Electronic Supplementary Information

Membrane-localized magnetic hyperthermia promotes intracellular delivery of cell-impermeant probes

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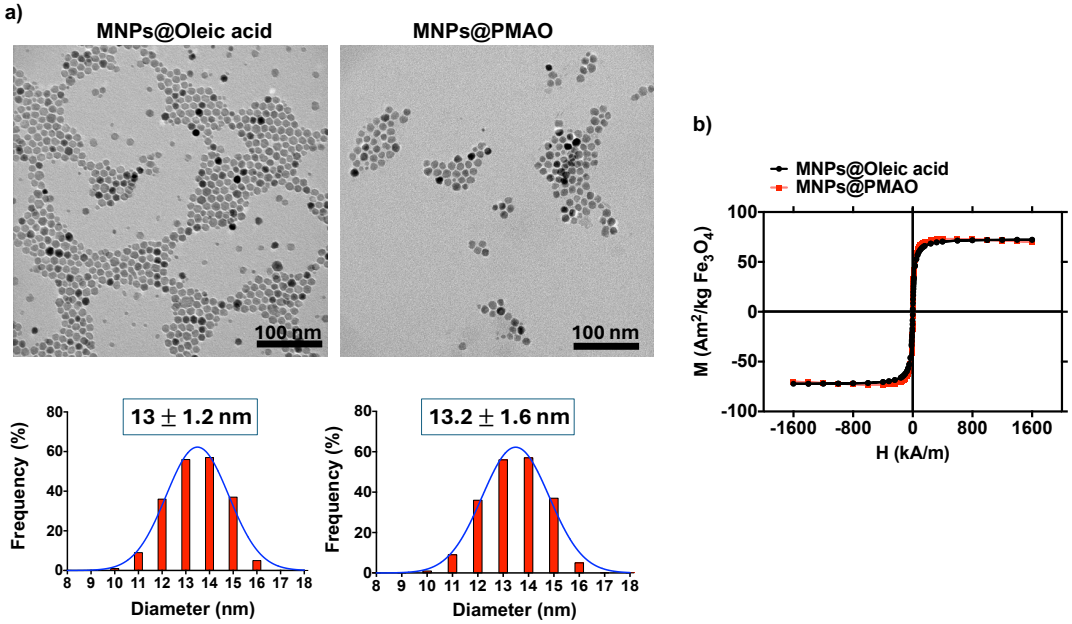
1. MNP characterization



c)

MNP	Hydrodynamic diameter (nm, intensity)	ζ potential (mV)
MNPs@PMAO	106 ± 5	-32.6 ± 0.8
MNPs@PMAO@PEG	91 ± 2	-21.1 ± 1.5
MNPs@PMAO@PEG@DBCO	82 ± 3	-8.4 ± 0.4

Figure S1. a) TEM images and size distribution histograms of the hydrophobic MNPs obtained by thermal decomposition (MNPs@Oleic acid) and the hydrophilic MNPs after polymer coating (MNPs@PMAO). b) Field-dependent magnetization of the two types of MNPs. c) Hydrodynamic diameters and ζ potential values of the MNPs@PMAO, MNPs@PMAO@PEG and MNPs@PMAO@PEG@DBCO in water.

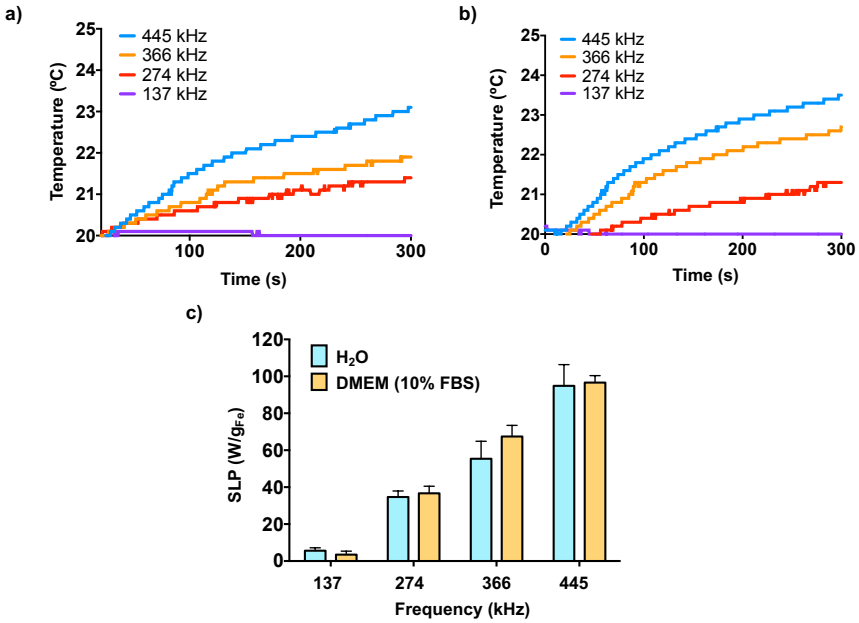


Figure S2. SLP measurements of MNPs@PMAO@PEG@DBCO (1 mg Fe/mL) in H_2O (a) and supplemented DMEM (b) at a field amplitude of 16.9 kA/m and different frequencies. c) Comparison of SLP values in H_2O and supplemented DMEM. Results represent the mean ± standard deviation of two different measurements.

2. *In vitro* magnetic hyperthermia

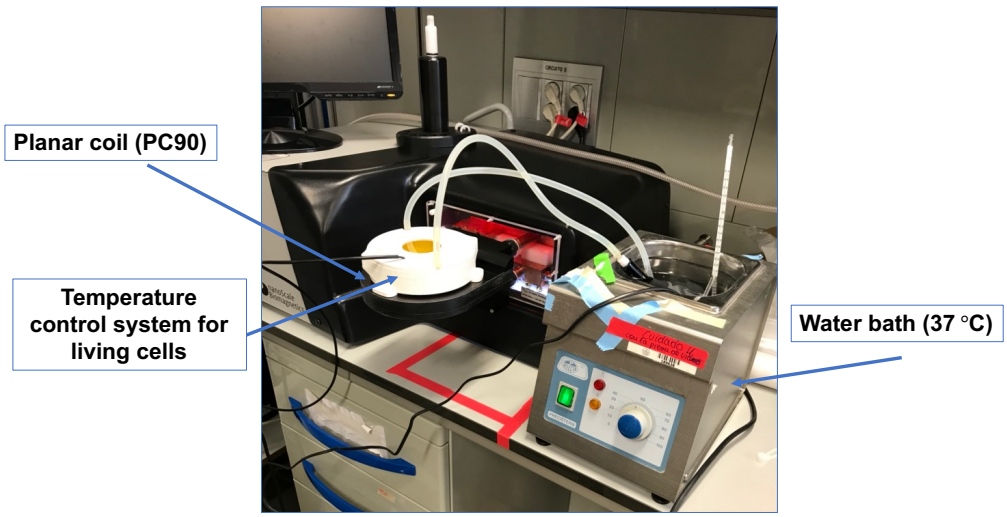


Figure S3. AMF setup for the application of MH in cell cultures.

