Supporting Information

Combination of live cell surface-enhanced Raman scattering imaging with chemometrics to study intracellular nanoparticle dynamics

Elisa Lenzi$^{1,2}$, Malou Henriksen-Lacey$^{1,2}$, Beatriz Molina$^1$, Judith Langer$^{1,2}$, Carlos D. L. de Albuquerque$^{1,2}$, Dorleta Jimenez de Aberasturi$^{1,2,3}$* and Luis Liz-Marzán$^{1,2,3}$*.

$^1$CIC biomaGUNE, Basque Research and Technology Alliance (BRTA), 20014 Donostia-San Sebastián, Spain

$^2$Centro de Investigación Biomédica en Red, Bioingeniería, Biomateriales, y Nanomedicina (CIBER-BBN), 20014 Donostia-San Sebastián, Spain

$^3$Ikerbasque, Basque Foundation for Science, 48009 Bilbao, Spain

Corresponding Authors

*email addresses: djimenezdeaberasturi@cicbiomagune.es (D.J.A.);
llizmarzan@cicbiomagune.es (L.M.L.-M.)
Simulation of 2D SERS mapping

To determine the descriptive parameters of the mapping, such as surface occupancy by SERS tags inside the mapping or average signal intensity, we created SERS tag mapping simulations. The simulations represent SERS mapping of a pre-defined number of SERS tags inside a fixed area of 400 × 400 µm, with a step size of 8 µm in x and y (i.e., in total each area contains 50 × 50 points, see Figure S1A). SERS tags were randomly positioned inside the selected area, following a certain percentage of covered surface. Each point inside the map can contain more than one SERS tag, but also none. Simulations were prepared assuming two conditions; a) each SERS tag shines with a similar SERS intensity, and b) the agglomeration of SERS tags does not lead to collective signal enhancement. These hypotheses were based on previous findings using similar SERS tags.¹ The intensities at the main peaks for each SERS tag signal were distributed around typical values obtained from real experiments. The background noise level was also defined from real measurements, see Figure S1B. Simulations were devised with different total amounts of SERS tags and different surface coverages, reproducing different mapping configurations.

The simulations were first used to verify that the SERS tags counting algorithm (see Materials and Methods) worked properly to identify all SERS tags inside the maps. This control was carried out by calculating the descriptive parameters (Table S1) of each simulation and comparing them with the parameters used to build the simulation itself (Figure S1C). Moreover, it was possible to define the relative intensity to the area (R) as a descriptive parameter of the systems, which was constant among maps with the same number of SERS tags.
Table S1. Descriptive parameters for a simulated map. The surface coverage (σ) is the number of selected spectra (N) in the SERS tags counting algorithm, over the total number of points inside the area (A). The average intensity (I) is defined as the sum of the intensities at one of the typical wavenumbers of the selected spectra (Iᵈ), divided by the number of the selected spectra. The relative intensity to the area (R) is calculated as the sum of the intensities at one of the main peaks, divided by the total number of points inside the area.

| Number of selected spectra | Total number of spectra | Surface coverage (σ) | Intensity at one of the typical SERS tags wavenumber | Average Intensity (Iᵈ) | Relative Intensity (R) |
|---------------------------|------------------------|---------------------|----------------------------------------------------|------------------------|------------------------|
| N                         | A                      | σ = N / A           | Iᵈ                                                 | Iᵈ = Σ Iᵈ / N          | R = Σ Iᵈ / A           |

Figure S1. A) Simulated 2D SERS map showing the SERS intensity at a hypothetical wavenumber of 1070 cm⁻¹. B) SERS spectra of all xy coordinates shown in A. C) Relative intensity-surface coverage graph for the intrinsic quantities (in green), and for the values calculated by the program with two different filters (in blue and red). Each point represents a virtual map. The orange square marks virtual maps with the same number of NPs: R is constant when the surface coverage is variable. The two different filters depend on the selection rules: the first one (as labeled) depends on the intensity value at a specific wavelength in a spectrum, while the second one is related to the b values assigned by linear regression analysis to each spectrum, with respect to the chosen reference. The second type of filter is independent of the intensity value, thus focused on the full shape and therefore more accurate than the first filter.
Application of SA analysis to experimental data

The SA analytical method was applied to SERS data, to determine the dilution of 2NAT-AuNR SERS tags in MCF7 cells over time. First, the number of cells per imaging area (shown by the red-dashed lines in Figure 1A) was determined using optical images showing the outline of individual cells (Figure S2A). SERS mapping spectra obtained on days 1, 2, 3 and 7 post-seeding, shown in Figure 1A, were used to determine the cell surface coverage of SERS tag-positive pixels. As shown in Figure S2B,C, a decrease in surface coverage and an increase in cell number were observed over the studied timeframe.

