Electronic Supplementary Information (ESI)

Surface-Enhanced Raman Spectroscopy of Microorganisms: Limitations and Applicability on the Single-Cell Level

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Cultivation of microorganisms

For 1 L of LB medium 6.25 g yeast extract, 12.5 g tryptone and 6.25 g NaCl were added to water and autoclaved. For a total volume of 1 L of the minimal medium 6.78 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 28.6 mg boric acid, 15.5 mg $MnSO_4 \cdot H_2O$, 19.2 mg ethylenediaminetetraacetate dihydrate, 10.2 mg $MgSO_4 \cdot 7H_2O$, 4.5 mg $CaCl_2$, 3.9 mg $Na_2MoO_4 \cdot 2H_2O$, 3 mg $FeSO_4 \cdot 7H_2O$, 2.2 mg $ZnSO_4 \cdot 7H_2O$, 0.8 mg $CuSO_4 \cdot 5H_2O$ and 0.5 mg $Co(NO_3)_2 \cdot 6H_2O$ were added to 200 mL water and autoclaved. Separately, a 0.5 M D-glucose solution was autoclaved which served as sole nutrient and a 3 mM thiamine solution was sterile filtrated with 0.22-µm pore size polyethersulfone syringe filters (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). 40 ml of the glucose and 1 ml of the thiamine solution were added to 200 ml of the salt solution. The concentrated medium was diluted with autoclaved water to the total volume of 1 L to achieve a final glucose concentration of 18 mg/L. For deuterated samples, the medium preparation was downscaled and performed with 32.9% (v/v) D_2O to achieve a final volume ratio of 25%. For cultivations with fully ^{13}C-labelled glucose only a fraction of the listed volumes was used with the same concentration resulting in a final $^{13}C_6$-glucose concentration of 18.6 mg/L. Cultures were incubated depending on the total volume in culture tubes (5 ml) or 250-ml Erlenmeyer flasks (25 ml) for 16 h at 37 °C and 100 rpm to the stationary phase (Figure S1a). *N. inopinata* and *N. gargensis* were incubated at 46 °C and without shaking with 0.5 mM NH_4Cl in a medium containing (per litre): 50 mg KH_2PO_4; 75 mg KCl; 50 mg $MgSO_4 \cdot 7H_2O$; 584 mg NaCl; 4 g $CaCO_3$; 1 ml of trace element solution; and 1 ml of selenium-tungsten solution. These solutions were added to the autoclaved medium by sterile filtration using 0.2-µm cellulose acetate filters. The trace element solution contained (per litre): 34.4 mg $MnSO_4 \cdot H_2O$; 50 mg H_3BO_3; 70 mg $ZnCl_2$; 72.6 mg $Na_2MoO_4 \cdot 2H_2O$; 20 mg $CuCl_2 \cdot 2H_2O$; 24 mg $NiCl_2 \cdot 6H_2O$; 80 mg $CoCl_2 \cdot 6H_2O$; 1 g $FeSO_4 \cdot 7H_2O$ and 2.5 ml of 37% (m/m) HCl. The selenium-tungsten solution contained (per litre): 0.5 g NaOH; 3 mg $Na_2SeO_3 \cdot 5H2O$; 4 mg $Na_2WO_4 \cdot 2H_2O$.

Viability Analysis

The viability of *E. coli* was measured with the Colilert test and by flow cytometry with the LIVE/DEAD *Bac*Light kit according to the user instructions. Briefly, for the Colilert test cultures were diluted to the factor of 10^{-9}, 10^{-10}, 10^{-11} and the labelled nutrient mixture added to a total volume of 100 ml. These dilutions were filled into the corresponding plastic trays, sealed and incubated at 35 °C for 18 h. The number of coloured or fluorescent wells was converted according to the 'Most Probable Number Table' provided by the supplier (IDEXX, Netherlands). For flow cytometric analysis 1.5 µl of a 3.34 mM SYTO 9 solution as well as 1.5 µl of a 20 mM propidium iodide in dimethyl sulfoxide were added to 996 µl of a 154 mM sodium chloride solution and mixed with 1 µl of the washed *E. coli* sample. This washed sample was obtained by centrifugation of 1 ml of the original *E. coli* culture (5500×*g*, 1 min, 20 °C), subsequently the supernatant was discarded and the cell pellet was resuspended in 1 ml of a 154 mM sodium chloride solution. This washing step was repeated twice. The stained sample was analysed with a CyFlow Cube 6 flow cytometer (Sysmex Partec GmbH, Germany).

