

Electronic Supplementary Information

Selective fluorescent-free detection of biomolecules on nanobiochips by wavelength dependent-enhanced dark field illumination

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The AVI movie “M1” shows color images of the PRS signal of a gold-nanopatterned biochip (Fig. S4A). The AVI movie “M2” shows color images of the PRS signal after SNP-cTnI antibody immuno-reactions on gold-nanopatterned biochips as a function of wavelength (Fig. S4B).

Experimental section

Reagent preparation. 11-mercaptoundecanoic acid (MUA, 95%), 6-mercapto-1-hexanol (MCH, 97%), 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (EDC), dimethyl sulfoxide (DMSO, 99.5%), 2-(morphozlino)ethanesulfonic acid (MES), glycine, and phosphate buffered saline (PBS) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Sulfo-NHS (*N*-hydroxysulfosuccinimide, NHSS), dithiobis(succinimidyl propionate) (DSP), and Protein A/G were purchased from Pierce (Rockford, IL, USA). Tris(base) was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). StabilGuard was purchased from SurModics (Eden Prairie, MN, USA). The 80 nm silver nanoparticles (1.83 pM, 1.10×10^9 particles/mL) were obtained from BBI Life Sciences (Cardiff, UK). Monoclonal mouse anti-cardiac troponin I antibody (clone 19C7 and 16A11) and standard human cardiac troponin I (cTnI, clone 8T53) were purchased from HyTest (Turku, Finland). Prior to use, 1× PBS (pH 7.4; 138 mM NaCl, 2.7 mM KCl) and 50 mM MES buffer were filtered through a 0.2- μ m membrane filter and photobleached overnight using a UV-B lamp (G15TE, 280-315 nm, Philips, Netherlands).

Fabrication of the 100 nm and 500 nm gold-patterned biochips. The gold-

nanopatterned substrate was fabricated by the National Nanofab Center (Daejeon, South Korea) (Figs. S1A and S5A). Briefly, the glass wafer was cleaned using piranha solution (1:1 = H₂SO₄:30% H₂O₂). The glass wafer was coated with an electron-sensitive photoresist, such as a 150 nm-thick polymethylmethacrylate (PMMA) layer. An electron-beam (Elionix E-beam system, 100 KeV/100 pA) was then used to burn off the polymer in the desired pattern. Au/Cr (20/5 nm thickness) was deposited by thermal evaporation, and PMMA was removed by dichloromethane to form array patterns with a pitch of 10 μm and diameters of 100 and 500 nm. The pattern consisted of 4 × 5 Au spots on a 10 mm² glass wafer.

Immobilization of the antibody on the silver nanoparticle surface. The strategy for bioconjugation of the SNPs to antibody is shown in Fig. S2A. Ten mM MUA and 30 mM MCH dissolved in ethanol were added to a nanoparticle solution suspended in water, followed by sonication for 10 min and reaction for 2 h 30 min to form a self-assembled monolayer (SAM) of MUA-MCH on the SNP surface, then washed with ultra-pure water by centrifugation (12,000 rpm, 90 min, 4°C). The SNP-MUA-MCH was resuspended in 50 mM MES and 0.1 M NaCl (pH 6.0). These procedures allowed the formation of a carboxylic acid-terminated alkanethiol monolayer on the surface.

Conjugation of SNPs with antibody was carried out using EDC and Sulfo-NHS. Forty μg of EDC (2 mg/mL in 50 mM MES pH 6.0) and 196 μg of Sulfo-NHS (2 mg/mL in 1 \times PBS pH 7.4) were added to SNPs-MUA-MCH solution. After stirring at room temperature for 40 min, SNP-MUA-MCH-NHSS was washed by centrifugation (17,000 rpm, 30 min, 4°C), and then re-dissolved in PBS. 10 $\mu\text{g}/\text{mL}$ monoclonal mouse anti-cTnI (clone 16A11) solution in PBS (pH 7.4) was added to the activated SNPs, allowed to react using a rotary shaker for 4 h at room temperature, and stored for 12 h at 4°C. Ten mM Tris (pH 7.5) and 1 M glycine were added and incubated for 15 min to quench the excess hydroxylamine. The final SNP-antibody suspension was centrifuged (17,000 rpm, ~90 min, 4°C) to remove any unbound antibody. The SNP-antibody was resuspended in PBS buffer and stored at 4°C.

