Supporting Information

for

Live Chicken Egg Embryos as an Alternative *In Vivo* Tumour Model for Deep Surface Enhanced Raman Spectroscopy

1. Materials and Methods

All materials used were purchased from Sigma-Aldrich Ltd (Dorset, UK), unless stated otherwise. The U87-MG glioblastoma cancer cell line was purchased from Caliper Life Sciences (Waltham, Massachusetts, US). Eagle's minimum essential medium (EMEM), Foetal bovine serum (FBS), Amphotericin-B, Penicillin/Streptomycin, Matrigel, Trypsin-EDTA, Histoplast and pelletised paraffin wax were purchased from Thermo Fisher Scientific (Leicestershire, UK). Histoclear clearing agent was purchased from National Diagnostics (Hull, UK). Bovine serum albumin was purchased from Fisher bioreagents, Fisher Scientific (Ottawa, Canada). Rabbit monoclonal to tenascin-C antibody (ab108930) and goat anti-rabbit IgG (ab6702), were purchased from Abcam (Cambridge, UK). White Leghorn fertilised eggs were purchased from the Roslin Institute (University of Edinburgh, UK). The Ova-Easy Advance Egg incubators were purchased from Brinsea (Weston-Super-Mare, UK).

2. Nanoparticle Synthesis and Characterisation

Shell isolated AuNPs (AuNP-SHIN) were formed using AuNP seeds created using a slightly modified Turkevich method,[1] with a final size of 58 nm. The Raman reporter 4-(1H-pyrazol-4-yl) PPY was added electrostatically to the surface and left for 2 minutes for slight aggregation to take place. Sodium silicate (1.5 mL, 1 mM) and (3-aminopropyl)trimethoxysilane (APTMS) (150 µL, 32 µM) were added to form the silica shell. Characterisation of extinction spectroscopy, dynamic light scattering, and zeta potential was carried out and revealed an LSPR of 530 nm and 82 nm average diameter.

Antibody addition to the shell of the PPY-AuNP-SHINs was carried out using passive adsorption by altering the pH of the NP suspension to 9. For antibody conjugation, 50 µL of borate buffer comprised of 34.4 mg phosphate buffer, 100 mg bovine serum albumin (BSA) and 20 mL of dH_2O at a pH of 9 was added to 500 µL of PPY-AuNP-SHINs. A monoclonal

antibody to tenascin-C (5 µL, 0.5 mg/mL) was added to the above mixture and shaken for 2 hours on a shaking plate at room temperature (RT). Bovine serum albumin (BSA) (100 mg/mL, 40 µL) was added as a protection layer to the surface of the AuNP-SHINs with shaking for 30 minutes at RT. The resulting NP conjugates were concentrated by centrifugation (1500 RCF, 20 min) and resuspended in 450 µL of dH_2O. Extinction spectroscopy, dynamic light scattering (DLS), zeta potential analysis and lateral flow immunoassays confirmed addition of the antibody to the PPY-AuNP-SHINs. The lateral flow nitrocellulose strips were pre-conjugated with a goat anti-rabbit IgG prior to running the samples. Therefore, if any binding was observed it was due to the interaction between the rabbit tenascin-C antibody on the nanotags and the goat anti-rabbit IgG antibody on the strip. A control strip was also run in parallel, without addition of the targeting antibody.

2.1 Extinction spectroscopy: Extinction spectroscopy was carried out using an Agilent, Cary 60 UV-Visible spectrophotometer. A 1 cm pathlength poly(methylmethacrylate) (PMMA) plastic cuvette was used, and a baseline was obtained prior to running the samples using dH_2O.

2.2 Size and Zeta Potential measurements: Dynamic light scattering (DLS) and zeta measurements were obtained using a Malvern Zetasizer, Nano ZS system (Malvern, UK). Before sample analysis, a 40 nm polystyrene latex bead standard was run to calibrate the instrument. To measure the size, the nanotags were placed into a 1 cm PMMA cuvette. The samples were measured in triplicate and the mean and standard deviation was calculated. The zeta potential of the samples was measured using a dip cell placed into the 1 cm PMMA cuvette.

