**SUPPLEMENTARY INFORMATION**

# Design of SERS nanotags for multiplexed lateral flow immunoassays

Maria Sánchez-Purrà,[1] Biel Roig-Solvas,[2] Alice Versiani,[3,4] Cristina Rodríguez-Quijada,[1] Helena de Puig,[5] Irene Bosch,[4] Lee Gehrke,[4,6] Kimberly Hamad-Schifferli[1,5,*]

[1]Department of Engineering, University of Massachusetts Boston, Boston, MA

[2]Department of Electrical Engineering, Northeastern University, Boston, MA

[3]Department of Microbiology, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil

[4]Institute of Medical Engineering and Science and [5]Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, MA

[6]Department of Microbiology and Immunology, Harvard Medical School, Boston, MA

Contact information: kim.hamad@umb.edu

## EXPERIMENTAL

**Reagents.** Gold chloride trihydrate (CAS: 16961– 25–4), N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) (CAS:7365-45-9), 1,2-bis(4-pyridyl)ethylene (BPE) (CAS:3362-78-2) , 4-mercaptobenzoic acid (MBA) (CAS:1074-36-8), Sucrose (CAS:57-50-1) and Tween-20 (CAS:9005-64-5) were purchased from Sigma-Aldrich. 5kD thiolated mPEG was from nanocs. Goat anti-mouse IgG, Fc, was purchased from Millipore (AQ127). ZIKV and DENV-1 NS1 native protein was from Native Antigen. Phosphate buffer saline (1x PBS, pH 7.4) was from Gibco (CAT: 10010– 049). Filtered human serum was obtained by filtering 1 mL of human serum from Sigma-Aldrich (H4522)

through a 0.2 μm cellulose acetate syringe filter (Pall, Acrodisc 25 mm Syringe Filter, with 0.2 μm HT Tuffryn Membrane). Sodium Citrate was purchased from Mallinckrodt Chemicals (CAS: 6132-04-3).

**Antibodies.** Hybridoma cells producing antibodies against ZIKV NS1 were obtained by injection of mice with recombinant native NS1 protein of ZIKV and DENV-1. After hybridomas were screened using ELISA and FACS analysis of ZIKV and DENV infected cells, selected cell cultures were harvested and concentrated using Millipore centrifugal units (30 kDa MW). Protein L columns were used to purify the kappa light chain mouse antibodies that were specific to ZIKV and DENV NS1. After purification, the antibodies were buffer-exchanged into PBS, concentrated, and stored at 4 °C. A NanoDrop 2000 UV−vis spectrophotometer at 280 nm was used to calculate the concentration of the purified antibody, and a TapeStation with a P200 ScreenTape from Agilent Technologies was used to confirm the purity of the monoclonal antibodies.

**Synthesis and conjugation of GNP.** To synthesize gold nanostars (GNS900 and GNS400), 900 μL and 400 μL of 140 mM HEPES (pH 7.4) were mixed with 100 μL and 600 μL of 18 MΩ deionized (Milli-Q) water, for GNS900 and GNS400, respectively, followed by the addition of 16 μL/sample of 10 mg/ml $HAuCl4 \cdot 3H_2O$ and further vortexing. The solution sat undisturbed for 1 h for the nanostar formation. Afterwards, GNS were separated from excess reagents by centrifugation at 4000 rcf for 20 min. Supernatant was then removed, and the nanostar pellet resuspended in 1 ml Milli-Q water. To synthesize gold nanospheres (GNSph), 45.5 mL of 18 MΩ deionized (Milli-Q) water was boiled under reflux while stirring with 0.5 mL of 10 mg/ml HAuCl4 · 3H2O. Then, 0.5 mL of 10 mg/ml solution of sodium citrate was added and allowed to stir for 15 min under boiling reflux. Afterwards, heating was stopped and stirring was kept for 45 min more. The solution was centrifuged for

20 min at 12000 rcf. Supernatant was removed and the nanospheres pellet resuspended in 50 ml 18 MΩ deionized (Milli-Q) water.

The Raman reporter molecule of interest was added and vortexed. For Z-nanotags, the amount of BPE added for GNS900, GNS400 and GNSph, was 4, 1.85 and 0.6 μL, respectively. For D-nanotags, the amount of MBA added was 10.4, 4.8 and 1.5 μL, respectively. These amounts were calculated in order to have a reporter monolayer on the nanoparticles' surface, assuming a maximal footprint of 70.18 and 49.89 Å for each reporter, respectively, calculated with MarvinSketch, and considering the nanoparticles free surface. The solution was left undisturbed for 30 min, and was further centrifuged for 20 min at 4000, 6000, 8000 rcf for GN2900, GNS400 and GNSph, respectively. The supernatant was removed and the pellet resuspended in 100 μL 40 mM HEPES and 300 μL Milli-Q water. For antibody conjugation, 10 μL of 1 mg/ml Ab were added to 1 ml of GNSs solution and 2.5 μL of 1 mg/ml Ab were added to 500 μL of GNSph, vortexed and further shaken overnight at 4°C. Afterwards, 10 μL of 0.01 mM mPEG 5 kDa were added, vortexed and further shaken for 30 min. Lastly, samples were centrifuged at 4000 rcf for 20 min to remove excess reagents and were ready to use.