Growth and viability of *E. coli* (strain W)

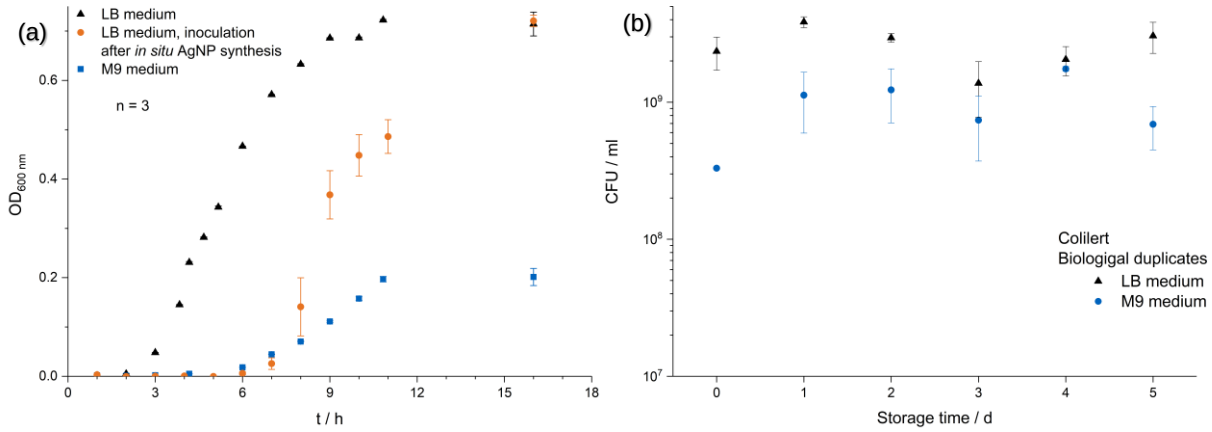

Figure S1. (a) Growth curve of *E. coli* in LB/M9 medium and with inoculation after *in situ* AgNP synthesis detected by UV-VIS spectroscopy; (b) viability of *E. coli* stored for 0 – 5 d detected by Colilert test.

UV-VIS spectroscopy of silver nanoparticles (AgNP)

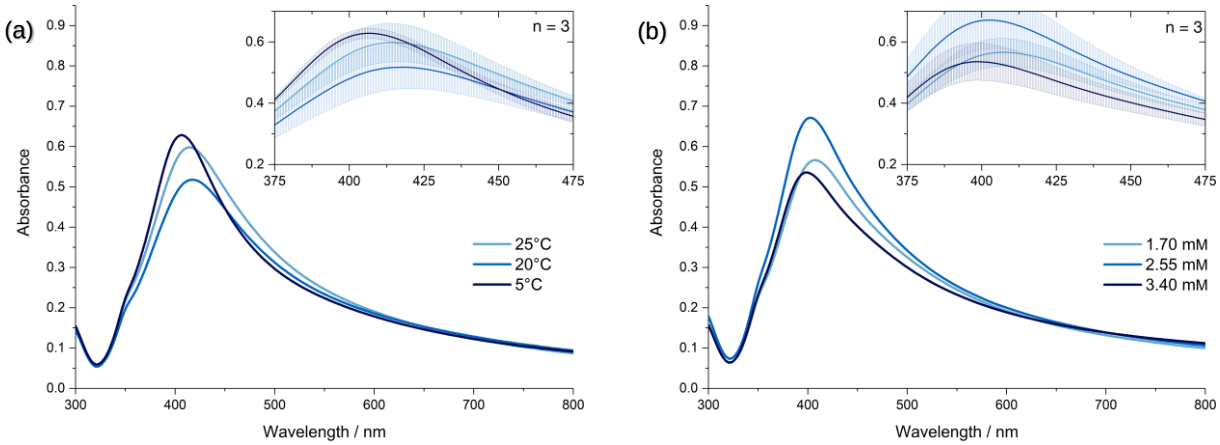

Figure S2. (a) UV-Vis spectroscopy of AgNP synthesised at different temperatures; (b) UV-Vis spectroscopy of AgNP synthesised with different reagent concentrations.