2.3 Nanoparticle Tracking Analysis (NTA)

A Nanosite 300 instrument with NTA software (Malvern Panalytical, UK) was used to assess the concentration of the PPY-AuNP-SHIN nanotags. The measurements were performed at RT and 60 second movies were created for each sample, which was repeated 5 times. The camera level was set to 6 (a.u.) and the nanotags were focused on the screen. The sample addition was by a 1 mL syringe set in a syringe pump, with a flow rate of 10.4 µL/min. The number of nanotags over 20 frames of a 60 second video were counted and the average number of particles per frame was calculated, giving the number of nanoparticles per mL.

3. Cell Culture

This work used human U87-MG glioblastoma cells cultured in Eagle's minimum essential medium (EMEM) with L-glutamine (2 mM) supplemented with 10% FBS, 100 µg/mL of penicillin/streptomycin and 2 µg/mL of amphotericin B. Cells were cultured in a T75 tissue culture flask and incubated at 37 ºC with 5% CO_2 in a humidified incubator. Flasks with a cell confluency of 70-80% were detached using 0.05% trypsin-EDTA and re-suspended in complete EMEM media and counted using a haemocytometer before reseeding for future experiments.

4. Microtome Sectioning

The tumours were sectioned using a microtome (Leica, RM2125RTF). Firstly, the tumours were dissected from the chorioallantoic membrane (CAM) of the chicken egg embryos using precise, sharp, and sterile laboratory scissors. The dissected tumour was then fixed in 4% paraformaldehyde (PFA) for 2 hours at RT. It was washed in phosphate buffered saline (PBS) and then added to a bio-wrap (Leica Biosystems Richmond, USA) and placed in a plastic cassette (Simport, Canada). The cassette was then placed into increasing concentrations (70%, 90% and 100%) of ethanol for 1 hour each, then into histoclear for 1 hour. The fixed tumour was then placed into paraffin wax at approximately 60 ºC for 6 hours to allow the wax to penetrate the tumour. After 6 hours, the tumour in wax was placed into a mould and allowed to cool to RT and placed at -20 ºC for at least 1 hour before being sectioned. The sections were added to polysine adhesion glass slides (Epredia, Fisher Scientific, UK) and baked at 60 ºC for 2 hours for drying. The slides with the sections were then stored in PBS at 4 ºC for mapping.

5. SERS mapping

An InVia Raman instrument was used for all SERS mapping, with 785 nm laser excitation, using 100% laser power at the sample (20 mW) unless otherwise stated, and a 1 second integration time. The images of the tumour were taken using a 5× NA 0.12 N PLAN EPI microscope objective lens and the sections with 63× NA 1.20 HC PL water immersion objective lens. SERS images were obtained by taking a series of 2D XY maps across the intact tumour every z = 100 µm (x = 75 µm, y = 75 µm) and the sections (x = 30 µm, y = 30 µm).

6. Chicken Embryo Tumour Model

The use of the chicken egg embryo model does not require a Home Office Project and Personal License under The Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 (ASPA) https://www.legislation.gov.uk/ukdsi/.
According to these regulations, a license is not required to work with this species for up to 2/3 of the gestation period (14 days).

7. Data Processing

All spectra were processed using Python 3. For each SERS spectrum, a 3rd order polynomial baseline was determined and subtracted from the original spectrum, yielding baseline corrected spectra. No smoothing was applied to the spectra. The resulting SERS maps were generated by finding the maximum peak intensity within a narrow range surrounding the peak of interest (~ ± 20 cm^{-1}) for each pixel in the map. Using a narrow range as opposed to the same Raman shift accounts for any slight fluctuation in the spectral position of the maximum intensity. The map was then reconstructed using Matlab (R2020b, The MathWorks, USA) software. A similar approach was used for the SORS spectra, with the additional step of subtracting the baseline corrected "Ambient" spectrum from the baseline corrected "Offset" spectrum. Once again, no smoothing was applied. The resulting spectra were imported into Matlab (R2020b, The MathWorks, USA) and plotted.

