Supplementary Materials

Confocal Microscopy and 3D Image Analysis – Relative Quantitative 3D-Microscopy

Vybrant DiI was imaged by an exciting argon laser-line at λ 514 nm and emission was collected between 533-577 nm. CellTracker Green was imaged by an exciting argon laser-line at λ 488 and emission was collected at λ 510-550 nm. AuNPs were visualised in reflection mode with a HeNe λ 594 nm laser-line and emission was collected at λ 592-597 nm (setup 1: AuNP-concentration experiments) and HeNe λ 633 nm laser (collected at λ 631-636 nm) (setup 2: CPZ experiments). Samples were imaged from approximately 2 µm below the surface found by maximum intensity auto-focus in reflection mode at λ 488 (collected at λ 486-491 nm). Image background normalisation was performed as follows: image background intensity was quantified with region of interest (ROI) analysis at sample height = 5µm) by LAS AF Lite (v.2.4.1 build 6384, Leica Microsystems). Individual image stack intensity values were divided by their individual background intensities and multiplied by a common value reflecting the optimal gray-scale maxima (to avoid digital saturation) by ImageJ (64bit v.1.47v) with Loci Tools plug-in (v. 4.4.6, Bio-Formats). Sample volume trimming: to ensure that the image stacks were comparable they were trimmed to match z-stack-start 1 µm below the sample surface. The position of the surface (in the z-stack) was determined and defined at the maximum intensity in the reflection channel (image average intensity analysis, LAS AF Lite).

Focused Ion Beam Scanning Electron Microscopy (FIB/SEM) – Detailed Experimental Procedure and Specimen Preparation

One day prior to AuNP-exposure (5µg/ml, 24 h) a 160 µl drop of HUVECs ($6.6x10^4$ cells) were seeded on ø13 mm Nunc™ Thermanox™ Coverslips (Thermo Fisher Scientific Inc., USA) placed in Nunc™ 12 well plates (Thermo Fisher Scientific Inc.). After 1½ h 1 ml of pre-heated HUVEC-medium were added the wells. One day after cell-seeding the cells were exposed to AuNPs (5 µg/ml) for 24 h. The cells were washed (x3) in PBS and fixed with glutaraldehyde fixation (2% in 0.5 M phosphate buffer pH 7.4). SEM specimen preparation: specimens were washed twice with 0.15 M phosphate buffer and once with 0.15 M sodium cacodylate buffer (pH 7.4) for 30 min. Post-fixation in 1% OsO_4 and 0.05 M K_3FeCn_6 in 0.12 M sodium cacodylate buffer for 2 h followed by short wash in 0.15 M sodium cacodylate buffer and distilled H_2O. Dehydration was by step-wise substitution with ethanol in three steps of 2x 15 min in: 70%, 96% and absolute ethanol. Epon-embedding was done in 1:1 propylenoxide/epon overnight and in pure epon for 2 h the following day. Specimens were mounted on a pin mount stub with the cover-slip facing upwards. The specimen were frozen in liquid nitrogen and the coverslip was then gently broken. This is necessary since the ion-beam has a limited milling

depth, meaning that the actual cell-sample needs to be exposed. Specimens were spotter coated by a Leica EM ACE600 (Leica Microsystems, Germany) with 2 nm Au before imaging, and platinum coated before milling to protect against unwanted beam-damage. Image reconstruction was done by standard methods (segmentation tool) in Amira.

Single Particle Inductively Coupled Plasma Mass Spectrometry (spICP-MS) – Additional Experimental Procedure and NP-Characterization