Surface-enhanced Raman spectroscopy of single microbial cells

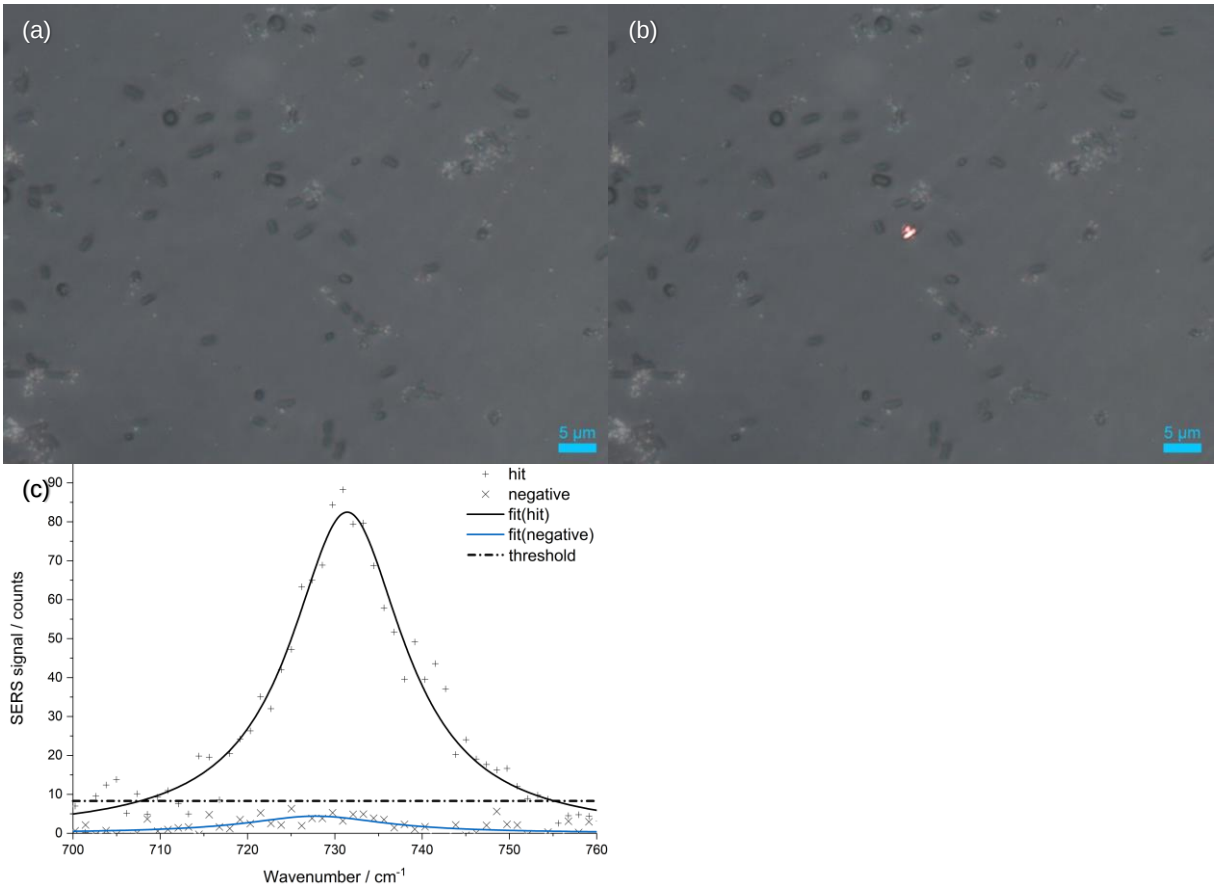

Figure S3. Optical microscope image of *E. coli* cells with AgNP, prepared *in situ*, without (a) and with (b) laser illumination; (c) automated hit detection of SERS signal at 733 cm^{-1}.

Raman analysis of deuterated *E. coli*

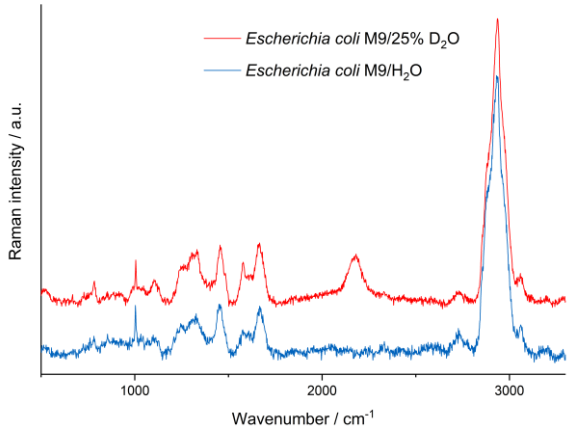

Figure S4. Raman spectra of *E. coli* cultivated with M9 medium of the natural isotopic abundance and with 25%(v/v) D_2O.

