Published in final edited form as:
Anal Chem. 2009 November 1; 81(21): 9183–9187. doi:10.1021/ac9018389.

A Microarray-based Multiplexed Scanometric Immunoassay for Protein Cancer Markers Using Gold Nanoparticle Probes

Dongwoo Kim[†], **Weston L. Daniel**[†], and **Chad A. Mirkin**[*]

Abstract

We report the use of electroless gold deposition as a light scattering signal enhancer in a multiplexed, microarray-based scanometric immunoassay using gold nanoparticle probes. The use of gold development results in greater signal enhancement than the typical silver development, and multiple rounds of metal development were found to increase the resulting signal compared to one development. Using these conditions, the assay was capable of detecting 300 aM (~ 9000 copies) of prostate specific antigen in buffer and 3 fM in 10% serum. Additionally, the highly selective detection of 3 protein cancer markers at low pM concentrations in buffer and 10% serum was demonstrated. The use of gold deposition may have significant utility in scanometric detection schemes and broader clinical and research applications.

Introduction

Sensitive, rapid, and selective immunoassays capable of multiplexed protein detection are critical for clinical applications.[1] For instance, in many kinds of cancers, following the disease during the course of and after treatment requires the detection of multiple protein markers.[2, 3] Despite the importance of these assays, the standard for protein detection, the enzyme-linked immunosorbent assay (ELISA), is often not sensitive enough to diagnose some diseases.[4, 5] In addition, multiplexed detection with ELISA has drawbacks such as overlapping spectral features and the need for complex instrumentation for signal readout.[6]

Antibody microarrays have emerged as a promising method for multiplexed detection of protein biomarkers.[6-8] Typically, these microarrays are functionalized with capture antibodies, which bind the protein targets. Next, a second fluorophore-labeled antibody binds the targets forming a sandwich structure detectable with typical DNA microarray detection instrumentation. One limitation of the technique is its sensitivity.[9] The use of amplification methods, such as immuno-PCR or rolling circle amplification, have been used to enhance sensitivity,[9] but require complicated, multistep protocols.[10]

Polyvalent oligonucleotide gold nanoparticle (Au NP) conjugates[11] have been utilized as probes for nucleic acids,[12-14] proteins,[15-17] metal ions,[18-20] and cancerous cells.[21] In addition, these conjugates are extraordinarily sensitive and selective labels for microarray-based DNA detection.[22-24] This assay, called the scanometric assay, has since become an FDA-approved detection method, and has spurred the development of many related assays.[16, 25, 26] The key to its high sensitivity is the ability to amplify the light scattering of

[*]To whom correspondence should be addressed. Department of Chemistry and International Institute for Nanotechnology, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208-3113 Phone: 847-467-7302 Fax: 847-467-5123 chadnano@northwestern.edu .
[†]These authors contributed equally to this work.

Supporting information available. Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

the Au NP probes with electroless silver deposition. In separate but related experiments, immunoblots using antibody Au NP conjugates as probes have shown that gold deposition gives greater signal amplification than silver.[27] Given these advances, we sought to combine the multiplexing utility of protein microarrays, the high sensitivity of Au NP conjugate-based detection systems, and the signal amplification of Au NP initiated gold reduction and subsequent deposition. Herein, we present a simple, rapid, and extremely sensitive microarray-based protein detection method called the scanometric immunoassay, that uses the light scattering of antibody-oligonucleotide Au NP conjugates and Au NP initiated gold deposition for signal readout.

Experimental section

General

The Verigene Reader™ light scattering reader system, silver enhancing solutions, and 10-well manual hybridization chambers were purchased from Nanosphere, Inc. Gold(III) chloride trihydrate (520918, Aldrich) and hydroxylamine hydrochloride (159417, Aldrich) were used for preparing gold enhancing solution. Normal donkey serum (Chemicon International, Temecula, CA) was used as received.

