Supplementary information:

Other CLEM protocols attempted:

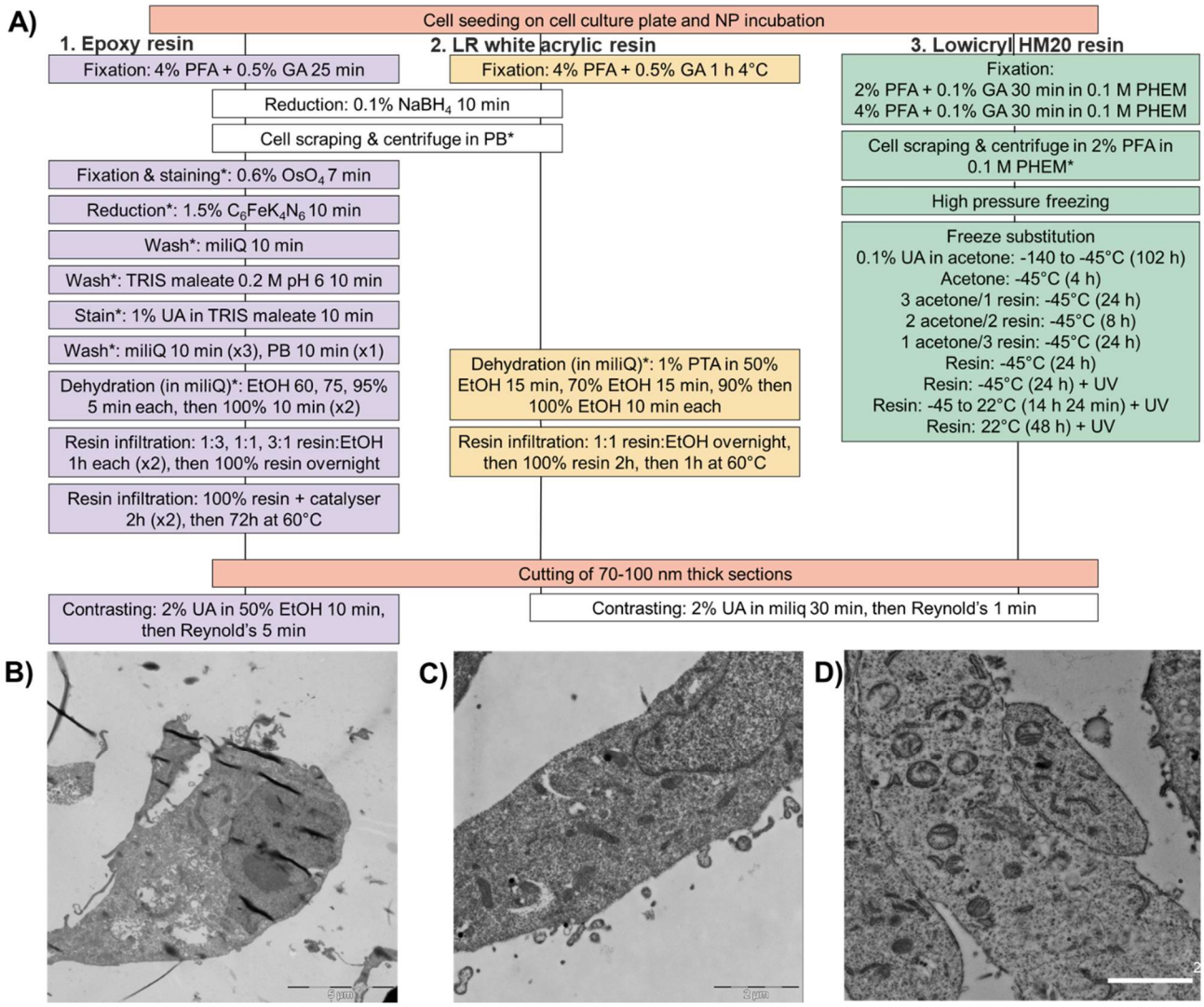


Figure S1. Summary of CLEM protocols attempted and resulting TEM morphology. A) Methodology used for 1. an epoxy resin protocol (Embed-812; Electron Microscopy Sciences), 2. a LR white acrylic resin (medium viscosity, catalysed; Agar Scientific) and 3. a lowicryl HM20 resin (Electron Microscopy Sciences). Unless stated otherwise, the steps were carried out at R.T. * denotes a centrifugation step at 2500 rpm at R.T. (Eppendorf 5804 R). B) TEM image of cell ultrastructure using the epoxy resin protocol; structure seems damaged with many empty cytoplasmic spaces (scale bar = 5 µm. C) TEM image of cellular ultrastructure using the LR white acrylic resin protocol; structure is better preserved than with the latter resin, but organelles are not easily distinguishable (scale bar = 2 µm). D) TEM image of cellular ultrastructure using Lowicryl HM20 resin; structure is well preserved with organelles clearly distinguishable (scale bar = 2 µm).

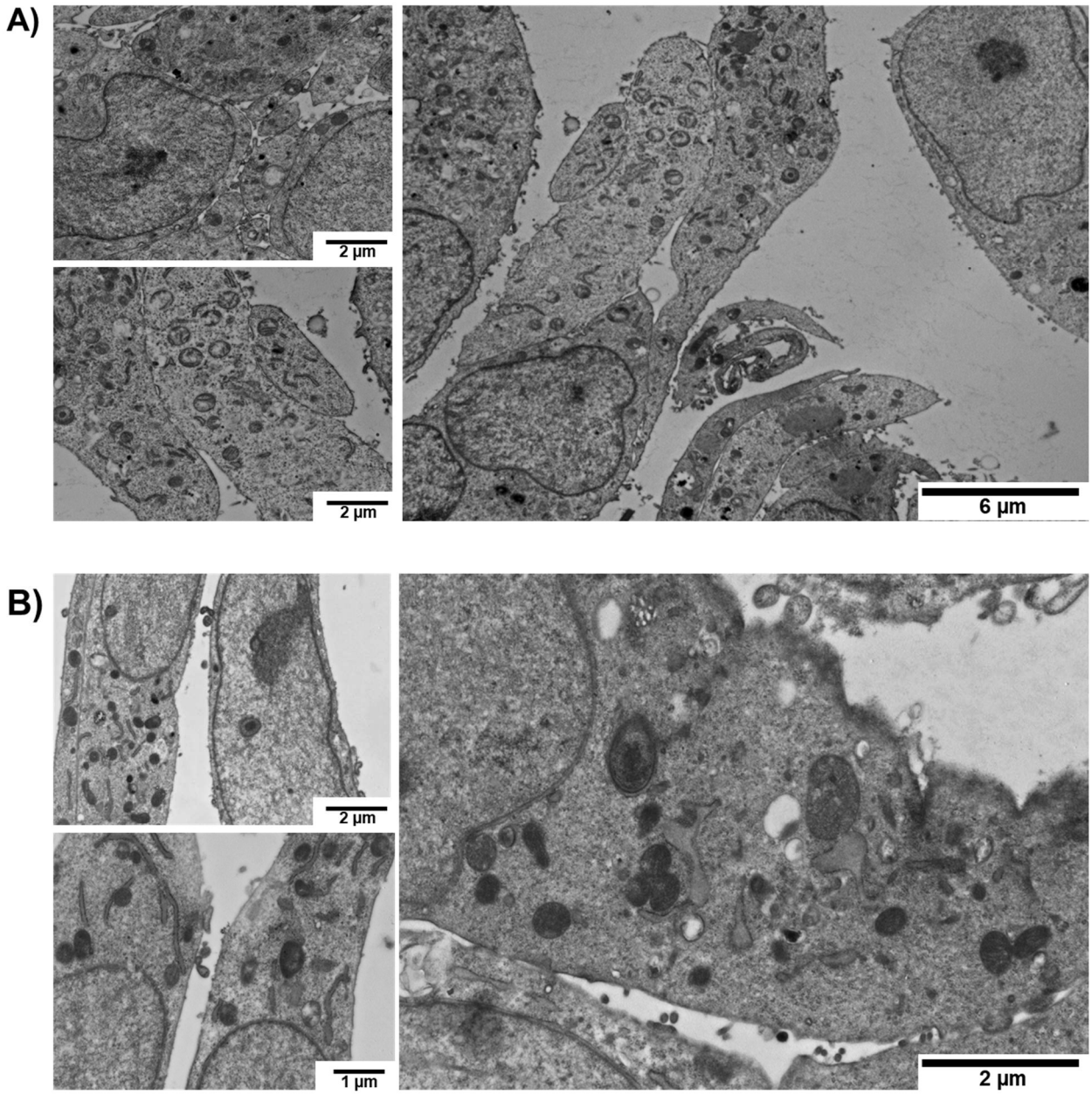


Figure S2. Ultrastructural preservation after STORM-TEM protocol treatments. A) Ultrastructure of HeLa cells incubated with PLGA-PEG DiI loaded NPs and imaged with STORM. B) ultrastructure of HeLa cells without NP incubation and without STORM imaging (control).

