Supporting Information

3D Optical Imaging of Multiple SERS Nanotags in Cells

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Nanoparticle and Nanotag Preparation

Nanoparticle Synthesis

Citrate reduced silver (Ag) colloid was prepared according to the Lee and Meisel method[1] whereby 90 mg of silver nitrate was added to 500 mL of distilled water (dH_2O) and heated until boiling. Upon boiling a 1% aqueous solution of sodium citrate (100 mg in 10 mL dH_2O) was added and boiling was maintained for 1h. The solution was then allowed to cool and continuous stirring was maintained throughout.

Nanotag Preparation

The nanotag stock solution was prepared by reacting 10 mL of Ag citrate colloid with 10 µL of 1,6 – hexamethylene diamine (1,6-HMD, 0.2 M - 0.0232 g in 1 mL dH_2O) for 3 mins. 2 mL of polyvinylpyrrolidone (PVP, 250 µM - 100 mg in 10 mL dH_2O) was then added to quench the aggregation induced by the 1,6-HMD. 1 mL aliquots were removed and labelled with either 10 µM (final concentration), NBT (10 mM, 0.00775 g in 5 mL MeOH) or 2-NPT (10 mM, 0.008012 g in 5 mL MeOH) or 50 µM (final concentration) MPY (10 mM 0.00556 g in 5 mL MeOH) or DTNB (10 mM, 0.0198 g in 5 mL MeOH).

Cell Preparation

Chinese Hamster Ovarian (CHO) cells were routinely grown in Ham's F12 nutrient mixture with heat-inactivated fetal bovine serum (10%), penicillin (1% v/v, 10.000 IU mL^{-1}) and streptomycin (10 mg mL^{-1}) at 37°C in a humidified 5% CO_2 atmosphere. After reaching confluence, cells were harvested and seeded at $2x10^5$ cells / mL on 25 mm x 1 mm CaF_2 windows. The cells were incubated overnight at 37°C, 5% CO_2 in order to allow the cells to

adhere to the windows. Ag nanotags were then added to the cells and incubated for 1 h. Following incubation and in order to remove any extracellular material the cells were washed four times with phosphate buffered saline (PBS) before fixation with paraformaldehyde (4% v/v). After 15 mins the windows were washed consecutively with PBS and dH_2O. After fixation the windows were air dried for ~2 h.

Analysis of cell samples

2D SERS imaging

Fixed cell samples were initially imaged in 2D (StreamLine™, StreamLineHR™ - Renishaw inVia Raman spectrometer / Leica DMI 5000 M microscope, Renishaw plc, Gloucestershire, UK.). A 633 nm laser (HeNe) excitation source was used and cell samples were imaged under immersion in a saline solution using an Olympus 60x (N.A. 1) water immersion objective. A grating of 1800 lines / mm was used with a RenCam charge-coupled device (CCD) (1040 x 256 pixels). Line mapping and high resolution images were performed with a StreamLine and StreamLineHR Raman mapping system and the following conditions were used – 633 nm line focus, spectral range 934.7 – 1720.8 cm^{-1}, ~8 mW laser power, acquisition time 5 s and 633 nm spot focus, spectral range 934.7 – 1720.8 cm^{-1}, 0.4 mW laser power, acquisition time 3 s, respectively. Following data collection a multiple component positive cell was identified by performing multivariate analysis in the form of component DCLS.

Volume 3D Raman imaging of cells

Once a multiple component cell was identified the cell was imaged in 3D (Volume Viewer™ using the system described above). A 532 nm laser (Cobalt) excitation source was used and cell samples were imaged under immersion as described above. A grating of 1800 lines / mm was used with a RenCam charge-coupled device (CCD) (1040 x 256 pixels). The following conditions were used – 532 nm volume mapping, spectral range 743.4 – 1600.7 cm^{-1}, ~ 40 mW laser power, acquisition time 2 s. The step sizes in the x and y directions were set at 0.8 µm and in the z direction it was set at 1µm. The z range was initially defined as ± 3 µm from the point of focus when the cell was observed under white light illumination (set as z = 0). It was apparent that the spectra from above z-slice +2.00 and below +0.00 were not relevant thus the 3D cell map was recollected from z-slice +2.00 - +0.00.

