Supplementary Results

Selection of potential iron oxide nanoparticles for breast cancer treatment based on in vitro cytotoxicity and cellular uptake

Johanna M Poller[1, 2], Jan Zaloga[1], Eveline Schreiber[1], Harald Unterweger[1], Christina Janko[1], Patricia Radon[3], Dietmar Eberbeck[3], Lutz Trahms[3], Christoph Alexiou[1], Ralf P Friedrich[1]

[1] Department of Otorhinolaryngology, Head and Neck Surgery, Section for Experimental Oncology & Nanomedicine (SEON), Else Kröner-Fresenius-Stiftung Professorship, Universitätsklinikum Erlangen, Erlangen, Germany; [2]Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Germany; [3]Physikalisch-Technische Bundesanstalt Braunschweig und Berlin, Berlin, Germany.

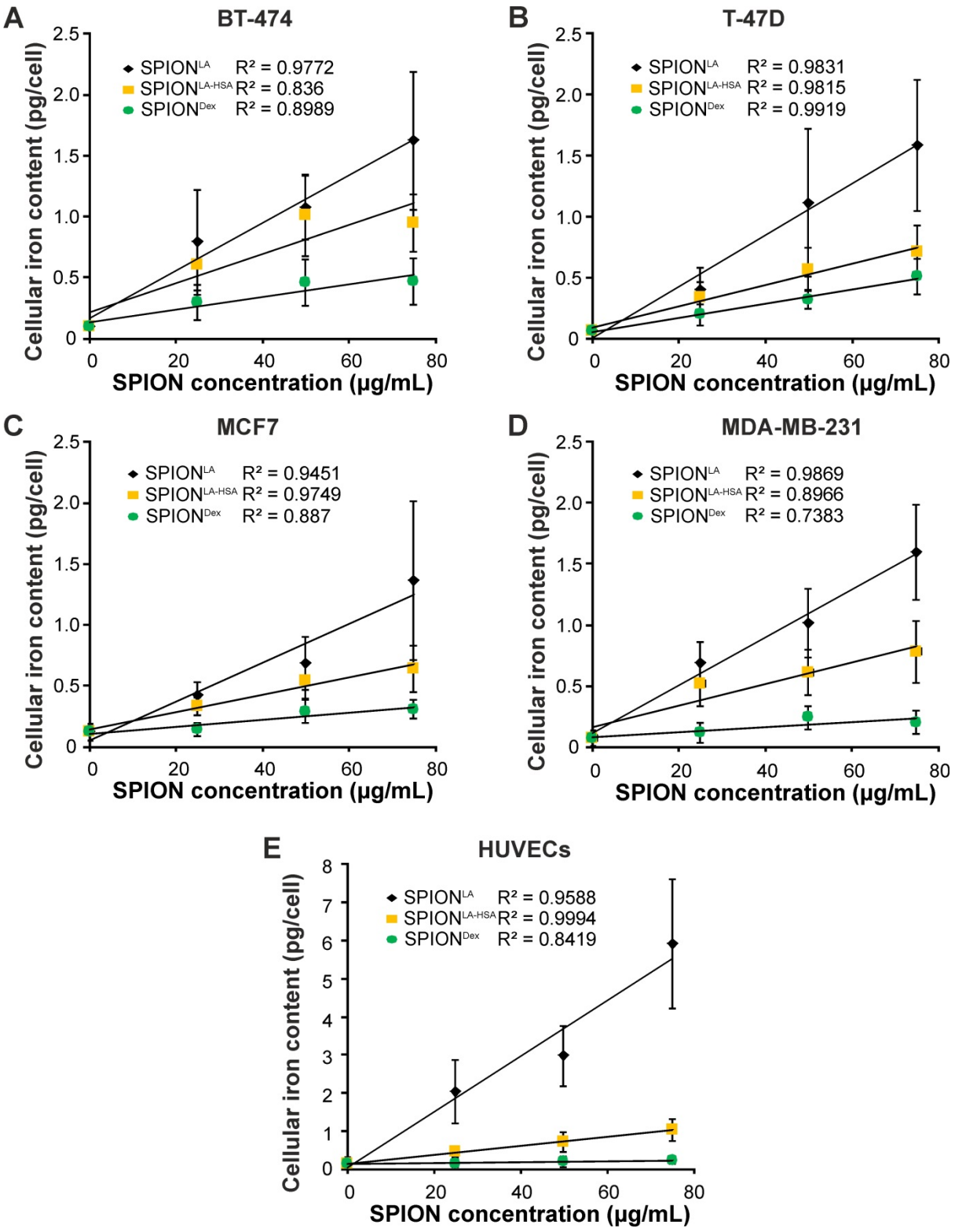
Correspondence: Christoph Alexiou.

Department of Otorhinolaryngology, Head and Neck Surgery, Section for Experimental Oncology & Nanomedicine (SEON), Else Kröner-Fresenius-Stiftung Professorship, Universitätsklinikum Erlangen, Glückstrasse 10a, 91054 Erlangen, Germany.