cTnI sandwich immunoassay on the gold-nanopatterned chips. The sandwich immunoassay process of cTnI on the gold substrate was prepared following a previously reported procedure (Talanta, 2013, **104**, 32–38). The gold-nanopatterned chip was immersed in 4 mg/mL DSP in DMSO for 30 min, then rinsed with DMSO and distilled water. A quantity of 0.1 mg/mL of Protein A/G in PBS was added to the activated gold surface for 1 h. Unreacted succinimide groups were blocked by reacting with 10 mM

Tris (pH 7.5) and 1 M glycine for 30 min. The chips were incubated with StabilGuard for 30 min to stabilize the bound proteins and then rinsed briefly with a few drops of distilled water. The chips were incubated with 20 μ L of 2 μ g/mL mouse anti-cTnI antibody (clone 19C7) in PBS (pH 7.4) for 1 h. After washing, 20 μ L of cTnI standard protein (85 aM - 35 fM) was incubated on a biochip for 1 h. Twenty μ L of monoclonal mouse anti-cTnI antibody (clone 16A11)-SNPs was reacted for 2 h to measure the dark field scattering image (Fig. S5B). Antibodies conjugated to SNPs are typically incubated for longer periods of time than molecular antigens, owing to their larger size. The biochip was washed by dipping in 1 \times PBS at each step. All of the reactions were carried out at room temperature with agitation.

The wavelength dependent-enhanced dark field microscopic (WD-EDFM) system.

SNP-cTnI antibodies on the gold-nanopatterned biochips were visualized *via* their resonant light scattering using a lab-made WD-EDFM system (Fig. S5C), which was an enhanced dark field (EDF) illumination system (CytoViva Inc., Auburn, AL, USA) attached to an upright Olympus BX51 microscope (Olympus Optical Co., Ltd., Tokyo, Japan). The system consisted of a CytoViva 1250 dark field condenser in place of the microscope's original condenser, attached *via* a fiber optic light guide to a Solarc 24 W

metal halide light source (Welch Allyn, Skaneateles Falls, NY, USA). A 100×/1.3 oil iris objective (UPLANFLN, adjustable numerical aperture, from 0.6 to 1.3) was also employed for dark field light scattering imaging. Filter cubes to select wavelength were composed of bandpass filters of various wavelength (406/15 nm, 473/10 nm, 520/15 nm, 575/15 nm, 610/10 nm, 670/30 nm) purchased from Semrock (Rochester, NY, USA). Colorful dark field light scattering photographs and high sensitivity dark field light scattering imaging of SNPs-cTnI antibodies on the gold-nanopatterned chips were captured with a Nikon D3S digital camera (Tokyo, Japan) and with an electron-multiplying, cooled charge-coupled device (EM-CCD) camera (QuantEM 512SC, Photometrics, AZ, USA). In addition, the hyperspectral imaging system (HSI) from CytoViva was used for spectral analysis of metals and biological samples.

Data analysis. The relative intensity of the PRS signal was corrected background and filtered for non-valid spots. The relative intensity of the PRS signals before and after SNPs-cTnI antibody immuno-reactions on gold nanopatterned chip as a function of wavelength were calculated by the average of corrected PRS intensity for antigen concentration. In addition, we quantitatively analyzed images from the wavelength dependent-enhanced dark field microscopic (WD-EDFM) system as follows: We first

selected signal regions and background regions with the same area. Then, we calculated the sum of PRS intensities of occupied pixels per one spot corrected by background subtraction. The calibration curves and quantified value of standard cTnI were calculated by sum of PRS intensities of 20 spots on 4×5 gold nanopatterned chip. Calibration curves were fitted using Excel software. Images were acquired and data were analyzed using MetaMorph 7.1 software.