**GNS Characterization.** Optical absorption spectra of the GNPs were obtained on a Varioskan Flash plate reader (Thermo Scientific). Morphology of the GNS was characterized with a FEI Tecnai G2 TEM at 120 kV. ImageJ was used to process TEM images and measure the dimensions of the GNP. In addition, a Zetasizer Nano ZS from Malvern Instruments was used to measure the hydrodynamic diameter ($D_H$) and the ζ of plain GNS and their Ab-conjugates. An ELISA test was performed to quantify the antibody attached to GNS and was used to quantify the amount of antibody bound per nanoparticle.

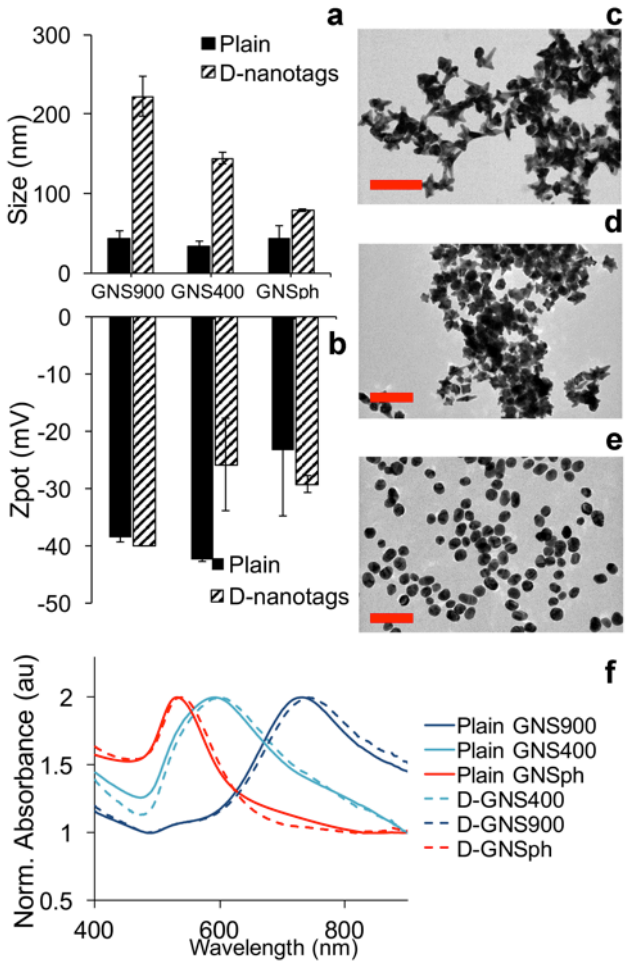
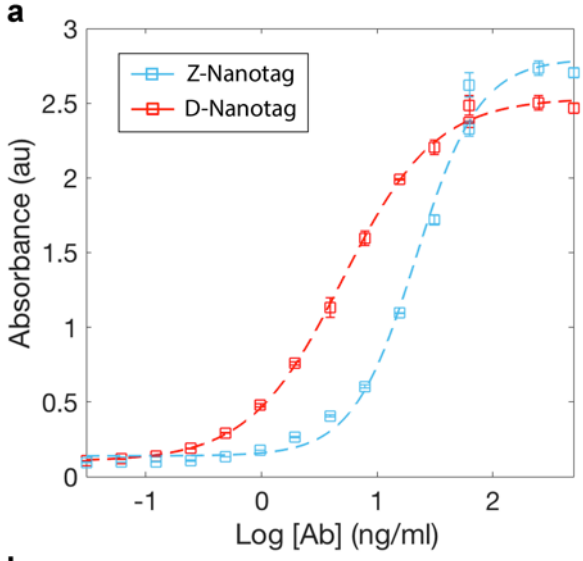



Figure S1: GNP characterization of D-nanotags. a) Size of GNS900, GNS400 and GNSph. b) Zeta potential of GNS900, GNS400 and GNSph. TEM images of (c) GNS900, (d) GNS400 and (e) GNSph, scale bar=100 nm. f) UV-vis spectra of plain GNP (solid line) and Ab-conjugated GNP (dashed line).

