Supporting Information

Chronic Inflammation Prediction for Inhaled Particles, the Impact of Material Cycling and Quarantining in the Lung Epithelium

*Hana Kokot‡, Boštjan Kokot‡, Aleksandar Sebastijanović‡, Carola Voss, Rok Podlipec, Patrycja Zawilska, Trine Berthing, Carolina Ballester López, Pernille Høgh Danielsen, Claudia Contini, Mikhail Ivanov, Ana Krišelj, Petra Čotar, Qiaoxia Zhou, Jessica Ponti, Vadim Zhernovkov, Matthew Schneemilch, Zahra Doumandji, Mojca Pušnik, Polona Umek, Stane Pajk, Olivier Joubert, Otmar Schmid, Iztok Urbančič, Martin Irmler, Johannes Beckers, Vladimir Lobaskin, Sabina Halappanavar, Nick Quirke, Alexander P. Lyubartsev, Ulla Vogel, Tilen Koklič *‡‡, Tobias Stoeger *‡‡, Janez Štrancar *‡‡*

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A word on supplement arrangement

The first part of the supplement (S0a to S0h, pages 11 - 62) contains general supporting material for the main paper ("Materials, methods and nanomaterial characterisation") as well as complementary data used in the main text but not shown in Figures in the main text ("Complementary experiments, not included in figures in the main text").

From S2 (page 63) onwards, detailed supplementary material for each image from the main text is shown ("Supplementary information for experiments in Figures 2 – 6 in the main text"). It contains details on experimental design, controls and repetitions (fluorescence images shown both in separate channels and overlayed), and names (cyphers) of experiments to ease locating them in the depository/database.

We first supply the main text image duplicates for easier orientation. The supplement sections correspond to the panels in the main text. For example, section S3c in the supplement corresponds to the experiment in Figure 3C in the main text.

--- Materials, methods and nanomaterial characterisation ---

S0a – General materials and methods

Materials

Animals and cells

- LA-4 murine alveolar epithelial cells (ATCC)
- MH-S murine alveolar macrophages (ATCC)
- NR8383 rat alveolar macrophages (ATCC)
- C57BL/6jBomtac mice (Taconic, Ejby, Denmark)

Chemicals

- LCIS: Live Cell Imaging Solution (Invitrogen)
- PBS: phosphate buffer saline (Gibco)
- 100x dcb: 100-times diluted bicarbonate buffer, pH 10, osmolarity 5 miliosmolar, mixed in-house
- F-12K: cell culture medium for LA-4 (Gibco)
- RPMI 1640: cell culture medium for MH-S (Gibco)
- Trypsin (Sigma)
- Penicillin-Streptomycin (Sigma)
- Non-essential amino acids (Gibco)
- Beta mercaptoethanol (Gibco)
- dH2O: deionised water
- glucose (Kemika)
- BSA: bovine serum albumin (Sigma)
- Hydrogen peroxide (Merck)
- Chlorpromazine (Alfa Aesar)
- MBCD: Metyl-Beta-Cyclodextran (Acros organics)
- Resveratrol (Sigma)
- KCl (Kemika)
- HCl (Merck)
- KOH (Carlo Erba)
- Limulus Amebocyte Lysate Assay (Lonza, Walkersville, MD, USA)
- 10% neutral buffered formalin (CellPath Ltd, UK)
- DMEM (Sigma-Aldrich, France, Saint-Quentin-Fallavier)
- heat-inactivated FBS (Sigma-Aldrich, France, Saint-Quentin-Fallavier)
- 4 mM L-glutamine (SIGMA-G7513)
- antibiotic/antimycotic composed of 100 U/mL of penicillin, 100 µg/mL of streptomycin (SIGMA-P0781) and 0.25 µg/mL of amphotericin B (SIGMA-A2942)
- LDH assay (Roche-4744934001, Germany)
- Triton (Sigma-Aldrich, France, Saint-Quentin-Fallavier)
- WST-1 assay (MV Berridge, AS Tan, KD McCoy, 1996) (Roche, 11644807001, USA)
- WST-1 kit (Roche, Germany)
- GeneChip® WT PLUS Reagent Kit (Thermo Fisher/Affymetrix)
- RNeasy Plus Mini Kit (Qiagen)
- WT PLUS Reagent Kit (Thermo Fisher Scientific Inc., Waltham, USA)

Materials

- #1.5H µ-Dish (Ibidi)
- #1.5H µ -Slide 8-well (Ibidi)
- Pelcotec™ SFG12 Finder Grid Substrate - Si wafers for HIM (Ted Pella)
- Aeroneb®Pro nebulizer (from VITROCELL® Cloud 6 system)
- Mouse Clariom S arrays (Thermo Fisher Scientific)

Nanomaterials used in this study

Synthesized in-house by P. Umek:
TiO_2 nanotubes (PU-nTOX-01-03) and TiO_2 nanocubes (PU-nTOX-01-21);

Kind gift from U. Vogel:
carbon black (Printex 90), TiO_2 MKNA015 (MKN- TiO_2 -A015), TiO_2 MKNA100 (MKN-TiO_2 -A100) and quartz silica (SiO_2 DQ12);

Kind gift from JRC Nanomaterial Repository:
NM-101 TiO2 anatase (TiO2-NM101-JRCNM01001a), NM-105 TiO2 rutil-anatase (TiO2-NM105-JRCNM01005a), NM-110 ZnO (ZnO-NM110-JRCNM62101a), and NM 111 ZnO (ZnO-NM111-JRCNM01101a), NM-200 SiO2 (SiO2-NM200-JRCNM02000a), NM-401 MWCNT (MWCNTs-NM401-JRCNM04001a), NM-402 MWCNT (MWCNTs-NM402-JRCNM04002a).

Software

- Imspector (version 16.2.8282-metadata-win64-BASE), provided by Abberior
- SPCImage 7.3 (Becker & Hickl)
- Fiji, ImageJ 1.52p (NIH)
- syGlass (http://www.syglass.io/, RRID:SCR_017961)
- Mathematica 12.0, licence L5063-5112 (Wolfram)
- genomics software: GSEA by Broad Institute
- modelling: GROMACS 2018.3 (calculation), VMD (visualisation)

Chemicals for imaging

Staining for *ex vivo* imaging:

- haematoxylin and eosin (H&E)

Fluorescent probes used for TiO_2 labelling:

- Alexa Fluor 647 NHS ester (Termo Fisher), $\lambda_{Ex/Em}$: 651/672 nm
- Star 520 SXP NHS ester (Abberior), $\lambda_{Ex/Em}$: 520/640
- ATTO 594 NHS ester (Atto-tec), $\lambda_{Ex/Em}$: 603/626 nm

Fluorescent probes used for cell labelling:

- CellMask Orange (Invitrogen)
- SiR Actin (Cytoskeleton)
- Priopidium Iodide, PI (Sigma)
- Hoechst 33342 (Sigma)
- pHrodo Red Transferrin conjugate (Invitrogen)
- Lysotracker Red (Invitrogen)
- MitoTracker™ Orange CMXRos (Invitrogen)
- Star Red-DPPE (Abberior)

Non-commercial fluorescent probes

Main message

Characterisation of used home-made fluorescent probes SHE-2N and SAG-38. SHE-2N labels plasma membrane, SAG-28 labels lipids droplets.
Supporting raw and analysed data:

Figure S1 - Figure S3

Characterisation

Spectra were recorded in toluene at 10^{-7} *M* concentration on Perkin Elmer LS-55 spectrofluorometer. For information regarding the probe synthesis contact the authors.

- 4-(8,9-Dimethyl-6,8-dinonyl-2-oxo-8,9-dihydro-2H-pyrano[3,2-g]quinolin-3-yl)-1-(3-(trimethylammonio)propyl)pyridin-1-ium dibromide (**SHE-2N**)

SHE-2N is an amphiphilc fluorescent probe designed for labelling of plasma membrane. Lipophilic tails and coumarin moiety are incorporated into lipid bilayer, while two positive charges at the headgroup reduce distribution to other membranes of the cell and thus provide stable labelling of plasma membrane. Additionally, labelling of plasma membrane is quick and with low photobleaching.

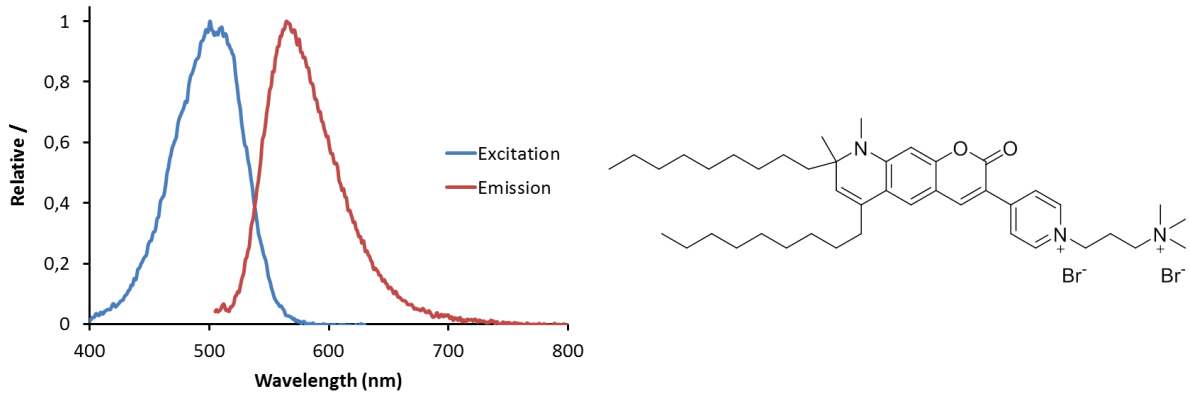


*Figure S1: Left: normalized fluorescence excitation (blue line) and emission (red line) spectra of **SHE-2N** recorded in toluene at 10^{-7} M concentration. Right: structure of membrane probe **SHE-2N**.*

- 3-(Benzo[d]thiazol-2-yl)-6,8,8,9-tetramethyl-2-oxo-8,9-dihydro-2H-pyrano[3,2-g]quinoline-4-carbonitrile (**SAG-38**)

SAG-38 is a lipophilic coumarin-based probe designed as an alternative to nile red for labelling of lipid droplets. Emission of **SAG-38** is red-shifted in comparison to emission of nile red and ideal for recording of emission within 580–625 nm.

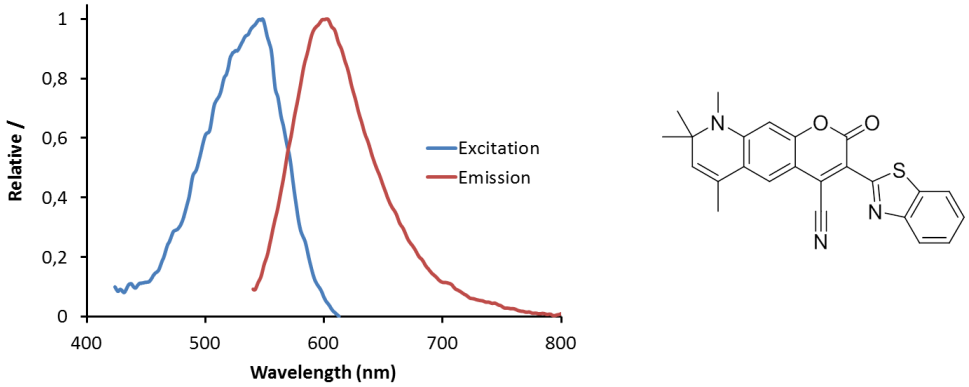


*Figure S2: Left: normalized fluorescence excitation (blue line) and emission (red line) spectra of **SAG-38** recorded in toluene at 10^{-7} M concentration. Right: structure of probe **SAG-38**.*

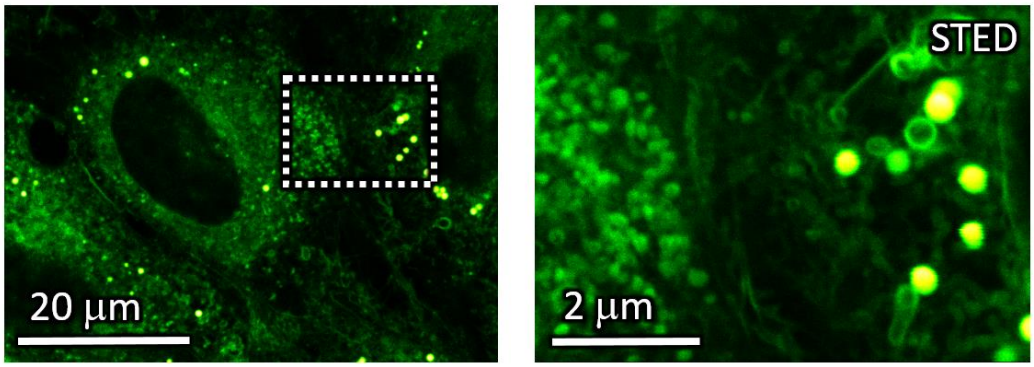


Figure S3: A confocal (left) and STED (right) image of a non-exposed LA-4 cell labelled with SAG-38 – the probe labels various vesicles, including lamellar bodies (bright, full vesicles).

- experiment 20190607/e03_s02_t05_SAG-38_STED.msr :
  - LA-4 cells were seeded @30% confluence in an Ibidi #1.5H µ-Slide 8-well
  - after 2 days, 10 µM probe slightly lipophilic probe SAG-38 was freshly diluted to 10 µM in LCIS, added to cells and incubated for 10 minutes, after which the image was taken
- analysis:
  - rescaling of green channel

Cell line	LA-4 (membranes and lipid droplets, SAG-38)	pixelsize (x,y)	50 nm	561nm	2%
NPs		FOV (x,y)	60 µm	640nm	
exposure		pixelsize (z)		STED	
imaging	xy confocal	FOV (z)		filter sets	605 nm – 625 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA1.2)

Cell line	LA-4 (membranes and lipid droplets, SAG-38)	pixelsize (x,y)	20 nm	561nm	20%
NPs		FOV (x,y)	15x11 µm	640nm	
exposure		pixelsize (z)		STED	25%
imaging	xy STED	FOV (z)		filter sets	605 nm – 625 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA1.2)

Cell culture

Murine epithelial lung tissue cell line (LA – 4; cat. no. ATCC CCL-196) and murine alveolar lung macrophage (MH-S; cat. No. CRL2019) cell line were purchased from and cultured according to American Type Culture Collection (ATCC) instructions. Cells were cultured in TPP cell culture flasks at 37 °C in a 5% CO_2 humidified atmosphere until monolayers were 80% confluent, at which time they were subcultured or seeded for experimentation. All experiments were performed with cells before the twentieth subculture. For long–term live cell experiments, a homemade incubator which maintains a humidified atmosphere with a 5% CO_2 and is heated to 37 °C.

Medium used for culturing of the epithelial LA-4 cells is Ham's F-12K (Kaihn's) medium, produced by Gibco, supplemented with 15% FCS (ATCC), 1% P/S (Sigma), and 1% NEAA (Gibco), 2 mM L-gln. For the specific composition of Ham's F-12K medium, look for details on the TermoFisher webpage under the catalogue number 21127022.

For Alveolar macrophages, MH-S, cell line we used RPMI 1640 (Gibco, ATCC modification) medium, supplemented with 10% FCS (ATCC), 1% P/S (Sigma), 2 mM L-gln, and 0.05 mM

beta mercapthoethanol (Gibco). For the specific composition of this medium, look for details on the TermoFisher webpage under the catalogue number A1049101.

Nanomaterial synthesis and labelling

The TiO_2 anatase nanotubes used in this paper were synthesized, functionalized with AEAPMS, and labelled with STED-compatible fluorescent probes via a covalent reaction between the AEAPMS and ester functional group on the probe. All this was done in-house [1]. Labelled TiO_2 was then stored suspended in 100x diluted bicarbonate buffer. For the multi-nanomaterial exposure experiments we used other nanomaterials as well. In this case, the nanomaterials were suspended in PBS and sonicated in ice bath using a tip sonicator (Sonicator 4000, Misonix, with 419 Microtip probe) for 15 min with 5s ON/ 5s OFF steps.

General *in vitro* sample preparation and exposure

Because nanomaterial sample preparation and administration method can influence experimental outcomes, especially cellular adsorption and uptake of nanoparticles[2], which is why we have used a sample preparation protocol which mimics physiological conditions in an alveolus as closely as possible and, most importantly, reproduces the phenomenon of nanoquarantining observed *in vivo* (Figure 2, comparing cauliflowers *in vivo* B and *in vitro* D&E&F). To this end, the usage of albumin in the nanomaterial dispersion protocol was omitted and nanoparticles were administered as a concentrated dose to cells in media and then mixed via pipetting in our *in vitro* model (mixed method as explained in Moore et al.[2]). To enable the inter-laboratory reproducibility of our work and to foster understanding of fundamental bio-nano interactions, we included general sample preparation in this section below and detailed sample preparation and experimental setup for each experiment separately at the appropriate sections of this Supplementary Information.

LA-4 and MH-S cells were seeded in Ibidi 1.5H dishes of various surface area, depending on the experiment. After 24 h, nanomaterial (c=1mg/mL) was added at an appropriate surface dose (SNP:Scells), according to the experiment needs (listed for each experiment in sections S2 – S6). Before exposure, nanomaterial suspension was sonicated for 10 s in an ultrasonic bath (Bransonic ultrasonic cleaner, Branson 2510EMT). Cells were then incubated at 37 °C and 5% CO2 atmosphere with the nanomaterial for the desired time in order to observe the cells at the post-exposure time points of interest. If the experiment required monoculture of either cell line, sample were prepared as described above, if however, we experimented with the co-cultures, sample preparation differed slightly. For co-cultures, we grew LA-4 and MH-S in separate dishes up to desired confluency (lower than for monocultures) and then mixed them together by adding MH-S in the LA-4 dish at a ratio of 1 : 40. Co-cultures were then incubated for 24 h more, exposed to nanomaterial as described above and incubated for additional desired amount of time. Growth medium for co-cultures was mixture of equal volumes of F12K and RPMI 1640. Cells were then labelled with fluorescent dyes according to the manufacturer's recommendations. Right before observing the live cells, unbound fluorescent label was washed and medium was exchanged for LCIS.
In some experiments we used different chemicals for modulation of the cell metabolism. For blocking the Clathrin-mediated endocytosis, cells were treated with 100 µm Chlorpromazine for 15 min. Membrane cholesterol was extracted with a 24 h incubation with 0.5 - 1 mM MBCD. FAS was inhibited with overnight 100 µM Resveratrol incubation. Finally, for actin stabilization, we used higher concentration (≥1mM) of Sir-Actin Label based on

Jasplankinolide. All the chemical modulators were added before exposure to nanomaterial and continued to be incubated with the cells even after during incubation with the nanomaterial for abovementioned time periods.

For the reuptake experiments different cell lines were grown separately, and washed with PBS before adding MH-S to LA-4.

HIM, SEM
Samples were prepared as usual but we grew them on Si-wafers. After reaching desired confluency samples were freeze-dried with metal mirror freezing technique.

Imaging *in vitro*

STED

Super-resolution and confocal fluorescence micrographs were acquired using custom build STED microscope from Abberior with an Olympus IX83 microscope and two avalanche photodiodes as detectors (APDs). The microscope is equipped with two 120 picosecond pulsed laser sources (Abberior) with excitation wavelengths 561 and 640 nm and maximal power of 50 µW in the sample plane. Pulse repetition frequency for experiments was 40 - 80 MHz, depending on the experiment. STED depletion laser wavelength is 775 nm with same repetition frequency as excitation lasers, pulse length of 1.2 ns and maximal power of 170 mW in the sample plane. Filter sets used for detection were either 605–625 nm (green channel) or 650–720 nm (red channel). Images were acquired using Imspector (version 16.2.8282-metadata-win64-BASE) software also provided by Abberior. All microscope settings were tuned separately for maximal resolution during each of the experiments and are listed with alongside the recorded images in Supplement.

FLIM

Fluorescence lifetime images (FLIM) were obtained on the same custom-built STED microscope (Abberior instruments) as confocal and STED fluorescence images in this study. This time, the emitted fluorescence was detected using PMT detectors and TCSPC technology developed by Becker & Hickl. 16-channel GaASP PMT detectors attached to a spectrograph with diffraction grating 600 l/mm were used to measure fluorescence lifetime of emitted photons with wavelengths ranging from 560 to 760 nm. Spectral information was discarded and the lifetimes were gathered in Imspector 16.2 (Abberior Instruments).

The fluorescence lifetime data was analysed with SPCImage 7.3 software (Becker & Hickl), where the Decay matrix was calculated from the brightest pixel in the image (monoexponential fitting), binning was set to 3 and threshold to 5. The rainbow LUT was rescaled to range from 500 ps to 1000 ps for all images and both intensity and contrast of the lifetime-coded image were adjusted for easier comparison of lifetimes between samples.

Imaging of nanomaterial in backscatter mode

In Figure 1c, simultaneously with measuring fluorescence from CellMask Orange in the cell membrane (as described in STED section), backscattered light was detected as well to locate the nanomaterial in the sample. A tuneable Chameleon Discovery laser (Coherent) with 100 fs long pulses, pulse repetition frequency 80 MHz, and maximal average power of 1.7 W at 850 nm was used as the scattering light. The pre-attenuated laser light with a wavelength of 750 nm first passed through a 785 nm built-in dichroic where a fraction of the power was directed onto

the sample through the same 60x WI objective (NA 1.2) as the excitation light for fluorescence imaging. The light scattered off the nanomaterial and passed back through the same objective and dichroic, now mostly passing through the dichroic towards the detectors. After passing through a pinhole (0.63 A.U.), the backscattered light was spectrally separated from the fluorescence by a short-pass 725 nm dichroic, afterwards being detected on the same PMT, as described in the FLIM section, this time set to collect light with wavelengths above 725nm.
Due to the large coherence of the laser, the backscattered light exhibited a strong speckle pattern, which was diminished by a 100-nm-wide Gaussian blur on the scattering image, thus decreasing false negative colocalisation of nanomaterial on account of spatial resolution.

SEM

SEM imaging has been performed on MIRA3 Flexible FE-SEM produced by TESCAN, by detection of secondary electrons. Beam powers used have been between 5.0 kV and 15 kV with variable field of view 1.8 µm to 180 µm. All samples have been measured under high pressure vacuum (HiVac). All analysis has been performed in Tescan developed software.

HIM

Super-resolution imaging on the nanoscale was carried out using Helium Ion Microscope (Orion NanoFab, Zeiss) available at IBC at the Helmholtz-Zentrum Dresden - Rossendorf e. V., a member of the Helmholtz Association. Microscope equipped with GFIS injection system and additional in-situ backscatter spectrometry and secondary ion mass spectrometry can achieve 0.5 nm lateral resolution imaging using 10-35 keV He ion beams. Measurements of secondary electrons (Se) emitted from the first few nm of the sample were done by He ion acceleration of 30 keV, current of 1.7 pA and were acquired under high vacuum inside the sample chamber (3x10-7 mBar). Field-of-view was varied from 60 µm x 60 µm down to 1 µm x 1 µm, with pixel steps small as 2nm. Imaging was performed on non-tilted and tilted sample stage (45 degrees) for better 3-D visualization.

FMS

Fluorescence micrographs of cell viability have been acquired on inverted fluorescence Nikon Eclipse TE 2000-E microscope with Xe-Hg source (Sutter Lambda LS, Novato, CA). Filter sets which have have been used were manufactured by BrightLine from Semrock, Rochester, NY. Images were taken with EMCCD camera (iXon3 897 from Andor, Belfast, UK).

TEM

See S0b – Nanomaterial .

Transcriptomics *in vitro*

Cells were grown in 6-well plates and exposed to TiO_2 nanotubes for 4 h and 48 h, control samples were taken at 0 h and 48 h. Samples were prepared as described above. Briefly, growth medium was removed and the 6-well plates containing cells only were frozen at -70°C. Total RNA was isolated employing the RNeasy Plus Mini Kit (Qiagen). The Agilent 2100 Bioanalyzer was used to assess RNA quality and RNA with RIN>7 was used for microarray analysis.
Total RNA (120 ng) was amplified using the WT PLUS Reagent Kit (Thermo Fisher Scientific Inc., Waltham, USA). Amplified cDNA was hybridized on Mouse Clariom S arrays (Thermo Fisher Scientific). Staining and scanning (GeneChip Scanner 3000 7G) was done according to manufacturer`s instructions.

Statistical analysis for all probe sets included limma t-test and Benjamini-Hochberg multiple testing correction. Raw p-values of the limma t-test were used to define sets of regulated genes ($p<0.01$). Detection Above Background (dabg) p-values were used to exclude background signals: significant genes were filtered for $p<0.05$ in more than half of the samples in at least one group. Array data has been submitted to the GEO database at NCBI (GSE146036).
In the arrow graphs, only genes which were up- or down-regulated more than two times compared to non-exposed cells are shown. The signal (x axis) is drawn in logarithmic scale. Expression is normalized to expression of control samples.

Transcriptomics *in vivo*

Microarray mRNA analysis was performed using Agilent 8 × 60 K oligonucleotide microarrays (Agilent Technologies Inc., Mississauga, ON, Canada) as described previously [3] with six replicas for each condition. Bioinformatics analysis of the row data: signal intensities were Loess normalized using the limma package in R/Bioconductor [4]. Analysis of differentially expressed genes (DEGs) was performed using the limma package. The genes were considered as significantly differentially expressed if the BH-adjusted p-values were less than or equal to 0.1. Statistical analysis is same as for the *in vitro* transcriptomics above.

Comparison of transcriptomics *in vitro* and *in vivo*

Mice were exposed to 18, 54 or 162 µg of TiO_2 nanotubes per mouse and lungs were harvested on 1st and 28th day post exposure for transcriptomic analysis to evaluate overlapping sets of genes differentially expressed in the *in vivo* and *in vitro* experimental data. The goal of the analysis is to determine and compare alterations in lipid metabolism, immune response in terms of proinflammatory signalling and cholesterol metabolism between two experimental systems. For the assessment of the monocyte influx, all genes encoding monocyte chemoattractive (C-C motif) chemokines were selected and their expression evaluated.

In vivo experiments

See S2b – *In vivo* data.

Modelling

See S3e – *In silico* data – atomistic molecular dynamics simulation, S6a – Model of chronic inflammation following nanomaterial exposure and determination of its parameters and S6b – Phase space of chronic inflammation

S0b – Nanomaterial characterization

Nanomaterials used in this study

Main message

Characteristics of nanomaterials used in this study presented in a table with additional information on chronic lung inflammation, either measured or adapted from the literature. Additional calculations about doses at which chronic inflammation occurs is included in the table.

Table S1

Nanomaterial name (chemistry and morphology)	ID	Dimensions (width x length in nm)	BET surface (m^2/g)	Chronic lung inflammation (<5% BAL PMN, day28)	Ref.	BET (cm2/ug)	dose (cm2/g-lung) by Instillation/mouse: 2ug	dose (cm2/g-lung) by Instillation/mouse: 18ug	dose (cm2/g-lung) by Instillation/mouse: 54ug	dose (cm2/g-lung) by Instillation/mouse: 162ug	NOAEL (>5%PMN) cm^2/g-lung	LOAEL (>5%PMN) cm^2/g-lung	surface area dose / surface area lung for 2ug	surface area dose / surface area lung for 18ug	surface area dose / surface area lung for 54ug	surface area dose / surface area lung for 162ug	NOAEL (>5%PMN) SA/SA-lung	LOAEL (>5%PMN) SA/SA-lung	NOAEL (>5%PMN) SA/SA-lung	LOAEL (>5%PMN) SA/SA-lung	ID
TiO2 (anatase) nanotubes	JSI: TiO2-NTs	10 x 100	152	YES	Danielsen et al., Toxicol Appl Pharmacol. 2020	1,52		152	456	1385	<152	152		0,05	0,16	0,49	<0.05	0,05		1:20	tubes
TiO2 (anatase) nanocubes	JSI: TiO2-NCs	15 x 20	97	NO	Danielsen et al., Toxicol Appl Pharmacol. 2020	0,97		97	291	883	883	n.d.		0,03	0,10	0,31	0,31	>0.31	1:3	>1:3	cubes
TiO2 (anatase) nanospheres small	MN-1_TiO2-A015	16-28[2]	74	NO	Danielsen et al., Toxicol Appl Pharmacol. 2020	0,74		74	222	674	674	n.d.		0,03	0,08	0,24	0,24	>0.24	1:4	>1:4	MN-1_TiO2
TiO2 (anatase) nanospheres large	MN-2_TiO2-A100	12-50[2]	85	NO	Danielsen et al., Toxicol Appl Pharmacol. 2020	0,85		85	255	774	774	n.d.		0,03	0,09	0,28	0,28	>0.28	1:4	>1:4	MN-2_TiO2
TiO2 rutile-anatase	NM-105	20	46	NO	Rahman et al., Mutagenesis. 2017	0,46		46	138	419	138	419		0,02	0,05	0,15	0,05	0,15	1:20	>1:7	NM-105
TiO2 anatase	NM-101	5	230	NO	Rahman et al., Mutagenesis. 2017	2,30		230	690	2096	2096	n.d.		0,08	0,25	0,75	0,75	>0.75	1:1.3	>1:1.3	NM-101
SiO2 crystalline	DQ12	960	10	YES	Danielsen et al., Toxicol Appl Pharmacol. 2020	0,10		10	30	92	30	92		0,00	0,01	0,03	0,01	0,03	1:100	1:33	DQ12
SiO2 amorphous	NM-200	20	189	n.d.	n.d.	1,89		189	567	1722				0,07	0,20	0,61					NM-200
ZnO uncoated	NM-110	158	12	NO	Hardup et al., Nanotoxicology, 2019	0,12	1,3	exp. discontinued because of systemic effects			1,3	>1.3	0,0005	exp. discontinued because of systemic effects			0,005	n.d.	1:200	n.d.	NM-110
ZnO coated	NM-111	152	15	NO	Hardup et al., Nanotoxicology, 2019	0,15	1,7	exp. discontinued because of systemic effects			1,7	>1.7	0,0006	exp. discontinued because of systemic effects			0,006	n.d.	1:167	n.d.	NM-111
Multiwall carbon nanotubes - thin	NM-402	11 x 1400	226	YES	unpublished data SNT consortium	2,26		226	678	2059	<226	226		0,08	0,24	0,73	<0.08	0,08		1:13	NM-402
Multiwall carbon nanotubes - thick	NM-401	67 x 4000	18	YES	Poulsen, Toxicol. Appl. Pharmacol., 2015	0,18		18	54	164	<18	18		0,01	0,02	0,06	<0.02	0,01		1:100	NM-401
Carbon black		14	300	NO	Bourdon, Part. Fibre Toxicol., 2012	3,00		300	900	2733	900	2733		0,11	0,32	0,97	0,32	0,97	1:3	1:1	Ptx90
				[1] see LOAEL for SA/SA-lung: <1:10							highest dose with no observed effect	lowest dose with observed effect		500cm2 SA for mouse lung							

surface area dose calculations

lung	weight (g)	surface area (m2)	surface area (cm2)
man	1300	140	1400000
rat	1,3		
mouse	**0,18**	**0,05**	**500**

Table S1 Extended version of the nanomaterial characteristics table presented in the main paper. Additionally, surface of nanomaterial to surface of lung doses at NOAEL (highest dose with no observed effect) and LOAEL (lowest dose with observed effect) have been calculated (third and second to last column in the table). The mouse lung surface used in the calculations is given bellow the tables on the left-hand side.

Zeta potential

Main message

Surface charge characterisation by measurement of ζ-potential on all our nanomaterials used for exposure in experiments presented in this paper.

Supporting raw and analysed data:

Figure S4

Materials and methods

All ζ-potential measurements were carried out on the NanoBrook ZetaPALS Potential Analyzer (Brookhaven) with Biolab ζ-potential electrode for non-organic solvents (AQ-1203, Brookhaven) (Holtsville, NY). Samples were prepared and measured as follows:

Samples for measurements were prepared as 10x dilutions of TiO2 nanotubes (1 mg/mL) in dH2O with 10 mM KCl (Kemika). Desired initial pH values were adjusted with HCl (Merck) or KOH (Carlo Erba) and measured with Seven Multi, Mettler Toledo pH meter equipped with 1 mm thick pH electrode (Inlab ExpertPro, Mettler Toledo). ζ-potential has been measured across the range of 8 or more pH values. Analyser was used according to the manufacturers recommendations under the following parameters:

- Size: 10-100 nm
- Concentration: 0.1 mg/mL
- Cycles: 10
- Runs: 10
- Mode of measurement: Smoluchowski
- Temperature: 20°C - 23°C
- Media: Aqueous

Samples for ζ-potential measurement are prepared from batches synthesised under different conditions (look under S0a – General materials and methods, Nanomaterial synthesis and labelling):

- TiO_2-17-non-labeled
- TiO_2-17-Alexa 647
- TiO_2-17-Star 520S
- TiO_2-40-non-labeled
- TiO_2-40-Alexa 647
- TiO_2-03-non-labeled

Results

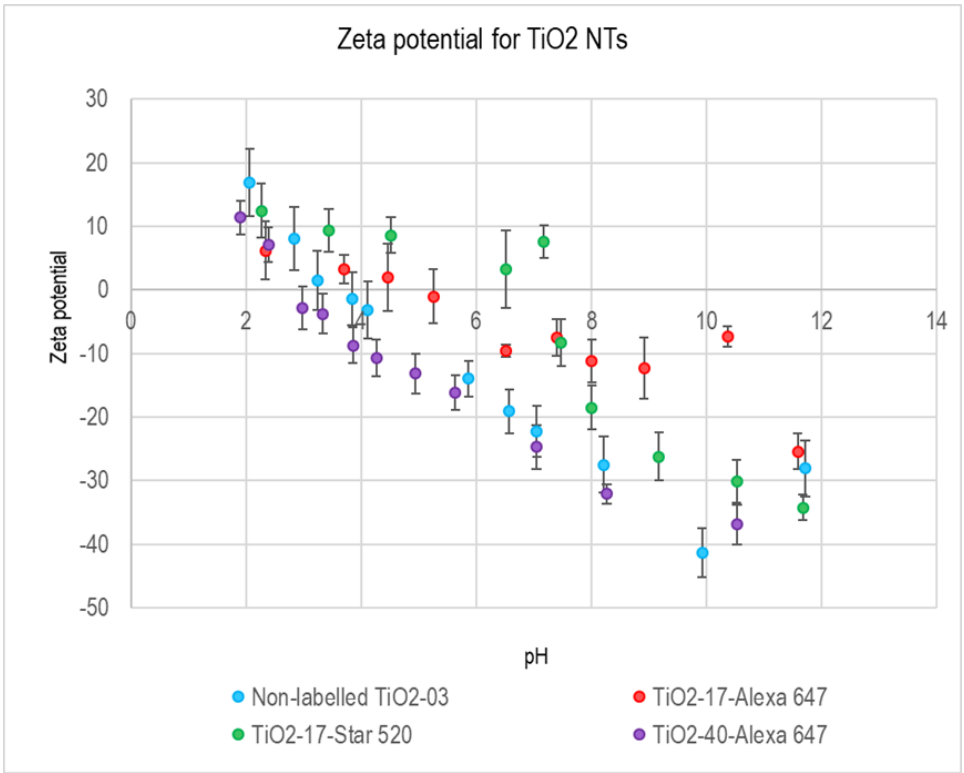


Figure S4 Measured ζ-potential of AEAPMS functionalized TiO_2 nanotubes from the batch 40. Error bars represent the standard error.

DLS, TEM of TiO_2 nanotubes

Detailed nanomaterial characterisation of TiO_2 nanotubes can be found in the recently submitted paper "Effects of physicochemical properties of TiO_2 nanomaterials for pulmonary inflammation, acute phase response and alveolar proteinosis in intratracheally exposed mice [5].The length of TiO_2 nanotubes was found to be between 40-500 nm and diameter 6-11 nm, based on TEM images. The DLS showed a bimodal distribution with a minor peak at 21 nm and a major peak at 60 nm.

TEM of TiO_2 nanotubes and other nanomaterials, used in this study

Main message

Nanomaterial structure is determined with transmission electron microscope with nm spatial resolution.

Materials and methods

Samples for TEM images of nanomaterials NM101, NM105, NM200, NM402 were prepared using the following protocol.
Of each material 1 mg was dispersed in 1 mL MilliQ water, except CNTs in 1 mL tannic acid solution 300 mg/L, using a vial tweeter for 15 min. Each suspension was diluted 1/10 and 3 µL drop deposited on Formvar Carbon coated 200 mesh copper grids (Agar Scientific, USA) and dehydrated overnight in a desiccator before analysis. Images were collected by JEOL JEM-2100 HR-transmission electron microscope at 120kV (JEOL, Italy).

Samples for TEM images of nanomaterials DQ-12, TiO_2 A015, TiO_2 A100, TiO_2 nanotubes, TiO_2 nanocubes were prepared using the following protocol.
The nanoparticles were dispersed in water and the dispersion sonicated in water bath for ~3h before use.
Of each sample 5 µL was deposed onto glow-discharged copper grid (Agar scientific Ltd, UK) for one minute and the excess of sample was removed blotting with filter paper. After shortly washing with one drop of water, the grid was therefore immersed into a 2% uranyl acetate (UA) solution for 20 s and blotted again with filter paper. The grids were imaged using a JEOL JEM-2100F fitted with a Gatan Orius SC 1000 camera (2x4k).

TEM images of carbon black nanoparticles (Printex90) were reprinted with permission from reference [6].

Images and statistics

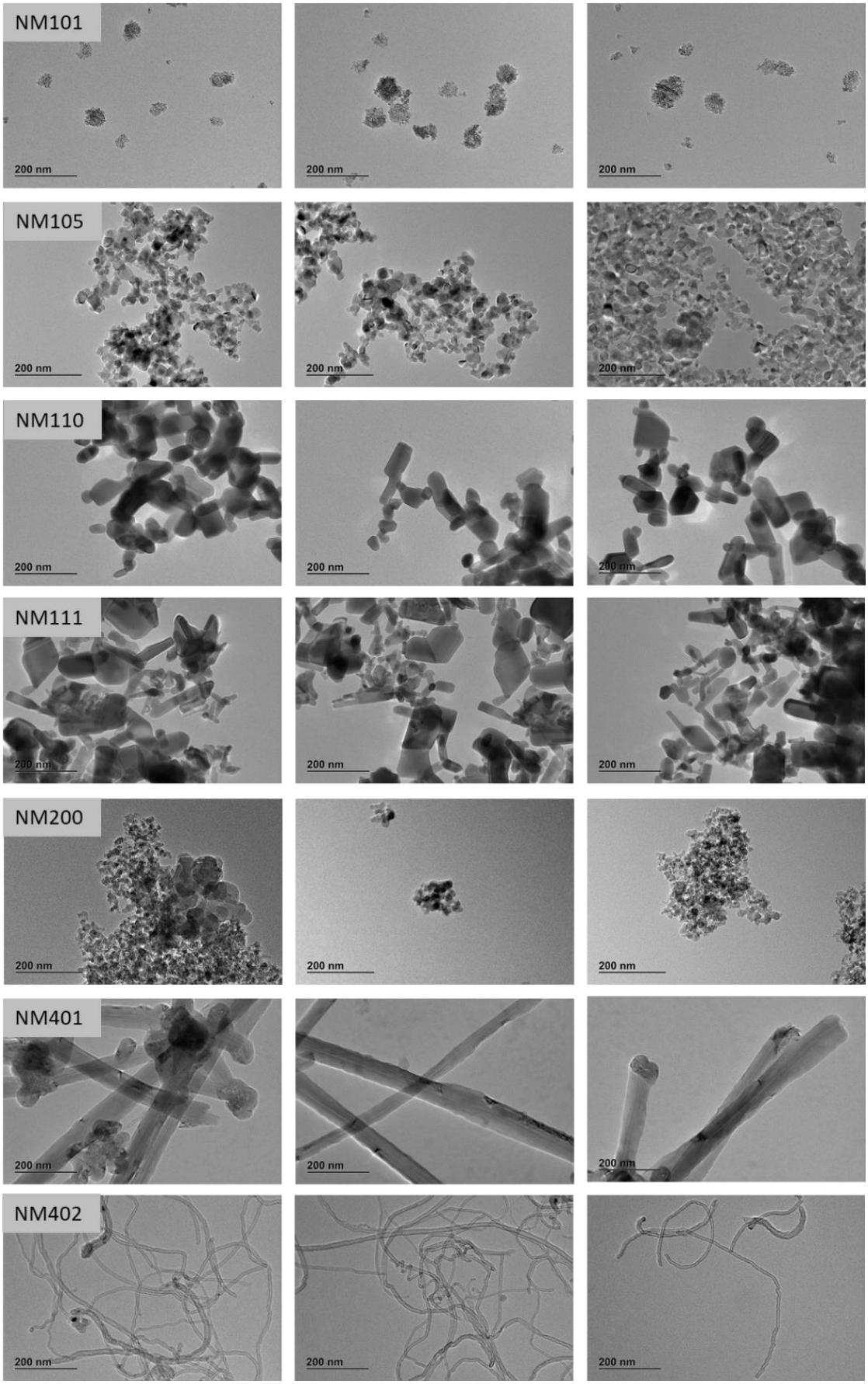


Figure S5: TEM images of NM101, NM105, NM110, NM11, NM200, NM401 and NM402.

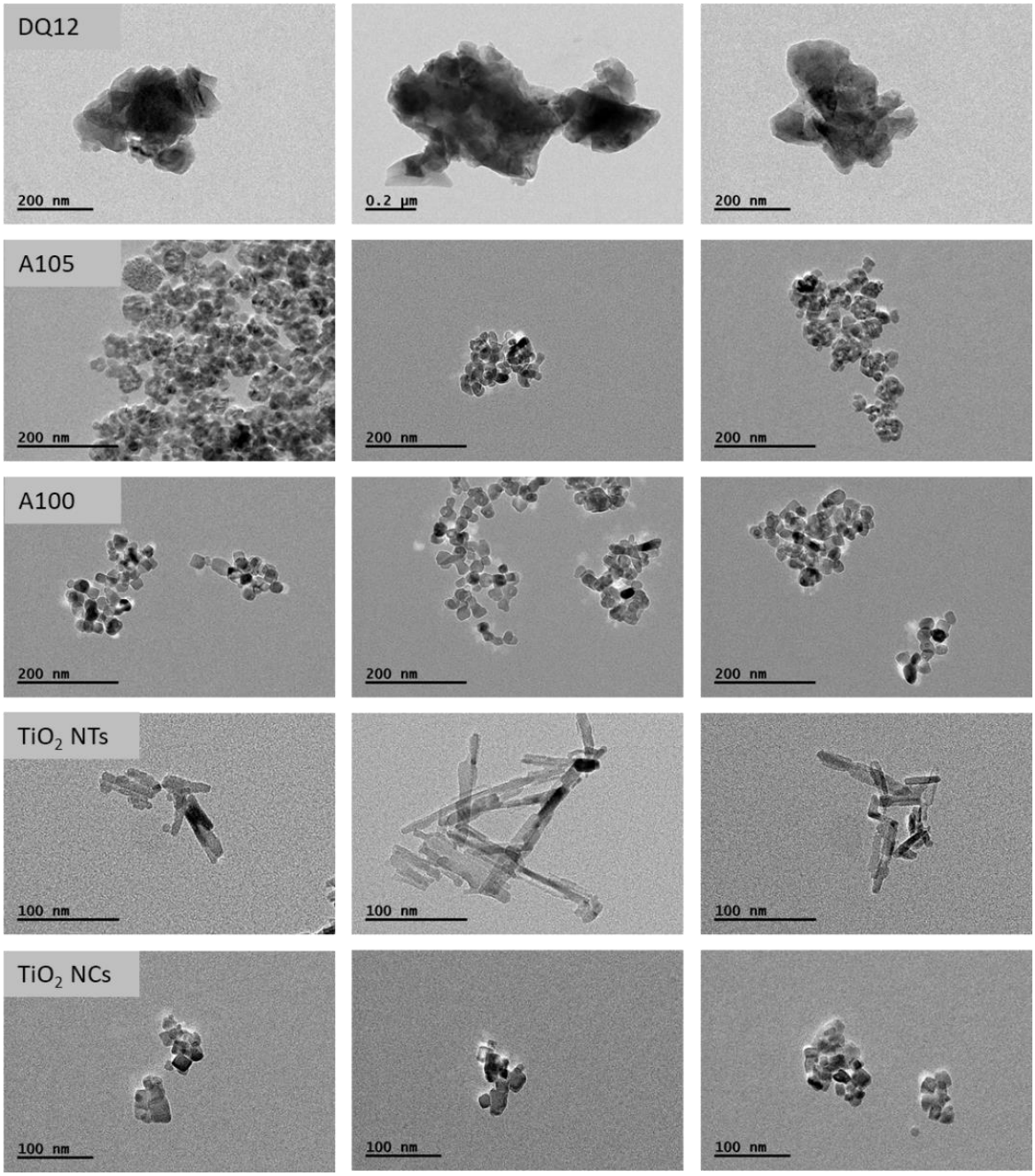


Figure S6: TEM images of DQ12, A105, A100, TiO_2 nanotubes and TiO_2 NCs.

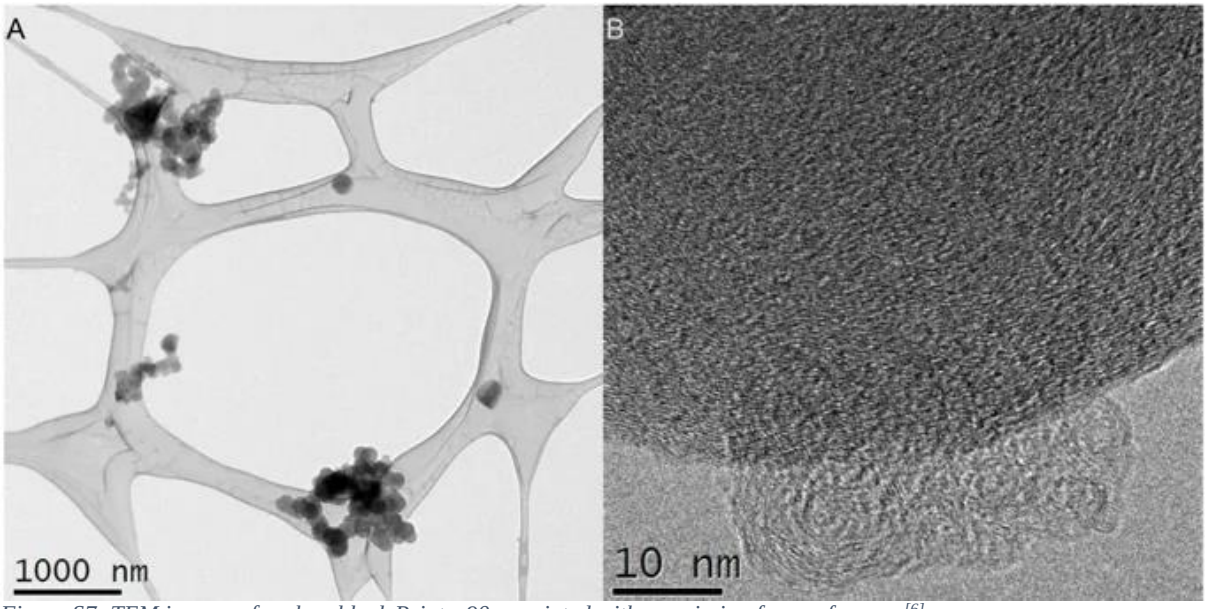


Figure S7: TEM images of carbon black Printex90, reprinted with permission from reference [6].

--- Complementary experiments, not included in figures in the main text ---

S0c – Interaction between TiO_2 nanotubes and lipid model membranes

Main message

Non-specific interaction between TiO_2 nanotubes and liposomes as lipid model membrane.

Short description

The interaction between TiO_2 nanotubes and lipid membranes has been evaluated employing liposomes as lipid model system. The TEM characterisation shown a clear interaction between the nanotubes and liposomes.

Materials and methods

All materials were purchase from Sigma-Aldrich unless stated otherwise. DOPC lipids were purchased from Avanti Polar Lipids Inc. (USA) in frozen powder form. They are manufactured to 99.9% purity and dissolved in chloroform (CHCl3) for easy stock dilution and use. Extrusion kit including polycarbonate filters were also purchased from Avanti Polar Lipids Inc. (USA).
Liposomes were fabricated using Freeze-Thawing cycles and extrusion. A lipid film (~ 5 mg) has been deposited into a glass vial and dried in N_2 stream and placed in a vacuum chamber overnight for complete CHCl3 removal. The film was hydrated the following day with DI water to a final concentration of 2 mg mL^{-1}. The lipid dispersion underwent freezing-thawing cycles for 5 times and then extruded 21 times using a 200 nm polycarbonate filter.
The TiO_2 nanotubes were dispersed in water and the dispersion sonicated in water bath for ~3h before use.
5 µL of sample was deposed onto glow-discharged copper grid (Agar scientific Ltd, UK) for one minute and the excess of sample was removed blotting with filter paper. After shortly washing with one drop of water, the grid was therefore immersed into a 2% uranyl acetate (UA) solution for 20 s and blotted again with filter paper. The grids were imaged using a JEOL JEM-2100F fitted with a Gatan Orius SC 1000 camera (2x4k).

Results

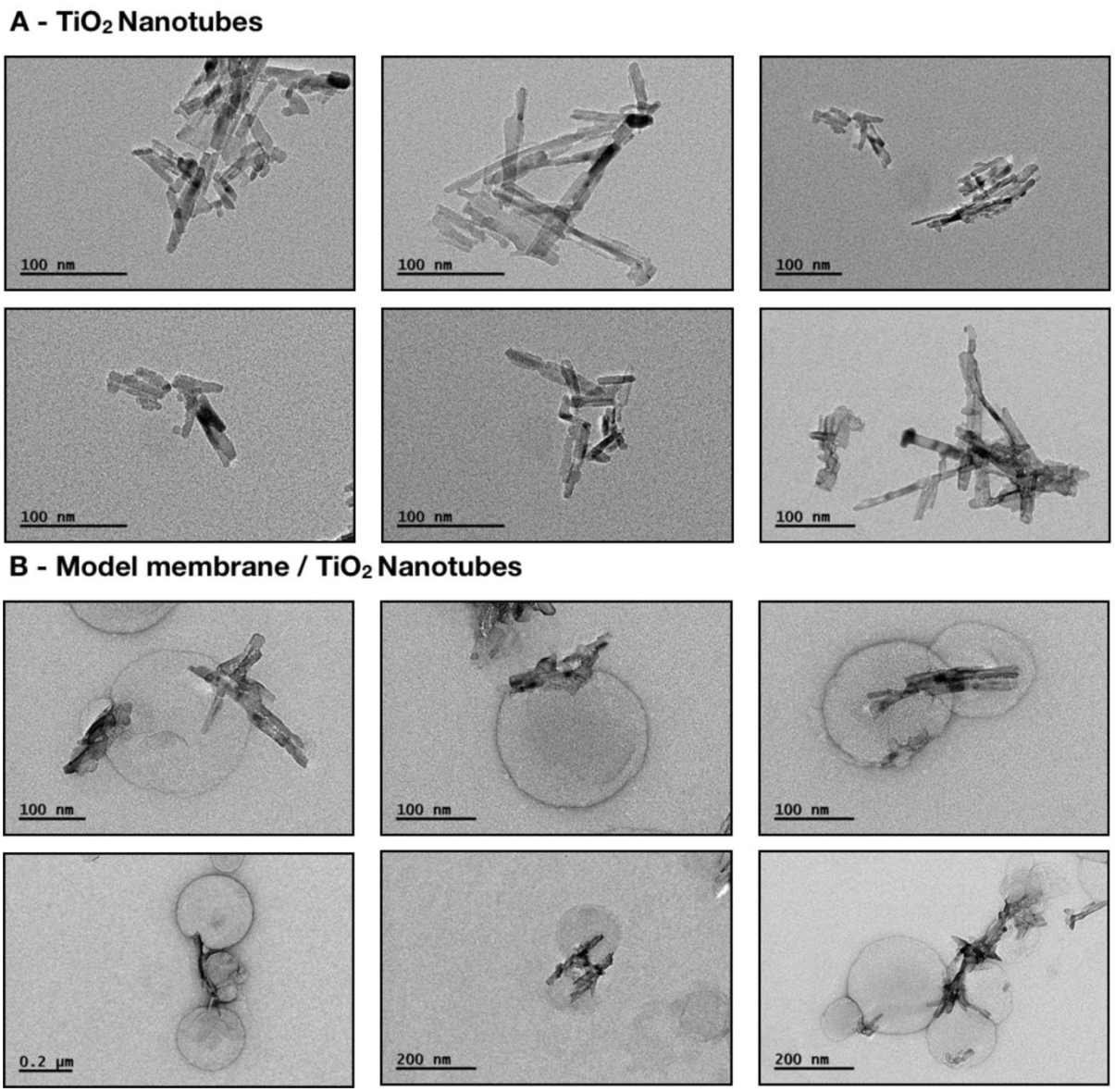


Figure S8: TEM micrographs of (A) TiO_2 nanotubes and (B) lipid model membranes interacting with TiO_2 nanotubes.

S0d – Uptake of nanomaterial

Main message

After exposure, the TiO_2 nanotubes are taken up into the LA-4 cells and have been colocalised with endosomes, lysosomes and other vesicles.

Supporting raw and analysed data:

main images:

Figure S9-Figure S10

controls and statistics:

Figure S11-Figure S14

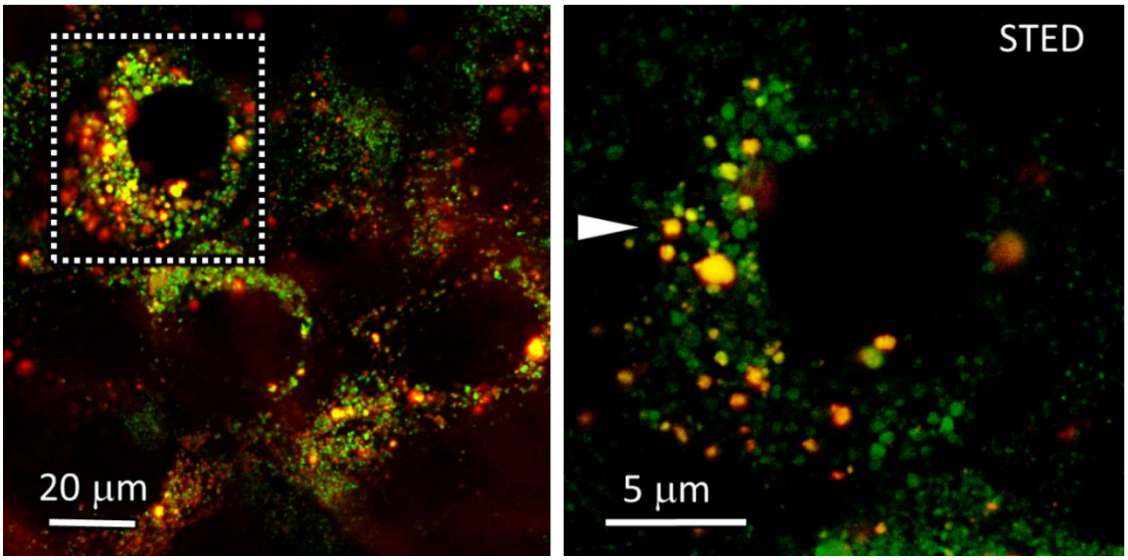


Figure S9: Confocal (left) and STED (right) colocalisation of TiO_2 nanotubes (Alexa Fluor 647, red) and endosomes (green, pHrodo Red Transferrin conjugate) after 30 minutes incubation

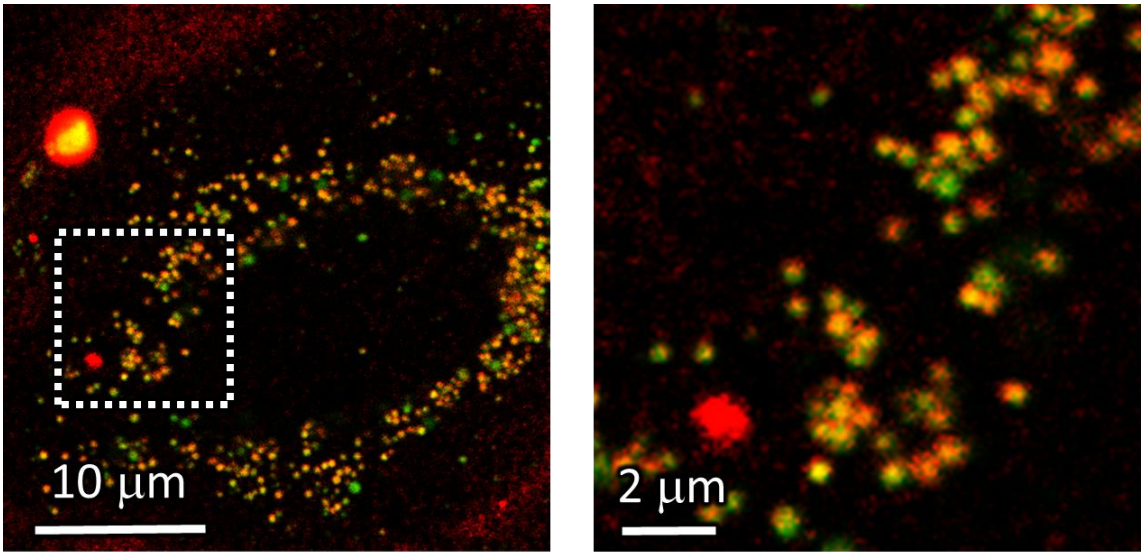


Figure S10: Colocalisation of TiO_2 nanotubes (Alexa Fluor 647, red) and lysosomes (Lysotracker Red, green) after 70 minutes of incubation.

Co-localisation of endosomes and TiO_2

controls and statistics for endosome colocalisation:
Figure S11-Figure S13

Materials and methods

- Experiment:
  - LA-4 cells were seeded @30% confluence in an Ibidi 1.5H µ-Dish.
  - After 48 hours LA-4 cells were washed with warm F12-K medium and placed on ice for 10 minutes.
  - Next, cells were washed 3 times with cold Live Cell Imaging Solution (LCIS) containing 20 mM glucose and 1% BSA.
  - 400 µL pHrodo Red Transferrin conjugate at 50 µg/mL in LCIS containing 20 mM glucose and 1% BSA was added.
  - After 30 minutes incubation at 37 °C, 35 µL freshly filtered 1 mg/mL TiO_2-Alexa647 in 100x dcb was added directly to the cells and mixed to achieve a 10:1 surface dose

- Analysis:
  - Confocal: logarithmic scale in red channel, cut-off at 5 counts; set maximum to 100 counts on green channel
  - STED: logarithmic scale in both channels, in red channel cut-off at 2 counts, set maximum to 50 counts; on green channel cut-off at 2 counts, set maximum to 75 counts;

Cell line	LA-4 (endosomes, pHrodo Red Transferrin Conjugate)
NPs	TiO2 (Alexa647)
exposure	10:1, 0min-60min
imaging	0min-30min xyz confocal, 30min

pixelsize (x,y)	100 nm
FOV (x,y)	80 µm

561nm	30%
640nm	30%
STED	-
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	wi 60x (NA0.3)

pixelsize (x,y)	30 nm
FOV (x,y)	37 µm
pixelsize (z)	
FOV (z)	
imaging time	
number of frames	

561nm	40%
640nm	40%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	wi 60x (NA1.1)

Experiment names

- Main experiment name:
  - 2018_12_06/e01_s02_t03_POSTIVE_CONTROL_LA-4_50ug_ml_pHrodo_30min_NP-Alexa647_1to10.msr
- Supplement names:
  - 2018_12_06/e01_s02_t03_POSTIVE_CONTROL_LA-4_50ug_ml_pHrodo_30min_NP-Alexa647_1to10.msr

    - 2018_12_06/e01_s02_t03_POSITIVE_CONTROL_LA-4_50ug_ml_pHrodo_30min_washed_NP-Alexa647
    - 2018_12_06/e01_s03_t04_POSITIVE_CONTROL_LA-4_50ug_ml_pHrodo_30min_washed_NP-Alexa647
    - 2018_12_06/e01_s05_t05_POSITIVE_CONTROL_LA-4_50ug_ml_pHrodo_30min_washed_NP-Alexa647
    - 2018_12_06/e04_s01_t01_POSTIVE_CONTROL_LA-4_50ug_ml_pHrodo_30min_washed_NP-Alexa647_1to10
- *Control:*
    - 2019_11_22/e03_s02_t03_LA-4_pHrodo_unwashed_25min

Controls and statistics

Colocalisation of nanomaterial with endosomes after 30 minutes incubation
Figure S11-Figure S12

Control for crosstalk of the pHrodo probe into the nanomaterial channel
Figure S13

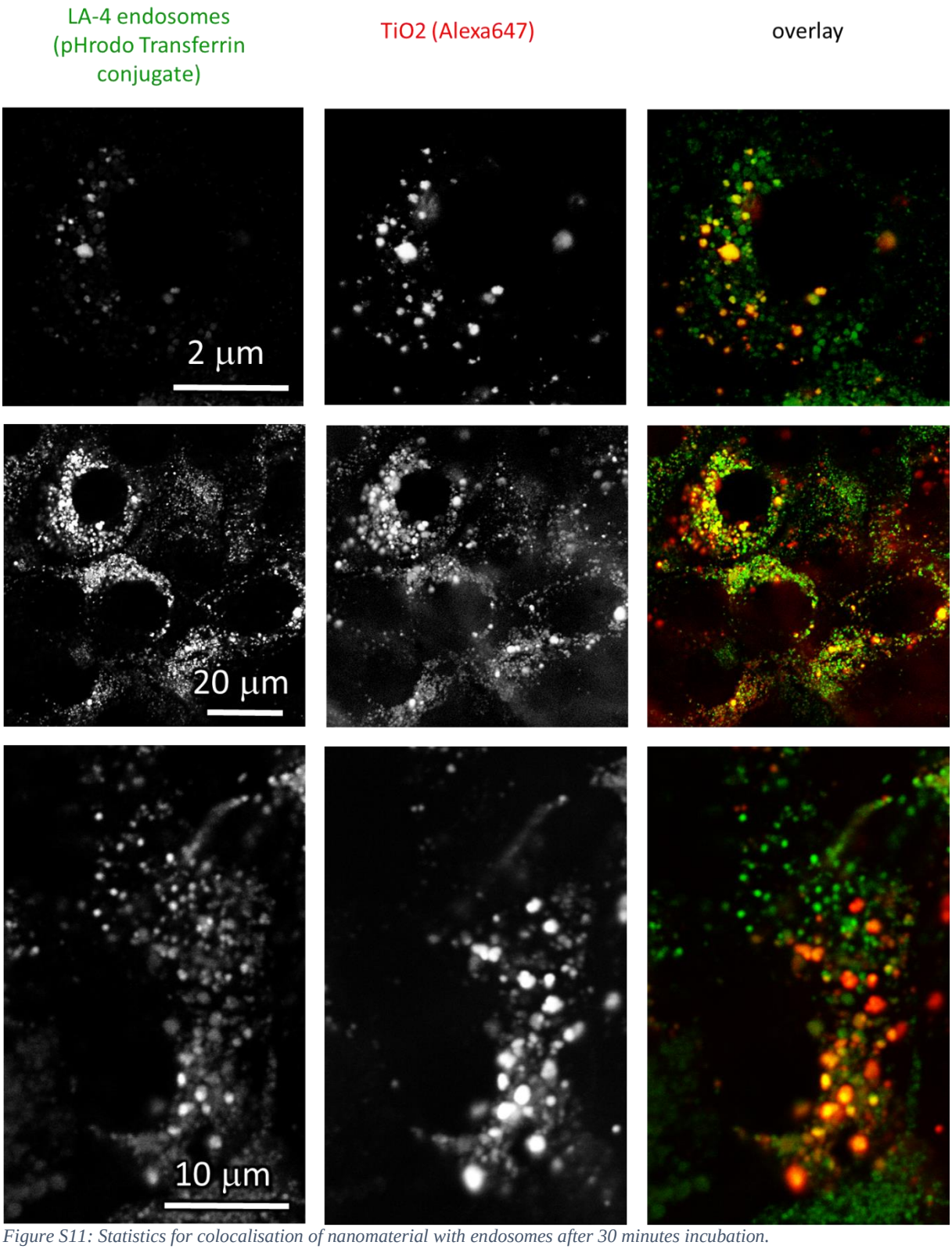


Figure S11: Statistics for colocalisation of nanomaterial with endosomes after 30 minutes incubation.

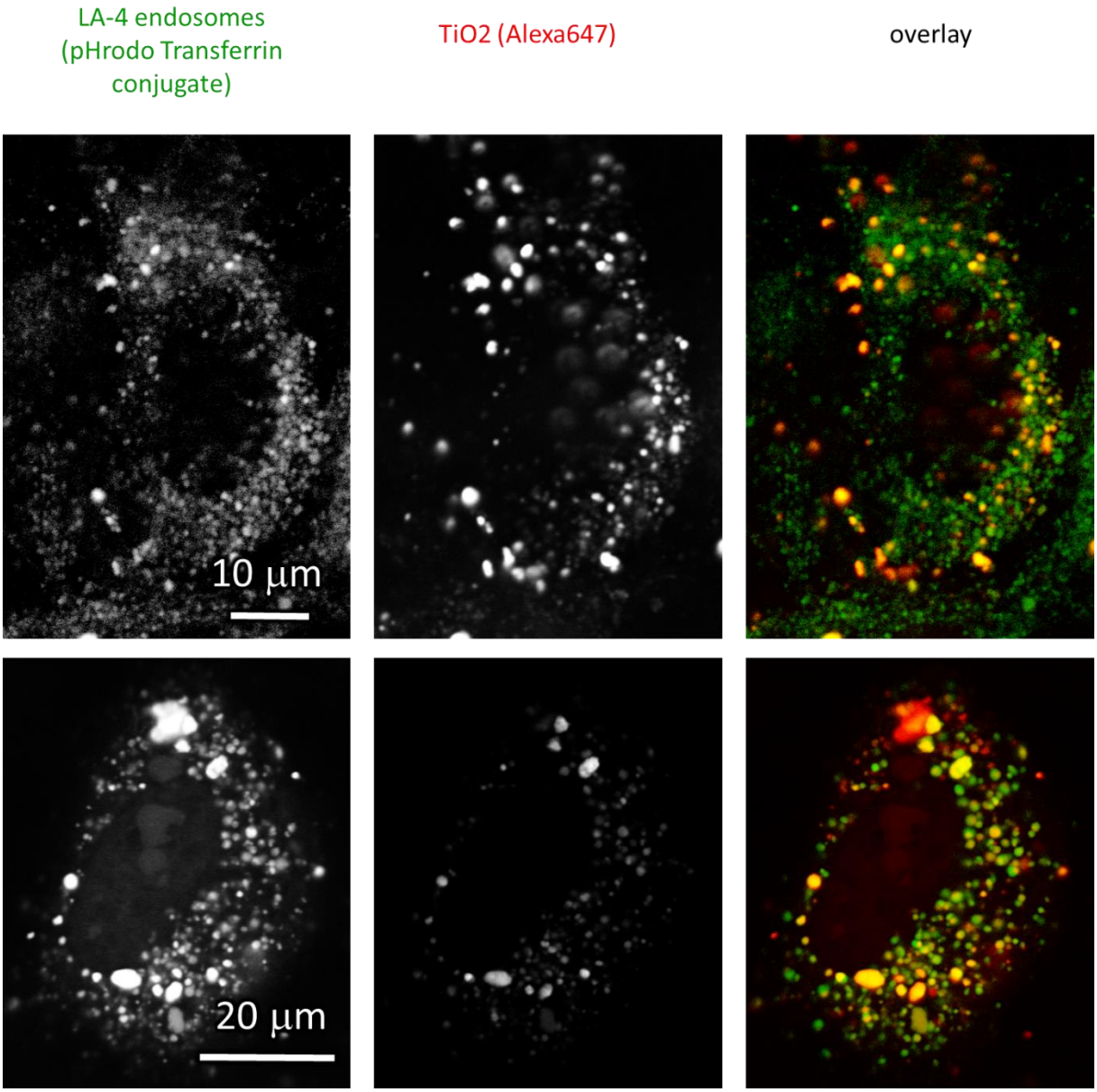


Figure S12: Statistics for colocalisation of nanomaterial with endosomes after 30 minutes incubation.

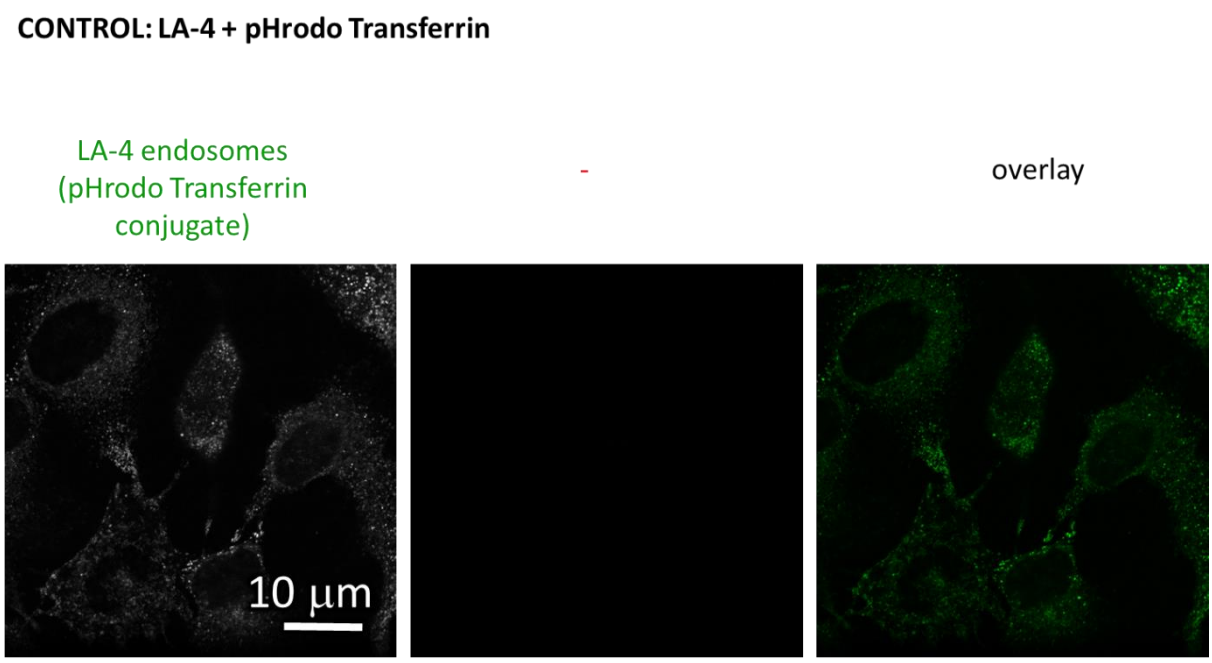


Figure S13: Control for possible false colocalisation of nanomaterial with endosomes after 30 minutes incubation due to crosstalk of the pHrodo probe into the nanomaterial channel.

Colocalisation of lysosomes and TiO_2 nanotubes

Lysosome colocalisation:

Figure S14

Materials and methods

- experiment 20181115/e03_s01_t04_LA-4 lysotracker TiO2Alexa647.msr:
  - LA-4 cells were seeded @30% confluence in an Ibidi #1.5H µ-Slide 8-well
  - after 3 days the cells were incubated with 150 µL 50nM Lysotracker in LCIS for 2h 20minutes. Afterwards, the medium was exchanged with 200 µL LCIS
  - 1 µL freshly filtered 1mg/mL TiO_2-40-Alexa647 in 100x dcb was added to cells to achieve a 1:1 surface dose of nanomaterial. The cells were continuously filmed on a heated insert at 32C for 1h 30 minutes
- analysis:
  - rescaling of green channel (max G = 200 counts)
  - rescaling of red channel (max R = 12 counts)

Cell line	LA-4 (lysosomes, Lysotracker)
NPs	TiO2 (Alexa647)
exposure	1:1, 0h-1h
imaging	xyt confocal, 0h-1h

pixelsize (x,y)	100 nm
FOV (x,y)	45 µm
pixelsize (z)	
FOV (z)	
imaging time	1h
number of frames	932

561nm	20%
640nm	20%
STED	
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	60x wi (NA1.2)

Experiment names

- Main experiment name:

- 20181115/e03_s01_t04_LA-4 lysotracker TiO2Alexa647.msr

Controls and statistics

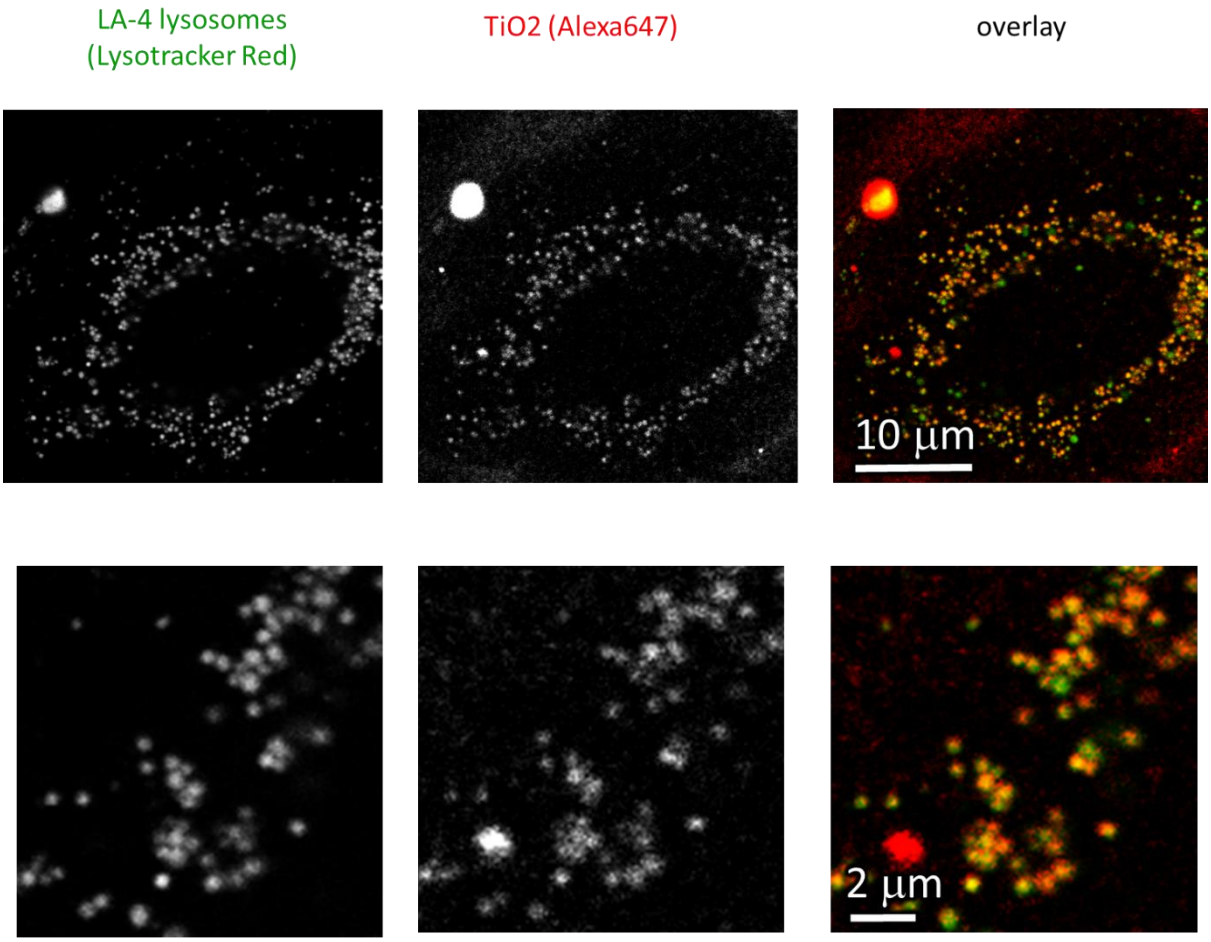


Figure S14: Colocalisation of lysosomes and nanomaterial after 1 hour of incubation.

S0e – Effect of TiO_2 nanotubes on the mitochondrial morphology of the epithelial lung cells

Main message

Mitochondria fragment in 3h after exposure to TiO_2 nanotubes. After two days the fragmentation is reverted to a state of normal, pre-exposure morphology.
Supporting raw and analysed data:

Figure S15-Figure S29

Short description

We evaluated the effects of titanium dioxide nanotubes on fluorescent labelled mitochondrial network of LA-4 murine lung epithelial cells, with fluorescent confocal microscopy and mitochondrial network analysis. LA-4 cells were treated with nanoparticles in 1:1/ 10:1/ 100:1 nanoparticle surface to cell surface ratio and then incubated for 3h, 1 and 2 days. 3h after incubation LA-4 cells with different concentrations of TiO2, the mitochondria were fragmented and closely resembled to positive control (H2O2), in comparison to negative control, where the length of mitochondria were significantly longer. 24 hours after of incubation TiO2NT with LA-4 cells we noticed slightly longer and less fragmented mitochondria in comparison to 3h incubation, which indicates that the mitochondrial morphology started to recover. That was seen especially at low concentration (1:1), while at higher concentration still prevailed fragmented mitochondria. 48 hours after incubation of LA-4 with TiO2NT, mitochondrial morphology became even more similar to negative control, indicating that low concentrations of TiO2NT altered the mitochondrial morphology only transiently. Only when treated with the highest concentration (100:1), the cells still exhibited fragmented mitochondria, but they were longer than those incubated 24h.

Materials and methods: Confocal imaging of LA-4 cells

The LA-4 murine lung epithelial cells were seeded into a 35 mm Ibidi µ-Dish and incubated in the complete culturing medium for a day (F12K medium, 15% FCS, 1% P/S (antibiotics), 1% NEAA (nonessential amino acids)). The powder of TiO_2 nanotubes was resuspended in distilled water, with addition of 2% of fetal calf serum and 0.5 % absolute ethanol, to the final concentration of 3,24 mg/mL, on ice and in sterile conditions (MISONIX Ultrasound Liquid Processor with 419 MicrotipTM probe for 16 minutes in an ice-bath, setting to the 10 % of the power). Just before treating the cells with nanoparticles in 1:1/ 10:1/ 100:1 nanoparticle surface to cell surface ratio, suspension of nanoparticles was diluted in the cell medium to desire concentration. After 3 hours/ 1 day/ 2 days of incubation, the samples were washed, incubated with 50 nM MitoTracker™ Red CMXRos (ThermoFisher Scientific) for 30 min, followed by observation in the Live cell imaging solution. For positive control 16,3 mM hydrogen peroxide (Merck) was used with incubation time 10 min. For imaging, an Abberior Instruments STED microscope equipped with a 60× water immersion objective was used. Images were acquired at 50 nm pixel size and 0.63 to 1.1 pinhole, depending on the sample. Mitrotracker was excited with the pulsed laser at 561 and fluorescence recorded with an avalanche photodiode within 580−625 nm (filters by Semrock).

Experiment names

- Main experiment:
  - 20180925/e01_s01_t01_LA-4_50nM_Mitotracker_3h2D.msr
  - 20180925/e01_s01_t01_LA-4_50nM_Mitotracker_1day_2D.msr
  - 20180925/e01_s01_t01_LA-4_50nM_Mitotracker_2days_2D.msr
- *Controls:*
  - 20180925/e01_s01_t01_LA-4_50nM_Mitotracker_16,3mM_H2O2_10 min_2D.msr

Controls and statistics

Representative image for each dose and time point:

Figure S15-Figure S16: Analysis of mitohondria fragmentation through mitochondria size and distribution estimation.

3h incubation:

Figure S17-Figure S21

12h incubation:

Figure S22-Figure S25

48h incubation:

Figure S26-Figure S29

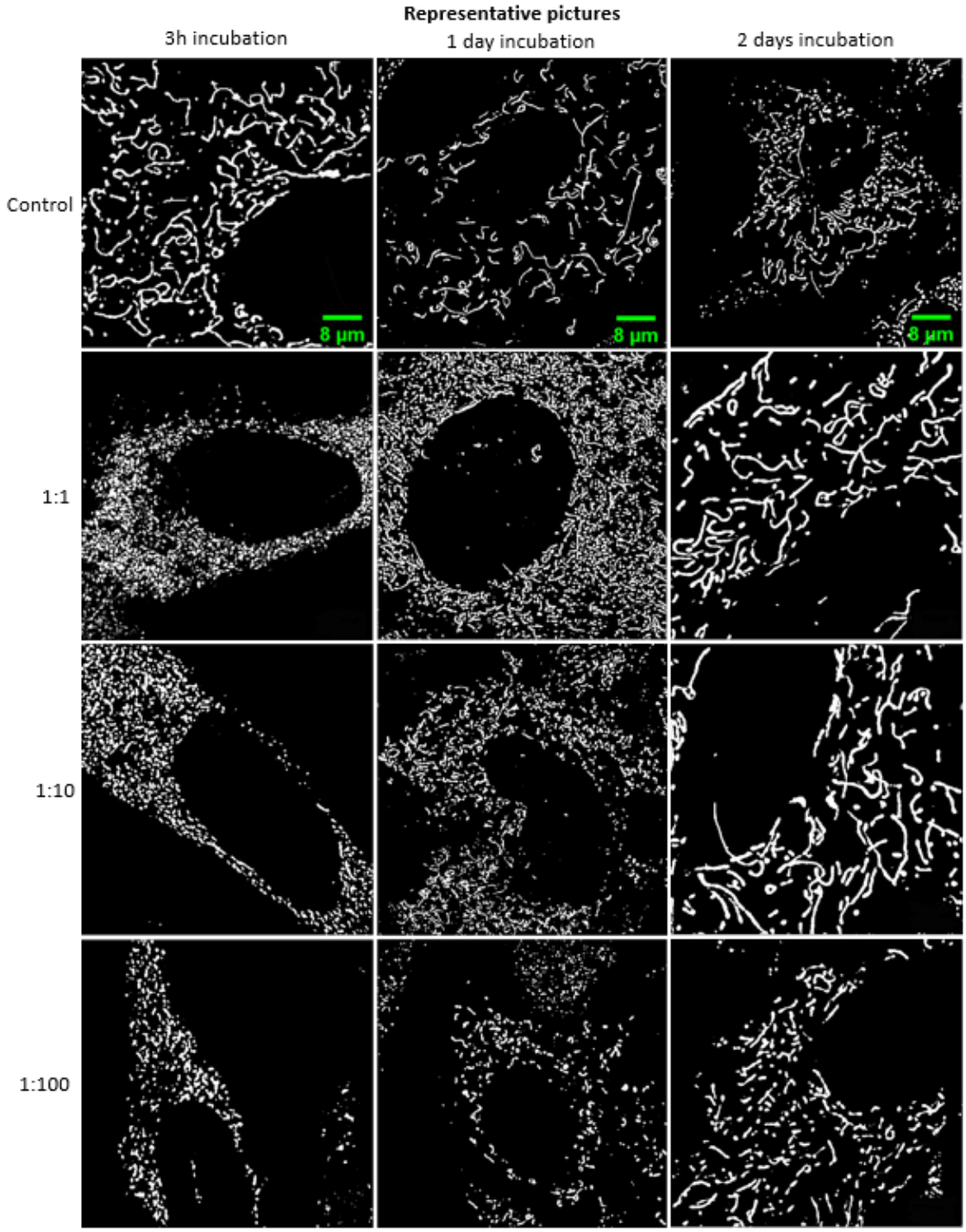


Figure S15: Representative image for each dose and time point together with control.

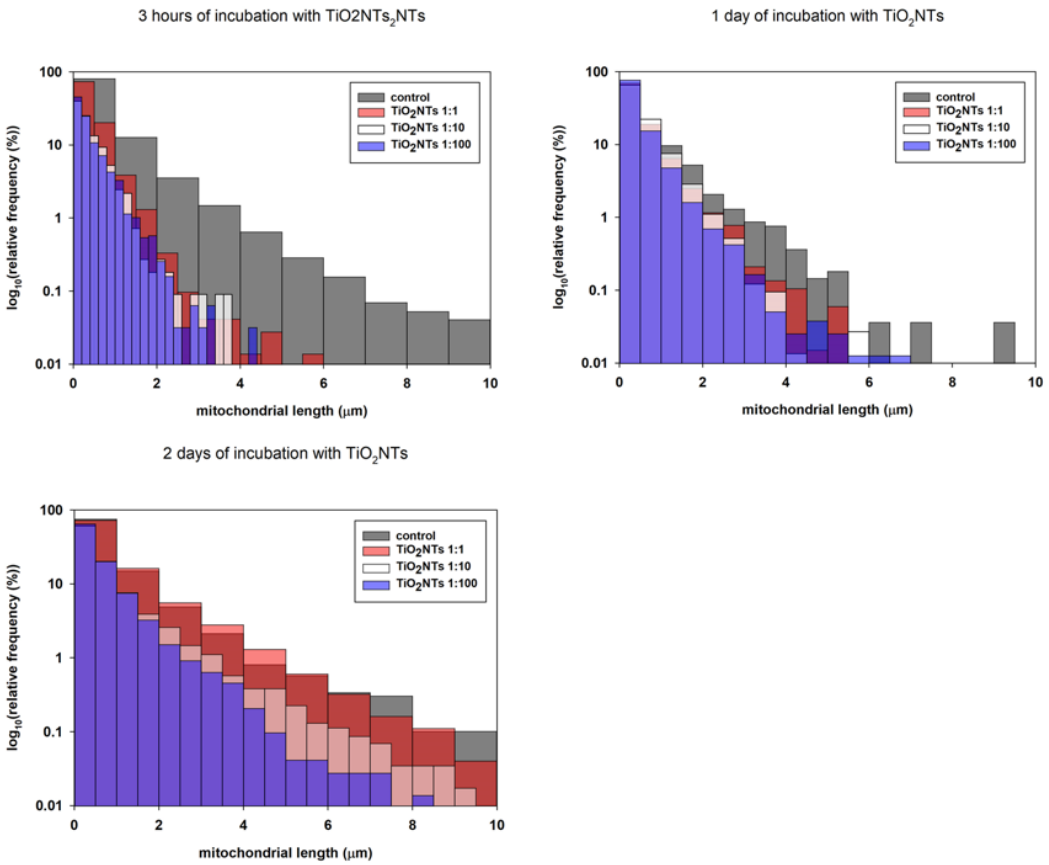


Figure S16: Analysis of mitohondria fragmentation through mitochondria size and distribution estimation.

3 hour incubation of LA-4 cells with TiO_2NT

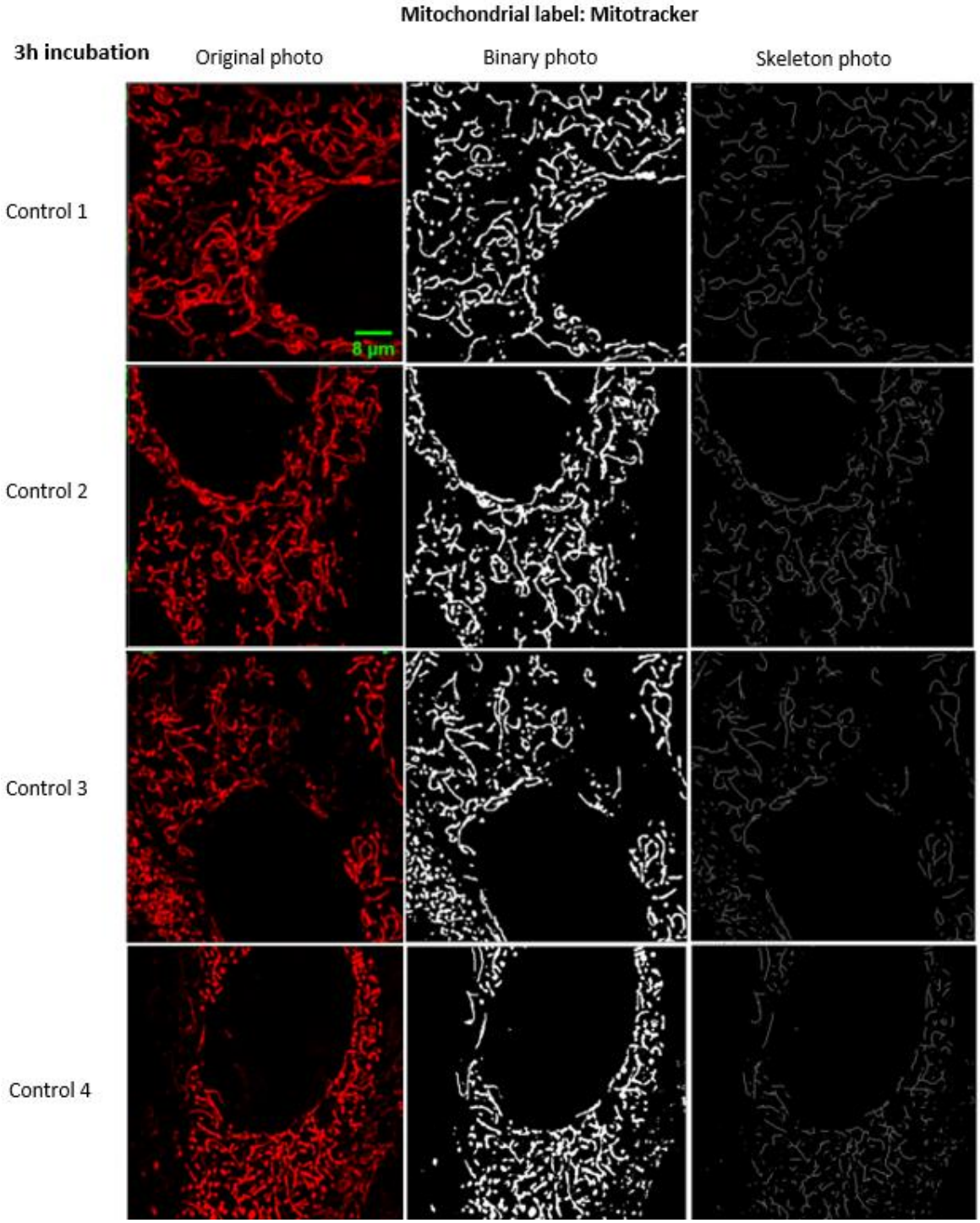


Figure S17: Statistics for 3h incubation control (non exposed sample) with underlying analysis. Left column is one-channel raw data.

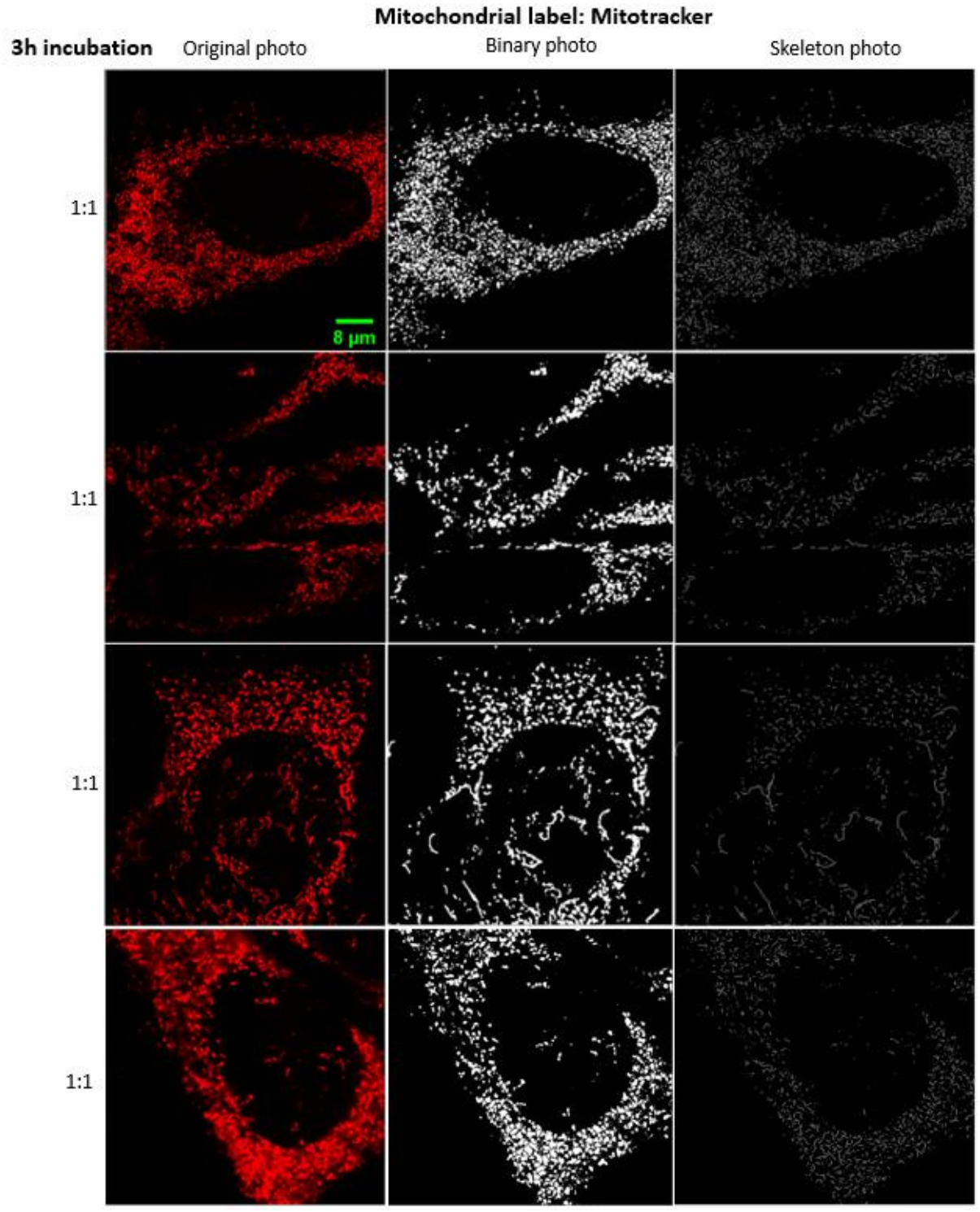


Figure S18: Statistics for 3h incubation 1:1 (surface of nanoparticles: surface of cells) dose with underlying analysis. Left column is one-channel raw data.

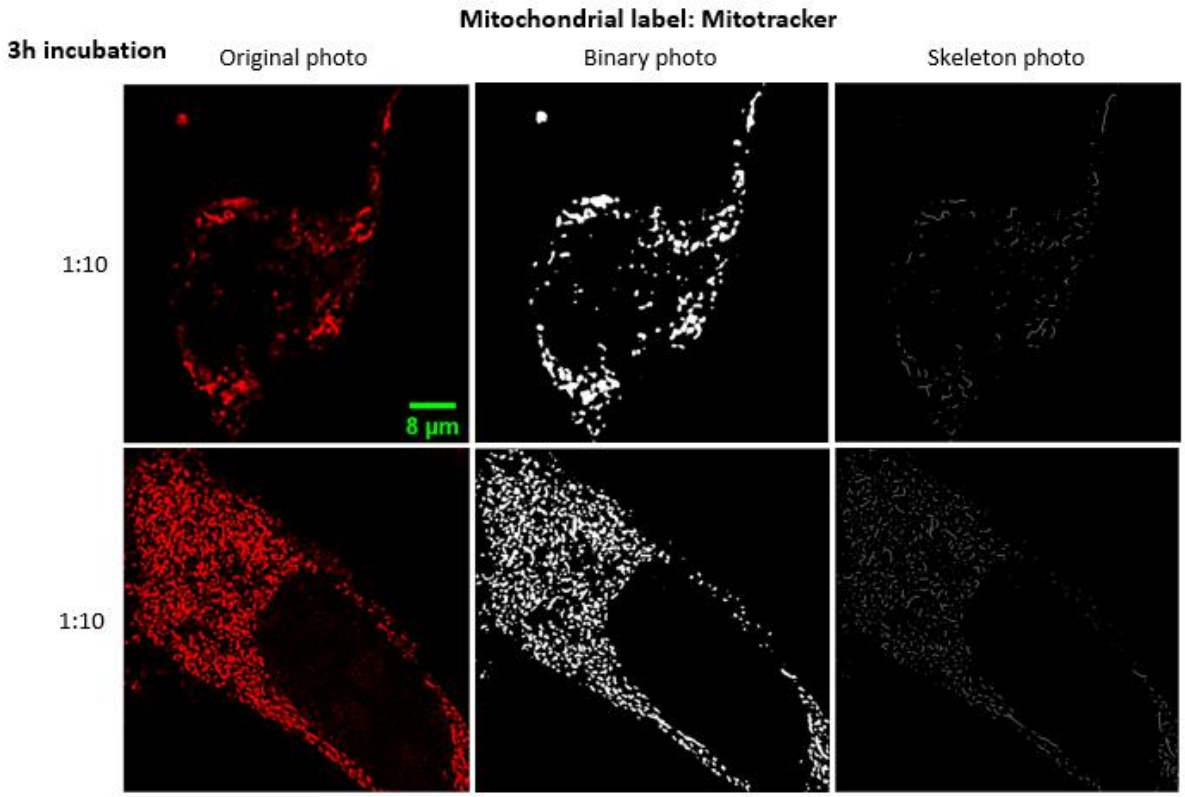


Figure S19: Statistics for 3h incubation 10:1 (surface of nanoparticles: surface of cells) dose with underlying analysis. Left column is one-channel raw data.

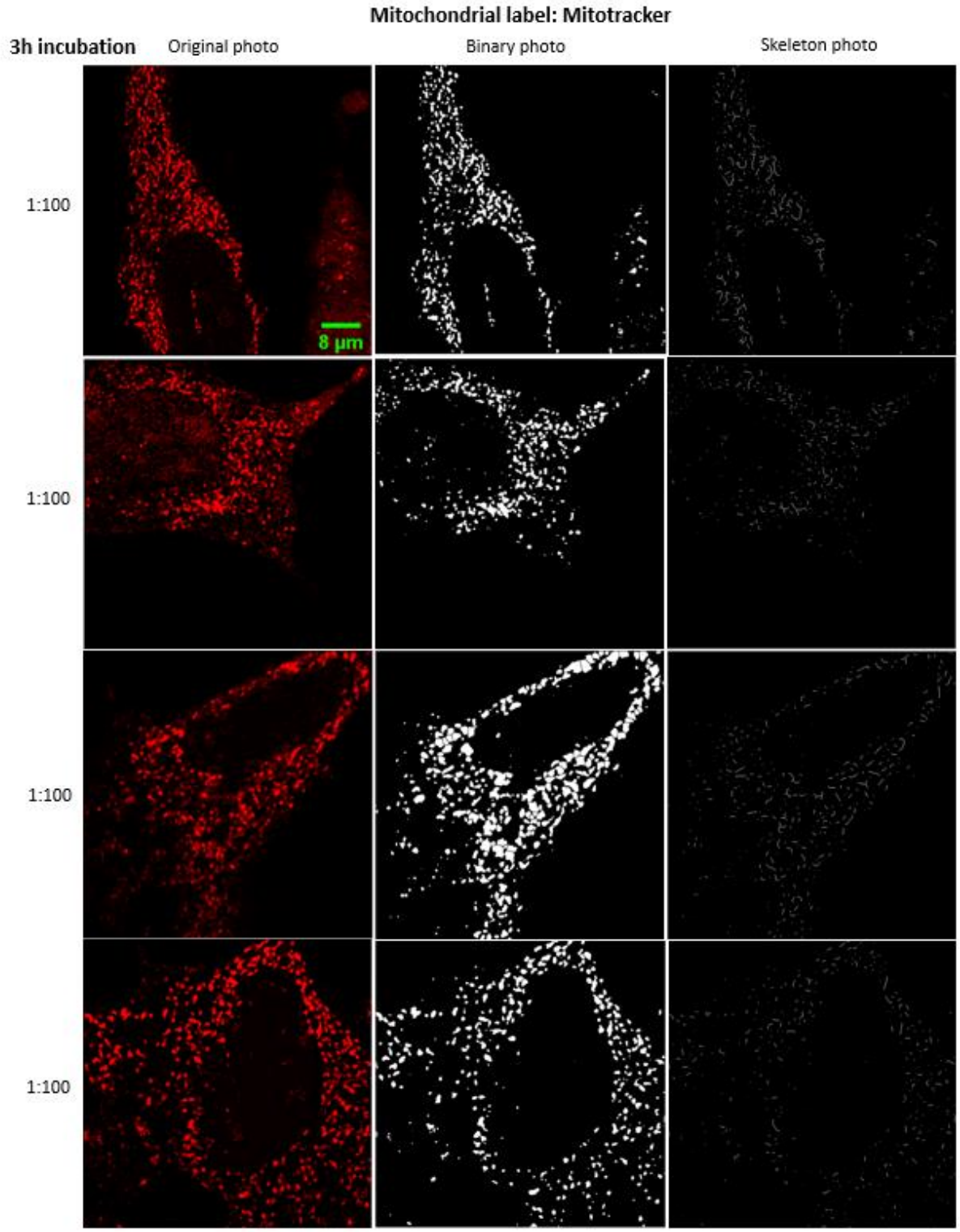


Figure S20: Statistics for 3h incubation 100:1 (surface of nanoparticles: surface of cells) dose with underlying analysis. Left column is one-channel raw data.

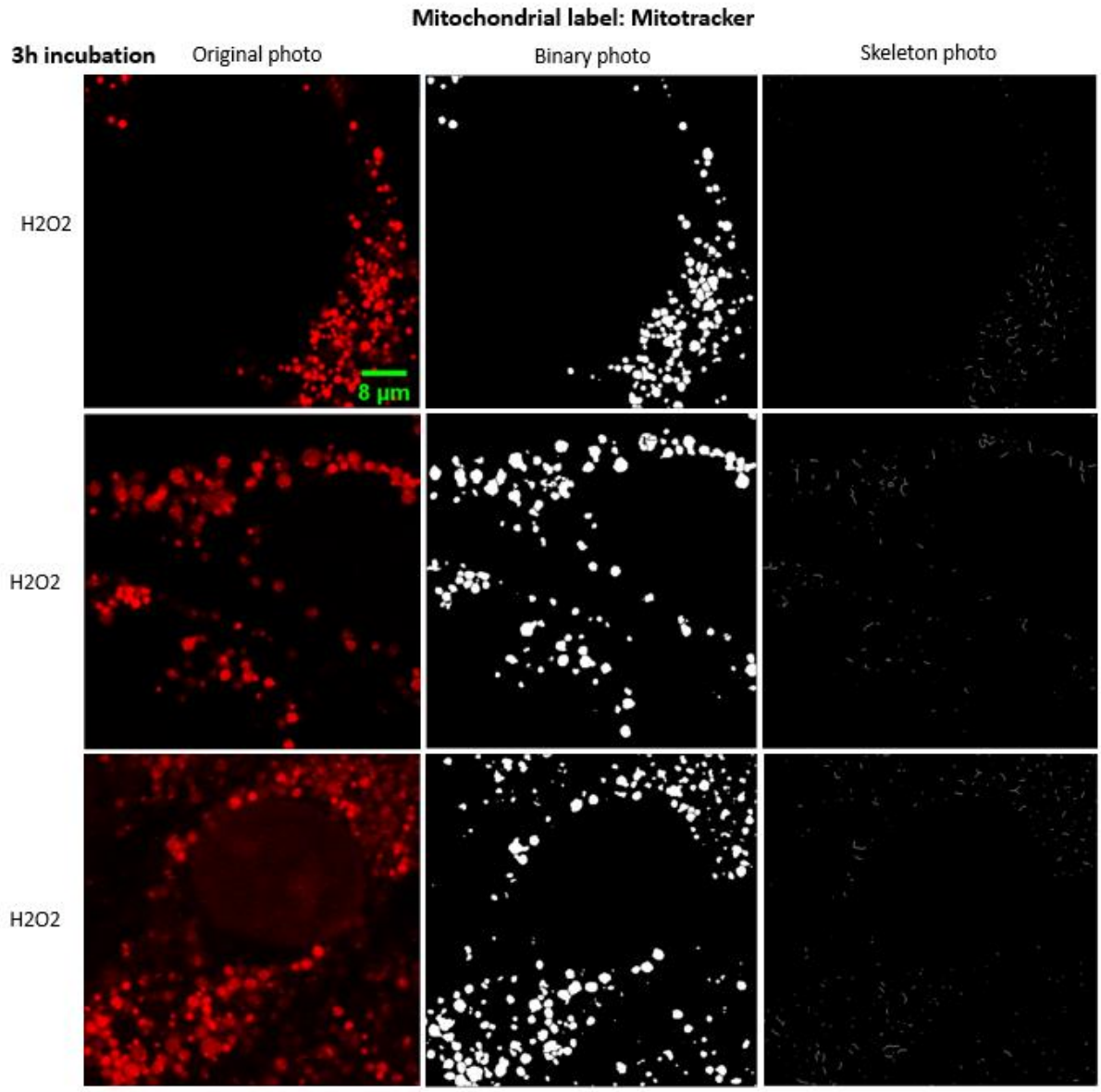


Figure S21: Statistics for 3h incubation control with H_2O_2 with underlying analysis. Left column is one-channel raw data.

