

Supplementary Information to

Sources of Variability in Nanoparticle Uptake by Cells

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Supplementary Tables

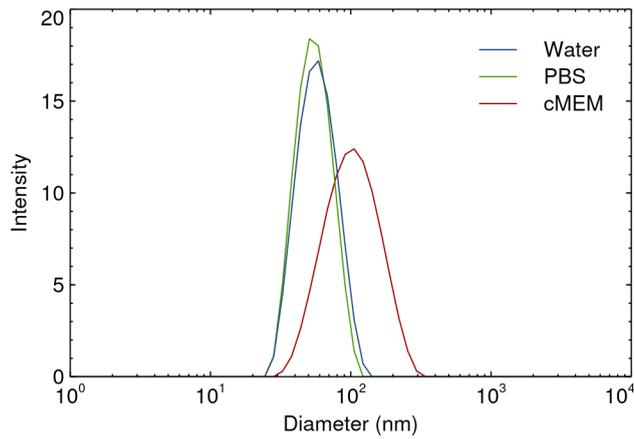
Supplementary Table S1. 40 nm polystyrene nanoparticle characterisation in relevant media. The diameter is the z-average hydrodynamic diameter extracted by cumulant analysis and the polydispersity index is from cumulant fitting of dynamic light scattering data. It should be noted that cumulant analysis assumes a single population to be present in the sample which is not the case in complete medium (cMEM) where also proteins are present. Therefore, we also show corresponding size distributions by CONTIN analysis in Supplementary Figure S1. We did not include the ζ potential in water due to the low conductivity of the dispersion in the absence of salt. The results are presented as the mean \pm the standard deviation over three replicate measurements.

Medium	Diameter (nm)	Polydispersity index	ζ (mV)
Water	55 \pm 1	0.09 \pm 0.02	-
PBS	53 \pm 1	0.08 \pm 0.01	-36 \pm 1
cMEM	84 \pm 1	0.25 \pm 0.00	-7 \pm 1

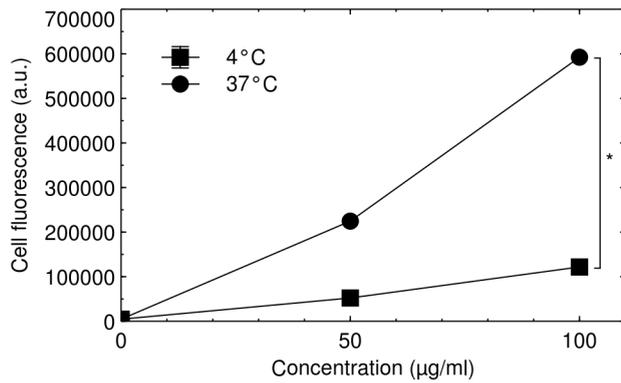
Supplementary Table S2. Enrichment after each subsequent sorting procedure of the 5% of HeLa cells exhibiting the lowest and highest uptake of 40 nm polystyrene nanoparticles. Cells were exposed to 100 µg/ml of the nanoparticles for 2 h and the 5% of cells taking up the least and most nanoparticles, respectively, were collected. The cells were then sorted another 4 (low) or 5 (high) times. At the beginning of each such sorting procedure, the fluorescence of unsorted cells was assessed and used to set the fluorescence thresholds that correspond to the 5% low and high uptakers, respectively. Subsequently, the fluorescence of the sorted cells was measured and the proportion of cells within the limits quantified, as reported in the table.

	2nd	3rd	4th	5th	6th sort
Low	5.1%	5.4%	5.4%	5.1%	
High	9.1%	9.2%	16.2%	11.8%	12.7%

