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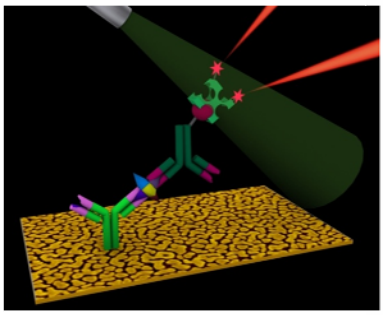

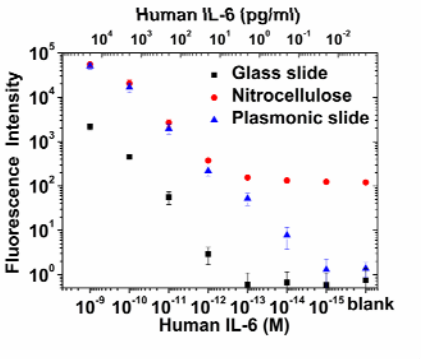

Fluorescence-enhanced microarray by plasmonic gold films was developed for multiplexed cytokine detection with up to three orders of magnitude higher sensitivity than on conventional nitrocellulose, glass substrates and ELISA. A panel of 6 cytokines (VEGF, IL-1β, IL-4, IL-6, IFN-γ, and TNF) were detected in the culture media of cancer cells. This approach could pave a new way for better proteomic analysis and disease diagnosis.

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ABSTRACT

Protein microarrays based on fluorescence detection have been widely utilized for high-throughput functional proteomic analysis. However, a drawback of such assays has been low sensitivity and narrow dynamic range, limiting their capabilities especially for detecting low abundance biological molecules such as cytokines in human samples. Here, we present fluorescence-enhanced microarrays by plasmonic gold films for multiplexed cytokine detection with up to three orders of magnitude higher sensitivity than on conventional nitrocellulose and glass substrates. Cytokine detection on the gold plasmonic substrate is about 1 to 2 orders of magnitude more sensitive than enzyme-linked immunosorbent assay (ELISA) and can be multiplexed. A panel of 6 cytokines (VEGF, IL-1β, IL-4, IL-6, IFN-γ, and TNF) are detected in the culture media of cancer cells. This work establishes a new method of high throughput multiplexed cytokine detection with higher sensitivity and dynamic range than ELISA.

KEYWORDS

microarray · cytokine · plasmonic · multiplex· near infrared fluorescence

Cytokines are low molecular weight proteins that act as mediators for communication between immune cells. Defects in the regulatory networks may cause abnormal expression of cytokine levels and result in a number of diseases, suggesting that cytokine sensing may be a valuable tool for diagnosis and prognosis of human diseases including the detection of cancer at early stages [1]. Currently, enzyme-linked immunosorbent assay (ELISA) has been the gold standard for protein quantification with sensitivity down to pg/ml level [2-3]. However, ELISA requires relatively large sample volumes and cannot be used for multiplexed high-throughput screening. Luminex xMAP, a bead array coupled with discrete fluorophores has been applied for cytokine

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profiling and can theoretically detect up to 100 cytokines with a detection limit similar to ELISA [4-6].

Protein microarray assays through fluorescence detection have found numerous applications [7-14], which has been constructed on various substrates, including nitrocellulose [15], glass slides [16], aldehyde modified glass [17], epoxy coated glass [18] and BSA-NHS coated glass [19]. However, such microarrays have afforded lower sensitivity and dynamic range over ELISA thus far.

Here, we demonstrate multiplexed cytokine detection on plasmonic gold films for vastly improving the sensitivity of cytokine measurement. Due to the vast library of cytokines and their low level in human serum, which is near or below the detection limit for ELISA [20], development of a high-throughput cytokine microarray assay with improved sensitivity for cytokine detection represents a significant step forward for both research and diagnostic applications.

A tortuous gold film comprised of gold nano-islands with abundant nanogaps (Figure 1a) was synthesized on glass slide using a solution phase method [21]. The resulting gold film exhibited surface plasmon resonances in the NIR region (Figure 1b), capable of enhancing the fluorescence intensity of several NIR fluorophores placed atop including Cy5, IRDye800 and single-walled carbon nanotubes [21-25]. For cytokine detection, we chemically modified the plasmonic gold film by covalently conjugating 6-arm branched poly(ethylene glycol) (PEG)-amine polymer stars to a self-assembled monolayer of mercaptohexadecanoic acid on the gold surface. The remaining free amine groups on the PEG star were converted to carboxylic acid groups through modification by succinic anhydride, and were subsequently activated through reaction with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS), resulting in a layer of NHS groups for conjugating to the amine groups and immobilization of capture antibodies specific for cytokines (Figure 1c). This was a novel functionalization method capable of efficient immobilization of capture antibodies while increasing the hydrophilicity of the plasmonic gold film through PEG-stars to minimize nonspecific binding (NSB) effects. Enhancement of specific binding signals through a physical principle, i.e., plasmonic fluorescence enhancement and chemical minimization of background signals by PEG-star blocking of NSB are important factors contributing to sensitive cytokine detections (Figure S1).