Briefly, ELISA was performed by incubating 96-well plates with ZIKV and DENV NS1 (at 1 µg/ml and 100 µL/well) separately overnight at room temperature. After washing free NS1, wells were blocked with 300 µL/well of Blotto 5% (2g non-fat milk, 2 ml washing solution 20X and 38 ml $H_2O$) for 1 hr at room temperature. Then, solution was discarded and wells were incubated for 2 hr with conjugated GNSs samples at 20% (v/v) in blotto 5%. Standard curves were performed with initial concentrations of 500 ng/ml of viral NS1 with subsequent 2-fold dilutions to get 10 points. Samples were discarded and washed 3 times with washing buffer (for 20 ml of buffer mix 1 ml of washing solution 20X and 19 ml $H_2O$). Then 100 µL/well of HRP- anti-Fc was added and incubated for 1h. After washing 3 times, 100 µL/well of TMS was added and let react till blue color appeared. Then, 50 µL/well 0.1 M

$H_2SO_4$ were added to quench the reaction and absorbance was measured at 450 nm with a TriStar Berthold Technologies plate reader. Figure S2a shows calibration curves for both Z- and D-nanotags, whereas table in Figure S2b shows Ab/GNP ratio for each type of GNP.

a




b

| | Ab/GNPs ratio | | |
|---|---|---|---|
| | GNS900 | GNS400 | GNSph |
| Z-Nanotags | 31.86 | 0.26 | 0.16 |
| D-Nanotags | 79.39 | 0.2 | 1.36 |

Figure S2: Ab coverage quantification by ELISA. a) Data fitting a sigmoidal curve for both nanonatgs. b) Ab/GNP ratio for the different GNP tested. Error bars represent mean ± SD, n=3.

Difference in Ab/GNP ratio of both GNS900 compared to other types of GNP is due to the nanostar concentration. GNS concentration in GNS900 samples was much lower, as they are centrifuged at a lower centrifuge speed to avoid aggregation, and thus for the same amount of Ab used for conjugation the ratio Ab/GNP is higher. This is the reason why spot intensity in Figure 5 is similar for all GNP for ZIKV although they have different Ab coverage.

**Dipstick LFA**. Antibodies were immobilized on the nitrocellulose strip by manually pipetting 0.3 µL of a 2 mg/mL solution of antibody onto the nitrocellulose paper and further allowed to dry for at least 30 min. In the test line, monoclonal antibodies against ZIKV or DENV NS1 protein were immobilized. The control line was spotted with goat antibody that

could bind to the Fc fragment of the mouse IgG antibodies on the GNS. To run the test, the strip was submerged at its lower end in the test solution containing 4 μL of 50% w/v sucrose in water, 8 μL of 1% v/v Tween 80 in PBS, 1 μL of the GNS-Ab conjugates, 30 μL of human serum and the analyte (NS1), rendering a total of 45 μL. Then, the solution migrated through the strip upwards via capillary action to the absorbent pad attached to the upper end of the strip. When all the solution had been absorbed, the strip was washed with 80 μL of 0.1% v/v Tween 80 in PBS through the same procedure to eliminate unbound conjugates, and allowed to dry.

**SERS measurements.** Raman and SERS spectra were acquired using a Raman Senterra II microscope (Bruker Optiks GmbH, Germany). A Ne laser with a power of 1 mW operating at λ = 785 nm was utilized as the excitation source. A thermoelectrically cooled CCD detector was coupled to a spectrograph. SERS mapping images were obtained using a point-by-point mapping mode. A computer-controlled translational stage was used to scan an area of 2 by 2 mm in 130 $\mu$m x 130 $\mu$m steps with a 20x objective lens. The data integration time at each point was 5 s with 5 co-additions. The numerical aperture of the objective lens used was 50 x1000 μm. The spectra acquired for each spot were decoded using OPUS software v 7.0 (Bruker Optiks GmbH, Germany). The baselines of each spectra were corrected by concave Rubberband correction method using 15 iterations and 64 baseline points. Mathematical calculations on the spectra such as spectra averaging, intensity, area or peak shift measurements were performed in Matlab.

The Enhancement Factor (EF) was calculated using the following equation[17,18]:

$$EF = \frac{I_{SERS}}{I_{Raman}} \times \frac{N_{Raman}}{N_{SERS}} \times \frac{P_{Raman}}{P_{SERS}} \times \frac{t_{Raman}}{t_{SERS}} \qquad \text{(Equation 1)}$$

where $I$ is the intensity of the same band, $N$ the number of contributing reporter molecules, $P$ the laser power, $t$ the integration time and subscripts Raman and SERS stand for measurements performed on the reporter molecule or on the GNS, respectively.