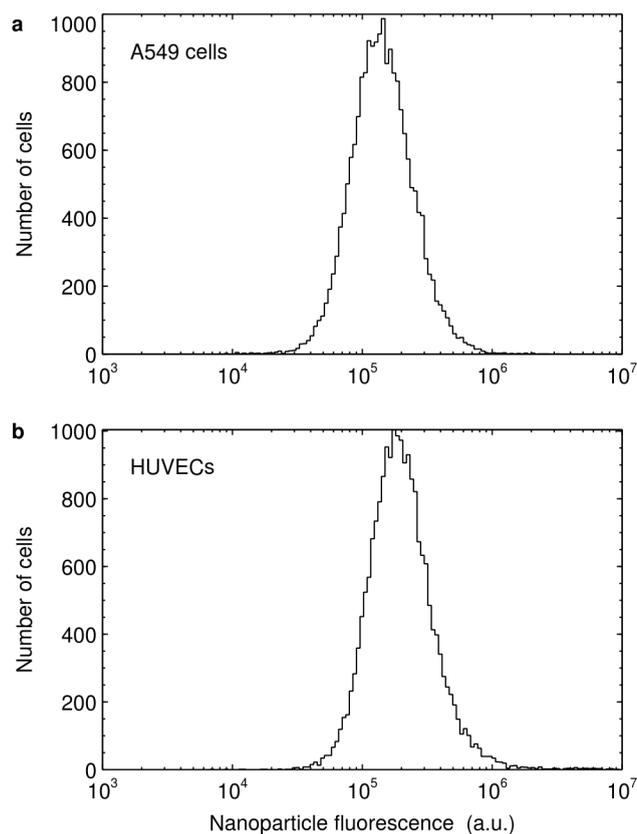
Supplementary Figures



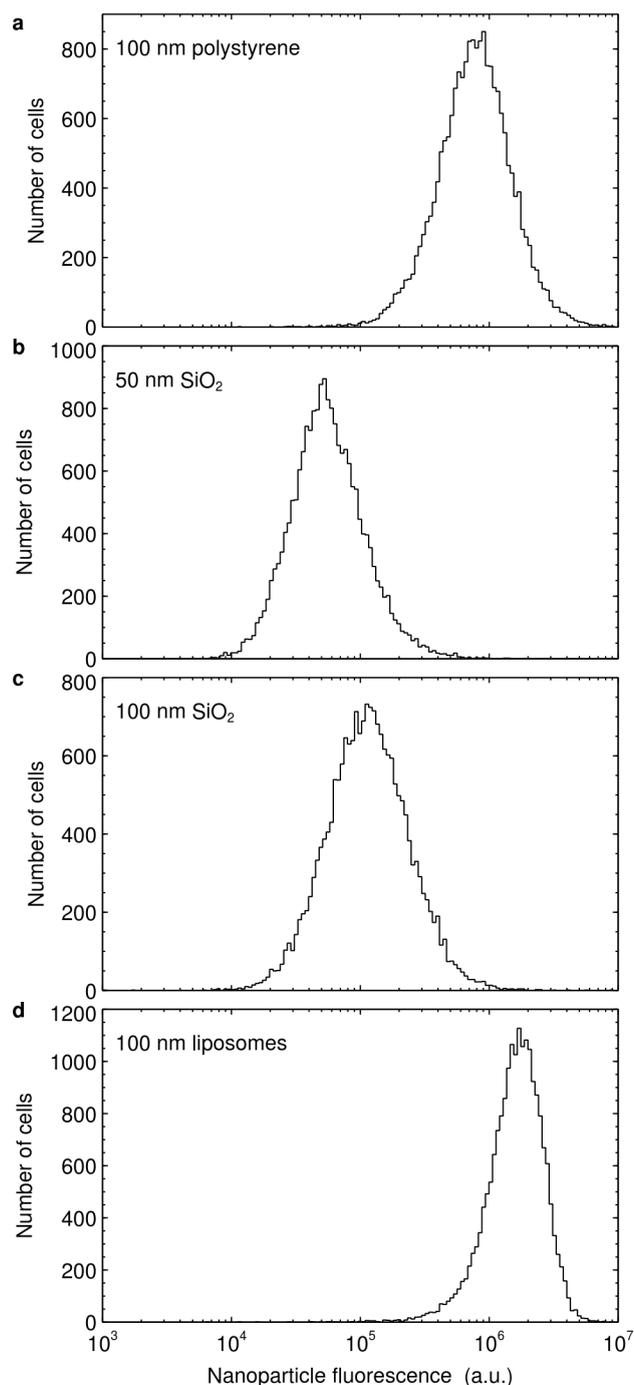
Supplementary Figure S1. 40 nm polystyrene nanoparticle size distribution by CONTIN analysis of dynamic light scattering data. The particles disperse well in water and PBS, consistent with the low polydispersity index (Supplementary Table S1) and previous literature.¹⁻⁶ In complete medium (cMEM) a shift of the distribution to larger sizes is observed, as expected upon protein adsorption from the medium and corona formation. However, the magnitude of the size shift and width of the distribution should be interpreted with some caution as free proteins (and other biomolecules) in the medium also contribute to scattering, a known limitation of using dynamic light scattering on complex samples.



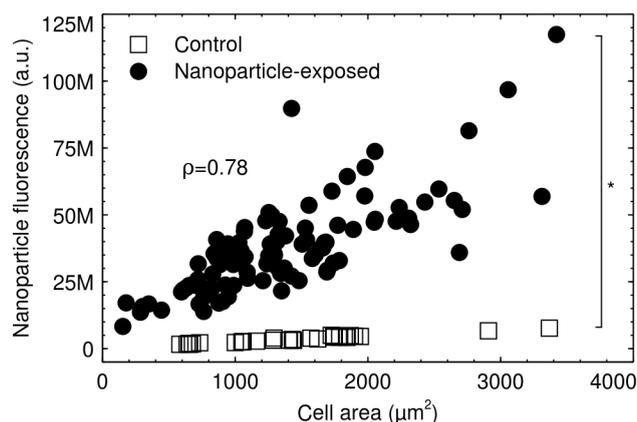
Supplementary Figure S2. Assessment of the fraction of 40 nm polystyrene nanoparticles adsorbed to the outer cell membrane, rather than internalised, in HeLa cells. The cells were exposed to the indicated concentrations of 40 nm polystyrene yellow/green nanoparticles for 2 h at 4 and 37 °C, respectively, and the fluorescence of the cells subsequently assessed by flow cytometry. Uptake occurs at 37 °C (*i.e.*, under “normal” conditions), but is blocked at 4 °C, since nanoparticle uptake is a cell-energy-dependent process.^{2,7,8} The fluorescence that, nevertheless, is measured under 4 °C-conditions thus mainly represents nanoparticles stuck on the outer cell membrane (as well as some residual dye leaking out from the particles²). Datapoints and error bars represent the mean and standard deviation over three replicate samples. A Friedman test with concentration as blocking factor shows a statistically significant difference between the two conditions. The results show that the majority of fluorescence stems from nanoparticles within the cells already after 2 h of uptake (at longer timescales the proportion will be even higher^{1,8}). For a more extended study of cellular adsorption of the same nanoparticles, we refer to our previous work.⁸