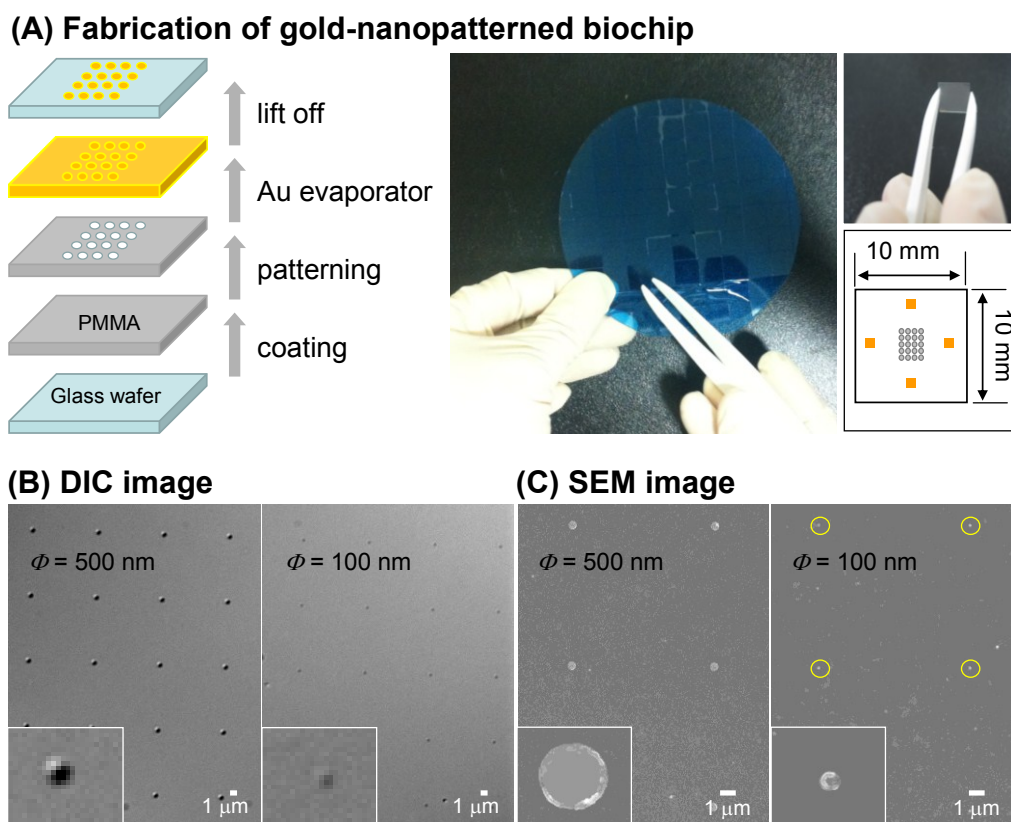
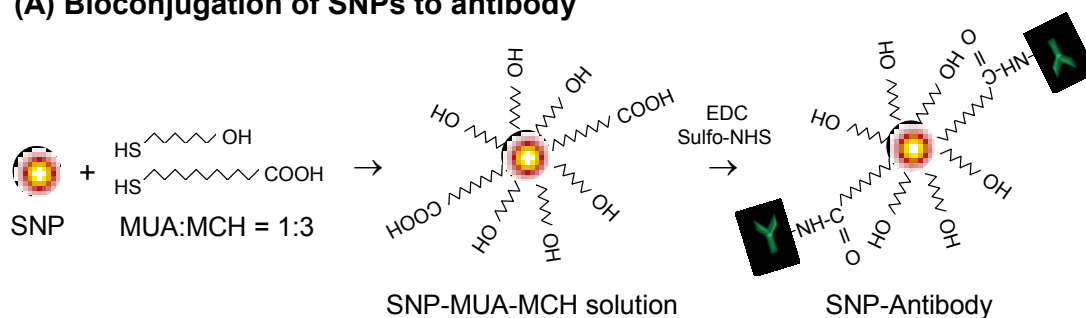
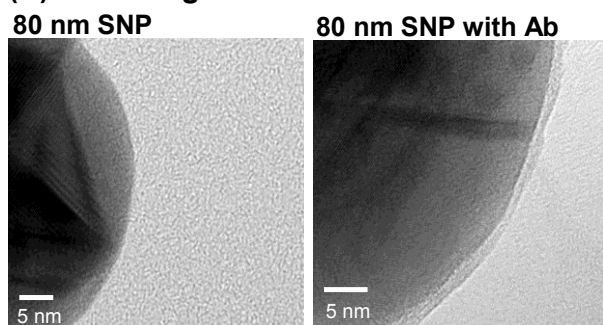


Fig. S1 (A) Photograph and schematic diagram of the fabrication of gold-nanopatterned chips *via* e-beam lithography. (B) DIC and (C) SEM images of gold-nanopatterned chips with spot diameters of 100 nm and 500 nm on glass substrates.

