Supporting information for:

Phenotypic profiling reveals polystyrene nanoplastics elicit sublethal and lethal effects on cellular morphology in rainbow trout gill epithelial cells

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Supplementary Table S1: Cell Painting Modified Protocol (3 channels)

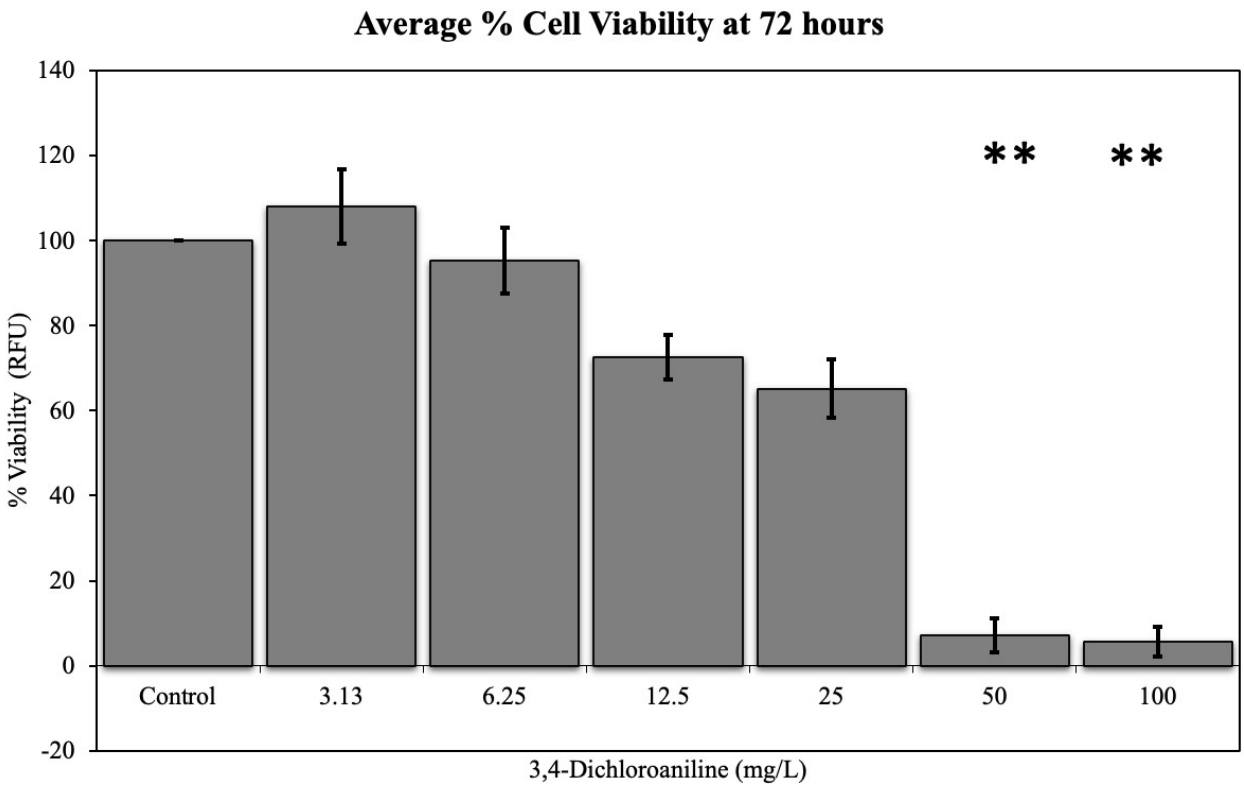
Step 1	Seed cells at 50,000 per well in 96-well plate
Step 2	Grow cells near 85% confluence in U-75 culture vessel
Step 3	Treat cells with particles for 24-hours
Step 4	Prior to take down, prepare live-cell MitoTracker staining solution. **Final concentration of 500 nM.**
Step 5	Remove cell solution from plates, and leave enough residual volume if about 60-70uL to prevent disturbing the cells
Step 6	Add 200uL of MitoTracker to each well.
Step 7	Incubate the plate at 19^{o}C in the dark for 30 min
Step 8	Prepare the phalloidin and Hoesct staining solution in 50 mL of 1% (wt/vol) of BSA in PBS(1X) solution. **5 ul/ml phalloidin and 5 ug/ml Hoesct.** Also prepare **4% Parafomaldehyde in PBS.**
Step 9	Remove Mitotracker from plates, and leave enough residual volume if about 60-70uL to prevent disturbing the cells.
Step 10	Add ~200uL of 4% PFA to each well for a **final concentration of ~3.2% PFA.**
Step 11	Incubate cells in the dark at RT for 20 min
Step 12	Wash cells once with 200uL of 1xPBS
Step 13	To permeabilize the cells, remove the PBS and add 200uL of 0.1% (v/v) Triton X-100 solution for each well
Step 14	Incubate for 10-20 min at RT in the dark
Step 15	Wash cells with 200 uL 2x with 1xPBS
Step 16	Remove PBS and add 200uL of staining solution from step 8.
Step 17	Incubate the plates in the dark for 30 min at RT
Step 18	Wash cells with 200uL of 1xPBS, with no final aspiration. Image immediately or store at 4^{o}C wraped in foil for up to 2 wks