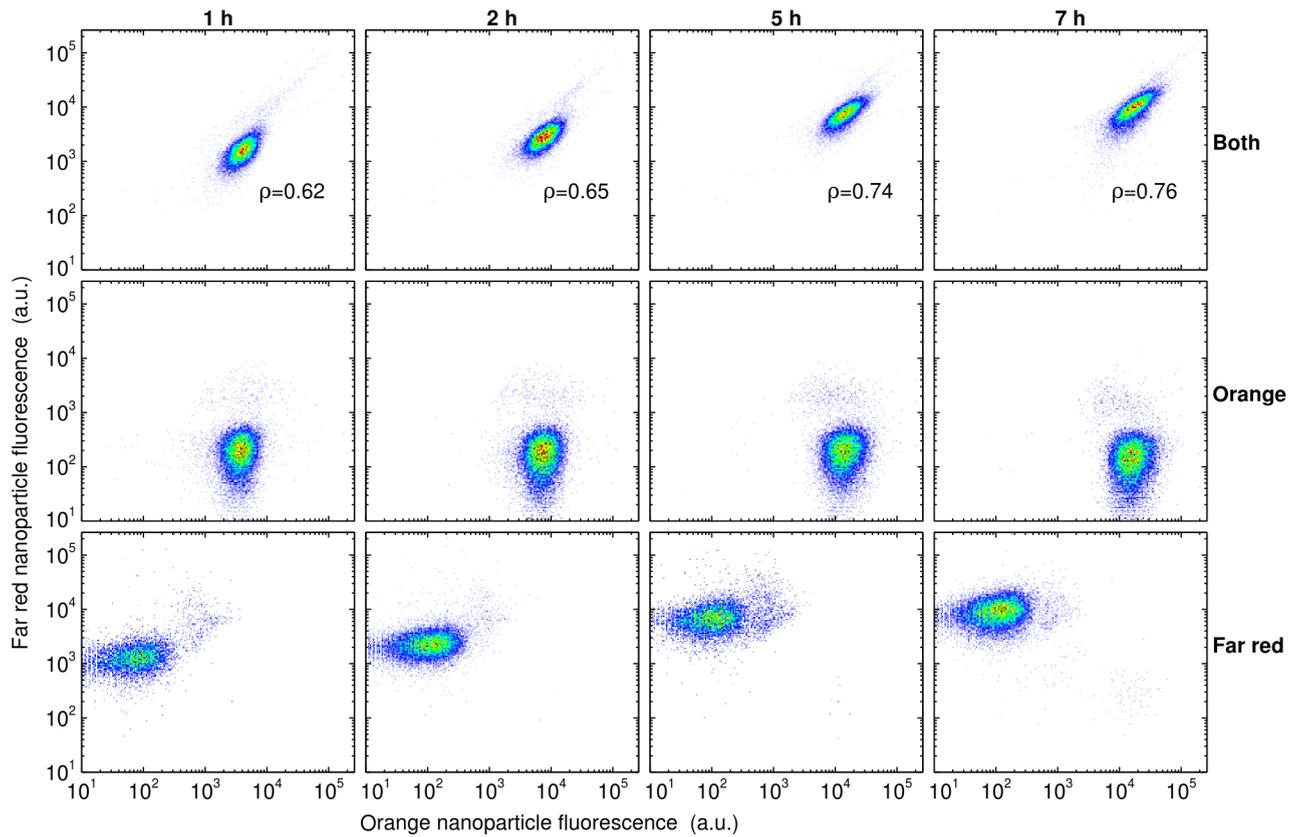
Supplementary Figure S3. Cell-to-cell variability in 40 nm polystyrene nanoparticle uptake by A549 cells and HUVECs. Cells were exposed to 40 nm yellow/green polystyrene nanoparticles and the fluorescence of the cells assessed by flow cytometry. The panel shows the number of cells which exhibit a given nanoparticle fluorescence. *N.B.* nanoparticle fluorescence is presented in log scale. **a**, A549 cells (25 $\mu\text{g}/\text{ml}$ for 2 h). **b**, HUVECs. (2.5 $\mu\text{g}/\text{ml}$ for 5 h). The results generalise the observation of a large variability in uptake observed for HeLa cells (Fig. 1a) to other cell types.



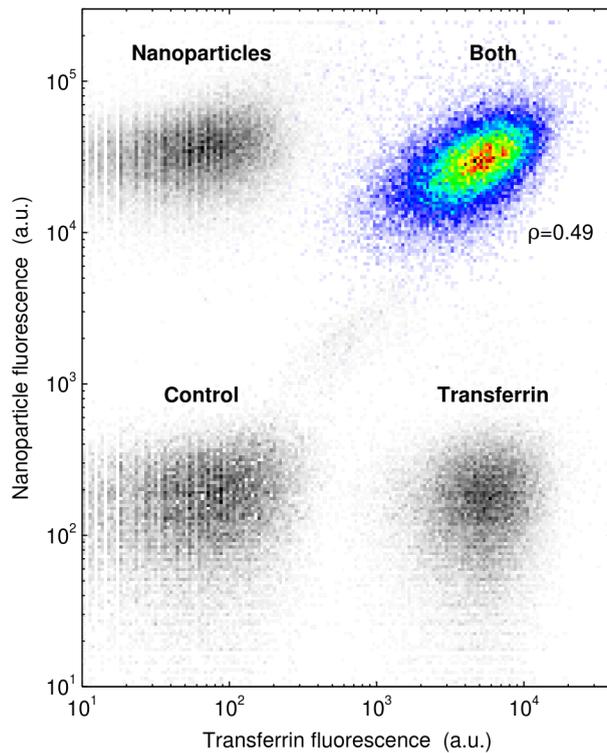
Supplementary Figure S4. Cell-to-cell variability in nanoparticle uptake by HeLa cells exposed to various types of nanoparticles. HeLa cells were exposed to the various nanoparticles and the fluorescence of the cells assessed by flow cytometry. The panel shows the number of cells which exhibit a given nanoparticle fluorescence. *N.B.* nanoparticle fluorescence is presented in log scale. **a**, 100 nm polystyrene nanoparticles (50 $\mu\text{g}/\text{ml}$ for 6 h). **b**, 50 nm silica nanoparticles (100 $\mu\text{g}/\text{ml}$ for 6 h). **c**, 100 nm silica nanoparticles (100 $\mu\text{g}/\text{ml}$ for 6 h). **d**, 100 nm liposomes (50 $\mu\text{g}/\text{ml}$ for 1 h). The results generalise the observation of a large variability in nanoparticle uptake by HeLa cells observed for 40 nm polystyrene nanoparticles (Fig. 1a) to other nanoparticles.