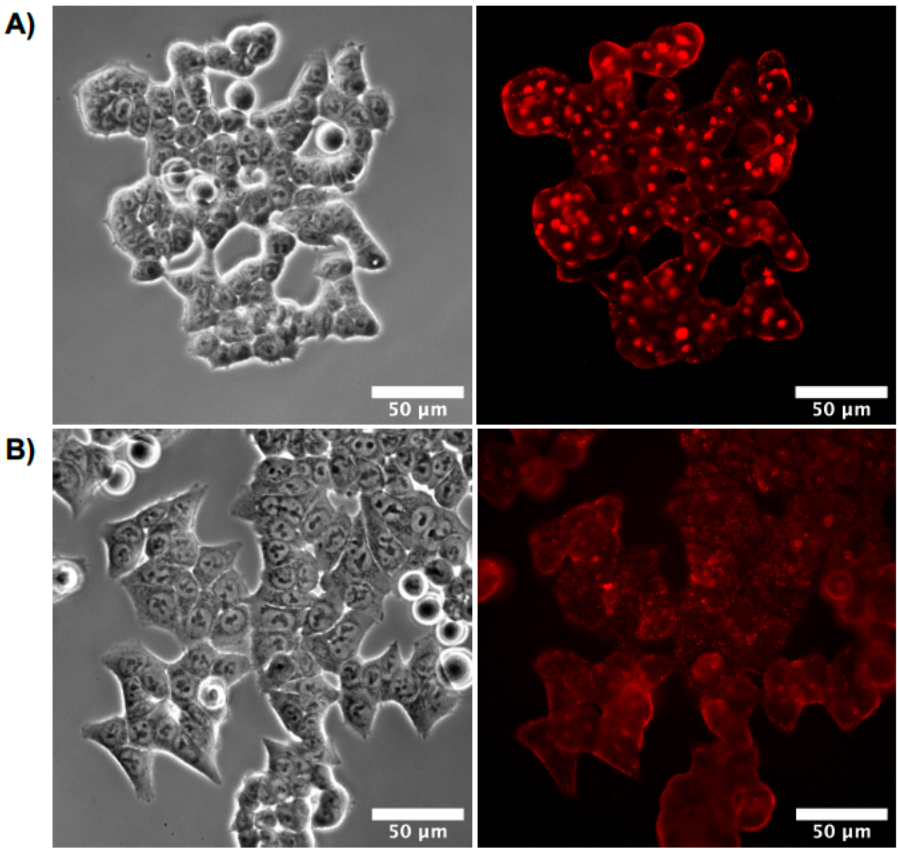


Figure S4. Brightfield and fluorescence microscopy images of HCT116 cells: a) 30 min after the AMF application (30 min, 425 kHz, 16.9 kA/m) and b) 1 h after incubation with MNPs but without AMF application.

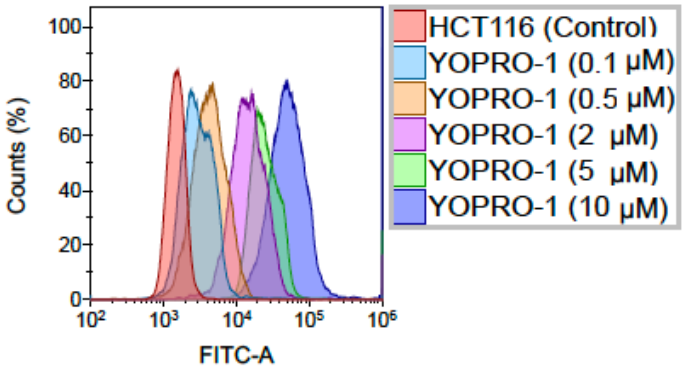


Figure S5. Flow cytometry optimization of the YO-PRO®-1 concentration for MH experiments. HCT116 cells were incubated with different concentrations of YO-PRO®-1 (0.1 to 10 µM) for 30 min at 37 °C.

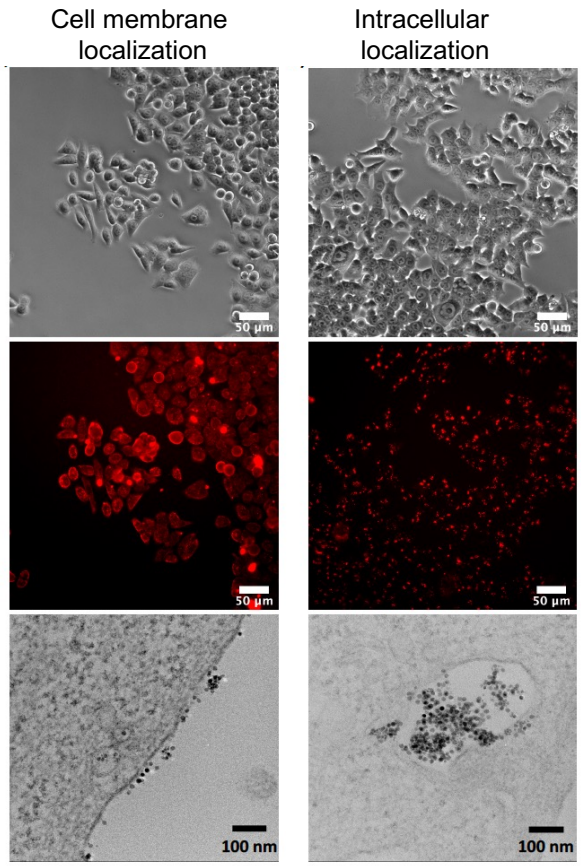


Figure S6. Fluorescence microscopy and transmission electron microscopy images of HCT116 cells with MNPs@PMAO@PEG@DBCO immobilized on the membrane (left) or internalized (right). Scale bars are 50 µm for the fluorescence microscopy images and 100 nm for the TEM images.

3. Cell viability studies

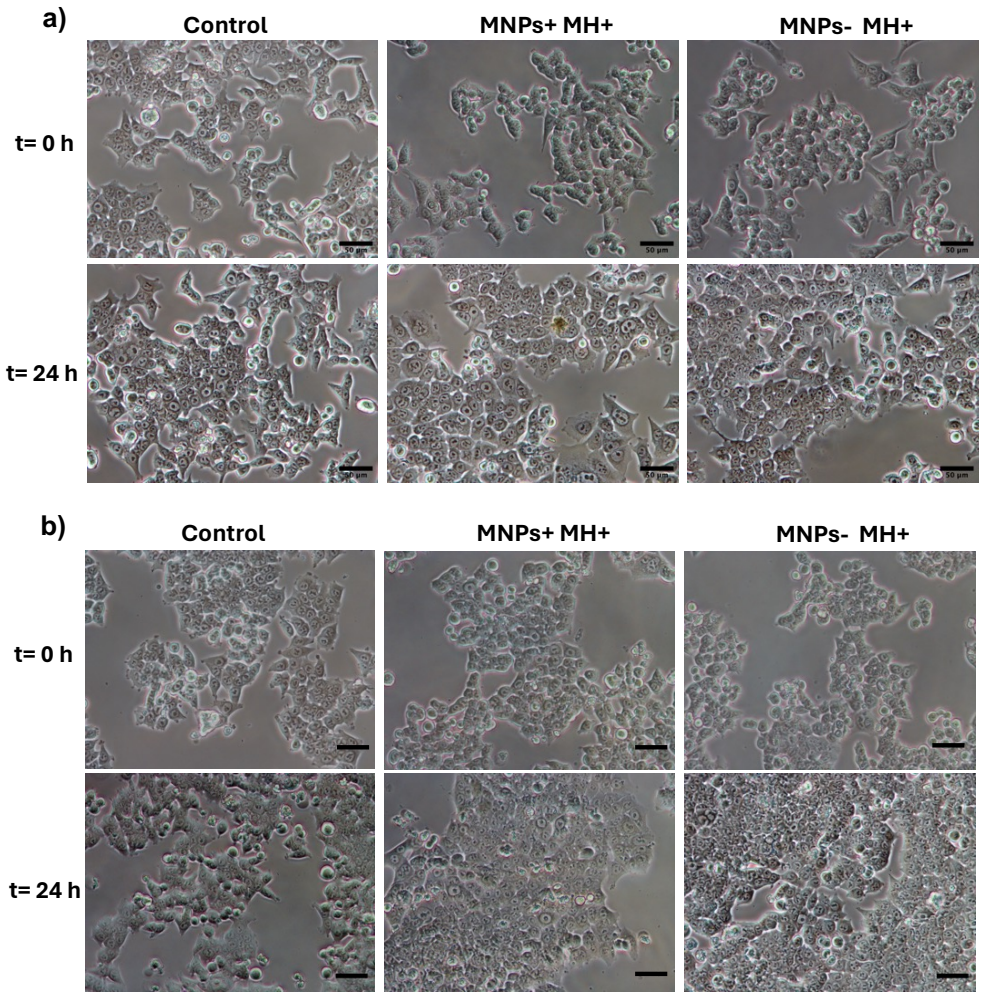


Figure S7. a) Evolution of cell growth after MH (425 kHz, 16.9 kA/m, 30 min) in HCT116 cells with MNPs bound to the membrane (center) or without MNP treatment (right). b) Evolution of cell growth after MH (425 kHz, 16.9 kA/m, 30 min) in HCT116 cells with MNPs internalized (center) or without MNP treatment (right). Scale: 50 μm.