SERS mapping data from OPUS was treated and plotted with Matlab.

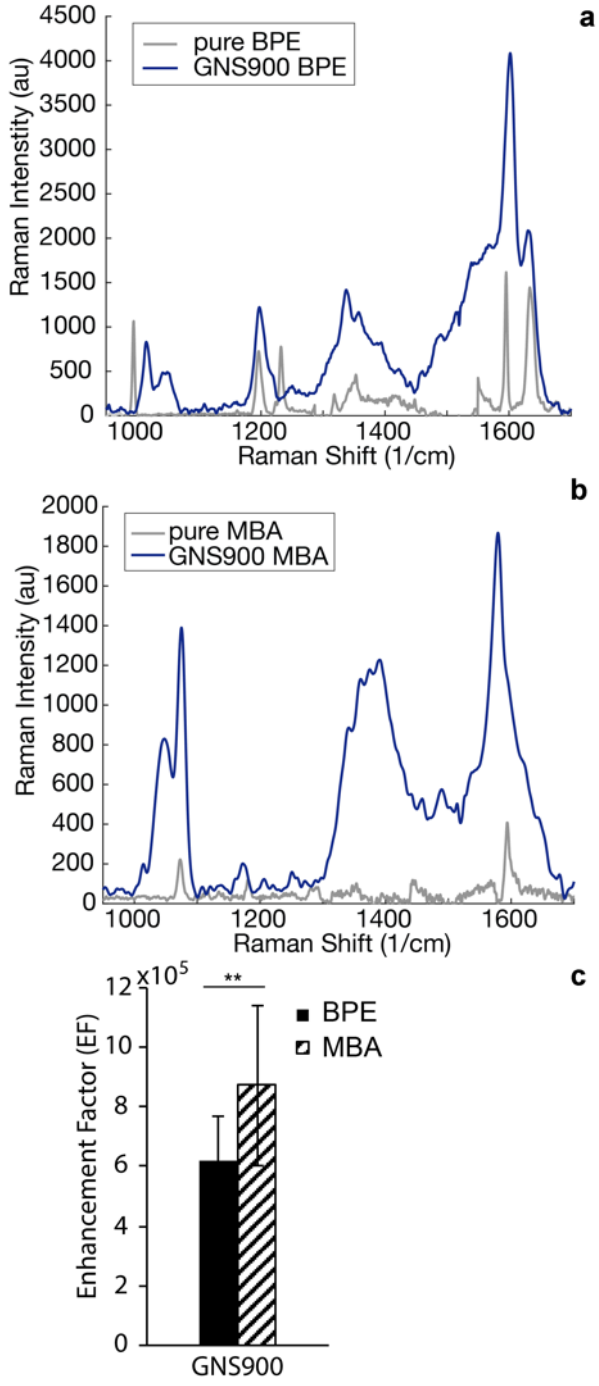



Figure S3: SERS enhancement by nanospheres and nanostars from another batch. a) Raman spectrum of pure BPE (grey) and SERS spectra of BPE-encoded samples: GNS900 (dark blue), GNS400 (light blue) and GNSph (red). b) Raman spectrum of pure MBA (grey) and SERS spectra of MBA-encoded samples: GNS900 (dark blue), GNS400 (light blue) and GNSph (red). c) Enhancement factor for GNS900, GNS400 and GNSph encoding BPE (solid) and MBA (dashed), error bars represent mean ± SD, n=25, **p<0.01, two-sample Student's t-test.

Controls for each of the GNPs tested were run (0 ng/ml of NS1) and analyzed by SERS. Figure S7 shows control images of samples from Figure 5. All bright field images

showed negative test with no GNP spot on it. SERS mapping images showed no signal for all samples.