NP analysis:

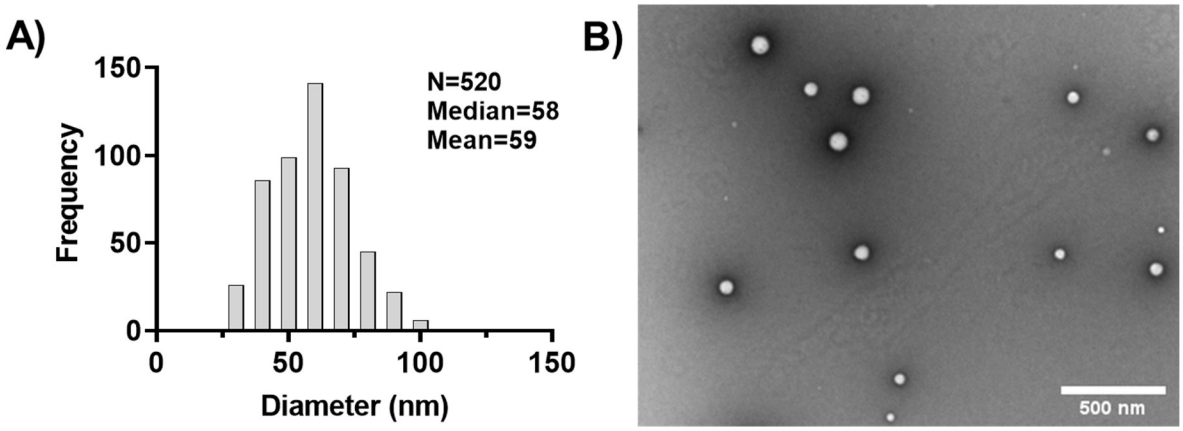
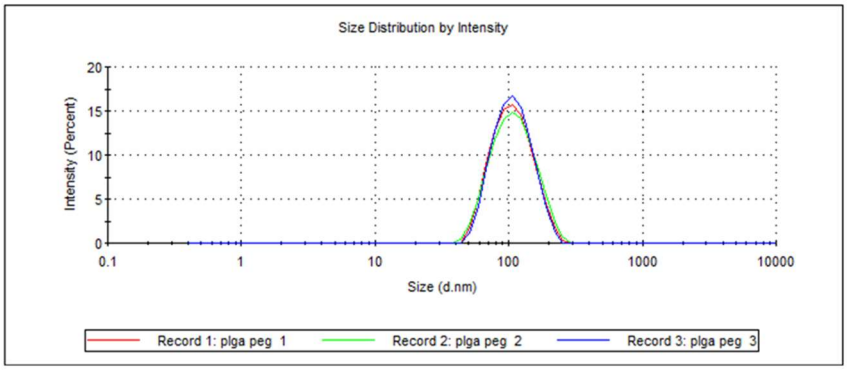


Figure S3. TEM characterization of PLGA-PEG NPs.

For negative staining, a 200-mesh carbon-layered copper grid was treated with UV-glow discharge (BAL-TEC CTA 005 Glow Discharge Unit) for 1 min to improve attachment. Using a fine tweezer, the grid was placed on top of a ~ 40 µL drop of NP solution 1mg/mL for 2 min. The grid was then washed to remove buffer salts using milliQ water drops for 1 min then 30 sec, then negatively stained using filtered 2% uranyl acetate (UA 2%, in milliQ water) for 1 min. Excess UA was removed by tapping the edge of the grid on Whatman filter paper. The grid was then allowed to dry overnight in a desiccator before TEM imaging.



	Zeta average (nm)	STDEV	PdI	STDEV	ZP (mV)	STDEV
PLGA-PEG 30 µM DiI	100	(+/-) 0.6	0.099	(+/-) 0.009	-20	(+/-) 0.3

Figure S4. DLS characterization of PLGA-PEG NPs**.** Analysis of the hydrodynamic radius (Zeta average nm) by intensity and polydispersity index (PdI) using Dynamic Light Scattering (DLS) at 25°C in milliQ water pH 7.0. Analysis of zeta potential (ZP, Mv) using a Zetasizer Nano ZS (Malvern Panalytical) at 25°C in milliQ water pH 7.0. Measurements were taken the following day after formulation. The standard deviation (STDEV) is for 3 measurement repeats.

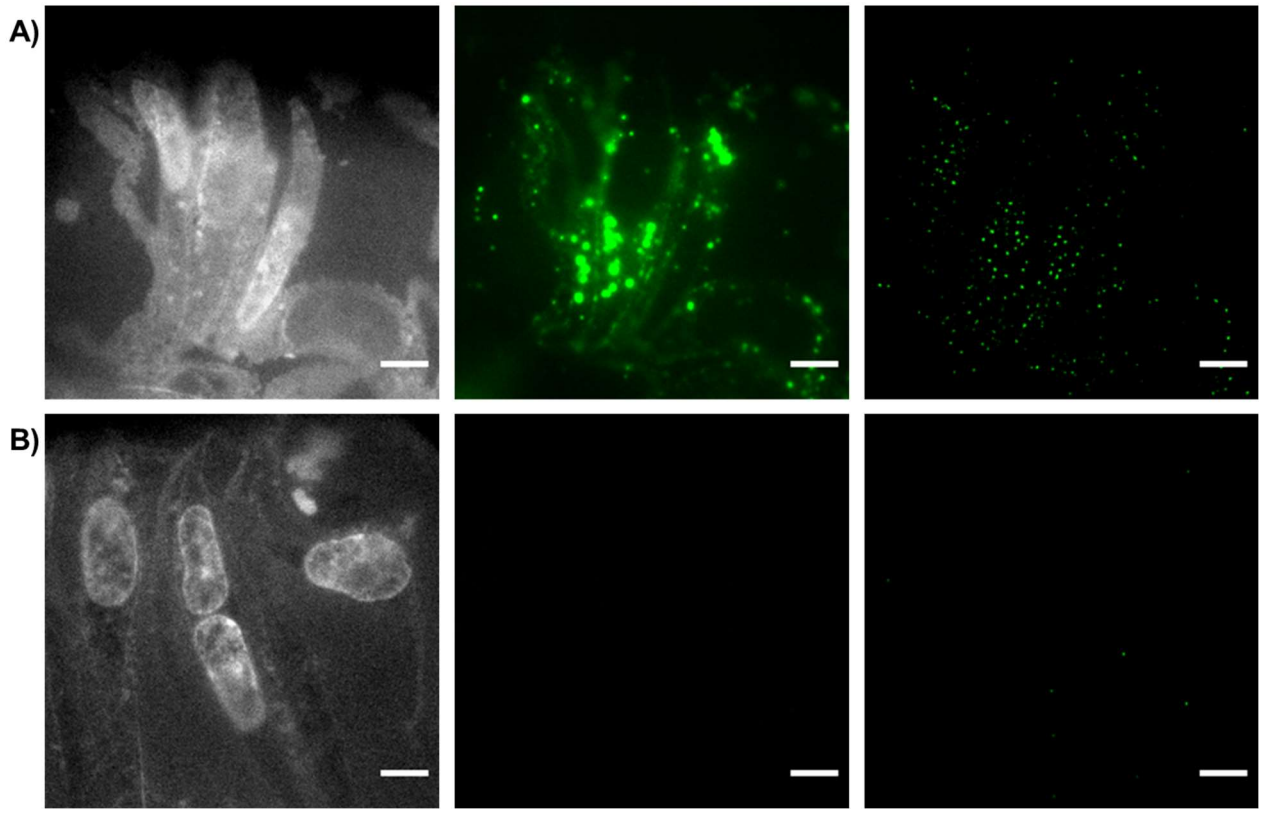


Figure S5. Fluorescence signal after mild fixation, HPF and FS in preparation for STORM-TEM protocol on 70-100 nm ultrathin sections for A) HeLa cells incubated with PLGA-PEG DiI loaded NPs for 8 h and for B) HeLa cells without any NP incubation. Left panel is of nuclei, middle panel is the low-resolution signal of NPs and right panel is the respective STORM image. Contrast for middle and right panels was kept the same. No significant background fluorescence was detected. Scale bars = 5 µm.