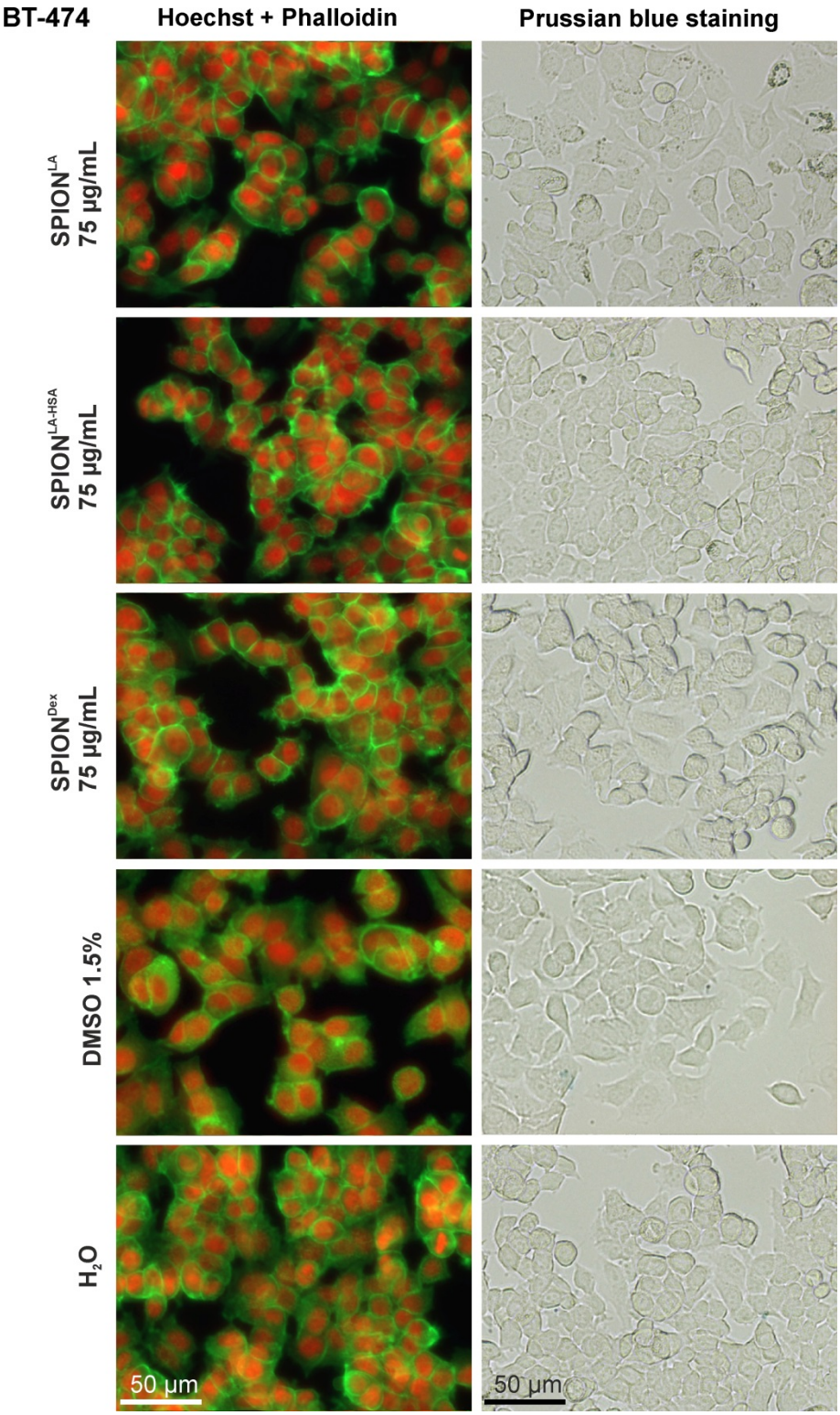
Tel +49 9131 85 33142

Fax +49 9131 85 34828

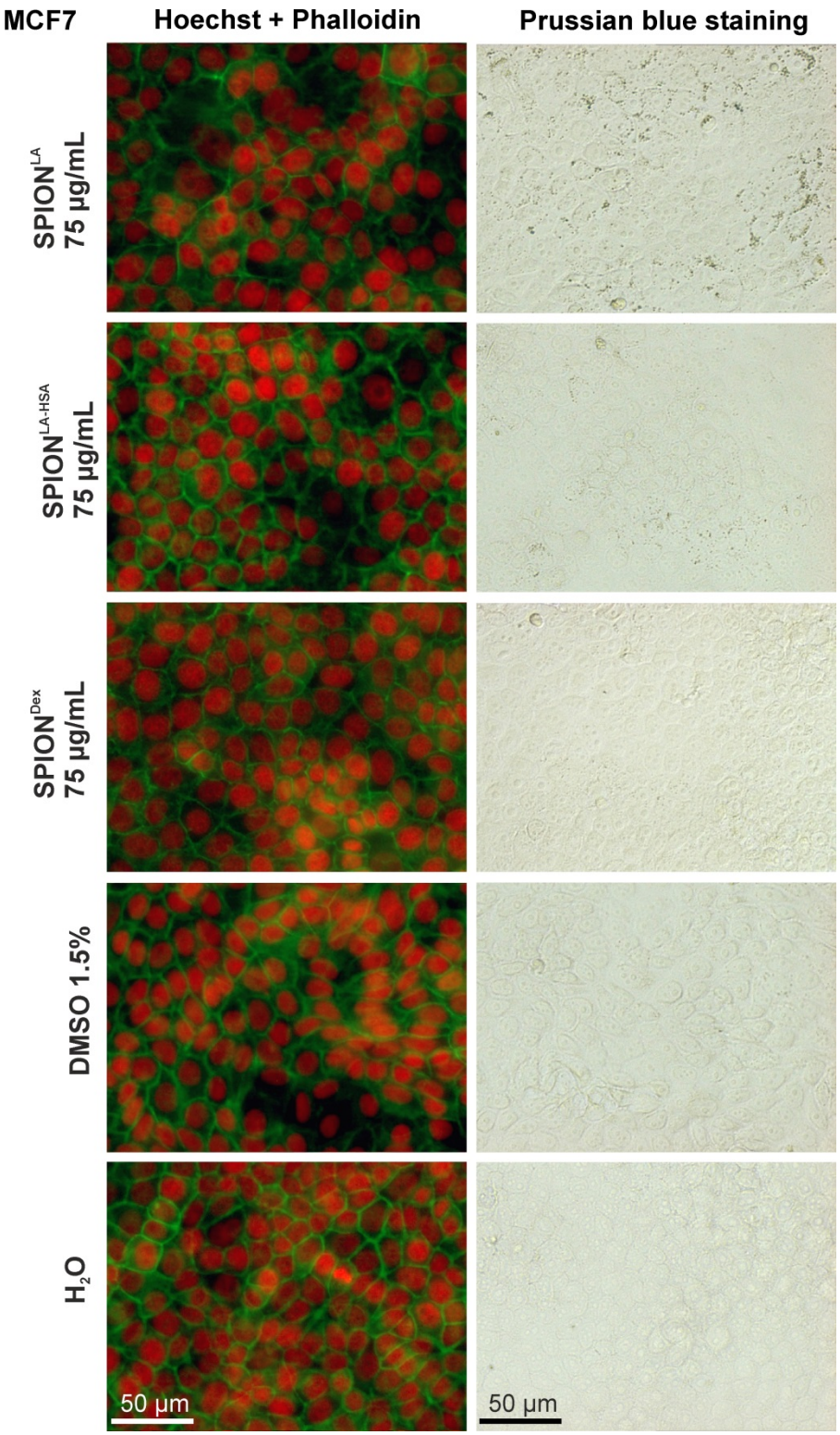
Email christoph.alexiou@uk-erlangen.de



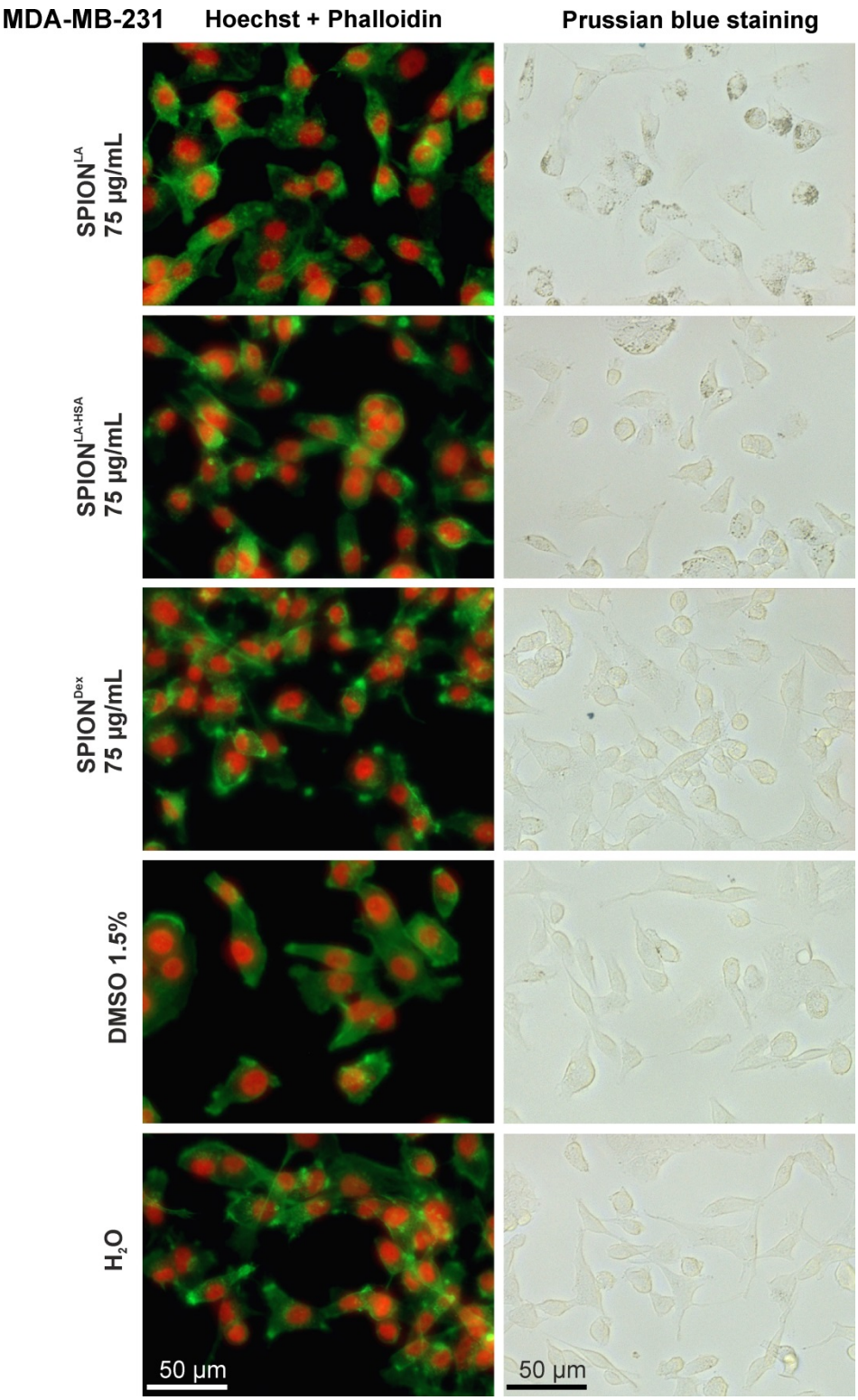
Supplementary Figure 1: **Correlation between Cellular Iron Content and Applied SPION Concentration in the Cell Culture Media.** Cells were incubated for 48 h with different concentrations of $SPION^{LA}$, $SPION^{LA\text{-}HSA}$ and $SPION^{Dex}$, followed by iron content analysis in cell lysates using microwave plasma-atomic emission spectrometer (MP-AES). MP-AES measurements with increasing SPION concentrations are shown for **(A)** BT-474, **(B)** T-47D, **(C)** MCF7, **(D)** MDA-MB-231 and **(E)** HUVECs. The data are expressed as the mean ± standard deviation (n=4 with technical triplicates). R^2 represents the coefficient of determination.