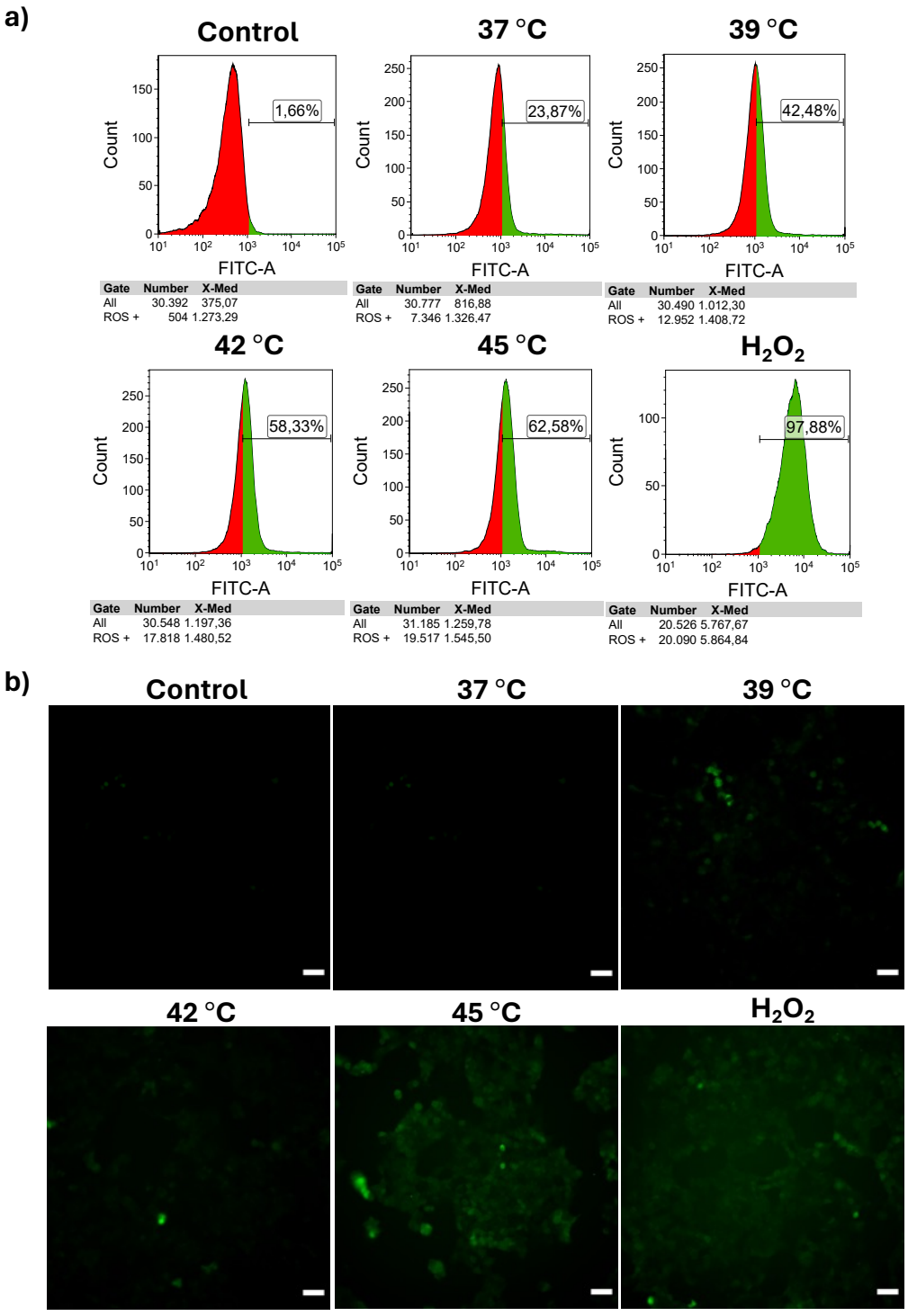


Figure S8. Study of ROS generation at different temperatures (external heating). HCT116 cells were treated with CM-H_2DCFDA (2 μM) and incubated at different temperatures for 30 min. Hydrogen peroxide (H_2O_2) was used as positive control for ROS production. a) Flow cytometry analysis. b) Fluorescence microscopy images. Scale bar: 50 μm.

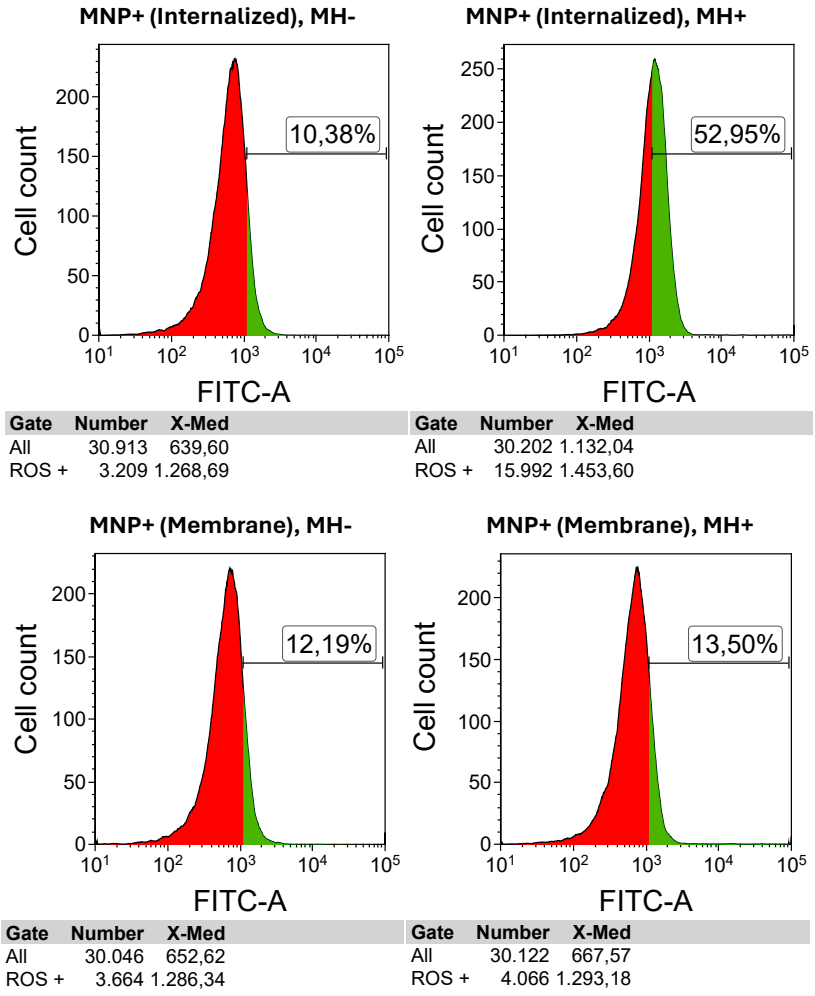
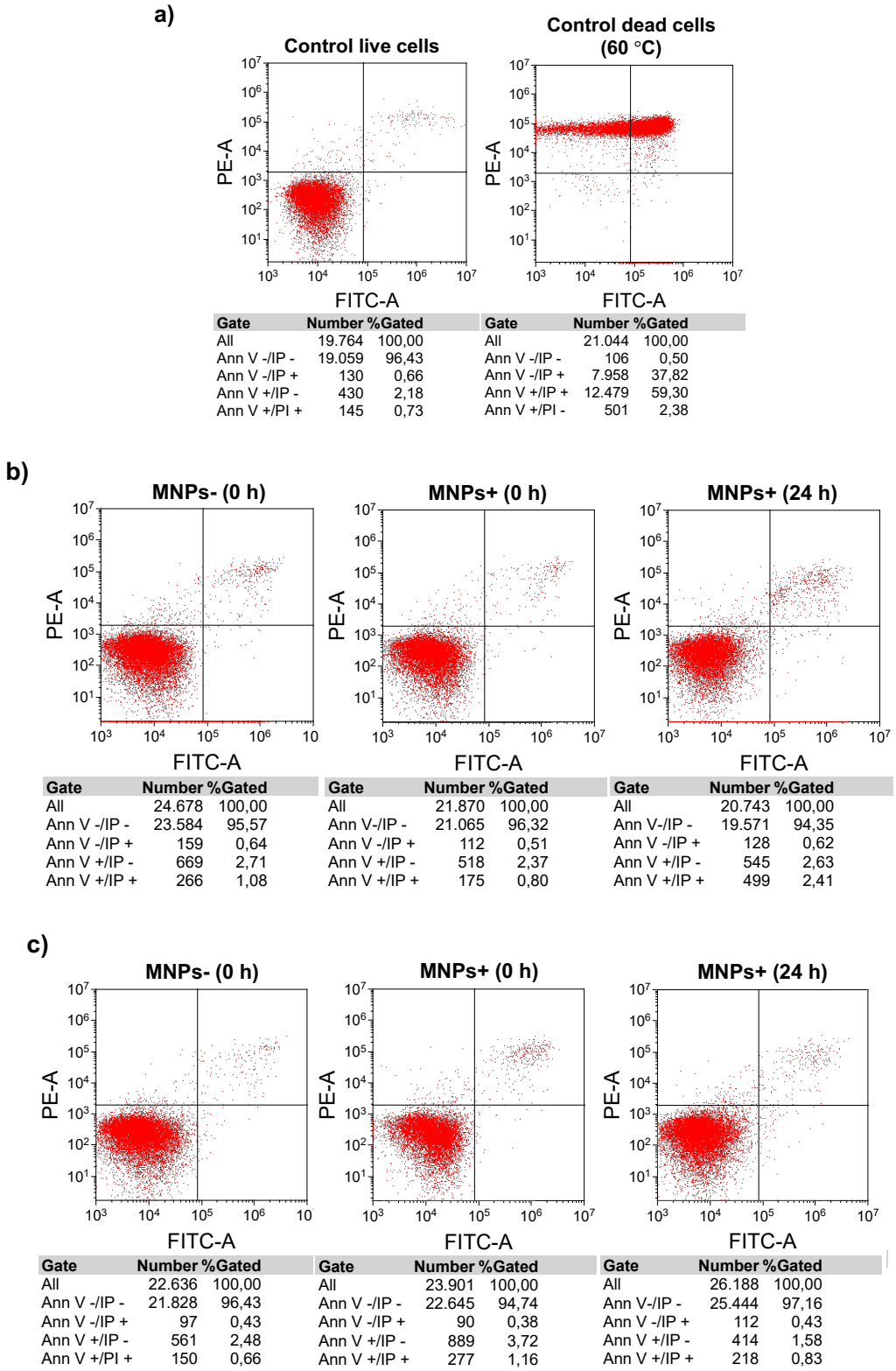