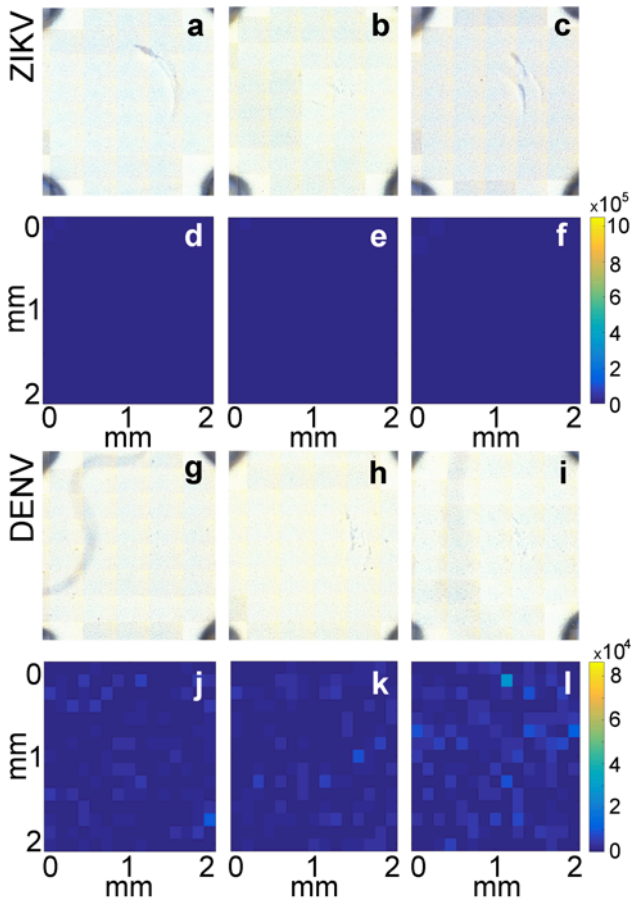



Figure S4: Distribution of signal in sandwich immunoassays of control samples. Bright field images of test area for GNS900-ZIKV (a), GNS400-ZIKV (b), GNSph ZIKV (c), GNS900-DENV (g), GNS400-DENV (h), GNSph DENV (i). SERS mapping images for GNS900-ZIKV (d), GNS400-ZIKV (e), GNSph ZIKV (f) at 1609 $cm^{-1}$ and GNS900-DENV (j), GNS400-DENV (k), GNSph DENV (l) at 1584 $cm^{-1}$. Color bars show Raman Intensity (au).

Vertical profile plots were analyzed for each sample from Figure 5, both in Imagej and Matlab. Plot profiles were taken from a vertical section of 0.13 mm of width in the middle of the spot, from the lowest point to the upper one (Figure S5). For imagej analysis, images were converted into grayscale 8-bit and images were inverted, so that white areas correspond to the highest grayscale intensity.