Supplementary Figures

PPY Raman reporter was added to the surface of the AuNPs prior to the formation of the silica shell of the PPY-AuNP-SHINs (Fig. S1).

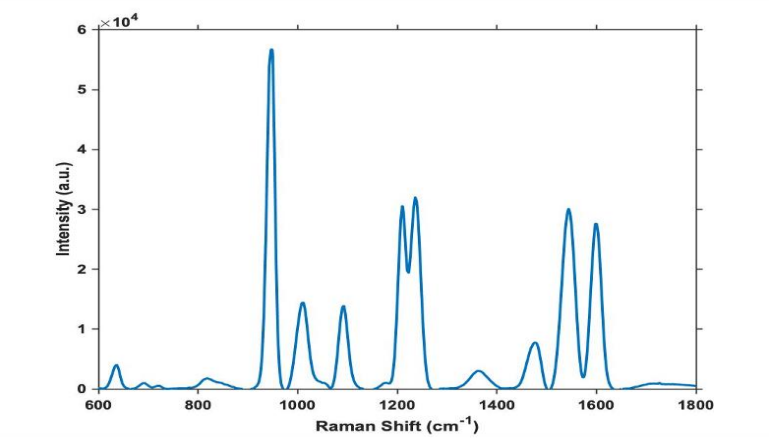

Fig. S1: SERS spectrum of 4-(1H-pyrazol-4-yl) pyridine (PPY) obtained using a handheld CBex Snowy Range Raman spectrometer. A 785 nm laser excitation was used with a laser power of 75 mW and an integration time of 0.2 seconds.

Characterisation of PPY-AuNP-SHINs and Ab-nanotags was carried out using extinction spectroscopy (Fig. S2), DLS and zeta potential (Table S1).

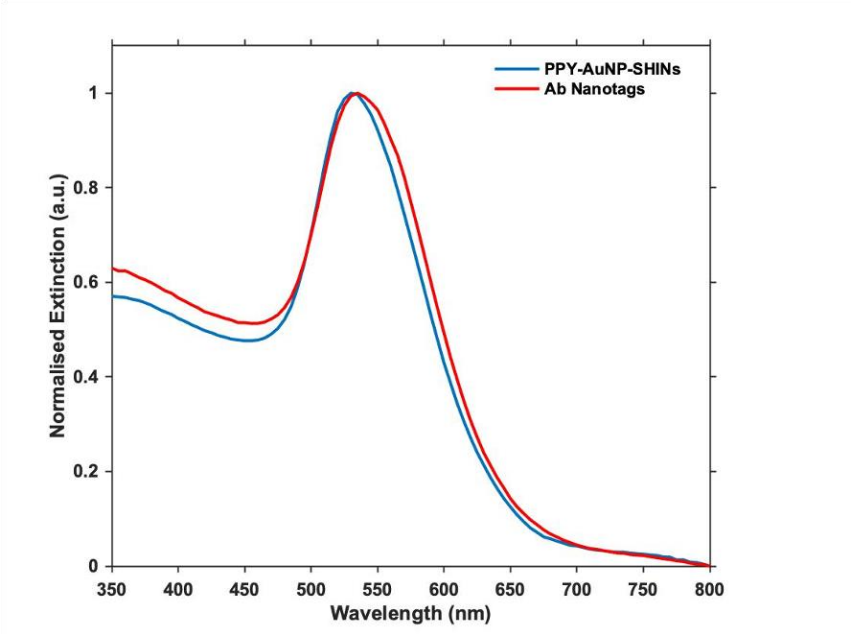

Fig. S2: Normalised extinction spectra of PPY-AuNP-SHIN nanotags (blue, diluted 1 in 4), and Ab nanotags (red, undiluted).