Figure S9. Flow cytometry analysis of ROS generation after MH stimulus in HCT116 cells with MNPs internalized or immobilized on the cell membrane. HCT116 cells were treated with CM-H_2DCFDA (2 µM) and exposed to the AMF (425 kHz, 16.9 kA/m) for 30 min. Hydrogen peroxide (H_2O_2) was used as positive control for ROS production. a).



Gate	Number	%Gated
All	19.764	100,00
Ann V -/IP -	19.059	96,43
Ann V -/IP +	130	0,66
Ann V +/IP -	430	2,18
Ann V +/PI +	145	0,73

Gate	Number	%Gated
All	21.044	100,00
Ann V -/IP -	106	0,50
Ann V -/IP +	7.958	37,82
Ann V +/IP +	12.479	59,30
Ann V +/PI -	501	2,38

Gate	Number	%Gated
All	24.678	100,00
Ann V -/IP -	23.584	95,57
Ann V -/IP +	159	0,64
Ann V +/IP -	669	2,71
Ann V +/IP +	266	1,08

Gate	Number	%Gated
All	21.870	100,00
Ann V-/IP -	21.065	96,32
Ann V -/IP +	112	0,51
Ann V +/IP -	518	2,37
Ann V +/IP +	175	0,80

Gate	Number	%Gated
All	20.743	100,00
Ann V-/IP -	19.571	94,35
Ann V -/IP +	128	0,62
Ann V +/IP -	545	2,63
Ann V +/IP +	499	2,41

Gate	Number	%Gated
All	22.636	100,00
Ann V -/IP -	21.828	96,43
Ann V -/IP +	97	0,43
Ann V +/IP -	561	2,48
Ann V +/PI +	150	0,66

Gate	Number	%Gated
All	23.901	100,00
Ann V -/IP -	22.645	94,74
Ann V -/IP +	90	0,38
Ann V +/IP -	889	3,72
Ann V +/IP +	277	1,16

Gate	Number	%Gated
All	26.188	100,00
Ann V-/IP -	25.444	97,16
Ann V -/IP +	112	0,43
Ann V +/IP -	414	1,58
Ann V +/IP +	218	0,83

Figure S10. Flow cytometry analysis of cell viability using the annexin V/propidium iodide method. a) Negative control of live cells and positive control of cell death (incubation for 30 min at 60 °C). b) Cell viability at 0 h and 24 h after cell membrane localized MH. c) Cell viability at 0 h and 24 h after intracellular MH. The FITC-A channel corresponds to Annexin V labeling and the PE-A channel to propidium iodide labeling.