This table contains *Bray et al., 2016 Cell Painting, a high-content image-based assay for morphological profiling using multiplexed fluorescent dyes* protocol.

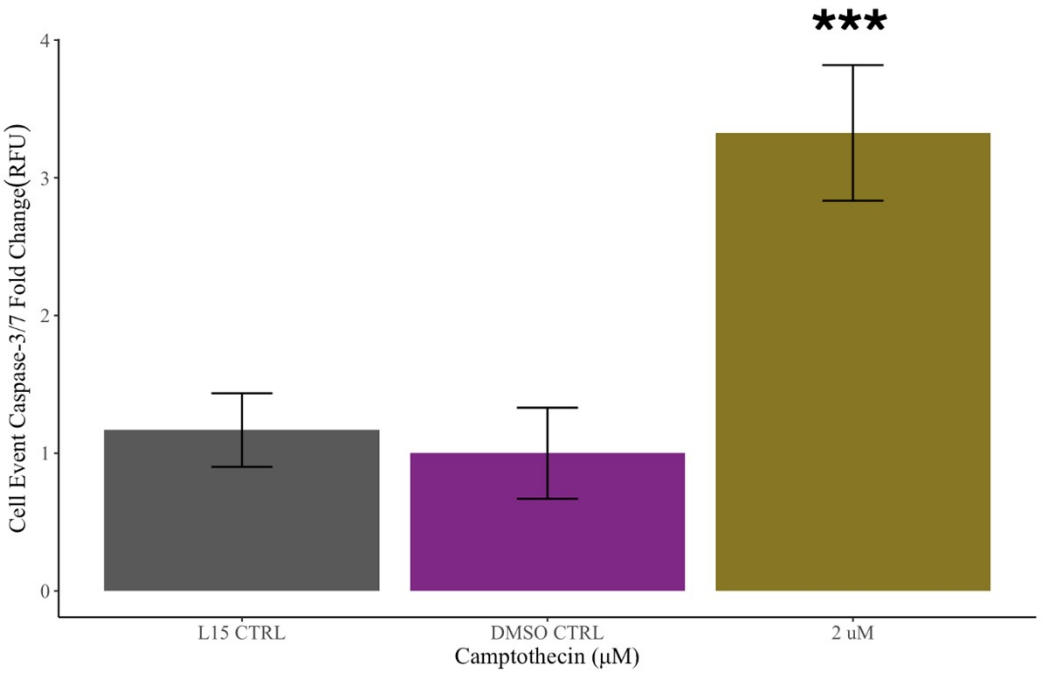
Supplementary Table S2: VIP Scores of Reference Chemicals

