Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Supporting information

Method

LMDFA procedure

Protein A/G functionalized glass slides (1.1×10^{10} molecules/mm^2) covered with a hydrophobic mask containing 192 sample loading spots were purchased from Arrayit Inc. (Sunnyvale, USA). Slides were loaded with a 1 μL aliquot of antibody (0.025 mg/mL) in PBS per spot and incubated (below-mentioned). Following this and all other incubation steps, all loading spots were vacuum-aspirated, then loaded with PBS and aspirated to wash each loading spot. Following antibody binding, loading spots were incubated for 1 hr at room temperature with 1 μL per spot of Protein-Free Pierce blocking buffer (Thermo Scientific, USA) to block all remaining potential binding sites. Slide loading spots were then loaded with 1 μL of the analyte solution, washed and loaded with 1 μL antibody conjugated nanoparticles. For each step involving particle immobilization and immunoreaction, slides were sonicated for 8 min at 37.5°C using a 5 s on/off cycle at 80% amplitude using a Q500 Sonicator (Qsonica) to accelerate binding. Following particle binding, slides were washed with 0.01% Tween-20 in PBS (PBST, pH 7.0) for 10 min followed by DI water for 10 min and then air dried before DFM observation. Unless otherwise specified, all samples were analyzed using 6 replicates.

Signal amplification assay

Biotin functionalized (AuNR-Biotin) and Streptavidin-functionalized gold nanorods (AuNR-AV) were purchased from Nanopartz (CZ12-25-650-BIOTIN-50, CZ12-25-650-NEUT-50, 5.1×10^{12}/mL). For the direct binding amplification assay (**Figure 2a**), 100× diluted AuNR-Biotin and AuNR-AV (0.5mL) samples

were mixed 1:1, and incubated 2 hrs at room temperature. For the linker-based amplification assay (**Figure 2**), biotinylated polyethylene glycol polymers (biotin-PEG-biotin, 400 Da and 2000 Da) and Methoxyl PEG (PEG2000, 2000Da) purchased from Nanocs were mixed at the indicated concentrations with 10× diluted AuNR-AVs for 2 hrs at room temperature. After AuNR incubation, samples were centrifuged at 1000g for 10min at room temperature, aspirated, and AuNRs were resuspended 100 μL PBS (pH 7.4) before analyzing their UV−visible absorption spectrum using a SpectraMax spectrophotometer (Molecular Device, USA). Sample absorbance versus linker concentration was fitted to a linear regression using Origin Pro software (OriginLab, USA) using the formula $R^2 = \frac{\sum_1^n (y_i - f(x_i))^2}{\sum_1^n (y_i - \bar{y})^2}$ to evaluate the fit to this line, where $f(x)$ is the linear fit function. For linker-amplified LMDFA assays (**Figure 3b**), 1 μL of biotinylated anti-CD9 antibody was loaded on each loading spot and incubated 1hr before loading 1 μL Protein-Free Pierce blocking buffer (Thermo Scientific, USA) and incubating 1 h at room temperature. Spots were then loaded with 1 μL of an AuNR- AV dilution, washed, loaded with 1 μL of 5 μg/μL biotin-PEG-biotin, washed and loaded with 1 μL of 40x diluted AuNR-AV secondary nanoparticles. For each step involving particle immobilization, slides were sonicated (Q500 Sonicator, Qsonica) for 8 min at 80% amplitude using a 5 s on/off cycle to accelerate binding at 37.5°C. Between each solation addition, the solution present at each spot was aspirated, and replaced with 1 μL PBS, which was aspirated before addition of the next solution. After assay completion, slides were rinsed with 0.01% Tween-20 in PBS (PBST, pH 7.0) for 10 min, followed by de-ionized water for 10 min, then air-dried before observation by DFM. Unless otherwise specified, all samples were analyzed

using 6 replicates. Morphological studies were performed using a scanning electron microscope (FEI Nova NanoSEM 230) operating at 20 keV.

DFM imaging and processing.