1 day incubation of LA-4 cells with TiO_2NT

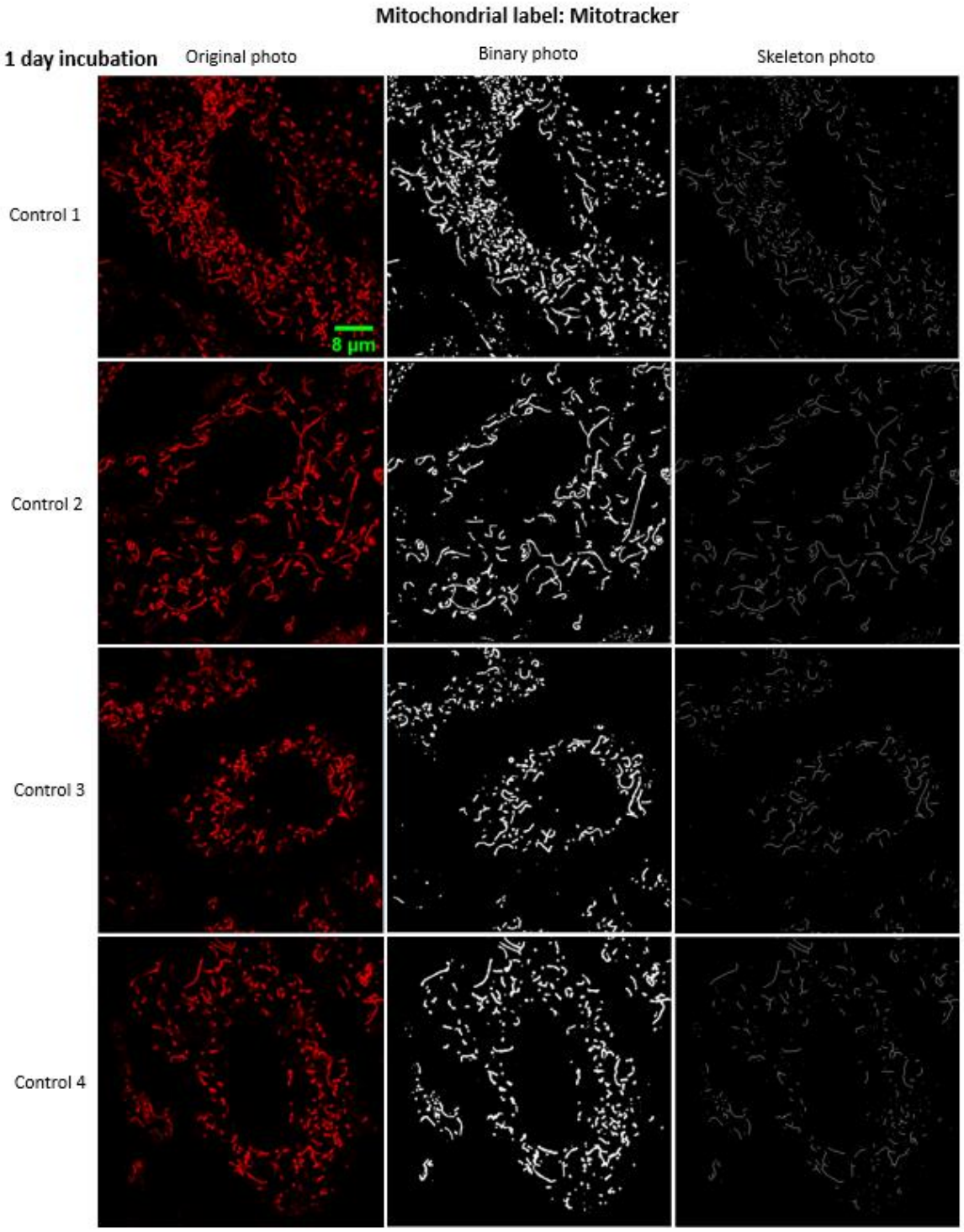


Figure S22: Statistics for 24h incubation control (non exposed sample) with underlying analysis. Left column is one-channel raw data.

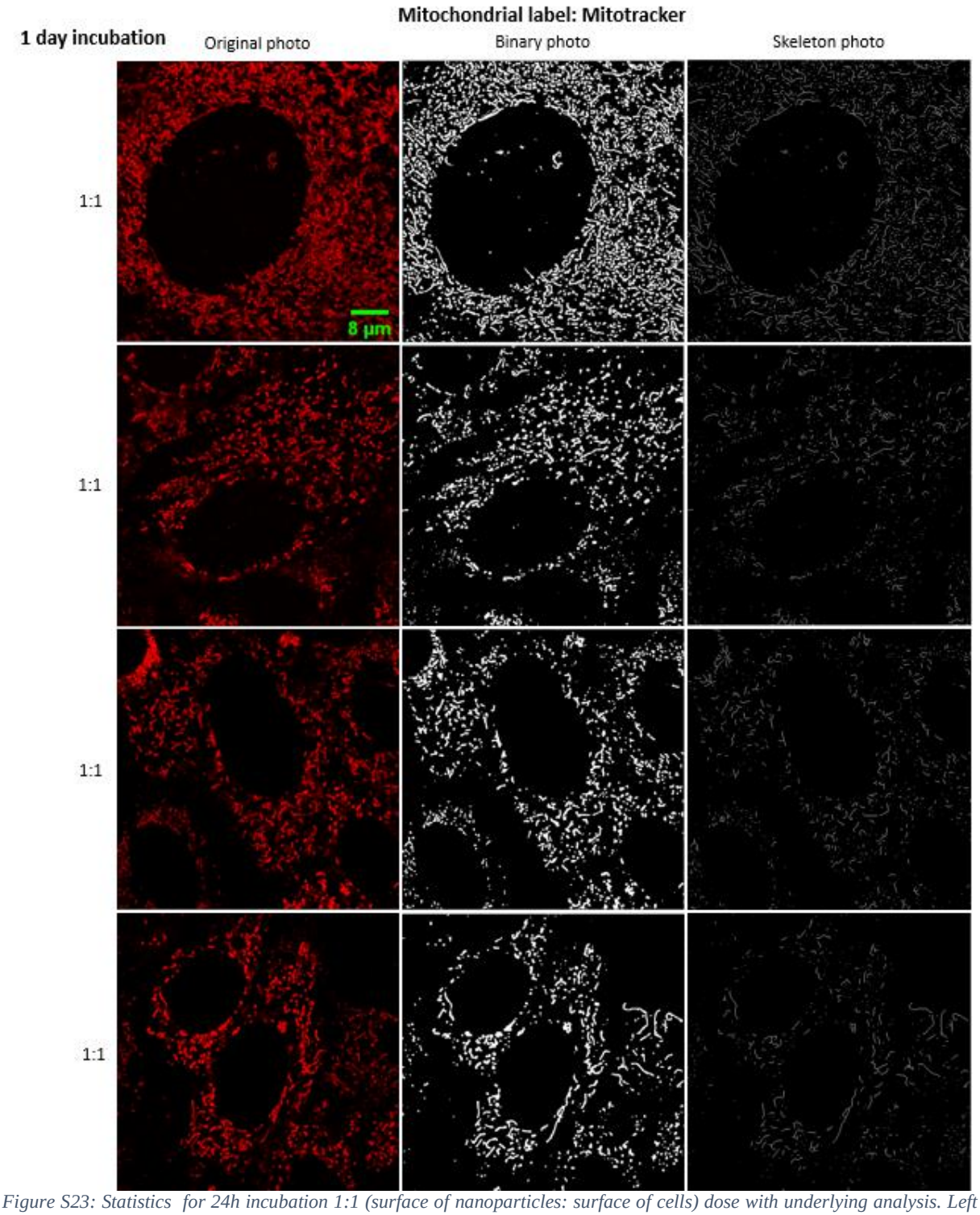


Figure S23: Statistics for 24h incubation 1:1 (surface of nanoparticles: surface of cells) dose with underlying analysis. Left column is one-channel raw data.

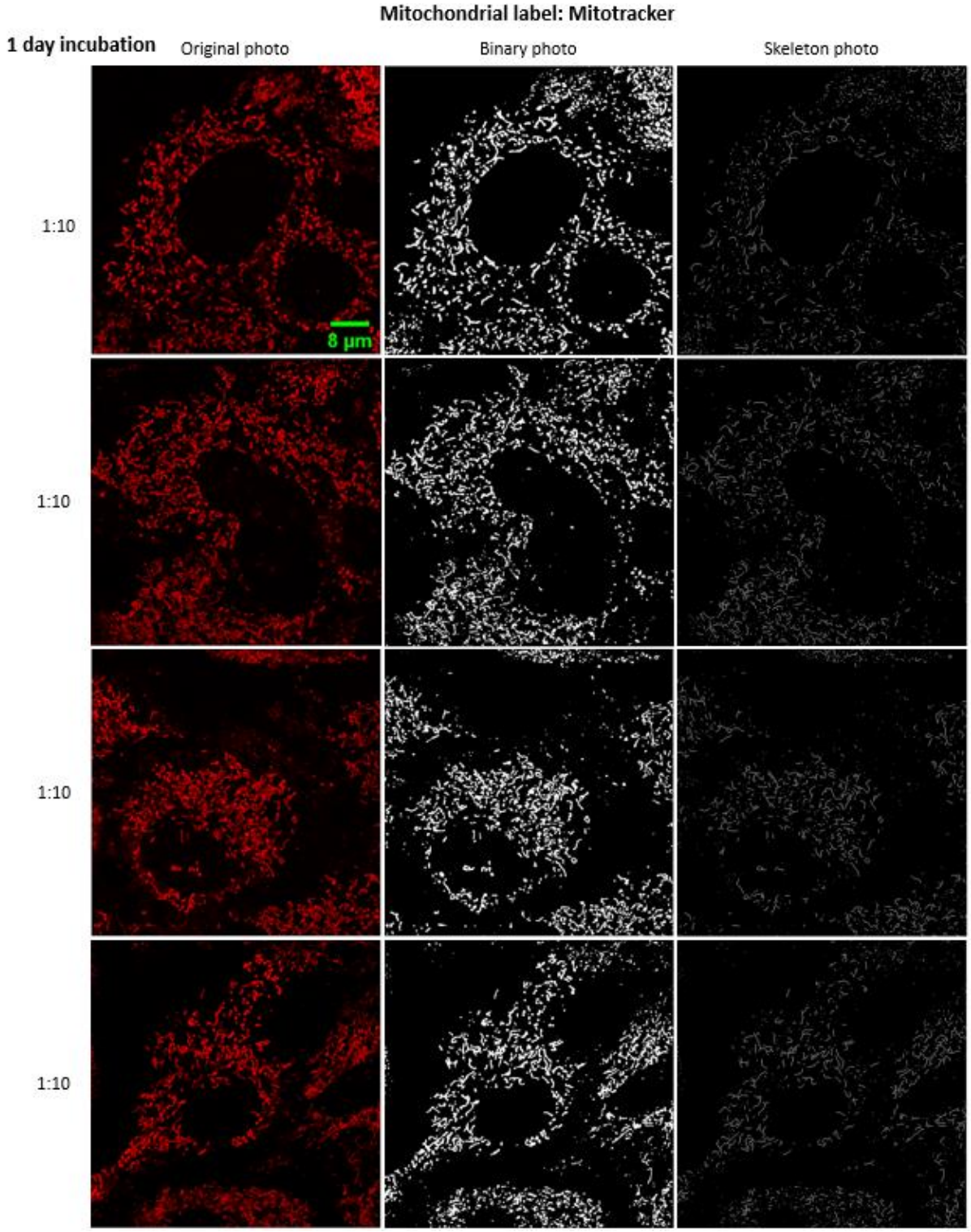


Figure S24: Statistics for 24h incubation 10:1 (surface of nanoparticles: surface of cells) dose with underlying analysis. Left column is one-channel raw data.

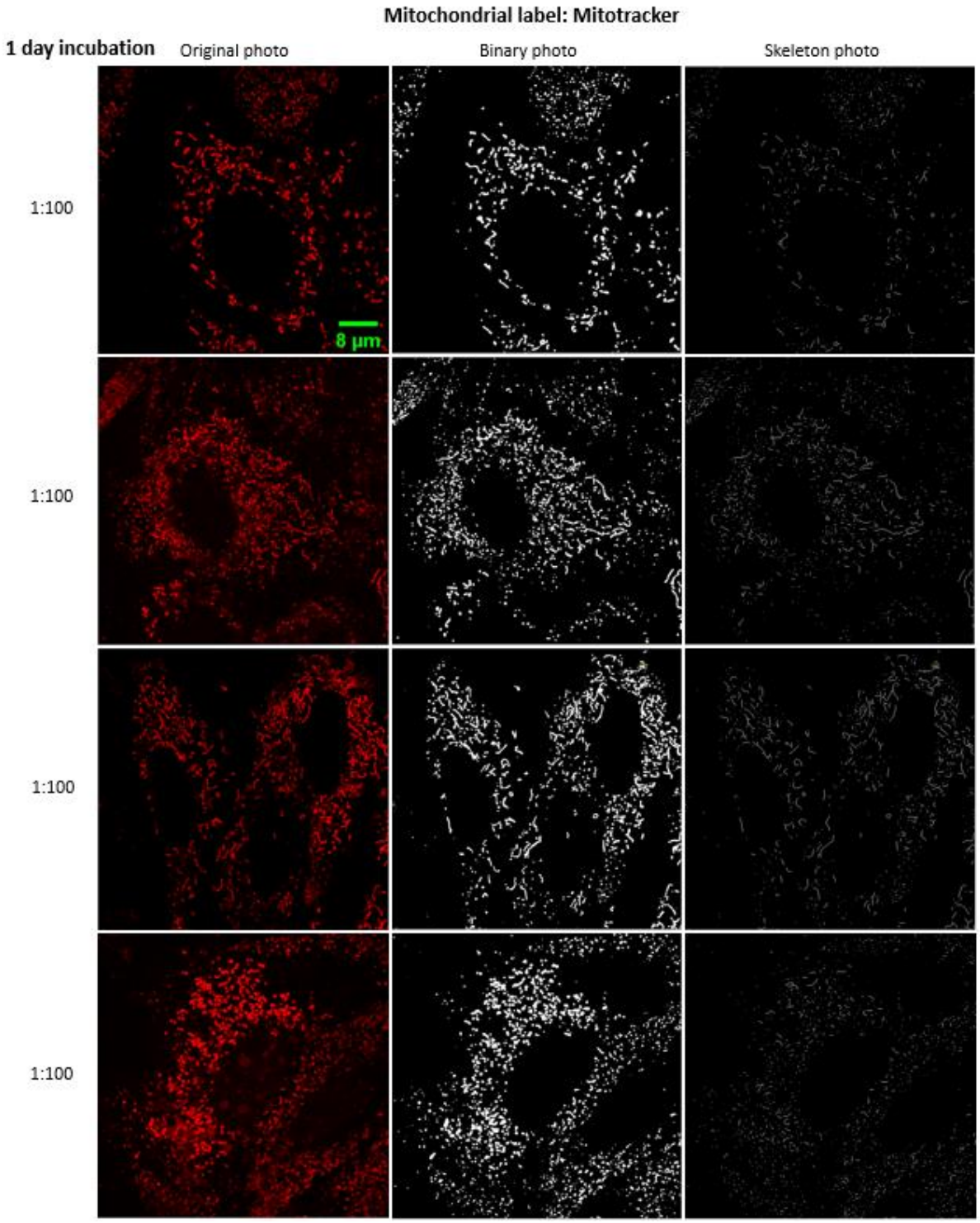


Figure S25: Statistics for 24h incubation 100:1 (surface of nanoparticles: surface of cells) dose with underlying analysis. Left column is one-channel raw data.

2 days incubation of LA-4 cells with TiO_2NT

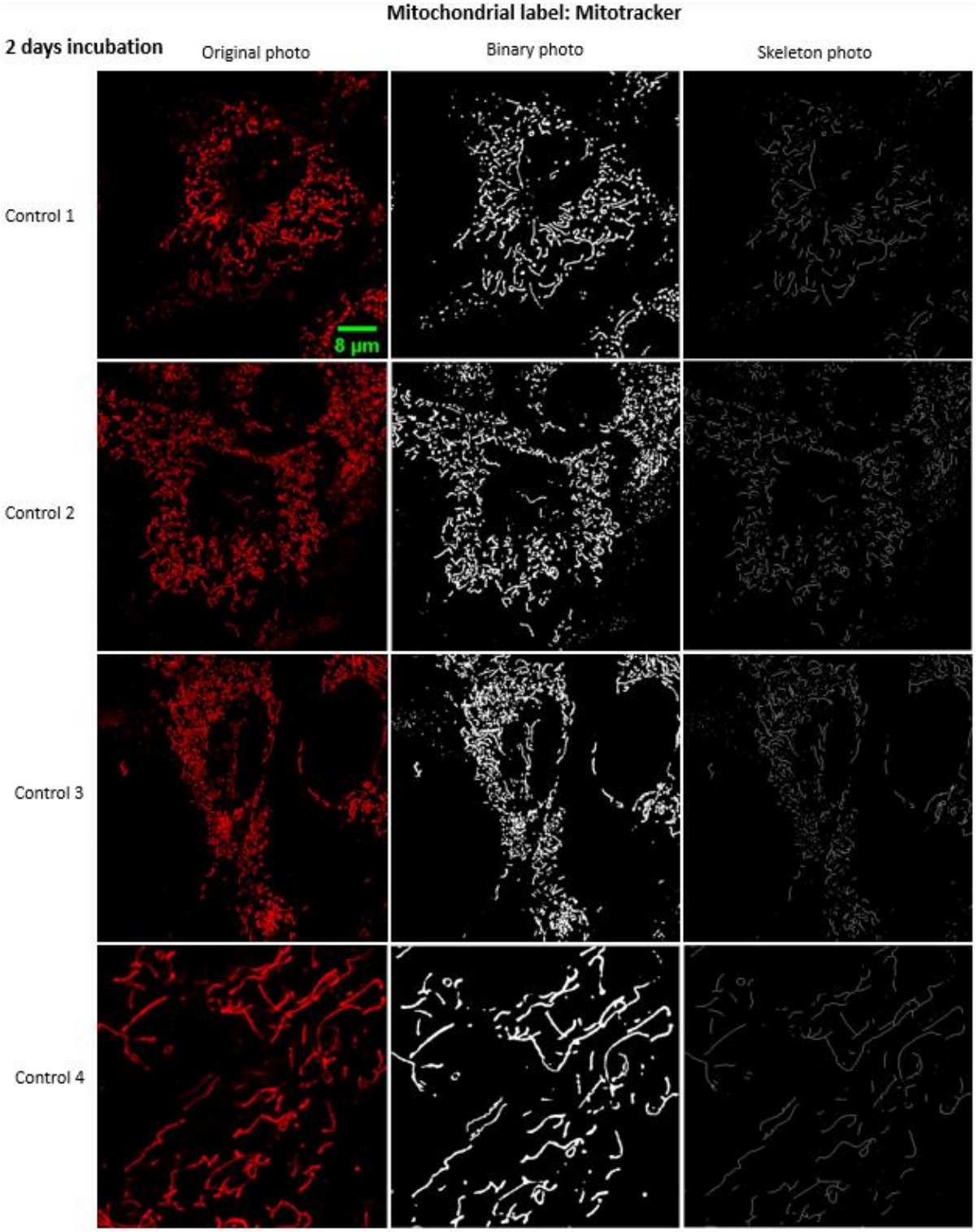


Figure S26: Statistics for 48h incubation control (non exposed sample) with underlying analysis. Left column is one-channel raw data.

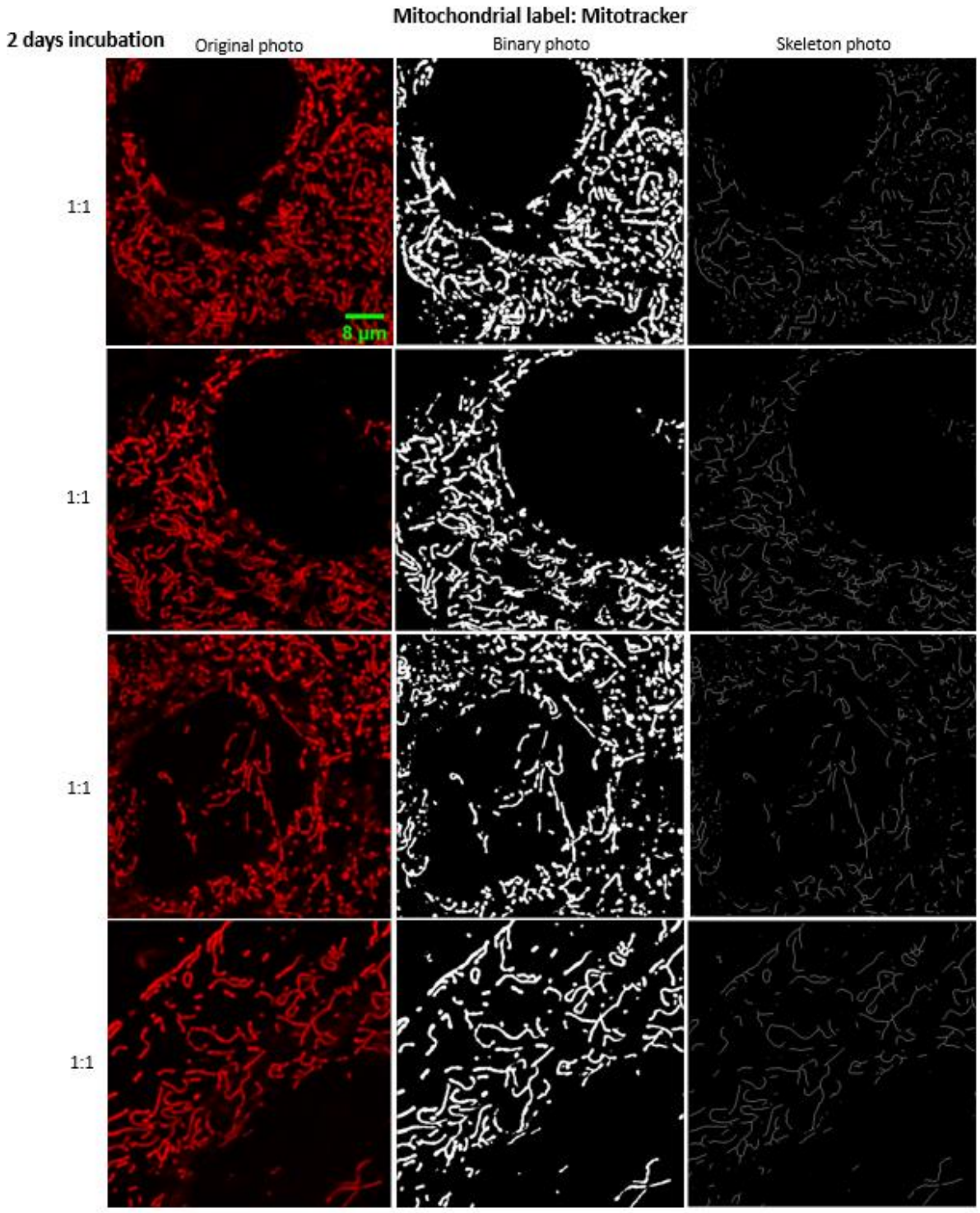


Figure S27: Statistics for 48h incubation 1:1 (surface of nanoparticles: surface of cells) dose with underlying analysis. Left column is one-channel raw data.

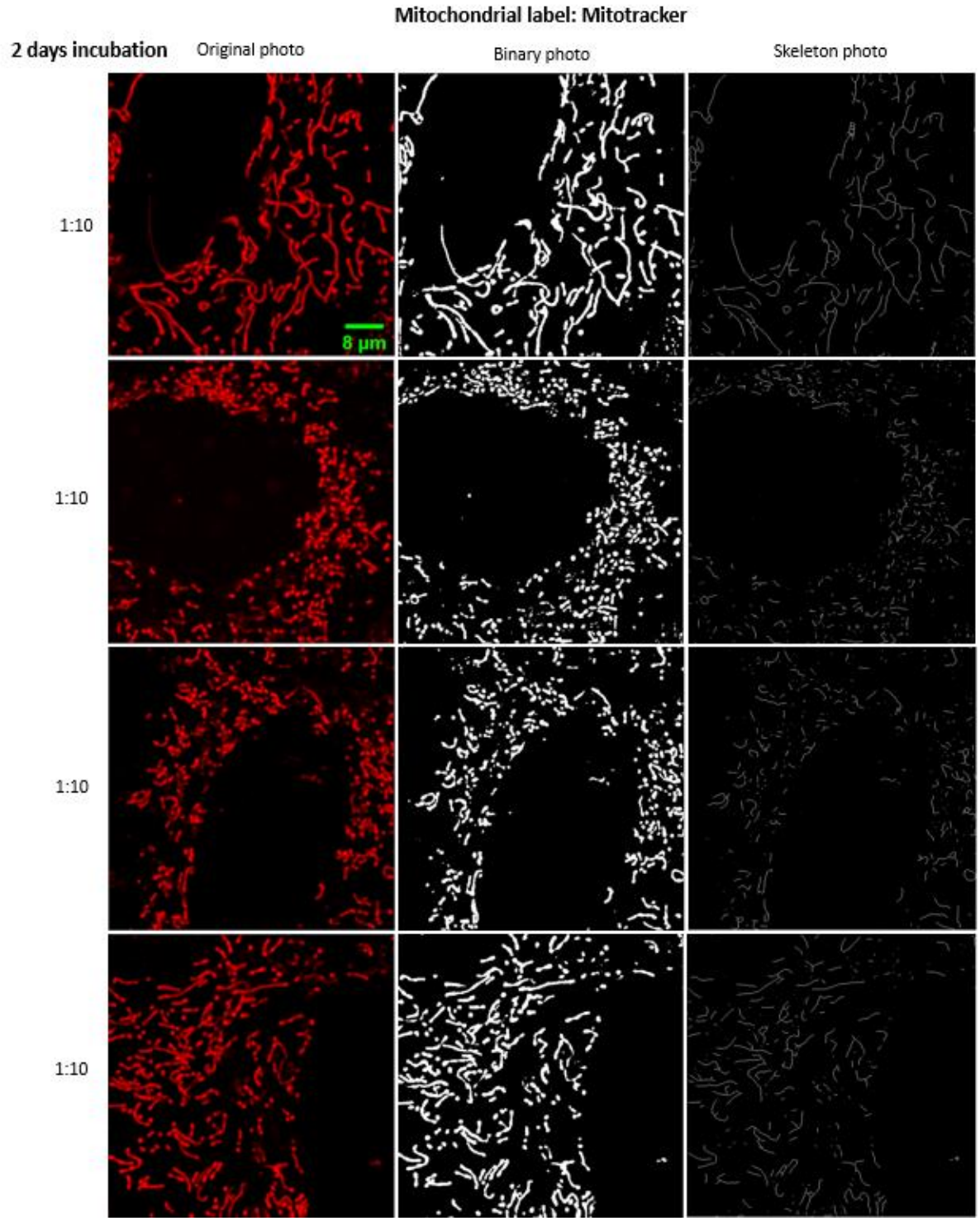


Figure S28: Statistics for 48h incubation 10:1 (surface of nanoparticles: surface of cells) dose with underlying analysis. Left column is one-channel raw data.

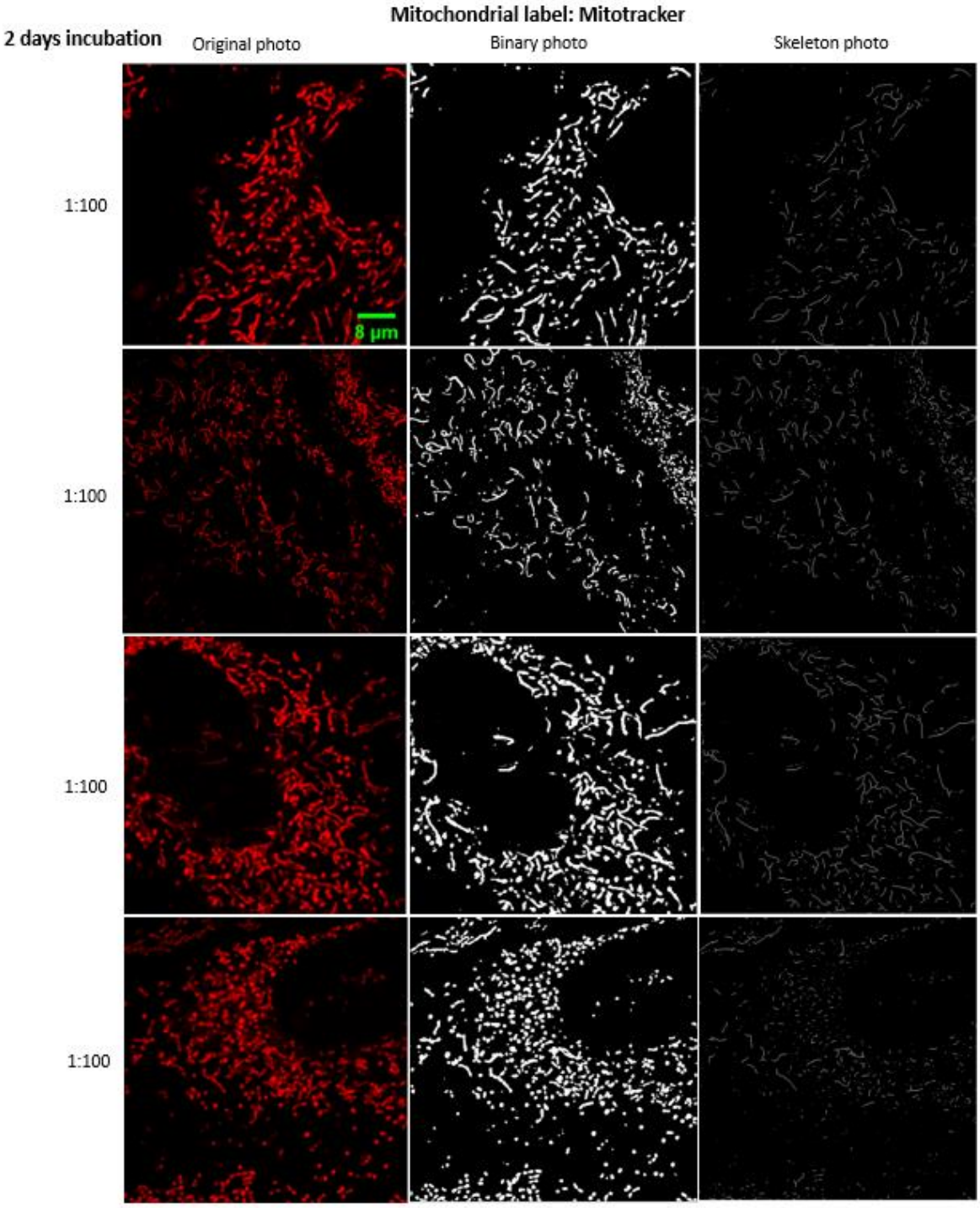


Figure S29: Statistics for 48h incubation 100:1 (surface of nanoparticles: surface of cells) dose with underlying analysis. Left column is one-channel raw data.

S0f – Cell viability – TiO_2 nanotubes, dose dependence

The cell viability after exposure to increasing doses of TiO_2 nanotubes was tested on LA-4 murine epithelial cells and MH-S murine alveolar macrophages using the WST-1 assay as well as on NR8383 alveolar rat macrophages using the LDH and WST-1 assay.

Effect of TiO_2 nanotubes on the cell viability of lung epithelial cells and alveolar macrophages

Main message

Dose response for cell viability was observed in LA-4 and MH-S cells 24h after exposure to TiO_2 nanotubes. For higher concentrations of TiO_2 nanotubes, for LA-4 cells the cell viability exhibited a plateau effect while MH-S show a continuous decline of metabolic activity. This indicates that epithelial LA-4 cells cope with higher doses compared to the macrophages. The same effect was observed also with crystalline silica (DQ12), and multiwall carbon nanotubes NM-401.

Short description

The effects of titanium dioxide nanotubes on viability of LA-4 lung epithelial cells and MH-S alveolar macrophages were compared using WST-1 assay. LA-4 cells and MH-S cells were exposed to titanium dioxide nanotubes in submerged conditions and incubated for 24h with the nanoparticle surface to cell surface ratio of 1:0.39, 1:3.85, 1:19.25, 1:38.5, and 1:385, respectively. 24h after incubation cells with different concentrations of TiO_2, the cell viability was measured by WST-1 assay. A significant dose-response was observed with the increased concentration of particles, and there was a plateau effect in the metabolic activity of LA-4 with the nanoparticle surface to cell surface ratio higher than 1:19.25 while MH-S shows a continuous decline of metabolic activity.

Materials and methods: Cell viability test of LA-4 and MH-S cells

LA-4 (ATCC# CCL-196) murine lung epithelial cells were cultured in HAMs F12 cell culture medium with stable Glutamin, 1% NEAA (nonessential amino acids), 15% FCS, and 1% Penicillin/Streptomycin. MH-S (ATCC# CRL-2019) murine alveolar macrophages were cultured in RPMI 1640 cell culture medium with stable Glutamin, 0.05mM 2ME (2-Mercaptoethanol), 10% FBS, and 1% Penicillin/Streptomycin. Cells were seeded at a density of $1x10^5$ cells/well into a 24-well plate and incubated in complete culture medium overnight. TiO_2 nanotubes were dispersed in distilled water, to the final concentration of 5 mg/mL. The suspension was sonicated on ice with a probe sonifier for 2 min 40 sec at 30% power and 100% cycle time. The nanoparticle suspension was diluted in cell culture medium to concentration of 1, 10, 50, 100, and 1000 ug/mL, which refer to the nanoparticle surface to cell surface ratio of 1:0.39, 1:3.85; 1:19.25; 1:38.5; and 1:385, respectively. Experiments using crystalline silica (DQ12), ZnO and Printex 90 were performed in a similar manner with varying dose ranges. Multiwall carbon nanotubes NM-401 were dispersed in distilled water with 0.2 mg/mL real lung surfactant from pig, to the final concentration of 1.5 mg/mL. The suspension was sonicated in ultrasonic water bath for 5 min and then sonicated on ice with a probe sonifier for 30 seconds at 30% power and 100% cycle time. The nanoparticle suspension was diluted in cell culture medium to desire concentrations just before treating the cells.

WST-1 assay was carried out 24h after the particle exposure using cell proliferation reagent WST-1 kit (Roche, Germany) for cell viability measurement. WST-1 solution was either 1:15 (LA-4 cells) or 1:10 (MH-S cells) diluted in complete cell culture medium and incubated with

the cells at 37 °C for 40 min (LA-4 cells) or 15 min (MH-S cells). 200ul of the culture medium supernatant were removed and centrifuged at 14000 rpm for 10 min after the incubation, and the absorbance (OD value) of the solution was determined at 450nm by microplate reader (Infinite®F200, Tecan). Cell viability of the samples were calculated as followed: cell viability = (sample OD – blank OD)/(control OD – blank OD)*100%.

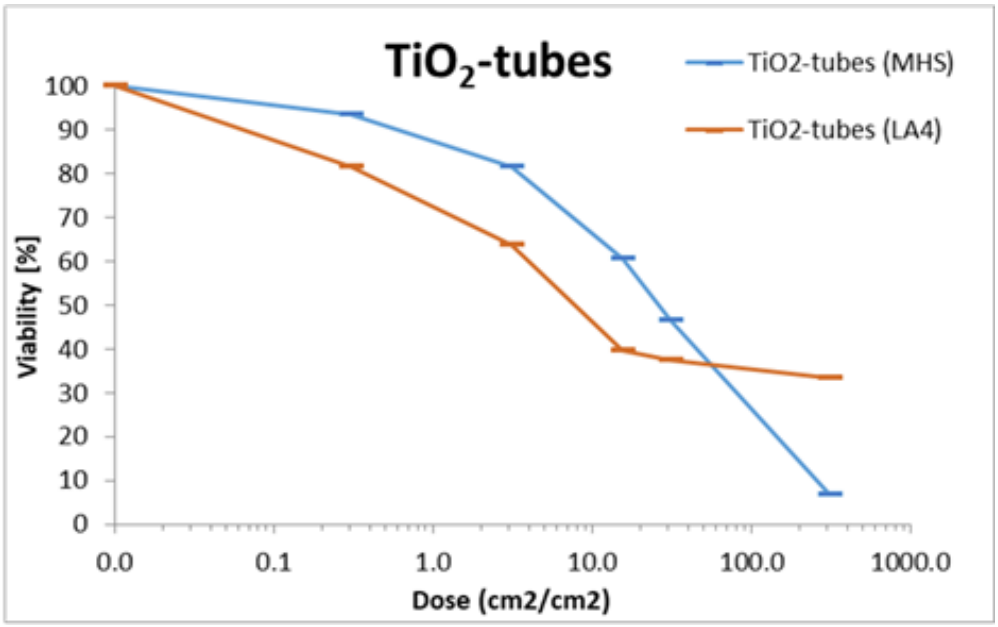


Figure S30: Dose dependant viability data of exposed MH-S alveolar macrophages and LA-4 alveolar epithelial cells to TiO_2 nanotubes in monoculture. Error bars represent the standard error.

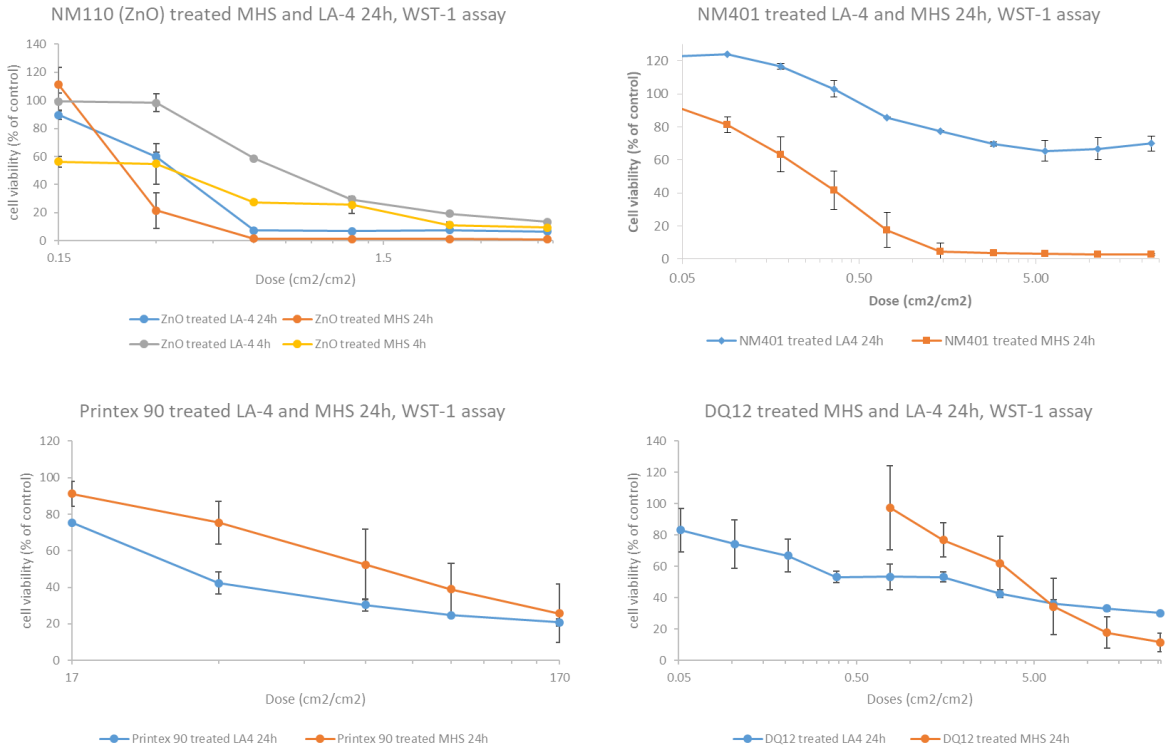


Figure S31: Dose dependent viability data of exposed MH-S alveolar macrophages and LA-4 alveolar epithelial cells to ZnO (NM 110), multi wall carbon nanotubes (NM401), carbon black (Printex90) and silica (DQ12) in monoculture. Error bars represent the standard error.

NR8383 alveolar rat macrophages, LDH and WST-1 assay, TiO_2 nanotubes

Main message

TiO_2 nanotubes don't show toxicity, even at the highest concentration tested. Cellular metabolism is slightly affected for concentrations until 100 µg/mL.

Supporting raw and analysed data:

Figure S32

Table S2

Materials and methods

Cell culture and exposure

NR8383 alveolar rat macrophages cell line was obtained from the American Type Culture Collection (ATCC, USA) and were grown in DMEM supplemented with 15 % heat-inactivated FBS, 4 mM L-glutamine (SIGMA-G7513) and a mixture of antibiotic/antimycotic composed of 100 U/mL of penicillin, 100 µg/mL of streptomycin (SIGMA-P0781) and 0.25 µg/mL of amphotericin B (SIGMA-A2942), at 37 °C in a humidified mixture of air (95%) and CO2 (5%). For all experiments, cells were seeded 24 h before exposure to nanoparticles at a density of 5 x 10^4 cells/mL. Cells were exposed to nanoparticles in cell media without FBS. The different concentrations of nanoparticles were mixed at room temperature to ensure homogeneity of the samples before exposure to cells. Cells not exposed to nanoparticles were served as controls in each experiment. Each experiment was conducted on 4 independent replicates.

Cell viability

Lactate dehydrogenase (LDH) leakage was analyzed using the LDH assay (Roche-4744934001, Germany) following the manufacturer's instruction. Briefly, NR8383 cells were seeded at 5 x 10^4 cells/mL in 96-well plates and exposed to different concentrations ranging 2.87 to 91.84 cm^2 of TiO_2 nanotubes per cm^2 of cells (cm^2/cm^2). These specific surface concentrations are equivalent of mass concentration of 6.25 and 200 µg/mL. After 24 h of exposure, plates were centrifuged at 800 xg for 10 min and 100 µL of each supernatant were transferred to a new 96-well plate with black bottom that already prefilled with 100 µL of the LDH reaction mixture. Extracellular media was incubated for 30 min at room temperature, then, 50 µL of a stop solution was added and the absorbance was measured at 490 nm on a microplate reader. NR8383 cells treated with 5 % Triton was considered as positive control. Unexposed NR8383 cells were considered as negative control. Nanoparticles cytotoxicity was expressed as the percent of LDH leakage measured in positive control cells. Dose-effect relationships were assessed by ANOVA and Dunett's test. p values < 0.05 were considered significant.

Metabolic activity

Metabolic activity was assessed using the WST-1 assay [7] (Roche, 11644807001, USA), according to manufacturer's protocol. NR8383 cells were seeded at 5 x 10^4 cells/mL in 96-well plates and exposed to different concentrations (6,25 to 200 µg/mL, corresponding to 2.87 to 91.84 cm^2/cm^2) of TiO_2 nanotubes. After 24 h of exposure, WST-1 reagent was added to each well. Cells were incubated at 37 °C for 2 h. The absorbance of the solution was determined at 480 nm on microreader (BioRad-iMARK). IC50 was measured for each nanoparticle according to Reed-Muench method [8] from WST-1 results.

Experiment names

- Main experiment name: 2019 09 30 - Oliver - Copie de NanoTube TiO2

Controls and statistics

Characterisation of TiO_2 nanotubes:

Table S2

Viability of rat macrophages in dependence of dose:

Figure S32

Hydrodynamic Radius	PDI	Specific area BET (m^2/g) (2)
371± 3	0.623 ± 0.2	152 m^2/g

Table S2: Main characteristics of NanoTube TiO_2

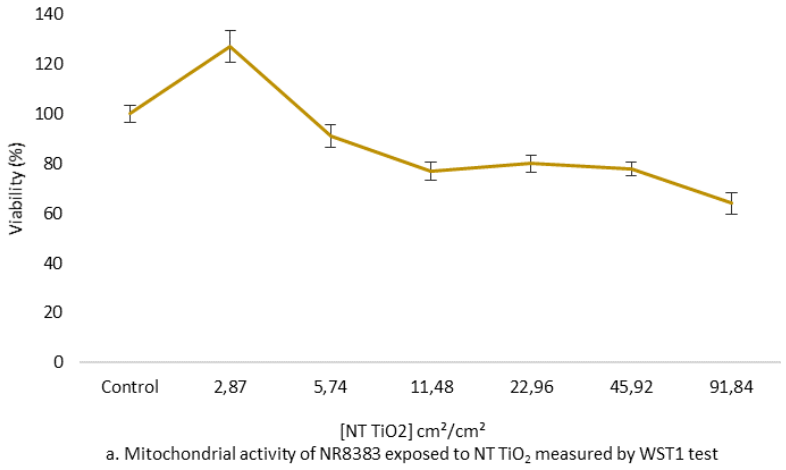


Figure S32: Viability of NR8383 exposed to nanoparticles of NanoTube TiO_2 in both tests of WST1 (top) and LDH (bottom) Cells exposed to TiO_2 nanotubes showed a significant decrease of viability from 5.74 cm²/cm². IC50 > 91.84 cm²/cm² (200 µg/mL).

S0g – Cell viability LA-4 – various nanomaterials, same surface dose

LA-4 murine alveolar cells, PI and Hoechst assay, various nanomaterial

Main message

We tested the toxicity of various nanomaterials to the LA-4 alveolar epithelial cells using the PI/Hoechst assay. ZnO exhibits the highest toxicity on LA-4 cell line. Cell viability after exposure to other nanomaterials remains high, but morphological changes are evident in comparison with the control samples.

The cauliflower-forming potential of the same nanomaterials is examined in "S2c – Cauliflowers with various nanomaterial" and the TEM micrographs are in section "S0b – Nanomaterial ".

Supporting raw and analysed data:

Figure S33

Materials and methods

- cell preparation and exposure was performed simultaneously and using the same nanomaterials and protocol as in S2c.
- preparation for imaging:
    - Propidium Iodide (PI) (Sigma, final concentration 0.3 µg/mL) was used to label the nuclei of dead cells and Hoechst 33342 (Sigma, final concentration 10 µg/mL) to label the nuclei of all cells. Cells were imaged in LCIS without washing
- Cells have been imaged with the FMS setup: inverted fluorescence Nikon Eclipse TE 2000-E microscope with Xe-Hg source (Sutter Lambda LS, Novato, CA). Excitation 352-402, dichroic 409 and emission 417-477 broad-band pass filter set have been used to image Hoechst 33342 labelled cells and excitation 503-538, dichroic 560 and emission 596-664 filter set have been used to image PI labelled cells (BrightLine from Semrock, Rochester, NY). Images were taken with EMCCD camera (iXon3 897 from Andor, Belfast, UK)
- Analysis:
    - Each well of the 8-well Ibidi, was imaged on three random locations to get the unbiased overview of the sample. Absolute number of cells in each frame was then determined manually as Hoechst 33342 + and number of dead cells as PI+ and Hoechst 33342 +
    - For the analysis median number of cells per well was used

Experiment names

- Main experiment name:
    - 2019_11_29 LA-4 Multiple NP exposure Viability

Results

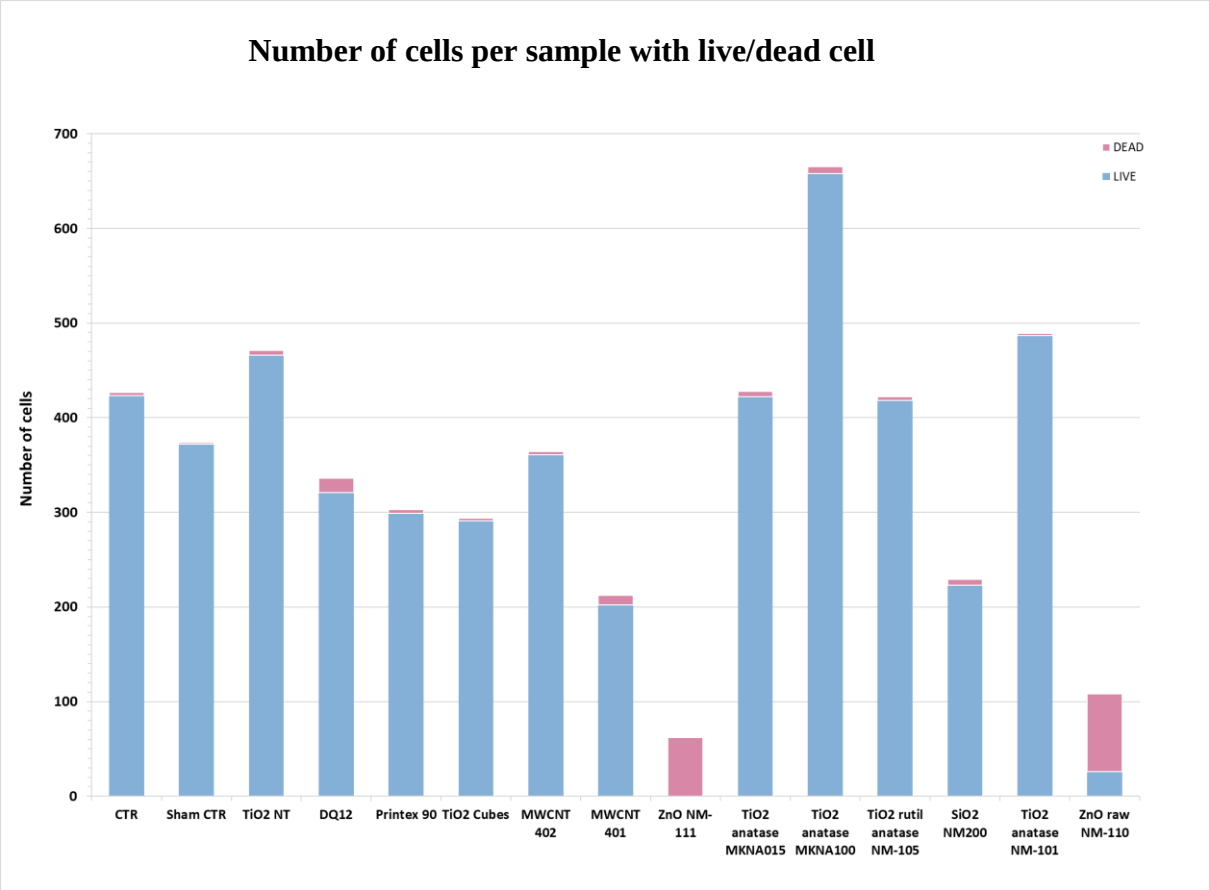
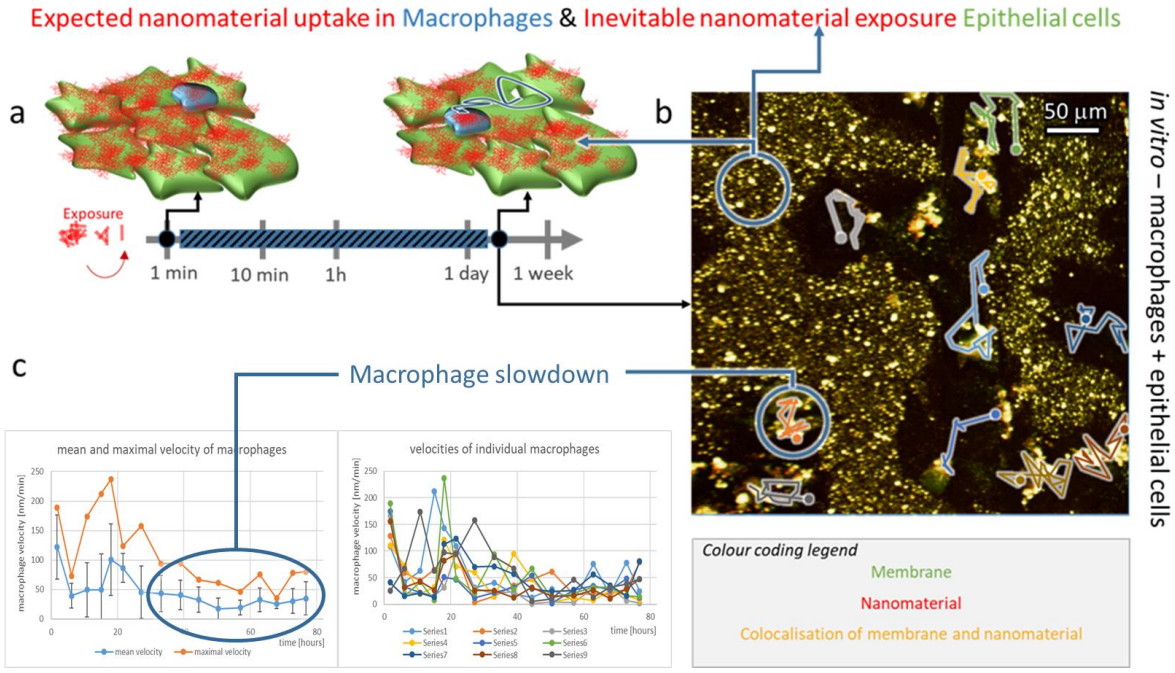


Figure S33: Median number of LA-4 per field of view in the sample with viability after a 2-day exposure to 10:1 surface dose of nanomaterials, as determined by PI-Hoechst 33342 staining.

S0h – *In vitro* exposure of cocultures of MH-S&LA-4 to TiO_2

Main message

Tracking of macrophages grown on top of epithelial cell layer through the first 3 days after exposure to see the relative surface cleaned by the macrophages as well as their slow down.



Calculations and reasoning

As depicted in graph (figure above, c), the maximal velocity of the macrophages decreases from 200 to 100 nm/min during the exposure to nanomaterials. Even at full speed, the non-exposed macrophages need a day to fully scan the alveolus surface (see calculation below). Even the fastest measured animal cell (human neutrophil, source [9]) with the migration speed of 9 µm/min would need 30 minutes to fully scan the alveolar surface, inevitably leaving enough time for the epithelium to internalize some of the nanomaterial.

The data and reasoning in the estimation are as follows:

Velocity of macrophages = v = 200 nm/min (measured from time-series, shown in figure above c);

Ratio between surface of macrophages (SM) and surface of alveoli in mice (SA) = α = 0.03;[10]

Diameter of macrophage = 2*r = 10 µm (estimated from microscopy images);

The time in which macrophages fully scan the alveolar surface = path / velocity =

$$\frac{\left(\frac{S_A}{2r}\right)}{v} = \frac{\frac{S_M}{\alpha}}{2rv} = \frac{\frac{\pi r^2}{\alpha}}{2rv} = \frac{\pi * r}{2\alpha v} = \frac{3.14 * 5\ \mu m}{2 * 0.03 * 0.2\ \mu m/min} = 1300\ minutes = 22\ hours$$

Portion of internalized material is further decreased due to slowing down of the macrophages originating from nanomaterial disruption of the cell cytoskeleton (as shown in Figure 4). This first order correction can depend on the total amount of nanomaterial delivered as well as on the nanomaterial biopersistence. Such an effect would thus be observed also with longer exposures at lower doses – as is the case with relevant environmental exposure (discussed in section S0i). Dose as used in this experiment (S_{NM} /$S_{lung\ epithelial\ cells}$ = 10:1) is therefore completely realistic.

Materials and methods

- experiment:
    - LA-4 cells were seeded @30% confluence in an Ibidi #1.5H µ-Dish
    - MH-S cells were seeded @30% confluence in a separate Ibidi #1.5H µ-Dish
    - After 48 hours of separate incubation, LA-4 and MH-S were mixed together. Growth media for cocultures was mixture of F12K and RPMI-1640 in 1:1 ratio
    - after 72 hours 35 µL freshly filtered 1 mg/mL TiO_2-17-Alexa647 in 100x dcb was added directly to the cocultures of LA-4 and MH-S cells (in 400 µL mixed medium) and mixed to achieve 10:1 surface dose
    - After approximately 96 hours exposed cocultures of LA-4 and MH-S was incubated with 1 um SHE-2N membrane label for 5 minutes in incubator at 37 °C and 5% CO2, afterwards they were flushed with 1x400 mL LCIS and observed in 400 µL mixture of F-12K and RPMI-1640 in 1:1 ratio (200 µL) and Live cell imaging solution (LCIS – 200 µL) in the home-made incubator at 37 °C for following 72 h

- analysis:
    - The colour intensity scale is changed from linear to logarithmic scale in both green and red channel to get better contrast. Images were analysed in Photo Shop.

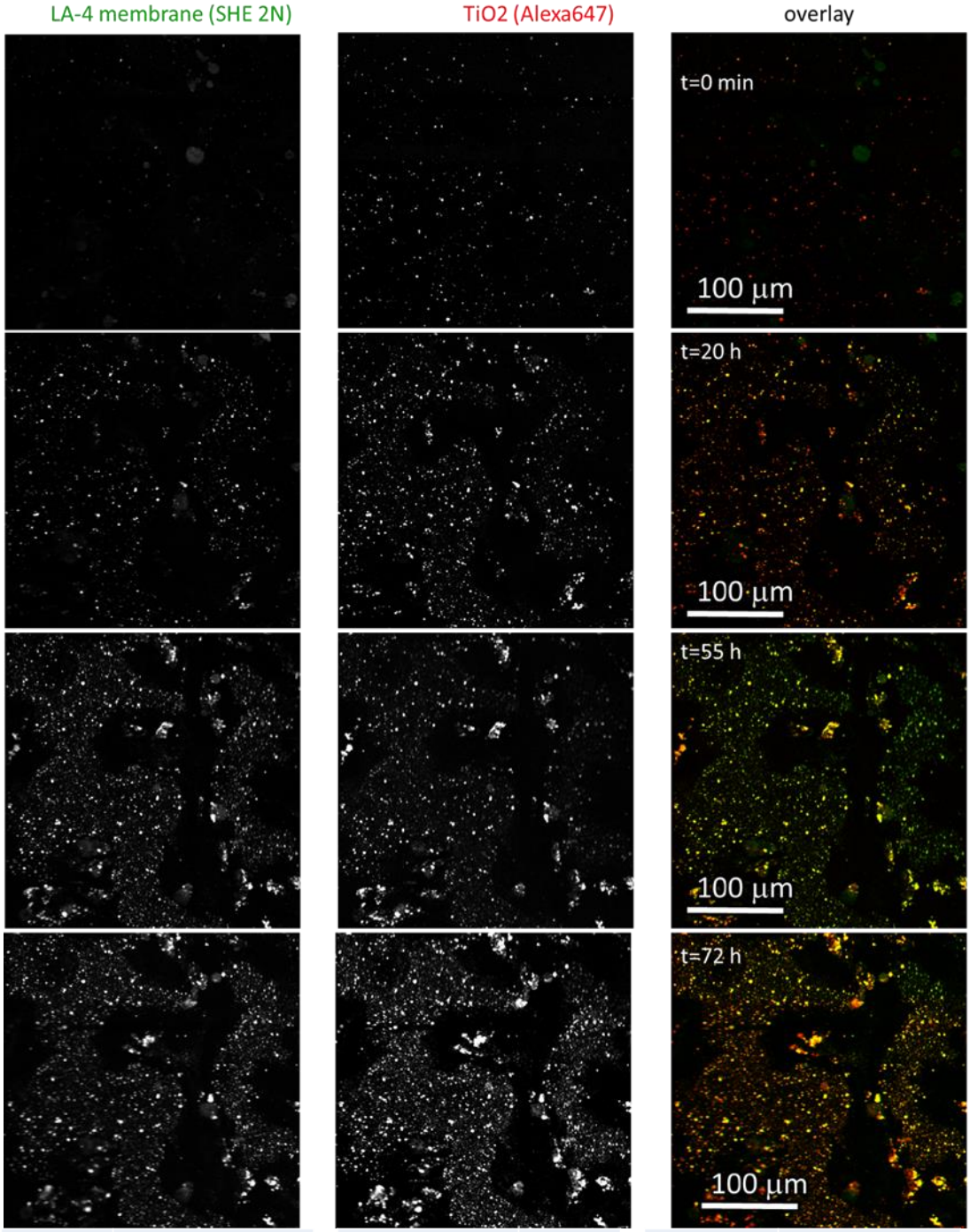
    - For the dynamics of movement analysis seventeen images with peak intensity and approximately five hour increments have been chosen for the analysis. They have been aligned in order to perform single cell tracking experiment with ImageJ' Manual tracking software. All data have been exported and analysed in excel (Fig 1.). Manual tracking plug-in enables users to overlay lines over chosen dots (MH-S in our case) and thus renders cell trajectories.

Experiment names

- 20190517_ e01 m01 s01 LA-4 & MH-S_SHE 2N exposed to TiO2 Alexa 647_72h_FRI-SAT_24h
- 20190517_ e01 m02 s01 LA-4 & MH-S_SHE 2N exposed to TiO2 Alexa 647_72h_SAT-SUN_48h
- 20190517_ e01 m03 s01 LA-4 & MH-S_SHE 2N exposed to TiO2 Alexa 647_SUN_48h_before crash
- 20190517_ e01 m03 s01 LA-4 & MH-S_SHE 2N exposed to TiO2 Alexa 647_MON_72h_ after the crash
- Seventeen representative images, which can be found in 20190517_e01 m01 s01 LA-4 & MH-S_SHE 2N exposed to 10 to 1 TiO_2 Alexa 647_72h_Raw folder are: 0, 43, 103, 156, 206, 226, 293, 359, 435, 500, 566, 643, 725, 784, 846, 902, 940

Controls and statistics

Figure S34



Cell line	LA-4 & MHS (membrane, SHE 2N)
NPs	TiO2 (Alexa647)
exposure	10:1, 0h-72h
imaging	xyz confocal, 72h

pixelsize (x,y)	300 nm
FOV (x,y)	400 µm
pixelsize (z)	/ nm
FOV (z)	/ µm
imaging time	72 hours
number of frames	1015

561nm	15%
640nm	15%
STED	%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	10x (NA0.3)

Figure S34: Co-culture of LA-4 and MH-S exposed to TiO_2 nanotubes for 72 h. MH-S are unable to phagocyte and remove all nanotubes from the epithelial layer after 72 h. See also Movie S1.

Link to time-lapse

- Macrophage activity in a coculture with epithelial cells over the course of 72h:
    - *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190517__e01_m01_s01_LA-4_&_MH-S_SHE_2N_exposed_to_TiO2_Alexa_647_72h_MH-S_tracking.gif*

Movie S1: Co-culture of LA-4 and MH-S exposed to TiO_2 nanotubes for 72 h. MH-S are unable to phagocyte and remove all nanotubes from the epithelial layer after 72 h. See also Figure S34.

S0i – Relevance of used exposure doses

In an occupational setting of the worst-case scenario, for example during an accidental release of powder material from bags of kg to tens of kg of material, an acute exposure to more than 100 mg is very likely to occur. To understand what kind of dose gets delivered to the lungs, we firstly need to understand the specific surface area of nanomaterials, which is often in the range of 100 m^2/g[11]. Taking into account the average human lung surface of about 100 m^2[12], uniform delivery of 100 mg of nanomaterial would thus correspond to a the ratio of Surface of NM to Surface of alveoli (S_{NM} /$S_{alveoli}$) being about 0.1:1. Due to the non-uniform distribution of material[13], local surface dose can easily reach 10:1 triggering strong inflammation response of entire organ and explaining why *in vitro* assays show comparable results to human exposure at 100-times greater dosages[13]. Therefore, the 10:1 surface dose used in our *in vitro* study corresponds to one-time inhalation of 100 mg of nanomaterials by human.

On the other hand, the same doses appear also during chronic exposures to polluted air due to accumulation and slow clearance of nanoparticles form the alveoli[14,15]. Namely, the WHO Air quality guideline values for Annual average exposure to fine particulate matter (PM2.5) is 10 $\mu g/m^3$[16], significantly exceeded in many cities around the globe[17,18]. With 6 to 8 liters of inhaled air per minute[19], and slow clearance of nanoparticles form the alveoli[14,15], this would result in human exposure of S_{NM} /$S_{alveoli}$ = 0.03 per year of constant exposure. As already stated, this would correspond to *in vitro* surface dose of 3:1 (reference) with local dose being even much higher, easily exceeding the dose of 10:1 used in this manuscript. 3

Instillation vs. inhalation

Although it can't be expected that single high dose applied by instillation will cause identical effects as a long-term inhalation exposure, there is good agreement between instillation and inhalation of nanoparticles with regards to pulmonary inflammation. Namely, in a recent rat study where we compared intratracheal instillation or 4-week inhalation, the dose-dependent inflammatory responses observed 28 days after exposure to different MWCNTs were very similar and much more material than application dependent[20]. Moreover, when comparing carbon black (Printex90) short term inhalation to instillation, the level and resolution kinetics of the inflammatory response coincide well (data not shown, manuscript in preparation). For CNTs, even slightly more severe effects have been described after inhalation as compared to aspiration[21]. We therefore believe that instillation exposure experiments are well-suited for mechanistical studies. Single exposures can lead to long lasting effects. For example, even after one single administration of different types of CNT (100 µg/mouse) the development of chronic granulomatous lung inflammation after 3 or 4 months has been described [22]. In addition, sub-acute inhalation and a single instillation of multi wall carbon nanotubes, which caused fibrosis at the highest dose, produced very similar inflammation and DNA damage responses[20].

Although dosages used in our *in vivo* data of about 50 µg/mouse, might be considered a high dose regarding its IT delivery within seconds. Nevertheless, a 5 day and 8 h per day exposure at a carbon black occupational exposure limit of 3.5 mg/m^3 could result in pulmonary carbon dose of 80 µg/mouse [23]. Considering the low alveolar clearance rate of less than 3% per 24 h for inhaled nanoparticles would result in an accumulated dose of about 50 µg/mouse after 5 days.[24] In general, we believe that IT dosage usually exceeds that of inhalation, however, long term response might be differently affected and Shvedova et al. demonstrated that aspiration of

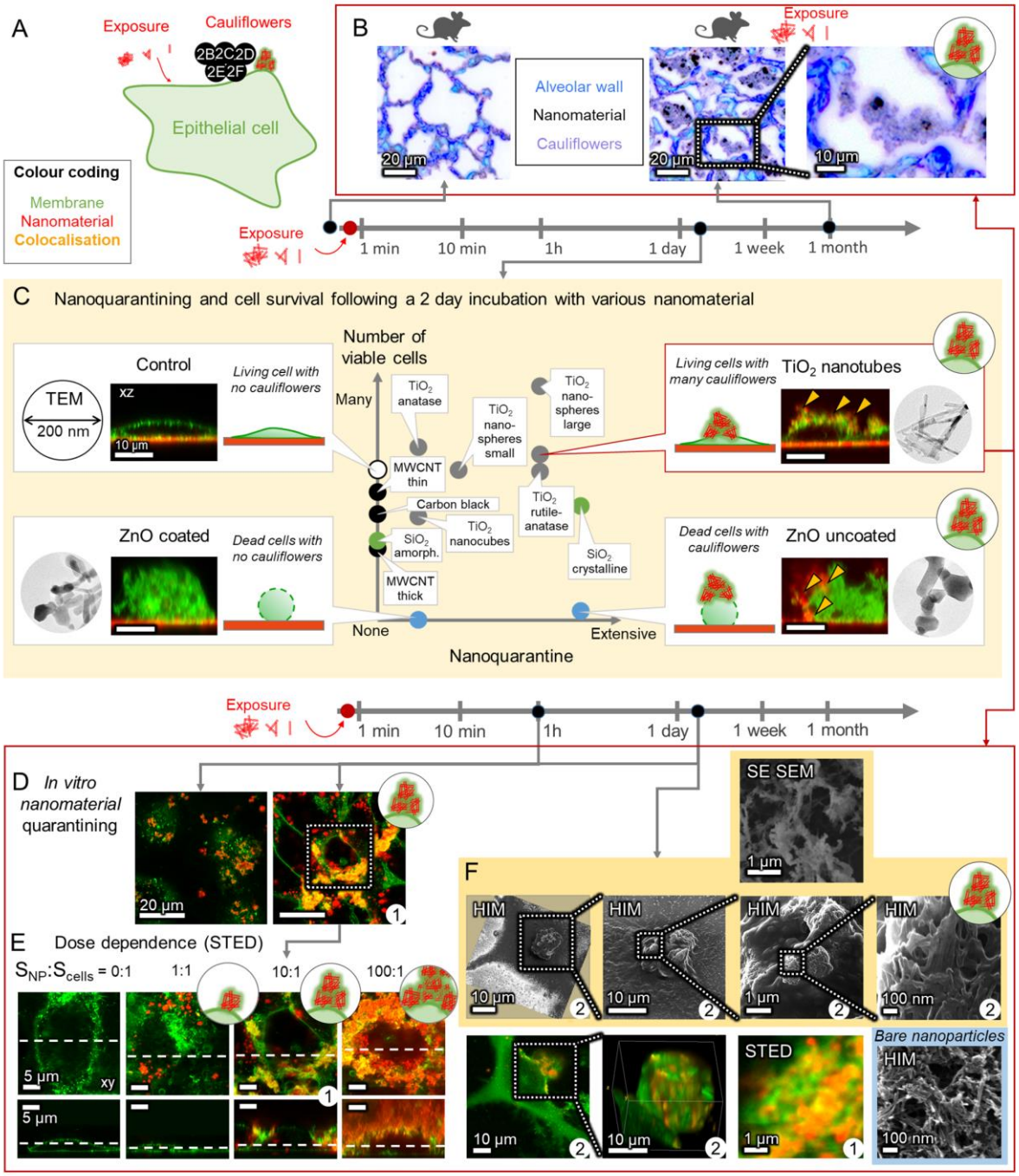
40 µg SWCNT induces a by far lower inflammatory response than inhalation of 5 µg of the same material one-year post exposure.[25] Moreover, the highest IT dose we used *in vivo* - 162 µg per mouse corresponds to an average surface dose of 3:1 $S_{nanomaterials}$:S_{cells} and is equivalent to 15 working days at the 8-h time-weighted average occupational exposure limit for TiO_2 by Danish Regulations (6.0 mg/m^3 TiO_2).

--- Supplementary information for experiments in Figures 2 – 6 in the main text ---

From here on, detailed supplementary material for each image from the main text is shown. It contains details on experimental design, controls and repetitions (fluorescence images shown both in separate channels and overlayed), and names (cyphers) of experiments to ease locating them in the depository/database.

We first supply the main text image duplicates for easier orientation. The supplement sections correspond to the panels in the main text. For example, section S3c in the supplement corresponds to the experiment depicted in Figure 2c in the main text.

S2 – Quarantining of nanomaterials



S2b – *In vivo* data

Main message

One month after intratracheal instillation in mice of TiO_2 tube, nanomaterial was mainly seen in bio-nano composites in alveolar spaces and in macrophages.

Supporting raw and analysed data:
For 28 days:

Figure S35

Materials and methods

The materials and methods used for intratracheal instillation of mice with TiO_2 tube are described in detail by Danielsen et. al[5] and included here in an abbreviated version.

Preparation and characterization of TiO_2 tube suspensions

For TiO_2 tube characterization see nanomaterial characterization section in S0b – Nanomaterial and [1].

The TiO_2 tube was suspended in nanopure water with 2 % v/v mouse serum (prepared in-house) to a final concentration of 3.24 mg/mL. The suspension was probe sonicated on ice for 16 min with 10 % amplitude. 3.24 mg/mL corresponds to a dose of 162 µg TiO_2 tube per 50 µL instillation volume per mice. The vehicle of nanopure water with 2 % v/v mouse serum was probe sonicated using the same protocol. The dose of 162 µg/mouse corresponds to an average surface dose of 3:1 $S_{nanomaterials}$:S_{cells} and is equivalent to 15 working days at the 8-h time-weighted average occupational exposure limit for TiO_2 by Danish Regulations (6.0 mg/m^3 TiO_2).

The average hydrodynamic particle size of the TiO_2 tube in suspension (3.24 mg/mL) was determined by Dynamic Light Scattering (DLS). The TiO_2 tube suspension had a bimodal size distribution with a major peak at 60 nm and a narrow peak at 21 nm[5]. The intensity-based z-average size was 168.7 nm and the polydispersity index (PI) was 0.586, indicating some polydispersity in the suspensions. Endotoxin levels were measured using the Limulus Amebocyte Lysate Assay. The level of endotoxins was low in TiO_2 tube suspensions (0.095 endotoxin units (EU)/mL), and in nanopure water with 2 % mouse serum (0.112 EU/mL).

Animal handling and exposure

Seven-week-old female C57BL/6jBomtac mice (Taconic, Ejby, Denmark) were randomized in groups for TiO_2 tube exposure (N=5 mice/group for histology) and vehicle controls (N = 2-4 mice/group). At 8 weeks of age the mice were anaesthetized and exposed to 0 µg or 162 µg TiO_2 tube in 50 µL vehicle by single intratracheal instillation. In brief, the mice were intubated in the trachea using a catheter. The 50 µl suspension was instilled followed by 200 µL air. The mouse was transferred to a vertical hanging position with the head up. This ensures that the administered material is maintained in the lung. Animal experiments were performed according to EC Directive 2010/63/UE in compliance with the handling guidelines established by the Danish government and permits from the Experimental Animal Inspectorate (no. 2015-15-0201-00465 and 2010/561-1779). Prior to the study, the experimental protocols were approved by the local Animal Ethics Council.

More details regarding the animal study can be found in Danielsen et al.[5].

Histology and enhanced darkfield imaging

At 28, 90 or 180 days post-exposure mice were weighed and anesthetized. Lungs were filled slowly with 4% formalin under 30 cm water column pressure. A knot was made on the trachea to secure formaldehyde in lungs to fixate tissue in “inflated state”. Lungs were then removed and placed in 4% neutral buffered formaldehyde for 24 hours. After fixation the samples were trimmed, dehydrated and embedded in paraffin. 3 µm thin sections were cut and stained with haematoxylin and eosin (H&E). Cytoviva enhanced darkfield hyperspectral system (Auburn, AL, USA) was used to image particles and organic debris in the histological sections of mouse lungs. Enhanced darkfield images were acquired at 100x on an Olympus BX 43 microscope with a Qimaging Retiga4000R camera.

Controls and statistics

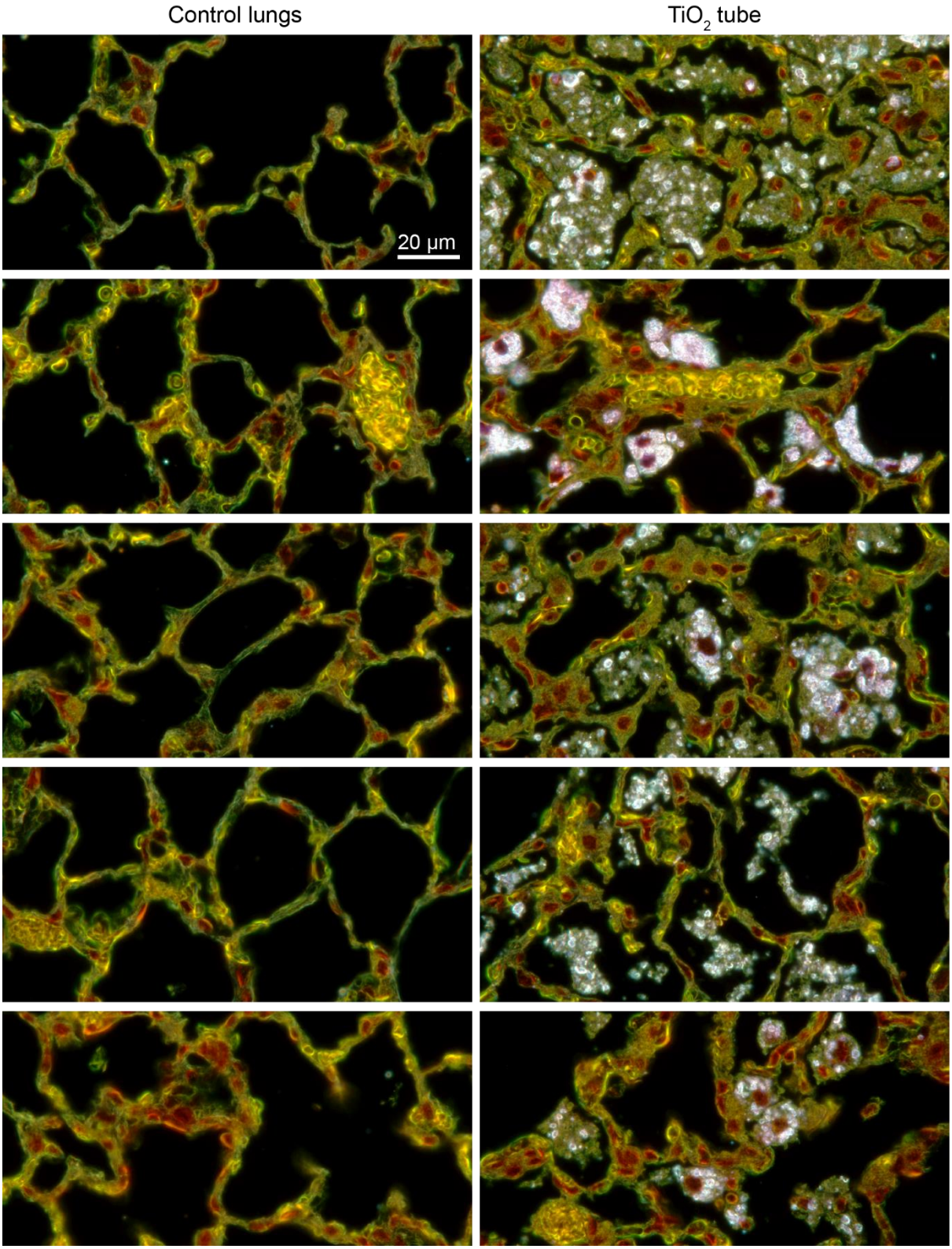


Figure S35: Alveoli of murine lungs one month after intratracheal instillation of control vehicle or TiO_2 tube nanomaterial (white). TiO_2 tube nanomaterial was mainly seen in macrophages and in bio-nano composites in alveolar spaces and often close to epithelial cells of the alveolar wall. Enhanced darkfield microscopy of H&E stained histological tissue sections.

S2c – Cauliflowers with various nanomaterial

Main message

Different nanomaterials exhibit different surface structures and viability of LA-4 cells – from large surface structures to none. Toxicity of the same nanomaterials is examined in "S0g – Cell viability LA-4 – various nanomaterials, same surface dose" and the TEM images of the nanomaterials are shown in section "S0b – Nanomaterial ".

Materials and methods

- Experiment
  - Cells were grown up to 80% confluency in Ibidi #1.5H µ-Slide 8-well chambers for 24h which was followed by exposure to various nanomaterials at a 10 : 1 (S_{nano}:S_{cells}) surface dose
  - nanomaterials used in experiment are in the Table S2
  - detailed information on used nanomaterial is provided in SXy

Table S3: List of nanomaterials exposed on LA-4 cells in this study with their specific β-BET surface [m^2/g]. Detailed information about used materials is available in section S0b in Table S1.

Name	official ID - name	Nanomaterial code	β-BET surface [m^2/g]
TiO_2 nanotubes	TiO_2 nanotubes	PU-nTOX-01-03	152
SiO_2 DQ12	Quartz DQ12	/	10,1
Printex 90	Printex 90, carbon black	/	300
TiO_2 nanocubes	TiO_2 nanocubes	PU-nTOX-01-21	97
TiO_2 rut-an NM-105	NM105 TiO2 rut-anat	TiO2-NM105-JRCNM01005a	46
TiO_2 anat MKNA015	MKNA015	MKN-TiO2-A015	74
TiO_2 anat MKNA100	MKNA100	MKN-TiO2-A100	85
TiO_2 an NM-101	NM101 TiO2 anatase	TiO2-NM101-JRCNM01001a	230
SiO_2 NM-200	NM200 Silica	SiO2-NM200-JRCNM02000a	189
MWCNT 402	NM402 MWCNT	MWCNTs-NM402-JRCNM04002a	226
MWCNT 401	NM401 SWCNT	MWCNTs-NM401-JRCNM04001a	18
ZnO NM-111	NM111 ZnO	ZnO-NM111-JRCNM01101a	12
ZnO raw NM-110	noncoated ZnO	ZnO-NM110-JRCNM62101a	15

- cells were incubated with nanomaterials for additional 48h
- preparation for imaging:
  - after incubation cell media was exchanged for 150 µL of fresh medium with 5 µg mL^{-1} of Cell Mask Orange and incubated for additional 10 min
  - cells were then washed 1x200 µL LCIS and mixture of 50%LCIS and 50%F12-K medium was added for imaging
  - samples were held on the microscope stage heated at 37 °C for 2h

- Imaging:
  - To get an unbiased overview of the sample we used a home-made script which semi-randomly selected all the imaging places. On each well, we'd select three regions of interest. In these regions, the first image was of our choosing remaining five images were randomly selected by our script. Each well is thus, represented with 18 images.
  - The extent of the surface structures was determined according to the cauliflower forming potential scale (CFP) devised in our lab (Table S3). Samples were evaluated during the imaging.

Table S4: Cauliflower forming potential scale used for sample description

CFP	Description
0	no cauliflowers in 1 cm^2
1	few cauliflowers per 1 cm^2
2	up to 1 % cells have small cauliflowers
3	up to 10% cells have small cauliflowers
4	most cells have small cauliflowers OR up to 10% cells have large cauliflowers
5	most cells have large cauliflowers

According to the CFP scale all evaluated nanomaterial have been given a CFP score presented in the Table S4

Table S5: CFP score of exposed LA-4 cells for all the nanomaterials used in this study

Name	**β-BET surface [m^2/g]**	**min CFP [/5]**	**Max CFP [/5]**	**total CFP**

SiO2 DQ12	10,1	2	5	5
ZnO raw NM-110	12	x	x	x
ZnO NM-111	15	x	x	x
TiO2 anatase MKNA100	85	4	4	4
MWCNT 401	18	0	0	0
TiO2 rut-anat NM-105	46	4	4	4
TiO2 NQs	97	1	1	1
TiO2 NTs	152	2	4	4
SiO2 NM-200	189	0	0	0
MWCNT 402	226	0	0	0
TiO2 anatase NM-101	230	1	1	1
TiO2 anatase MKNA015	74	2	2	2
Printex 90	300	0	0	0
Sham Control	-	-	-	-
Control	-	-	-	-

Experiment names

CTRL >

20191129_exp08_LA-4-CMO_ctrl-48h-3-pos_0_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp08_LA-4-CMO_ctrl-48h-3-pos_0_xzy_MC-PMT_AC_8bit_ovrly1
20191129_exp08_LA-4-CMO_ctrl-48h-2-pos_0_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp08_LA-4-CMO_ctrl-48h-2-pos_0_xzy_MC-PMT_AC_8bit_ovrly1
20191129_exp08_LA-4-CMO_ctrl-48h-2-pos_5_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp08_LA-4-CMO_ctrl-48h-2-pos_5_xzy_MC-PMT_AC_8bit_ovrly1

Sham >

20191129_exp15_LA-4-CMO_sham-48h-1-pos_4_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp15_LA-4-CMO_sham-48h-1-pos_4_xzy_MC-PMT_AC_8bit_ovrly1
20191129_exp15_LA-4-CMO_sham-48h-1-pos_5_xzy_MC-PMT_AC_8bit_ovrly1
20191129_exp15_LA-4-CMO_sham-48h-3-pos_0_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp15_LA-4-CMO_sham-48h-3-pos_0_xzy_MC-PMT_AC_8bit_ovrly1

TiO2 NTs >

20191129_exp01_LA-4-CMO_TiO2-NT-N-48h-4-pos_4_xy_MC-PMT_AC_8bit_ovrly1

20191129_exp01_LA-4-CMO_TiO2-NT-N-48h-4-pos_4_yzx_MC-PMT_AC_8bit_ovrly1

20191129_exp01_LA-4-CMO_TiO2-NT-N-48h-4-pos_3_xy_MC-PMT_AC_8bit_ovrly1

20191129_exp01_LA-4-CMO_TiO2-NT-N-48h-4-pos_3_xzy_MC-PMT_AC_8bit_ovrly1

20191129_exp01_LA-4-CMO_TiO2-NT-N-48h-6-pos_0_xy_MC-PMT_AC_8bit_ovrly1

20191129_exp01_LA-4-CMO_TiO2-NT-N-48h-6-pos_0_xzy_MC-PMT_AC_8bit_ovrly1

DQ12 >

20191129_exp02_LA-4-CMO_DQ12-N-48h-1-pos_0_xy_MC-PMT_AC_8bit_ovrly1

20191129_exp02_LA-4-CMO_DQ12-N-48h-1-pos_0_xzy_MC-PMT_AC_8bit_ovrly1

20191129_exp02_LA-4-CMO_DQ12-N-48h-3-pos_5_xy_MC-PMT_AC_8bit_ovrly1

20191129_exp02_LA-4-CMO_DQ12-N-48h-3-pos_5_xzy_MC-PMT_AC_8bit_ovrly1

20191129_exp02_LA-4-CMO_DQ12-N-48h-1-pos_1_xy_MC-PMT_AC_8bit_ovrly1

20191129_exp02_LA-4-CMO_DQ12-N-48h-3-pos_5_yzx_MC-PMT_AC_8bit_ovrly1

Printex 90 >

20191129_exp03_LA-4-CMO_Printex90-N-48h-3-pos_0_xy_MC-PMT_AC_8bit_ovrly1

20191129_exp03_LA-4-CMO_Printex90-N-48h-3-pos_0_xzy_MC-PMT_AC_8bit_ovrly1

20191129_exp03_LA-4-CMO_Printex90-N-48h-1-pos_4_xy_MC-PMT_AC_8bit_ovrly1

20191129_exp03_LA-4-CMO_Printex90-N-48h-1-pos_4_xzy_MC-PMT_AC_8bit_ovrly1

20191129_exp03_LA-4-CMO_Printex90-N-48h-2-pos_1_xy_MC-PMT_AC_8bit_ovrly1

20191129_exp03_LA-4-CMO_Printex90-N-48h-2-pos_1_xzy_MC-PMT_AC_8bit_ovrly1

TiO2 NQs >

20191129_exp04_LA-4-CMO_TiO2-NQs-N48h-3-pos_3_xy_MC-PMT_AC_8bit_ovrly1

20191129_exp04_LA-4-CMO_TiO2-NQs-N48h-3-pos_3_xzy_MC-PMT_AC_8bit_ovrly1

20191129_exp04_LA-4-CMO_TiO2-NQs-N48h-2-pos_3_xy_MC-PMT_AC_8bit_ovrly1

20191129_exp04_LA-4-CMO_TiO2-NQs-N48h-2-pos_3_yzx_MC-PMT_AC_8bit_ovrly1

20191129_exp04_LA-4-CMO_TiO2-NQs-N48h-4-pos_3_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp04_LA-4-CMO_TiO2-NQs-N48h-4-pos_3_xzy_MC-PMT_AC_8bit_ovrly1

MWCNT-401 >
20191129_exp06_LA-4-CMO_MWCNT-NM401-N-48h-1-pos_0_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp06_LA-4-CMO_MWCNT-NM401-N-48h-1-pos_0_xzy_MC-PMT_AC_8bit_ovrly1
20191129_exp06_LA-4-CMO_MWCNT-NM401-N-48h-2-pos_5_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp06_LA-4-CMO_MWCNT-NM401-N-48h-2-pos_5_yzx_MC-PMT_AC_8bit_ovrly1
20191129_exp06_LA-4-CMO_MWCNT-NM401-N-48h-3-pos_4_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp06_LA-4-CMO_MWCNT-NM401-N-48h-3-pos_4_yzx_MC-PMT_AC_8bit_ovrly1

MWCNT-402 >
20191129_exp05_LA-4-CMO_MWCNT-NM402-N-48h-3-pos_1_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp05_LA-4-CMO_MWCNT-NM402-N-48h-3-pos_1_xzy_MC-PMT_AC_8bit_ovrly1
20191129_exp05_LA-4-CMO_MWCNT-NM402-N-48h-2-pos_0_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp05_LA-4-CMO_MWCNT-NM402-N-48h-2-pos_0_xzy_MC-PMT_AC_8bit_ovrly1
20191129_exp05_LA-4-CMO_MWCNT-NM402-N-48h-1-pos_1_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp05_LA-4-CMO_MWCNT-NM402-N-48h-1-pos_1_yzx_MC-PMT_AC_8bit_ovrly1

ZnO-NM111 >
20191129_exp07_LA-4-CMO_ZnO-coat-NM111-N-48h-1-pos_0_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp07_LA-4-CMO_ZnO-coat-NM111-N-48h-1-pos_0_xzy_MC-PMT_AC_8bit_ovrly1
20191129_exp07_LA-4-CMO_ZnO-coat-NM111-N-48h-2-pos_0_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp07_LA-4-CMO_ZnO-coat-NM111-N-48h-2-pos_0_xzy_MC-PMT_AC_8bit_ovrly1
20191129_exp07_LA-4-CMO_ZnO-coat-NM111-N-48h-2-pos_3_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp07_LA-4-CMO_ZnO-coat-NM111-N-48h-2-pos_3_xzy_MC-PMT_AC_8bit_ovrly1

ZnO-Raw >
20191129_exp14_LA-4-CMO_ZnOraw-NM110-N-48h-3-pos_2_xy_MC-PMT_AC_8bit_ovrly1

20191129_exp14_LA-4-CMO_ZnOraw-NM110-N-48h-3-pos_2_xzy_MC-PMT_AC_8bit_ovrly1
20191129_exp14_LA-4-CMO_ZnOraw-NM110-N-48h-3-pos_0_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp14_LA-4-CMO_ZnOraw-NM110-N-48h-3-pos_0_xzy_MC-PMT_AC_8bit_ovrly1
20191129_exp14_LA-4-CMO_ZnOraw-NM110-N-48h-1-pos_2_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp14_LA-4-CMO_ZnOraw-NM110-N-48h-1-pos_2_yzx_MC-PMT_AC_8bit_ovrly1

TiO2-anatase L MKNA 100 >
20191129_exp10_LA-4-CMO_TiO2anatL-MKNA100-N-48h-3-pos_2_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp10_LA-4-CMO_TiO2anatL-MKNA100-N-48h-3-pos_2_xzy_MC-PMT_AC_8bit_ovrly1
20191129_exp10_LA-4-CMO_TiO2anatL-MKNA100-N-48h-3-pos_1_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp10_LA-4-CMO_TiO2anatL-MKNA100-N-48h-3-pos_1_xzy_MC-PMT_AC_8bit_ovrly1
20191129_exp10_LA-4-CMO_TiO2anatL-MKNA100-N-48h-3-pos_4_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp10_LA-4-CMO_TiO2anatL-MKNA100-N-48h-3-pos_4_yzx_MC-PMT_AC_8bit_ovrly1

TiO2-tutile-anatase-MKNA 105 >
20191129_exp11_LA-4-CMO_TiO2rut-anat-NM105-N-48h-2-pos_3_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp11_LA-4-CMO_TiO2rut-anat-NM105-N-48h-2-pos_3_xzy_MC-PMT_AC_8bit_ovrly1
20191129_exp11_LA-4-CMO_TiO2rut-anat-NM105-N-48h-1-pos_2_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp11_LA-4-CMO_TiO2rut-anat-NM105-N-48h-1-pos_2_xzy_MC-PMT_AC_8bit_ovrly1
20191129_exp11_LA-4-CMO_TiO2rut-anat-NM105-N-48h-1-pos_0_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp11_LA-4-CMO_TiO2rut-anat-NM105-N-48h-1-pos_0_xzy_MC-PMT_AC_8bit_ovrly1

TiO2-anatase-NM101 >
20191129_exp13_LA-4-CMO_TiO2-anat-NM101-N-48h-3-pos_4_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp13_LA-4-CMO_TiO2-anat-NM101-N-48h-3-pos_4_xzy_MC-PMT_AC_8bit_ovrly1
20191129_exp13_LA-4-CMO_TiO2-anat-NM101-N-48h-1-pos_4_xy_MC-PMT_AC_8bit_ovrly1
20191129_exp13_LA-4-CMO_TiO2-anat-NM101-N-48h-1-pos_3_yzx_MC-PMT_AC_8bit_ovrly1
20191129_exp13_LA-4-CMO_TiO2-anat-NM101-N-48h-1-pos_0_xy_MC-PMT_AC_8bit_ovrly1

20191129_exp13_LA-4-CMO_TiO2-anat-NM101-N-48h-1-pos_0_yzx_MC-PMT_AC_8bit_ovrly1

SiO2 NM200 >

20191129_exp12_LA-4-CMO_SiO2-NM200-N-48h-3-pos_0_xy_MC-PMT_AC_8bit_ovrly1

20191129_exp12_LA-4-CMO_SiO2-NM200-N-48h-3-pos_0_yzx_MC-PMT_AC_8bit_ovrly1

20191129_exp12_LA-4-CMO_SiO2-NM200-N-48h-2-pos_0_xy_MC-PMT_AC_8bit_ovrly1

20191129_exp12_LA-4-CMO_SiO2-NM200-N-48h-2-pos_0_xzy_MC-PMT_AC_8bit_ovrly1

20191129_exp12_LA-4-CMO_SiO2-NM200-N-48h-1-pos_4_xy_MC-PMT_AC_8bit_ovrly1

20191129_exp12_LA-4-CMO_SiO2-NM200-N-48h-1-pos_4_xy_MC-PMT_AC_8bit_ovrly1

Control and statistics

LA-4 cells exposed to 10 to 1 surface dose of various nanomaterials.