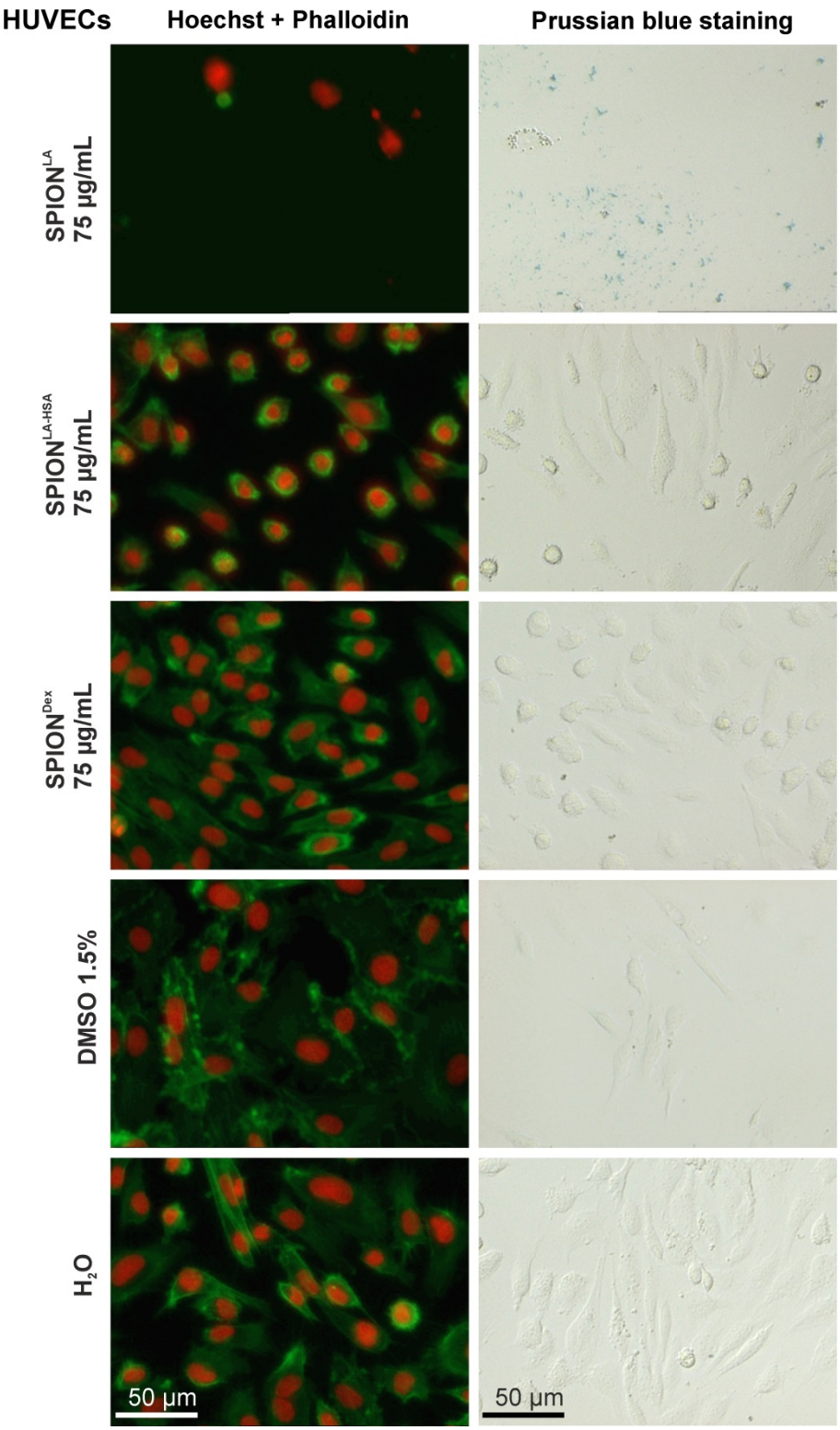
Supplementary Figure 2: **Images of the Cellular Particle-load and Cell Morphology after SPION Treatment.** BT-474 cells were incubated for 48 h with 75 µg/ml SPION^{LA}, SPION^{LA-HSA} and SPION^{Dex}. Images were taken with a fluorescence microscope. Cells stained with Alexa Fluor 488 Phalloidin (green) and Hoechst 33342 (red) images are shown in the left column, Prussian blue staining is shown in the right column.



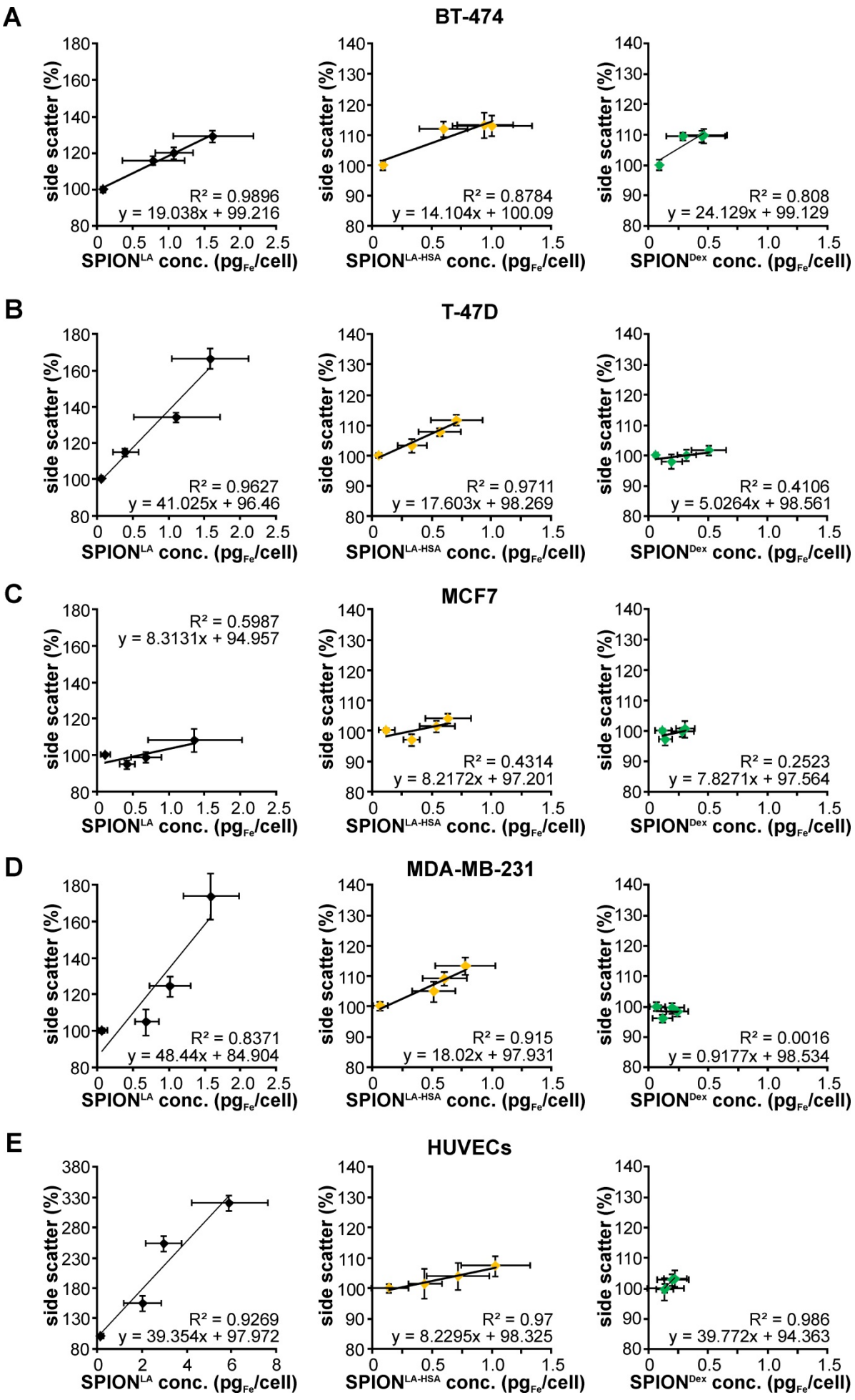
Supplementary Figure 3: **Images of the cellular Particle-load and Cell Morphology after SPION Treatment.** MCF7 cells were incubated for 48 h with 75 µg/ml $SPION^{LA}$, $SPION^{LA-HSA}$ and $SPION^{Dex}$. Images were taken with a fluorescence microscope. Cells stained with Alexa Fluor 488 Phalloidin (green) and Hoechst 33342 (red) images are shown in the left column, Prussian blue staining is shown in the right column.



