Supporting Information:

Plasmonic Micro-Beads for Fluorescence Enhanced, Multiplexed Protein Detection with Flow Cytometry†

Bo Zhang,[ac] Jiang Yang,[ac] YingpingZou,[bc] Ming Gong,[a] Hui Chen,[a] Guosong Hong,[a] Alexander L. Antaris,[a] Xiaoyang Li,[a] Chien-Liang Liu,[a] Changxin Chen[a] and Hongjie Dai*[a]

[a]Department of Chemistry, Stanford University, Stanford, California 94305, USA. E-mail: hdai@stanford.edu

[b]College of Chemistry and Chemical Engineering, Central South University, Changsha 410083, China

[c]These authors contribute equally to the work.

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Experimental Section

Materials. [3-(2-aminoethylamino)propyl]trimethoxysilane, chloroauric acid trihydrate, hydroxylamine HCl, sodium borohydride, Cysteamine, Avidin were purchased from Sigma-Aldrich. Ammonium hydroxide (30% ammonia) and Hyclone fetal bovine serum were purchased from Fisher Chemicals. Purified cytokine antigen standards for VEGF (Cat # 293-VE-010), IL-1β (Cat # 201-LB-005), IL-6 (Cat # 206-IL-010) and IFN-γ (Cat # 285-IF-100) were purchased from R&D systems. Purified CA-125 (Cat # 30-AC11) antigen was purchased from Fitzgerald industries. Sandwich Antibody pairs for IL-1β (Cat # BAF201 and MAB601), IL-6 (Cat # BAF206 and MAB610), and IFN-γ (Cat # BAF285 and MAB2852) were purchased from R&D systems. Sandwich antibody pairs for VEGF (Cat # 500-M88 and 500-P10Bt) were purchased from Peprotech, inc. Sandwich antibody pair for CA-125 was purchased from Fitzgerald industries. Cy5-NHS and Cy3-NHS esters were purchased from GE-healthcare. Alexa fluor 488-NHS ester was purchased from Invitrogen Life Technologies Corporation. 6-armed poly(ethylene glycol)-amine was purchased from SunBio. Sulfo-SMCC and Biotin-NHS ester were purchased from Thermo Scientific.

Synthesis of plasmonic gold nano-island coated glass bead. 800 mg 8 micron glass beads were dispersed in 10 mL ethanol with 10% [3-(2-aminoethylamino)propyl]trimethoxysilane and stirred overnight, resulting in amine modified glass beads. The beads were washed 3 times with centrifugation at 1000 rcf and resuspension in 50 mL DI water, resulting in a glass bead solution at 16 mg/ml. 2.5 mL of the bulk solution was diluted with 7.5 mL water, and 100 μL 0.1 M $HAuCl_4$ and 15 μL Ammonium hydroxide were added successively. The reaction last for 20 min with stirring. After this, the beads were washed 3 times with centrifugation at 150 rcf and resuspension in water. The glass beads with Au clusters (1.6 mg/ml, 10 mL) were diluted to 1.6

mg/ml and 24 μL freshly prepared $NaBH_4$ (0.1 M) was introduced into the solution. The reduction process lasted for 10 min with stirring. The beads with reduced gold seeds were washed for 3 times with centrifugation at 3000 rcf and resuspension in water. The beads were diluted to 8 mL solution with bead concentration at 0.8 mg/ml. The same amount of $HAuCl_4$ and Hydroxylamine was added to the solution, resulting in $HAuCl_4$ concentrations at 45 - 160 uM. The reaction was kept for 10 min with stirring to allow gold nano-island growth on Au seeds. The gold beads were washed 3 times with centrifugation at 3000 g and resuspension in water.