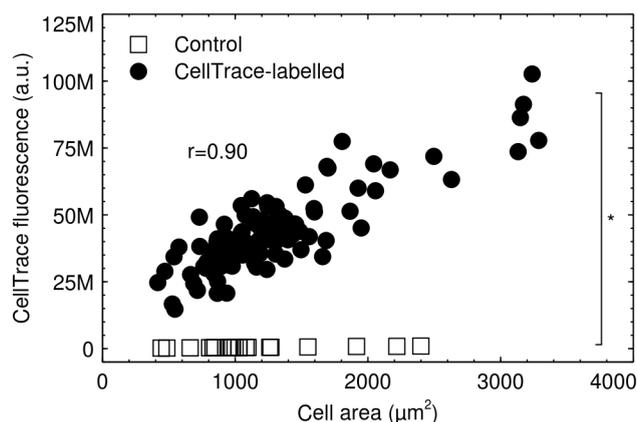
Supplementary Figure S5. Cell-to-cell variability in 40 nm polystyrene nanoparticle uptake by HeLa cells assessed using fluorescence microscopy. Cells were exposed to 50 μg/ml 40 nm yellow/green nanoparticles for 5 h and then imaged using fluorescence microscopy. Cell area was quantified by identification of the outline of cells using a cell membrane stain (CellMask far red); nanoparticle fluorescence was quantified as the total nanoparticle fluorescence within the cell outline. Each datapoint in the figure corresponds to one cell. (Control) Nanoparticle fluorescence of cells not exposed to nanoparticles; (Nanoparticle-exposed) Nanoparticle fluorescence of cells exposed to nanoparticles. A one-sided Mann-Whitney U test shows a statistically significant difference between control and nanoparticle-exposed cells (30 and 100 cells, respectively). The results show that the background fluorescence is much lower than the signal measured for nanoparticle-exposed cells. The correlation between nanoparticle fluorescence and cell area was quantified in terms of Spearman's correlation coefficient, ρ , as indicated in the figure. The results show a positive correlation between nanoparticle uptake and cell (cross-sectional) area. Nevertheless, there is still a degree of cell-to-cell variability that is not explained by cell (cross-sectional) area.



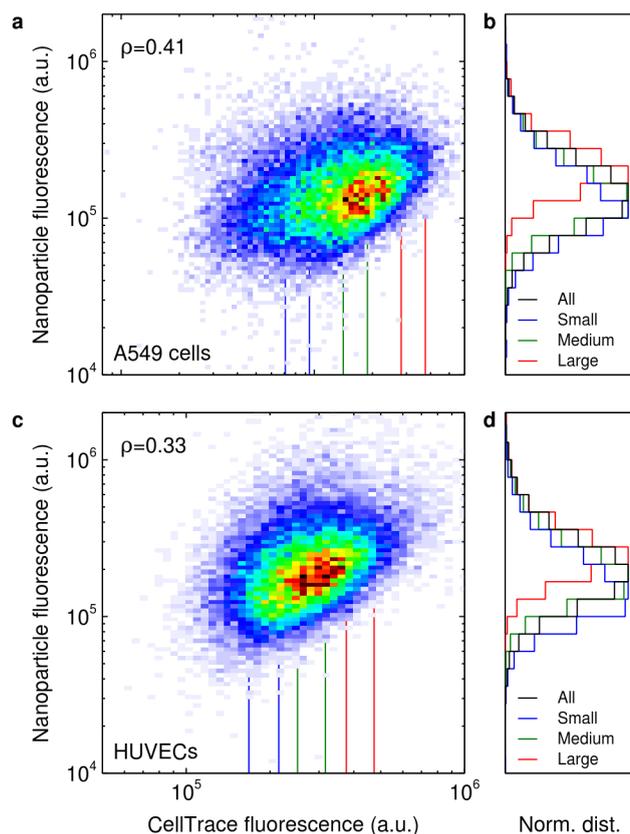
Supplementary Figure S6. Cell-to-cell variability in 40 nm polystyrene nanoparticle uptake by HeLa cells as a function of time. Cells were exposed to either 40 nm far-red nanoparticles, 40 nm orange nanoparticles or to both nanoparticle colours at the same time. The nanoparticle concentration was 5 $\mu\text{g/ml}$ for each colour (so the cells exposed to both colours were exposed to 10 $\mu\text{g/ml}$ nanoparticles in total) and the exposure lasted for the times indicated in the figure. Afterwards the fluorescence of the cells was assessed by flow cytometry. The rows denote cells exposed to both nanoparticle colours, orange nanoparticles and far-red nanoparticles, respectively. The columns denote different exposure times. The 5 h sample is the one presented in Fig. 1. The correlation between uptake of the two nanoparticles was quantified in terms of Spearman's correlation coefficient, ρ , as indicated in the upper panels.