Additional experimental procedure: Ultrapure water (18.2mΩ/cm) was obtained from a Millipore Element apparatus (Millipore, USA) and used throughout the work. Bovine serum albumin (BSA) containing ≈ 98 % protein monomer was obtained from Sigma Aldrich (USA). A 25 % (v/v) aqueous solution of electronic grade tetramethylammonium hydroxide (TMAH) was obtained from Alfa Aesar (USA). After HUVECs were exposed they were thoroughly washed to ensure loosely attached AuNPs were removed and to obtained the same numbers of washes after exposure of samples for confocal microscopy (before sample fixation): x6 in medium and x2 in HEPES buffer. BSA was added to each sample before TMAH-treatment to avoid agglomeration of the AuNPs in the sample solution. Each sample contained on average $3x10^3$ cells and BSA was added in increasing mass following the exposure concentration (AuNPs 0 and 1.25 µg/ml: 0.73 ng; AuNPs 5 µg/ml: 1.93 ng; AuNPs 10 µg/ml: 4.35 ng). NP characterization: For each sample the 197Au signal intensity was recorded for 180 s with a dwell time of 10 ms. Following the analysis of each sample, ultrapure water was analysed to control if carry-over from the previous measurement could be detected. For all analyses, raw signal intensity data were plotted versus number of events to create a signal distribution histogram using a spread sheet routine (Microsoft Excel). Very low signal intensities (less than 10 counts per 3 ms) were considered to be instrument background. Slightly higher signal intensities were considered as incomplete events, i.e. partial gold ion plumes that were detected during two consecutive dwell times. Instrument calibration was achieved by analysis of ultrapure water as blank (mass zero) and 30 nm AuNPs (RM8012, average mass per particle = 0.21 fg) as well as 60 nm AuNPs as calibrants (RM8013, average mass per particle = 1.78 fg). The average intensity for the blank was calculated. For the AuNP calibrants the mean peak intensity corresponding to particle events was determined from the frequency distribution. The intensity values were plotted against mass. The slope was used to convert the measured intensities of AuNPs in the samples into particle masses. By assuming a spherical particle shape and a particle density of 19.3 g/cm^3 (density of gold), the masses were finally converted into particle diameters. The transport efficiency, which was necessary to determine the particle concentration in the samples was determined according to the "particle frequency" method [Pace HE, Rogers NJ, Jarolimek C, Coleman VA, Higgins CP, and Ranville JF (2011) Anal. Chem. 83:9361-9369] by measuring RM 8013. The transport efficiency, which was determined daily, was calculated as number

of particles detected by spICP-MS in percentage of the theoretical (calculated) particle number in the aspirated AuNP suspension. The sample flow rate was accurately determined daily by weighing the amount of water that was delivered by the peristaltic pump of the sample introduction system during one minute.

Table S1 Settings for the spICP-MS instrument.

Parameter (unit)	**Value**
Plasma power (W)	1550
Plasma gas flow rate (L/min)	14
Nebulizer flow rate (L/min)	0.96 - 0.99
Axillary gas flow rate (L/min)	0.8
Sample uptake flow rate (mL/min)	0.352 - 0.362*
Monitored isotope (m/z)	^{197}Au
Dwell time (ms)	10
Analysis time (s)	180
Wash time (s)	120
Spray chamber type	Quartz cyclonic type, Peltier-cooled
Nebulizer type	Micro flow PFA nebulizer

File S1 Movie of 3D-rendered HUVECs after 3 h AuNP-exposure (5µg/ml). The cell **(**CellTracker) is visualized in red and the AuNPs in green. In the movie it can be seen that the signal of the AuNPs are elongated due to the reduced resolution in the z-direction compared to x-y. The grid length unit is 15.39 µm.

File S2 Movie of a z-stack of HUVEC after 3 h AuNP-exposure (5 µg/ml). The cell (CellTracker) is visualized in grayscale and AuNPs are in green. The movie is showing the localization of AuNPs in the perinuclear region and in possible association with endosomes and lysosomes. The vesicle structures are not stained directly but is visible due to reduced staining (scale-bar = 20 µm).

File S3 Movie showing the FIB/SEM image-stack of HUVECs after exposure to 5µg/ml AuNPs (yx-z and xz-y). The movie shows the aligned FIB/SEM image-stack (~730 images) first from the xy-view going through the z-axis followed by the xz-view going through the y-axis.

File S4 Movie of 3D-redered HUVECs after exposure to 5µg/ml AuNPs by FIB/SEM. The outer limits of the segmented cell (magenta), the nucleus (purple), the AuNPs (yellow), endosomes (green), and lysosomes (red) are visualised and rotated. The 3D visualisation is done by orthogonal view so the scale bar represents all depths of view.