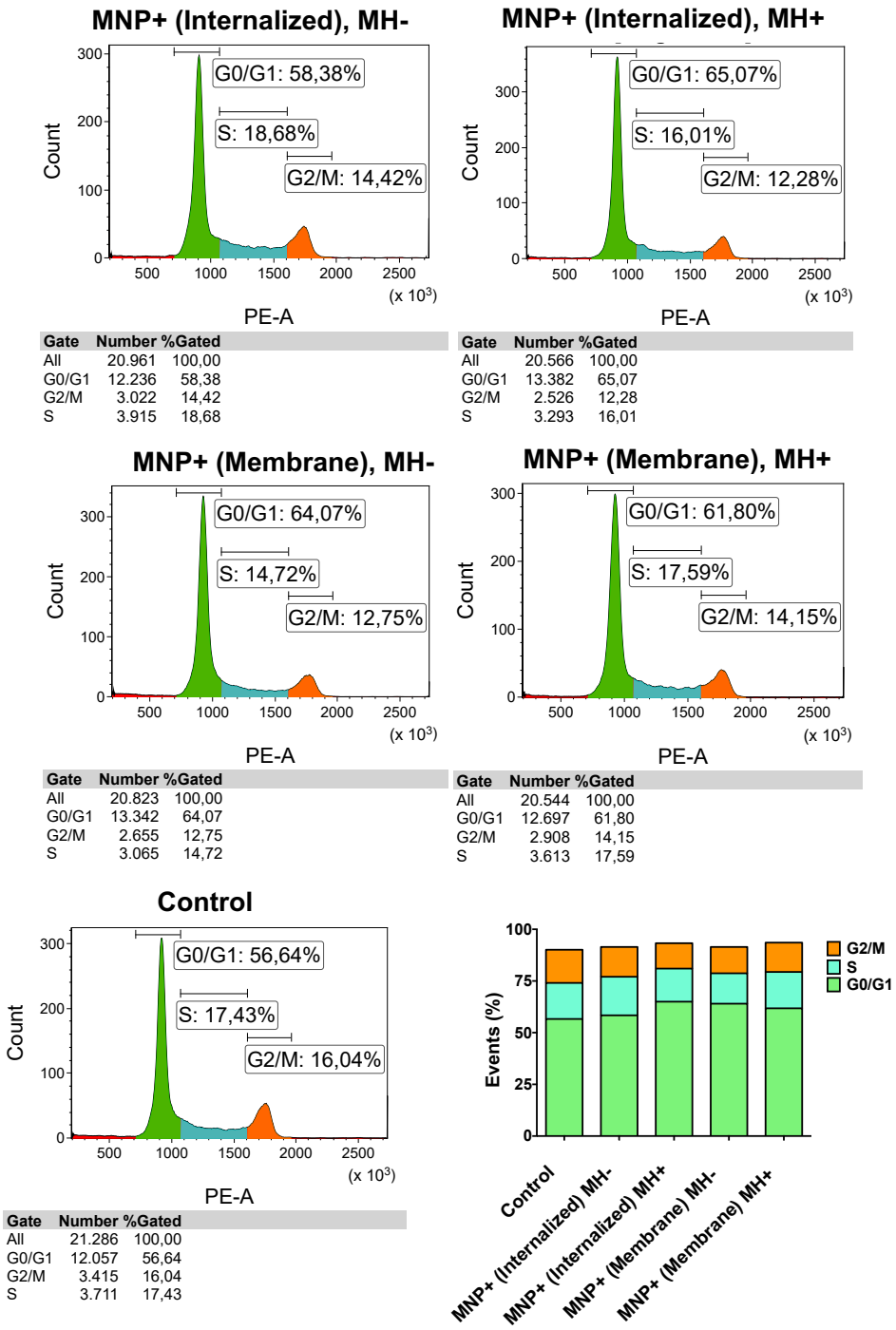


Figure S11. Cell cycle analysis of HCT116 cells after cell membrane localized or intracellular MH (AMF conditions: 425 kHz, 16.9 kA/m, 30 min).

4. Optimization of cell transfection experiments

Plasmid transfection with Lipofectamine 2000

MCF7 cells were seeded at a density of 150000 cells per well in 24-well plates and cultured in supplemented DMEM for 24 h prior to transfection at 37 °C, under standard cell culture conditions. A plasmid that encodes a green fluorescent protein (λ_{ex} 475 nm/λ_{em} 505 nm) optimized for high expression in mammalian cells (pAcGFP1-Nuc Vector 4.8 kb), was added (0.25, 0.5, and 1 μg/well) to cells using a 1:2 ratio plasmid:Lipofectamine 2000 (m/m) and incubated in DMEM with 2% of FBS (v/v) without antibiotics for approximately 5 h at 37 ºC, according to the manufacturer's recommendation. After 5 h of incubation, the medium was replaced with fresh-supplemented DMEM. At 24 h post-transfection, GFP expression was confirmed by fluorescence microscopy.

Optimization of GFP silencing using siRNA and Lipofectamine RNAiMax

GFP silencing efficacy was optimized with 10 nM and 20 nM of siRNA (using Lipofectamine RNAiMax) prior to the magnetic hyperthermia experiments. MCF7 cells were seeded in a 24 well-plate (150000 cells per well) 24 h before plasmid transfection. pAcGFP1 Nuc-vector (1 μg/well) was added to cells using a 1:2 ratio of Lipofectamine 2000, DMEM with 2% of FBS (v/v) without antibiotics. After 5 h of incubation, the medium was replaced with fresh-supplemented DMEM. Twenty-four hours after pDNA transfection, cells were visualized using a fluorescence microscope for GFP green signal detection. Afterward, 10 nM or 20 nM of siRNA was mixed with the cationic lipid reagent Lipofectamine RNAiMax and added to the cells. GFP expression was evaluated at 0 h, 24 h and 48 h after transfection. Moreover, 48 h post-transfection, MCF7 cells were detached for RNA extraction. The RNA integrity was confirmed, and cDNA synthesis was performed prior to RT-qPCR analysis. GFP mRNA expression was analyzed with the $2^{-\Delta\Delta ct}$ method. Following RT-qPCR data analysis, 20 nM of siRNA demonstrated an efficient silencing of GFP. This condition was therefore selected for silencing experiments using MH.

Magnetic hyperthermia – mediated siRNA transfection (silencing of GFP)

MCF7 cells were seeded in a 24 well-plate at a density of 150000 cells per well. After 24 h, cells were treated with 100 μM of Ac_4ManNAz for 48 h prior to magnetic hyperthermia experiments. On the second day after transfection with AcGFP1 vector (carried out as previously described), cells were washed twice with PBS and incubated for 10 minutes with MNPs@PMAO@PEG@DBCO (10 μg_{Fe}/mL) in DMEM without FBS at 37 ºC. Cells were then washed twice with PBS and detached with Versene solution, centrifuged, and resuspended

in fresh medium. The cell suspension was introduced into an adapted glass vial of 2 mL, 12 x 32 mm in size, suitable for the magnetic hyperthermia equipment, and 20 nM antisense siRNA was added. The AMF (23.9 kA/m, 418 kHz) was applied for 30 minutes with five-minute pulses and 60-second pauses between pulses, using a magnetic hyperthermia applicator (D5 Series device). As a control for gene silencing experiments, transfection with Lipofectamine RNAiMax was used, according to the manufacturer's instructions. After transfection, cells were counted and seeded in a 96 well-plate for cell viability assay and in a 24 well-plate for fluorescence microscopy and RNA extraction and grown under standard cell culture conditions.

AcGFP1 expression analysis

AcGFP1 expression was verified by fluorescence microscopy following 24 h and 48 h of gene silencing experiment. Images of cells were acquired using a Nikon Eclipse T*i*-U inverted microscope (Nikon TMS, Tokyo, Japan). The cell images were analyzed using the open access software platform FIJI (ImageJ). 48 h after transfection, RNA was extracted from samples using the Nzyol reagent according to the manufacturer's guidelines. Total RNA extracted from MCF7 cells was reverse transcribed to cDNA using the NZY M-MuLV First-Strand cDNA Synthesis kit. RT-qPCR was performed using NZYSupreme qPCR Green Master Mix (2x) according to the manufacturer's protocol in a Qiagen Rotor-Gene Q cycler (Qiagen, Hilden, Germany). The following conditions were used: initial denaturation at 95 °C for 5 min; 30 cycles of 95 °C for 30 s, Tm 52 °C for 30 s, 72 °C for 45 s; and a final extension step at 72 °C for 7 min. *AcGFP1* gene expression was evaluated according to the $2^{-\Delta\Delta Ct}$ method, using *18S* ribosomal gene as reference.

Cell viability post-transfection

Cells were plated at approximately 50000 cells per well in 96-well plate and incubated for 24 h at 37 °C, under standard cell culture conditions. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was performed to determine cytotoxicity following transfection with magnetic hyperthermia and with Lipofectamine RNAiMax using the MTS assay kit to the manufacturer's instructions. At 24 h post-transfection the cell culture medium was removed, MTS solution was added to the cells and incubated for 1 h. The absorbance was measured at 490 nm using a microplate reader Infinite M200 (Tecan, Männedorf, Switzerland). Cell viability was normalized to control cells (cells with culture medium).

Figure S12. pAcGFP1-Nuc Vector design.

