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Supporting information for: Gold-nanodome patterned microchips for intracellular surface-enhanced Raman spectroscopy

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Methods

Fabrication of nanodome-patterned microchips A layer of 200 nm Si_3N_4 was deposited on a 4" (100) Si wafer through plasma-enhanced chemical vapour deposition (Advanced Vacuum Vision 310-PECVD), out of which 2 cm x 2 cm pieces were cleaved for further processing. Next, a 5 w/v% colloidal solution of mono-disperse 448 nm (±3%) polystyrene beads in water (Microparticles GmbH) was densified to 15 w/v% by centrifugation for 15 minutes at 4300 g. This solution was re-suspended in a Triton-X (Sigma):Methanol (Sigma)

solution (1:400). The final ratio of H_2O to Triton-X:Methanol was 1/2, again resulting in a 5 w/v% solution. Possible clusters formed upon centrifugation were broken by sonication in an ultrasonic bath for 20 minutes. Prior to spin coating, the Si_3N_4/Si substrates were rendered hydrophilic in an O_2 plasma for 30 minutes (PVA-TEPLA GIGAbatch 310 M, 6000 sccm O_2, 600 W, 750 mTorr)). Next, a monolayer of polystyrene beads was formed by spin coating 50 µl of the polystyrene-beads solution on a 4 cm^2 chip at approximately 1400 rpm for 40 seconds with an acceleration of 648 rpm/s. In a second spin coating step, excess polystyrene beads at the edges were spun off at 4000 rpm for 40 seconds. After visual inspection of the monolayer coverage, spin speed and acceleration were slightly adjusted to improve the monolayer quality of subsequent chips,[S1,S2] see also figure S2. Five different samples with polystyrene beads were thinned down in an O_2 plasma for respectively 30, 35, 40, 45 and 50 seconds (Advanced Vacuum Vision 320-RIE, 50 sccm O_2, 75 W, 100 mTorr), where the exact time defined the final gap size in between the nanodomes . Next, the pattern was transferred into the Si_3N_4 layer by a highly anisotropic reactive-ion etch (80 sccm CF_4 / 3 sccm H_2, 210 W, 20 mT, 160 s), as shown in figure S1. Specific details on the anisotropic etching of Si_3N_4 can be found in.[S3] Briefly, the low pressure results in a more physical etching which simultaneously thins down the polystyrene beads while etching the Si_3N_4 surface in between the beads. The addition of H2 further increases anisotropic etching due to the formation of a polymer protective layer on the Si_3N_4 side walls. Afterwards, partially-etched beads were washed off in an ultrasonic bath with ethanol, although dichloromethane (carcinogenic, Sigma) was sometimes required for a complete removal of the PS beads. Subsequently, 6 µm disks were patterned through contact-lithography (resist: AZ400-K, positive, Microchemicals) and etched into the Si_3N_4 layer by the same anisotropic CF_4 H_2 etch for 350 s followed by an isotropic under-etch of the Si substrate (80 sccm SF_6 / 3 sccm H_2, 210 W, 20 mTorr, 160 s). Next, the chips were cleaned in acetone and isopropanol, followed by a O_2 plasma for 30 minutes (PVA-TEPLA GIGAbatch 310 M, 6000 sccm O_2, 600 W, 750 mTorr)) in order to remove photo-resist and other organic contaminants. This under-etch is required to enable

the lift-off of the microchips after the subsequent evaporation of a 2-5 nm Ni + 200 nm Au layer. The patterned chips were further cleaved into 1 cm x 2 cm pieces and inserted into a 2 ml centrifugation tube (Eppendorf) to which a 30 w/v% potassium-hydroxide (*KOH*, VWR)/water solution was added at 70 ℃ to under-etch the Si substrate. After 300 seconds of etching, the tube was cooled for two minutes in a cold water bath at 5 ℃. Finally the released microchips were sedimented in the tube by centrifugation for 6 minutes at 2300 g. The remaining KOH-etchant was washed out with water in at least five centrifugation steps. The colloidal microchip solution was stored at 4 ℃.

