Electronic supplementary information

Mapping intracellular thermal response of cancer cells to magnetic hyperthermia treatment

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Experimental Section

Synthesis of Fe_3O_4@PAA MNPs

Iron (II) chloride ($FeCl_2.4H_2O$, 8 mmol) and iron (III) chloride ($FeCl_3.6H_2O$, 14 mmol) were dissolved in 10 mL of Milli-Q® water. In a separate vial, poly(acrylic acid sodium salt) (MW 5100 Da) was dissolved in 5 mL of Milli-Q® water. Both solutions were mixed together in a polytetrafluoroethylene (PTFE) reactor and 15 mL of concentrated (28-30%) ammonium hydroxide solution were added into the reactor. The PTFE reactor was closed with a PTFE lid and sealed in a general-purpose Parr autoclave reactor. The solution was hand shaken and the reactor was placed in an oven at 150 ˚C for 24h. After 1 day the autoclave was allowed to cool down to room temperature and the solution was diluted down with acetone (approximately 13 mL sample to 50 mL with acetone) and centrifuged at 8500 rpm for 5 min. The supernatant was discarded, and the pellet resuspended in 15 mL of Milli-Q® water. This water solution was centrifuged at 3000 rpm for 3 min to remove large aggregates, the pellet was discarded, and the solution stored until further use in a glass vial.

Inductively Coupled Plasma - Optical Emission Spectroscopy (ICP-OES)

The quantitative analysis of cell internalized Fe (from natural sources plus Fe_3O_4 nanoparticles) was performed by ICP-OES measurements. Cells were plated in T25 cell culture flasks, at a concentration of 3 * 10^5 cells/mL and incubated at 37 ˚C with 5 % of CO_2 flow during 24 h. After, two different concentrations of Fe_3O_4@PAA MNPs, 50 and 100 µg/mL (plus control without Fe_3O_4@PAA MNPs), for three different time points, 4, 8, 24 h were incubated with HeLa cells. Afterwards, cells were detached by the addition of 2 mL of trypsin-EDTA followed by 5 min incubation. Cells were recovered into a 15 mL falcon tube and centrifuged at 1200 rpm in order to remove trypsin-EDTA.
Finally, the formed cell pellet was loaded with 1 mL of 37 % HCl to digest the cell membrane and Fe_3O_4@PAA MNPs overnight. Final solutions were diluted with 7 mL of Milli-Q®-water.

The Fe concentration was determined by ICP-OES (ICPE-9000, Shimadzu). Measurements were performed in triplicates, for standard deviation analysis.

Cell culture

HeLa cells were plated and grown in T75 flasks (ThermoFisher Scientific) with 10 mL cell culture medium, composed of Dulbecco Dulbecco's Modified Eagle's Medium (DMEM) from with 4.5 g/L glucose (Lonza) with additional 10 % Fetal Clone® III (GE Healthcare, US) and 1 % penicillin/streptomycin (SigmaAldrich). Medium replacement was performed twice per week, during which washing steps were done with 5 mL Phosphate Buffer Saline (PBS) (CORNING) and detached from the flask surface with 2 mL of trypsin-EDTA (Gibco®). All cell sample preparations were done under a laminar flow hood (Telstar – Bio II Advance).
For actin-GFP transfection, CellLight™ Actin-GFP, (ThermoFisher) reagent was added 24 h after plating.

Cell viability assay

The cell viability studies were performing using the resazurin assay. HeLa cells were plated in a 96 wells plate, at a cell density of 5 * 10^3 cells/mL and incubated for 24h.
A concentration-dependent study was performed with a sequential dilution of the magnetic nanoparticles and fixed incubation time of 24h. 20 µL of Resazurin aqueous solution at a concentration of 0.05 mg/mL were added to the cells and incubated 1h before measurements.
Fluorescence intensity measurements were performed in a microtiter plate reader (Synergy H1MFD, BioTek) with excitation wavelength at 560 nm and fluorescence signal collected at 590 ± 8 nm.