The dark-field images were acquired on an inverted microscope (TiS Eclipse, Nikon) equipped with a 4x objective lens (NA=0.13), a dark-field condenser (1.2< NA< 1.43), and a motorized stage. Slides were illuminated with 100 W halogen light source and scattered light was recorded by a digital camera (DS-Ri2, Nikon), using the motorized stage and image stitching function of NIS-Elements (Nikon) to capture a dark-field color image of the entire slide. Lighting and magnification conditions were held constant between all analyses. Images were processed and quantified using our previously reported "DarkScatterMaster" DSM algorithm[11] with the following software input parameters: contour threshold (Ct) = 253.020, center scale (S) = 0.8, type = Red, Low (Lt)/High (Ht) quantification limit: 0/62.

Simulation

Finite-difference time-domain (FDTD) simulations of signal enhancement resulting from the proximity of 25 × 60nm AuNRs (aspect ratio ~2.5) were performed by calculating scattering cross sections using commercial software available from Lumerical FDTD Solutions. The layout of the simulation is shown in Figure S1 (Supporting Information). The complex permittivity of AuNR ($\varepsilon(\lambda) = \varepsilon_r(\lambda) + \varepsilon_i(\lambda) \cdot i$) as function of wavelength (λ) was determined by FMPS (Fast Multi-Particle Scattering) Software[12], and listed in Table S1. Nanorods were illuminated from above with a 650 nm plane wave, applying a nonuniform mesh with a minimum grid size of 2 nm.

Modeling of various geometries and conditions was conducted using the finite element analysis (FEM)

software of the COMSOL Multiphysics software package. Near- and far-field optical properties of the AuNR were solved numerically in the frequency domain using the scattered field formulation. The 3D simulation space was composed of three spherical volumes: AuNR, an embedding medium, and a perfectly matched layer (PML), where the air was set as the embedding medium. AuNRs were seated on a glass of sides with a thickness of 100 nm, and surface dimensions starting at 250 nm × 250 nm and increasing to 5000 nm × 5000 nm, according to the number of AuNRs and assigned distance between them. A plane wave used for excitation was inserted within the PML surrounding the embedding medium. Meshing elements were restricted to one tenth the illumination wavelength. Using parameters similar to those in the experiment (650 nm excitation, AuNR with 25 nm diameter and 60 nm height), and literature values for the permittivity of air (ε=1.00) and glass (ε = 2.09), field distributions of scatter at the detector were obtained using excitation from beneath the glass slide. This simulation (Figure 1f) employed a detector set perpendicular to the slide to collect only scatter from various wavelengths of plane wave incident light introduced from below the slide.

