Supporting Information

Multiplexed Detection of Protein Cancer Markers with Biobarcoded Nanoparticle Probes

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Antigens and Antibodies

a) PSA

Prostate specific antigen (P3235, Sigma-Aldrich), MMP-monoclonal PSA antibody (ab403, Abcam), Au NP-polyclonal PSA antibody (AF1344, R&D Systems)

b) AFP

α-Fetoprotein antigen (A32260H, Biodesign International), MMP-monoclonal AFP antibody (10-A05, clone M19301, Fitzgerald Industries International, Inc.), Au NP-polyclonal AFP antibody (70-XG05, Fitzgerald Industries International, Inc.)

c) HCG

Human Chorionic Gonadotropin (A81355M, Biodesign International), MMP-monoclonal HCG antibody (E20579, Biodesign International), Au NP-monoclonal HCG antibody (E20106, Biodesign International)

Preparation of Au NP Probes

a) Preparation of 30 nm coloaded Au NP probes

Gold nanoparticles with an average diameter of 30 nm were purchased from Ted Pella, Inc. and used as received at a concentration of approximately 330 pM (~2 x 10^{11} particles/mL). Prior to functionalization, the pH of the Au colloid was adjusted to 9.2 using 0.2 M NaOH. First, the 30 nm Au particles were functionalized with antibodies by adding each antibody at 0.1 μg/μL starting concentration to each of the Au colloids and incubating the solutions for 30 min at 10 ^{o}C under slow vortex. The final concentrations of the antibodies in the Au colloids are the following: 2 μg/mL for PSA, 4 μg/mL for AFP, and 4 μg/mL for HCG. Next, freshly deprotected thiol-functionalized oligonucleotides (Integrated DNA Technologies, Inc.) were added to the Au colloids (final concentration of oligonucleotides 3-4 μM) and the solutions were shaken gently for 5 min at 10 ^{o}C. The disulfide bonds in all oligonucleotides were reduced prior to the addition to the Au colloids by soaking in 0.1 M dithiothreitol (DTT, Pierce) in 0.17 M phosphate buffer, pH=8 for 2 h (10 OD of lyophilized DNA is typically reduced with 150 μL 0.1 M DTT solution). The deprotected DNA solutions were purified through desalting NAP-5 columns (GE Healthcare), and the amount of DNA from each column was determined by reading the absorbance of the solutions at 260 nm.

The colloids were first buffered to 10 mM phosphate concentration, pH=7.2 including 0.02 % Tween 20 (Sigma-Aldrich), and the NaCl concentration was brought to 0.15 M in one step under vortex. The solutions were incubated for 30 min at 10 ^{o}C, purified from excess DNA by repeated centrifugation at 10,000 rpm at 10 ^{o}C for 10 min, and washed with 0.15 M NaCl, 0.025 % Tween 20, 0.1 % BSA, 10 mM phosphate buffer, pH=7.2 (assay buffer). As a final step, all Au NP probes were re-dispersed in assay buffer at a concentration of ~ 6 nM. The exact Au nanoparticle concentration was determined by reading the absorbance of the colloid at 530 nm. The molar extinction coefficient for 30 nm particles at 530 nm is 3 x 10^{9}

$M^{-1}cm^{-1}$ (calculated from the measured UV-Vis absorbance of a colloid with a known particle concentration). The Au NP probes were stored at 4 ^{o}C prior to use.

b) Preparation of 13 nm Au NP chip-probes

Gold nanoparticles with a 13 nm average diameter were synthesized by citrate reduction of $HAuCl_4$. The 13 nm Au particles were functionalized with freshly deprotected thiol-functionalized oligonucleotides and salted in a step-wise fashion to 0.3 M NaCl, 10 mM phosphate buffer, 0.01 % SDS, pH=7.2 over one day. Each 4 hour, the salt concentration was increased by 0.1 M. The 13 nm Au probe was purified from excess DNA by centrifugation at 14,000 rpm at 20 ^{o}C for 25 min, which was repeated three more times. The particles were washed and finally resuspended in 0.5 M NaCl, 10 mM phosphate buffer, 0.01 % Tween 20, pH=7.2 (scanometric buffer) at a concentration of ~ 10 nM, and stored at 4 ^{o}C prior to use. The exact Au nanoparticle concentration was determined by reading the absorbance of the colloid at 525 nm. The molar extinction coefficient for 13 nm particles at 525 nm is 2.4 x 10^8 $M^{-1}cm^{-1}$ (calculated from the measured UV-Vis absorbance of a colloid with a known particle concentration).