Single-plex immunoassay on plasmonic bead. 100000 plasmonic beads (counted by flow cytometry) were coated with avidin by soaking the beads in 1 μM avidin/PBS solution at 4C overnight, followed by washing with PBS solution for 3 times. The avidin coated plasmonic beads were incubated with 100 nM biotinylated mouse anti IL-6 capture antibody for 3 h, followed by washing twice with PBST and once with PBS. The beads were later blocked with biotinylated branched PEG and then 20 % FBS in PBS. 5000 IL-6 capture antibody labeled beads were distributed into each well, and 100 μL serial dilution of IL-6 antigen from 1 nM to 10 fM in PBS solution with 20% FBS was added to each well, incubated for 2 h at room temperature and washed twice with PBST and once with PBS. 100 μL 10 nM Cy5 labeled mouse anti IL-6 detection antibody in PBS with 20% FBS was added to each well, incubated at room temperature for 1h in dark and washed twice with PBST and once with PBS.

Construction of 6-plex plasmonic bead system for multiplexed protein sensing. Plasmonic beads were mixed with 10 μM fluorophore-NHS ester in DMSO and incubated at room temperature for 2 h in dark, during incubation the fluorophore was linked with free amine groups between the gaps of the gold island on each bead. The fluorophore labeled beads were then coated with avidin by soaking the beads in 1 μM avidin/PBS solution at 4C overnight, followed

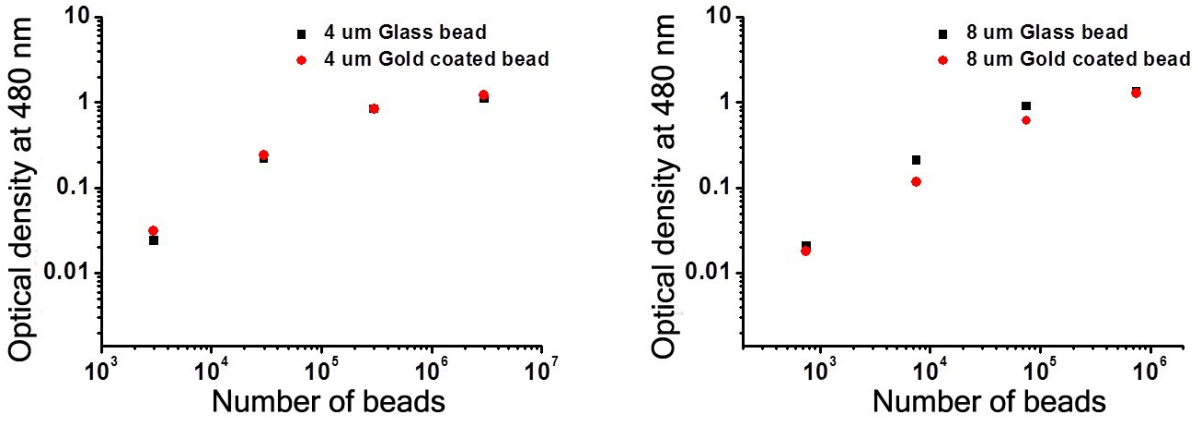
by washing with PBS solution. The avidin coated plasmonic beads were incubated with 100 nM biotinylated capture antibody for 3h, washed and blocked with biotinylated branched PEG and 20 % FBS in PBS. 8 micron beads with CA-125 capture antibody, Alexa 488 coded 8 micron beads with IFN-gamma capture antibody, Cy3 coded 8 micron beads with IL-6 capture antibody, 4 micron beads with VEGF capture antibody, Alexa 488 coded 4 micron beads with IL-1 beta capture antibody and Cy3 coded 4 micron beads with PEG for negative control were constructed by following the procedure above and were mixed together to form the 6-plex plasmonic bead system.

Fluorescence quantification of bead based immunoassay by flow cytometry. Cy5 fluorescence distribution for plasmonic beads of each well was quantified with Scanford flow cytometry system from Stanford Shared FACS Facility. Scanford flow cytometry system was equipped with 3 lasers: 488 nm blue laser (30 mW), 640 nm red laser (100 mW) and 561 nm yellow /green laser (100 mW). Front scattering and side scattering of 488 nm laser by the bead was used to correlate with bead size. A combination of 488 nm laser (gain setting at 600) and 525/50 nm band pass filter was used to quantify Alexa 488 fluorescence. A combination of 561 (gain setting at 600) nm laser and 590/20 nm band pass filter was used to quantify Cy3 fluorescence. A combination of 640 nm laser (gain setting at 600) and 710/50 nm band pass filter was used to quantify Cy5 fluorescence.