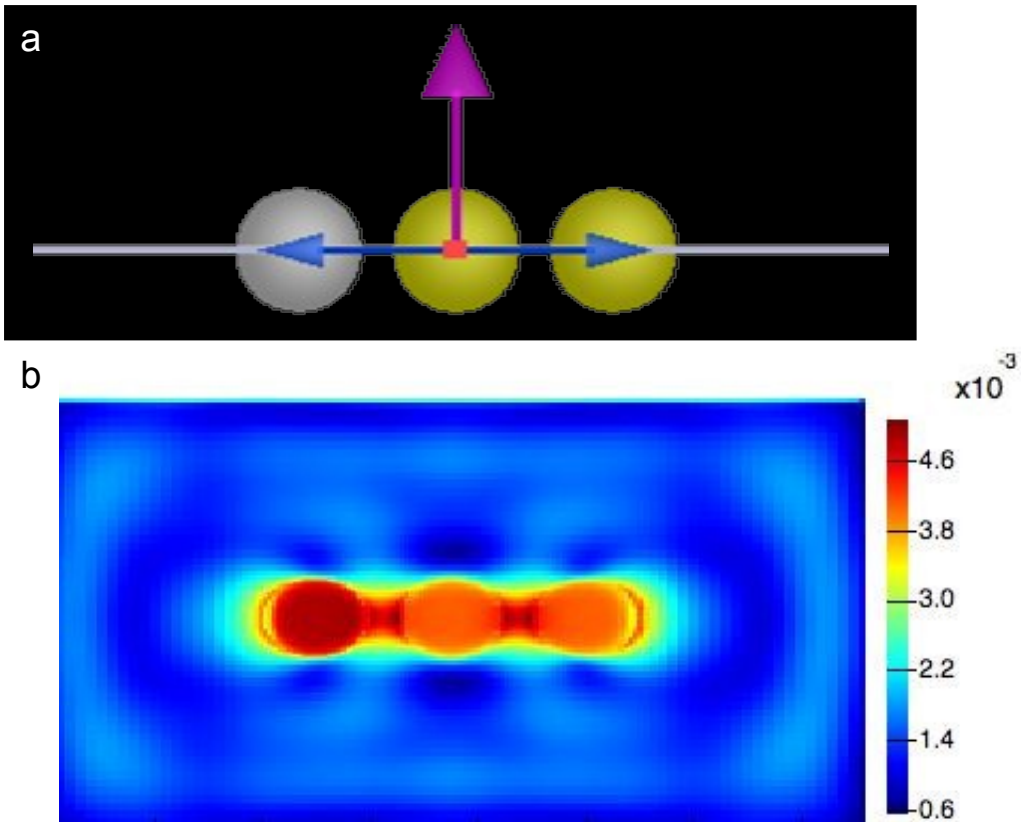

Figure S1 a) FDTD simulation scheme (Ag-Au-Au) with the center-to-center distance of 60nm between the interacting nanorods. b) Scattering intensity distribution by simulation.

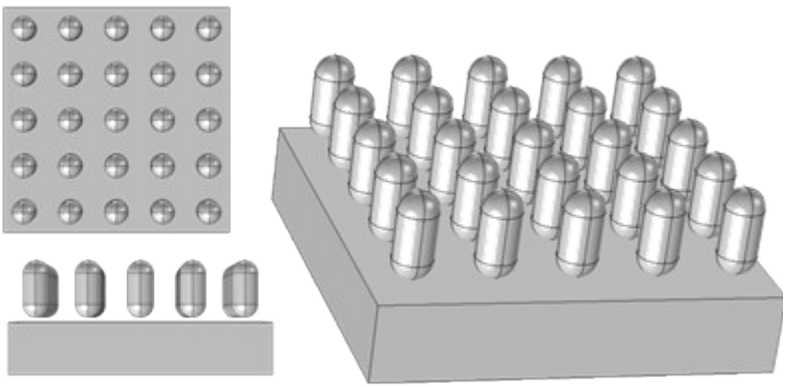

Figure S2. 3D profile for FEM simulation (nanorods on glass slide).