Figures S 36-49

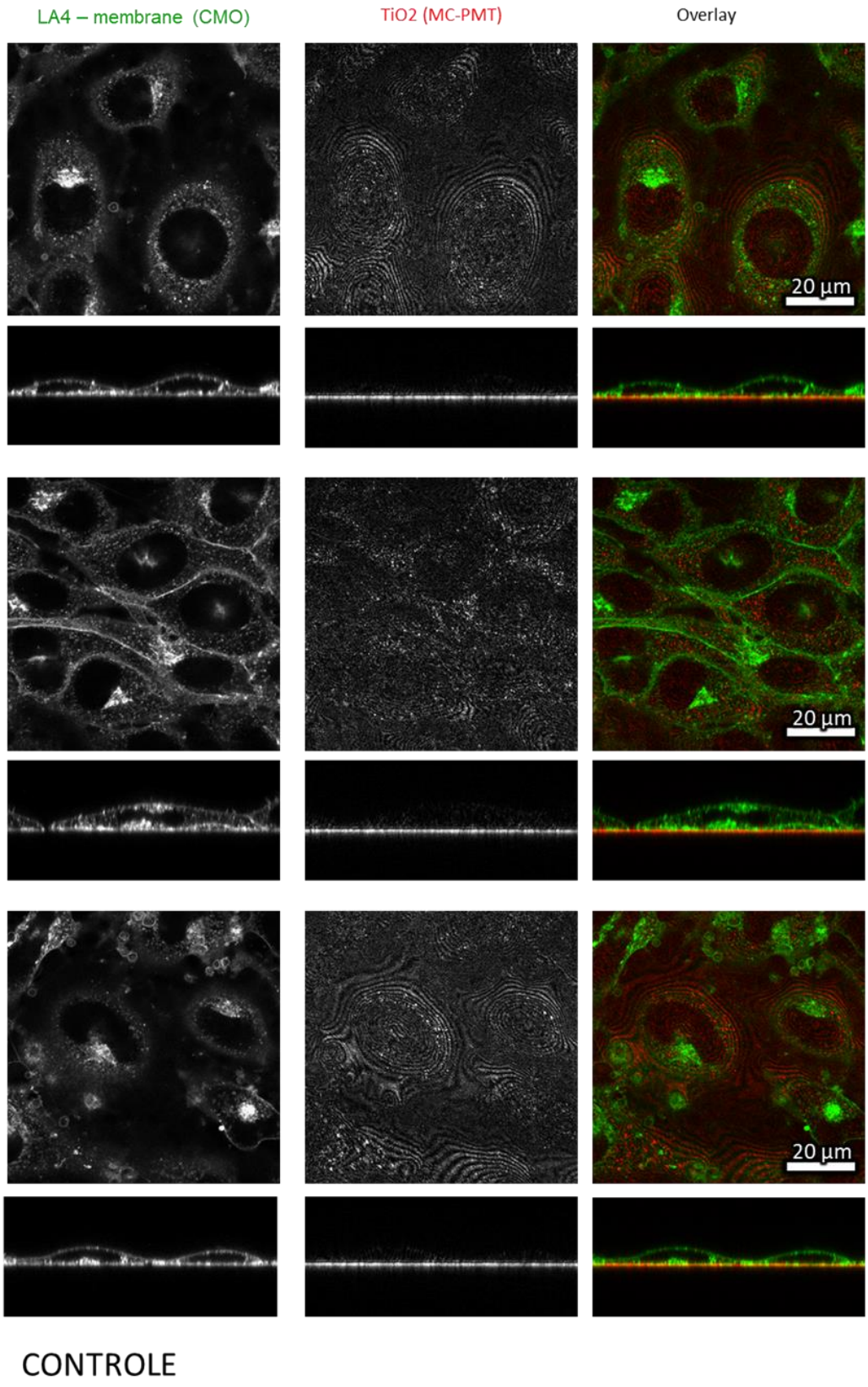


Figure S36: Confocal images of unexposed LA-4 cells after 48 h of incubation in xy plane (upper windows) and xz plane (lower windows)

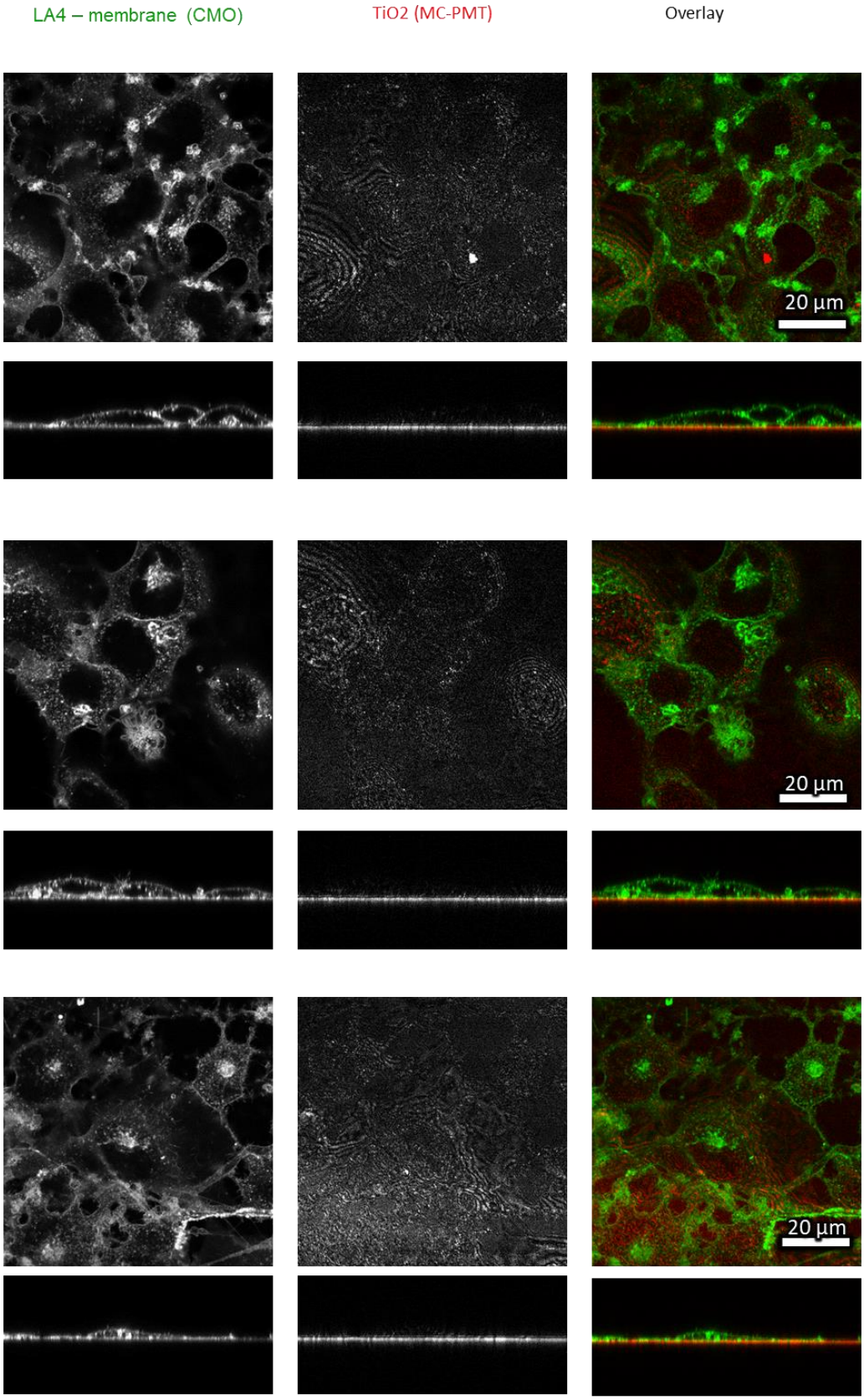


Figure S37: Confocal images of unexposed LA-4 cells after 48 h of incubation in tip sonicated medium in xy plane (upper windows) and xz plane (lower windows)

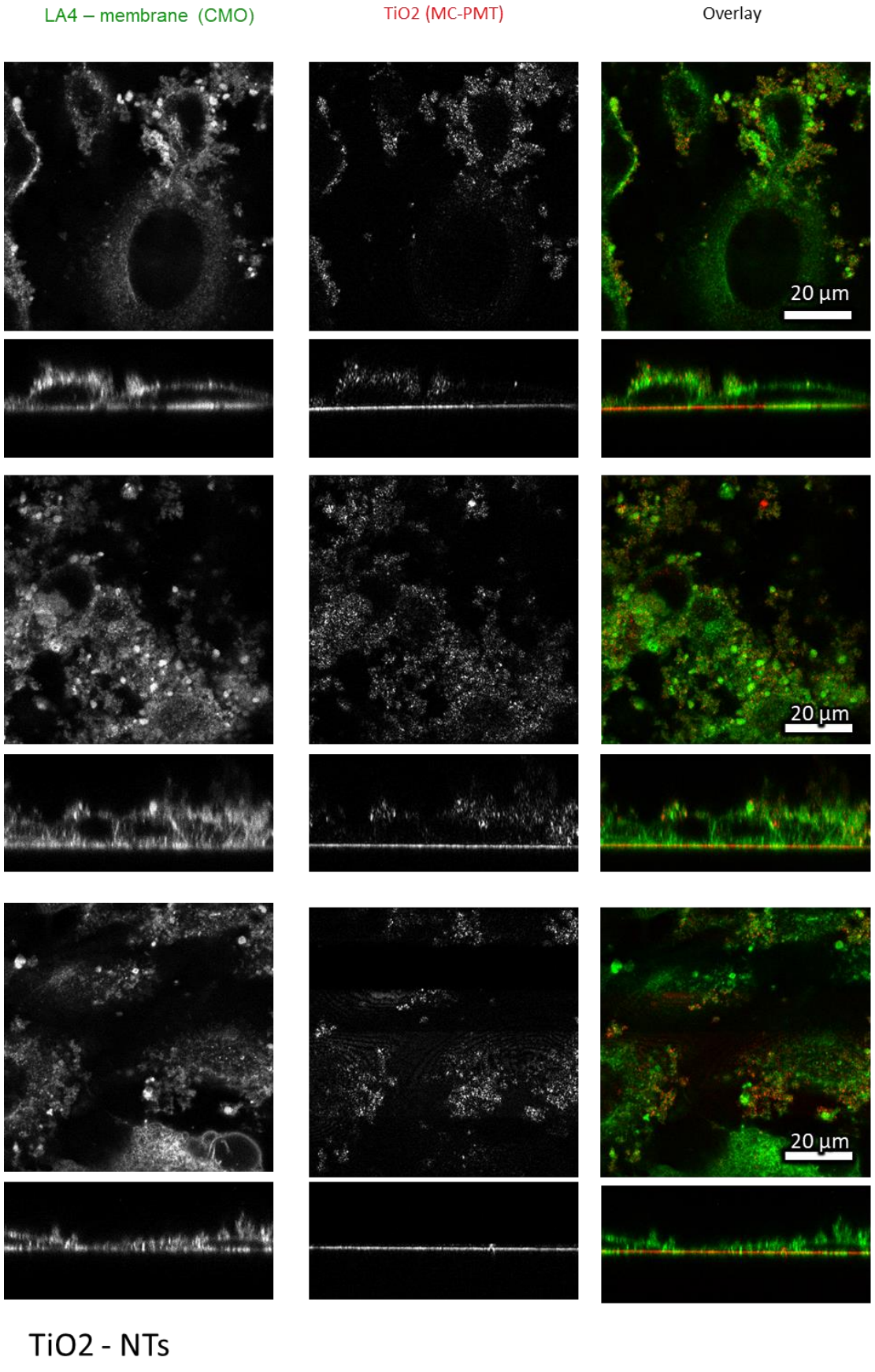


Figure S38: Confocal images of LA-4 cells exposed to 10 to 1 surface dose of TiO_2 nanotubes for 48 h in xy plane (upper windows) and xz plane (lower windows)

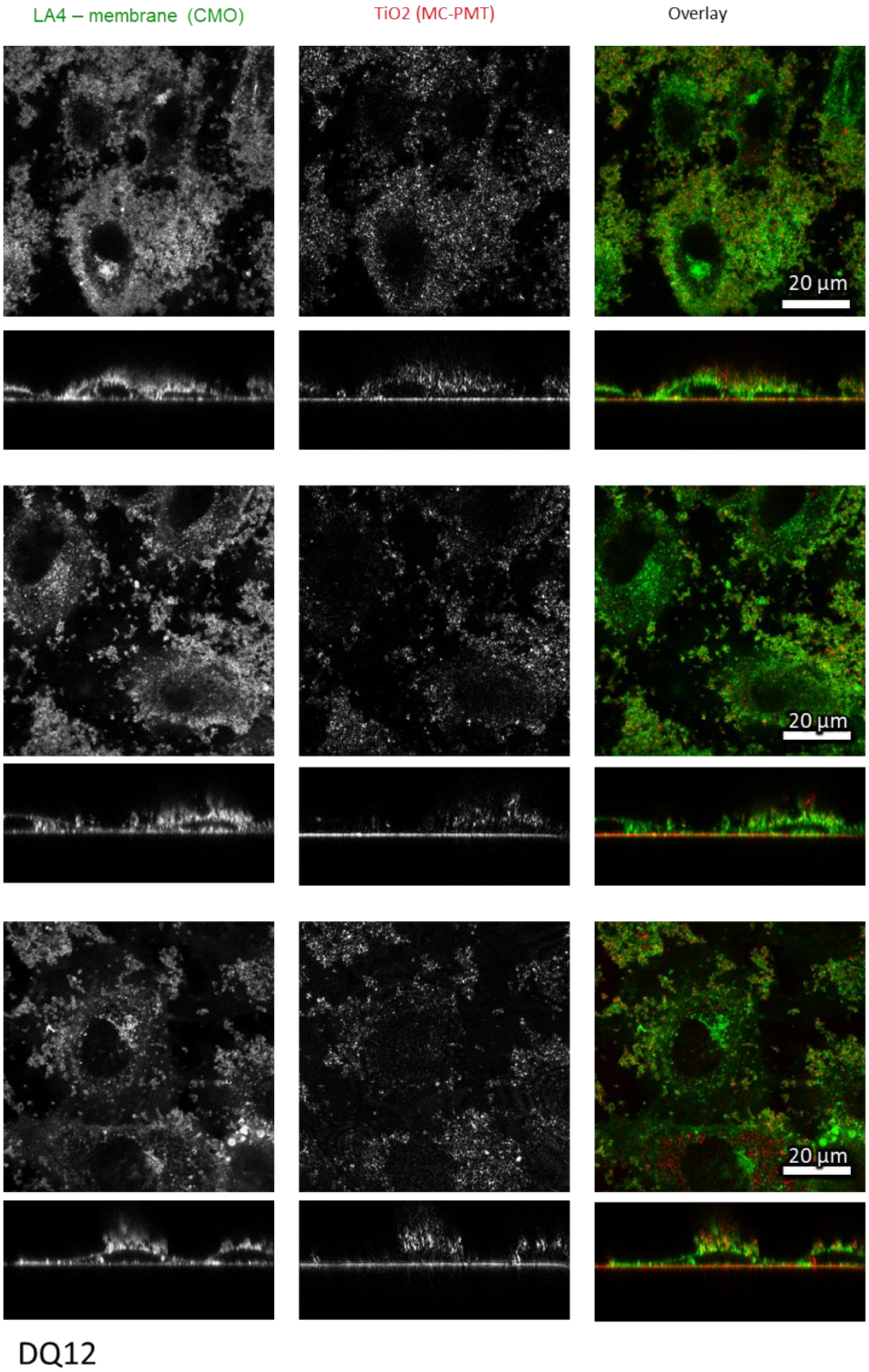


Figure S39: Confocal images of LA-4 cells exposed to 10 to 1 surface dose of DQ12 for 48 h in xy plane (upper windows) and xz plane (lower windows)

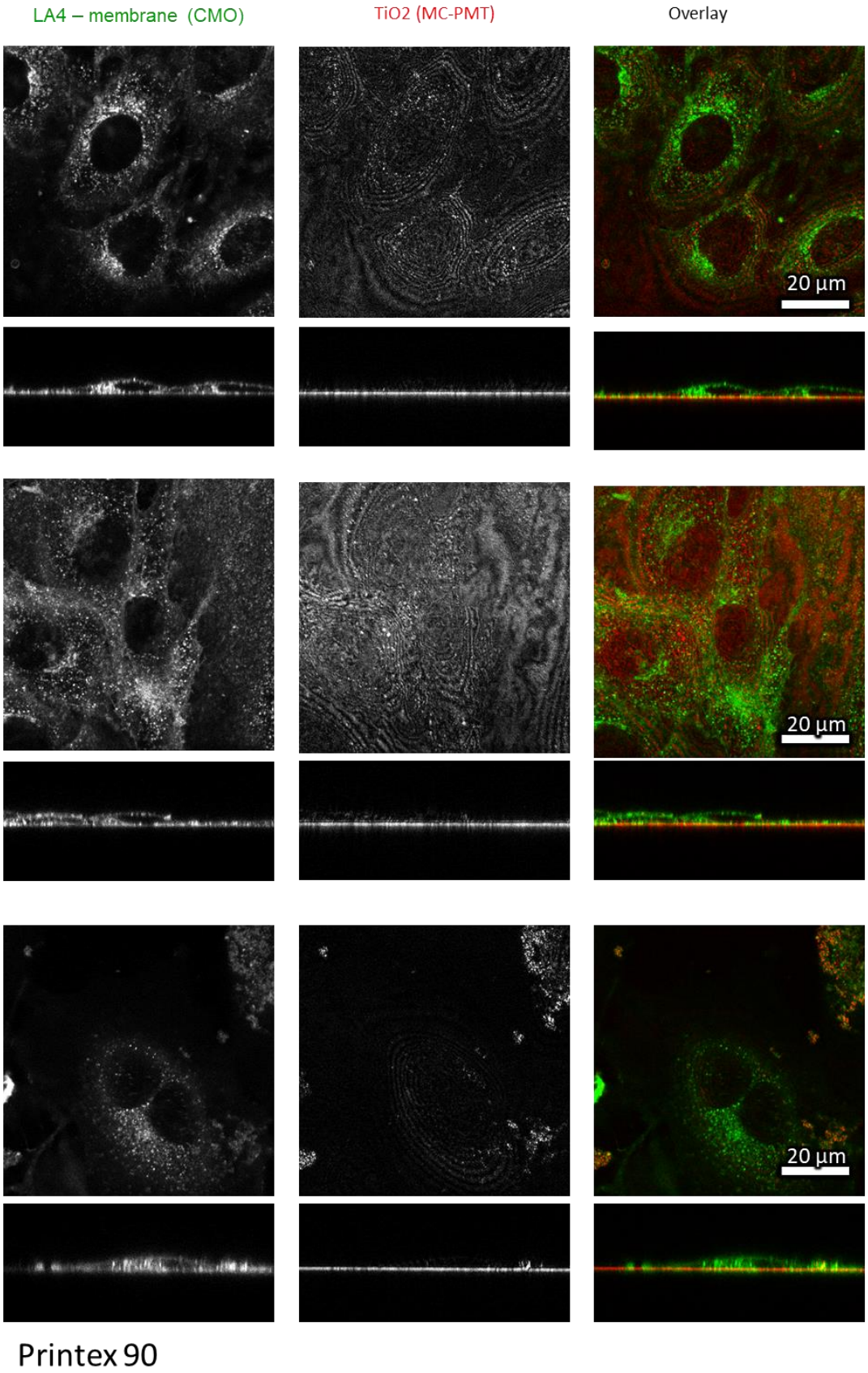


Figure S40: Confocal images of LA-4 cells exposed to 10 to 1 surface dose of Printex 90 for 48 h in xy plane (upper windows) and xz plane (lower windows)

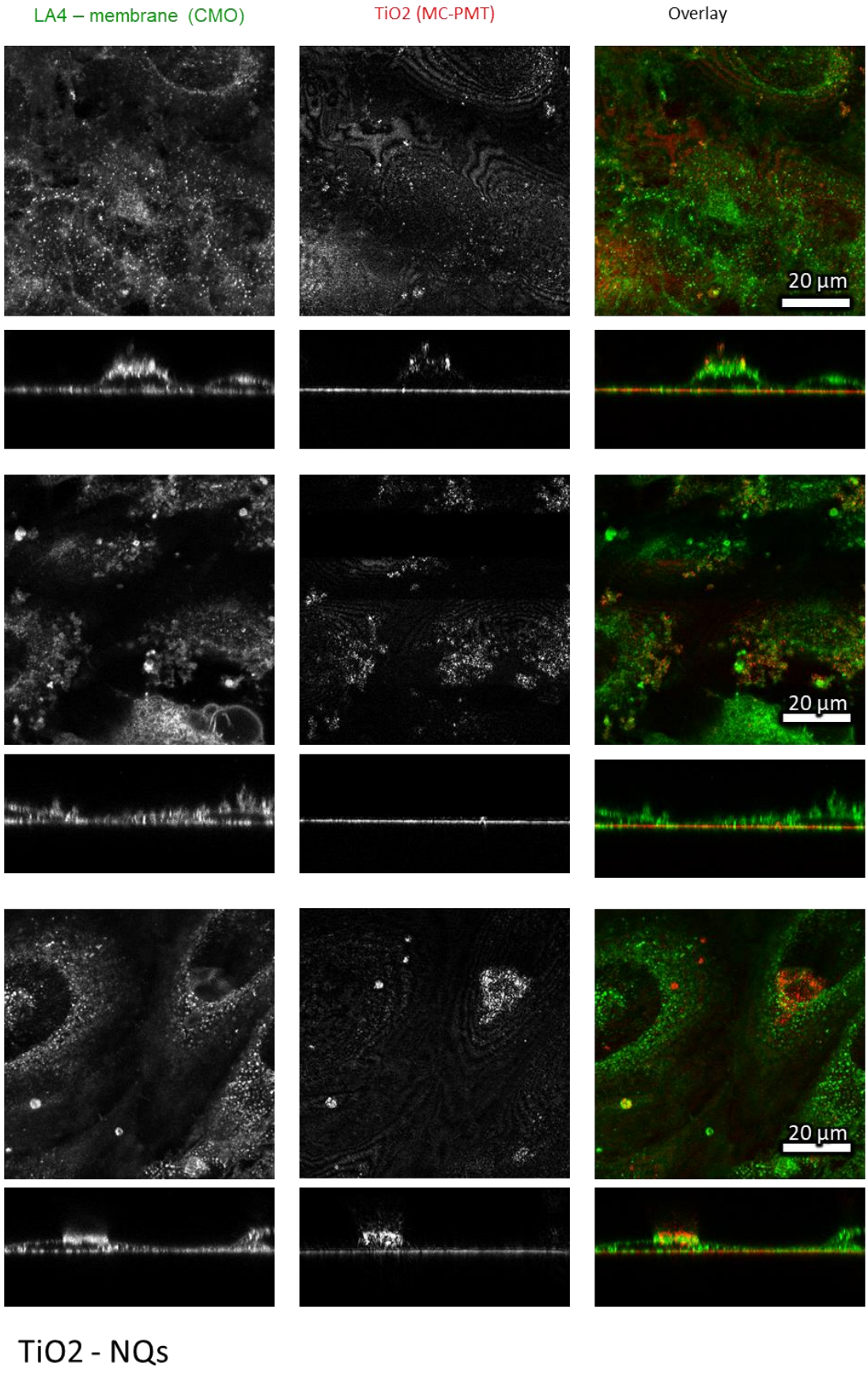


Figure S41: Confocal images of LA-4 cells exposed to 10 to 1 surface dose of TiO_2 nanoqubes for 48 h in xy plane (upper windows) and xz plane (lower windows)

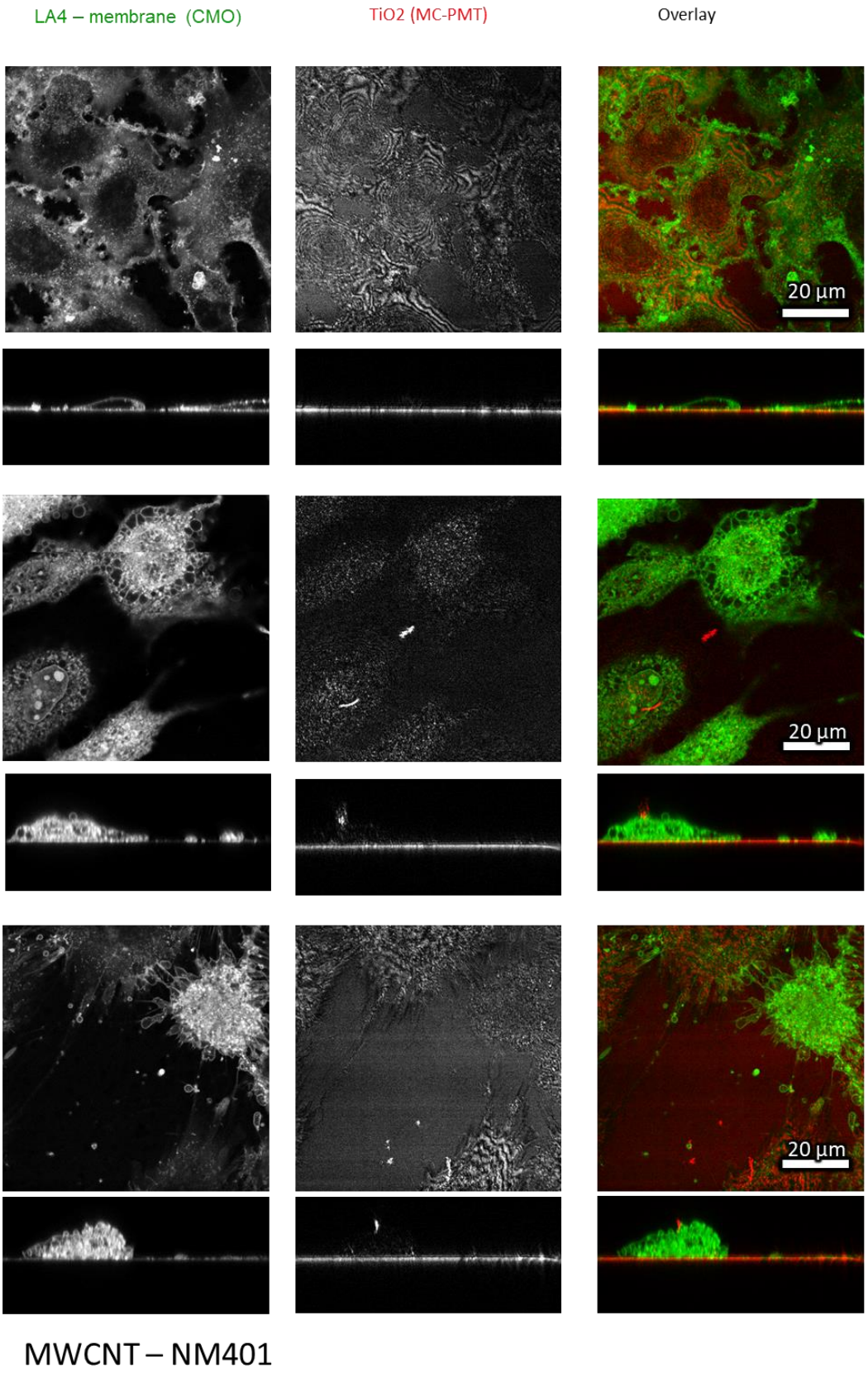


Figure S42: Confocal images of LA-4 cells exposed to 10 to 1 surface dose of MWCNT-NM401 for 48 h in xy plane (upper windows) and xz plane (lower windows)

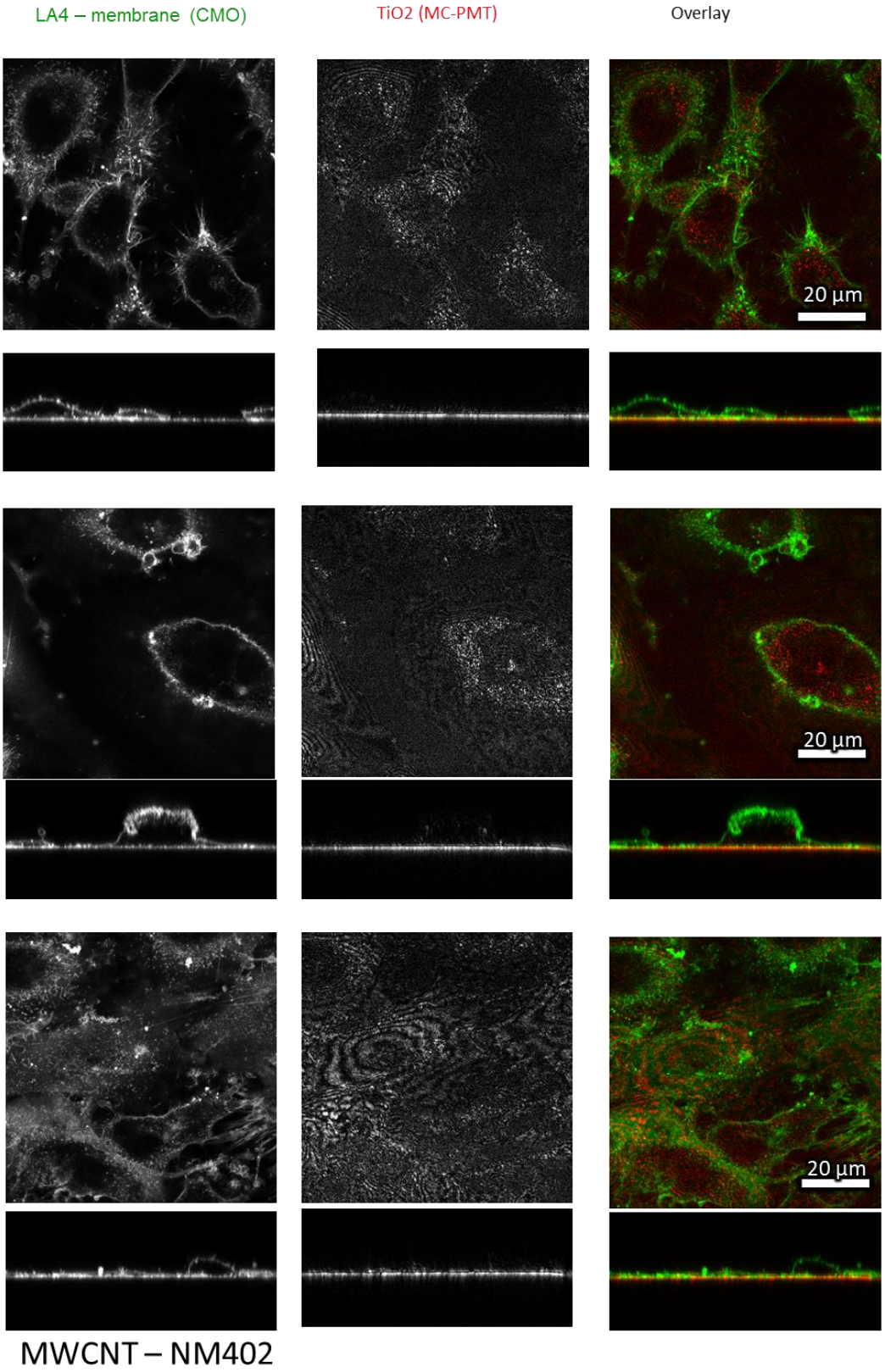


Figure S43: Confocal images of LA-4 cells exposed to 10 to 1 surface dose of MWCNT-NM402 for 48 h in xy plane (upper windows) and xz plane (lower windows)

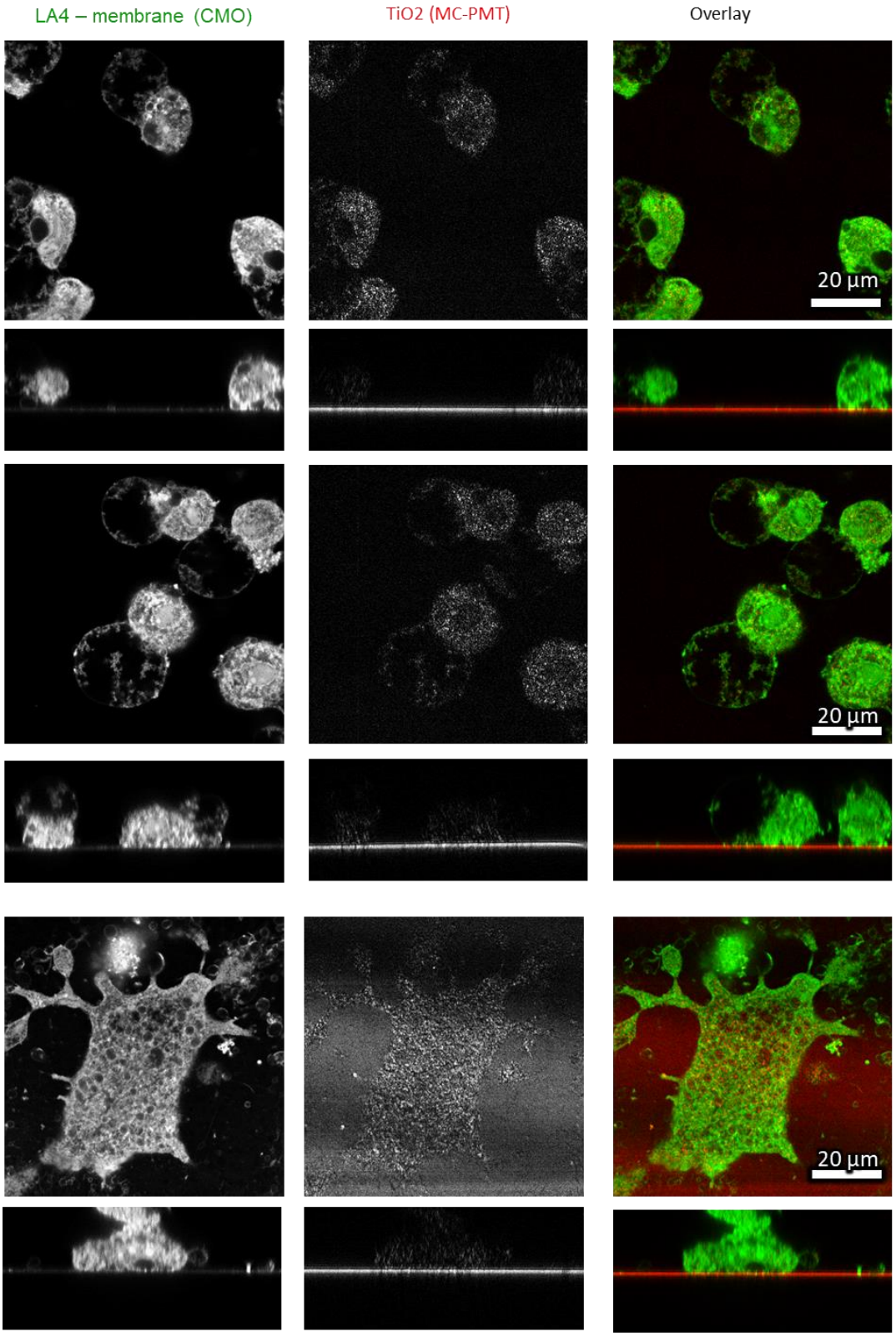
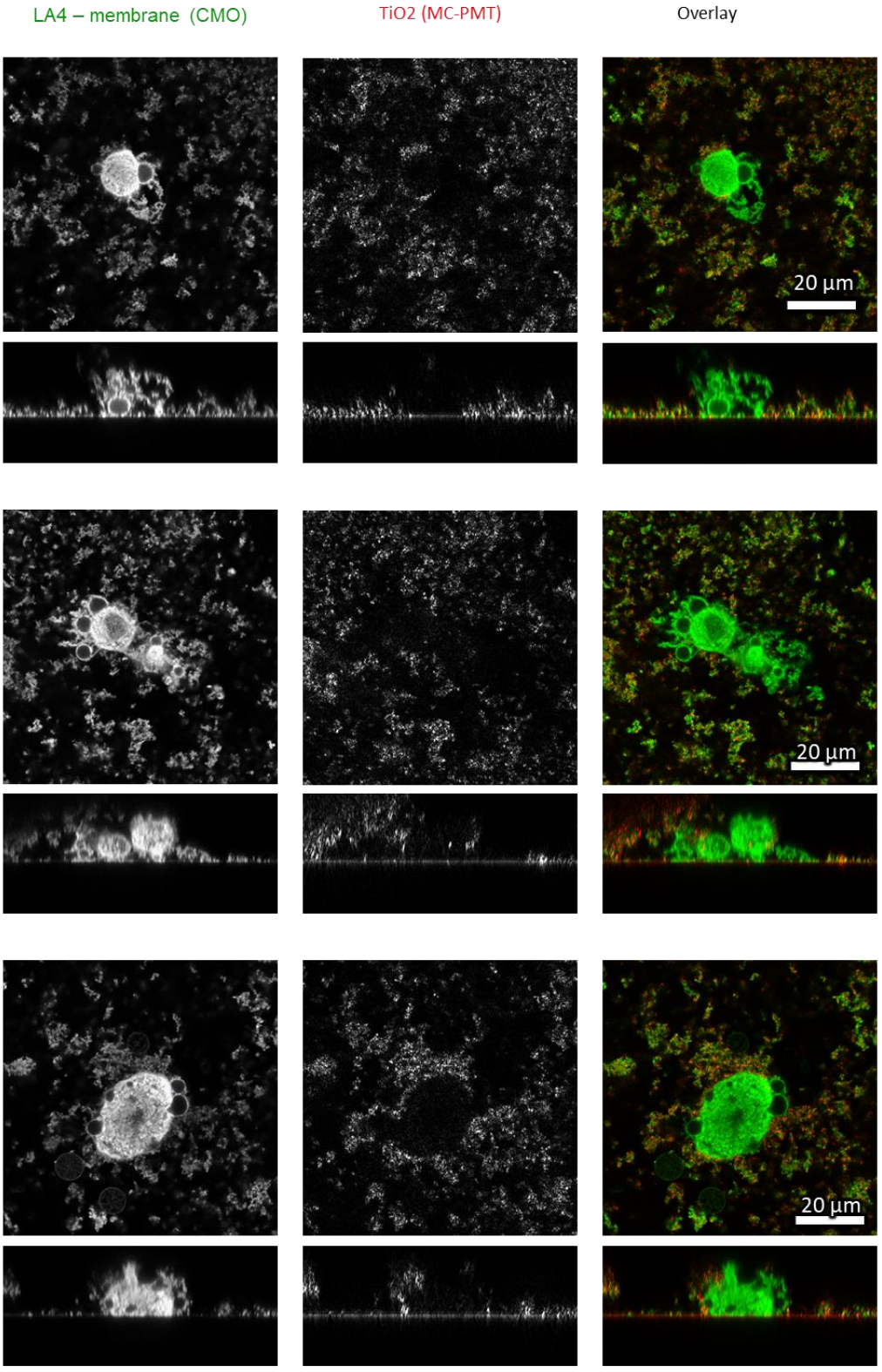


Figure S44: Confocal images of LA-4 cells exposed to 10 to 1 surface dose of ZnO-NM111 for 48 h in xy plane (upper windows) and xz plane (lower windows)



ZnO - Raw

Figure S45: Confocal images of LA-4 cells exposed to 10 to 1 surface dose of ZnO-Raw for 48 h in xy plane (upper windows) and xz plane (lower windows)

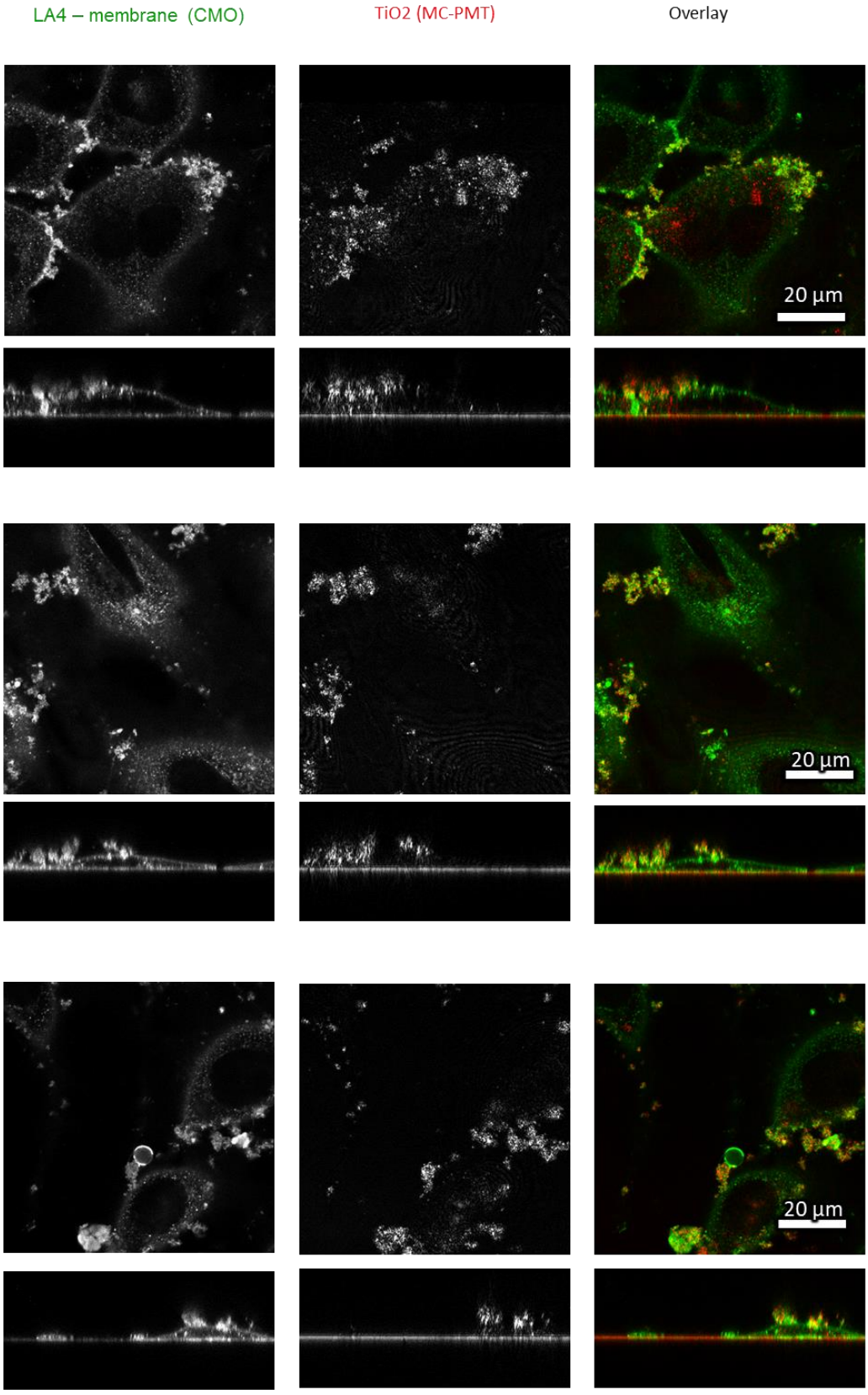


Figure S46: Confocal images of LA-4 cells exposed to 10 to 1 surface dose of TiO_2 anatase MKNA015 for 48 h in xy plane (upper windows) and xz plane (lower windows)

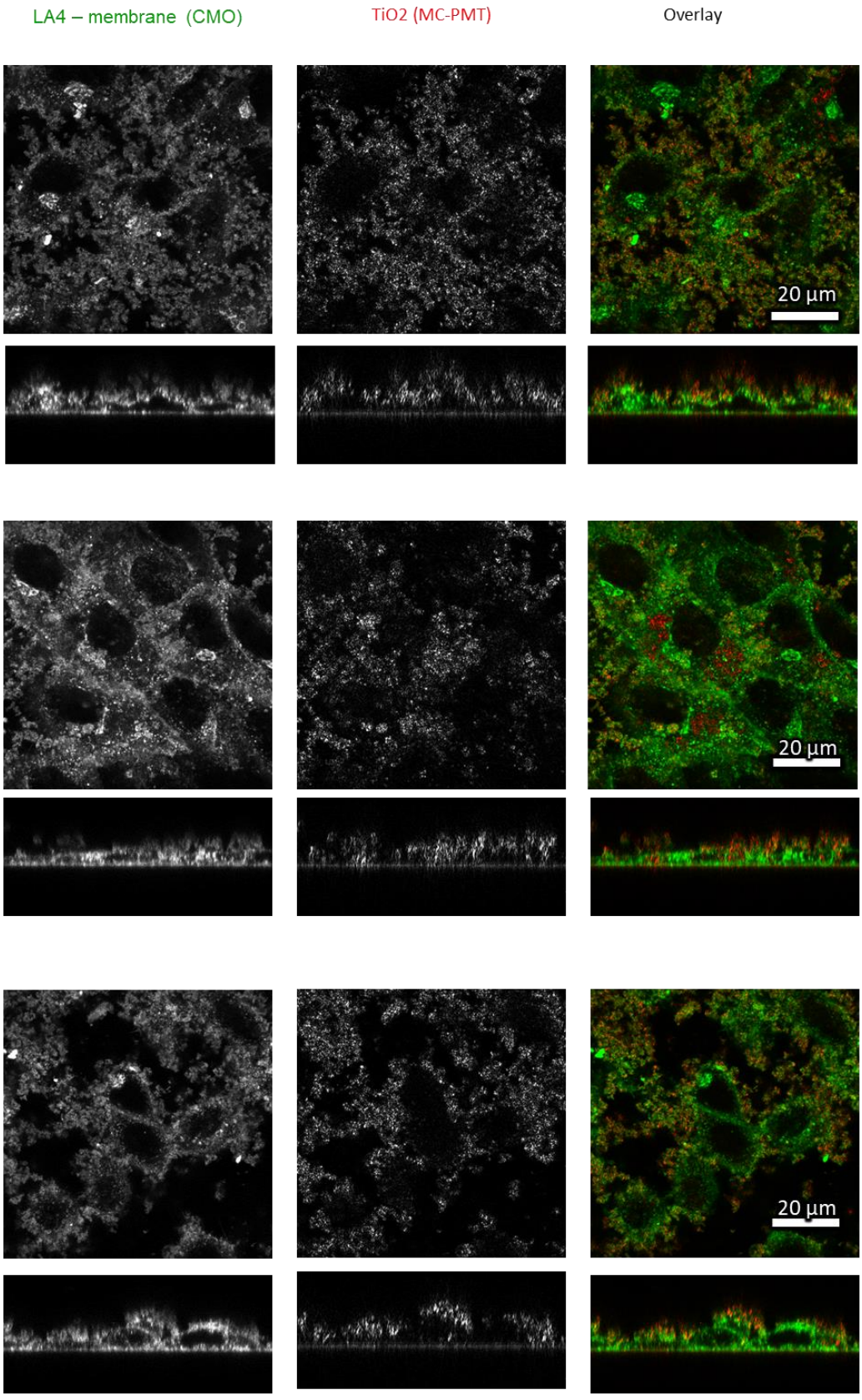
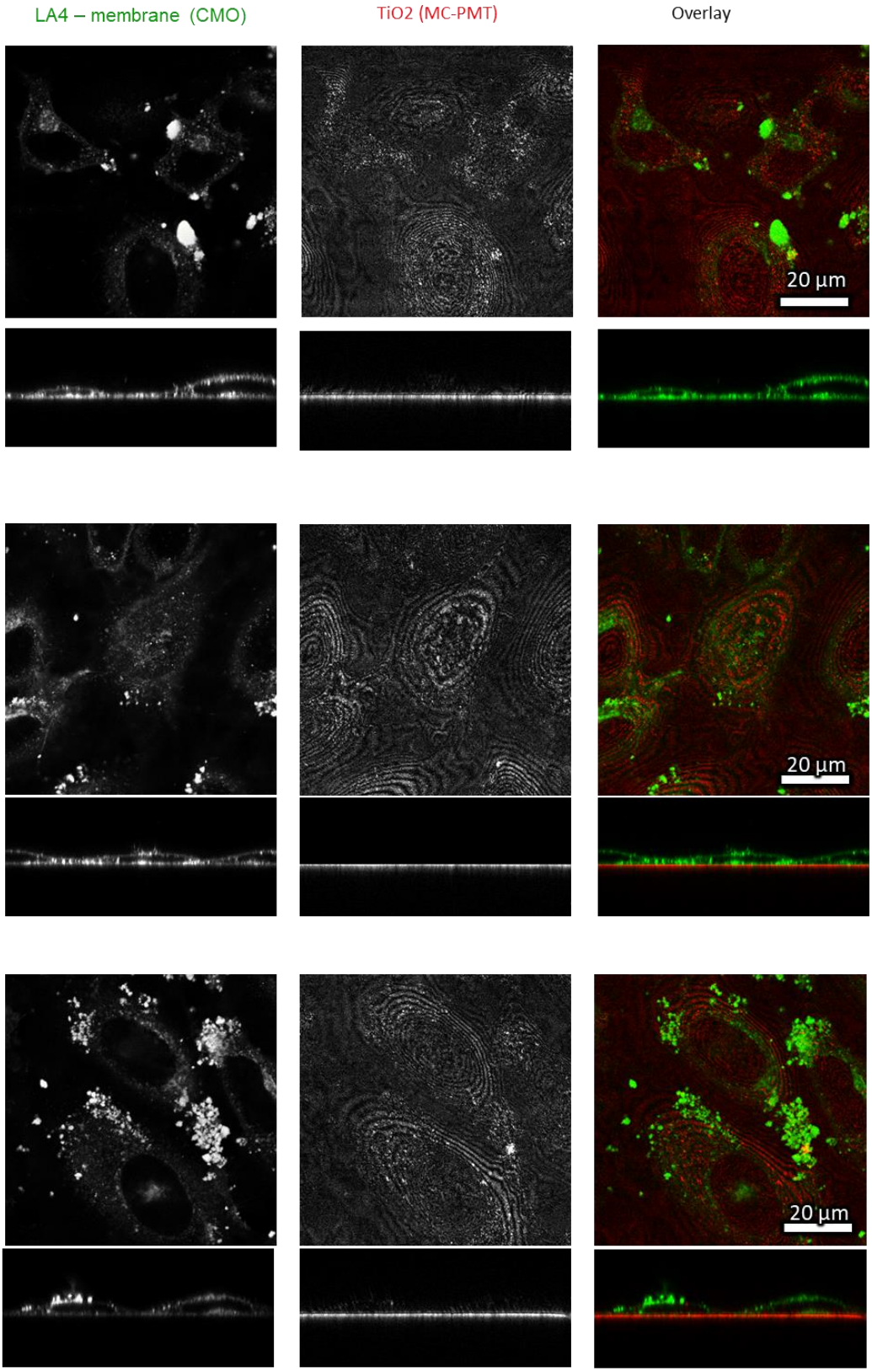


Figure S47: Confocal images of LA-4 cells exposed to 10 to 1 surface dose of TiO_2 rutile-anatase for 48 h in xy plane (upper windows) and xz plane (lower windows)



SiO2 – NM200

Figure S48: Confocal images of LA-4 cells exposed to 10 to 1 surface dose of SiO_2 for 48 h in xy plane (upper windows) and xz plane (lower windows)

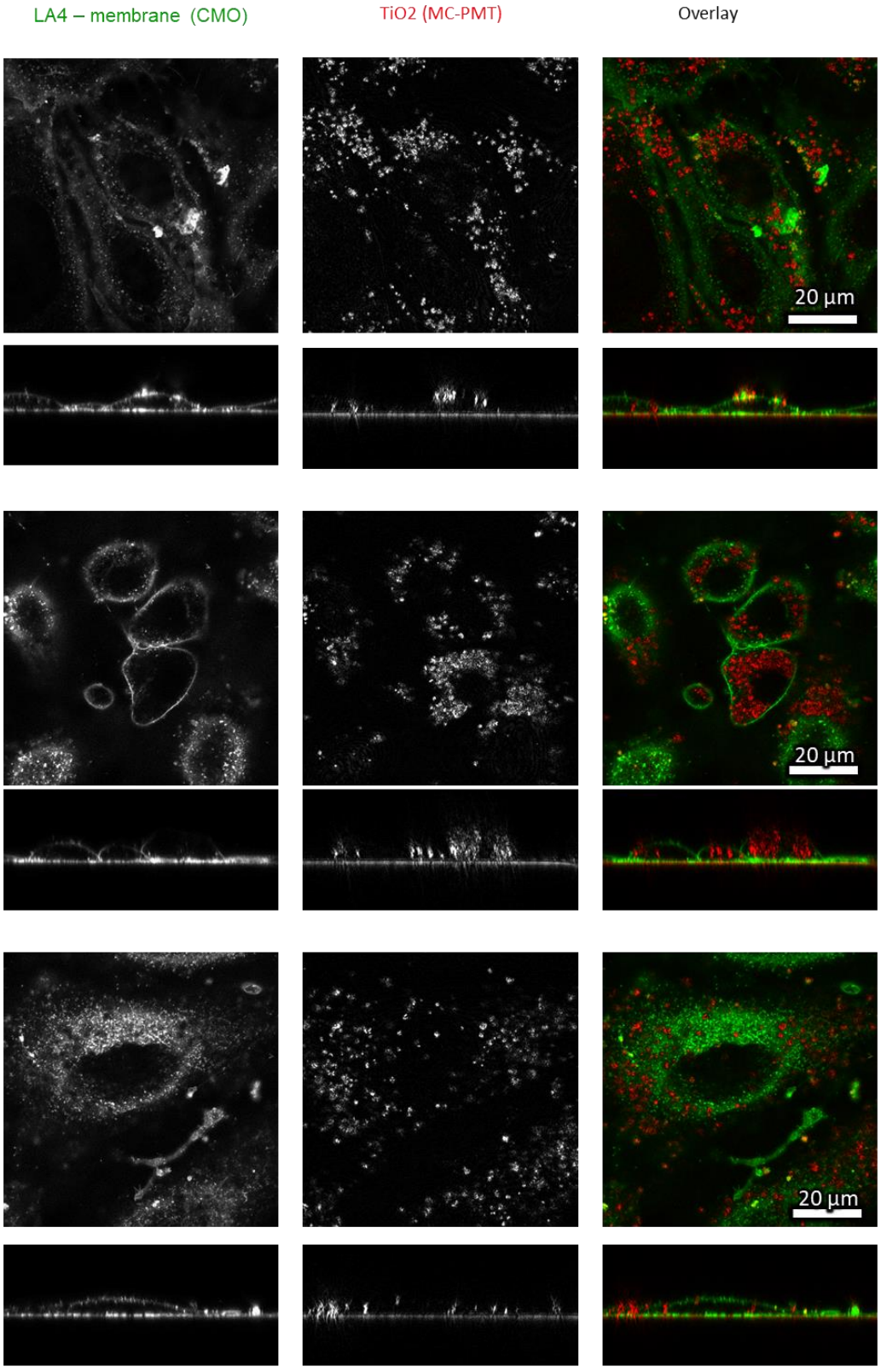


Figure S49: Confocal images of LA-4 cells exposed to 10 to 1 surface dose of TiO_2 anatase NM101 for 48 h in xy plane (upper windows) and xz plane (lower windows)

Cell line	LA-4 (membrane, CMO)
NPs	TiO2 (Star 520)
exposure	10:1, 48 h
imaging	xz confocal, 48 h

pixelsize (x,y)	100 nm
FOV (x,y)	80um
pixelsize (z)	100nm
FOV (z)	35um
imaging time	/min
number z-stacks	/

561nm	100%
640nm	0%
Discovery 2PE 999nm	ON
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 ms
objective	wi60x (NA1.2)

Cell line	LA-4 (membrane, CMO)
NPs	TiO2 (Star 520)
exposure	10:1, 48 h
imaging	xy confocal, 48 h

pixelsize (x,y)	100 nm
FOV (x,y)	80x80um
pixelsize (z)	/nm
FOV (z)	/um
imaging time	/min
number z-stacks	/

561nm	100%
640nm	0%
Discovery 2PE 999nm	ON
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 ms
objective	wi60x (NA1.2)

S2d – Time evolution of cauliflower growth

Main message

Proto-cauliflowers start forming after 1h, after 48h big cauliflower-like structures can be observed at dose 10:1 (surface of TiO_2-NTs: surface of the cells).

Supporting raw and analysed data:
For 1h, 10:1 :
Figure S50-Figure S53
For 25h, 10:1 :
Figure S54-Figure S56

Materials and methods

See supplement S2e – Dose-dependent exposure of LA-4 to TiO2 nanotubes.

Experiment names

See supplement S2e – Dose-dependent exposure of LA-4 to TiO2 nanotubes.

Controls and statistics

For 1h, 10:1 :
Figure S50-Figure S53
For 25h, 10:1 :
Figure S54-Figure S56

control: 10:1, 1h exposure confocal xy

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	10:1,1h incubaton
imaging	xy confocal, @1h

pixelsize (x,y)	100 nm
FOV (x,y)	80 µm
pixelsize (z)	/
FOV (z)	/
imaging time	/
number of frames	/

561nm	30%
640nm	30%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	20 µs
objective	wi60x (NA1.2)

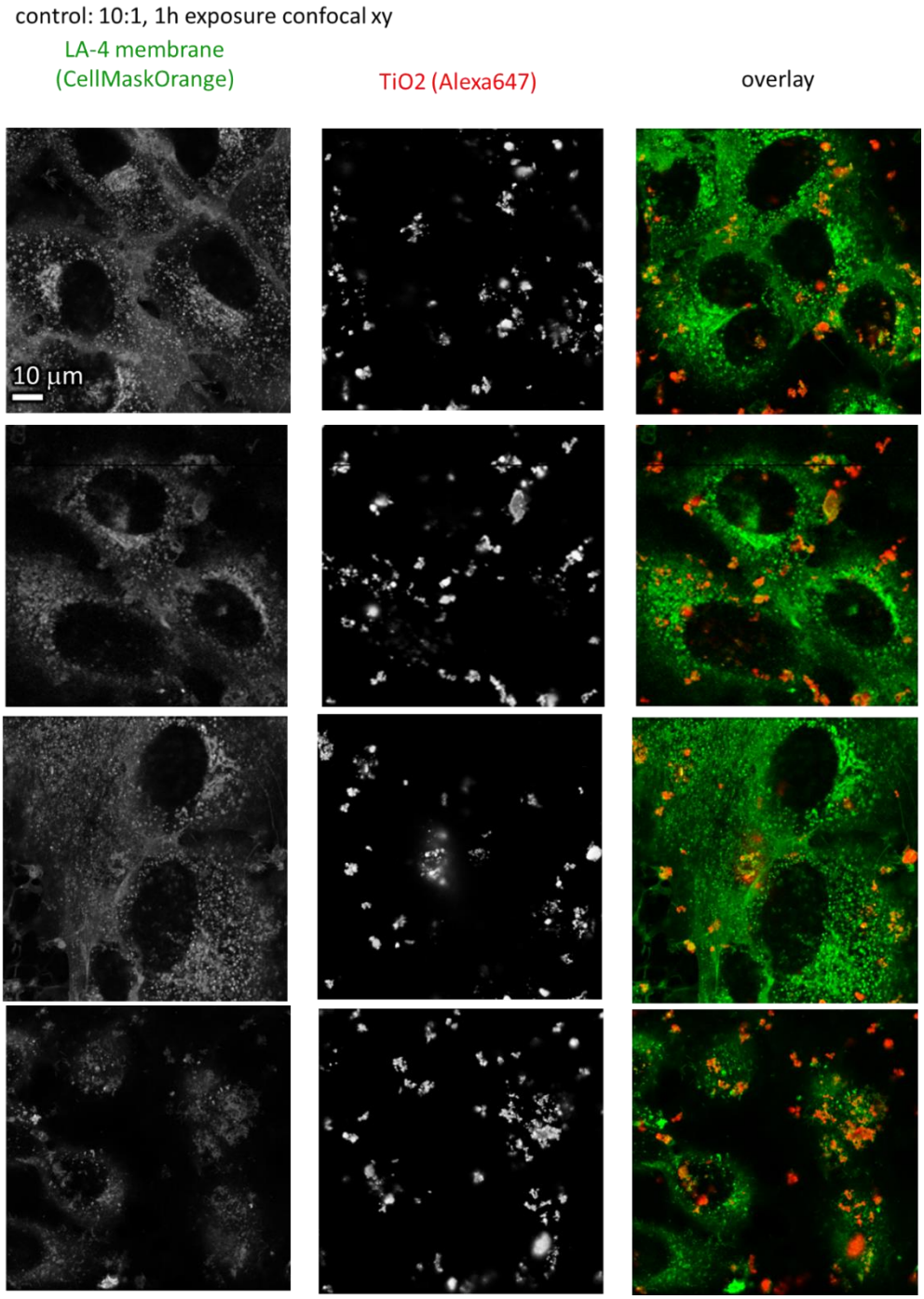


Figure S50: Statistics for time point 1h – confocal xy recordings.

control: 10:1, 1h exposure STED xy

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	10:1,1h incubaton
imaging	xy confocal, @1h

pixelsize (x,y)	30 nm
FOV (x,y)	25 µm
pixelsize (z)	62 nm
FOV (z)	12 µm
imaging time	/
number of frames	/

561nm	30%
640nm	30%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	20 µs
objective	wi60x (NA1.2)

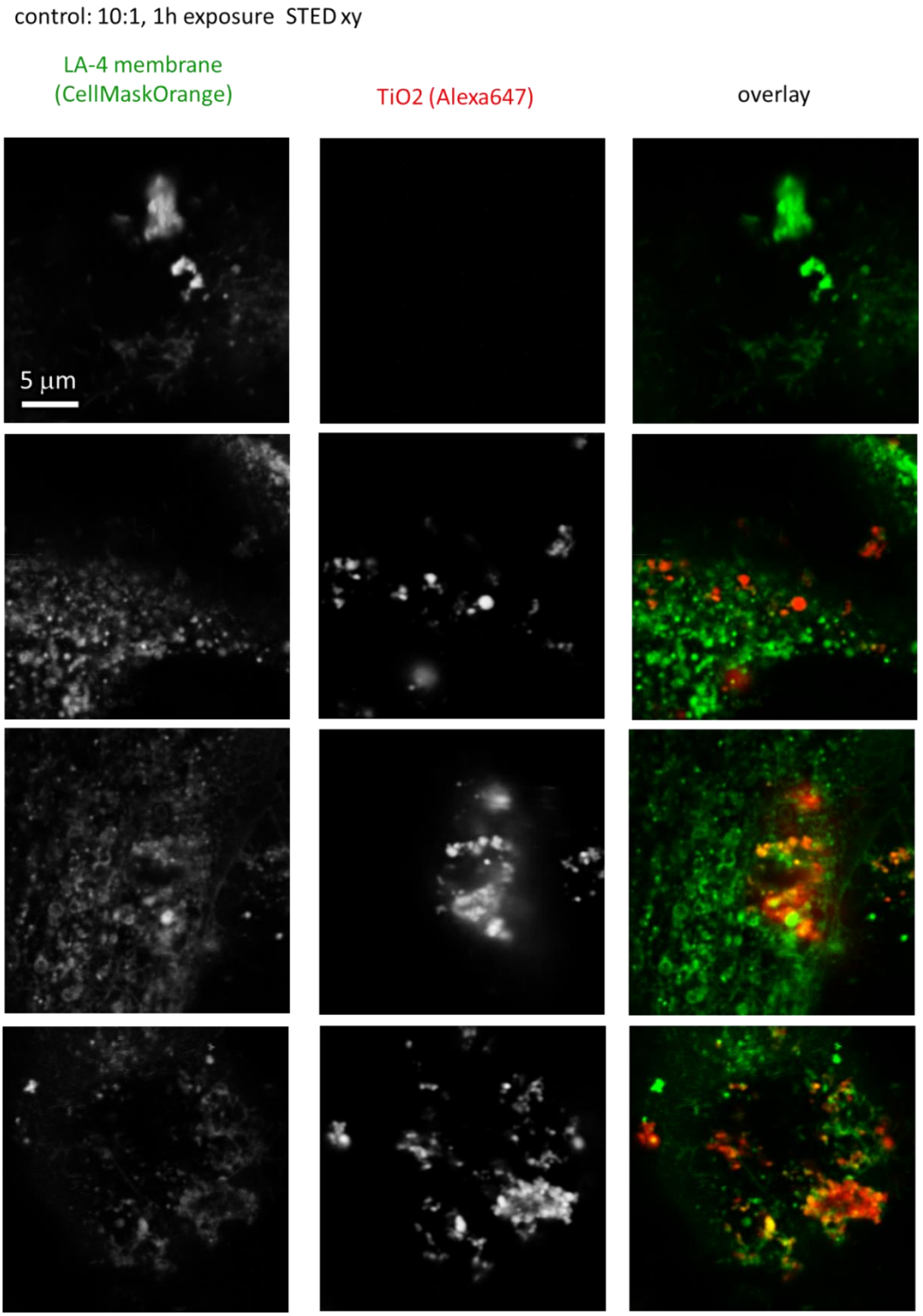


Figure S51: Statistics for time point 1h – STED xy recordings.

control: 10:1, 1h exposure confocal xz

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	10:1,1h incubaton
imaging	xy confocal, @1h

pixelsize (x,y)	100 nm
FOV (x,y)	70 µm
pixelsize (z)	131 nm
FOV (z)	25 µm
imaging time	/
number of frames	/

561nm	30%
640nm	30%
STED	0
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	20 µs
objective	wi60x (NA1.2)

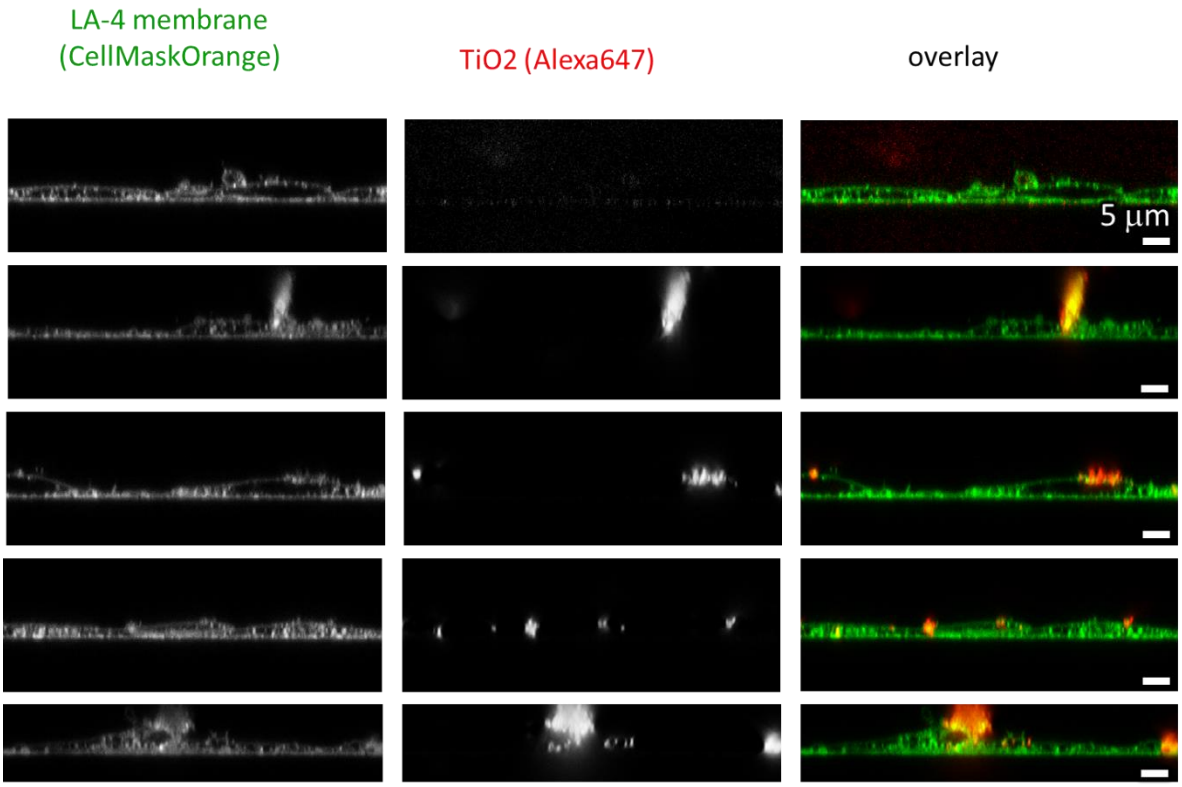


Figure S52: Statistics for time point 1h – confocal xz recordings.

control: 10:1, 1h exposure STED xz

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	10:1,1h incubaton
imaging	xy confocal, @1h

pixelsize (x,y)	100 nm
FOV (x,y)	70 µm
pixelsize (z)	131 nm
FOV (z)	25 µm
imaging time	/
number of frames	/

561nm	30%
640nm	30%
STED	0
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	20 µs
objective	wi60x (NA1.2)

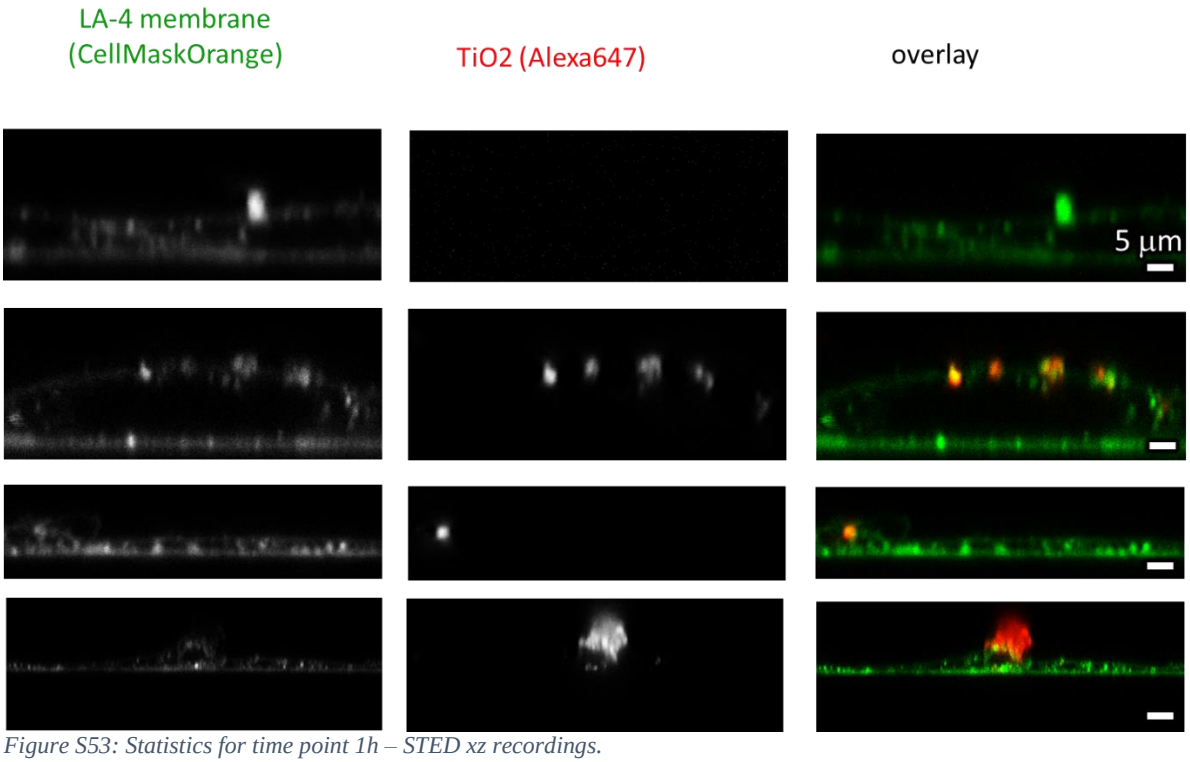


Figure S53: Statistics for time point 1h – STED xz recordings.

confocal xy – statistics over sample

Cell line	LA-4 (membrane, CellMaskOrange)	pixelsize (x,y)	100 nm	561nm	20%
NPs	TiO_2 (Alexa647)	FOV (x,y)	70 µm	640nm	20%
exposure	10:1, 25h incubaton	pixelsize (z)	/	STED	/
imaging	xy confocal, @25h	FOV (z)	/	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	20 µs
		number of frames	/	objective	wi60x (NA1.2)

❄

Cell line	LA-4 (membrane, CellMaskOrange)	pixelsize (x,y)	300 nm	561nm	20%
NPs	TiO_2 (Alexa647)	FOV (x,y)	70 µm	640nm	20%
exposure	10:1, 25h incubaton	pixelsize (z)	/	STED	/
imaging	xy confocal, @25h	FOV (z)	/	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	20 µs
		number of frames	/	objective	wi60x (NA1.2)

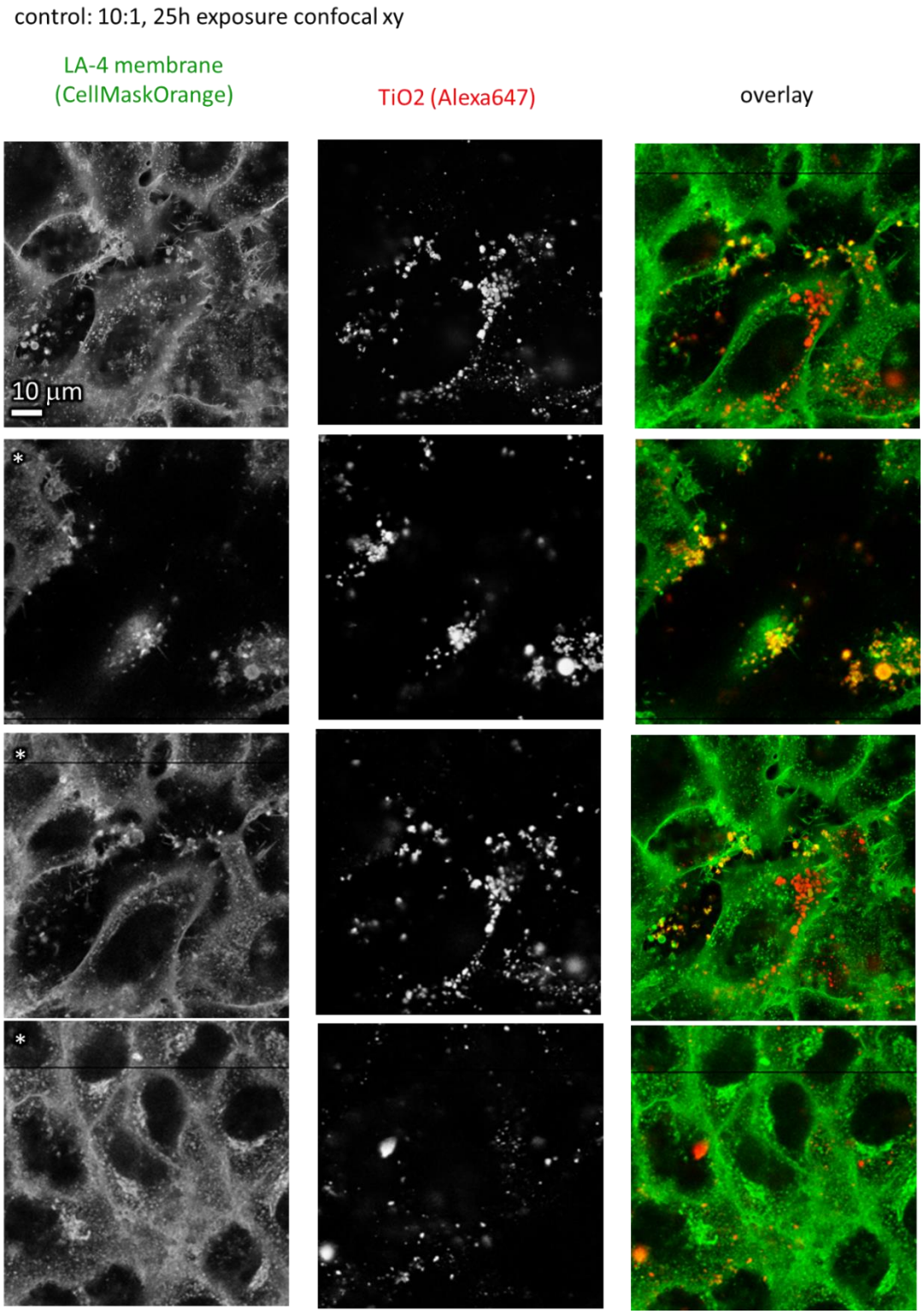


Figure S54: Statistics for time point 25h – confocal xy recordings.

control: 10:1, 25h exposure confocal xz

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	10:1,25h incubaton
imaging	xy confocal, @1h

pixelsize (x,y)	100 nm
FOV (x,y)	70 µm
pixelsize (z)	131 nm
FOV (z)	25 µm
imaging time	/
number of frames	/

561nm	30%
640nm	30%
STED	0
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	20 µs
objective	wi60x (NA1.2)

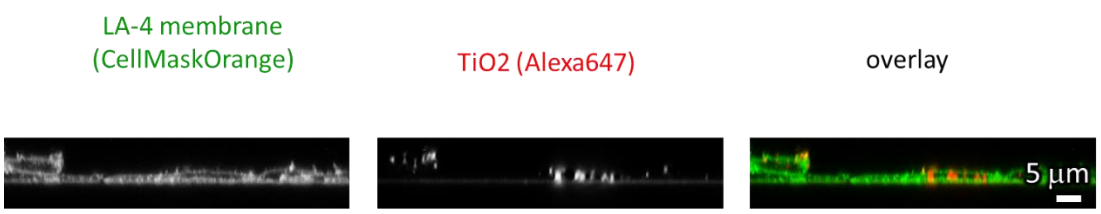


Figure S55: Statistics for time point 25h – confocal xz recordings.

control: 10:1, 25h exposure STED xz

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	10:1,25h incubaton
imaging	xy confocal, @25h

pixelsize (x,y)	100 nm
FOV (x,y)	70 µm
pixelsize (z)	131 nm
FOV (z)	25 µm
imaging time	/
number of frames	/

561nm	30%
640nm	30%
STED	0
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	20 µs
objective	wi60x (NA1.2)

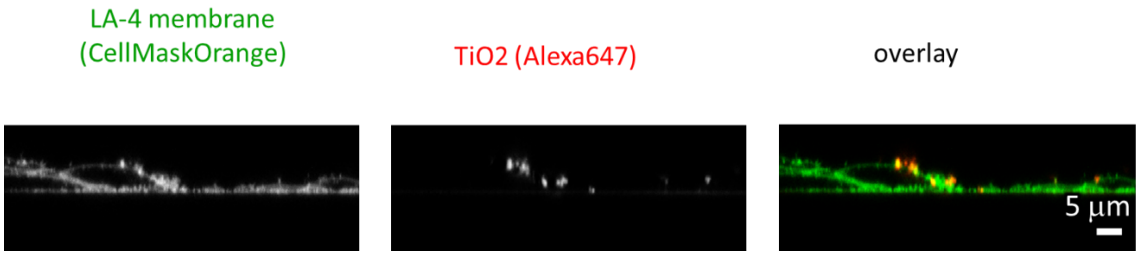


Figure S56: Statistics for time point 25h – STED xz recordings.

S2e – Dose-dependent exposure of LA-4 to TiO_2 nanotubes

Main message

With increasing dose, the amount of nanomaterial in cauliflower-like structures increases. At low doses the amount of nanomaterial in the cells also increases but remains constant from the dose 10:1 upwards (surface of TiO_2-NTs: surface of the cells).

Supporting raw and analysed data:

Figure S57-Figure S76

Materials and methods

- experiment (dose dependence):
  - LA-4 cells were seeded @30% confluence in an Ibidi Ibidi #1.5H µ-Dish
  - after 48 hours freshly filtered 1 mg/mL TiO_2-17-Alexa647 in PBS was mixed into 315 µL fresh media, PBS was added to the final volume of 350 µL and added to the cells. The volume of added nanoparticles for each sample was 3.5 µLfor 1:1 surface dose (S_{NPs}:$S_{cellsurface}$) and 35 µL for 10:1 surface dose. For surface dose 100:1, the nanoparticles were filtered directly into full cell medium.
  - after exposure for 53 hours, media with nanoparticles was slowly removed and the cells were incubated with 5 µg/mL CellMaskOrange for 6 minutes at 37 °C, afterwards media was carefully exchanged with 100 µl LCIS, cells have been observed at room temperature.
- experiment (1 h incubation):
  - LA-4 cells were seeded @60% confluence in an Ibidi #1.5H µ-Dish
  - after 48 hours, the cells were incubated with 1.6 µg/mL CellMaskOrange in PBS for 6 minutes at 37 °C and 5% CO2, afterwards they were flushed with LCIS and observed in 315 µL LCIS on a heated insert on the microscope (28C)
  - on the microscpe, 35 µL freshly filtered 1 mg/mL TiO_2-40-Alexa647 in PBS were added to cells to achieve a 10:1 surface dose. Cells were observed the first 3 hours after incubation
  - experiment (25 h incubation):
  - LA-4 cells were seeded @60% confluence in an Ibidi #1.5H µ-Dish
  - after 23 hours 35 µL freshly filtered 1 mg/mL TiO_2-40-Alexa647 in 100x dcb was mixed into 350 µL fresh media on cells to achieve 10:1 surface dose
  - after exposure for 25 hours, the cells were incubated with 1.6 µg/mL CellMaskOrange in PBS for 6 minutes at 37 °C and 5% CO2, afterwards they were flushed with LCIS and observed in 315 µL LCIS on a heated insert on the microscope (28C)
- Analysis:
  - threshold of both channels in all STED images has been set to 1 count, since this is the noise of our detector
  - maximum in all STED images in both channels has been adjusted for maximal visibility
  - all STED images in the supplement have been analyzed in same manner
  - brightness of all confocal overlays has also been adjusted for maximal visibility (in supplement)
  - all images in supplement are represented with brightness/threshold adjusted overlay and non-contrasted raw (.png) of both channel separately

Experiment names

- Main experiment name:
  - 20191104/e03_s01_t01_LA-4 CellMaskOrange_TiO2 Alexa647_STED.msr
  - 20180202/e03_s04_t04_LA-4 CellMask TiO2 Alexa647-wellLabeled - 1 to 1 _ SNPs to Scells - cellsB.msr
  - 20180202/e04_s02_t03_LA-4 CellMask TiO2 Alexa647-wellLabeled - 10 to 1 _SNPs to Scells - cellsG.msr
  - 20180202/e05_s06_t07_LA-4 CellMask TiO2 Alexa647-wellLabeled - 100to 1 _SNPs to Scells - cellsH-biggerZ.msr
  - 20180202/e06_s02_t02_LA-4 CellMask - control - cellsA.msr
  - 20180202/e04_s03_t04_LA-4 CellMask TiO2 Alexa647-wellLabeled - 10 to 1 _SNPs to Scells - cellsG.msr
  - 20190308/e02_t08_LA-4 CellMask TiO2Alexa647 10 to 1.msr
  - 20190308/e03_t01_LA-4 CellMask TiO2Alexa647 10 to 1_25h incubation.msr

- Supplement experiment names:
  - dose dependence - 20180202
    - STED and confocals:
      - e06_s05_t05_LA-4 CellMask - control - cellsA.msr
      - e06_s04_t04_LA-4 CellMask - control - cellsA.msr
      - e06_s03_t03_LA-4 CellMask - control - cellsA.msr
      - e05_s06_t06_LA-4 CellMask TiO2 Alexa647-wellLabeled - 100to 1 _SNPs to Scells - cellsH.msr
      - e05_s05_t05_LA-4 CellMask TiO2 Alexa647-wellLabeled - 100to 1 _SNPs to Scells - cellsH.msr
      - e05_s04_t04_LA-4 CellMask TiO2 Alexa647-wellLabeled - 100to 1 _SNPs to Scells - cellsH.msr
      - e05_s03_t03_LA-4 CellMask TiO2 Alexa647-wellLabeled - 100to 1 _SNPs to Scells - cellsH.msr
      - e04_s04_t05_LA-4 CellMask TiO2 Alexa647-wellLabeled - 10 to 1 _SNPs to Scells - cellsG.msr
      - e03_s04_t05_LA-4 CellMask TiO2 Alexa647-wellLabeled - 1 to 1 _ SNPs to Scells - cellsB.msr
      - e03_s03_t03_LA-4 CellMask TiO2 Alexa647-wellLabeled - 1 to 1 _ SNPs to Scells - cellsB.msr
      - e03_s02_t02_LA-4 CellMask TiO2 Alexa647-wellLabeled - 1 to 1 _ SNPs to Scells - cellsB.msr
      - e03_s01_t01_LA-4 CellMask TiO2 Alexa647-wellLabeled - 1 to 1 _ SNPs to Scells - cellsB.msr
      - e03_s01_t01_LA-4 CellMask TiO2 Alexa647-wellLabeled - 1 to 1 _ SNPs to Scells - cellsB - time.msr
    - confocals:
      - e06_s01_t01_LA-4 CellMask - control - cellsA - time.msr
      - e03_s01_t01_LA-4 CellMask TiO2 Alexa647-wellLabeled - 1 to 1 _ SNPs to Scells - cellsB - time.msr
      - e04_s01_t01_LA-4 CellMask TiO2 Alexa647-wellLabeled - 10 to 1 _SNPs to Scells - cellsG - time.msr
      - e05_s01_t01_LA-4 CellMask TiO2 Alexa647-wellLabeled - 100to 1 _SNPs to Scells - cellsH - time.msr
      - e05_s02_t02_LA-4 CellMask TiO2 Alexa647-wellLabeled - 100to 1 _SNPs to Scells - cellsH - time.msr

Controls and statistics

control – no exposure to nanomaterial:

Figure S57-Figure S59

Figure S71-Figure S72

1:1 surface to surface dose:

Figure S58-Figure S62

Figure S71-Figure S72

10:1 surface to surface dose:

Figure S63-Figure S65

Figure S73-Figure S74

100:1 surface to surface dose:

Figure S66-Figure S68

Figure S75-Figure S76

control: 0:1 exposure STED xz

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	/
exposure	/
imaging	xz STED, @48h

pixelsize (x,y)	30 nm
FOV (x,y)	30 µm
pixelsize (z)	85 nm
FOV (z)	20 µm
imaging time	/
number of frames	/

561nm	30%
640nm	3%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	80 µs
objective	wi60x (NA1.2)

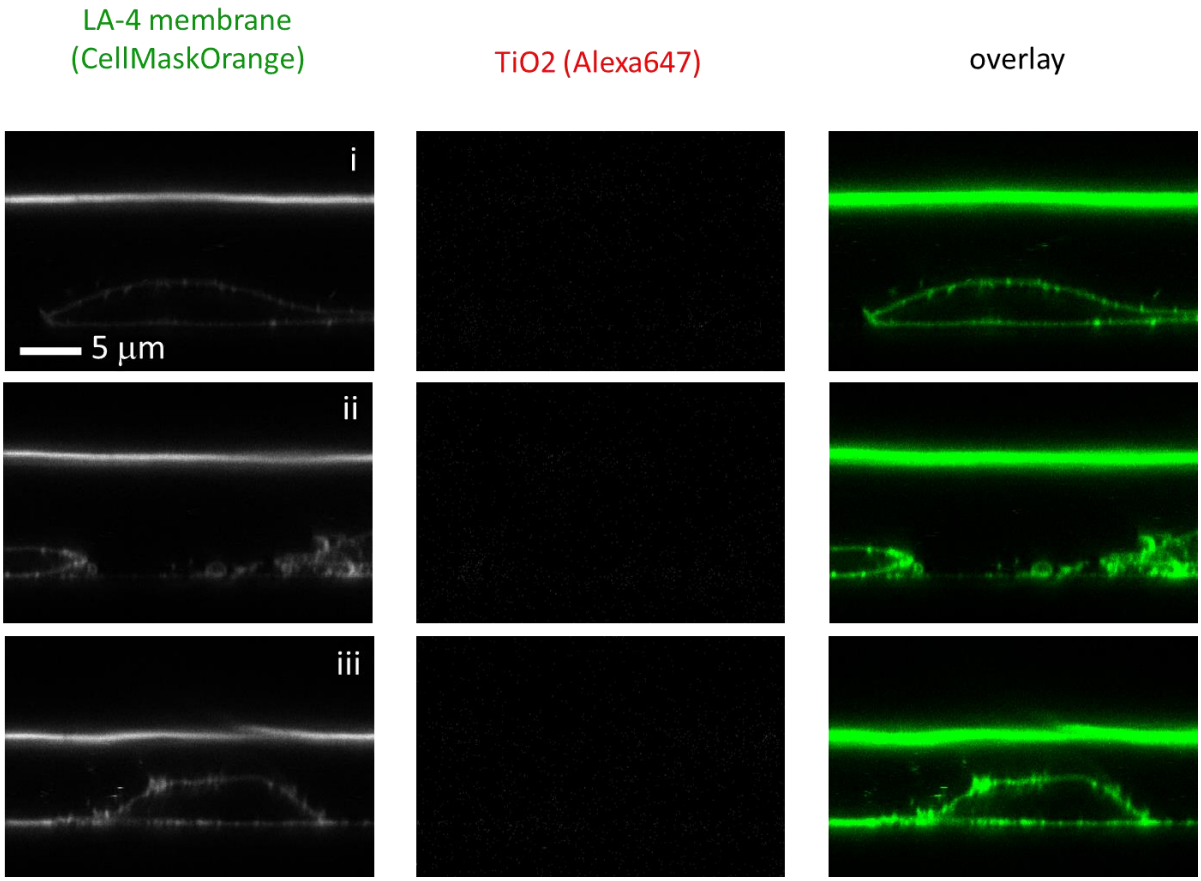


Figure S57: Statistics for control with no exposure to nanomaterial – STED xz recordings.

control: 0:1 exposure STED xy

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	/
exposure	/
imaging	xy STED, @48h

pixelsize (x,y)	30 nm
FOV (x,y)	30 μm
pixelsize (z)	/
FOV (z)	/
imaging time	/
number of frames	/

561nm	30%
640nm	3%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	80 μs
objective	wi60x (NA1.2)

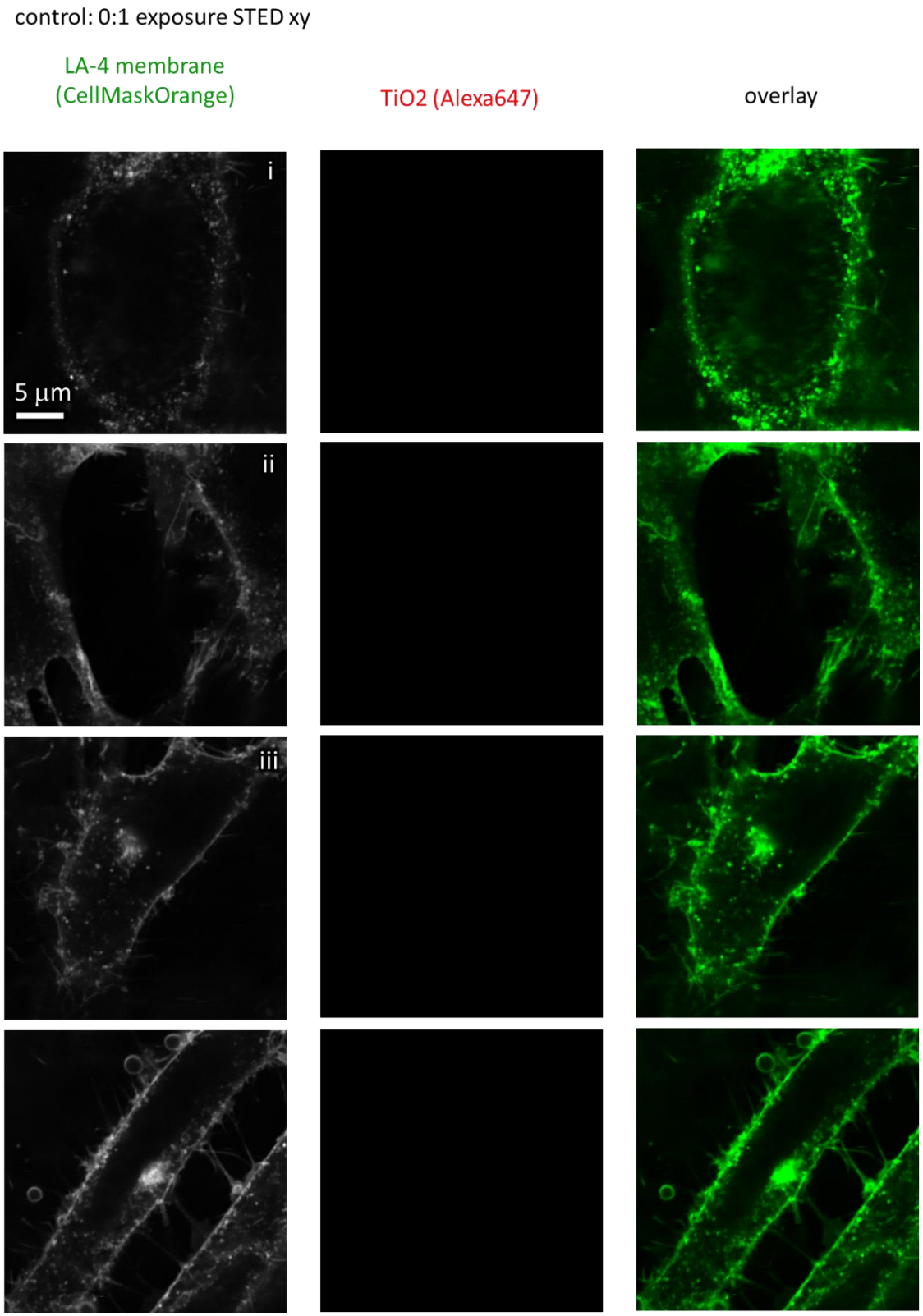


Figure S58: Statistics for control with no exposure to nanomaterial – STED xy recordings.

control: 0:1 exposure confocal xy (STED is a zoom)

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	/
exposure	/
imaging	xy confocal, @48h

pixelsize (x,y)	100 nm
FOV (x,y)	80 µm
pixelsize (z)	/
FOV (z)	/
imaging time	/
number of frames	/

561nm	30%
640nm	3%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	80 µs
objective	wi60x (NA1.2)

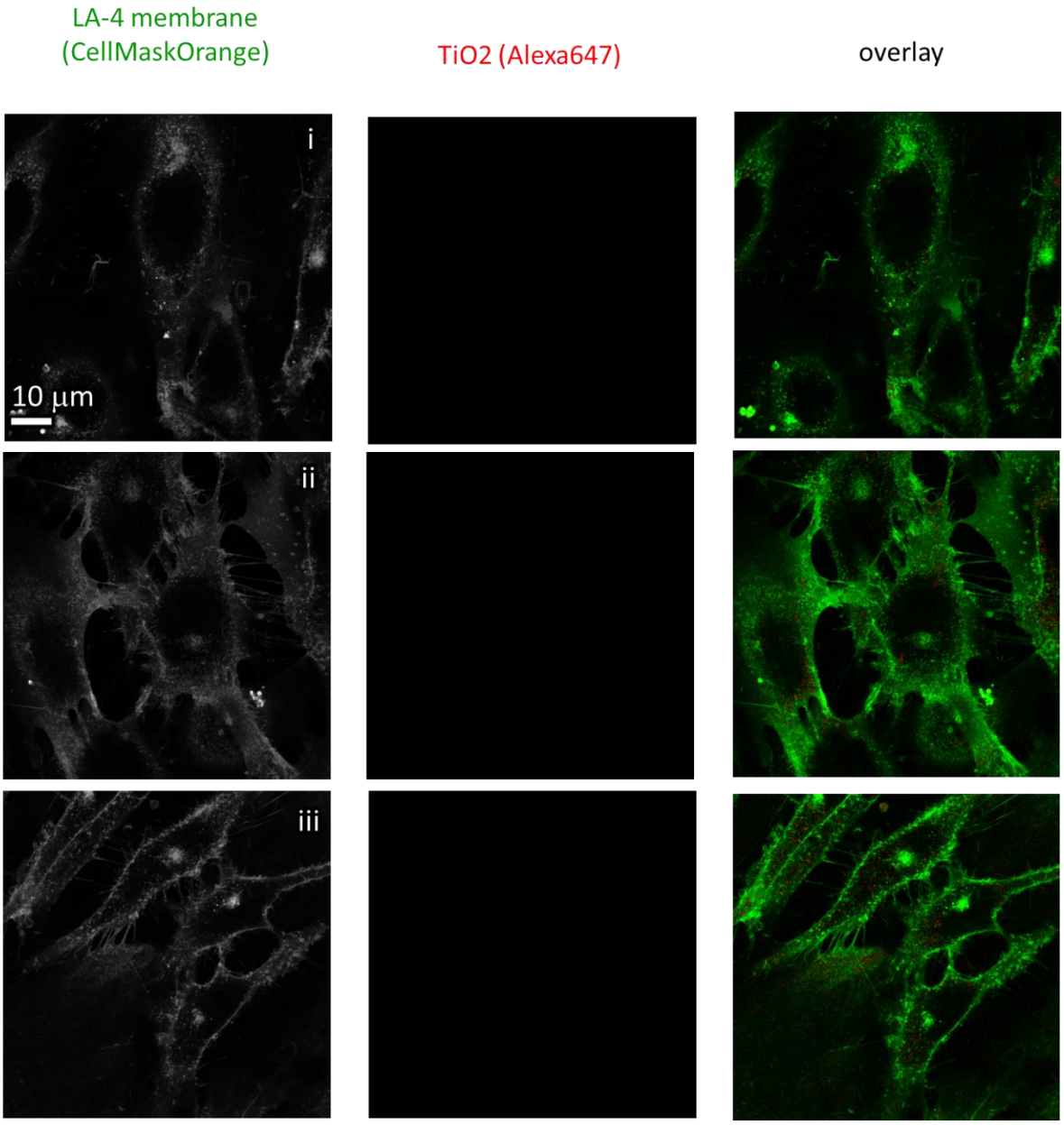


Figure S59: Statistics for control with no exposure to nanomaterial – confocal xy recordings.

exposure: 1:1 exposure STED xz

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	1:1, 48h incubaton
imaging	xz STED, @48h

pixelsize (x,y)	30 nm
FOV (x,y)	30 µm
pixelsize (z)	85 nm
FOV (z)	20 µm
imaging time	/
number of frames	/

561nm	30%
640nm	3%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	80 µs
objective	wi60x (NA1.2)

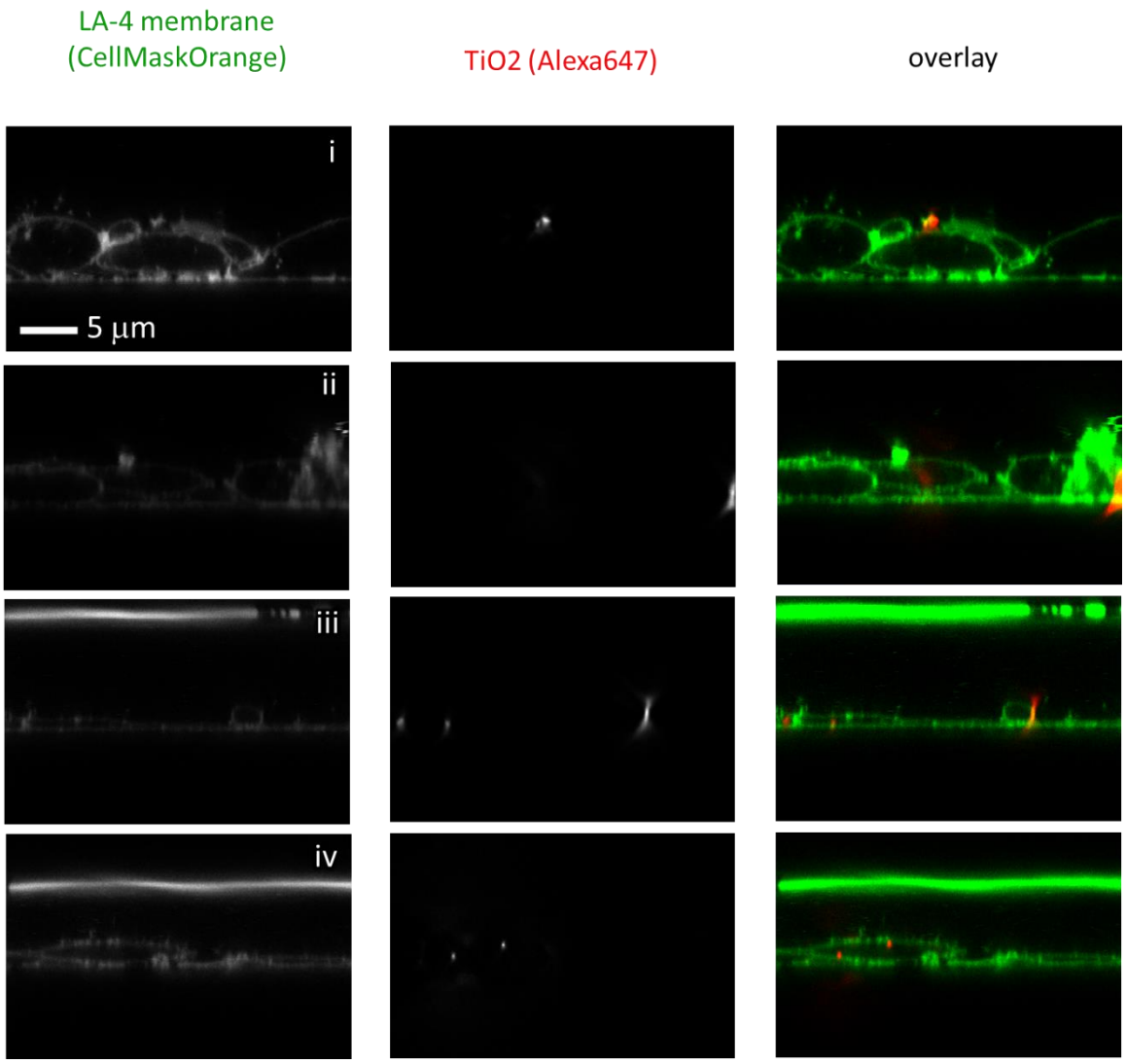


Figure S60: Statistics at surface nanoparticles to surface cells dose 1:1 – STED xz recordings.

control: 1:1 exposure STED xy

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	1:1, 48h incubaton
imaging	xy STED, @48h

pixelsize (x,y)	30 nm
FOV (x,y)	30 µm
pixelsize (z)	/
FOV (z)	/
imaging time	/
number of frames	/

561nm	30%
640nm	3%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	80 µs
objective	wi60x (NA1.2)

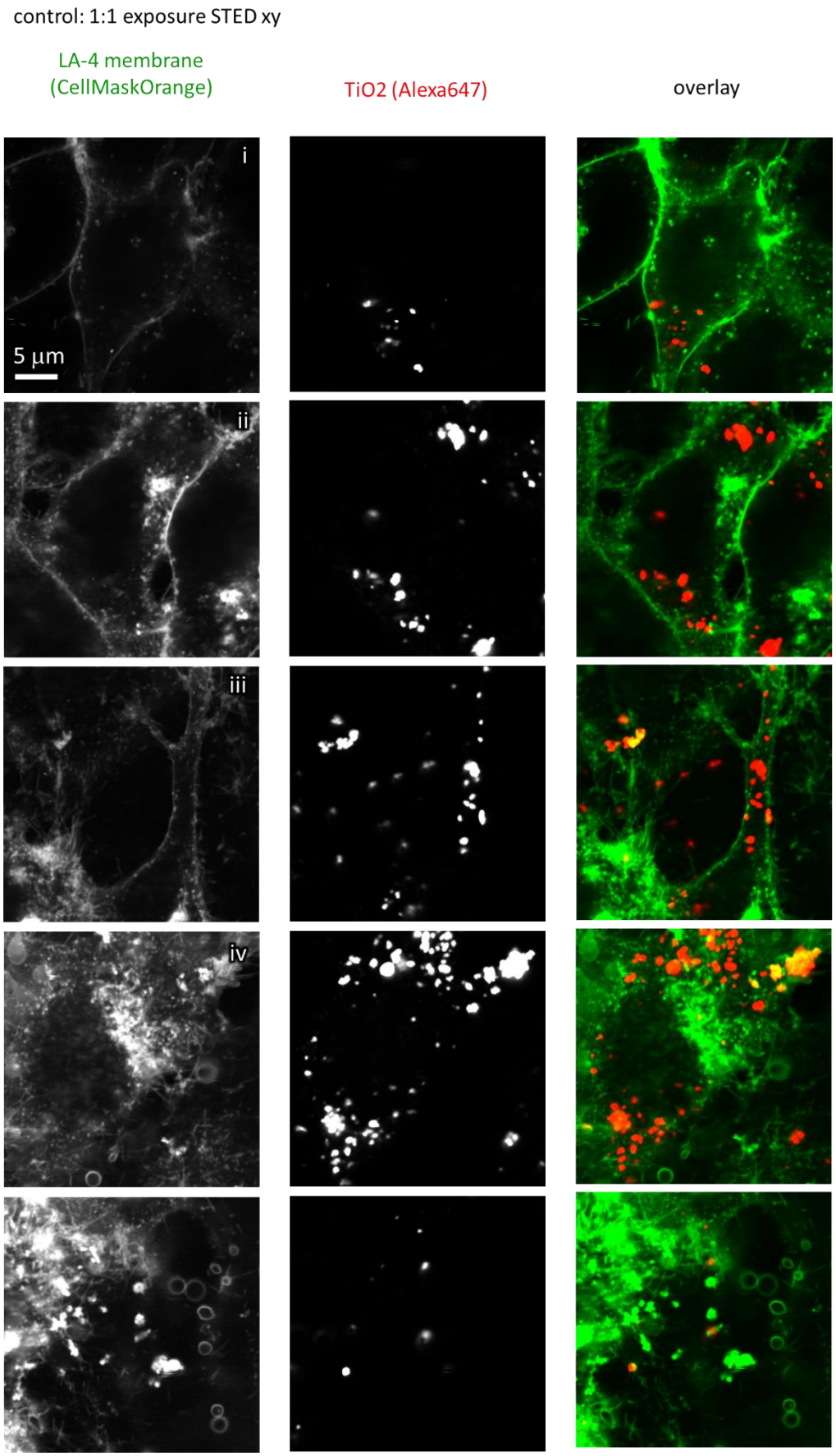


Figure S61: Statistics at surface nanoparticles to surface cells dose 1:1 – STED xy recordings.

control: 1:1 confocal xy

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	1:1, 48h incubaton
imaging	xy confocal, 48h

pixelsize (x,y)	100 nm
FOV (x,y)	80 µm
pixelsize (z)	/
FOV (z)	/
imaging time	/
number of frames	/

561nm	30%
640nm	3%
STED	/
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	80 µs
objective	wi60x (NA1.2)

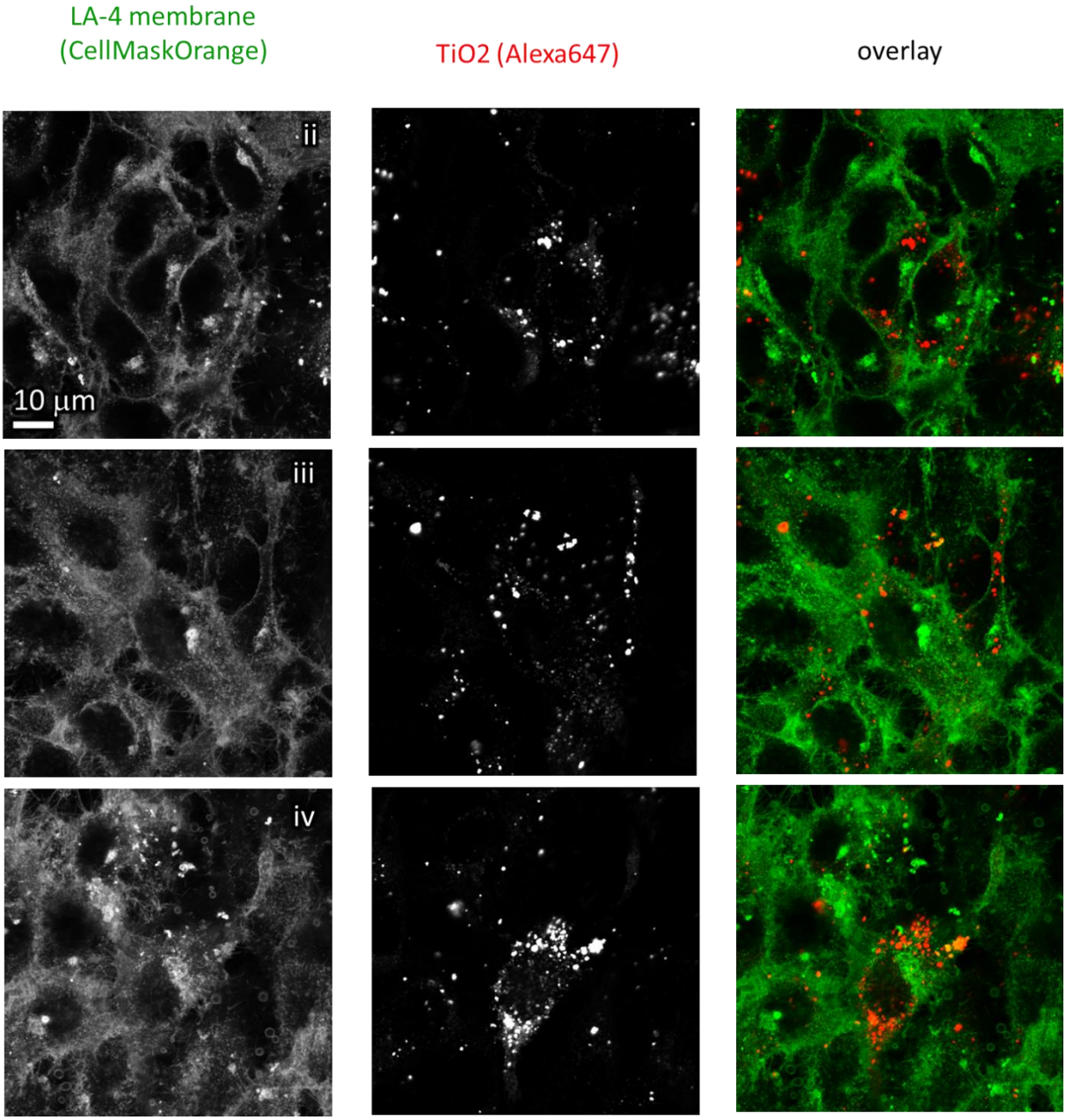


Figure S62: Statistics at surface nanoparticles to surface cells dose 1:1 – confocal xy recordings.

exposure: 10:1 exposure STED xz

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	10:1, 48h incubaton
imaging	xz STED, @48h

pixelsize (x,y)	30 nm
FOV (x,y)	30 µm
pixelsize (z)	85 nm
FOV (z)	20 µm
imaging time	/
number of frames	/

561nm	30%
640nm	3%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	80 µs
objective	wi60x (NA1.2)

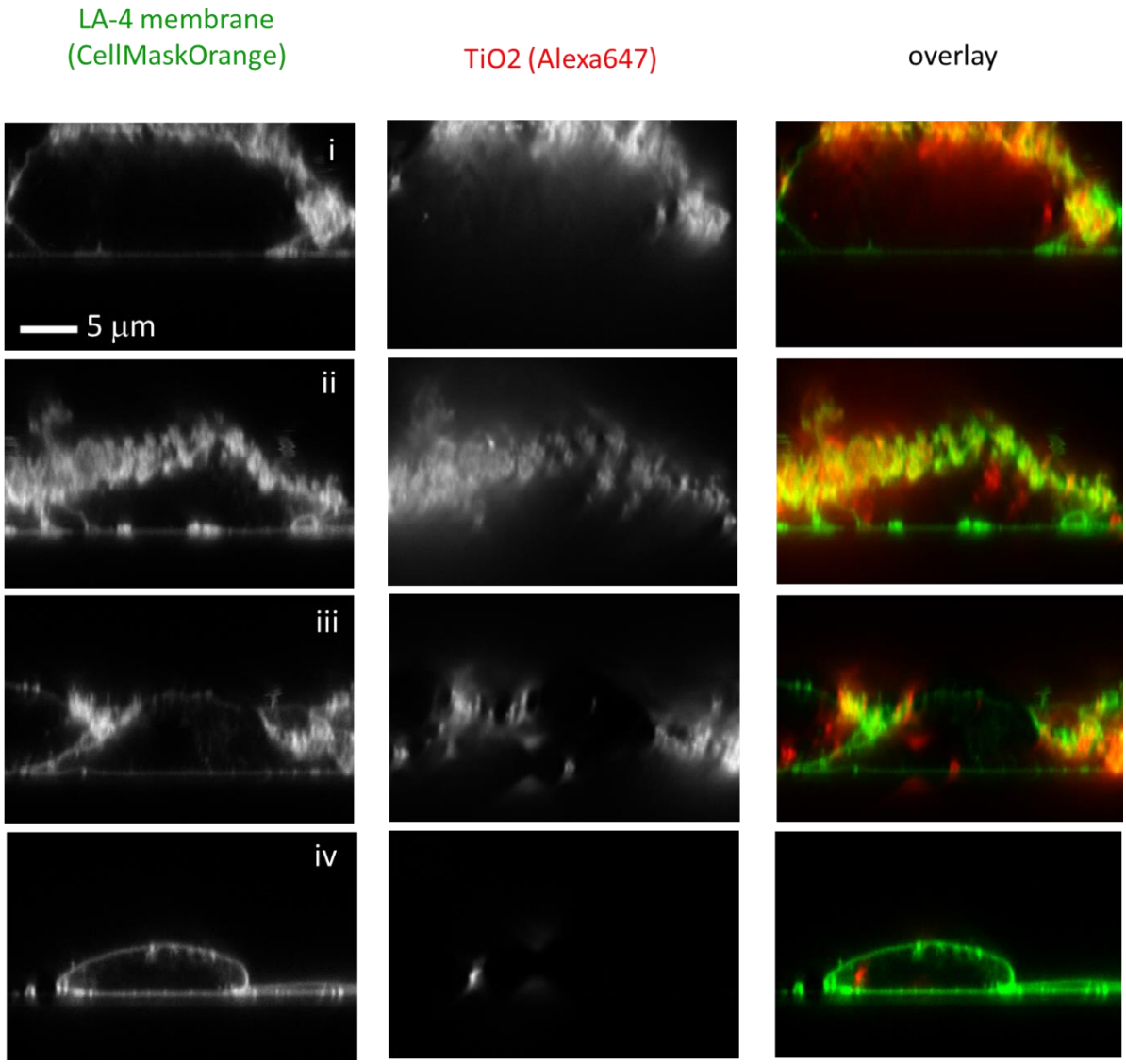


Figure S63: Statistics at surface nanoparticles to surface cells dose 10:1 – STED xz recordings.