Preparation of MMP Probes

Tosyl-functionalized MMPs (1 μm diameter, Cat. No. 655-01, Invitrogen) were covalently linked to the primary amino groups of the corresponding antibodies (*see Antigens and Antibodies Section*) at 37 ^{o}C in borate buffer solution at pH=9.5. First, 100 μL of tosyl-functionalized MMPs (100 mg/mL) was washed twice with 1 mL of borate buffer while physically retaining the particles with a magnetic field. They were then re-suspended in 100 μL of borate buffer. The conjugation solution in each case was prepared by mixing 66 μL of borate buffer, 84 μL of 3 M $(NH_4)_2SO_4$ in borate buffer, 20 μL of the pre-washed magnetic beads (*vide supra*) and 80 μL of each MMP-monoclonal antibody solution (1.0 μg/μL in borate buffer). The conjugation was carried out in 200 μL PCR tubes for 24 hours under vortex at 1,400 rpm at 37 ^{o}C. Next, the MMP solutions were placed on a magnet, the

supernatant aspirated, and the MMPs were passivated by adding 250 μL of blocking buffer (0.15 M NaCl, 10 mM phosphate, 0.025 % Tween 20, 0.5 % BSA, pH=7.4). The passivation step proceeded for 24 hours under vortex at 1,400 rpm at 37 ^{o}C. The MMP probes were washed three times, each with 1 mL of storage buffer (0.15 M NaCl, 10 mM phosphate, 0.025 % Tween 20, 0.1 % BSA, pH=7.4), resuspended in 200 μL of storage buffer (10 mg/mL final concentration), and stored at 4 ^{o}C. The loading of antibodies on the MMP probes is ~1x10^{4} mAbs/bead from UV/Vis measurement at 288 nm before and after the coupling reaction.

Multiplexed Biobarcode Assay

a) In buffer

Prior to the experiment, each MMP probe was washed twice with assay buffer and MMP probe solution composed of all three MMP probes (MMP multiplexing solution) was prepared by diluting equal volumes of each MMP probe (10 mg/mL) in assay buffer to a final total MMP concentration of 0.0125 mg/mL. The assay was initiated by mixing 150 μL of assay buffer, 40 μL of MMP multiplexing solution and 10 μL of the appropriately mixed target solution in a PCR tube. The concentrations of the target antigens were kept constant for all experiments (each 170 fM final concentration in the assay). The system was incubated at 25 ^{o}C for 1.5 hours under constant vortex to allow binding between the MMP probes and the target antigens. The antigen capturing time can be reduced in the future. For example, we have found that a 30 min time period results in efficient capturing for a single PSA detection. In the case of multiplexed detection, one has to consider the diversity of target antigens and the different binding constants of the capturing antibodies. Next, the reaction tube was placed on a 96-well plate magnet (Invitrogen), and the MMP/target complexes were washed twice with 200 μL of assay buffer. A NP probe solution, composed of all three NP probes (NP multiplexing solution), was prepared by diluting equal volumes of each NP probe (6 nM) in assay buffer to a final total NP concentration of 240 pM in the presence of 6.6 mg/mL tRNA