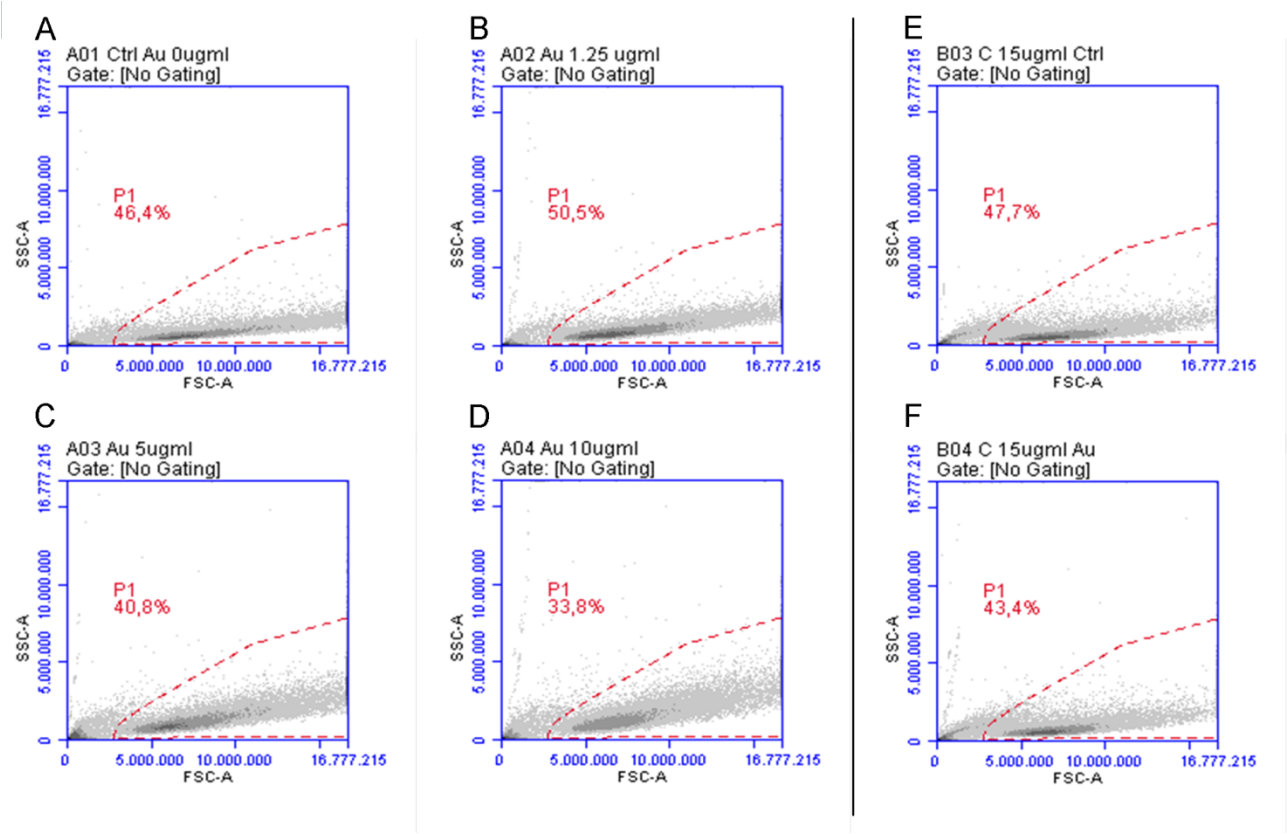

Figure S1 Flow cytometry raw data and gating strategy. Forwards-scatter (FSC-A) and side-scatter (SSC-A) used for gating a data analysis for HUVECs. These are representative examples of: control without AuNP-exposure (A), and 3 h exposure to AuNPs with 1.25 µg/ml (B), 5 µg/ml (C) and 10 µg/ml (D). Furthermore HUVECs modulated with chlorpromazine (15 µg/ml = 42 µM) (E and D); control without AuNPs (E) and 3 h exposure to 5 µg/ml AuNPs (D).