Assay buffer preparation

The assay buffer was prepared by adding 500 μL of a 10% bovine serum albumin (BSA) solution (DY995, R&D Systems), 500 μL of an aqueous 10% Tween 20 solution (Sigma), and 500 mg of poly(acrylic acid) (420344, Sigma) to Dulbecco's PBS buffer (Invitrogen) in a final volume of 50 mL.

Antibodies and antigens

The proteins used in the study were prostate specific antigen (PSA) (P3338, Sigma-Aldrich), the spotted PSA antibody (ab403, Abcam), the Au NP PSA antibody (AF1344, R&D Systems), α-fetoprotein antigen (APF) (A32260H, Biodesign International), the spotted AFP antibody (10-A05, clone M19301, Fitzgerald Industries International, Inc.), the Au NP AFP antibody (70-XG05, Fitzgerald Industries International, Inc.), human chorionic gonadotropin (HCG) (A81355M, Biodesign International), the spotted HCG antibody (E20579, Biodesign International), and the Au NP-monoclonal HCG antibody (E20106, Biodesign International).

Preparation of antibody and oligonucleotide modified gold nanoparticles (Au NPs)

13 ± 1 nm Au NPs were synthesized by the Frens method,[28] resulting in ~ 10 nM solutions. The 3′ - propylthiol - T_{24} - decanoic acid oligonucleotide was synthesized with standard phosphoramidite chemistry reagents purchased from Glen Research and purified with ion exchange HPLC. The oligonucleotide Au NP conjugates were synthesized by incubating 3 μM of the oligonucleotide with the as-synthesized Au NPs. The conjugates were salted using literature procedures[29] to a final concentration of 1.0 M NaCl and purified via repeated centrifugation and resuspension in 0.01% Tween 20 in water. The antibodies were conjugated to the oligonucleotide modified Au NPs with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and sulfo *N*-hydroxysuccinimide (NHS). In this procedure, 10 μL of 0.01% Tween 20 solutions containing 0.5 pmol of the particles were prepared. 5 μL of a 30 mM sulfo-NHS solution in a 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer at pH 5, followed by 5 μL of 15 mM EDC solution in 0.1 M MES were added to these particles. This mixture was agitated for 15 minutes, and then the particles were purified from excess reagent via centrifugation and resuspension three times in 5 mM MES buffer supplemented with 0.01% Tween 20. After the final centrifugation and

supernatant removal, the particles were isolated in 10 μL of oily suspension. To this solution, 5 μg of antibodies in 10 mM PB were added from a 1 mg/mL solution. Last, 5 μL of 0.1 M phosphate buffer (PB), pH 7.4 were added to the mixture, and the solution was agitated overnight at room temperature. The conjugates were purified by repeated centrifugation and resuspension in Dulbecco's PBS with 0.025% Tween 20 and 0.1% BSA. Finally, BSA was added to a final concentration of 1%, and the conjugates were passivated overnight.

Microarray preparation

An arrayer equipped with 125 μm diameter pins (GMS 417, Affymetrix) was used for the preparation of the antibody microarrays. The microarrays were fabricated by spotting 250 μg/mL solutions of the antibodies in 0.1 M phosphate buffer (PB), pH 8.0 with 150 mM NaCl and 0.001% Tween 20 on to the surface of NHS ester-activated Codelink slides (SurModics Inc.). Six replicate spots for single analyte detection or three replicate spots of each antibody for multiplexed detection were arrayed at defined locations. The slides were then incubated overnight at 4 °C under an N_2 atmosphere. They were then passivated by incubating them with a 0.2 % (v/v) solution of ethanolamine (411000, Aldrich) in 50 mM borate buffer, pH = 9.5 overnight at 4 °C. Finally, they were then washed with Nanopure™ water (>18 MΩ, Barnstead International) and spin-dried for one minute.