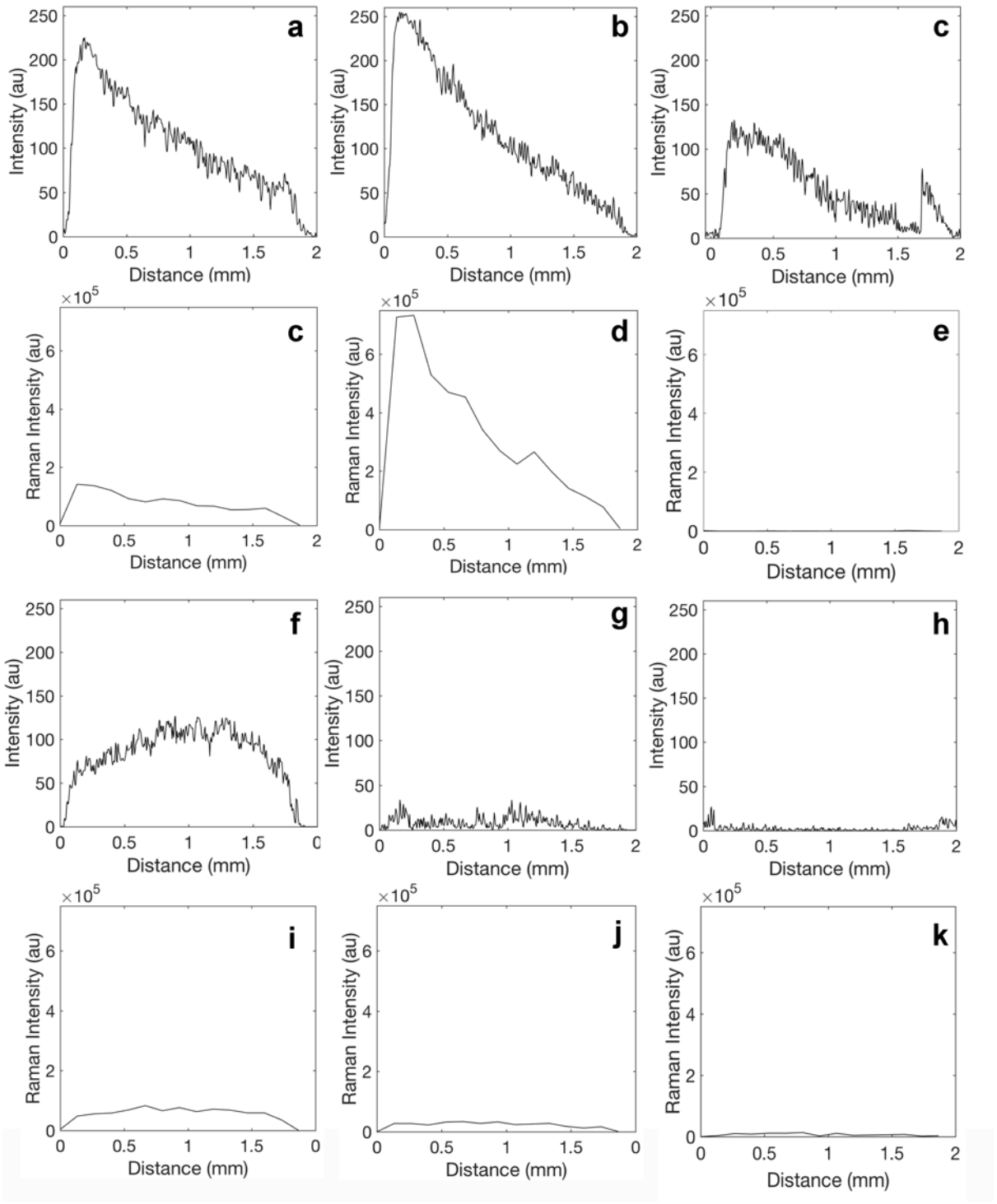


Figure S5: Vertical profile plots of images from Figure 5. Plot profiles from bright field images for GNS900 (a), GNS400 (b) and GNSph (c) for ZIKV and GNS900 (f), GNS400 (g) and GNSph (h) for DENV. Plot profiles from SERS mapping images for GNS900 (c), GNS400 (d) and GNSph (e) for ZIKV and GNS900 (i), GNS400 (j) and GNSph (k) for DENV.

**Quantitative spectral analysis.** NNLS finds the weights of the linear combination of spectra from the pure components contained in the sample that minimizes the squared difference with the Raman spectrum of the sample. In this work, the SERS signal was considered to have 4 components: BPE, MBA, nitrocellulose (strip) and glass (glass microscope slide onto nitrocellulose was placed) (Figure S6). SERS spectra for Z-nanotag and D-nanotag were considered as the BPE and MBA components, respectively, and were acquired from 0.3 µL of nanotags solution in water at the same test concentration deposited directly on a nitrocellulose strip. Nitrocelulose and glass raman spectra were acquired under the same conditions. The NNLS of the 4-components from 800 $cm^{-1}$ to 1800 $cm^{-1}$ was performed in Matlab.

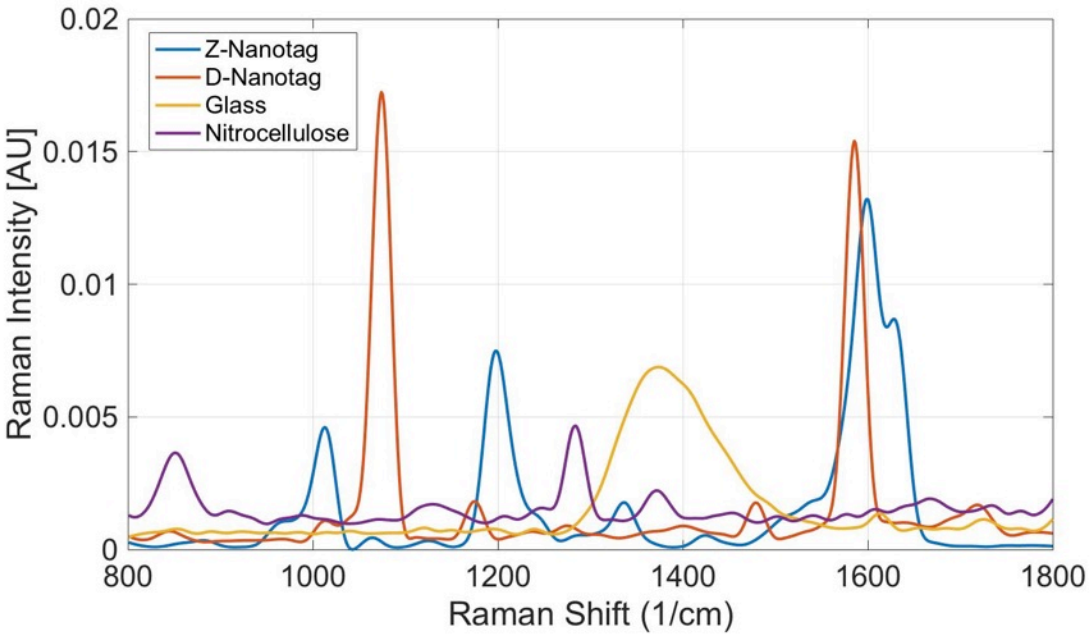



Figure S6: SERS spectra of Z-Nanotag (BPE) and D-Nanotag (MBA) and Raman spectra of glass and nitrocellulose.