Efficient plasmid transfection and expression of the encoded protein are crucial to ensure a suitable and robust starting point for the analysis of the MH-promoted transfection effects. Low levels of AcGFP1 expression could lead to a misleading interpretation of the MH transfection effects; moreover, large heterogeneity between cells should be avoided, as this would imply many cells lacking the target to be silenced. The protein expression can be easily monitored by fluorescence microscopy upon the accumulation of the AcGFP1 inside the nuclei of the cells. In this regard, three main parameters play a key role in the optimization of the transfection of cells with the selected plasmid: cell confluency, pAcGFP1-Nuc plasmid quantity, and gene expression times. These parameters were optimized for an efficient plasmid transfection based on the use of fluorescence microscopy and flow cytometry (Figure S13). First, cell confluency was adapted to be between 60 to 70%, as lower confluency percentages (< 50%) compromise cell transfection due to Lipofectamine toxicity, and higher confluency (> 80%) reduces the surface area for an efficient transfection. Once the cell confluency was optimized, three different concentrations of plasmid (0.25, 0.5 and 1 µg per/well) were evaluated for transfection with Lipofectamine 2000. A standard protocol was followed, based on a 1:2 plasmid:Lipofectamine 2000 ratio in DMEM with a low concentration of FBS (2%) to avoid the interference of the cationic lipids with the serum proteins and without antibiotics to minimize cytotoxicity effects derived from the increase of cell permeability provided by the cationic lipids. After 5 h of incubation of the cells with plasmid-Lipofectamine 2000 complexes, the cell culture medium was replaced with fresh supplemented DMEM, and the analysis of AcGFP1 expression was performed by fluorescence microscopy at three three-time points post-transfection (24 h, 48 h and 72 h) (Figure S13 a-c). Results revealed a minimal AcGFP1 expression using 0.25 µg of plasmid for the three expression times tested. However, by increasing the plasmid concentration to 0.5 and 1 µg, each expression rate was almost double when compared to the previous one, confirming a plasmid dose-dependent transfection

efficiency. Using 1 µg of plasmid and 48 h of expression, green cells were counted in four aleatory fluorescence microscopy images, we could estimate a transfection efficiency of approximately 28%. Additionally, flow cytometry analysis of transfected cells revealed that 72 h post-transfection the AcGFP1 expression was slightly reduced in comparison with the 48 h post-transfection levels, for all the plasmid concentrations tested (for example, from 33.5% to 26% at 1 µg and from 16.3% to 11% at 0.5 µg, respectively) (Figure S13 d). These results indicate that the optimal AcGFP1 expression was reached at 48 h post-transfection with 1 µg of plasmid; thus, this condition was selected for the subsequent experiments further studies with siRNA. Concentrations above 1 µg of plasmid were discarded because of the inherent toxicity of the Lipofectamine 2000 (the higher the plasmid concentration, the higher the concentration of Lipofectamine 2000 required) and to ensure a cost-effective experiment, due to the high price of the transfection reagent.

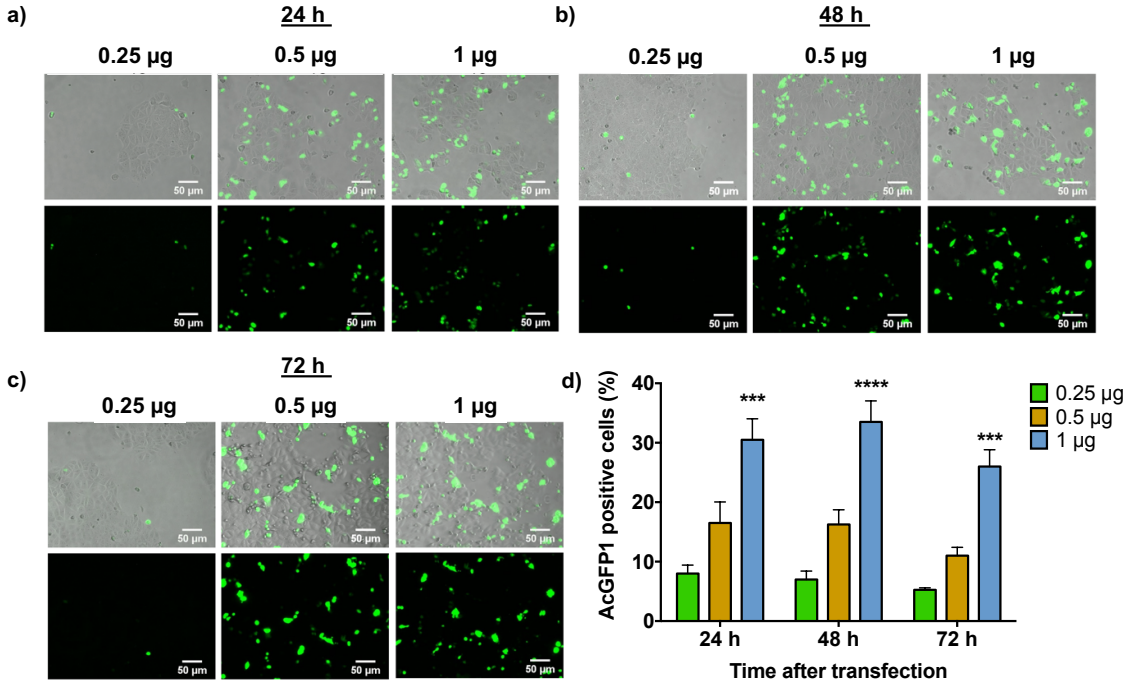


Figure S13. Optimization of the quantity of pAcGFP1-Nuc vector (0.25, 0.5 and 1 µg) for MCF7 cell transfection using Lipofectamine 2000. a-c) Fluorescence microscopy images obtained for each quantity of pAcGFP1 Nuc-vector at 24, 48 and 72 h post-transfection, respectively. d) Flow cytometry analysis of the expression of AcGFP1-positive cells 24, 48 and 72 h post-transfection with 0.25, 0.5 and 1 µg of pAcGFP1-Nuc plasmid. Black asterisks indicate statistical difference with respect to cells transfected with the lowest concentration of plasmid (**$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; one-way ANOVA, Tukey's multiple comparisons test). Data analyses are expressed as mean ± standard deviation of two independent experiments.

Before testing the potential of localized MH for siRNA transfection, a standard Lipofectamine transfection of siRNA anti-*AcGFP1* (10 and 20 nM) was used to optimize and confirm an effective silencing of the target gene. After the optimal expression of AcGFP1-Nuc in MCF7 cells, two different concentrations of siRNA were tested (10 and 20 nM), using a commercially available Lipofectamine adapted for short RNA strands (Lipofectamine RNAiMax). The silencing effect was followed by fluorescence microscopy after 24 and 48 h (Figure S14 a-c). Due to the high levels of AcGFP1 protein expression induced by the human cytomegalovirus (CMV) promoter encoded in the pAcGFP1-Nuc vector, the silencing effect of the siRNA was masked from a protein fluorescence perspective. For this reason, the quantification of the silencing effect was performed at mRNA levels by using RT-qPCR, which allows a higher sensitivity and accuracy. The results revealed a higher degree of silencing using 20 nM of siRNA 48 h post-transfection (Figure S14 d). Therefore, this condition was selected for the subsequent siRNA transfection *via* MH.