Feature	VIP
Image_Granularity_13_Mitochondria1	**2.30930532**
Mean_Cell_AreaShape_CentralMoment_1_2	**2.256167586**
Image_Granularity_9_Mitochondria1	**2.145745084**
Mean_Mit_per_cell_AreaShape_NormalizedMoment_0_3	**1.878118247**
Mean_Nucleus_AreaShape_BoundingBoxMinimum_Y	**1.819675791**
Image_Granularity_10_Mitochondria1	**1.810699511**
Mean_Mit_per_cell_AreaShape_Zernike_6_0	**1.775395641**
Mean_Mit_per_cell_AreaShape_Zernike_3_3	**1.774584718**
Mean_Mit_per_cell_AreaShape_Zernike_5_3	**1.769501322**
Mean_Mit_per_cell_AreaShape_Zernike_4_0	**1.765831758**

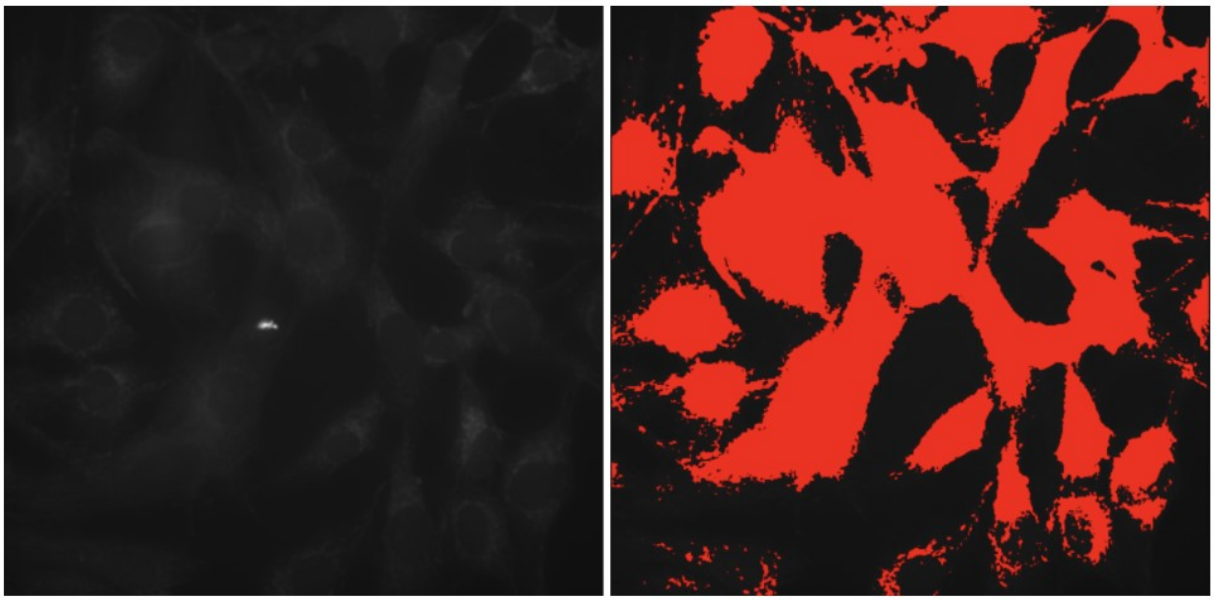
This table contains the top 10 variable importance in projection (VIP) scores for the PS NPs. These VIP scores were derived from the PLS-DA plots indicating features that influenced the separation between the response groups.