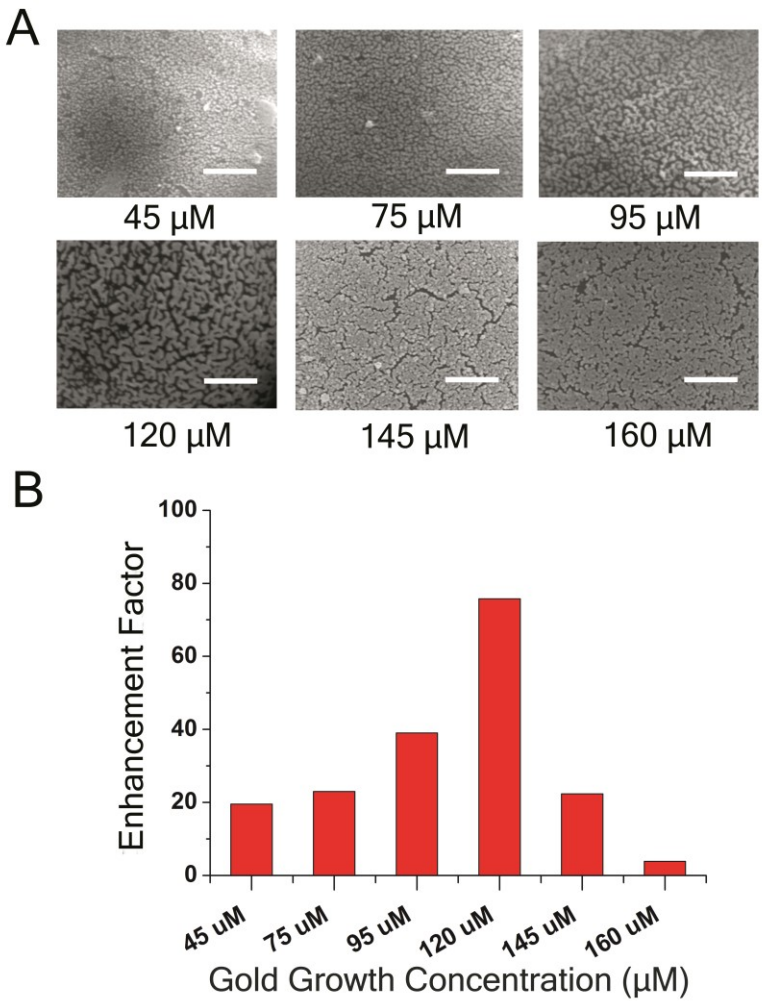
OVCAR-3 and SKOV-3 cell culture. SKOV-3 cells were cultured in McCoy's 5A Medium with L-glutamine, and OVCAR-3 cells were cultured in RPMI Medium 1640 with L-glutamine. Both culture media were supplemented with 10 % fetal bovine serum, 100 $IU \bullet mL^{-1}$ penicillin

and 100 μg/mL streptomycin. Cells were maintained in a 37°C incubator with 5% CO_2 for 48 h at 50~60 % confluency, before the supernatant was used for cytokine quantification. As a control, fresh cell medium without cell growing was also used for cytokine quantification.