(A) Bioconjugation of SNPs to antibody



(B) TEM image



(C) UV-Vis spectra

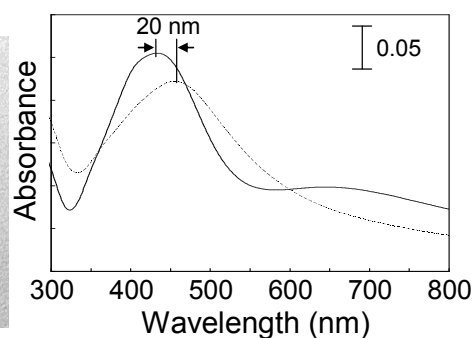


Fig. S2 (A) Schematic illustration for modification of silver nanoparticles (SNPs) with 11-mercaptoundecanoic acid (MUA) and 6-mercapto-1-hexanol (MCH) and conjugation with antibody. (B) TEM images of the bare SNPs (left) and antibody-labeled SNPs (right). (C) UV/Vis spectra of 80 nm SNP-cTnI antibody dispersion (dashed line, $\lambda_{\text{max}} = 453$ nm) and 80 nm SNP solution (solid line, $\lambda_{\text{max}} = 433$ nm).

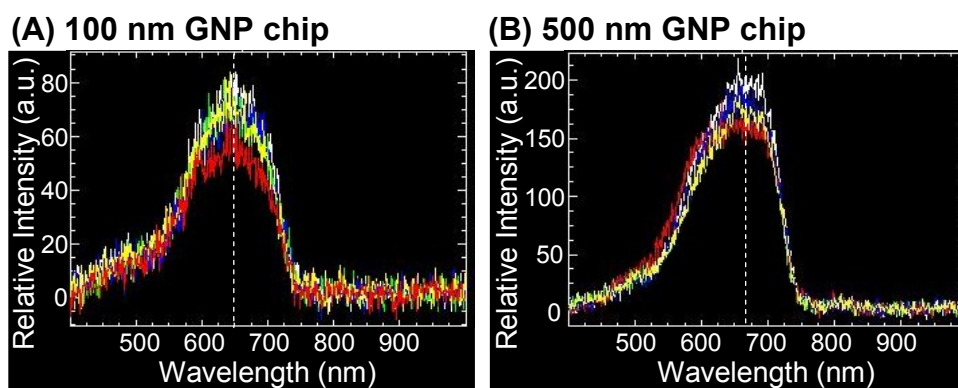
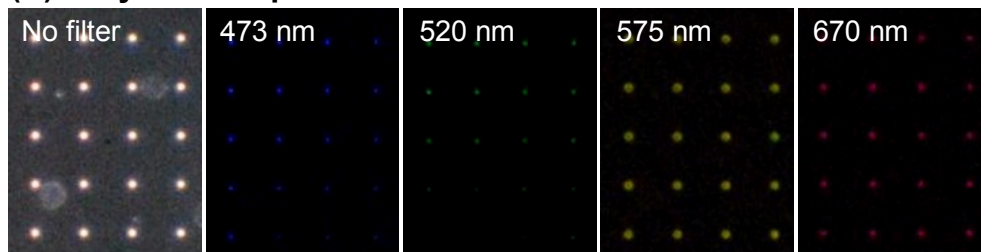


Fig. S3 The resonant scattering peaks of the several (A) 100 nm and (B) 500 nm gold-nanopatterned chips.