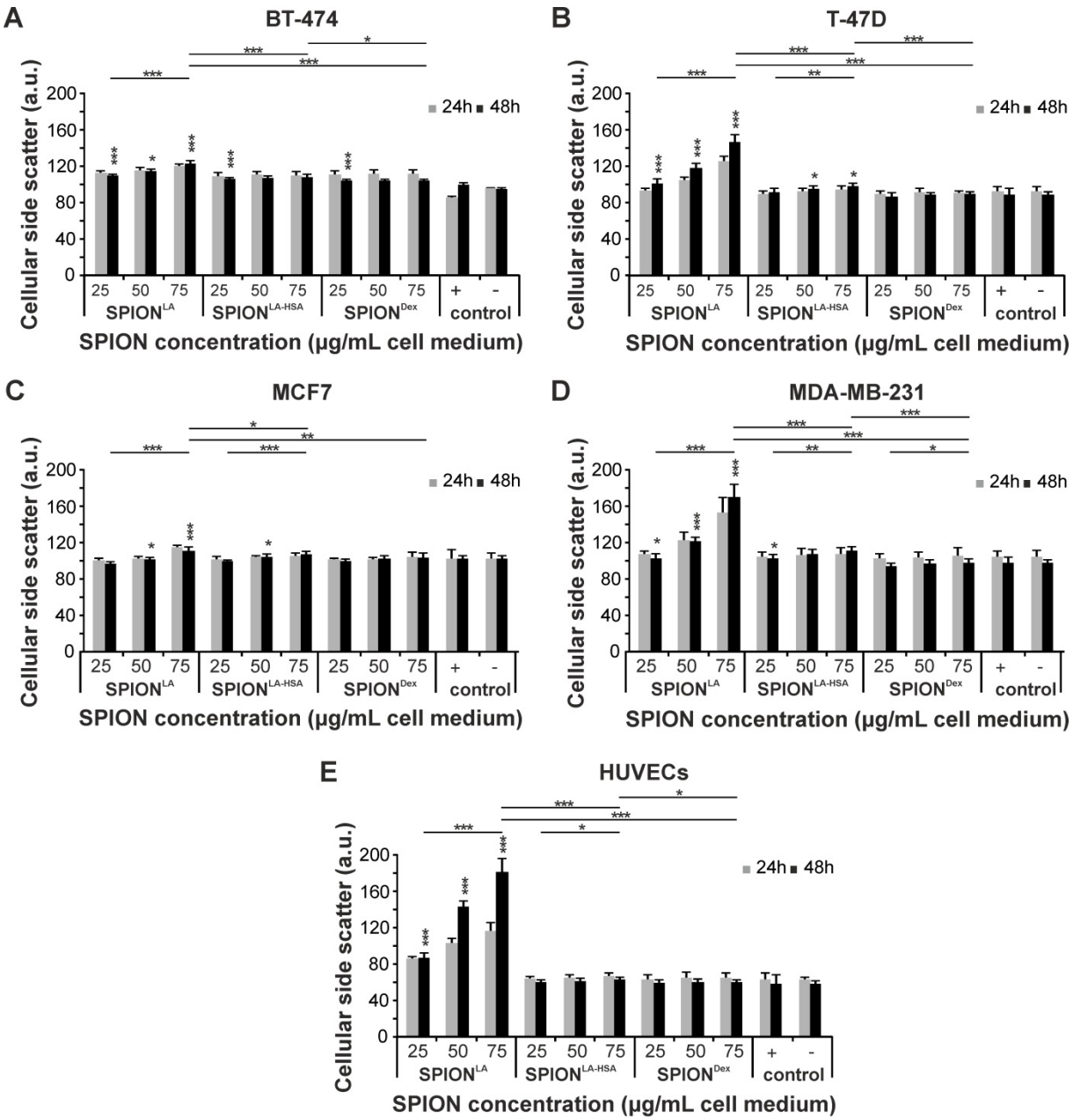
Supplementary Figure 4: **Images of the cellular Particle-load and Cell Morphology after SPION Treatment.** MDA-MB-231 cells were incubated for 48 h with 75 µg/ml $SPION^{LA}$, $SPION^{LA\text{-}HSA}$ and $SPION^{Dex}$. Images were taken with a fluorescence microscope. Cells stained with Alexa Fluor 488 Phalloidin (green) and Hoechst 33342 (red) images are shown in the left column, Prussian blue staining is shown in the right column.



