are placed in close proximity to a layer of metallic nanoparticles [8–12]. The enhancement is due to the interaction of excited-state fluorophores with mobile electrons on the surface of the metallic nanoparticles that results in an increase in the rate of radiative decay [6,13], shorter fluorescence lifetimes and faster turnover of excited states [3,4,8,9]. This phenomenon is known as “surface plasmon resonance”. There have been many studies of potential bioanalytical applications [11,14]. More recently, it was demonstrated that silicon wafers are a suitable solid phase for observing metal-enhanced fluorescence [15]. Thus, it was anticipated that the use of fluorescence with SIF on silicon chips would improve the assay sensitivity and signal-to-noise ratio.

In this study, we applied SIF to a microtransponder p-Chip-based platform in a DNA hybridization assay and a cytokine IL-6 immunoassay. This platform has been developed and is used in both genomics and proteomics research [16–18]. The key feature of the platform is its applicability to multiplex assays, in which concentrations of many analytes are determined simultaneously. In the recently described cystic fibrosis (CF) DNA assay [19], p-Chips were used to determine 50 mutations related to CF. Up to 300 p-Chips were used in a single assay for one DNA sample.

The p-Chip is a monolithic, integrated semiconductor device with standard dimensions of 500 μm $\times$ 500 μm $\times$ 100 μm . The electronic side of the p-Chip is composed of photocells, read-only memory (ROM), control electronics, and an antenna loop. The photocells, when illuminated, provide power for the logic circuitry. The circuitry accesses the ROM contents and modulates current through the antenna. The antenna transmits the ID digitally through a varying magnetic field in the vicinity of the chip that can be decoded by the radio frequency readout system to provide the specific serial number. The current p-Chip design allows 2^{10} (1,024) unique IDs to be encoded; however, the ROM contains additional 48 unused bits and the encoding scheme can easily be expanded for up to 2^{30} ($\sim 10^9$) unique IDs. In bioassay applications, p-Chips are coated with a polymer layer containing both hydroxyl and amino groups that allow conjugation of oligonucleotide or protein probes to the surface. Probe identity is thus associated with a unique ID for each p-Chip. The p-Chip-based platform is designed to increase the flexibility and throughput of bioassays and has been successfully applied in multiplexed genotyping [19], proteomic and cytotoxicity assays [20].

There are two advantages associated with using p-Chips in a bioassay: first, p-Chips are individually identifiable, thus traceability of p-Chips throughout manufacturing is maintained and the manufacturing process itself is easier to control. Secondly, it is easy to modify the multiplex panel configurations by adding p-Chips derivatized with additional oligonucleotides or antibody probes (this task might be much more difficult in alternative approaches, e.g., microarrays).

The goal of this study was to demonstrate that silver nanoparticles can increase the sensitivity of p-Chip-based bioassays by enhancing the fluorescence signal. Towards this goal, we developed a method to coat p-Chips with silver nanoparticles. Coating small chips or beads requires a different approach than coating glass slides and involves many challenges including: uniform adherence to the chip surface (a silicon or silicon dioxide surface) under conditions during which the chips are suspended in a solution and are being agitated; controlling the size of silver nanoparticle being deposited; and assuring of the thickness and uniformity of the

polymer and silver film layers on the chip; drying of the chips. It should be noted that spin coating techniques cannot be used with these applications. The two coating methods available to us: immersing the silver nanoparticles in the polymer coating of the p-Chips, or forming a separate silver nanoparticle layer on surface of the polymer layer, were tested, and physical measurements of the surface properties using atomic force microscopy (AFM) and scanning electron microscopy (SEM), were performed.

In this study, fluorescence enhancement for four commonly used fluorophores was investigated, namely green fluorescent protein (GFP), Alexa Fluor 555 (AF555), phycoerythrin (PE) and Cy3. In addition, to demonstrate potential applications for diagnostics in vitro, two types of biological assays were performed on p-Chips coated with silver nanoparticles: an interleukin IL-6 enzyme-linked immunosorbent assay (ELISA); and a DNA hybridization assay in which a fluorescently labeled DNA probe was allowed to bind to a DNA target synthesized on the p-Chip.