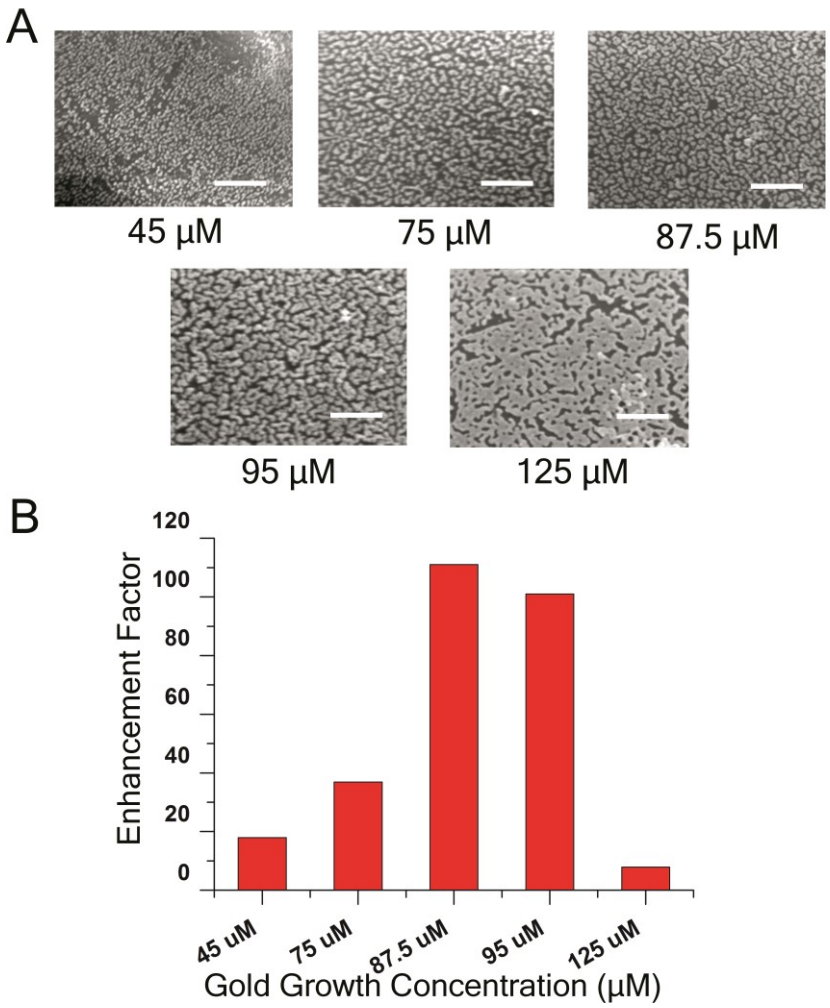
Detection of IL-6 in cell culture media by ELISA. Capture antibodies for IL-6 at concentration of 1.5 μg/ml in sodium carbonate buffer (pH 8.5) were coated on Nunc-Immuno Maxisorp 96-well plates (Thermo Scientific, Rochester NY) at 4C overnight. The plates were washed with PBST for three times and blocked with 10 % FBS/PBST solution for 30 min at room temperature. The plates were washed again with PBST for three times and a two-fold serial dilution of cytokine IL-6 standards, starting from 2500 pg/ml, were loaded in replicates of two in a volume of 50 μl and incubated for 2h at RT, together with cell culture media. After washing the plates with PBST for five times, biotin-conjugated detection antibodies against IL-6 (1.5 μg/mL) and streptavidin conjugated horseradish peroxidase (1:1000 dilution) (BD # 51-9000209) in 10% FBS/PBST solution were loaded into the wells and incubated for 1 h at RT. Plates were washed with PBST for five times and then TMB One-Step Substrate (Dako, Carpinteria, CA) was added for 30 min. The reaction was terminated with 2 M sulfuric acid (Sigma) and Colorimetric values were obtained on a SpectraMAX 190 plate reader at 450 nm (Molecular Devices, Sunnyvale , CA)."