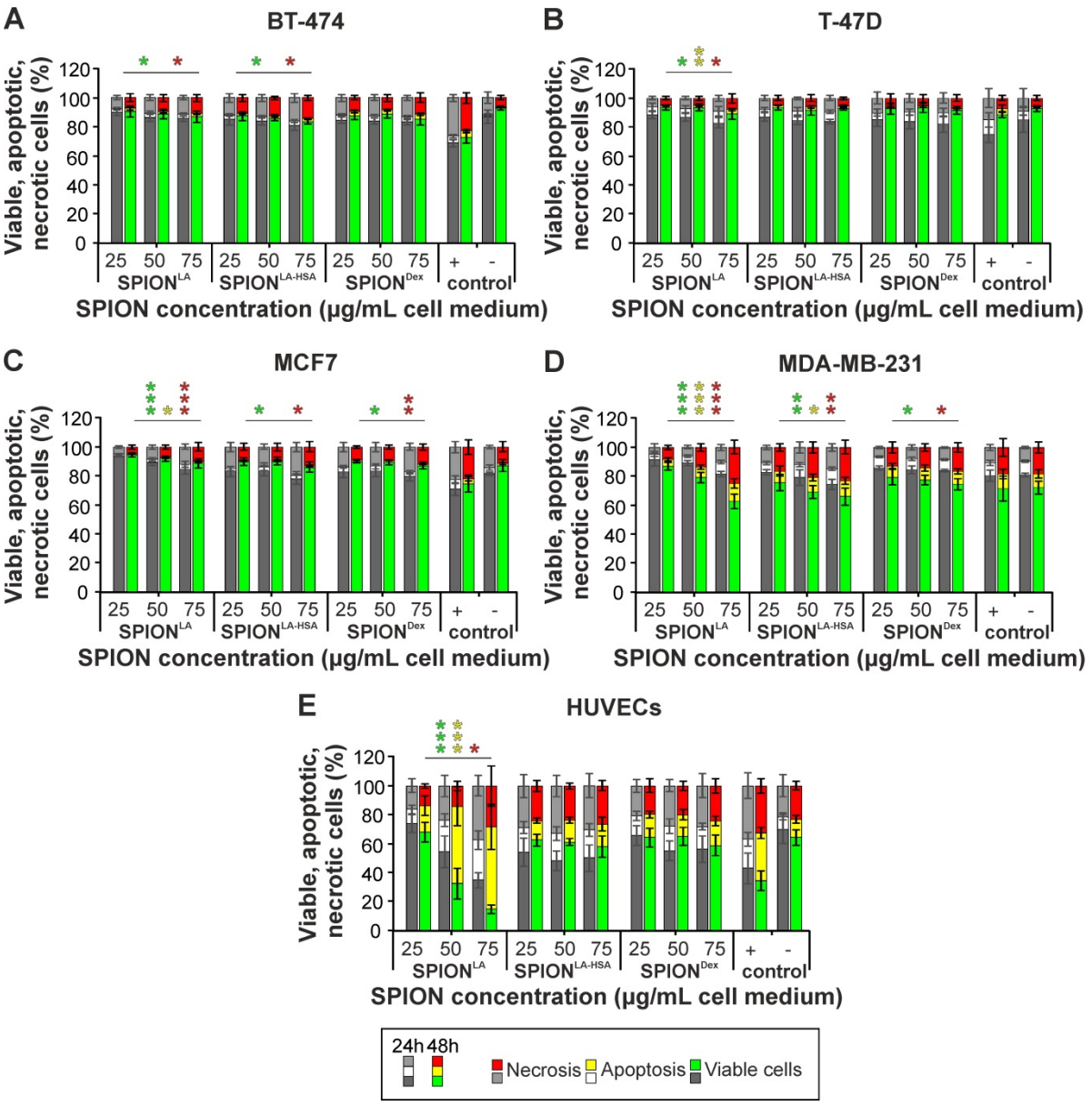
Supplementary Figure 5: **Images of the cellular Particle-load and Cell Morphology after SPION Treatment.** HUVECs were incubated for 48 h with 75 µg/ml $SPION^{LA}$, $SPION^{LA\text{-}HSA}$ and $SPION^{Dex}$. Images were taken with a fluorescence microscope. Cells stained with Alexa Fluor 488 Phalloidin (green) and Hoechst 33342 (red) images are shown in the left column, Prussian blue staining is shown in the right column.


A
BT-474
side scatter (%)
R² = 0.9896
y = 19.038x + 99.216
SPION^LA conc. (pg_Fe/cell)
R² = 0.8784
y = 14.104x + 100.09
SPION^LA-HSA conc. (pg_Fe/cell)
R² = 0.808
y = 24.129x + 99.129
SPION^Dex conc. (pg_Fe/cell)
B
T-47D
R² = 0.9627
y = 41.025x + 96.46
R² = 0.9711
y = 17.603x + 98.269
R² = 0.4106
y = 5.0264x + 98.561
C
MCF7
R² = 0.5987
y = 8.3131x + 94.957
R² = 0.4314
y = 8.2172x + 97.201
R² = 0.2523
y = 7.8271x + 97.564
D
MDA-MB-231
R² = 0.8371
y = 48.44x + 84.904
R² = 0.915
y = 18.02x + 97.931
R² = 0.0016
y = 0.9177x + 98.534
E
HUVECs
R² = 0.9269
y = 39.354x + 97.972
R² = 0.97
y = 8.2295x + 98.325
R² = 0.986
y = 39.772x + 94.363