The microarray of the oligonucleotide-modified Au NP conjugates for SEM imaging was prepared by spotting ~ 400 conjugates to the surface of glass slides (Codelink, SurModics).[30] Three replicate spots were arrayed at defined locations. The glass slides were dried and the diameters of Au NP probes were increased with silver or gold staining solution, gently washed with Nanopure™ water and spin-dried. The slides were sputtered with a 20 nm of Au/Pd before imaging. All scanning electron microscopy (SEM) images were obtained using a LEO Gemini SEM.

Scanometric immunoassay protocol

The antibody microarray was assembled with 10-well manual hybridization chamber. Antibody spots on the microarray were arrayed at defined locations across the glass slides so that multiple tests could be performed on the single slide by isolating reaction sites with silicone gaskets to create individual wells. Each well of the chamber was filled with 50 μL of antigen solution, and allowed to incubate for 1 hr at room temperature with shaking at 1200 rpm. After washing the chambers three times with assay buffer, 50 μL of 150 pM Au NP probes in assay buffer were incubated with the slides for 1 hr at room temperature. The concentration of each of the Au NP probes was 150 pM in multiplexed detection experiments. The chamber was again washed three times and then disassembled. The slide was rinsed with Dulbecco's PBS with 0.1% Tween 20 and Nanopure™ water, and spin-dried for one minute. The slide was then developed with silver or gold enhancing solution (1:1 (v:v) mixture of 5 mM $HAuCl_4$ and 10 mM NH_2OH) for 5 mins and imaged with a Verigene Reader™ system.

Results and Discussion

As a proof-of-concept detection experiment, we created a microarray sandwich assay for prostate specific antigen (PSA), Scheme 1. PSA was chosen as an initial analyte because of its importance as a prostate cancer marker,[31] and since many assays have been developed for this analyte,[16, 32-36] there is a good basis for comparison. In a typical experiment, an antibody microarray was fabricated by spotting monoclonal capture antibodies to the surface of *N*-hydroxysuccinimide-activated glass slides (CodeLink, SurModics). Six spots, all with antibodies for PSA, were used in each assay well. The use of six spots allow one to obtain

statistically significant data in each assay. The slides were then passivated with ethanolamine. Probes were prepared by first modifying 13 nm diameter Au NPs with 3′-propylthiol and 5′-decanoic acid modified oligonucleotides and then covalently immobilizing antibodies for PSA via carbodiimide coupling.[37] The assay began by incubating the test solution with PSA at a designated concentration for 1 h at room temperature on the chip with capture antibodies (assay buffer: Dulbecco's PBS with 0.1 % Tween 20, 0.1 % BSA and 1 % poly(acrylic acid)). Since each chip had ten different wells (in addition to six capture spots in each well), multiple separate assays can be carried out at once (top to bottom, Figure 1). After washing the slide with assay buffer, 150 pM of the Au NP probes in assay buffer were incubated with the microarray-bound targets for 1 hr at room temperature. The slides were washed again. To increase the light scattering signal of the immobilized Au NP probes, gold or silver was catalytically deposited on them using electroless deposition techniques (left to right, Figure 1). Finally, the light scattering was quantified with a Verigene Reader™ system, which is a device that captures evanescent wave-induced light scattering from the amplified Au NPs.

In a conventional scanometric detection experiment, electroless silver deposition is used to grow Au NP probes on oligonucleotide microarrays,[23] Figure 1A. When PSA was used as the analyte under the conditions described above, the limit of detection (LOD) is 3 pM when silver was the amplifying agent. Interestingly, the silver-plated Au NP conjugates could be used as nucleation agents to perform a second silver deposition on the same microarray, which improves the LOD to 30 fM, Figure 1B. Others have shown that a second round of silver development increases the limit of detection of immunoblots[27] and immunosorbent assays.[38] The increase in signal arises from particle growth (*vide infra*), because on the nano- and microscale light scattering intensity increases dramatically with particle diameter.[39] A third round of silver deposition did not significantly improve the assay LOD due to increased background signal (data not shown).