SERS analysis of different microorganisms

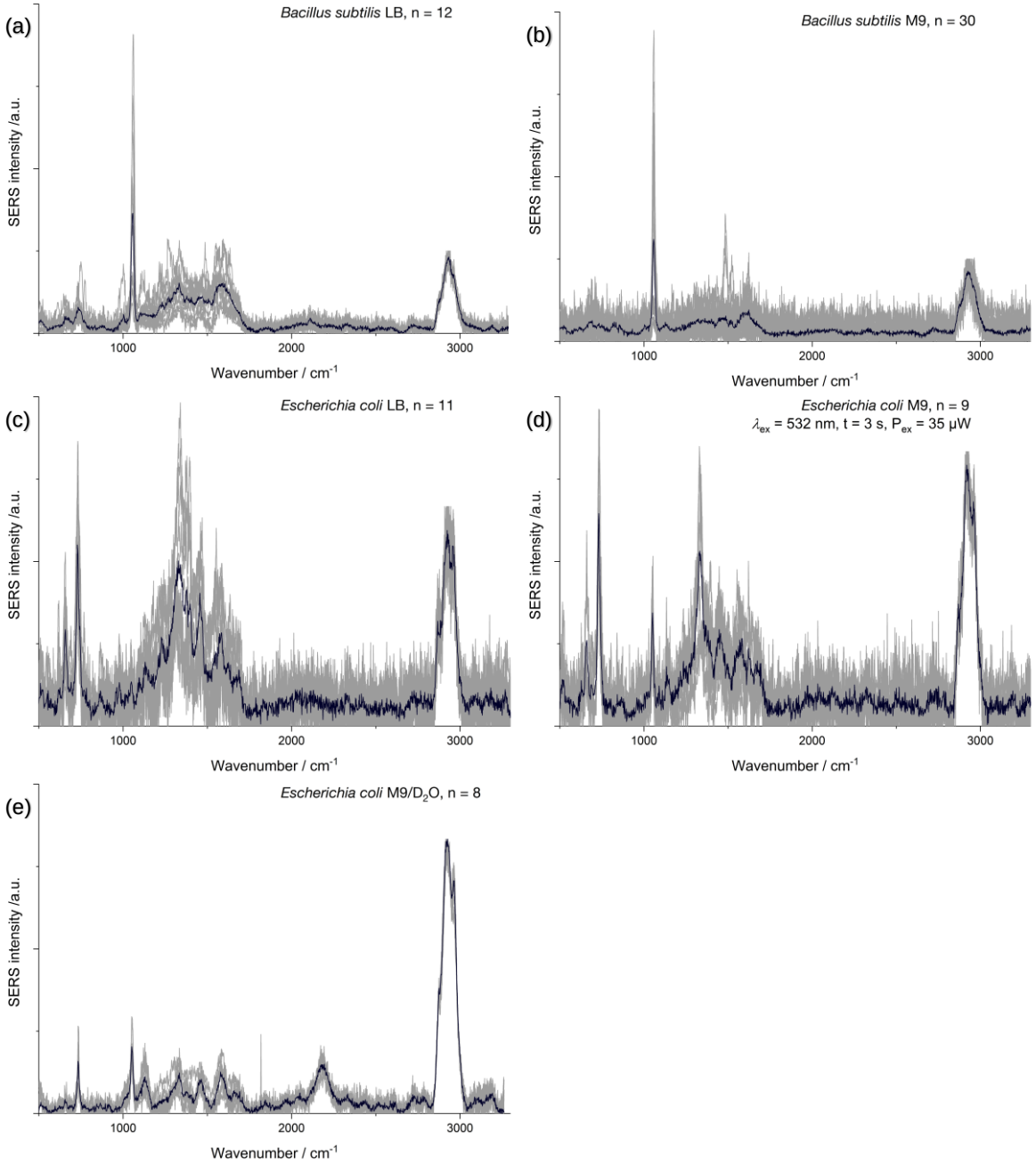

Figure S5. SERS single cell spectra of *B. subtilis* cultured with LB (a), M9 (b) and *E. coli* with LB (c), M9 (d), M9 25% D2O (e).