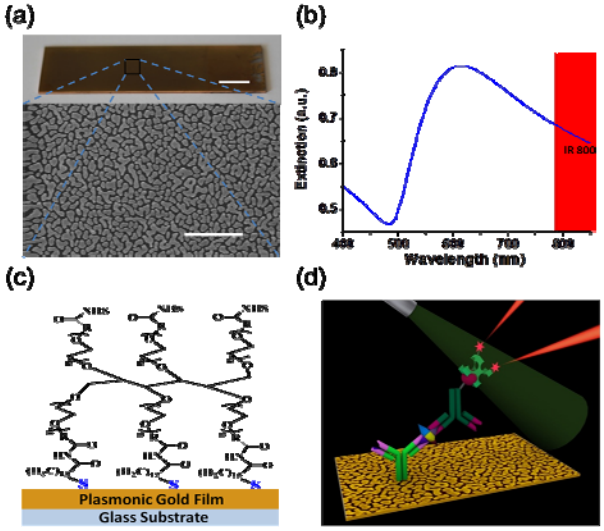


Figure 1 Develop plasmonic gold substrate for antibody based cytokine microarray assay. (a) Scanning electron micrograph (scale bar, 1 μm) of a solution-phase grown gold film on glass showing the nanoscopic gold island morphology. Top inset shows digital photograph of an plasmonic Au film-coated glass slide with scale bar, 1 cm. (b) Extinction spectrum of plasmonic gold substrate overlaid with the excitation (line) and emission (shaded area) regions of IR800 dye. (c) Schematic of multilayer surface chemistry on gold film, which affords biocompatibility of the substrate and binding sites for immobilizing capture antibodies. (d) Schematic of 4 layer antibody based cytokine microarray assay strategy.

Cytokine measurement was performed using a 4-layer immunoassay approach (Figure 1d). First, capture antibodies specific for human cytokines (VEGF, IL-1β, IL-4, IL-6, IFN-γ or TNF) were immobilized by contact printing onto a PEG-star coated plasmonic gold substrate through the

formation of covalent amide bonds between the amine groups on the capture antibodies and EDC/NHS activated carboxylic acid group on the PEG-stars. A blocking step was added by treating the substrate with a fetal bovine serum (FBS) solution in PBS solution. Second, the substrate was exposed to PBS solutions containing 10% FBS and serially diluted cytokine in the 1 fM (~0.02 pg/ml) -1 nM (~20000 pg/ml) concentration range together with a blank control. For the third and fourth layers, biotin conjugated antibodies specific for the cytokine was incubated followed by incubation with IRDye800 labeled streptavidin. Fluorescence detection of IRDye800 was then performed using a commercial scanner. For each of the VEGF, IL-1β, IL-4, IL-6, IFN-γ and TNF cytokines, we observed a dynamic range > 5 orders of magnitude with excellent linearity (Figure 2 and S2-S6). Cytokine measurement reached down to ~ 1 pg/ml detection limit defined as the concentration corresponding to the blank signal plus two standard deviations. The cytokine measurement results were consistent from batch to batch (Figure S7). Note that the sensitivity and specificity of cytokine detection depends highly on the antibodies used. We have selected the antibody pairs (from R&D systems and Peprotech Inc.) with the minimum non-specific binding effects and cross-reactivity for the current work.

The same assays were performed on commercial nitrocellulose slides and glass slides to compare with the plasmonic gold substrate. Owing to higher fluorescence of the IRDye800 by up to ~100-fold, cytokine assays on gold afforded 2-3 order broader dynamic range compared to assays on glass (Figure 2). Nitrocellulose has been the substrate of choice for biological assays due to high surface area and porous structures, affording higher protein immobilization and binding capacity and thus increasing signal intensities. However, the relatively high background on nitrocellulose caused by autofluorescence of nitrocellulose and the lack of chemistry for blocking nonspecific binding limited the dynamic range and sensitivity of cytokine detection (Figure 2 and Figure S8). Notably, switching from the widely used Cy5 dye to IRDye800 for fluorescence labeling of cytokines on nitrocellulose improved the sensitivity by nearly an order of magnitude due to reduced autofluorescence in the NIR region (Figure S8). However, the detection sensitivity for cytokine was still at least an order of magnitude lower than on the plasmonic gold substrate (Figure 2).