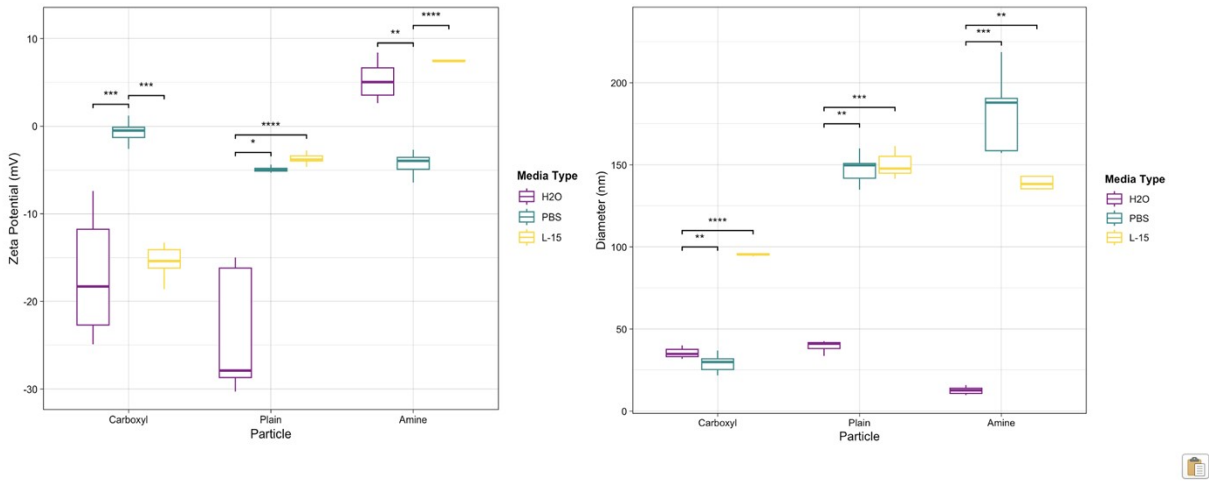
Supplementary Figure S1: 3,4-Dichloroaniline (3,4-DCA) was used to demonstrate the sensitivity of the RTgill-W1 cell line to AB (p-value < 0.05, n=3) according to the OECD Test No. 249: Fish Cell Line Acute Toxicity (OECD, 2021).



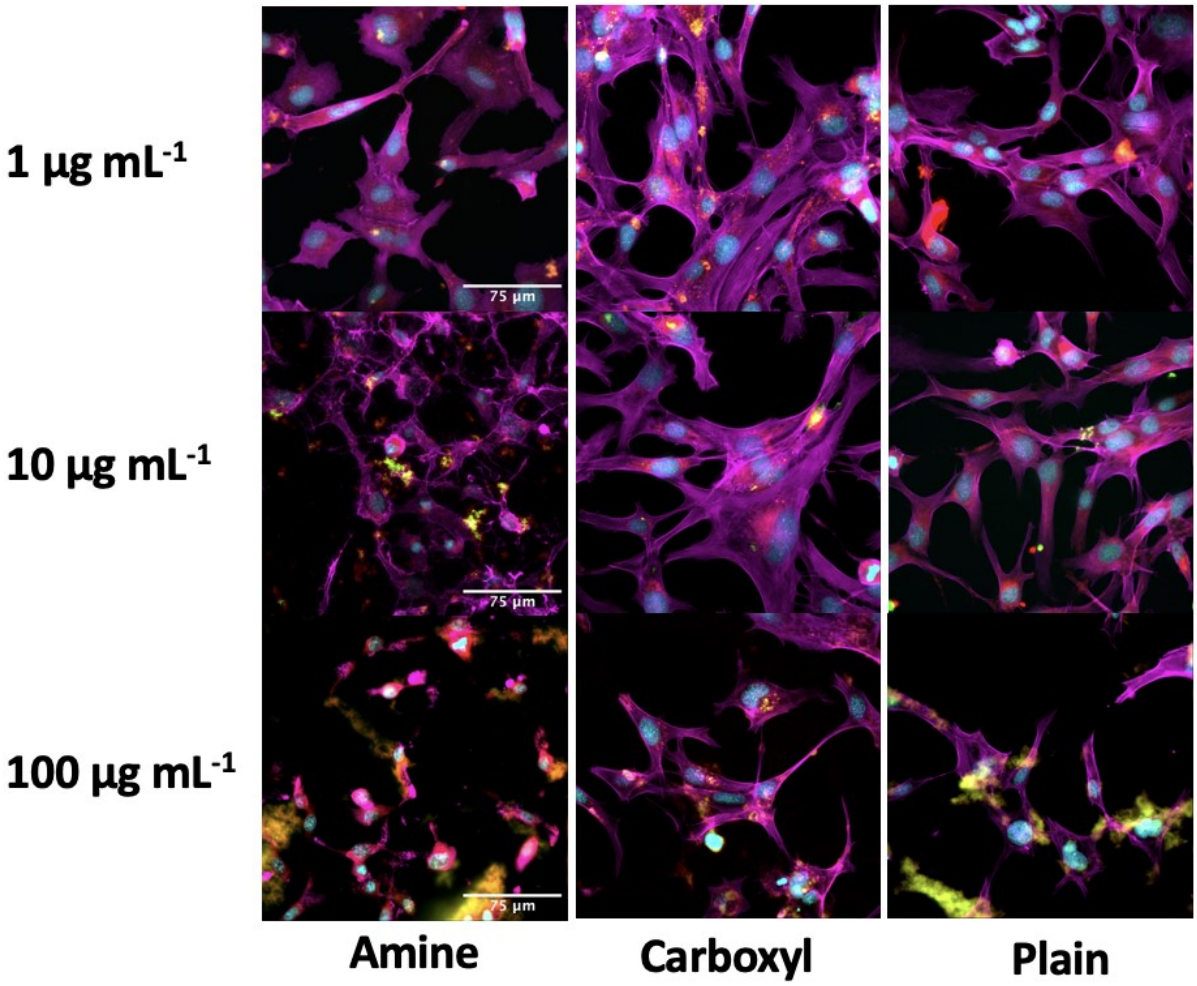
Supplementary Figure S2: Validation of Caspase-3/7 Red CellEvent Assay using 2 µM of Camptothecin (p-value < 0.001) positive control using 0.1% DMSO control and L-15 Media as negative controls (n=3).