Volume 3D SERS imaging of nanotags within cells

Following cell imaging with a 532 nm laser excitation the same area and cells were imaged in 3D (Volume Viewer™ using the system described above) using a 633 nm laser (HeNe) excitation source to determine the localisation of the SERS nanotags. Cell samples were imaged under immersion as described above. A grating of 1800 lines / mm was used with a RenCam charge-coupled device (CCD) (1040 x 256 pixels). The following conditions were used – 633 nm volume mapping, spectral range 934.7 – 1720.8 cm^{-1}, ~ 0.4 mW laser power, acquisition time 0.5 s. The step sizes in the x and y directions were set at 0.5 µm and in the z direction it was set at 1µm. The z range was defined as ± 3 µm from the point of focus when the cell was observed under white light illumination (set as z = 0).

Production of the volume 3D images and the 2D z-slice images.

The volume 3D Raman cell images of the cell and the nuclei (Fig. 1) were taken directly from the WiRE 3 Volume Viewer software. Images were cropped with Microsoft Office Picture Manager and the original labels on the on the x, y and z axes were replaced using textboxes in Microsoft Office PowerPoint simply to increase clarity.

The 2D slices for the Raman images of the cell and the nuclei, and the 2D slices for the SERS images of the nanotags (Figs. 3, 4) were taken directly from the Volume Viewer software. Images were cropped with Microsoft Office Picture Manager and the original labels on the x, y and z axes were replaced using textboxes in Microsoft Office PowerPoint to increase the clarity. The individual 2D slices were then combined into a single image using a GNU image manipulation programme. The y-axis for the Raman cell and SERS nanotag images were measured from -22.00 – 20.40 and -22.50 –20.00 respectively. In order to account for this 0.5 µm discrepancy the SERS image was offset from the Raman image by 0.5 µm. This was accurately determined by pixel count.

Characterisation Data

UV-Visible Absorption Spectroscopy

UV-visible absorption spectra were recorded on a Varian Cary® 300 Bio UV-visible spectrophotometer using 1 cm path length cells. A distilled water blank was run prior to any sample analysis to establish a baseline. Sample analysis was carried out with 150 µL of the

colloid, unlabelled and labelled nanotags diluted to 2 mL with distilled water; all UV-visible spectra were normalised.

Scanning Electron Microscopy (SEM) Imaging

Silicon wafers were cleaned using water and ethanol before being dried under nitrogen flow. Wafers were placed in an oxygen plasma cleaner for 60 s before treatment with poly(diallyldimethylammonium) chloride (PDDA) (50 µL) – (30 µL PDDA in 1 mL 1 mM NaCl). The PDDA provides a positive surface to which the negatively charged nanoparticles can adhere. Wafers were left for 30 mins before being washed with distilled water and dried under nitrogen flow. 50 µL of sample was deposited on to the individual wafers and allowed to rest for 30 mins. The solution was then removed and the wafers were again washed with distilled water and dried under nitrogen flow. Imaging was carried out on a Sirion 200 Schottky field emission electron microscope (FEI) operating at an accelerating voltage of 5 kV.