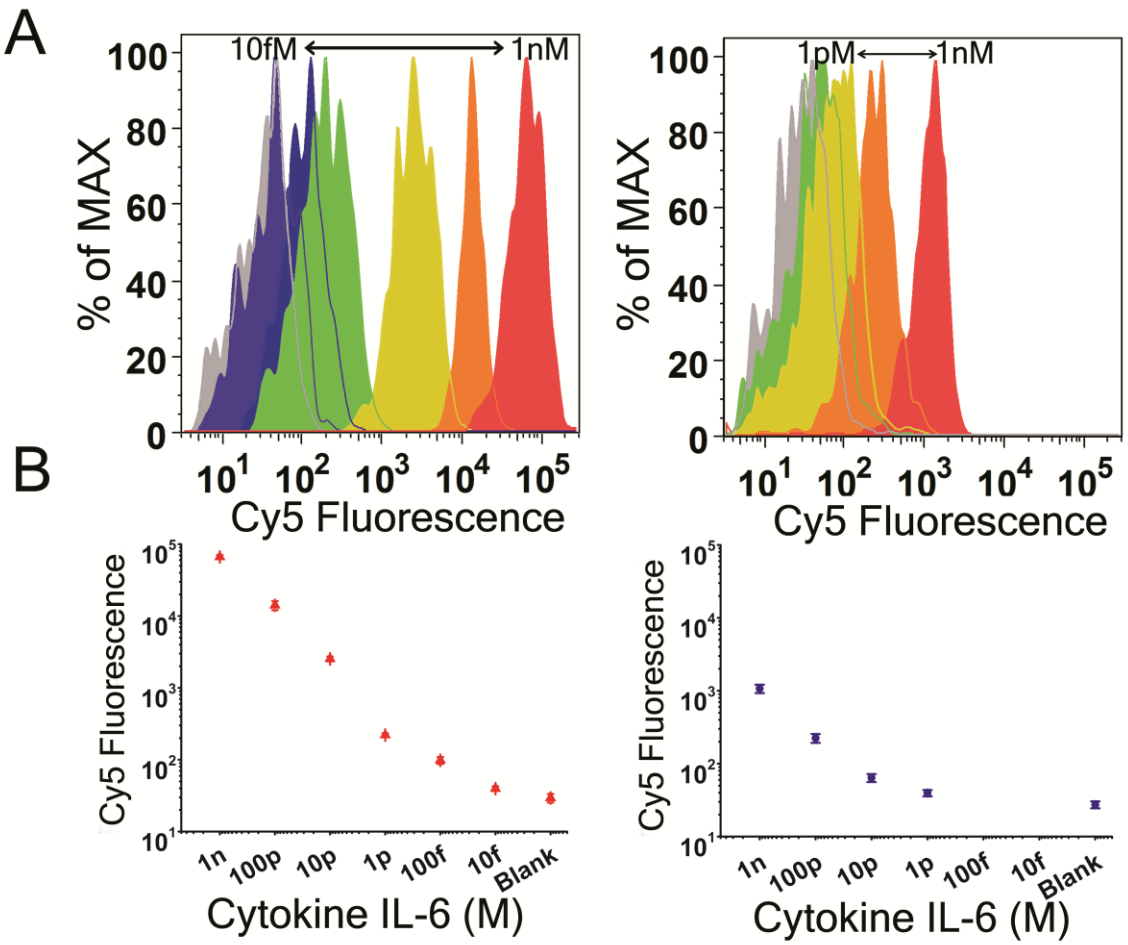
Supplemental Figure 1. Enzymatic activity of biotin-HRP conjugated avidin coated gold bead and glass bead. Left: Avidin coated 4 micron gold bead and glass bead was soaked in PBS solution with 200 nM biotin labeled HRP overnight at 4^{o}C, and washed with PBS for 3 times. HRP labeled 4 micron gold bead and glass bead was each serial diluted by 10 fold to form 4 bead samples with bead number vary from 3 million to 3 thousand. Enzymetic activity of HRP labeled gold bead and glass bead was tested adding 3,3',5,5'-Tetramethylbenzidine (TMB) substrate to each bead sample. Reaction was later stopped by 0.5M H_2SO_4 solution and optical density at 480 nm was recorded for the 8 samples with Tecan spectrafluor plus plate reader. Similar titration curve of 480 optical density for HRP conjugated gold bead and glass bead suggested similar amount of HRP conjugated to the two types of avidin coated bead, which is reflective of similar amount of avidin coating on gold bead and glass bead. **Right:** Same procedure was applied also for 8 micron gold bead and glass bead, except the bead number varied from 750,000 to 750 for HRP test (total surface area is same to 4 micron bead for each dilution).