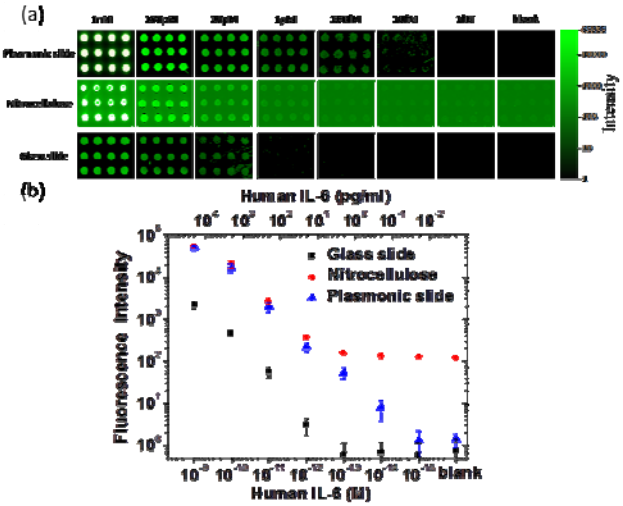


Figure 2 Antibody based cytokine microarray assays on plasmonic gold film, nitrocellulose slide and glass slide. (a) Top panel: fluorescence maps **(in** log **scale for the intensities)** for microarray on plasmonic substrate generated by integration of streptavidin-IRDye800 fluorescence emission under a 785 nm excitation for different concentrations (12 duplicate spots for each concentration) of human Interleukin-6 spiked into PBS solution with 10% FBS. Middle Panel: fluorescence maps on the same intensity scale as the top panel for comparison, generated in an identical fashion on a nitrocellulose slide. Lower panel: fluorescence maps on the same intensity scale as the top panel for comparison, generated in an identical fashion on a glass substrate. (b) Calibration curves for IL-6 quantification generated by averaging the integrated fluorescence intensity of streptavidin-IRDye800 emission over the 12 duplicate microarray spots for each of the cytokines at various concentrations on plasmonic gold slide, nitrocellulose slide and glass slides respectively. Error bars represent the standard deviation of the mean over the 12 duplicate assay features.

Cytokine assays on plasmonic Au substrates (Figure 2 and Figure S2-S6) were about an order of magnitude more sensitive than ELISA data provided by the ELISA kit manufacturer (Table 1). Nevertheless, we performed our own ELISA measurements in parallel with the assay on gold using the same reagents as used for the Au-arrays for each cytokine and obtained somewhat lower sensitivity for ELISA than data provided by the manufacturer (Table 1 and Figure S9).

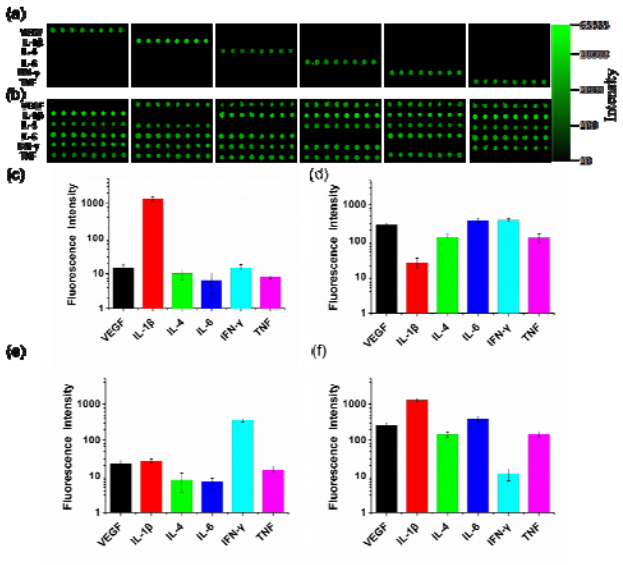
Table 1. Detection limit of cytokine sensing through different approaches

Cytokine	ELISA sensitivity (pg/ml)	Nitrocellulose sensitivity (pg/ml)	Plasmonic gold slide sensitivity (pg/ml)	Glass slide sensitivity (pg/ml)
VEGF	100 (vendor) 331 (this work)	91	4.8	192
IL-1β	1 (vendor) 4 (this work)	0.8	0.07	9
IL-4	10 (vendor) 29 (this work)	7	1.3	52
IL-6	0.7 (vendor) 19(this work)	2.3	0.06	10.15
IFN-γ	8 (vendor) 36.1 (this work)	3.2	0.25	27
TNF	4.4	3.67	0.47	41