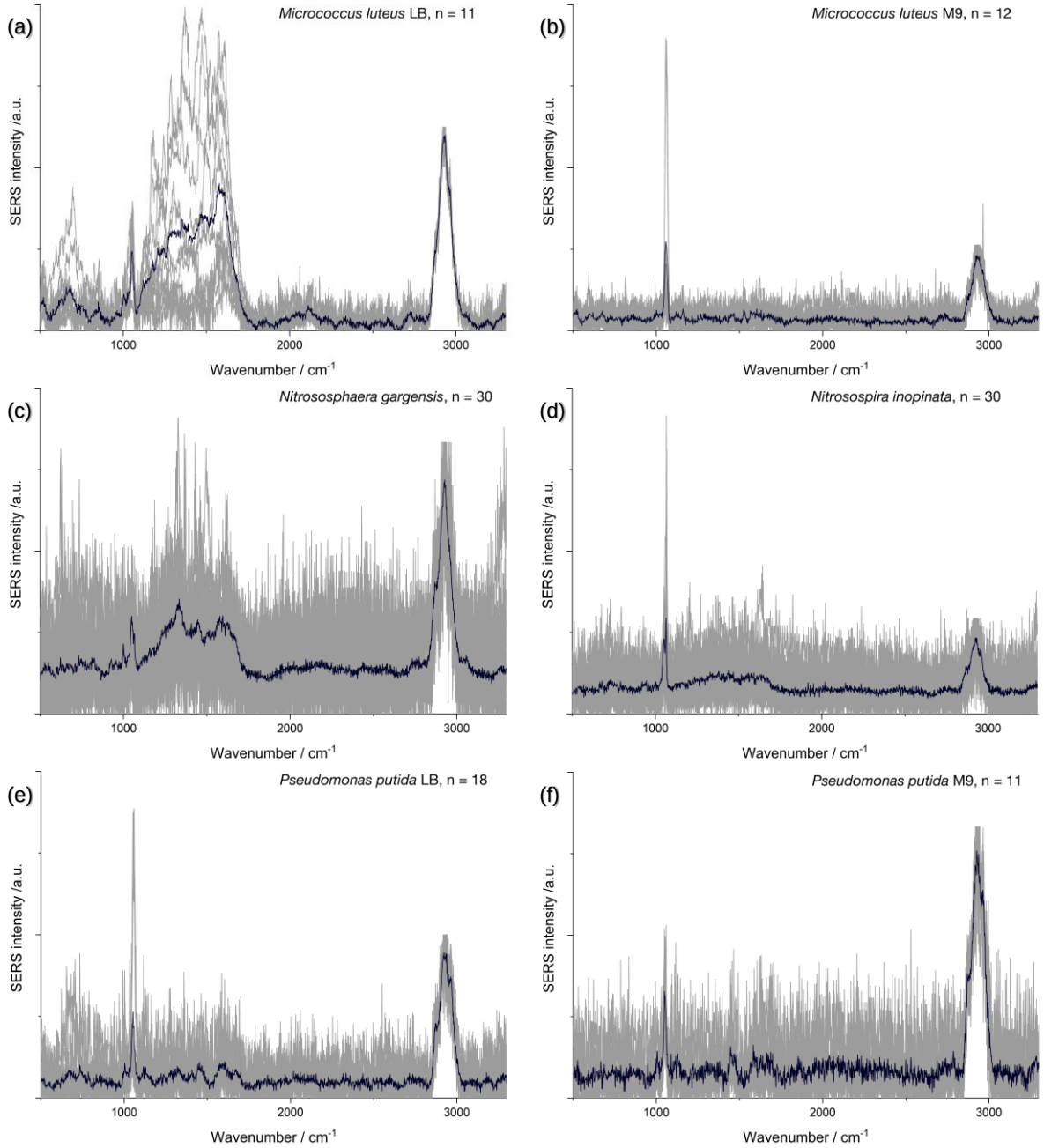

Figure S6. SERS single cell spectra of *M. luteus* cultured with LB (a), M9 (b), *N. gargensis* (c), *N. inopinata* (d), *P. putida* with LB (e) and M9 medium (f).

Mass spectrometric analysis of cell supernatants

The supernatant of washed *E. coli* cells was analysed by mass spectrometry to test for decreasing concentrations of the metabolite adenine (ESIMS $[M+H]^+$ *m/z* 136.06 (calculated for $C_5H_5N_5$, 136.06)) in case of stored cultures. The sample preparation was executed as similar as possible to the SERS procedure. Therefore, 1 ml of the freshly harvested or stored culture was centrifuged (5500×*g*, 1 min, 20 °C), the supernatant discarded and the cell pellet resuspended with 1 ml H_2O. This washing step was repeated twice. After the final centrifugation step the cell pellet was resuspended with 0.5 ml H_2O. After an interaction time of 1 h the sample was vortexed for 10 s and sterile filtrated with 0.22-µm pore size polyethersulfone syringe filters (Carl Roth GmbH &Co. KG, Karlsruhe, Germany). The sample was acidified with 0.1% formic acid and directly injected at a flow rate of 25 µL/min into an Orbitrap-based Exactive mass spectrometer equipped with an electrospray ionisation source (Thermo Fisher Scientific, Bremen, Germany). The system was operated in the positive ion mode, acquiring data in the range of 50–999 m/z and averaging over a time interval of 2 min. Experiments were performed in biological triplicates for freshly harvested and for 5 d at 5 °C stored cultures. The corresponding signal of adenine decreases for the supernatant of freshly harvested cells from 10508 ± 1041 a.u. to 6660 ± 1002 a.u. for the stored samples. This drop of 37% shows the same trend as the results of the SERS analysis.