This shows a slight redshift in the wavelength due to the presence of the antibody on the surface of the PPY-AuNP-SHINs, along with very slight peak broadening, demonstrating an increase in overall size of the nanotags after antibody functionalisation.

Table S1: Dynamic light scattering (DLS) and zeta potential measurements for PPY-AuNP-SHINs and Ab nanotags.

	PPY-AuNP-SHIN	Ab Nanotags
Size ± Standard Deviation (nm)	66 ± 2	81.8 ± 2
Zeta Potential ± Standard Deviation (mV)	-41 ± 1	-25 ± 1

To create the Ab nanotags, a monoclonal antibody to tenascin-C was immobilised onto the surface of the PPY-AuNP-SHINs. A lateral flow immunoassay was carried out as a further characterisation step to ensure the antibody was successfully conjugated onto the SHINs (Fig. S3).

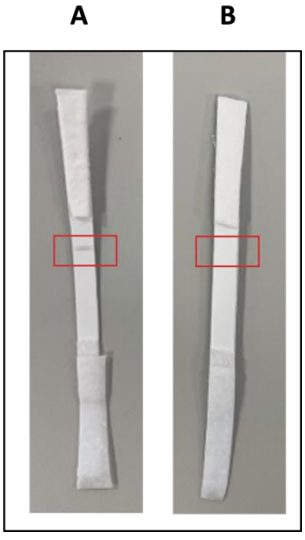

Fig. S3: Lateral flow immunoassay where (A) shows the presence of a line and therefore binding of the Ab nanotags, (B) shows the absence of a line and therefore the lack of binding of the nanotags with no Ab attached, indicated by the red box.

This demonstrates the presence and absence of the antibody due to the line on the lateral flow strip. Where the antibody is conjugated to the nanotags, there is a binding interaction between the antibody bound to the strip (goat anti-rabbit IgG, ab6702) and the rabbit monoclonal tenascin-C antibody on the nanotags. In the absence of the antibody on the nanotags, run as a control with BSA alone, there is no binding observed.

SESORS

SESORS was carried out on the live glioblastoma tumours grown on the CAM of the chicken egg embryo models. This was carried out on three separate occasions, on three separate tumours grown on three different chicken embryo models. Fig. S4 shows the second (A) and third (B) replicates for the stacked plots of the Ab nanotags in the tumours using SESORS.