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	10:1, 48h incubaton
imaging	xy STED, 48h

pixelsize (x,y)	30 nm
FOV (x,y)	30 μm
pixelsize (z)	/
FOV (z)	/
imaging time	/
number of frames	/

561nm	30%
640nm	3%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	80 μs
objective	wi60x (NA1.2)

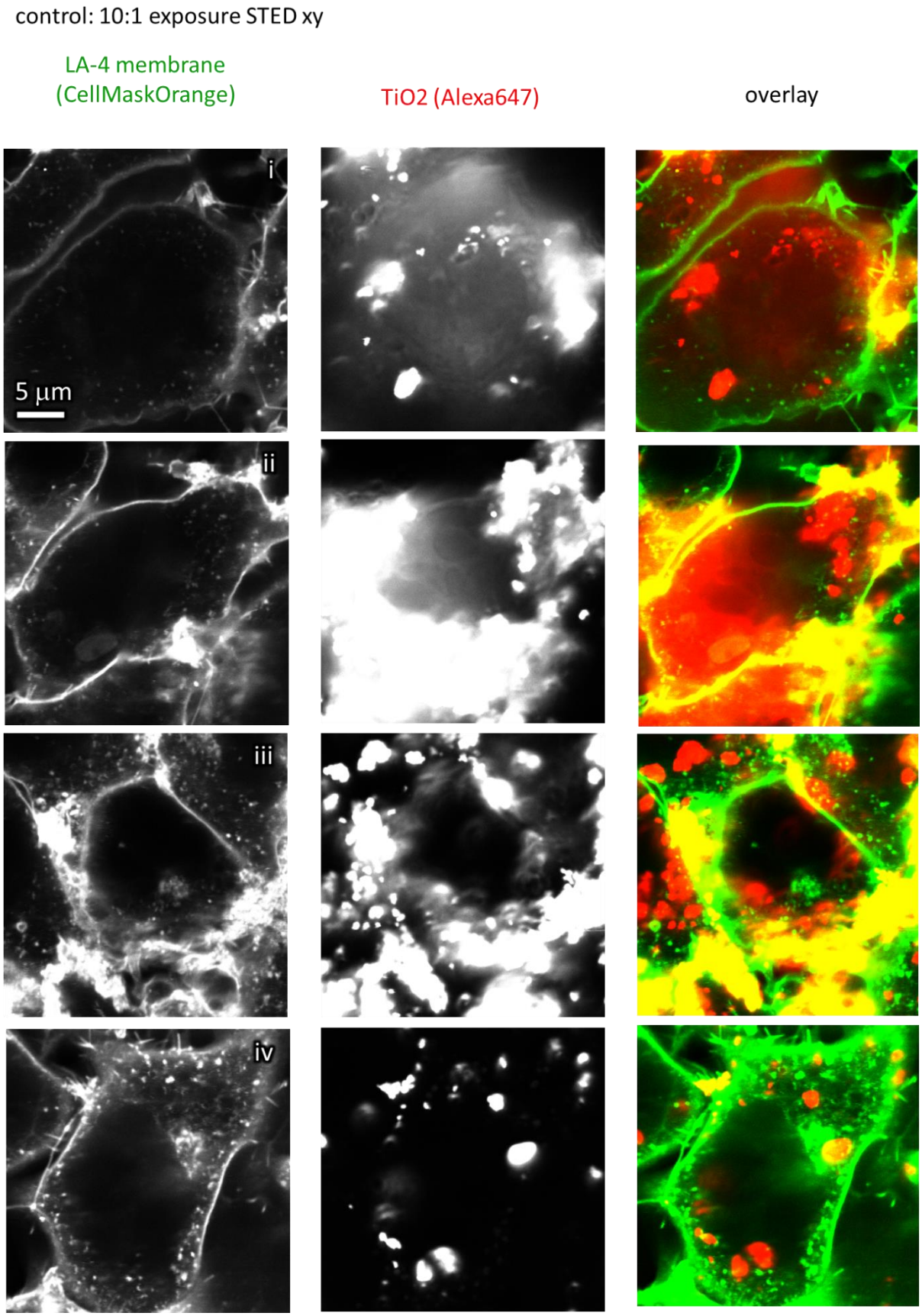


Figure S64: Statistics at surface nanoparticles to surface cells dose 100:1 – STED xy recordings.

control: 10:1 exposure confocal xy (STED is a zoom)

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	10:1, 48h incubaton
imaging	xy confocal, 48h

pixelsize (x,y)	100 nm
FOV (x,y)	80 µm
pixelsize (z)	/
FOV (z)	/
imaging time	/
number of frames	/

561nm	30%
640nm	3%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	80 µs
objective	wi60x (NA1.2)

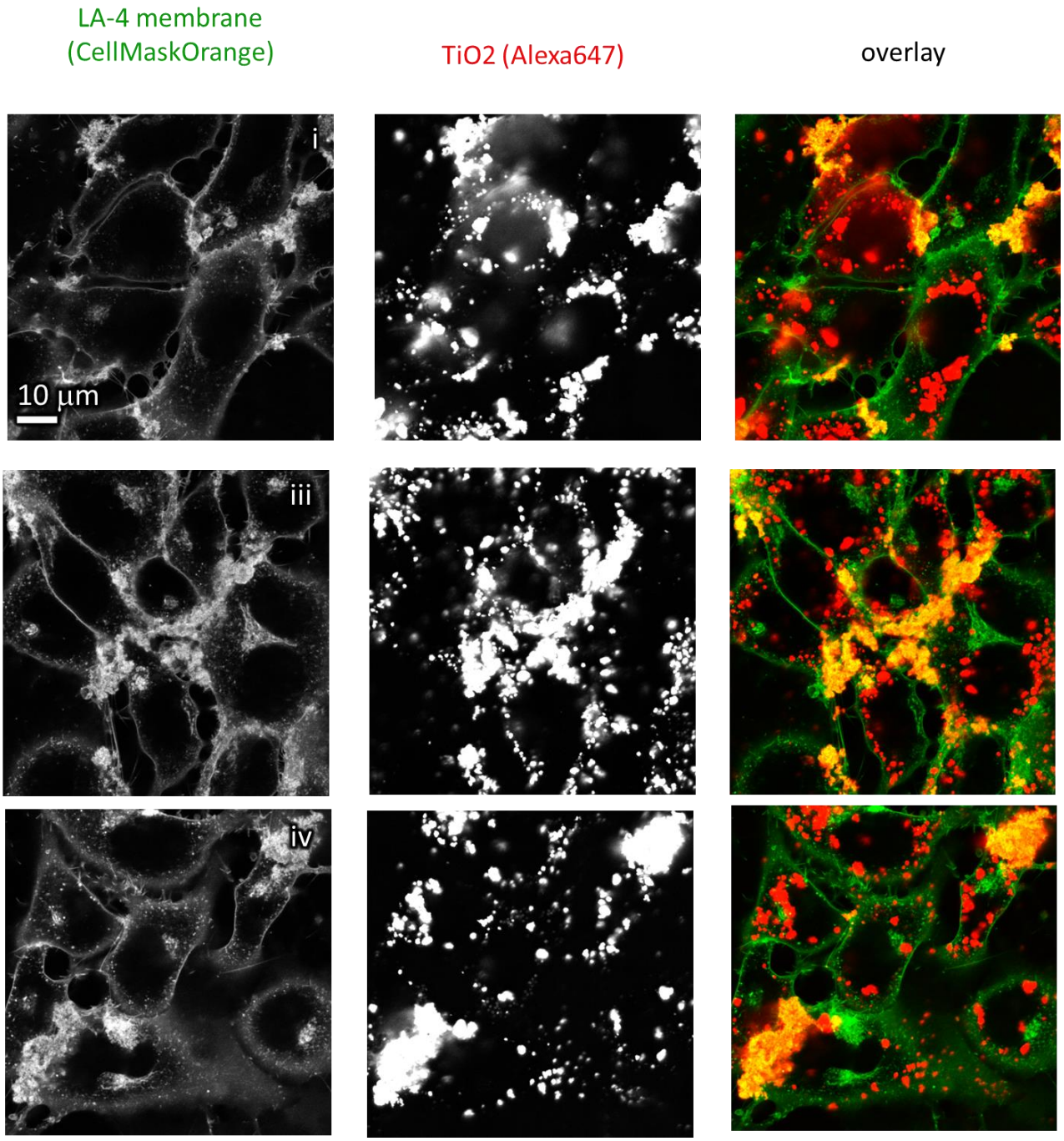


Figure S65: Statistics at surface nanoparticles to surface cells dose 10:1 – confocal xy recordings.

exposure: 100:1 exposure STED

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	100:1, 48h incubaton
imaging	xz STED, @48h

pixelsize (x,y)	30 nm
FOV (x,y)	30 µm
pixelsize (z)	85 nm
FOV (z)	20 µm
imaging time	/
number of frames	/

561nm	30%
640nm	3%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	80 µs
objective	wi60x (NA1.2)

iv pixelsize (x,y)	30 nm
FOV (x,y)	30 µm
pixelsize (z)	104 nm
FOV (z)	50 µm
imaging time	/
number of frames	/

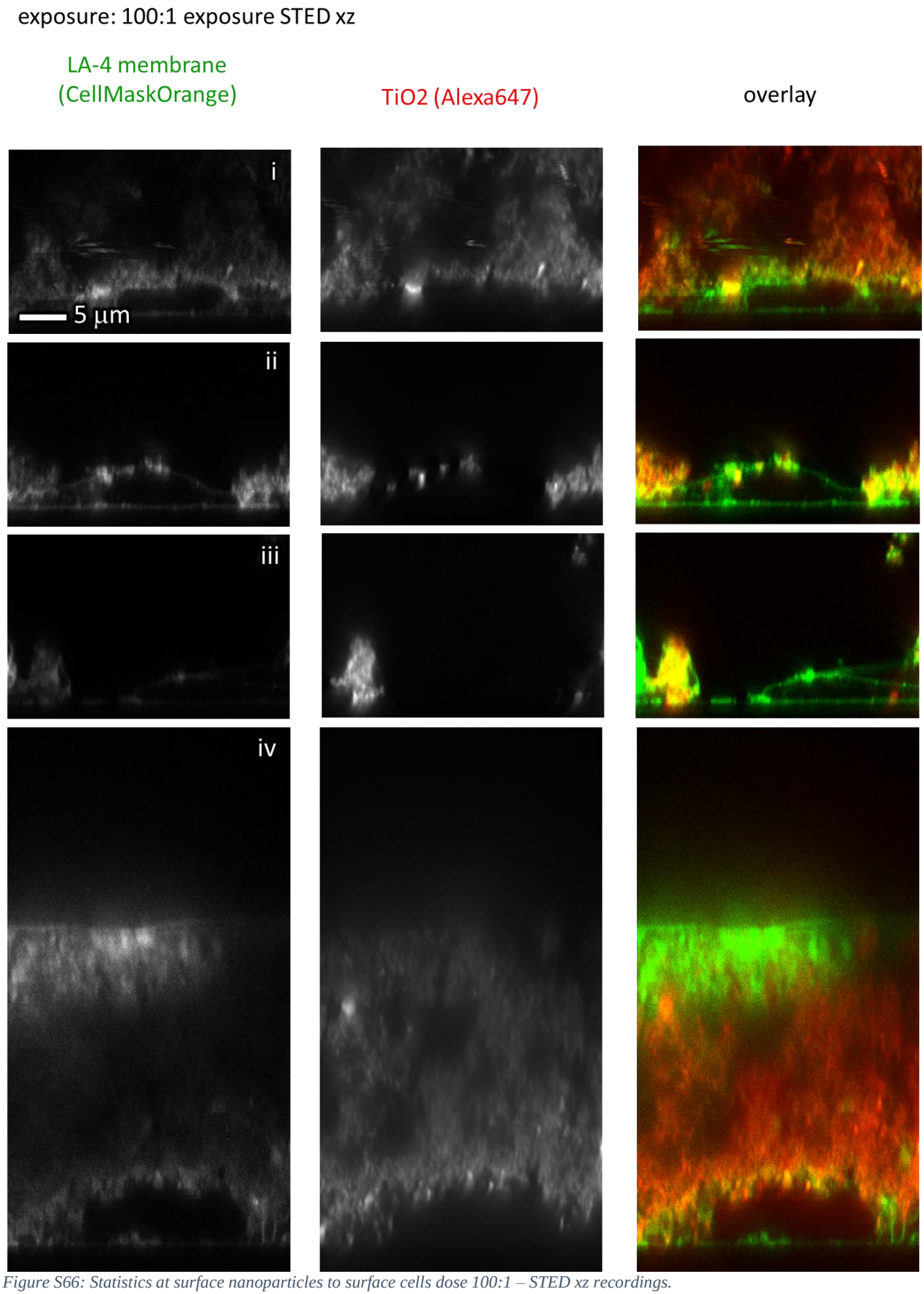


Figure S66: Statistics at surface nanoparticles to surface cells dose 100:1 – STED xz recordings.

control: 100:1 exposure STED xy

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	100:1, 48h incubaton
imaging	xy STED, @48h

pixelsize (x,y)	30 nm
FOV (x,y)	30 µm
pixelsize (z)	/
FOV (z)	/
imaging time	/
number of frames	/

561nm	30%
640nm	3%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	80 µs
objective	wi60x (NA1.2)

iv pixelsize (x,y)	30 nm
FOV (x,y)	30 µm
pixelsize (z)	/
FOV (z)	/
imaging time	/
number of frames	/

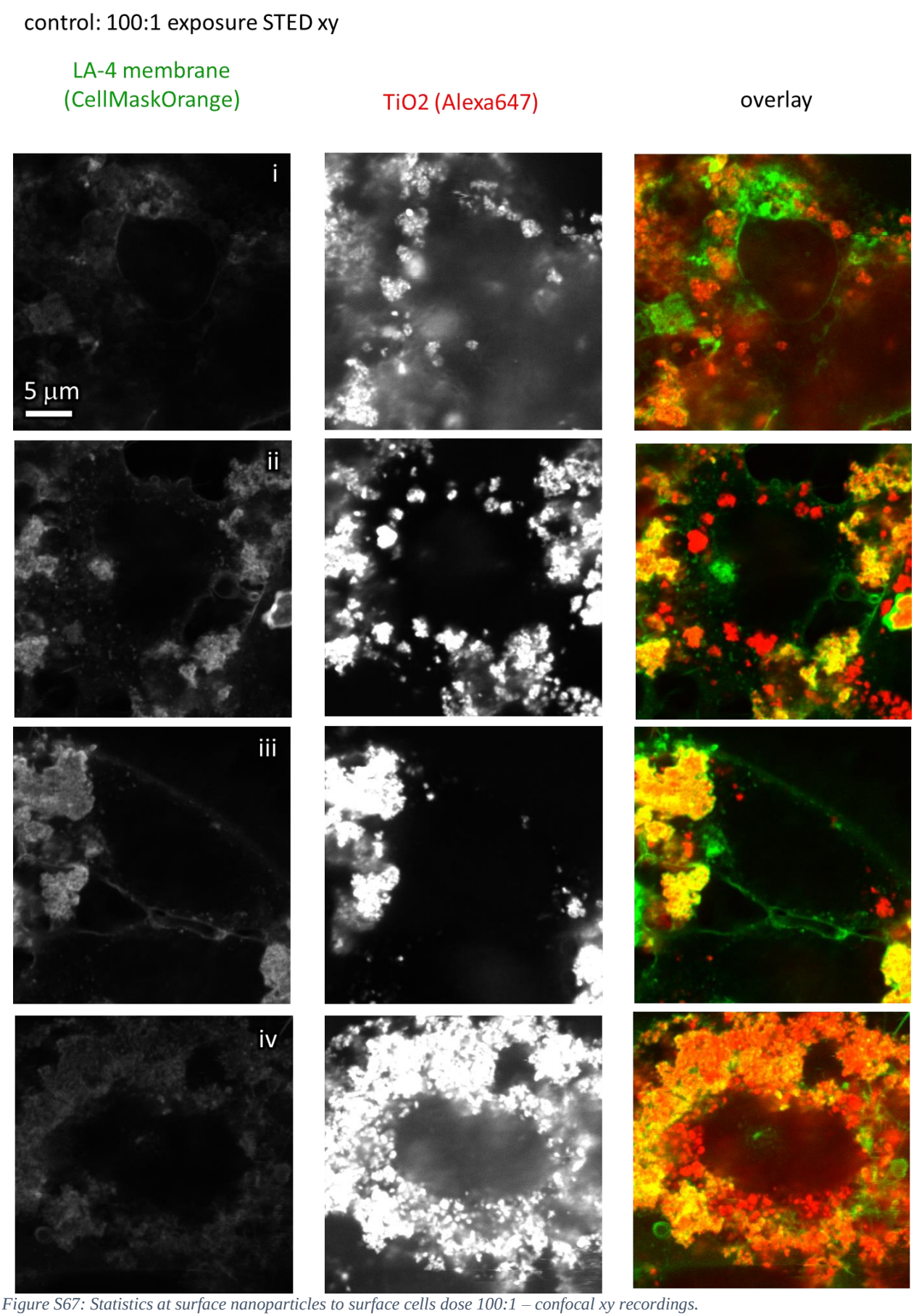


Figure S67: Statistics at surface nanoparticles to surface cells dose 100:1 – confocal xy recordings.

control: 100:1 exposure confocal xy

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	100:1, 48h incubaton
imaging	xy confocal, @48h

pixelsize (x,y)	100 nm
FOV (x,y)	80 µm
pixelsize (z)	/
FOV (z)	/
imaging time	/
number of frames	/

561nm	30%
640nm	3%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	80 µs
objective	wi60x (NA1.2)

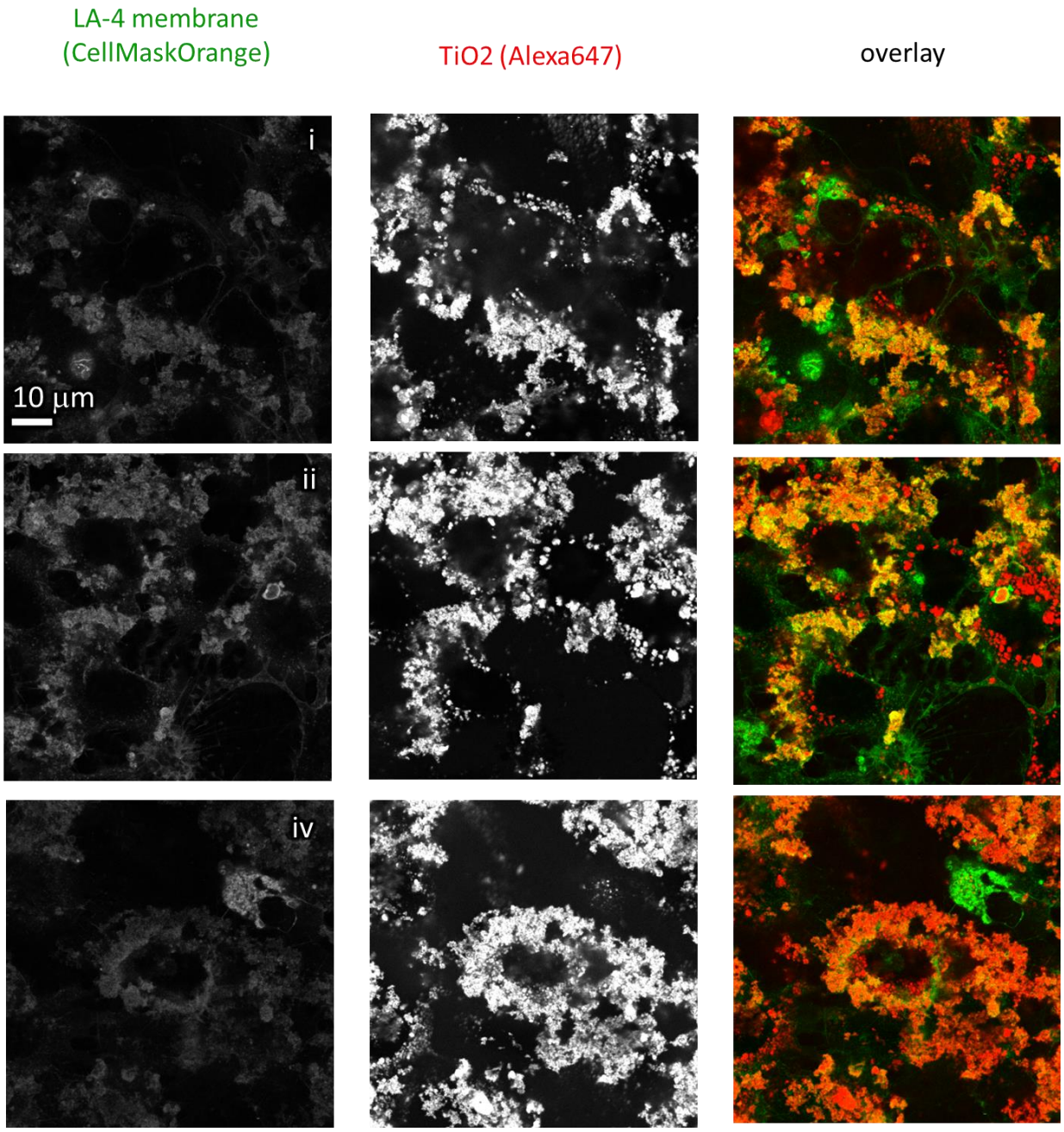


Figure S68: Statistics at surface nanoparticles to surface cells dose 100:1 – confocal xy recordings.

confocal xy – statistics over sample

LA-4 membrane (CellMaskOrange)

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	/
exposure	/
imaging	xy confocal, @48h

TiO2 (Alexa647)

pixelsize (x,y)	100 nm
FOV (x,y)	80 µm
pixelsize (z)	/
FOV (z)	/
imaging time	/
number of frames	/

overlay

561nm	30%
640nm	30%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	20 µs
objective	wi60x (NA1.2)

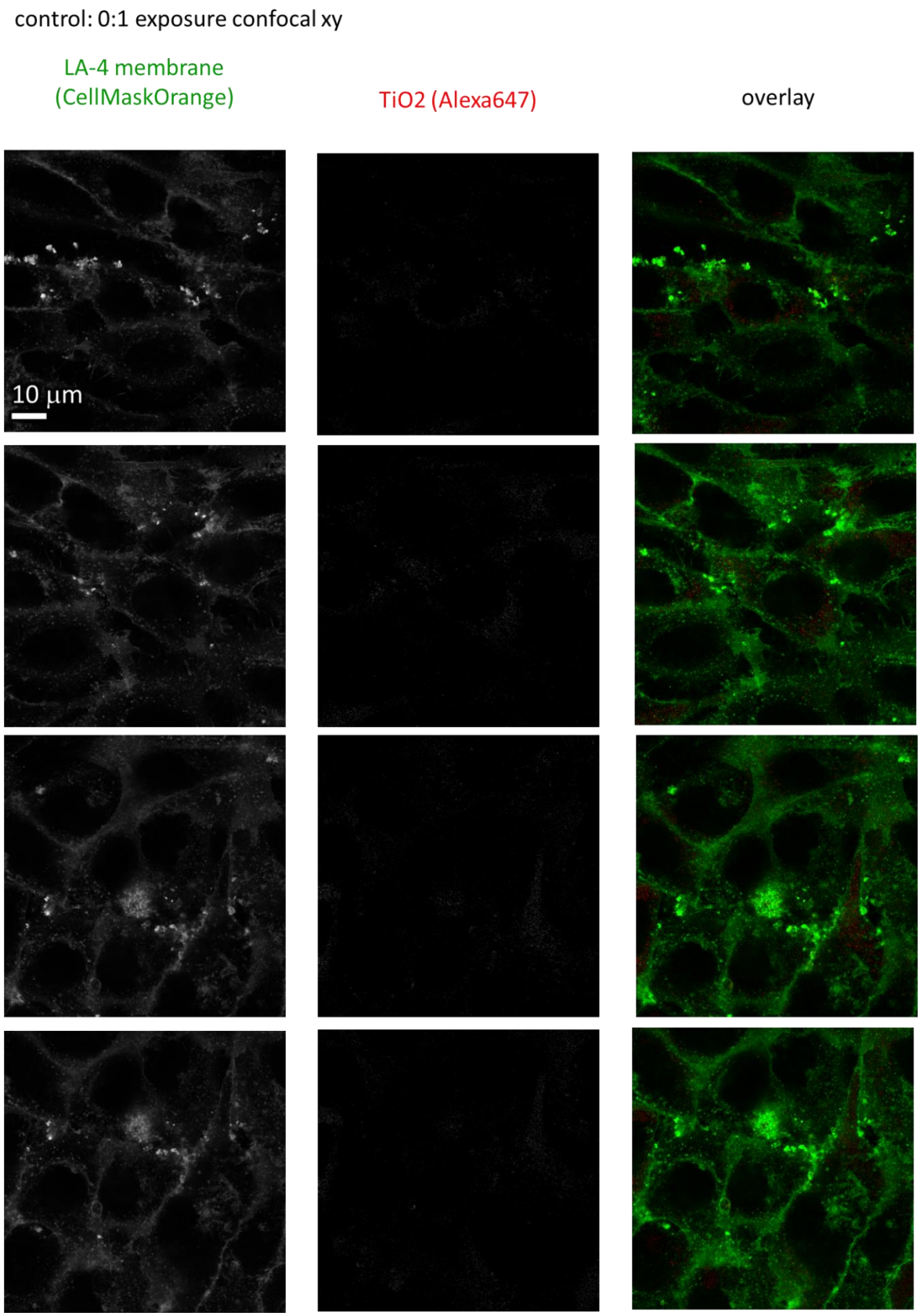
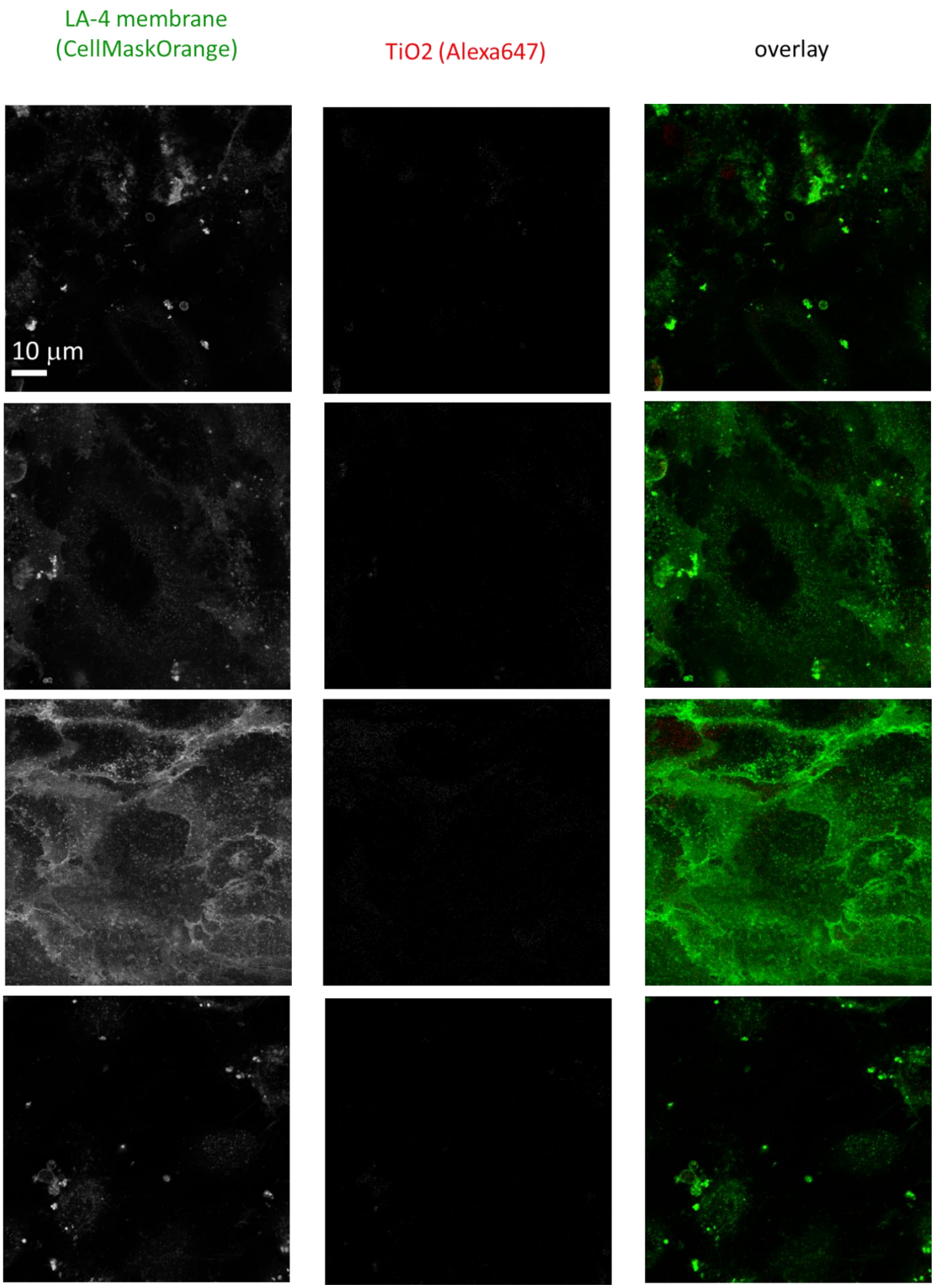


Figure S69: Statistics for control with no exposure to nanomaterial – confocal xy recordings.

control: 0:1 exposure confocal xy



control: 0:1 exposure confocal xy

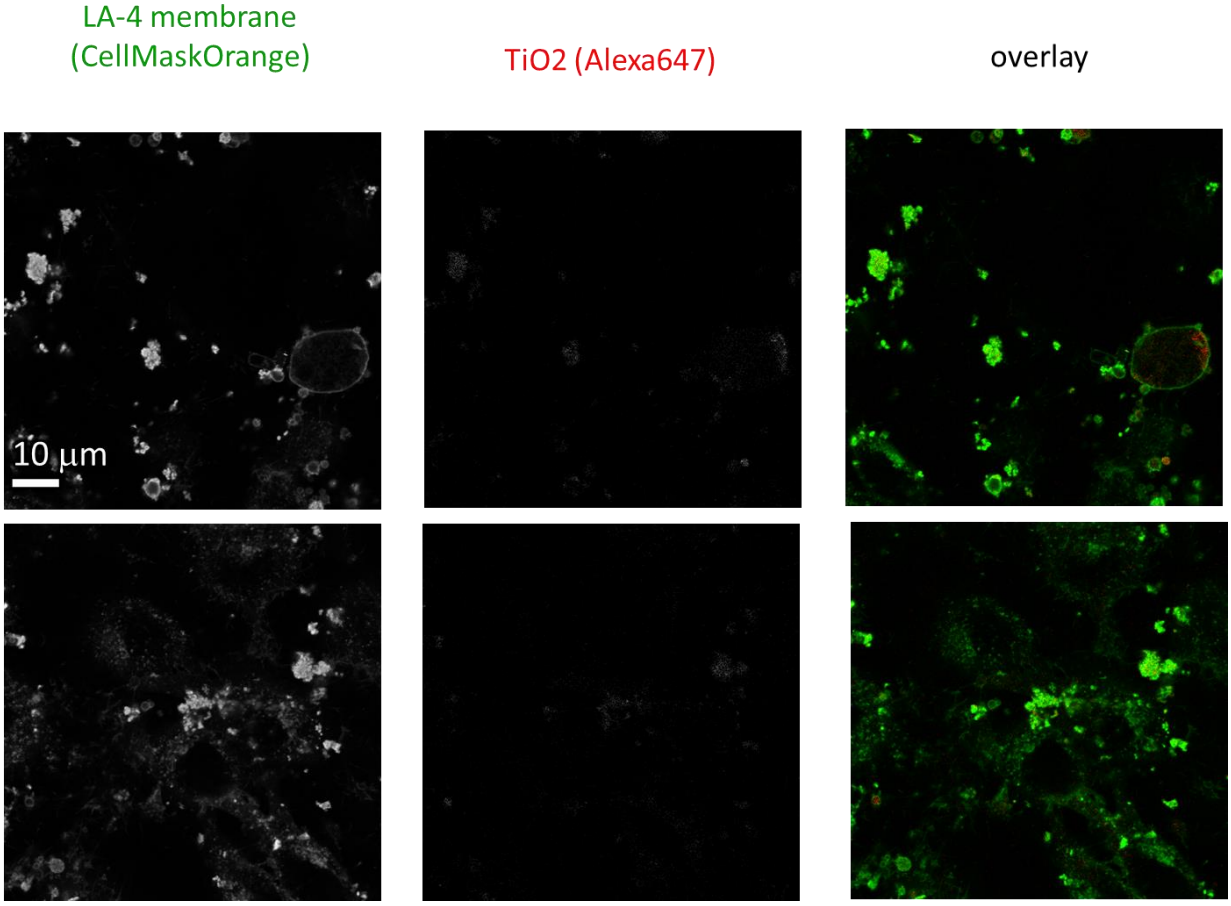


Figure S70: Statistics for control with no exposure to nanomaterial – confocal xy recordings.

control: 1:1 exposure confocal xy

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	1:1, 48h incubation
imaging	xy confocal, @48h

pixelsize (x,y)	100 nm
FOV (x,y)	80 µm
pixelsize (z)	/
FOV (z)	/
imaging time	/
number of frames	/

561nm	30%
640nm	30%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	20 µs
objective	wi60x (NA1.2)

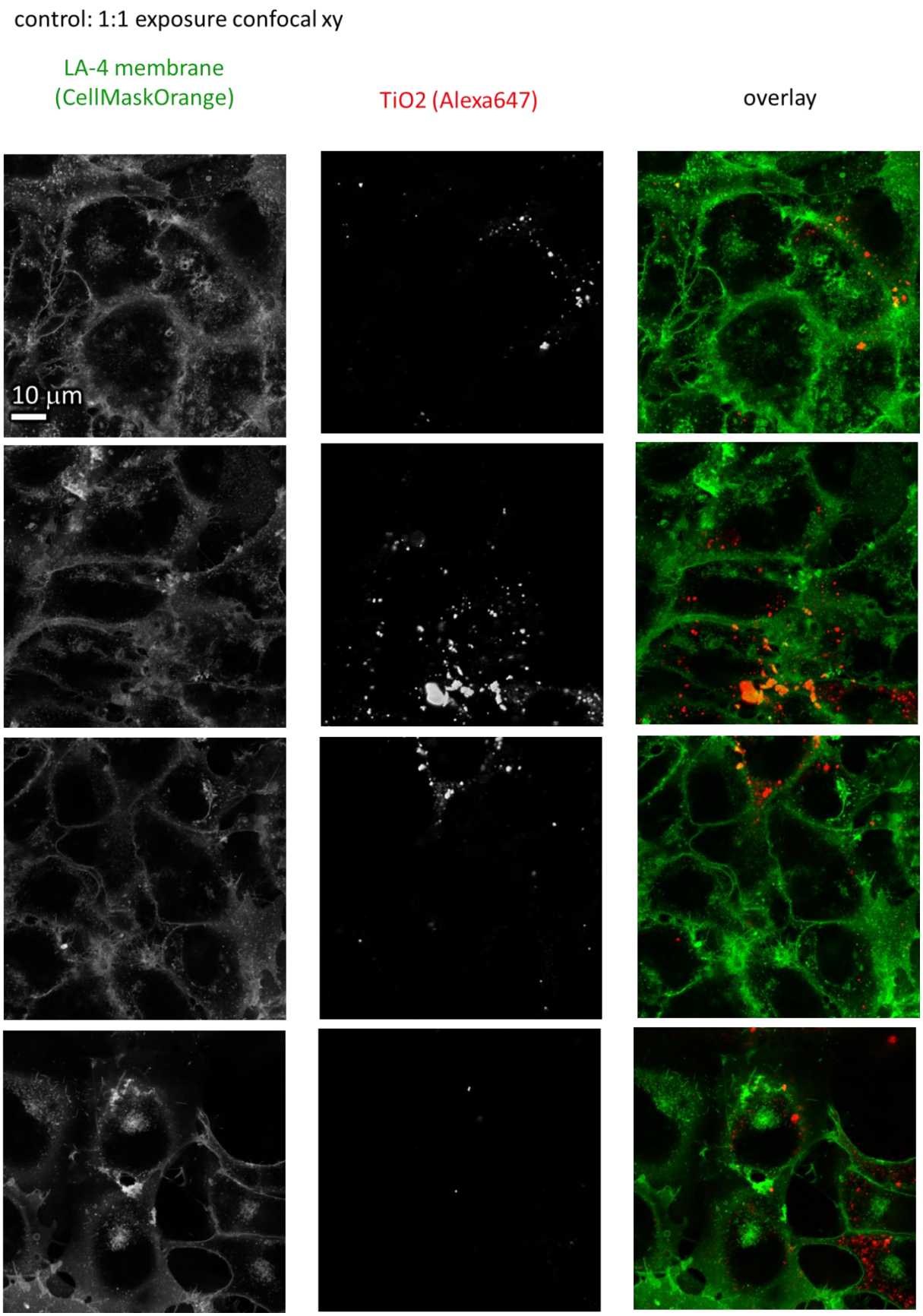
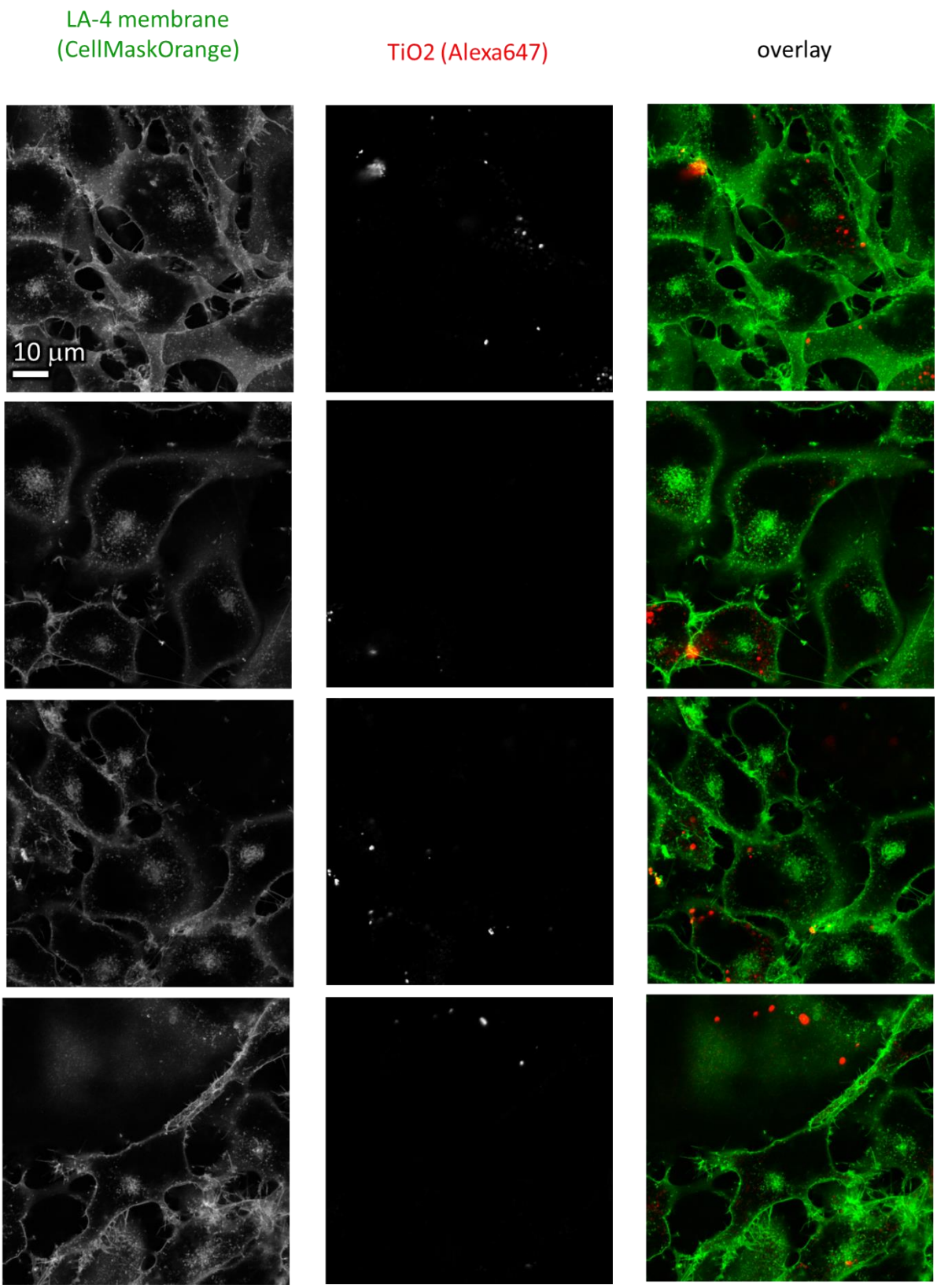


Figure S71: Statistics at surface nanoparticles to surface cells dose 1:1 – confocal xy recordings.

control: 1:1 exposure confocal xy



control: 1:1 exposure confocal xy

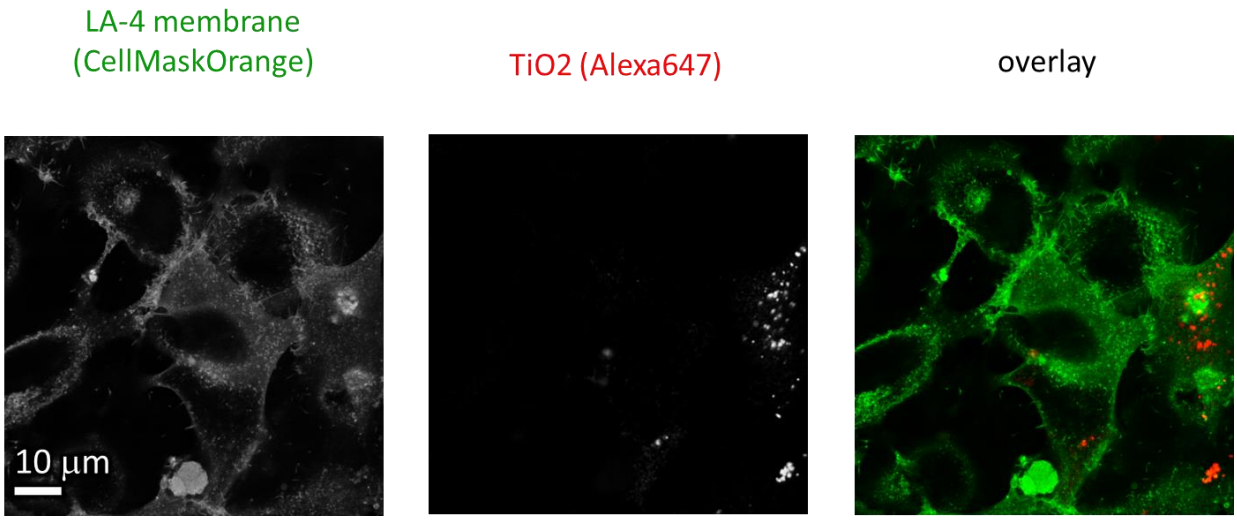


Figure S72: Statistics at surface nanoparticles to surface cells dose 1:1 – confocal xy recordings.

control: 10:1 exposure confocal xy

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	10:1, 48h incubation
imaging	xy confocal, @48h

pixelsize (x,y)	100 nm
FOV (x,y)	80 µm
pixelsize (z)	/
FOV (z)	/
imaging time	/
number of frames	/

561nm	30%
640nm	30%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	20 µs
objective	wi60x (NA1.2)

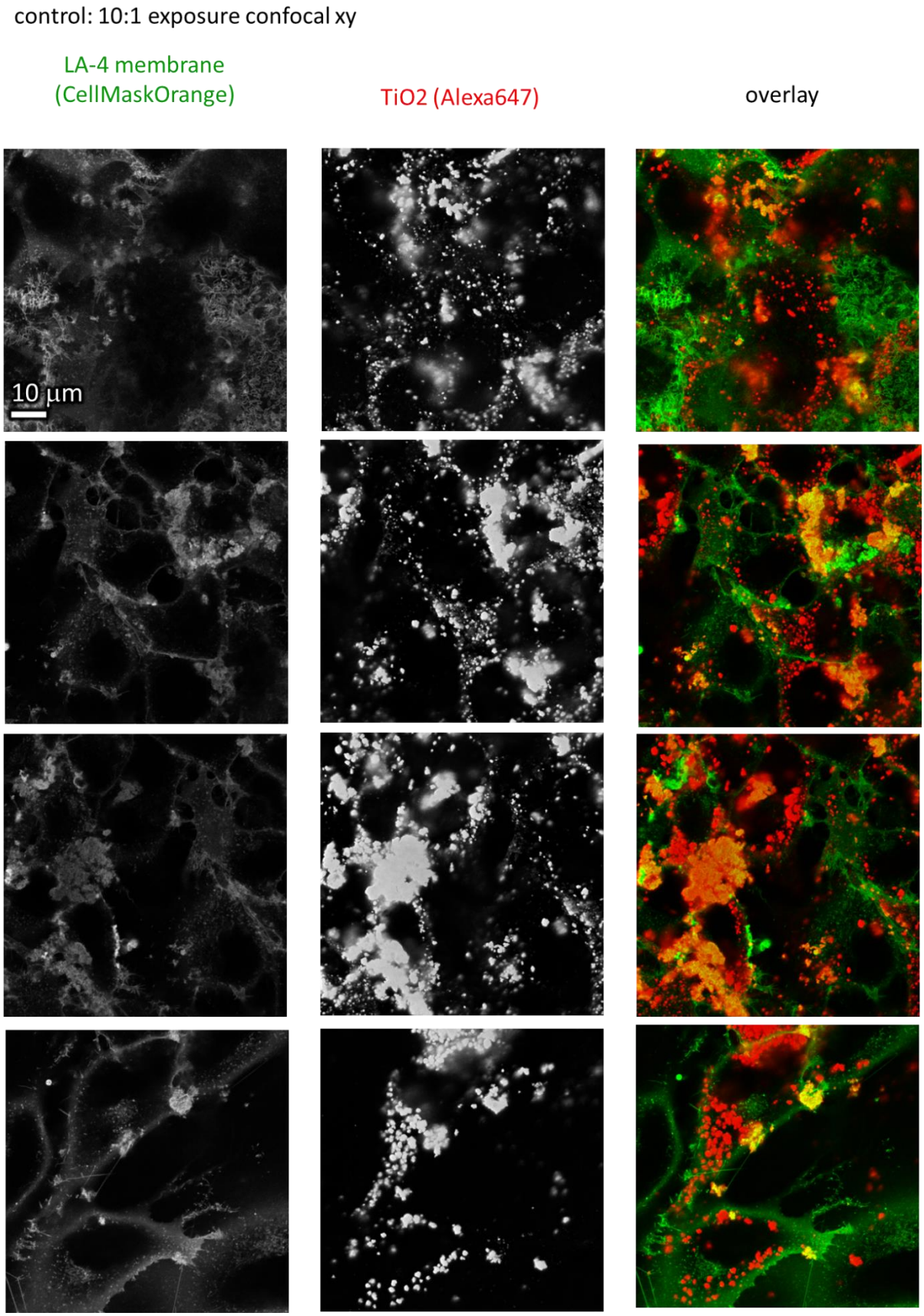


Figure S73: Statistics at surface nanoparticles to surface cells dose 10:1 – confocal xy recordings.

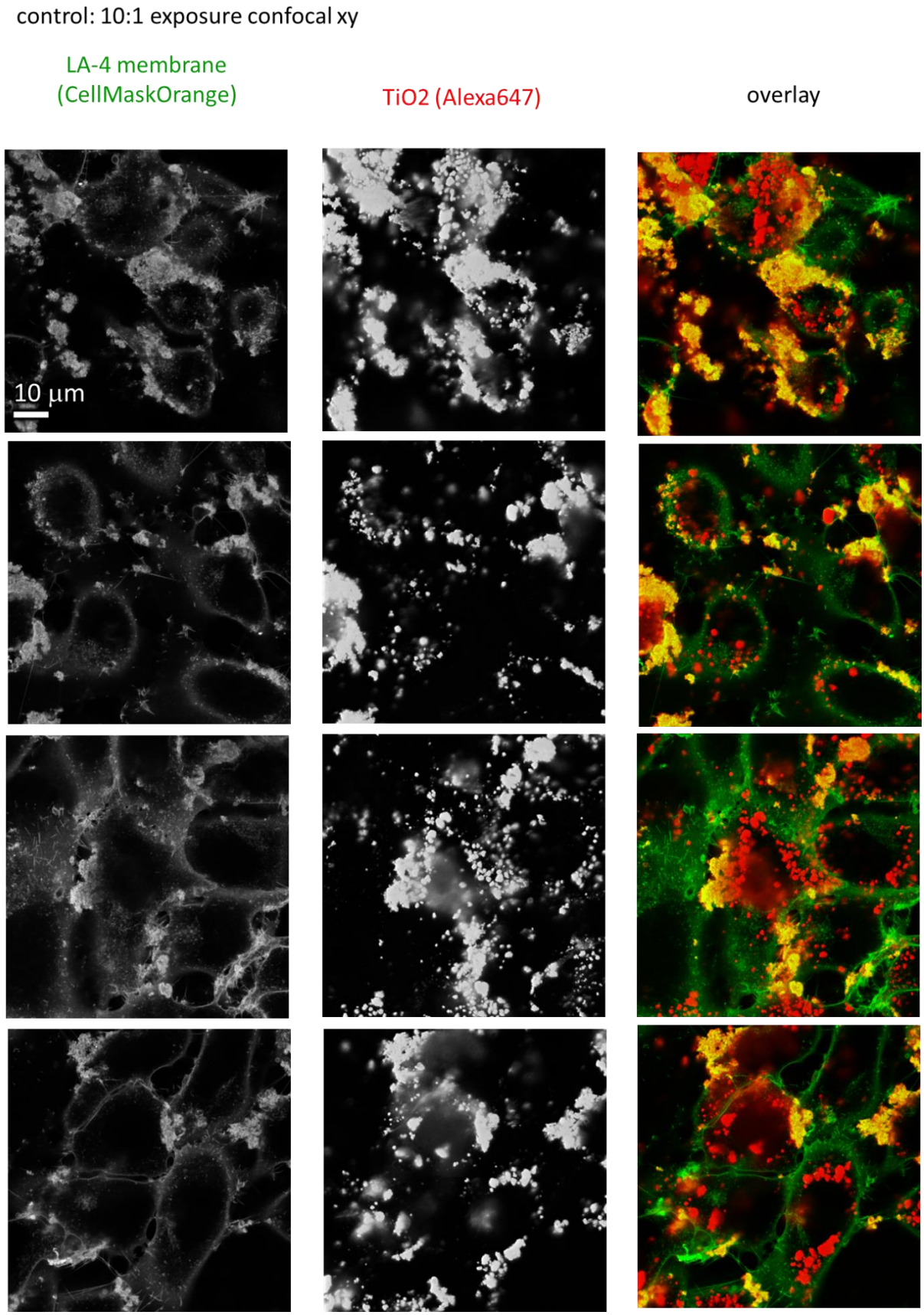


Figure S74: Statistics at surface nanoparticles to surface cells dose 10:1 – confocal xy recordings.

control: 100:1 exposure confocal xy

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	100:1, 48h incubation
imaging	xy confocal, @48h

pixelsize (x,y)	100 nm
FOV (x,y)	80 µm
pixelsize (z)	/
FOV (z)	/
imaging time	/
number of frames	/

561nm	30%
640nm	30%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	20 µs
objective	wi60x (NA1.2)

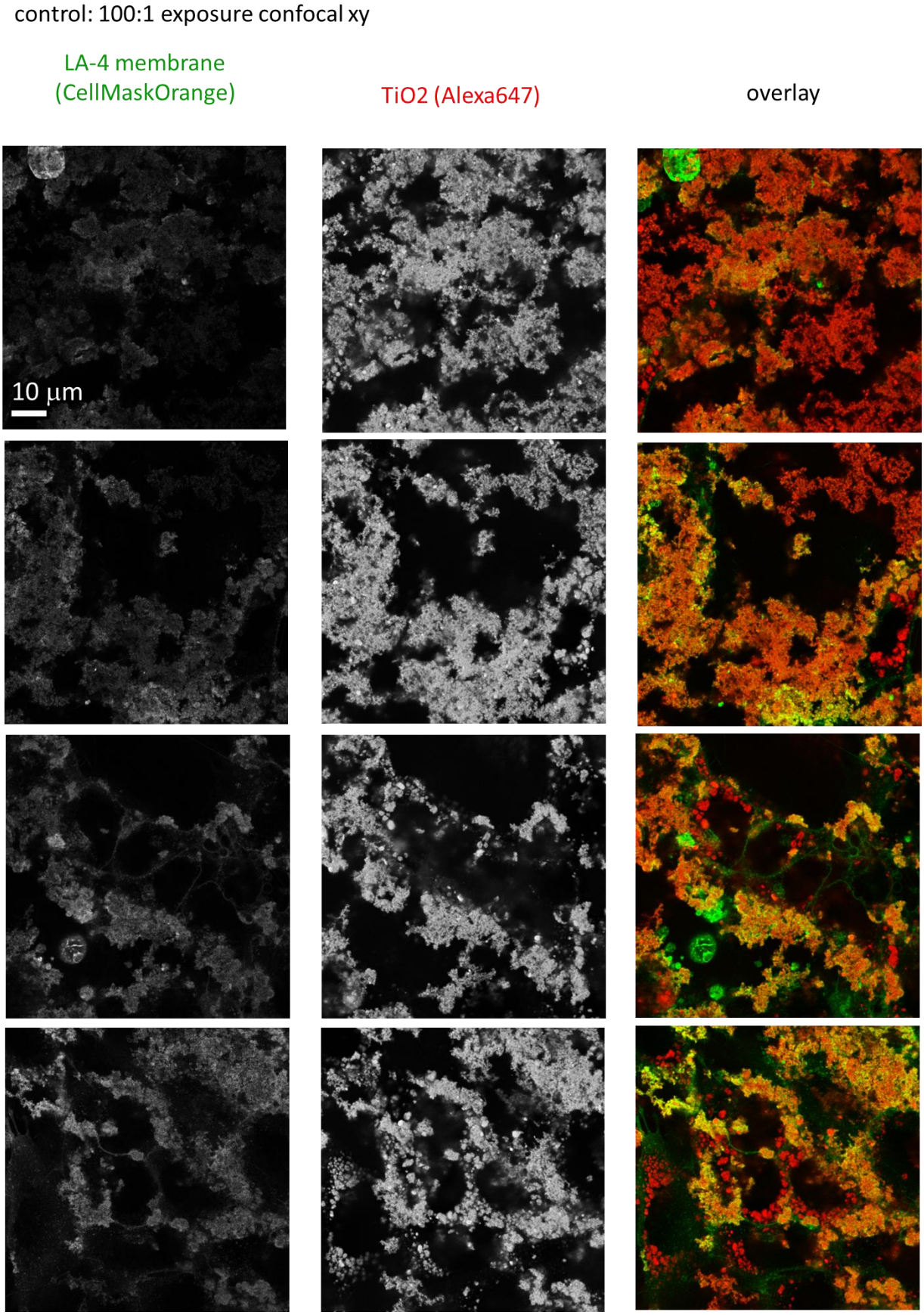


Figure S75: Statistics at surface nanoparticles to surface cells dose 100:1 – confocal xy recordings.

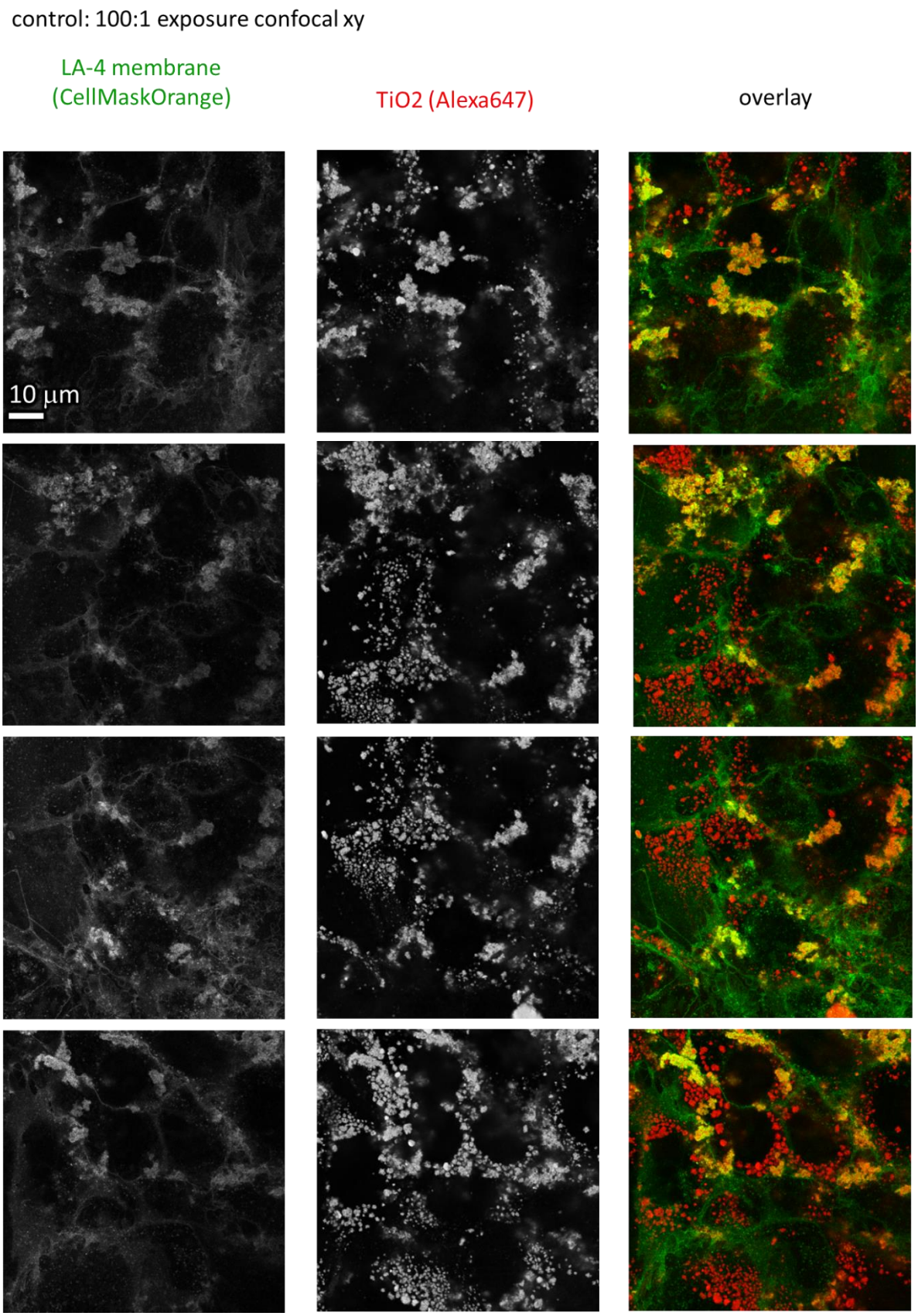


Figure S76: Statistics at surface nanoparticles to surface cells dose 100:1 – confocal xy recordings.

S2f – STED, HIM, SE SEM resolution comparison

For STED supplement see

S2d – Time evolution of cauliflower growth and S2e – Dose-dependent exposure of LA-4 to TiO2 nanotubes.

Main message

Large magnification high-resolution HIM and SE SEM show that cauliflower-like bio-nano composites really are composed of small tubes and lipids as we claim based on STED high-resolution images.

Supporting raw and analysed data:

Figure S77-Figure S84

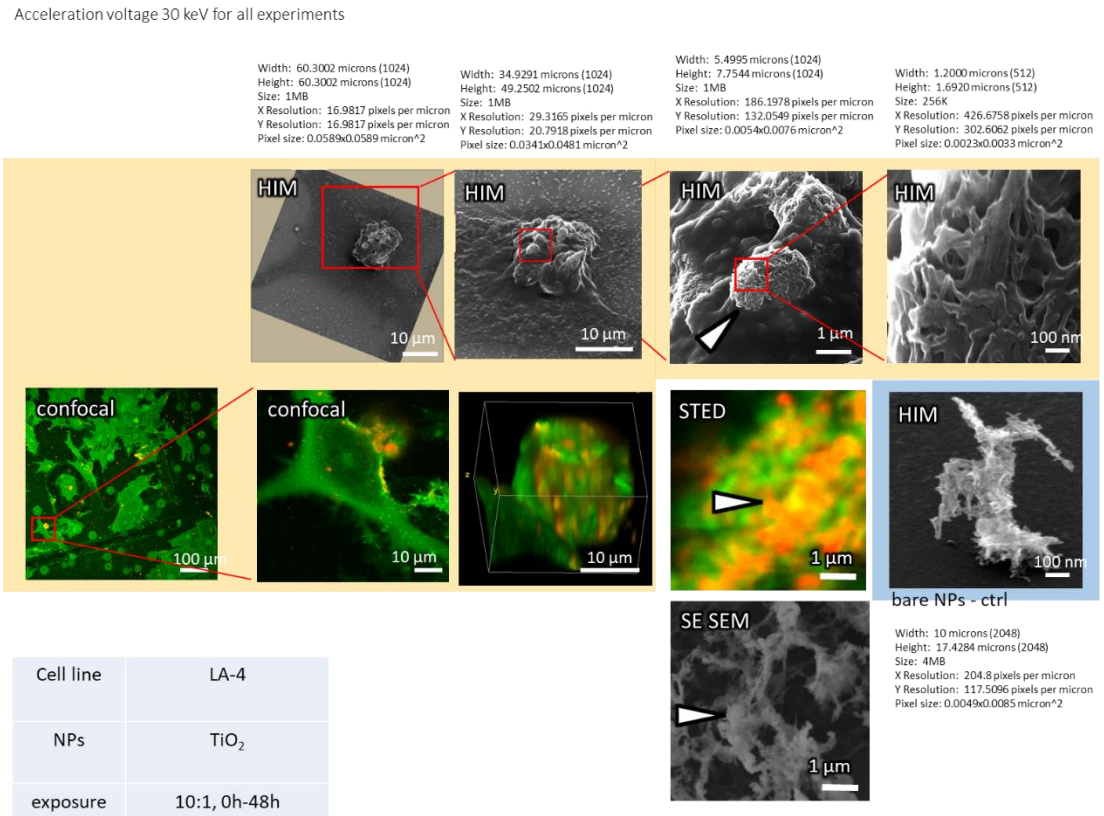
Materials and methods

In both HIM and SEM experiments samples were prepared in the same way. LA-4 cells were seeded in flasks with Si wafers (Pelcotec™ SFG12 Finder Grid Substrate, Ted Pella) placed on the bottom. After 48h Si wafers with cells on the surface were transferred to the 8-well holders. In this stage, Si wafers were approximately 70% confluent. Fresh medium was added together with 10:1(surface:surface) TiO_2 nanotubes. Samples where incubated for following 24 h before imaging or freezing. Freezing has been performed by freeze drying.

Controls and statistics – HIM

Several positions in several samples:

Figure S77-Figure S83



Cell line	LA-4
NPs	TiO_2
exposure	10:1, 0h-48h
imaging	HIM, @ 24h

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2-Alexa647
exposure	/
imaging	xy confocal wide, @48h xy confocal, @48h xy STED, @48h xy 3D confocal, @48h

xy

image	xy conf wide/ conf/ STED
pixelsize (x,y)	100 nm/100 nm/30 nm
FOV (x,y)	80 µm/40 µm/10 µm

xyz – 3D

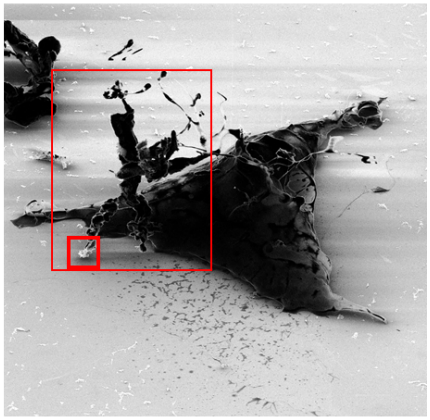
pixelsize (x,y)	100 nm
FOV (x,y)	30 µm
pixelsize (z)	500 nm
FOV (z)	30 µm

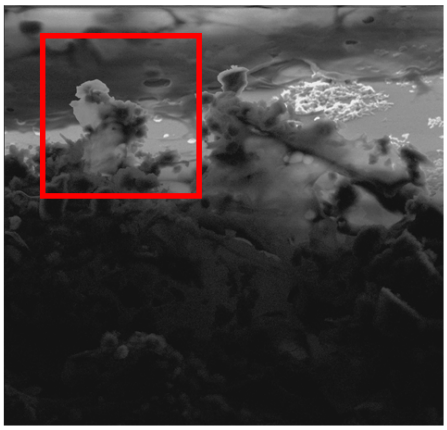
561nm	30%
640nm	3%
STED	20%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	80 µs
objective	wi60x (NA1.2)

Figure S77: Fluorescence and HIM correlation micorscopy with 3D scan as well as higher magnifications.

Cauliflover formation (LA4)

Title: A3-cell-2.czi
Width: 85.0000 microns (2048)
Height: 85.0001 microns (2048)
Size: 4MB
X Resolution: 24.0941 pixels per micron
Y Resolution: 24.0941 pixels per micron
Pixel size: 0.0415x0.0415 micron^2





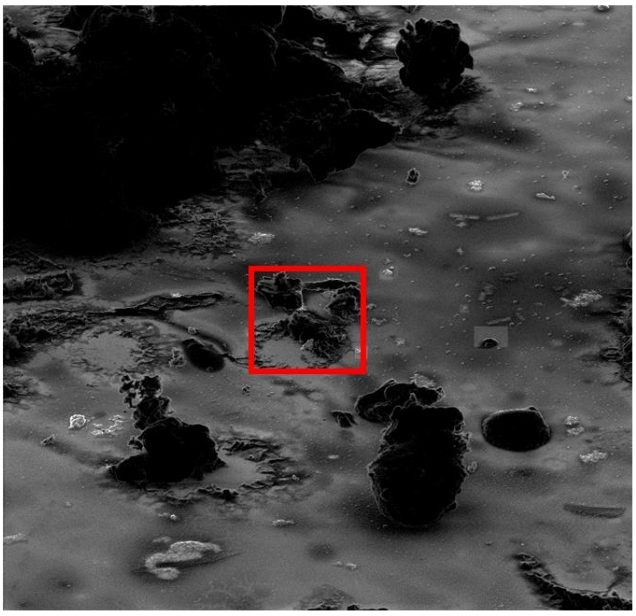
LA4 cell with destroyed part of a membrane which is ful of TiO2 nanoparticles

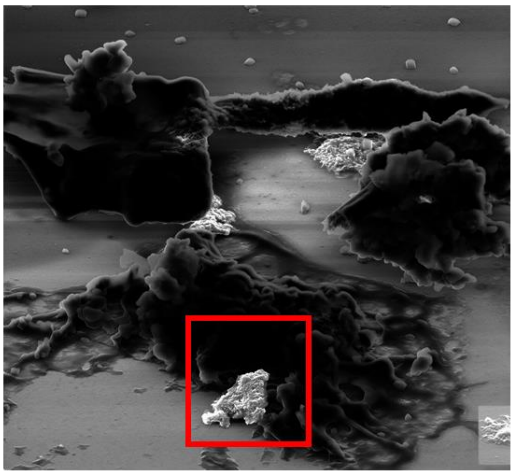
Cell line	LA-4
NPs	TiO_2
exposure	10:1, 0h-48h
imaging	HIM, @ 24h

Figure S78: Different magnifications of samples exposed to 10:1 (surface of nanomaterial: surface of cell) dose using HIM.

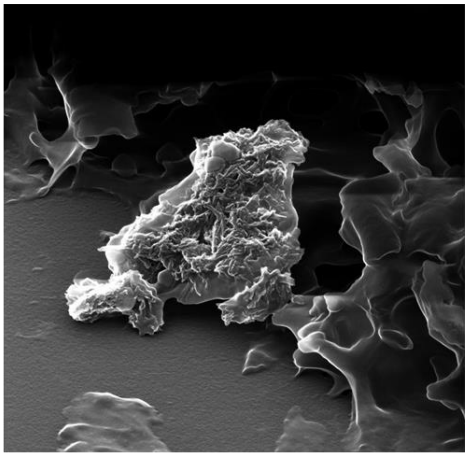
Cauliflover formation (LA4)

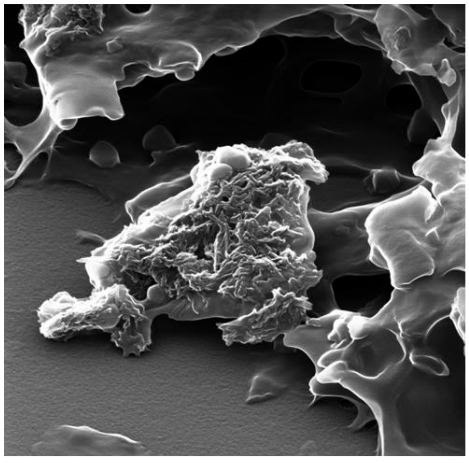
Title: C3-5.czi
Width: 75 microns (1024)
Height: 130.7127 microns (1024)
Size: 1MB
X Resolution: 13.6533 pixels per micron
Y Resolution: 7.8340 pixels per micron
Pixel size: 0.0732x0.1276 micron^2





Title: C3-9.czi
Width: 2.5 microns (2048)
Height: 4.3571 microns (2048)
Size: 4MB
X Resolution: 819.2 pixels per micron
Y Resolution: 470.0384 pixels per micron
Pixel size: 0.0012x0.0021 micron^2



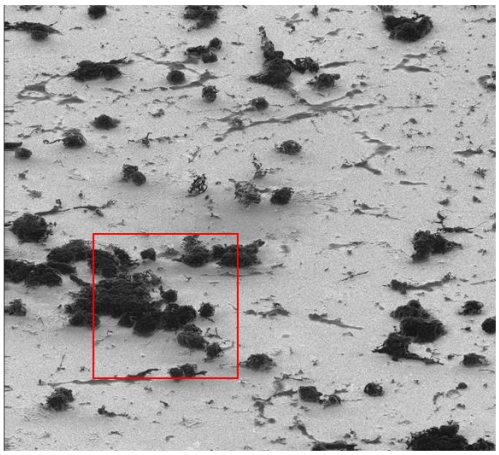


Cell line	LA-4
NPs	TiO_2
exposure	10:1, 0h-48h
imaging	HIM, @ 24h

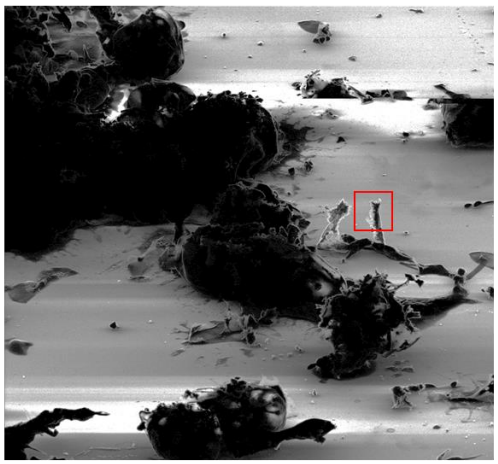
Figure S79: Different magnifications of samples exposed to 10:1 (surface of nanomaterial: surface of cell) dose using HIM.

Destroyed MHS, debris of membranes with NPs

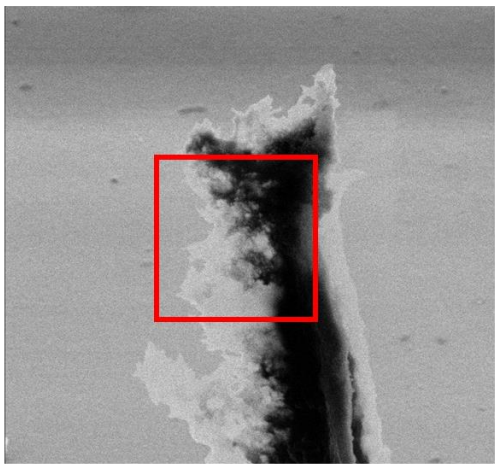
Title: A8-4.czi
Width: 350 microns (2048)
Height: 609.9927 microns (2048)
Size: 4MB
X Resolution: 5.8514 pixels per micron
Y Resolution: 3.3574 pixels per micron
Pixel size: 0.1709x0.2978 micron^2



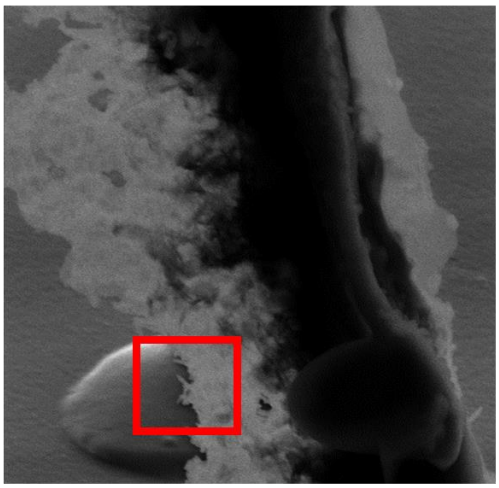
Title: A8-6.czi
Width: 70 microns (2048)
Height: 121.9985 microns (2048)
Size: 4MB
X Resolution: 29.2571 pixels per micron
Y Resolution: 16.7871 pixels per micron
Pixel size: 0.0342x0.0596 micron^2



Title: A8-11.czi
Width: 4.5 microns (1024)
Height: 7.8428 microns (1024)
Size: 1MB
X Resolution: 227.5556 pixels per micron
Y Resolution: 130.5662 pixels per micron
Pixel size: 0.0044x0.0077 micron^2



Title: A8-13.czi
Width: 1.9986 microns (1024)
Height: 3.4833 microns (1024)
Size: 1MB
X Resolution: 512.3530 pixels per micron
Y Resolution: 293.9765 pixels per micron
Pixel size: 0.0020x0.0034 micron^2



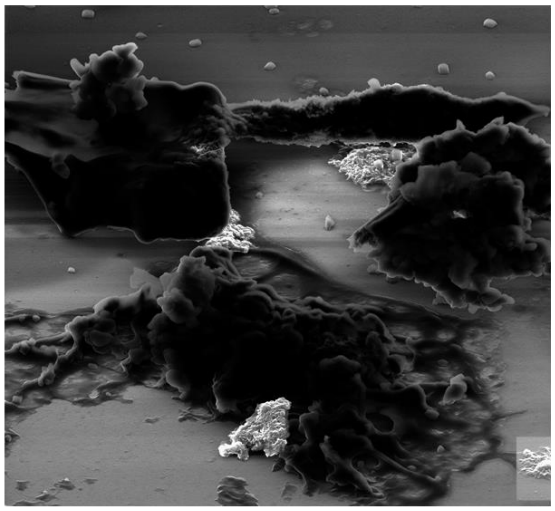
Single TiO2 nanotubes nicely observed at the surface
Whole image is just 2µm * 2µm

Cell line	MHS
NPs	TiO_2
exposure	10:1, 0h-48h
imaging	HIM, @ 24h SE SEM, @ 24h

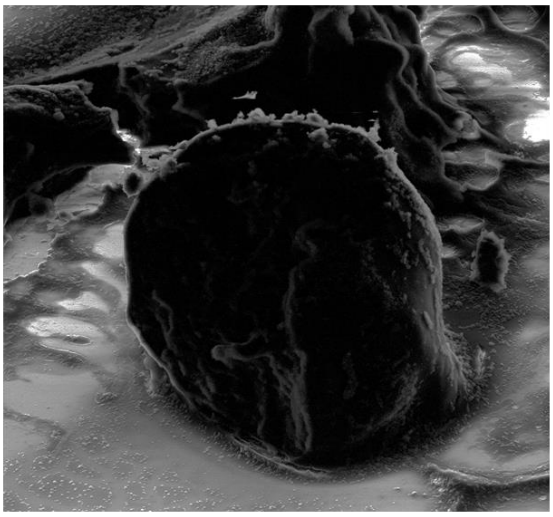
Figure S80: Different magnifications of samples exposed to 10:1 (surface of nanomaterial: surface of cell) dose using HIM.

MHS, short term exposure with NPs

Title: C3-7.czi
Width: 12 microns (2048)
Height: 20.9140 microns (2048)
Size: 4MB
X Resolution: 170.6667 pixels per micron
Y Resolution: 97.9247 pixels per micron
Pixel size: 0.0059x0.0102 micron^2



Title: C3-11.czi
Width: 15.0000 microns (2048)
Height: 26.1425 microns (2048)
Size: 4MB
X Resolution: 136.5333 pixels per micron
Y Resolution: 78.3397 pixels per micron
Pixel size: 0.0073x0.0128 micron^2

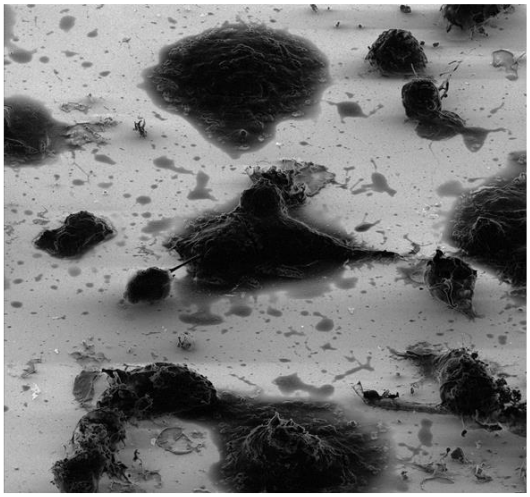


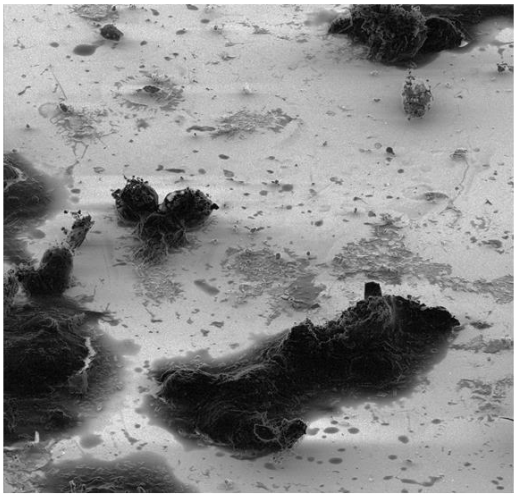
Cell line	MHS
NPs	TiO_2
exposure	10:1, 0h-48h
imaging	HIM, @ 1h

Figure S81: Different magnifications of samples exposed to 10:1 (surface of nanomaterial: surface of cell) dose using HIM.

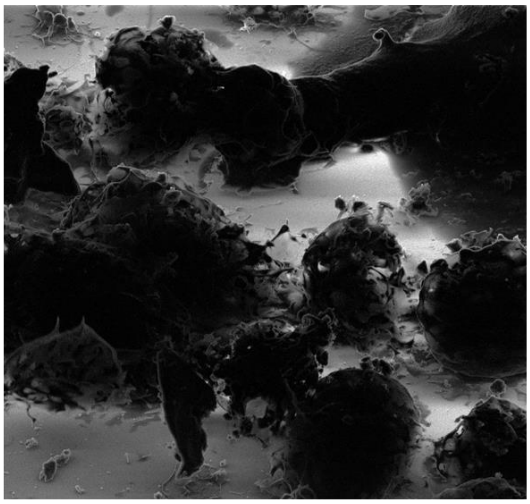
MHS, long term exposure with NPs

Title: B3-cell-20-tilted.czi
Width: 125 microns (2048)
Height: 193.2184 microns (2048)
Size: 4MB
X Resolution: 16.384 pixels per micron
Y Resolution: 10.5994 pixels per micron
Pixel size: 0.0610x0.0943 micron^2





Title: B3-cell-24-tilted.czi
Width: 40 microns (2048)
Height: 61.8299 microns (2048)
Size: 4MB
X Resolution: 51.2 pixels per micron
Y Resolution: 33.1231 pixels per micron
Pixel size: 0.0195x0.0302 micron^2



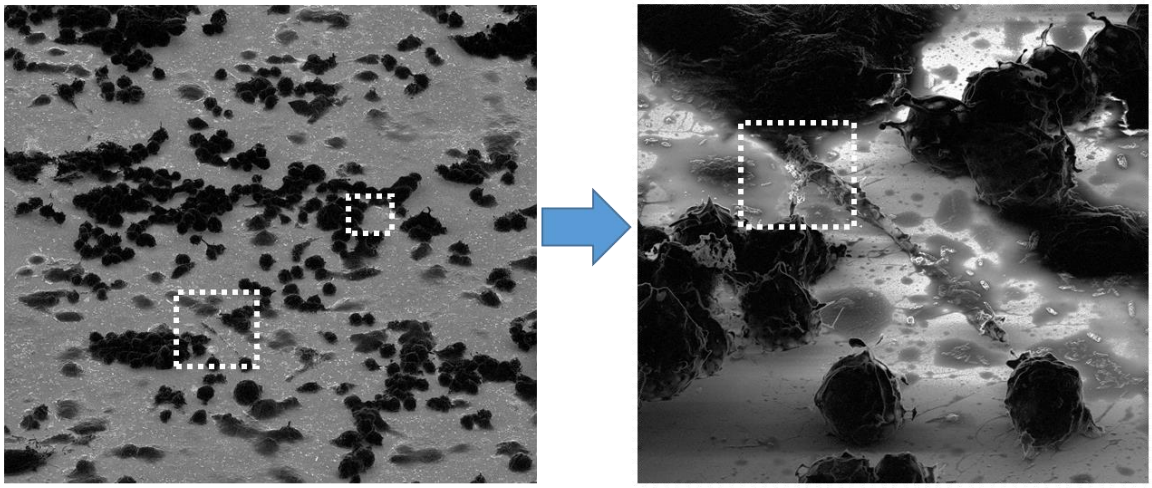
Cell line	LA-4
NPs	TiO_2
exposure	10:1, 0h-48h
imaging	HIM, @ 24h

Figure S82: Different magnifications of samples exposed to 10:1 (surface of nanomaterial: surface of cell) dose using HIM.

MHS, long term exposure with NPs

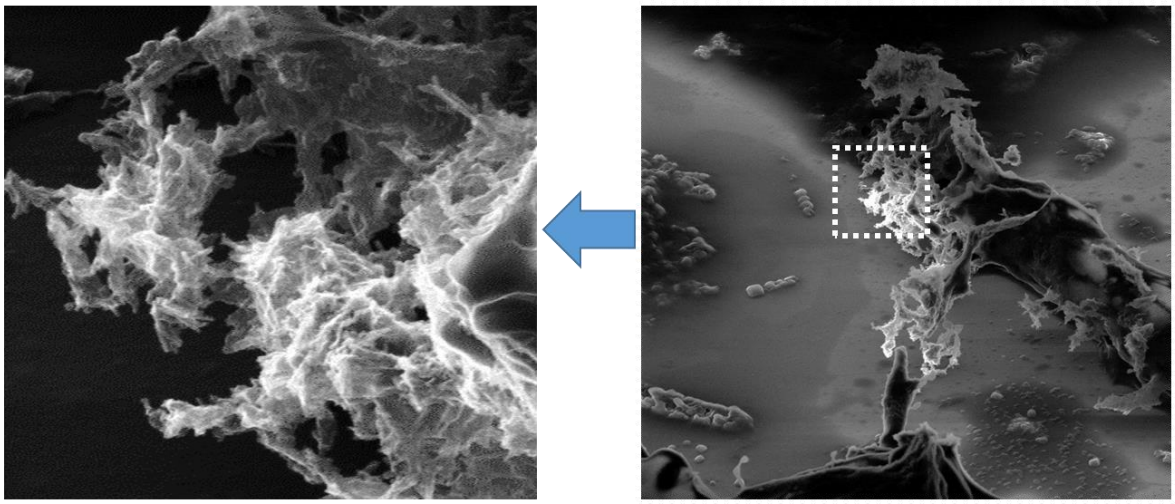
Title: A7-cell-14-tilted.czi
Width: 425 microns (2048)
Height: 656.9427 microns (2048)
Size: 4MB
X Resolution: 4.8188 pixels per micron
Y Resolution: 3.1175 pixels per micron
Pixel size: 0.2075x0.3208 micron^2

Title: A7-cell-15-tilted.czi
Width: 90.0000 microns (2048)
Height: 139.1173 microns (2048)
Size: 4MB
X Resolution: 22.7556 pixels per micron
Y Resolution: 14.7214 pixels per micron
Pixel size: 0.0439x0.0679 micron^2



Title: A7-cell-17-tilted.czi
Width: 15.0000 microns (2048)
Height: 23.1862 microns (2048)
Size: 4MB
X Resolution: 136.5333 pixels per micron
Y Resolution: 88.3284 pixels per micron
Pixel size: 0.0073x0.0113 micron^2

Title: A7-cell-19-tilted.czi
Width: 1.4999 microns (1024)
Height: 2.3185 microns (1024)
Size: 1MB
X Resolution: 682.6956 pixels per micron
Y Resolution: 441.6605 pixels per micron
Pixel size: 0.0015x0.0023 micron^2



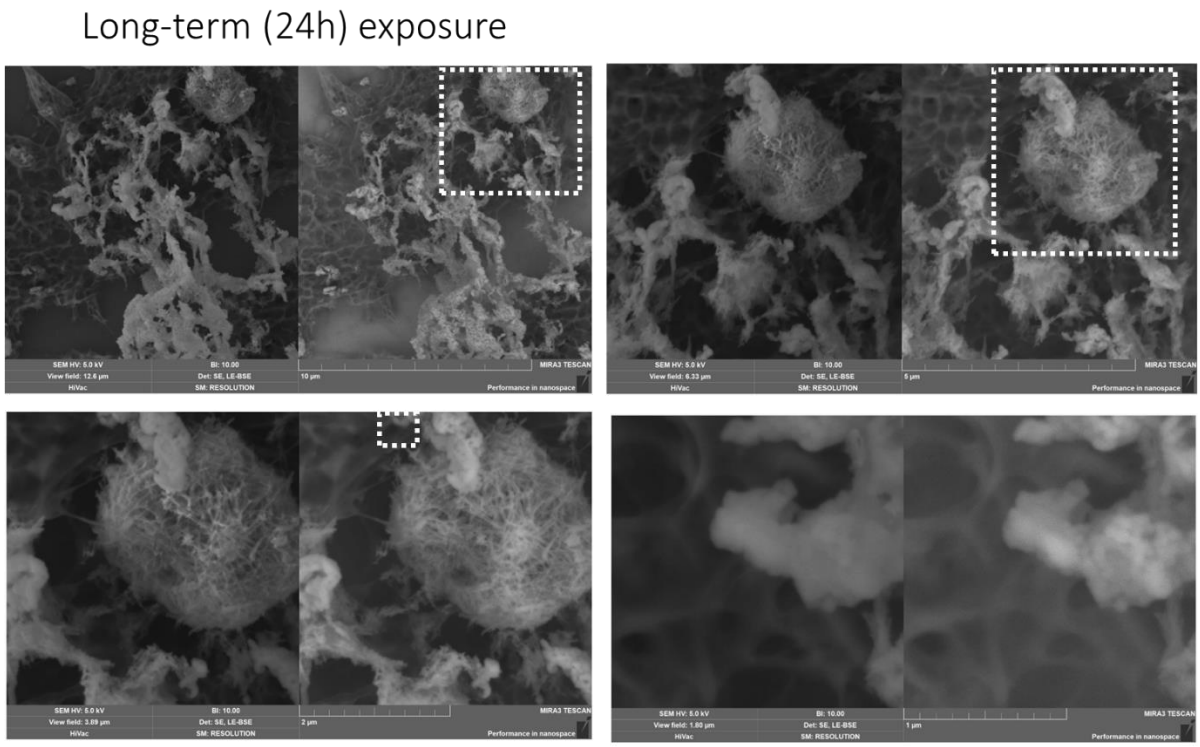
Cell line	LA-4
NPs	TiO_2
exposure	10:1, 0h-48h
imaging	HIM, @ 24h

Figure S83: Different magnifications of samples exposed to 10:1 (surface of nanomaterial: surface of cell) dose using HIM.

Controls and statistics – SE SEM

Larger field of view on the sample with several zoom-ins:

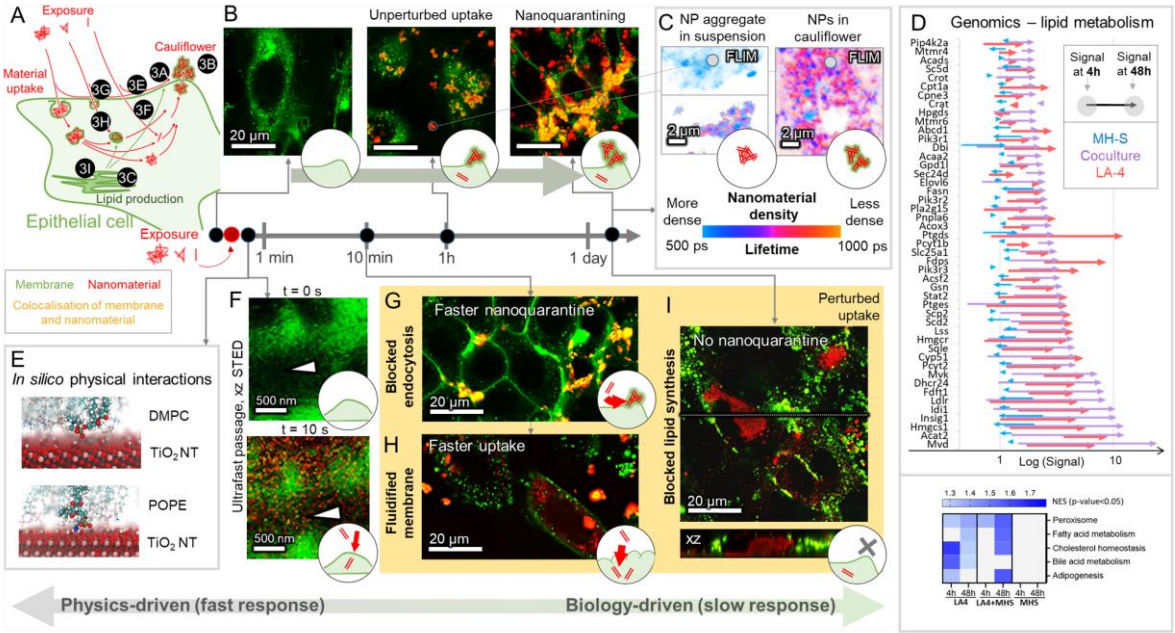
Figure S84



Cell line	LA-4
NPs	TiO_2
exposure	10:1, 0h-24h
imaging	SE SEM, @ 24h

Figure S84: Different magnifications of sample exposed to 10:1 (surface of nanomaterial: surface of cell) dose using SE SEM.

S3 – The role of lipids



S3b – Time evolution of cauliflower growth

See supplement section

S2d – Time evolution of cauliflower growth.

S3c – FLIM of cauliflowers

Main message

The fluorescence lifetime of labelled TiO_2 nanotubes in cauliflowers is longer than their lifetime in aggregates and shorter than the lifetime of free-floating labelled TiO_2 nanotubes, indicating that the density of nanomaterial in cauliflowers is somewhere in between dense aggregates and free-floating nanomaterial. Also, the bleed-through from the membrane labels into the FLIM channel shown in this paper is negligible.

Supporting raw and analysed data:

Figure S85-Figure S94

Materials and methods

- experiment 20190419_e06_s02_t02 (upper left image - giant aggregate FLIM):
  - 11 µl labelled TiO_2-40-Alexa647 in 100x dcb was added to 200 µl LCIS in an Ibidi #1.5H µ-Slide 8-well to achieve an effective surface dose 10:1 (in regard to the bottom surface of the well)
  - after 4 hours, the sample was observed on the heated stage at 32C

Cell line	
NPs	TiO2 (Alexa647)
exposure	10:1, 4h
imaging	xy confocal FLIM

pixelsize (x,y)	100 nm
FOV (x,y)	51,2 µm
TCSPC pixelsize	122 ps
TCSPC FOV	19.5 ns

561nm	
640nm	10%
STED	
diffraction grating	523 – 722 nm
dwell time	50 µs
objective	60x wi (NA1.2)

- experiment 20190628_fc04_s01_t01 (lower left image - small aggregate FLIM)
  - 10 µl labelled TiO_2-17-Alexa647 in 100x dcb was added to 200 µl LCIS in an Ibidi #1.5H µ-Slide 8-well to achieve an effective surface dose 10:1 (in regard to the bottom surface of the well)
  - after 1 hour, the sample was observed on the heated stage at 32C

Cell line	
NPs	TiO2 (Alexa647)
exposure	10:1, 1h
imaging	xy confocal FLIM

pixelsize (x,y)	100 nm
FOV (x,y)	51,2 µm
TCSPC pixelsize	122 ps
TCSPC FOV	19.5 ns

561nm	
640nm	30%
STED	
diffraction grating	523 – 722 nm
dwell time	200 µs
objective	60x wi (NA1.2)

- experiment 20190621_e01.100_s02_t04 (right image - cauliflower FLIM):
  - LA-4 cells were seeded @50% confluence in an Ibidi #1.5H µ-Dish with a 4-Well Culture-Insert (Ibidi)
  - after 36 hours, 35 µL freshly filtered 1 mg/mL TiO_2-17-Alexa647 in 100x dcb was mixed into 130 µL fresh F12K medium and added to cells in one of the inserts to achieve 100:1 surface dose

- 29 hours later, the cells in the well were first incubated with 1.6 µg/mL CellMaskOrange in LCIS for 5 minutes, then incubated 15 minutes with freshly diluted 5 µM SAG-38 in LCIS at room temperature, and finally flushed with LCIS and observed in 150 µL LCIS in the home-made incubator at 37 °C

Cell line	LA-4 (membrane, CellMaskOrange and lipid bodies, SAG-38)
NPs	TiO2 (Alexa647)
exposure	100:1, 29h
imaging	xy confocal FLIM

pixelsize (x,y)	100 nm
FOV (x,y)	51,2 µm
TCSPC pixelsize	122 ps
TCSPC FOV	19.5 ns

561nm	
640nm	10%
STED	
diffraction grating	523 – 722 nm
dwell time	100 µs
objective	60x wi (NA1.2)

- analysis:
  - the fluorescence lifetime data was sent from Imspector 16.2 (Abberior Instruments) to SPCImage 7.3 (Becker & Hickl), where the Decay matrix was calculated from the brightest pixel in the image (monoexponential fitting), binning was set to 1 and threshold to 5. In the supplement, wherever the lifetime of non-agglomerated nanomaterial was determined, the binning was set to 10 (also noted in the image itself)
  - the rainbow LUT was rescaled to range from 500 ps to 1000 ps and intensity and contrast of the lifetime-coded image were adjusted for easier comparison between experiments
  - the lifetime-coded image and color legend were exported as a TIF and imported into IrfanView, where the negative was obtained for more clear presentation in the paper (green color was avoided in the FLIM image to prevent confusing it with the green-coded membranes in ordinary fluorescence images)

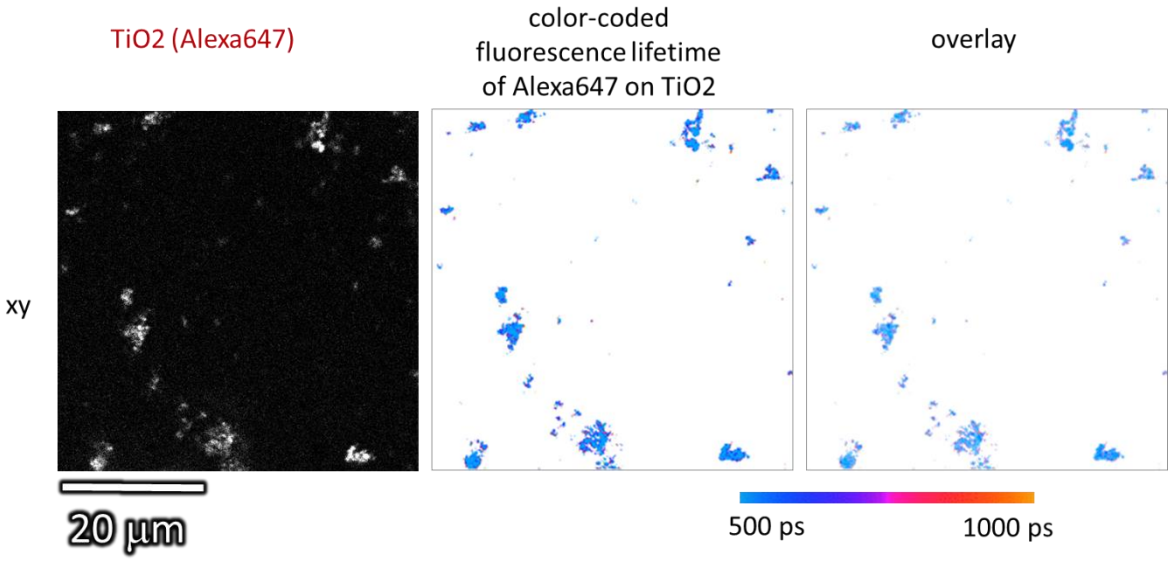
Experiment names

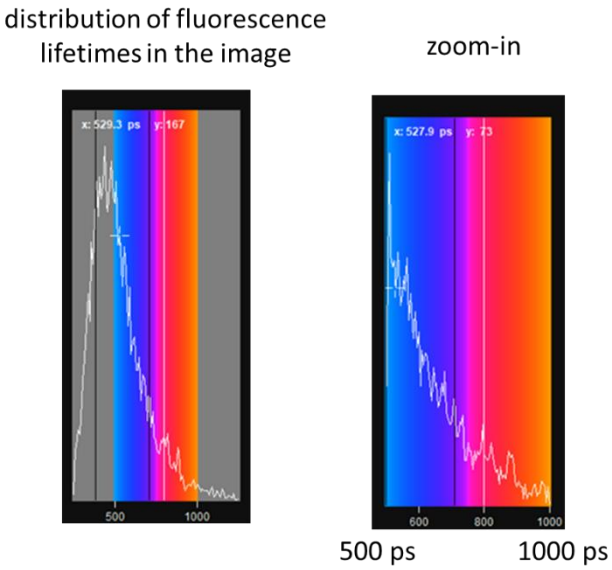
- Main experiment name:
  - 20190419_e06_s02_t02_TiO2-40-Alexa647 in LCIS 10.1_aggregate.img
  - 20190628_fc04_s01_t01_TiO2-17-Alexa647 in LCIS_FLIM.img
  - 20190621_e01.100_s02_t04_LA-4CellMask TiO2-17-Alexa647 100.1_cauliflower.img

- Supplement Experiment names:
  - 20190419/e06_s01_t01_TiO2Alexa647 before autoclaving in LCIS 10.1_bottom_FLIM_noSpectralFlim.msr
  - 20190419/e12_s01_t01_Alexa647 before autoclaving in LCIS_middle_noSpectralFlim.msr
  - 20190607/e03_s02_t02_SAG-38_conf.msr
  - 20190628/fc04_s01_t01_TiO2-17-Alexa647 in LCIS_FLIM.msr
  - 20190628/fc01_s01_t03_LA-4 free Alexa647 1h_FLIM.msr
  - 20190808/e01_s01_LA-4 CMO C75 c0.msr

Controls and statistics

experiment 20190419_e06_s01_t01 (smaller aggregates of TiO2-Alexa647 in LCIS):





parameters of the distribution [ps]

µ	528,28
µ-sig	384,7
µ+sig	710,65

Cell line	
NPs	TiO2 (Alexa647)
exposure	10:1, 1h
imaging	xy confocal FLIM

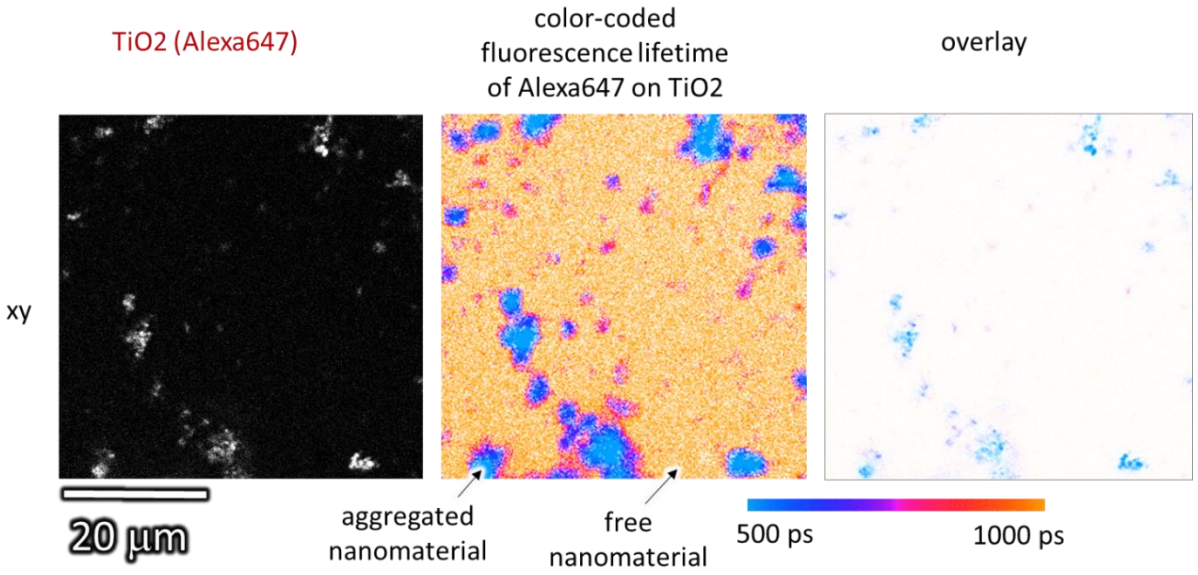
pixelsize (x,y)	100 nm
FOV (x,y)	51,2 µm
TCSPC pixelsize	122 ps
TCSPC FOV	19.5 ns

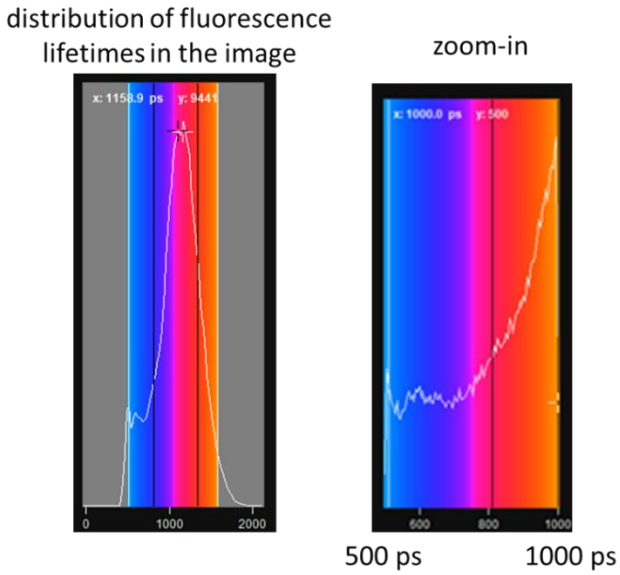
561nm	
640nm	10%
STED	
diffraction grating	523 – 722 nm
dwell time	200 µs
objective	60x wi (NA1.2)

Figure S85: Analysis of fluorescence lifetimes of agglomerated labelled nanomaterial – fluorescence image, fluorescence-lifetime-color-coded image and distribution of fluorescence lifetimes in the image.

experiment 20190419_e06_s01_t01 (smaller aggregates of TiO2-Alexa647 in LCIS):

use binning of 10 pixels to measure the free-floating nanomaterial:





parameters of the distribution [ps]

µ	1097,19
µ-sig	809,08
µ+sig	1337,21

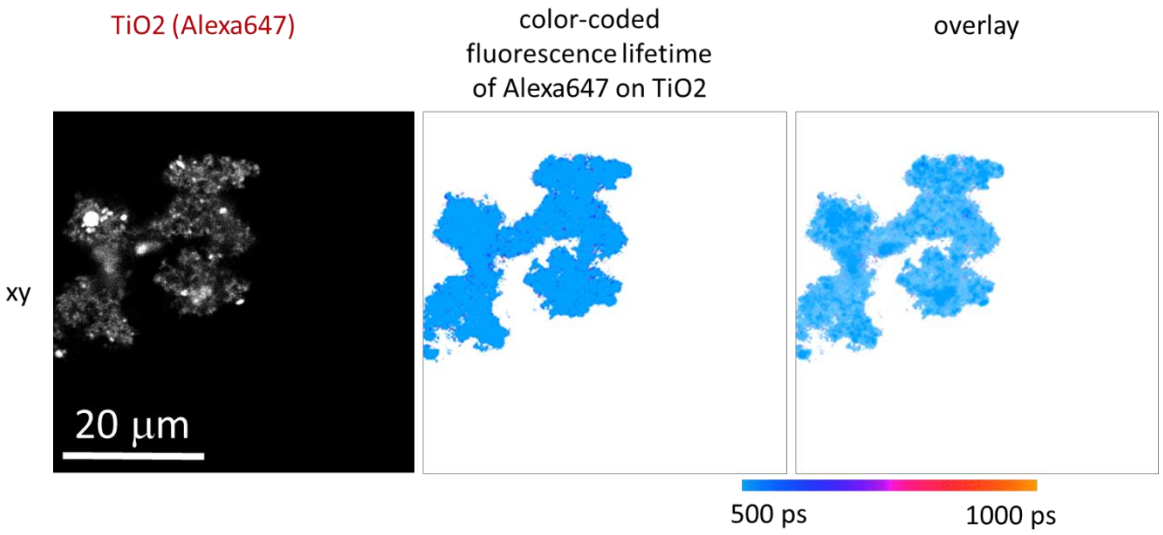
Cell line	
NPs	TiO2 (Alexa647)
exposure	10:1, 1h
imaging	xy confocal FLIM

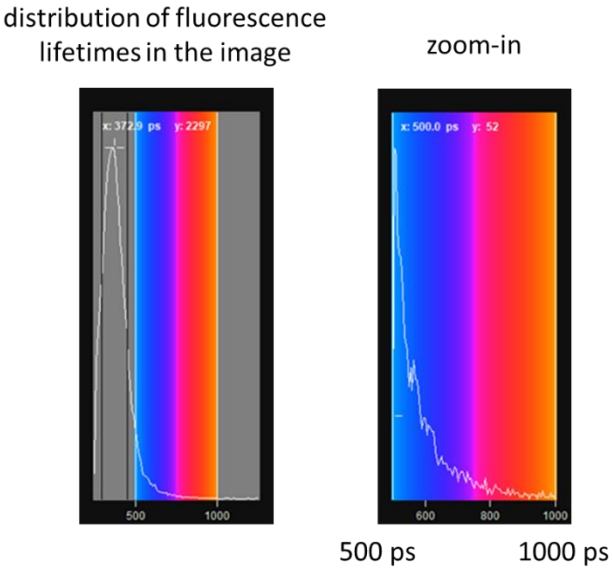
pixelsize (x,y)	100 nm
FOV (x,y)	51,2 µm
TCSPC pixelsize	122 ps
TCSPC FOV	19.5 ns

561nm	
640nm	10%
STED	
diffraction grating	523 – 722 nm
dwell time	200 µs
objective	60x wi (NA1.2)

Figure S86: Analysis of fluorescence lifetimes of free-floating and agglomerated labelled nanomaterial – fluorescence image, fluorescence-lifetime-color-coded image and distribution of fluorescence lifetimes in the image.

experiment 20190419_e06_s02_t02 (giant aggregate of TiO2-Alexa647 in LCIS):





parameters of the distribution [ps]