Materials and methods

Fluorescent dyes and reagents

GFP was purchased from BioVision. AF555 labeled bovine serum albumin (BSA-AF555) and streptavidin-R-phycoerythrin (SA-PE) were purchased from Invitrogen. Cy3-labeled oligonucleotides were synthesized by MWG Biotech. All of the chemicals used for polymer coating and silver deposition were purchased from Sigma.

Coating p-Chips with polymer

p-Chips were pretreated with 99.5% methyl alcohol at room temperature (RT) for 10 min, and repeated three times. The p-Chips were then rinsed with 0.01% distilled water and 0.9% aminopropyltriethoxysilane (APTS) in dry toluene/dimethylformamide (DMF) mixture at RT, and repeated four times. After rinsing, p-Chips were immediately treated with a coating solution (mixture of 0.01% distilled water, 0.9% APTS, and 0.3% 3-glycidoxypolytrimethoxysilane (GPTS) in dry toluene and DMF) at 80 °C for 45 min and repeated once. After the coating reaction, p-Chips were washed once with toluene, three times with DMF, and three times with acetonitrile at RT, followed by air drying. The procedure placed both amino and hydroxy groups on the surface of p-Chips.

Conversion of amino group to carboxylic acid

Amino-derivatized p-Chips were treated with 10% succinic anhydride in dry pyridine:DMF (1:9) on a tissue culture rotator at RT for 30 min. This step was repeated once using fresh reagents. After the reaction, the carboxylated p-Chips were washed with DMF four times and acetonitrile twice, followed by air drying.

Deposition of SIF on p-Chips

SIF was deposited on the surface of carboxylated p-Chips as reported previously [14] with several modifications. Two drops of 5% NaOH was progressively added to 6 ml of 0.83% AgNO₃ solution with intensive stirring at RT in a 15-ml reaction tube. A 0.2-ml of 30% NH₄OH was subsequently added with intensive stirring at RT. The clear solution was incubated in an ice bath for 10 min, followed by the addition of 1.5 ml of a 4.8% fresh glucose solution with intensive stirring. Carboxylated p-Chips were incubated in this solution in an ice bath for 2 min, and then on a tissue culture rotator at RT for 20 min. After the silver deposition, p-Chips were immediately washed with distilled water three times followed by air drying.

To test the effectiveness of the SIF-polymer layer on p-Chip in fluorescence enhancement, the p-Chips were incubated with 50 µg/ml GFP for 1 h at RT and then washed with distilled water

three times followed by air drying. The GFP fluorescence enhancement was measured using a fluorescent microscope (Nikon Eclipse E600 with Y-FL EPI fluorescence attachment and IP Lab v. 3.55 software [Scanalytics, Inc.]).

Atomic force microscopy

AFM images were collected by scanning the surface of a p-Chip on a TMX2100 Explorer SPM atomic force microscope (Veeco). This instrument is equipped with a dry scanner calibrated using a standard calibration grid as well as 100 nm gold nanoparticles (Ted Pella). Images were analyzed using SPMLAB software.

Scanning electron microscopy

Joel JSM-6510LV scanning electron microscope (Joel Ltd., Japan) was used for fast characterization and imaging of silver structures deposited on the p-Chips. All images were taken at high vacuum at the potential of 20 kV applied to the tungsten filament. Magnification was $\times 15,000$ with the beam width 20 mm.

Measurement of fluorescence lifetimes

Fluorescence decay of AF555 deposited on p-Chips was measured on a FluoTime 200 fluorometer (PicoQuant) using excitation from a pulsed picosecond 475 nm solid state laser. The instrument was equipped with a microchannel plate photomultiplier ultrafast detector, a monochromator and a polarizer in the detection path. Two 550 nm long wave pass filters were used on the emission optics for observation at 600 nm to eliminate scattered excitation light. For measurements, p-Chip were placed between two cover slips and mounted in a front face attachment. Fluorescent lifetime data were analyzed with a FluoFit version 4 software (Picoquant) and fitted to a multi-exponential model.