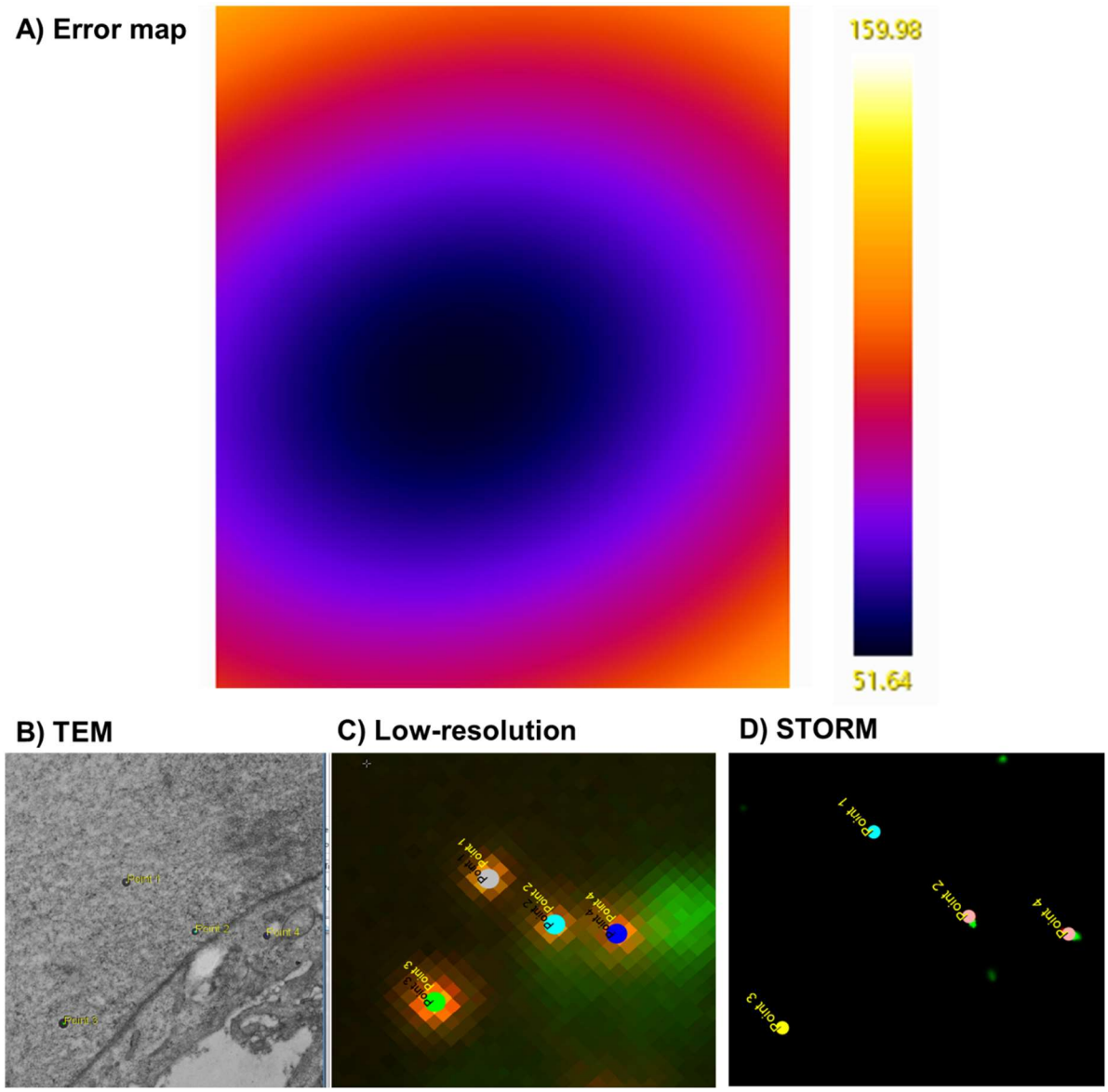


Figure S6. The error map resulting from the correlation of a FM/STORM image with a TEM image as calculated by the open source ec-CLEM (ICY) software. Colour gradient indicates an error of 51.64 nm in the centre of the image to 159.98 nm on the extremities of the image. Tetraspeck fiducial markers were used for the correlation, as they are visible in TEM (B) and in FM (C)/STORM(D).

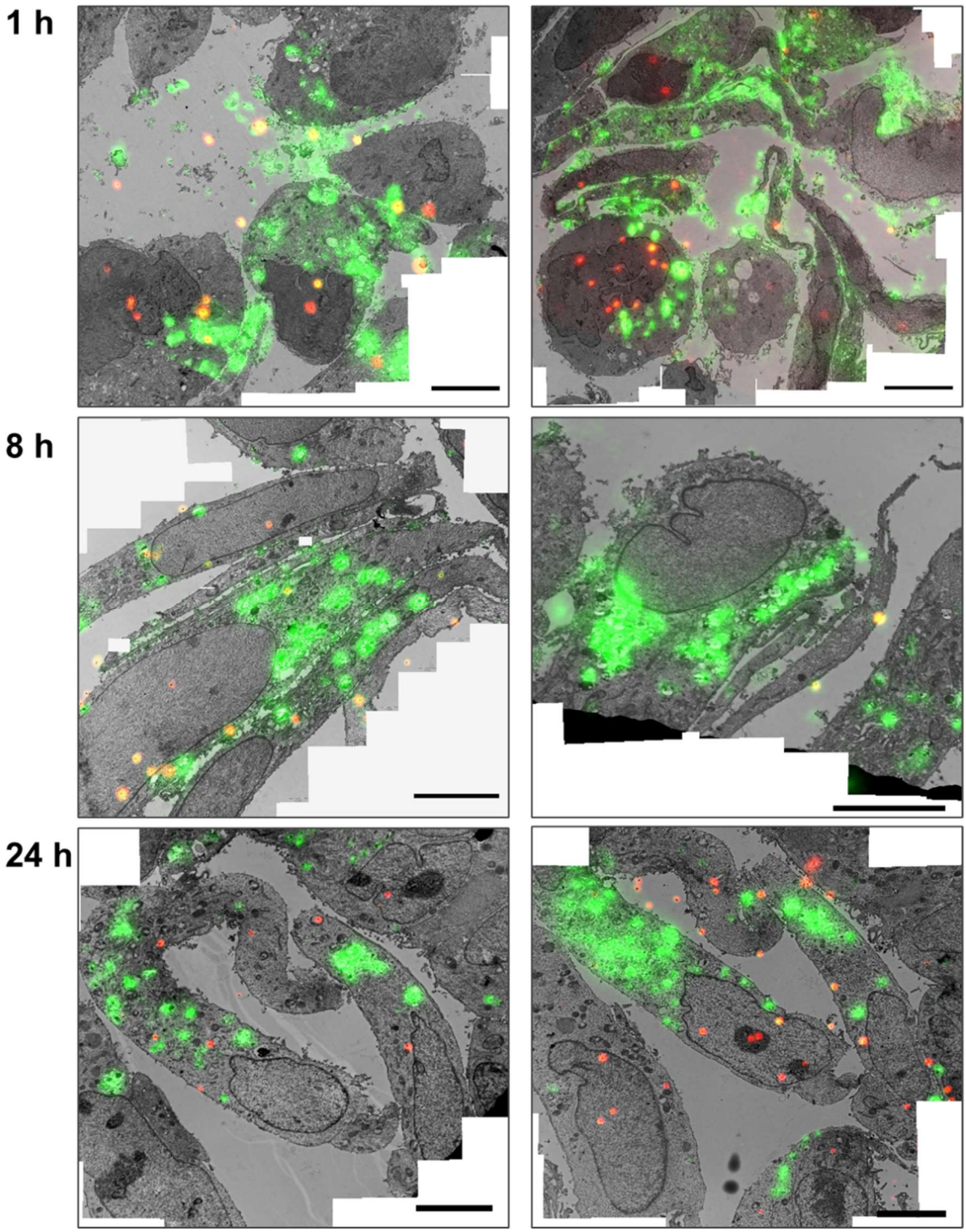


Figure S7. Correlative light and electron microscopy (low resolution-TEM) images of PLGA-PEG NPs incubated with HeLa cells at different time points (1 h, 8 h and 24 h). NP signal is seen in green and fiducial marker (Tetraspeck) signal is seen in red/orange. NPs incubated for 1 h are mainly internalized within intracellular compartments, but a high population is found outside the cell/on cell membrane. NPs incubated for 8 h appear more internalized within intracellular compartments, although some signal is found near the cell membrane. At 24 h incubation, the signal seems to be almost completely internalized within the cell. A 4 h pulse chase was carried out for 8 h and 24 h incubation time points. Scale bars = 5 µm.