Figure S2: (A) Optical images of an area selected for SERS mapping (red dotted square), with cell borders outlined and counted. (B) Cell surface coverage of SERS tag-positive pixels. (C) Number of cells counted by optical imaging. In (B) and (C), data were obtained on days 1, 2, 3 and 7 post-seeding.
ICP-MS study of SERS tag concentrations

We prepared MCF7 cells labeled with SERS tags at five different gold concentrations (30, 6.0, 1.0, 0.2 and 0.04 µM), to study the expected number of SERS tags over time. The highest concentration is close to the common value used to label cells, whereas the lowest value is the concentration expected after 3 weeks of cellular division.

![Graph showing the number of SERS tags per cell for different initial concentrations.](image)

**Figure S3.** Number of SERS tags per cell for different initial concentrations. Considering the standard incubation conditions (100 µM NPs), the smallest concentration represents the amount of gold inside cells after three weeks of incubation. 4BPT-AuNS SERS tags were employed.
SERS tag uptake confirmation via TEM

Figure S4: TEM images showing 4BPT-AuNS SERS tags uptake in an MCF7 cell. Cells were incubated with SERS tags overnight (final concentration ca. 20 µM) before being washed to remove non-endocytosed SERS tags. (A-C) Higher magnification images from the areas labeled in the left-most panel.
SERS tag cytotoxicity under laser irradiation

Figure S5. A) Average signal of a fixed area (shown in B) exposed to increasing laser powers (fixed step size of 5 μm and integration time of 0.02 s), containing SERS-labeled MCF7 cells ([Au] = 0.1 mM). 4BPT-AuNS SERS tags were employed. C) Same area after all the scans: changes in cell shape and an increase in the intensity of dark areas inside cells (accumulations of SERS tags) indicates an excessive exposition to the laser. Scale bars = 20 μm.

Figure S6. A) Optical image of non-labeled MCF7 cells. The squares indicate areas measured under different conditions to check laser irradiation cytotoxicity: light blue is the control, non-irradiated zone; yellow is an area measured with 30 mW laser power (9.6 mW μm²) and 20 ms accumulation time; magenta is an area measured with 30 mW (9.6 mW μm²) and 50 ms; purple is an area measured with 80 mW (25.6 mW μm²) and 20 ms. B, C) Fluorescence imaging showing live (B) and dead (C) cells. The corresponding percentages of dead cells in the coloured squares are indicated in A, as calculated from the ratio between live and dead cells.
Figure S7. A) SERS mapping of MCF7 cells labeled with 0.1 mM SERS tags, performed with 5 mW laser power (1.6 mW μm⁻²), 0.02 s accumulation time and two different xy step sizes (5 μm and 1 μm), to study the influence of measurement conditions. 4BPT-AuNS SERS tags were employed. B,C) Average spectra from SERS mappings (B) and optical images (C) of cells before and after scanning, showing no considerable differences in SERS intensity resulting from the decrease in step size. Scale bars: 100 μm.
Redefinition of analysis parameters

We redefined the quantity $R$ as the product of $\overline{I}_c$ and $\sigma_c$, which are respectively the average intensity per cell and the cellular surface coverage. The cellular surface coverage is calculated as the number of cells divided by the total area, while the average intensity per cell is defined as the sum of the intensities divided by the number of cells. If the number of cells remains constant, $\sigma_c$ can also be considered as constant. In this case, the average intensity and the relative intensity have the same trend and it is possible to use directly the average intensity per cell ($\overline{I}_{c\tilde{v}}$).