Conjugation of antibody to p-Chips

In order to protect SIF from scratches, a thin layer of polymer (20 min polymer coating, see above) was deposited onto SIF-p-Chip and carboxylated (see the "Conversion of amino group to carboxylic acid" section above) prior to performing the immunoassays. The antibody was conjugated to p-Chips by incubating the carboxylated p-Chips with 50 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 50 mg/ml *N*-hydroxysuccinimide in 0.1 M HEPES buffer (pH 7.5) on a rotator for 30 min at RT. The p-Chips were then washed with 60 μ l PBS three times and incubated with 200 μ g/ml monoclonal anti-human IL-6 antibody (R&D Systems) for 30 min at RT on a tissue culture rotator. The p-Chips were washed with PBS three times and blocked with SuperBlock solution (Thermo Scientific) for 5 min at RT on a rotator. This blocking step was repeated twice. The p-Chips were then washed with PBS three times (2 min each) and stored in PBS with 1% BSA at 4 °C.

Immunoassay

Anti-IL-6-conjugated p-Chips were incubated with 50 μ l recombinant human IL-6 standard (R&D Systems) in PBS with 1% BSA or 50 μ l diluted normal human serum (United States Biological) spiked with recombinant human IL-6 for 1.5 h at RT on a rotator. After incubation, the p-Chips were washed with Tris-Buffered Saline Tween-20 (TBST) three times.

The detection antibody solution was prepared by diluting biotinylated anti-human IL-6 antibody (R&D System) to 5.0 μ g/ml with 1% BSA in PBS. The p-Chips were then incubated with 50 μ l of detection antibody for 1 h, followed by washing with TBST three times. The p-Chips were pooled and incubated with 50 μ l of 8 μ g/ml SA-PE in PBS for 15 min at RT in the dark. After incubation, the p-Chips were washed with TBST three times and distilled water twice, and air dried. PE fluorescence was confirmed using a fluorescent microscope (Nikon

Eclipse E600 with Y-FL EPI fluorescence attachment) and quantified with Image J software (NIH).

The assay sensitivity is defined as the minimum IL-6 concentration producing a signal equal to three deviations (SD) from the standard zero. This represents the lowest value read from the standard curve that can be statistically distinguished from zero. To determine the assay sensitivity, a standard curve was generated using Prism 5.0 software: three standard SDs for the standard zero were added to the mean fluorescence for the standard zero replicates (six replicates for non-SIF and nine replicates for SIF) and the corresponding concentration was determined from the standard curve.

DNA assay

Carboxylated SIF or non-SIF-p-Chips were conjugated with 50 µg/ml avidin (see the “Immunoassay” section above) and incubated with 10 µM 5'-biotinylated probe oligonucleotides on a tissue culture rotator at RT for 1 h. The p-Chips were then washed with TE buffer three times at RT, and stored in TE buffer at 4 °C. The sequence of the oligonucleotide probe was: 5'-biotin-TTTTTTTTTGCCTTCCTTCACTG-3'. As a negative control, another probe with a point T/C mutation (underlined) was used: 5'-biotin-TTTTTTTTTCTTCCTCCACTG T-3'. In the alignment of the two sequences, the 14 nt section within the negative control sequence is shifted 1 nt to the 3' end compared to the probe oligo to ensure that the control and the probe oligo have similar T_m .

The hybridization process was done as previously reported [19]. In brief, oligonucleotide-linked p-Chips were incubated in 1× pre-hybridization buffer at 45 °C for 10 min. After removing the pre-hybridization buffer, the p-Chips were incubated in 1× hybridization solution that contained 5'-Cy3-labeled target oligonucleotides for 2 h at 45 °C in a hybridization oven (Bambino, Boekel Scientific), in the dark. The sequence of the target oligonucleotide was: 5'-Cy3-AATAACTTTGCAACAGTGAAGGAAAGCCTTTGG A-3'. The target and probe oligonucleotides contained a perfectly matched sequence of 14 nt (underlined). After hybridization, p-Chips were rinsed twice with a pre-warmed (45 °C) 1× washing buffer and incubated in a fresh 1× washing buffer at 45 °C for 30 min. Then the p-Chips were washed with distilled water three times at RT and air dried. Images of fluorescent p-Chips were taken with a digital camera attached to the fluorescent microscope and the intensity quantified.