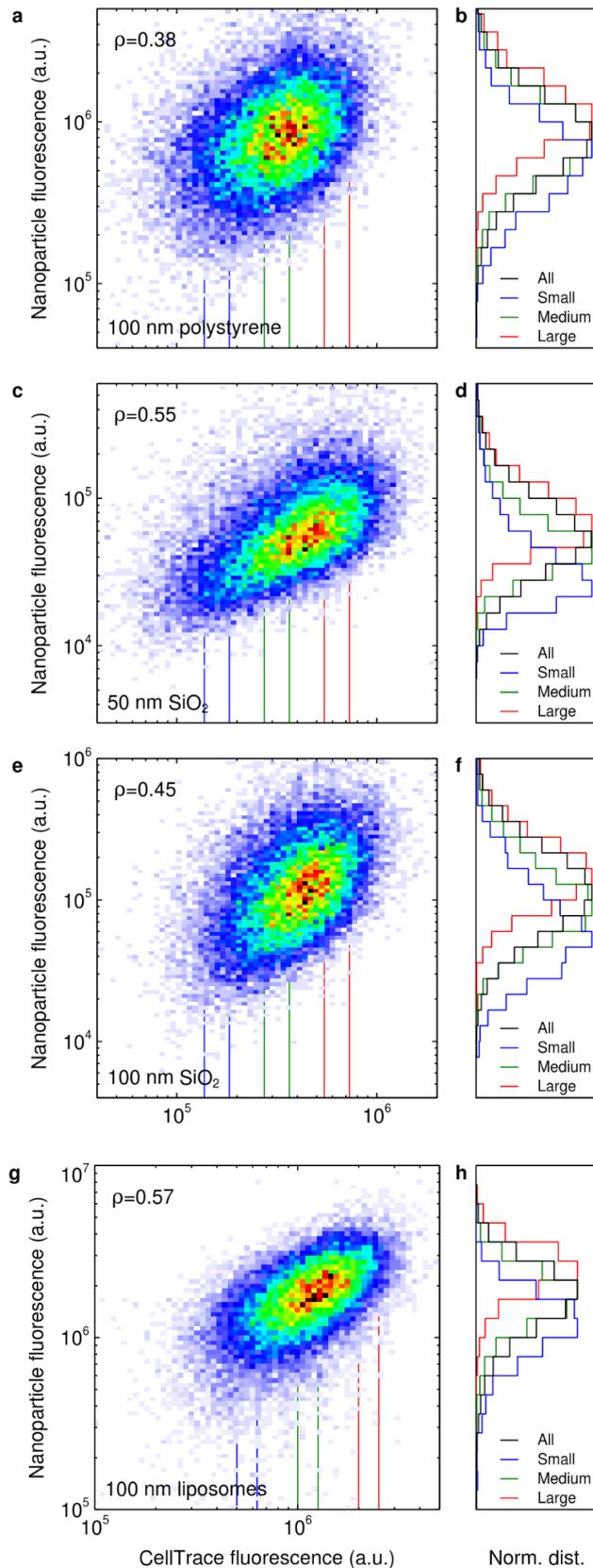
Supplementary Figure S7. Cell-to-cell variability in 40 nm polystyrene nanoparticle and transferrin uptake in HeLa cells. Cells were exposed to 20 $\mu\text{g}/\text{ml}$ of 40 nm far-red polystyrene nanoparticles and/or 15 $\mu\text{g}/\text{ml}$ of transferrin for 5 h. Unlabelled control cells were also left for 5 h. Afterwards the fluorescence of the cells was assessed by flow cytometry. The panel shows all the different samples (including also control cells) as heat maps. The correlation between nanoparticle and transferrin accumulation was quantified in terms of Spearman's correlation coefficient, ρ , as indicated in the figure.



Supplementary Figure S8. Relationship between CellTrace fluorescence and cell size in HeLa cells assessed using fluorescence microscopy. Cells were labelled with CellTrace and then imaged using fluorescence microscopy. Cell area was quantified by identification of the outline of cells using a cell membrane stain (CellMask orange); CellTrace fluorescence was quantified as the total fluorescence within the cell outline. Each datapoint in the figure corresponds to one cell. (Control) CellTrace fluorescence of cells not labelled by CellTrace; (CellTrace-labelled) CellTrace fluorescence of cells labelled by CellTrace. A one-sided Mann-Whitney U test shows a statistically significant difference between control and CellTrace-labelled cells (20 and 107 cells, respectively). The results show that the background fluorescence is much lower than the signal measured for CellTrace-labelled cells. The correlation between CellTrace fluorescence and cell area was quantified in terms of Pearson's correlation coefficient, r , as indicated in the figure. We used Pearson's correlation coefficient, because it gives a measure of the *linear* correlation; a strong linear correlation would justify the usage of CellTrace fluorescence as a proxy for cell size, without further data treatment. Indeed, the results show a strong linear correlation between CellTrace fluorescence and cell size.

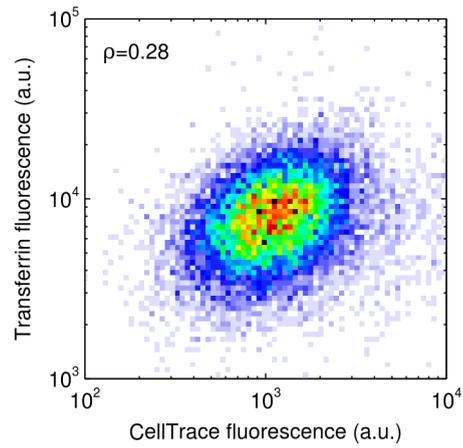


Supplementary Figure S9. 40 nm polystyrene nanoparticle uptake by A549 cells and HUVECs as a function of cell size. Cells were labelled with CellTrace, exposed to 40 nm yellow/green polystyrene nanoparticles and the fluorescence of the cells assessed by flow cytometry. **a-b**, A549 cells (25 $\mu\text{g}/\text{ml}$ for 2 h). **c-d**, HUVECs (2.5 $\mu\text{g}/\text{ml}$ for 5 h). **a, c**, Cellular nanoparticle fluorescence vs. cell size measured in terms of CellTrace fluorescence. The correlation between the two variables was quantified in terms of Spearman's correlation coefficient, ρ , as indicated in the figure. **b, d**, The distribution of cellular nanoparticle fluorescence for the total cell population as well as three subpopulations corresponding to small, medium and large cells. The distributions have been normalised such that the peak corresponds to 1 in order to present all results on the same scale. The subpopulations were defined in terms of the CellTrace fluorescence as indicated in panel a and c, respectively (vertical lines). The results generalise the observation in HeLa cells of a correlation between cell size and nanoparticle uptake (Fig. 2a), as well as the residual variability not explained by size (Fig. 2b), to other cell types.