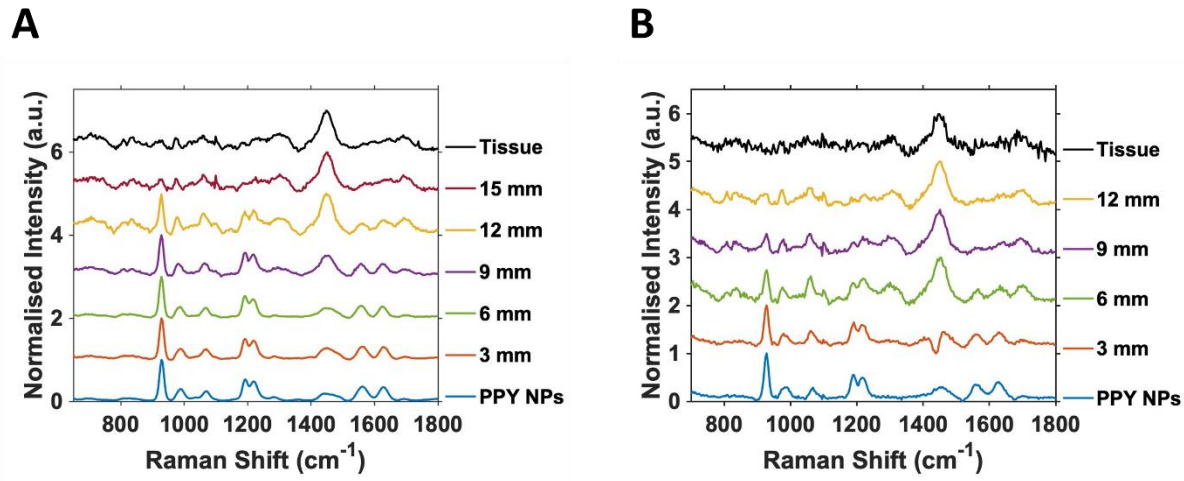

Fig. S4: Normalised stacked SESORS plot of the Ab nanotags with increasing depth. All measurements were carried out using an 8 mm offset and a total exposure time of 10 seconds (2 s integration time, 5 accumulations) at a laser excitation wavelength of 830 nm, with a maximum laser power at the source of 475 mW.

This was also carried out on chicken egg embryos that did not have any Ab nanotags added to the tumours and were used as controls. Fig. S5 shows the lack of the Ab nanotag signal that is observed in Fig. 2 and Fig. S4 and therefore further proves the presence of the Ab nanotags within the tumours in these figures.

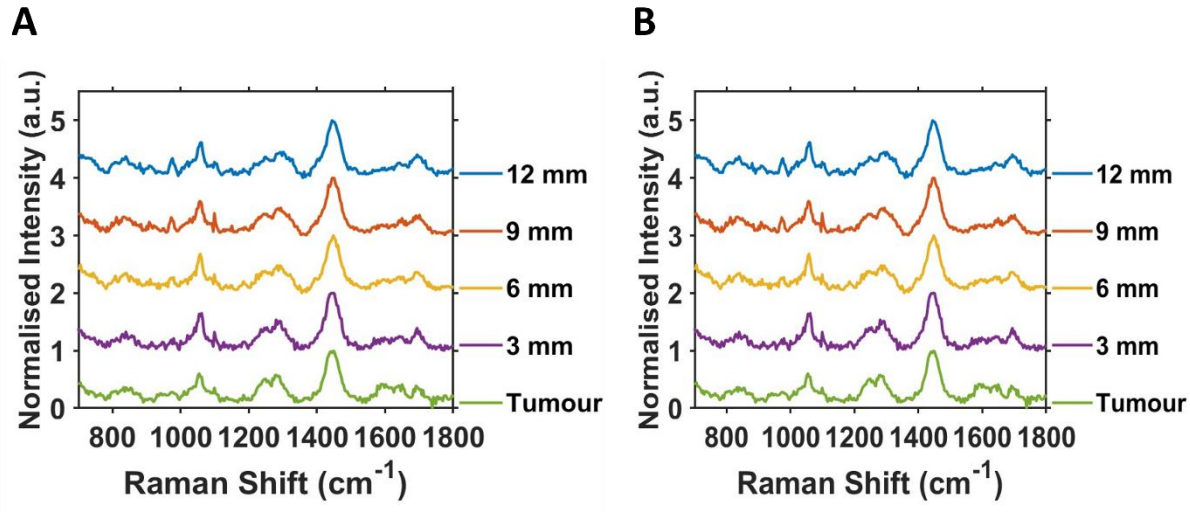

Fig. S5: Normalised stacked SESORS plot of the absence of Ab nanotags with increasing depth. All measurements were carried out using an 8 mm offset and a total exposure time of 10 seconds (2 s integration time, 5 accumulations) at a laser excitation wavelength of 830 nm, with a maximum laser power at the source of 475 mW.

SERS on Intact Tumour

The U87-MG tumours grown on the chicken embryo were dissected in full and fixed using paraformaldehyde (PFA, 4%) and placed onto a microscope slide for SERS mapping to determine the localisation of the Ab nanotags throughout the full, intact tumour (Fig. S6).