The pre-hybridization buffer contained 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% Ficoll (type 400), 5 mM EDTA, 200 µg/ml sheared, denatured salmon sperm DNA and 1 µg/µl BSA. The 1× hybridization solution contained 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 5 mM EDTA (pH 8.0) and 50 µg/ml sheared, denatured salmon sperm DNA. Washing buffer was 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl and 0.1% SDS.

Results and discussion

Deposition of silver nanoparticles on p-Chips: surface properties

Silver nanoparticles were deposited on both sides of p-Chips using a reduction reaction with AgNO₃, as described in the “Materials and methods” section. The surface of a SIF-deposited p-Chip appeared granular in bright field microscopy images (Fig. 1a). In contrast, the surface of a bare p-Chip was smooth showing only the fine lines resulting from the wafer thinning process. To determine the size of silver nanoparticles the surfaces of SIF on bare p-Chips and polymer-coated p-Chips were scanned by AFM and SEM (Fig. 1b–c). The size of most silver particles was between 100 and 200 nm. However, aggregated silver particles (larger than 1 µm) were also observed occasionally (Fig. 1b–c). Such inhomogeneity of the particles may limit the maximum fluorescence enhancement and cause a batch-to-batch variation (as

discussed in the next section). This can be minimized in the future by further improvements of the method to coat p-Chips with SIF.

We also tried to form the SIF by mixing silver nanoparticles (~100 nm) with the APTS/GPTS polymer-forming solution during the p-Chip coating process. The latter approach was not satisfactory. The silver nanoparticles formed aggregates, especially on the edges of p-Chips and were not evenly distributed on the surface. As a result, the fluorescence enhancement was low (data not shown). Therefore, the chemical reduction method was used for all subsequent experiments.

Fluorescence enhancement by SIF and lifetime measurements for various fluorophores

Silver nanostructures have been shown to be able to enhance fluorescence of nearby fluorophores in many recent studies [3–12]. Such a phenomenon is due to the interactions between the excited-state fluorophores and free electrons in the metal, the so-called surface plasmon electrons, which lead to enhanced local fluorescence intensity and increased radiative decay rates. Geddes et al. estimated that the radiative rate could be theoretically increased 1,000-fold when a fluorophore is placed near a silver nanoparticle [21]. In practice the fluorescence enhancement has been observed in less than ideal conditions under which an aggregate fluorescence of a surface is measured. For instance, Matveeva et al. reported a four- to fivefold fluorescence enhancement in an immunoassay on flat glass [14]. Lukomska et al. reported an eightfold enhancement when SIF was deposited on quartz slides using Texas Red-labeled BSA [22].

To test the fluorescence enhancement by SIF on silicon surfaces, p-Chips were coated with the APTS/GPTS polymer and the SIF was deposited on the p-Chips. The purpose of polymer coating on the surface of p-Chips was to provide functional groups for conjugation of fluorescent dyes or charged groups to facilitate non-covalent protein binding to the chips. Different fluorescent probes were placed on the surface of the chips, namely GFP (Fig. 2a), AF555 (Fig. 2b), PE (Fig. 4), and Cy3 (Fig. 5). SIF and non-SIF-p-Chips were incubated with GFP (50 µg/ml) or AF555-labeled BSA (10 µg/ml) for 1 h at RT. We observed a 23.7-fold enhancement of the signal for GFP and 14.4-fold for AF555 on SIF-p-Chips compared with non-SIF-p-Chips (Fig. 2). In the test of PE (Fig. 4) and Cy3 (Fig. 5) in immunoassay and DNA hybridization assay, a fluorescence enhancement of 5.5 to 50-fold was consistently observed. All four fluorophores tested showed significant enhancement which varied from five to 50-fold. This means that the SIF-based enhancement is not dye-specific and, thus, has potential for broad use.