Sample preparation for Transmission Electron Microscopy (TEM) imaging

Cells were platted in two T75 cell culture flasks with 1 * 10^6 cells/mL each and incubated for 24 h. 200 µg/mL of Fe_3O_4@PAA MNPs were then added in order to have a higher concentration of nanoparticles in the cells and incubated for 24 h. After, the cells were washed three times with 5 mL of PBS and detached with 2 mL of trypsin-EDTA. Trypsin-EDTA was removed after centrifugation and the cells were fixed with a solution of 3 % glutaraldehyde, at 0.12 M and pH 7.4, for 30 min.
To remove the glutaraldehyde, a 5 min centrifugation at 12000 rpm was performed, followed by three times washing step with a 0.12 M PBS, with 5 min centrifugation in-between at 7000 rpm. The following step was to stain the sample with a 1 % of osmium tetroxide, followed by stirring during 3 h wrapped with aluminum foil to prevent light exposure.
To deplete aqueous cytoplasm from the cells, they were washed with solutions of increased acetone content starting at 30 % acetone in PBS until a final solution of 100 % acetone and twice for each solution. Between each washing step there was a 10 min waiting period.
The intracellular environment was replaced by a resin. The resin was prepared with 21 mL of Durcupan™ ACM single component A, M epoxy resin (Sigma-Aldrich) and 180 µL of Durcupan™ ACM single component C, accelerator 960 (DY 060) (Sigma-Aldrich). Cells were mixed in resin with decreasing concentrations of acetone with a waiting time of 1h between each use. The 100 % resin was added to the cells and left overnight. After, it was removed and replaced by

another 100 % resin. Finally, the sample was centrifuged, in order to create a pellet of cells, which was incubated at 65 ˚C for 48 h, to harden the resin.
The cell pellet was sliced in 70 nm thickness layer and deposited in a copper grid for the TEM imaging.

TEM imaging

In order to increase the contrast, cellular slice was stained with lead uranyl acetate. TEM (JEM2100-HT, JEOL) providing electron energies of 80 kV was used for image acquisition. Fiji software was used for image analysis allowing contrast enhancement.

Magnetization characterization

Magnetic measurements were carried out on powder samples using a superconducting quantum interference device SQUID-VSM magnetometer from Quantum Design. Prior to the measurements, the particles were dried overnight in vacuum at room temperature, grounded using an agate mortar, and finally placed in gelatin capsules for the measurements. Hysteresis loops were obtained at 300 K by applying a magnetic field up to ±20 kOe.

Fluorescence lifetime spectroscopy on purified GFP in solution

In order to test the fluorescence lifetime of GFP in a larger temperature range, an ISS ChronosBH fluorescence lifetime spectrometer based on TCSPC and temperature controlled sample chamber was used for a sample containing 10 µL of a 1 µg/µL GFP (#14392, Chemicon/Millipore) in 1000 µl of MilliQ water solution. A 467 nm picosecond pulsed laser (PLP-10, Hamamatsu) was coupled to the spectrometer, equipped with a 473 LP filter and a PMT output recorded by a B&H SPC130 TCSPC card. The temperature controlled sample chamber contains a Peltier-heating/cooling element and a temperature sensor located at the bottom of the cuvette (Quantum Northwest, TC425). A 1 cm per 3 mm quartz cuvette with inserted magnetic stirrer was used, and the signal was recorded at 90 ˚detection geometry. Each temperature was equilibrated for 10 min, and acquisition time was 3s. The Vinci software (ISS) was used for fluorescence lifetime determination using a single exponential model.

Sample preparation for Fluorescence Lifetime Imaging Microscopy (FLIM) measurements

FLIM measurements were performed in HeLa cells plated at a density of approximately 10^4 cells/mL inside two wells glued to the bottom of 35 mm diameter 1.5# cover glass petri dish (MatTek).
After 24 h of transfection, Fe_3O_4@PAA MNPs were added to the sample, with a concentration of 50 µg/mL, and incubated overnight. Finally, sample was washed with PBS and loaded with fresh culture media.

FLIM setup

A custom-built FLIM inverted microscope was used for fluorescence lifetime imaging. A scheme of the setup can be found in the **SI Fig. S 13**. In short, a vertically polarized 467 nm picosecond pulsed laser (PLP-10, Hamamatsu). To clean-up the laser profile, the beam passes a spatial filter composed of two lenses (f1=50 mm and f2= 50 mm, LA4148-A, Thorlabs), and a 100 µm pinhole.
Additionally, a 470 nm ± 25 nm clean-up filter (470FS10-25, Thorlabs) is used for securing the monochromaticity of the laser source, removing potential luminescence background. A shutter is used to block the laser between experiments. The laser was set to a repetition rate of 20 MHz and attenuated to a power of approximately 9 µW at the entry of the microscope.
For alignment of the laser two steering mirrors are used. The laser is then reflected to a beam expander system composed of a pair of lenses (f1=40 mm and f2= 250 mm, AC254-040-B and AC254-250-B, Thorlabs) which is integrated into a cage system, and which is directly connected with the inverted microscope body (RM21, MadCityLabs). A 480LP Dichroic (F38-482, AHF Analysentechnik) is used to guide the excitation towards the sample. A 20x objective with 0.4 NA (M-20X, Newport) is used for focusing the excitation and signal collection. Scanning is performed by micro- and nanopositioning systems. The nanopositioner being a closed loop piezo-controlled system (NanoLPS200, MCL). The signal is collected in epifluorescent detection mode. For confocal detection a spatial filter with a 25 µm pinhole is used mounted between a pair of lenses (f1 = 50 mm and f2 = 50 mm), after a 473 nm long-pass filter (AHF, F76-472). The signal