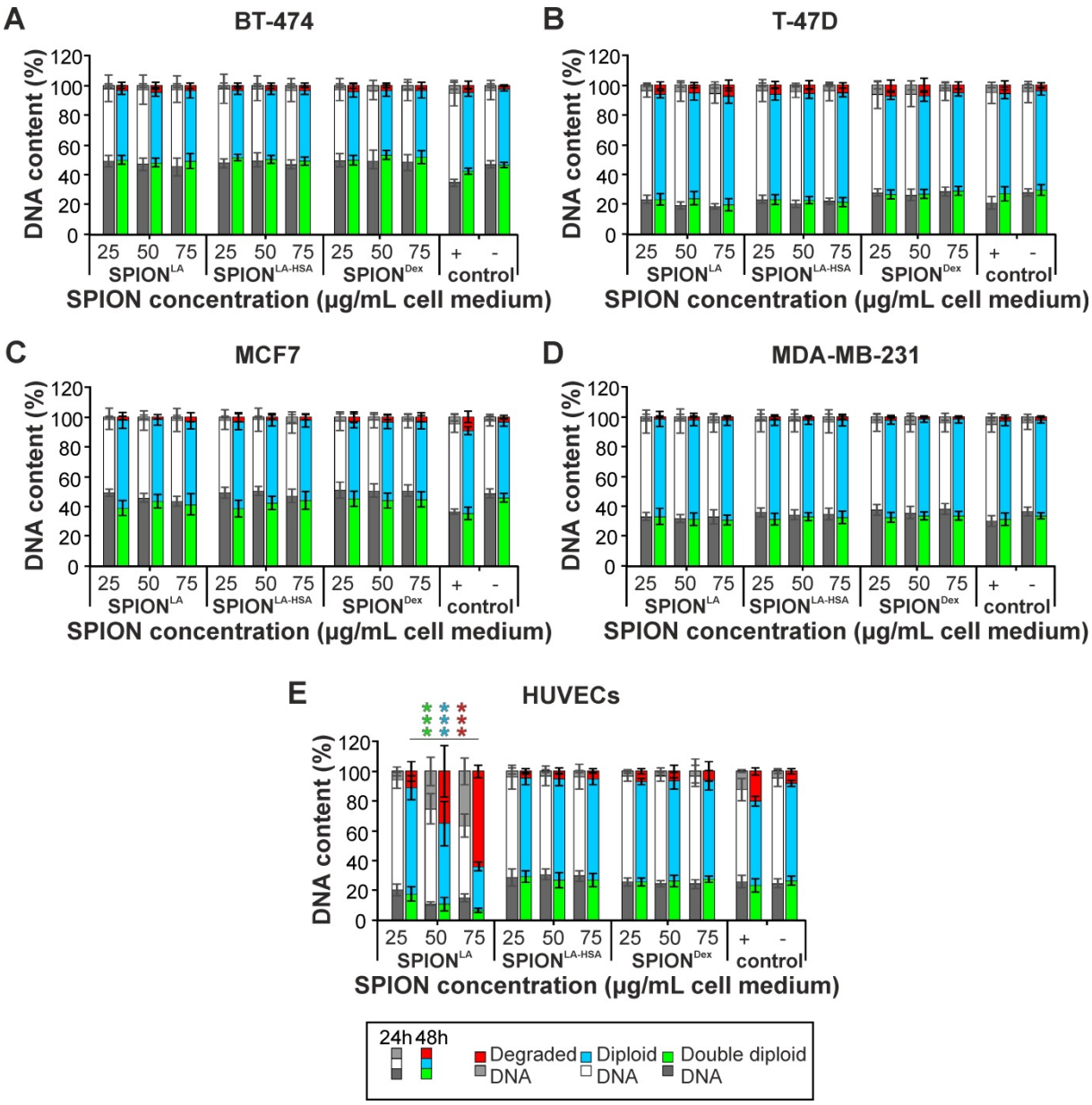
Supplementary Figure 6: **Correlation between Flow Cytometric Side Scatter Data (SSc) and Quantitative Cellular Iron Measurements by MP-AES.** Cells were incubated for 48 h with 0 - 75 µg/ml $SPION^{LA}$, $SPION^{LA-HSA}$ and $SPION^{Dex}$. For SSc determination, it was gated on phenotypically viable cells (Ax-PI-) **(A-E)** Relationship between the cellular SPION load as measured by MP-AES and the normalized flow cytometric side scatter data. Correlations were shown for **(A)** BT-474, **(B)** T-47D, **(C)** MCF7, **(D)** MDA-MB-231 and **(E)** HUVECs incubated with different concentrations of $SPION^{LA}$ (left panel), $SPION^{LA-HSA}$ (middle panel) and $SPION^{Dex}$ (right panel). The data are expressed as the mean ± standard deviation (n=4 with technical triplicates). R^2 represents the coefficient of determination. y describes the mathematic relationship between side scatter and cellular iron content.