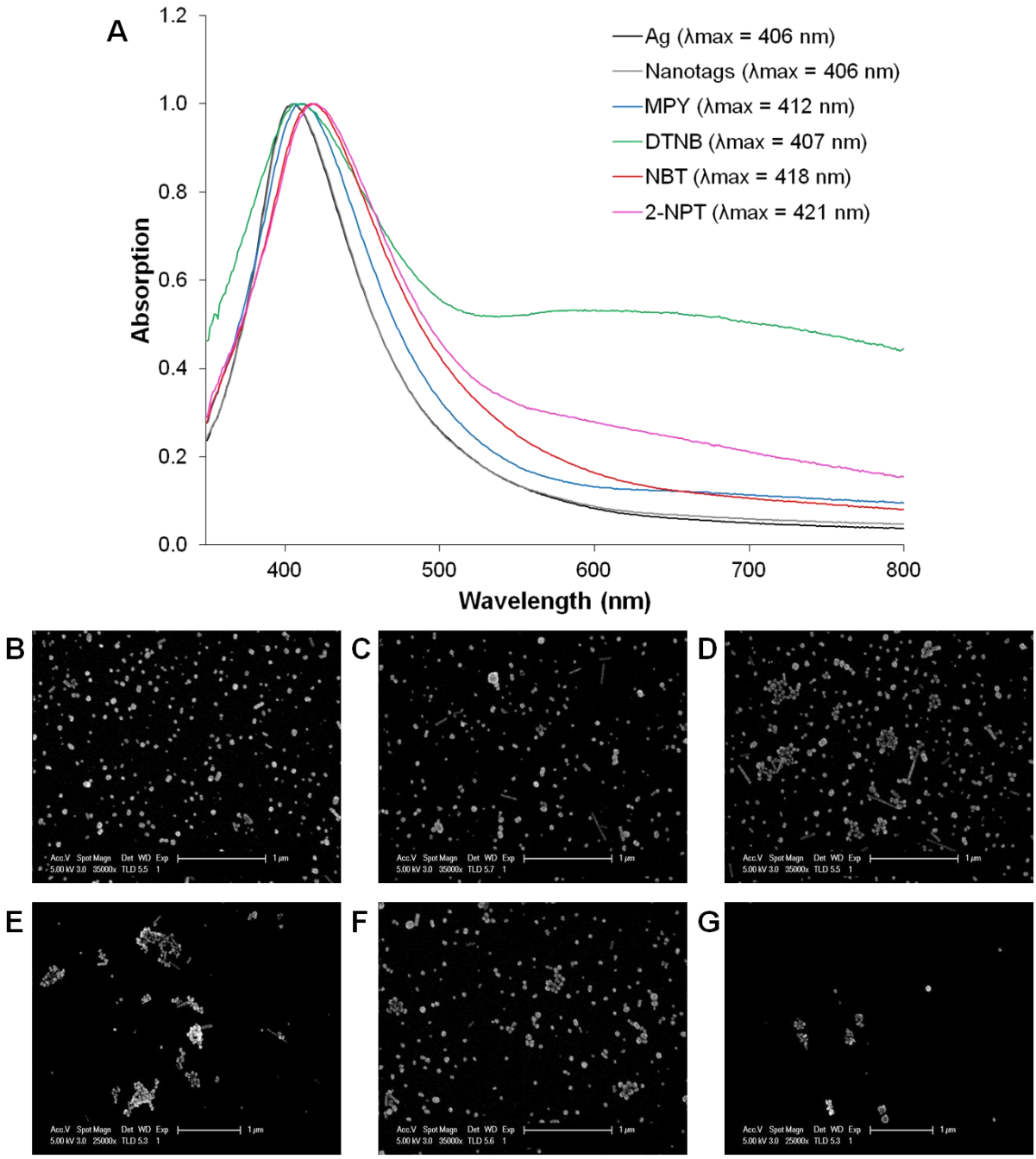

Fig. S1 UV-Visible absorption spectroscopy and SEM characterisation of bare Ag colloid, unfunctionalised and small molecule functionalised nanotags. **(A)** Absorption spectra of bare Ag colloid and nanotags, **(B-G)** SEM was used to characterise **(B)** bare Ag colloid, **(C)** unfunctionalised nanotags, **(D)** MPY functionalised nanotags, **(E)** DTNB functionalised nanotags, **(F)** NBT functionalised and **(G)** 2-NPT functionalised nanotags.

Dynamic Light Scattering (DLS) and Zeta potential measurements

DLS was used to determine the size of the nanoparticles within the suspension and observe the size effect of functionalising the nanoparticle surface. Sample analysis was carried out with 1 mL of the colloid, unlabelled and labelled nanotags diluted to 2 mL with distilled water. DLS measurements were carried out on a Malvern Zetasizer Nano Zs using 1 cm path length cells.

Zeta potential measurements were carried out in an identical manner to the DLS measurements except a 1 cm path length dip cell was used with 600 µL of the appropriate sample.

Table S1 DLS size data for bare Ag citrate colloid, unlabelled and small molecule labelled nanotags.

Solution measured	Mean Size (nm)	Standard Deviation (nm)	Relative Standard Deviation (RSD, %)
Ag colloid	42.00	0.5046	1.2
Unlabelled nanotags	41.55	0.1626	0.391
MPY labelled nanotags	42.91	0.6001	1.4
DTNB labelled nanotags	91.62	0.3695	0.403
NBT labelled nanotags	92.42	0.1804	0.195
2-NPT labelled nanotags	82.79	0.6716	0.811

Table S2 Zeta potential data for bare Ag citrate colloid, unlabelled and small molecule labelled nanotags.