Methods of electroless deposition using $HAuCl_4$ and NH_2OH have been used to increase the diameter of Au NPs in solution[40] and in immunoblots.[27] Interestingly, a microarray developed with these reagents resulted in an LOD of 30 fM, comparable to that of two sequential silver depositions, Figure 1C. An additional treatment with the gold development solution improved the LOD to 300 aM, Figure 1D. A third deposition of gold increased the light scattering signal, but did not improve the LOD due to increased background signal, Figure 1E. Experiments where combinations of gold and silver deposition were used resulted in limits of detection less than that of two gold depositions (data not shown).

As one moves to more complex matrices, assay LODs are often challenged due to increased background. When this assay was carried out in 10% serum, the LOD was 3 fM with two gold depositions, Figure S1**.** This LOD is approximately three orders of magnitude lower than that of commercially available ELISA assays for PSA (~ pM).[41]

One of the unique features of a multi-stage development is that it allows for quantification over a large concentration range in addition to increased sensitivity. With one gold deposition, the dynamic range of this assay in buffer is between 30 fM and 3 pM, and with two, it is between 300 aM and 300 fM, Figure 1. Therefore, with two serial gold depositions, this scanometric assay is capable of PSA detection over a 4 order of magnitude concentration range.

To better understand the reason why multiple gold depositions provide better signal than one silver deposition, the growth of Au NP probes were investigated by scanning electron microscopy (SEM) after various metal deposition procedures. In a typical experiment, a microarrayer was used to deposit ~ 400 Au NPs per spot on glass slides[30] and then the size

of the Au NP probes were measured after silver or gold development. With silver, the average diameters of the probes were 100 ± 25, 270 ± 130, 550 ± 140 nm after one, two and three developments, respectively. With gold, the developed probe diameters are 420 ± 100, 1400 ± 470 and 2700 ± 710 nm after one, two and three depositions, respectively. These data indicate that repeated metal depositions increase the average probe diameter. Larger nano- and microstructures scatter light better than smaller ones,[39, 42, 43] which correlates with increased light scattering intensity as seen in Figure 1.

The greater signal amplification observed when using gold deposition versus silver likely arises from their different growth mechanisms. Typically, silver deposition causes the autocatalytic reduction of silver on the Au NPs,[23] increasing the size of the structure, which results in signal enhancement, Figure 2a. The gold development solution, however, likely leads to the continuous nucleation of new Au NPs by the probe Au NPs in addition to autocatalytic growth. These newly nucleated particles aggregate on the probe Au NPs, resulting in signal enhancement and gold microstructures that are larger than those developed by silver, Figure 2b. The nucleation of new particles by existing Au NPs has been observed in the seed-mediated synthesis of Au NPs.[44]

Having determined the origin of the increased signal using gold development, the scanometric immunoassay was challenged with detecting three protein cancer markers using multiple gold depositions. Multiplexed protein analysis is becoming increasingly important for disease diagnosis and high selectivity is critical for the success of multiplexing assays.[2] In a typical experiment, antibodies to PSA, human chorionic gonadotropin (hCG), a testicular cancer marker, and α-fetoprotein (AFP), a hepatic cancer marker, were spotted onto a microarray chip. Next, the target antigens were incubated in the wells. After washing, antibody modified oligonucleotide Au NPs specific for PSA, hCG or AFP were used to sandwich the antigens. The selectivity of the system was tested by detecting eight different combinations of antigens. In the first well, all three antigens are present. In the next seven wells, different combinations of targets were mixed. The concentrations of each of the target antigens were kept constant at 1.4 pM. In the first well, all three antigens were present. In the next seven wells, different combinations of targets were mixed. After two serial gold depositions the presence of the target in each combination was clearly indicated by the high intensity signal, Figure 3. In the absence of the protein cancer marker, little signal was observed. This indicates that the assay is capable of highly selective antigen detection. The differences in spot intensity for the different antigens are likely a result of differences in the binding affinity of the antibodies.[45] Finally, the assay demonstrated high selectivity in 10% serum, Figure S2.