is then separated in two detection arms dependent on its polarization using a polarizing beam splitter (CCM1-PBS251/M, Thorlabs) and two lenses are used to focus the signal down to the active area of the two avalanche photodiode detectors (APDs) (PD50CTD, MPD). The Time Correlated Single Photon Counting (TCSPC) is implemented using a single-photon counting card (SPC130, Becker&Hickl) to which the sync of the pulsed laser source and the output of the router (HRT-81, Becker&Hickl) that combines the signal from the two APDs is fed. The custom-build FLIM setup is controlled via custom-built LabView programmed software (LabView, National Instruments).
An AMF sample holder with incubator (MagneTherm ™ with Live Cell Exposure Option, nanoTherics) was used for calibration experiments and hyperthermia applications.
The incubator was flushed with premixed gas of 5 % of CO2 and 95 % of oxygen for live cell imaging. A circulating water bath (Recirculating Heater/Chiller F25, Julabo) enables setting different environment temperatures that are monitored by an immersed optical fiber attached to a temperature reader (Multi Channel Optical Sensing Signal Conditioner, Luxtron 812).

***In vitro* hyperthermia FLIM experimental settings**

Temperature of the sample was cooled from 29 to 18 ˚C by a water circulating system and controlled by temperature sensor immersed inside the sample. In order to stabilize the temperature between each measurement an equilibration time of about 20 min was carried out, after which intracellular temperatures were assumed to be equal to the one measured in the medium.
We perform the calibration and magnetic hyperthermia treatment on same healthy cells. By applying magnetic hyperthermia treatment to the same HeLa cell, we avoid fluorescence lifetime differences that could be promoted by other changes of intracellular microenvironments, such as pH, even though it was reported with GFP-S65T variant that fluorescence lifetime is stable in a wide range of pH values.[70]
In vitro magneto thermal therapy was performed with 20 mT of magnetic field using following configurations: 224.53 ± 20 kHz, 27.7 ± 1 V and 13.1 ± 1 A for frequency, voltage and current, respectively. FLIM images were recorded at different time points during the continuous AMF exposure, while the focus needed to be re-adjusted between images.
All FLIM images were recorded with resolution of 1 µm/px and 20 ms collection time.

FLIM data analysis

For FLIM analysis, the FLIM images detected with parallel and orthogonal polarization with respect to the laser excitation are first summed to achieve balanced polarizing detection according to equation: $I_{total} = I_{II} + 2I_{\perp}$. The resulting fluorescence lifetime images were then calculated by performing a multi-exponential fit described elsewhere.[71] Also, deconvolution with an instrument response function (IRF), measured on a highly quenched Atto 488 on graphene sample, was applied to account for the delay that the setup may induce. Resulting FLIM images were analyzed in selected regions of interest using a script based on a freehand MATLAB R2017b function.

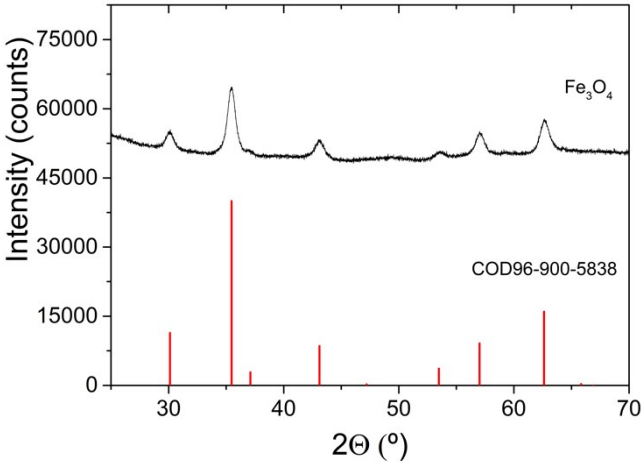

Fig. S 1.. X-ray diffraction pattern of the Fe_3O_4@PAA nanoparticles used in this study. All the observed diffraction peaks were indexed according to the pattern of the Crystallographic Open Database COD96-900-5838, which corresponded to a magnetite (Fe_3O_4) phase. No reflections from secondary phases or impurities were observed.