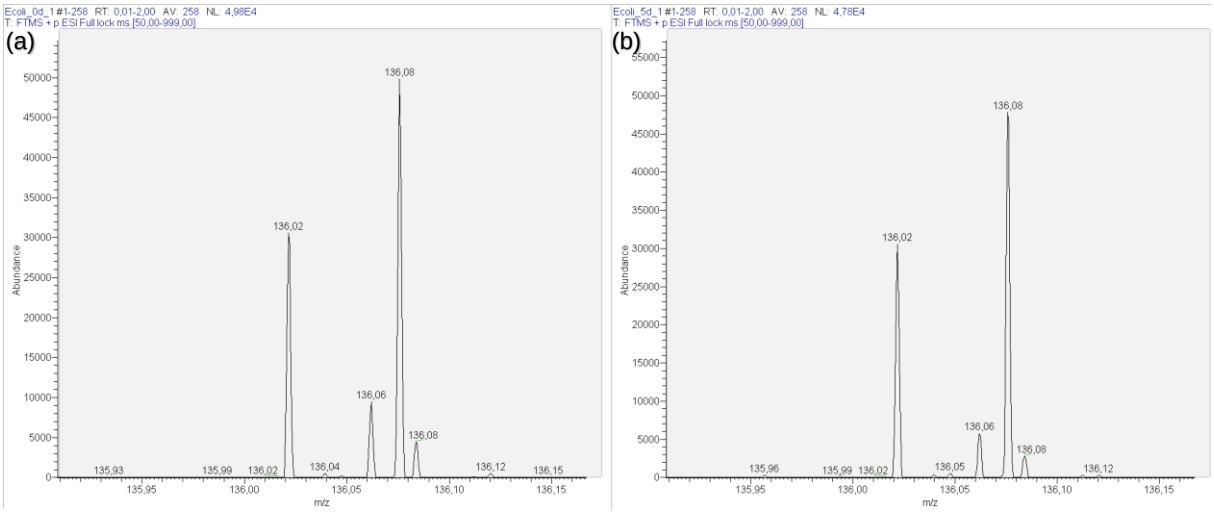

Figure S7. Mass spectrogram of *E. coli* supernatant of freshly harvested, washed cells (a) and of stored, washed cells (b).

SERS for cell tweezing

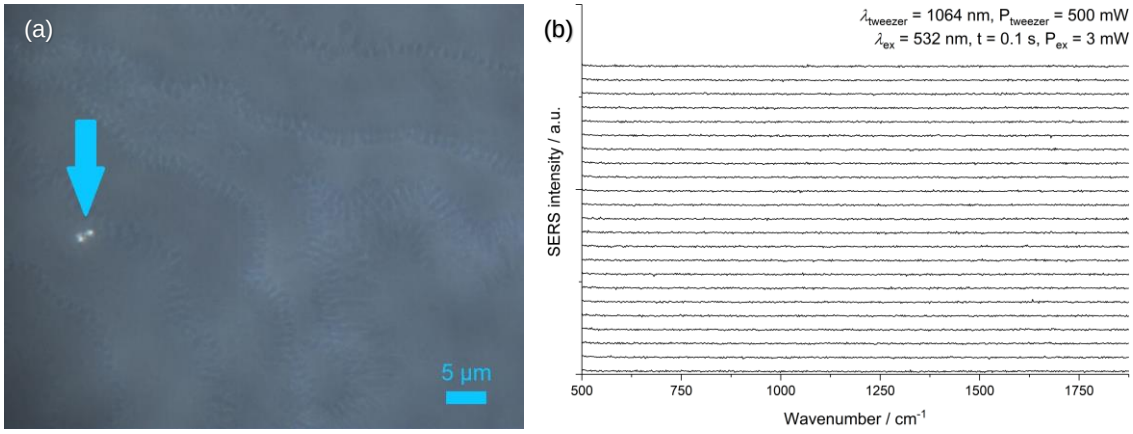

Figure S8. (a) Microscopic image during optical tweezing with AgNP@*E. coli* agglomerate outside of laser focus; (b) 23 consecutively acquired spectra of AgNP@*E. coli* agglomerate outside of laser focus.

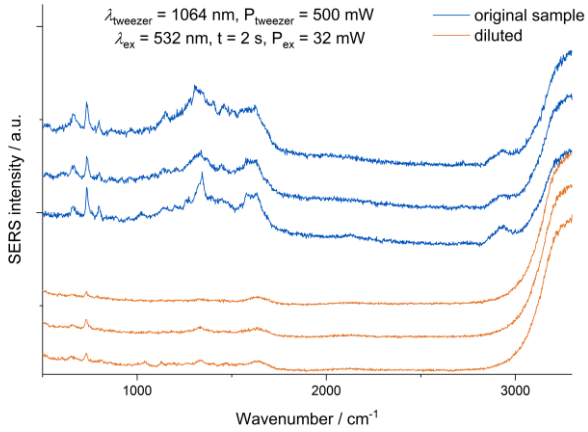

Figure S9. Continuously recorded SERS spectra of *E. coli* in fluid with *in situ* synthesised AgNP under tweezing conditions (500 mW 1064-nm laser, 32 mW 532-nm laser) but without centrifugal preparation step (blue) and diluted 1:9 (orange).