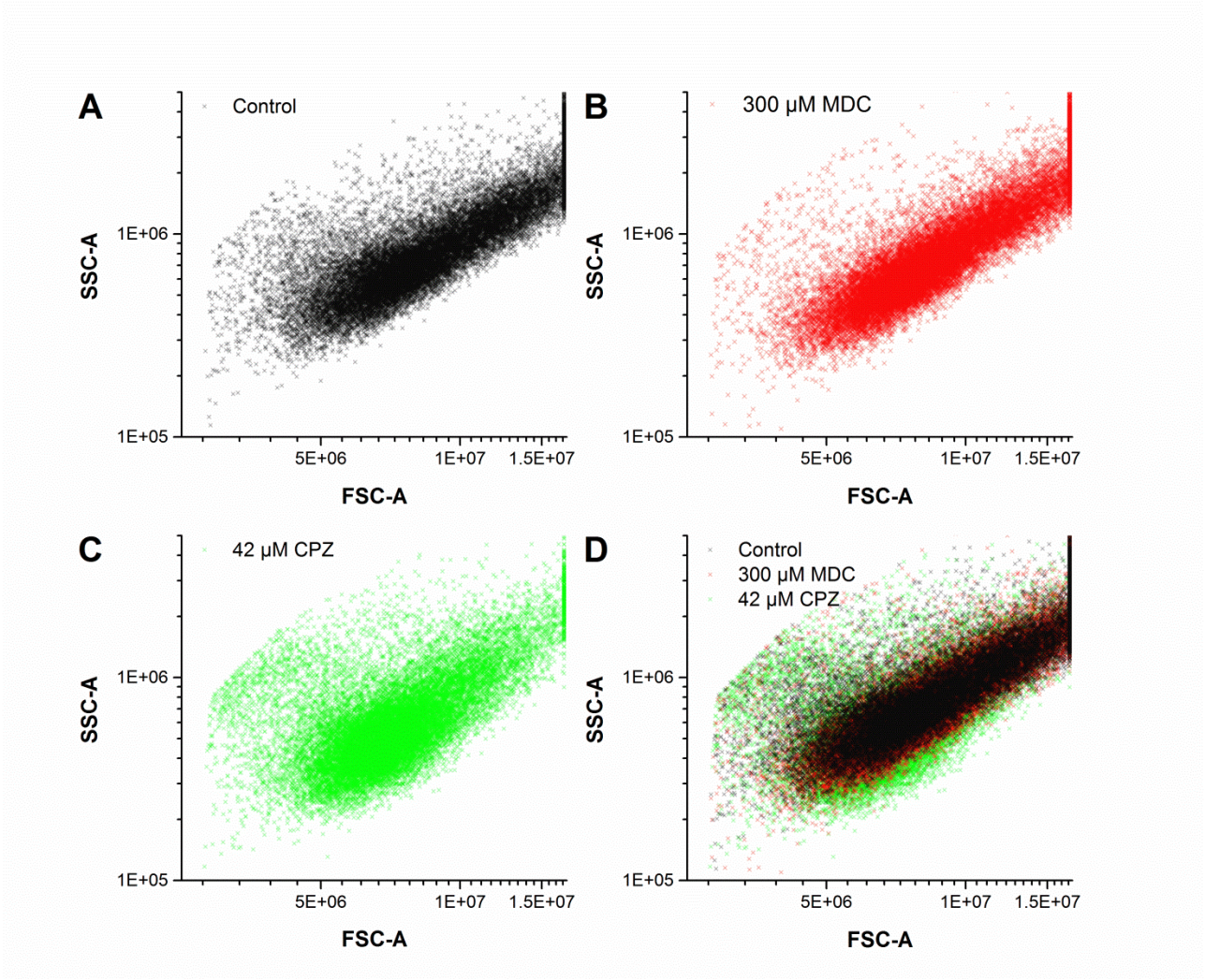

Figure S2 Forward- and side-scatter of HUVECs after modulation with monodansyl cadaverine (MDC) and chlorpromazine (CPZ). Representative data from the same experiment showing scatter-plots of unmodulated control cells (A, black), MDC modulated cells (B, red), and CPZ modulated cell (C, green), and a scatter-plot overlaying the previous three plots (D).