For each strip, the aforementioned NNLS analysis was carried out to estimate the contribution of the reporters in each scanning measurement. However, some of these scanning measurements are outside the "spot" and should not be taken into account when analysing the presence of reporter in the test line. In order to keep only the measurements relevant to the analysis, the reporter NNLS components of all scan measurements were sorted in ascending order and the first measurements accounting for 20% of the overall reporter NNLS component were discarded.

The rest of the measurements were taken to be the relevant reporter measurements and their mean reporter NNLS component was used as the NNLS reporter feature that describes the whole test line.

Langmuir isotherms for each biomarker were estimated by running samples at a fix nanotag concentration at decreasing concentration of NS1 (1000-10 ng/ml). Experimental (solid line) and fitted (dashed line) Langmuir isotherms are plotted in Figure S7a for ZIKV NS1 and Figure S7 for DENV NS1.

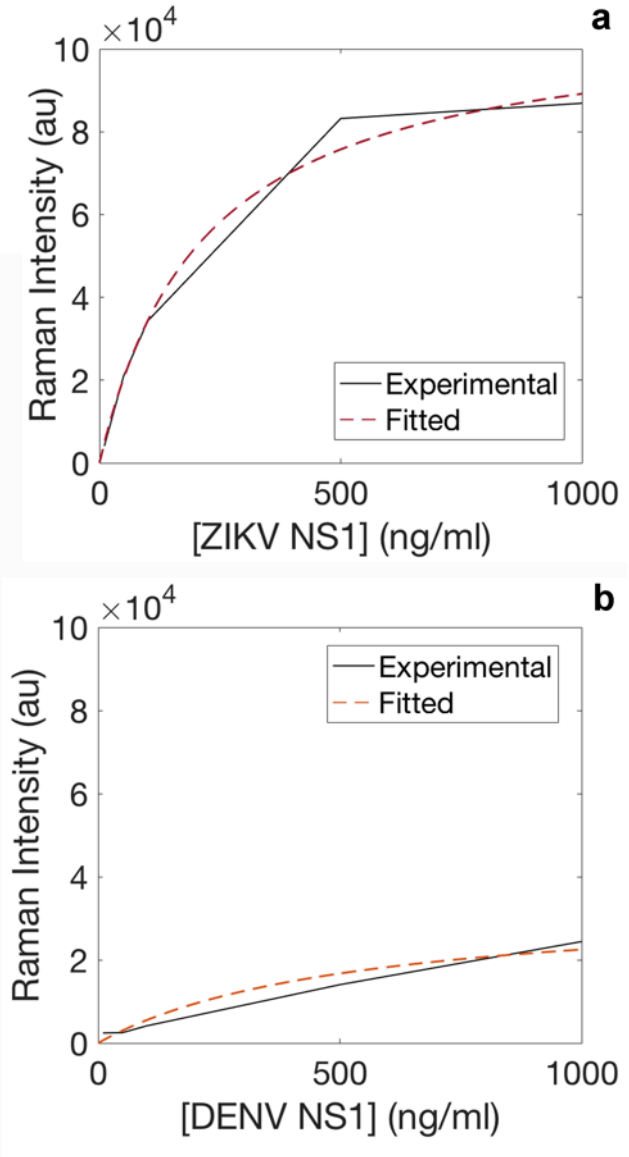



Figure S7: Experimental (solid) and fitted (dashed) Langmuir isotherm for ZIKV NS1 (a) and DENV NS1 (b).

Experimental data in Figure S7a was taken from SERS mapping images in Figure S8a, shown at increasing concentration of ZIKV NS1 (10-1000 ng/ml). Figure S8b shows BPE contribution of multiplex assay from Figure7 (p-t) in the manuscript, at decreasing concentration of ZIKV NS1.

