Supporting Information

Multiplex Cancer Cell Detection by SERS Nanotags with Cyanine and Triphenylmethine Raman Reporters

Kaustabh Kumar Maiti, Animesh Samanta, Marc Vendrell, Kiat-Seng Soh, Malini Olivo, Young-Tae Chang*

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Synthetic Materials and Methods

Analytical grade reagents and solvents were purchased from Sigma Aldrich, Alfa Aesar, Fluka, MERCK or Acros, and used without further purification. EGFR-IgG_{2a} (sc-120), HER2 (sc-71667) were supplied by Santa Cruz Biotechnology, Inc. Cell culture materials (FBS, antibiotics, and 0.25% (v/v) Trypsin-EDTA) were purchased from Invitrogen. Normal-phase purifications were carried out using SiliCycle 60 (particle size: 0.040-0.063 mm, 230-400 mesh). Analytical characterization was performed on a 6130 Agilent HPLC-MS with a DAD detector and a single quadrupole mass spectrometer with an ESI probe. Analytical method, unless indicated: eluents: A: H_2O (0.1% HCOOH), B: ACN (0.1% HCOOH), gradient from 5 to 100% B in 4 min; C_{18}(2) Luna column (4.6 × 50mm^2, 5 μm particle size). High resolution mass spectrometry (HRMS) data was recorded on a Micromass VG 7035 (Mass Spectrometry Laboratory at National University of Singapore (NUS)). NMR spectra were recorded on Bruker Advance 300 MHz and 500 MHz spectrometers. Surface plasmon absorption spectra were measured on a SpectraMax M2 spectrophotometer (Molecular Devices). SERS measurements were carried out in a Renishaw InVia Raman microscope with a laser beam (633 nm excitation wavelength) directed to the sample through 20× objective lens and a Peltier cooled CCD detector. Prior to every measurement, a calibration with a silicon standard (Raman peak centered at 520 cm^{-1}) was performed, and WiRE 3.0 software package was used for data acquisition and analysis.

1. Synthetic procedures and characterization of 1, 2, 3, Cy3LA and Cy5LA.

Synthesis of **1**.

To a solution of 2,3,3-trimethyl-3*H*-indole (2 g, 12.5 mmol, 1 eq.) in ACN, 1-iodopropane (10.6 mL, 62 mmol, 5 eq.) was added, and refluxed with continuous stirring for 15 h. The mixture was dried in high vacuum and washed by Et_2O. The resulting solid was re-crystallized in acetone to obtain **1** as a white solid (3.9 g, yield 95%).

^{1}H-NMR (300 MHz, DMSO-d_6): 1.04 (t, 3H, *J* = 7.2 Hz), 1.64 (s, 6H), 2.67 (s, 3H), 1.34 (m, 2H), 4.17 (t, 2H, *J*=7.8 Hz), 7.63 (d, 2H, *J* = 7.2 Hz), 7.82 (m, 2H).

tR: 2.46 min, ESI *m/z* ($C_{14}H_{20}N^+$) calc: 202.4; found: 202.1.

Synthesis of **2**.

3-bromopropylamine hydrobromide (2.74 g, 12.5 mmol, 1 eq.) was added in a seal tube containing 2,3,3-trimethyl-3*H*-indole (2 mL, 12.5 mmol, 1 eq.) under N_2 atmosphere, and was gently heated up to 110 ^{o}C in an oil bath. The mixture was kept at 120 ^{o}C for 10 h with stirring. After the reaction was completed, the mixture was cooled down to r.t. to form a solid cake that was washed with Et_2O and a chloroform-Et_2O (1:1) solution. The resulting solid was then dried under high vacuum to obtain **2** as a white solid (4.25 g, yield 85%).

^{1}H-NMR (300 MHz, DMSO-d6): 1.55 (s, 6H), 2.16-2.21 (m, 2H), 2.50 (s, 3H), 3.05-3.07 (m, 2H), 4.60 (t, 2H, *J* = 7.5 Hz), 7.61-8.08 (m, 4H).

tR: 2.10 min, ESI-MS m/z ($C_{14}H_{22}BrN_2^+$) calc: 217.2, found: 217.1.

Synthesis of **3**.