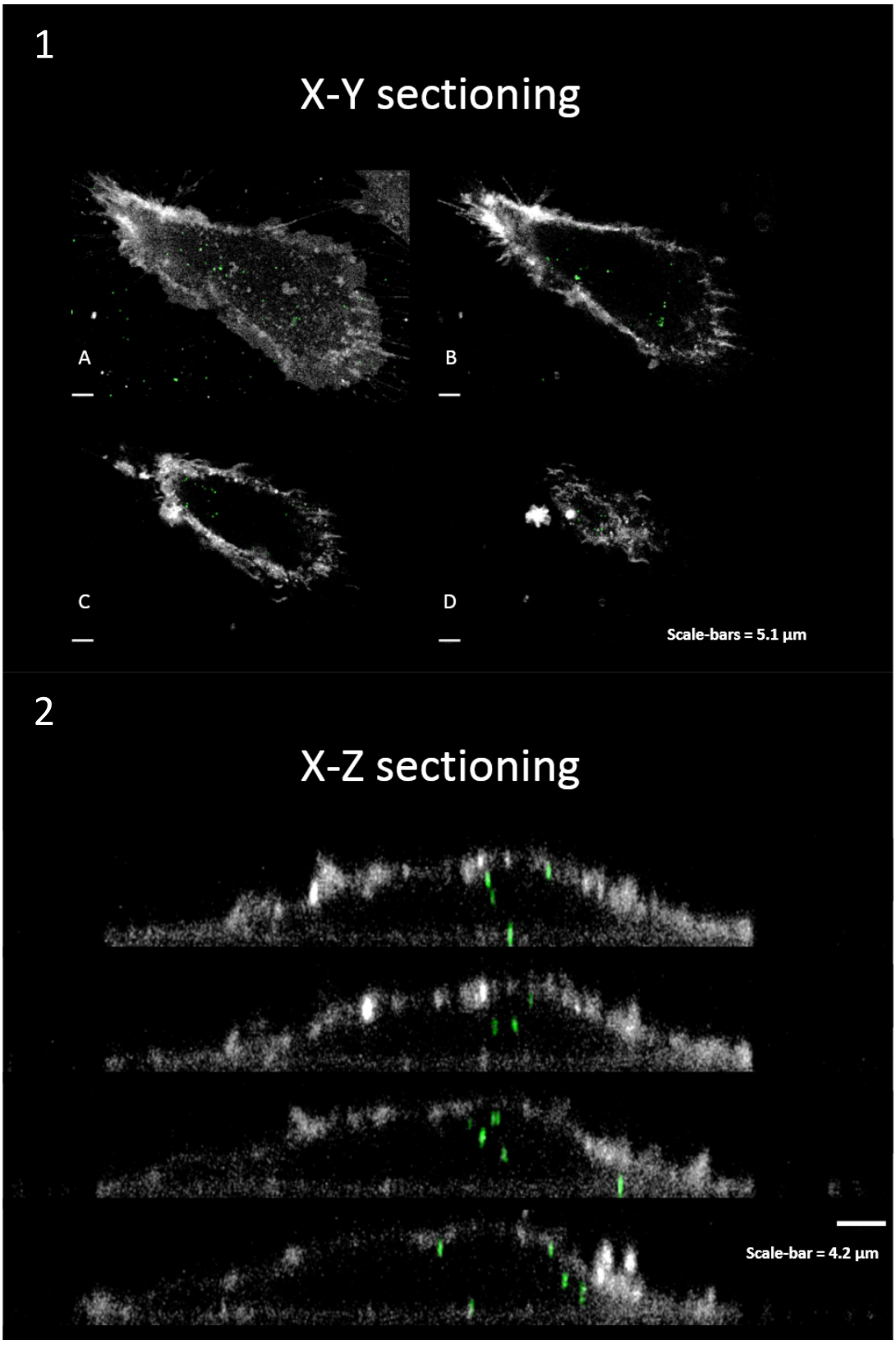

Figure S3 Confocal images of HUVEC stained with plasma membrane stain vibrant DiI. Grayscale is DiI and green is AuNP- reflection. Panel 1 shows selected x-y sections from the adhering surface to top of the cell (A-D) (scale bar = 5.1 µm). Panel 2 shoes generated z-x planes of the same cell (scale bar = 4.2 µm).