Fabrication of nanotriangle-patterned substrates For the fabrication of nanotriangle substrates, the chip with a HCP-monolayer of PS beads was first cleaned for 30 seconds in O_2 plasma to remove organic residues (Advanced Vacuum Vision 320-RIE, 50 sccm O_2, 75 W, 100mTorr). Next, a 2 to 5 nm thick Ni adhesion layer and a 70 nm thick Au layer were thermally evaporated on the substrate (Leybold Univex). Finally, the PS beads were lifted off in an ultrasonic bath of ethanol or dichloromethane.

UV-Vis characterisation UV/Vis spectra were acquired on a PerkinElmer Lambda 950 UV/Vis spectrometer. The nanodome substrates, which are not transparent, were characterized in specular reflection under an 8° angle with a 4 mm x 4 mm spot size and a 10 nm resolution in the 600-1200 nm wavelength range. A 150 nm uniform gold film on a 200 nm Si_3N_4/Si substrate was used as reference. Reflection spectra in water were acquired by squeezing a drop of water in between the substrate and a 170 µm microscope slide.

Cell Culture and Reagents Normal Human Dermal Fibroblast (NHDF, PromoCell), HeLa cells (ATCC) and HeLa-H2B cells (a kind gift from Prof. Manders, UvA, The Netherlands) were cultured in Advanced Dulbecco's Modified Eagle's Medium (GIBCO-Life Technologies 12491-015) supplemented with 2% fetal bovine serum (GIBCO-Life Technologies 10500-056) and 1 % penicillin-streptomycin-L-glutamine (GIBCO-Life Technologies 10378-

016), and grown in culture flasks in an incubator at 37 ℃ and 5% CO_2. HeLa-H2B is a transgenic HeLa derivative, stably expressing the DNA binding histone protein H2B fused with a green fluorescent protein (GFP). One day before Raman or fluorescence imaging, cells were seeded on a 35 mm gridded glass-bottom dish (MatTek). Prior to imaging, cells were washed three times with Hanks Balanced Salt Solution (HBSS, GIBCO-Life Technologies 14025-050) and imaged in HBSS supplemented with 25 mM HEPES. For the 12 h monitoring of cell division, pure HBSS was added .

Confocal fluorescence microscopy Image acquisition was done using a Nikon A1r confocal microscope (Nikon Instruments, Apris, France), mounted on a Nikon Ti body and equipped with a 60x Plan Apo oil immersion objective (Numerical Aperture 1.4). For visualizing the nano-dome microchips we took advantage of their strongly reflective gold layer, ideal for reflectance detection. The intracellular localization of the microchips was verified by labelling the cell membrane with a 10 µg/ml solution of Wheat Germ Agglutinin, Alexa Fluor 488 Conjugate (WGA-AF488, Life Technologies) dissolved in PBS. A single 488 nm Ar laser line was used to simultaneously acquire confocal fluorescence, transmission and reflectance signals. Using an 80/20 beam splitter, microchip reflectance was captured through a 488/20 nm band pass filter and WGA-AF488 fluorescence was detected through a 525/50 nm band pass filter. For live cell imaging, the microscope incubator was equilibrated at 36.5 ℃, while a dedicated stage holder was used for maintaining 5% CO_2. and 90% relative humidity. To minimize photo-damage during time-lapse acquisitions, laser power was kept low (¡2%) and temporal sampling was reduced to a minimum (15 min intervals). Image representation and annotation was performed using Nikon NIS Elements software and Fiji open source freeware (http://fiji.sc).