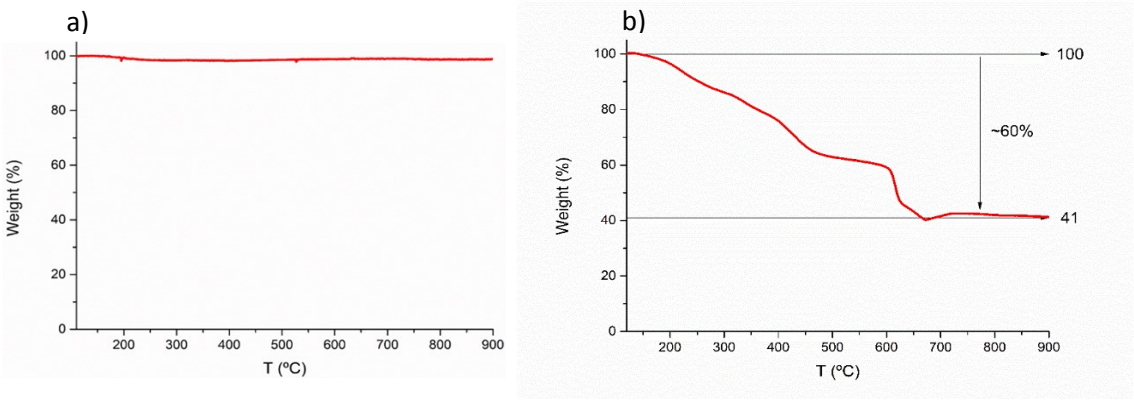

Fig. S 2. Thermogravimetric analysis. a) Bare Fe_3O_4 nanoparticles prepared by coprecipitation; b) Fe_3O_4@PAA nanoparticles used in this study showing a content of organic matter close to 60 %.

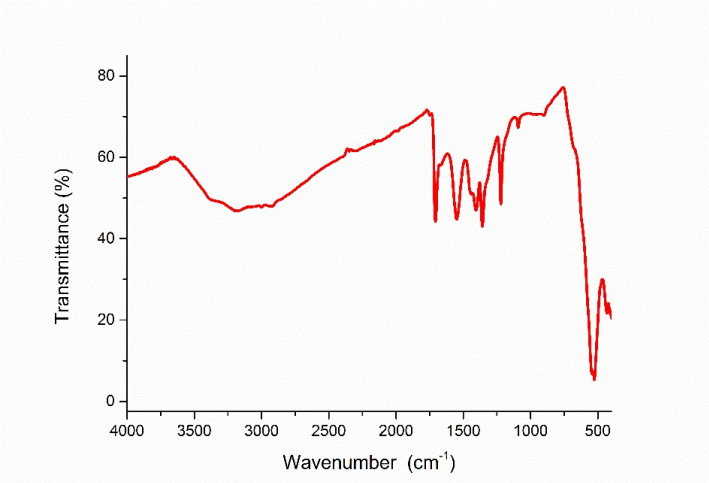

Fig. S 3. Fourier-transform infrared spectrum of the Fe_3O_4@PAA nanoparticles used in this study showing the expected peaks coming from the organic coating of the nanoparticles (νC-H below 3000 cm-1, νC=O over 1700 cm^{-1}), and the nanoparticles themselves (νFe-O around 500 cm^{-1}).

Table S1. Hydrodynamic size and Zeta potential of the Fe_3O_4@PAA nanoparticles used in this study as measured in MilliQ water at 37 ˚C.

Sample	***D*$_h$ (nm)**	**ζpot (mV)**
Fe_3O_4@PAA	39.4 ± 8.5	-78.1 ± 2.2

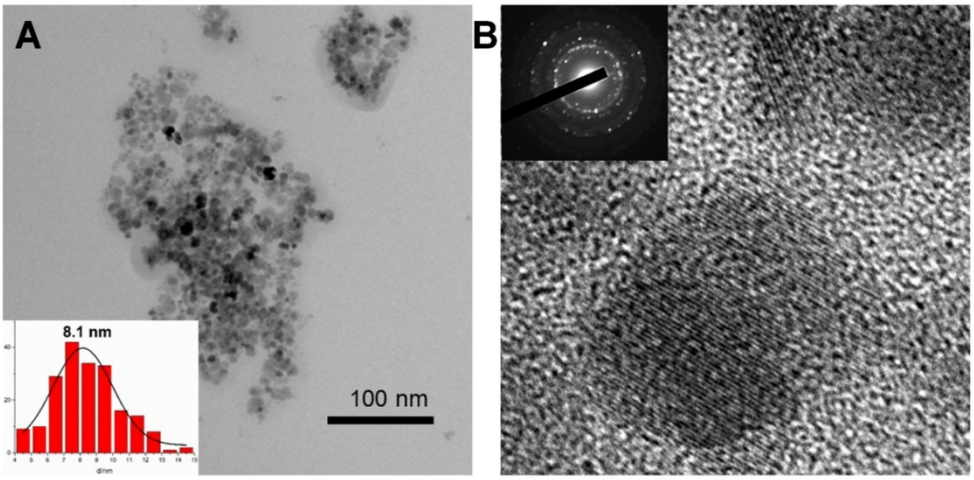

Fig. S 4. A) Overview TEM micrograph of Fe_3O_4@PAA nanoparticles. Inset, size distribution of the NPs. B) HR-TEM micrograph of Fe_3O_4@PAA nanoparticles showing the crystal lattice of these structures. Inset, electron diffraction pattern obtained from this sample corroborating the Fe_3O_4 nature of the nanoparticles.