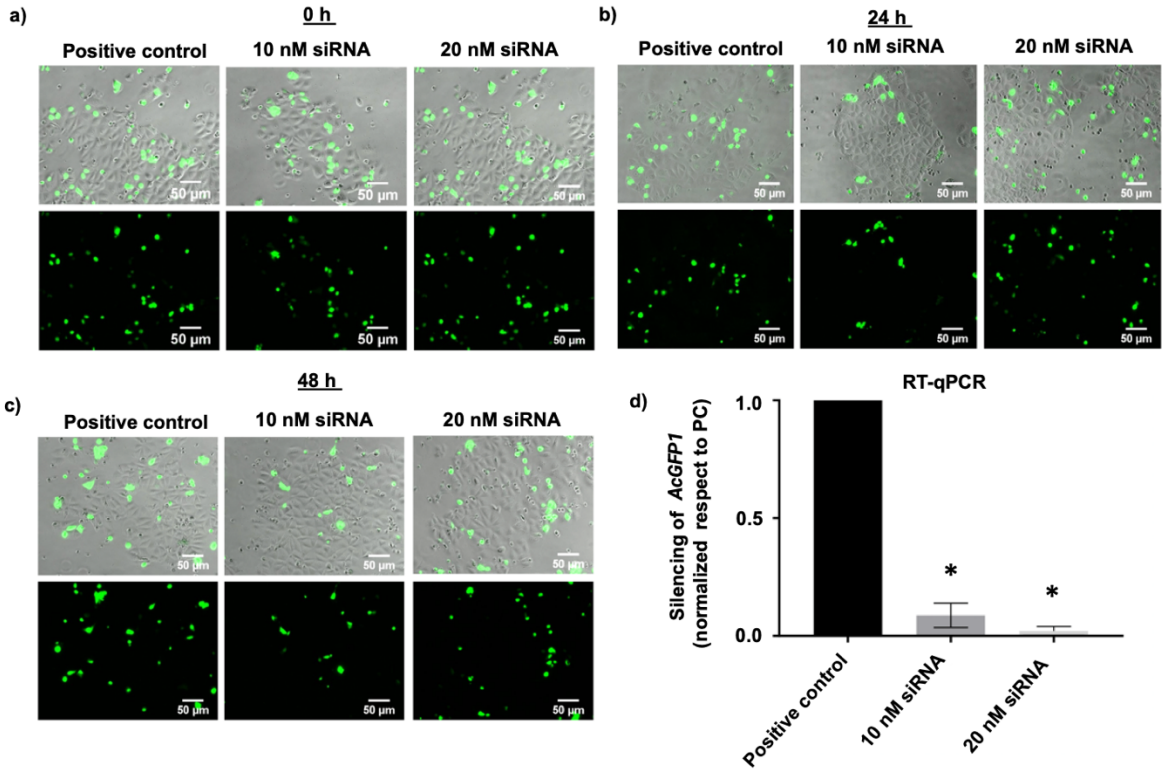


Figure S14. Fluorescence microscopy images and RT-qPCR analysis of the optimization of siRNA transfection using Lipofectamine RNAiMax. a-c) Silencing effect at 0, 24 h, and 48 h post-transfection with 10 and 20 nM of siRNA against *AcGFP1*. d) RT-qPCR data analysis of 48 h post-siRNA transfection with Lipofectamine RNAiMax reagent and *AcGFP1* mRNA expression (*$p < 0.05$ each condition related to the positive control using unpaired parametric t-test with Welch's correction). Data represents the mean value ± the standard error mean of at least two independent experiments.

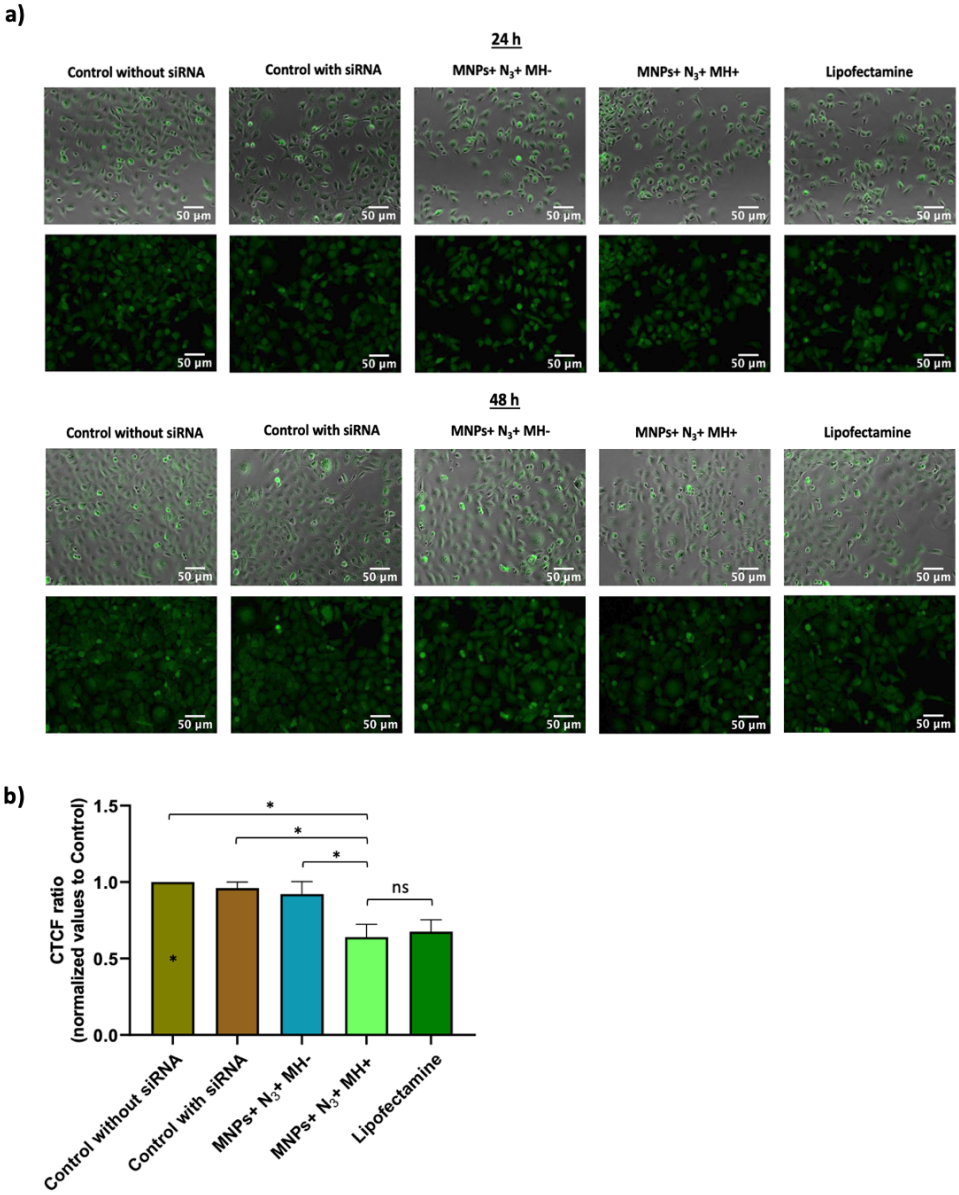


Figure S15. Fluorescence microscopy images of MCF7/GFP cells and CTCF evaluation after MH experiment. a) Fluorescence microscopy images of 24 and 48 h after MH experiment in the different conditions tested. b) CTCF analysis to evaluate copGFP protein expression at 48 h post transfection. Black asterisks indicate statistical differences between MNPs+ N_3+ MH+ and the controls (cells with and without siRNA) and MNPs+N_3+MH- (*$p < 0.05$; Unpaired parametric t-test with Welch's correction). Data represent the mean value ± the standard error mean of three biologically independent experiments with two technical replicates for each.

5. Transfection experiments in MCF7 cells with transient GFP expression

The results obtained for siRNA transfection *via* MH were similar to the ones attained in MCF7 cells constitutively expressing copGFP described in the main text. This confirms the effect of MNPs+N_3+MH+ (50% of *AcGFP1* silencing) on the transfection of silencing oligos comparable to Lipofectamine reagent, but with less impact on cell viability (Figure S16 and S17). Besides, for the MH-treated cells (MH+), although there were no statistical differences in the effect observed between cells expressing azide reporters (N_3+) and cells without azides (N_3-), expression of *AcGFP1* for MNPs+ N_3+MH+ was 5% lower than for MNPs+N_3-MH+ (Figure S17 a). This confirms that the non-specific attachment of MNPs to cells without azide groups on the membrane also contributes to permeabilization and promotes the intracellular delivery of siRNA by AMF. Finally, cells (both N_3+ and N_3-) incubated with MNPs but without MH application do not show significant inhibition of the expression of *AcGFP1*.