Extra CLEM images:

1h

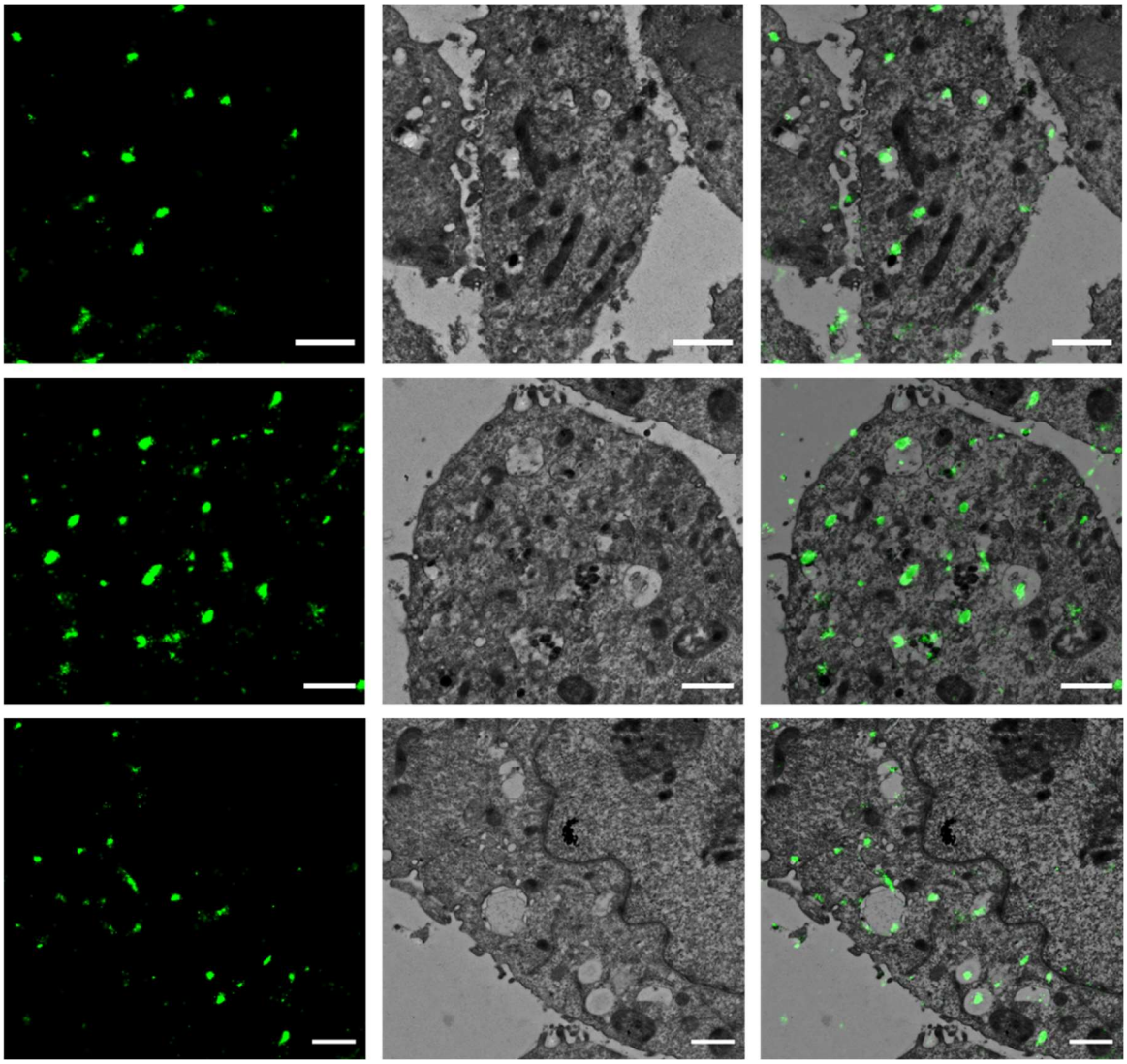


Figure S8. CLEM (STORM-TEM) images of PLGA-PEG NPs incubated with HeLa cells for 1 h. Left panels are STORM images, middle panels are TEM images of the same region, and the right panels are the overlay of STORM and TEM images. Scale bars = 1 μm

8 h

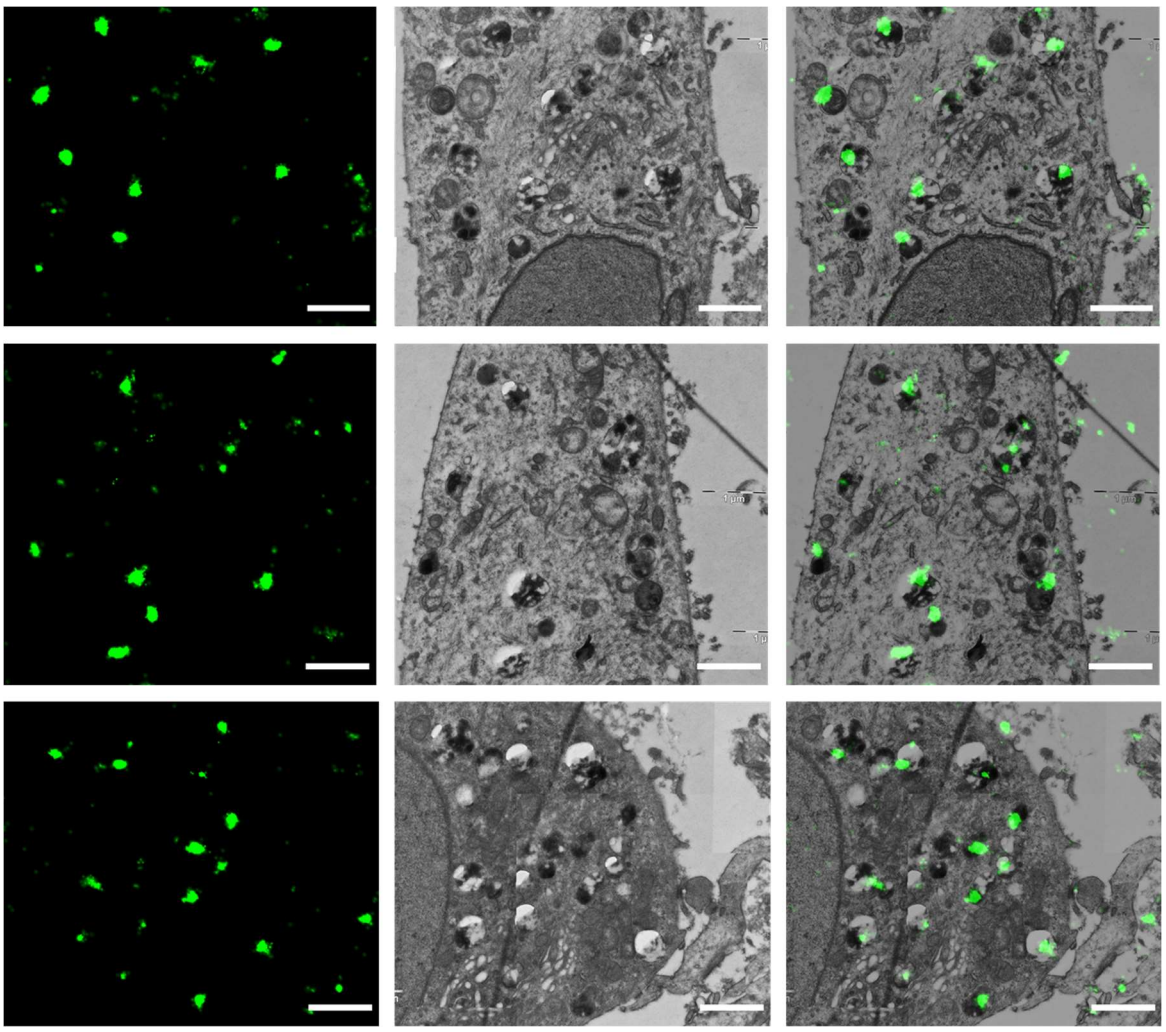


Figure S9. CLEM (STORM-TEM) images of PLGA-PEG NPs incubated with HeLa cells for 8 h. A pulse-chase was done after 4 h of incubation. Left panels are STORM images, middle panels are TEM images of the same region, and the right panels are the overlay of STORM and TEM images. Scale bars = 1 μm