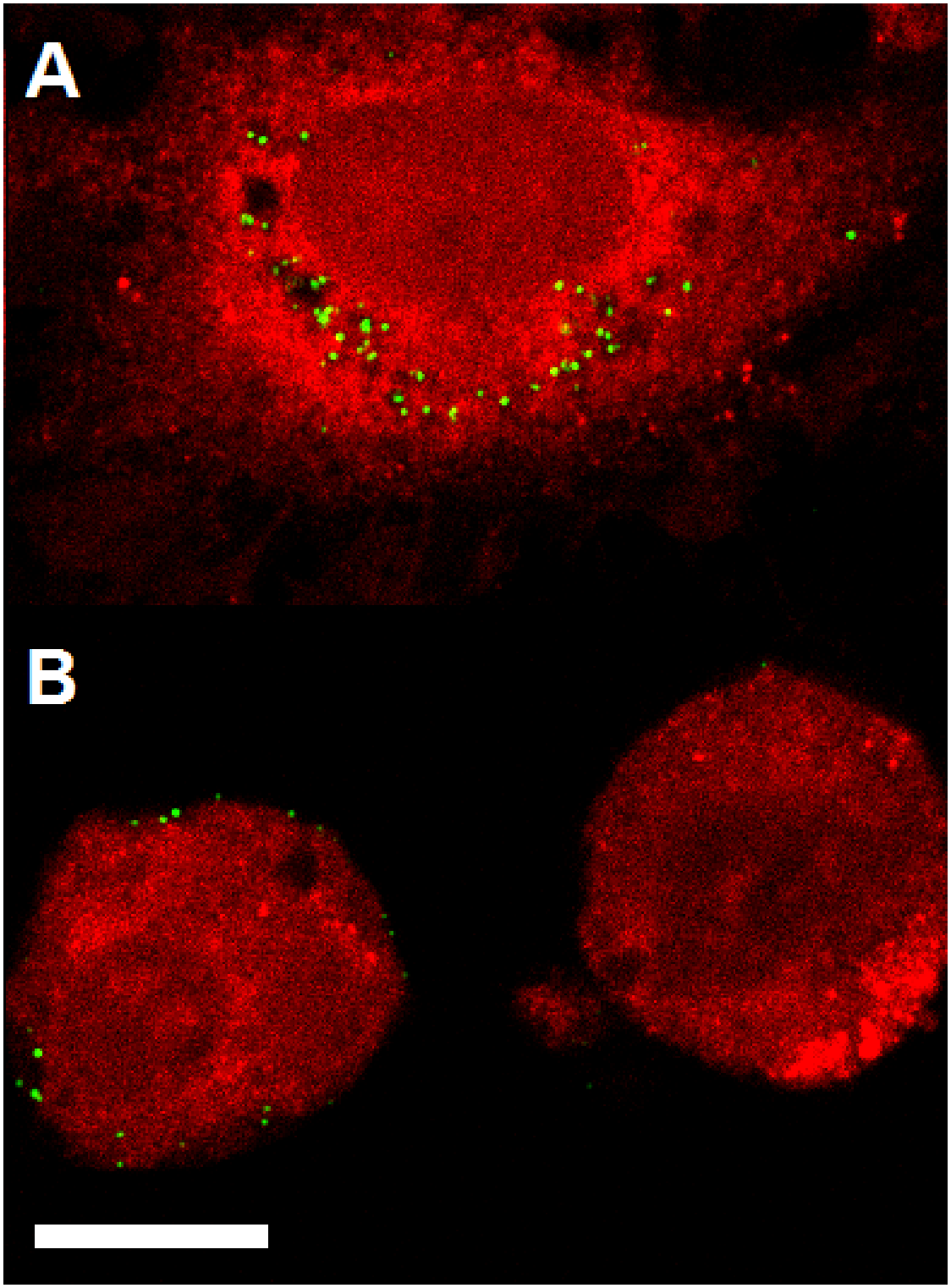

Figure S4 Confocal images of HUVECs after 3 h AuNP-exposure (5 µg/ml) with or without pre-treatment with chlorpromazine. Panel A and B shows selected confocal slices (single planes) of an unmodulated HUVEC (A) and two HUVECs modulated with 42 µM chlorpromazine (B), cells are stained with CellTracker (red) and AuNPs is shown in reflection mode (green). It can be observed that AuNP are being internalized into intracellular compartments by unmodulated cells while most of the AuNPs appear to be confined to the cortical cytosol or plasma membrane of the modulated cells (scale bar = 13 µm).