Conclusions

In conclusion, we have used multiple gold depositions as a signal enhancing mechanism in a simple, rapid and ultrahigh sensitivity scanometric assay based on antibody microarrays and Au NP probes. Multiple gold depositions are an alternative light scattering amplification tool for scanometric assays that provide greater signal than the typical single silver deposition. This greater signal arises because the developed probe diameters are much larger, and thus scatter light better, than probes developed by one silver deposition. Gold-developed structures are likely larger than silver due to the unique growth mechanism of gold deposition. Although this work focused on the detection of protein cancer markers, the use of multiple gold developments should improve the signal from any scanometric assay, including those for DNA[23] and metal ions,[46] and the bio-barcode assay.[16] Ultimately, gold-based signal enhancement could have significant utility in detection schemes as well as in broader clinical and research applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

C.A.M. acknowledges the NCI-CCNE and the NSF for support of this work and he is also grateful for a National Security Science and Engineering Faculty Fellows award. D.K. acknowledges the Korea Research Foundation Grant (KRF-2007-357-C00053) funded by the Korean Government (MOEHRD) for postdoctoral fellowship support.

References

(1). Kodadek T. Chem. Biol. 2001; 8:105–115. [PubMed: 11251285]

(2). Ferrari M. Nat. Rev. Cancer. 2005; 5:161–171. [PubMed: 15738981]

(3). Sidransky D. Nat. Rev. Cancer. 2002; 2:210–219. [PubMed: 11990857]

(4). Barletta JM, Edelman DC, Constantine NT. Am. J. Clin. Path. 2004; 122:20–27. [PubMed: 15272526]

(5). Maia M, Takahashi H, Adler K, Garlick RK, Wands JR. J. Virol. Methods. 1995; 52:273–286. [PubMed: 7601903]

(6). MacBeath G. Nat. Genet. 2002; 32(Suppl):526–532. [PubMed: 12454649]

(7). Angenendt P. Drug Discov. Today. 2005; 10:503–511. [PubMed: 15809196]

(8). Ekins RP. Clin. Chem. 1998; 44:2015–2030. [PubMed: 9733000]

(9). Schweitzer B, Roberts S, Grimwade B, Shao WP, Wang MJ, Fu Q, Shu QP, Laroche I, Zhou ZM, Tchernev VT, Christiansen J, Velleca M, Kingsmore SF. Nat. Biotechnol. 2002; 20:359–365. [PubMed: 11923841]

(10). Niemeyer CM, Adler M, Wacker R. Trends Biotechnol. 2005; 23:208–216. [PubMed: 15780713]

(11). Mirkin CA, Letsinger RL, Mucic RC, Storhoff JJ. Nature. 1996; 382:607–609. [PubMed: 8757129]

(12). Elghanian R, Storhoff JJ, Mucic RC, Letsinger RL, Mirkin CA. Science. 1997; 277:1078–1081. [PubMed: 9262471]

(13). Storhoff JJ, Elghanian R, Mucic RC, Mirkin CA, Letsinger RL. J. Am. Chem. Soc. 1998; 120:1959–1964.

(14). Seferos DS, Giljohann DA, Hill HD, Prigodich AE, Mirkin CA. J. Am. Chem. Soc. 2007; 129:15477–15479. [PubMed: 18034495]

(15). Nam JM, Park SJ, Mirkin CA. J. Am. Chem. Soc. 2002; 124:3820–3821. [PubMed: 11942805]

(16). Nam J-M, Thaxton CS, Mirkin CA. Science. 2003; 301:1884–1886. [PubMed: 14512622]

(17). Zheng GF, Daniel WL, Mirkin CA. J. Am. Chem. Soc. 2008; 130:9644–9645. [PubMed: 18597453]

(18). Lee JS, Han MS, Mirkin CA. Angew. Chem. Int. Ed. 2007; 46:4093–4096.

(19). Liu J, Lu Y. J. Am. Chem. Soc. 2003; 125:6642–6643. [PubMed: 12769568]

(20). Li D, Wieckowska A, Willner I. Angew. Chem. Int. Ed. 2008; 47:3927–3931.