24 h

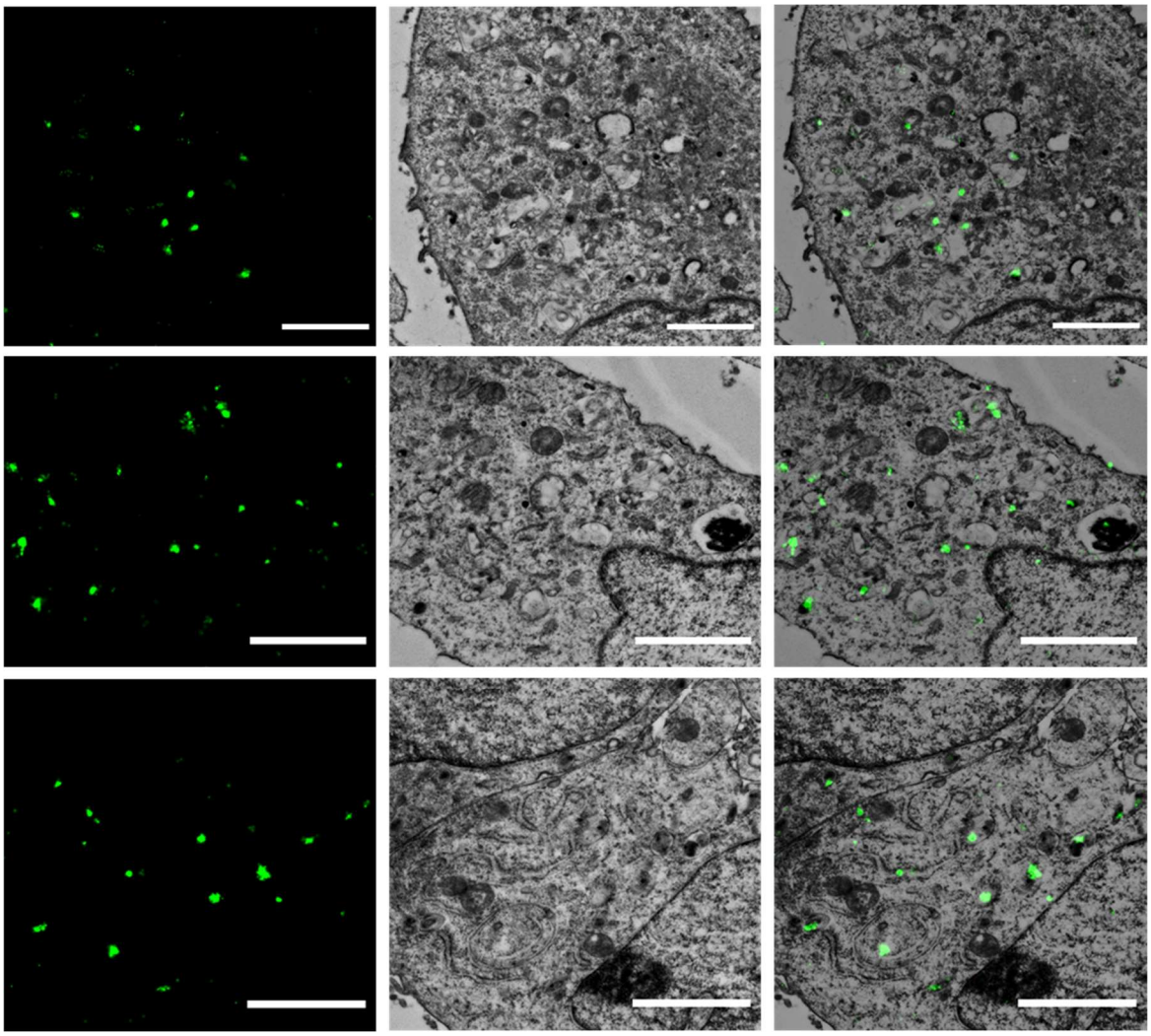


Figure S10. CLEM (STORM-TEM) images of PLGA-PEG NPs incubated with HeLa cells for 24 h. A pulse-chase was done after 4 h of incubation. Left panels are STORM images, middle panels are TEM images of the same region, and the right panels are the overlay of STORM and TEM images. Scale bars = 1 μm

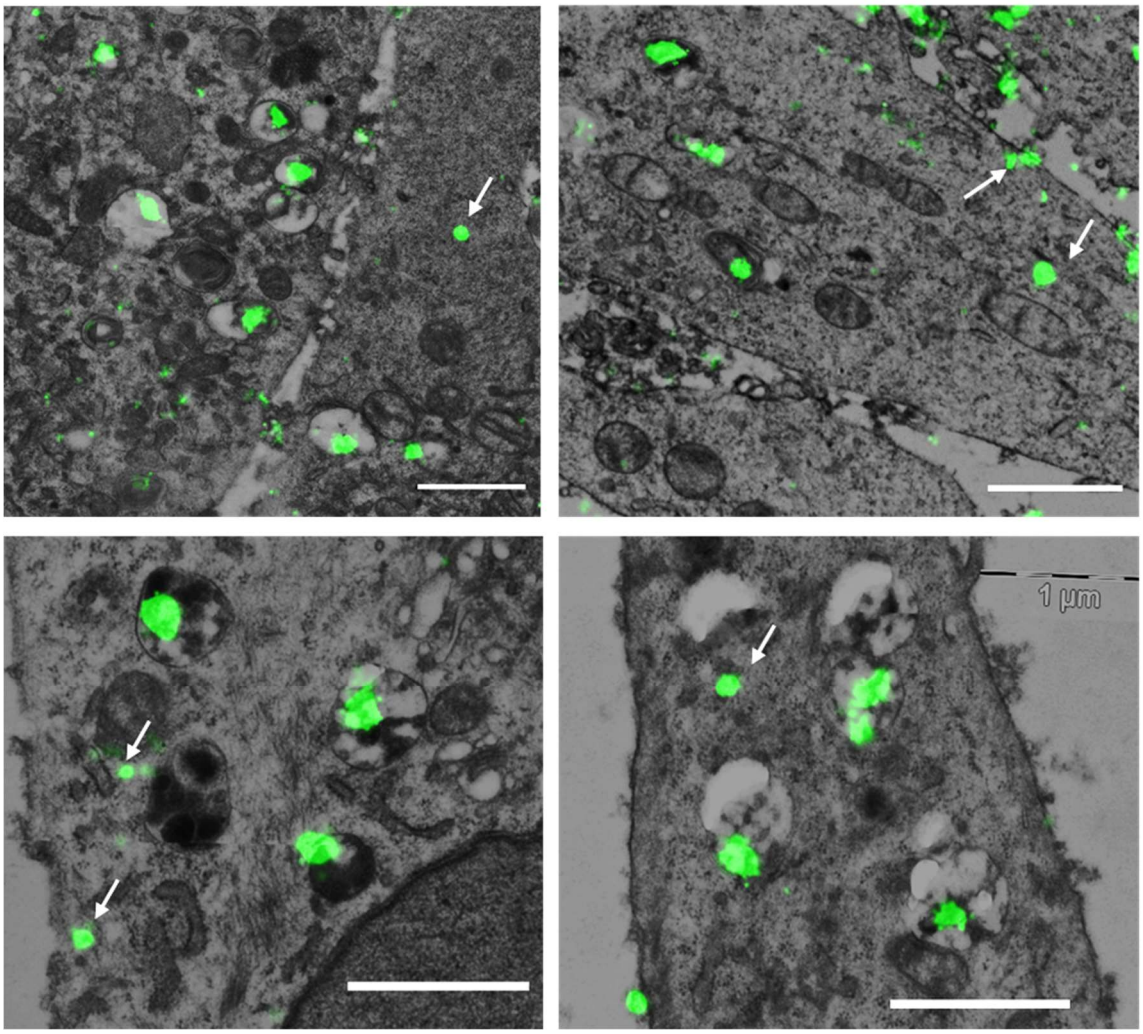


Figure S11. CLEM (STORM-TEM) images of PLGA-PEG NPs incubated with HeLa cells. White arrows highlight the NPs found outside of endo-lysosomal compartments and denoted as ‘non-endosomal (NE)’ throughout the main article. NPs found on the cell membrane were not included in this group. Scale bars = 1 µm