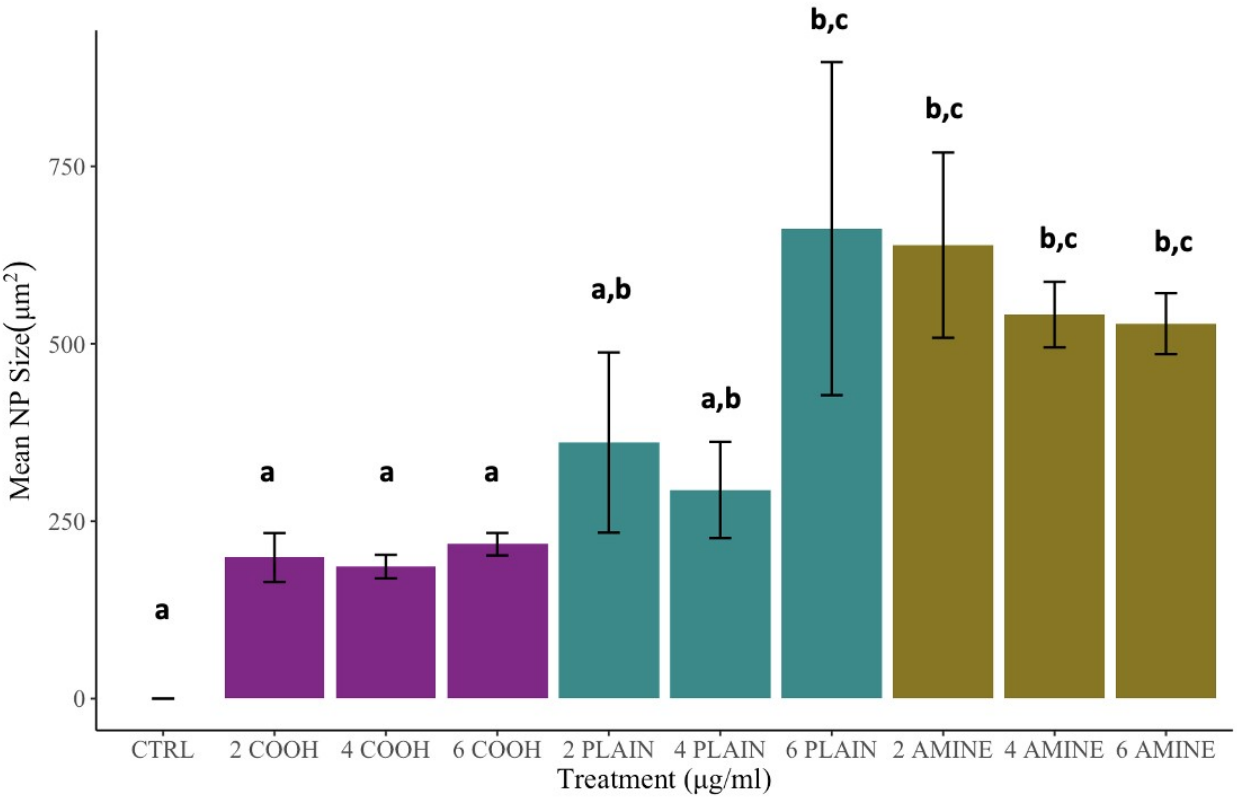
Supplementary Figure S3: Example of a threshold mask applied to mitochondria to include all areas of biological interest across the z-stacks by choosing a pixel cutoff to quantify colocalization on.