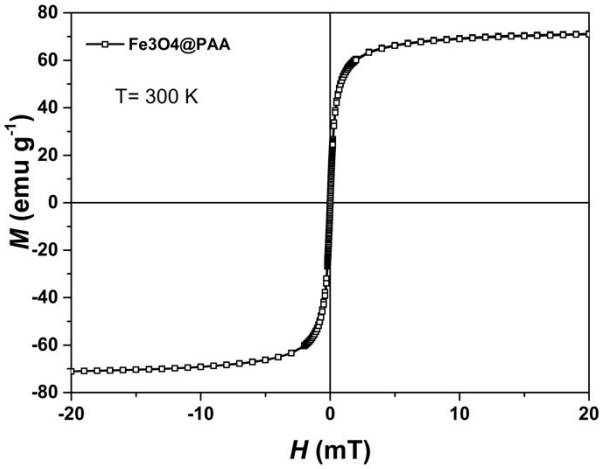

Fig. S 5. Magnetization curve as a function of the magnetic field at T=300 K for the Fe_3O_4@PAA nanoparticles used in this study showing superparamagnetic behavior.

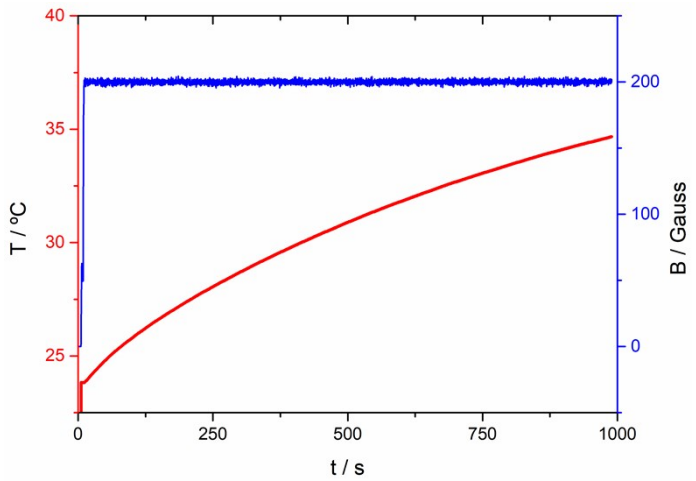

Fig. S 6. Heating profile of a water sample of the Fe_3O_4@PAA nanoparticles (0.5 mg/mL) used in this study under an alternating magnetic field. The conditions of the AMF application were 499 kHz and 20 mT.

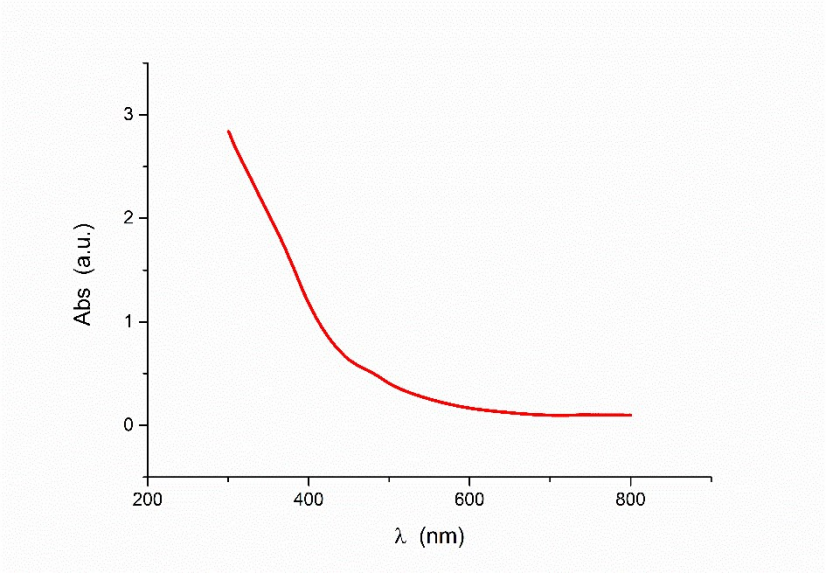

Fig. S 7. UV-Vis spectrum of the Fe_3O_4@PAA nanoparticles used in this study. The spectrum shows the expected profile from non-plasmonic subwavelength dielectric nanoparticles.