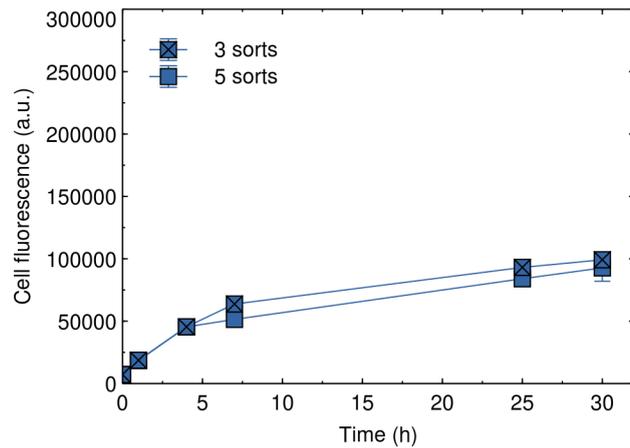


Supplementary Figure S10. Nanoparticle uptake by HeLa cells as a function of cell size for various types of nanoparticles. Cells were labelled with CellTrace, exposed to the various nanoparticles and the fluorescence of the cells assessed by flow cytometry. **a-b**, 100 nm polystyrene nanoparticles (50 $\mu\text{g/ml}$ for 6 h); **c-d**, 50 nm silica nanoparticles (100 $\mu\text{g/ml}$

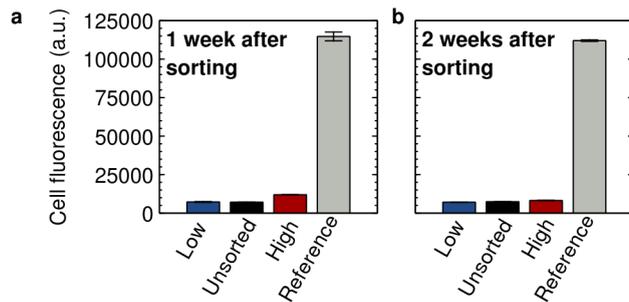
for 6 h); **e-f**, 100 nm silica nanoparticles (100 µg/ml for 6 h); **g-h**, 100 nm liposomes (50 µg/ml for 1 h). **a, c, e, g**, Cellular nanoparticle fluorescence vs. cell size measured in terms of CellTrace fluorescence. The correlation between the two variables was quantified in terms of Spearman's correlation coefficient, ρ , as indicated in the figure. **b, d, f, h**, The distribution of cellular nanoparticle fluorescence for the total cell population as well as three subpopulations corresponding to small, medium and large cells. The distributions have been normalised such that the peak corresponds to 1 in order to present all results on the same scale. The subpopulations were defined in terms of the CellTrace fluorescence as indicated in panels a, c, e and g, respectively (vertical lines). The results generalise the observation for 40 nm polystyrene nanoparticles of a correlation between cell size and nanoparticle uptake (Fig. 2a), as well as the residual variability not explained by size (Fig. 2b), to other nanoparticles.



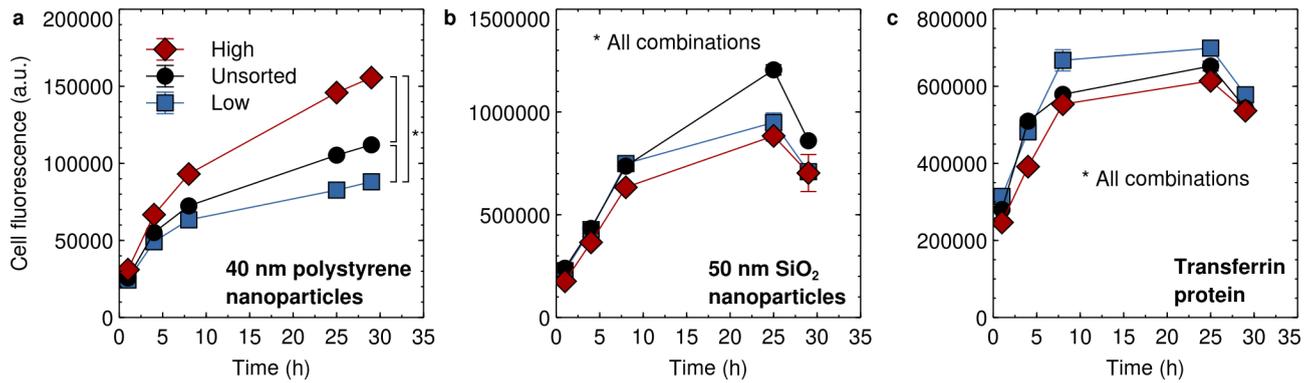
Supplementary Figure S11. Transferrin uptake as a function of HeLa cell size. Cells were labelled with CellTrace, after which they were exposed to 15 $\mu\text{g/ml}$ of Alexa Fluor 546-conjugated transferrin for 5 h and the fluorescence of the cells assessed by flow cytometry. The results are presented as cellular transferrin fluorescence vs. cell size measured in terms of CellTrace fluorescence. The correlation between the two variables was quantified in terms of Spearman's correlation coefficient, ρ , as indicated in the figure.