2 (0.38 g, 1 mmol, 1 eq.) and di-tert-butyl dicarbonate (0.55 g, 2.5 mmol, 2.5 eq.) were added to a mixture of dry $CHC1_3$ (30 mL) and DIEA (0.88 mL, 5 mmol, 5 eq.). The reaction mixture was gently heated to reflux temperature and stirred for 4 h. Afterwards, the organic layer was extracted with Et_2O, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. Purification of the crude residue on a silica gel column (elution with CH_2C1_2-MeOH 50:l) rendered 3 as a light brown liquid (1.26 g, yield 60%).

^{1}H-NMR (300 MHz, $CDCl_3$): 1.33 (s, 9H), 1.44 (s, 6H), 1.84-1.87 (m, 2H), 2.27(s, 3H), 3.42 (t, 2H, *J* = 6.5 Hz), 3.55 (t, 2H, *J* = 7.0 Hz), 6.52-7.12 (m, 4H).

tR: 5.21 min, ESI-MS m/z ($C_{19}H_{29}N_2O_2^+$) calc: 317.2, found: 317.1.

Synthesis of **6**.

N,*N*-diphenylformamidin hydrochloride **4**, (0.39 g, 2 mmol, 1 eq.) was condensed with **1** (0.66 g, 2 mmol, 1 eq.) in a solution of ACOH:Ac_2O (1:1) at 110 ^oC for 20 min, and cooled down to r.t. Then **3** (1.2 g, 3 mmol, 1.5 eq.) and pyridine were added to the mixture and stirred under reflux. After 1 h, the reaction mixture was poured into water and $NaHCO_3$ was slowly added with stirring until complete neutralization was reached. After diluting with CH_2Cl_2 the organic layer was washed, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Purification of the crude residue on a silica gel column (elution with CH_2C1_2-MeOH 50:2) rendered **6** as a blue solid (0.57 g, yield 50%).

ESI-MS m/z ($C_{33}H_{44}N_3O_2^+$) calc: 528.35, found: 528.2.

Synthesis of **7**.

Malonaldehyde bis(phenylimine) hydrochloride **5**, (0.52 g, 2 mmol, 1 eq.) was condensed with **1** (0.66 g, 2 mmol, 1 eq.) in a solution of ACOH:Ac_2O (1:1) at 110 ^oC for 20 min, and cooled down to r.t. Then **3** (1.2 g, 3 mmol, 1.5 eq.) and pyridine were added to the mixture and stirred under reflux. After 1 h, the reaction mixture was poured into water and $NaHCO_3$ was slowly added with stirring until complete neutralization was reached. After diluting with CH_2Cl_2 the organic layer was washed, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Purification of the crude residue on a silica gel column (elution with CH_2C1_2-MeOH 50:2) rendered **7** as a blue solid (0.66 g, yield 54%).

ESI-MS m/z ($C_{36}H_{48}N_3O_2^+$) calc: 554.4, found: 554.2.

Synthesis of lipoic acid nitrophenol resin.

Aminomethyl nitrophenol polystyrene resin was prepared according to reported procedures.[1] The nitrophenol resin (2 g, 2.9 mmol, 1 eq.) was swollen in 10 mL of DMF, and lipoic acid (2 g, 10 mmol, 3.3 eq.), *N*,*N*'-diisopropylcarbodiimide (1.2 mL, 12 mmol, 4 eq.) and a catalytic amount of DMAP (20 mg) were added to the resin, which was continuously shaken for 24 h at r.t. Subsequently, the resin was washed with DCM (10 × 25 mL) and dried under vacuum until use.

Synthesis of **Cy3LA** and **Cy5LA**.

For the preparation of **Cy3LA**, **6** (0.10 g, 0.19 mmol) was treated with a solution of TFA-DCM (1:9) at r.t. overnight, washed with a solution of $NaHCO_3$, and the organic layer was dried over anhydrous Na_2SO_4 and

concentrated under reduced pressure. The resulting solid was dissolved in a solution of CH_2Cl_2-CH_3CN (9:1), added to the lipoic acid nitrophenol resin and shaken for 16 h at r.t. After the reaction, the resulting filtrates were combined and dried under pressure to render **Cy3LA** as a blue solid (92 mg, yield 85%). The same procedure was used starting from **7** to obtain **Cy5LA** (95 mg, yield 78%).