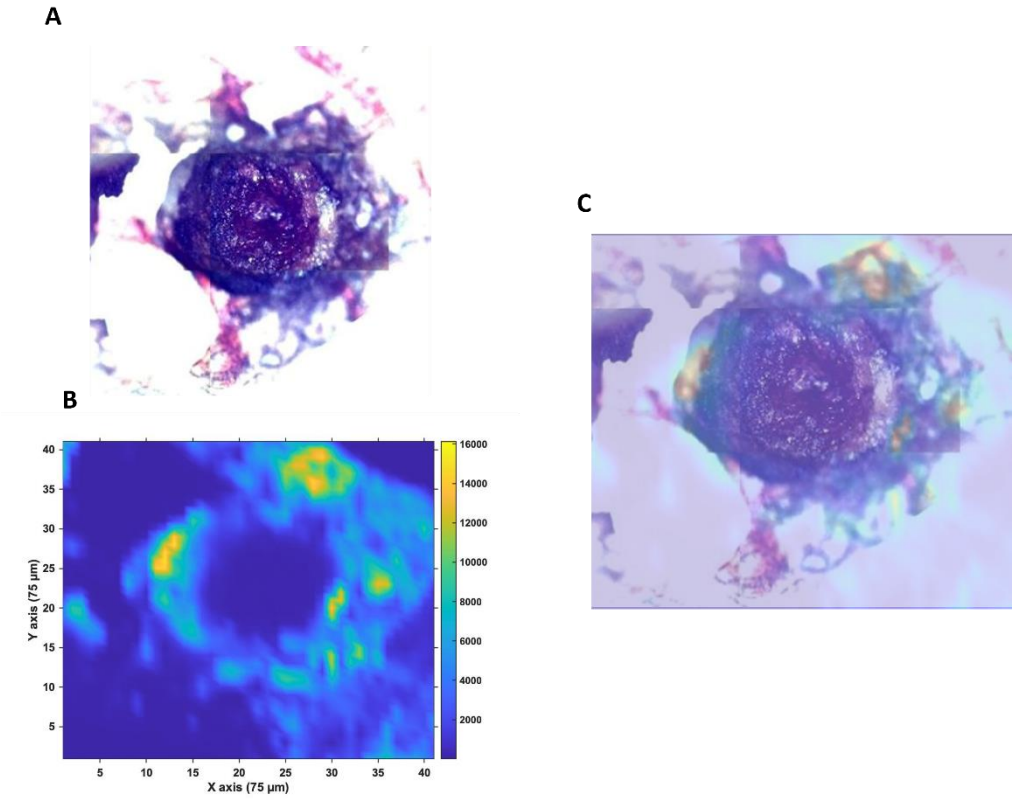

Fig. S6: Overlay of the white light image of the U87-MG tumour with the SERS intensity. (A) White light image (B) SERS map of the Z = 0 of the full tumour (C) Overlay of (A) and (B) to show the distribution of the Ab nanotags from the SERS map on the tumour and CAM.

The overlay indicates that the Ab nanotags are not only in the CAM, but they are also around the periphery of the tumour. The SERS map of the PPY signal from the Ab nanotags shows the lack of signal in the main bulk of the tumour, however this area is smaller than that of the tumour in the white light image. To further support this finding, SERS line scans at different Z depths were taken across the full tumour (Fig. S7).