(21). Medley CD, Smith JE, Tang Z, Wu Y, Bamrungsap S, Tan WH. Anal. Chem. 2008; 80:1067–1072. [PubMed: 18198894]

(22). Taton TA, Lu G, Mirkin CA. J. Am. Chem. Soc. 2001; 123:5164–5165. [PubMed: 11457374]

(23). Taton TA, Mirkin CA, Letsinger RL. Science. 2000; 289:1757–1760. [PubMed: 10976070]

(24). Cao YWC, Jin RC, Mirkin CA. Science. 2002; 297:1536–1540. [PubMed: 12202825]

(25). Xu XY, Georganopoulou DG, Hill HD, Mirkin CA. Anal. Chem. 2007; 79:6650–6654. [PubMed: 17663531]

(26). Niemeyer CM, Ceyhan B. Angew. Chem. Int. Ed. 2001; 40:3685–3688.

(27). Ma ZF, Sui SF. Angew. Chem. Int. Ed. 2002; 41:2176–2179.

(28). Frens G. Nature-Phys. Sci. 1973; 241:20–22.

(29). Hurst SJ, Lytton-Jean AKR, Mirkin CA. Anal. Chem. 2006; 78:8313–8318. [PubMed: 17165821]

(30). Andreeva LV, Koshkin AV, Letiedev-Stepanov PV, Petrov AN, Alfimov MV. Colloid. Surface. A. 2007; 300:300–306.

(31). Lilja H, Ulmert D, Vickers AJ. Nat. Rev. Cancer. 2008; 8:268–278. [PubMed: 18337732]

(32). Oh BK, Nam JM, Lee SW, Mirkin CA. Small. 2006; 2:103–108. [PubMed: 17193564]

(33). Schweitzer B, Wiltshire S, Lambert J, O'Malley S, Kukanskis K, Zhu ZR, Kingsmore SF, Lizardi PM, Ward DC. Proc. Natl. Acad. Sci. USA. 2000; 97:10113–10119. [PubMed: 10954739]

(34). Yu X, Munge B, Patel V, Jensen G, Bhirde A, Gong JD, Kim SN, Gillespie J, Gutkind JS, Papadimitrakopoulos F, Rusling JF. J. Am. Chem. Soc. 2006; 128:11199–11205. [PubMed: 16925438]

(35). He L, Musick MD, Nicewarner SR, Salinas FG, Benkovic SJ, Natan MJ, Keating CD. J. Am. Chem. Soc. 2000; 122:9071–9077.

(36). Goluch ED, Nam JM, Georganopoulou DG, Chiesl TN, Shaikh KA, Ryu KS, Barron AE, Mirkin CA, Liu C. Lab Chip. 2006; 6:1293–1299. [PubMed: 17102842]

(37). Hermanson, GT. Bioconjugate Techniques. Academic Press; San Diego, CA: 1996.

(38). Shim SY, Woo JR, Nam EJ, Hong HJ, Mook-Jung I, Kim YH, Nam JM. Nanomedicine. 2008; 3:485–493. [PubMed: 18694311]

(39). Jain PK, Lee KS, El-Sayed IH, El-Sayed MA. J. Phys. Chem. B. 2006; 110:7238–7248. [PubMed: 16599493]

(40). Brown KR, Natan MJ. Langmuir. 1998; 14:726–728.

(41). Ward AM, Catto JWF, Hamdy FC. Ann. Clin. Biochem. 2001; 38:633–651. [PubMed: 11732646]

(42). Yguerabide J, Yguerabide EE. Anal. Biochem. 1998; 262:157–176. [PubMed: 9750129]

(43). Yguerabide J, Yguerabide EE. Anal. Biochem. 1998; 262:137–156. [PubMed: 9750128]

(44). Jana NR, Gearheart L, Murphy CJ. Chem. Mater. 2001; 13:2313–2322.