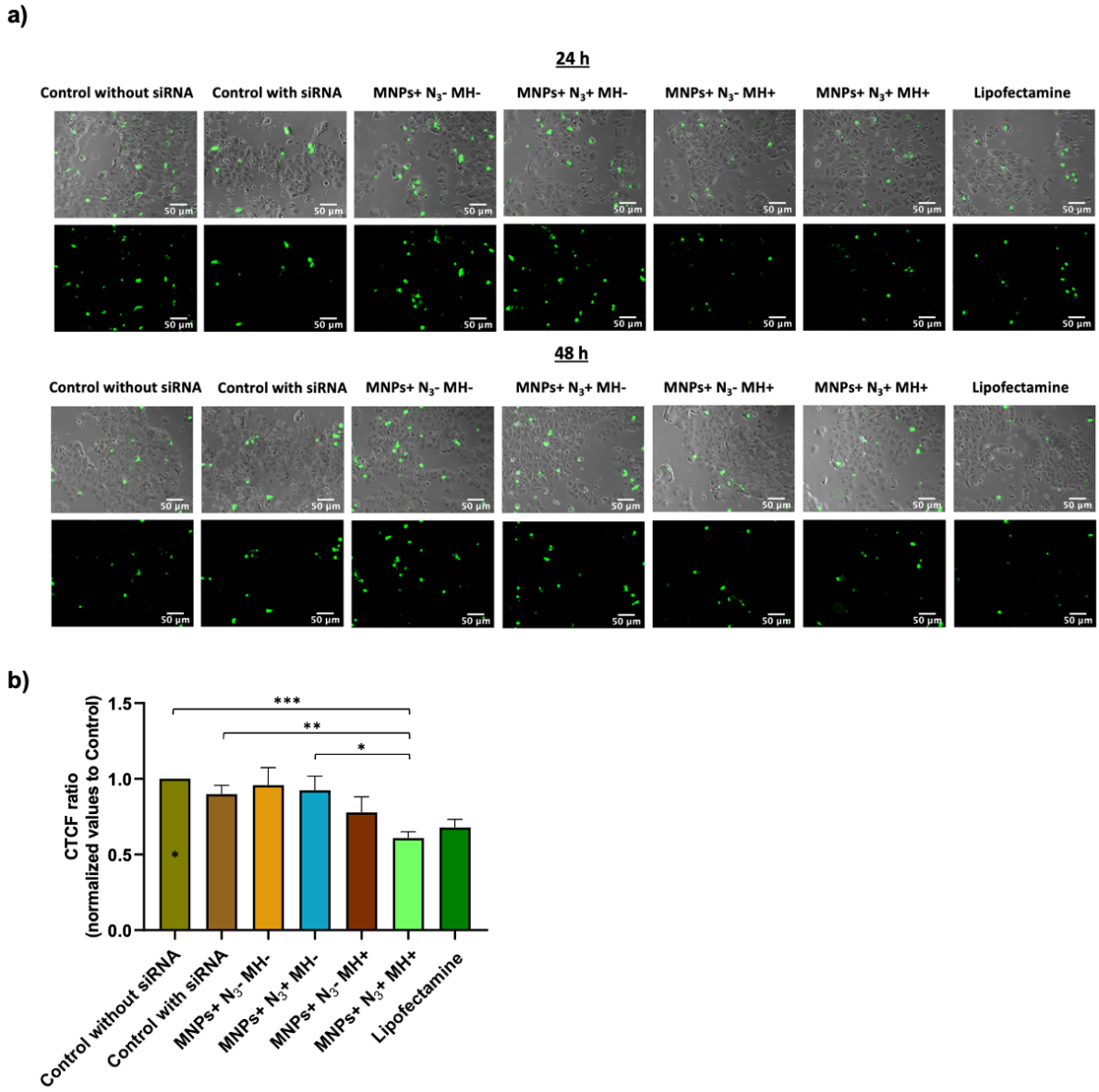


Figure S16. Fluorescence microscopy images of MCF7 cells and CTCF evaluation after MH experiment. a) Fluorescence microscopy images of cells 24 and 48 h after the MH experiment for the different conditions tested. b) CTCF analysis to evaluate AcGFP1 protein expression at 48 h post-siRNA transfection. Black asterisks denote statistically significant differences between MNPs+N_3+MH+ and the control without siRNA, control with siRNA, and MNPs+N_3+MH- (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$, respectively; unpaired parametric t-test with Welch's correction). Data represent the mean value ± the standard error mean of three biologically independent experiments with two technical replicates for each.

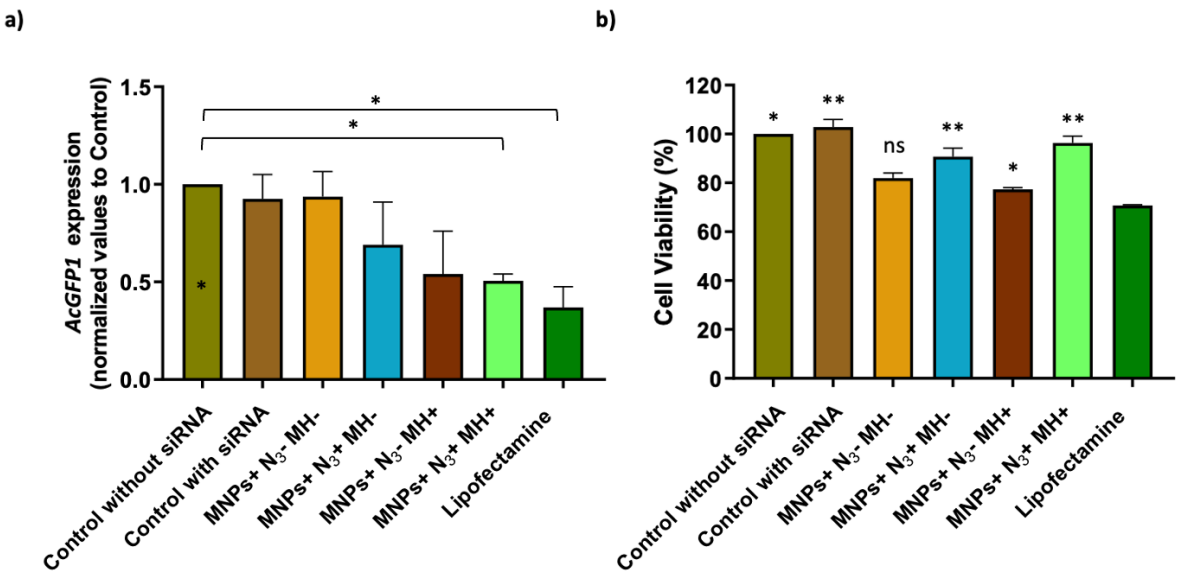


Figure S17. RT-qPCR analysis and cell viability assessment 48 h post-transfection in MCF7 cells. a) RT-qPCR to evaluate *AcGFP1* gene expression in different conditions tested. Expression levels were normalized to unchallenged cells. Black asterisks indicate statistical differences between MNPs+N_3+MH+ and Lipofectamine with the control without siRNA (*$p < 0.05$; unpaired parametric t-test with Welch's correction). Data represents the mean value ± the standard error mean of three biologically independent experiments with two technical replicates for each. b) Cell viability analysis of the different samples tested after MH via MTS assay. The statistical differences between the cells treated with Lipofectamine and other samples are represented (*$p < 0.05$; **$p < 0.01$; unpaired parametric t-test with Welch's correction).

Primers and siRNA used in this work:

18S (housekeeping gene) primers

Forward primer: 5'-GTAACCCGTTGAACCCCATT-3'

Reverse primer: 5'-CCATCCAATCGGTAGTAGCG-3'

HSPA1A primers

Forward primer: 5'-ACCACTTCGTGGAGGAGGAGTTCAAGA-3'

Reverse primer: 5'-ACGTGTAGAAGTCGATGCCCTCAA-3'

HSPA8 primers

Forward primer: 5'-ACCTACTACTCTCTTGTGTGGGTGTT-3'

Reverse primer: 5'-GACATAGCTTGGAGTGGTTCG-3'

HSPA9 primers

Forward primer: 5'-CTTGTTTCAAGGCGGGATTATGC-3'

Reverse primer: 5'-GCAGGAGTTGGTAGTACCCAAA-3'

AcGFP1 primers

Forward primer: 5'-ATGGTGAGCAAGGGCGAGGA-3'

Reverse primer: 5'-CTTGTACAGCTCGTCCATGC-3'

copGFP primers

Forward primer: 5'-TTCTACCACTTCGGCACCTA-3'

Reverse primer: 5'-TCCACCACGAAG CTG TAGTA-3'

AcGFP1 antisense siRNA

5'-GCAAGCUGACCCUGAAGUUC-3'

copGFP antisense siRNA

5'-CACCCGCAUCGAGAAGUACG-3'