(A) Only GNP chip



(B) Sandwich immunoassay on GNP chip

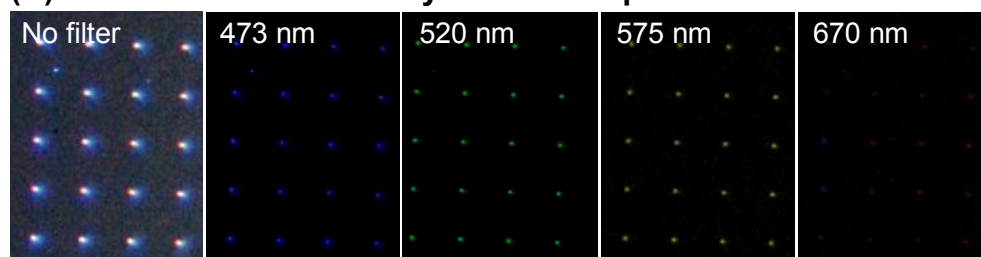


Fig. S4 Color images of PRS signals (A) before and (B) after SNP-cTnI antibody immuno-reactions on a 500 nm gold-nanopatterned biochip as a function of wavelength.

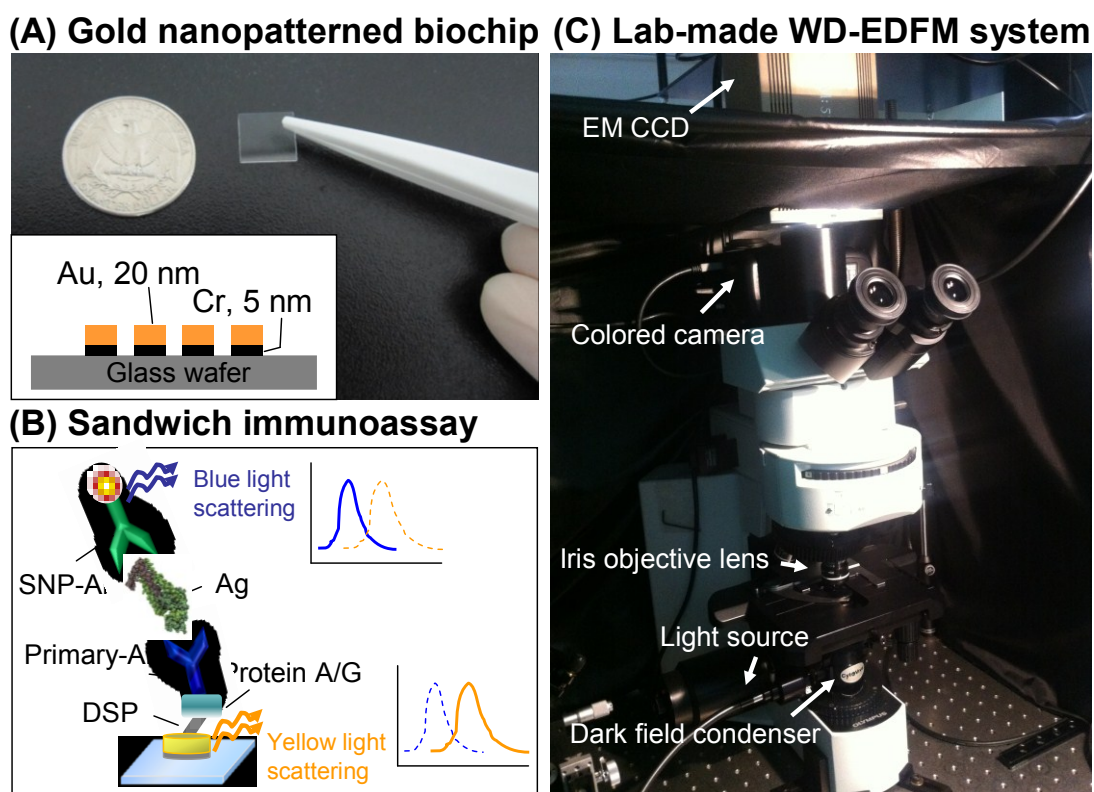


Fig. S5 (A) Photograph of a gold-nanopatterned chip patterned with a 5 nm layer of chromium and a 20 nm layer of gold. (B) Schematic diagram of the sandwich fluorescence immunoassay on the gold-nanopatterned protein chip. (C) Physical layout of the lab-made WD-EDFM system. The following acronyms are used: DSP, dithiobis(succinimidyl propionate); Ag, antigen; Ab, antibody; SNP, silver nanoparticle; WD-EDFM, wavelength dependent-enhanced dark field microscopy.