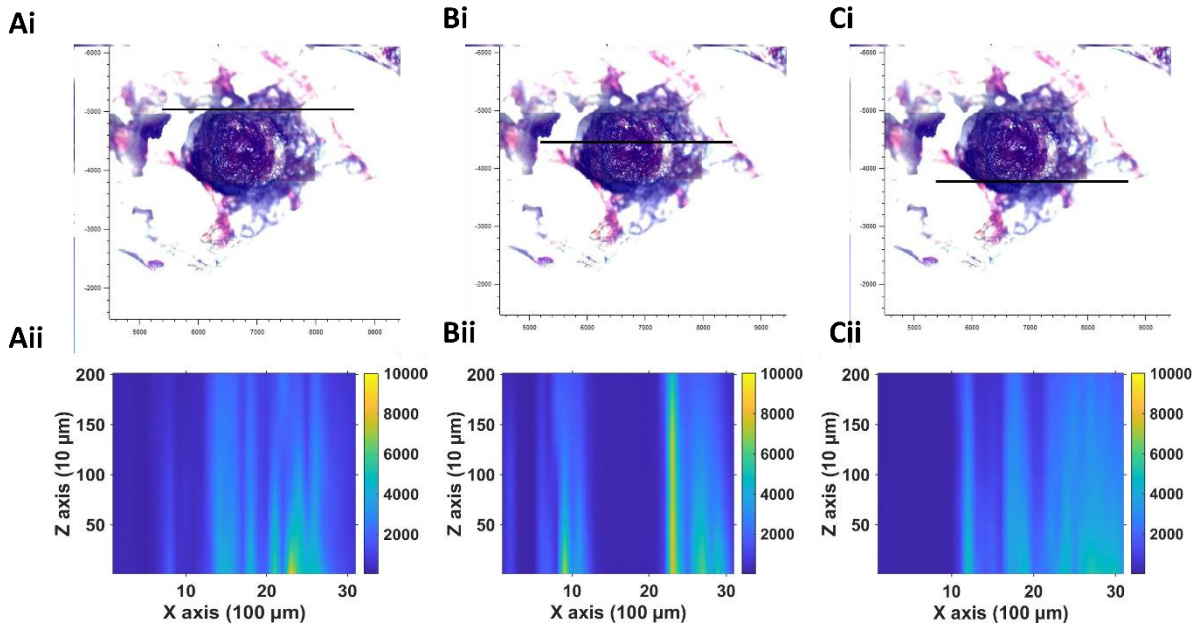

Fig. S7: (A) White light images of the tumour with the line across which the SERS intensity was measured indicated. (B) Resulting SERS response of the Ab nanotags within the tumour.

These data support that of Fig. 3 as it indicates that the majority of the Ab nanotags are found around the CAM and periphery of the tumour and very little have been able to migrate into the bulk of the tumour. To understand the distribution, XZ line scans were carried out at the top, middle and bottom of the tumour. Fig. S7Bii indicates a clear lack of nanotag signal in the bulk area of the tumour, whereas Fig. S7Aii and Cii show more signal in the tumour because these scanned pixels have by-passed the main bulk of the tumour and are only determined around the periphery and CAM. Due to the large size (3 mm × 3 mm) of the tumour and to further understand the uptake of the nanotags, sections were taken using a microtome (Fig. S8).

Sectioning of Glioblastoma Tumours

The U87-MG glioblastoma tumours grown on the chicken egg embryo models were dissected on day 14, fixed and subsequently sectioned using a microtome (details in section 4 above). The two additional replicates of the sections mapped using SERS are shown in Fig. S8 where Ai-Fi is a replicate of one U87-MG tumour and Aii-Fii is another replicate of a different U87-MG tumour, grown on the chicken embryo models.