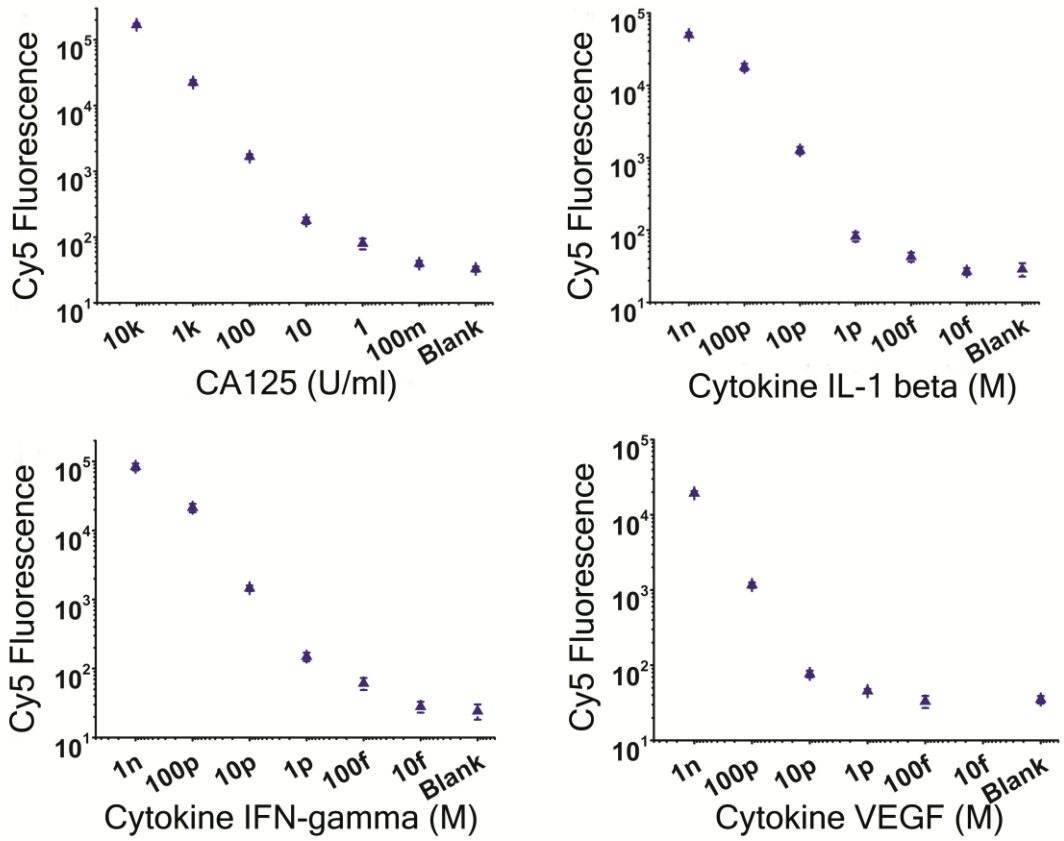
Supplemental Figure 2. Gold growth morphology is dependent on growth concentration of hydrochloroauric acid. A) SEM images (scale bar = 500 nm) of gold island coating on 4 µm glass bead with different hydrochloroauric acid concentration from 45 µM to 160 µM. Higher concentration of $HAuCl_4$ facilitates the growth of each gold seed nanoparticle into gold nano-islands, which ultimately coalesce with each other to form continuous gold film. **B)** Cy5 fluorescence enhancement over glass bead for the gold coated bead in A, reflecting gold island structure with fine-tuned island size and gap afford optimal Cy5 enhancement.