µ	374,11
µ-sig	295,27
µ+sig	454,31

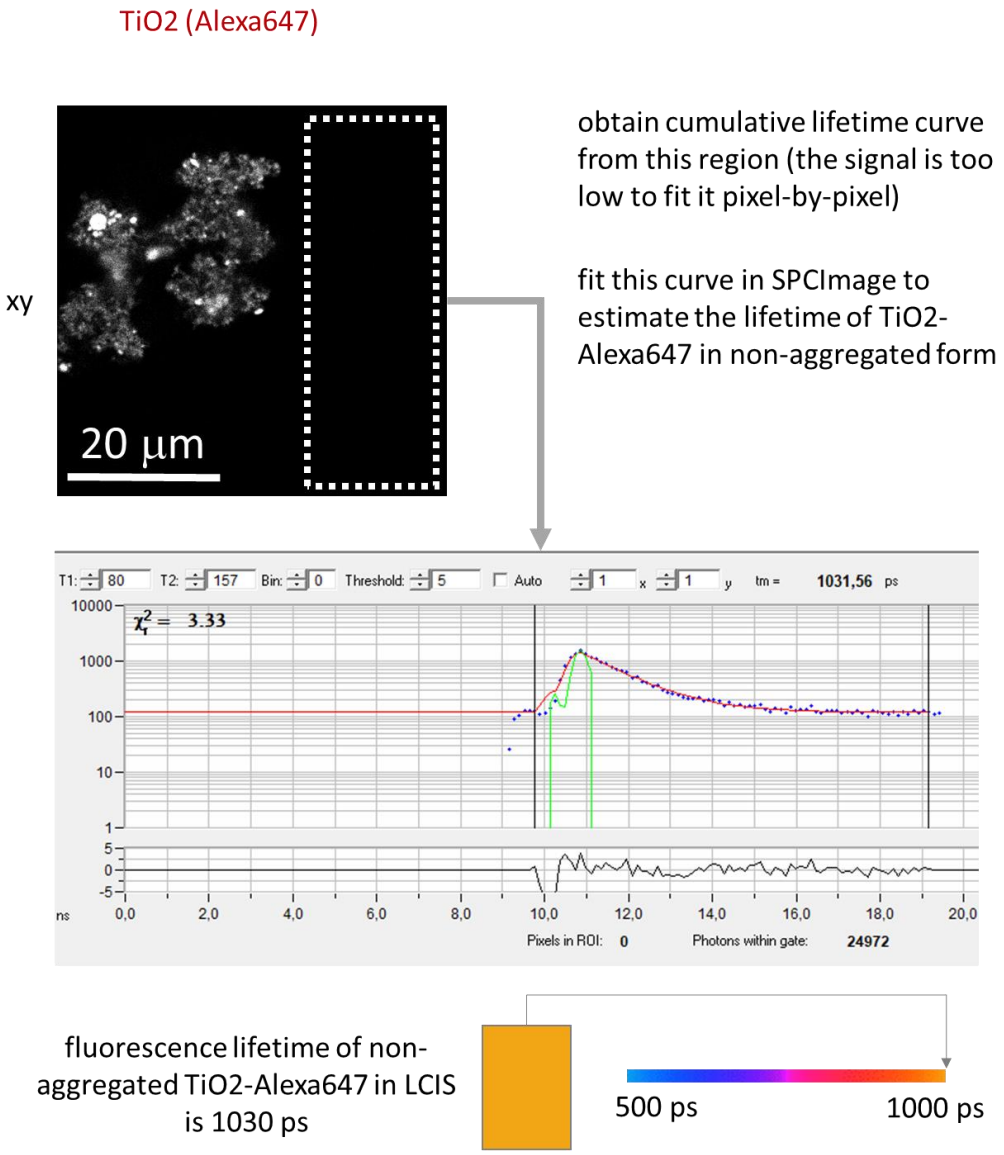
Cell line	
NPs	TiO2 (Alexa647)
exposure	10:1, 4h
imaging	xy confocal FLIM

pixelsize (x,y)	100 nm
FOV (x,y)	51,2 µm
TCSPC pixelsize	122 ps
TCSPC FOV	19.5 ns

561nm	
640nm	10%
STED	
diffraction grating	523 – 722 nm
dwell time	50 µs
objective	60x wi (NA1.2)

Figure S87: Analysis of fluorescence lifetimes of aggregated labelled nanomaterial – fluorescence image, fluorescence-lifetime-color-coded image and distribution of fluorescence lifetimes in the image.

experiment 20190419_e06_s02_t02 (giant aggregate of TiO2-Alexa647 in LCIS):



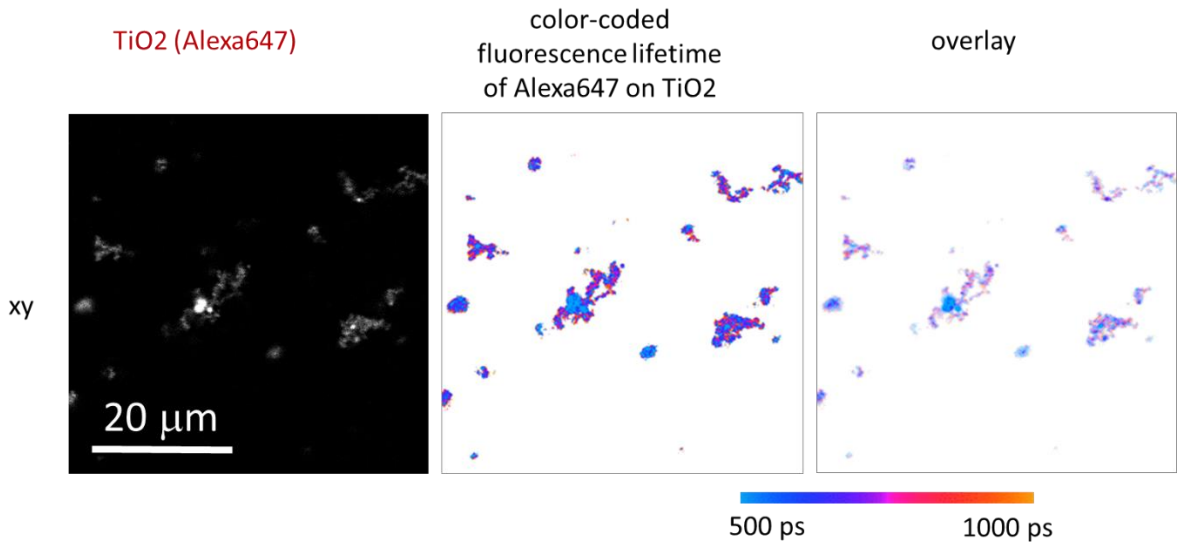
Cell line	
NPs	TiO2 (Alexa647)
exposure	10:1, 4h
imaging	xy confocal FLIM

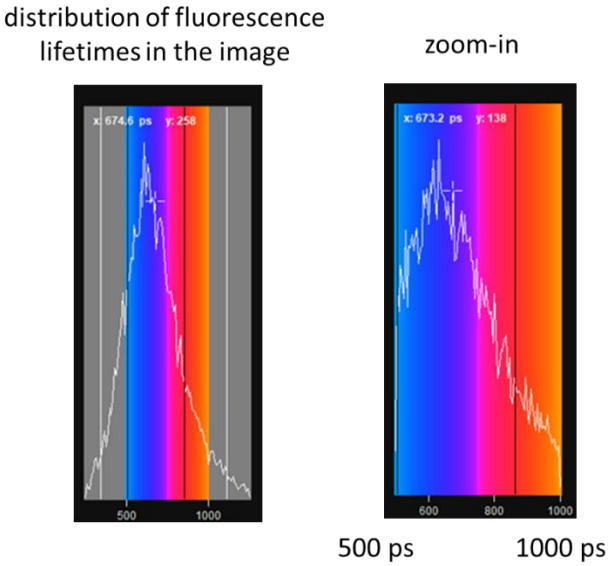
pixelsize (x,y)	100 nm
FOV (x,y)	51,2 µm
TCSPC pixelsize	122 ps
TCSPC FOV	19.5 ns

561nm	
640nm	10%
STED	
diffraction grating	523 – 722 nm
dwell time	50 µs
objective	60x wi (NA1.2)

Figure S88: Analysis of fluorescence lifetime of free-floating labelled nanomaterial – fluorescence image and mean fluorescence lifetime in the marked area.

experiment 20190628_fc04_s01_t01 (smaller aggregates of TiO2-Alexa647 in LCIS):





parameters of the distribution [ps]

µ	673,64
µ-sig	506,83
µ+sig	862,69

Cell line	
NPs	TiO2 (Alexa647)
exposure	10:1, 1h
imaging	xy confocal FLIM

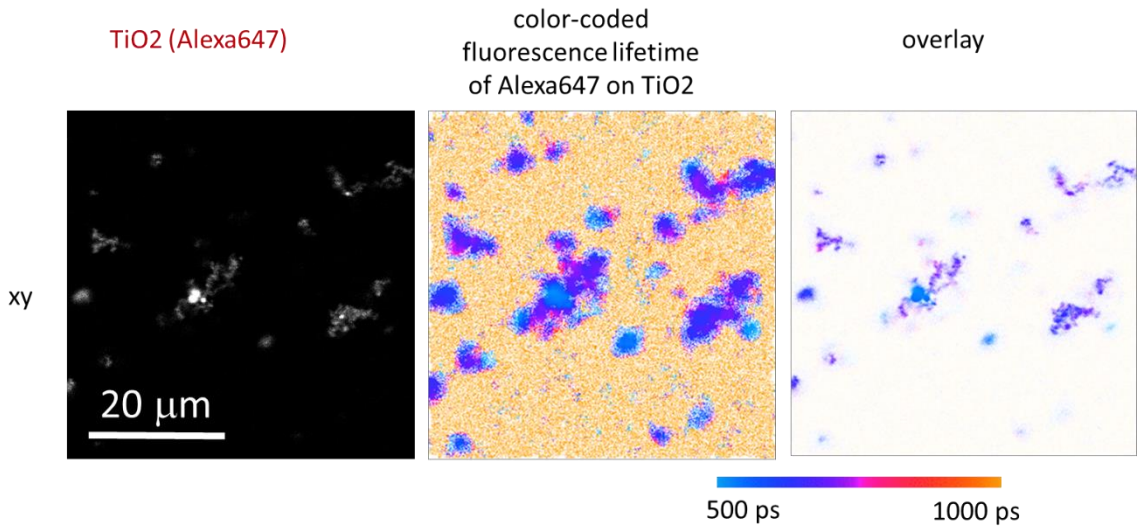
pixelsize (x,y)	100 nm
FOV (x,y)	51,2 µm
TCSPC pixelsize	122 ps
TCSPC FOV	19.5 ns

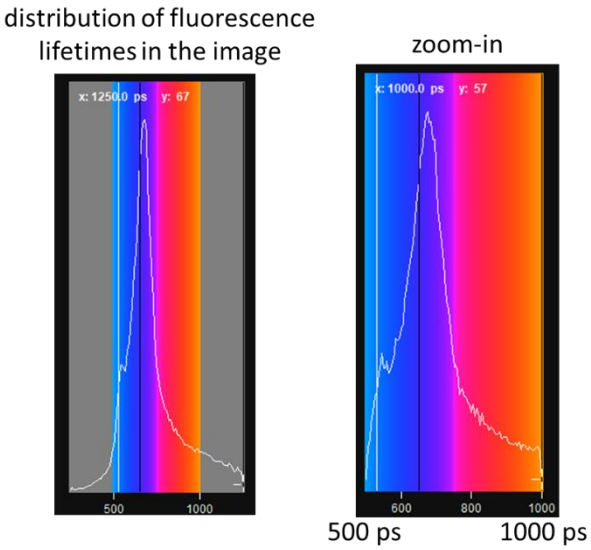
561nm	
640nm	30%
STED	
diffraction grating	523 – 722 nm
dwell time	200 µs
objective	60x wi (NA1.2)

Figure S89: Analysis of fluorescence lifetimes of aggregated labelled nanomaterial – fluorescence image, fluorescence-lifetime-color-coded image and distribution of fluorescence lifetimes in the image.

experiment 20190628_fc04_s01_t01 (smaller aggregates of TiO2-Alexa647 in LCIS):

use binning of 10 pixels to measure the free-floating nanomaterial:





parameters of the distribution

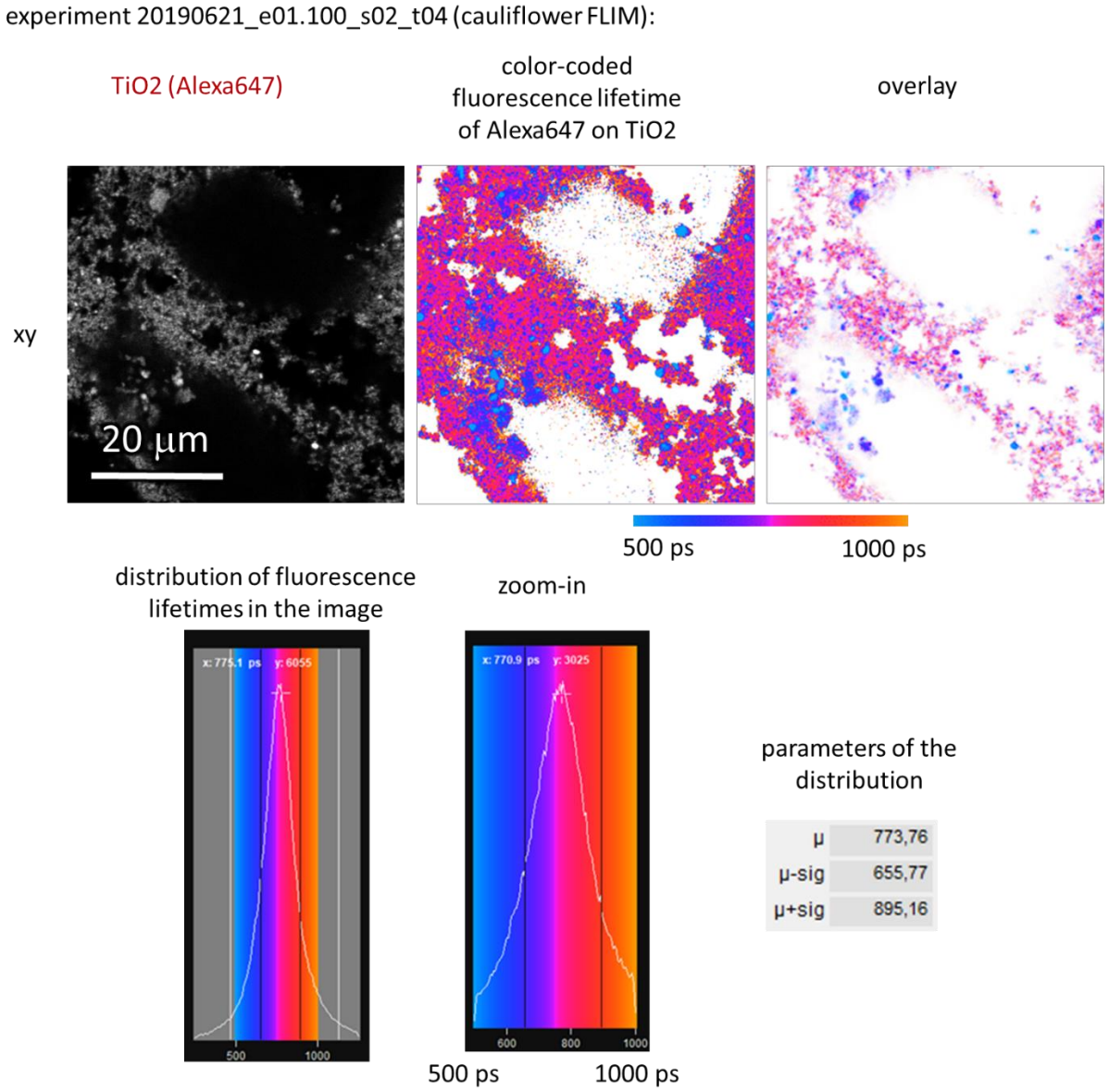
µ	2099,66	57
µ-sig	651	1559
µ+sig	2723	57

Cell line	
NPs	TiO2 (Alexa647)
exposure	10:1, 1h
imaging	xy confocal FLIM

pixelsize (x,y)	100 nm
FOV (x,y)	51,2 µm
TCSPC pixelsize	122 ps
TCSPC FOV	19.5 ns

561nm	
640nm	30%
STED	
diffraction grating	523 – 722 nm
dwell time	200 µs
objective	60x wi (NA1.2)

Figure S90: Analysis of fluorescence lifetimes of aggregated and free-floating labelled nanomaterial – fluorescence image, fluorescence-lifetime-color-coded image and distribution of fluorescence lifetimes in the image.



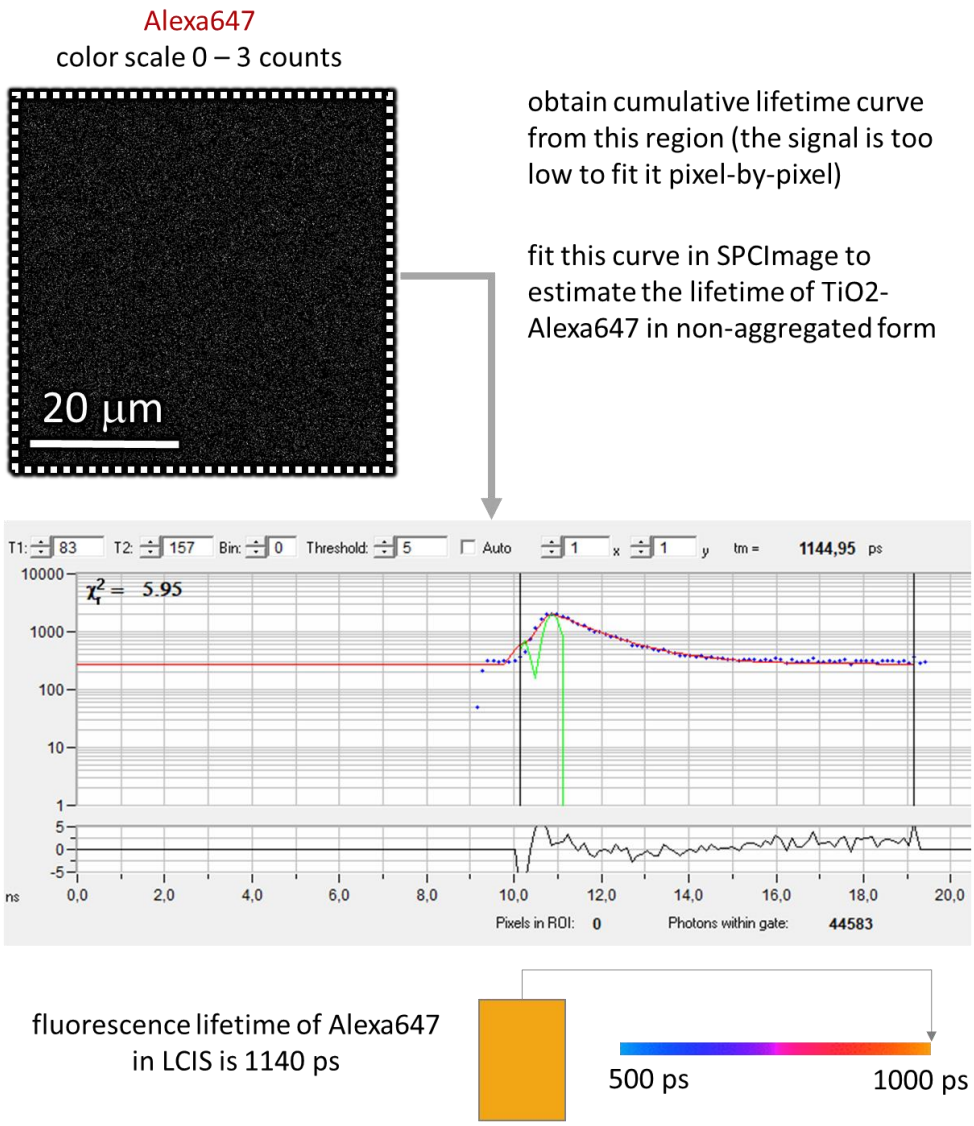
Cell line	LA-4 (membrane, CellMaskOrange and lipid bodies, SAG-38)
NPs	TiO2 (Alexa647)
exposure	100:1, 29h
imaging	xy confocal FLIM

pixelsize (x,y)	100 nm
FOV (x,y)	51,2 µm
TCSPC pixelsize	122 ps
TCSPC FOV	19.5 ns

561nm	
640nm	10%
STED	
diffraction grating	523 – 722 nm
dwell time	100 µs
objective	60x wi (NA1.2)

Figure S91: Analysis of fluorescence lifetimes of nanomaterial in cauliflowers – fluorescence image, fluorescence-lifetime-color-coded image and distribution of fluorescence lifetimes in the image.

experiment 20190419_ee12_s01_t01 (free Alexa647 in LCIS):



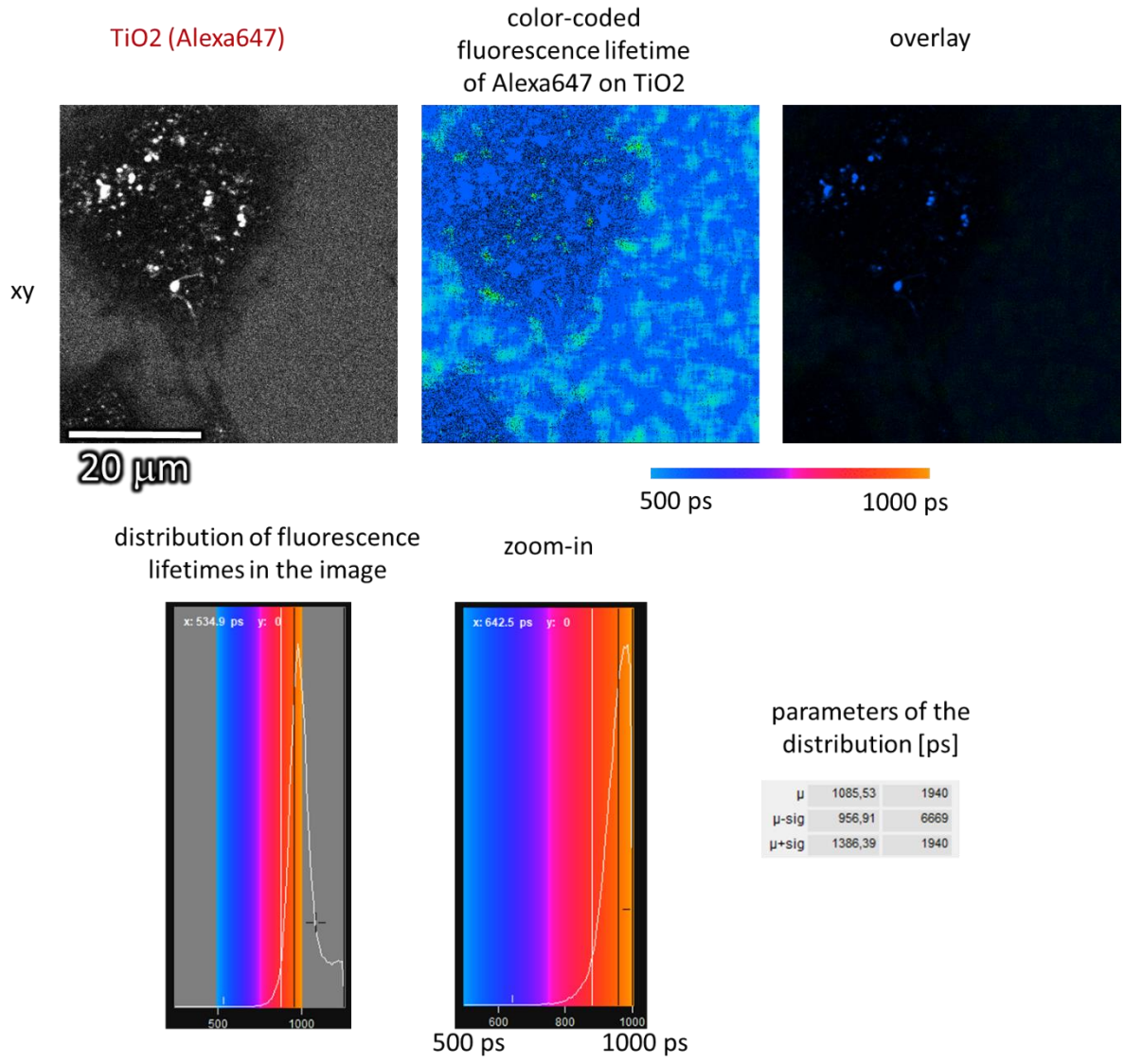
Cell line	
NPs	Free Alexa647
exposure	
imaging	xy confocal FLIM

pixelsize (x,y)	100 nm
FOV (x,y)	51,2 µm
TCSPC pixelsize	122 ps
TCSPC FOV	19.5 ns

561nm	
640nm	10%
STED	
diffraction grating	523 – 722 nm
dwell time	50 µs
objective	60x wi (NA1.2)

Figure S92: Determination of fluorescence lifetime of free-floating labelled nanomaterial – fluorescence image and mean fluorescence lifetime in the marked area.

experiment 20190628_fc02_s02 (FLIM of free Alexa647 on LA4 cells):



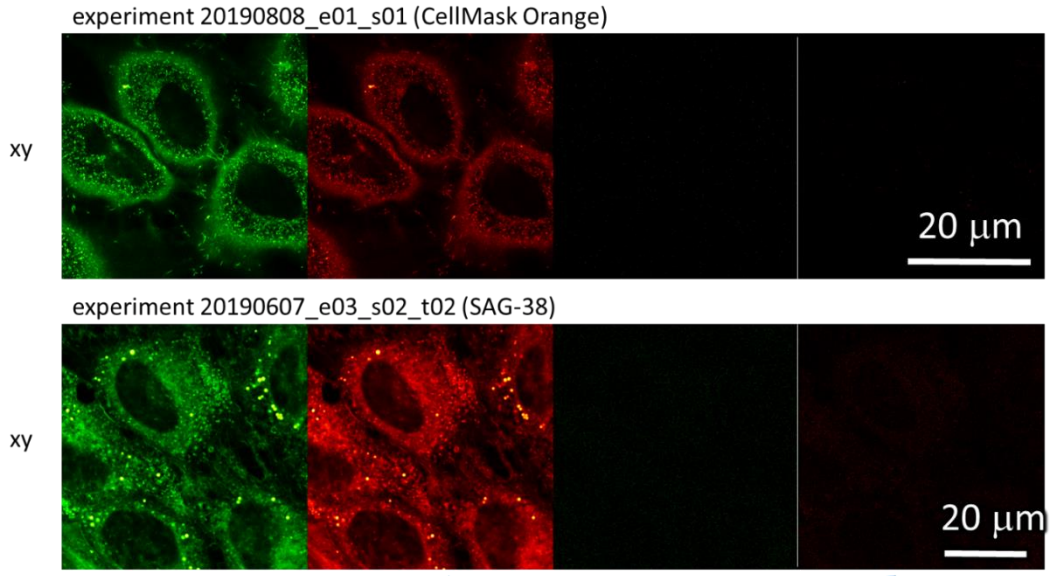
Cell line	LA-4 (Alexa647, 2uM, 1h incubation)
NPs	
exposure	
imaging	xy confocal FLIM

pixelsize (x,y)	100 nm
FOV (x,y)	51,2 µm
TCSPC pixelsize	122 ps
TCSPC FOV	19.5 ns

561nm	
640nm	30%
STED	
diffraction grating	523 – 722 nm
dwell time	600 µs
objective	60x wi (NA1.2)

1hour 2uM Alexa647 >> c(Alexa) on 1:1 NPs

Figure S93: Analysis of fluorescence lifetimes of free Alexa Fluor 647 if exposed to LA-4 cells for 1 hour at 2uM concentration – fluorescence image, fluorescence-lifetime-color-coded image and distribution of fluorescence lifetimes in the image.



excitation	561nm	561nm	640nm	640nm
detector	605-625 nm	650-720 nm	605-625 nm	650-720 nm
CellMask Orange	204	69	2	3
SAG-38	180	151	3	3

# of counts on the detector

640 nm laser does not excite CellmaskOrange or SAG-38
-> the signal of these probes is not seen in FLIM images when the sample is excited with 640 nm laser
-> all FLIM histograms, excited with 640nm laser can be attributed to Alexa647

Cell line	LA-4 (membrane, CellMaskOrange)	pixelsize (x,y)	100 nm	561nm	5%
		FOV (x,y)	80.0 µm	640nm	5%
NPs	TiO2 (Alexa647)	pixelsize (z)		STED	
exposure	10:1, 2 days	FOV (z)		filter sets	605 nm – 625 nm, 650 nm – 720 nm
imaging	xy confocal	imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA 1.2)

Cell line	LA-4 (lipid bodies, SAG-38)	pixelsize (x,y)	50 nm	561nm	2%
		FOV (x,y)	60.0 µm	640nm	2%
NPs	TiO2 (Alexa647)	pixelsize (z)		STED	
exposure	10:1, 2 days	FOV (z)		filter sets	605 nm – 625 nm, 650 nm – 720 nm
imaging	xy confocal	imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA 1.2)

Figure S94: Crosstalk reference for CellMaskOrange and SAG-38 – these two probes are not excited by 640 nm laser. Since all FLIM measurements in this paper are done by 640 nm excitation, they represent the fluorescence lifetimes of solely the nanomaterial with no artefacts from the membrane label signal.

S3d – Transcriptomics *in vitro* and *in vivo* after exposure to TiO_2 and comparison of both

In vitro

Main Message

The transcriptome profile of LA-4 cells exposed to TiO_2 nanotubes is analysed in terms of gene sets and pathways, which are mechanistically involved in cauliflower formation: lipid metabolism, immune system, vesicular trafficking and actin cytoskeleton organization.

Results

Gene analysis

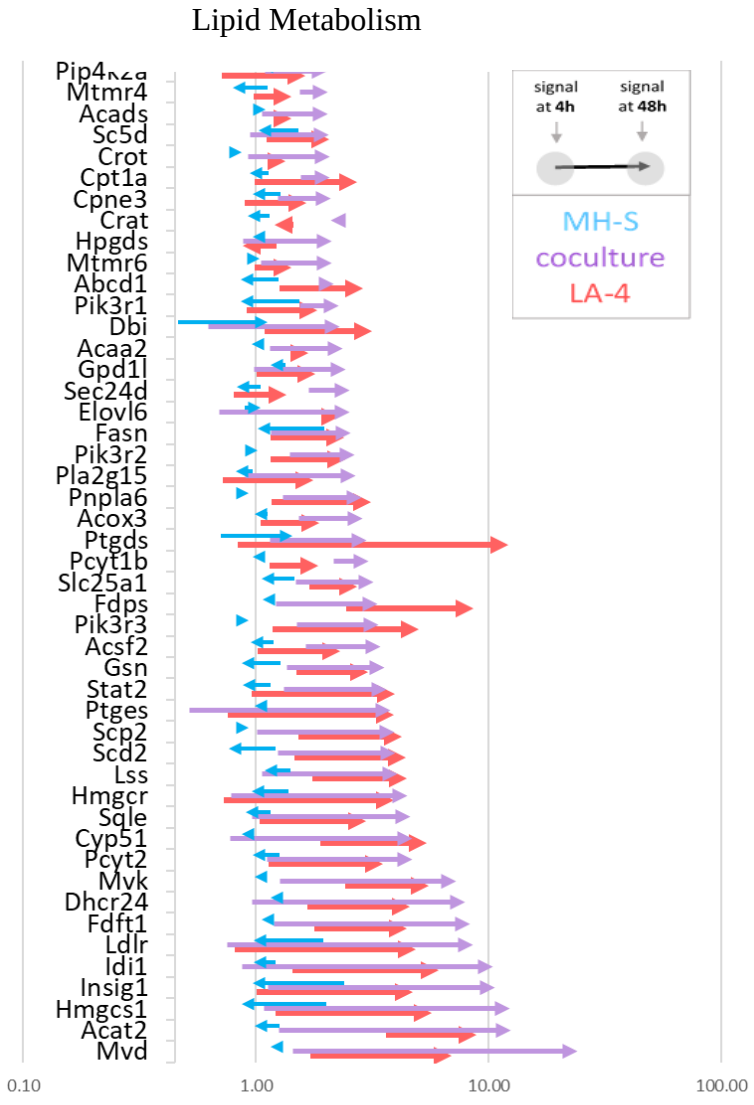
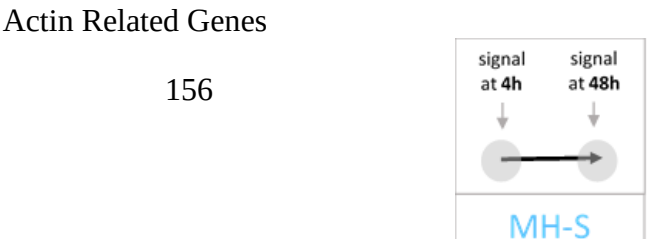


Figure S95: Lipid metabolism related genes with more than two fold increased expressions. Fold increase in expression is determined according to the non-exposed control sample at the same time point. Arrows represent the time evolution of gene expression so that the beginning of the arrow shows expression at 4 h and an arrowhead shows the expression at the 48 h time point



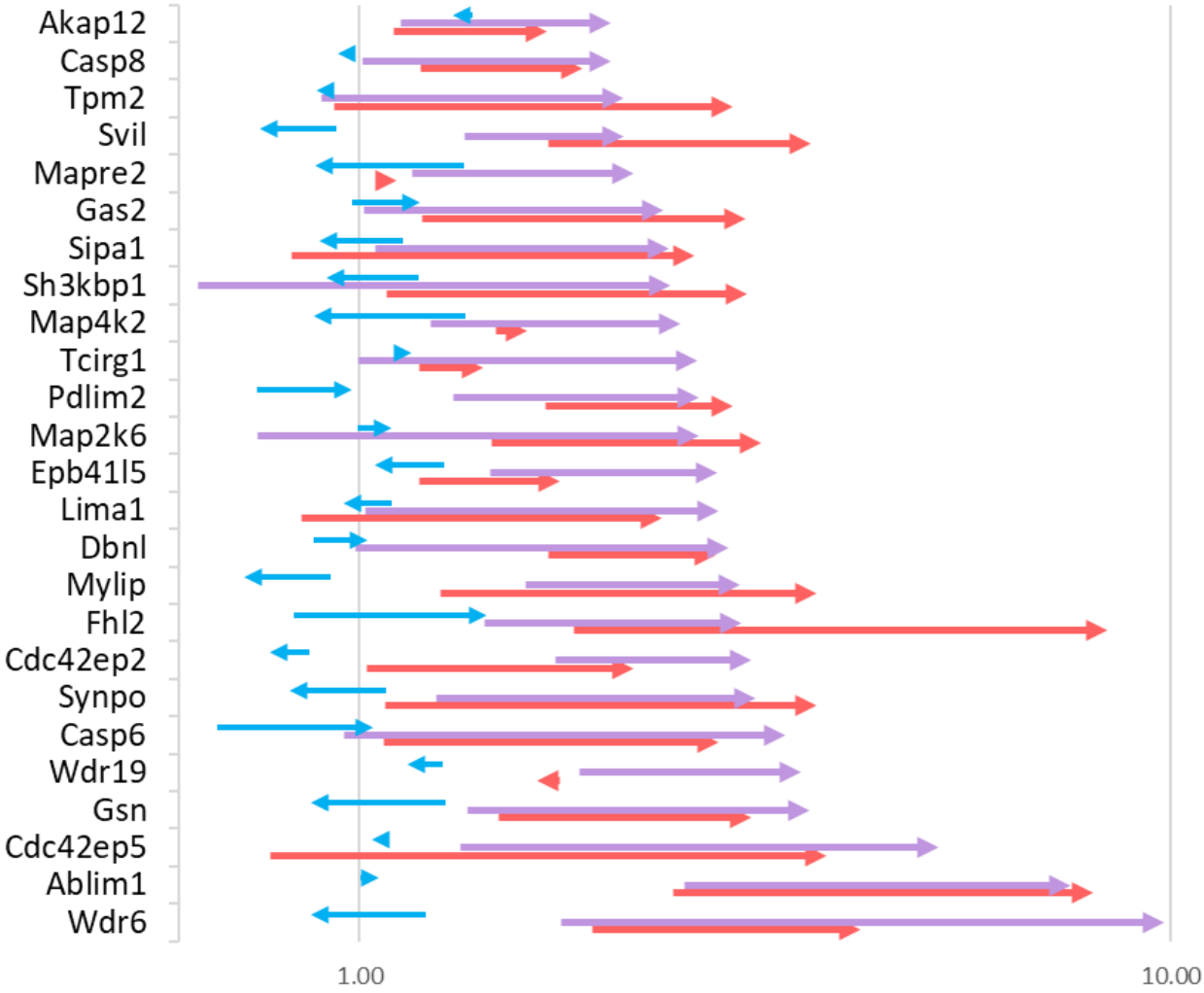


Figure S96: Actin cytoskeleton related genes with more than two fold increased expressions. Fold increase in expression is determined according to the non-exposed control sample at the same time point. Arrows represent the time evolution of gene expression so that the beginning of the arrow shows expression at 4 h and an arrowhead shows the expression at the 48 h time point

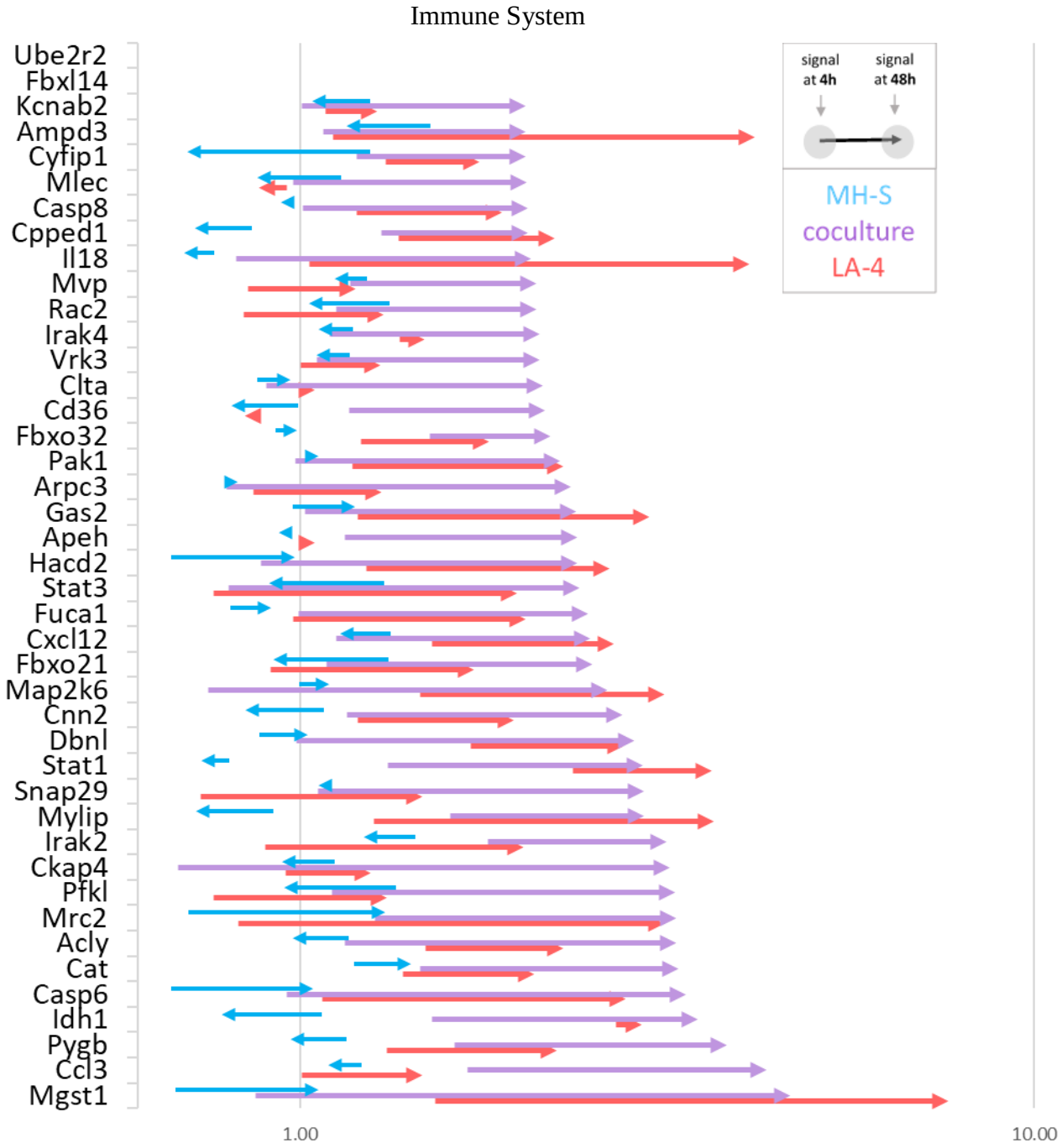


Figure S97: Immune response related genes with more than two fold increased expressions. Fold increase in expression is determined according to the non-exposed control sample at the same time point. Arrows represent the time evolution of gene expression so that the beginning of the arrow shows expression at 4 h and an arrowhead shows the expression at the 48 h time point.

Pathway analysis

To further analyse the cellular response to the TiO_2 exposure, gene expression analysis was performed and related pathways were assessed by gene set enrichment analysis of mono and co-cultures of epithelial cells (LA-4) and macrophages (MH-S) at different time points. As

described for cauliflower formation, also lipid metabolism and actin-dependent pathways were significantly enriched in epithelial cells and co-cultures with macrophages, but not in single cultures of macrophages. This supports the idea that upon uptake, nanoparticle excretion and cauliflowers formation is specific for epithelial cells but for professional phagocytes. Most of the pathways are abundantly enriched during the early phase 4h after nanomaterial exposure, such as cholesterol homeostasis, bile acid metabolism, from the lipid pathways, while others like peroxisome regulation show a stronger enrichment over time. This time dependence can be taken from the colour coded NES values (Figure S98 left) or the respective enrichment plots (Figure S98 right). Interestingly, only macrophages monocultures showed an inflammatory signature evidenced by the enrichment of TNFα/NFkB and IL-2/STAT5 signalling pathways, as well as for alveolar macrophages typical oxidative phosphorylation. None of these showed enrichment in epithelial cells single cultures (Figure S98 left).

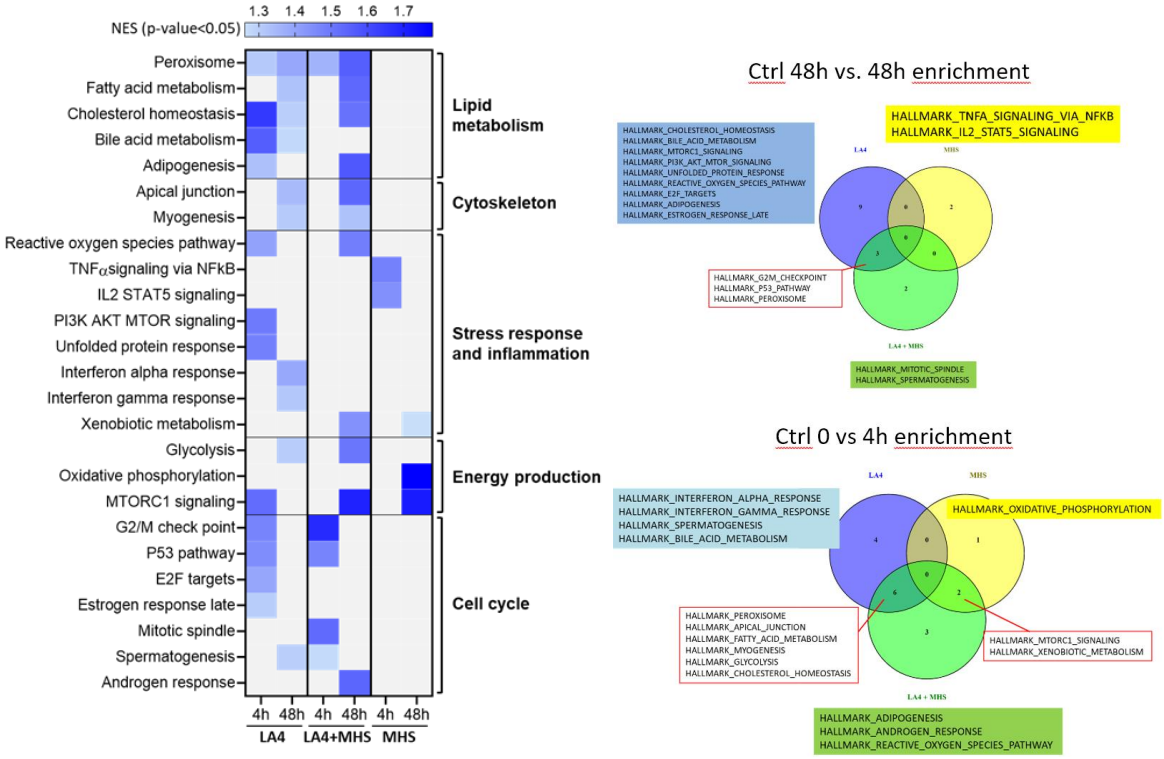


Figure S98: Left: Heat maps for enrichment of hallmark pathways: lipid metabolism, cytoskeleton stress response and inflammation, energy production and cell cycle, (NES: normalized enrichment score) gene expression on a pathway level for for genes increased in two-fold. Right: Venn diagrams.

4h 48h

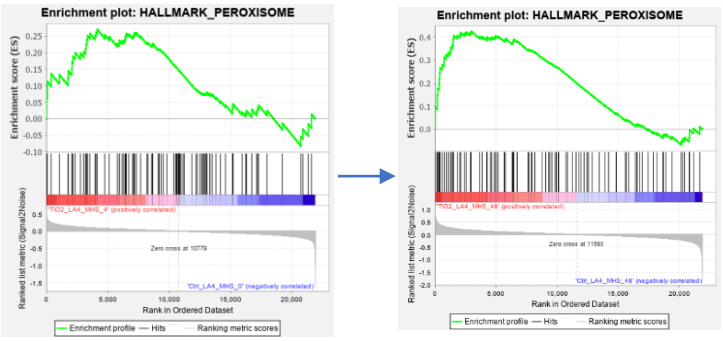


Figure S99: Enrichment plots of time progression of the peroxisome pathway (co-cultures)

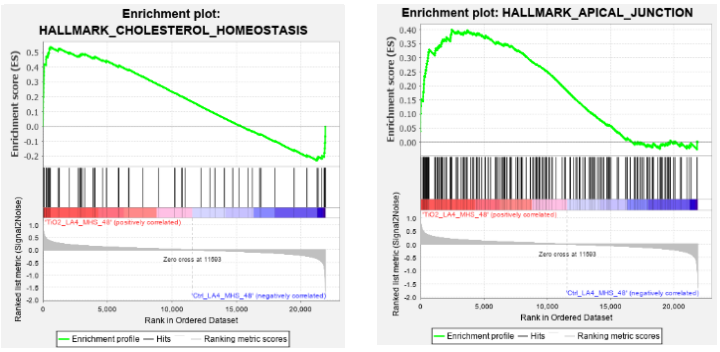


Figure S100: Enrichment plots of lipid metabolism and cytoskeleton pathways (co-cultures, 48h)

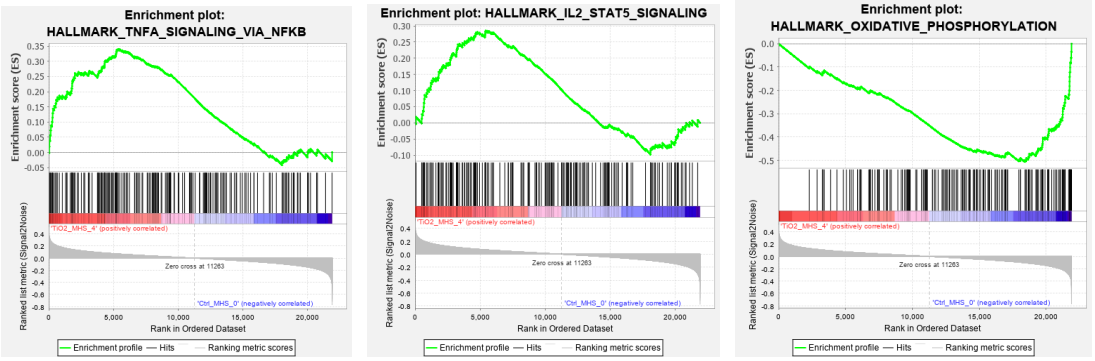


Figure S101: Enrichment plots of pro-inflammatory and oxidative phosphorylation pathways (MH-S mono-culture, 4h)

Materials and Methods

Sample preparation

Samples for the experiments, LA-4, MH-S, and cocultures were grown in 6-well plates until desired confluency. Cocultures were seeded so that the ratio of LA-4:MH-S was approximately 40:1 which reflects the physiological conditions of human alveoli. Cells were exposed to nanoparticles (TiO_2 or MWCNT) at a 10:1 surface dose ($NP_{surface}$ to $Cell_{surface}$ ratio) when they reached 90 % confluency. Cells were exposed to TiO_2 nanotubes and MWCNT for 4 h and 48 h and control samples were taken at 0 h and 48 h. Samples were prepared as described above. Briefly, growth medium was removed and 6-well plates containing cells only were frozen at -70°C. Detailed preparation of samples is given in the following tables:

Table S6: Sample preparation for the 4 h exposure of LA-4, MH-S and their co-culture to TiO_2 nanotubes

4h exposure	LA-4	Coculture	MH-S
1st day	25 % LA-4	25 % LA-4	/
3rd day	Δ medium	Δ medium + 2 % MH-S	MH-S 50 %
5th day	Δ medium + TiO_2		
5th day	HARVEST DAY		

Table S7: Sample preparation for the 48 h exposure of LA-4, MH-S and their co-culture to TiO_2 nanotubes

48h exposure	LA-4	Coculture	MH-S
1st day	15 % LA-4	15 % LA-4	/
3rd day	Δ medium	Δ medium + 1 % MH-S	MH-S 20 %
5th day	Δ medium + TiO_2		
7th day	HARVEST DAY		

Control samples were prepared in the same manner without exposure to nanoparticles.

RNA isolation

Total RNA was isolated employing the RNeasy Plus Mini Kit (Qiagen). The Agilent 2100 Bioanalyzer was used to assess RNA quality and RNA with RIN>7 was used for microarray analysis.

Total RNA (120 ng) was amplified using the WT PLUS Reagent Kit (Thermo Fisher Scientific Inc., Waltham, USA). Amplified cDNA was hybridized on Mouse Clariom S arrays (Thermo Fisher Scientific). Staining and scanning (GeneChip Scanner 3000 7G) was done according to manufacturer`s instructions.

Statistical analysis

Statistical analysis for all probe sets included limma t-test and Benjamini-Hochberg multiple testing correction. Raw p-values of the limma t-test wereused to define sets of regulated genes ($p<0.01$). Detection Above Background (dabg) p-values were used to exclude background signals: significant genes were filtered for $p<0.05$ in more than half of the samples in at least one group. Array data has been submitted to the GEO database at NCBI (GSE146036).

Gene Set Enrichment Analysis

GSEA software from the Broad Institute (*http://www.gsea-msigdb.org/gsea/index.jsp*) (*Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles.Proc Natl Acad Sci U S A. 2005; 102: 15545-15550*) was used to identify enrichment of defined gene sets in our microarray data (GSE146036). Among the molecular signature databases offered by the GSEA collection, The Hallmark (H) database was selected due its consistent representation of specific biological processes and lack of redundancy (*Liberzon, Arthur, et al. "The molecular signatures database hallmark gene set collection." Cell systems 1.6 (2015): 417-425.).* Analysis was run under default settings of the GSEA software.

Arrow graphs

In the arrow graphs, only genes which were up- or down-regulated more than two-fold compared to non-exposed cells are shown. The signal (x axis) is drawn in logarithmic scale. Expression is normalized to expression of control samples. Genes in arrow charts are ordered according to their fold increase at 48 hours (fold increase was calculated in regard to non-exposed cells at the same time-point). Gene sub-sets were selected manually.

Experiment names

20200117_genomics_TiO2_results.xlsx

In vivo

Main Message

The transcriptome profile of female C57BL/6 mice at 1 and 28 days post-exposure to TiO_2 nanotubes shows enriched gene sets involved in fatty acid metabolism after 28 days thus supporting our findings in *in vitro* system. Furthermore, it shows a strong immune response, in terms of chemokine signaling, additionally strengthening our hypothesis.

Table S8: Lipid-related pathways from the KEGG pathway analysis. The analysis was performed using the GSEA method and the KEGG signalling pathways database. Pathways were selected if they were identified with FDR corrected p-value < 0.05. The values in the table indicate -log10(FDR corrected p-value).

	in vivo					
KEGG term name	18 µg d1	18 µg d28	54 µg d1	54 µg d28	162 µg d1	162 µg d28
Glycosphingolipid biosynthesis - ganglio series	1.18	1.19	1.01	1.15	-	-
Sphingolipid signaling pathway	2.13	-	4.54	-	1.71	-
Glycerophospholipid metabolism	-	-	-	-	-	-
Ether lipid metabolism	1.56	-	-	1.73	-	-
Fatty acid degradation	-	5.63	1.10	2.40	-	-
Fatty acid metabolism	-	4.31	-	1.95	-	-
Fatty acid elongation	-	1.52	-	1.46	-	-
Biosynthesis of unsaturated fatty acids	-	-	-	-	-	-
Glycosaminoglycan biosynthesis - keratan sulfate	1.40	-	-	1.46	-	-
Non-alcoholic fatty liver disease (NAFLD)	-	10.52	1.80	2.55	-	-

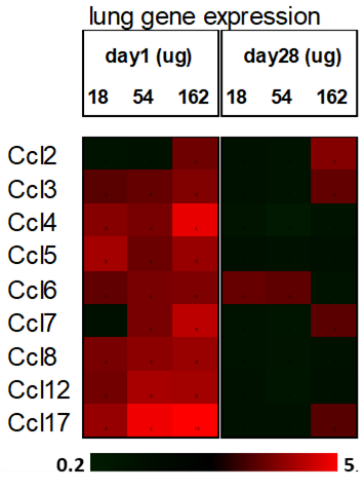


Figure S102: Genes encoding monocyte chemoattractive (C-C motif) chemokines upregulated in mice exposed to TiO_2 nanotubes after 1 and 28 days.

Cell composition in bronchoalveolar lavage fluid (BAL)

Danielsen et al. [5] assessed inflammatory cells recruitment in BAL fluid at 1, 3, 28, 90 and 180 days post-exposure as a pulmonary inflammatory response marker. For the TiO_2 tube, the number of neutrophils and macrophages was statistically significant increased at day 28 post-exposure. As a response to acute inflammation number of neutrophils was, in the dose dependent matter, highest after the first day of exposure and kept decreasing towards 28th day, but remained elevated nevertheless. Number of macrophages was elevated on the 1st day post-exposure and kept increasing until the 28th day in a dose dependent matter, indicative of chronic inflammation. The cell dynamics reflects the transcriptomics data both *in vitro* and *in vivo*, where we can see acute response transitioning into a chronic one.

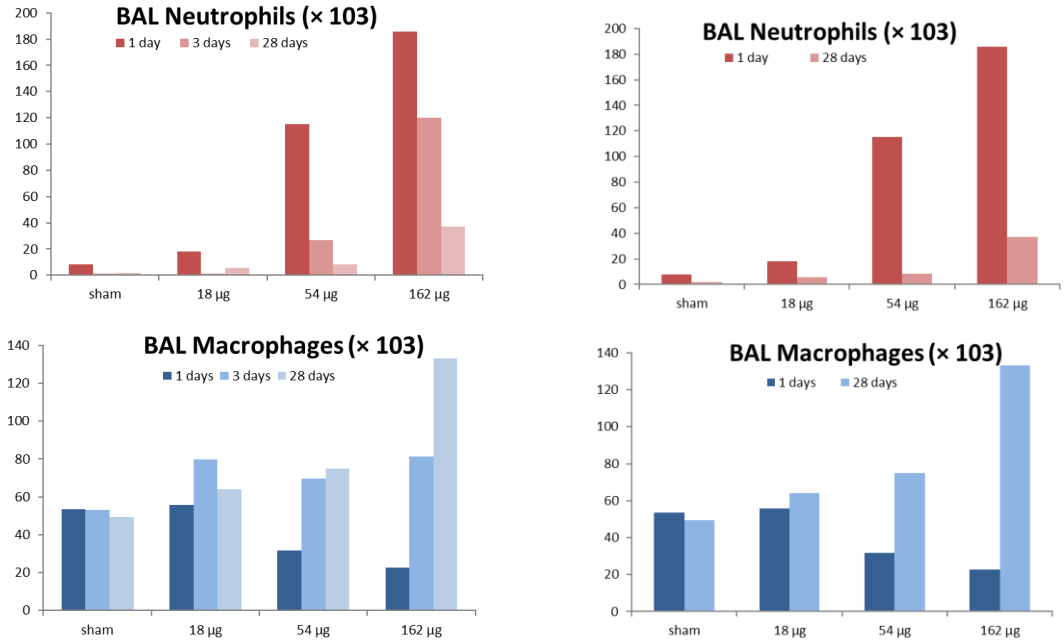


Figure S103: Neutrophil and macrophage recruitment in BAL fluid at 1, 3, 28, 90 and 180 days post-exposure

Materials and Methods

Sample preparation

The materials and methods used for intratracheal instillation of mice with TiO2 tube are described in detail by Danielsen et. al [5] and included in this document (S2b – *In vivo* data) in a short version. Female C57BL/6 mice were exposed by single intratracheal instillation to 18, 54 or 162 µg/mouse of a TiO2 tube. Lung tissues were harvested on day 1 and 28 after exposure. Microarray mRNA analysis was performed using Agilent 8 × 60 K oligonucleotide microarrays (Agilent Technologies Inc., Mississauga, ON, Canada) as described previously [3] with 6 replicas for each condition. Bioinformatics analysis of the row data: signal intensities were Loess normalized using the limma package in R/Bioconductor [4]. Analysis of differentially expressed genes (DEGs) was performed using the limma package. The genes were considered as significantly differentially expressed if the BH-adjusted p-values were less than or equal to 0.1.

RNA isolation

RNA was isolated and treated in the same manner as in the *in vitro* experiment described above.

Statistical analysis

Statistical analysis for all probe sets included limma t-test and Benjamini-Hochberg multiple testing correction. Raw p-values of the limma t-test were used to define sets of regulated genes

(p<0.01). Detection Above Background (dabg) p-values were used to exclude background signals: significant genes were filtered for p<0.05 in more than half of the samples in at least one group. Array data has been submitted to the GEO database at NCBI (GSE146036).

Gene Set Enrichment Analysis

The KEGG gene set enrichment analysis was performed using camera function from the limma R package [4]. Pathways were selected if they were identified with FDR corrected p-value < 0.05. For the assessment of the monocyte influx, all genes encoding monocyte chemoattractive (C-C motif) chemokines were selected and their expression evaluated (Figure S102).

Experiment names

KEGG_pathway_analysis.xlsx

Comparison *in vivo* and *in vitro*

Table S9: Lipid-related pathways from the KEGG pathway analysis. The analysis was performed using the GSEA method and the KEGG signalling pathways database. Pathways were selected if they were identified with FDR corrected p-value < 0.05. The values in the table indicate -log10(FDR corrected p-value).

	in vitro						*in vivo*					
KEGG term name	LA-4 4h	LA-4 48h	LA-4 / MH-S 4h	LA-4 / MH-S 48h	MH-S 4h	MH-S 48h	18 µg d1	18 µg d28	54 µg d1	54 µg d28	162 µg d1	162 µg d28
Glycosphingolipid biosynthesis - ganglio series	-	1.35	-	-	-	-	1.18	1.19	1.01	1.15	-	-
Sphingolipid signaling pathway	-	-	1.57	-	1.81	-	2.13	-	4.54	-	1.71	-
Glycerophospholipid metabolism	-	-	-	-	1.76	-	-	-	-	-	-	-
Ether lipid metabolism	-	-	-	-	-	-	1.56	-	-	1.73	-	-
Fatty acid degradation	-	1.17	-	1.57	-	-	-	5.63	1.10	2.40	-	-
Fatty acid metabolism	1.14	2.49	-	3.75	-	-	-	4.31	-	1.95	-	-
Fatty acid elongation	1.04	-	-	-	-	-	-	1.52	-	1.46	-	-
Biosynthesis of unsaturated fatty acids	-	-	-	2.89	-	-	-	-	-	-	-	-
Glycosaminoglycan biosynthesis - keratan sulfate	-	-	1.17	-	-	-	1.40	-	-	1.46	-	-
Non-alcoholic fatty liver disease (NAFLD)	-	-	1.29	-	10.64	-	-	10.52	1.80	2.55	-	-
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	-	-	-	-	1.31	-	1.65	-	2.33	1.70	-	-
Fatty acid biosynthesis	-	-	-	-	-	-	-	2.03	-	-	-	-

Comparison of transcriptome response to TiO2 perturbation for *in vivo* and *in vitro* conditions

Mice were exposed to 18, 54 or 162 µg of TiO_2 nanotubes per mouse and lungs were harvested on 1st and 28th day post exposure for transcriptomic analysis to evaluate overlapping sets of genes differentially expressed in the *in vivo* and *in vitro* experimental data. The goal of the analysis is to determine and compare alterations in lipid metabolism, immune response in terms of proinflammatory signalling and cholesterol metabolism between two experimental systems.

Experiment names

KEGG_pathway_analysis.xlsx

S3e – *In silico* data – atomistic molecular dynamics simulation

Pool of lipids

Main message

From a pool of lipids (non-bilayer) the POPE and DMPC lipids attach to TiO_2 surface by lipid head-group. Due to less dense packing on the surface than in a bilayer a interleaved bi-layer is formed (with thickness between 2 and 3 nm (instead of usual 4 nm for bilayer).

Supporting raw and analysed data:

Figure S104-Figure S105

Table S10-Table S11

After a few tens of nanosecond from the simulations start, the lipids formed bilayer-like patches on the TiO_2 surface (Figure S105), binding to the surface by the headgroups, see Figure 2e (main text) and Figure S104. We found that PC and PE lipids bind to TiO_2 differently: while PE lipids bind by amino-group directly to the TiO_2 surface, PC lipids bind by the phosphate group through the intermediate layer of water molecules. These binding modes are shown in Figure S105 (A,B), and density profiles of N and P atoms of lipids are shown in Figure S105 (C,D). One can see that in the case of PE lipids the density maxima are sharper, and density profile of N-atoms is on a shorter distance from the bilayer surface compared to PC lipids, which is indication that PE lipids bind to anatase (101) surface stronger than PE lipids. In the both cases, one can see formation of the second lipid layer with lipids having hydrophobic tails contacting lipid tails of the first layer, and with lipid headgroups exposed to solvent. It would be naturally to suggest that headgroups of lipids of the second layer can bind to the surface of another TiO_2 nanoparticle, thus building lipid-nanoparticles composites.

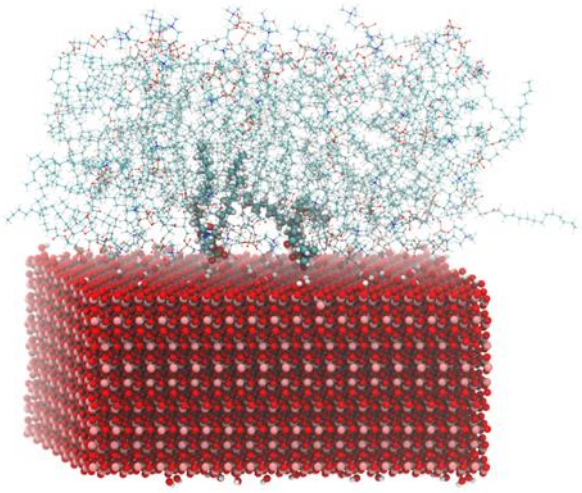


Figure S104: DMPC lipid bilayer formed on TiO_2 anatase(101) surface. Two lipid molecules are highlighted; water molecules are not shown. In the beginning of simulations the lipids were dispersed in the solution

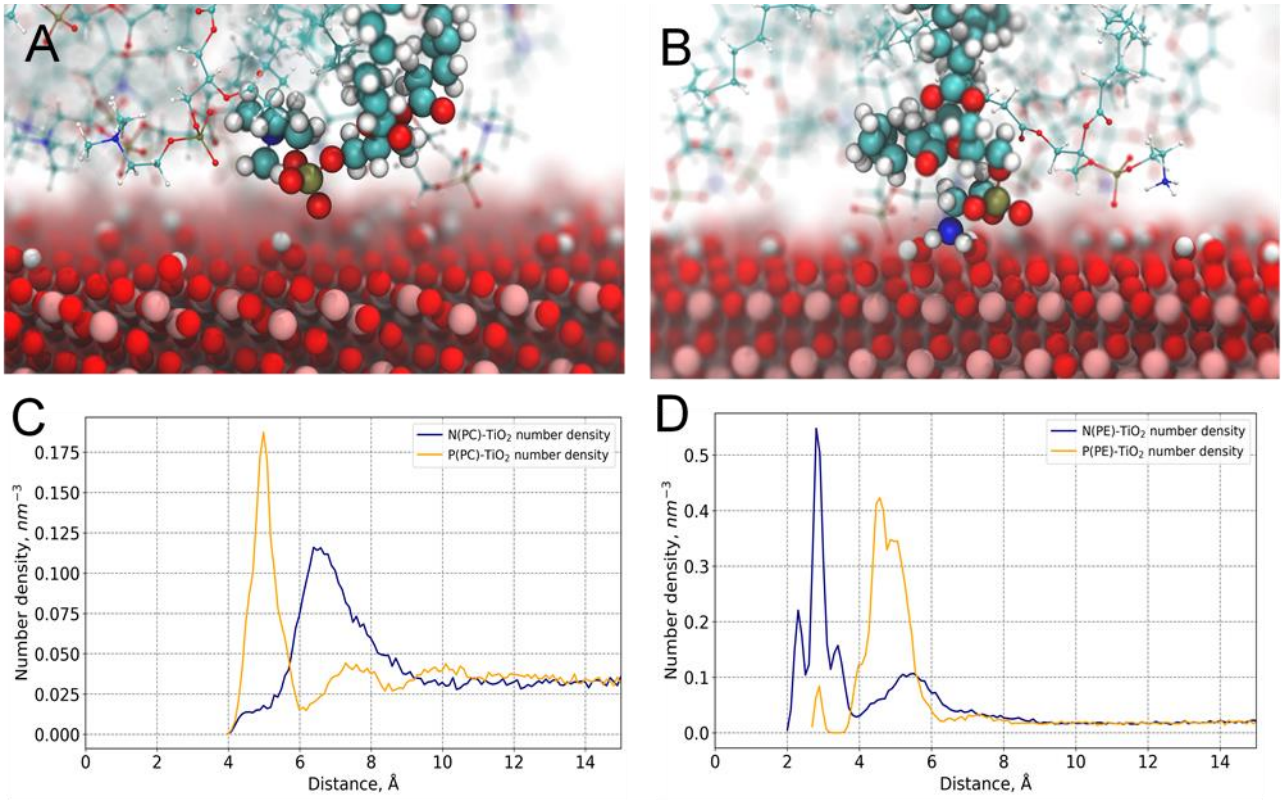


Figure S105: Binding modes of DMPC (A) and POPE (B) lipids to anatase (101) TiO_2 surface, density profiles (in terms of number density) of DMPC choline and phosphate groups (C), and of POPE amino- and phosphate groups (D) near anatase (101) TiO_2 surface. The density profiles were computer after 50 ns of equilibration simulations. The distance is counted from the outmost layer of Ti atoms in the slab.

Link to time-lapse

- *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/Fig2_atomistic_molecular_dynamics_binding_anatase-101-DMPC(40ns).mp4*

Movie S2: Time-lapse of the first 40 ns of DMPC lipids binding to anatase (101) TiO_2 surface

- *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/Fig2_atomistic_molecular_dynamics_binding_anatase-101-POPE(17.5ns).mp4*

Movie S3: Time-lapse of the first 17.5 ns of POPE lipids binding to anatase (101) TiO_2 surface

Link to 3D

- *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/Fig2_atomistic_molecular_dynamics_binding_anatase-101-DMPC-rotation.mp4*

Movie S4:Final state of the atomistic molecular dynamics simulation of DMPC lipids binding to anatase (101) TiO_2 surface

- *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/Fig2_atomistic_molecular_dynamics_binding_anatase-101-POPE-rotation.mp4*

Movie S5: Final state of the atomistic molecular dynamics simulation of POPE lipids binding to anatase (101) TiO_2 surface

Atom type	Comment	q (e)	□ (Å)	□ (kJ/mol)

Ti(O6)	Bulk Ti	2.248	1.9	13.79
Ti(O5)	Surface Ti	2.159	1.9	13.79
O(Ti3)	Oxygen in TiO_2 bulk	-1.124	3.51	0.409
O(Ti2)	Bridge oxygen on TiO_2	-1.035	3.42	0.401
O(Ti,H)	Hydroxyl oxygen	-0.913	3.29	0.389
H (O)	Hydrogen	0.417	0	0

Table S10: Non-bonded force field parameters for TiO_2. For each atom type, coordinated atoms are given in parenthesis

Bond type	b_o (Å)	k_b (kJ/mol Å^2)
Ti-O(Ti3) bulk	1.9	8000.
Ti-O(Ti2) bridge	1.9	8000.
Ti-O(H) hydroxyl	1.9	8000.
O-H hydroxyl	1.0	3267.
Angle type	□$_0$ (deg)	k□ (kJ/mol deg^2)
Ti-O-H hydroxyl	114.85	5433.

Table S11: Bonded parameters for TiO_2

Materials and methods

System composition

Atomistic molecular dynamics simulations have been carried out for DMPC and POPE lipids near anatase (101) TiO_2 surface in water environment. Anatase slab (71.8 x 68.2 x 30.5 Å) with (101) surface normal to the z axis is used as a model of a nanoparticle surface. The slab contains 4536 Ti atoms of which 504 are five-fold coordinated atoms on the surface. (101) anatase surface was chosen as a surface of the lowest energy. At neutral pH TiO_2 surface is covered by hydroxyl groups and is negatively charged. In our model we bind hydroxyl groups to 5-coordinated surface Ti atoms so that the surface charge density is close to the experimental value at neutral pH. Thus we add 151 hydroxyl groups to randomly picked Ti surface atoms (which constitutes 30% of their total amount) which results in a surface charge density of -0.62 electrons/nm^2, which is in line with the experimental results[26].

The TiO_2 slab is then placed in the middle of the simulation box with 3D periodic boundary conditions. The box size in X and Y directions is defined by the slab length and width so that the slab is periodic in those directions. The height of the box is set to 130 Å to accommodate the TiO_2 slab (thickness of 30.5 Å), eventual formed lipid bilayer on the both sides (2 x 40 Å) as well as their hydration layers (2 x 10 Å). 82 lipid molecules (POPE or DMPC) are inserted at random unoccupied positions in the box in random orientations, after that the box is filled with water molecules (about 12000). Then, a small number of water molecules are picked at

random and are substituted with Na^+ and Cl^- ions to balance the negative surface charge of the slab and provide NaCl concentration of 0.15 M in the water phase of the simulated system.

Simulation protocol

First, energy minimization of the simulated systems using the steepest gradient descent method is performed, followed by a short 100 ps pre-equilibration run at constant volume and temperature. After that, the pressure in the system is equilibrated to 1 bar using anisotropic Berendsen barostat[27] with relaxation time of 5 ps during 10 ns, which is finally followed by 1 μs production run in the NVT ensemble. Leap-frog algorithm with time step 1 fs is used to integrate the equations of motion. Center-of-mass motion is removed every 100 steps. Verlet cut-off scheme[28] with the buffer tolerance of 0.005 kJ x mol^{-1} x ps^{-1} per atom is used to generate the pair lists. Minimum cut-off of 1.4 nm is used for both short ranged electrostatic and VdW interactions. Long range electrostatics are calculated using PME[29] with the grid spacing of 0.12 nm and cubic interpolation. Long range dispersion corrections are applied to both energy and pressure. Velocity rescaling thermostat[30] is used to control the temperature, which is set to 303 K with the relaxation time of 1 ps. All bonds with hydrogen atoms are constrained using the LINCS algorithm[31]. Atom coordinates and energies are saved every 5 ps. All simulations were performed by the Gromacs 2019 software package[32]. Visualization of the simulations is done by VMD[33].

Models used

Lipids are described by the Slipids force field[34]. For TiO_2, we use parameters optimized to fit results on charge density distributions and water-TiO_2 surface coordination obtained in *ab-initio* simulations of TiO_2-water interface[35]. These parameters are listed in tables in supplement S6a and S6b. Water molecules are represented by the TIP3P model[36], and for Na^+ and Cl^- ions Yoo and Aksimentiev ion parameters is used[37]. Lorentz-Berthelot rules are applied to determine Lennard-Jones parameters for cross-interactions.

Modelling of bilayer adhesion to TiO_2 surface

Main message

Anatase TiO_2 tube (cylinder) with radius of 10 nm cannot wrap into a non-perturbed bilayer.

Supporting raw and analysed data:

Figure S106

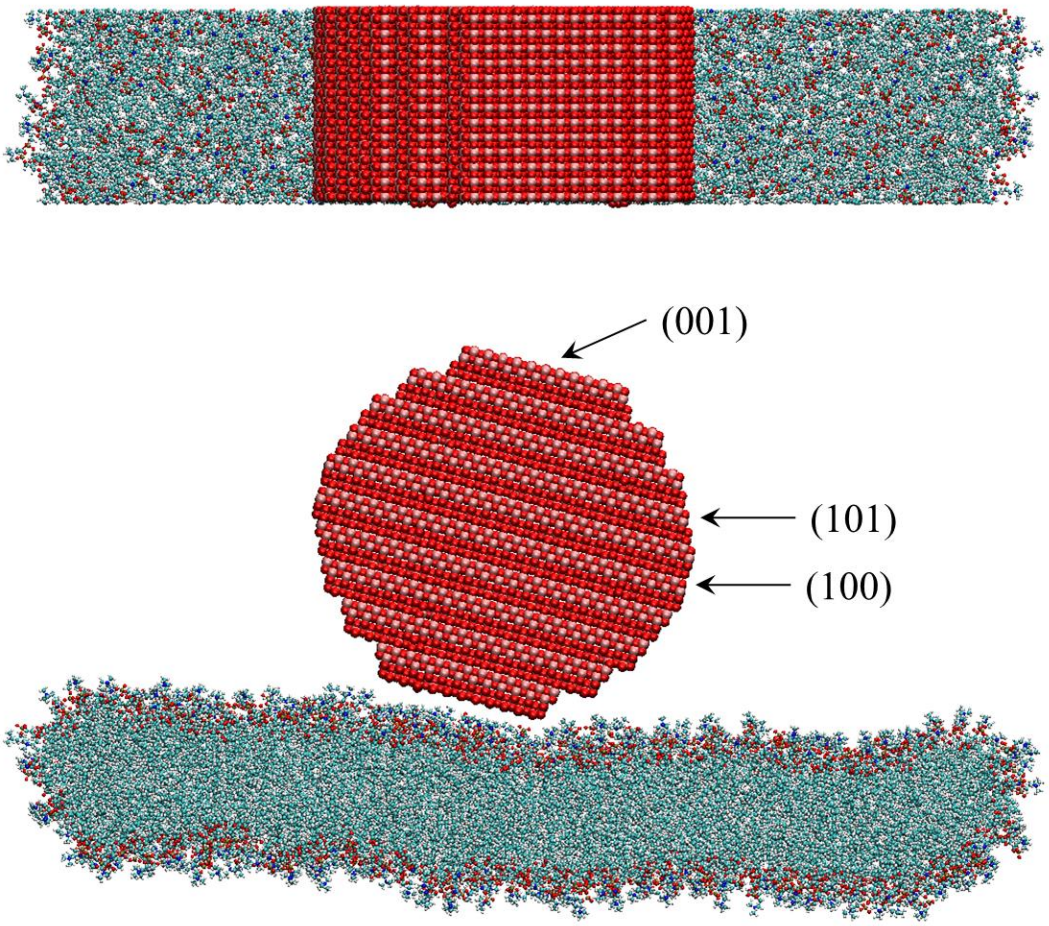


Figure S106: Top and side views of a 10 nm cylinder undergoing wrapping by a CG bilayer in the ribbon geometry. Red arrows show the direction of restraining potentials.

Materials and methods

The simulation was performed using GROMACS 2018.3 software with a 2 fs time step. Temperatures were maintained at 310 K using the Nose-Hoover thermostat with 5 ps time constant. Pressure was maintained at 1 atm using an anisotropic Parrinello-Rahman barostat with 10 ps time constant. Electrostatic interactions were calculated with a Particle Mesh Ewald (PME) summation. Lennard-Jones and real space electrostatic interaction potentials were truncated at 1.4 nm. The DOPC lipids were modeled using the fully atomistic 118 site Slipids force field which was developed for use in conjunction with TIP3P water[34,38,39]. This system was shown to reproduce many experimentally observed properties of bilayers including the lipid specific area and volume, bilayer thickness, isothermal area compressibility, and nuclear magnetic resonance (NMR) order parameters and scattering form factors. The titania force field was derived from electron densities generated by DFT simulation and was able to reproduce the DFT water density profiles at six low-energy titania cleavage planes. The axis of the cylinder lies along the [010] crystallographic direction, which is perpendicular to three of the four anatase cleavage planes with least surface energy, as determined by DFT simulation[40]. The surface thus comprises cleavage planes from the {001}, {101} and {100} families, as indicated in the diagram.

S3f – Ultrafast passage of nanomaterial of cell membrane

Main message

Nanoparticles passively (by physical interaction driven) pass exposed LA-4 membrane with only thin layer of water on top, with a 1s time-scale.

Supporting raw and analysed data:

Figure S107-Figure S111

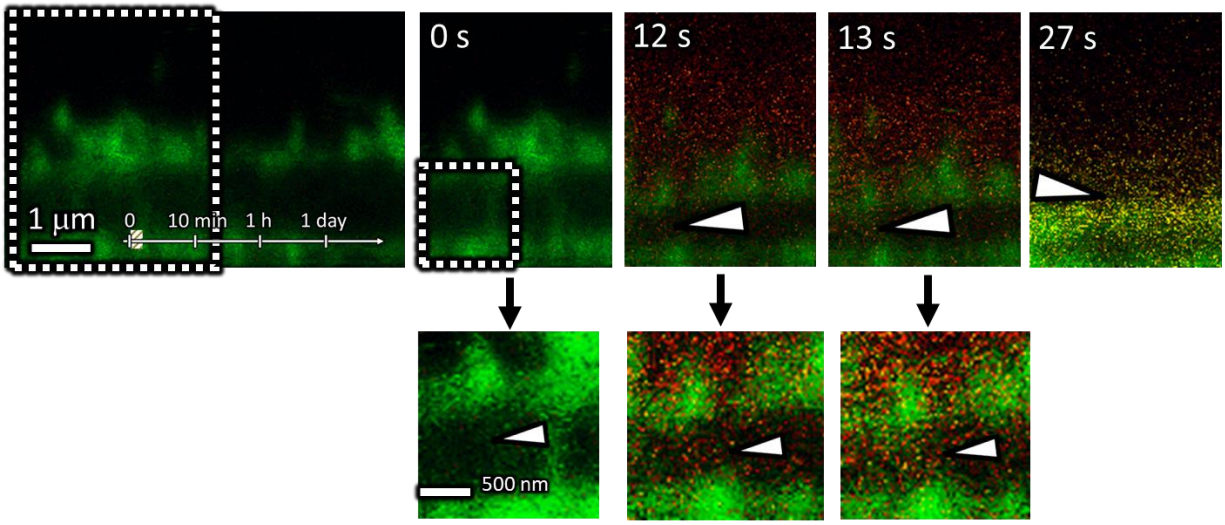


Figure S107: Composition of three times points alongside time point 0s wth additional zoomin to region of interest. Note that the nanomaterial passes through the membrane on 1s time scale (between frame 12s and 13s). At the end of exposure a lot nanomaterial is stuck to the top of the membrane and some nanomaterial can be seen inside. See also Movie S6.

Link to time-lapse

- *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/movie-NanoRain-TiO2_NT_nebulization_obto_LA-4_-_40s_duration_filtered_scaled_fliped-gif_40s.gif*

Movie S6: Nanoparticles (in red TiO_2 – Alexa 647) are nebulized directly to LA-4 epithelial cell membrane (in green – Cellmask Orange) and imaged in time with 1s time scale between frames. Nanoparticle signal was thresholded to 2 counts (there is one count noise on the detector) and membrane signal has been adjusted between frames for visibility due to bleaching of the signal. Whole movie is 40s long. See also Figure S107.

Materials and methods

- experiment:
  - LA-4 cells were seeded @60% confluence in an Ibidi #1.5H µ-Dish
  - after 24 hours, cells were checked to be @ approx. 100% confluency (bright field microscope), cells were incubated with 1.6 µg/mL CellMaskOrange for 5 minutes at room 37 °C in the incubator, afterwards they were flushed with 3x400 µl LCIS and in 300 µL LCIS at room temperature
  - right prior exposure LCIS has been almost completely removed leaving just thin water layer on top
  - Ibidi #1.5H µ-Dish has been kept @ 37 °C during measurement, but not the tubing
  - about 30 s after LCIS removal cells have been exposed to nebulized (sprayed on top) nanomaterial

        - 3 µl of 33 mg/mL TiO_2 (Alexa Fluor 647 labeled) has been added to Aeroneb®Pro nebulizer taken from VITROCELL® Cloud 6 system and mounted to standard plastic 50 mL centrifuge with a diameter of 2.75 cm. Nebulizer has been 10 cm above cell surface.
    - cells have been observed 50s in total after the start of experiment
- analysis:
    - images were analyzed to present co-localization on each image separately
        - contrast on each image has been adjusted for maximal visibility due to bleaching in green channel or appearance of more signal due to nanoparticle signal rise in time in red channel – bleaching rate of CellMask™ Orange can be seen on raw images of green signal
        - contrast has been re-scaled to maximum value for each image seprately
        - contrast was adjusted for yellow color to appear where both colors have been present in one pixel above noise (noise = 1 count) – using standard gamma correction
        - bottom threshold has been set to 1 count or above in both channels to reduce noise enough for the desired features to be seen
    - top of the images has been cropped for maximal visibility of desired phenomena on paper

Experiment names

- Main experiment name:
    - 20180206 /e02_s01_t01_LA-4 CellMask TiO2Alexa647_after nebulization_xztSTED.msr (date and name of .msr file so it can be easily found)

Controls and statistics

All separate channels and overlays for all time points:

Figure S108-Figure S111

Cell line	LA-4 (membrane, CellMaskOrange)
NPs	TiO_2 (Alexa647)
exposure	1:1, 3s-50s (live nebulization)
imaging	xzt STED, 0-50s

pixelsize (x,y)	30 nm
FOV (x,y)	7.95 µm
pixelsize (z)	33 nm
FOV (z)	6.58 µm
imaging time	50 s
number of frames	50

561nm	20%
640nm	20%
STED	13%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	60 µs
objective	wi60x (NA1.2)

TiO2 (Alexa647)

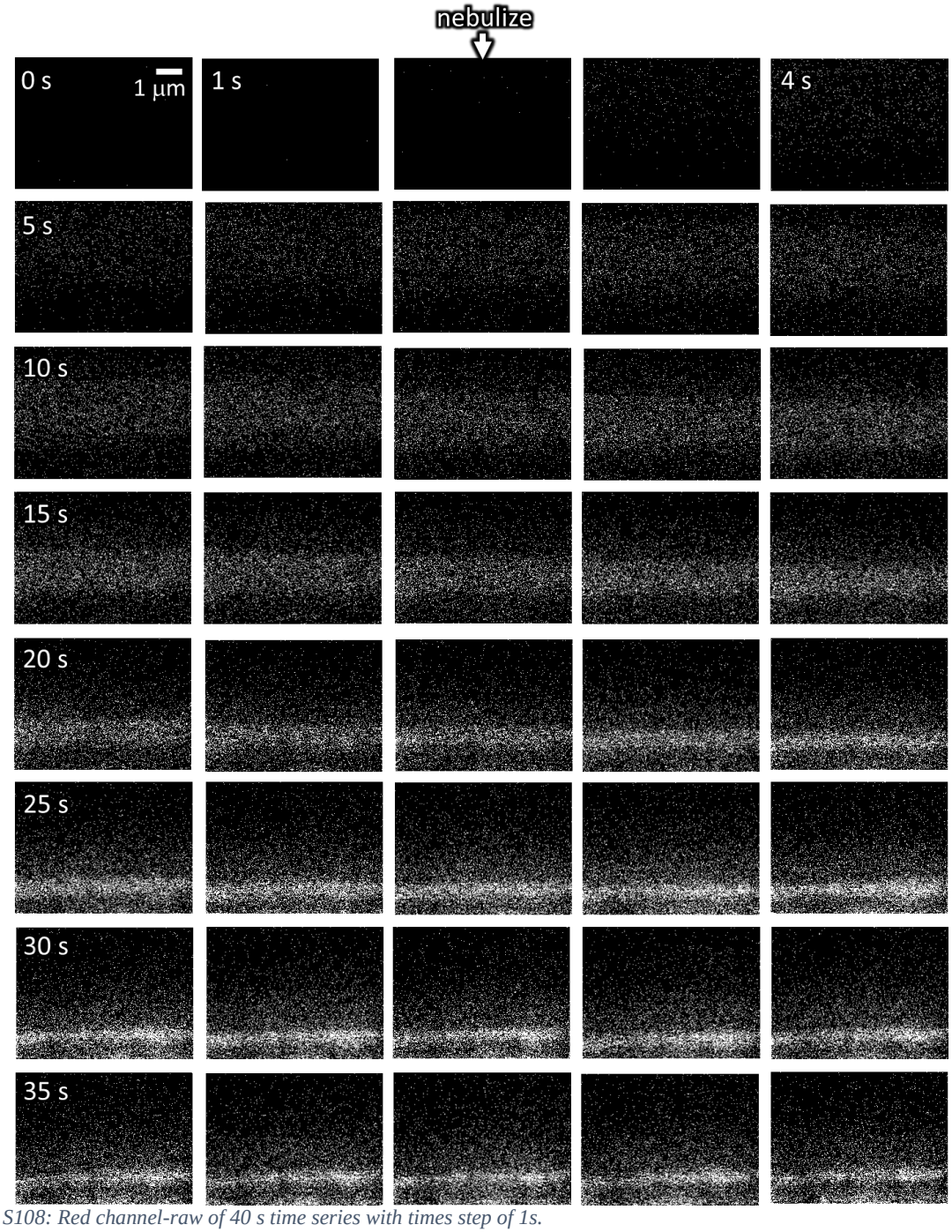


Figure S108: Red channel-raw of 40 s time series with times step of 1s.

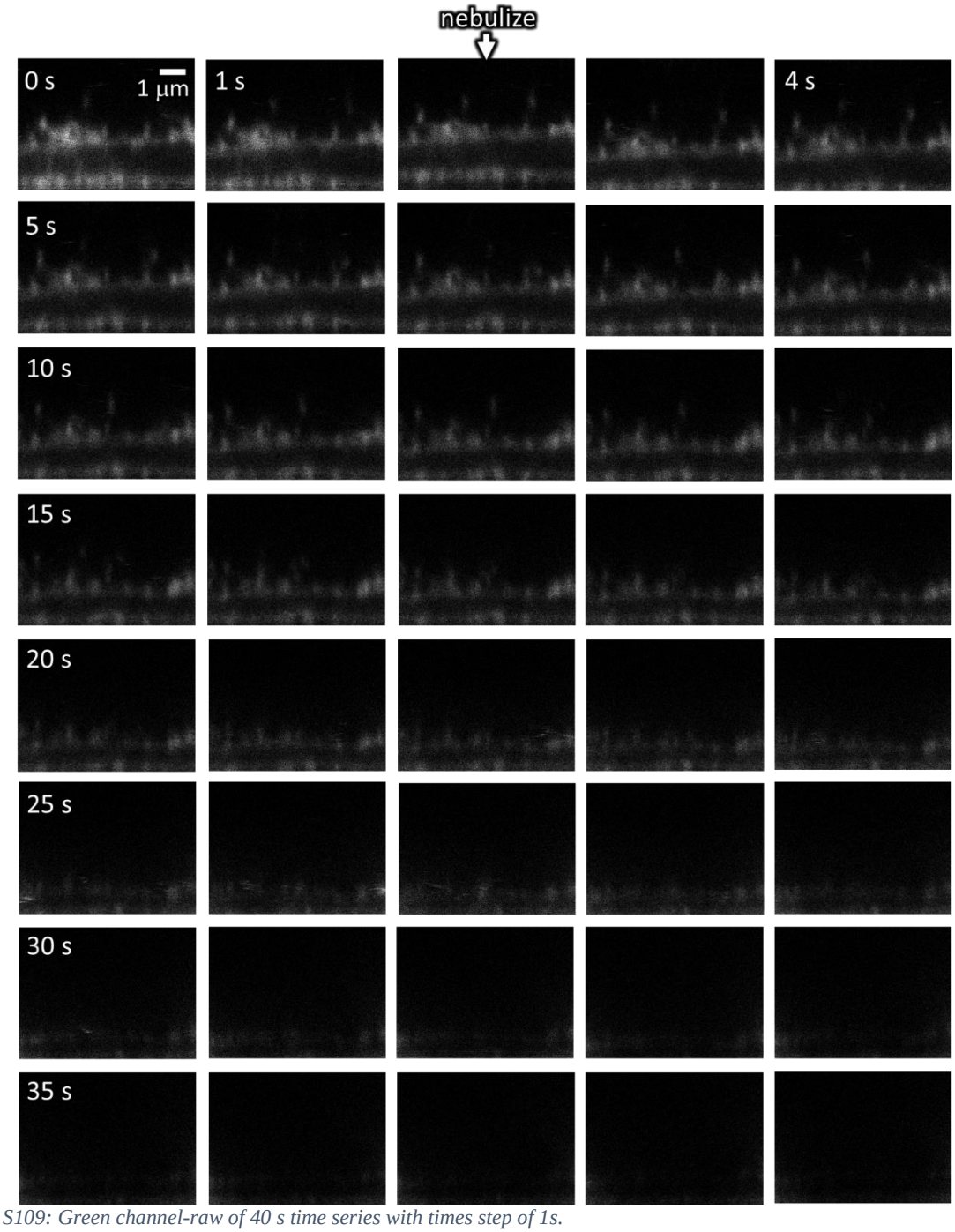


Figure S109: Green channel-raw of 40 s time series with times step of 1s.

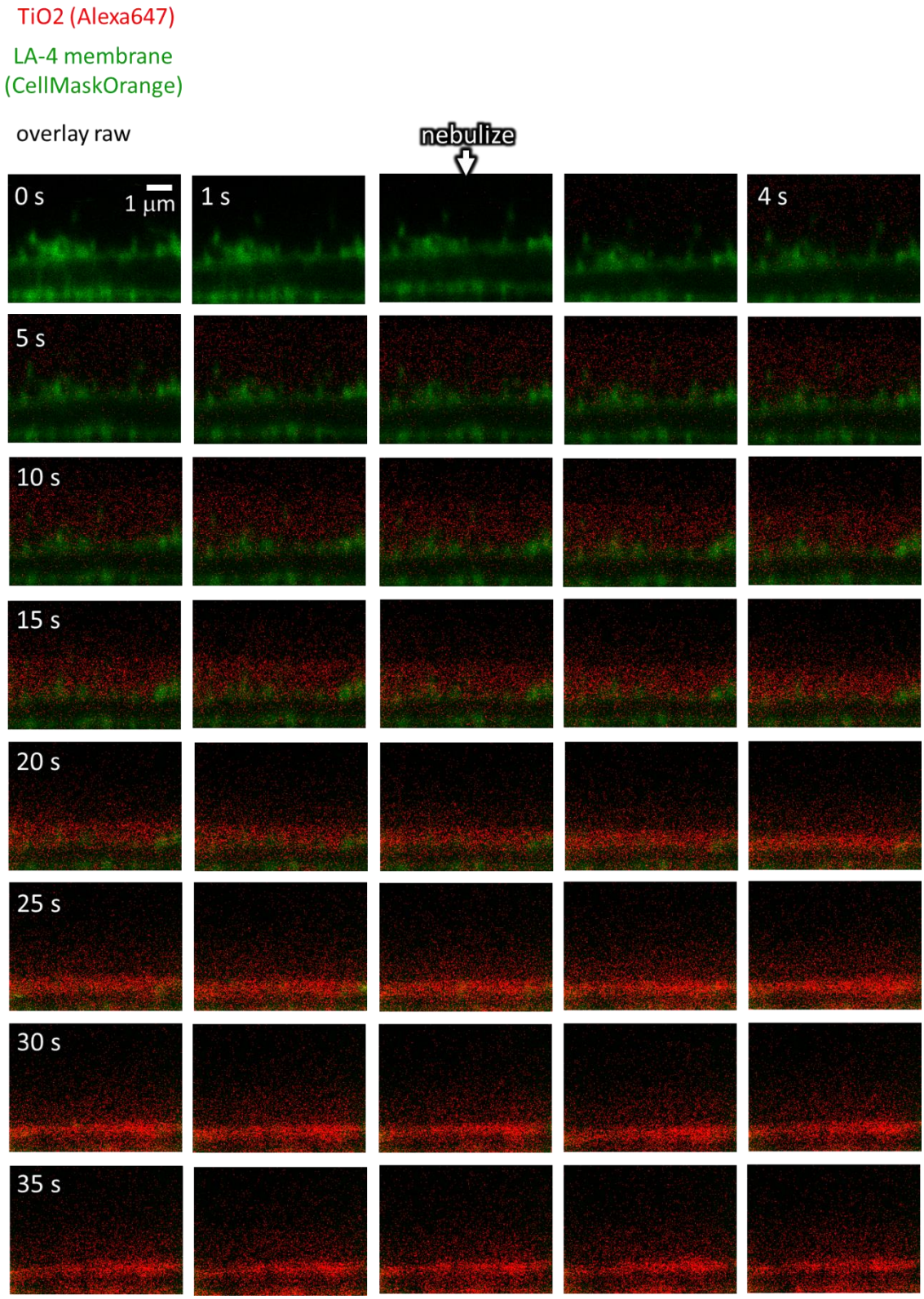


Figure S110: Overlay-raw of 40 s time series with times step of 1s.

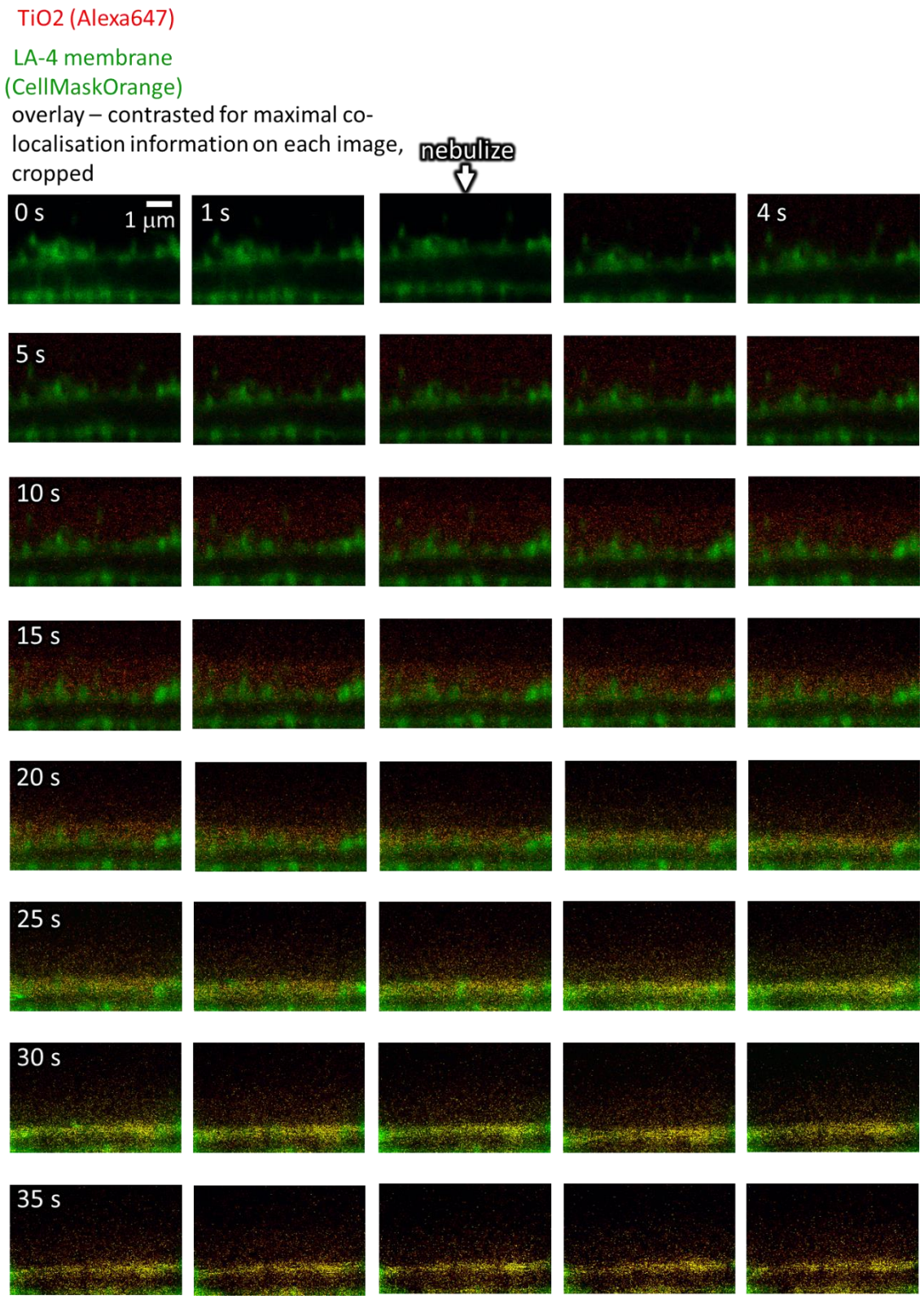


Figure S111: Contrasted overlay of 40 s time series with times step of 1s.

S3g – Blocking clathrin-mediated endocytosis

Main message

When blocking clathrin-mediated endocytosis using chlorpromazine, no nanomaterial enters the cell in the first 4 hours. However, at this timepoint, cauliflowers can be observed on the surface of the cell. They are smaller than fully-grown cauliflowers at day 2, but noticeably larger than the cauliflowers in control cells without blocked endocytosis.

Supporting raw and analysed data:

Figure S112-Figure S121

Materials and methods

- experiment **LA-4 + CellMask + TiO_2-Alexa 647 + 100 µm Chlorpromazine**
  - LA-4 cells were seeded @30% confluence in an Ibidi #1.5H µ-Dish.
  - After 48 hours LA-4 cells were washed with warm F12-K medium and placed on ice for 10 minutes.
  - Next, cells were washed 3 times with cold Live Cell Imaging Solution (LCIS) containing 20 mM glucose and 1% BSA .
  - 400 µL of 100 µm chlorpromazine in LCIS containing 20 mM glucose and 1% BSA was added and incubated at 37 °C for 15 minutes.
  - Then 1.5 µg/mL of CellMask was added to medium.
  - After 15 minutes incubation at 37 °C, 35 µL freshly filtered 1 mg/mL TiO_2-Alexa647 in 100x dcb was added directly to the cells and mixed to achieve 10:1 surface dose
- analysis:
  - Confocal: logarithmic scale on red channel, cut-off at 2 counts; set maximum to 350 counts on red channel; set maximum to 50 counts on green channel
  - STED: logarithmic scale on red channel, cut-off at 2 counts, set maximum to 500 counts on red channel; set maximum to 50 counts on green channel

pixelsize (x,y)	50 nm
FOV (x,y)	100 µm
pixelsize (z)	50 nm
FOV (z)	30 µm

561nm	30%
640nm	20%
STED	-
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	wi 60x (NA1.1)

Cell line	LA-4 (membrane, CellMask)
NPs	TiO2 (Alexa647)
exposure	10:1, 0h-4h
imaging	0min-5h xyz confocal, h

pixelsize (x,y)	30 nm
FOV (x,y)	26x33 µm
pixelsize (z)	
FOV (z)	
imaging time	-
number of frames	-

561nm	40%
640nm	20%
STED	15%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	wi 60x (NA1.1)

- experiment **LA-4 + DPPE Star580 + TiO_2-Alexa 647 + 200 µm Chlorpromazine**:
  - LA-4 cells were seeded @30% confluence in an Ibidi #1.5H µ-Dish

- After 48 hours LA-4 cells were washed with warm F12-K medium and placed on ice for 10 minutes.
- Next cells were washed 3 times with cold Live Cell Imaging Solution (LCIS) containing 20 mM glucose and 1% BSA .
- 400 µL of 200 um chlorpromazine in LCIS containing 20 mM glucose and 1% BSA was added and incubated at 37 °C for 15 minutes.
- Then 4 µL of 1mM DPPE-Star580 was added to medium
- After 15 minutes incubation at 37 °C, 35 µL freshly filtered 1 mg/mL TiO_2-Alexa647 in 100x dcb was added directly to the cells and mixed to achieve a 10:1 surface dose

- analysis:
  - Confocal: logarithmic scale on red channel, cut-off at 3 counts; set maximum to 300 counts on both channels
  - STED: logarithmic scale on red channel, cut-off at 3 counts; set maximum to 175 counts on both channels

Link to time-lapse

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190613_e01_s02_t01_LA-4_1uM_DPPEstar580_200uM_chlorpromazine_TiO2Alexa_1to10_1s_is_20min.gif

Movie S7: First 4 hours following exposure of LA-4 (membranes labelled with DPPEstar580, green) with chlorpromazine-blocked clathrin-mediated endocytosis to 10:1 TiO2 (Alexa 647, red), first 4 hours following exposure. 1 second in the movie corresponds to 20 minutes in real time. See also Figure S113.

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190613_e02_s01_t01_LA-4_1uM_DPPEstar580_200uM_chlorpromazine_TiO2Alexa_1to10_1s_is_20min.gif

Movie S8: First 3.5 hours following exposure of LA-4 (membranes labelled with DPPEstar580, green) with chlorpromazine-blocked clathrin-mediated endocytosis to 10:1 TiO2 (Alexa 647, red). 1 second in the movie corresponds to 20 minutes in real time. See also Figure S118.

Link to 3D

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190613_e01_s02_t04_LA-4_1uM_DPPEstar580_200uM_chlorpromazine_TiO2Alexa_10to1_4h_xyz_FOV_100_x_100_x_30_um.gif

Movie S9: Final state after 4 hours of exposure of LA-4 (membranes labelled with Star580 DPPE, green) with chlorpromazine-blocked clathrin-mediated endocytosis to 10:1 TiO2 (Alexa 647, red). Field-of-view is 100 x 100 x 30 µm. See also Figure S113.