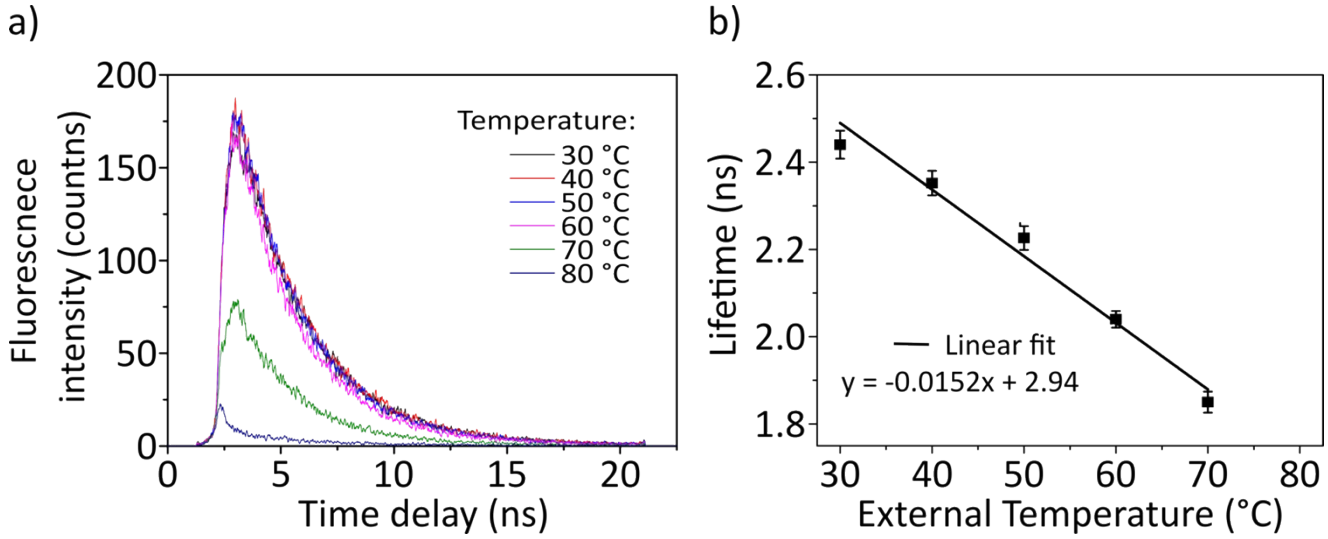

Fig. S 8. Raw data of the photon arrival time histograms and deduced fluorescence lifetimes for GFP in solution. **a)** The decay curves show an intensity decrease at higher temperatures, with relevant decrease at 70˚C and with nearly vanishing signal at 80˚C. **b)** The curves were fitted with a single exponential and a linear dependency is found for the temperature range from 30 to 70 ˚C. The 80 ˚C curve could not be fitted with such model.