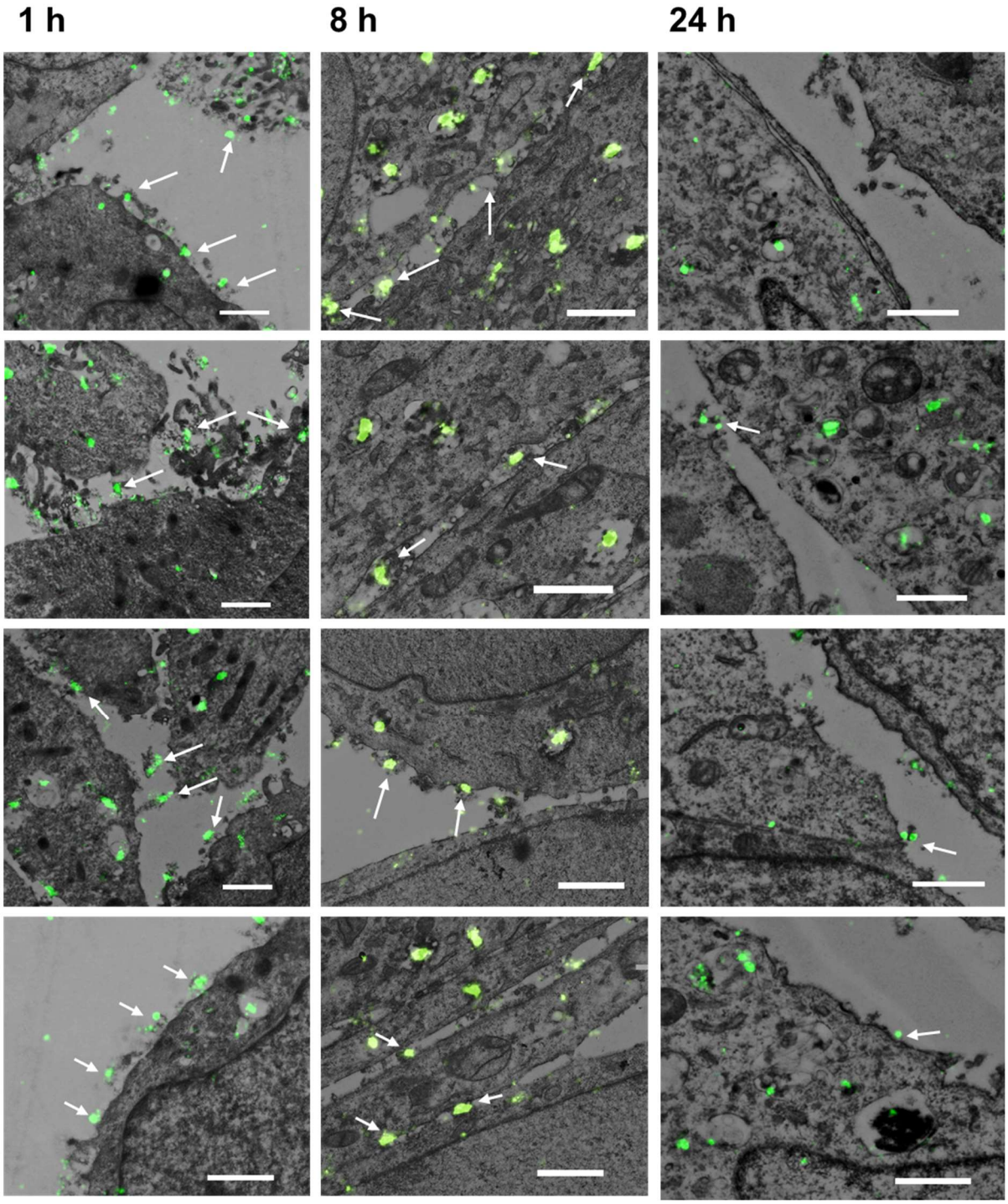


Figure S12. CLEM (STORM-TEM) images of PLGA-PEG NPs on the surface or outside of HeLa cells, after incubation at different time points (1 h, 8 h and 24 h). STORM NP signal is observed in green. At 1 h, many NPs can be observed on the surface of cells, followed by less at 8 h, with 24 h showing the lowest number. White arrows are used to direct the reader to extracellular NPs that are not easily visible. Scale bars = 1 μm

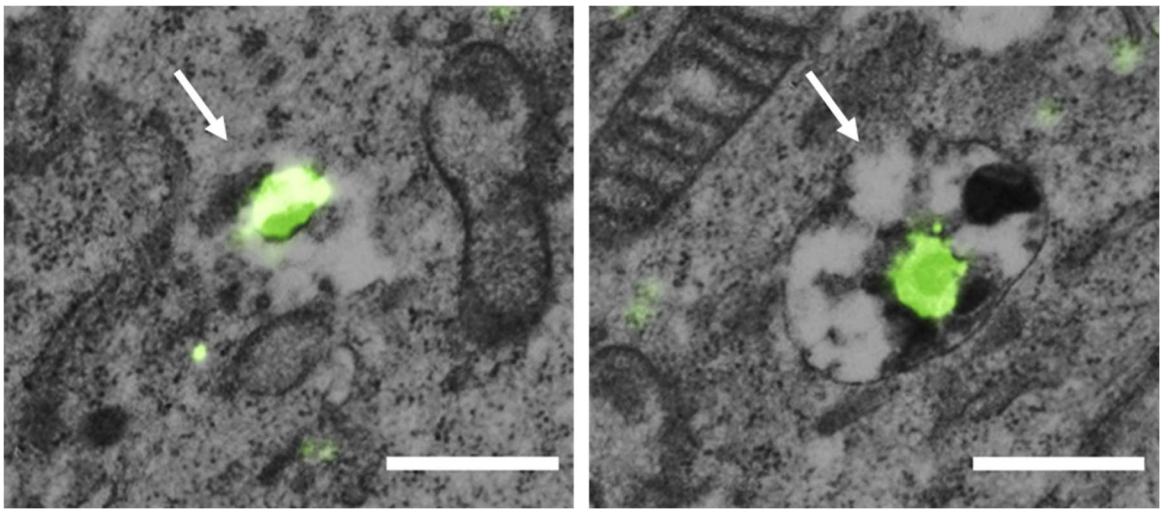


Figure S13. STORM-CLEM images of PLGA-PEG NPs (green) in ruptured cellular compartments. Scale bar = 500 nm.

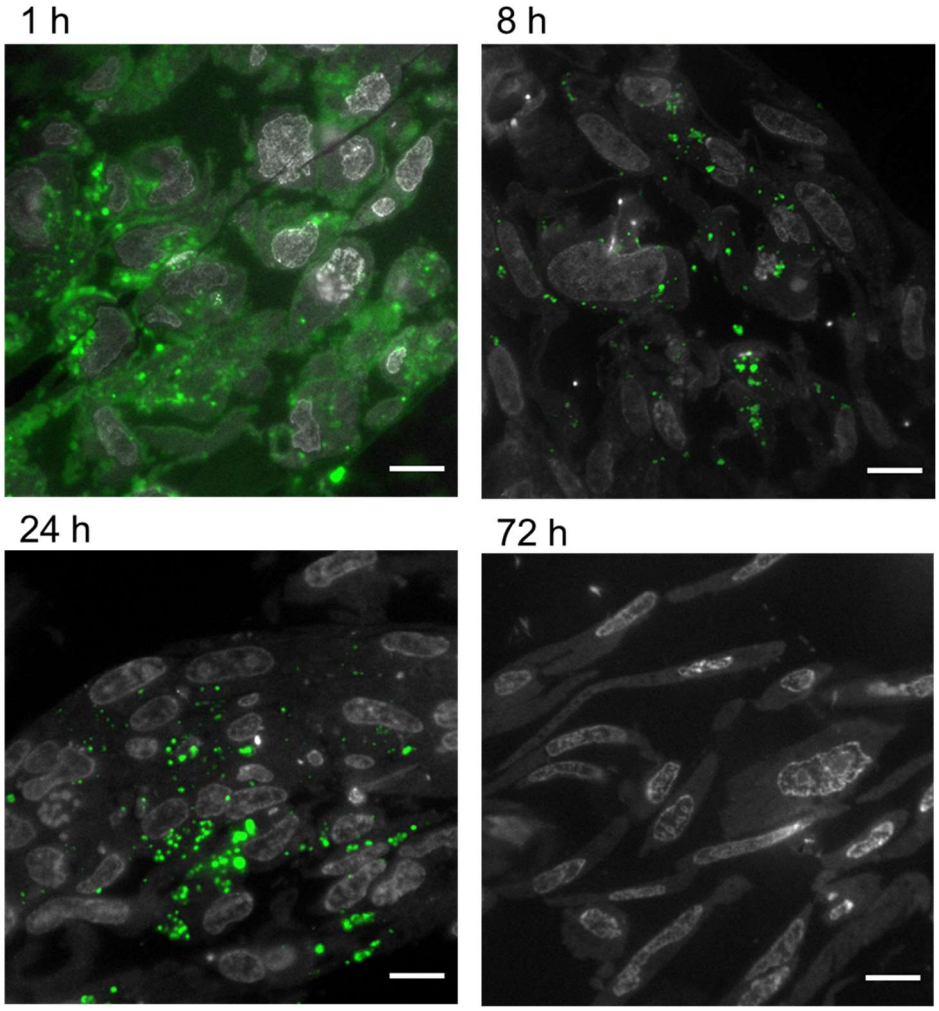
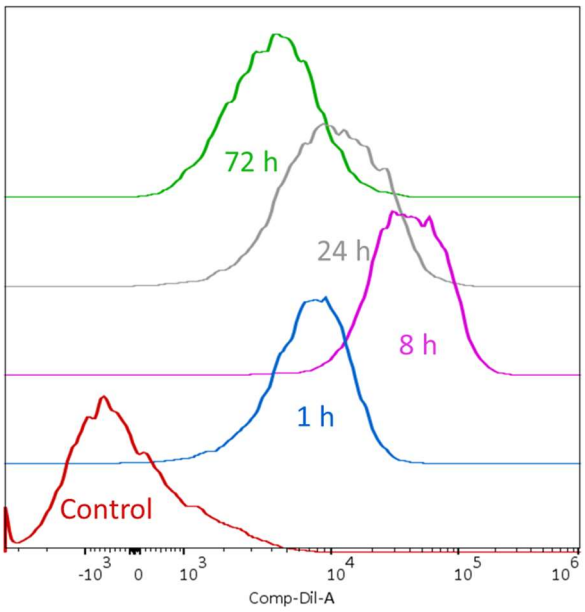


Figure S14. Fluorescence signal of PLGA-PEG NPs incubated for different time points (1 h, 8 h, 24 h and 72 h) with HeLa cells in 500 nm semithick sections after CLEM protocol. At 1 h signal looks diffuse around the cells, with some localized signal inside cells. At 8 h and 24 h the signal looks more localized in specific intracellular compartments. At 72 h there is a lack of signal. A 4 h pulse chase was carried out for 8 h and 24 h incubation time points. Scale bar = 10 μm.