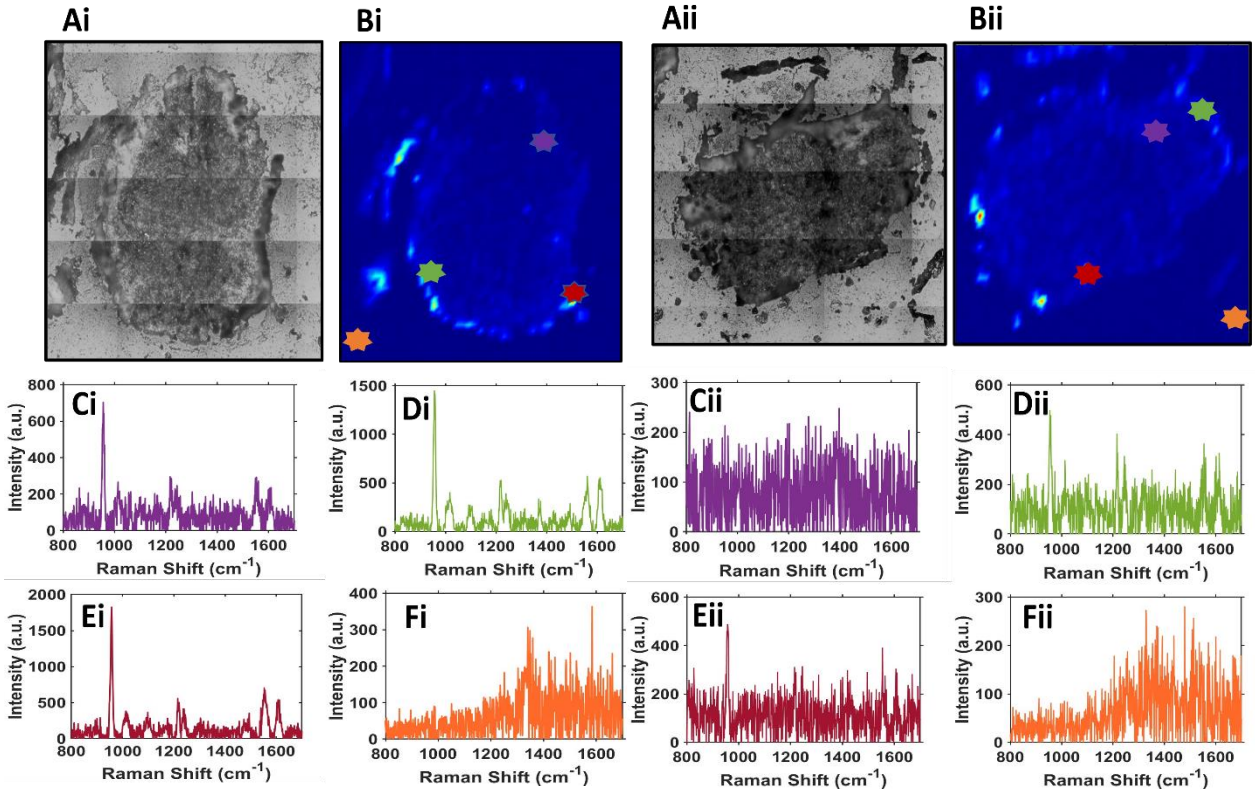

Fig. S8: Section of the U87-MG tumour with SERS spectra shown at random locations to illustrate the nanotag localisation. (Ai and Aii) White light image of the tumour section. (Bi and Bii) SERS intensity map of the PPY 956 cm^{-1} peak of the tumour section, (Ci and Cii) PPY spectrum of a point in the inside of the section, (Di and Dii) PPY spectrum of a point on the edge of the section, (Ei and Eii) highest intensity PPY spectrum, (Fi and Fii) lack of PPY spectrum due to being off the section. Each coloured spectrum corresponds to the location of the same-coloured star indicated on the SERS intensity image. All sections were 10 µm thick. The step size used was 30 µm in both *x* and *y* axes. All spectra were taken using a 785 nm excitation wavelength, 1 s integration time and 50% of the instrument's laser power at the sample equating to 10 mW.

As observed within the three replicates of the sectioned tumours (Fig. 4 and Fig. S8) the Ab nanotags have accumulated around the outside layer of the tumours and can be observed within the tumours. The points at which the SERS intensity was plotted was chosen at random and therefore this could be why there is slight variation within the sections. Since these are derived from different tumours, and are random sections from each tumour, there is biological variability. There is higher signal found from the Ab nanotags within one section compared to

the next, which is expected due to different sizes, shapes, and morphology of each tumour. Nevertheless, they all show that there are Ab nanotags present within the sections presented and there is no signal found when the spectrum was taken on the glass slide i.e., off the section. Therefore, it is reasonable to suggest that the Ab nanotags can penetrate the outer periphery of the tumour, however become unable to penetrate deep into the tumour tissue due to the lack of the nanotag signal.