3D Raman imaging of plastic beads

An agarose gel mixed with beads of poly(methyl methacrylate) (PMMA), melamine formaldehyde (MF), and polystyrene (PS) was analysed by Raman microspectroscopy to test the depth resolution of the used system setup (Horiba LabRAM HR800, Olympus LUMPlan FL N NA = 1.0, λ_0 = 532 nm, confocal pinhole: 100 µm). The monodisperse beads have a diameter of 3.8 µm (PMMA), 4.4 µm (MF), or 5.2 µm (PS), respectively. The different polymers are detected by means of the characteristic peaks at 813 cm^{-1} (PMMA), 977 cm^{-1} (MF), or 1001 cm^{-1} (PS). The intensity of these signals at each grid point is depicted in Figure S10 by spheres with the colours red, green, or blue, respectively. The size of these signal spheres and the saturation of its colour depend on the intensity of the corresponding signal. The signal spheres at the scanning points of the total scanned volume add up to three PMMA, one MF, and two PS beads. The depth resolution is inferior to the lateral resolution but is sufficient to reproduce microscopic particles with a diameter in the single-digit micrometre-range.

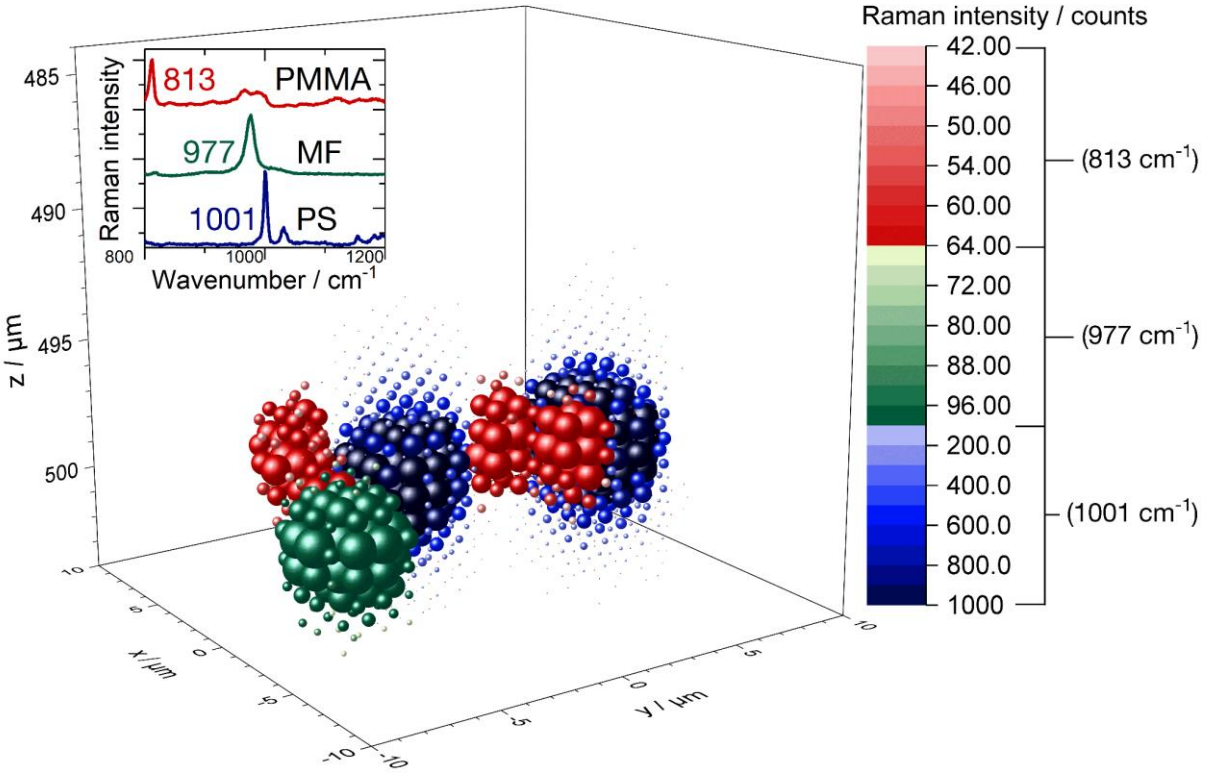

Figure S10. 3D Raman image of an agarose gel with 3 PMMA (d = 3.8 µm), 1 MF (d = 4.4 µm), and 2 PS beads (d = 5.2 µm) measured with a water-immersion objective (NA = 1.0) at an excitation wavelength of 532 nm.