Batch-to-batch variation in fluorescence enhancement was noticed for p-Chips coated with polymer and having a SIF deposited on them. We implemented a routine test to evaluate the variation. The test involved incubating the derivatized p-Chips with GFP and measuring fluorescence enhancement. The observed enhancement was in the range from 8.1- to 23.7-fold (the number is based on three determinations). Despite the fact that the amount of enhancement varied, the enhancement was consistently observed. To minimize the effects of the batch-to-batch variation on the conclusions reached, the p-Chips used in the same experiment came from the same batch in our study. The current protocol can be further optimized to reduce the variation.

Previous studies have shown that the enhanced fluorescence intensity by SIF is associated with decreased fluorophore lifetimes [3,4,8,9]. Therefore, we also measured the radiative decay rate of AF555 on the surface of SIF-p-Chips after the chips were incubated with AF555-labeled BSA (Fig. 3). We tested both high (50 µg/ml) and low (0.08 µg/ml) BSA-AF555 concentrations. In both cases, the lifetime of AF555 was significantly shorter on SIF-p-Chips compared to non-SIF-p-Chips (Fig. 3). When 50 µg/ml AF555-BSA was used, the half-life of

AF555 was 0.19 ns on non-SIF-p-Chips compared to 0.02 ns on SIF-p-Chips, a 9.5-fold reduction. When 0.08 $\mu\text{g/ml}$ AF555-BSA was used, the half-life of AF555 was 1.15 ns on non-SIF-p-Chips compared to 0.05 ns on SIF-p-Chips, a 23-fold reduction.

Immunoassay

To test the effects of SIF in bioassays, we performed IL-6 immunoassays. The cytokine IL-6 is a pleiotropic protein that is critical in acute-phase reactions, inflammation, hematopoiesis, bone metabolism, and cancer progression [23–25]. In our test, a monoclonal anti-human IL-6 antibody was conjugated to SIF-p-Chips or non-SIF-p-Chips. The antibody conjugation involved a two-step carbodiimide reaction (see the “Materials and methods” section). The carboxyl groups on the surface of the polymer-coated p-Chips were first activated with a carbodiimide derivative prior to coupling with the primary amines on the antibody. The anti-IL-6 conjugated p-Chips were used to capture recombinant human IL-6. To detect IL-6, a biotinylated anti-human IL-6 antibody was incubated with the p-Chips, followed by incubation with SA-PE (Fig. 4a). We observed that the SIF-induced fluorescence enhancement ranged from 8.1 to 15.5-fold (Fig. 4b). The assay sensitivity was initially calculated using normalized fluorescence intensity units, then interpolated from the standard curve and expressed as pg/ml. The IL-6 curve indicates that the sensitivity for IL-6 standard is 4.3 pg/ml for SIF-p-Chips and 107 pg/ml for non-SIF-p-Chips, a 25-fold improvement. In recent studies, Liao et al. reported that the serum level of IL-6 in healthy people is 4.08 ± 0.28 pg/ml [26] and Edgell reported a level of 31 ± 9 pg/ml in healthy people [27]. In addition, the IL-6 level can be elevated up to tenfold in cancer patients [26,27]. The assay sensitivity with SIF-p-Chips in our study is 4.3 pg/ml. Thus, it is in the range of detecting serum IL-6 in healthy individuals and even more suitable for cancer patients.