### Table S2. Real map descriptive parameters defined from cells properties.

| Number of cells | Total area | Surface coverage ($\sigma_c$) | Intensity at a typical wavenumber ($\tilde{\nu}$) | Average intensity per cell ($\overline{I}_{c\tilde{v}}$) | Relative intensity ($R$) |
|-----------------|------------|-------------------------------|-----------------------------------------------|-------------------------------------------------|--------------------------|
| $\eta$          | $A$        | $\sigma_c = \frac{\eta}{A}$ | $I_{\tilde{\nu}}$ | $\overline{I}_{c\tilde{v}} = \frac{\Sigma I_{\tilde{\nu}}}{\eta}$ | $R = \overline{I}_{c\tilde{v}}\sigma_c$ |
**Individual SERS tag signal calibration**

To calculate the signal for a single SERS tag, in the same conditions as for the analysis in cells, we followed the protocol described in ref. 1, performing SERS measurements in water. To ensure no changes in the NPs on the TEM grid after immersion, TEM images were taken before and after the procedure. The SERSTEM application was used to count the tags on the grid and make sure that there are no relevant changes (Figure S8). After this control, the signal for a single SERS tag was estimated. 4BPT-AuNS SERS tags were employed. Raman and TEM images were correlated in three different areas of the TEM grid (Figure S9A). All such associations were then used to generate the signal distribution for a single SERS tag, from which the median could be estimated, as well as the 25th and 75th percentiles values, as shown in Figure S9B.

**Figure S8.** A) TEM image of the inner part of the letter P in the labeled grid, measured after preparation; the inset shows a zoom of the area marked in green. B) The same area imaged by TEM after immersion in water. The inset shows a zoom of the area marked in blue, equivalent to the one in green in A.
Figure S9. A) Three different measured areas in a TEM grid: inner parts of the letters G, P, Q, with blue squares signaling those spots where the SERSTEM App found a correlation between the presence of non-clustered NPs and SERS signal. Red spots indicate the found NPs. The area inside P presents a low number of squares because it is small, and the presence of clustered NPs resulted in the elimination of the majority of the area from the analysis. All maps were analyzed using the following parameters: Grey Limit Value (GLV) = 0.2, square size = 2 µm per length, Maximum pixel number = 300, b threshold = 6. B) Histogram and boxplot for the SERS signal per particle (SSpP) found in all three regions. Reported in the boxplot are the median signal per particle (red bar) and the 25th and 75th percentiles (lower and upper edges of the blue box). Outliers of the distribution are marked as red dots.
Estimation of the number of NPs in 3D in live cells with SA

Figure S10. A) Average intensity per cell ($I_c$) obtained by applying the SERS tags voxels sum algorithm to the cellular measurements taken over time. 4BPT-AuNS SERS tags were employed. B) Zoom over the last three days in vitro (DIV). C) Number of NPs per cell, calculated from SERS analysis, using the SERS signal per particle (median value reported with a red bar, 25 % and 75 % are represented by a grey area), and ICP-MS, considering the average dimensions of the corresponding SERS tags.
Figure S11. Outline of Cells determined from brightfield image. Cell outlines were manually drawn to highlight the number of cells per imaging zone.
Estimation of the number of NPs in 3D live cells with SA for \([\text{Au}^0] = 0.05 \text{ mM} \ (1.9 \times 10^9 \text{ NP/mL})\)

**Figure S12.** A) 3D reconstructions of the selected SERS signal at 3 time points: 1DIV, 6DIV, 13DIV and 17DIV. Colored boxes measure ca. \(84 \times 84 \times 25 \ \mu\text{m}\). 4BPT-AuNS SERS tags were employed. B) Evolution of SERS spectra over time, obtained via 3D sum (orthogonal projection) of the selected SERS signals. C) Images of cells at the first and last days in vitro, showing labeled
cells in the measured areas. The optical images were used to count the cells and obtain the SERS signal per cell. D) Cell outlines were manually drawn to highlight the number of cells per imaging zone. E) Comparison with ICP-MS results, which yield the amount of gold (μg) per cell. F) Number of NPs per cell calculated from both techniques: for SERS analysis, the number of NPs was estimated using the SERS signal per particle (median value reported with a red bar, 25% and 75% are represented by a grey area), while for ICP-MS we considered the average dimensions of the corresponding SERS tags.