SERS experiments Raman experiments were conducted on a WITec Alpha300R+ confocal Raman microscope equipped with a 785 nm excitation diode laser (Toptica) and an UHTS 300 spectrometer with a -60 ℃ cooled CCD camera (ANDOR iDus 401 BR-DD). To

compare the enhancement across different substrates, they were immersed overnight in a 1 mM solution of 4-nitrothiophenol (4-NTP, Sigma), thoroughly rinsed with ethanol and dried with a nitrogen air gun. The substrates were immersed in a plastic dish with Mili-Q water. SERS spectra were collected with an upright low NA objective (Nikon PlanFluor 10x/0.3), resulting in a beam diameter of approximately 4 µm (characterized by scanning laterally over a Si edge). Spectra were acquired with a laser power of approximately 1 mW on sample and a 0.12 s integration time. 400 different spectra were collected in a 20 µm x 20 µm square, the average spectra and the 2σ interval is shown in figure 2(e). We did not observe significant changes in the 4-NTP spectra resulting form the laser illumination at this power. The enhancement of the different substrates was compared by integrating the intensity of the cm^{-1} peak from 1313 cm^{-1} to 1373 cm^{-1} after subtraction of the background. The relative standard deviation of the signal along the substrate was calculated by acquiring a total of 1600 spectra over an area of 1600 μm^2 and dividing the standard deviation of the integrated peak intensity by the mean integrated peak intensity.

For live cell SERS experiments, the microscope was used in inverted mode. Cells were cultured on a gridded glass-bottom dish (MatTek), which allowed correlation of Raman and fluorescence signals of the same cell on different microscopes. Cells were kept at 36.5 ℃ during experiments. Raman spectra were collected with a Nikon PlanFluor 20x/0.5 objective at a laser power of approximately 100 µW on sample with a 0.1 s integration time. Cells were imaged in HBSS, which was replaced by a 2 µM Rhodamine 6G (Sigma) solution in HBSS for intracellular R6G detection. The reference R6G spectrum was recorded on a nanodome SERS surface with a 10 µM R6G concentration in water and 2 s integration time over 25 averages and a laser power of approximately 1 mW. Spectra were processed using *Project FOUR* (WITec GmbH) to remove cosmic rays and the MATLAB package *COBRA*[S4] to subtract the SERS background with a polynomial and wavelet-based fit.

Uniformity of the monolayer and chip to chip variability

Ideally, all microchips are identical. However, the reproducibility of the microchips is limited by the uniformity of the spin coated polystyrene monolayer (figure 1 (a-1)). We currently achieve an area fraction of roughly 85% hexagonally close-packed monolayers on a 7.5 cm^2 chip (figure S2(a-d)). The remaining 15% consists of double- or multi layers or loosely packed regions. Furthermore the packing of monolayer itself is not perfect, but contains line and point defects (figureS2 (e)). A meticulous optimization of spin speed, acceleration, concentration of beads and ratio of surfactants under a controlled temperature and humidity may result in an increased monolayer coverage with big defect-free regions. Over 90% monolayer coverage with 400 μm^2 defect free areas have been reported.[S1,S2] To achieve a 100 % reproducibility, techniques like e-beam-, deep-UV- or nano-imprint lithography should be used.

Characterisation of nanosphere-lithography fabricated nanotriangle surface

Figure S3 (a-b) describes the fabrication and characterisation of nano-triangle substrates. These substrates have been well characterized in literature,[S5] and serve as a benchmark for the nano-dome substrates (figure 2). The far-field properties of the nanotriangle substrates were characterized by UVVisNIR transmission measurements (figure S3 (c)). For this purpose, a Si_3N_4/Glass substrate with nanotriangles was patterned in along an identical procedure to the Si_3N_4/Si chips. From these graphs, the dependence of the plasmonic resonance on the geometric properties of the gold nanotriangles is clearly visible. Higher aspect ratios and wider particles result in red-shifted resonances. The substrate from the left curve (red, w=150 nm, h=70 nm) was used for comparing the SERS enhancement in figure 2. Figure S4(a) characterizes both AuFON, nanotriangle and nanodome surfaces by

their UVVisNIR reflection/transmission spectra. As the nanotriangle substrates show an optimal localized surface plasmon resonance in air, the near-field enhancement from 4-NTP spectra in air is shown in figure S4 to demonstrate the superior performance of nanodome- and AuFON substrates.