Cy3LA: ^{1}H-NMR (500 MHz, $CDCl_3$): 1.08 (t, 3H, *J* = 7.0 Hz), 1.69-1.72 (m, 4H), 1.84 (s, 6H), 1.85 (s, 6H), 1.87-1.91 (m, 4H), 2.40-2.44 (m, 2H), 3.08-3.10 (m, 2H), 3.14-3.17 (m, 2H), 3.48-3.52 (m, 2H), 3.58-3.62 (m, 2H), 4.17 (t, 2H, *J* = 7.5 Hz), 4.25 (t, 2H, *J* = 7.5 Hz), 5.29 (s, 3H), 7.06-8.42 (m, 8H), 8.39 (bs, 1H).

^{13}C-NMR (126 MHz, $CDCl_3$): 11.25, 20.97, 25.59, 26.45, 28.08, 28.12 (2C), 28.87(2C), 29.62, 30.85, 34.58, 35.83, 36.45, 40.08, 42.91, 45.89, 53.34, 56.53, 104.43, 110.98, 121.92, 121.99, 125.14, 125.26, 128.75, 128.96, 140.49, 140.55, 141.77, 150.89, 173.62, 173.80, 174.43.

tR: 3.98 min, ESI-HRMS m/z ($C_{37}H_{50}N_3O\ S_2^+$) calc: 616.3390, found: 616.3388

Cy5LA: ^{1}H-NMR (500 MHz, $CDCl_3$): 1.09 (t, 3H, *J* = 7.0 Hz), 1.59-1.62 (m, 4H), 1.69 (s, 6H), 1.72 (s, 6H), 1.84-1.95 (m, 4H), 2.45-2.49 (m, 3H), 3.08-3.10 (m, 2H), 3.14-3.17 (m, 2H), 3.48-3.52 (m, 2H), 3.58-3.62 (m, 2H), 3.94 (t, 2H, *J* = 7.5 Hz), 4.40 (t, 2H, *J* = 7.5 Hz), 6.17 (d, 2H, *J* = 7.2 Hz), 6.98 (d, 1H, *J* = 8.0 Hz), 7.06-8.42 (m, 8H), 8.61 (bs, 1H).

^{13}C-NMR (126 MHz, $CDCl_3$): 11.43, 20.69, 25.67, 26.78, 28.04, 28.28, 28.89(2C), 28.92 (2C), 30.37, 34.63, 36.12, 36.59, 38.40, 38.76, 43.58, 45.49, 48.83, 49.40, 56.62, 102.74, 106.02, 110.11, 111.44, 122.02, 122.94, 124.55, 125.59, 127.84, 128.97, 140.67, 141.61, 141.82, 142.43, 151.90, 153.85, 171.22, 173.68, 174.47.

tR: 4.07 min, ESI-HRMS m/z ($C_{39}H_{52}N_3O\ S_2^+$) calc: 642.3546, found: 642.3555.

2. Surface plasmon absorption spectra.

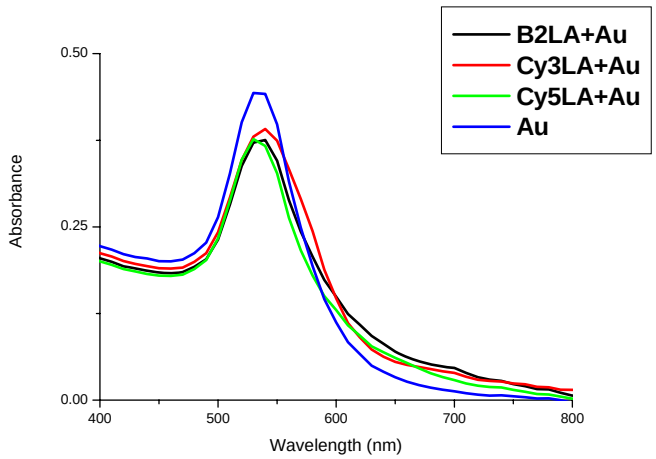

Figure S1. Surface plasmon absorption spectra of Au-colloid and **B2LA**, **Cy3LA** and **Cy5LA**-nanotags.