**Methods to improve NP detection at low concentrations**

*Figure S13.* A) Average SERS spectra from the mappings in B), acquired with two different laser powers, namely 5 mW and 80 mW (equivalent to 1.6 and 25.6 mW μm$^2$, respectively), after 17 DIV. C) Decreasing trend of the SERS signal/cell (red squares) calculated for the measurement performed with 5mW laser power; and SERS signal/cell (blue dots) for those time points measured with higher laser power (80 mW).
DUA for quantification of SERS tags

**Supervised algorithm**

1. Baseline subtraction
2. Smoothing
3. 3D SERS mappings of labeled cells
4. Spectral selection
5. Multiple Linear Regression using reference spectra
6. Filter by the goodness of fit (p-value)
7. Filter by spectral similarity (b-value)
8. Sum of intensity over different planes
9. Average intensity per cell
10. Ratio with SERS signal of a single particle
11. Quantification

**Digital Unsupervised algorithm**

1. Unfolding spectra + pre-processing
2. 3D SERS mappings of labeled cells
3. Spectral selection I
4. Singular value decomposition
5. Pre-processed 2D SERS spectra
6. Number of factors \( (\uparrow R^2 \text{ and } \downarrow \text{LoF}) \)
7. IV. Non-negative Matrix Factorization
8. SERS spectra selection \( (\rho^2 \geq 0.75) \)
9. Spectral selection II
10. V. Digital calibration curve with ICP-MS data
11. Comparison SERS vs ICP-MS
12. Quantification

* explained variance \(*\) lack of fit (error) \(*\) correlation coefficient

**Figure S14.** Comparison between the two algorithm workflows.
Shown in Figure S15 is an example of application of the DUA on SERS maps. In detail, first (A) the raw data from the SERS map were treated with the NMF algorithm to extract the signal of the SERS tags in (B) (blue spectrum). The latter was then used to filter from the initial map only those spectra with a Pearson’s linear correlation coefficient $\rho \geq 0.75$ (C). Shown in Figure S16 are the selected SERS signals for SERS tags dried onto a TEM grid, showing good agreement between the selected area by SERS and the nanoparticle positions recorded by TEM. After validation, we applied the SERS picker method to select the signal of SERS tags from cells labeled with a SERS tags concentration of 0.1mM. Our method was able to detect a single SERS tag spectrum, even in the presence of a strong and stochastic background (Figure S16D,E). The sensitivity of our method can be further increased by enlarging the size of the map and the number of volumes, but this would come at the expense of an increase in experiment time. More details are provided in Materials and Methods.
Figure S15. DUA approach: A) SERS maps, B) components obtained from the NMF decomposition algorithm, showing in blue the signal corresponding to SERS tags and in orange the background. C) Selected spectra from the initial map with correlation level $\rho \geq 0.75$, with respect to the reference identified in the previous step.
**Figure S16.** A) SERS mapping (light blue square) analyzed with the NFM procedure, showing the selected points reported in a color scale of intensity. B) TEM image overlapped with SERS mapping: green dots show co-localization of SERS signal and NPs in the TEM image. C) Selected SERS spectra, corresponding to the points in A. D) Distribution of intensity at the three major peaks of the SERS tags (1080, 1282 and 1590 cm⁻¹). E) Diameter of selected spots from A), obtained by treating the spectra with a bicubic interpolation.
Figure S17. A) SERS spectra from a mapping of a single area of MCF7 cells labeled with SERS tags after 17 DIV. B) Spectra selected by DUA with correlation level $\rho \geq 0.75$.

Dimensions of the holder

Figure S18. Illustration (A) and dimensions in mm (B) of the in-house holder designed with Autodesk Inventor Software (Autodesk Inc. CA, USA). Cells were seeded inside an area of 30.25 mm$^2$, in a minimum volume of 100 μL. Once adhered, additional media (<1.5 mL) could be added to perform SERS measurements with immersion objectives.
References:

(1) Lenzi, E.; Litti, L.; Jimenez de Aberasturi, D.; Henriksen-Lacey, M.; Liz-Marzán, L. M. SERSTEM: An App for the Statistical Analysis of Correlative SERS and TEM Imaging and Evaluation of SERS Tags Performance. *J. Raman Spectrosc.* 2021, 52, 355–365. https://doi.org/10.1002/jrs.6043.