Intracellular localization of the microchips

The intracellular fate of the microchips determines the applications for which they can be used. Therefore it is important to know whether they are freely available in the cytosol or end up in lipid-membrane bound vesicles like phagosomes or lysosomes. We investigate the colocalization of the microchips with these vesicles using two different fluorescent dyes, Vybrant DiI (Molecular Probes) and Lysotracker Red DND-99 (Life Technologies). In this experiment, NHDF were incubated with microchips in a 96-well plate (Cellstar 96-Well uClear-flatbottom, Greiner Bio-One) for 24 hours and subsequently washed and stained. Images were acquired on a confocal microscope (see Methods) with a Nikon CFI Plan Apo VC 20X objective (NA 0.75), except for figures S5(d) and S6(c), where a Nikon CFI Plan Apo VC 60XWI (NA 1.2) objective was used. Vybrant DiI is a lipophilic membrane stain which shows increased fluorescence when incorporated in membranes. Figure S5 does not indicate colocalization between this dye and the microchips. Lysotracker is a probe which selectively accumulates in cellular compartments with low internal pH, Figure S6 indicates that there is no colocalization of the microchips with acidic compartments like lysosomes. A number of papers report the free localisation of microparticles - or capsules in the cytosol based on fluorescence experiments.[S6,S7] However, the fluorescent stains may not label all vesicles. Furthermore the localization of the microparticle in the cell can strongly depend on time after incubation, as well as surface properties and shape.[S7] For these reasons, additional experiments are required to confirm the exact intracellular localization of the chips.

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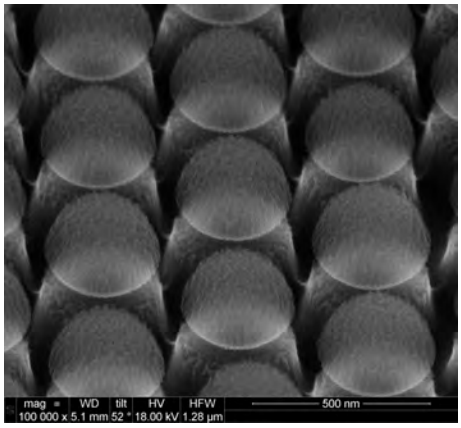

Figure S1: Tilted SEM view showing the remainder of polystyrene beads on top of the nanodome-patterned Si_3N_4 surface after reactive ion-etching and prior to lifting off the beads.