(R9001, Sigma-Aldrich). The NP multiplexing solution (50 μL) was added to the MMP/target complexes, and the solution was incubated under constant vortex for 1 hour at 25^{o}C. The system was washed seven times with 200 μL of assay buffer each time to remove non-specifically bound NPs and any free barcode DNA. The barcode DNA strands were released from the NP probes by the addition of 75 μL scanometric buffer (*vide supra*). The DTT-induced release of the barcodes was allowed to proceed for 10 min at 50 ^{o}C and 50 min at 25 ^{o}C under vortex. Magnetic field introduction removed all MMP probes from the solution, leaving barcode DNA for detection with the chip-based scanometric method. The signal was identified with a Verigene ID system (Nanosphere, Inc.) that measured the scattered light from the developed spots after silver amplification.

b) In goat serum

The barcode assay was initiated by mixing 140 μL of assay buffer, 10 μL of 10 % goat serum (G9023, Sigma-Aldrich) in assay buffer, 40 μL of MMP multiplexing solution and 10 μL of the appropriate mixed target solution in a PCR tube. The concentration of each target in undiluted goat serum corresponded to 33 pM. Further, the multiplexed barcode assay was carried out under conditions similar to the ones used in buffer solution (*vide supra*). The scanometric results are presented in Figure 1S.

Scanometric Detection of the DTT-Released Barcodes

For scanometric barcode DNA detection, N-hydroxysuccinimide-activated Codelink glass microarray slides (GE Healthcare) were spotted at Nanosphere, Inc. with amine-terminated barcode capture and control capture DNA sequences of the type 5'-H_2N-$(PEG_{18})_2$-DNA (Integrated DNA Technologies) following the manufacturer's protocol. Each DNA was spotted six times. DTT-released barcodes in scanometric buffer (20 μL) were injected into hybridization chambers (Nanosphere, Inc.) attached to a microarray slide. The barcodes were hybridized to the slide for 15 min at 60 ^{o}C, 30 min at 37 ^{o}C, and 15 min at 25 ^{o}C under mild shaking in an incubator. The gaskets were disassembled, the slides were copiously rinsed

with scanometric buffer, spin-dried and reassembled with new hybridization gaskets. The universal Au NP chip-probe solution (20 μl, 500 pM Au NP, 10 % formamide in scanometric buffer) was introduced into the hybridization chambers and allowed to hybridize for 45 min at 37 ^{o}C. The gaskets were disassembled, washed three times in 0.5 M $NaNO_3$ solution, quickly dipped in cold 0.1 M $NaNO_3$, and spin-dried. Silver enhancement was performed by applying 2 mL of silver staining solution (Nanosphere, Inc.) on the dried chips for 5.5 min. The reaction was terminated by washing the slides with Nanopure water (18 MΩ, Barnstead International). The slides were spin-dried and imaged with the Verigene ID system (Nanosphere, Inc.) that measures the scattered light from the developed spots.