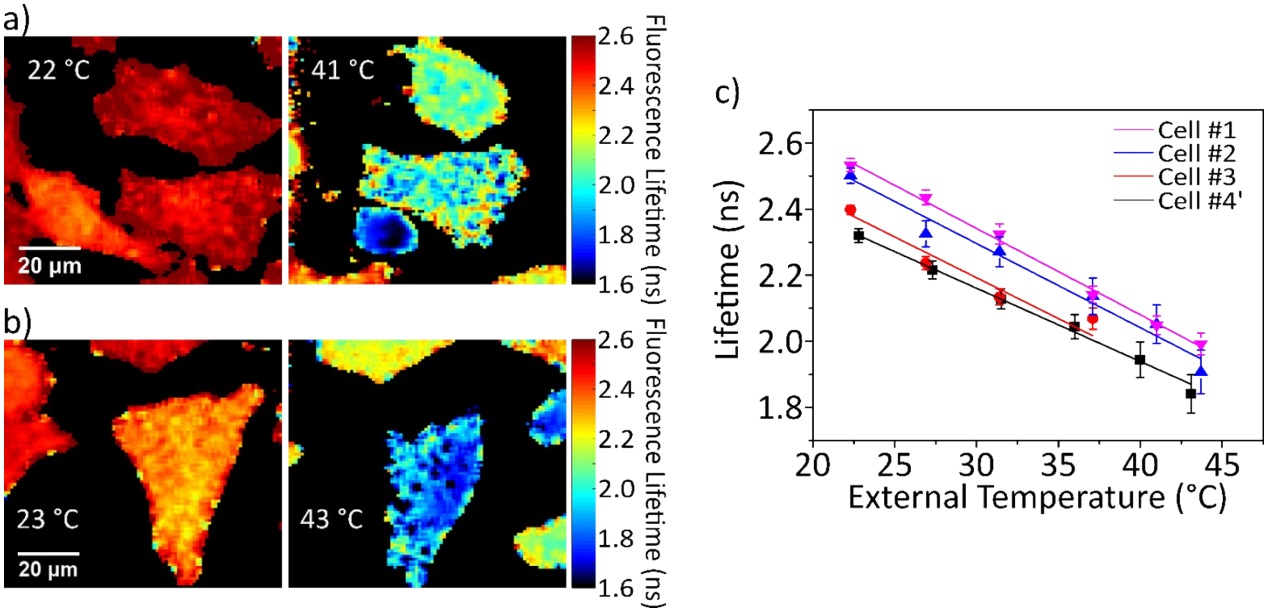

Fig. S 9. Fluorescence lifetime-dependent on external temperature measured for several individual HeLa cells expressing actin-GFP. **a)** FLIM images taken at various temperatures in a scan range containing 3 entire cells, one of them died during the study. **b)** Temperature dependent FLIM images on an independent sample where a 4th cell as was measured. An equilibration time of 20 min was chosen for each external temperature set point. c**)** Temperature-dependent average fluorescence lifetime of actin-GFP per cell, for the cells shown in a) and b).

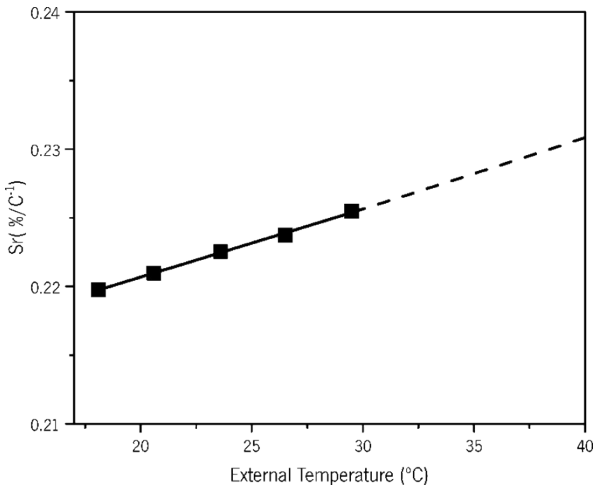

Fig. S 10. Relative thermal sensitivity of actin-GFP fluorescence lifetime in the recorded and extrapolated (dotted line) temperature range.

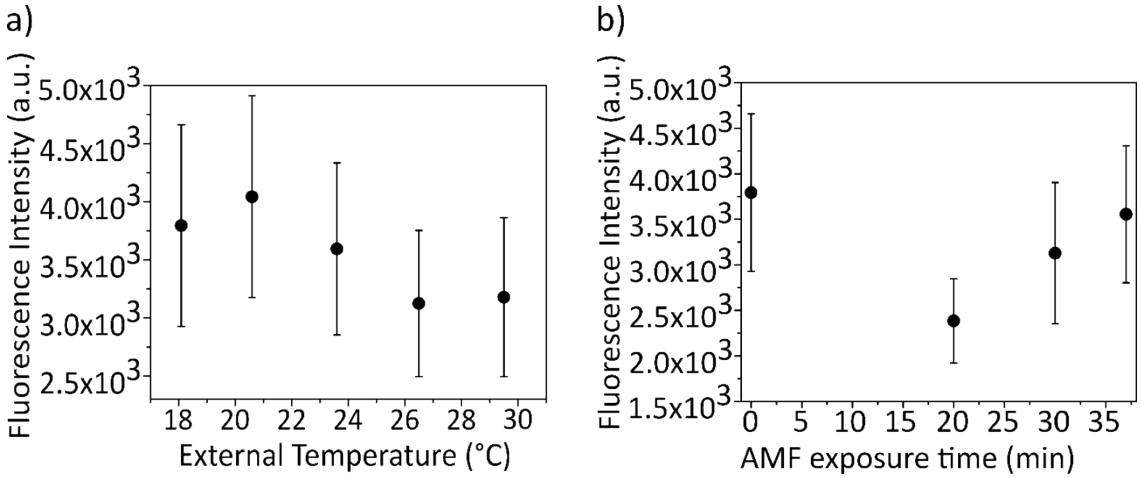

Fig. S 11. Temperature dependence of actin-GFP intensity and during magnetothermal treatment. **a)** Temperature-dependent fluorescence intensity of actin-GFP **b)** Fluorescence intensity change observed during the magnetothermal treatment.