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190613_e02_s01_t03_LA-4_1uM_DPPEstar580_200uM_chlorpromazine_TiO2Alexa_10to1_3.5h_xyz_FOV_100_x_100_x_30_um.mp4

Movie S10: Final state after 3.5 hours of exposure of LA-4 (membranes labelled with Star580 DPPE, green) with chlorpromazine-blocked clathrin-mediated endocytosis to 10:1 TiO2 (Alexa 647, red). Field-of-view is 100 x 100 x 30 µm. See also Figure S118.

pixelsize (x,y)	50 nm
FOV (x,y)	100 µm
pixelsize (z)	50 nm
FOV (z)	30 µm

561nm	30%
640nm	30%
STED	-
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	wi 60x (NA0.3)

Cell line	LA-4 (membrane, DPPE-Star580)
NPs	TiO2 (Alexa647)
exposure	10:1, 0h-4h
imaging	0min-4h xyz confocal, 4h

pixelsize (x,y)	20 nm
FOV (x,y)	50 µm
pixelsize (z)	
FOV (z)	
imaging time	3.5h
number of frames	150

561nm	20%
640nm	30%
STED	15%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	wi 60x (NA1.1)

Experiment names

- Main experiment name:
  - e02_s03_t01_LA-4_1,5ug_ml_CellMask_100um_chlorpromazine_TiO2Alexa_1to10_4h

- Experiment names:

  CellMask:
  - e02_s01_t01_LA-4_1,5ug_ml_CellMask_100um_chlorpromazine_TiO2Alexa_1to10_3D
  - e02_s02_t01_LA-4_1,5ug_ml_CellMask_100um_chlorpromazine_TiO2Alexa_1to10_3h
  - e02_s03_t01_LA-4_1,5ug_ml_CellMask_100um_chlorpromazine_TiO2Alexa_1to10_4h
  - e02_s04_t01_LA-4_1,5ug_ml_CellMask_100um_chlorpromazine_TiO2Alexa_1to10_5h
  - e02_s01_t02_LA-4_1,5ug_ml_CellMask_100um_chlorpromazine_TiO2Alexa_1to10_timeLapse
  - 20190611/e03_s01_t01_LA-4_1,5ug_ml_CellMask_200uM_chlorpromazine_TiO2Alexa_1to10

  DPPE-Star580:
  - 20190613/e01_s02_t04_LA-4_1um_DPPEstar580_200um_chlorpromazine_TiO2Alexa_1to10_3D_4h.msr
  - 20190613/e01_s02_t02_LA-4_1um_DPPEstar580_200um_chlorpromazine_TiO2Alexa_1to10_1.5h.msr
  - 20190613/e01_s02_t03_LA-4_1um_DPPEstar580_200um_chlorpromazine_TiO2Alexa_1to10_3h.msr
  - 20190613/e01_s02_t01_LA-4_1um_DPPEstar580_200um_chlorpromazine_TiO2Alexa_1to10_0.5h.msr
  - 20190613/e02_s01_t01_LA-4_1um_DPPEstar580_200um_chlorpromazine_TiO2Alexa_1to10_0.5h
  - 20190613/e02_s01_t02_LA-4_1um_DPPEstar580_200um_chlorpromazine_TiO2Alexa_1to10_3.5h_3D

- 20190613/e02_s01_t03_LA-4_1um_DPPEstar580_200um_chlorpromazine_TiO2Alexa_1to10_3.5h_3D

Controls:

- 20190618/e01_s01_t01_LA-4_1um_DPPEstar580_200um_chlorpromazine_0.5h_3D
- 20190121/e01_s01_t01_LA-4_50ug_ml_pHrodo_200um_chlorpromazine_TiO2-Alexa647_steps_30minInk
- 20190121/e01_s02_t02_LA-4_50ug_ml_pHrodo_200um_chlorpromazine_TiO2-Alexa647_steps_30minInk
- 20190611/e01_s01_t02_LA-4_1ug_ml_CellMask_100uM_chlorpromazine_test3hInk3D

Controls and statistics

Time-course of cauliflower growth in cells with inhibited endocytosis
Figure S112-Figure S118

control – cells with blocked endocytosis, not exposed to nanomaterial
Figure S119 - Figure S120

control – colocalisation of TiO_2 with endosome probe and chlorpromazine
Figure S121

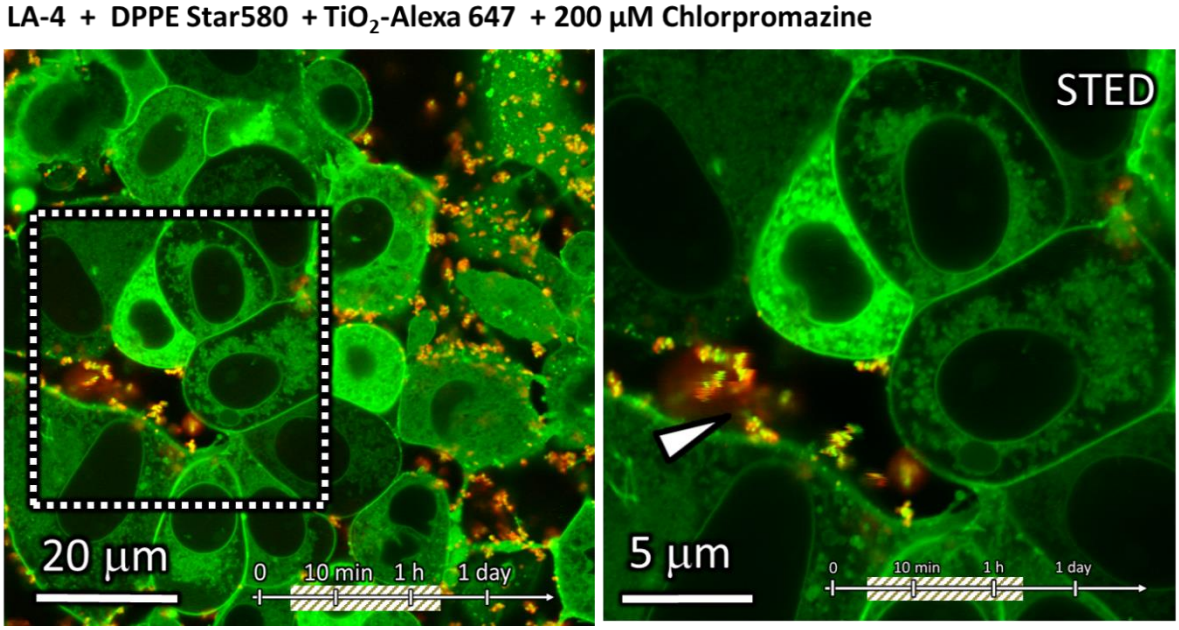


Figure S112: Cauliflower growth in cells with inhibited endocytosis (time-point 2.5 hours)

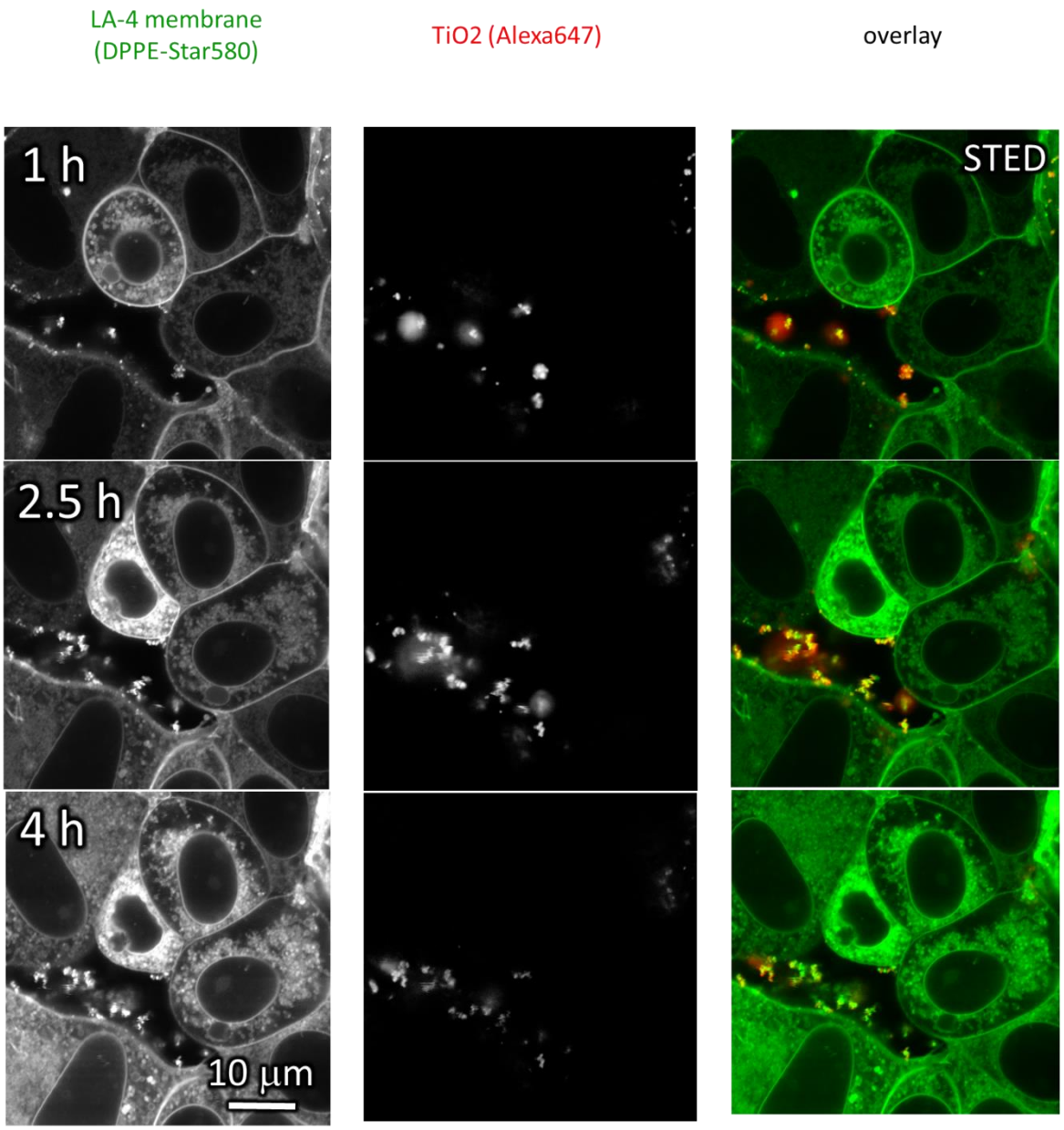


Figure S113: Time-course of cauliflower growth in cells with inhibited endocytosis – xy cross-sections. See also Movie S7 and Movie S9

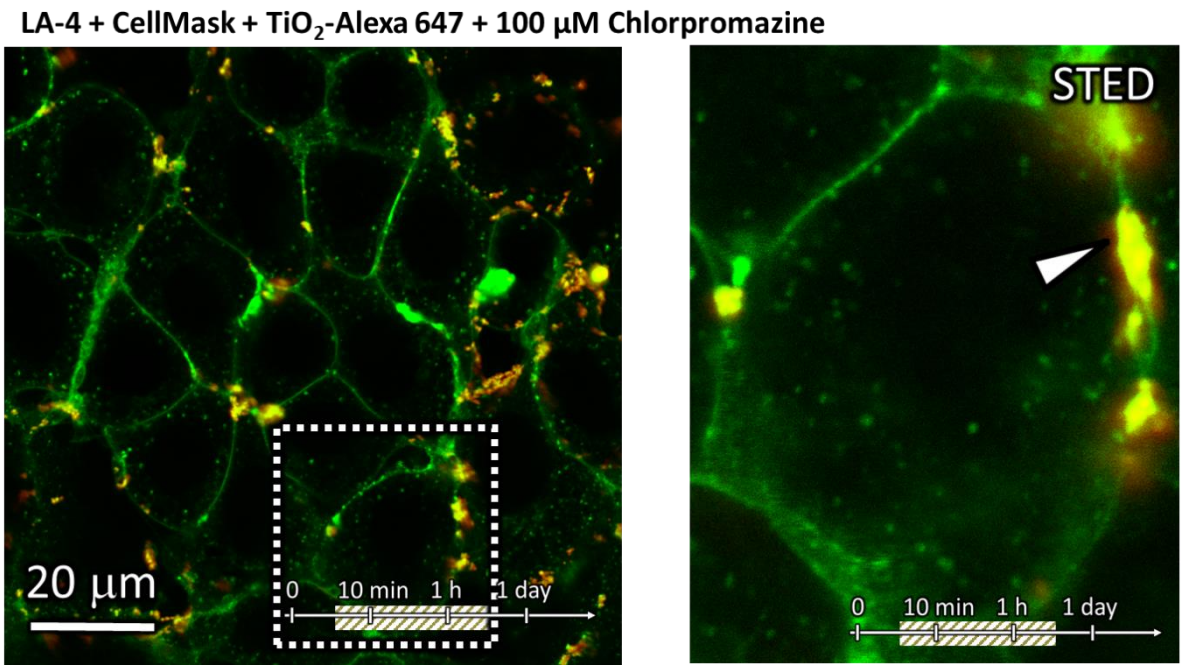


Figure S114: Cauliflower growth in cells with inhibited endocytosis (time-point 5 hours)

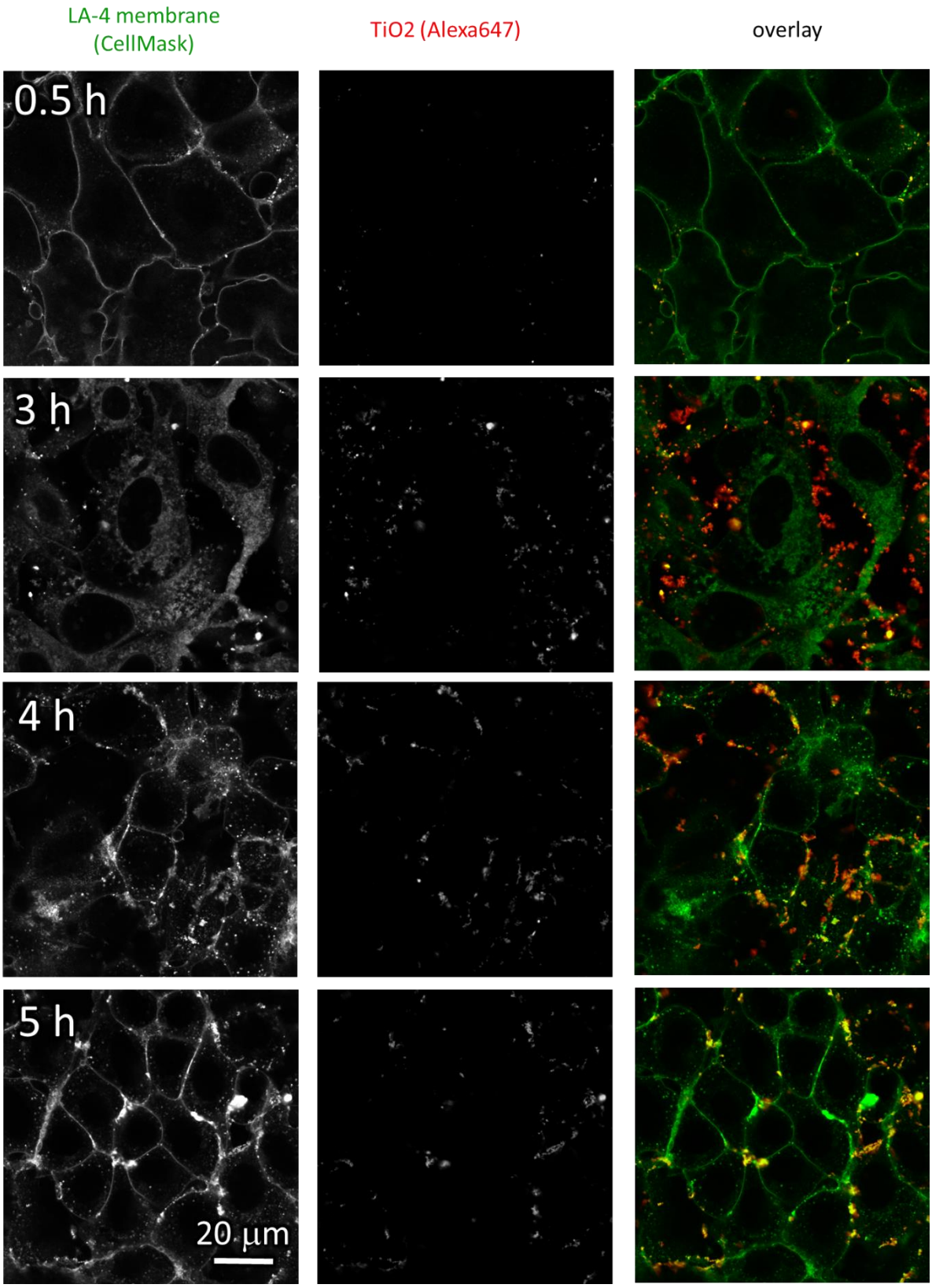


Figure S115: Time-course of cauliflower growth in cells with inhibited endocytosis – xy cross-sections.

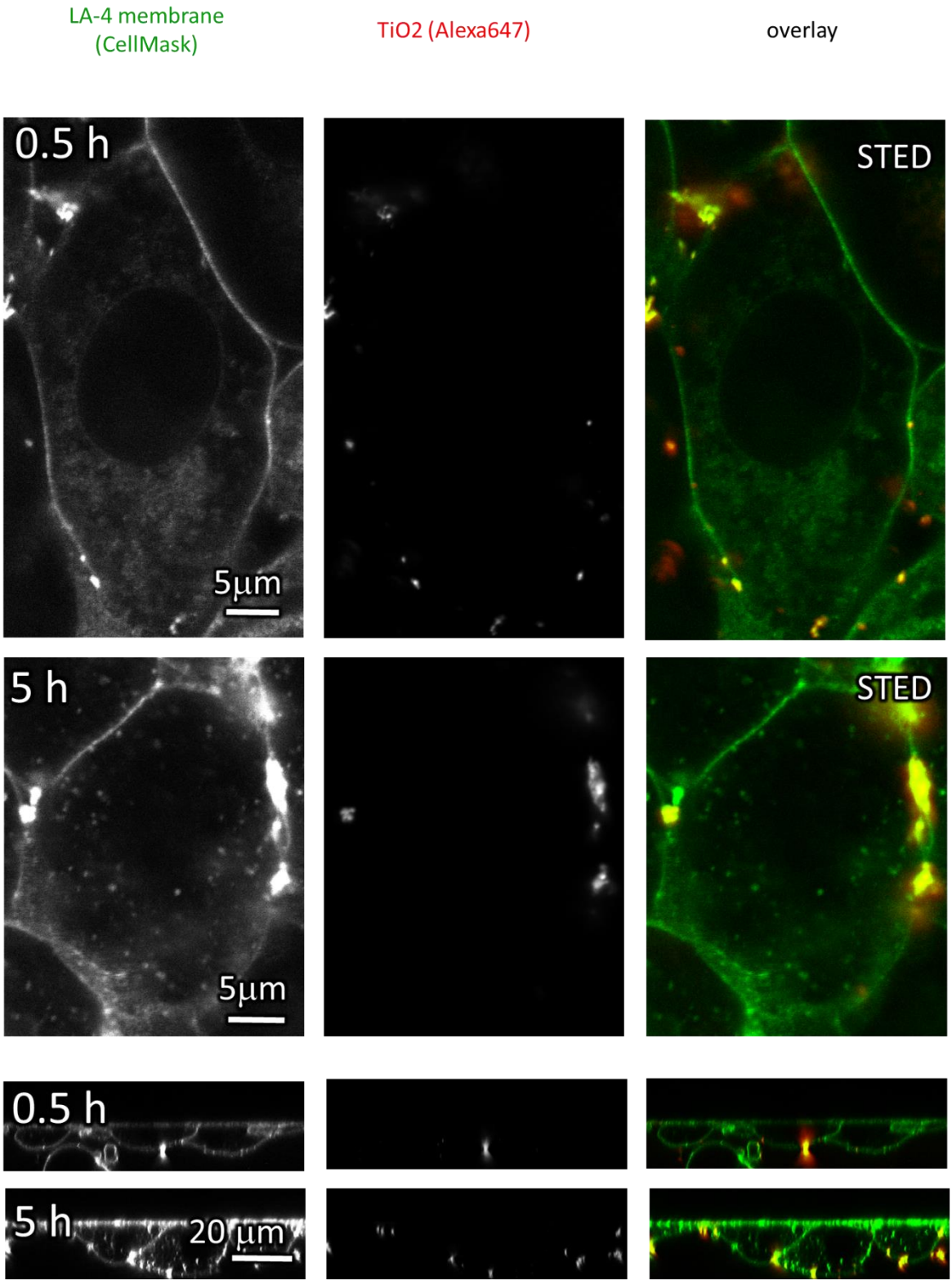


Figure S116: Time-course of cauliflower growth in cells with inhibited endocytosis – xy and xz cross-sections.

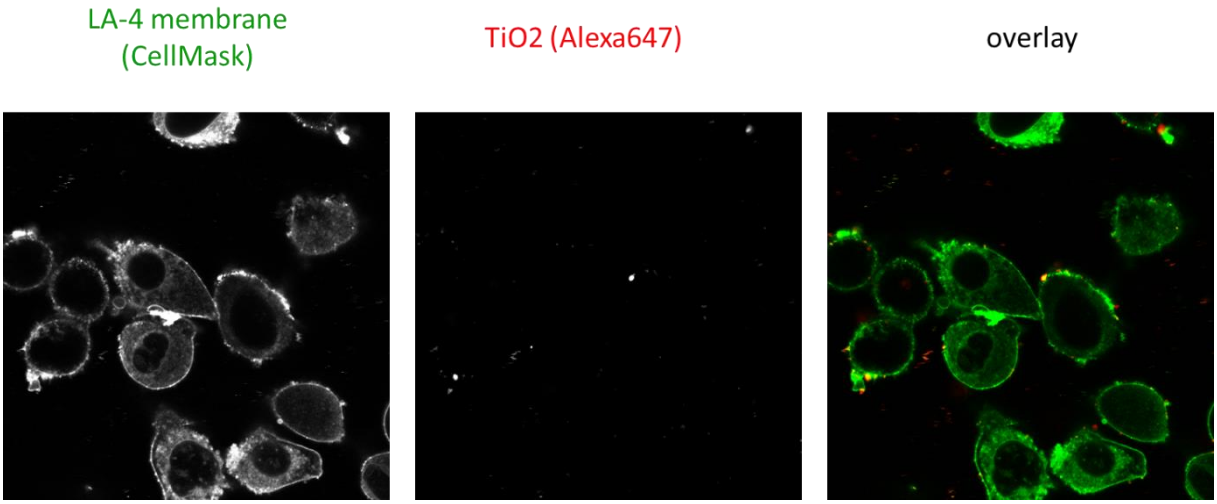


Figure S117: Cauliflower growth in cells with inhibited endocytosis (time-point 0.5 hours).

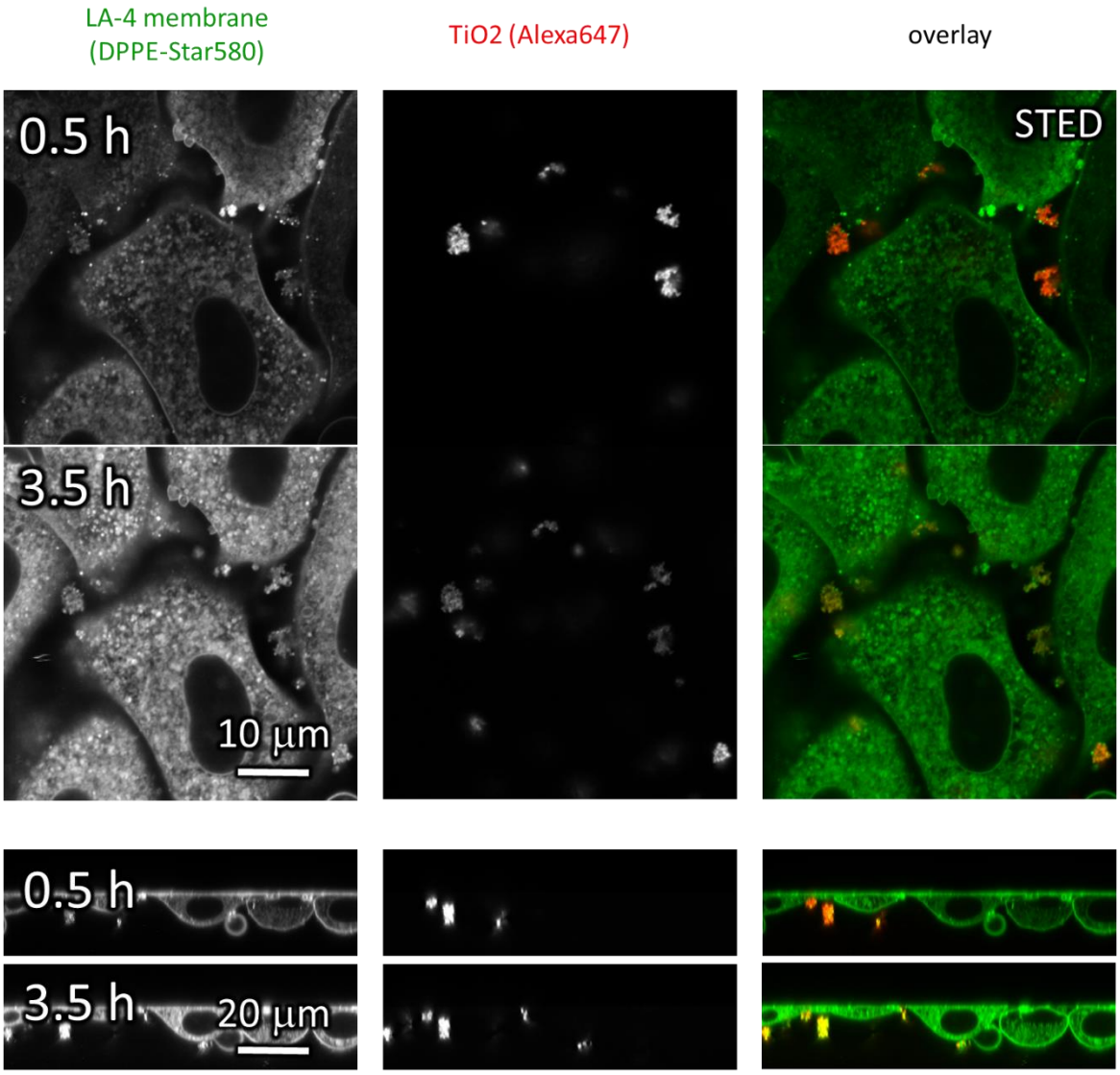


Figure S118: Time-course of cauliflower growth in cells with inhibited endocytosis – xy and xz cross-sections. See also Movie S8 and Movie S10.

Figure S119: Control –labelled cells with blocked endocytosis, not exposed to nanomaterial.

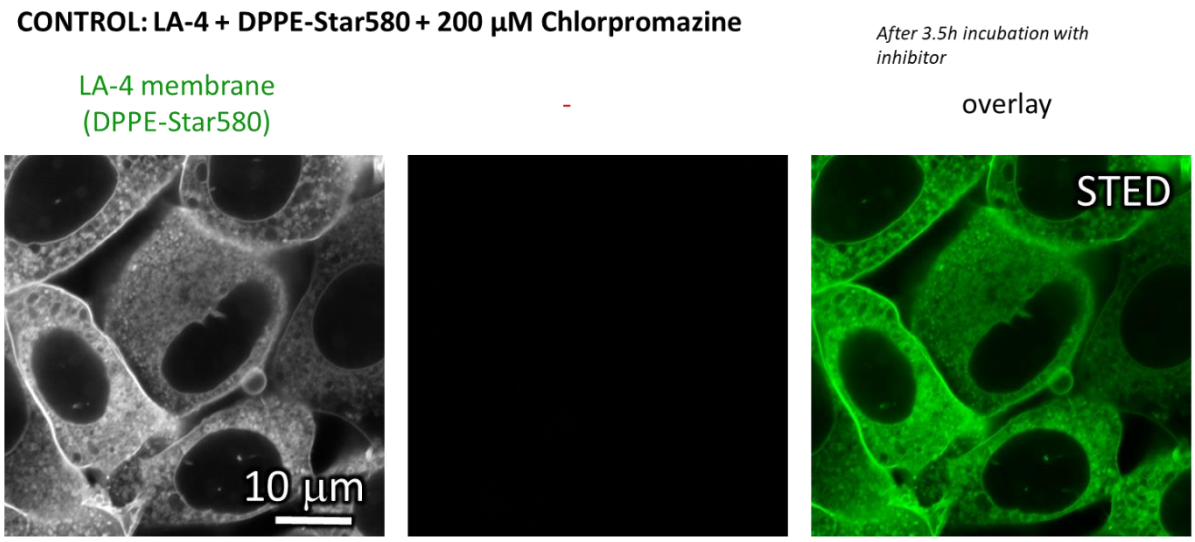


Figure S120: Control –labelled cells with blocked endocytosis, not exposed to nanomaterial.

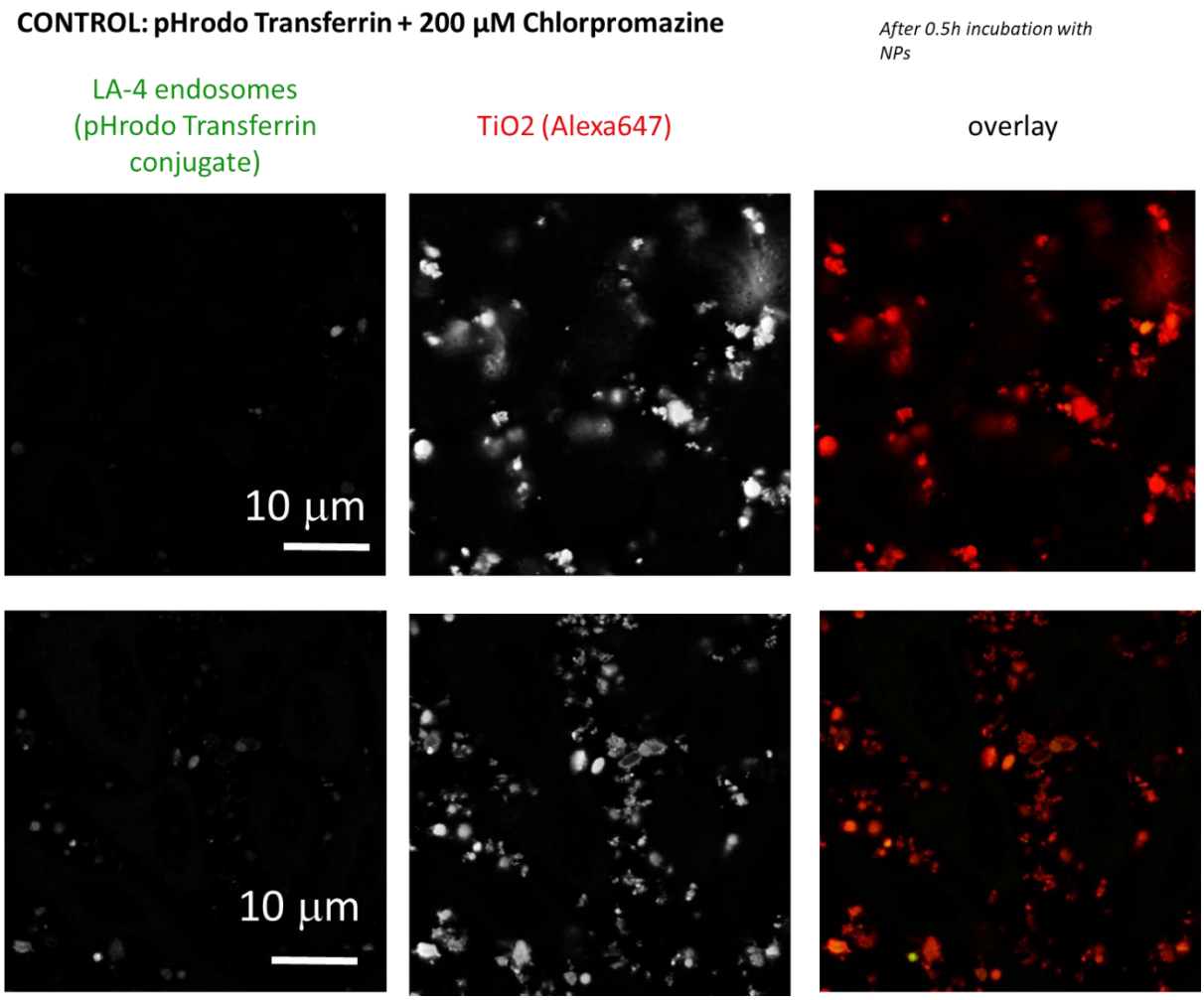


Figure S121: Control – colocalisation of labelled TiO_2 with endosome probe (pHrodo Transferrin) when incubated with chlorpromazine.

S3h – Membrane cholesterol extraction with Methyl-Beta-Cyclodextran

Main message

Observing formation of cauliflower-like structures after the extraction of cholesterol from plasma membrane with Metyl – Beta – Cyclodextrin.

Supporting raw and analysed data:

Figure S122-Figure S125

Materials and methods

- experiment:
  - LA-4 cells were seeded @60% confluence in an Ibidi #1.5H µ-Dish
  - after 24 hours medium was exchanged for fresh mixture of LCIS and medium with 1 mM Metyl-Beta-Cyclodextran (MBCD). Immediately after 35 µL of freshly filtered 1 mg/mL TiO_2-40-ATTO 594 in 100x dcb was added directly to the cells (in 400 µL medium with 1 mM MBCD) and mixed to achieve 10:1 surface dose. Cells were incubated for additional 24h
  - After 48 hours cells were incubated with 1 um Star Red DPPE for 5 min in incubator at 37 °C, 5% CO2. Cells were not washed with LCIS in order to not wash away MBCD. Cells were imaged on the microscope stage heated on 37 °C.
- analysis:
  - Sharpening algorithm (σ = 2, c = 0,3; described below) was applied in red channel and 1 pixel Gaussian blur in green
  - Contrast is adjusted to achieve better visibility

    To reduce the noise and accentuate fine details on some images we used a sharpening algorithm for subtraction of a blurred image (Gaussian low-past filter) from the original image. This can be considered as a convolution operation on an image with a kernel mask that is a two-dimensional Gaussian function:

$$g(x,y) = \frac{1}{\sigma\sqrt{2\pi}} e^{-(x^2+y^2)/2\sigma^2}$$

    Where σ represents the size of the Gaussian kernel mask which determines the range of frequencies removed by the Gaussian filter. This blurred image is then subtracted from the original image as:

$$F(x,y) = \frac{c}{2c-1} I(x,y) - \frac{(1-c)}{2c-1} U(x,y)$$

    where the F(x, y) represents the brightness value of a pixel at the coordinate (x, y) in the filtered image, and I(x, y) and U(x, y) represent the brightness values of the corresponding pixels in the original and blurred images, respectively. The constant c controls the relative weightings of the original and blurred images in the difference equation.
    Such a subtraction enhances high-frequency spatial detail at the expense of low-frequency spatial information in the image.

Experiment names

- Main experiment name:
  - 20190612_e01 m02 s01_LA-4_SR DPPE_TiO2 Star520S_1 mM MBCD_overnight incubation_NM in the cell

- Supplement Experiment names:
  - 20190612_e03 m03 s01 _LA-4_SR DPPE_TiO2 Star520S_1 mM MBCD_overnight incubation_uptake of TiO2
  - 20190612_e01 m04 s02 LA-4_SR DPPE TiO2 Star 520 0.5mM MBCD overnight much NM in the cell
  - 20190612_e01 m02 s01_LA-4_SR DPPE_TiO2 Star520S_1 mM MBCD_overnight incubation_NM in the cell_STED
  - 20190612_e01 m03 s01 LA-4_SR DPPE TiO2 Star 520 0.5 mM MBCD overnight much NM in the cell_3D.msr

Controls and statistics

Shortly after treatment with 1 mM MBCD cells show increased uptake of single and small aggregates of nanomaterial. We see less large cauliflower structures on the surface of the cells:

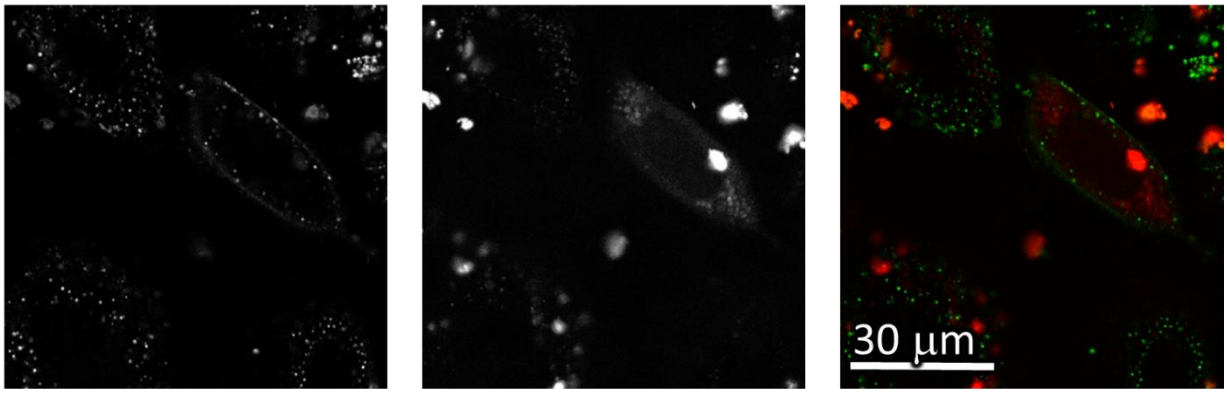
Figure S122-Figure S124

Four time points from a time lapse movies of the cell rapidly uptaking TiO_2 nanotubes:

Figure S125

Main Experiment

LA-4 membrane (SR DPPE) TiO2 (ATTO 594) overlay

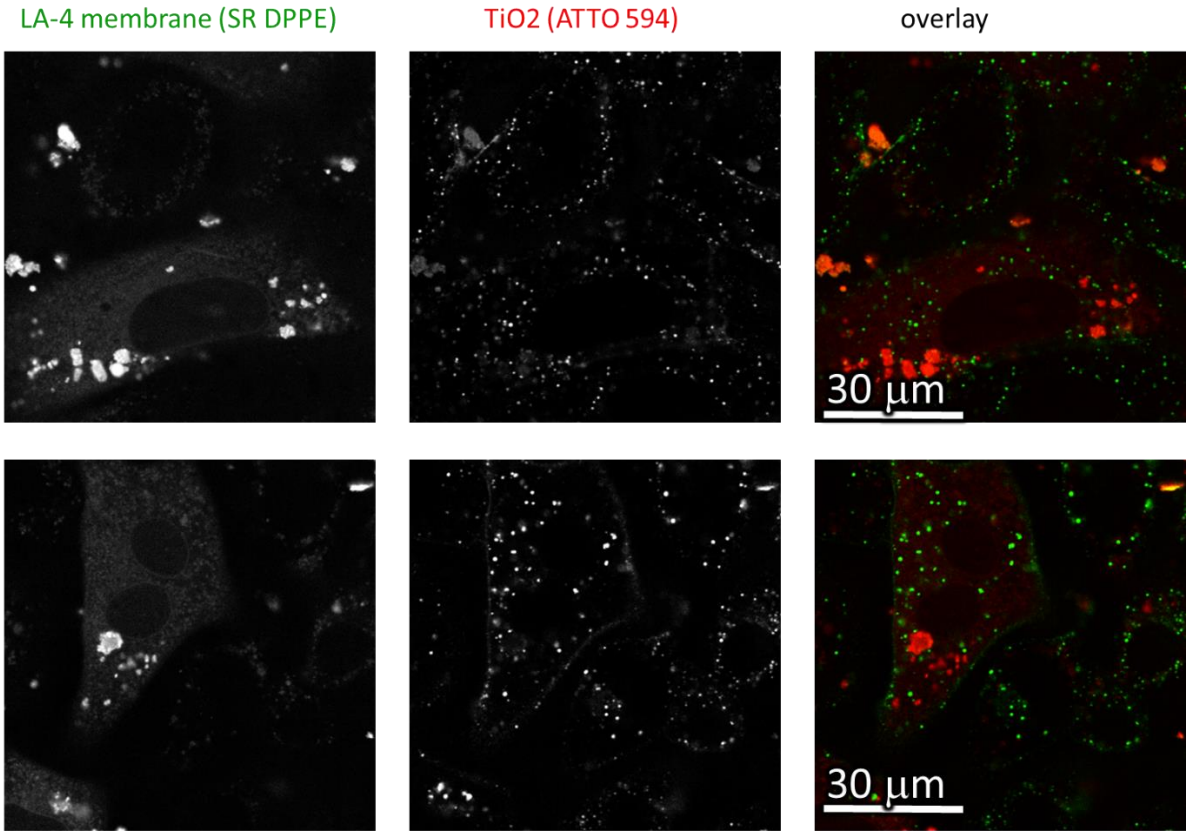


Cell line	LA-4 (membrane, StarREd-DPPE)
NPs	TiO2 (ATTO 594)
exposure	10:1, 24h-48h 1 mM MBCD
imaging	xy confocal, 48h

pixelsize (x,y)	200
FOV (x,y)	80 µm
pixelsize (z)	/ nm
FOV (z)	/ µm
imaging time	/
number of frames	/

561nm	30 %
640nm	40 %
STED	/ %
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	6wi0x (NA1.2)

Figure S122: Confocal images of LA-4 in green and TiO_2 nanotubes in red channel. Cells treated with 1 mM MBCD for 48 hours. No collocalization and no cauliflower-like formations. Cells have been stained with Star Red-DPPE dye which tends to redistribute inside the cell into membranes of different vesicles. For that reason we can't see clear outlines of all the cells. Note how cells are full of individual nanotubes.



Cell line	LA-4 (membrane, StarREd-DPPE)
NPs	TiO2 (ATTO 594)
exposure	10:1, 24h-48h 1 mM MBCD
imaging	xyt STED /, xy confocal, 48h

pixelsize (x,y)	200
FOV (x,y)	80 µm
pixelsize (z)	/ nm
FOV (z)	/ µm
imaging time	/
number of frames	/

561nm	30 %
640nm	40 %
STED	/ %
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	6wi0x (NA1.2)

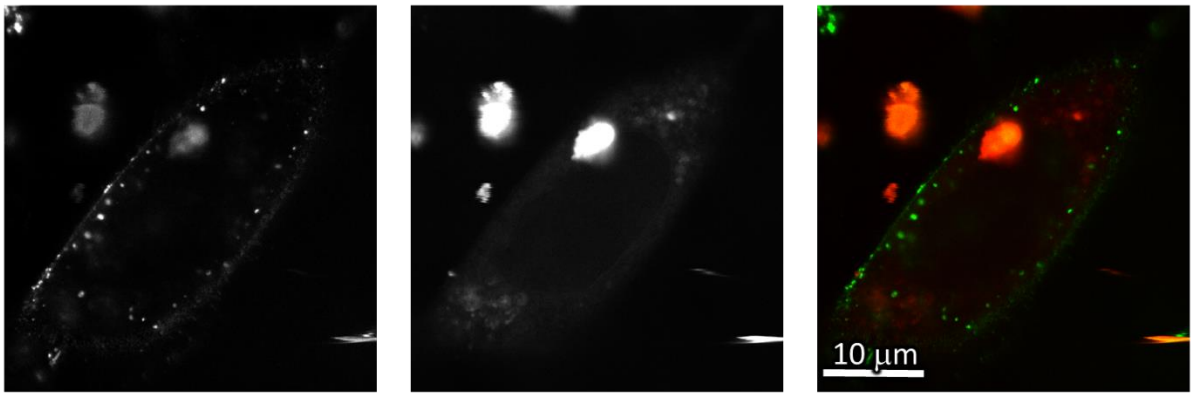
Cell line	LA-4 (membrane, StarREd-DPPE)
NPs	TiO2 (ATTO 594)
exposure	10:1, 24h-48h 1 mM MBCD
imaging	xz STED 48h, xz confocal /

pixelsize (x,y)	40
FOV (x,y)	80 µm
pixelsize (z)	40 nm
FOV (z)	12 µm
imaging time	/
number of frames	/

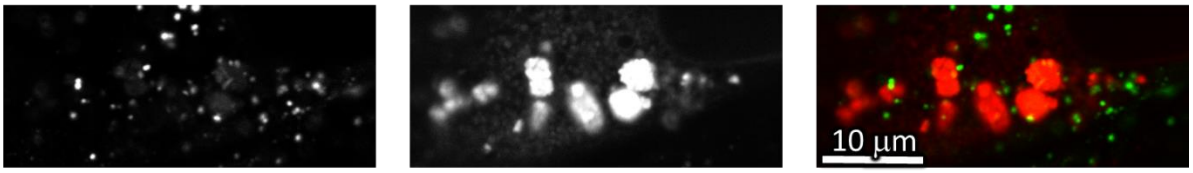
561nm	30 %
640nm	30 %
STED	20 %
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	6wi0x (NA1.2)

Figure S123: Confocal images of LA-4 in green and TiO_2 nanotubes in red channel. Cells treated with 1 mM MBCD for 48 hours. No collocalization and no cauliflower-like formations. Cells in first two windows are XY shots of different spots on the sample. In 3rd window we see STED of the XZ plane of the large cell in the first window. Note how cells are full of individual nanotubes.

LA-4 membrane (SR DPPE) TiO2 (ATTO 594) overlay

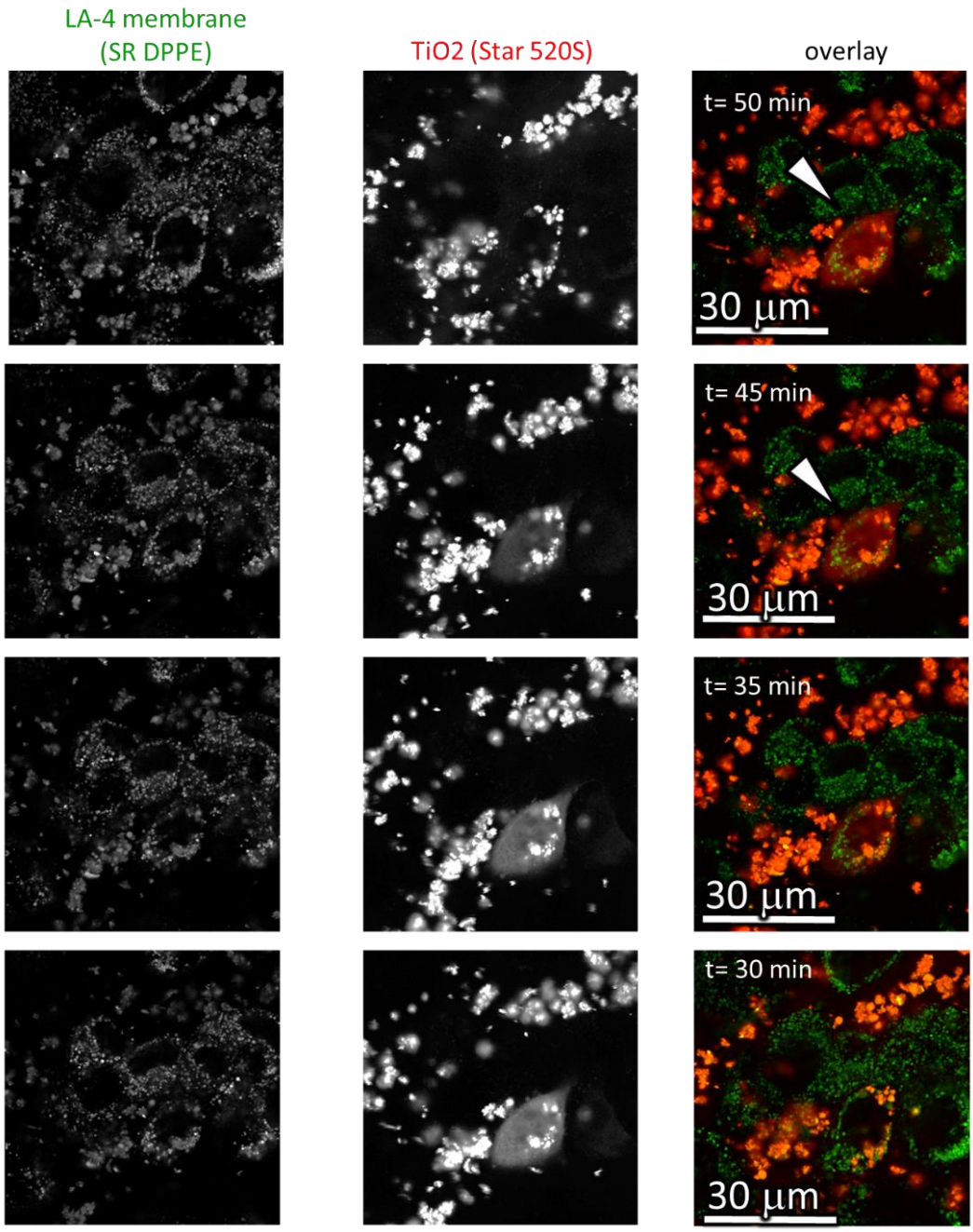


Cell line	LA-4 (membrane, StarRed-DPPE)	pixelsize (x,y)	30	561nm	30 %
NPs	TiO2 (ATTO 594)	FOV (x,y)	39 x 40,4 µm	640nm	30 %
exposure	10:1, 24h-48h	pixelsize (z)	/ nm	STED	20 %
imaging	xy STED, 48h	FOV (z)	/ µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 µs
		number of frames	/	objective	6wi0x (NA1.2)



Cell line	LA-4 (membrane, StarRed-DPPE)	pixelsize (x,y)	40	561nm	30 %
NPs	TiO2 (ATTO 594)	FOV (x,y)	37 x 16 µm	640nm	30 %
exposure	10:1, 24h-48h	pixelsize (z)	/ nm	STED	/ %
imaging	xy confocal, 48h	FOV (z)	/ µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 µs
		number of frames	/	objective	6wi0x (NA1.2)

Figure S124: Confocal images of LA-4 in green and TiO_2 nanotubes in red channel. Cells treated with 1 mM MBCD for 48 hours. No collocalization and no cauliflower-like formations. Confocal images of a XY plane in the 1st window and XZ plane in the 2nd rst window. Note how cells are full of individual nanotubes. See also Movie S11.



Cell line	LA-4 (membrane, StarREd-DPPE)
NPs	TiO2 (ATTO 594)
exposure	10:1, 24h-48h
imaging	xy confocal, 48h

pixelsize (x,y)	80
FOV (x,y)	80 µm
pixelsize (z)	/ nm
FOV (z)	/ µm
imaging time	1h
number of frames	61

561nm	30 %
640nm	40 %
STED	/ %
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	6wi0x (NA1.2)

Figure S125 Confocal images of LA-4 in green and TiO_2 nanotubes in red channel. Cells treated with 1 mM MBCD for 48 hours. No collocalization and no cauliflower-like formations. Confocal images of XY planes of different time points in a time lapse video. Filling of the cell with nanotubes in course of 20 min. See also Movie S12.

Link to 3D

- *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190612_e01_m03_s01_LA-4_SR_DPPE_TiO2_Star_520_0.5mM_MBCD_overnight_much_NM_in_the_cell__3D.mp4*

Movie S11: 3D representation of the LA-4 cells (StarRed DPPE, green) filled with TiO_2 nanotubes (Star 520 SXP, red). Cells were treated with 1 mM MBCD for 48 hours beforehand to decrease cholesterol concentration in membrane. See also Figure S124

Link to time-lapse

- *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/e03_m03_s01_LA-4_SR_DPPE_TiO2_Star_520_added_1_mMMBCD_NM_uptake.gif*

Movie S12: Time-lapse of filling of the LA-4 cells (StarRed DPPE, green) with TiO_2 nanotubes (Star 520 SXP, red) in course of 20 min. Cells were treated with 1 mM MBCD for 48 hours beforehand to decrease cholesterol concentration in membrane. See also Figure S125.

S3i – Block of lipid synthesis by FAS inhibition

Main message

Formation of cauliflower-like structures after the chemical inhibition of fatty acid synthase (FAS) enzyme complex by Resveratrol after 2 days of incubation with TiO_2 nanotubes.

Supporting raw and analysed data:
Figure S126-Figure S131

Materials and methods

- Main experiment:
  - LA-4 cells were seeded @60% confluence in an Ibidi #1.5H µ-Dish in cell medium
  - After 24 hours medium was exchanged for fresh medium with 100 µM Resveratrol and 35 mL of freshly filtered 1 mg/mL TiO_2-17-Alexa 647 in 100x dcb was added directly to the cells (in 400 µL medium/100 µM Resveratrol) and mixed to achieve 10:1 surface dose. Cells were incubated for additional 24h.
  - After incubation with TiO_2 and Resveratrol cells were incubated with 1 µM SHE-2N for 5 min in incubator at 37 °C, 5% CO2. Cells were not washed with LCIS in order not to wash away Resveratrol. Cells were imaged on the microscope stage heated on 37 °C.
- Supplement experiments:
  - Sample preparation was the same for supplement experiments. Only difference was labelling of the cells where we stained one sample with 1 µm CellMask and another with 1 µm SirActin in the incubator at 37 °C, 5% CO2 for 5 min and 2 hours respectively. Cells were not washed with LCIS in order not to wash away Resveratrol. Cells were imaged on the microscope stage heated on 37 °C.

Analysis:

- Images analysed with ImageJ
  - Signal count was multiplied 1.5 times to gain better visibility
  - Sharpening algorithm (σ = 2, c = 0,6) was applied on a red channel and 1 pix Gaussian Blur on a green channel

Experimental names

- Main experiment name:
  - 20190614_e02 m05 s03 CM 100 µM Resveratrol LA-4 SHE 2N and 10 - 1 S520 TiO2_larger FOV

- Supplement Experiment names:
  - 20190619_e06 m01 s01_LA-4_CellMask_ NEG CNOTROLE for 100 µM Resveratrol and 10 - 1 S520 TiO2_3D
  - 20190619_e05 m05 s04 LA-4_CM 100 µM Resveratrol and 10 - 1 S520 TiO2_Alexa 647_larger feed of view
  - 20190619_e04 m08 s07 LA-4_1 um SA_10 to 1_ TiO2_Star520S_100 µM Resveratrol 24h incubation
  - 20190619_e05 m05 s05 LA-4_CM 100 µM Resveratrol and 10 - 1 S520 TiO2_Alexa 647_larger feed of view

- 20190619_e05 m09 s07 LA-4_CM 100 µM Resveratrol and 10 - 1 S520 TiO2_Alexa 647_larger feed of view
- 20190614_e01 m05 s05_ LA-4 SHE 2N and 10 - 1 S520 TiO2_controle
- 20190614_e02 m05 s03 CM 100 µM Resveratrol LA-4 SHE 2N and 10 - 1 S520 TiO2_larger FOV
- 20190614_e02 m04 s03 LA-4 treated with 100 µM Resveratrol and 10-1 TiO2 3D

Controls and statistics

Absence of large cauliflower-like structures on the surface of the cells treated with 100 µM Resveratrol after 2 days of incubation with TiO_2 nanotubes:

Figure S126

Control experiment where the cells were not treated with the Resveratrol and were exposed to TiO_2 under the same conditions. We see formation of cauliflower-like structures on the surface on the cells:

Figure S127-Figure S129

Cells treated with 100 µM Resveratrol and exposed to TiO_2 nanotubes for 2 days. Different labels were used, as described in boxes underlying the images:

Figure S130-Figure S131

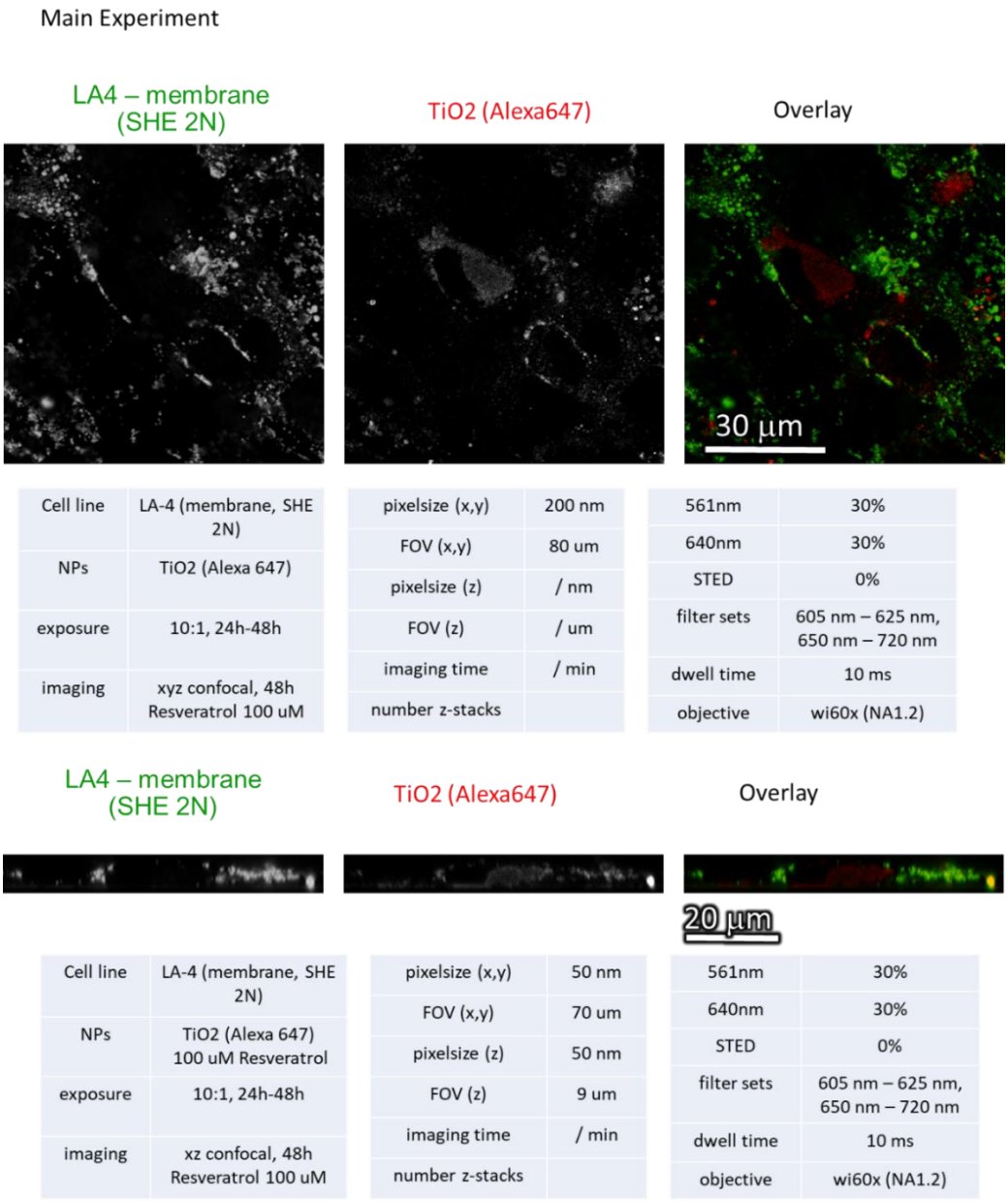
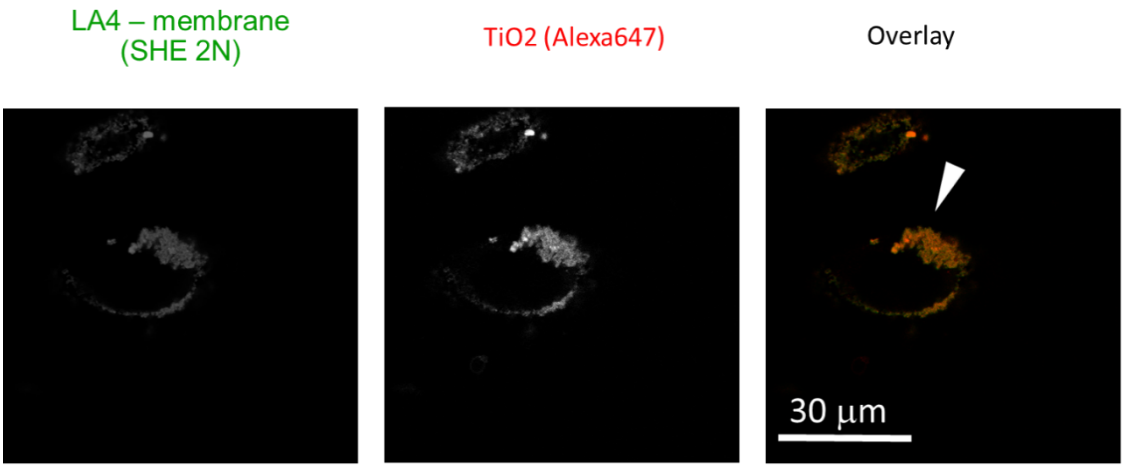
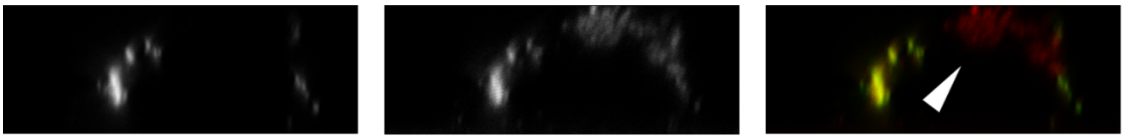


Figure S126: Confocal images of LA-4 in green and TiO_2 nanotubes in red channel. Cells treated with 100 µM Resveratrol for 48 hours. No collocalization and no cauliflower-like formations. XY on upper window and XZ on lower.

Supplement Experiments

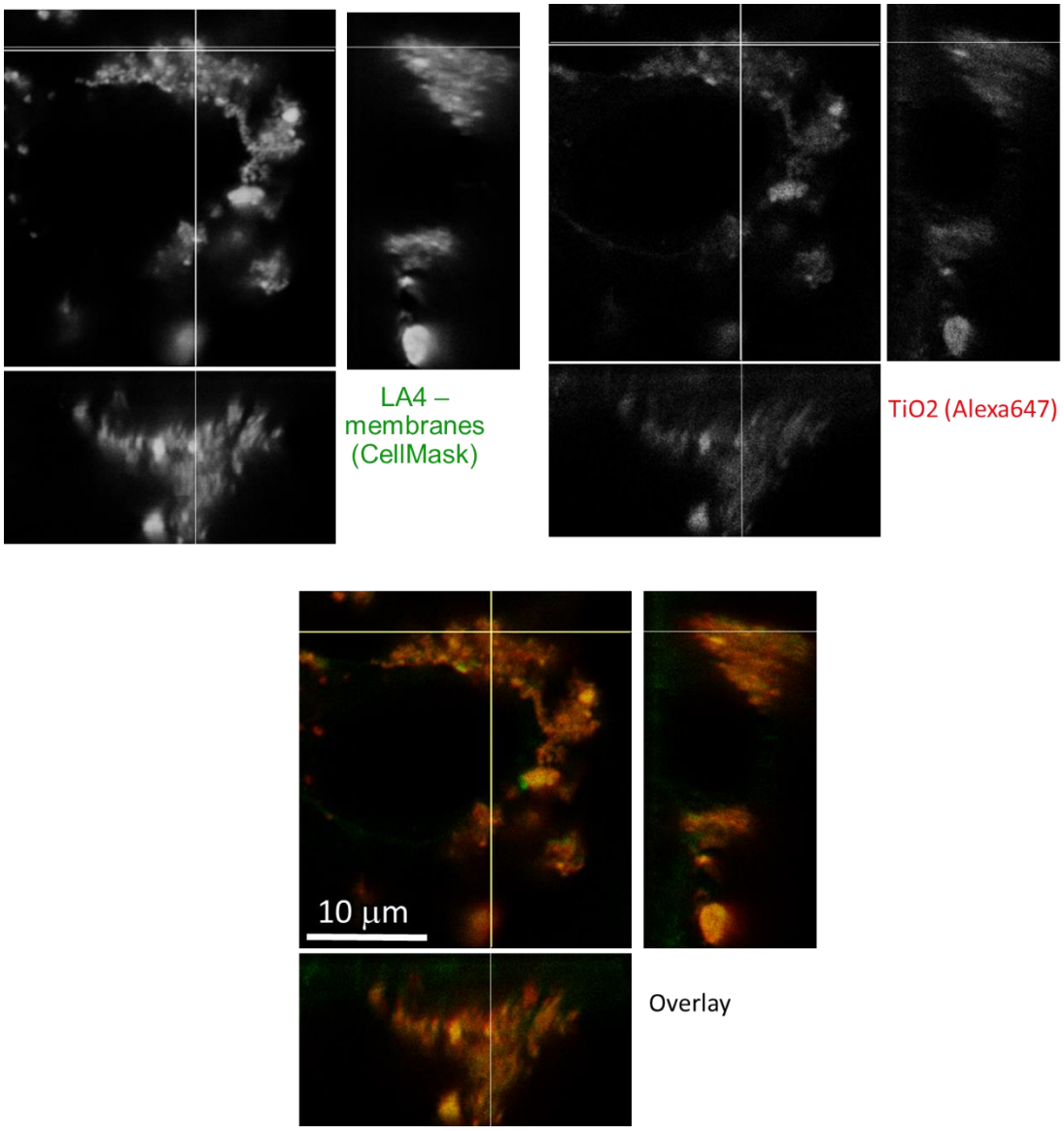


Cell line	LA-4 (membrane, SHE 2N)	pixelsize (x,y)	200 nm	561nm	30%
NPs	TiO2 (Alexa 647)	FOV (x,y)	80 x 80 um	640nm	30%
exposure	10:1, 24h-48h	pixelsize (z)	/	STED	0%
imaging	xz confocal, 48h CONTROLE	FOV (z)	/	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 ms
		number z-stacks	/	objective	wi60x (NA1.2)



Cell line	LA-4 (membrane, SHE 2N)	pixelsize (x,y)	50 nm	561nm	30%
NPs	TiO2 (Alexa 647)	FOV (x,y)	36 um	640nm	30%
exposure	10:1, 24h-48h	pixelsize (z)	50 nm	STED	0%
imaging	xz confocal, 48h CONTROLE	FOV (z)	13 um	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 ms
		number z-stacks		objective	wi60x (NA1.2)

Figure S127: Confocal images of LA-4 in green and TiO_2 nanotubes in red channel. Cells not treated with Resveratrol. We can see collocalization and cauliflower-like formations on the topp of the cell. XY on upper window and XZ on lower.

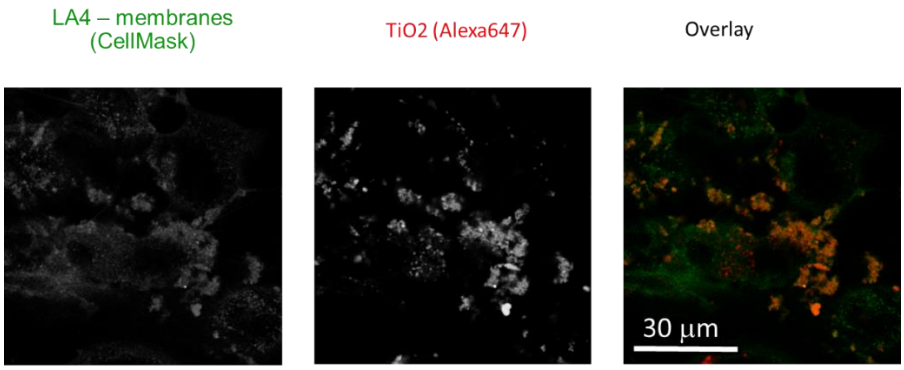


Cell line	LA-4 (membrane, CellMask)
NPs	TiO2 (Alexa 647) CONTROLE
exposure	10:1, 24h-48h
imaging	xyz STED, 48h CONTROLE

pixelsize (x,y)	100 nm
FOV (x,y)	28 x 31 um
pixelsize (z)	100 nm
FOV (z)	15 um
imaging time	10 min
number z-stacks	150

561nm	50%
640nm	50%
STED	10%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 ms
objective	wi60x (NA1.2)

Figure S128: Confocal images of LA-4 in green and TiO_2 nanotubes in red channel. Image is just one z-slice from a whole z-stack. Cells not treated with Resveratrol. We can see collocalization and cauliflower-like formations on the topp of the cell. XY on upper window and XZ on lower.

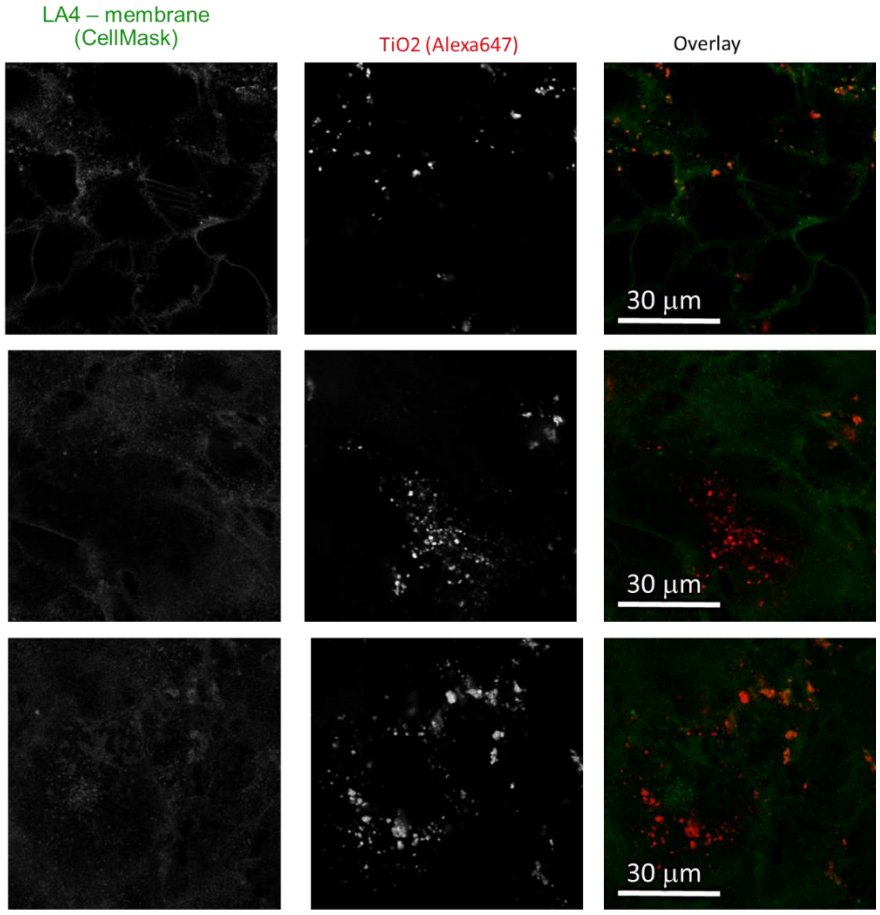


Cell line	LA-4 (membrane, CellMask)
NPs	TiO2 (Alexa 647)
exposure	10:1, 24h-48h CONTROLE
imaging	xy confocal, 48h CONTROLE

pixelsize (x,y)	200 nm
FOV (x,y)	80x 80 um
pixelsize (z)	/
FOV (z)	/
imaging time	/
number z-stacks	/

561nm	30%
640nm	30%
STED	0%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 ms
objective	wi60x (NA1.2)

Figure S129: Confocal images of LA-4 in green and TiO_2 nanotubes in red channel. Cells not treated with Resveratrol. We can see collocalization and cauliflower-like formations on the topp of the cell. XY on upper window and XZ on lower.

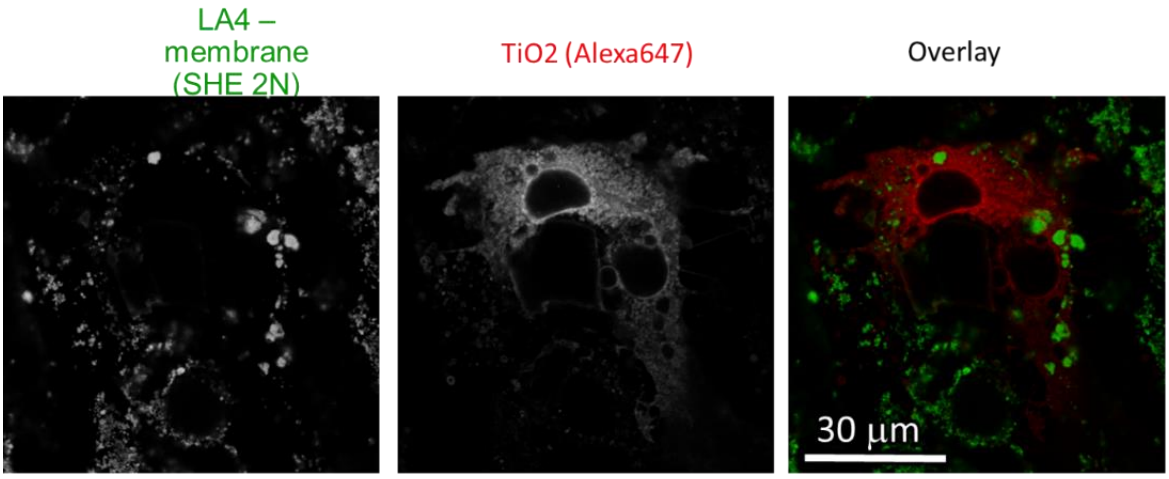


Cell line	LA-4 (membrane, CellMask)
NPs	TiO2 (Alexa 647)
exposure	10:1, 24h-48h
imaging	xy confocal, 48h Resveratrol 100 uM

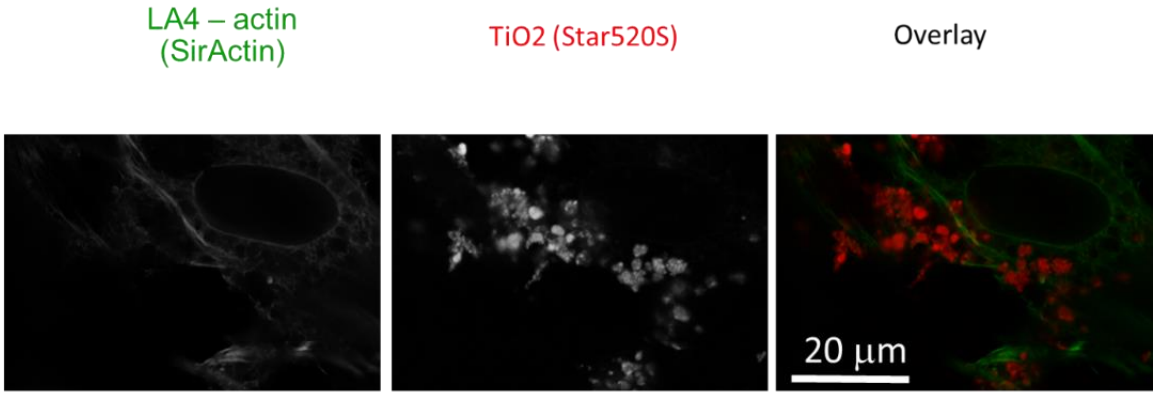
pixelsize (x,y)	200 nm
FOV (x,y)	80x 80 um
pixelsize (z)	/
FOV (z)	/
imaging time	/
number z-stacks	/

561nm	30%
640nm	30%
STED	0%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 ms
objective	wi60x (NA1.2)

Figure S130: Confocal images of LA-4 in green and TiO_2 nanotubes in red channel. Cells treated with 100 µM Resveratrol for 48 hours. No collocalization and no cauliflower-like formations. XY shots of three different FoVs.



Cell line	LA-4 (membrane, CellMask)	pixelsize (x,y)	200 nm	561nm	30%
NPs	TiO2 (Alexa 647)	FOV (x,y)	80x 80 um	640nm	30%
exposure	10:1, 24h-48h	pixelsize (z)	/	STED	0%
imaging	xy confocal, 48h Resveratrol 100 uM	FOV (z)	/	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 ms
		number z-stacks	/	objective	wi60x (NA1.2)



Cell line	LA-4 (actin, SirActin)	pixelsize (x,y)	60 nm	561nm	50%
NPs	TiO2 (Star 520S)	FOV (x,y)	65 x 45 um	640nm	50%
exposure	10:1, 24h-48h	pixelsize (z)	/	STED	20%
imaging	xy confocal, 48h Resveratrol 100 uM	FOV (z)	/	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 ms
		number z-stacks	/	objective	wi60x (NA1.2)

Figure S131: Confocal images of LA-4 in green and TiO_2 nanotubes in red channel. Cells treated with 100 µM Resveratrol for 48 hours. No collocalization and no cauliflower-like formations. In upper window we can see LA-4 cells stained with plasma membrane dye Cell Mask Orange. In the lower window LA-4 cells have been stained with Sir Actin dye which stains F-actin.

Link to 3D

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190619_Treated_with_100_mM_Resveratrol_overnight_overview_NM_in_the_cell_XYZ.mp4

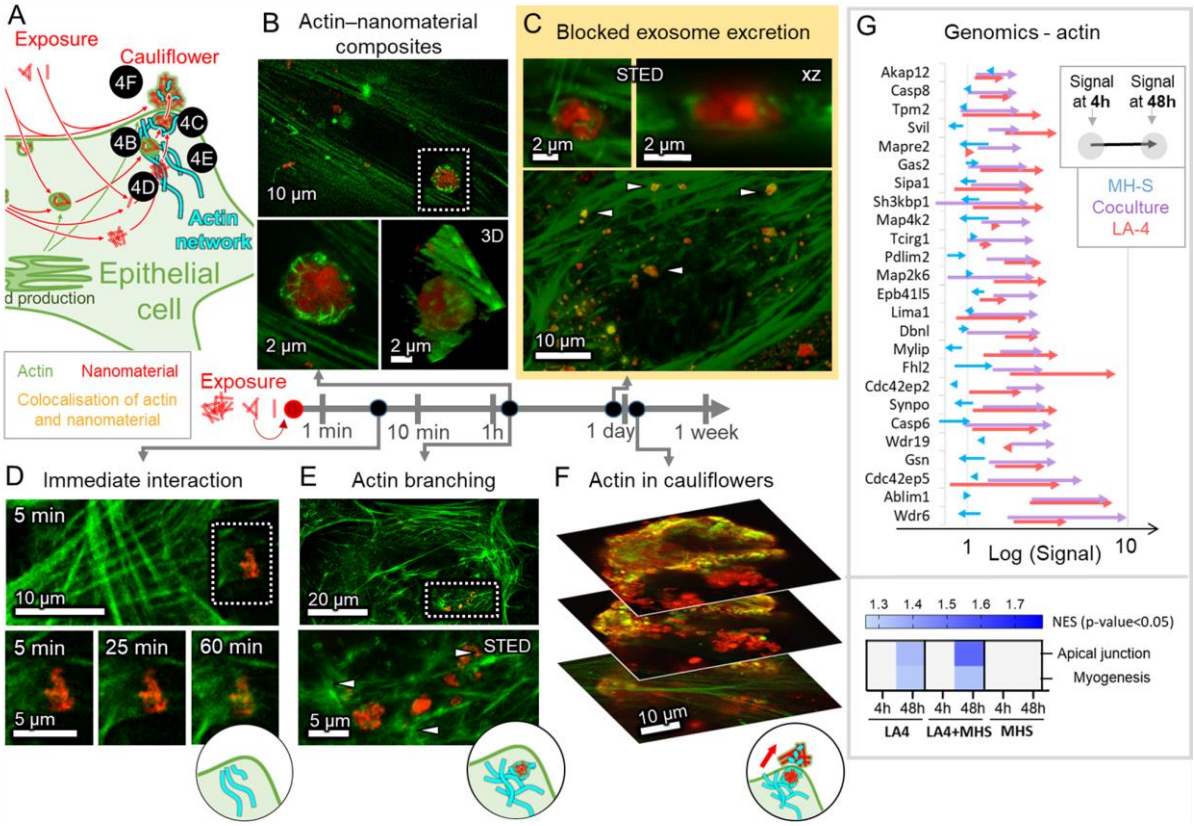
Movie S13: Cells treated with 100 µM Resveratrol, show reduced cells cauliflower formation and more nanomaterial in the cytosolic side of the cell.

Link to time-lapse

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190619_e06_m01_s01_LA-4_CellMask__NEG_CNOTROLE__for_100mM_Resveratrol_and_10_-_1_S520_TiO2_3D.mp4

Movie S14: Cells not treated with Resveratrol grow huge cauliflowers on the surface of membranes outside of the cell

S4 – The role of actin



S4b – Actin – nanomaterial interaction

Main message

Fluorescent micrographs of cytoskeleton after few hours of incubation with the TiO_2 nanotubes.

Supporting raw and analysed data:

Figure S132-Figure S134

Materials and methods

- Main experiment:
  - LA-4 cells were seeded @70% confluence in an Ibidi #1.5H µ-Dish and incubated at 37 °C and 5% CO2
  - after 24 hours fresh 400 µL medium with 1 µM of SirActin (Spirochrome Probes for Bioimaging) was added and incubated for additional 2 hours
  - After incubation cells were washed 3x400 µL LCIS and exposed to 35 µL freshly filtered 1 mg/mL TiO_2-40-ATTO 594 in 100x dcb (in 400 µL LCIS), mixed to achieve 10:1 surface dose
  - Cells were observed in 400 µL LCIS with label on the microscope stage heated at 37 °C for 2h

- Supplement experiments:
  - 20190122_e01_m05_LA-4_SA_actin interaction with TiO2_ATTO 594 has the same sample preparation procedure
  - 20190711_e01 m16 s10_LA-4_1 um SA & TiO2_S520_1 - 10_24h incubation _3D_faberges sample was seeded @40% confluency in an Ibidi #1.5H µ-Dish and incubated at 37 °C and 5% CO2
  - After 24 hours after 24 hours fresh 400 µL medium with 1 µM of Sir Actin was added and exposed to 35 µL freshly filtered 1 mg/mL TiO_2-17-Star 520S and incubated for further 24h
  - After incubation cells were washed 3x400 µL LCIS and observed in 400 µL LCIS on the microscope stage heated at 37 °C for 2h

- Analysis (Image J):
  - 3D STED: in red channel signal was multiplied to gain maximal visibility and noise was reduced by Gaussian Blur (1 pix); in green channel only Gaussian Blur (1 pix) was applied
  - Confocal: red channel has logarithmic scale, and Gaussian filter (1 pix) was used
  - For STED image in red channel Gaussian Blur was applied; in green to better visualize fine actin strands we sharpened the image ($\sigma = 2$, $c = 0{,}4$) using following:

Experiment names

- Main experiment name:
  - 20190122_e02_m05_Aleksandar_LA-4_SA and TiO2_ATTO 594_ Faberge_exp09_01
  - 20190122_e02_m05_Aleksandar_LA-4_SA and TiO2_ATTO 594_ Faberge_exp09_02

- 20190122_e02_m05_Aleksandar_LA-4_SA and TiO2_ATTO 594_ Faberge_exp09_03

- Supplement Experiment names:
  - 20190122_e01_m05_LA-4_SA and TiO2_ATTO594_interaction_ after 120_min.msr
  - 20190122_e01_m06_LA-4_SA and TiO2_ATTO594_interaction_ after 120_min.msr
  - 20190711_e01 m16 s10_LA-4_1 um SA and TiO2_S520_1 - 10_24h incubation _3D_faberges

Controls and statistics

Four vertical (z) sections of a 3D stack of images showing one large TiO_2 nanotubes -actin structure:

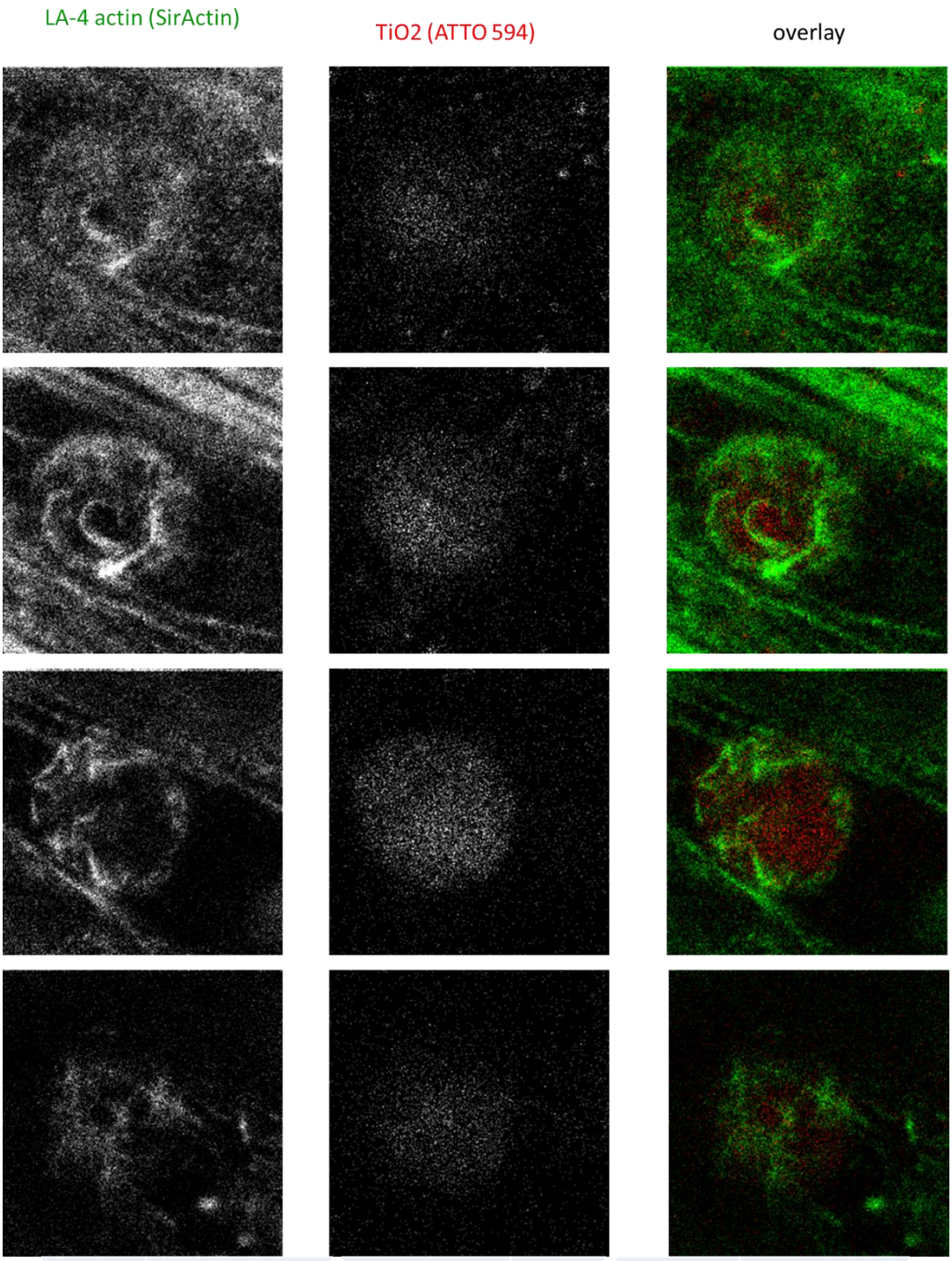
Figure S132

STED zoom in of the 3D stack from previous figure and large field of view of the same structure:

Figure S133

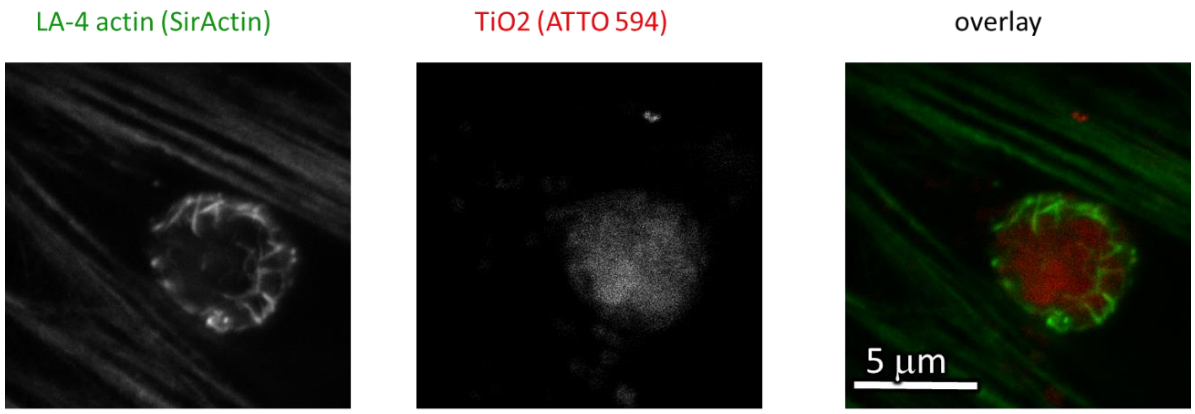
STED time lapse of an actin engulfing a TiO_2 agglomerate:

Figure S134

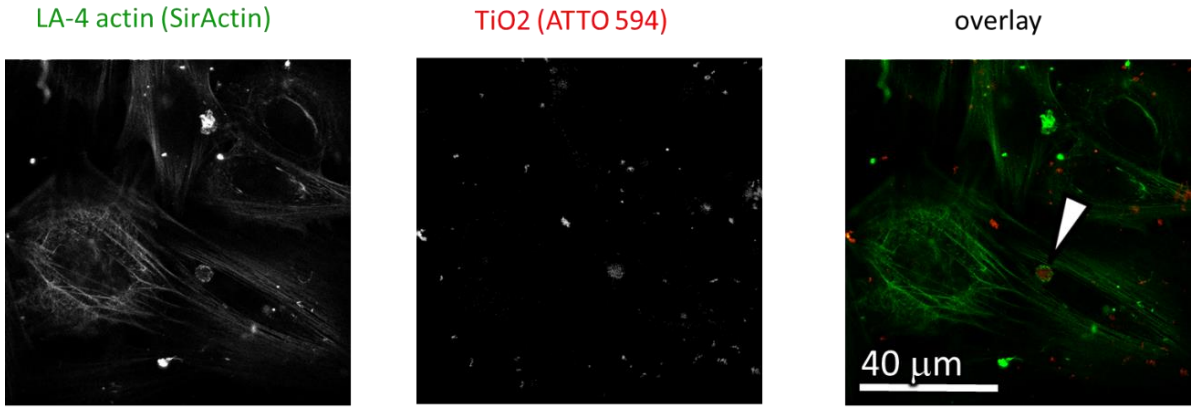


Cell line	LA-4 (actin, SirActin)	pixelsize (x,y)	40 nm	561nm	26%
NPs	TiO2 (ATTO 594)	FOV (x,y)	9 µm	640nm	15%
exposure	10:1, 26h-28h	pixelsize (z)	60 nm	STED	10%
imaging	XYZ STED, 26h	FOV (z)	4 µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 µs
		number of frames	68	objective	wi60x (NA1.2)

Figure S132: Confocal images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. STED images of XY planes of different slices of a whole Z-stack.

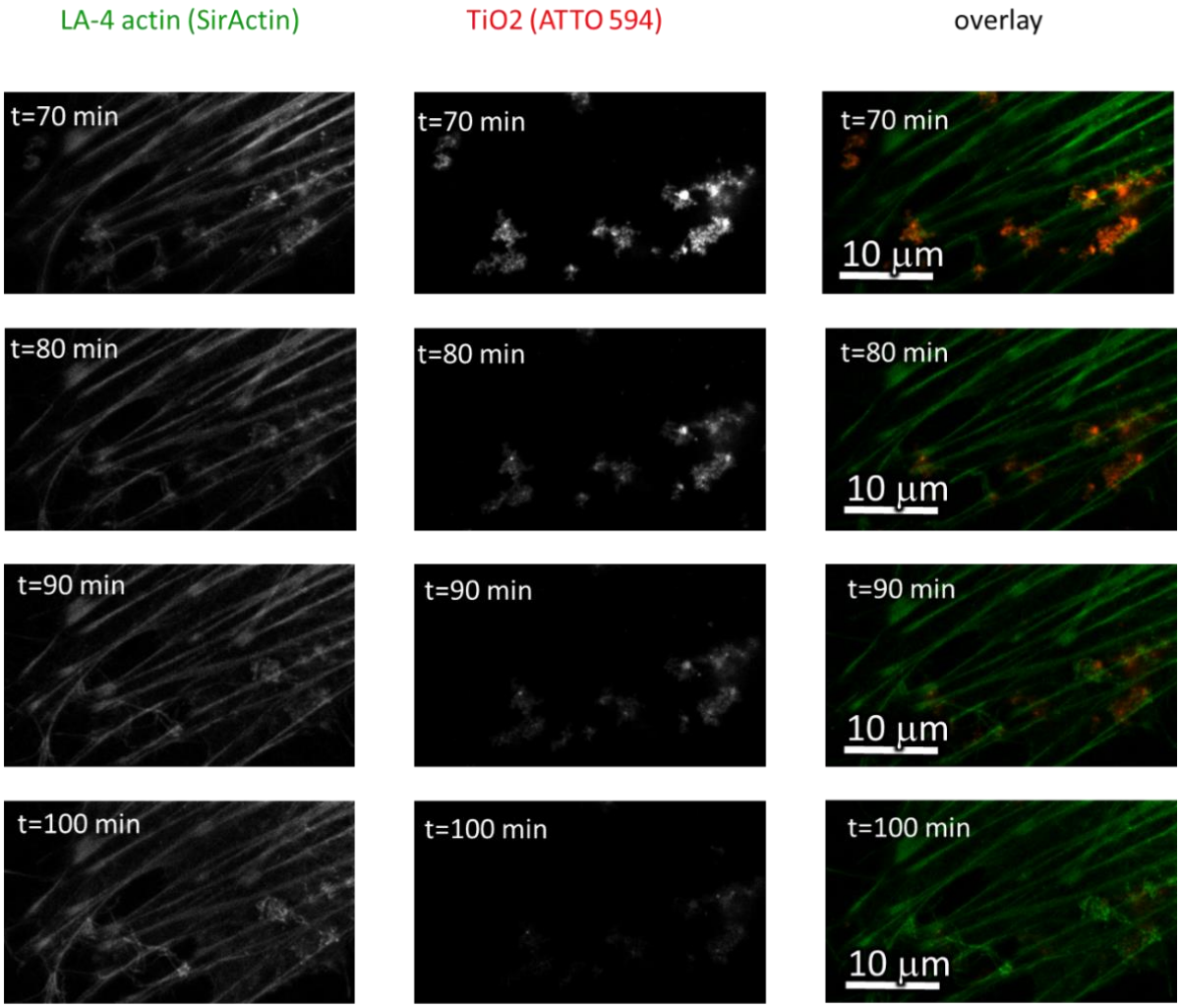


Cell line	LA-4 (actin, SirActin)	pixelsize (x,y) (STED)	15nm	561nm	30%
NPs	TiO2 (ATTO 594)	FOV (x,y)	14 µm	640nm	15%
exposure	10:1, 26h-28h	pixelsize (z)	/ nm	STED	10%
imaging	xy STED, 26h xy confocal, 26h	FOV (z)	/ µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 µs
		number of frames	/	objective	wi60x (NA 1.2)



Cell line	LA-4 (actin, SirActin)	pixelsize (x,y) (confocal)	200 nm	561nm	5%
NPs	TiO2 (Alexa647)	FOV (x,y)	100 µm	640nm	10%
exposure	10:1, 26h-28h	pixelsize (z)	/ nm	STED	/
imaging	xy STED, 26h xy confocal, 26h	FOV (z)	/ µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 µs
		number of frames	/	objective	wi60x (NA 1.2)

Figure S133: STED and confocal images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. STED zoom – in (XY plane) the upper window shows the same actin-NTs agregat from the previous figure. In lower window is the confocal image (XY plane) where the specific location of the actin-NTs agregate in the cell is clearly visible.



Cell line	LA-4 (actin, SirActin)	pixelsize (x,y)	70 nm	561nm	40%
NPs	TiO2 (ATTO 594)	FOV (x,y)	37 µm	640nm	25%
exposure	10:1, 26h-27h	pixelsize (z)	/	STED	15%
imaging	xyt STED, 27h-27h	FOV (z)	/	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	1 hour	dwell time	10 µs
		number of frames	611	objective	wi60x (NA1.2)

Figure S134: STED time lapse of LA-4 in green and TiO_2 nanotubes in red channel. Shown are time points from 70 – 100 min with 10 min increments. Actin fibers are completely engulfing TiO_2 nanotubes.

Link to 3D

- *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190122_e02_m05_Aleksandar_LA-4_Sir-Actin_Fabrege_exp09_03_.gif*

Movie S15: Interaction of actin fibres with TiO_2 agglomerates in the cell after few hours - Faberge egg

- *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190122_e02_m05_Aleksandar_LA-4_Sir-Actin_Fabrege_exp09_03_.mp4*

Movie S16: Interaction of actin fibres with TiO_2 agglomerates in the cell after few hours - Faberge egg

- *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190122_e02_m05_Aleksandar_LA-4_Sir-Actin_Fabrege_exp09_03_1.mp4*

Movie S17: Interaction of actin fibres with TiO_2 agglomerates in the cell after few hours - Faberge egg

- *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190711_e01_m16_s10_LA-4_1_uM_SA_&_TiO2_S520_1_-_10_24h_incubation__3D_faberges.gif*

Movie S18: Interaction of actin with uptaken nanomaterial after 24h - Faberge egg pattern

S4c – Inhibition of exosome excretion by high concentrations of Jaspakinolide

Main message

Upon stabilisation of actin fibres, cell is incapable to compress formed actin rings around to-be-exported cargo, which we see as red globes of nanomaterial, unable to be extruded, wrapped by green actin filaments close to the surface of the cell.

Supporting raw and analysed data:

Figure S135-Figure S140

Materials and methods

- Main experiment:
  - LA-4 cells were seeded @30% confluence in an Ibidi #1.5H µ-Dish
  - A) 24 hours later, the cells were incubated with 100 nM SirActin and Jaspakinolide overnight at the incubator at 37 °C and 5% CO2. On the 3rd day the cells were washed with 1x400 µL LCIS and observed in 400 µL LCIS at microscope stage heated on 37 °C
  - B) 24 hours later, the cells were incubated with 1 µM SirActin and Jaspakinolide overnight at the incubator at 37 °C and 5% CO2. On the 3rd day the cells were washed with 1x400 µL LCIS and observed in 400 µL LCIS at microscope stage heated on 37 °C
  - C) After 24 hours 35 mL freshly filtered 1 mg/mL TiO_2-17-Alexa647 in 100x dcb was added directly to the cells (in 400 µL medium with 1 um SirActin) and mixed to achieve 10:1 surface dose
  - With A and B setup we allowed the cauliflowers to grow and observe what role actin has with already formed cauliflowers as opposed with C setup, where stabilisation of actin would weaken cauliflower forming potential

- Main experiment (Fig 4c) has been performed as described in A, in supplement we can see same effect of C
  - Supplement experiments have been prepared in the same way

- Analysis:
  - Confocal images are just exported in raw format
  - 0,5 pixel Gaussian blur was applied on STED images

Experiment names

- Main experiment name:
  - 20190627_e08 m13 s03 LA-4_1um SA & 10 - 1 TiO2_S520 3DI_omega vesicles
  - 20190627_e08 m13 s12 LA-4_1um SA & 10 - 1 TiO2_S520 3DI_omega vesicles_xy STED

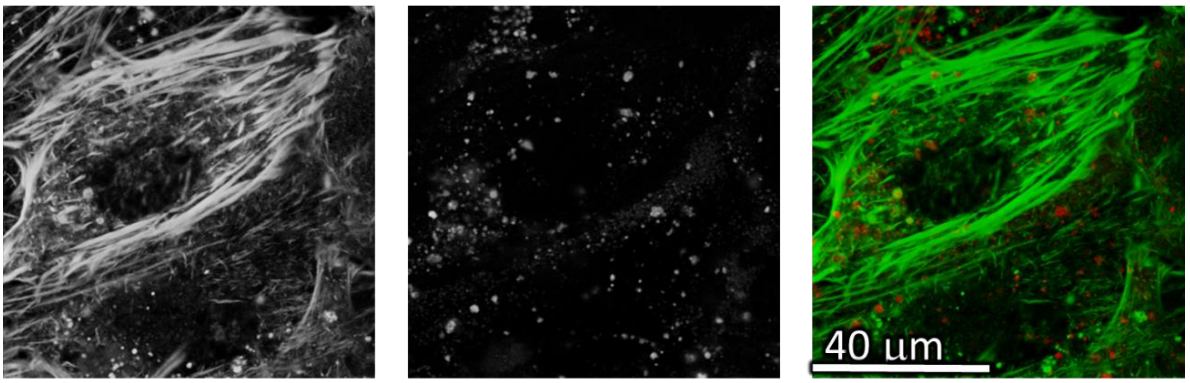
- Supplement Experiment names:
  - 20190627_e01 m04 s03 LA-4_1um SA & 10 - 1 TiO2_S520 3DI_confocal
  - 20190627_e01 m01 s01 LA-4_1um SA & 10 - 1 TiO2_S520 3DI_confocal

- 20190627_e01 m03 s02 LA-4_1um SA & 10 - 1 TiO2_S520 3DI_confocal
- 20190627_e01 m06 s05 LA-4_1um SA & 10 - 1 TiO2_S520 3DI_confocal
- 20190711_e01m09s05_LA-4 1um SA and 10 to 1 TiO2 Star520S_3D
- 20190711_e01 m16 s10_LA-4_ 1um SA and 10 to 1 TiO2_Star520s_Omega vesicles_3D

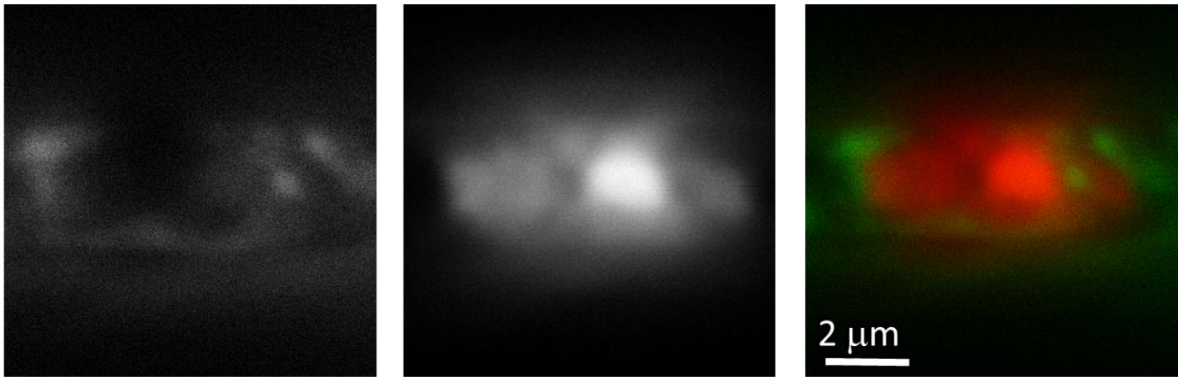
Controls and statistics

Confocal and STED images of actin rings after Jaspankinolide treatment:
Figure S135-Figure S139

LA-4 actin (SirActin) TiO2 (Star 520S) overlay



Cell line	LA-4 (actin, SirActin)	pixelsize (x,y)	200 nm	561nm	30%
NPs	TiO2 (Star 520S)	FOV (x,y)	80 µm	640nm	50%
exposure	10:1, 24h-60h C	pixelsize (z)	/ nm	STED	20%
imaging	xy confocal, 60h	FOV (z)	/ µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 µs
		number of frames	/	objective	60wi0x (NA1.2)



Cell line	LA-4 (actin, SirActin)	pixelsize (x,y)	10 nm	561nm	30 %
NPs	TiO2 (Star 520S)	FOV (x,y)	8,5 µm	640nm	35 %
exposure	10:1, 24h-60h C	pixelsize (z)	18 nm	STED	15 %
imaging	xz STED, 60h	FOV (z)	20 µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 µs
		number of frames	/	objective	60wi0x (NA1.2)

Figure S135: Confocal image and STED images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. Confocal image of large FoV in upper window and STED zoom – in (XZ plane) in the lower window showing representative actine rings, unable to compress and excrete nanomaterial outside the cell due to Jaspankinolide treatmant.