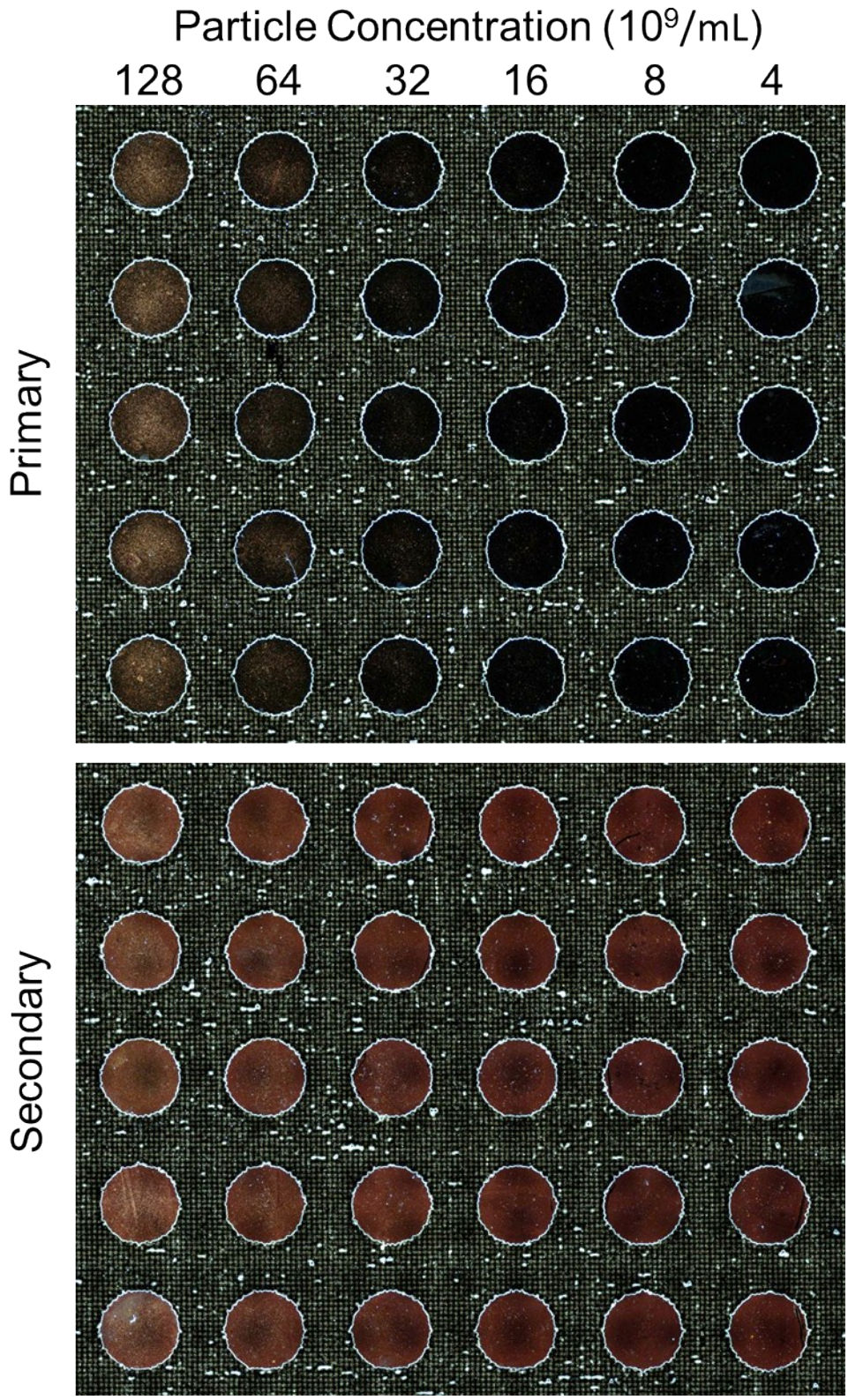

Figure S3. Dark field image for signal amplification (Figure 3d-e).

Table S1 Permittivity of AuNR in simulation

$\boldsymbol{\lambda}$	ε_r	ε_i
1215.686275	-66.218525	5.7015
1087.719298	-51.0496	3.861
984.1269841	-40.2741	2.794
892.0863309	-32.040669	1.92542
821.192053	-25.811289	1.62656
756.097561	-20.610164	1.27176
704.5454545	-16.817709	1.06678
659.5744681	-13.648209	1.03516
616.9154229	-10.661884	1.37424
582.1596244	-8.112669	1.66054
548.6725664	-5.842125	2.1113
521.0084034	-3.946161	2.58044
496	-2.278289	3.81264
471.4828897	-1.702701	4.84438
450.9090909	-1.758996	5.28264
430.5555556	-1.692204	5.6492
413.3333333	-1.702164	5.71736
397.4358974	-1.649404	5.73888
381.5384615	-1.604889	5.64436
367.9525223	-1.400625	5.6092
354.2857143	-1.231956	5.598
342.5414365	-1.310241	5.53816
331.5508021	-1.355289	5.57368
320.4134367	-1.230804	5.84584
310.7769424	-1.242549	5.79258
300.9708738	-1.227421	5.78034
292.4528302	-1.306784	5.59644
284.4036697	-1.332261	5.49486
276.169265	-1.366509	5.28242