3. Procedures for nanotag labeling and conjugation, cell culture, Raman spectra determination and mapping experiments.

Nanotag labeling and antibody conjugation

The reporter molecules (**B2LA** and **Cy3LA**, 10 μM) were incubated with citrate-stabilized Au-colloid for 10 min before the heterofunctional linker HS-PEG-CO_2H (10 μM) was added to both solutions (volume: 2.6 mL). After shaking for 15 min, the Au-colloid was exposed to excess PEG-SH (1.8 mL, 10 μM) to maximize the surface coverage and stabilize the PEG and the chemisorbed reporter molecule. After 4 h, free PEG-SH was removed incubation by 3 rounds of centrifugation (4000 rpm, 15 min), and the colloid was re-suspended in PBS for bioconjugation. The carboxylic acids groups were activated with *N*-(3-(dimethylamino)-propyl)-*N*'-ethylcarbodiimide (EDC) (125 nmol) and *N*-hydroxysuccinimide (NHS) (125 nmol). After 30 min incubation, excess of EDC and NHS was removed by 3 rounds of centrifugation (8000 rpm, 10 min), and re-suspended in PBS using Amicon Ultra 3K centrifuge filters (Milipore). The activated particles were then reacted with two different antibodies at 25°C for 2 h and then overnight at 4 °C: for B2LA nanotags, a mouse monoclonal anti-EGFR IgG_{2a} (12 nM); for Cy3LA nanotags, a mouse monoclonal anti-HER2. Further non-specific binding chemicals and antibodies were removed by centrifugation (8000 rpm, 10 min) and the final nanotags were re-suspended in PBS and stored at 4 °C.

Thiolated PEG encapsulation

Freshly prepared reporter solutions at different concentrations (e.g. 5 to 30 μM) were mixed with Au colloid in a 1:9 ratio (v/v) to optimize the reporter concentration. Maximum SERS intensities and minimum colloidal aggregation were obtained at 10 μM concentration of reporter. After 10 min incubation, a 100 μM solution of thiolated PEG (PEG-SH, M.W $_{PEG}$: 5 kDa) was added in 10 to 20-fold excess and incubated overnight incubation. Excess of PEG-SH was removed by 3 rounds of centrifugation (5000 rpm for 6 min) and re-suspension in water.

Cell culture

OSCC and SKBR-3 cells were grown in RPMI1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 U mL^{-1} penicillin/100 μg mL^{-1} streptomycin) in a humidified atmosphere at 37 °C with 5% (v/v) CO_2. Approximately 5×10^5 cells per well were seeded on 12-well culture plates the day before SERS measurements.

SERS measurements of **B2LA**, **Cy3LA** and **Cy5LA**-gold colloid mixtures

20 μM solutions of **B2LA**, **Cy3LA** and **Cy5LA** in deionized water were mixed with Au colloid (2.6×10^{10} particles/mL) in a 1:9 ratio (v/v). 20 μL of the reporter-Au colloid mixture solutions were placed on a glass slide with cover slip, and their Raman spectra (range: 400 to 2000 cm^{-1}, resolution: 1 cm^{-1}, acquisition time: 10 s) were measured in a Renishaw InVia Raman microscope under excitation with a 633 nm excitation wavelength laser (6.2 mW power). The results are plotted as average intensities of three independent experiments.

SERS spectra determination and SERS mapping experiments in OSCC, SKBR-3 cells and co-cultured cells

OSCC, SKBR-3 and co-cultures cells were grown as mentioned above in 12-well culture plates. For co-culture experiments, an equal amount (aprox. 5×10^5 cells) of OSCC and SKBR-3 cells were plated in the same well 24 h before the measurements. Antibody-conjugated **B2LA** and **Cy3LA**-nanotags (450 pM) were incubated with OSCC, SKBR-3 or co-cultured cells for 1 h at 25 °C. Afterwards, the cells were washed with cold PBS (×3), gently scrapped and re-suspended in PBS (every well of a 12 well-plate containing approximately 5×10^5 cells was re-suspended in 100 μL PBS). Raman experiments were performed in a Renishaw InVia Raman microscope with a laser beam directed to the sample through 20× objective lens and a Peltier cooled CCD detector. Samples were excited with a 633 nm excitation wavelength laser, and Raman spectra were collected in the range of 400 to 2000 cm^{-1} with 1 cm^{-1} resolution. Acquisition time for all spectra was 10 s.