Solution measured	Zeta Potential (mV)	Standard Deviation (mV)	Relative Standard Deviation (RSD, %)
Ag colloid	-38.3	1.18	3.07
Unlabelled nanotags	-24.0	1.70	7.09
MPY labelled nanotags	-36.4	3.19	8.76
DTNB labelled nanotags	-24.2	0.976	4.03
NBT labelled nanotags	-23.8	0.662	2.78
2-NPT labelled nanotags	-13.1	0.474	3.62

Solution measurements of the SERS nanotags

The small molecule functionalised nanotags were prepared according to the protocol described (Nanoparticle and Nanotag preparation – nanotag preparation). 50 µL aliquots were analysed using a Renishaw inVia Raman spectrometer / Leica DMI 5000 M microscope and the following conditions were used –633 nm spot focus, 5x (N.A. 0.12) spectral range 99.2 – 3198.7 cm^{-1}, ~10 mW laser power, acquisition time 10 s, respectively. No peaks were found to occur below 900 cm^{-1} or above 1700 cm^{-1} hence the spectra are only shown within the range 900 – 1700 cm^{-1}.

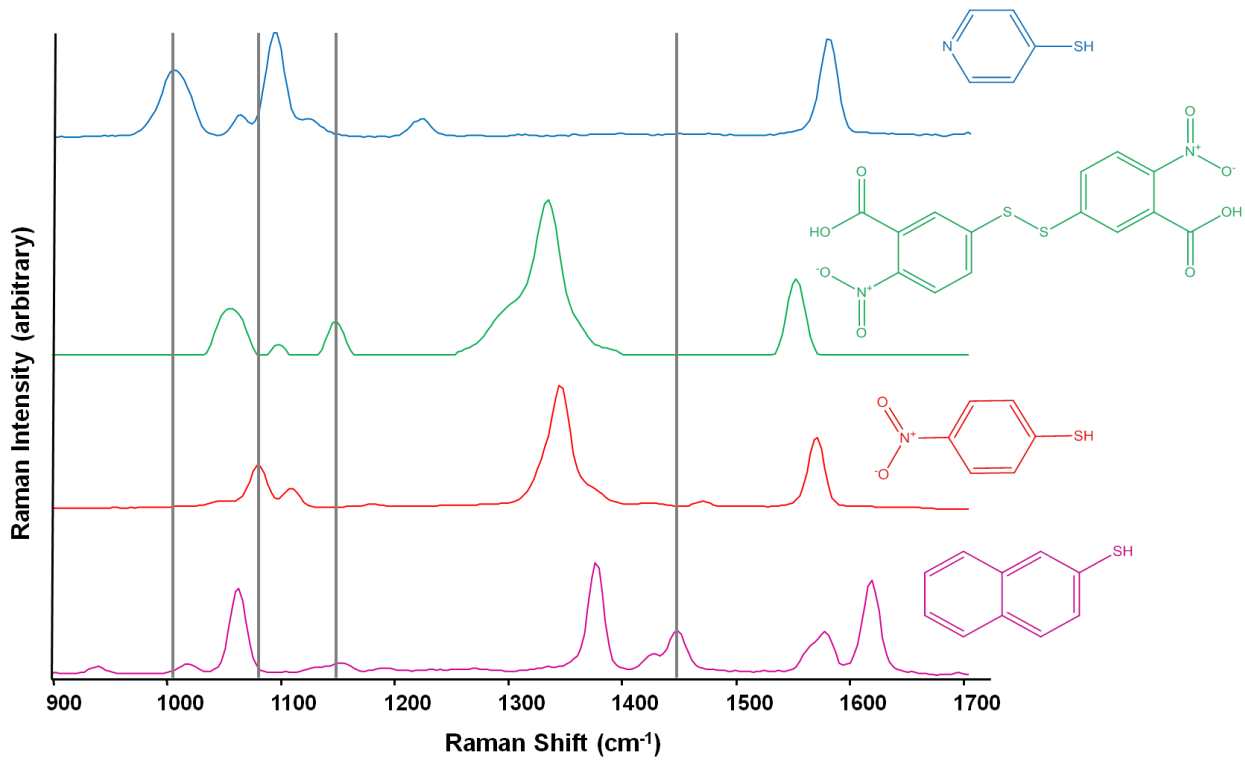

Fig. S2 Suspension SERS spectra of the prepared nanotags and corresponding structures for the small molecule reporters - 4-mercaptopyridine (MPY), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 4-nitrobenzenethiol (NBT) and 2-naphthalenethiol (2-NPT) are shown in blue, green, red and magenta respectively. Unique peaks were found at ~ 1004, 1150, 1084 and

1450 cm^{-1} for MPY, DTNB, NBT and 2-NPT respectively. (λ_{ex} = 633 nm, extended scan, 10 s, 900-1700 cm^{-1}).

References

1. P. C. Lee and D. Meisel, *J. Phys. Chem.*, 1982, **86**, 3391-3395.