	Sample Name	Subset Name	Count	Freq. of Parent	Mean : Comp-DiI-A
☐	E2 72.fcs	cells	9917	74.8	5299
☐	D2 24.fcs	cells	9959	68.4	14316
☐	C2 8.fcs	cells	9928	69.5	47709
☐	B2 1.fcs	cells	9897	70.0	8452
☐	A2 C.fcs	cells	9832	69.6	-128

Figure S15. Cellular uptake using flow cytometry of PLGA-PEG NPs in HeLa cells at different incubation times. The highest number of NPs within/on the cells is found at 8 h (purple) > 24 h (grey) > 1h (blue) > 72 h (green). The red colour indicated background fluorescence due to cells only.

A 12-well Nunc™ cell culture plate (dDBiolab) was seeded with HeLa cells (ATCC) and incubated for 24 h in Dulbecco's Modified Eagle Medium (DMEM, with L-Glutamine 10 mM, 4.5 g/L D-glucose and pyruvate, Gibco) supplemented with FBS 5% (Gibco) and penicillin/streptomycin 1% (Biowest), at 37˚C and 5% CO_2, to achieve an approximate confluency of 100 000 cells/well. After the incubation, the medium was removed and the cells washed x3 with warm PBS (1x, pH 7; Thermo Fisher). Then, the PLGA-PEG NPs were diluted as to achieve the original concentration of NPs per number of cells in the CLEM experiments (20 µg/mL, 1 mL per well) in OptiMEM™ (Thermo Fisher). NPs were added to the wells and incubated for either 1 h, 8 h, 24 h or 72 h at 37 ˚C and 5% CO_2. For the latter 3 time points, the NP solution was removed after 4 h and washed x3 with PBS (at 37 ˚C), then replaced with full medium for the remaining incubation time. One well was kept as control (no NP incubation). Next, the medium was removed, and the cells were washed x3 with PBS (at 37 ˚C). Then, cells were detached by incubating with 500 uL of trypsin-EDTA (0.25%; Thermo Fisher) for 5 min at 37 ˚C. 1.5 mL of full medium was added per well to inactivate the trypsin. The cells were then centrifuged at 5000 rpm (Eppendorf 5415 R) for 3 min at 4 ˚C, and resuspended in cold PBS, followed by two more rounds of centrifugation and resuspension. The final resuspension was done in 400 µL cold PBS and the samples were stored on ice until analysed. Data was analysed using a SA3800 (Sony) spectral flow cytometer equipped with 4 lasers (405, 488, 561, and 635-640 nm), using the 561 laser for NPs. A total of approx.10 000 counts were acquired per sample.

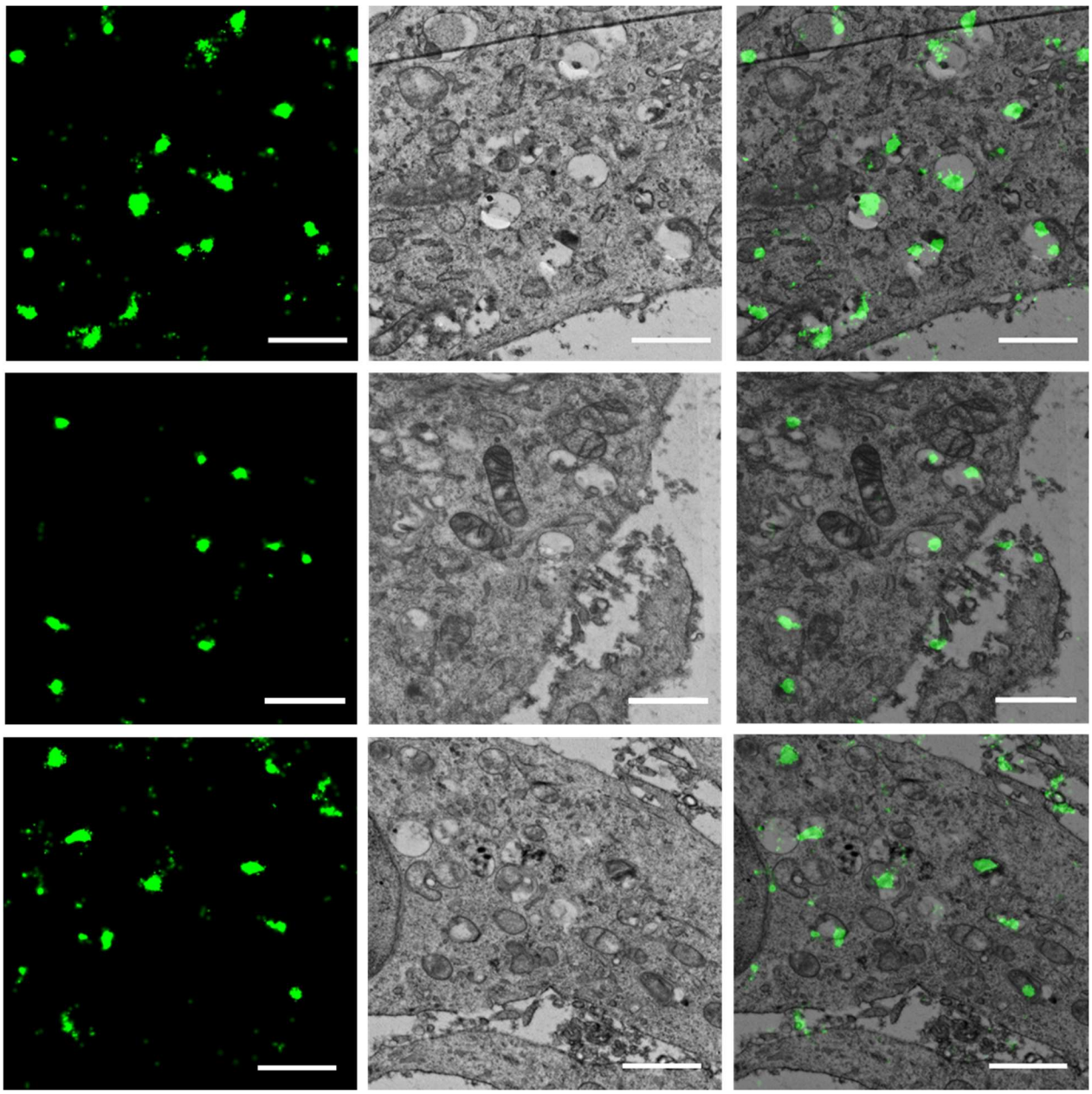


Figure S16. CLEM (STORM-TEM) images of HeLa cells incubated with 100 μM chloroquine for 4 h, followed by a 4 h pulse and a 4 h chase incubation with PLGA-PEG DiI loaded NPs. Left panels are STORM images, middle panels are TEM images of the same region, and the right panels are the overlay of STORM and TEM images. Scale bars = 1 μm.

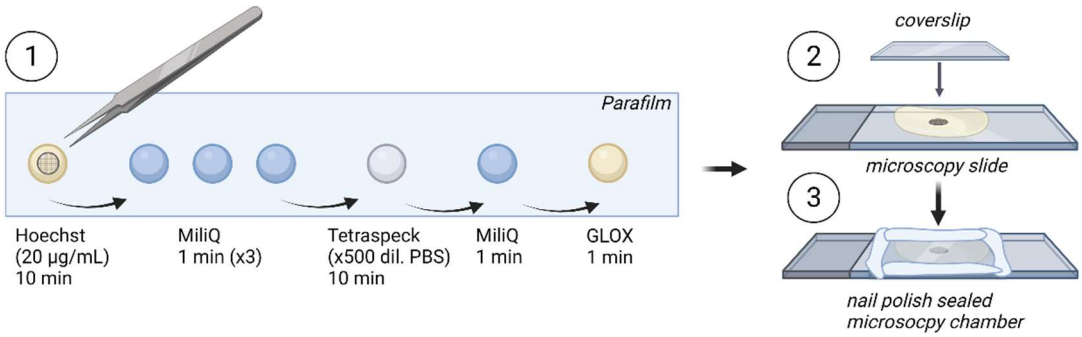


Figure S17. Preparation of grids for STORM imaging.

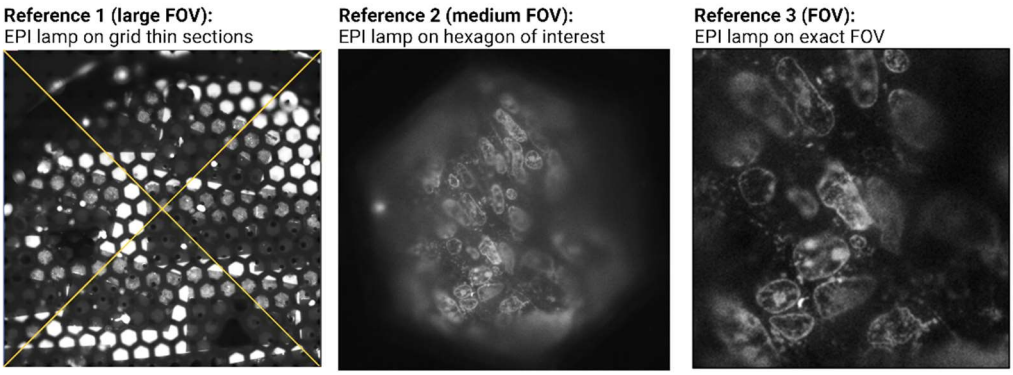


Figure S18. Reference image acquisition in STORM. Where FOV is field of view.