To test the SIF-p-Chip-based immunoassay in a complex environment, we used normal human serum spiked with 50 ng/ml recombinant human IL-6. The spiked serum was then serially diluted (1:5, 1:10, 1:20 and 1:40, respectively) in PBS. For both the non-SIF and SIF-p-Chip-based assays, the linearity of dilution was confirmed with a R^2 greater than 0.99 (Fig. 4c). In addition, the fluorescence enhancement by SIF was consistently observed in the range from 10.7 to 14.9-fold (Fig. 4d). To determine the recovery rate after spiking, the IL-6 level was calculated based on the IL-6 standard curve (Fig. 4a) and compared to the expected level. For non-SIF chips, the recovery rate was in the range from 105% to 128%, while for SIF chips the recovery rate was between 97% and 114% (Table 1). The results suggest that the p-chip-based IL-6 assay works well in a complex environment such as spiked serum.

It should be noted that the assay sensitivity is influenced by signal strength, affinities of binding molecules used, fluorescence background, structure of the polymer on the solid phase, design of the analytical instrument to measure the fluorescence intensity, and others factors. In this report, we only concentrated on enhancing the fluorescence signal strength. Thus, it is anticipated that the sensitivity of the assays can be further improved by using a more sensitive detection method, reducing fluorescence background by optimizing the polymer surface on SIF-p-Chips and screening for antibodies demonstrating the highest affinity in p-Chip-based assays.

DNA hybridization assay

We also performed a simple DNA-based assay on SIF-derivatized p-Chips. The assay involved a determination of whether the target DNA (here: an oligonucleotide) carried a mutation in the human cystic fibrosis transmembrane conductance regulator gene. The target oligonucleotide contained the mutant allele of the W1282X mutation, one of the 23 mutations in the basic panel used in many CF tests [19]. The probe oligonucleotide-linked on p-Chips is specific to the

target oligonucleotide. We also used a control oligonucleotide designed to be only specific to the wild type allele.

First, a protein layer of avidin was built by conjugation to the polymer surface of SIF-p-Chips or non-SIF-p-Chips. The biotinylated oligonucleotide probe was then bound to the avidin protein layer, and the Cy3-labeled target oligonucleotide hybridized to the probe-linked p-Chips. We tested different concentrations of the target oligonucleotide to derive a standard curve (Fig. 5a). The fluorescence enhancement was in the range from 5.5 to 50-fold (Fig. 5b). The assay sensitivity was determined to be 1.7 nM for the non-SIF-p-Chips and 0.05 nM for the SIF-p-Chips, a 38-fold enhancement (Fig. 5a).

The purpose of using the avidin layer was to create a short distance between the oligonucleotides and SIF to avoid quenching effects when the reporting fluorophores are too close to the SIF [28]. In our test, we observed that SIF-induced fluorescence enhancement is significantly reduced without the avidin layer (data not shown). We also noticed that the fluorescence enhancement is dependent on the concentration of target oligonucleotide with the greatest enhancement observed at 0.5 nM (Fig. 5b). The mechanism responsible for this observation is still under investigation.

Conclusions

The present study was focused on testing colloidal silver nanoparticles as a vehicle to enhance fluorescence intensity on a flat surface through plasmon resonance. We tested various fluorophores and showed that the application of SIF to the p-Chip-based assay enhanced fluorescence intensity from five to 50-fold. The increase of fluorescence intensity of the p-Chips led to a clear improvement in the assay sensitivity: a 25-fold increase over the method not involving a SIF in the IL-6 immunoassay, and a 38-fold increase in the DNA hybridization assay. The SIF-p-Chip-based assay has clearly great potential in extending multiplex assays to higher sensitivities. It is expected that the methods developed here are applicable to other types of multiplex assays, such as those based on microarrays or microbeads both in basic research as well as clinical applications.

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Fig. 1. Silver nanoparticles on the surface of bare p-Chip coated and not coated with SIF. Non-electronic sides of the p-Chips are shown. No polymer coating was applied to the p-Chips in panels **a** and **b**. **a** Bright field images of p-Chips. **b** AFM images of p-Chips. The sizes of most silver particles are in the range from 100 to 200 nm. Aggregates of silver particles can also be seen. **c** SEM image of an APTS/GPTS-coated p-Chip deposited with SIF

Fig. 2.