SERS reference spectra (λ_0 = *532 nm and 633 nm)*

Reference spectra of riboflavin, flavin adenine dinucleotide (FAD) and adenine were recorded to exclude the interference of a resonance effect of riboflavin and FAD at about 500 nm leading to surface-enhanced resonance Raman scattering (SERRS). This could fundamentally impair the comparison of SERS with the used AgNP recorded at the excitation wavelength of 532 nm or 633 nm. While spectra of *E. coli* at both excitation wavelengths have distinct similarities with SERS spectra of adenine, they do not exhibit peaks at 531 cm^{-1}, 552 cm^{-1}, 619 cm^{-1}, 805 cm^{-1}, 838 cm^{-1}, 1087 cm^{-1} or 1629 cm^{-1} corresponding to riboflavin or FAD.

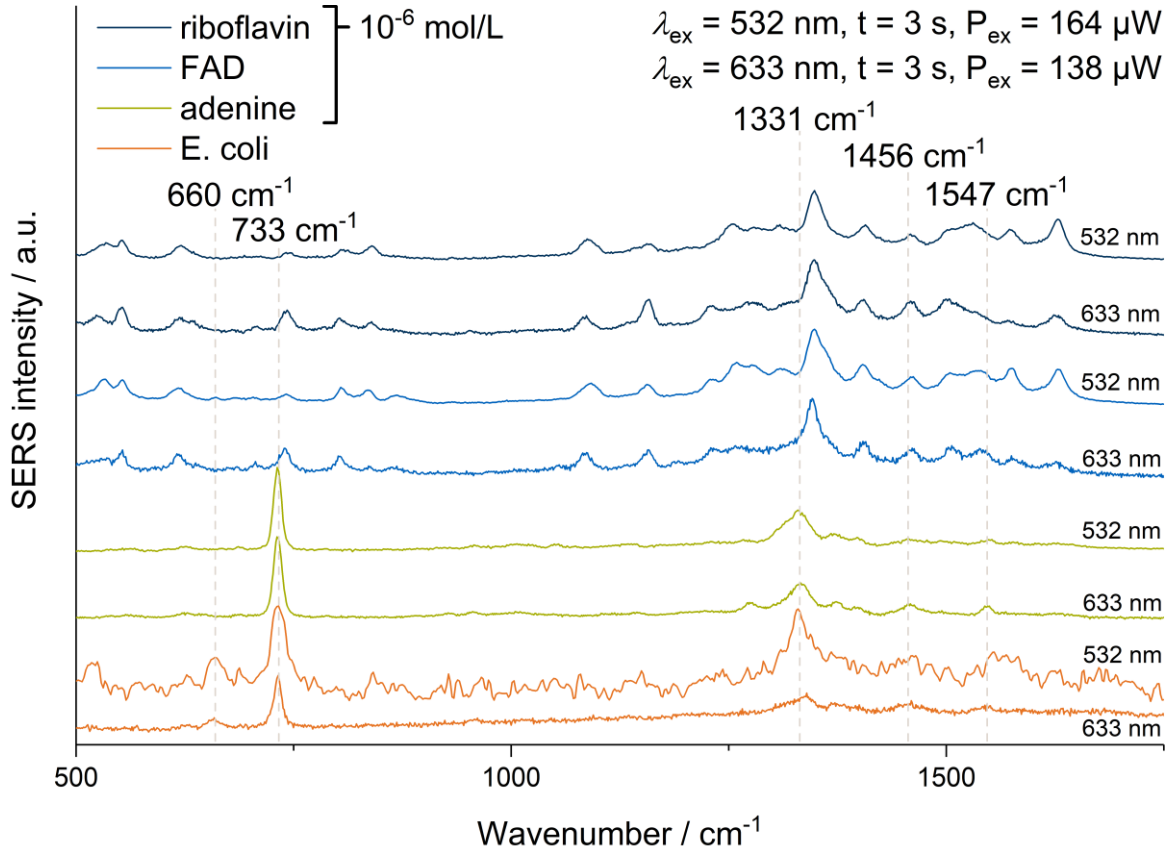

Figure S11. Normalised SERS spectra of riboflavin, FAD, adenine (separate AgNP synthesis, AgNP mixed with 10^{-6} M solution) and *E. coli* (*in situ* AgNP synthesis) at 532 nm and 633 nm excitation wavelength.