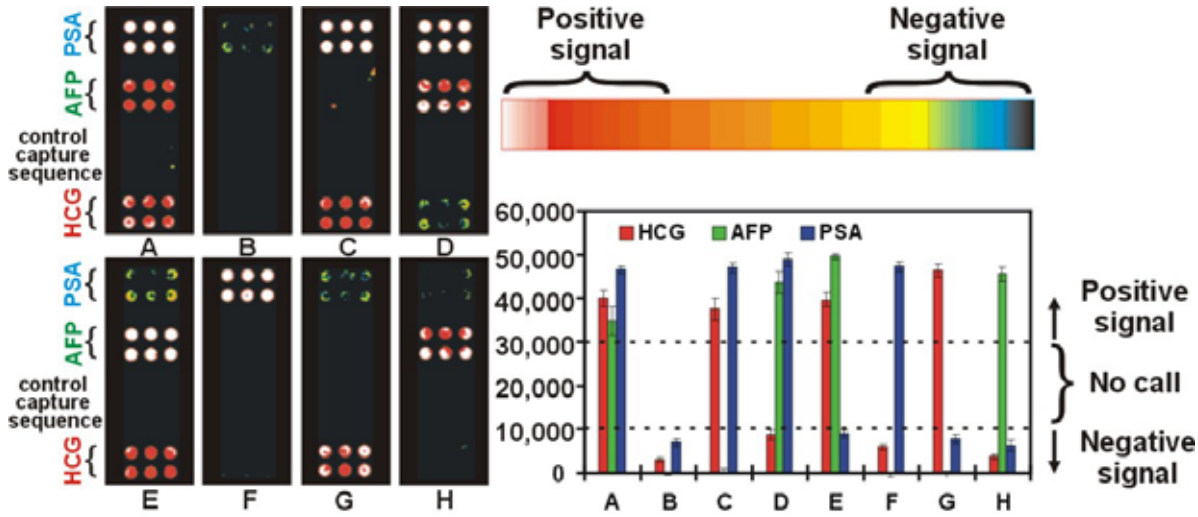

Figure 1S. Scanometric identification of the barcodes released from the 30 nm Au NPs in buffer and the corresponding quantitation of the net signal intensities (determined with a GenePix Pro 6 software, Molecular Devices). The minimum signal-to-noise ratio is 3 based on the absolute signal net intensity. If we have a net signal intensity of more than 30,000, we consider it a positive signal, and in case of less than 10,000, it is considered a negative signal. Any net signal intensity between 10,000 and 30,000 is considered a “no call”, and it indicates that we need to repeat the assay.

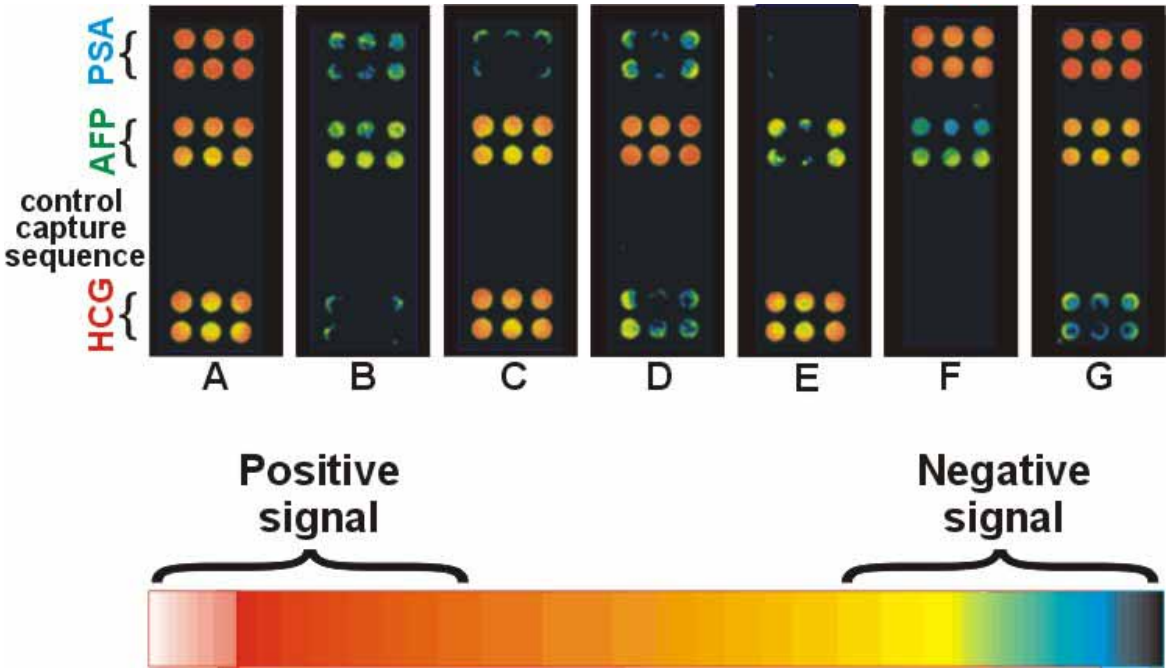

Figure 2S. Scanometric detection of the barcode DNA strands from an assay carried out with goat serum. (A) All targets are present. (B) No target. (C) AFP and HCG. (D) AFP. (E) HCG. (F) PSA. (G) PSA and AFP. The gray scale images from Verigene ID system are converted into colored ones using GenePix Pro 6 software (Molecular Devices).