For SERS mapping experiments, cells were plated in a 8-well glass slide at a density of 10^6 cells/mL, and after incubation with antibody-conjugated **B2LA** and **Cy3LA**-nanotags (450 pM) for 1 h at 25 °C, cells were rinsed with cold PBS (×3) and subsequently placed on a cover slip with mounting media. Raman experiments were performed in a Renishaw InVia Raman microscope with a laser beam directed to the sample through 20× objective lens and a Peltier cooled CCD detector. Samples were excited with a 633 nm excitation wavelength and a laser power of 6.2 mW, and measurements were carried out as raster scans in 2 μm steps over OSCC and SKBR-3 cells with a computer-controlled *xy*-stage.

4. TEM images of B2LA and Cy3LA nanotags.

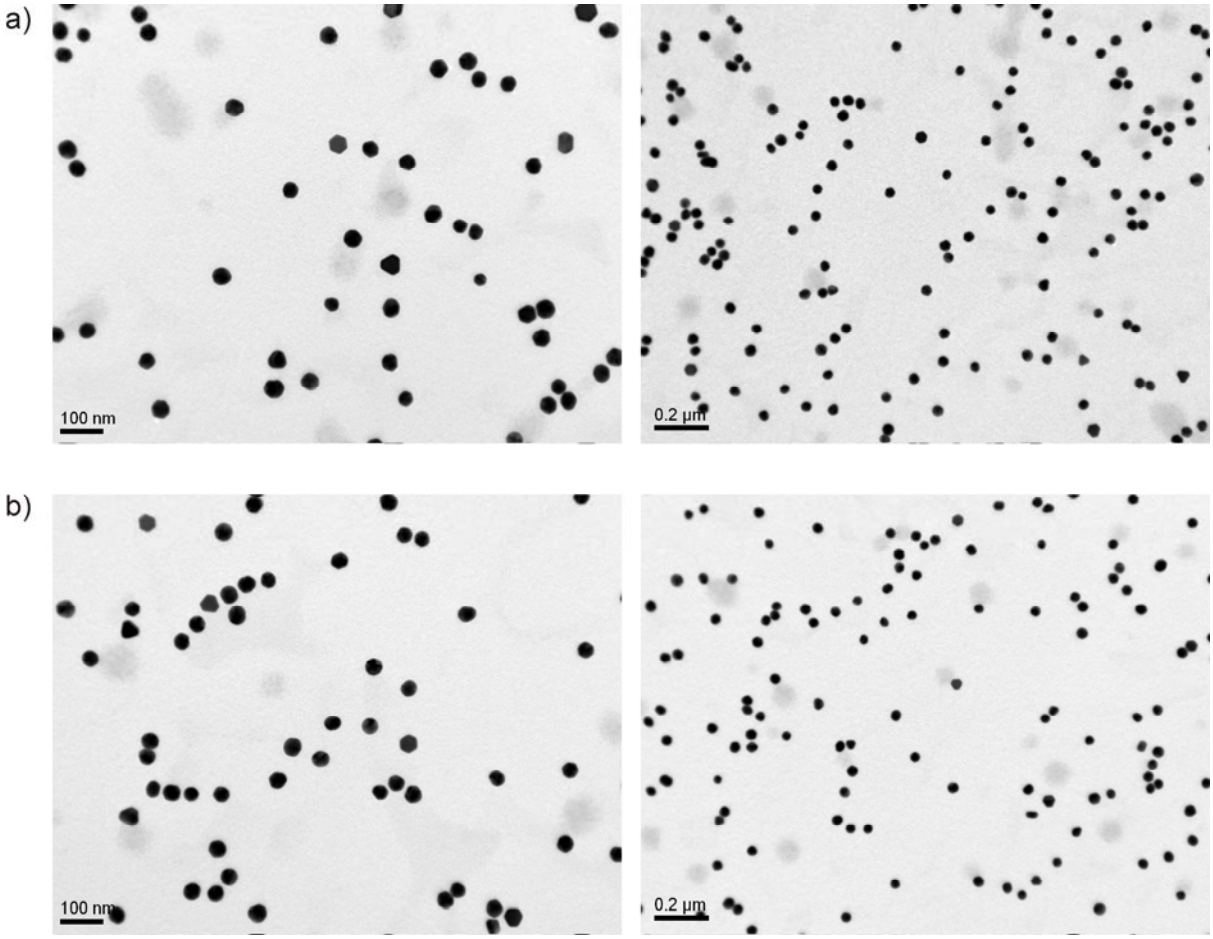

Figure S2. Transmission Electron Microscopy (TEM) images of nanotags derivatized with: (a) **B2LA**, (b) **Cy3LA**.

5. Stability of the SERS signals for B2LA and Cy3LA-nanotags.

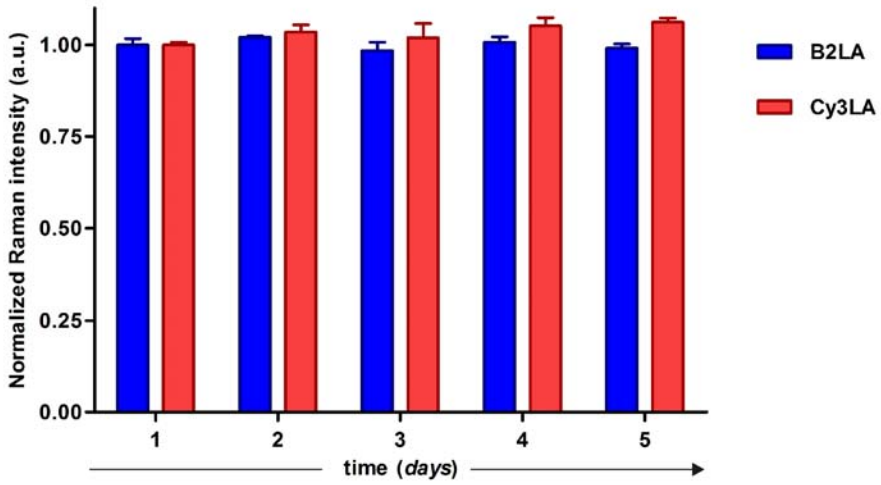

Figure S3. Evaluation of the stability of the SERS signals for **B2LA** anti-EGFR and **Cy3LA** anti-HER2 nanotags. Values are represented as means ± SD (*n*=3).