Next, we performed multiplexed cytokine detection on plasmonic gold substrates and focused on investigating the selectivity of detection. Capture antibodies for VEGF, IL-1β, IL-4, IL-6, IFN-γ, and TNF were printed in a 6×8 spot matrix format with each row containing one type of capture antibody in replicates of 8 (Figure 3). Following incubation of a single cytokine or a cocktail of mixed cytokines spiked into 10% FBS/PBS solution as analytes, a mixture of biotin conjugated detection antibodies for VEGF, IL-1β, IL-4, IL-6, IFN-γ and TNF were incubated on the cytokine chip. IRDye800-labeled streptavidin was then applied as the last layer for fluorescence labeling and detection.

To demonstrate specificity of the multiplexed cytokine array, solutions containing a single cytokine were first measured at the ~10 pg/ml (1 pM) level (near the detection limit of ELISA). Only the corresponding row of the microarray showed bright fluorescence signal (Figure 3a) after exposure to the cytokine, a mixture of all six biotinylated detection antibodies and IRDye800 labeled streptavidin. This clearly suggested high specificity of the array. A high positive to negative signal ratio of 10 -100 at ~10 pg/ml (1 pM) cytokine concentration (Figure 3c and Figure 3e) were measured, outperforming on nitrocellulose or glass slides where positive signals were only slightly higher than the negative signal or the background level (Figure S10).

Figure 3 Specificity test for multiplexed cytokine assay. (a)



From left to right: fluorescence images showing spots at a certain row emitting clear fluorescent signals when a multiplexed antibody chip (6 rows, 6 different antibodies against cytokines labeled at the left of the image) was incubated in a solution containing the corresponding cytokine at 1 pM (10 pM for VEGF) without any other cytokines. (b) From left to right: images of the same multiplexed antibody array as in (a) showing 5 brightly fluorescent rows with one dark row after the array was incubated in a solution containing corresponding 5 cytokines at 1 pM (10 pM for VEGF) to the bright rows. (c) Averaged fluorescence intensity over spots in each row of capture antibodies when only IL-1β was incubated on the antibody microarray at 1 pM. (d) Averaged fluorescence over spots in each row when a cocktail (without IL-1β) of VEGF, IL-4, IL-6, IFN-γ, and TNF were incubated on the microarray at 1pM each (10 pM for VEGF). (e) Averaged fluorescence intensity over spots in each row of capture antibodies when only IFN-γ was incubated on the antibody microarray at 1 pM. (f) Averaged fluorescence over spots in each row when a cocktail (without IFN-γ) of VEGF, IL-1β, IL-4, IL-6, TNF were incubated on the microarray at 1 pM each (10 pM for VEGF).

An additional specificity test was performed by incubation of the multiplexed microarrays on gold to a mixture of five cytokines spiked into 10% FBS/PBS with the sixth cytokine excluded. In this case, five rows of the spotted capture antibodies exhibited bright fluorescent spots with one missing row corresponding to the excluded sixth cytokine (Figure 3b, Figure 3d and Figure 3f). These results clearly demonstrated the capability of performing cytokine assays on plasmonic gold substrates in a multiplexed format with high specificity.

As an application of the arrays on plasmonic gold substrates, we measured cytokine expression levels in conditioned media collected from the culture medium of several cancer cell lines. The ovarian cancer OVCAR3 cell line was cultured for 48 hours, after which the cell culture medium was collected, and the concentration of each secreted cytokine was measured with an antibody-based cytokine microarray on a plasmonic gold slide against a calibration curve obtained with each cytokine spiked into fresh cell medium in 100 pM - 1 pM (2000-20 pg/ml) concentrations.

Out of the 6 cytokines, VEGF, IL-6 and TNF were clearly detected in the OVCAR3 cell conditioned medium compared to fresh cell medium as blank control (Figure 4a). Concentrations of the 3 cytokines were ~770 pg/ml (40 pM) for VEGF, ~510 pg/ml (25 pM) for IL-6 and ~32 pg/ml (1.8 pM) for TNF based on the calibration curves simultaneously obtained on the same chip (Figure S11). Detection of cytokine expression levels in the cell culture medium from the ovarian cancer SKOV3 cell line were also performed in a similar manner. In this case, only VEGF and IL-6 expressions were detected at ~1030 pg/ml (53 pM) and ~890 pg/ml (44 pM) respectively (Figure S12).

Microarrays on nitrocellulose slides and glass were also constructed for cytokine detection in cell media. Compared to the arrays on plasmonic gold substrate, the background intensity on nitrocellulose was higher, and the spots corresponding to fresh medium negative control showed non-specific signals higher than the background (Figure 4a and Figure 4f). Due to the low signals on glass slides, only IL-6 can be quantitatively measured in the cell media (Figure S13).