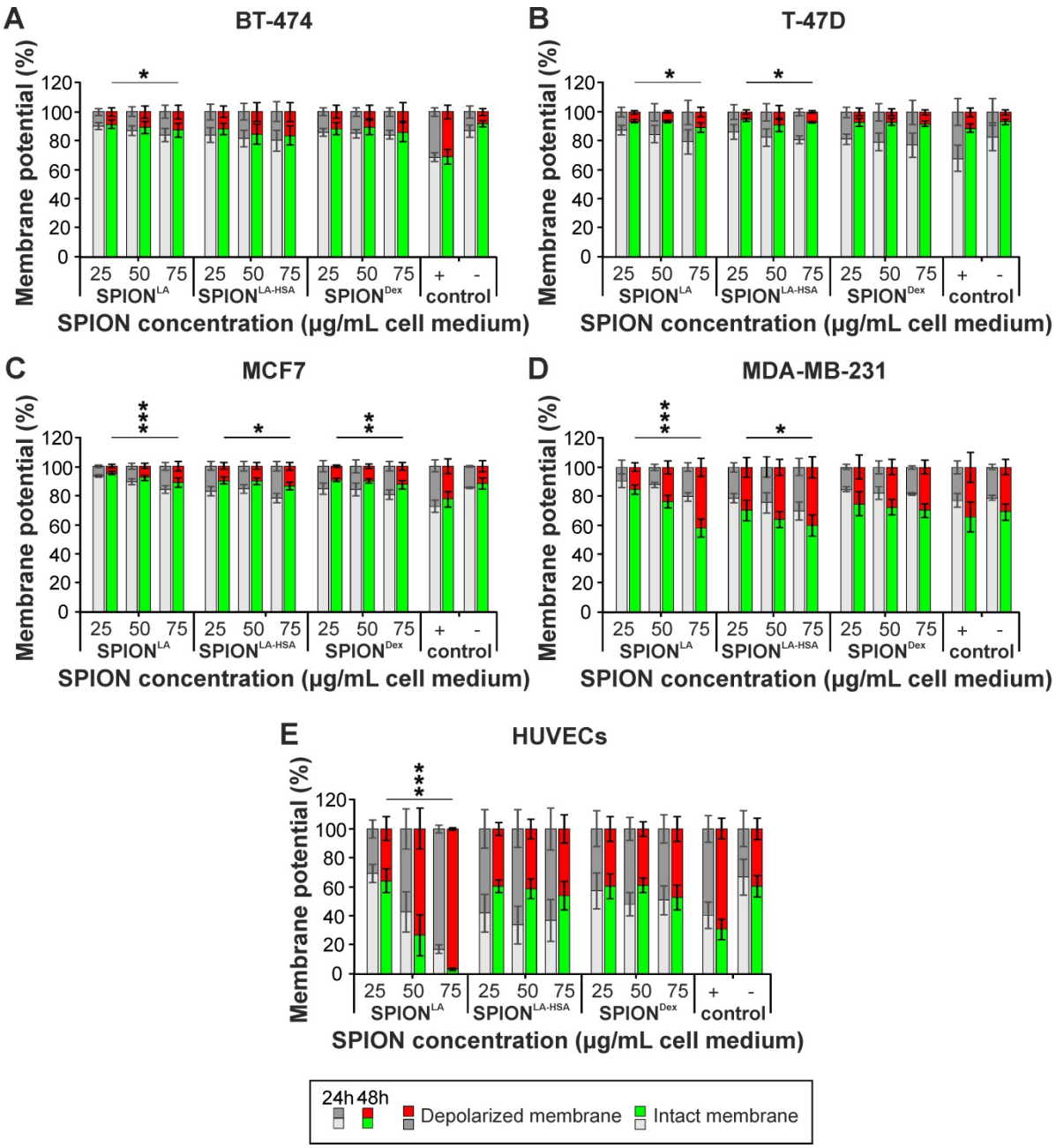
Supplementary Figure 7: **Evaluation of the Cellular Nanoparticle Load via SSc Measurements using Flow Cytometry.** Cells were incubated for 24 h and 48 h with different concentrations of $SPION^{LA}$, $SPION^{LA\text{-}HSA}$ and $SPION^{Dex}$ and investigated by flow cytometry. For SSc determination, it was gated on phenotypically viable cells (Ax-PI-). SSc data are shown for **(A)** BT-474, **(B)** T-47D, **(C)** MCF7, **(D)** MDA-MB-231 and **(E)** HUVECs. Positive controls contain 2% DMSO, negative controls represent the corresponding amount of H_2O instead of water-based ferrofluid. The data are expressed as the mean ± standard deviation (n=4 with technical triplicates). Statistical significance of 48 h data sets are indicated with *p<0.05, **p<0.001 and ***p<0.0001 and were calculated via Student's t-test. Asterisks shown directly on bars indicate dose-dependent significance to the next lower SPION concentration. Asterisks over the respective lines indicate dose-dependent significance between lowest and highest SPION concentration and between the highest SPION concentrations of different SPIONs. Abbreviations: SSc, side scatter; a.u., arbitrary unit.



Supplementary Figure 8: **Time- and Dose-Dependent Viability of Different Cells after SPION Treatment.** Cells were incubated for 24 h and 48 h with increasing amounts of $SPION^{LA}$, $SPION^{LA\text{-}HSA}$ and $SPION^{Dex}$. Cell viability was determined by Annexin A5-Fitc/propidium iodide staining and analyzed by flow cytometry. The amount of viable (Ax-PI-), apoptotic (Ax+PI-) and necrotic (PI+) cells are shown for **(A)** BT-474, **(B)** T-47D, **(C)** MCF7, **(D)** MDA-MB-231 and **(E)** HUVECs. Positive controls contain 2% DMSO, negative controls represent the corresponding amount of H_2O instead of water-based ferrofluid. Data are expressed as the mean ± standard deviation (n=4 with technical triplicates). Statistical significance of 48 h data sets are indicated with *p<0.05, **p<0.001 and ***p<0.0001 and were calculated via Student's t-test. Colored asterisks indicate dose-dependent significance between lowest and highest SPION concentrations on necrosis (red asterisks), apoptosis (yellow asterisks) and viability (green asterisks).



Supplementary figure 9: **Time- and Dose-Dependent DNA Degradation and Cell Cycle Analysis by Propidium Iodide-Triton X (PIT) Staining.** Cells were incubated for 24 h and 48 h with increasing amounts of SPION^{LA}, SPION$^{LA\text{-}HSA}$ and SPION^{Dex} and analyzed by flow cytometry. The DNA conditions are shown for **(A)** BT-474, **(B)** T-47D, **(C)** MCF7, **(D)** MDA-MB-231 and **(E)** HUVECs as the amount of degraded DNA, diploid DNA (G1-phase) and double diploid DNA (synthesis/G2-phase). Positive controls contain 2% DMSO, negative controls represent the corresponding amount of H_2O instead of water-based ferrofluid. Data are expressed as the mean ± standard deviation (n=4 with technical triplicates). Statistical significance of 48 h data sets are indicated with *p<0.05, **p<0.001 and ***p<0.0001 and were calculated via Student's t-test. Colored asterisks indicate dose-dependent significance between lowest and highest SPION concentrations on degraded DNA (red asterisks), diploid DNA (blue asterisks) and double diploid DNA (green asterisks).



Supplementary Figure 10: **Time- and Dose-Dependent Changes of the Mitochondrial Membrane Potential in Different Cells after SPION Treatment.** Cells were incubated for 24 h and 48 h with increasing amounts of SPION^{LA}, SPION$^{LA\text{-}HSA}$ and SPION^{Dex}. Membrane integrity was determined by 1,1′,3,3,3′,3′-hexamethylindodicarbocyanine iodide (DiIC1(5)) staining and analyzed by flow cytometry. The amount of cells with intact (DiIC1(5) positive) and depolarized (DiIC1(5) negative) membrane potential are shown for **(A)** BT-474, **(B)** T-47D, **(C)** MCF7, **(D)** MDA-MB-231 and **(E)** HUVECs. Positive controls contain 2% DMSO, negative controls represent the corresponding amount of H_2O instead of water-based ferrofluid. Data are expressed as the mean ± standard deviation (n=4 with technical triplicates). Statistical significance of 48 h data sets are indicated with *p<0.05, **p<0.001 and ***p<0.0001 and were calculated via Student's t-test. Asterisks indicate dose-dependent significance between lowest and highest SPION concentration on mitochondrial membrane integrity.