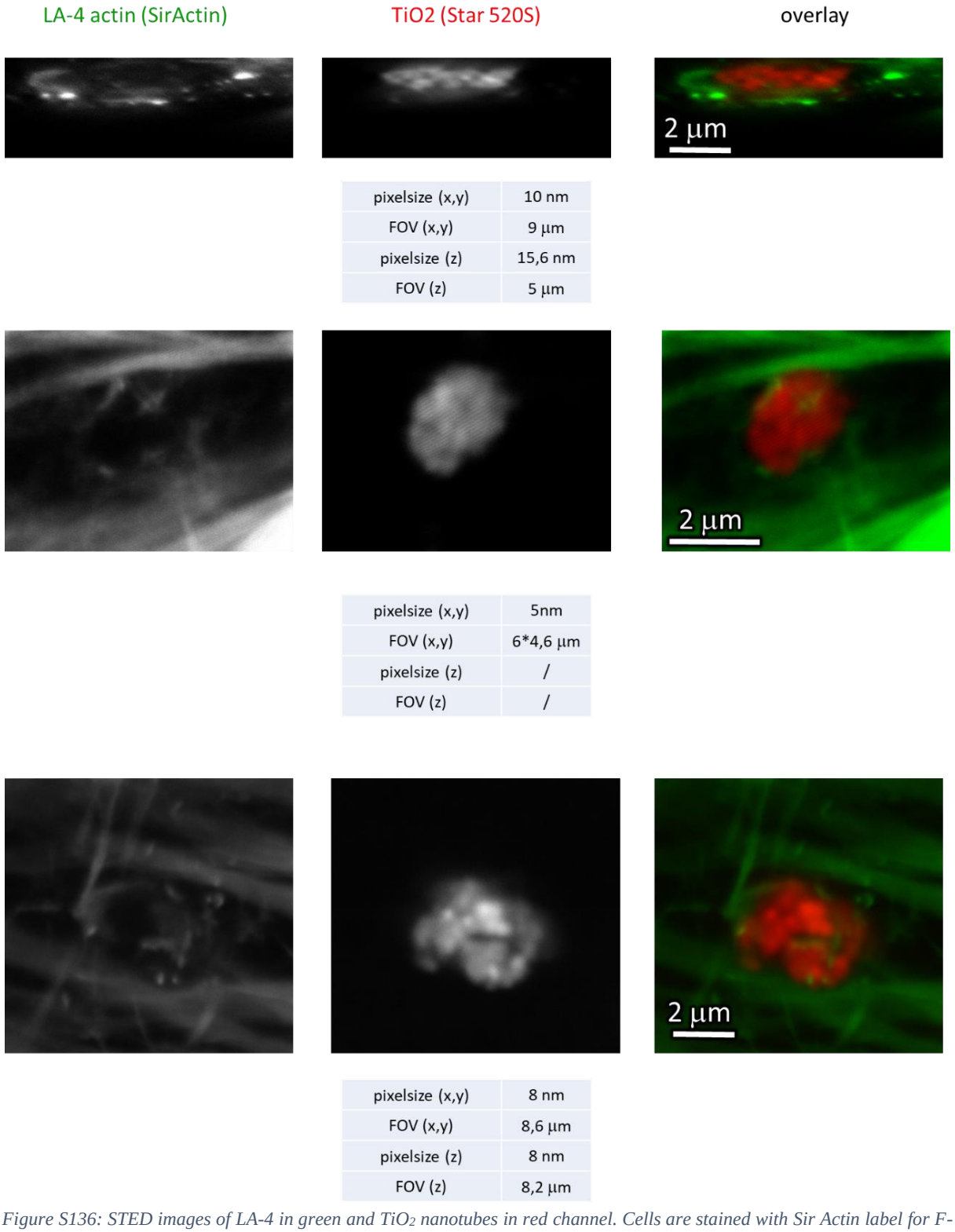
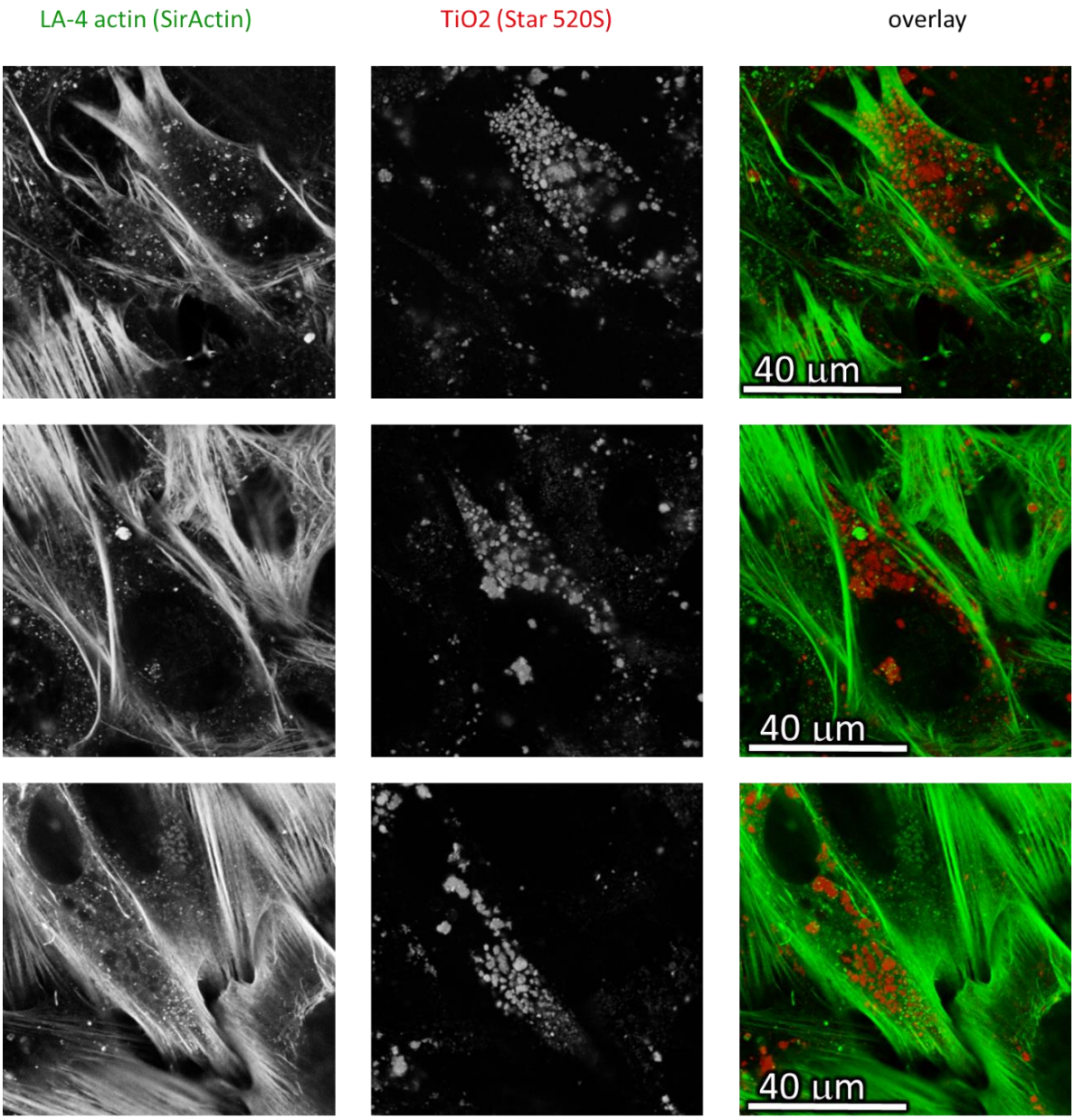


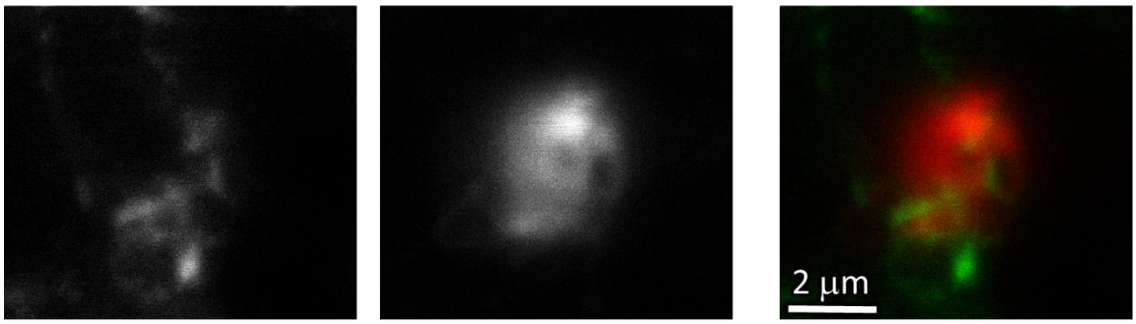
Figure S136: STED images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. STED zoom – ins (XZ plane in upper window and two XY planes in middle and lower window) showing representative actine rings, unable to compress and excrete nanomaterial outside the cell due to Jaspankinolide treatmant. All parameters which are left out from boxes are the same as for the above images.



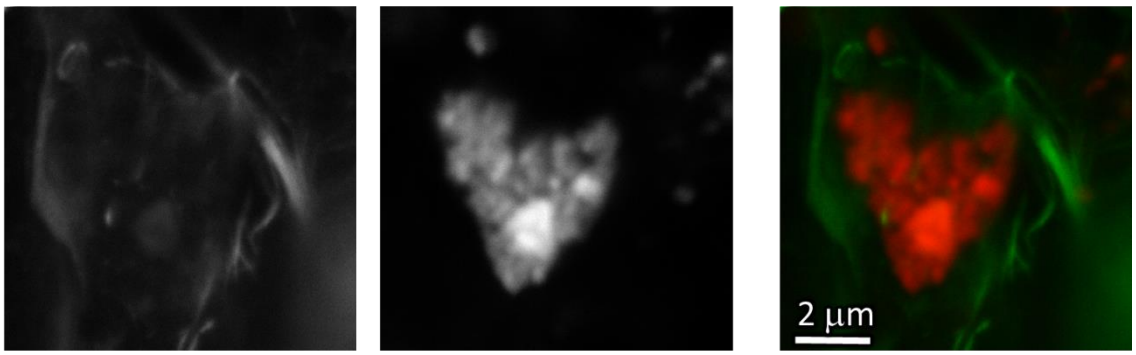
Cell line	LA-4 (actin, SirActin)	pixelsize (x,y)	200 nm	561nm	30%
NPs	TiO2 (Star 520S)	FOV (x,y)	80 µm	640nm	50%
exposure	10:1, 24h-60h	pixelsize (z)	/ nm	STED	/
imaging	xy confocal, 60h	FOV (z)	/ µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 µs
		number of frames	/	objective	60wi0x (NA1.2)

Figure S137: Confocal images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. Confocal image of large FoVs in all window showing representative actine rings, unable to compress and excrete nanomaterial outside the cell due to Jaspankinolide treatmant.

LA-4 actin (SirActin) TiO2 (Star 520S) overlay



Cell line	LA-4 (actin, SirActin)	pixelsize (x,y)	10 nm	561nm	50%
NPs	TiO2 (Star 520S)	FOV (x,y)	7,4 x 6,6 µm	640nm	65%
exposure	10:1, 24h-60h	pixelsize (z)	/ nm	STED	30%
imaging	xy STED, 60h	FOV (z)	/ µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 µs
		number of frames	/	objective	60wi0x (NA1.2)

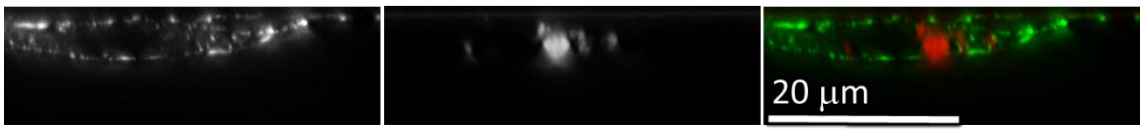


Cell line	LA-4 (actin, SirActin)	pixelsize (x,y)	10 nm	561nm	30%
NPs	TiO2 (Star 520S)	FOV (x,y)	9 x 9 µm	640nm	50%
exposure	10:1, 24h-60h A	pixelsize (z)	/ nm	STED	15%
imaging	xy STED, 60h	FOV (z)	/ µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 µs
		number of frames	/	objective	60wi0x (NA1.2)

Figure S138: STED images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. STED zoom – ins (XY planes in both windows) showing representative actine rings, unable to compress and excrete nanomaterial outside the cell due to Jaspankinolide treatmant.

Cell line	LA-4 (actin, SirActin)	pixelsize (x,y)	40 nm	561nm	30%
NPs	TiO2 (Star 520S)	FOV (x,y)	50 µm	640nm	50%
exposure	10:1, 24h-60h	pixelsize (z)	40 nm	STED	15%
imaging	xz STED, 60h	FOV (z)	15 µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 µs
		number of frames	/	objective	60wi0x (NA1.2)

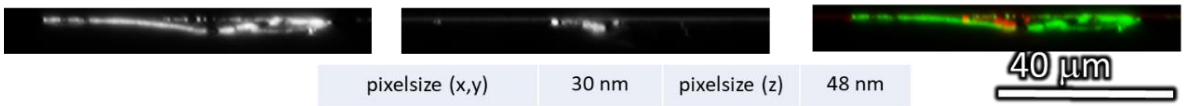
Cell line	LA-4 (actin, SirActin)	pixelsize (x,y)	30 nm	561nm	30%
NPs	TiO2 (Star 520S)	FOV (x,y)	70 µm	640nm	50%
exposure	10:1, 24h-60h	pixelsize (z)	50nm	STED	15%
imaging	yz STED, 60h	FOV (z)	30 µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 µs
		number of frames	/	objective	60wi0x (NA1.2)



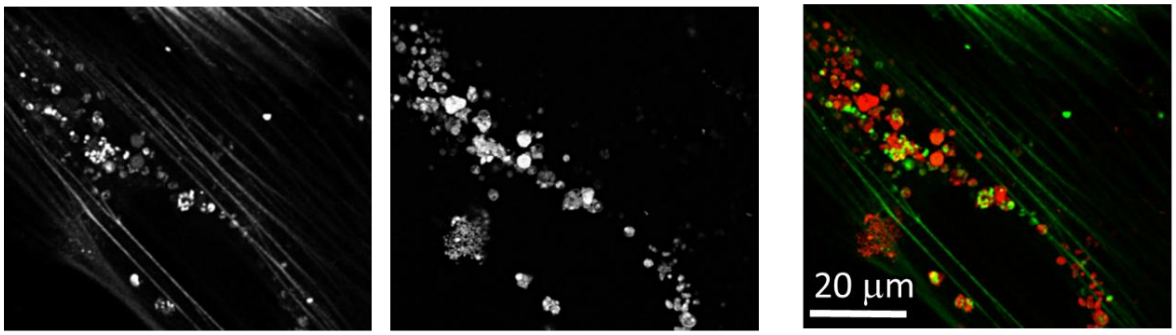
Cell line	LA-4 (actin, SirActin)	pixelsize (x,y)	40 nm	561nm	30%
NPs	TiO2 (Star 520S)	FOV (x,y)	40 µm	640nm	50%
exposure	10:1, 24h-60h	pixelsize (z)	40 nm	STED	15%
imaging	yz STED, 60h	FOV (z)	13 µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 µs
		number of frames	/	objective	60wi0x (NA1.2)

Figure S139: STED images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. STED images (XZ plane in upper window and two YZ planes in middle and lower window) showing representative actine rings, unable to compress and excrete nanomaterial outside the cell due to Jaspankinolide treatmant.

Cell line	LA-4 (actin, SirActin)	pixelsize (x,y)	150 nm	561nm	30%
NPs	TiO2 (Star 520S)	FOV (x,y)	80 µm	640nm	30%
exposure	10:1, 24h-48h A	pixelsize (z)	/ nm	STED	/%
imaging	XY confocal, 48h	FOV (z)	/ µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 µs
		number of frames	/	objective	60wi0x (NA1.2)



pixelsize (x,y)	30 nm	pixelsize (z)	48 nm
FOV (x,y)	60 µm	FOV (z)	12 µm



Cell line	LA-4 (actin, SirActin)	pixelsize (x,y)	210 nm	561nm	30%
NPs	TiO2 (Star 520S)	FOV (x,y)	70 x 62 µm	640nm	30%
exposure	10:1, 24h-48h A	pixelsize (z)	30 nm	STED	/%
imaging	XYZ confocal, 48h	FOV (z)	7 µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 µs
		number of frames	/	objective	60wi0x (NA1.2)

Figure S140: STED images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. Confocal image of large FoV in upper window, STED zoom – in (XZ plane) in the middle window and one sXY plane of a whole Z-stack (3D)in the lower window showing representative actine rings, unable to compress and excrete nanomaterial outside the cell due to Jaspankinolide treatmant

Link to 3D

- *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190711_e01_m16_s10_LA-4__1um_SA_and_10_to_1_TiO2_Star520s_Omega_vesicles_3D.mp4*

Movie S19: Representative actine rings, unable to compress and excrete nanomaterial outside the cell due to Jaspankinolide treatmant. Cells are stained with Sir Actin label for F-actin (green) and were exposed to 10:1 surface dose of TiO_2 nanotubes (Star 520 SXP, red).

S4d – Nanoparticle actin interaction

Main message

Uptake of a bigger nanomaterial agglomerate by the cell within one hour of exposure.

Supporting raw and analysed data:

Figure S141-Figure S144

Materials and methods

- Main experiment:
  - LA-4 cells were seeded @60% confluence in an Ibidi #1.5H µ-Dish
  - after 24 hours cells were incubated with 1 µm Sir Actin for 2 hours in the incubator at 37 °C, 5% CO2 afterwards they were washed with 3x400 mL LCIS
  - After 2h of incubation 35 µL freshly filtered 1 mg/mL TiO_2-40-ATTO 594 in 100x dcb was added directly to the cells (in 400 µL medium) and mixed to achieve 10:1 surface dose and observed in 400 µL LCIS at microscope stage heated on 37 °C

- Analysis:
  - threshold has been set to 1 count and brightness has been adjusted for maximal visibility
  - 0,5 pixel Gaussian blur and sharpening algorithm were applied as well

Experiment names

- Main experiment name:
  - 20190122_e01_m02_LA-4_SirActin and 10 to 1_ TiO2_ATTO 594_uptake of NM

- Supplement Experiment names:
  - 20190127_e01 m02_LA-4_SA and 10 to 1 TiO2_ATTO 594_uptake
  - 20190129_e01m01s01_Native LA-4_SA and 10 to 1 TiO2_ATTO 594_large FOV
  - 20190129_e01m01s04_Native LA-4_SA_static and parallel filaments
  - 20190129_e01m01s05_Native LA-4_SA_filaments_STED

Controls and statistics

Uptake of TiO_2 nanotubes immediately after exposure of the cells:

Figure S141

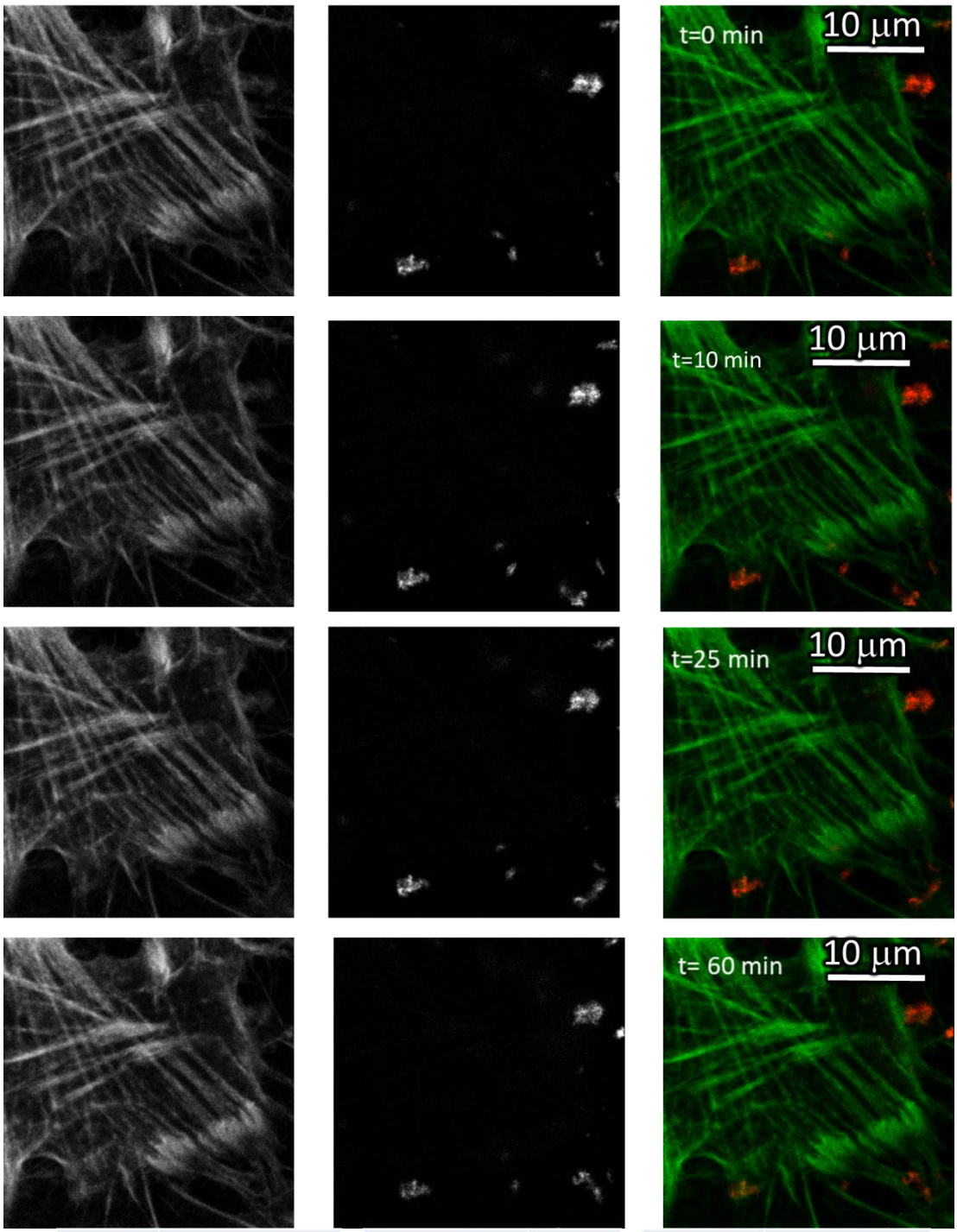
Control, unexposed cells labelled with Sir-Actin:

Figure S142-Figure S143

Uptake of TiO_2 nanotubes immediately after exposure of the cells:

Figure S144

LA4 – actin (SirActin) TiO2 (ATTO 594) Overlay



Cell line	LA-4 (actin, Sir Actin)
NPs	TiO2 (ATTO 594)
exposure	10:1, 0h-1h
imaging	xyt STED, 0h-1h

pixelsize (x,y)	100 nm
FOV (x,y)	30 mm
pixelsize (z)	/ nm
FOV (z)	/ mm
imaging time	20 min
number of frames	511

561nm	25%
640nm	25%
STED	14%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 ms
objective	wi60x (NA1.2)

Figure S141: STED images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. Images are four different time points with 10, 15 and 25 min increments, respectively. Actin actively engulfs TiO_2 nanotubes and pulls them towards cell interior.

Cell line	LA-4 (actin, Sir Actin)
NPs	TiO2 (ATTO 594)
exposure	CONTROLE
imaging	Xy confocal, 0h-1h

pixelsize (x,y)	200 nm
FOV (x,y)	100 mm
pixelsize (z)	/ nm
FOV (z)	/ mm
imaging time	/ min
number of frames	/

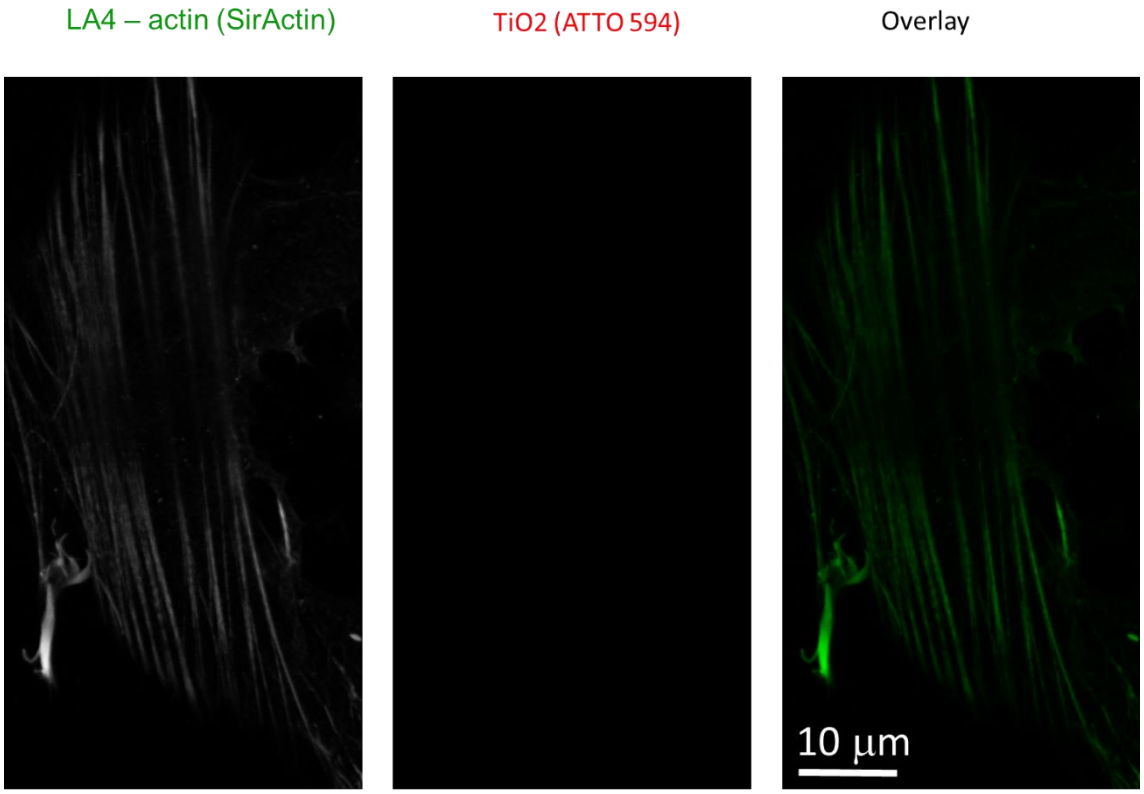
561nm	/%
640nm	13%
STED	/%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 ms
objective	wi60x (NA1.2)

Cell line	LA-4 (actin, Sir Actin)
NPs	TiO2 (ATTO 594)
exposure	CONTROLE
imaging	xy STED

pixelsize (x,y)	15 nm
FOV (x,y)	20 x 14 mm
pixelsize (z)	/ nm
FOV (z)	/ mm
imaging time	/ min
number of frames	/

561nm	/%
640nm	25%
STED	15%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 ms
objective	wi60x (NA1.2)

Figure S142: STED images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. Cells which have been grown under the same conditions as all experimental cells but haven't been exposed to TiO_2 nanotubes.

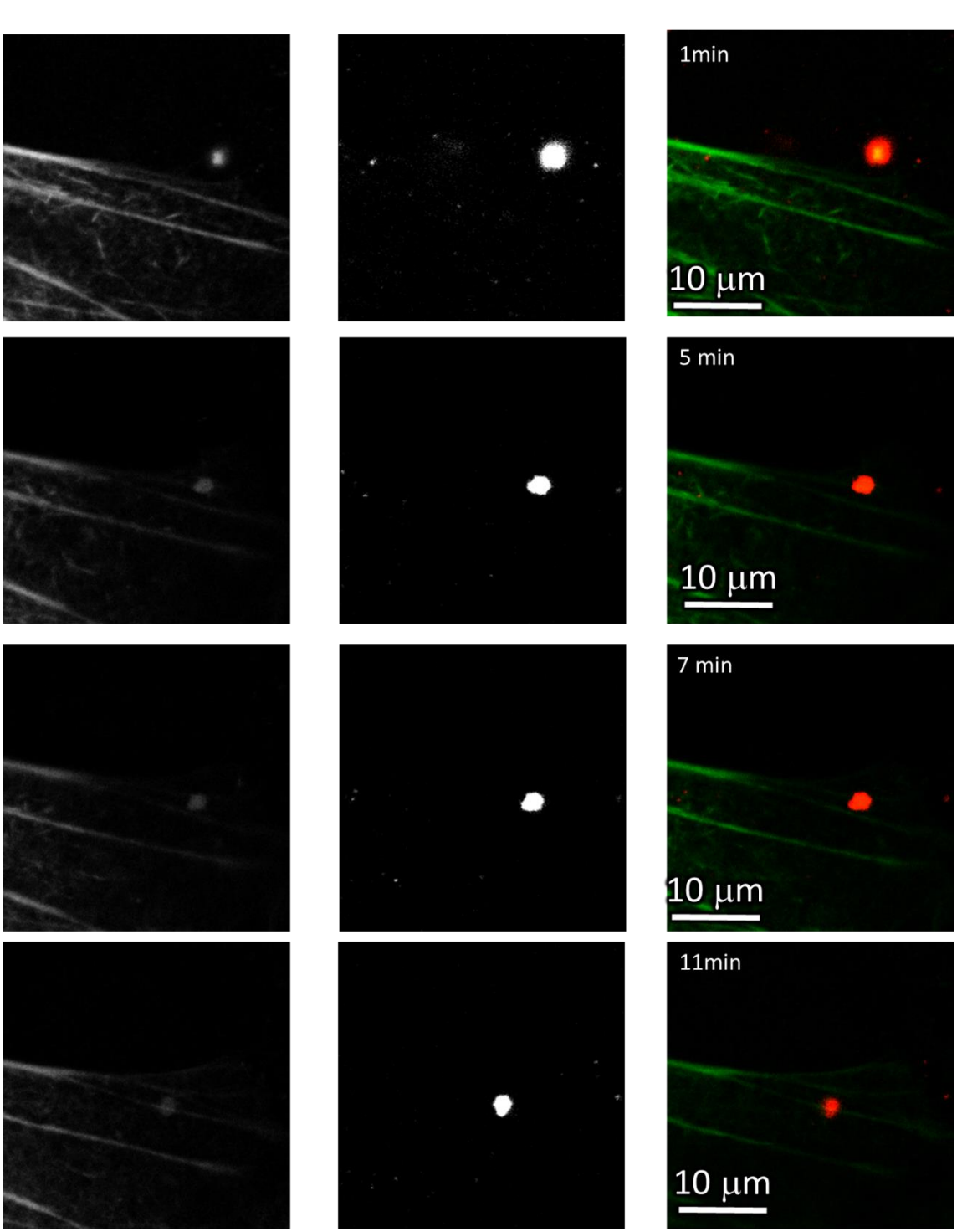


Cell line	LA-4 (actin, Sir Actin)
NPs	TiO2 (ATTO 594)
exposure	CONTROLE
imaging	xy STED

pixelsize (x,y)	30 nm
FOV (x,y)	36 x 72 mm
pixelsize (z)	/ nm
FOV (z)	/ mm
imaging time	/ min
number of frames	/

561nm	/%
640nm	15%
STED	9%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 ms
objective	wi60x (NA1.2)

Figure S143: STED images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. Cells which have been grown under the same conditions as all experimental cells but haven't been exposed to TiO_2 nanotubes.



Cell line	LA-4 (actin, Sir Actin)
NPs	TiO2 (ATTO 594)
exposure	10:1, 0h-1h
imaging	xyt STED, 0h-1h

pixelsize (x,y)	80 nm
FOV (x,y)	33 x 23 mm
pixelsize (z)	/ nm
FOV (z)	/ mm
imaging time	25 min
number of frames	221

561nm	20%
640nm	25%
STED	12%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 ms
objective	wi60x (NA1.2)

Figure S144: STED images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. Images are four different time points with 5, 2 and 4 min increments, respectively. Actin actively orients towards the agregate of TiO_2 nanotubes, which is then pulled into the cell interior.

Link to time-lapse

- Main experiment
  - *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/e01_m02_LA-4_SA_uptake_of_TiO2_ATTO_594_25us.gif*

Movie S20: Actin filaments (green) folding and uptaking large TiO_2 agglomerate (red)

- Supplementary experiment
  - *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190127_e01_m02_LA-4_SA_uptake_TiO2_ATTO_594_50us.gif*

Movie S21: Uptake of a single TiO_2 agglomerate (red). Observe dynamics of small actin filaments (green) branching from the main fibres.

S4e – Actin branching

Main message

Branching of actin filaments (usually parallel in cells which are not engaged in any activity) after incubation with TiO_2 for more than 24h.

Supporting raw and analysed data:

Figure S145-Figure S147

Materials and methods

- Main experiment:
  - LA-4 cells were seeded @60% confluence in an Ibidi #1.5H µ-Dish and incubated at 37 °C and 5% CO2 for 24 hours
  - After incubation 100 nM of SirActin and 35 µL freshly filtered 1 mg/mL TiO_2-40-ATTO 594 in 100x dcb was added directly to the cells (in 400 µL medium) and mixed to achieve 10:1 surface dose and returned into the incubator
  - Cells were observed 12 hours later in 400 µL medium with label at the microscope stage heated at 37 °C for 2h

- Supplement experiments
  - Samples were prepared the same way and images were processed the same way

- Analysis (Image J):
  - For STED images in both channels sharpening algorithm (σ = 2, c = 0,5) was used to gain For confocal images Gaussian Blur (0,5 pixel) was applied to reduce grains

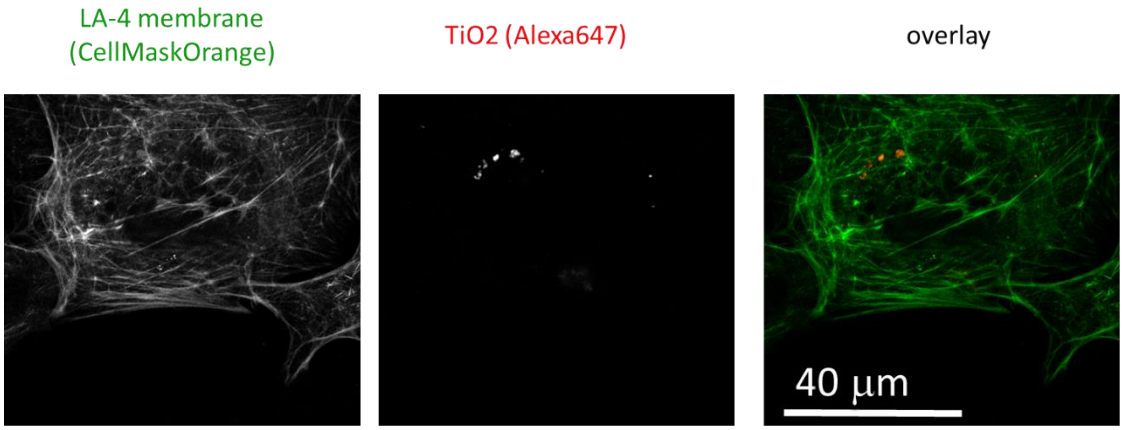
Experiment names

- Main experiment name:
  - 20190118_e01_m03_LA-4_0.1 um SA and 10 to 1TiO2_ATTO 594_overnight incubation_Actin branching_STED and confocal
  - Supplement Experiment names:
  - 20190118_e01_m01_LA-4_0.1 um SA and 10 to 1TiO2_ATTO 594_overnight incubation_Actin branching_STED and confocal
  - 20190118_e01_m07_LA-4_0.1 um SA and 10 to 1TiO2_ATTO 594_overnight incubation_Actin branching_STED and confocal

Controls and statistics

Branching of actin cytoskeleton after the exposure and incubation with TiO_2 nanotubes:

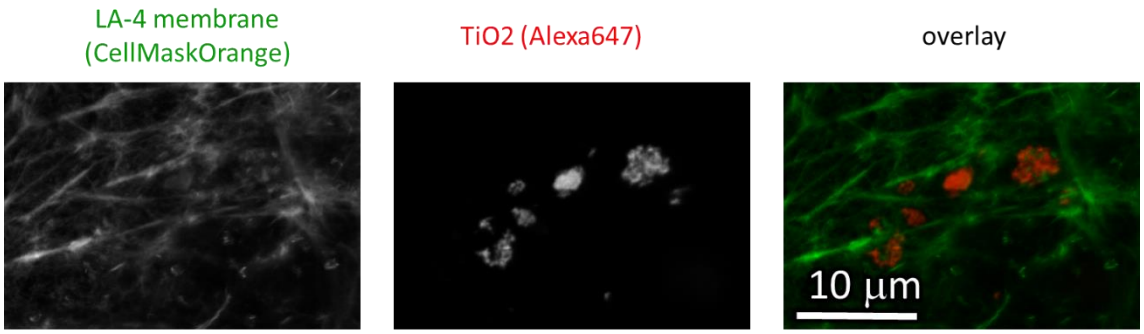
Figure S145-Figure S147



Cell line	LA-4 (actin, SirActin)
NPs	TiO2 (ATTO 594)
exposure	10:1, 24h-36h
imaging	xy confocal, 36h

pixelsize (x,y) (confocal)	200 nm
FOV (x,y)	80 µm
pixelsize (z)	/ nm
FOV (z)	/ µm
imaging time	/
number of frames	/

561nm	5%
640nm	10%
STED	/
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	wi60x (NA 1.2)



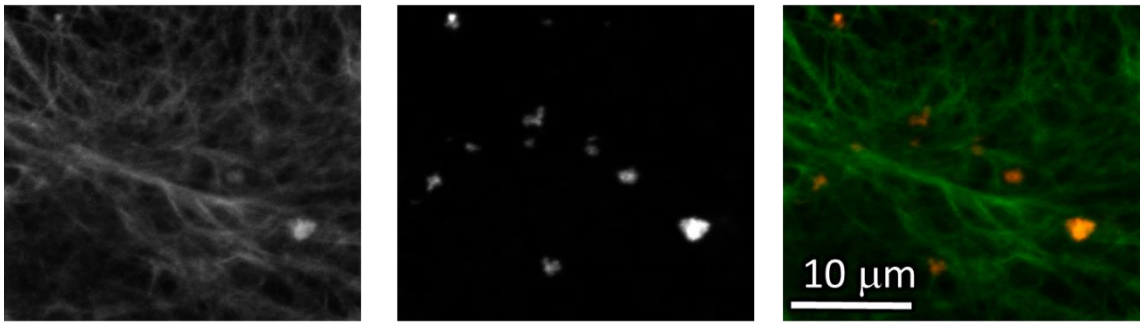
Cell line	LA-4 (actin, SirActin)
NPs	TiO2 (ATTO 594)
exposure	10:1, 24h-36h
imaging	xy STED, 36h

pixelsize (x,y) (confocal)	25 nm
FOV (x,y)	29 * 22 µm
pixelsize (z)	/ nm
FOV (z)	/ µm
imaging time	/
number of frames	/

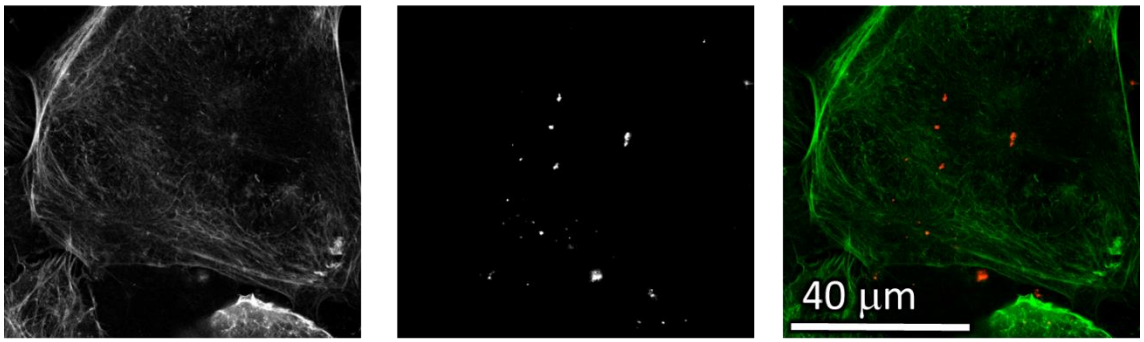
561nm	10%
640nm	15%
STED	15%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	wi60x (NA 1.2)

Figure S145: Confocal and STED images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. Confocal image of larger FoV in the upper window shows branching of actin filaments after overnight incubation with TiO_2 nanotubes. In the lower window is the STED zoom-in of the former confocal image, showing more intricately actine structure and interaction with TiO_2 agglomerates.

LA-4 membrane (CellMaskOrange) | TiO2 (Alexa647) | overlay



Cell line	LA-4 (actin, SirActin)	pixelsize (x,y) (confocal)	25 nm	561nm	10%
NPs	TiO2 (ATTO 594)	FOV (x,y)	29 * 22 µm	640nm	15%
exposure	10:1, 24h-36h	pixelsize (z)	/ nm	STED	15%
imaging	xy STED, 36h	FOV (z)	/ µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 µs
		number of frames	/	objective	wi60x (NA 1.2)



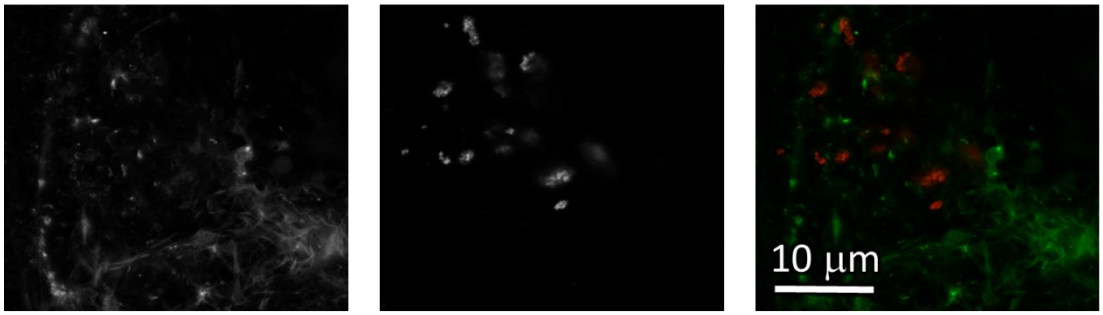
Cell line	LA-4 (actin, SirActin)	pixelsize (x,y) (confocal)	200 nm	561nm	5%
NPs	TiO2 (ATTO 594)	FOV (x,y)	80 µm	640nm	10%
exposure	10:1, 24h-36h	pixelsize (z)	/ nm	STED	/
imaging	xy confocal, 36h	FOV (z)	/ µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/	dwell time	10 µs
		number of frames	/	objective	wi60x (NA 1.2)

Figure S146: Confocal and STED images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. Confocal image of larger FoV in the lower window shows branching of actin filaments after overnight incubation with TiO_2 nanotubes. In the upper window is the STED zoom-in of the former confocal image, showing more intricately actine structure and interaction with TiO_2 agglomerates.

Cell line	LA-4 (actin, SirActin)
NPs	TiO2 (ATTO 594)
exposure	10:1, 24h-36h
imaging	xy STED, 36h

pixelsize (x,y) (confocal)	25 nm
FOV (x,y)	29 * 22 µm
pixelsize (z)	/ nm
FOV (z)	/ µm
imaging time	/
number of frames	/

561nm	10%
640nm	15%
STED	15%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	wi60x (NA 1.2)



Cell line	LA-4 (actin, SirActin)
NPs	TiO2 (ATTO 594)
exposure	10:1, 24h-36h
imaging	xy STED, 36h

pixelsize (x,y) (confocal)	25 nm
FOV (x,y)	35 * 31 µm
pixelsize (z)	/ nm
FOV (z)	/ µm
imaging time	/
number of frames	/

561nm	10%
640nm	15%
STED	15%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	wi60x (NA 1.2)

Figure S147: STED images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. In both windows are STED images showing branched actine filaments and interaction with TiO_2 bio-nano composites after the exposure and incubation with TiO_2 nanotubes. For non-branched, control, experiment see S4d – Nanoparticle actin interaction.

S4f – Actin in cauliflowers

Main message

After exocytosis of nanomaterial, some remnants of actin can be seen in cauliflower-like formations.

Supporting raw and analysed data:

Figure S148-Figure S151

Materials and methods

- Main experiment:
  - LA-4 cells were seeded @60% confluence in an Ibidi #1.5H µ-Dish in the cell medium
  - after 24 hours 35 mL of freshly filtered 1 mg/mL TiO_2-17-Alexa 647 in 100x diluted bicarbonate buffer was added directly to the cells (in 400 µL medium) and mixed to achieve 10:1 surface dose.
  - After 48 hours cells were incubated with 100 nM Sir Actin overnight in the incubator at 37 °C, 5% CO2. According to manufacturer of the label, it is not necessary to wash it if there is no background, so we imaged the cells without washing them. Cells were imaged on the microscope stage heated on 37 °C.

- Images analysed with ImageJ
  - Signal was multiplied by 1.8x
  - Sharpening algorithm ($\sigma = 3$, $c = 0{,}3$) was used

Experiment names

- Main experiment name:
  - 20190627_e05 m02 s02_1um SA & TiO2 S520_Remnants of actin in cauliflower_ STED and confocal

- Supplement Experiment names:
  - 20190619_e01 m01 s01 NEG CONTROLE for10 mM MBCD LA-4 Actin and 10 - 1 S520 TiO2_actin in cauliflowers_STED
  - 20190619_e01 m02 s01 NEG CONTROLE for10 mM MBCD LA-4 Actin and 10 - 1 S520 TiO2_actin in cauliflowers_confocal
  - 20190619_e07 m01 s01_LA-4 SA and 10 - 1 S520 TiO2_actin in cauliflowers_confocal and STED
  - 20190619_e04 m01 s01_100 µM Resveratrol LA-4_SA and 10 - 1 S520 TiO_actin in caulflowers_STED and confocal
  - 20190627_e05 m03 s03 LA-4 0.1um SA & 10 - 1 TiOs S520 overnightincubation 3D and STED

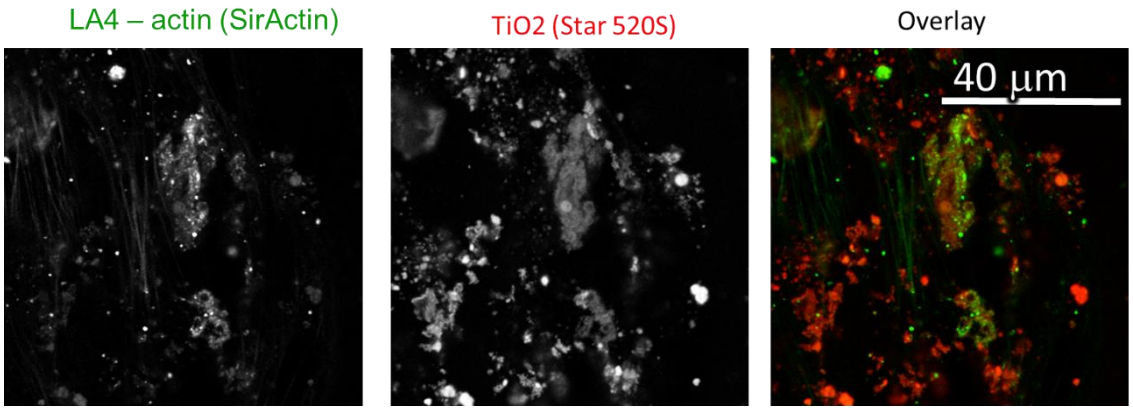
Controls and statistics

Actin filaments are exocytosed together with the nanomaterial:

Figure S148-Figure S150

Actin filaments are exocytosed together with the nanomaterial even in cells treated with Resveratrol

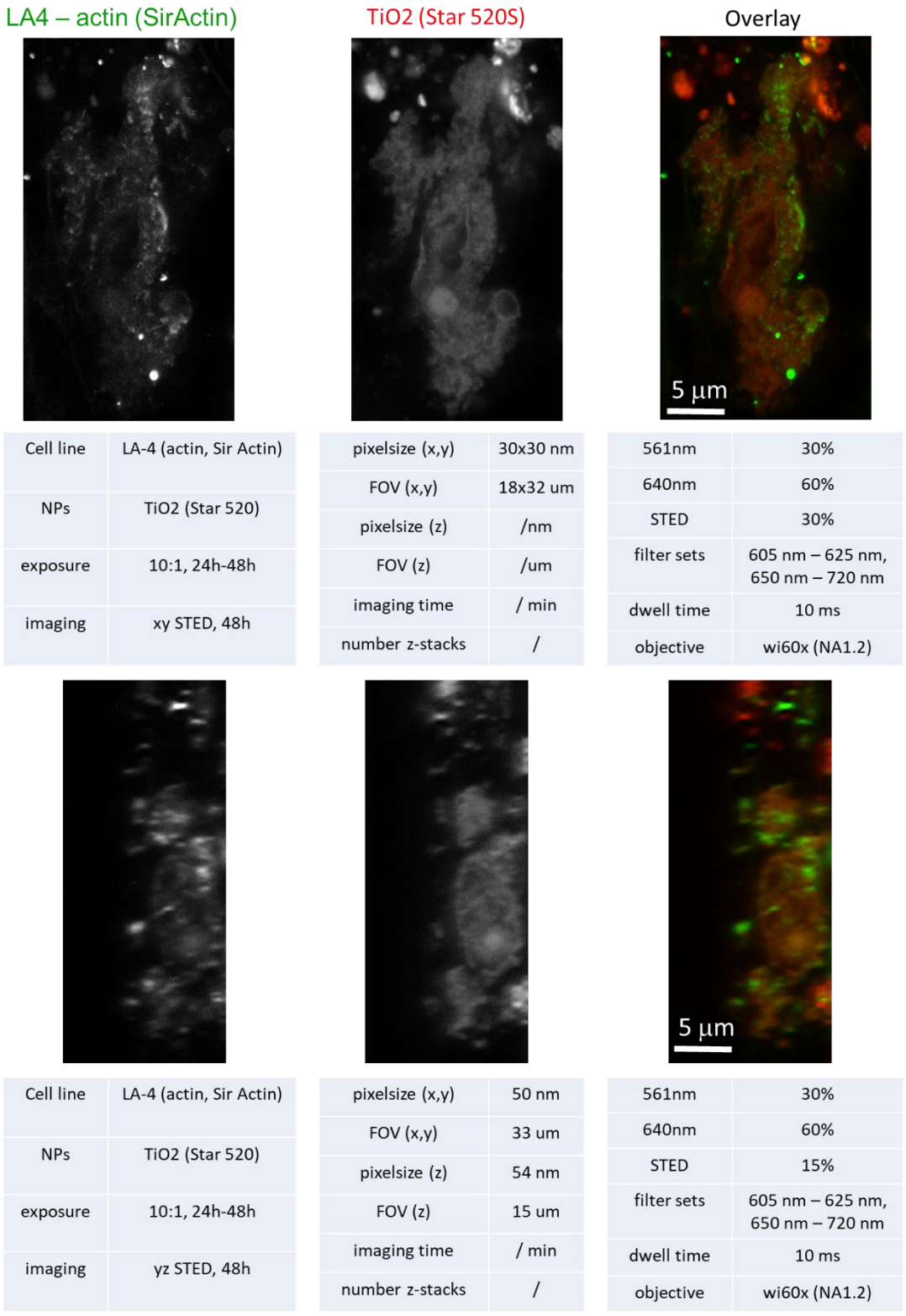
Figure S151



Cell line	LA-4 (actin, Sir Actin)
NPs	TiO2 (Star 520)
exposure	10:1, 24h-48h
imaging	xy confocal, 48h

pixelsize (x,y)	200 nm
FOV (x,y)	80 um
pixelsize (z)	/nm
FOV (z)	/um
imaging time	/min
number z-stacks	

561nm	30%
640nm	50%
STED	0%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 ms
objective	wi60x (NA1.2)



Cell line	LA-4 (actin, Sir Actin)
NPs	TiO2 (Star 520)
exposure	10:1, 24h-48h
imaging	xy STED, 48h

pixelsize (x,y)	30x30 nm
FOV (x,y)	18x32 um
pixelsize (z)	/nm
FOV (z)	/um
imaging time	/ min
number z-stacks	/

561nm	30%
640nm	60%
STED	30%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 ms
objective	wi60x (NA1.2)

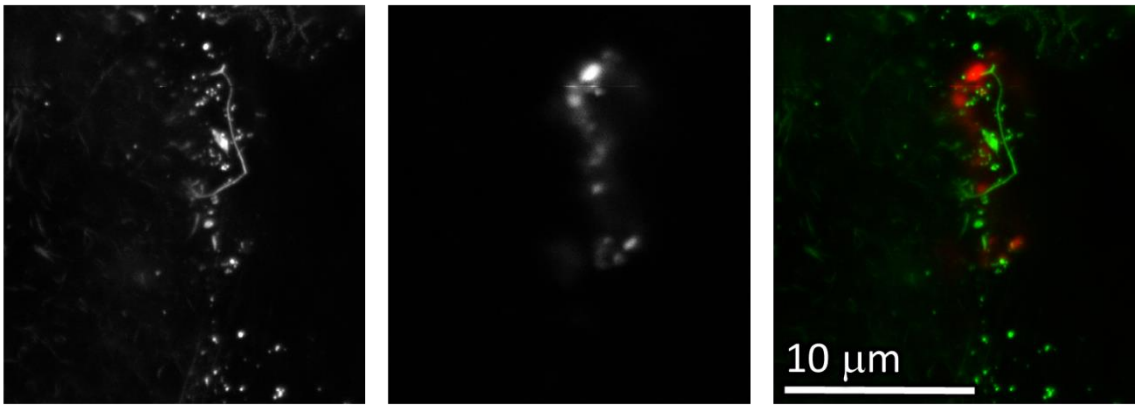
Cell line	LA-4 (actin, Sir Actin)
NPs	TiO2 (Star 520)
exposure	10:1, 24h-48h
imaging	yz STED, 48h

pixelsize (x,y)	50 nm
FOV (x,y)	33 um
pixelsize (z)	54 nm
FOV (z)	15 um
imaging time	/ min
number z-stacks	/

561nm	30%
640nm	60%
STED	15%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 ms
objective	wi60x (NA1.2)

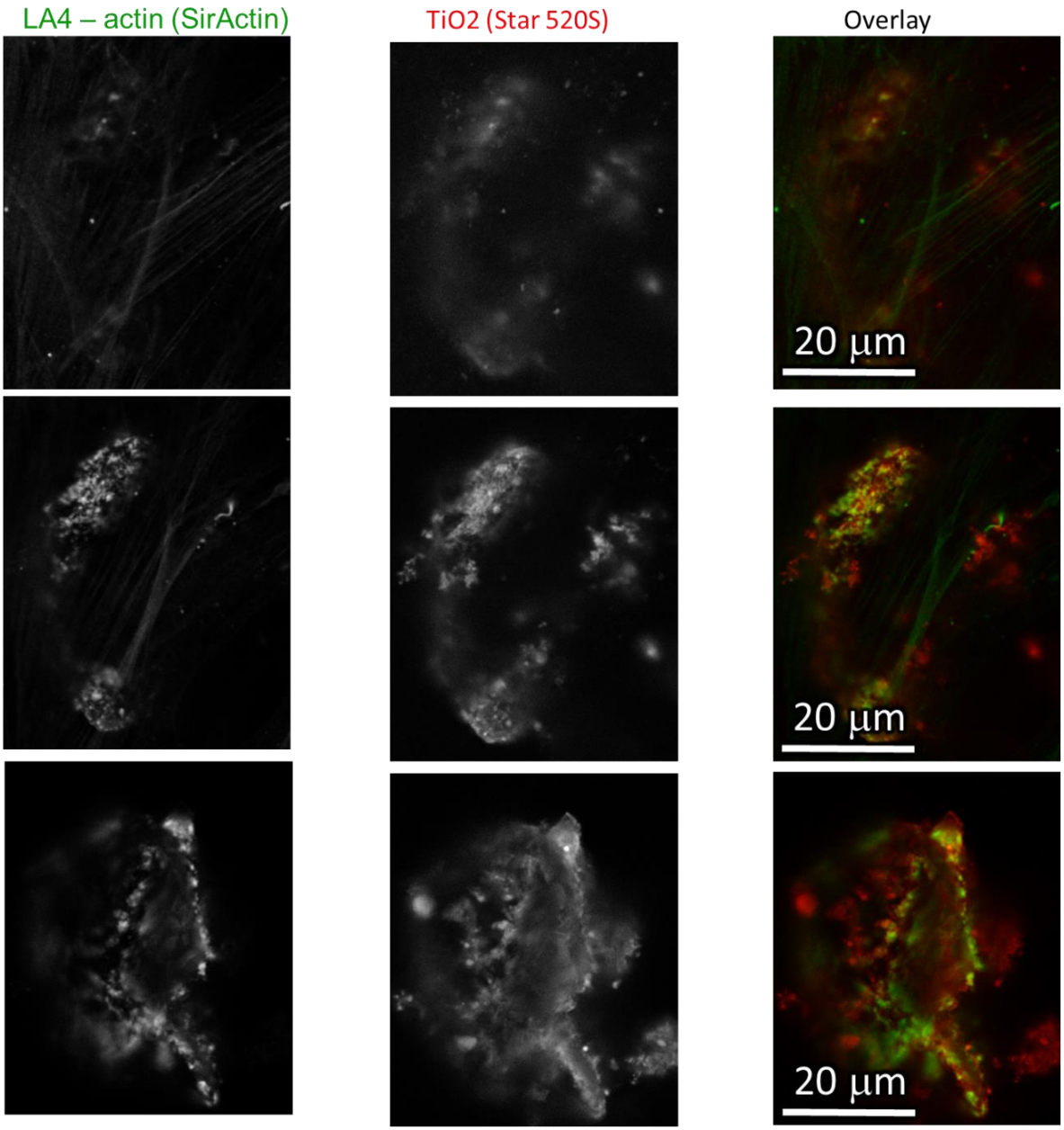
Figure S148: Confocal and STED images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. Confocal image of larger FoV in the upper window shows actin cytoskeleton of few cells with a lot of nanomaterial on the surface and one large cauliflower-like structure. In the middle window is STED zoom-in (XY plane) of the cauliflower-like structure form the upper window where we can clearly see actin filaments (green) intertwined with nanomaterial (red). In the lower window we see the STED zoom-in (YZ plane) of the same cauliflower-like structure shown in the middle window. Again, actin filaments are noticable in the cauliflower-like structure bulk.

Cell line	LA-4 (actin, 0,1 µM SirActin 12h)	pixelsize (x,y)	200 nm	561nm	20%
NPs	TiO2 (Star 520)	FOV (x,y)	80 um	640nm	20%
exposure	10:1, 24h-48h	pixelsize (z)	/nm	STED	0%
imaging	xy confocal, 48h	FOV (z)	/um	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/min	dwell time	10 ms
		number z-stacks	/	objective	wi60x (NA1.2)



Cell line	LA-4 (actin, Sir Actin)	pixelsize (x,y)	15 nm	561nm	30%
NPs	TiO2 (Star 520)	FOV (x,y)	19x21 um	640nm	40%
exposure	10:1, 48h-24h	pixelsize (z)	/nm	STED	15%
imaging	xy STED, 48h	FOV (z)	/um	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/min	dwell time	10 ms
		number z-stacks	/	objective	wi60x (NA1.2)

Figure S149: Confocal and STED images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. Confocal image of larger FoV in the upper window shows actin cytoskeleton of two cells with some nanomaterial on the surface and one small cauliflower-like structure (red agglomerate). In the lower window is STED zoom-in (XY plane) of the cauliflower-like structure form the upper window where we can clearly see actin filaments (green) sitting on the top of nanomaterial (red).



Cell line	LA-4 (actin, Sir Actin)
NPs	TiO2 (Star 520)
exposure	10:1, 24h-48h
imaging	xyz STED, 48h

pixelsize (x,y)	100 nm
FOV (x,y)	43x53 um
pixelsize (z)	100 nm
FOV (z)	15 um
imaging time	/min
number z-stacks	149

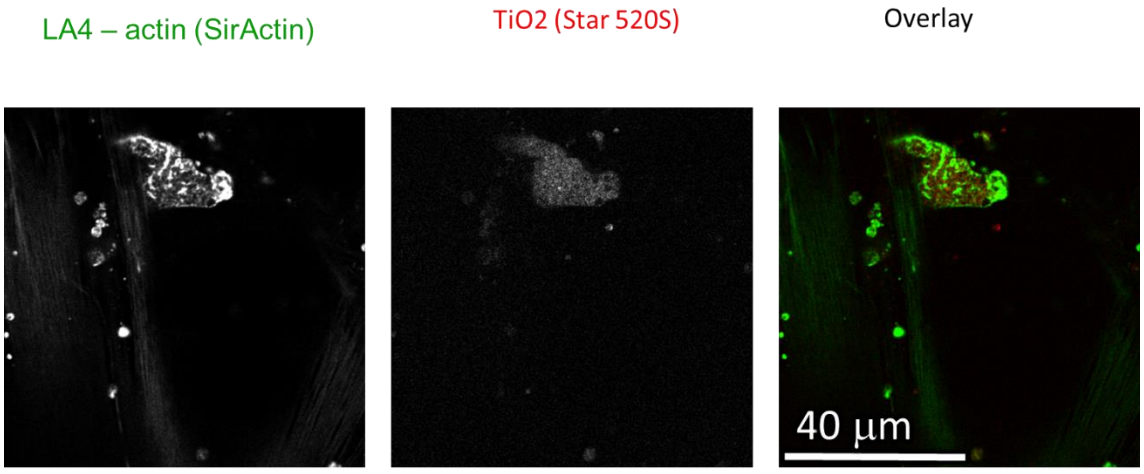
561nm	30%
640nm	40%
STED	15%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 ms
objective	wi60x (NA1.2)

Figure S150: STED images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. Three different XY plane slices from a whole Z-stack (3D) of a huge cauliflower-like structure sitting on the surface of the LA-4 cell (green cytoskeleton).

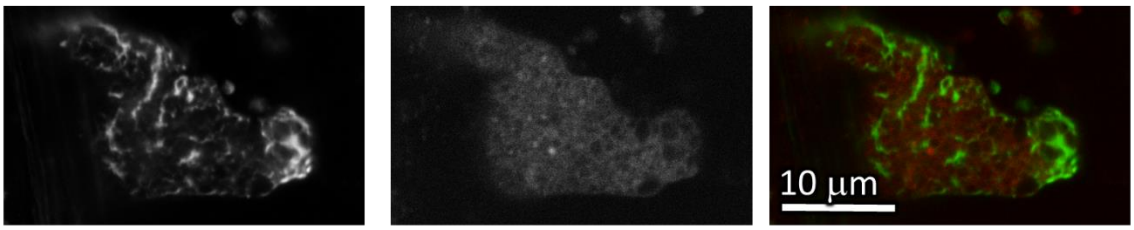
Link to 3D

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190627_e05m03s03_LA-4_0.1_uM_SA_and_10_to_1_TiO2_StarS520_remnants_of_actin_in_cauliflower_from_3D.mp4

Movie 22: 3D rendering of the actin remains (green) in a large cauliflower on top of LA-4 epithelial cell:



Cell line	LA-4 (actin, Sir Actin)	pixelsize (x,y)	200 nm	561nm	20%
NPs	TiO2 (Star 520)	FOV (x,y)	80x80um	640nm	20%
exposure	10:1, 24h-48h & 100 mM Resveratrol	pixelsize (z)	/nm	STED	0%
imaging	xy confocal, 48h	FOV (z)	/um	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/min	dwell time	10 ms
		number z-stacks	/	objective	wi60x (NA1.2)



Cell line	LA-4 (actin, Sir Actin)	pixelsize (x,y)	30 nm	561nm	50%
NPs	TiO2 (Star 520)	FOV (x,y)	32x20 um	640nm	50%
exposure	10:1, 24h-48h & 100 mM Resveratrol	pixelsize (z)	/nm	STED	20%
imaging	xy STED, 48h	FOV (z)	/um	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	/min	dwell time	10 ms
		number z-stacks		objective	wi60x (NA1.2)

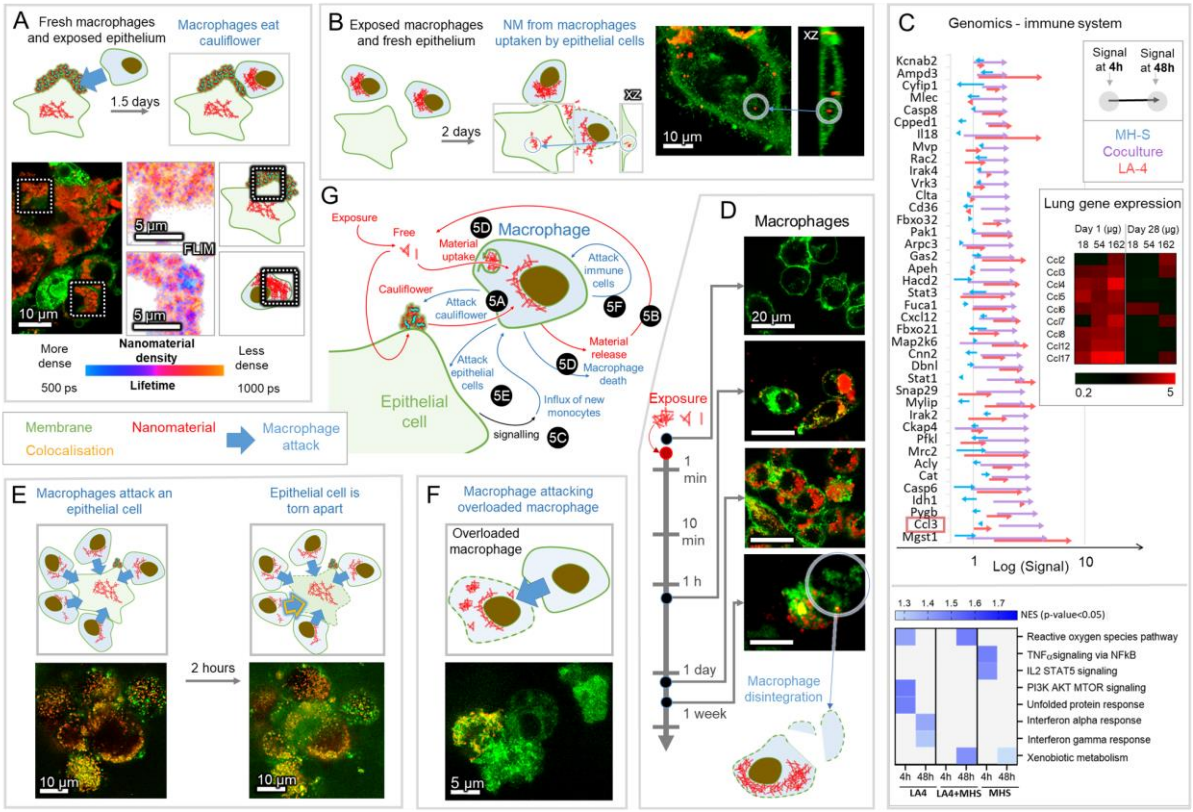
Figure S151: Confocal images of LA-4 in green and TiO_2 nanotubes in red channel. Cells are stained with Sir Actin label for F-actin. Confocal image of larger FoV in the upper window shows actin cytoskeleton of few cells with a lot of nanomaterial on the surface and one large cauliflower-like structure. In the lower window is STED zoom-in (XY plane) of the cauliflower-like structure form the upper window where we can clearly see actin filaments (green) with nanomaterial (red). Cells in this experiment have been treated with 100 µM Resveratrol in order to inhibit the activity of a FAS enzyme complex. During the

FAS inhibition we don't see big cauliflowers when we stain for plasma membrane (there is no colocalisation of plasma membrane and nanomaterial), but here we do observe cauliflowers and actin fibers excreted together with nanomaterial.

S4g – Genomics – actin related expressions

See supplement S3d – Transcriptomics *in vitro* and *in vivo* after exposure to TiO2.

S5 – Macrophage action against epithelial defence



S5a – MH-S eat cauliflowers

Main message

2 days after previously non-exposed macrophages are added to a LA-4 culture with cauliflowers, they are seen filled up with nanomaterial. Since the LA-4 culture was washed prior to adding the macrophages to remove all free-floating nanomaterial, the only available source of such a huge amount of nanomaterial is from the cauliflowers. The fluorescence lifetime of the nanomaterial in macrophages lower than the fluorescence lifetime of nanomaterial in cauliflowers. Since lower lifetime corresponding to a more agglomerated form of nanomaterial, the nanomaterial in macrophages is more tightly-packed than in the cauliflowers.

Supporting raw and analysed data:

Figure S152-Figure S155

Materials and methods

- experiment 20190621_e03.100_s01_FLIM:
  - LA-4 cells were seeded @50% confluence in an Ibidi #1.5H µ-Dish with a 4-Well Culture-Insert (Ibidi)
  - after 24 hours, 35 µL freshly filtered 1 mg/mL TiO_2-17-Alexa647 in 100x dcb was mixed into 130 µL fresh F12K medium and added to cells in one of the inserts to achieve 100:1 surface dose
  - 48 hours later, medium was removed from the cells and cells were washed with 2x 100 µl PBS to remove freely floating nanomaterial.
  - fresh (nonexposed) macrophages MH-S were added at 30% confluency to the exposed and washed LA-4, and were kept in a 1:1 mixture of media (total volume 140 µL) until observation
  - 34 hours later, the cells in the well were first incubated with 1.6 µg/mL CellMaskOrange in LCIS for 5 minutes, then incubated 15 minutes with freshly diluted 5 mM SAG-38 in LCIS at room temperature, and finally flushed with LCIS and observed in 150 µL LCIS in the home-made incubator at 37 °C

Cell line	LA-4 and MH-S (membrane, CellMaskOrange and lipid bodies, SAG-38)
NPs	TiO2 (Alexa647)
exposure	100:1, 48h LA-4, observed after 34h with MH-S
imaging	xy confocal

pixelsize (x,y)	100 nm
FOV (x,y)	70.0 µm
pixelsize (z)	
FOV (z)	
imaging time	
number of frames	

561nm	10%
640nm	10%
STED	
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	60x wi (NA 1.2)

Cell line	LA-4 and MH-S (membrane, CellMaskOrange and lipid bodies, SAG-38)
NPs	TiO2 (Alexa647)
exposure	100:1, 48h LA-4, observed after 34h with MH-S
imaging	xy confocal FLIM

pixelsize (x,y)	100 nm
FOV (x,y)	51.2 µm
TCSPC pixelsize	122 ps
TCSPC FOV	19.5 ns

561nm	(10%)
640nm	10%
STED	
diffraction grating	523 – 722 nm
dwell time	100 µs
objective	60x wi (NA1.2)

- fluorescence lifetime measurement and analysis:
  - see S3c – FLIM of cauliflowers

Experiment names

- Main experiment name:
  - 20190621_e03.100_s01_LA-4 MH-S CellMask TiO2-17-Alexa647 100.1_FLIM.msr

- Supplement Experiment names:
  - 20190621_e03.100_s01_LA-4 MH-S CellMask TiO2-17-Alexa647 100.1_FLIM.msr
  - 20190621_e03.100_s0X_cellsB.msr

Controls and statistics

Cross-sections of the cocultures of MH-S and LA-4 after the previously nonexposed MH-S have been in a coculture with LA-4 with already formed cauliflowers:
Figure S152-Figure S154

Fluorescence lifetime mapping of Figure S154:
Figure S155

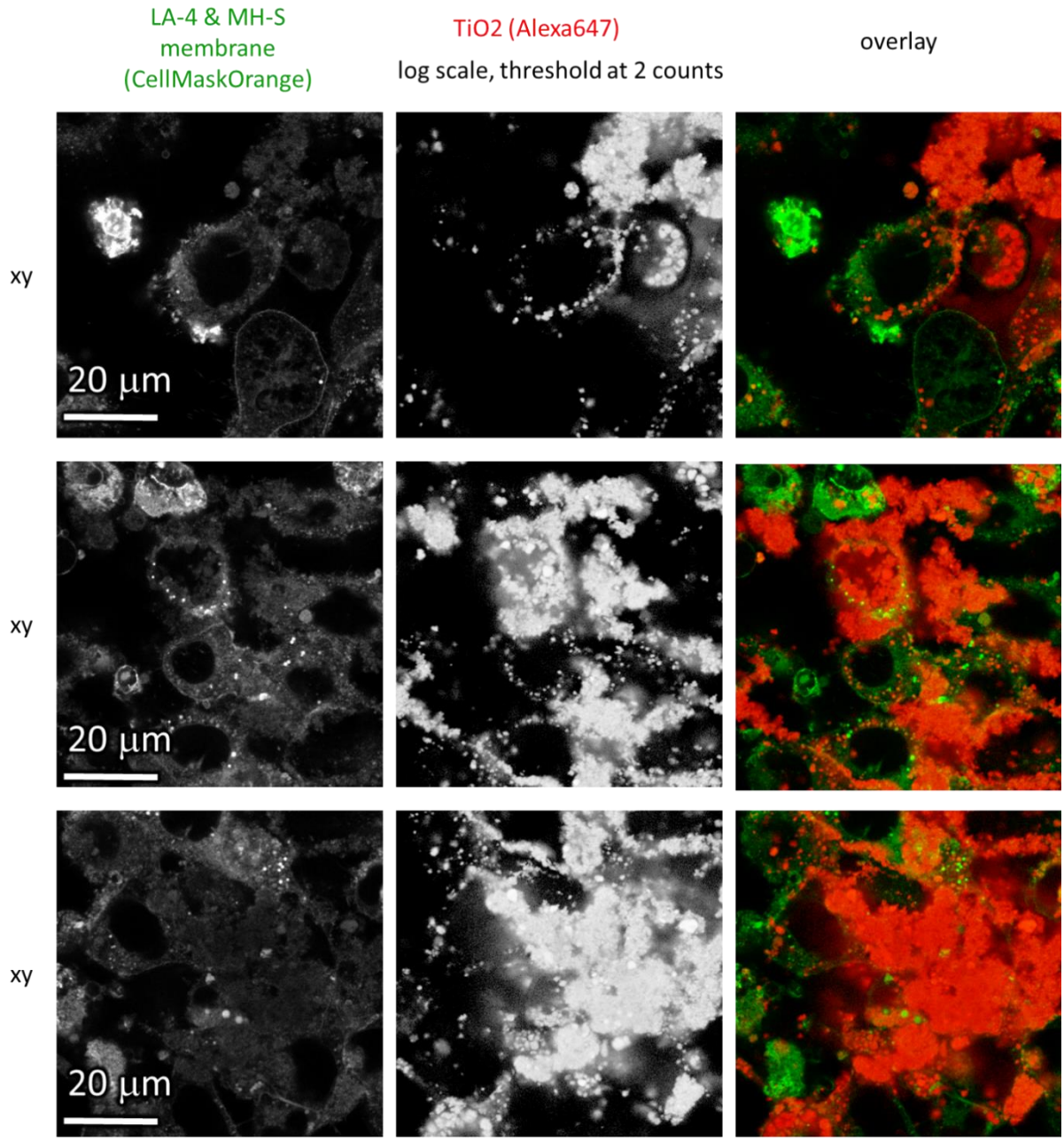
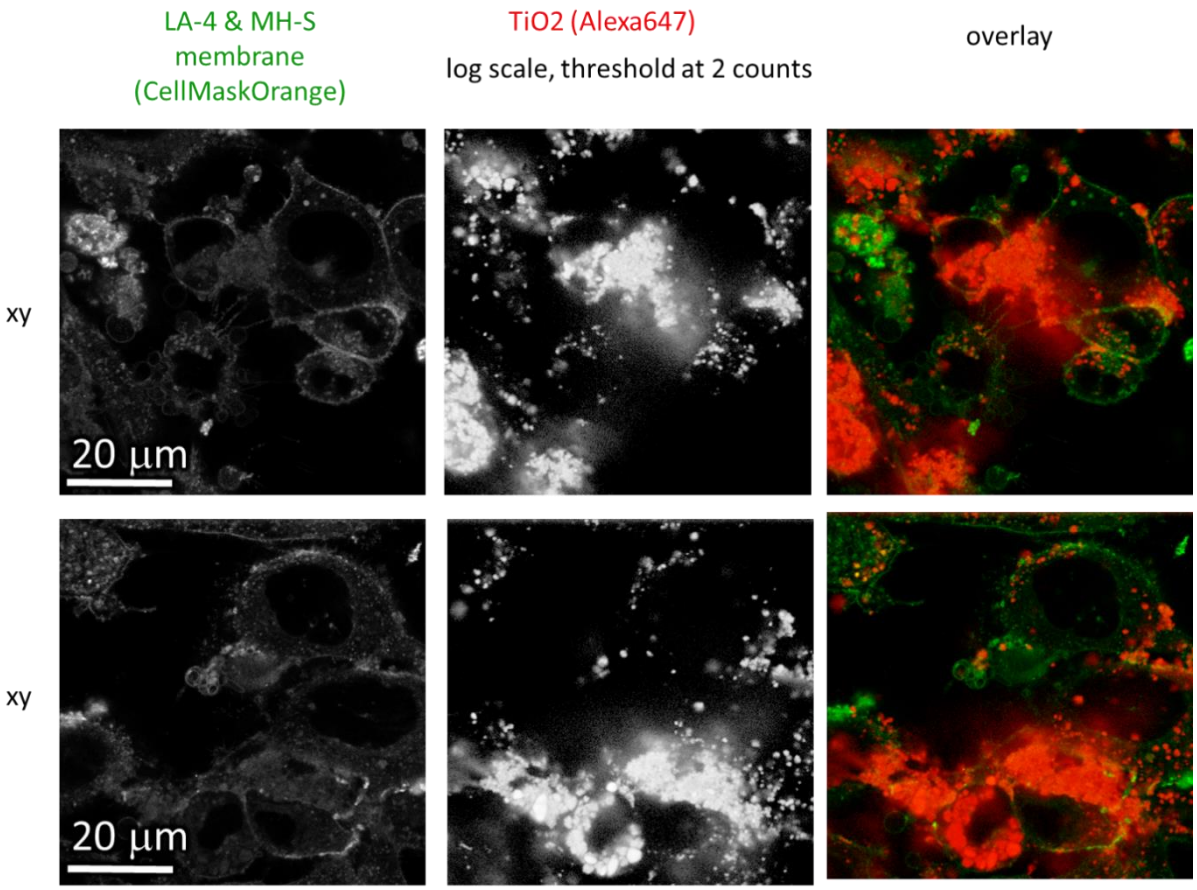


Figure S152: Statistics for xy cross-sections of the cocultures of MH-S and LA-4 after the previously nonexposed MH-S have been in a coculture with LA-4 with already formed cauliflowers for 34 hours.

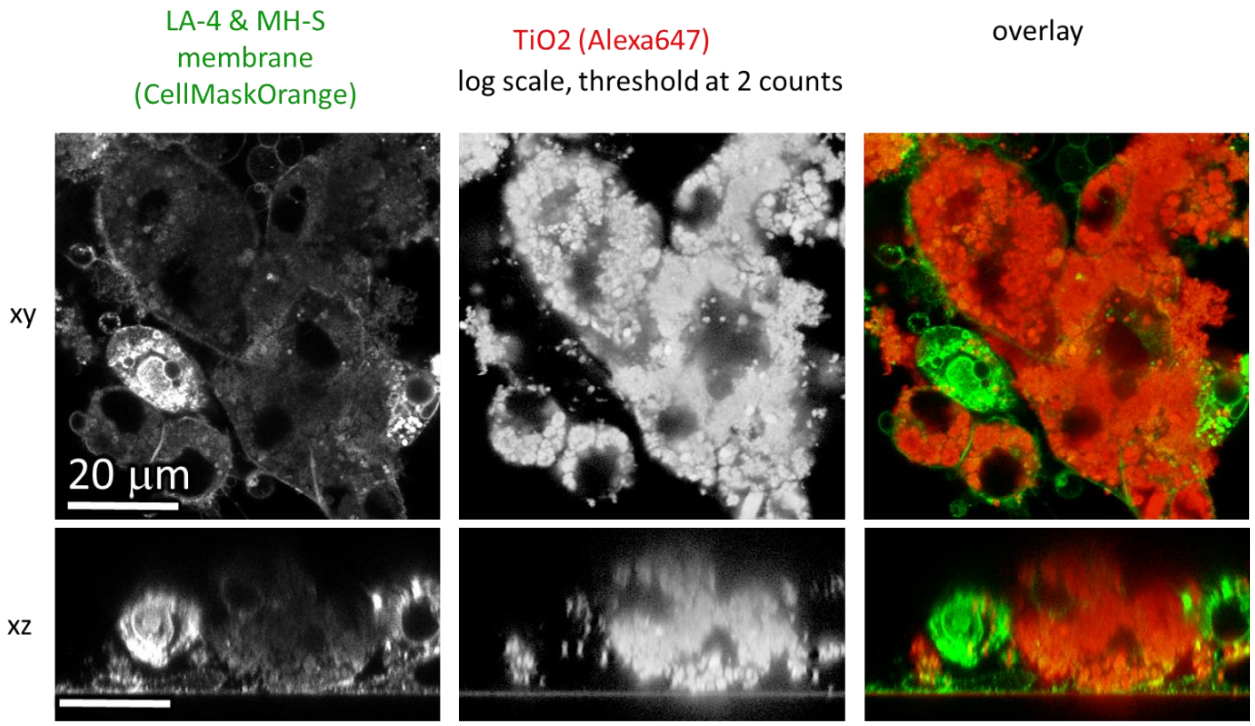


Cell line	LA-4 and MH-S (membrane, CellMaskOrange and lipid bodies, SAG-38)
NPs	TiO2 (Alexa647)
exposure	100:1, 48h LA-4, observed after 34h with fresh MH-S
imaging	xy confocal

pixelsize (x,y)	150 nm
FOV (x,y)	70.0 µm
pixelsize (z)	
FOV (z)	
imaging time	
number of frames	

561nm	10%
640nm	10%
STED	
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	60x wi (NA 1.2)

Figure S153: Statistics for xy cross-sections of the cocultures of MH-S and LA-4 after the previously non-exposed MH-S have been in a coculture with LA-4 with already formed cauliflowers for 34 hours.



xy

Cell line	LA-4 and MH-S (membrane, CellMaskOrange and lipid bodies, SAG-38)
NPs	TiO2 (Alexa647)
exposure	100:1, 48h LA-4, observed after 34h with fresh MH-S
imaging	xy confocal

pixelsize (x,y)	100 nm
FOV (x,y)	70.0 µm
pixelsize (z)	
FOV (z)	
imaging time	
number of frames	

561nm	10%
640nm	10%
STED	
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	60x wi (NA 1.2)

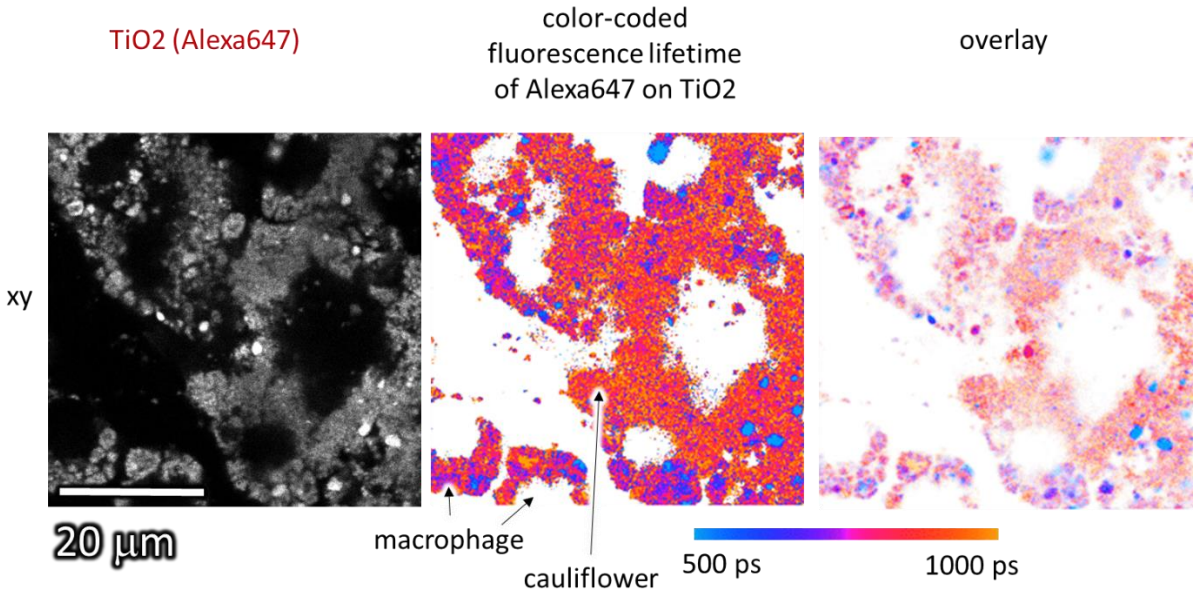
xz

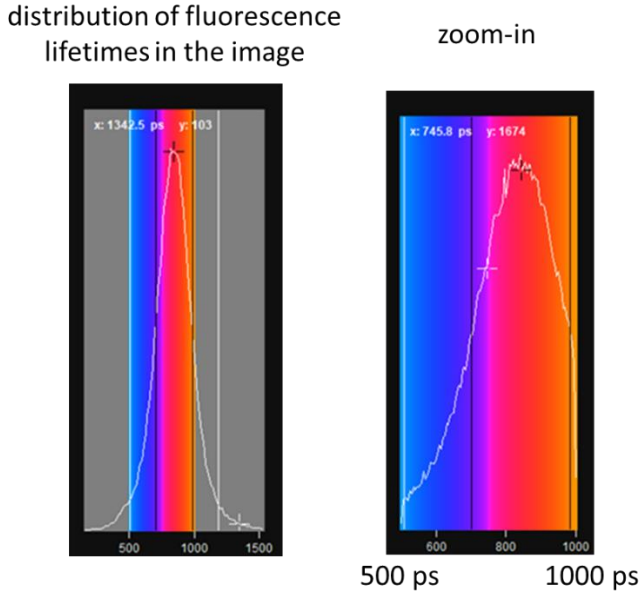
Cell line	LA-4 and MH-S (membrane, CellMaskOrange and lipid bodies, SAG-38)
NPs	TiO2 (Alexa647)
exposure	100:1, 48h LA-4, observed after 34h with fresh MH-S
imaging	xz confocal

pixelsize (x,y)	100 nm
FOV (x,y)	70.0 µm
pixelsize (z)	131 nm
FOV (z)	35.0 µm
imaging time	
number of frames	

561nm	10%
640nm	10%
STED	
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	60x wi (NA 1.2)

Figure S154: Xy and xz cross-sections of the cocultures of MH-S and LA-4 after the previously non-exposed MH-S have been in a coculture with LA-4 with already formed cauliflowers for 34 h.





parameters of the distribution [ps]

µ	843,44
µ-sig	701,32
µ+sig	983,39
	x

Cell line	LA-4 and MH-S (membrane, CellMaskOrange and lipid bodies, SAG-38)
NPs	TiO2 (Alexa647)
exposure	100:1, 48h LA-4, observed after 34h with fresh MH-S
imaging	xy confocal FLIM

pixelsize (x,y)	100 nm
FOV (x,y)	51.2 µm
TCSPC pixelsize	122 ps
TCSPC FOV	19.5 ns

561nm	
640nm	10%
STED	
diffraction grating	523 – 722 nm
dwell time	100 µs
objective	60x wi (NA1.2)

Figure S155: Analysis of fluorescence lifetimes of the coculture of MH-S and LA-4 after the previously nonexposed MH-S have been in a coculture with LA-4 with already formed cauliflowers. The fluorescence image, fluorescence-lifetime-color-coded image and distribution of fluorescence lifetimes in the image are shown.

S5b – Re-uptake of nanomaterial into LA-4

Main message

MH-S macrophages, which were exposed to a 10:1 surface dose of TiO_2 nanotubes for 2 days, were added to non-exposed epithelial LA-4 monoculture. After two days of coculture of exposed macrophages with nonexposed epithelial cells, some nanomaterial was found in the epithelial cells. Because the macrophages were thoroughly washed before they were added to the epithelial cells, one can conclude that the nanomaterial which was found inside the epithelial cells came from inside the macrophages.

Supporting raw and analysed data:

Figure S156-Figure S158

Materials and methods

- experiment setup:
  - LA-4 and MH-S cells were seeded separately @15% confluence in two wells on an Ibidi #1.5H µ-Slide 8-well
  - after 8 hours, 10 µL 1 mg/mL TiO_2-17-Alexa647 in 100x dcb was added to MH-S and mixed to achieve 10:1 surface dose
  - 47 hours later, the medium was removed from MH-S cells and they were flushed with 200 µl PBS to remove mobile nanomaterial from the sample. After adding 100 µl fresh MH-S medium, they were scraped and added to LA-4 with 100 µL freshly changed LA-4 medium.
  - after 45 hours, the cells were incubated with 1.6 µg/mL CellMaskOrange for 7 minutes at 37 °C, afterwards they were observed in 200 µL LCIS in the home-made stage-top incubator at 37 °C

- analysis:
  - logarithmic scale in red and green channel, threshold at 3 counts, saturation at 200 counts (green channel) or 10 counts (red channel)

Cell line	LA-4 and MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	50 nm	561nm	5%
NPs	TiO2 (Alexa647)	FOV (x,y)	70.2 µm	640nm	5%
exposure	10:1, 47h MH-S, observed after 45h with LA-4	pixelsize (z)		STED	5%, 71% 3D STED
imaging	xy STED	FOV (z)		filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA1.2)

Cell line	LA-4 and MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	50 nm	561nm	5%
NPs	TiO2 (Alexa647)	FOV (x,y)	69.6 µm	640nm	5%
exposure	10:1, 47h MH-S, observed after 45h with LA-4	pixelsize (z)	50 nm	STED	5%, 71% 3D STED
imaging	xz STED	FOV (z)	30.0 µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA1.2)

Experiment names

- Main experiment name:
  - 20190802_e10_s03_LA-4 MH-S CMO TiO2-17-Alexa647_0000 NN00 10.1_2 days nothing 2 days with full MH-S.msr

- Supplement experiment name:
  - 20190802/e10_s01_LA-4 MH-S CMO TiO2-17-Alexa647_0000 NN00 10.1_2 days nothing 2 days with full MH-S.msr
  - 20190802/e10_s02_LA-4 MH-S CMO TiO2-17-Alexa647_0000 NN00 10.1_2 days nothing 2 days with full MH-S.msr
  - 20190802/e10_s03_LA-4 MH-S CMO TiO2-17-Alexa647_0000 NN00 10.1_2 days nothing 2 days with full MH-S.msr
  - 20190802/e10_s04_LA-4 MH-S CMO TiO2-17-Alexa647_0000 NN00 10.1_2 days nothing 2 days with full MH-S.msr
  - 20190802/e10_s06_LA-4 MH-S CMO TiO2-17-Alexa647_0000 NN00 10.1_2 days nothing 2 days with full MH-S.msr

Controls and statistics

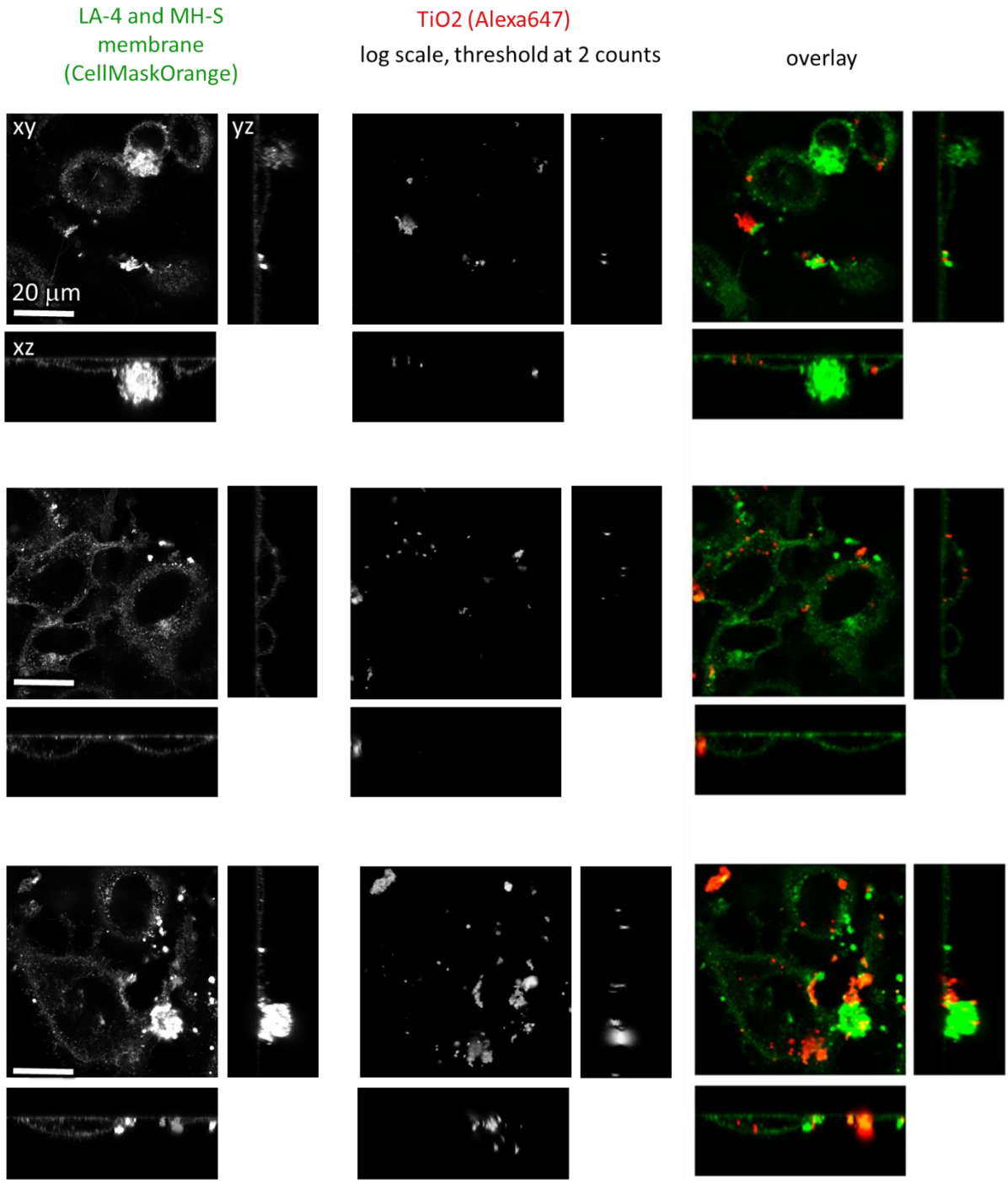


Figure S156: Localisation of nanomaterial inside LA-4, which were exposed to nanomaterial solely by exposure to nanomaterial-laden macrophages (xy, xz and yz cross-sections)

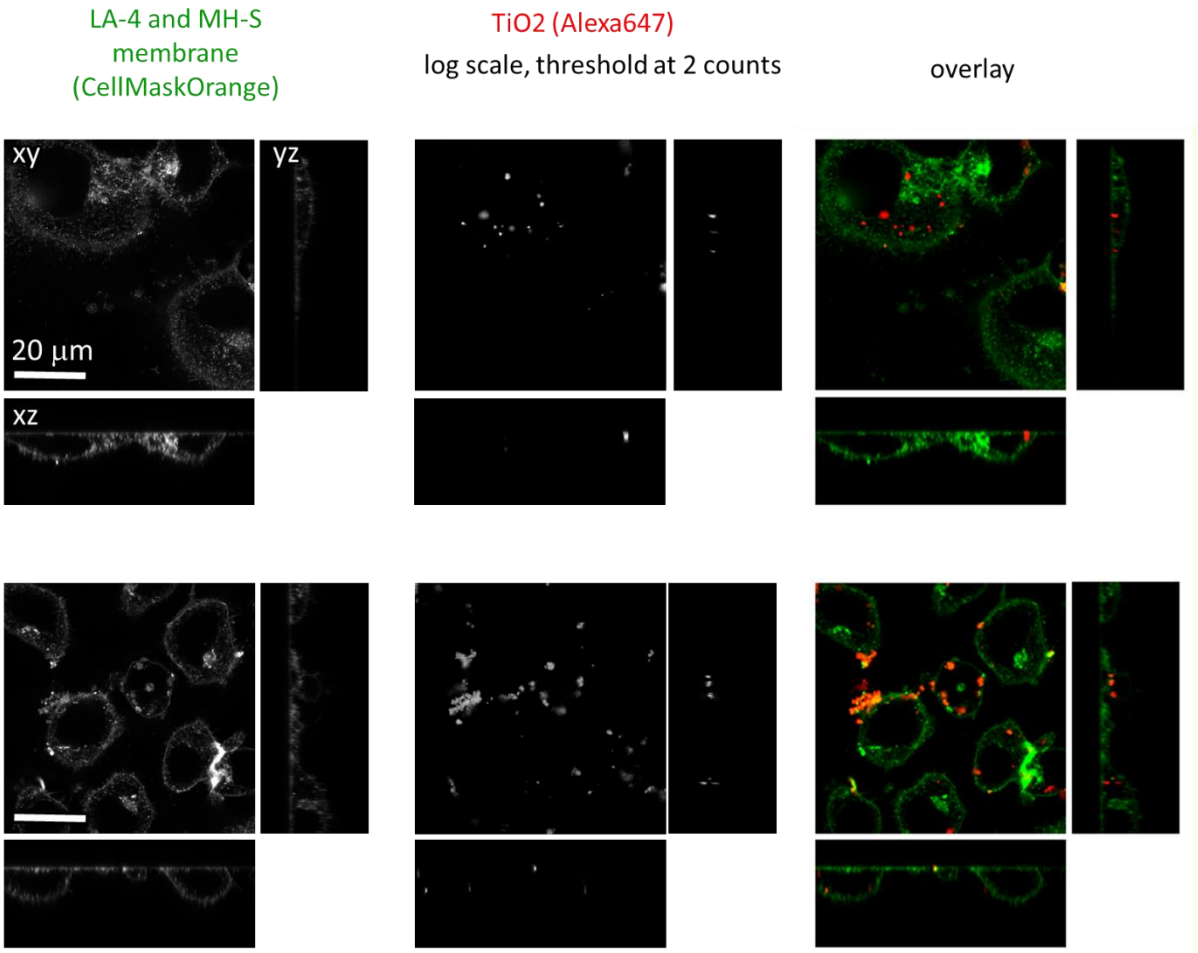


Figure S157: Localisation of nanomaterial inside LA-4, which were exposed to nanomaterial solely by exposure to nanomaterial-laden macrophages (xy, xz and yz cross-sections)

Cell line	LA-4 and MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	50 nm	561nm	5%
NPs	TiO2 (Alexa647)	FOV (x,y)	70.2 µm	640nm	5%
exposure	10:1, 47h MH-S, observed after 45h with LA-4	pixelsize (z)		STED	5%, 71% 3D STED
imaging	xy STED	FOV (z)		filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA1.2)

Cell line	LA-4 and MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	50 nm	561nm	5%
NPs	TiO2 (Alexa647)	FOV (x,y)	69.6 µm	640nm	5%
exposure	10:1, 47h MH-S, observed after 45h with LA-4	pixelsize (z)	50 nm	STED	5%, 71% 3D STED
imaging	xz STED	FOV (z)	30.0 µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA1.2)

Cell line	LA-4 and MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	50 nm	561nm	5%
NPs	TiO2 (Alexa647)	FOV (x,y)	69.6 µm	640nm	5%
exposure	10:1, 47h MH-S, observed after 45h with LA-4	pixelsize (z)	50 nm	STED	5%, 71% 3D STED
imaging	yz STED	FOV (z)	30.0 µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA1.2)

Figure S158: Imaging parameters for the upper two Figures.

S5c – Genomics – immune system

See supplement S3d – Transcriptomics *in vitro* and *in vivo* after exposure to TiO2.

S5d – MH-S eat nanomaterial

Main message

Macrophages quickly internalise nanomaterial and are full of nanomaterial after a few days. However, they are also seen to die after a few days of exposure to nanomaterial.

Supporting raw and analysed data:

Figure S159-Figure S169

Materials and methods

Experiment at time 0h:

- experiment: 20190412_e05_t01
- protocol:
  - MH-S cells were seeded @20% confluence in an Ibidi #1.5H µ-Dish
  - after 4 days 100 µl 5 µg/mL CellMaskOrange was added to 400 µl old cell medium on the cells. After 7 minutes at room temperature, the medium was removed and cells were observed in 400 µl LICS in the home-made stage-top incubator at 37 °C.
- analysis:
  - rescaling Green channel to 65 counts

Link to time-lapse

- *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190412_e05_t01b_MH-S_CellMask_xyt_10_minutes_1s_is_2minutes.gif*

Movie S23: Time-lapse of non-exposed macrophages (CellMask Orange, green), 1 s in movie corresponds to 2 minutes in real time.

- *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190412_e05_t04_xyt_MH-S_CellMask_MH-S_dividing_10minutes_1s_is_2minutes.gif*

Movie S24: Time-lapse of non-exposed macrophages and their division (CellMask Orange, green), 1 s in movie corresponds to 2 minutes in real time.

Link to 3D

- *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190412_e05_t01_MH-S_CellMask_xyz_FOV_70um_x_70um_x_30um.mp4*

Movie S25: 3D of non-exposed macrophages (CellMask Orange, green), field-of-view is 70 x 70 x 30 µm.

Cell line	MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	100 nm	561nm	5%
NPs		FOV (x,y)	70.2 µm	640nm	5%
exposure		pixelsize (z)		STED	
imaging	xy confocal	FOV (z)		filter sets	605 nm – 625 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA1.2)

Cell line	MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	200 nm	561nm	5%
NPs		FOV (x,y)	70.2 µm	640nm	5%
exposure		pixelsize (z)	1 µm	STED	
imaging	xyz confocal	FOV (z)	30 µm	filter sets	605 nm – 625 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA1.2)

Cell line	MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	200 nm	561nm	5%
NPs		FOV (x,y)	70.2 µm	640nm	5%
exposure		pixelsize (z)		STED	
imaging	xyt confocal	FOV (z)		filter sets	605 nm – 625 nm
		imaging time	10 minutes	dwell time	10 µs
		number of frames	60	objective	60x wi (NA1.2)

Experiment at time 2h:

- experiment: 20190412/e06_t03…t13

- protocol:
  - MH-S cells were seeded @20% confluence in an Ibidi #1.5H µ-Dish
  - after 4 days 100 µl 5 µg/mL CellMaskOrange was added to 400 µl old cell medium on the cells. After 7 minutes at room temperature, the medium was removed and cells were observed in 400 µl LICS in the home-made stage-top incubator at 37 °C.
  - just prior to filming, freshly filtered 5 mL 1 mg/mL TiO_2-40-Alexa647 in 100x dcb was added directly to the cells (in 400 µl LCIS) and mixed to achieve 2:1 surface dose

- analysis:
  - logarithmic scale in red channel, cut-off at 2 counts, saturation at 256 counts, linear scale on green channel, saturation at 150 counts

Link to time-lapse

- *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190412_e06_MH-S_CellMask_TiO2Alexa647_1.1_live_xyt_total_2_h_with_breaks_for_xyz_1s_is_2min.gif*

Movie S26: Live movie of macrophages (CellMask, green) exposed to 1:1 surface dose of TiO_2, from 0h – 2 hours (1 second corresponds to 2 minutes real time).

Link to 3D

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190412_e06_MH-S_CellMask_TiO2Alexa647_1.1_xyz_FOV_70_x_70_x_30_um_1.mp4

Movie S27: Comparison of 3D renders of macrophages (CellMask, green) exposed to 1:1 surface dose of TiO_2 nanotubes (Alexa 647, red) at different time-points from 0h – 2 hours, field-of-view is 70 x 70 x 30 µm.

http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190412_e06_MHS_CellMask_TiO2Alexa647_1.1_xyz_FOV_70_x_70_x_30_um.mp4

Movie S28: 3D movie of macrophages (CellMask, green) exposed to 1:1 surface dose of TiO_2 nanotubes (Alexa 647, red) at different time-points from 0h – 2 hours, field-of-view is 70 x 70 x 30 µm.

- separate timepoints:
  - http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190412_e06_t04_MH-S_CellMask_TiO2Alexa647_1.1_live_xyz_FOV_70_x_70_x_30_um_25min.gif

Movie S29: Macrophages (CellMask, green) exposed to 1:1 surface dose of TiO_2 nanotubes (Alexa 647, red) after 25 minutes

  - http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190412_e06_t06_MH-S_CellMask_TiO2Alexa647_1.1_live_xyz_FOV_70_x_70_x_30_um_45min.gif

Movie S30: Macrophages (CellMask, green) exposed to 1:1 surface dose of TiO_2 nanotubes (Alexa 647, red) after 45 minutes

  - http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190412_e06_t08_MH-S_CellMask_TiO2Alexa647_1.1_live_xyz_FOV_70_x_70_x_30_um_1h_25min.gif

Movie S31: Macrophages (CellMask, green) exposed to 1:1 surface dose of TiO_2 nanotubes (Alexa 647, red) after 1h 25 minutes

  - http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190412_e06_t09_MH-S_CellMask_TiO2Alexa647_1.1_live_xyz_FOV_70_x_70_x_30_um_1h_45min.gif

Movie S32: Macrophages (CellMask, green) exposed to 1:1 surface dose of TiO_2 nanotubes (Alexa 647, red) after 1h 45 minutes

  - http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190412_e06_t12_MH-S_CellMask_TiO2Alexa647_1.1_live_xyz_FOV_70_x_70_x_30_um_2h.gif

Movie S33: Macrophages (CellMask, green) exposed to 1:1 surface dose of TiO_2 nanotubes (Alexa 647, red) after 2 hours

  - http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190412_e06_t13_MH-S_CellMask_TiO2Alexa647_1.1_live_xyz_FOV_70_x_70_x_30_um_2h_10min.gif

Movie S34: Macrophages (CellMask, green) exposed to 1:1 surface dose of TiO_2 nanotubes (Alexa 647, red) after 2 h 10 minutes

Cell line	MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	200 nm	561nm	5%
NPs	TiO2 (Alexa647)	FOV (x,y)	70.2 µm	640nm	5%
exposure	2:1, 25min – 2h	pixelsize (z)		STED	
imaging	xyt confocal	FOV (z)		filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	95 min	dwell time	10 µs
		number of frames	491	objective	60x wi (NA 1.2)

Cell line	MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	200 nm	561nm	5%
NPs	TiO2 (Alexa647)	FOV (x,y)	70.2 µm	640nm	5%
exposure	2:1, 0min – 2h	pixelsize (z)	500 nm	STED	
imaging	xyz confocal	FOV (z)	30 µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA 1.2)

Experiment at time-point 2 days:

- experiment: 20190412/e07_t04

- protocol:
  - MH-S cells were seeded @20% confluence in an Ibidi #1.5H µ-Dish
  - after 1 day 30 µl freshly filtered 1 mg/mL TiO_2-40-Alexa647 in 100x dcb was added to 400 µL cell medium already on the cells and mixed to achieve 10:1 surface dose
  - 53 hours later, 100 µL 5 µg/mL CellMaskOrange was added to 400 µL old cell medium on the cells. After 9 minutes at 37 °C, the medium was removed and cells were observed in 400 µL LICS in the home-made stage-top incubator at 37 °C.

- analysis:
  - logarithmic scale in red channel, cut-off at 2 counts, linear scale on green channel, saturation at 40 counts

Link to 3D

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190412_e07_t04_MH-S_CellMask_TiO2-Alexa647_10.1_2_days_xyz_FOV_70_x_70_x_30_um.mp4

Movie S35: Macrophages (CellMask, green) after 2 days of exposure to 10:1 surface dose of TiO_2 nanotubes (Alexa 647, red), field-of-view is 70 x 70 x 30 µm.

Link to time-lapse

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190412_e07_t06_MH-S_CellMask_TiO2-Alexa647_10.1_2_days_xyt_11min_1s_is_1min.gif

Movie S36: Dynamics of macrophages (CellMask, green) after 2 days of exposure to 10:1 surface dose of TiO_2 nanotubes (Alexa 647, red), 1 second in movie corresponds to 1 minute real time.