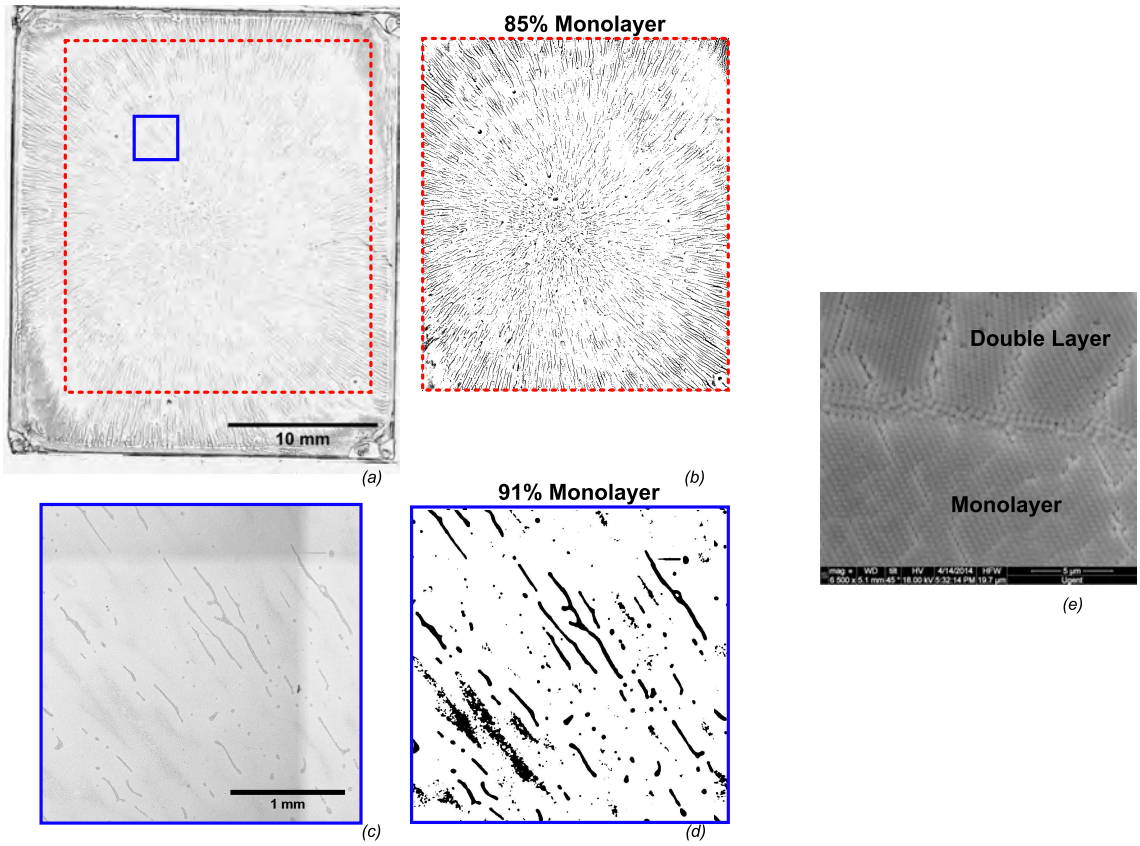

Figure S2: Spin coated layer of polystyrene beads on a 3 cm x 2.5 cm chip. (a) Microscope image, the lighter regions correspond to monolayers while the darker regions indicate double or multi-layers. (b) Tresholded image of red-dot marked area, with monolayer in white and multilayers or gaps in black, the area fraction of monolayer coverage is 85%. (c) Zoomed image of blue-solid marked area and (d) corresponding monolayer and multilayer regions, with a 91 % area fraction of monolayers. (d) Tilted SEM view showing a double- and monolayer of polystyrene beads)