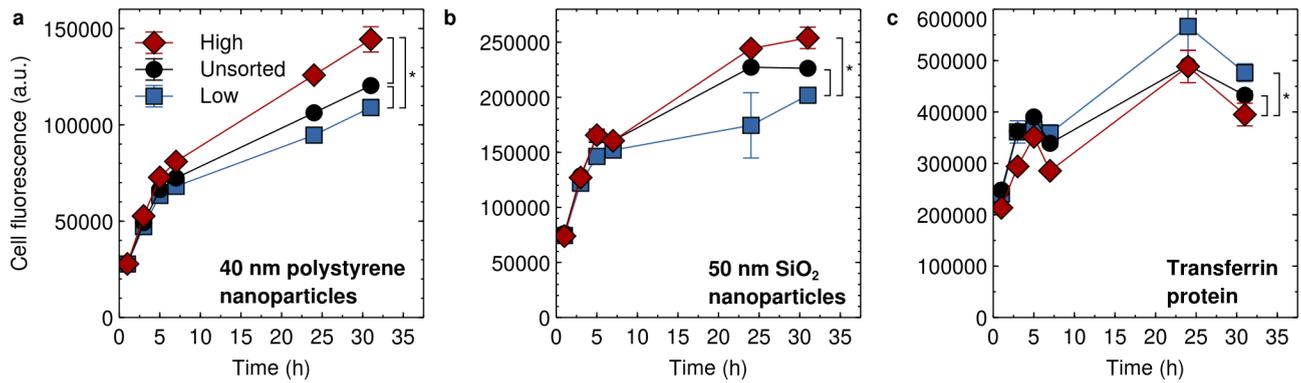
Supplementary Figure S12. 40 nm polystyrene nanoparticle uptake in HeLa cells subjected to different number of sorting occasions, sorted for low uptake. Cells were sorted the indicated number of times with the threshold set to separate the 1% of cells exhibiting the lowest nanoparticle uptake. The cells were exposed to 10 $\mu\text{g/ml}$ of 40 nm polystyrene yellow/green nanoparticles for the indicated time periods and their fluorescence assessed by flow cytometry. Datapoints and error bars represent the mean and standard deviation over two replicate samples. A Friedman test with time as blocking factor does not show a statistically significant difference between the cells sorted 3 and 5 times. We did not perform extensive experiments during the sorting procedure, in order to minimise the time between sorting occasions (see the main text for details). However, these results show that the number of sorting occasions do not matter (at least for the low uptakers and for 3 and 5 sorting occasions).



Supplementary Figure S13. Residual nanoparticle fluorescence after sorting of HeLa cells with a 1% threshold. Cells were sorted 5 times with the threshold set to separate the 1% of cells exhibiting the lowest and highest nanoparticle uptake, respectively. Unsorted cells subcultured an equivalent number of times were used as controls. The experiment was performed on all cell cultures simultaneously, which implies that it had passed a differing number of days after the last sorting session for the low and high uptakers, respectively (the two cultures ended up being sorted on different days). See the main text and Experimental section for details. To assess eventual residual fluorescence of the sorted cells, cell fluorescence was measured by flow cytometry after conclusion of the sorting procedure. As a comparison, the background fluorescence of unsorted cells that had never been exposed to nanoparticles was assessed. The result reported is the fluorescence corresponding to yellow/green polystyrene nanoparticles. The “reference” shows the fluorescence signal of unsorted cells that were exposed to nanoparticles for 30 h to set a relevant scale for comparison. **a**, Results corresponding to Fig. 4c-e (performed 9 and 7 days after the last sorting session for the low and high uptakers, respectively). **b**, Results corresponding to Fig. S13 below (performed 16 and 14 days after the last sorting session for the low and high uptakers, respectively). Datapoints and error bars represent the mean and standard deviation over two replicate samples. A Friedman test shows no statistically significant difference between the three different types of cells for either experiment. The results show that the cells sorted for both low and high uptake (which were exposed to nanoparticles during the sorting procedure) essentially overlap in fluorescence with unsorted cells (that were never exposed to nanoparticles). Thus, the sorted cells exhibit no major residual nanoparticle fluorescence, consistent with the cells dividing and diluting their nanoparticle load¹ multiple times in the period between the sorting procedure and the subsequent experiments on the sorted cells.



Supplementary Figure S14. Nanoparticle and protein uptake in sorted HeLa cells (repeat of the experiment shown in Fig. 4 one week later). Cells were sorted 5 times with the threshold set to separate the 1% of cells exhibiting the lowest and highest nanoparticle uptake, respectively. Unsorted cells subcultured an equivalent number of times were used as controls. The experiment was performed on all cell cultures simultaneously, which implies that it had passed 16 and 14 days after the last sorting session for the low and high uptakers, respectively (the two cultures ended up being sorted on different days). See the main text and Experimental section for details. The cells were exposed to **a**, 10 $\mu\text{g}/\text{ml}$ of 40 nm yellow/green polystyrene nanoparticles **b**, 100 $\mu\text{g}/\text{ml}$ of 50 nm SiO₂ nanoparticles and **c**, 15 $\mu\text{g}/\text{ml}$ transferrin for the indicated time periods and the fluorescence of the cells assessed by flow cytometry. Datapoints and error bars represent the mean and standard deviation over two replicate samples. A Friedman test with time as blocking factor shows a statistically significant difference between the three different types of cells in all cases. Comparison between individual curves shows statistically significant differences in all cases. The results show that for the particle used to define the sorted subpopulations (40 nm polystyrene; panel a) the high uptakers take up the most nanoparticles, the low the least and the unsorted cells in between. This ranking cannot be confirmed for the other nanoparticle (50 nm silica; panel b) in this experiment, though in the repeat experiment performed a week earlier (Fig. 4d) and when we used the 5% threshold (Supplementary Fig. S15 below) it partly could. For transferrin, the same ranking is not present at all, consistent with the other results (Fig. 4e and Supplementary Fig. S15 below).