Fluorescence enhancement for different fluorophores. SIF was deposited on APTS/GPTS-coated p-Chips. The p-Chips were incubated with 50 $\mu\text{g/ml}$ GFP (**a**) or 10 $\mu\text{g/ml}$ AF555-labeled BSA (**b**). Fluorescence was greatly enhanced on SIF-p-Chips (*p-Chips on the right*) compared to non-SIF-p-Chips (*p-Chips on the left*). In the control (*Ctrl*) group, SIF-p-Chips were incubated in PBS. Panel **a** magnification 40 $\times$, exposure time 15 s. Panel **b** magnification 40 $\times$, exposure time 50 ms. Experiments were repeated at least three times; representative p-Chips are shown

Fig. 3.

Fluorescence intensity decay rates of AF555 on SIF-p-Chips. SIF-p-Chips were incubated with 50 µg/ml AF555-BSA (**a**) or (**b**) 0.08 µg/ml AF555-BSA. *Left panels* no SIF was applied. *Right panels* SIF was deposited on the p-Chips. *Blue lines* represent the fluorescence intensity decay curves. *Black lines* are the best multi-exponential fits. *Red profile* is an instrument response function (IRF) taken from the scatter at the 470 nm excitation wavelength

Fig. 4.

The application of SIF in p-Chip-based IL-6 immunoassay. **a** Standard curve for the IL-6 assay. Anti-human IL-6 monoclonal antibody was conjugated to the polymer surface of SIF-p-Chips and non-SIF-p-Chips. The anti-IL-6 conjugated p-Chips were used to capture the recombinant human IL-6. The background signal was determined using PBS in the capture step. Biotinylated anti-human IL-6 antibody was then used for detection, followed by incubation with SA-PE. The background signal (PBS control) from non-SIF or SIF-p-Chips was subtracted. Error bar: standard deviation from $n=3$ or 4 p-Chips. **b** The fluorescence enhancement by SIF. **c** The linearity of dilution in spiked serum. Normal human serum was spiked with 50 ng/ml recombinant human IL-6 and serially diluted (1:5, 1:10, 1:20, and 1:40) in PBS. The IL-6 immunoassay on non-SIF and SIF-p-Chip was performed as described in (a). The linearity of dilution was then determined. **d** The fluorescence enhancement by SIF using the spiked human serum. ^aFluorescence enhancement is the ratio of fluorescence intensity of SIF-p-Chips to that of non-SIF-p-Chips

Fig. 5.

The effect of SIF in a p-Chip-based DNA assay. Avidin was conjugated to the polymer surface of SIF-p-Chips and non-SIF-p-Chips. Biotinylated oligonucleotide probe was then attached to the avidin-conjugated p-Chips, and a Cy3-labeled target oligonucleotide was hybridized to the conjugated p-Chips. A probe that had a single mismatch to the target oligo was used as a negative control (data not shown as fluorescence intensities were very low, typically less than 10% of that found in corresponding experimental groups). As a control to determine the background signal, no target oligonucleotide was used in the hybridization step. The Cy3 fluorescence intensity (a) and fluorescence enhancement (b) were determined and plotted. The background signal (no target control) from non-SIF or SIF-p-Chips has been subtracted from fluorescence intensity data. *Error bar* indicated the standard deviation calculated from fluorescence intensity measurements for $n=3$ or 4 p-Chips. ^aFluorescence enhancement is the ratio of fluorescence intensity of SIF-p-Chips to that of non-SIF-p-Chips

Table 1

Recovery of dilution experiment

	Dilution	Expected (ng/ml)	Observed (ng/ml)	Recovery (%)
non-SIF	1:5	10	10.47	105
	1:10	5	5.60	112
	1:20	2.5	3.19	128
	1:40	1.25	1.38	110
SIF	1:5	10	9.71	97
	1:10	5	5.64	113
	1:20	2.5	2.84	114
	1:40	1.25	1.32	106

Normal human serum was spiked with 50 ng/ml recombinant human IL-6. The observed value for the recovery of dilution was determined from the IL-6 standard curve (Fig. 4a)