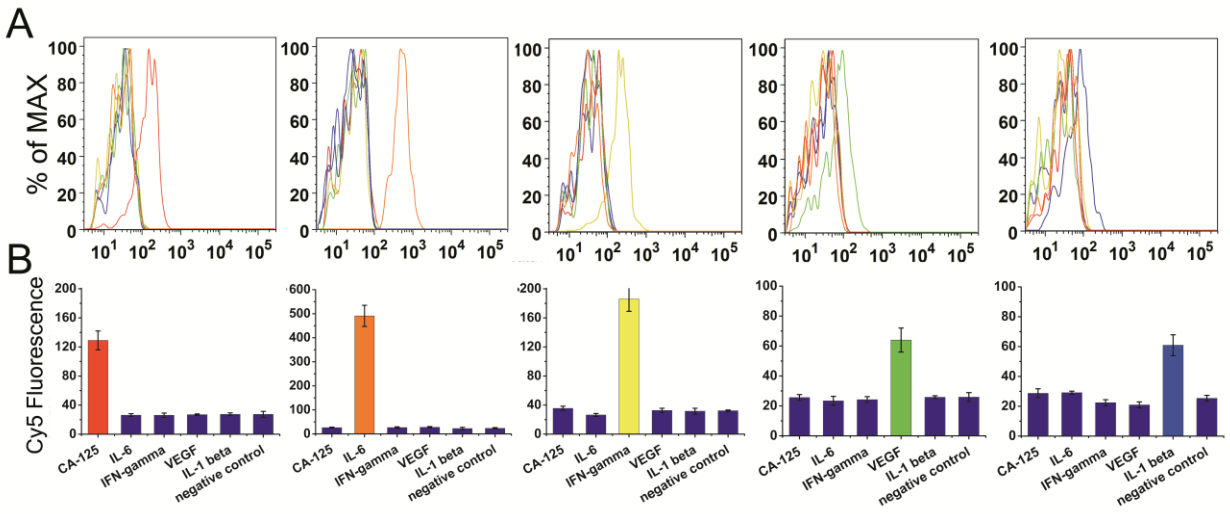
Supplemental Figure 3. Gold growth morphology is dependent on growth concentration of hydrochloroauric acid. A) SEM images (scale bar = 500 nm) of gold island coating on 8 µm glass bead with different hydrochloroauric acid concentration from 45 µM to 125 µM. **B)** Cy5 fluorescence enhancement over glass bead for the gold coated bead in A.



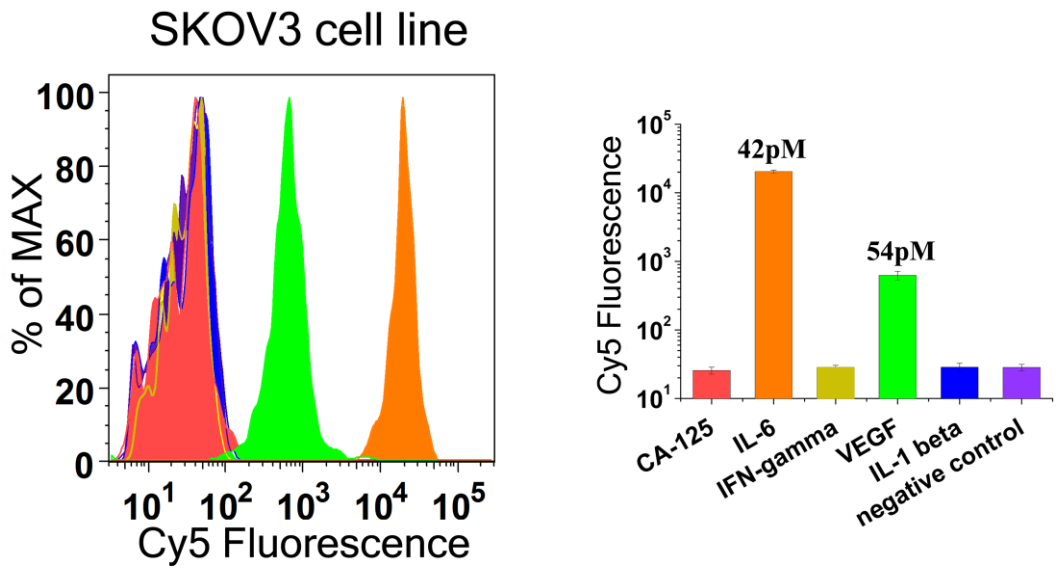
Supplemental Figure 4. Protein quantification on plasmonic bead. A) Human cytokine IL-6 calibration curve on 4 micron plasmonic bead (left) and glass bead (right), showing Cy5 fluorescence distribution when measuring: 1nM IL-6 (red curve), 100 pM IL-6 (orange curve), 10 pM IL-6 (yellow curve), 1pM IL-6 (green curve), 100 fM IL-6 (blue curve), 10 fM IL-6 (purple curve) and blank control (grey curve). **B)** Cy5 Fluorescence quantification curve for IL-6 detection on plasmonic beads (left) and glass beads (right).