Cell line	MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	150 nm	561nm	5%
NPs	TiO2 (Alexa647)	FOV (x,y)	70.1 µm	640nm	5%
exposure	10:1, 2 days	pixelsize (z)	500 nm	STED	
imaging	xyz confocal	FOV (z)	30 µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA 1.2)

Cell line	MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	150 nm	561nm	5%
NPs	TiO2 (Alexa647)	FOV (x,y)	70.1 µm	640nm	5%
exposure	10:1, 2 days	pixelsize (z)		STED	
imaging	xyt confocal	FOV (z)		filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	10 min	dwell time	10 µs
		number of frames	65	objective	60x wi (NA 1.2)

Experiment at time 4 days:

- 20190225_e02 m03 s03_dying MH-S_SR DPPE and TiO2_ATTO 594_ over 48h_1 to 10

- protocol:
  - MH-S cells were seeded @30% confluence in an Ibidi #1.5H µ-Dish
  - after 24 hours 35 mL freshly filtered 1 mg/mL TiO_2-40-ATTO594 in 100x dcb was added directly to the cells (in 400 µL medium) and mixed to achieve 1:1, 10:1 and 100:1 surface doses
  - 72 hours later, the cells were incubated with 1 µm StarRed-DPPE for 5 minutes at the room temperature, afterwards they were flushed with 1x400 mL LCIS (in order not to lose too many macrophages which are not completely adherent) and observed in 400 mL LCIS on the microscope stage heated at 37 °C

- analysis:
  - Green channel signal was multiplied by the factor of 1,5 and Gaussian Blur (0,5 pixel) was applied
  - Red channel was multiplied as much as the green in order to conserve colocalization signal and Unshrap Mask (3 pixels with weight 0,6) was applied to better visualise nanoparticle structure

Link to 3D

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190225_e02_m03_s03_dying_MH-S_SR_DPPE_and_TiO2_ATTO_594__over_48h_1_to_10_b.mp4

Movie S37:Macrophage (StarRed DPPE, green) after 72 h of exposure to 10:1 dose of TiO_2 nanotubes (ATTO594, red).

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190225_dying_MHS_SR_DPPE_and_TiO2_ATTO_594__over_48h_1_to_1.gif

Movie S38: Macrophage (StarRed DPPE, green) after 72 h of exposure to 1:1 dose of TiO_2 nanotubes (ATTO594, red).

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190225_e02_m02_s02_dying_MHS_SR_DPPE_and_TiO2_ATTO_594__over_48h_1_to_10.gif

Movie S39: Macrophage (StarRed DPPE, green) after 72 h of exposure to 10:1 dose of TiO_2 nanotubes (ATTO594, red).

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190225_e02_m03_s03_dying_MH-S_SR_DPPE_and_TiO2_ATTO_594__over_48h_1_to_10_b.mp4

Movie S40: Macrophage (StarRed DPPE, green) after 72 h of exposure to 10:1 dose of TiO_2 nanotubes (ATTO594, red).

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190225_e03_m01_s01_dying_MH-S_SR_DPPE_and_TiO2_ATTO_594__over_48h_1_to_100_agglomerats_of_NP_and_3D.gif

Movie S41: Macrophage (StarRed DPPE, green) after 72 h of exposure to 100:1 dose of TiO_2 nanotubes (ATTO594, red).

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/3D_72h_MH-S_SR_DPPE_&_TiO2_ATTO_594_dose_dependend_desintegration.mp4

Movie S42: Comparison of macrophages (StarRed DPPE, green) after 72 h of exposure to 1:1, 10:1, 10:1 and 100:1 dose of TiO_2 nanotubes (ATTO594, red).

Cell line	LA-4 (membrane, SR-DPPE)
NPs	TiO2_ATTO 594
exposure	1:10
imaging	xyz STED, @72h

pixelsize (x,y)	150 nm
FOV (x,y)	32x28 µm
pixelsize (z)	150 nm
FOV (z)	20 µm
imaging time	/
number of stacks	106

561nm	25%
640nm	40%
STED	23%
filter sets	605 nm – 625 nm, 400nm-780nm
dwell time	10 µs
objective	wi60x (NA1.2)

Experiment names

- Main experiment name:
  - **experiment at time 0h**
    - 2019_04_12/e05_t01_MH-S CellMask_xyt.msr
  - **experiment at time 2h**
    - 2019_04_12/e06_t03_MH-S CellMask TiO2-Alexa647_1.1 live_xyz xyt.msr
    - 2019_04_12/e06_t04_MH-S CellMask TiO2-Alexa647_1.1 live_xyz xyt.msr
    - 2019_04_12/e06_t06_MH-S CellMask TiO2-Alexa647_1.1 live_xyz xyt.msr
    - 2019_04_12/e06_t07_MH-S CellMask TiO2-Alexa647_1.1 live_xyz xyt.msr
    - 2019_04_12/e06_t08_MH-S CellMask TiO2-Alexa647_1.1 live_xyz xyt.msr
    - 2019_04_12/e06_t09_MH-S CellMask TiO2-Alexa647_1.1 live_xyz xyt.msr
    - 2019_04_12/e06_t10_MH-S CellMask TiO2-Alexa647_1.1 live_xyz xyt.msr
    - 2019_04_12/e06_t12_MH-S CellMask TiO2-Alexa647_1.1 live_xyz xyt.msr
    - 2019_04_12/e06_t13_MH-S CellMask TiO2-Alexa647_1.1 live_xyz xyt.msr

    - 2019_04_12/e06_t14_MH-S CellMask TiO2-Alexa647_1.1 live_xyz xyt.msr

- **experiment at time 2 days**
    - 2019_04_12/e07_t04_MH-S CellMask TiO2-Alexa647_10.1 2 days_xyz.msr

- **experiment at time 4 days**
    - 20190225_e02 m03 s03_dying MH-S_SR DPPE and TiO2_ATTO 594_ over 48h_1 to 10

- Supplement experiment name:

    - **experiment at time 0h**
        - 20190412/ e05_t01_MH-S CellMask_xyt.msr
        - 20190412/ e05_t04_MH-S CellMask_xyt.msr
        - 20190802/e03_s01_MH-S CMO 4 days nothing_happy MH-S.msr
        - 20190802/e03_s02_MH-S CMO 4 days nothing_happy MH-S.msr
        - 20190802/e03_s03_MH-S CMO 4 days nothing_happy MH-S.msr

    - **experiment at time 2h**
        - 2019_04_12/e06_t04_MH-S CellMask TiO2-Alexa647_1.1 live_xyz xyt.msr
        - 2019_04_12/e06_t06_MH-S CellMask TiO2-Alexa647_1.1 live_xyz xyt.msr
        - 2019_04_12/e06_t09_MH-S CellMask TiO2-Alexa647_1.1 live_xyz xyt.msr
        - 2019_04_12/e06_t13_MH-S CellMask TiO2-Alexa647_1.1 live_xyz xyt.msr

    - **experiment at time 2 days**
        - 2019_04_12/e07_t04_MH-S CellMask TiO2-Alexa647_10.1 2 days_xyz.msr
        - 20190712_MH-S NP_s03_t04_MH-S eating.msr
        - 20190712_MH-S NP_s03_t06_STED MH-S eating.msr
        - 20190712_MH-S NP_s04_t08_STED.msr
        - 20190712_MH-S NP_s07_t12_STED no cauliflowers.msr
        - 20190712_MH-S NP_s11_t16_glued MH-S.msr

    - **experiment at time 4 days**
        - 20190225_e01 m04 s05_dying MH-S_SR DPPE and TiO2_ATTO 594_ over 48h_1 to 1
        - 20190225_e02 m02 s02_dying MH-S_SR DPPE and TiO2_ATTO 594_ over 48h_1 to 10
        - 20190225_e03 m01 s01_dying MH-S_SR DPPE and TiO2_ATTO 594_ over 48h_1 to 100 agglomerates of NP and 3D

Controls and statistics

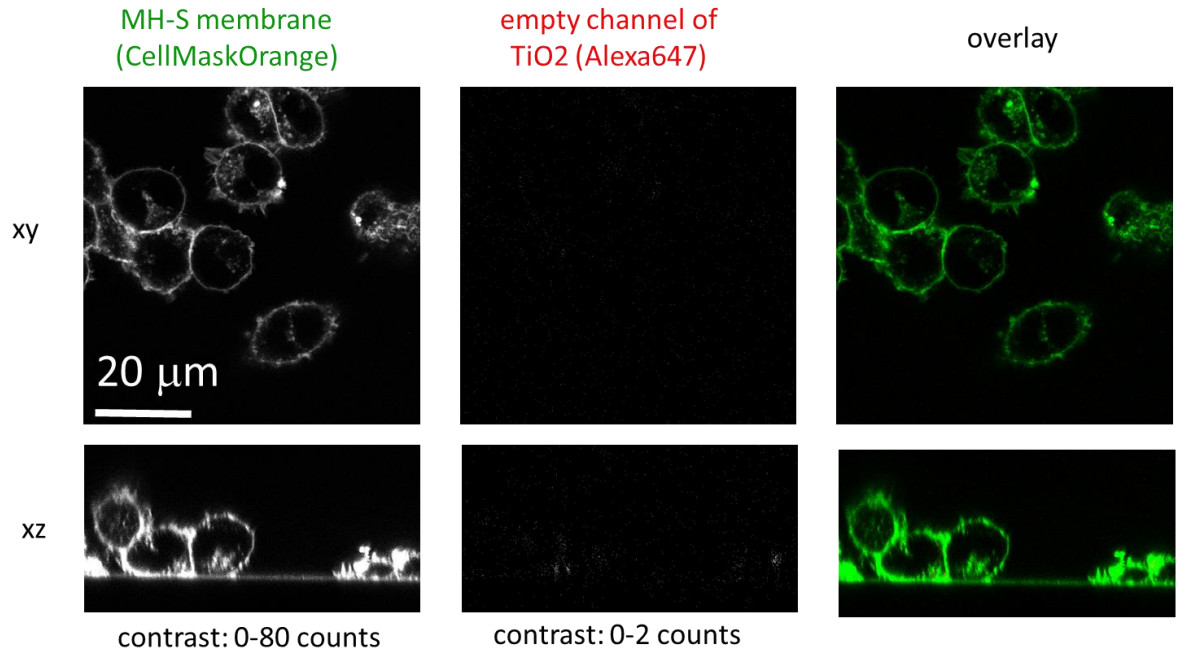
Non-exposed macrophages at time 0h:
Figure S159-Figure S161

Initial internalisation of nanomaterial in macrophages at time 0-2h:
Figure S162

Macrophages are full on nanomaterial at 2 days of exposure:
Figure S163-Figure S165

Experiment at time 0h

- 20190412_e05_t01



xy

Cell line	MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	100 nm	561nm	5%
NPs		FOV (x,y)	70.2 µm	640nm	5%
exposure		pixelsize (z)		STED	
imaging	xy confocal	FOV (z)		filter sets	605 nm – 625 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA1.2)

xz

Cell line	MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	100 nm	561nm	10%
NPs		FOV (x,y)	70.0 µm	640nm	10%
exposure		pixelsize (z)	131 nm	STED	
imaging	xz confocal	FOV (z)	34.9 µm	filter sets	605 nm – 625 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA1.2)

Figure S159: Non-exposed macrophages at time 0h

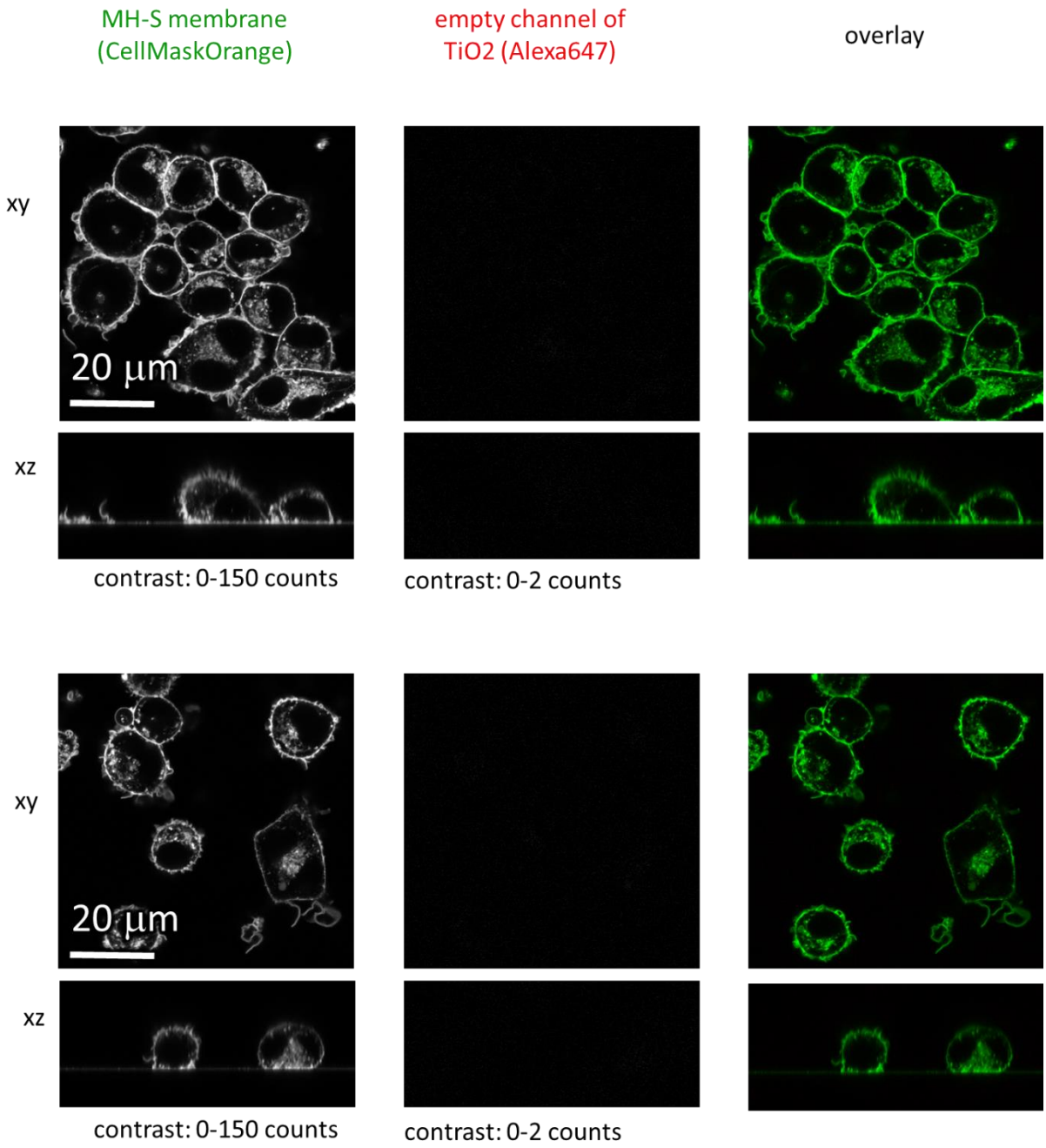
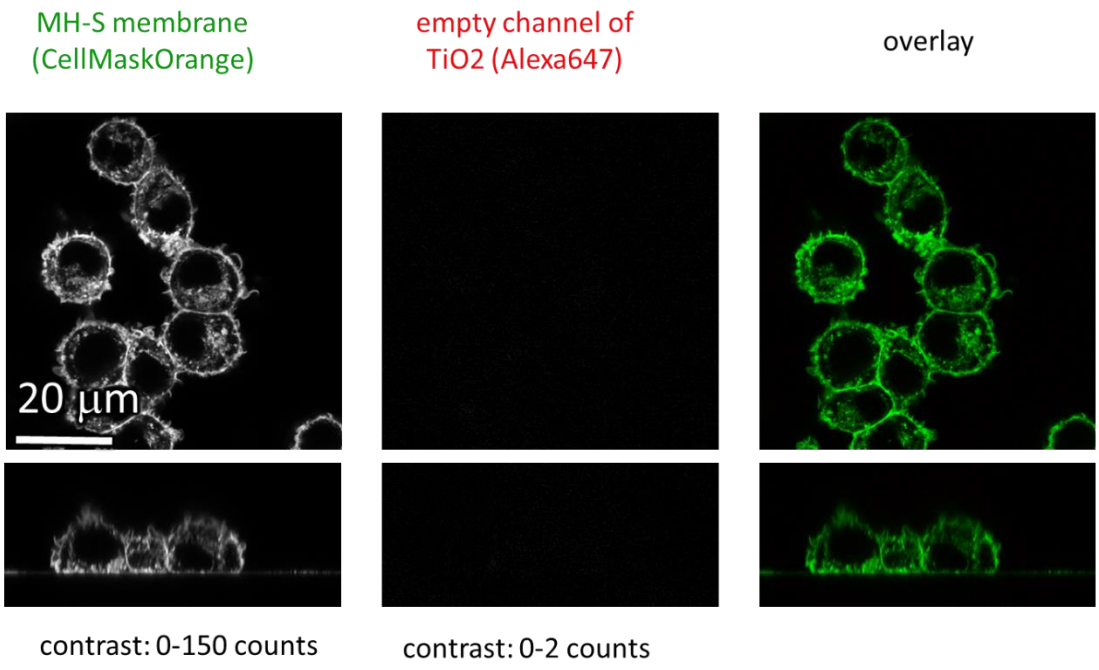


Figure S160: Non-exposed macrophages at time 0h



xy

Cell line	MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	50 nm	561nm	5%
NPs		FOV (x,y)	70.2 µm	640nm	5%
exposure		pixelsize (z)		STED	5%
imaging	xy confocal	FOV (z)		filter sets	605 nm – 625 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA1.2)

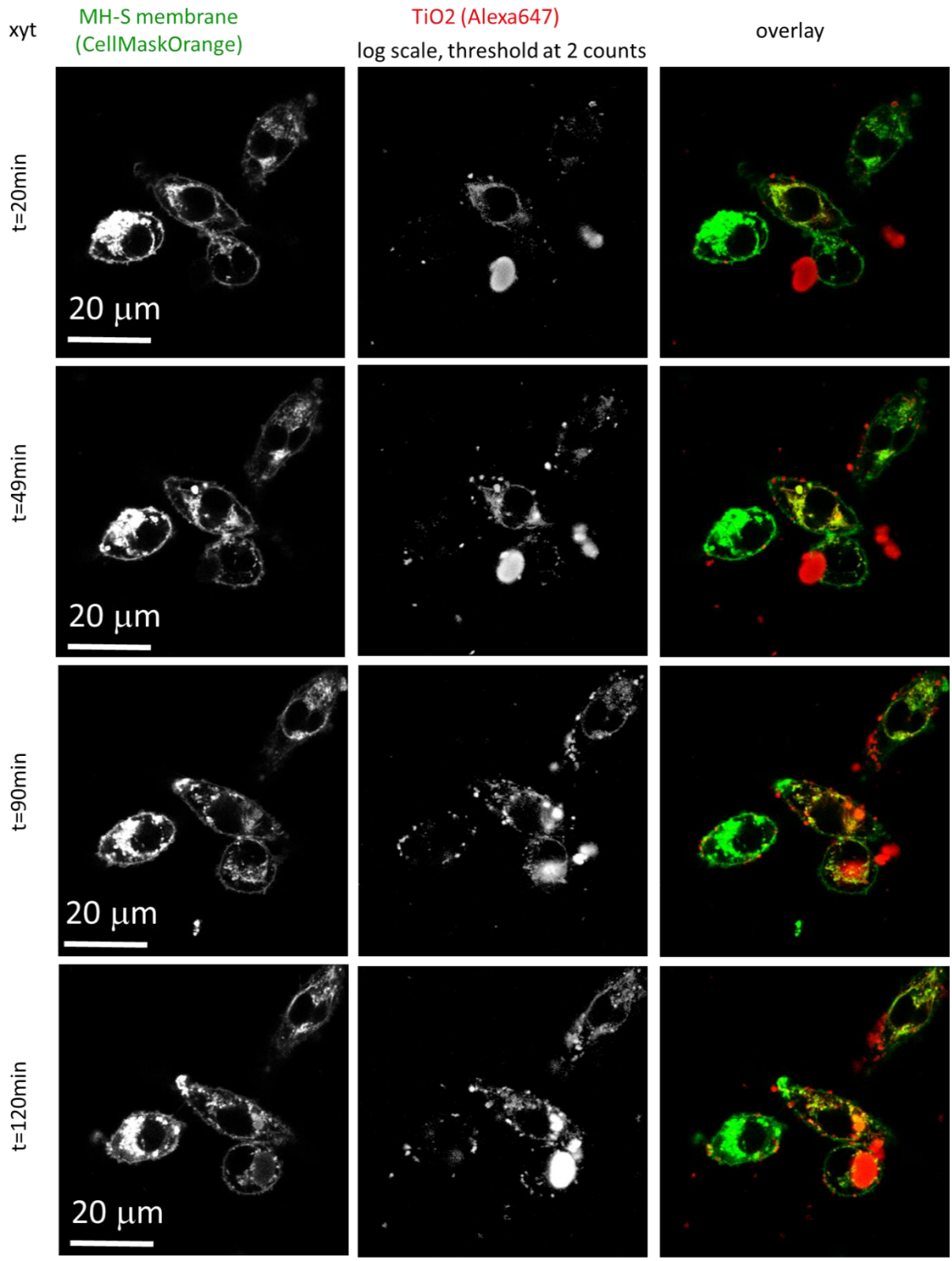
xz

Cell line	MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	50 nm	561nm	5%
NPs		FOV (x,y)	70.0 µm	640nm	5%
exposure		pixelsize (z)	50 nm	STED	5%
imaging	xz confocal	FOV (z)	30 µm	filter sets	605 nm – 625 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA1.2)

Figure S161: Non-exposed macrophages at time 0h

Experiment at time 0-2h

- 20190412/e06_t03…t13

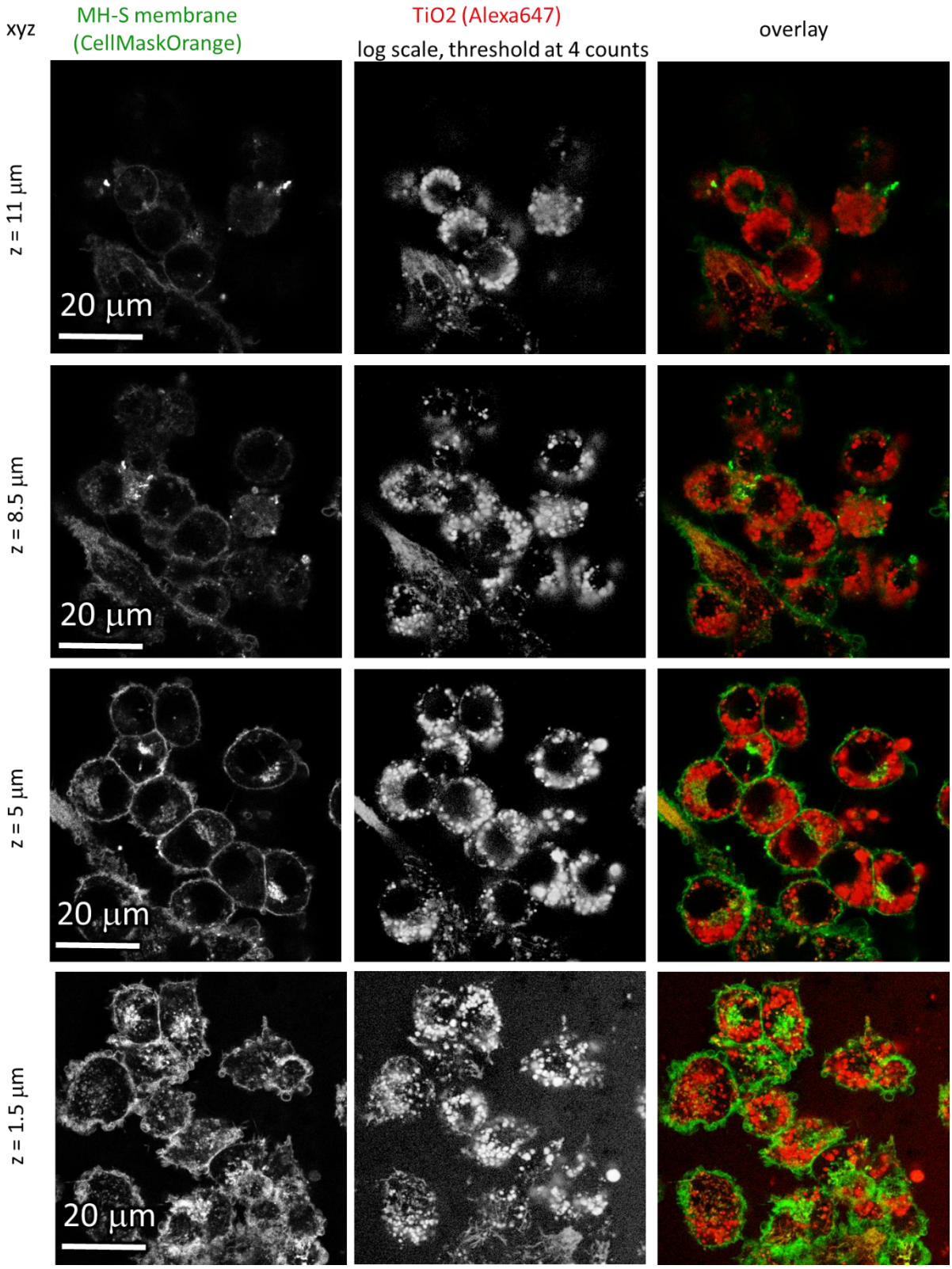

xyt
MH-S membrane
(CellMaskOrange)
TiO2 (Alexa647)
log scale, threshold at 2 counts
overlay
t=20min
t=49min
t=90min
t=120min
20 µm
20 µm
20 µm
20 µm

Cell line	MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	200 nm	561nm	5%
NPs	TiO2 (Alexa647)	FOV (x,y)	70.2 µm	640nm	5%
exposure	2:1, 0min – 2h	pixelsize (z)	500 nm	STED	
imaging	xyz confocal	FOV (z)	30 µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA 1.2)

Figure S162: Initial internalisation of nanomaterial in macrophages at time 0-2h

Experiment at time 2 days

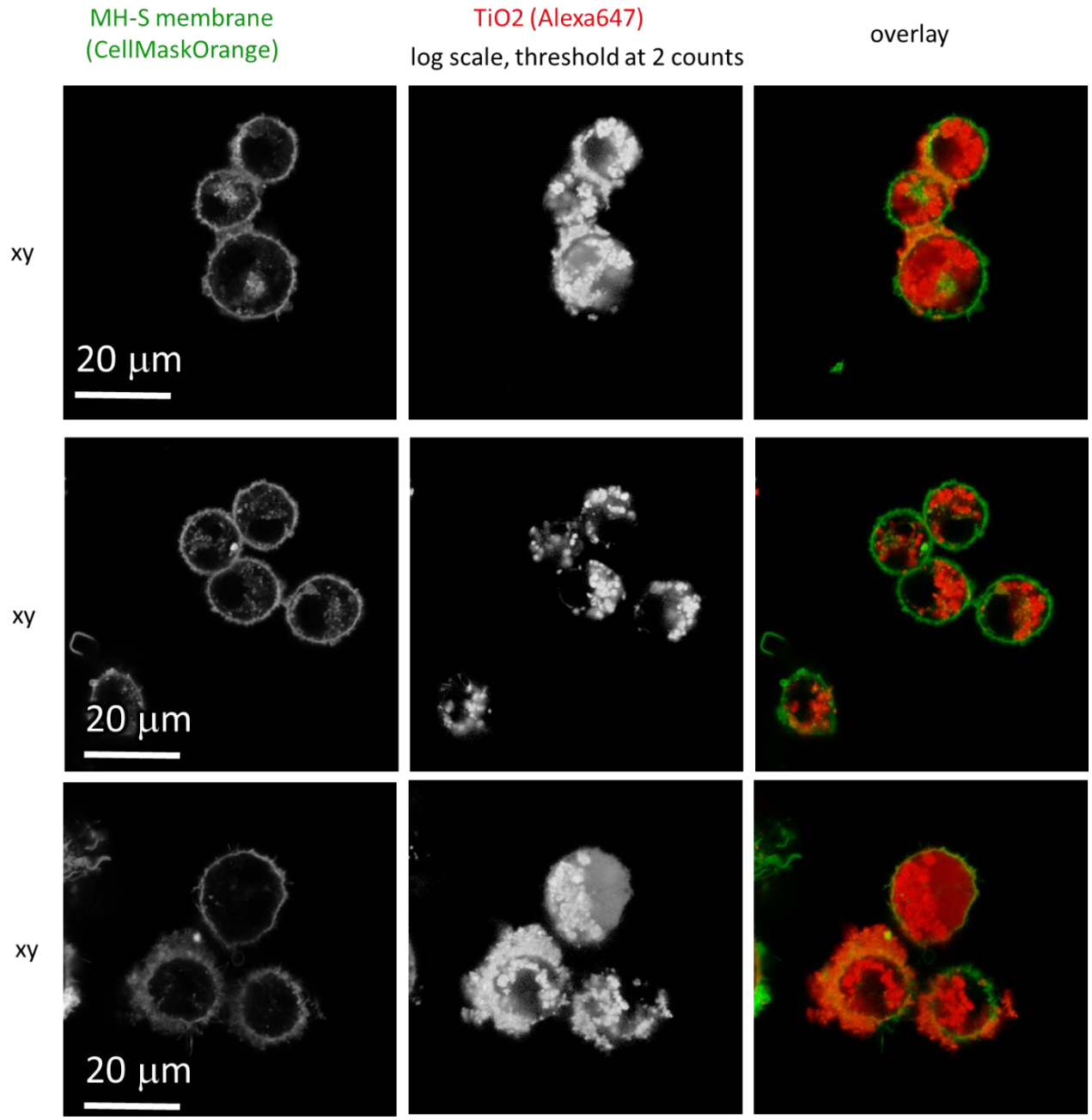
- 20190412/e07_t04


xyz
MH-S membrane
(CellMaskOrange)
TiO2 (Alexa647)
log scale, threshold at 4 counts
overlay
z = 11 µm
z = 8.5 µm
z = 5 µm
z = 1.5 µm
20 µm
20 µm
20 µm
20 µm

Cell line	MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	150 nm	561nm	5%
NPs	TiO2 (Alexa647)	FOV (x,y)	70.1 µm	640nm	5%
exposure	10:1, 2 days	pixelsize (z)	500 nm	STED	
imaging	xyz confocal	FOV (z)	30 µm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA 1.2)

Cell line	MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	150 nm	561nm	5%
NPs	TiO2 (Alexa647)	FOV (x,y)	70.1 µm	640nm	5%
exposure	10:1, 2 days	pixelsize (z)		STED	
imaging	xyt confocal	FOV (z)		filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	10 min	dwell time	10 µs
		number of frames	65	objective	60x wi (NA 1.2)

Figure S163: Macrophages are full on nanomaterial at 2 days of exposure

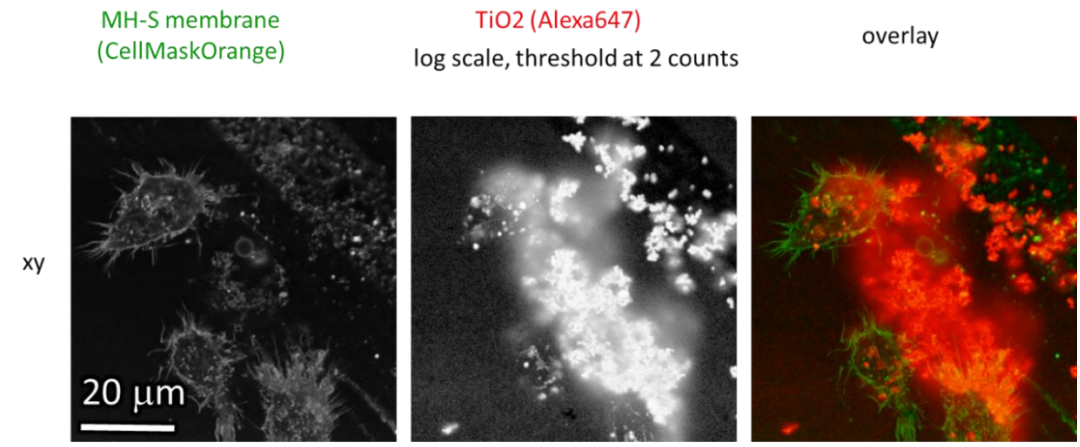


Cell line	MH-S (membrane, CellMaskOrange)
NPs	TiO2 (Alexa647)
exposure	10:1, 2 days
imaging	xy confocal

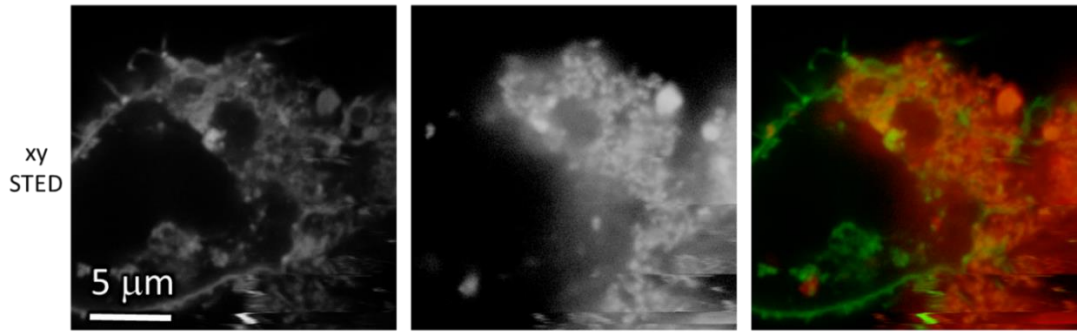
pixelsize (x,y)	100 nm
FOV (x,y)	70.0 µm
pixelsize (z)	
FOV (z)	
imaging time	
number of frames	

561nm	10%
640nm	10%
STED	
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	60x wi (NA 1.2)

Figure S164: Macrophages are full on nanomaterial at 2 days of exposure



Cell line	MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	100 nm	561nm	10%
NPs	TiO2 (Alexa647)	FOV (x,y)	70.0 µm	640nm	100%
exposure	10:1, 2 days	pixelsize (z)		STED	
imaging	xy confocal	FOV (z)		filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA 1.2)



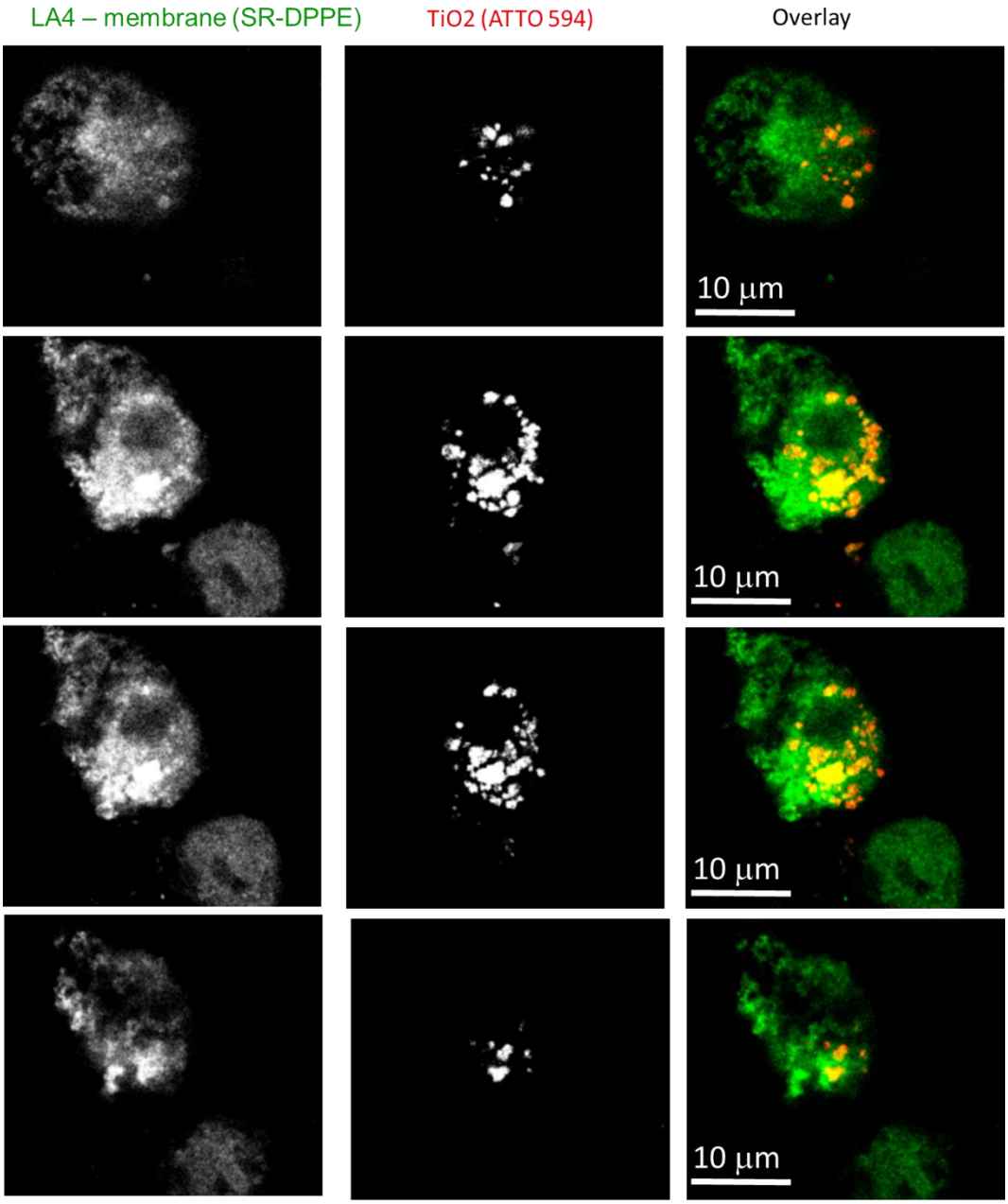
Cell line	MH-S (membrane, CellMaskOrange)	pixelsize (x,y)	30 nm	561nm	10%
NPs	TiO2 (Alexa647)	FOV (x,y)	20.0 µm	640nm	10%
exposure	10:1, 2 days	pixelsize (z)		STED	25%, 71% 3D STED
imaging	xy confocal	FOV (z)		filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time		dwell time	10 µs
		number of frames		objective	60x wi (NA 1.2)

Figure S165: Macrophages are full on nanomaterial at 2 days of exposure

Experiment at time 4 days

- 20190225_e02 m03 s03_dying MH-S_SR DPPE and TiO2_ATTO 594_ over 48h_1 to 10

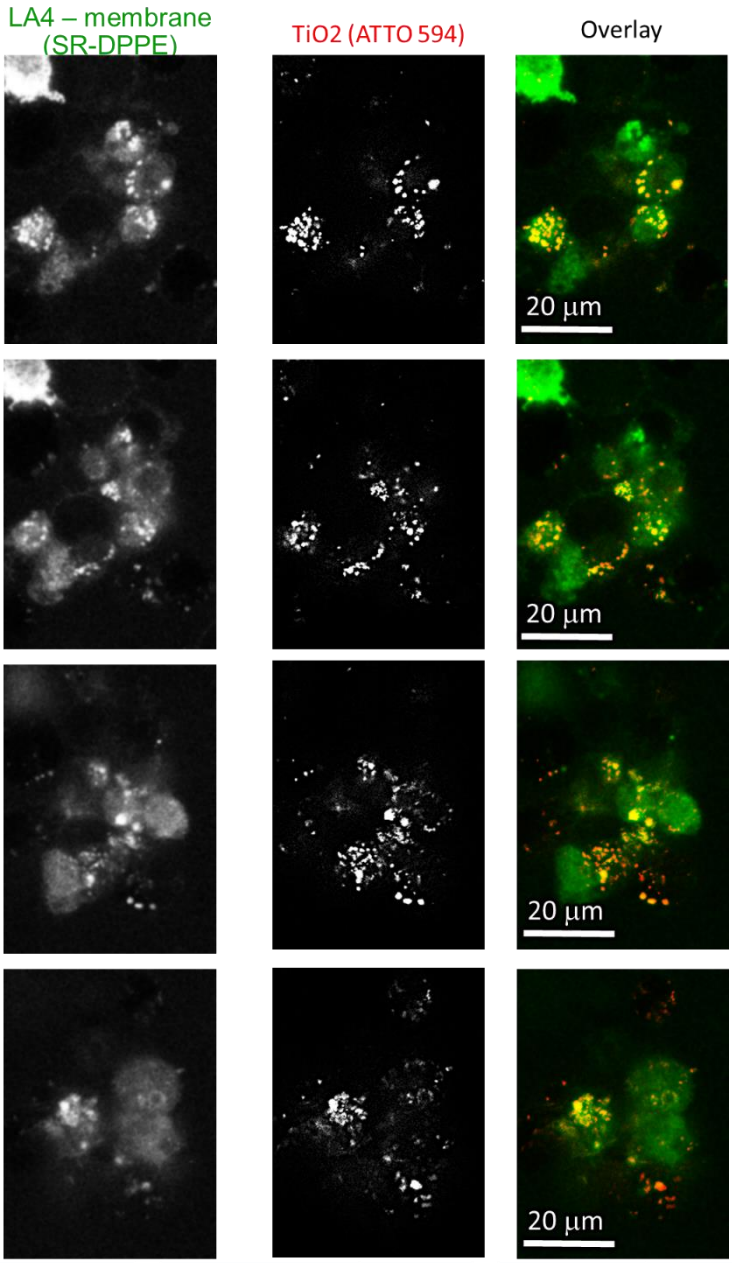
Macrophages exposed to different surface doses of TiO_2NTs.
Figure S166 1:1,
Figure S167-168 10:1 and
Figure S169 100:1 surface dose of TiO_2 nanotubes



Cell line	LA-4 (membrane, SR-DPPE)	pixelsize (x,y)	150 nm	561nm	25%
NPs	TiO2_ATTO 594	FOV (x,y)	32x28 µm	640nm	40%
exposure	1:10	pixelsize (z)	150 nm	STED	23%
imaging	xyz STED, @72h	FOV (z)	20 µm	filter sets	605 nm – 625 nm, 400nm-780nm
		imaging time	/	dwell time	10 µs
		number of stacks	106	objective	wi60x (NA1.2)

Figure S166: STED images of LA-4 in green and TiO_2 nanotubes in red channel. MH-S cells are labelled with Star Red – DPPE label for membrane. On figure are four representative XY plane slices from a whole Z-stack (3D). Macrophage (green) are devouring nanomaterial (red) which is seen as a colocalization of both signals in yellow color. When macrophages are full of nanomaterial we observe that they start to shring in size, dissintegrate and finally die. In this experiment surface dose of TiO_2 nanotubes is 10:1.

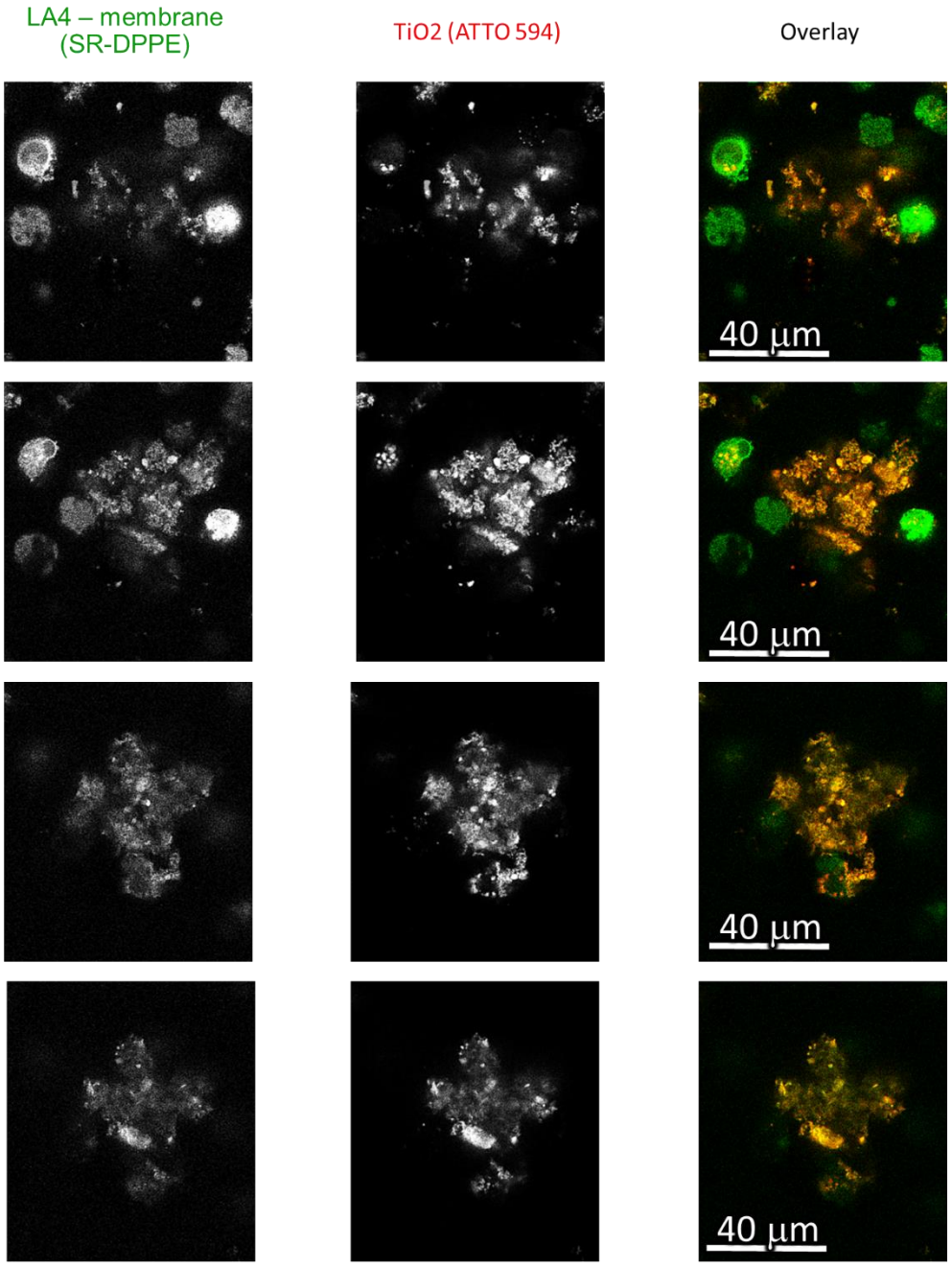
- 20190225_e01 m04 s05_dying MH-S_SR DPPE and TiO2_ATTO 594_ over 48h_1 to 1



Cell line	LA-4 (membrane, SR-DPPE)	pixelsize (x,y)	200 nm	561nm	25%
NPs	TiO2_ATTO 594	FOV (x,y)	47x65 µm	640nm	40%
exposure	1:1	pixelsize (z)	150 nm	STED	23%
imaging	xzz STED, @72h	FOV (z)	21 µm	filter sets	605 nm – 625 nm, 400nm-780nm
		imaging time	/	dwell time	10 µs
		number of stacks	140	objective	wi60x (NA1.2)

Figure S167: STED images of LA-4 in green and TiO_2 nanotubes in red channel. MH-S cells are labelled with Star Red – DPPE label for membrane. On figure are four representative XY plane slices from a whole Z-stack (3D). Macrophage (green) are devouring nanomaterial (red) which is seen as a colocalization of both signals in yellow color. When macrophages are full of nanomaterial we observe that they start to shring in size, dissintegrate and finally die. In this experiment surface dose of TiO_2 nanotubes is 1:1.

- 20190225_e02 m02 s02_dying MH-S_SR DPPE and TiO2_ATTO 594_ over 48h_1 to 10



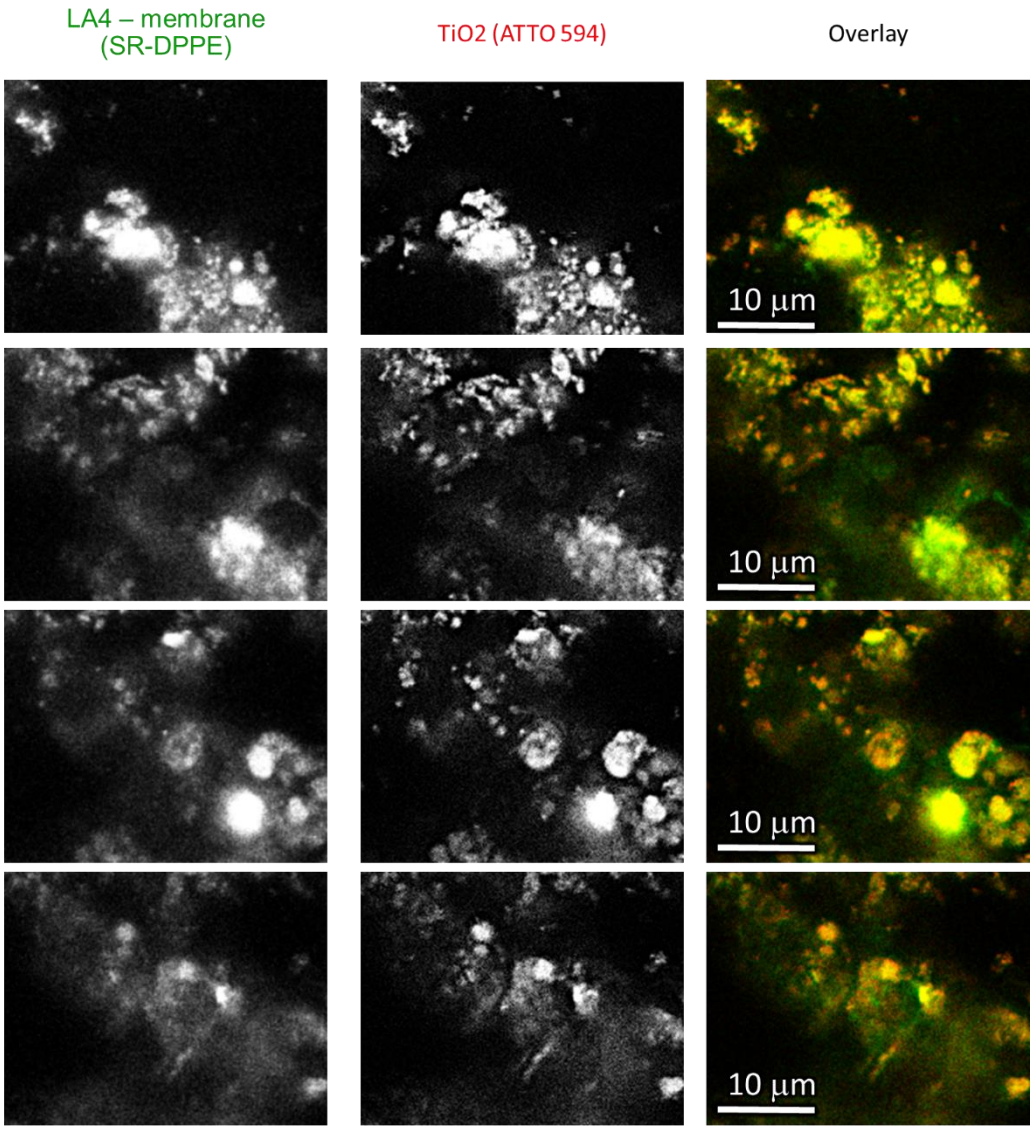
Cell line	LA-4 (membrane, SR-DPPE)
NPs	TiO2_ATTO 594
exposure	1:10
imaging	xzz STED, @72h

pixelsize (x,y)	260 nm
FOV (x,y)	80x90 µm
pixelsize (z)	150 nm
FOV (z)	25 µm
imaging time	/
number of stacks	87

561nm	25%
640nm	40%
STED	23%
filter sets	605 nm – 625 nm, 400nm-780nm
dwell time	10 µs
objective	wi60x (NA1.2)

Figure S168: STED images of LA-4 in green and TiO_2 nanotubes in red channel. MH-S cells are labelled with Star Red – DPPE label for membrane. On figure are four representative XY plane slices from a whole Z-stack (3D). Macrophage (green) are devouring nanomaterial (red) which is seen as a colocalization of both signals in yellow color. When macrophages are full of nanomaterial we observe that they start to shring in size, dissintegrate and finally die. In this experiment surface dose of TiO_2 nanotubes is 10:1.

- 20190225_e03 m01 s01_dying MH-S_SR DPPE and TiO2_ATTO 594_ over 48h_1 to 100 agglomerates of NP and 3D



Cell line	LA-4 (membrane, SR-DPPE)
NPs	TiO2_ATTO 594
exposure	1:100
imaging	xzz STED, @72h

pixelsize (x,y)	150 nm
FOV (x,y)	33x25 µm
pixelsize (z)	150 nm
FOV (z)	25 µm
imaging time	/
number of stacks	116

561nm	25%
640nm	40%
STED	23%
filter sets	605 nm – 625 nm, 400nm-780nm
dwell time	10 µs
objective	wi60x (NA1.2)

Figure S169: TED images of LA-4 in green and TiO_2 nanotubes in red channel. MH-S cells are labelled with Star Red – DPPE label for membrane. On figure are four representative XY plane slices from a whole Z-stack (3D). Macrophage (green) are devouring nanomaterial (red) which is seen as a colocalization of both signals in yellow color. When macrophages are full of nanomaterial we observe that they start to shring in size, dissintegrate and finally die. In this experiment surface dose of TiO_2 nanotubes is 1:100. Here macrophages are completely seald in a dense detritus of nanomaterial, live and dead macrophages resembling structures we've seen on the citoviva dark-field images (Fig.1a)..

Link to 3D

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190225_e02_m03_s03_dying_MH-S_SR_DPPE_and_TiO2_ATTO_594__over_48h_1_to_10_b.mp4

Movie S43: Macrophage (green) are devouring TiO_2 nanotubes (red), surface dose 10:1.

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190225_dying_MHS_SR_DPPE_and_TiO2_ATTO_594__over_48h_1_to_1.gif

Movie S44: Macrophage (green) are devouring TiO_2 nanotubes (red), surface dose 1:1.

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190225_e02_m02_s02_dying_MH-S_SR_DPPE_and_TiO2_ATTO_594__over_48h_1_to_10.gif

Movie S45: Macrophage (green) are devouring TiO_2 nanotubes (red), surface dose 10:1.

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190225_e03_m01_s01_dying_MH-S_SR_DPPE_and_TiO2_ATTO_594__over_48h_1_to_100_agglomerats_of_NP_and_3D.gif

Movie S46: Macrophage (green) are devouring TiO_2 nanotubes (red), surface dose 100:1.

S5e – Macrophages attacking epithelial cells

Main message

Several macrophages surround and attacked an epithelial cell in the middle (discernible by its bigger size) which is full of nanomaterial.

Supporting raw and analysed data:

Figure S170

Materials and methods

- Main Experiment:
  - LA-4 cells were seeded @60% confluence in an Ibidi #1.5H µ-Dish
  - MH-S were seeded at 20% confluence in an Ibidi #1.5H µ-Dish
  - after 24 hours cultures were mixed in a cocultures and left in the incubator for another 24h
  - After 48h 35 µL freshly filtered 1 mg/mL TiO_2-40-ATTO 594 in 100x dcb was added directly to the cells (in 400 µL medium) and mixed to achieve 10:1 surface dose
  - Next day, cells were incubated with 1 um Star Red-DPPE for 10-15 minutes 2 hours in an incubator at 37 °C, 5% CO2
  - afterwards they were washed with 1x400 µL LCIS and observed in 400 µL LCIS at microscope stage heated on 37 °C

- Images analysed with ImageJ
  - Signal was multiplied and 0,5 pix Gaussian blur was applied Gaussian Blur 0.5 pix
  - Red channel has been analysed in the same way, the only difference being is the sharpening algorithm (σ = 3, c = 0,5) to more accentuate the nanomaterial

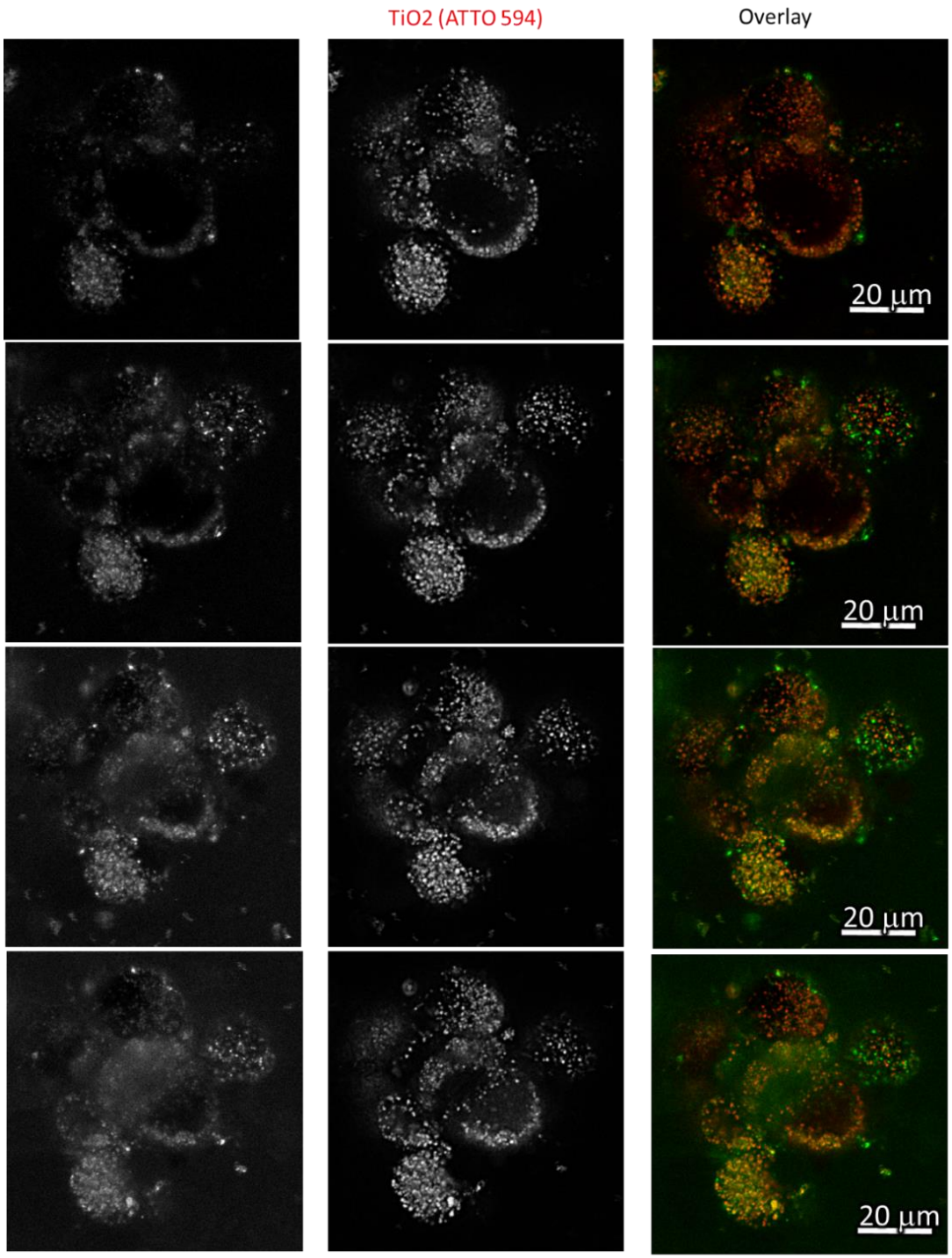
Experimental names

- Main Experiment:
  - 20190423_e01 m02 s02_LA-4 and MH-S_SR DPPE_1 and 10 to 1_ TiO_2 ATTO 594_ TL1
  - 20190423_e01 m02 s02_LA-4 and MH-S_SR DPPE_1 and 10 to 1_ TiO_2 ATTO 594_ TL2

Controls and statistics

Macrophages are attacking a LA-4 cell:

Figure S170



Cell line	LA-4 & MHS(membrane, SR-DPPE)	pixelsize (x,y)	80 nm	561nm	6%
NPs	TiO2 (ATTO 594)	FOV (x,y)	55 um	640nm	19%
exposure	10:1, 48h-72h	pixelsize (z)	/ nm	STED	20%
imaging	xyt STED, 0h-1h	FOV (z)	/ mm	filter sets	605 nm – 625 nm, 650 nm – 720 nm
		imaging time	2 hours	dwell time	10 ms
		number of frames	364	objective	wi60x (NA1.2)

Figure S170: STED images of LA-4 and MH-S in green and TiO_2 nanotubes in red channel. Cells are stained with Star Red – DPPE label for membrane. Four time points from a time lapse video. Five macrophages are surrunding and attacking one LA-4 cell, eventually braking the integrity on the LA-4 cell.

Link to time-lapse

- *http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190423_e01_m02_s02_LA-4_and_MH-S_SR_DPPE_1_and_10_to_1__TiO_2_ATTO_594__TL1_25_us.gif*

Movie S47: Time-lapse video of five macrophages (green, Star Red DPPE) surrunding and attacking one LA-4 cell, eventually braking the integrity on the LA-4 cell.

S5f – Macrophages attacking another macrophage

Main message

Macrophage is approaching and engulfing another macrophage which is completely filled with nanomaterial is already apoptotic and shrinking. Event takes place on the surface of the LA-4 cell.

Supporting raw and analysed data:

Figure S171

Materials and methods

- Main experiment:
  - LA-4 cells were seeded @30% confluence in an Ibidi #1.5H µ-Dish
  - MH-S cells were seeded @30% confluence in a separate Ibidi #1.5H µ-Dish
  - After 24 hours of separate incubation, LA-4 were labelled with 0,1 µM SirActin and MH-S were labelled with <1 µM cell mask and mixed together. Growth media for cocultures was mixture of F12K and RPMI-1640 in 1:1 ratio
  - after 48 hours 35 mL freshly filtered 1 mg/mL TiO_2-40-ATTO 594 in 100x dcb was added directly to the cells (in 400 µL medium) and mixed to achieve 10:1 surface dose and incubated overnight in the incubator at 37 °C and 5% CO2
  - Next day cells were flushed with 1x400 µL of LCIS and imaged on heated microscope stage in LCIS (37 °C)

- Analysis was performed in the ImageJ software:
  - Signal was multiplied in order to get adequate visibility for 3D rendering. Signal of stacks of images containing MH-S was multiplied 2x more because signal intensity was not the same. MH-S and LA-4 have been labeled with different labels and they did not yield the same number of counts on our detectors.
  - Additionaly 1 pixel Gaussian Blur was applied in a green channel and sharpening algorithm ($\sigma = 2.5$, $c = 0.6$) in the red channel

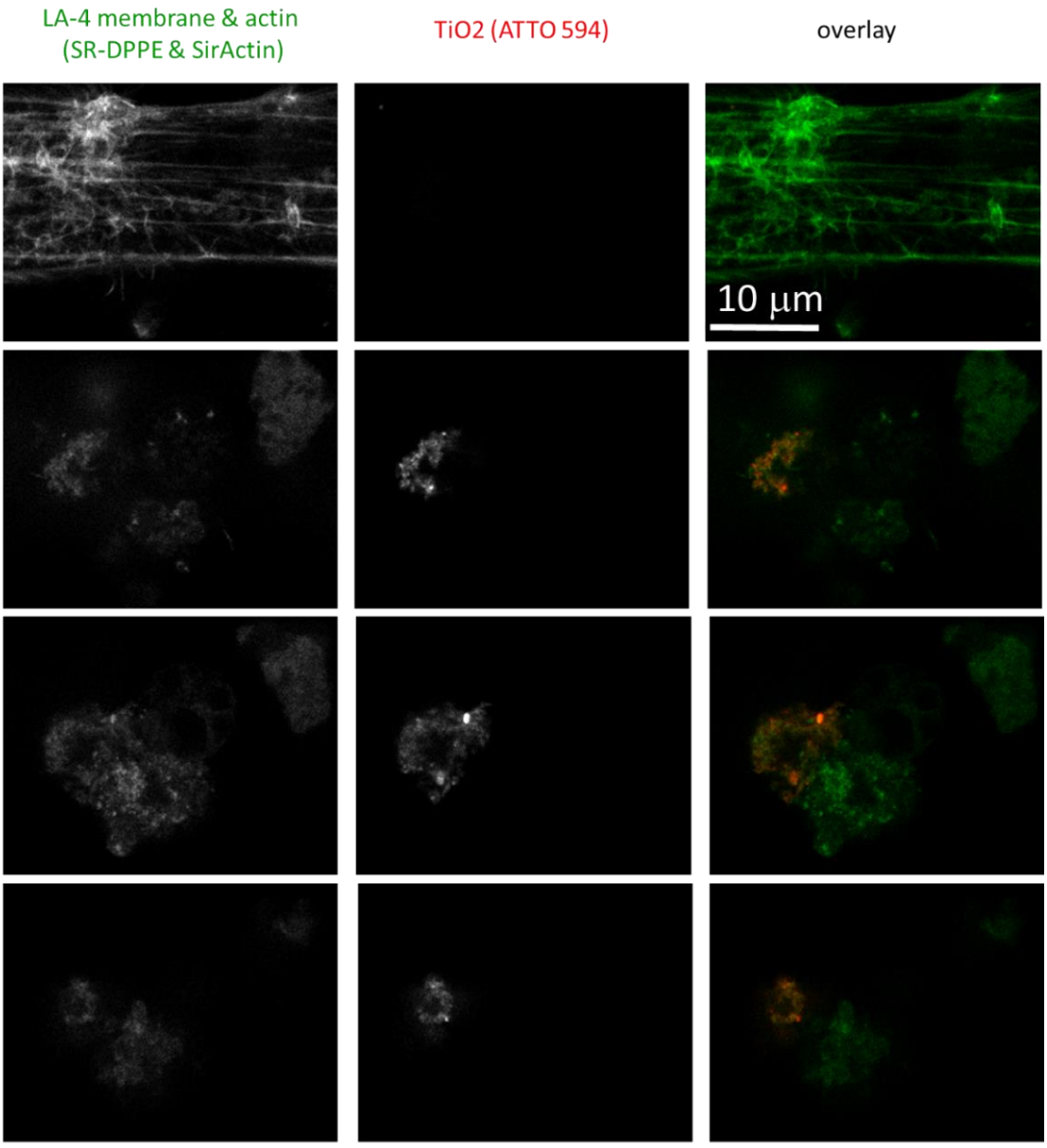
Experimental names

- Main experiment:
  - 20190204_LA-4_SA and MH-S_CM coculture_10 to 1 TiO2_ATTO 594_MH-S attacking another MH-S_3D

Controls and statistics

Macrophage devouring another macrophage, completely full of nanomaterial, on the top pf the LA-4 cell:

Figure S171



Cell line	LA-4 (actin, Sir Actin) & MHS (membrane, CellMaskOrange)
NPs	TiO2 (ATTO 594)
exposure	10:1, 48h-55h
imaging	xyt STED, 55h

pixelsize (x,y)	100 nm
FOV (x,y)	31 x 24 µm
pixelsize (z)	120 nm
FOV (z)	12 µm
imaging time	
number of z-sections	99

561nm	15%
640nm	11%
STED	9%
filter sets	605 nm – 625 nm, 650 nm – 720 nm
dwell time	10 µs
objective	60wix (NA1.2)

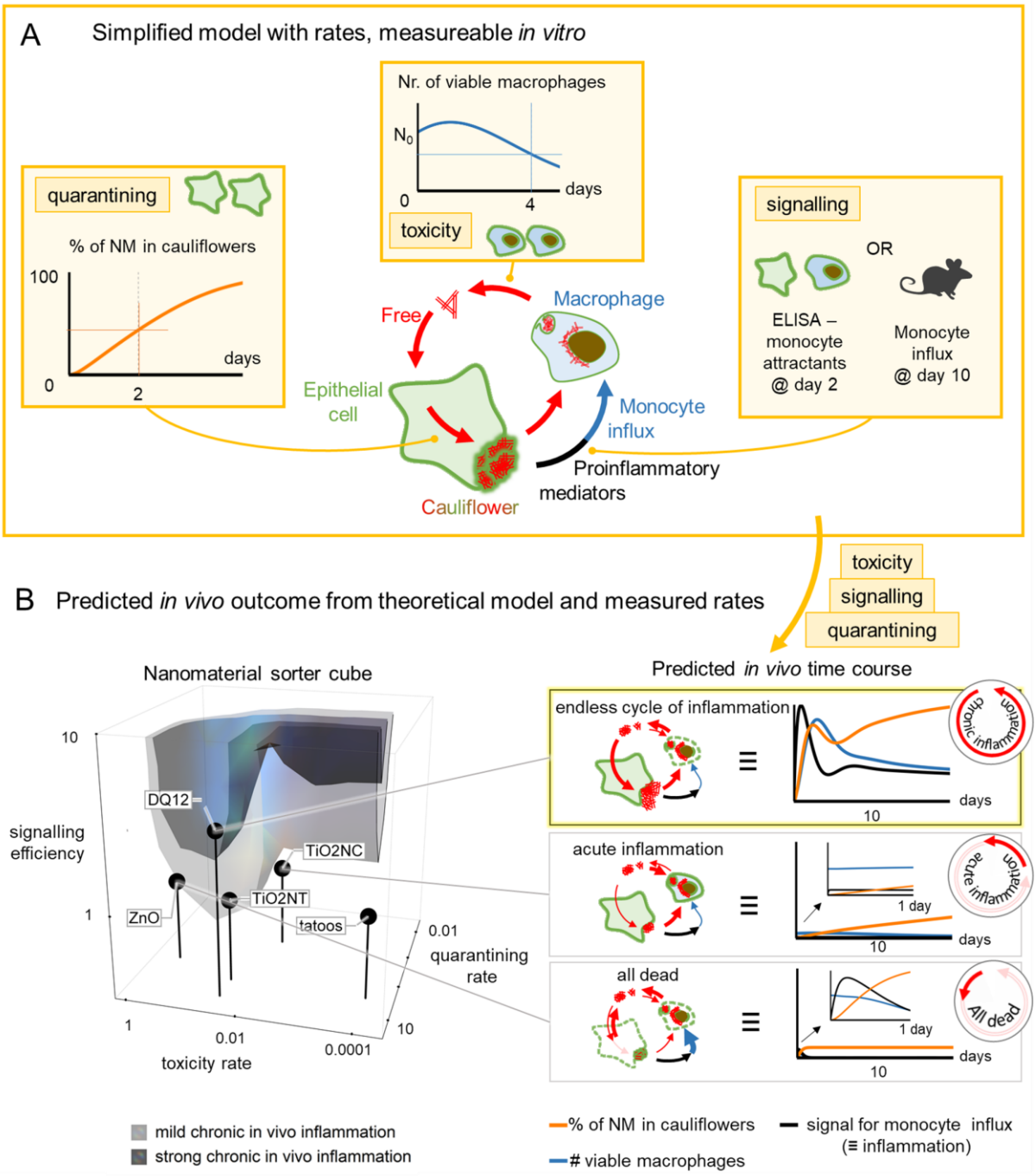
Figure S171: STED images of LA-4 and MH-S in green and TiO_2 nanotubes in red channel. LA-4 cells are stained with Sir-Actin (green cytoskeleton filaments) and MH-S are labelled with Star Red – DPPE label for membrane. On figure are four representative XY plane slices from a whole Z-stack (3D). One macrophage (green) on top of the LA-4 cell (green filaments) is devouring another macrophage (green, red, yellow colocalization) which is full of nanomaterial and possibly dying.

Link to 3D

- http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/20190204_LA-4_SA_and_MH-S_CM_coculture_10_to_1_TiO2_ATTO_594_MH-S_attacking_another_MH-S_3D.mp4

Movie S48: Macrophage (CellMask Orange, green) attacking another macrophage full of TiO_2 nanotubes (Atto 594, red) on top of epithelial cell.

S6 – Towards predictive toxicology



Main message

Based on the complex scheme in Figure5a, we designed a theoretical model of the events following exposure to nanomaterial with the least complexity possible (S6a). The rates and parameters in the model can be measured *in vivo* or in *in vitro* systems for any desired nanomaterial (S6a). Based on the time evolution of the theoretical model, outcomes such as chronic and acute inflammation may be predicted for a wide variety of nanomaterials (S6b).

S6a – Model of chronic inflammation following nanomaterial exposure and determination of its parameters

Model of chronic inflammation following nanomaterial exposure

Main message

The theoretical model of chronic inflammation following nanomaterial exposure is described by a series of differential equations, describing the events observed in *in vitro* and *in vivo* experiments in this work. This minimal-complexity *in vivo* model consists of 6 variables (surface of nanomaterial in epithelial cells *npLA4*, in cauliflowers *npCF*, in macrophages *npMHS* and freely-floating nanomaterial *npFree*, surface of macrophages *sMHS* and surface of epithelial cells *sLA4*), 4 fixed parameters which are calibrated for each model system and later locked (endocytosis rate *endo*, rate of cauliflower endocytosis *endo*cfuEff*, delay between cauliflower production and signalling for macrophage influx *delay*, and epithelial cell replication rate *LA4Rep*) and 3 nanomaterial-associated parameters (cauliflower formation rate *cff*, signalling efficiency *signEff*, and toxicity *tox*). Separate *in vitro* models were obtained from the *in vivo* model by swapping the macrophage influx with macrophage replication and leaving out non-existent cells for monocultures.

The system of equations was solved numerically using Wolfram Mathematica 12.0, licence L5063-5112 to obtain the time evolution of the model. The same software was also used for visualization of the results.

Supporting material:

Figure S172 - Figure S175

Rate equations and dynamic evolution of *in vivo* system and *in vitro* monocultures and cocultures:

In vivo model

rate equations:

$$D[npFree[t], t] == -endo\ sLa4[t] * npFree[t] - endo\ sMhs[t] * npFree[t] + tox\ npMhs[t] * npMhs[t] / sMhs[t] + tox\ npLa4[t] * npLa4[t] / sLa4[t]$$

change of free NM | free NM endocytosed by epithelial cells | free NM endocytosed by macrophages | released from dead macrophages | released from dead epithelial cells

$$D[npLa4[t], t] == endo\ sLa4[t] * npFree[t] - cff\ sLa4[t]\ Tanh[npLa4[t] / (sLa4[t] * cff * timecf)] - tox\ npLa4[t] * npLa4[t] / sLa4[t]$$

change of NM in epithelial cells | free NM endocytosed by epithelial cells | excreted into cauliflowers by epithelial cells | released from dead epithelial cells

$$D[npCF[t], t] == cff\ sLa4[t]\ Tanh[npLa4[t] / (sLa4[t] * cff * timecf)] - endo * cfuEff\ sMhs[t] * npCF[t]$$

change of NM in cauliflowers | excreted into cauliflowers by epithelial cells | cauliflowers endocytosed by macrophages

$$D[npMhs[t], t] == endo\ sMhs[t] * npFree[t] + endo * cfuEff\ sMhs[t] * npCF[t] - tox\ npMhs[t] * npMhs[t] / sMhs[t]$$

change of NM in macrophages | free NM endocytosed by macrophages | cauliflowers endocytosed by macrophages | released from dead macrophages

$$D[sMhs[t], t] == -tox\ npMhs[t] + signalEff\ cff\ sLa4[t - delay]\ Tanh[npLa4[t - delay] / (sLa4[t - delay] * cff * timecf)] * macrophageSurface$$

change of number of macrophages | dying macrophages | influx of new macrophages, signalled by cauliflower production (increased lipid production) in epithelial cells at an earlier time

$$D[sLa4[t], t] == -tox\ npLa4[t] + la4Rep\ sLa4[t]\ (1 - sLa4[t] / la4Max)$$

change of number of epithelial cells | dying epithelial cells | replication of epithelial cells

initial conditions:

```
npFree[0] = exposureSurfaceDose = 10;

npLa4[0] = 0,  npLa4[t /; t < 0] = 0,

npCF[0] = 0,

npMhs[0] = 0,

sMhs[0] = macrophageSurface = N[1 / 40];

sLa4[0] = 1    sLa4[t /; t < 0] = 1,

la4Max = 1;
```

At t = 0, we add nanomaterial to fully confluent epithelial cells and macrophages. The ratio surface of nanomaterial : surface of epithelial cells : surface of macrophages = 10 : 1 : 1/40

variables:

npFree[t] : surface of free-floating nanomaterial, normed to the surface of epithelial cells at t = 0
npLa4[t] : total surface of nanomaterial inside epithelial cells, normed to the surface of epithelial cells at t = 0
npCF[t] : surface of nanomaterial in cauliflowers, normed to the surface of epithelial cells at t = 0
npMhs[t] : total surface of nanomaterial inside macrophages, normed to the surface of epithelial cells at t = 0
sMhs[t] : surface of macrophages, normed to the surface of epithelial cells at t = 0
sLa4[t] : surface of epithelial cells, normed to the surface of epithelial cells at t = 0
t : time, measured in days

parameters – rate coefficients

endo : rate of endocytosis = 1
tox : toxicity of nanomaterial
cff : rate of cauliflower formation
timecf : norming factor for exocytosis saturation = 1
cfuEff : rate of cauliflower endocytosis compared to endocytosis of free NM = 1
signalEff : signalling efficiency – how many macrophages are infiltrated by the released signal
delay : delay between signal excretion and macrophage infiltration = 0.1
La4Rep : replication rate of epithelial cells = 0.3

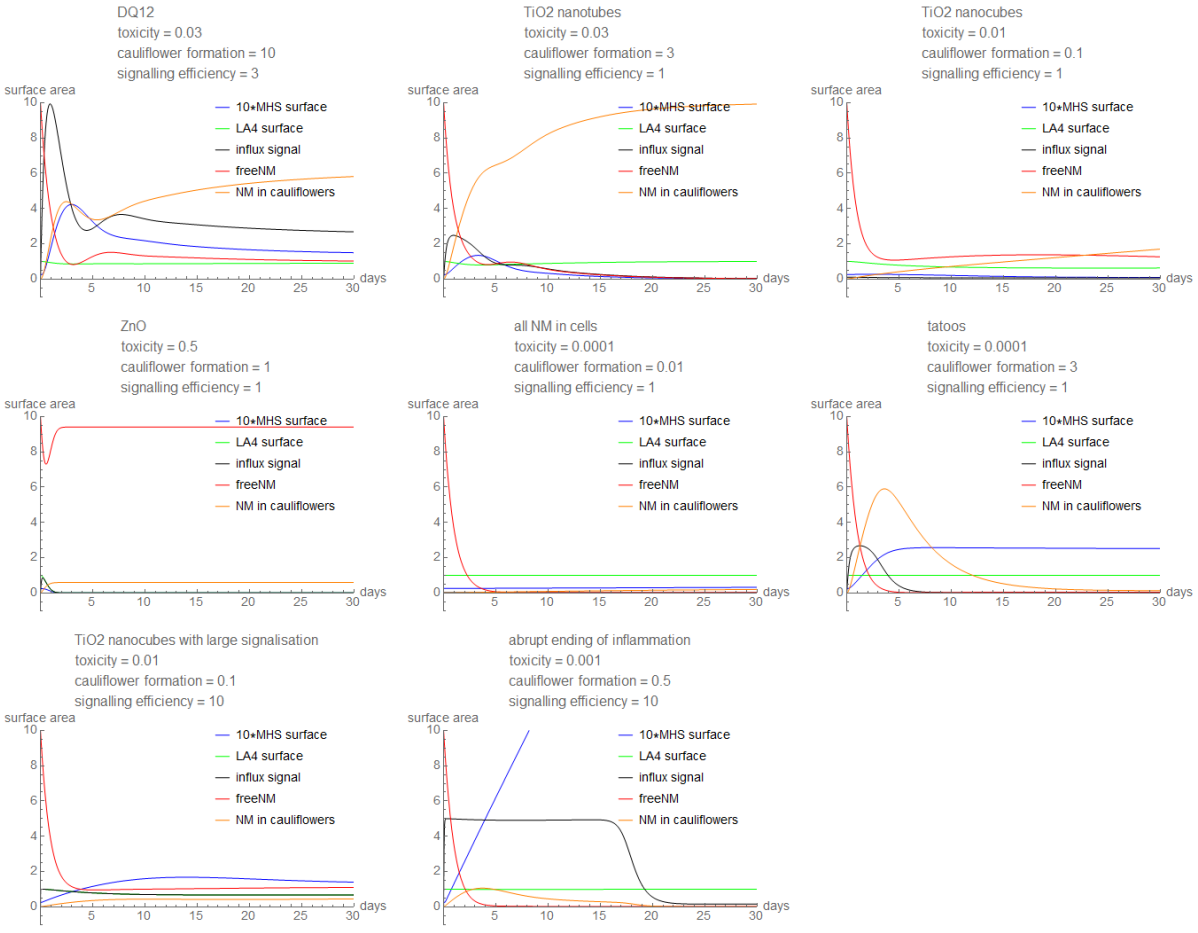


Figure S172: Time evolution of the in vivo theoretical model for 8 different nanomaterials.

In vitro coculture model

For the descriptions of the terms see the *in vivo* model - the rate equations are similar to the *in vivo* model, with some terms being left out and MH-S influx is swapped with MH-S replication.

rate equations:

```
enacbe[{endo_, tox_, cff_, timecf_, cfuEff_, delay_, signalEff_(*signal efficiancy*), la4Rep_(*replication rate of LA4*)}] :=
 {D[npFree[t], t] == -endo sLa4[t] * npFree[t] - endo sMhs[t] * npFree[t] + tox npMhs[t] * npMhs[t] / sMhs[t] + tox npLa4[t] * npLa4[t] / sLa4[t],

  D[npLa4[t], t] == endo sLa4[t] * npFree[t] - cff sLa4[t] Tanh[npLa4[t] / (sLa4[t] * cff * timecf)] - tox npLa4[t] * npLa4[t] / sLa4[t],

  D[npCF[t], t] == cff sLa4[t] Tanh[npLa4[t] / (sLa4[t] * cff * timecf)] - endo * cfuEff sMhs[t] * npCF[t] ,

  D[npMhs[t], t] == endo sMhs[t] * npFree[t] + endo * cfuEff sMhs[t] * npCF[t]  - tox npMhs[t] * npMhs[t] / sMhs[t],

  D[sMhs[t], t] == -tox npMhs [t] + la4Rep sMhs[t] (1 - sMhs[t] / la4Max),

  D[sLa4[t], t] == -tox npLa4[t] + la4Rep sLa4[t] (1 - sLa4[t] / la4Max),

  sLa4[t /; t < 0] == 1,
  npLa4[t /; t < 0] == 0,
```

initial conditions:

```
npFree[0] = exposureSurfaceDose = 10;

npLa4[0] == 0,  npLa4[t /; t < 0] == 0,

npCF[0] == 0,

npMhs[0] == 0,

sMhs[0] == macrophageSurface = N[1 / 40];

sLa4[0] == 1    sLa4[t /; t < 0] == 1,

la4Max = 1;
```

At t = 0, we add nanomaterial to fully confluent epithelial cells and macrophages. The ratio

surface of nanomaterial : surface of epithelial cells : surface of macrophages = 10 : 1 : 1/40

variables:

- npFree[t] : surface of free-floating nanomaterial, normed to the surface of epithelial cells at t = 0
- npLa4[t] : total surface of nanomaterial inside epithelial cells, normed to the surface of epithelial cells at t = 0
- npCF[t] : surface of nanomaterial in cauliflowers, normed to the surface of epithelial cells at t = 0
- npMhs[t] : total surface of nanomaterial inside macrophages, normed to the surface of epithelial cells at t = 0
- sMhs[t] : surface of macrophages, normed to the surface of epithelial cells at t = 0
- sLa4[t] : surface of epithelial cells, normed to the surface of epithelial cells at t = 0
- t : time, measured in days

parameters – rate coefficients

- *endo* : rate of endocytosis = 1
- ***tox*** : toxicity of nanomaterial
- ***cff*** : rate of cauliflower formation
- *timecf* : norming factor for exocytosis saturation = 1
- *cfuEff* : rate of cauliflower endocytosis compared to endocytosis of free NM = 1
- ***signalEff*** : signalling efficiency – how many macrophages are infiltrated by the released signal
- *delay* : delay between signal excretion and macrophage infiltration = 0.1
- *La4Rep* : replication rate of epithelial cells = 0.3

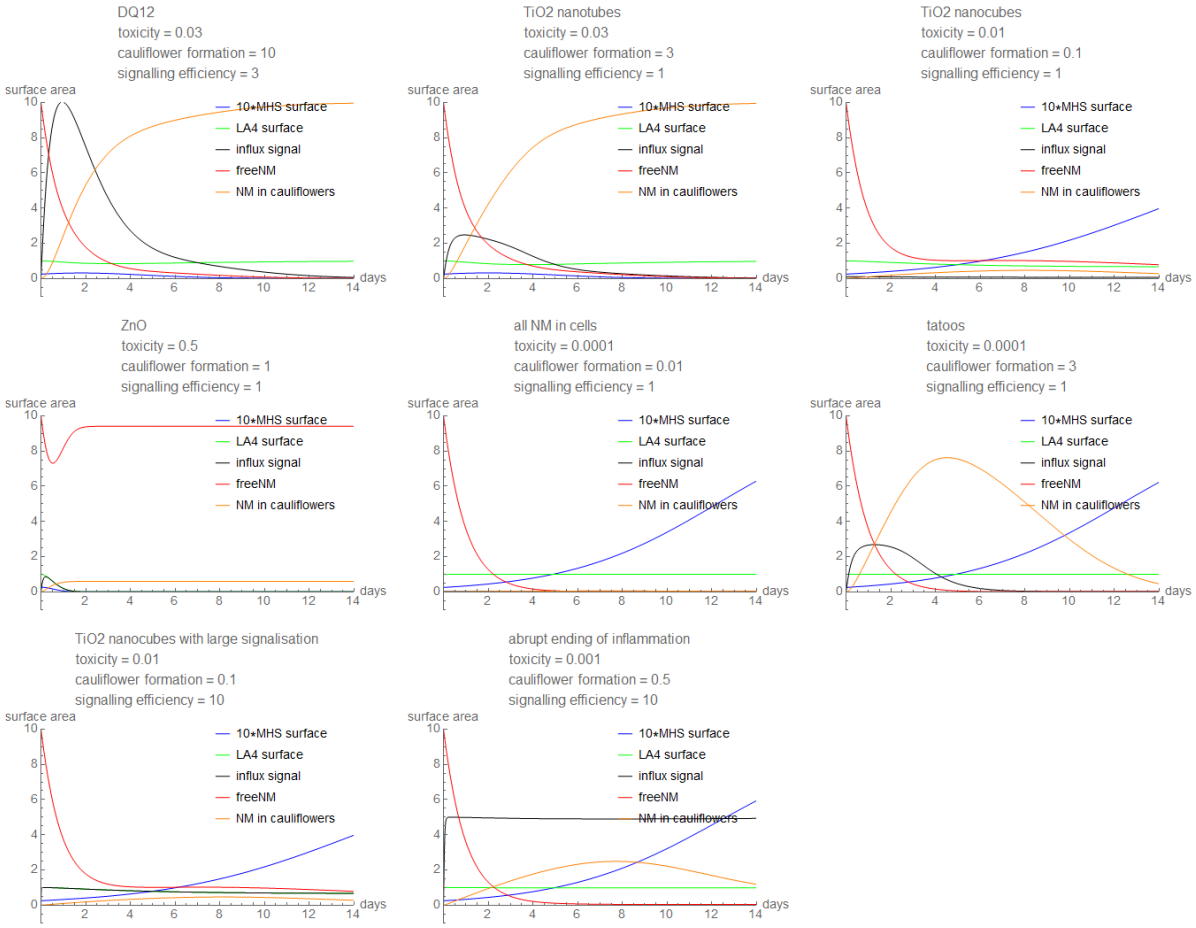


Figure S173: Time evolution of the in vitro coculture theoretical model for 8 different nanomaterials.

In vitro LA-4 monoculture model

For the descriptions of the terms see the *in vivo* model - the rate equations for LA-4 monoculture are similar to the *in vivo* model, with some terms being left out.

rate equations:

```
D[npFree[t], t] == -endo sLa4[t] * npFree[t] + tox npLa4[t] * npLa4[t] / sLa4[t],

D[npLa4[t], t] == endo sLa4[t] * npFree[t] - cff sLa4[t] Tanh[npLa4[t] / (sLa4[t] * cff * timecf)] - tox npLa4[t] * npLa4[t] / sLa4[t],

D[npCF[t], t] == cff sLa4[t] Tanh[npLa4[t] / (sLa4[t] * cff * timecf)] ,

D[sLa4[t], t] == -tox npLa4[t] + la4Rep sLa4[t] (1 - sLa4[t] / la4Max),
```

initial conditions:

```
npFree[0] == exposureSurfaceDose = 10;

npLa4[0] == 0,

npCF[0] == 0,

sLa4[0] == 1

la4Max = 1;
```

At t = 0, we add nanomaterial to fully confluent epithelial cells. The ratio surface of nanomaterial : surface of epithelial cells = 10 : 1 : 1/40

variables:

npFree[t] : surface of free-floating nanomaterial, normed to the surface of epithelial cells at t = 0
npLa4[t] : total surface of nanomaterial inside epithelial cells, normed to the surface of epithelial cells at t = 0
npCF[t] : surface of nanomaterial in cauliflowers, normed to the surface of epithelial cells at t = 0
sLa4[t] : surface of epithelial cells, normed to the surface of epithelial cells at t = 0
t : time, measured in days

parameters – rate coefficients

endo : rate of endocytosis = 1
tox : toxicity of nanomaterial
cff : rate of cauliflower formation
timecf : norming factor for exocytosis saturation = 1
La4Rep : replication rate of epithelial cells = 0.3

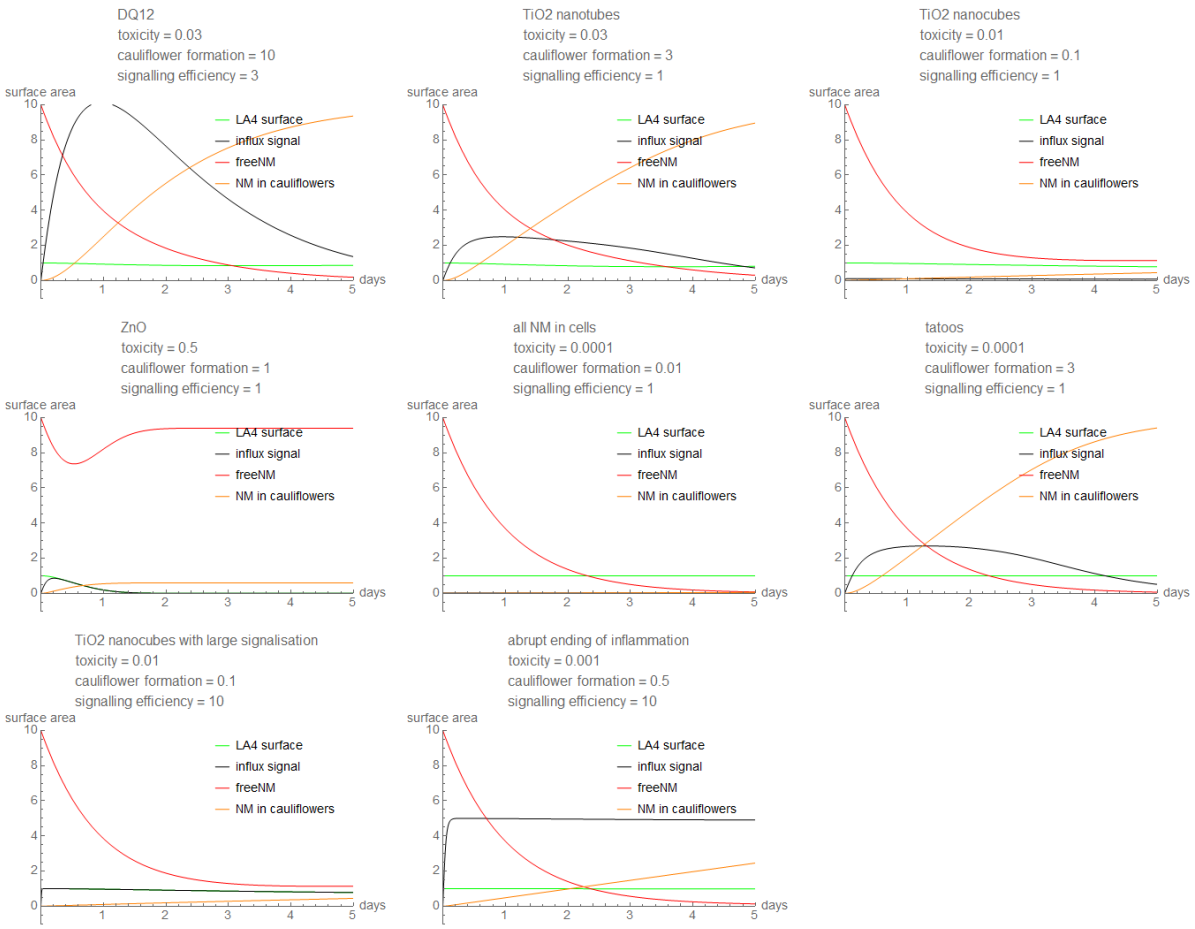


Figure S174: Time evolution of the in vitro LA-4 monoculture theoretical model for 8 different nanomaterials.

In vitro MH-S monoculture model

For the descriptions of the terms see the *in vivo* model - the rate equations for MH-S monoculture are similar to the *in vivo* model, with some terms being left out.

rate equations:

```
D[npFree[t], t] == -endo sMhs[t] * npFree[t] + tox npMhs[t] * npMhs[t] / sMhs[t],

D[npMhs[t], t] == endo sMhs[t] * npFree[t] - tox npMhs[t] * npMhs[t] / sMhs[t],

D[sMhs[t], t] == -tox npMhs[t] + la4Rep sMhs[t] (1 - sMhs[t] / la4Max),
```

initial conditions:

```
npFree[0] == exposureSurfaceDose = 10;
npMhs[0] == 0,
sMhs[0] == macrophageSurface = N[1 / 40];
la4Max = 1;
```

At t = 0, we add nanomaterial to macrophages. The ratio surface of nanomaterial : surface of the dish: surface of macrophages = 10 : 1 : 1/40

variables:

npFree[t] : surface of free-floating nanomaterial, normed to the surface of epithelial cells at t = 0
npMhs[t] : total surface of nanomaterial inside macrophages, normed to the surface of epithelial cells at t = 0
sMhs[t] : surface of macrophages, normed to the surface of epithelial cells at t = 0
t : time, measured in days

parameters – rate coefficients

endo : rate of endocytosis = 1
tox : toxicity of nanomaterial
La4Rep : replication rate of epithelial cells = 0.3

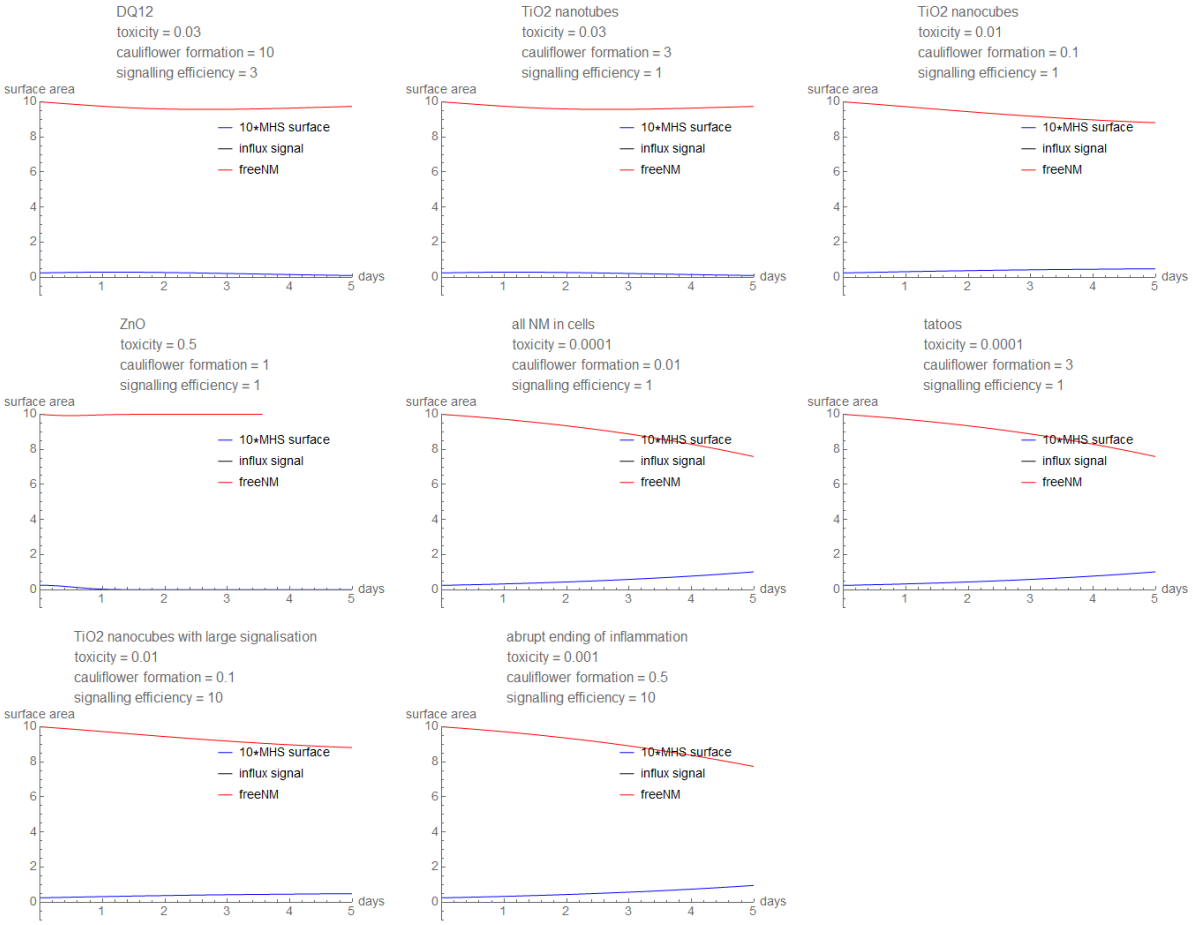


Figure S175: Time evolution of the in vitro MH-S monoculture theoretical model for 8 different nanomaterials.

Determination of rates for the theoretical model from *in vitro* assay results

Main message

In order to determine the nanomaterial-specific model parameters toxicity rate (*tox*), quarantining rate (*cff*) and signalling efficiency (*signalEff*) from the model in S6a for a desired nanomaterial, one must first determine the toxicity of the nanomaterial (*tox*) from real-life *in vitro* MH-S monoculture viability as shown below. By combining this with the observed magnitude of cauliflowers in a real-life *in vitro* LA-4 monoculture, the cauliflower formation rate (*cff*) may be determined as shown below. Since the amount of cauliflowers depends both on the toxicity of the nanomaterial as well as the exocytosis process itself, the *cff* cannot be measured directly. From there on, efficiency of signaling and monocyte influx replacing the dying macrophages *(signalEff)* is calculated from the rates *tox*, *cff* and either via measured polymorphonuclear cell influx *in vivo* after 10 days or measured macrophage attractants in *in vitro* co-culture of LA-4 and MH-S after 2 days (pre-calibrated with *in vivo*).
All analysis and visualisation in this section were done in Wolfram Mathematica 12.0, licence L5063-5112.

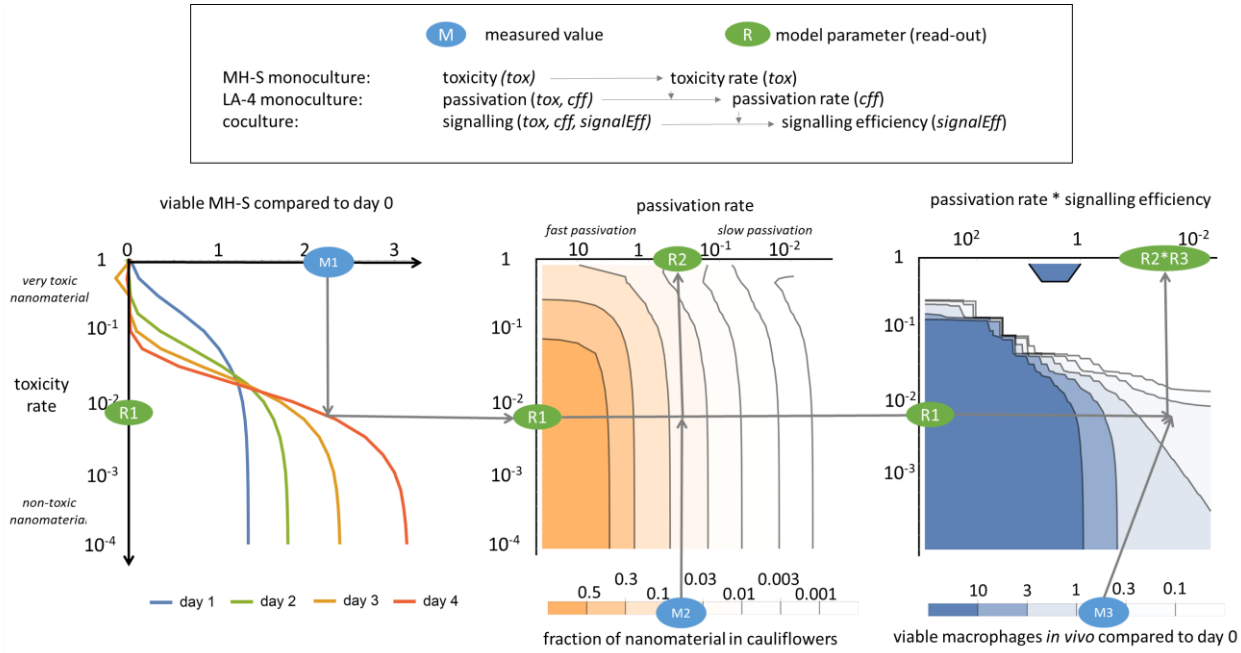


Figure S176: Schematic of workflow and determination of parameters of the model.

S6b – Phase space of chronic inflammation

Main message

The phase space was scanned by calculating the time evolution of the appropriate system of equations from section S6a for a set of nanomaterials with appropriately interspaced parameters: toxicity rate (*tox*), quarantining rate (*cff*) and signalling efficiency (*signalEff*). For each parameter, 30 logarithmically-equally-spaced values in a sensible range were chosen – the total amount of values in the grid was thus 30 x 30 x 30 = 27.000. The parameter values for plotted nanomaterials were approximated from the known behaviour of the *in vivo* and *in vitro* systems following exposure to these nanomaterials.
For three chosen nanomaterials, the time-courses were simulated.
All the analysis in this section was done in Wolfram Mathematica 12.0, licence L5063-5112.
More information can be found in S6a.

Supporting data:

Figure S177

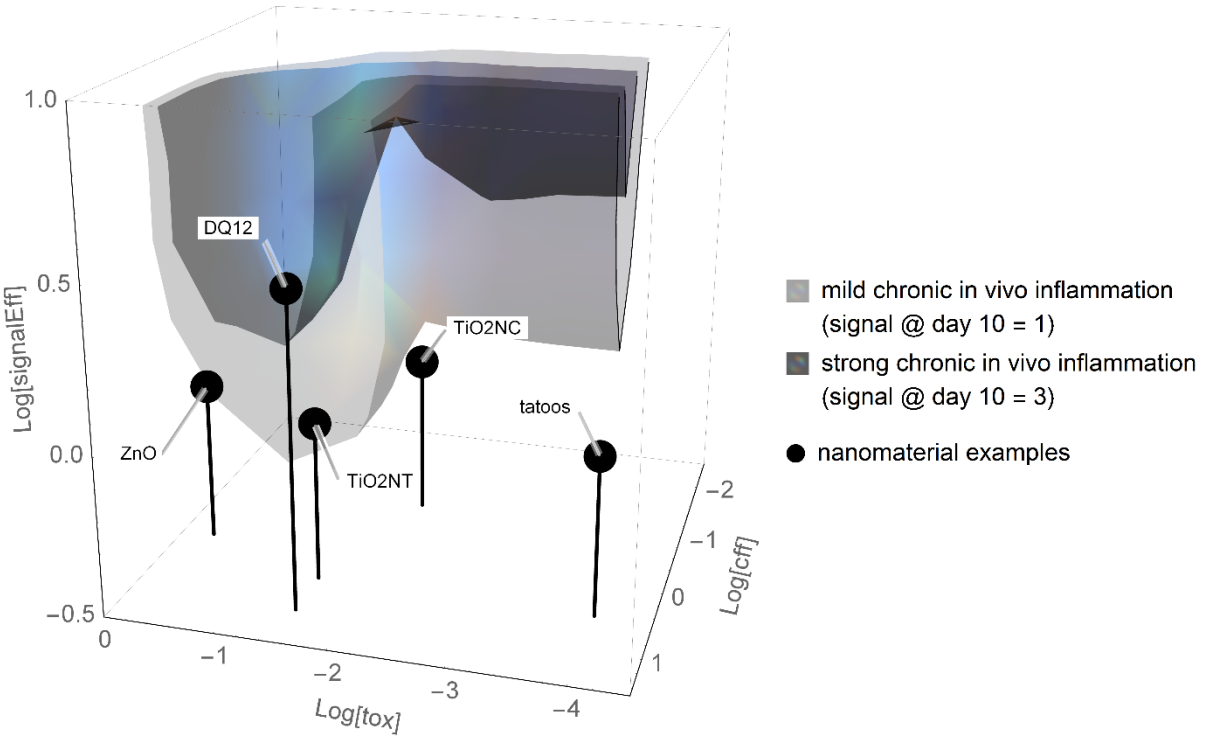


Figure S177: The black contour in the 3D parameter phase space plot represents the set of parameters with the predicted influx signal in vivo at day 10 equal to 3 (strong chronic inflammation) and the grey contour with the predicted influx signal in vivo at day 10 equal to 1 (weak chronic inflammation) (black line on Figure S172). Black spheres depict the estimated location of the nanomaterials in the cube.

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