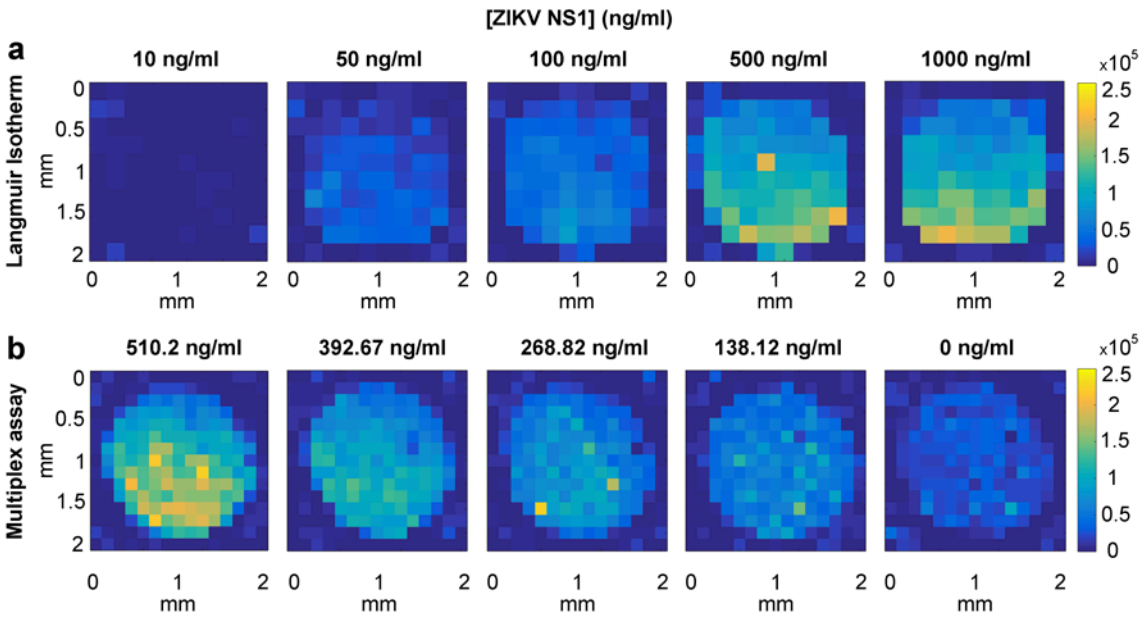



Figure S8: SERS mapping images at increasing concentration of ZIKV NS1 for the Langmuir isotherm estimation (a,1-5). BPE contribution of samples from multiplex assay (Figure 7f-j) at decreasing concentration of ZIKV NS1 (b,1-5).

Experimental data in Figure S7b was taken from SERS mapping images in Figure S9a, shown at increasing concentration of DENV NS1 (10-1000 ng/ml). Figure S8b shows MBA contribution of multiplex assay from Figure7 (p-t) in the manuscript, at increasing concentration of DENV NS1.

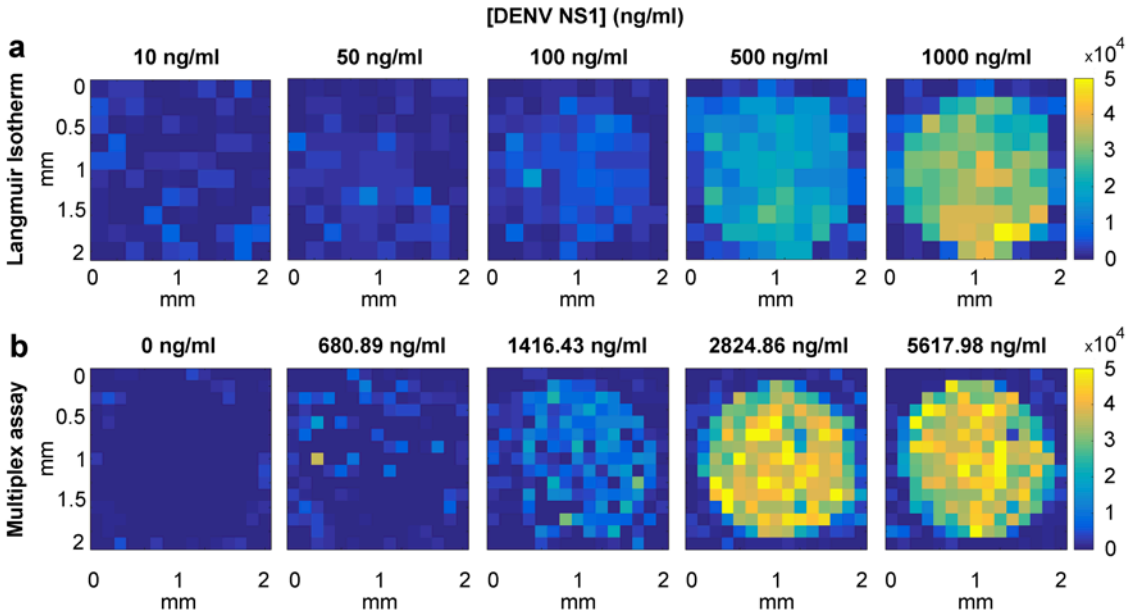



Figure S9: SERS mapping images at increasing concentration of DENV NS1 for the Langmuir isotherm estimation (a,1-5). MBA contribution of samples from multiplex assay (Figure 7p-t) at decreasing concentration of DENV NS1 (b,1-5).