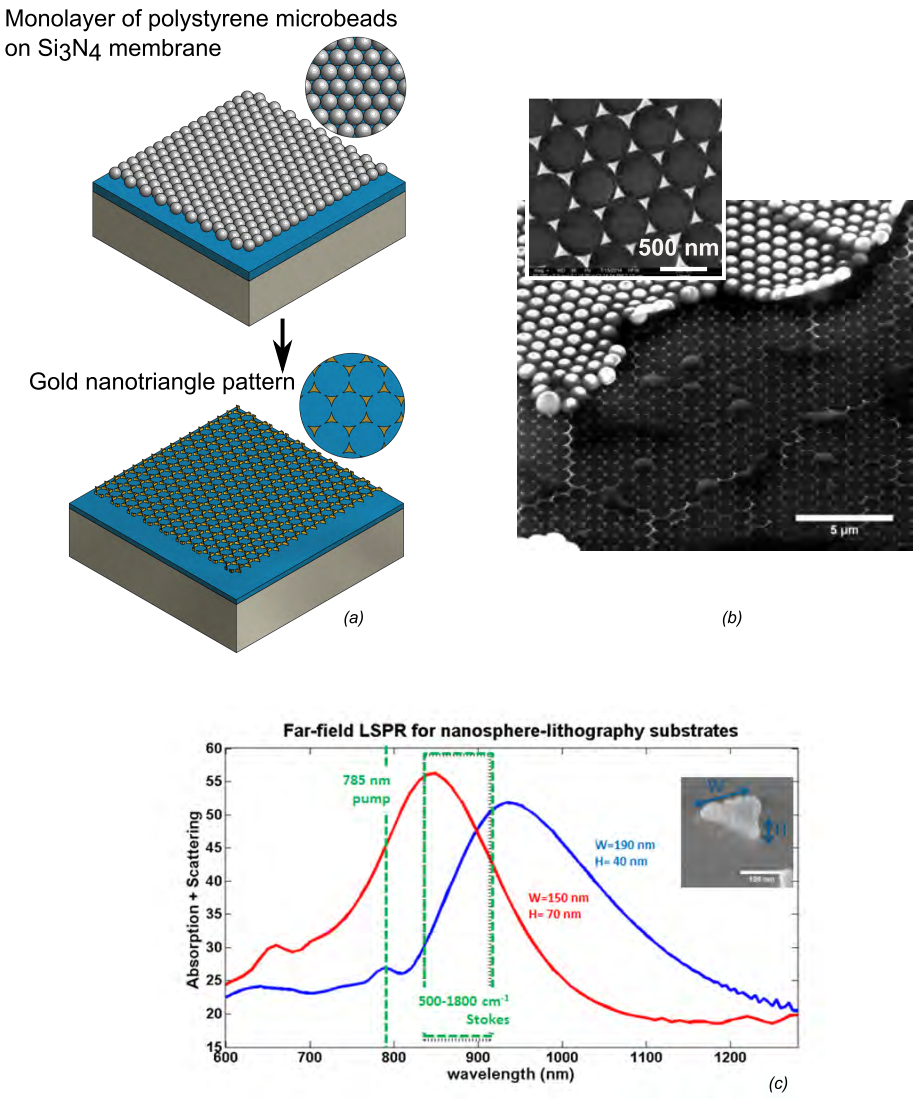

Figure S3: (a) Schematic of fabrication of nanotriangle pattern with nano-sphere lithography. Gold is deposited on a monolayer of 450 nm polystyrene beads, after which the beads are lifted off. (b) Tilted SEM showing a partially lifted-off substrate with PS beads (top) and nanotriangles (bottom), the inset shows a top view of the nano-triangle pattern. (c) UVVisNIR transmission spectra in air for nanotriangle-patterned substrates.

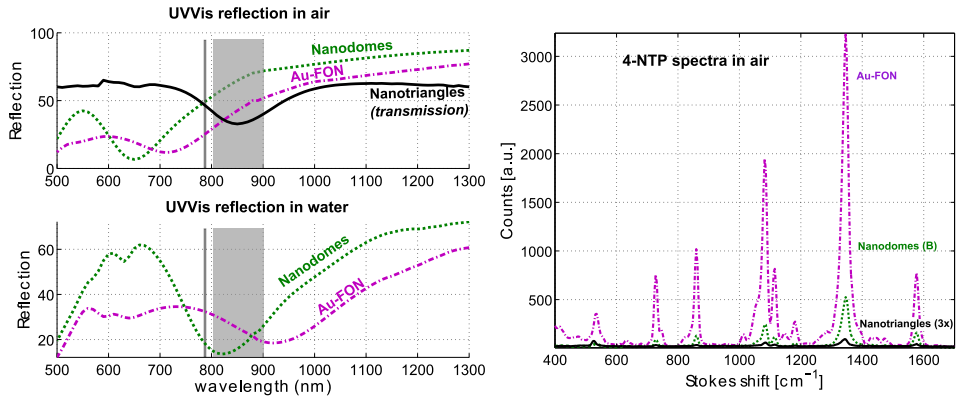

Figure S4: (a)UVVisNIR resonance comparison of AuFON, NSL and nanodome substrates. (b) Comparison of SERS spectra from monolayer of 4-NTP in air