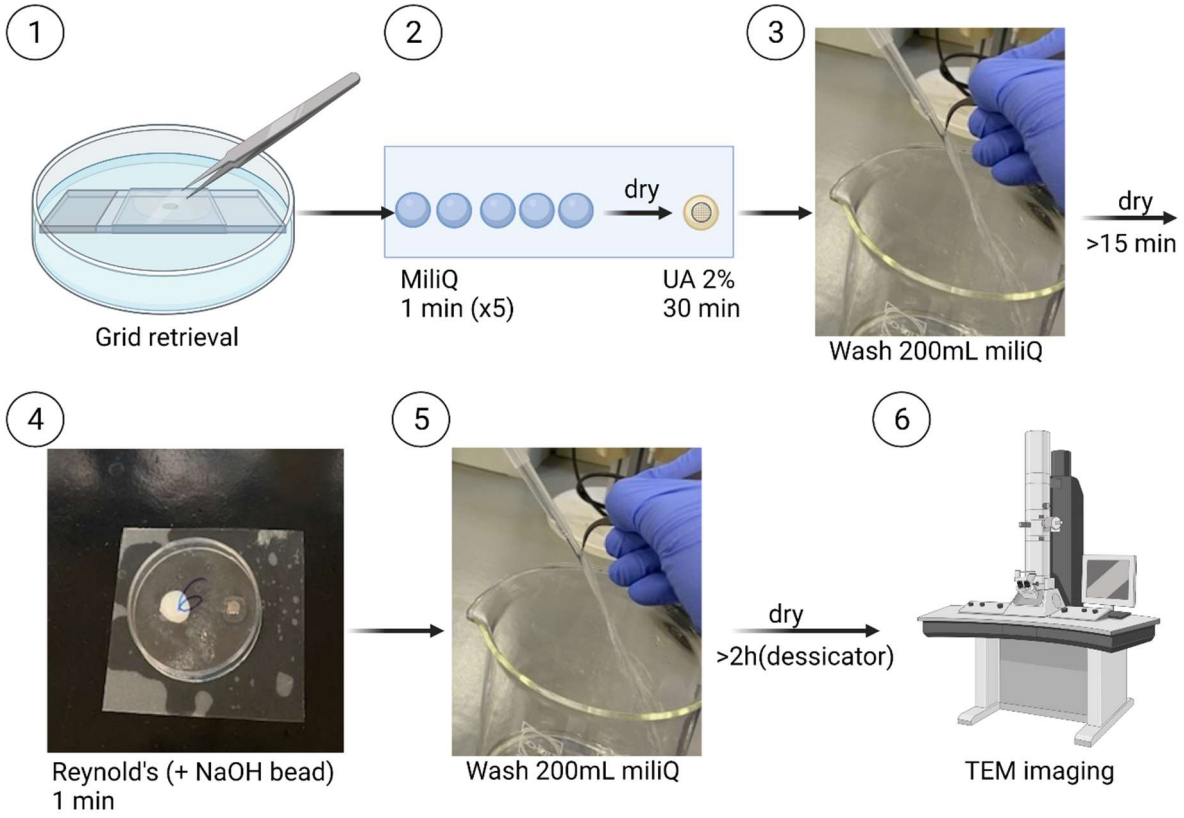


Figure S19. Grid retrieval and contrasting steps for TEM imaging.

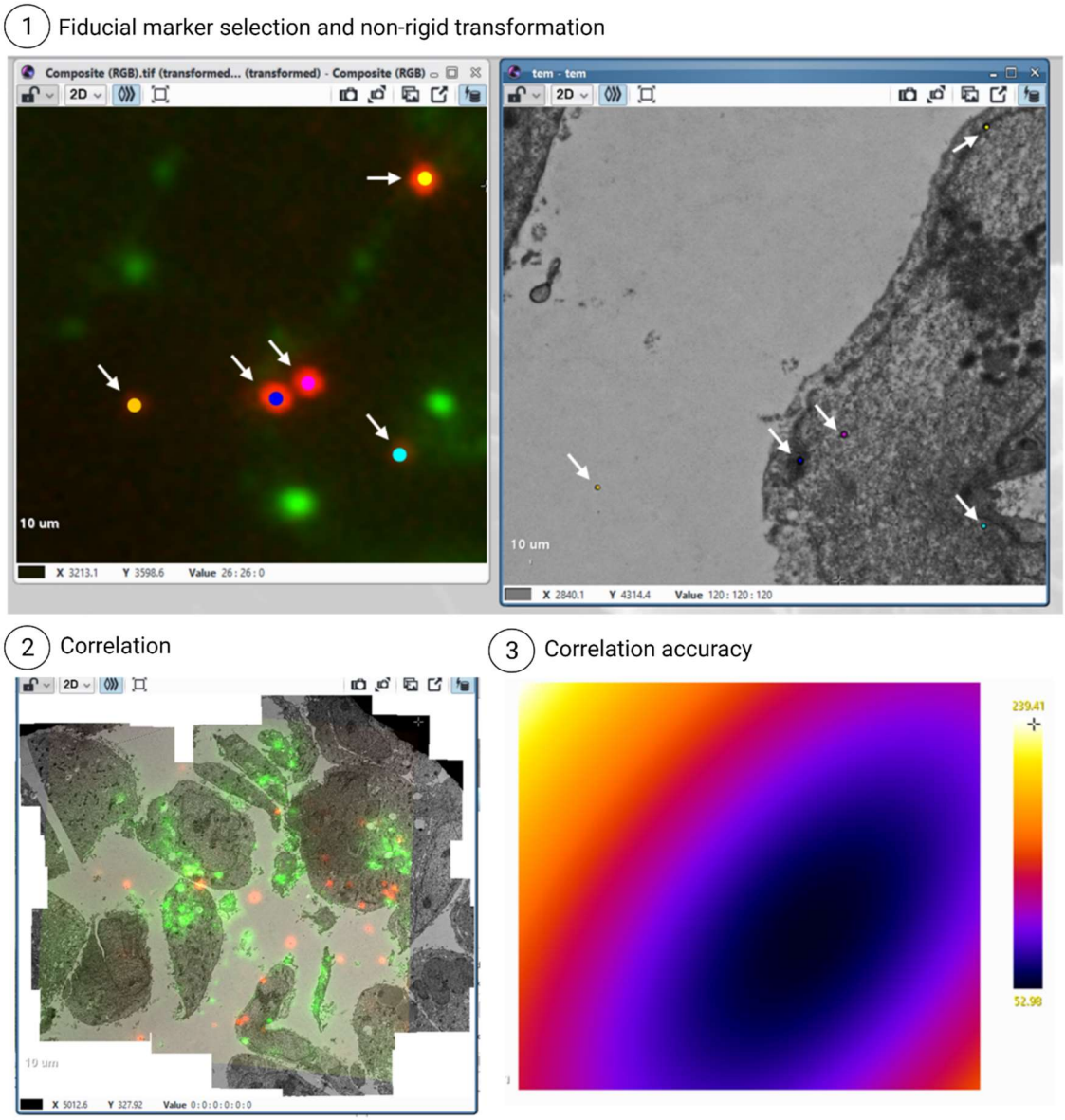


Figure S20. CLEM correlation using ec-CLEM.

Table S1. Freeze substitution steps.

Step	T_{start} (°C)	T_{end} (°C)	Slope (°C/h)	Time (hh:mm)	Reagent	%	UV
1	-140	-140	0	01:00	Acetone/UA 0.1%	100%	
2	-140	-90	50	09:00	Acetone/UA 0.1%	100%	
3	-90	-90	0	80:00	Acetone/UA 0.1%	100%	
4	-90	-45	5.6	08:00	Acetone/UA 0.1%	100%	
5	-45	-45	0	04:00	Acetone/UA 0.1%	100%	
6	-45	-45	0	01:00	Acetone	100%	
7	-45	-45	0	01:00	Acetone	100%	
8	-45	-45	0	01:00	Acetone	100%	
9	-45	-45	0	01:00	Acetone	100%	
10	-45	-45	0	24:00	3 Acetone/1 Lowicryl HM20	100%	
11	-45	-45	0	08:00	2 Acetone/ 2 Lowicryl HM20	100%	
12	-45	-45	0	24:00	1 Acetone/ 3 Lowicryl HM20	100%	
13	-45	-45	0	24:00	Lowicryl HM20	100%	
14	-45	-45	0	24:00	Lowicryl HM20	100%	+
15	-45	22	4.7	14:24	Lowicryl HM20	100%	+
16	22	22	0	48:00	Lowicryl HM20	100%	+