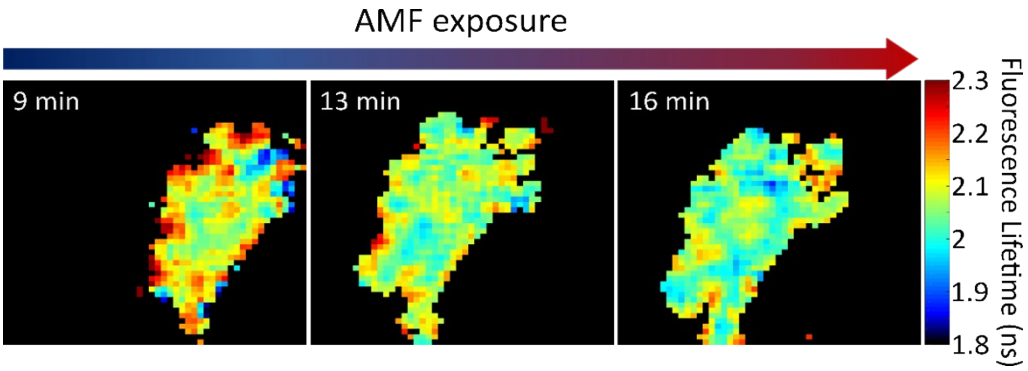

Fig. S 12. Additional time points-associated FLIM images of HeLa cells expressing actin-GFP recorded during magnetic hyperthermia treatment using the AMF exposure on Fe3O4@PAA MNPs -loaded HeLa live cells. Lower resolution images acquired with shorter exposure times.

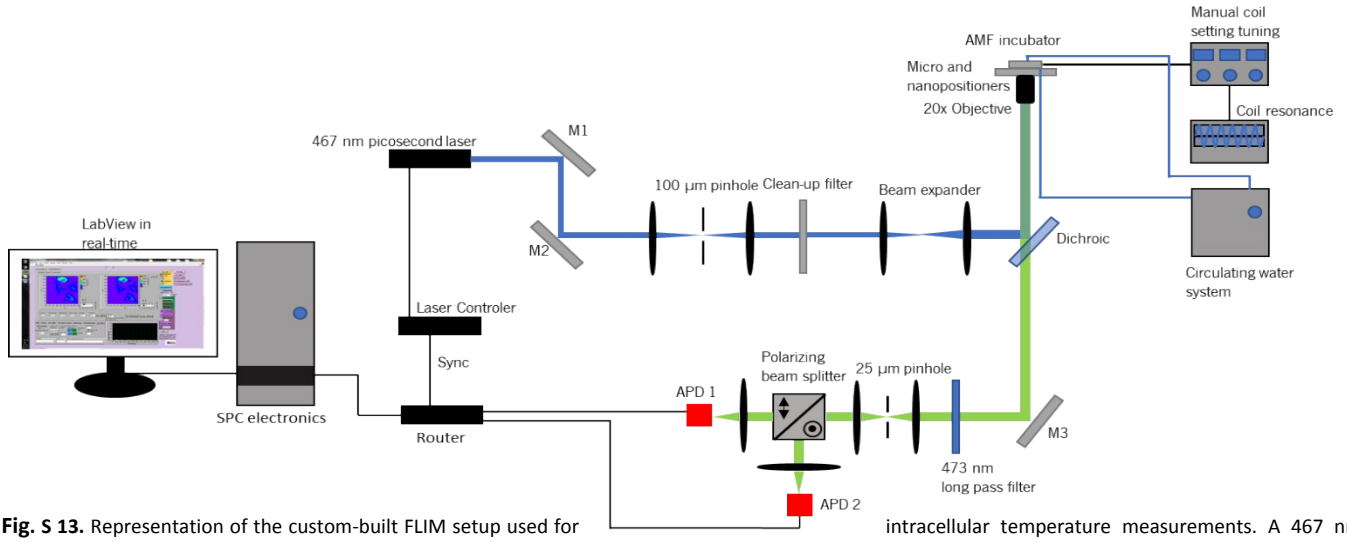

Fig. S 13. Representation of the custom-built FLIM setup used for intracellular temperature measurements. A 467 nm picosecond laser is guided by mirrors and a dichroic to a 0.4 NA 20x objective that focuses the laser beam in the sample and collects the fluorescence emission. This is then guided through a pinhole, to reduce emission from outside focal plane, into a polarizing beam splitter that splits fluorescence emission in vertical and horizontal polarization, before reaching two APDs. The signal from the APDs is read by SPC electronics that enable TCSPC. The sample is scanned with a micro and a nanopositioner, recording data in each pixel, allowing to build images in real-time by LabView based software