(45). Stoeva SI, Lee JS, Smith JE, Rosen ST, Mirkin CA. J. Am. Chem. Soc. 2006; 128:8378–8379. [PubMed: 16802785]

(46). Lee JS, Mirkin CA. Anal. Chem. 2008; 80:6805–6808. [PubMed: 18665608]

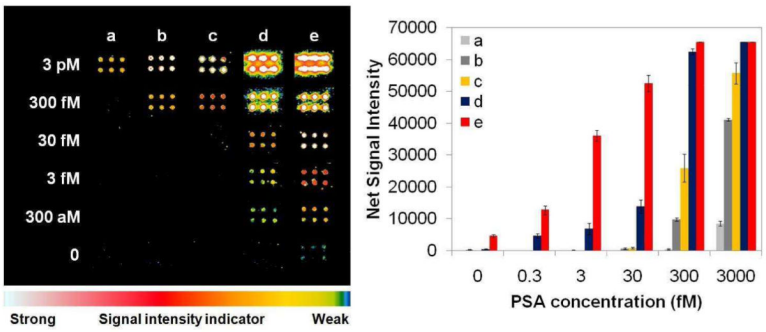


Figure 1.

Scanometric identification (left) and the corresponding quantization (right) of the net signal intensities of various concentrations of PSA in buffer after: a) one silver deposition, b) two silver depositions, c) one gold deposition, d) two gold depositions, and e) three gold depositions. The light scattering signal was saturated at 65536 (2^{16}) units. The gray scale images from the Verigene Reader™ system were converted into colored ones using GenePix Pro 6 software (Molecular Devices). The exposure time was 500 ms.

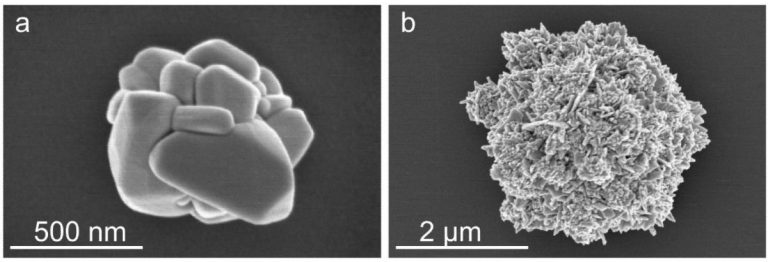


Figure 2.
Representative SEM images of Au NP probes developed with: a) three silver depositions and b) three gold depositions.

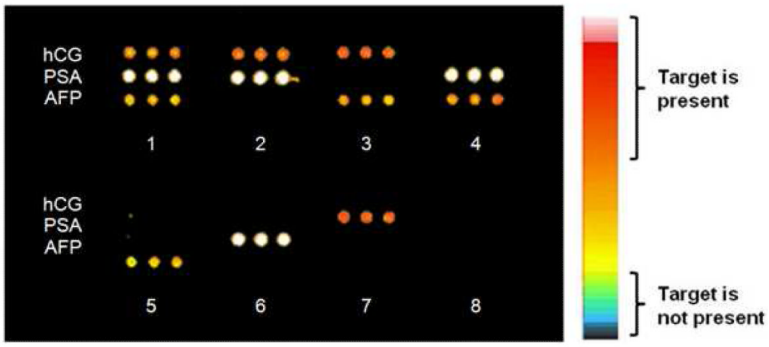
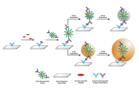


Figure 3.

Scanometric identification of three protein cancer markers for eight different samples in buffer after two gold depositions. The concentration of each antigen was 1.4 pM. 1) All targets present; 2) hCG and AFP; 3) PSA and AFP; 4) hCG and PSA; 5) AFP; 6) PSA; 7) hCG; 8) No targets present. The gray scale images from the Verigene Reader™ system were converted into colored ones using GenePix Pro 6 software (Molecular Devices) and the exposure time was 200 ms.



Scheme 1.
The scanometric immunoassay.