6. SERS measurements of antibody-free B2LA and Cy3LA-nanotags in OSCC or SKBR-3 cells.

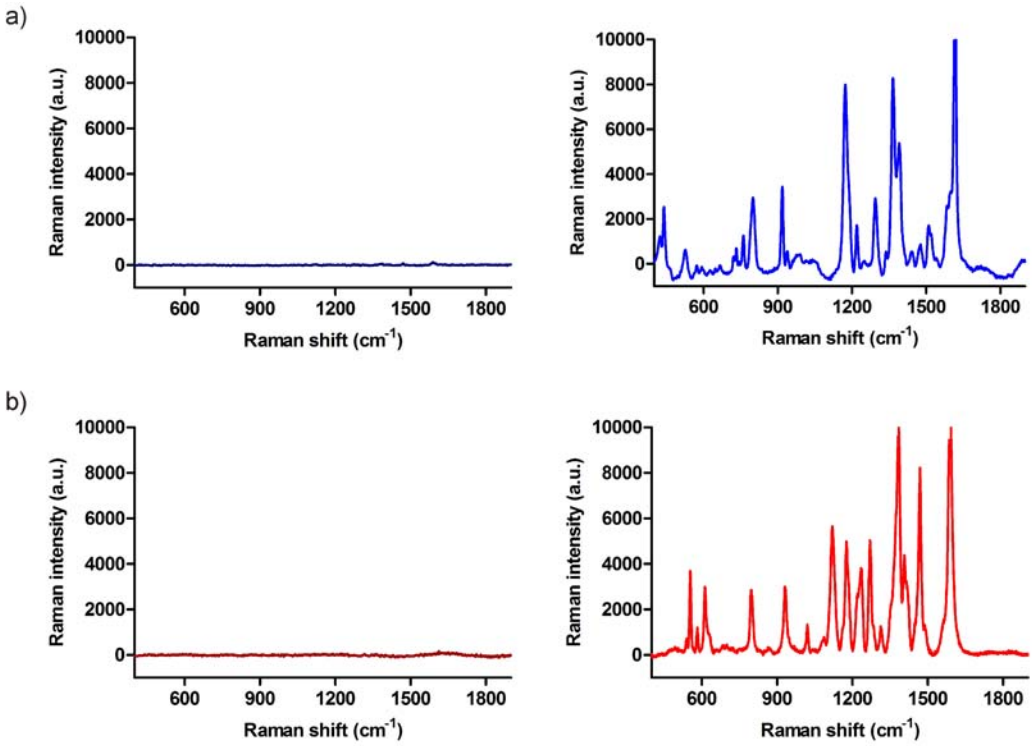

Figure S4. (a) SERS spectra of OSCC cells after incubation with antibody-free **B2LA**-nanotags (left) or anti-EGFR **B2LA**-nanotags (right); (b) SERS spectra of SKBR-3 cells after incubation with antibody-free **Cy3LA**-nanotags (left) or anti-HER2 **Cy3LA**-nanotags (right).

7. SERS mapping experiments in non-treated OSCC and SKBR-3 cells.

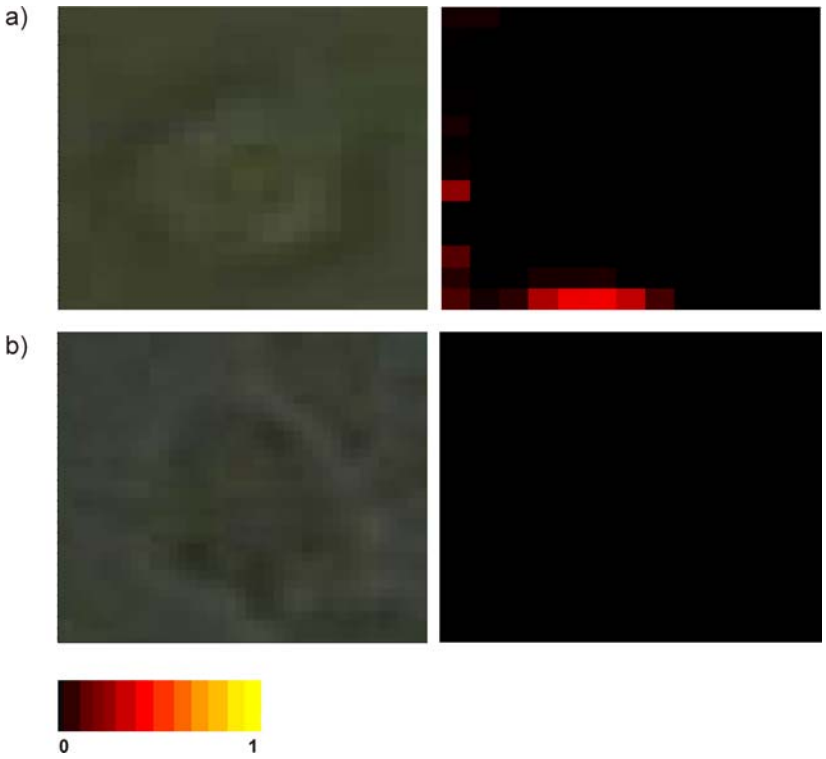

Figure S5. Bright field and SERS mapping images of: (a) non-treated OSCC cells (1615 cm^{-1}), (b) non-treated SKBR-3 cells (1468 cm^{-1}). All mapping images (size: 30 × 30 μm^2) were scanned at an interval of 2 μm (633 nm excitation wavelength) and the intensities were normalized between the lowest (0) and the highest color (1) values of the corresponding **B2LA** anti-EGFR nanotag-treated OSCC cells and **Cy3LA** anti-HER2 nanotag-treated SKBR-3 cells shown in Figure 3.

References

1. J. W. Lee, Y. Q. Louie, D. P. Walsh, and Y. T. Chang, *J. Comb. Chem.*, 2003, **5**, 330-335.