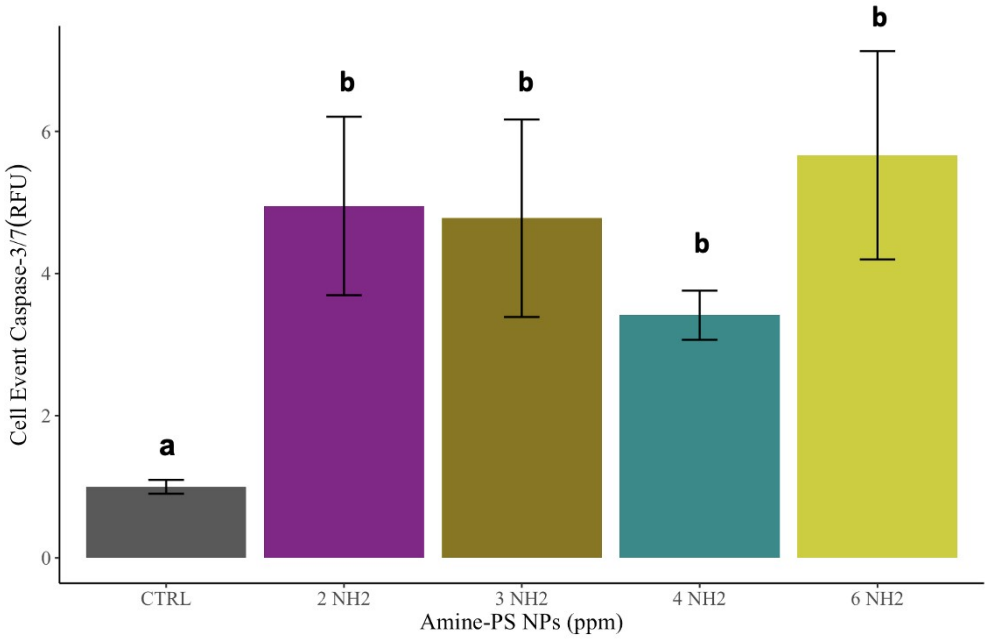
Supplementary Figure S4: (a) Zeta potential of PS NPs were measured in phenol red- and FBS-free L-15 growth media, UltraPure™ Distilled Water, and phosphate buffered saline (PBS). (b) Diameter of PS NPs were measured in both phenol red- and FBS-free L-15 growth media and UltraPure™ Distilled Water, and phosphate buffered saline (PBS). Asterisks indicate significant differences in size or zeta potential (p-value < 0.05).