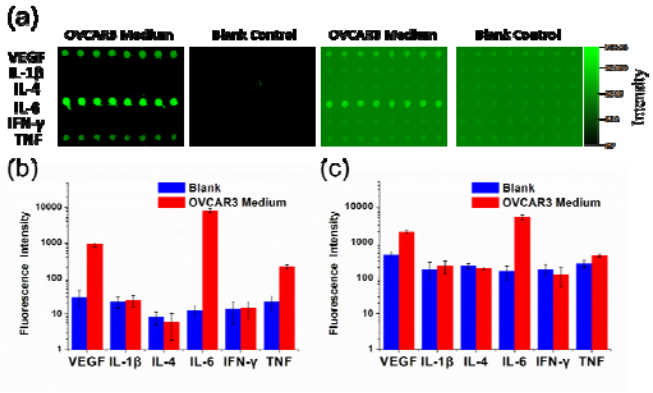


Figure 4 Multiplexed cytokine measurements in conditioned cell culture medium of ovarian cancer OVCAR3 cells. (**a**) Fluorescence maps generated by integration of streptavidin-IRDye800 fluorescence emission at 785 nm excitation for a 6-plexe antibody (against VEGF, IL-1β, IL-4, IL-6, IFN-γ and TNF) microarray after exposure to a culture medium conditioned with OVCAR3 cells for 48 h. A fresh cell medium without any cells was used as the blank control. Measurements on plasmonic gold slide (left two images) and nitrocellulose slide (right two images) were performed side by side. (**b**) Averaged fluorescence of each row of antibody spots corresponding to the images recorded on plasmonic gold substrate in (a). (**c**) Averaged fluorescence of each row of antibody spots corresponding to the images recorded on nitrocellulose substrate in (a).

Biomarkers shed from cancerous tissue as well as species inherent to a patient's immune response such as cytokines may provide information about disease progression and therapeutic efficacy. For example, carbohydrate antigen 125 (CA-125) [26] and carcinoembryonic antigen (CEA) [27] are clinically employed protein biomarkers for diagnosis and therapeutic monitoring of cancer. Angiogenesis is a key feature of tumor development in which multiple cytokines are involved. Cytokine profiling could be correlated to various cancer types and stages, making it potentially useful for early diagnosis of cancer [28]. It has been reported that detection of a panel of

cytokines including IL-6, VEGF, TNF, M-CSF, MCP-1 may present a diagnostic value for ovarian cancer detection [29-32]. For these reasons, highly sensitive, multiplexed cytokine detections on our plasmonic substrates could facilitate cancer research and diagnosis and prognosis.

Measurement of IL-6 levels in the cell culture medium for the OVCAR3 cell line after 48 hours has been measured by ELISA previously to be ~ 400 pg/ml [33], which agrees well with our measurement on gold (~500 pg/ml). The expression level of TNF in the OVCAR3 cell line has not been reported, while a low concentration of ~ 32 pg/ml (1 pM, near the ELISA detection limit) is detected on our gold substrates, which highlights the potential of our technique for sensitive cytokine detection in cancer. Multiplexed cancer biomarker panels may provide valuable diagnostic and staging information compared with assays for a single cancer biomarker [34-35].

In conclusion, we have developed a novel multiplexed cytokine detection method based on fluorescence enhancing plasmonic gold film with higher sensitivity and dynamic range than ELISA. Owing to the large fluorescence enhancement afforded by plasmonic gold substrate, pg/ml to sub-pg/ml sensitivity for multiplexed cytokine detection was realized. Microarray aimed at a panel of 6 cytokines (VEGF, IL-1β, IL-4, IL-6, IFN-γ, and TNF) was designed and applied for detection of cytokines concentration in the cell culture media of cancer cells, establishing multiplexed cytokine sensing on plasmonic gold slides in a wide dynamic range of 1 pg/ml -10^4 pg/ml (10 fM – 1 nM). The cytokine chips based on plasmonic gold substrate require no additional reagents, no specialized equipment, and no changes to currently adopted microarray protocols, which could be easily adopted by general scientists and clinicians. It could pave a new way for better proteomic analysis and disease diagnosis.

Acknowledgements

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Electronic Supplementary Material: Reagents, synthesis of gold substrate and following surface chemistry, microarray procedure for cytokine measurement, fluorescence mapping and analysis, culture of cancer cell lines, ELISA measurement result is available in the online version of this article at http://dx.doi.org/10.1007/s12274-***-****-* (automatically inserted by the publisher).

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