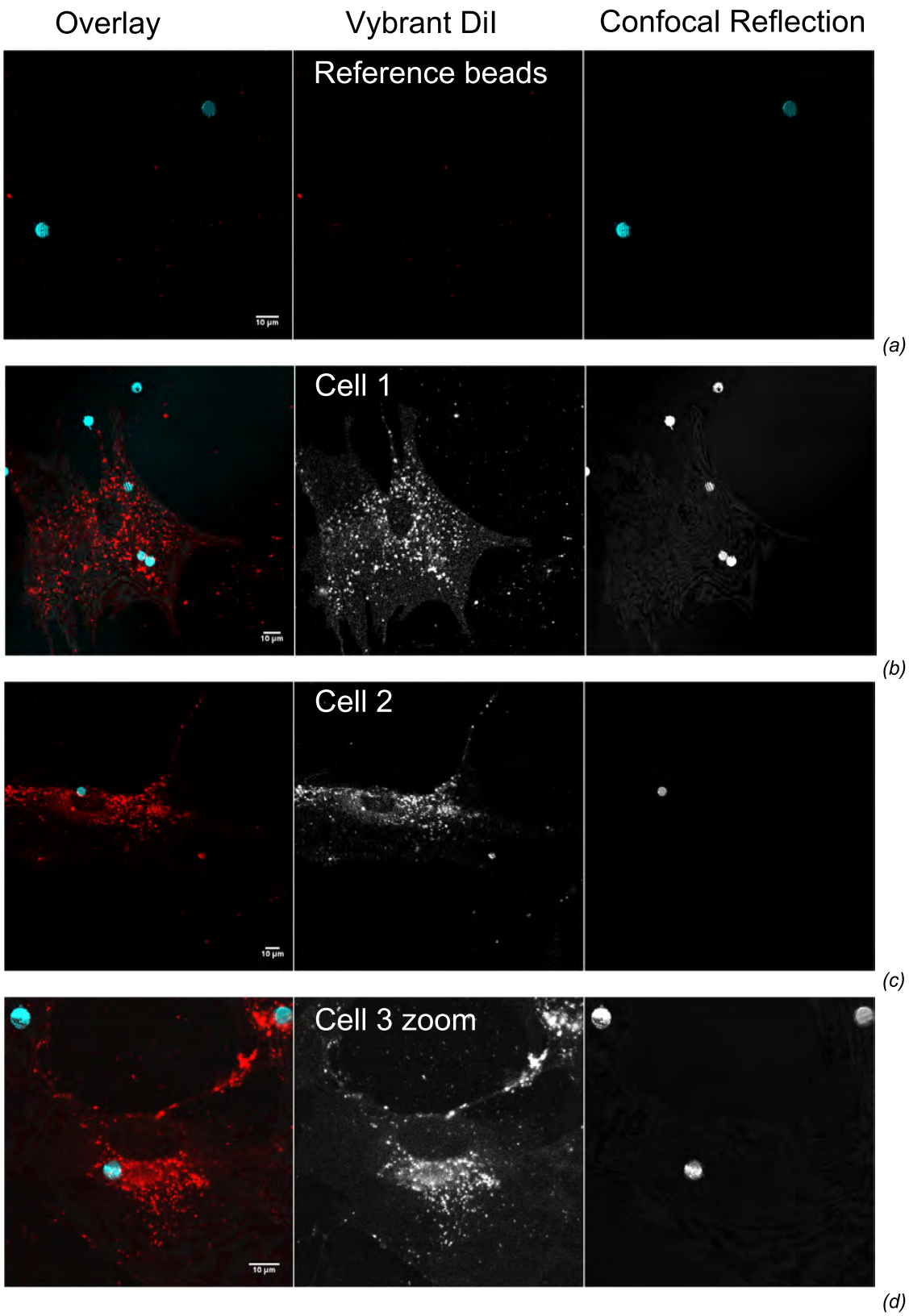

Figure S5: Staining NHDF cells containing microchips with the lipophilic dye *Vibrant DiI* does not indicate colocalization of the microchips (cyan) with lipid (red) membranes. (a) Reference measurement with only microchips and Vybrant DiI. (b-c) Cells with microchips and (d) zoomed image of a cell containing a microchip.