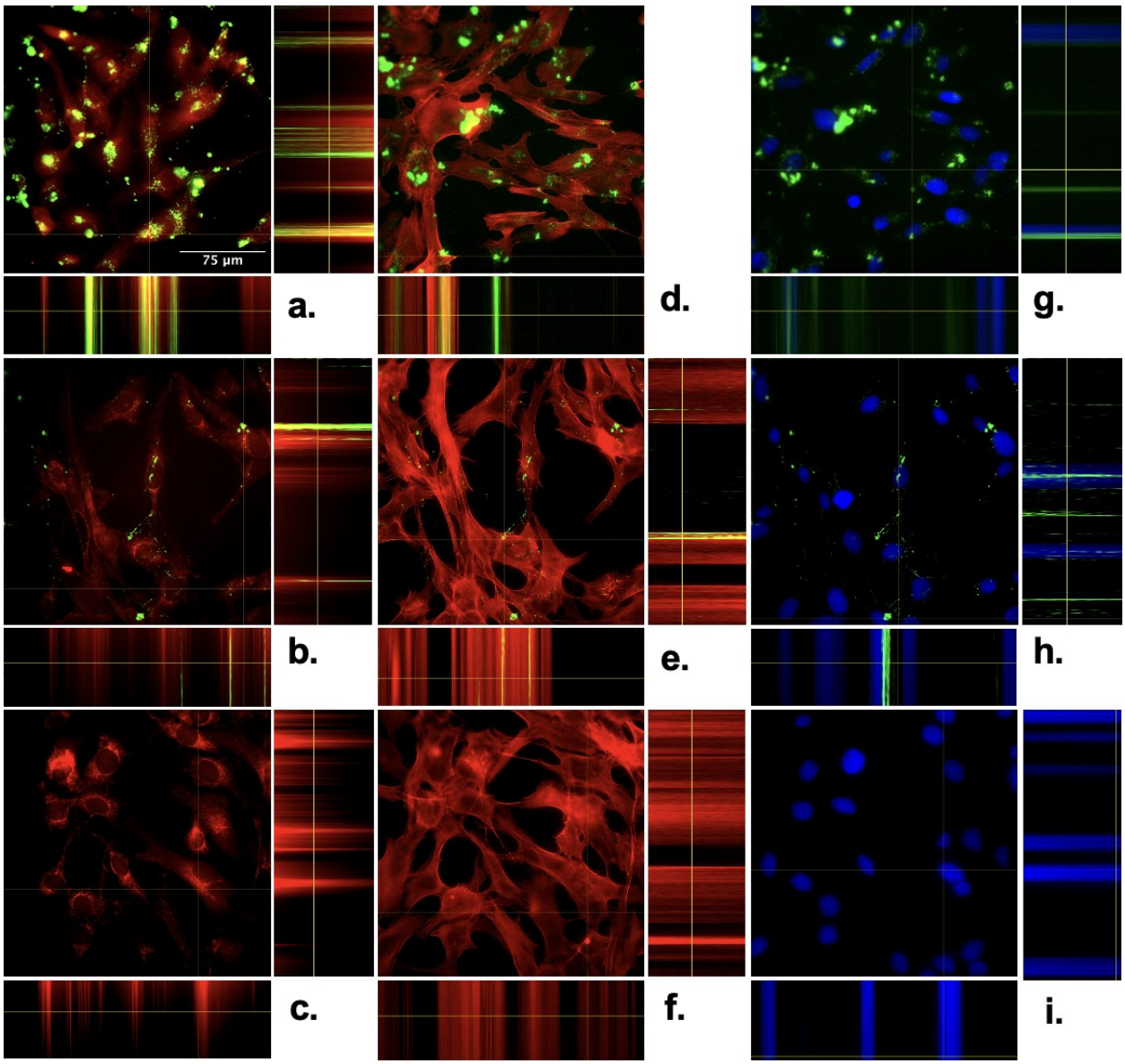
Supplementary Figure S5: after 24 hours exposure, PS NP agglomerates can be observed at larger concentrations on the fluorescent microscope at high concentrations of 100 µg mL^{-1} (Molecular Devices, California, USA). Scale bars are the same for all images.



Supplementary Figure S6: Size of particles post 24 hours quantified via HCI data. This further supports that both the aggregation of the particles and sedimentation have occurred (n=5).



Supplementary Figure S7: Caspase-3/7 Red CellEvent Assay with NH_2-PS NP treatments (n=4) via high-content imaging. Average Caspase Intensity.



Supplementary Figure S8: Orthogonal projections for RTgill-W1 colocalized with PS NPs. (a) PS-NH_2 vs mitochondria, (b) PS-COOH vs mitochondria, (c) PS-PLAIN vs mitochondria, (d) PS-NH_2 vs actin, (e) PS-COOH vs actin, (f) PS-PLAIN vs actin, (g) PS-NH_2 vs nuclei, (h) PS-COOH vs nuclei, (i) PS-PLAIN vs nuclei. Image contrast was increased for visualization purposes on ImageJ. Scale bars are the same for all images.