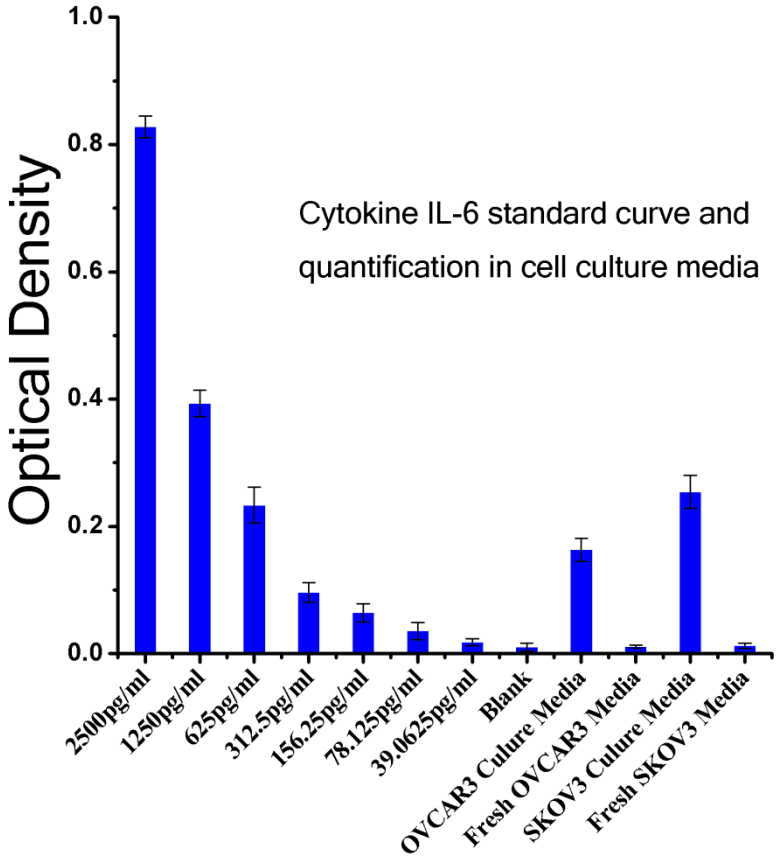
Supplemental Figure 5. Protein quantification on plasmonic bead in 6-plexed plasmonic bead system. Fluorescence quantification curve for serial dilution of human cytokine CA-125, IL-1 beta, IFN-gamma and VEGF.



Supplemental Figure 6. Selectivity for protein quantification on plasmonic bead in 6-plexed plasmonic bead system. A) Flow cytometry result for selectivity test of multiplexed plasmonic bead assay, reflecting fluorescence of each of the 6 sub-region of plasmonic beads where 10 U/ml CA125 (red), 1pM IL-6 (orange), 1pM IFN-gamma (yellow), 10 pM VEGF (green) or 1pM IL-1 beta (blue) was applied for biomarker quantification with the multiplexed plasmonic beads system. **B)** Bar graph for selectivity test of multiplexed plasmonic bead assay in A.



Supplemental Figure 7. Plasmonic beads for multiplexed protein biomarker quantification in cell culture medium. Left: Protein detection result for ovarian cancer cell line SKOV3 culture medium: IL-6 (orange curve) and VEGF (green curve) are identified in the cell culture medium. **Right:** Quantification of protein concentration in the cell culture medium of SKOV3 cell line in C by fitting Cy5 mean fluorescence into the calibration curve of each protein.



Supplemental Figure 8. ELISA measurement of Human Cytokine IL-6. IL-6 expression in OVCAR cell culture medium and SKOV cell culture medium were detected by ELISA and a standard curve of IL-6 was developed to quantify IL-6 level detected in cell culture media. 29 pM IL-6 in OVCAR3 culture media and 42 pM IL-6 in SKOV3 culture media were detected by ELISA.

Table S1. CV (%) of cytokine measurement at various concentrations within the dynamic range.

Concentration	IL-6	IFN-gamma	VEGF	IL-1 beta
1 nM	8.08	11.57	6.96	7.43
100 pM	6.35	14.09	8.41	11.64
10 pM	12.51	9.10	10.43	11.42
1 pM	8.70	14.09	6.62	14.70
100 fM	8.37	19.70	18.18	14.01
10 fM	17.96	17.85	14.70	11.15
Blank	21.81	24.79	11.42	20.83