Supplementary Figure S15. Nanoparticle and protein uptake in HeLa cells sorted with a 5% threshold after cryopreservation and subsequent reculture. Cells were sorted a number of times with the threshold set to separate the 5% of cells exhibiting the lowest (sorted 5 times) and highest (sorted 6 times) nanoparticle fluorescence, respectively. Unsorted cells subcultured an equivalent number of times were used as controls. See the main text for details. After sorting, the cells were cryopreserved and then brought back into culture. The cells were subsequently exposed to **a**, 10 $\mu\text{g/ml}$ of 40 nm yellow/green polystyrene nanoparticles **b**, 100 $\mu\text{g/ml}$ of 50 nm SiO₂ nanoparticles and **c**, 15 $\mu\text{g/ml}$ Alexa Fluor 647-conjugated transferrin for the indicated time periods and the fluorescence of the cells assessed by flow cytometry. Datapoints and error bars represent the mean and standard deviation over two replicate samples. A Friedman test with time as blocking factor shows a statistically significant difference between the three different types of cells in all cases. Comparison between individual curves (taking into account multiple comparisons) gives the statistically significant differences indicated by asterisks in the figure. The results show that for the particle used to define the sorted subpopulations (40 nm polystyrene; panel a) the high uptakers take up the most nanoparticles, the low the least and the unsorted cells in between. For the other nanoparticle (50 nm silica; panel b) the low uptakers take up fewer nanoparticles than the high uptakers and unsorted cells. For transferrin, the results do not show the same ranking at all.

Further discussion of the sorting experiments

There are several technical and interpretational issues with the sorting experiments which dictated our detailed choices and interpretation of the results:

One concern is residual nanoparticles remaining within the cells after the sorting procedure and hence accumulating with each exposure. This would skew both subpopulations into higher nanoparticle fluorescence. Indeed, it is most likely the case that the 40 nm polystyrene nanoparticles are not exported from HeLa cells, as we have previously shown conclusively for a different cell line (and over a limited 4-10 h time period post-exposure).¹ However, as the cells proliferate, the average number of nanoparticles per cell is reduced due to cell division and this effectively mitigates the issue.¹ In fact, the residual nanoparticle fluorescence of the sorted cells is essentially the same as cells that have never been exposed to nanoparticles (Supplementary Fig. S13).

A related issue is that the cell division cycle will inherently lead to cells at the beginning of the cell cycle being cells with low amounts of nanoparticles and cells about to divide being cells with high.^{1,9} The sorting procedure would then (partly) enrich for cells at two positions of the cell cycle, something which would subsequently be lost upon reculture and amplification. However, the cell cycle effect is only apparent at time-scales of the cell population doubling time (around a day for these cells). We consequently used a more limited exposure time of 2 h to mitigate this issue as much as possible.

One must also differentiate between nanoparticles adhered on the outside of the cell membrane vs. those actually internalised, while the cell fluorescence measurement actually includes both.⁸ Since we are interested in sorting for cells having a low/high uptake, only the ones inside matter. This becomes an issue, for example, since larger cells may adsorb more nanoparticles, without actually internalising more (or internalising a smaller proportion) and, similarly, cells about to divide are often larger cells. In both cases, larger cells and/or cells about to divide would then be (partly) enriched during sorting of the high uptakers and *vice versa* for the low uptakers. This is particularly an issue for shorter exposure times, where a larger portion of nanoparticles will be found on the outside, compared to for longer exposure times. Quenching of the fluorescence extracellularly would be ideal, but a quencher would be unlikely to make a difference for the nanoparticles we use, given that they are labelled in the core. In any case, we chose an exposure time of 2 h, which we have previously demonstrated is sufficient for the majority, though not all, of the measured fluorescence to stem from nanoparticles within the cells.⁸

Another issue is that since each sorting procedure takes several hours, the samples by necessity remain in the absence of nanoparticles for differing amounts of time. That is, the cells that were sorted in the beginning of a session had been left only a minimal time after nanoparticle exposure, while the cells that were sorted at the end of a session had been left for longer time after nanoparticle exposure. Nanoparticles adsorbed to the outer side of the cell membrane thus have differing times to desorb, which suggests that the nanoparticle fluorescence of cells will shift towards lower values during a sorting session. Furthermore, free fluorescence dye also has time to leave the cells.² Indeed a shift in fluorescence was observed. In order to accommodate for this effect, the definition of low and high uptakers was shifted throughout the experiment, in such a way that the proportion of cells within each subpopulation remained the same throughout.

When checking the purity of the sorted samples, there are also some issues to bear in mind. First, there is a certain variability associated with the fluorescence measured even if the cell was exactly the same (*e.g.*, due to the illumination being slightly different depending upon how the cell flows past the detectors). This variability will contribute to a widening of the fluorescence distribution and will give some “spill-over” across the threshold when checking the purity. The background signal could also be different between the original and

the sorted sample due to the different buffers used, as well as due to some dye leaking from the nanoparticles² which will be at a different concentration in the original highly concentrated cell suspension compared to the more dilute sorted one. Furthermore, nanoparticle desorption from the outer cell membrane could give rise to a loss of signal (as discussed above), something which could additionally be aggravated by shear stress during the actual sorting procedure as well as the high amount of serum (50% FBS) included in the buffer used to collect the cells after the sorting (serum can facilitate the desorption process).

References

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