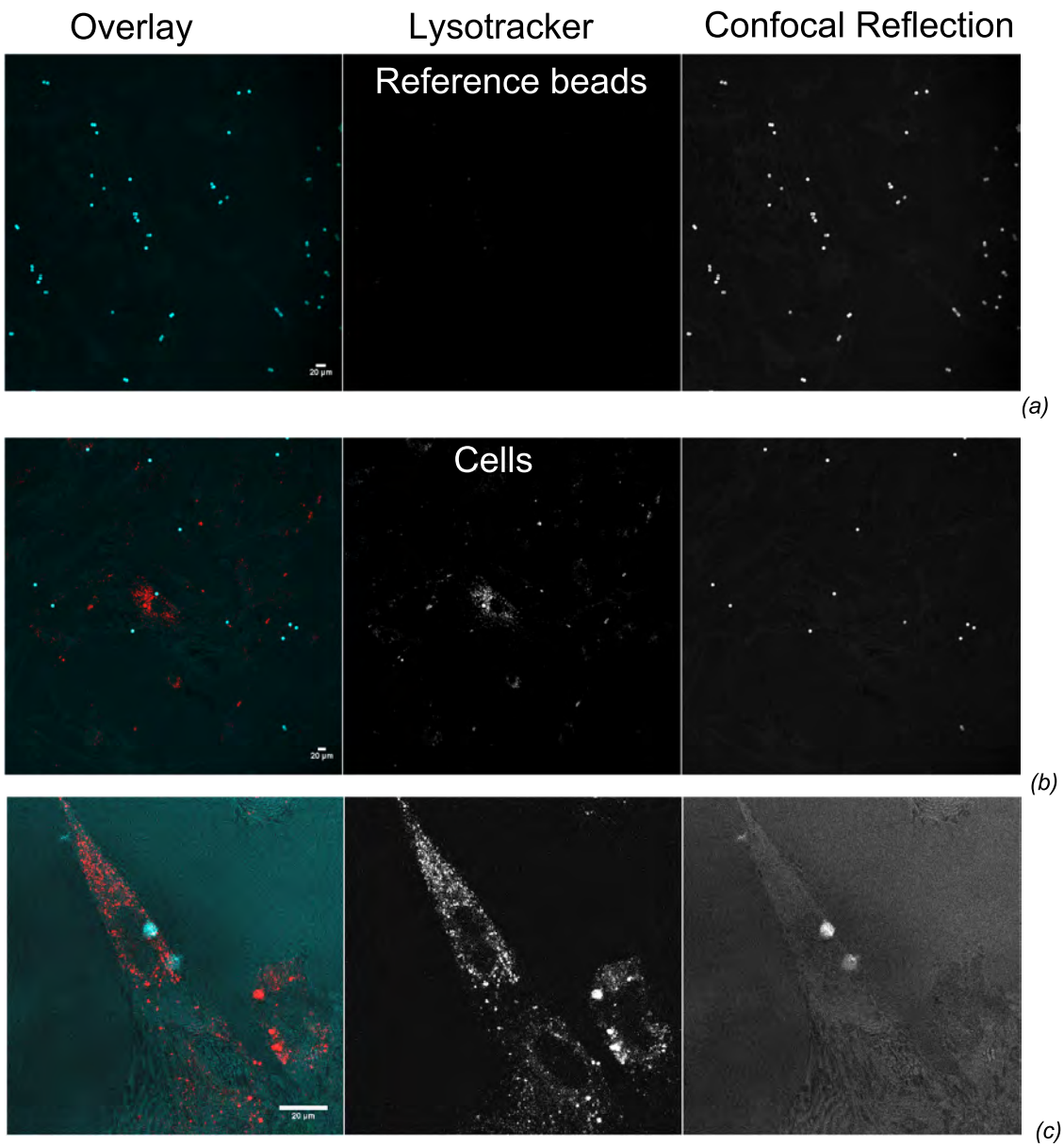

Figure S6: Staining acidic compartments of NHDF cells with *Lysotracker Red DND-99* does not indicate colocalization of the microchips (cyan) with lipid acid compartments like lysosomes (red). (a) Reference measurement with only microchips and Lysotracker. (b) Cells with microchips (c) Zoomed image of a cell containing a microchip.